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Evidence for conduction of protons along the interface between water and a polar lipid monolayer

(bioenergetics/microchemiosmosis)

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ABSTRACT **Movements of H^+ along the polar heads of phospholipids spread in monolayers were compared to movements of H^+ in the aqueous subphase. The probe for detecting H^+ movement along the monolayer was a pH-sensitive fluorescein chromophore covalently bound to the head group of phosphatidylethanolamine. The behavior of this probe was not affected by the electrical properties of the lipid/water interface. Lateral diffusion of H^+ along the phospholipid/water interface was then studied by acid-jump experiments in which advantage was taken of the large size of the monolayer. H^+ was injected a few centimeters away from the probe observation area. The time needed for H^+ diffusion to the probe was monitored by the change in the fluorescence signal, fluorescein being nonfluorescent in an acid medium. Diffusion of H^+ in the bulk phase was monitored by the fluorescence change of water-soluble fluorescein isothiocyanate. Diffusion along the lipid monolayer was found to be 20 times faster than in the bulk water phase and required a structured monolayer in order to occur, as revealed by variation of the molecular area occupied by the lipid molecules. The molecular basis of rapid H^+ transfer along the lipid monolayer may be the existence of a hydrogen-bond network along the polar heads, capable of supporting a rapid "hop and turn" of H^+.**

The coupling between electron transport and ATP synthesis catalyzed by membranes of mitochondria, chloroplasts, and bacteria is now recognized to be protonic in nature. This coupling is described as being localized (1), semilocalized (2), or delocalized (3). In the chemiosmotic or delocalized hypothesis, it is assumed that the difference in the electrochemical gradient of protons ($\Delta p = \Delta\Psi - z\Delta pH$, in which z is 59 mV), generated across the energy-transducing membrane by electron transport or by ATP hydrolysis, is the driving force for the synthesis of ATP from ADP and inorganic phosphate (P_i). In this hypothesis, which is phenomenological, the membrane plays no role in the storage and transmission of energy, which is assumed to take place via the bulk aqueous phases separated by the membrane.

Although much experimental evidence supports the delocalized chemiosmotic hypothesis, recent results from different laboratories appear to conflict with such a delocalized pathway of protons. Studies on the fast processes involved in oxidative photophosphorylation in *Rhodospirillum rubrum* show that the functional driving force is provided by the electrical term $\Delta\Psi$, as no proton ejection is observed (4). In the case of chloroplasts, photophosphorylation can be observed even in the presence of permeant buffers that abolish the proton gradient between the two bulk phases separated by the membrane (5). Both in chloroplasts (5–7) and in photosynthetic bacteria (8, 9), phosphorylation begins before proton ejection into the bulk phase occurs. In thylakoids, where localized domains exist in the membrane, in terms of both structure (10) and chemical composition (11), the effect of the replacement of protons by deuterons ($^2H^+$) on the control of electron flow and on the ratio of phosphorylation to electron transport (P/e^- ratio), as well as experimental evidence for a difference in the yield of phosphorylation for the same ΔpH between photosystem I (located in the stroma region) and photosystem II (located in the grana stacks), suggest the occurrence of microdomains in the membrane differently sensitive to the bulk phase pH gradients (12–14). Similarly, numerous studies on mitochondria indicate that the bulk phase Δp cannot account for energy transduction (15). Additionally, the alkalophilic bacteria appear capable of generating ATP at very low Δp, with inverted ΔpH (16); moreover, under some conditions halobacteria transduce energy for ATP synthesis in the absence of transmembrane Δp. In these membrane systems the transmembrane proton electrochemical potential as represented by $\Delta p = \Delta\Psi - z\Delta pH$ cannot be considered as the direct driving force for phosphorylation. It has therefore been suggested that proton movements may be localized in or on the membrane surface so that proton fluxes between the energy source and the ATP synthase system may occur laterally along the membrane, perhaps concurrently with the classical delocalized proton flux between the bulk aqueous phases.

Some theoretical and experimental considerations have suggested that polar lipid headgroups may be a pathway for proton transfer along the surface of a membrane (17, 18). In the present communication, direct experimental evidence for such a lateral conduction along the headgroups of a phospholipid monolayer is presented. We utilized a fluorescence probe for following H^+ changes in the membrane/water interface and in the bulk aqueous phase (19, 20). Local fluxes of protons close to the phospholipid monolayer were monitored with a pH-sensitive chromophore covalently bound to the lipid (21, 22).

MATERIALS AND METHODS

Chemicals. Fluorescein isothiocyanate (FITC) and phosphatidylethanolamine (PtdEtn) (from *Escherichia coli*) were obtained from Sigma. 12-(9-Anthroyloxy)stearic acid was purchased from Molecular Probes (Junction City, OR). Salts and solvents were of analytical grade.

Fluorescein phosphatidylethanol thiocarbamide (F-PtdEtn) was synthesized as described in ref. 23. The labeled lipid was purified by chromatography on a Sephadex G-25 coarse gel column (Pharmacia) that had been allowed to swell in methanol/water (1:1, vol/vol) and then equilibrated with chloroform saturated with the above methanol/water solution. The same chloroform phase was used as eluting

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Abbreviations: PtdEtn, phosphatidylethanolamine; FITC, fluorescein isothiocyanate; F-PtdEtn, fluorescein phosphatidylethanol thiocarbamide.

solvent. The labeled lipid was chromatographed twice on this column to remove unreacted isothiocyanate. It was purified to homogeneity by preparative thin-layer chromatography on silica gel G (chloroform/methanol/water, 65:25:4, vol/vol/vol).

Monolayer Preparation. Monolayer experiments were carried out with ultrapure water from Motorola (Toulouse, France). Lipids were spread in the form of chloroform/methanol solutions (5:1, vol/vol) and measurements on the lipid film (surface pressure π, surface potential ΔV, and fluorescence F) were made only after a 5-min period necessary for complete solvent evaporation. The film surface pressure was monitored by means of a platinum plate connected to a force transducer of our fabrication. Compression isotherms and surface potential data were continuously recorded with an automatic apparatus devised in our laboratory, using two americium electrodes.

Fluorescence Measurements. Fluorescence was measured with an interface fluorimeter of our fabrication in which front-face fluorescence from a small illuminated area was monitored for different compression states of the film (19, 20). In this apparatus the troughs were milled from Plexiglas to minimize light scattering. The excitation and observation beams were separated by 45°, the observation direction being perpendicular to the film surface (Fig. 1). The diameter of the illuminated spot was about 4 mm and its illumination was uniform, as in fluorescence recovery after photobleaching experiments. The light sources were an Osram HBO 100W/2 mercury arc lamp or an Osram XBO 75W/4 xenon lamp. Wavelengths were selected by means of filters. The 12-(9-anthroyloxy)stearic acid was excited by the mercury lamps. The filters used for excitation were Schott UG1 or Kodak 18A Colorglas, which selected the mercury lines around 360 nm. The filters for observation were MTO DH525c′ plus Kodak 2E broad-band filters transmitting light above 420 nm. The fluorescein derivatives were excited with the xenon lamp; the interference filters used were MTO 7984 (excitation at 462 nm) and MTO 10313 (emission at 519 nm). The signals were monitored by a photomultiplier tube linked to a data acquisition unit. The fluorimeter will be described in detail elsewhere.

Orientation of the probe was analyzed as described in ref. 19. The excitation beam was linearly polarized by means of a Glan prism. This device was tiltable between two crossed positions, giving light polarized parallel either to the monolayer or to the incident plane (Fig. 1). The ratio of the observed fluorescence with these crossed positions is a good indicator of orientation changes of the probe, due to the cylindrical symmetry of the monolayer.

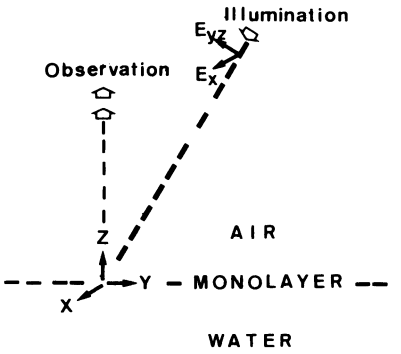

FIG. 1. Fluorescence measurements on monolayers: schematic drawing of the geometrical disposition. Orientation changes are obtained from the determination of the ratio of the fluorescence intensities observed when the electric field (E) of the incident light is parallel to E_x and when it is parallel to E_{yz}. Due to the 45° illumination, the fluorescence of the small volume of the subphase under the illuminated part of the monolayer is detected.

Fluorescence was measured as follows. The trough was first filled with the aqueous subphase. The signal was either set as zero fluorescence (when the fluorescence of F-PtdEtn was to be observed) or 100% fluorescence (when the signal of FITC dissolved in the subphase was of interest). The film was then built by spreading a small aliquot (about 10 μl) of the lipid mixture in chloroform/methanol (5:1, vol/vol). After 5 min for evaporation of the solvent, the fluorescence was monitored. Compression was then initiated when the characteristics of the probe were studied, or acid was injected when the lateral diffusion of protons was to be measured.

RESULTS

pH Dependence of the Emission of F-PtdEtn

The measurement of interfacial pH presents some difficulties (21, 22). It can be modulated by the electrical term Ψ_0 of the surface potential, which in turn depends upon the lipid ionization state and on the molecular packing (24). Furthermore, the response of a fluorescent pH probe at such an interface can depend on the nature of the polar head groups and on the lipid packing, probably via changes in the water dielectric constant at the interface (25). Before using F-PtdEtn for the proton conductivity experiments it was necessary to test its behavior as a pH indicator embedded in different lipid monolayers, under various ionic conditions in the subphase, and for various degrees of lipid packing.

Monolayers of PtdEtn (neutral) or of total lipid extract (acidic), both from *E. coli*, containing the pH indicator F-PtdEtn (2 mol %) were compressed on subphases of different pH or ionic strength. Fluorescence emission of the probe was observed to be altered by the pH of the subphase for all compression states of the lipid matrix. A maximum was observed above pH 7. The apparent pK of the F-PtdEtn probe, taken as the subphase pH giving a 50% change in the total emission, was observed to increase slightly with increasing surface pressure (from 5.7 at π = 0.2 mN/m to 6.2 at π = 30 mN/m) (Fig. 2).

No clear-cut effect of the nature of the lipid polar heads was observed. A small decrease (less than 0.3 pH unit) was noted on monolayers of acidic lipids spread on a high (100 mM) as compared to a low (10 mM) ionic strength buffer. No changes in orientation were detected for the different experimental conditions by using the linearly polarized incident light procedure described in *Materials and Methods*.

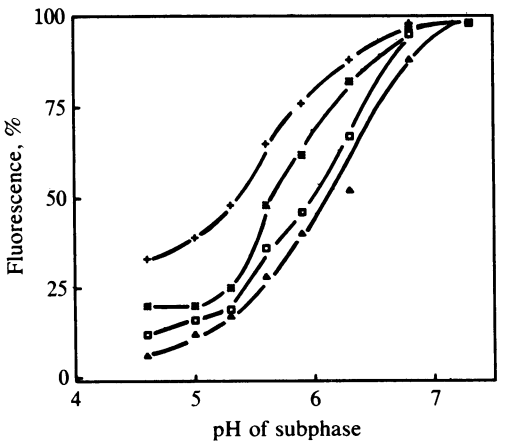

FIG. 2. Relative changes of F-PtdEtn fluorescence versus the pH of the subphase. The chromophore was embedded at 2% (mol/mol) in a PtdEtn monolayer. The 100% fluorescence was taken at pH 7.2 for the different surface pressures of the host monolayer. The changes are plotted for the following surface pressures: +, 0.2 mN/m; ✱, 10 mN/m; □, 20 mN/m; △, 30 mN/m.

Lateral Diffusion of Protons

Proton conduction along the lipid surface was detected by measurement of the time T_{H^+} between injection of protons at one position of the trough and the observation of a fluorescence intensity decrease at the observation area, a few centimeters distant from the injection compartment. The amplitude of this decrease ΔF was recorded (Fig. 3*A*). Since the probe FITC can detect the presence of protons in the aqueous subphase, owing to the geometry of the detection unit (Fig. 1), fluorescence changes of a small volume underneath the surface were measured. The F-PtdEtn probe would be affected only by protons in the lipid/water interface.

Two different types of proton jump, with different specific troughs, were employed (Fig. 4). Experiments were always carried out with films of constant surface area (fixed Teflon barrier). The lipid molecular area was varied by spreading different amounts of lipids. The subphase was always 5.0 mM potassium phosphate buffer, pH 7.0.

Proton "Wave" Jump. HCL solution (300 μl of 1 M) was injected in the part of the trough containing no film. The stirring was very gentle and was used only to equilibrate the subphase in the injection compartment. H^+ would then diffuse freely in the subphase, driven by its concentration gradient. In this case a wave of H^+ diffuses homogeneously across the whole thickness of the solution (Fig. 4*A*). Proton diffusion pathways would then be linear. The distance between the injection compartment and the observation area was about 10 cm.

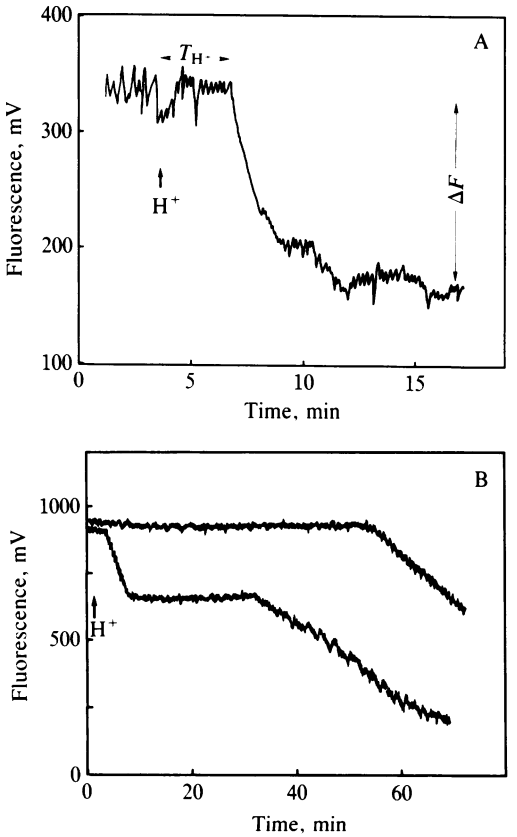

FIG. 3. Kinetics of the fluorescence changes of the fluorescein derivatives after an acid jump. (*A*) F-PtdEtn was embedded in a PtdEtn monolayer. The surface pressure was 20 mN/m and this value was kept during the experiment. The probe-to-lipid molar ratio was 2%. (*B*) FITC was present in the subphase. The upper line is the signal when no monolayer was present at the water surface. The lower line is observed when a PtdEtn monolayer is present at a surface pressure of 20 mN/m. In both *A* and *B* the proton "window" jump technique was used. At the time indicated by H^+, 150 μl of 3 M HCl was injected, giving a ΔpH of 4 units. T_{H^+} is the delay between H^+ injection and the beginning of the decrease in fluorescence. ΔF is the change in fluorescence when a plateau is again observed.

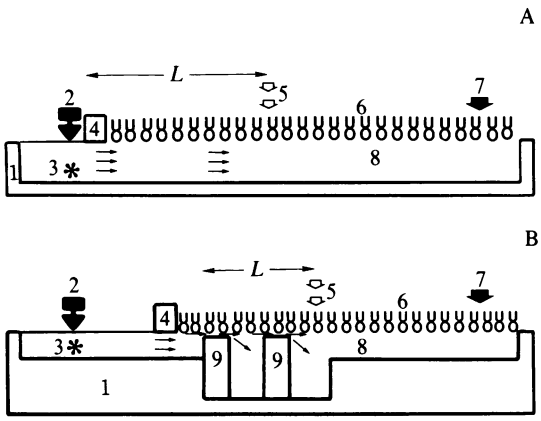

FIG. 4. Schematic drawing of the monolayer trough. (*A*) Proton "wave" jump experiment. 1, Plexiglas trough; 2, injection of acid; 3, stirrer (a very gentle stirring is used); 4, Teflon barrier; 5, fluorescence observation area; 6, monolayer; 7, surface pressure transducer; 8, aqueous subphase. The horizontal arrows indicate the preferential diffusion pathways of protons in this configuration. The distance *L* was 10 cm. (*B*) Proton "window" jump experiment. Features 1 to 8 are as in *A* except that the stirring at 3 was very strong. 9, Glass barriers that hinder the proton diffusion in the bulk phase and limit it to the "window" underneath the film. The arrows in the subphase indicate the preferential diffusion pathway of protons in this configuration. The distance *L* was 4.3 cm.

Results are given in Table 1. Values of T_{H^+} were clearly smaller when a lipid monolayer was present at the interface and H^+ fluxes were monitored by F-PtdEtn than when FITC was measured in the bulk phase without any spread film. Computer simulations (not shown) of the proton movement, using the assumption that the diffusion pathways are preferentially linear, show that these observations constitute evidence that the lateral diffusion of protons is faster along the interface than in the bulk phase.

Proton "Window" Jump. In this type of experiment, the injection compartment was separated from the observation area by means of two glass barriers (Fig. 4*B*). Proton injection was carried out as described above but strong stirring (90 rpm) was employed. Continuity of the subphase occurred via a very thin layer (1 mm) between its surface and the top of the barriers (the so-called windows). In this case, protons would first diffuse close to the interface and then be diluted in the bulk. Measurements of the surface pressure provided evidence that the geometry of the trough was of no consequence for the thermodynamic properties of the lipid monolayer.

The diffusion pathways of protons in this type of experiment were thus rather complicated compared with the previous configuration. Interference induced by stirring on the concentration-driven diffusion was very limited, as was directly observed by spreading talc on the aqueous surface under the same stirring conditions.

When no film was present with FITC in the bulk phase, the fluorescence kept a constant value for about 60 min (Fig.

Table 1. Proton "wave" jump experiments: Values of T_{H^+}

Conditions	Film	T_{H^+}, s	Comment
FITC (1 μM) in the subphase	None	450 ± 90	Diffusion in the bulk phase only
F-PtdEtn (2%) in the film	*E. coli* phospholipids, π = 20 mN/m	270 ± 30	Diffusion along the film

Results are mean ± SD for at least five measurements.

3*B*). When a film of lipids such as PtdEtn containing F-PtdEtn at a 2% molar ratio was present, the fluorescence decreased strongly after a T_{H^+} of 180 s. This value was not affected by the surface pressure of the film between 0.2 and 40 mN/m (Fig. 5*B*). The relative change in fluorescence intensity induced by the fast movement of protons was, on the contrary, strongly dependent on the packing of the lipid matrix (Fig. 5*C*). The relative change was larger when the film was tightly packed. When a film of the same lipid was spread on a FITC-containing subphase, the change in fluorescence was clearly biphasic (Fig. 3*B*). A small decrease was detected after a T_{H^+} of about 180 s, a larger one occurring thereafter over a much longer period (about 60 min). It should be noted that to be detected the fast process required a structured film, as shown by the surface potential measurements. At large molecular areas, the values for PtdEtn monolayers were zero, compared to about 250 mV observed for molecular areas smaller than 1 nm^2 (Fig. 5*A*). Such behavior is indicative of a loss of structure of the film. When experiments were performed with a film at a molecular area of 1.2 nm^2 ($\Delta V = 0$), we were unable to detect the fast change in fluorescence (Fig. 5 *D* and *E*).

The kinetics of H^+ movements, with either F-PtdEtn or FITC as a probe, were not affected by the ionic composition of the subphase. The measured T_{H^+} values were the same after addition to the subphase of NaCl at 100 mM or of chaotropic anions such as thiocyanate at 10 mM. It was also observed that the compression isotherms of PtdEtn were not modified by these different ions.

DISCUSSION

F-PtdEtn proved to be a good interfacial pH indicator since (*i*) its pK range is always close to 6, (*ii*) its pK values are only slightly affected by the packing of the lipids or by the electrical properties of the interface, and (*iii*) it probes only the domain close to the interface due to its amphiphilic properties. F-PtdEtn is thus a reliable probe of local variations of H^+ concentration at, near, or under the polar heads, since its fluorescence properties are independent of factors other than interfacial pH. Lateral diffusion of protons along the monolayer surface was thus reliably detected by the change in fluorescence intensity of F-PtdEtn. Both types of experimental measurements (wave jump and window jump) strongly indicated the occurrence of very fast proton conductance along the lipid monolayer. A direct comparison of the relative increase in T_{H^+} values (monolayer versus bulk) obtained by the two techniques shows an apparent difference. But one should take into account that the diffusion pathways are different in the two cases; it is obvious that the diffusion in the bulk is much faster in the wave jump, in which the protons are diffusing more freely than in the window jump, in which they are apparently blocked by the barriers. We observed that the T_{H^+} without a film is $\frac{1}{6}$ in the wave jump of what it is in the window jump, leading to an apparently smaller relative increase in the diffusion rate along the monolayer. The kinetics of proton transfer was unaffected by the film packing [for π values larger than 0.2 mN/m (Fig. 5 *B* and *D*)]. But the efficiency of H^+ conductance depends on molecular packing, as shown by the amplitude of the fluorescence change (Fig. 5 *C* and *E*).

From these observations, it can be concluded that in fact two processes are competing: a fast lateral conduction along the lipid layer and a proton loss to the aqueous subphase (Fig. 6). More direct evidence of such H^+ loss was provided by experiments with FITC as a probe. As described above, FITC signals the proton concentration in the subphase; thus the fast change in FITC fluorescence when a film is present is indicative of a leak of protons moving along the lipid/water interface. The experiments with FITC also provide further evidence that the efficiency of the fast proton transfer at the interface is determined by the packing arrangement of the lipid film. As soon as the surface potential decreases to zero (i.e., molecular areas larger than 1.2 nm^2), transfer

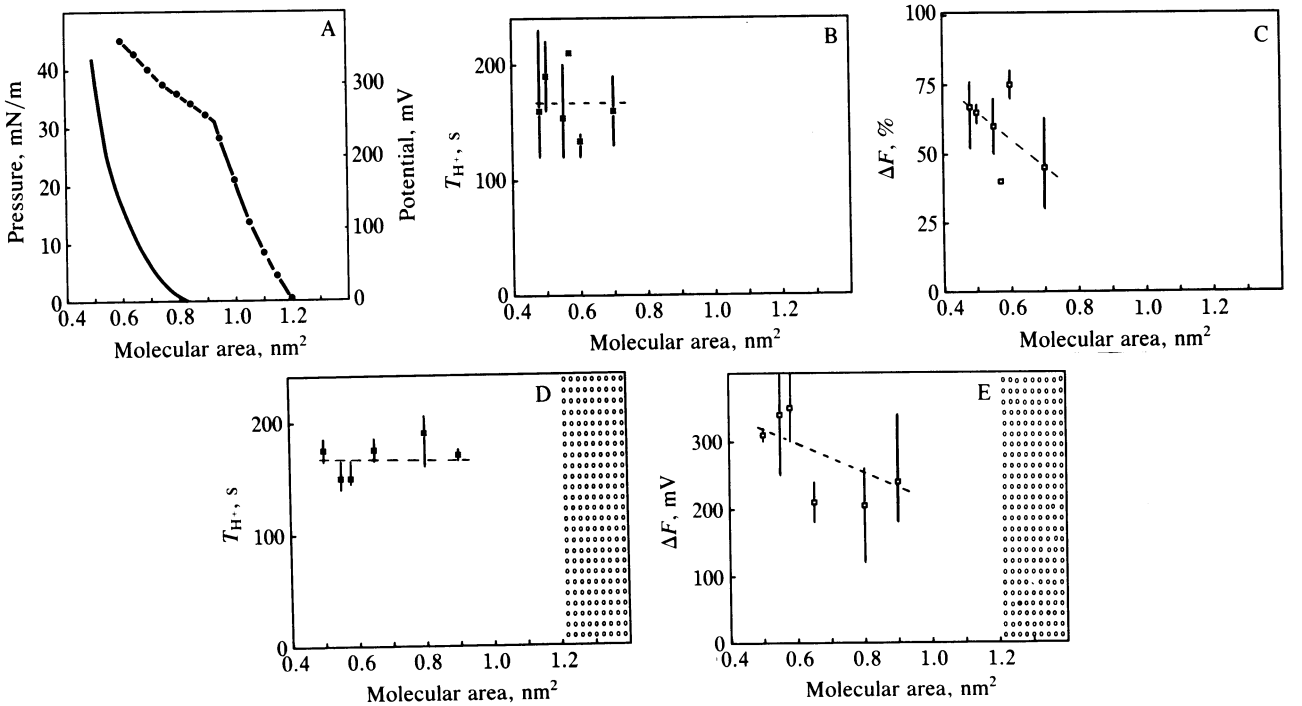

FIG. 5. Influence of the molecular packing of the PtdEtn monolayer on the proton diffusion. (*A*) Thermodynamic parameters of the monolayer: —, surface pressure; ●—●, surface potential. (*B*) Time of apparition of the fluorescence decrease of F-PtdEtn embedded in the matrix (2% molar ratio). The amplitude of the signal was too small to be reliably measured for molecular areas larger than 0.8 nm^2. Bars indicate SD. (*C*) Relative extent of the fluorescence decrease of F-PtdEtn embedded in the matrix (2% molar ratio). The signal observed before acid injection is 100% fluorescence. (*D*) Time of apparition of the "fast" fluorescence decrease of FITC (1 μM) in the subphase. This fast signal was never detected in the dotted area, where the surface potential is 0. (*E*) Amplitude of "fast" fluorescence decrease of FITC (1 μM) in the subphase.

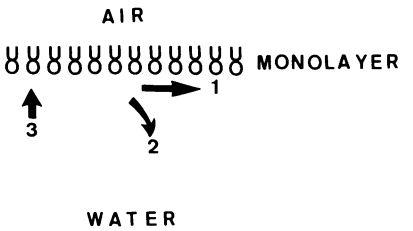

FIG. 6. Proton pathways at the lipid/water interface. 1, A fast conduction is present along the polar heads. Its efficiency is directly linked to the structure of the monolayer. 2, Protons diffusing along 1 may be trapped by the subphase. 3, Protons injected in the subphase are trapped by the interface and will diffuse along 1.

along the interface is no longer detected (Fig. 5 *D* and *E*). It should be emphasized that the decrease of the surface potential to zero is indicative of the absence of cooperative interactions among lipid molecules—i.e., the loss of monolayer structure (26).

In an attempt to analyze the molecular basis of the lateral proton conduction, we look for the possibility of a "carrier" mechanism. The proton would then be carried by a protonated lipid. In this case the lateral diffusion of the lipid would itself be the rate-limiting step in proton conductance. Lateral diffusion of the lipid was measured by fluorescence recovery after photobleaching (FRAP) as described in ref. 27, with 12-(9-anthroyloxy)stearic acid as a probe. A value of the diffusion coefficient $D = 7 \times 10^{-4}$ cm^2/s was found for PtdEtn as a host matrix below and above the critical molecular area of 1.2 nm^2. The Stokes–Einstein relationship $\langle L^2 \rangle = 2\,Dt$ (in which L is the average distance traveled by a molecule during the time t) was used to estimate the transit time of a lipid between the injection compartment and the observation area ($L = 4.3$ cm). This time would be 10^4 s and is far too long to account for our observations of H^+ transit ($T_{H^+} = 180$ s). Moreover, if lipid diffusion were the means of H^+ transfer it should take place even at very large lipid molecular areas, at which the proton conductance disappears in our experiments.

The requirement for a structured organization of the lipid layer for the proton conduction to be observed suggests that the "bound" water at the interface is involved in the process. But in this case chaotropic ions should affect proton conductance, for they are known to interact with these strongly attached water molecules (2); we found that SCN^- did not affect the proton conduction. Moreover the Gouy–Chapman layer of counterions appears not to be involved in proton conductance along the interface, since its thickness is decreased by an increase in the ionic strength. An increase in ionic strength did not affect proton conductance.

Other proton-conducting pathways along the surface of membranes have been recently suggested and offer theoretical explanations for our experimental results. Anionic lipid headgroups share protons as acid-anion dimers and can lead to the trapping and the conduction of protons along the lipid layer (17). But it should be noted that in our case H^+ conductance occurred with the zwitterionic lipid PtdEtn. Transmembrane proton conduction by proteins appears to occur by means of hydrogen-bonded chains (28–30). Such a concept of proton conduction is also valid in the case of the lipid/water interfaces studied here and could explain the lateral H^+ conductance. Hydrogen-bonded structures between lipid polar heads present in organic solvents have been shown not to be disrupted by water in lipid membrane surfaces (31). The formation of hydrogen bonds between polar heads of membrane lipids provides a network capable of supporting a "hop and turn" mechanism (30) of proton conduction along the interface. The compatibility of such a process with our experimental data is strengthened by results obtained by proton magnetic resonance studies of PtdEtn vesicles (18). The effective NH_2 to NH_3^+ transfer rate was observed to be 8×10^5 s^{-1}. Assuming a molecular area per lipid molecule of 0.65 nm^2 and a straight path, a proton would require 67 s to travel 4.3 cm, in complete agreement with our experimental observation of 180 s.

The lateral diffusion coefficient of protons along the headgroups may be evaluated by comparing the results for diffusion in the bulk phase (more than 60 min) and along the interface (3 min). The diffusion coefficient is at least 20 times larger along the film interface than in the bulk aqueous phase. Using a value of 10^{-4} cm^2/s in bulk water, we obtain a value of 2×10^{-3} cm^2/s along the lipid monolayer.

Our experiments thus provide direct experimental evidence of proton transport over long distances along the lipid monolayer water interface, sufficient to account for H^+ movements between source and sink as emphasized in localized theories of chemiosmosis (2, 4, 5, 15, 32). Direct energy coupling via H^+ moving along the membrane water interface is thus possible. The ejection of H^+ into the bulk phase, which is observed in experiments in the presence of K^+ valinomycin, Ca^{2+}, or permeant anions may be due to the induced loss of H^+ from the membrane interface to the bulk aqueous phase, such as observed in our experiments.

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