Dynamic studies of proton diffusion in mesoscopic heterogeneous matrix
II. The interbilayer space between phospholipid membranes

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ABSTRACT   The thin water layer, as found in chloroplast or mitochondria, is confined between low dielectric amphypathic surfaces a few nm apart.

The physical properties of this mesoscopic space, and how its dimensions affect the rate of chemical reactions proceeding in it, is the subject for this study.

The method selected for this purpose is time resolved fluorometry which can monitor the reversible dissociation of a proton from excited molecule of pyranine (8 hydroxy pyrene 1,3,6 tri sulfonate) trapped in thin water layers of a multilamellar vesicle made of neutral or slightly charged phospholipids.

The results were analyzed by a computer program of N. Agmon (Pines, E., D. Huppert, and N. Agmon. 1988. *J. Am. Chem. Soc.* 88:5620–5630) that simulates the diffusion of a proton, subjected to electrostatic attraction, in a thin water layer enclosed between low affinity, proton binding surfaces. The analysis determines the diffusion coefficient of the proton, the effective dielectric constant of the water and the water accessibility of the phosphomoieties of the lipids.

These parameters were measured for various lipids [egg-phosphatidylcholine (egg PC), dipalmitoyl phosphatidylcholine (DPPC), cholesterol + DPPC (1:1) and egg PC plus phosphatidyl serine (9:1)] and under varying osmotic pressure which reduces the width of the water layer down to ~ 10 Å across.

We found that: (a) The effective dielectric constant of the aqueous layer, depending on the lipid composition, is ~ 40. (b) The diffusion coefficient of the proton in the thin layer (30–10 Å across) is that measured in bulk water $D = 9.3 \cdot 10^{-5}$ cm^2/s, indicating that the water retains its normal liquid state even on contact with the membrane. (c) The reactivity of the phosphomoiety, quantitated by rate of its reaction with proton, diminishes under lateral pressure which reduces the surface area per lipid.

We find no evidence for abnormal dynamics of proton transfer at the lipid water interface which, by any mechanism, accelerates its diffusion.

INTRODUCTION

The intermembranal space between the mitochondrial cristea or tylakoids is a thin water layer, 30–300 Å across, which acts as sink and source for the massive proton flux associated with ATP synthesis by these organelles.

In the present communication we wish to evaluate to what extent the width of the aqueous space between the membrane affects the dynamics of the reaction proceeding between them. The model we selected for this study is the highly reproducible water layers of multilamellar vesicles made of pure phospholipid.

The width of the water layer in these structures is known and can be experimentally adjusted by application of external pressure (Rand and Parsegian, 1989; Lis et al., 1982; McIntosh et al., 1987, 1989).

This geometrically defined reaction space is made of high dielectric fluid held between low dielectric matter. Consequently not only the shape of the reaction space differs from bulk conditions, but also the intensity of the electrostatic interactions, which are amplified by image charges mirrored on the boundary of the dielectric discontinuity (Kjellander and Marcelja, 1988; Mathias et al., 1991).

To investigate how the environment affects the dynamics of a chemical reaction we monitor the behavior of a standard gauge particle, the hydrated proton. The reaction we monitor is the highly synchronized geminate recombination between the proton and the excited state pyranine anion (8 hydroxy pyrene 1,3,6 tri sulfonate, ΦOH^*) (Pines et al., 1988).

The reaction, monitored by time resolved fluorescence, is subjected to theoretical reconstruction by the formalism of Agmon, which propagates the Debye-Smoluchowski operator in time and space (Agmon and Szabo, 1990; Huppert et al., 1990). In our previous publication (Gutman et al., 1992) we have demonstrated the capacity of the method to quantitate the properties of water in solutions of increasing sucrose concentrations. In the present one we shall apply it for the study of the thin water layer of multilamellar structures.

A schematic presentation of the reaction space is seen in Scheme I. The proton, released from a dye adsorbed to the membrane, appears on the surface of the reaction sphere at the rate of k_f. At the first shell the proton is either reabsorbed into the reaction sphere (with rate constant k_r) or diffused away.

The diffusion is quantitated by a stepping frequency between adjacent shells, with a width of Δr each, given (in s^{-1} units) by the following expression

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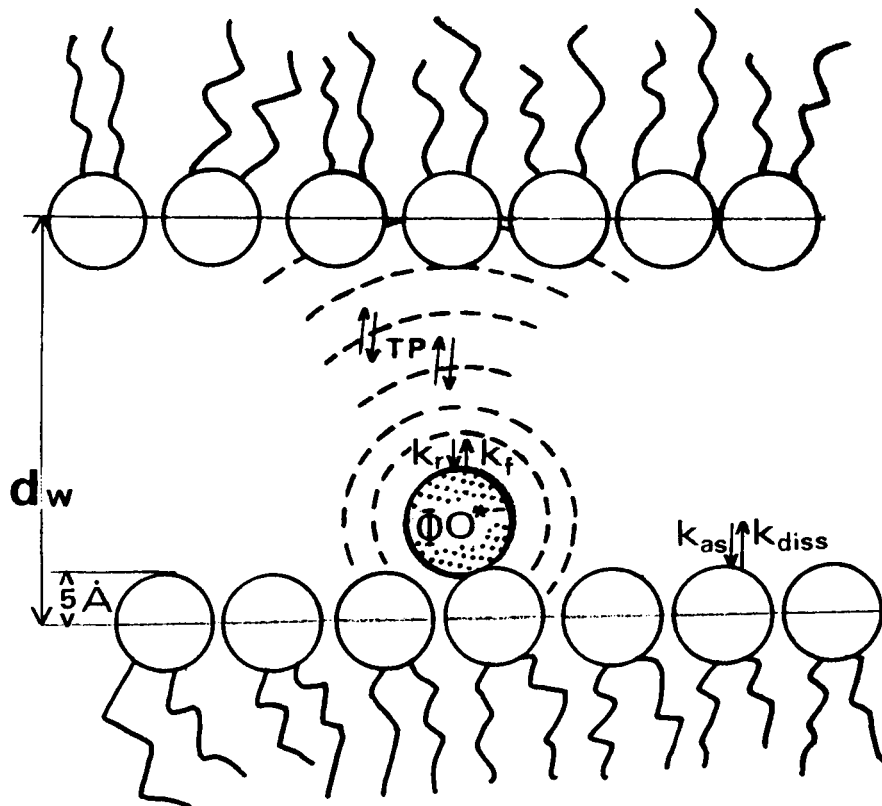
SCHEME 1   Schematic drawing of the reaction space for the proton-pyranine geminate recombination between uncharged phospholipid membranes. The dye, represented by the sphere on the surface, ejects a proton with rate constant k_f and reabsorbs it, with the rate k_r. The diffusion proceeds in hemispherical symmetry at $r < d_w$ and shifts to diffusion between two surfaces at $r > d_w$. The reaction of the proton at each concentric layer with the phosphomoieties is given by k_{as} and k_{dis} for binding and release respectively.

$$TP_{i/j} = \frac{D_H{}^+}{\Delta r^2} \cdot f_n(r_i/r_j) \cdot \exp - \left(\frac{R_D}{2} \left(\frac{1}{r_i} - \frac{1}{r_j} \right) \right). \qquad (1)$$

This expression accounts, in a quantitative way, for how the physical and geometrical characteristics of the diffusion space regulate the probability of a proton to escape from the vicinity of the anion. The first term states that all transition probabilities are a linear function of the diffusion coefficient (D_{H+}). The second term is a geometric parameter, indicating the incremental volume available for the proton on each diffusional step. For a three dimensional space the value of the function is $f_3(r_i/r_j) = r_i/r_j$, while for a two dimensional step $f_2(r_i/r_j) = (r_i/r_j)^{1/2}$. The change in the function is in accord with the milder concentration gradient of a two dimensional space (Hardt, 1979). The last term is the gradient of the electrostatic potential, which is quantitated by R_D, the Debye radius. This radius is the distance where ion pair electrostatic potential is reduced to the level of thermal energy.

When the proton is confined between membranes it can react with the phosphomoiety of the phosphocholine zwitterion with rate constant k_{as} and redissociate with k_{dis}. The various aspects of these conditions have been integrated by the following analytical considerations. (a) The intensity of electrostatic interactions: the dielectric attenuation of electrostatic forces operating at close proximity to the dielectric boundary of the water lipid interface has been the subject for many studies (Matthew and Richards, 1982; Kjellander and Marcelja, 1988; Gilson et al., 1985; Mathias et al., 1991; for review see Cevc, 1990).

Near a dielectric boundary one must describe the intensity of electrostatic forces by an effective dielectric constant (ϵ_{eff}), a value which varies sharply from $\epsilon \sim 2$ (in the lipid core) up to ~ 80 (of the bulk water) over a narrow layer of ~ 10 Å. At the level of the polar headgroups, a layer 5–6 Å deep, ϵ_{eff} is estimated to be 20–40 (Coster and Smith, 1974; Seelig et al., 1987). In our analysis we could not attain this discrimination, neither did we look for one. Our purpose was to monitor the dielectric property of the aqueous layer between the membranes, the physiological space in which ions diffuse. Experimentally we monitored a highly mobile ion, the hydrated proton, and the data we acquired is the effective dielectric constant averaged for the entire space probed by the proton.

The magnitude of ϵ_{eff} is derived from the Debye radius $R_{(D)}$ according to Eq. 2.

$$\epsilon_{eff} = z_1 z_2 e_o^2 / R_D kT. \qquad (2)$$

(b) Geometry of the diffusion space: as a proton diffuses away from the pyranine anion, its initial diffusional

steps proceed in a three dimensional space (see Scheme I). When the diffusion distance is larger than the intermembranal space ($r_i > d_w$), the second boundary becomes effective. From that distance and on, the diffusion space is approximated by a two dimensional space, a regime where the concentration gradient is less steep (Hardt, 1979). In our analysis we assumed that the proton can sample the whole width of the aqueous phase, as defined in the measurements of Rand, Parsegian (1980, 1989) and their co-workers. The values of d_w were taken from raw data which relates d_w as a function of the osmotic pressure (Lis et al., 1982). In the simulation program, we thus, shift the value of $f_n(r_i/r_j)$ at the geometrical transition point $r_i = d_w$.

(c) Protonation of surface groups: the zwitterionic surface of PC provides a multitude of phospho anions (pK = 2.25, Nachliel and Gutman, 1988) which rapidly binds a proton. In the macroscopic-steady state domain, the reversible binding of proton to immobile buffer reduces the apparent diffusion coefficient of a proton (Junge and McLaughlin, 1987). In our formalism, where the diffusion coefficient is not apparent (see Eq. 1), the binding of protons is introduced as a discrete stochastic effect. The proton reacts with the surface with a given probability (k_{as}) and dissociates with k_{dis}. Yet, considering the slow dissociation time ($\tau \sim 50$ ns, Gutman et al., 1989), the probability of a proton bound to the surface to redissociate within the lifetime of ΦO^{*-} ($\tau = 6$ ns) is rather small. Consequently, our calculations are sensitive to k_{as} but rather blind to k_{dis}.

(d) Detailed structure of the water-membrane surface: the lipid water surface is, on a molecular scale, a rough surface. The electron density maps of McIntosh and Simon (1986), McIntosh et al. (1987, 1989) clearly indicate that the phosphocholine groups of two facing surfaces can collide with each other, and in the presence of cholesterol (acting as a spacer) even to mesh. Consequently, the aqueous phase where the proton diffuses is not homogeneous; the protruding head group introduces a nonaqueous matter into the layer. This situation resembles the diffusion of proton in concentrated solution of sucrose (Gutman et al., 1992), where we found that the diffusion of proton can still be quantitated by continuum modeling of both ϵ and D_{H+}. The dielectric constant of sucrose solution, measured by the subnanosecond dynamics, was identical to that measured by classical methods. The diffusion coefficient was found to be identical to that measured in pure water, even in a 2 M solution of sucrose, where the partial volume of the sucrose is 45%. Apparently, a proton can diffuse in the aqueous phase between the sucrose molecules as freely as in bulk water and can "sneak" between sucrose molecules as they execute their Brownian motion.

In accord with this observation, we set in our computations the width of the proton permeable space as the full width of the aqueous layer (Lis et al., 1982) while we

allowed protons within the polar region to interact reversibly with the phosphomoieties.

As shown below, we used these guidelines to reconstruct the dynamics of proton diffusion between neutral, or slightly charged, phospholipid membranes under varying osmotic pressures. Under all conditions the solutions converged into a persistent set of values which characterize the physical and chemical properties of the thin water layer.

MATERIALS AND METHODS

Materials

Lipids (egg PC, brain PS, DPPC and cholesterol) at highest purity were obtained from Sigma Chemical Co. (St. Louis, MO). Pyranine, 8 hydroxy pyrene 1,3,6 trisulfonate, laser grade, was from Eastman Kodak Co. (Rochester, NY).

Preparation of vesicles

Large multilamellar vesicles were made as described by Rochel et al. (1990). The lipids (10 mg) were dissolved in chloroform and dried to a thin layer in a 100-ml round bottomed flask in a rotating evaporator. The swelling was carried out by the addition of 10 ml 2 mM pyranine in water (pH = 5.5) followed by slow overnight shaking (15 rpm) at 45°C. External pyranine was removed by repeated centrifugations and the vesicles were suspended in 5 mM Mes buffer at pH = 5.5.

Application of stress

Osmotic pressure was applied by addition of the vesicles to sucrose solution of known concentration. The change in fluorescence emission spectrum was practically immediate. By the time the sample was placed in the fluorometer, the emission spectrum already exhibited enhanced ΦOH^* emission and remained so without any change for days.

The osmotic pressure of the solution was calculated according to the data given in Appendix 8.6 of Robinson and Stokes (1954). The width of the aqueous layer (d_w) was taken from the d_w vs osmotic pressure measurements of Lis et al. (1982).

Determination of interbilayer space

The volume of the interbilayer space taken by the pyranin was measured by swelling the dried lipid in dilute solution containing C^{14} sucrose in a similar procedure to that described above. The swollen vesicles were collected on 0.2 μ millipore filter and rinsed by water. The amount of incorporated sucrose corresponded with 1.13 ± 0.19 μl/mg lipid. This volume is equated with the volume of the outermost interbilayer shells.

Penetration of sucrose into the MLV during dispersion of preswollen MLV in concentrated sucrose solution was measured by dilution of preswollen MLV in concentrated sucrose solution containing C^{14} sucrose. The vesicles were allowed to equilibrate for a period comparable with the handling time of fluorometric measurements (5–45 min) then collected by filtration, rinsed by unlabeled sucrose solution and amount of C^{14} retained on the filter paper was determined.

Time correlated single proton counting was measured by the instrumental setup described by Huppert et al. (1990).

Theoretical reconstruction of experimental curves was carried out by the method described by Huppert et al. (1990) using the computer program of N. Agmon, Department of Physical Chemistry, The Hebrew University of Jerusalem. The program solves the time dependent Smoluchowski equation for a relative translational diffusion of a proton attracted by Coulomb potential and confined between planar surfaces. The partial differential equations were converted to a Master equation with detailed balancing which was propagated in time using a Chebysheve expansion.

RESULTS

Binding of pyranine to lipid membranes

Pyranine is readily adsorbed to neutral phospholipid membranes (Clement and Gould, 1981). The association constant of the dye to outer surface of small unilamellar vesicles of egg PC is $K_{as} = 1 \ 10^4 \ M^{-1}$ (Gutman et al., 1988). The binding to DPPC or DPPC + cholesterol membrane is of the same order of magnitude (Nachliel and Kiryati, unpublished results). Based on these arguments, we depicted the reaction space as described in scheme I, where the dye is located at the interface.

Effect of osmotic pressure on the fluorescence of pyranin in multilamellar vesicles

The swelling procedure used in this experiment, where the dye's solution is added to the dry film of phospholipids, yields a rather low efficiency of trapping (Gutman et al., 1989). That is because the dye is excluded from the inner aqueous core of the MLV structure, occupying the outermost interbilayer shells. Under osmotic pressure the dye exhibits an enhanced steady-state emission of the undissociated form of pyranin, while the time resolved fluorescence revealed enhanced geminate recombination (Rochel et al., 1990). The osmosant selected in this study, as in previous one (Rochel et al., 1990) was sucrose. Attempts to replace it by Dextran failed for two reasons: the high viscosity of concentrated dextran solution and its endogenous fluorescence. Dextran, when excited by the pulse laser ($\lambda = 300$ nm) fluoresces with a broad waveband and a long lifetime emission ($\tau > 6$ ns) which interfered with the analysis of the ΦOH^* signal, especially in the time frame where geminate recombination is monitored (>500 ps).

Sucrose has the advantage of lower viscosity and its fluorescence (excitation wave length $\lambda = 300$ nm) is almost negligible. (Sucrose samples of the highest purity supplied by Sigma, BDH and other brand name companies all exhibit this emission). To ensure that the sucrose does not penetrate into the interbilayer space when MLV are mixed with sucrose we monitored the amount of C^{14} sucrose which is picked up by the vesicles (see Methods).

The amount of C^{14} sucrose that was incorporated corresponded with penetration of the sucrose to a volume which is less than 5% of the interbilayer space occupied by the dye.

Dynamics of fluorescence decay

The fluorescence decay curve of pyranine (in its ΦOH^* form) is depicted on a logarithmic Y scale in Fig. 1. Two curves are shown: one is for pyranine trapped in multilamellar vesicles, made of DPPC + cholesterol, at no applied pressure; the other is under osmotic pressure ($\log P = 7.53$ [dyn/cm^2]). In both cases the earliest phase
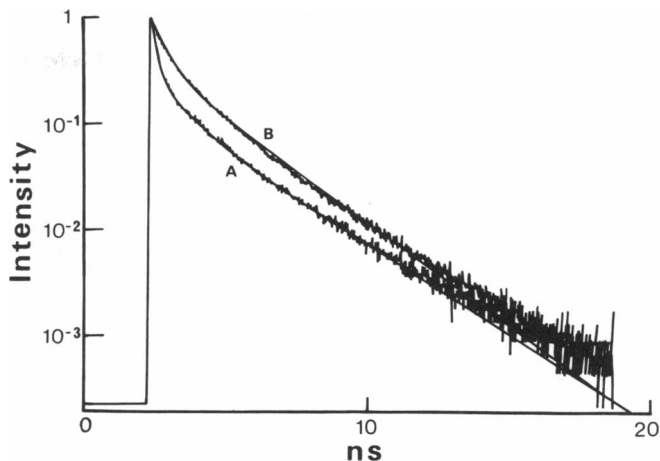

FIGURE 1   The fluorescence decay dynamics of the excited pyranine molecule trapped in the aqueous layer of a multilamellar liposome made of DPPC + cholesterol (1:1).

The fluorescence, measured at the wavelengths of the undissociated form (ΦOH^*, $\lambda = 435$ nm), is drawn on a logarithmic scale. The continuous curves are the reconstructed decay curves. A was measured in absence of external pressure, suspending the vesicles in 5 mM Mes pH 5.5. B was measured with vesicles suspended in 1 M sucrose + 5 mM Mes pH 5.5, a concentration which applies osmotic pressure of 3.3 × 10^7 dyn/cm^2.

of the decay is fast, dominated by the dissociation of the proton from the excited dye. In the later phase, decay is much slower, representing the repetitive dissociation recombination of proton with the excited anion.

The reconstruction of the observed dynamics by Agmon's procedure involves a methodical variation of the parameters defined in Scheme I, looking for a combination which will reproduce the experimental curve (see Fig. 1). The theoretical curve is affected by the various parameters in a selective manner. The most initial phase of the decay can be mimicked only by k_f. The timing of the appearance of the "tail" and the curvature at that region are modulated by the dielectric constant (ϵ_{eff}) (see Eqs. 1 and 2). The magnitude and curvature of the "tail" are shaped by D_{H+} and k_{as}. Thus, due to the complex features of the experimental signal, there is only one combination of parameters which reproduce it accurately.

The systematic analysis of a set of signals, obtained with a given lipid composition, started with the sample under minimal osmotic pressure (5 mM Mes log $P =$ 5.3). The parameters selected for this sample were then used as a first guess for a higher osmotic pressure. By this procedure we could detect trends in the variations, and used them as guidelines for simulating signals obtained at higher pressures.

In the remainder of this report we shall discuss the magnitude of these parameters.

Diffusion coefficient of H$^+$

Diffusion of proton at interface was measured by microscopic-kinetic methods using multilamellar systems

(Rochel et al., 1990) or by macroscopic techniques looking at the subphase of monolayers (Gabriel et al., 1991; Morgan et al., 1991). In contrast to monolayer systems, where the role of bulk conductance is overwhelmingly dominant, in our system the diffusion is exclusively between the lipid membrane, with no signal at all coming from the bulk.

The diffusion space is described in Scheme I. The protons can diffuse through the whole width of the aqueous phase (d_w). In case they penetrate the 5 Å layer of the polar region, they react reversibly with the phosphomoieties. The whole space is characterized by a single diffusion coefficient which directly modulates the magnitude of all transition probabilities (see Eq. 1). In our simulation, D_{H+} was varied widely around the value of proton diffusion in bulk water $(D_{H+} = 9.3 \ 10^{-5} \ cm^2/s)$. In all cases the fitting of the curve converged towards the value of bulk water. This observation is supported by the results of our studies with concentrated solutions of sucrose, where its presence did not hinder the diffusion of the proton. Our observations are in accordance with the transient Overhouser effect NMR studies of Milburn and Jeffrey (1990) reporting a very fast (picosecond) relative motion between the phosphate and the nearby water molecules.

Dielectric constant of the aqueous layer between the lipid membranes

At low ionic strength, the attenuation of electrostatic forces is carried out by the dielectric properties of the environment.

In Agmon's program the dielectric attentuation is introduced through the R_D term in Eq. 1, which for simulation purposes is an adjustable parameter. The formalism treats the dielectrics as a continuum parameter which is an average over the whole space probed by the proton.

The dependence of the effective dielectric constant on the membrane composition and the bilayer separation distance is given in Fig. 2. The magnitude of ϵ_{eff} seems to be specific for the lipid component and independent of the width of the aqueous layer in the range of 30 to 10 Å.

In the preceding publication (Gutman et al., 1992) we demonstrated that the kinetic analysis determines very accurately the dielectric constants of sucrose solutions which decrease with increasing sucrose concentrations. The fact that we did not obtain any change in ϵ_{eff} even at high osmotic stress is evidence that sucrose did not penetrate into the proton probed space.

Reactivity of the phosphomoieties with the solvated proton

The reversible protonation of the phosphomoiety of the phosphocholine head group was incorporated into the analytic program as a stochastic event quantitated by two stochastic parameters, k_{as} and k_{diss} for binding and release respectively. The binding of proton to the phos-
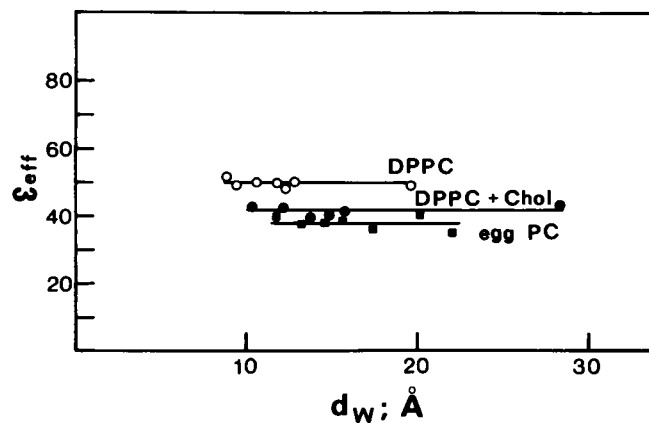

FIGURE 2 The effective dielectric constant of the thin water layer between phospholipid membranes. The value of ϵ_{eff} was deduced from dynamic analysis of fluorescence decay curves as exemplified in Fig. 1. The separation distance between the lipid membranes was determined in accordance with osmotic pressure using data given by Rand et al. (1980) and Lis et al. (1982).

phomoiety was calculated for each concentric shell at each time interval, according to the momentary probability density of protons. This function of bound proton was propagated in time and space together with the diffusion and the reactions on the surface of the reaction sphere. The rate of proton binding derived from this procedure, given in s^{-1} units, was normalized by the equivalent concentration of the phosphocholine moiety in the aqueous phase to obtain a second order rate constant $(M^{-1} \ s^{-1})$. Through this expression we compare the rates under conditions where both d_w and A (surface per phosphomoiety) vary under the applied pressure. Any effect on the rate which is only a function of concentration (or probability density) will be filtered by this expression, exposing structural effects caused by the stress and packing. Fig. 3 depicts the dependence of the normalized rate constant on the surface area per phosphocholine moiety as measured with three membrane preparations (DPPC + cholesterol membrane, egg PC and DPPC) under varying lateral pressure (0–4 dyn/cm). The rate behaves as a function of the surface area. Dense packing produces a partial masking of the phosphomoieties which reduce their rate of reaction with the proton.

Reaction of proton with the excited dye molecule

The analysis described above related to the properties of the diffusion space. We can also derive highly localized information by looking at the rates on the surface of the reaction sphere, the only place where proton and anion interact with each other.

The rates of proton production-consumption on the surface of the reaction sphere vary with the experimental conditions. The rate of proton dissociation varies with the activity of water in the solution (Huppert et al., 1982; Bardez et al., 1984; Politi and Chaimovich, 1986; Schul-
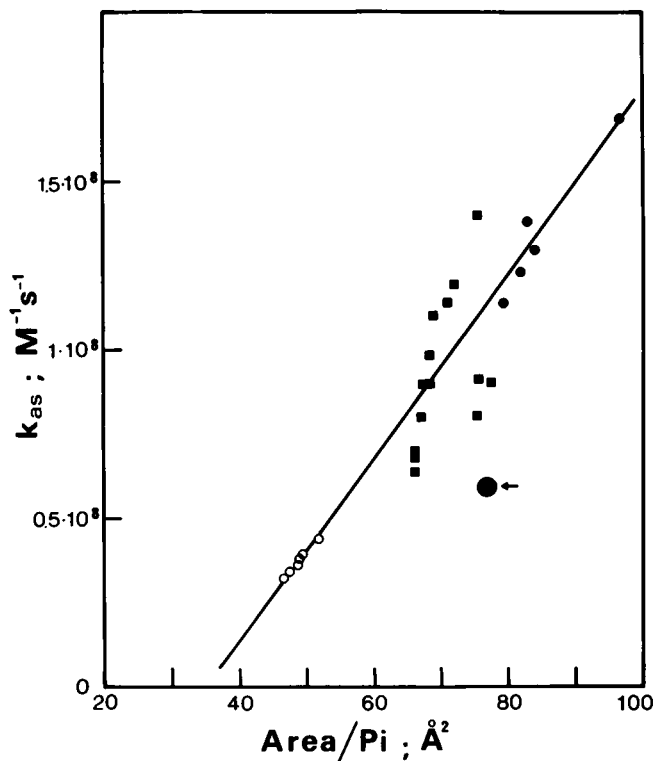
FIGURE 3 The variation of the rate constant of phosphatidylcholine protonation on the surface area of the lipid head group. The rate constants were obtained from the theoretical reconstructed dynamics and normalized for the aqueous volume per phosphomoiety (Lis et al., 1982; Parsegian et al., 1979; Rand and Parsegian, 1989). The area per phospholipid was taken from these sources too. (●) Data collected from DPPC + cholesterol vesicles at $5.3 < \log P < 7.8$. The point marked with arrow represents measurements at a pressure where the lipid and cholesterol undergo phase separation. (■) Data collected with egg PC vesicles $5.3 < \log P < 8.1$. (O) Data collected from DPPC vesicles under varying osmotic stress $5.3 < \log P < 8.1$.

man et al., 1990). It is also sensitive to masking of the reaction sphere by nonaqueous matter (Gutman et al., 1992). The latter parameter, quantitated by σ, is the ratio of k_f or k_r as measured under given conditions with the respective rate measured in pure water ($\sigma = k/k_0$). As the activity of water in sucrose solution (even at high concentration) is close to unity (Robinson and Stokes, 1954), the required correction of σ for a_{H_2O} was rather small.

Fig. 4 relates σ with the width of the aqueous layer. In the absence of applied pressure, the value of σ is ~50%, independent of the lipid composition. Application of pressure, causing the membranes to approach each other, impose a further reduction of σ, suggesting an enhanced masking of the pyranine.

Proton-pyranine recombination between negatively charged membranes

The binding of pyranine to the surface is prevented by incorporation of Phosphatidyl serine in the membranes

(Clement and Gould, 1981; Gutman et al., 1987). At a mole fraction of 10% the PS density on the surface is sufficient to prevent the adsorption of the dye, yet not too high to increase the separation distance to very large values. At osmotic pressure of $\log P = 5.3$ and low ionic strength, the separation distance is 50–60 A (Loosley-Millman et al., 1982). Fig. 5 depicts the fluorescence decay curve of pyranine enclosed between the lipid membranes of multilamellar vesicles made of PC + PS at 9:1 ratio maintained at the osmotic pressure of $\log P = 5.3$ and the ionic strength (of the aqueous phase between the membranes) $I = 0.012$ M.

The most initial decay of the signal is as fast as recorded for pyranine in bulk water (i.e., $\sigma = 1$). This is evidence that indeed the dye is not on the surface of the membrane.

The analysis of the observed dynamics was based on a geometry represented by Scheme II. The pyranine is depicted at the middle of the aqueous layer, where its electrostatic potential will be at the minimum. The proton diffuses in a three-dimensional spherical space until $r_i = \frac{1}{2}d_w$, and whenever $r_i > (\frac{1}{2}d_w - 5)$ it can also react with the surface groups of the membrane.

The rate of protonation of the surface groups k_{as} was taken as that determined for the major component of the membrane, Phosphatidylcholine ($k_{as} = 1.1 \pm 0.1 \ 10^8$ $M^{-1} \ s^{-1}$, Fig. 3). With all these self imposed restriction, we reconstructed the dynamics with only two adjustable parameters, ϵ_{eff} and d_w.
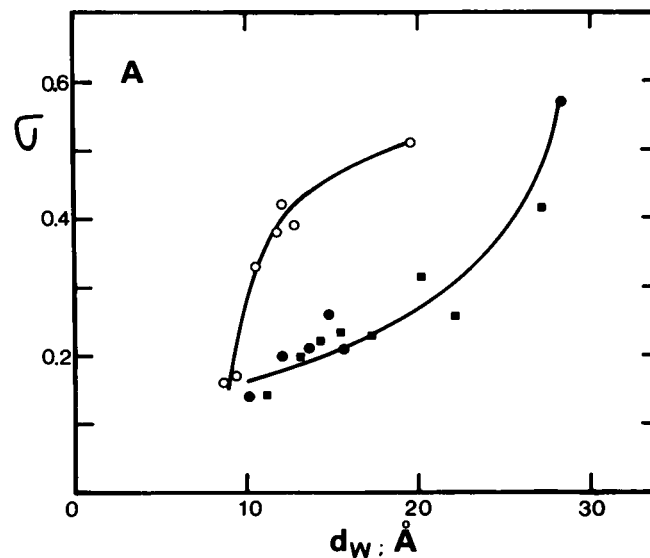

FIGURE 4 The dependence of the steric factor (σ) on experimental conditions. The steric factor is defined as the rate of proton dissociation (k_f) or recombination (k_r) measured under given conditions normalized to the rate measured with the dye dissolved in pure water. (The rate constant k_f was adjusted for the reduced water activity of sucrose using the activity Table of Robinson and Stokes [1965] and the rate vs a_{H_2O} relationship given by Huppert et al., [1982]).

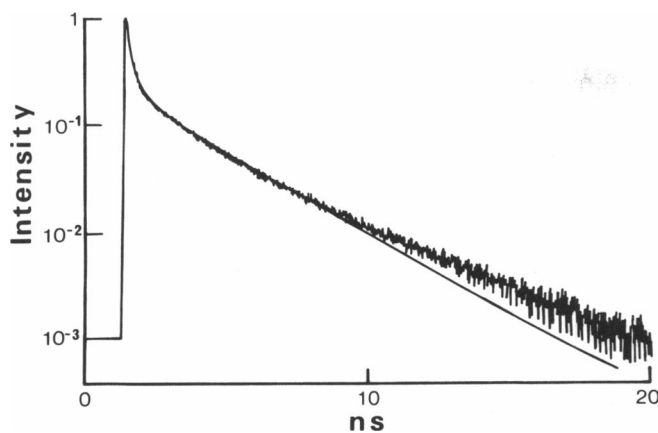(●) DPPC + cholesterol (1:1) vesicles, (■) egg PC vesicles, (O) DPPC.

The theoretical curve shown in Fig. 5 was fitted with $\epsilon_{eff} = 49$ and $d_w = 55$. The former is compatible with values measured for uncharged membranes, and the latter is within the range estimated by Loosley-Millman et al. (1982) for membranes containing one negative charge per ~ 500 Å^2.

DISCUSSION

In this study we investigated the aqueous phase between phospholipid membranes using the geminate recombination between a proton and pyranine anion (ΦO^{*-}) as a probe reaction. This well recognized reaction has been carried out in an unknown environment and through its analysis we gained information about the space probed by the proton.

On a molecular scale the aqueous phase consists of a thin water layer into which the phosphocholine moieties protrude (McIntosh et al., 1987, 1989). The width of the aqueous layer was calculated by Rand, Parsegian and their colleagues according to Luzzati (1968) which defines the water and lipid as nonpenetrating layers.

In our calculations, we combined the structural details of the two models. We allowed the proton to probe the whole width of the aqueous layer, according to the Luzzati (1968) formalism, and at the region of the polar headgroups (McIntosh et al., 1987; 1989) the proton was allowed to react chemically with the phosphomoiety.

The volume of the space probed by the proton is determined by the lifetime of the excited species ΦO^{*-}. We can detect the recombination only while ΦO^{*-} molecules are still in the excited state. Within the ~ 20 ns observation time, the population of ΦO^{*-} decays to $\sim 3\%$ of the initial value. Within this time the proton

diffuses an average distance of ~ 200 Å. This space is sufficiently large to contain $\sim 4,000$ phospho head groups and $\sim 1.5 \cdot 10^5$ water molecules. The homogeneous reaction space, defined by the equilibrium concentration of the proton, is much larger ($\sim 2,500$ Å for pH = 5.5). This assures us that the observed dynamics is free from events emerging from protons homogeneously distributed between the membranes.

Dielectric properties of the intermembranal space

The dielectric constant of a pure homogeneous medium is a physical property, directly interpretable in terms of the molecular structure of the matter. The dielectric constant of a homogeneous dispersion of two compounds is related to the properties of the constituent (Robinson and Stokes, 1954). In both cases, the dielectric constant is a continuum property and its value is the same all over. When a dielectric discontinuity is present as a boundary in the reaction space, the loss of spheric symmetry near the boundary generates image charges which alter the self energy of any charge approaching it. As a result the electrostatic potential at each point is determined by the position of other charges and their distance from the boundary. When one of the charges, as in our case, is a free diffusing species, ϵ_{eff}, the effective dielectric constant, is the average for the whole space probed by the proton (Gutman et al., 1991).

The dielectric properties of the lipid-water interface had been investigated by macroscopic observation (Coster and Smith, 1971) and microscopic evaluation of the fluorescence of dyes on liposomes (Kimura and Ikegami, 1985). Both methods focused their attention at the lipid surface, not the adjacent aqueous phase. To the best of our knowledge the data we presented is the first report of the dielectric properties of the aqueous phase in multilamellar structures. At present it should be taken as the best approximation for the dielectric properties of physiological structures of similar ordering like mitochondria, tylakoids and photoreceptors.

Head group organization

The rate of phosphocholine protonation varies with the surface area of the head group. It decreases at high packing density. This corresponds with diminished water accessibility of the phosphate probably being masked by the choline.

Diffusion of proton between the membranes

The diffusion of proton on a surface of a membrane has been a subject of scientific debate for more than 10 years. To explain some anbiguities concerning the efficiency of oxidative phosphorylation, Kell (1979) proposed that protons are confined in time and space to the Helemholtz layer of the membrane, where they diffuse faster
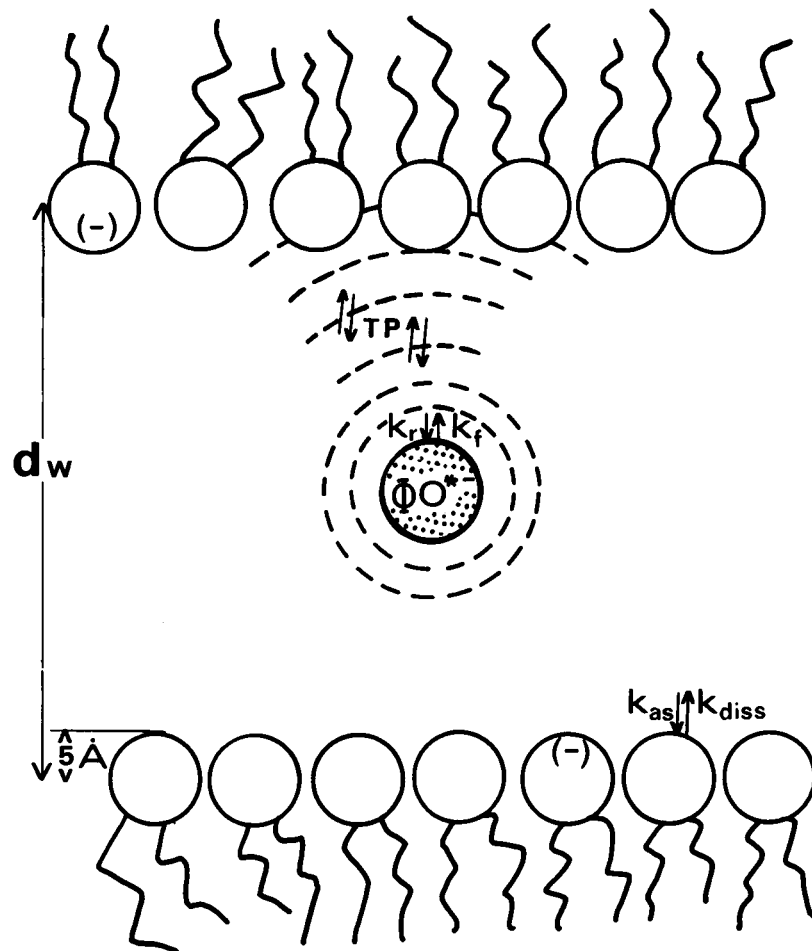
SCHEME II The reaction space for proton-pyranine geminate recombination in thin water layer between negative charged lipids. The dye is represented by a sphere in the middle of the aqueous layer, where its self energy is at minimum. The diffusion regime is spheric symmetric up to $r = \frac{1}{2}dw$. Beyond that distance it behaves as diffusion between parallel surfaces. The interaction of proton with the phosphomoiety begins at $r > \frac{1}{2}dw - 5$.

than in bulk water. This hypothesis was supported by Prats et al. (1985), which carried out experiments in the subphase of monolayer spread over water. The monolayer was marked with fluorophore and its acidification upon addition of acid in a remote place ($\sim$5 cm away from the point of fluorescence observation) was monitored. These results were analyzed by a computer simulation for the propagation of a solute in a very small square grid (30 × 6). The conclusions were that a proton in the subphase propagates $\sim$20 times faster than in the bulk. The reason that the proton remains in the subphase, without mixing with the huge bulk barrier, is an energy barrier. This barrier was assumed "to be abolished by strong stirring which provides proton with kinetic energy and reappears as soon as stirring is suppressed" (Prats et al., 1985).

Kasianowicz et al. (1987) pointed out that taking Prats' model by its face value implies that the diffusion coefficient of the proton at the interface is 10^6 larger than in bulk water.

A recent experiment by Gabriel et al. (1991) claimed to falsify Kasianowicz et al.'s argument (1987), yet the authors conceded that their observation may be due to a simple repulsion of protons from the monolayer interface due to the addition of large quantities of positively charged detergent.

Morgan et al. (1991) carried out conductivity measurements of pure water covered by a monolayer of phospholipids. They noticed that compression of the monolayer increased the overall conductance by $\sim$3% and attributed it to a rapid proton conductance of the monolayer's subphase. If we assume that the conducting layer has the width of the lipid hydration layer ($\sim$10 Å) and the total depth of the trough is $\sim$1 cm, then the 3% increase of total conductance implies that the 10 Å layer carries protons $\sim$3 10^4 times faster than the bulk water. What is more, Menger and co-workers' (Menger et al., 1989) experiments indicated the contrary: formation of a monolayer reduced the total conductance of the system.

In contrast with these macroscopic, time averaging ob-

servations, the experiments we describe are carried out in microscopic dimensions and in the time resolved domain. The diffusion proceeds in a thin water layer of known width, insulated from the bulk and immune against any stirring artifact. What is more, the diffusion coefficient is explicitly extracted from the analytic procedure. It was with surprise that we found that the diffusion coefficient of the proton was as measured in bulk water, even when the membranes were pressed together up to the level of meshing of the phosphocholine head groups of the DPPC-cholesterol vesicles. Apparently, the water molecules in the intermembranal space, which in some cases was as low as ~ 15 water molecules per lipid headgroup (Lis et al., 1982) still maintain sufficient connectivity to support an unhindered rate of proton propagation.

This observation is in accord with what we measured in concentrated solutions of sucrose: the value of D_{H^+} remained as high as in pure water, even in 2 M solution of sucrose. At this concentration 45% of the volume is taken up by sucrose molecules and the free water concentration is 16.8 M. We explained our observation by a rapid motion of the proton in the water interspaced between the nonaqueous matter and rapid transition between the foreign molecules as they exercise their Brownian motion. In the same sense we envision the diffusion of proton between the lipid bilayers as a propagation in a torturous path through the water, moving between and around the head groups. This unhindered diffusibility of proton between the lipid membrane is evidence that the diffusion still proceeds by the Grotthuuss mechanism, a rapid exchange of covalent and hydrogen bonds between the water molecules. This is in contrast with the self diffusion of cations (Rigaud and Gary-Bobo, 1977) or anions (BenYoucef et al., 1978) which exhibit reduced diffusion coefficient as the lipid membranes are pressed together. Thus, we conclude that the intermembranal water provides the protons with an efficient diffusion matrix where the proton propagates by the same mechanism as in bulk water.

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