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We monitored the reaction between proton and excited pyranin anion (ϕO^{*-}) as it proceeds in a thin water layer 30-10A confined between phospholipid membranes.

The reaction was followed within a 20ns time window, and analyzed by numerical integration of the Debye-Smoluchowski operator using a stochastic formalism.

By means of this analysis we determined the diffusion coefficient of the proton, the effective dielectric constant and the steric hindrance for proton approach due to the anion's adsorption to the membrane.

We have demonstrated that proton diffusion studies can be a tool for evaluating physical properties of microscopic and macroscopic aqueous systems.

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Our first attempts at a quantitation of the mechanism of proton propagation in thin water layers between phospholipid membranes was based on microsecond kinetic measurements supported by the steady state Debye Smoluchowski equation for diffusion controlled reactions as an analytic tool. These measurements revealed the effect of the width of the water layer on the rate of proton transfer reactions, but could not evaluate specifically the contribution of each of the following paramaters: diffusion coefficient, intensity of electrostatic attractions, steric hindrance and uptake of proton by the phosphomoiety of the membrane. Yet within these restrictions we concluded that the diffusion coefficient of proton in thin water layer is similar, or somehwat smaller than that in bulk water. We also quantitated the rate constant of proton capture by the phospho moiety of phosphatidyl-choline ($6 \times 10^9\ M^{-1}\ sec^{-1}$) which is ~3% of the rate measured with pyranin (1).

The lack of accurate expression of the contribution of electrostatic potential to the rate of the reaction forced us to look for a better resolving system.

The observation method we selected was the fluorimetric monitoring of the geminate recombination between H^+ and the excited pyranin anion (ΦO^{*-}) as it proceeds in the thin water layer. The dynamics were followed with a 20 ps resolution over very short time frame of 2 ns. The results were analyzed by a semiquantitative procedure which indicated that the average number of proton-anion recombinations is a function of the physical characteristics of the reaction space, mostly dominated by the activity of water (a_{H2O}) of the layer (2). As the activity of water and the average number of recombinations are macroscopic parameters, we looked for a better system for the analysis of the results.

The present procedure satisfies these demands. Experimentally we follow the dynamics of the proton ΦO^{*-} recombination over a longer time range (20 ns) where the intensity of the ΦOH^* fluorescence decreases by almost 4 orders of magnitude, and our analysis covers the whole dynamic range.

The analysis of the signal is achieved by N. Agmon's mathematical procedure (Department of Chemistry, the Hebrew University of Jerusalem; 3,4,5). The beauty and advantage of his analytic formalism is the usage of stochastic arguments for the description of the events. These stochastic values, given by the probabilities of events, are blended by the numeric treatment to reproduce the time resolved dynamics of the observed population. The stochastic parameters are an explicit function of physical parameters like dielectric constants, diffusion coefficients, size of the reaction sphere, the width of the aqueous layer and the release-capture of a proton from all reactants present in the system.

To evaluate the accuracy of the analysis, we tested the whole procedure, kinetic measurements and mathematical analysis, on the well defined system of pyranin dissolved in concentrated solutions of sucrose. The analysis was carried out using the dielectric constant of the solution as an adjustable parameter and was compared with the values determined by the classical techniques (6). The results of our calculations are depicted in Figure 1. Thus the fast kinetic measurements are capable of monitoring the dielectric constant of the immediate vicinity of the dye.

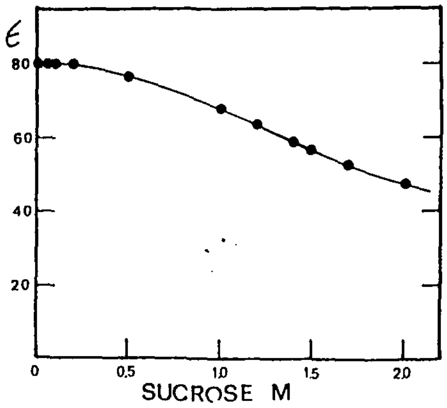


FIGURE 1

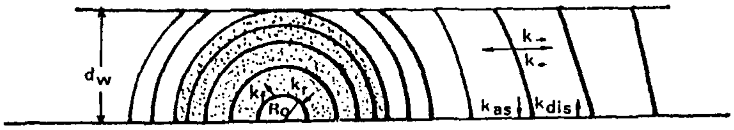
The dependence of the dielectric constant of sucrose solution on its molar concentration. The points shown in the graph were calculated from time resolved fluorescence dynamics. The continuous line is drawn through the values listed in the International Critical Tables (6).

The verification of the validity of the methodology, as tested in a spheric symmetric space, encouraged us to employ it for the study of the dynamics in the thin water layer between lipid membranes.

For the study of proton transfer in thin water layer, Agmon's program had to be adjusted to consider the following factors:

1. Distortion of the spheric symmetry of the electric field due to image charges formed on the dielectric boundary between water and lipid (7). To account for that unknown value, the effective dielectric constant (ϵ_{eff}) was treated as an adjustable parameter.

2. Temporary capture by the phosphomoeties of the membrane delays the propagation of the proton, leading to an apparent diffusion coefficient which is smaller than that of proton in water (8). To account for that reaction, the binding of proton to the membrane was expressed as a stochastic event (see Scheme I). That formalistic treatment segregated binding from diffusion, allowing us to quantitate the diffusion coefficient by its real value.



SCHEME I

Schematic presentation of the reaction space and definition of parameters.

The pyranin is adsorbed to the membrane and enclosed within the reaction sphere with radius Ro.

The proton ejected from the pyranin appears on the surface of the reaction sphere at the rate k_f, and is readsorbed with k_r. The propagation of proton between the concentric shells is given by the rate constants $k_{\rightarrow}$ and $k_{\leftarrow}$. The frequency element of the rates is controlled by the diffusion coefficient and their value is function of the gradient of the electrochemical potential of the proton (3). The binding of proton to the phosphomoiety is given by k_{ass} and redissociation by k_{diss}. Within the dotted space, the proton propagates in a 3-dimensional space where $dv/dr=f(r^2)$ beyond this range $dv/dr=f(r)$, as in a 2-dimensional space.

3. Geometric constraints of the reaction space limit the random walk of proton to a two dimensional space at the point where $r_i >$ dw (see Scheme). In that region the incremental volume of a shell varies as $dv/dr = f(r)$ while in the range ri<dw $dv/dr = f(r^2)$. This shift in volume increment was incorporated in the potential term of the Debye Smoluchowski operator used for the calculation of the transition probabilities between the concentric shells.

4) Steric restriction of approach trajectories due to partial masking of the reaction sphere's surface by contact with the membrane. The reactions on the surface of the reaction space (k_f and k_r) vary with the activity of water (9) and the masking of the surface by non aqueous solute. As a_{H_2O} is almost independent of sucrose concentration we had to evaluate independently the role of the masking of reaction sphere. Our studies with proton dissociation in sucrose solutions yielded an almost linear decrease of both k_r and k_f with the partial volume of sucrose in the solution (see figure 2). In accordance, the analysis of the dynamics we measured with the membranal system allowed both k_f and k_r to vary and the ratio of each, with respect to that measured in water, was taken as the value of the steric hindrance. $\sigma = k/k_o$

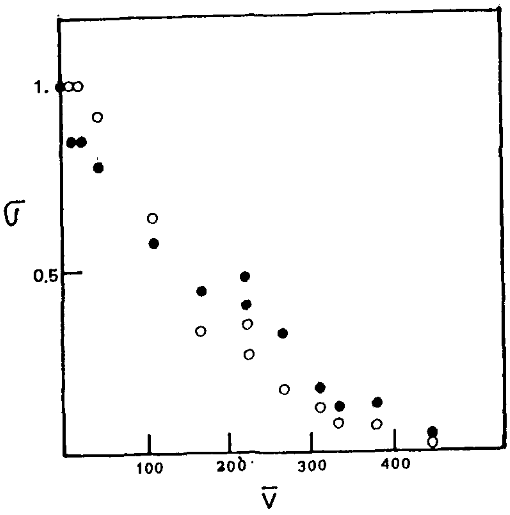


FIGURE 2

The dependence of steric hindrance fot proton approach to pyranin anion on the partial volume of sucrose in the solution. Open symbols are σ values calculated from k_f; solid symbols are for values calculated from k_r.

Experimental

Time resolved fluorescence of pyranin was recorded by a single photon counting at wavelength of ΦOH^* emission over a 20ns period. The number of photon counted at the maximum is 5000 and more than 10^6 were counted in each experiment.

Analysis

The signals were reconstructed by the program written by N. Agmon and adapted to our special conditions by Mrs. E. Nachliel of our group. These adjustments in the program were then chekced and approved by Dr. Agmon.

Results

Figure 3 depicts the signal of ΦOH^* emission over a 20 ns period over dynamic range of more than 3 orders of magnitude.

The reconstructed dynamics is shown on both linear and logarithmic presentation.

The experiments depicted in Figure 3 were repeated with DPPC and DPPC+cholesterol vesicles over a wide range of osmotic pressures. The results were analyzed and the dependence of the microscopic constants on the osmotic pressure and resulting value of dw (10) are given in Table I.

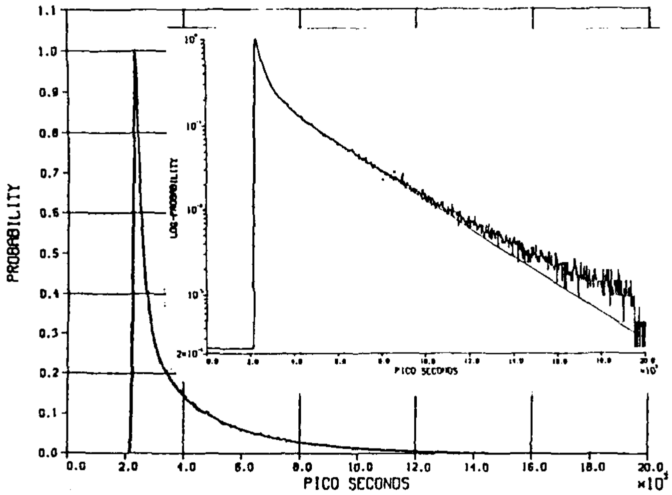


FIGURE 3

Fluorescence decay dynamics of excited pyranin (ϕOH^*) as measured for the dye trapped between DPPC membranes at a distance of 9.4A apart. The main frame depicts the signal on a linear scale. The insert presents the signal on a logarithmic Y scale. The continuous line is the reconstructed dynamics, calculated according to Agmon, as modified for a thin layer, using the values given in Table I.

Discussion

The diffusion coefficient of H^+

In our analysis, D_{H+} was treated as an adjustable parameter. Yet it converged in all samples to the value characterizing bulk water ($D_{H+} = 9.3 \times 10^{-5}$ cm^2/sec).

On the basis of the many experiments we have carried out, we reject the notion of Prats et al. (11) that the ordering of water on the surface of membrane (12) has any accelerating effect on the diffusion of proton (11).

Dielectric constant

The effective dielectric constant calculated for the diffusion space is significantly lower than that of bulk water, demonstrating the contribution of multiple image charges on the dielectric boundaries of the two membranes (7). Examination of the value implies that upon compression the intensivity of the charge-image charges is increased. We also note a marked difference between DPPC and DPPC+cholesterol surfaces. In the latter ϵ_{eff} is smaller, indicating a better adsorption of the dye to the surface (see also Gould, 13).

Proton binding to the membrane: The probability of proton capture by a phosphomoiety on DPPC membrane is ~2% with respect to the rate of recapture by pyranin anion. This ratio is in agreement

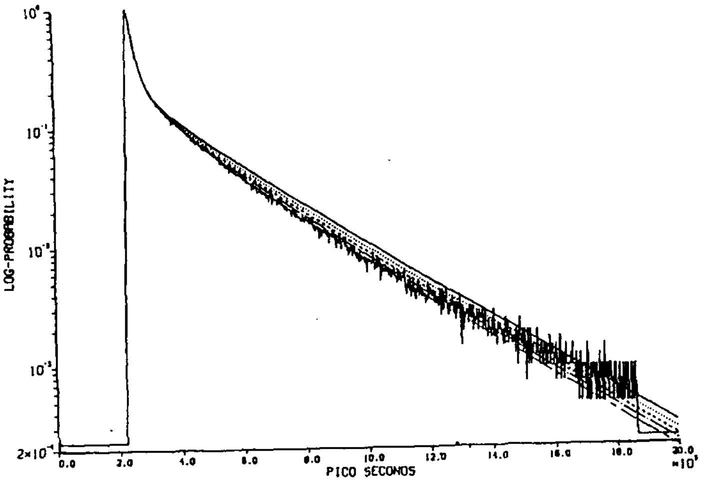


FIGURE 4

The effect of the geometry of reaction space on the dynamics of the reaction between H^+ and ϕO^{*-}.

The curves depict the expected fluorescence decay of ϕOH^* proceeding in a water layer between DPPC-cholesterol membranes (under no external pressure) as the width of the aqueous layer (d_w) is decreased from 30A (lowermost curve) to 20A (uppermost curve). As seen in the figure, even the first decrament in d_w (by 2.5A) already shifts the curve out of the range set by the experimental noise.

with our previous analysis, based on Debye Smoluchowski equation (1) which set the ratio as ~3%.

The reactivity of surface groups increases, as expected, with the constriction of the space.

The reactivity of DPPC moieties increases when separated by cholesterol molecules. This may be interpreted as a reflection of the larger separation between phospho moieties (14) which increase their accessibility to H^+.

<u>Geometry of the reaction space</u>. The geometry of the space is a built-in feature in our computations. A demonstration of its effect on the dynamics, insulated from the other variables, is attainable by simulated dynamics (see Figure 4). This figure demonstrates that even a 2.5A change in dw already distorts the dynamics beyond the margins set by the experimental noise.

It should be stressed that the width of the water layer, derived from X-ray studies of multilamellar vesicles, is still under debate (10,14). The estimation derived from the kinetics of reaction within the aqueous layer may provide an independent procedure to evaluate the physical characteristics of this ultrathin layer.

Steric restriction: The rate of proton production on the surface of the reaction sphere, and the rate at which it is adsorbed there, were introduced as independent variables in the program. As seen in Table I, both values are similarly affected by the osmotic constriction of the aqueous layer. We interpret this as enhanced "adhesion" of the pyranin molecule to the lipid membrane and masking the surface from the aqueous matrix.

The lower value of σ recorded for pyranin between DPPC+cholesterol membrane is thus in accord with the lower value of ϵ_{eff} measured with that system.

TABLE I

The effect of osmotic pressure on the dynamics of geminate recombination in water layer between phospholipid membranes

Sample	osmotic pressure (dyn/cm^2)	dw$^{(1)}$ (A)	k_f	k_r	k_{as}	$\sigma^{(2)}$	ϵ_{eff}
			(given in $10^9 \times S^{-1}$)				
Bulk water	NA	NA	7	5.5	NA	1	80
DPPC vesicles							
in water	0	19.6	3.6	2.8	0.15	0.48	50
0.4M sucrose	1.1×10^7	12.8	2.7	2.2	0.2	0.4	50
0.6M sucrose	1.8×10^7	11.8	2.7	2.1	0.2	0.4	50
1.0M sucrose	3.57×10^7	10.5	2.7	2.1	0.22	0.4	50
1.5M sucrose	6.35×10^7	9.4	1.2	1.0	0.22	0.18	50
1.75M sucrose	8.8×10^7	8.8	1.15	0.9	0.17	0.16	52
DPPC+cholesterol							
in water	0	28	5	4	0.15	0.71	54
0.5M sucrose	1.47×10^7	15.8	1.8	1.2	0.35	0.23	40
1.0M sucrose	3.57×10^7	12	1.4	1.1	0.38	0.20	42
1.5M sucrose	8.8×10^7	10.2	1.0	0.8	0.32	0.14	42

(1) Data of Parsegian (10) and refs. therein

(2) Taken as ratio of $k_r/k_{r(0)}$ or $k_f/k_{f(0)}$

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