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Rapid Report

Measurements of local pH changes near bilayer lipid membrane by means of a pH microelectrode and a protonophore-dependent membrane potential. Comparison of the methods

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Shifts of pH near the bilayer lipid membrane (BLM) were measured in the absence of pH difference between bulk solutions by two methods, i.e. pH microelectrode and membrane potential recordings in the presence of a protonophore. A quantitative agreement of the results of both methods was obtained. The kinetics of the generation of potential induced by the addition of ammonium chloride was accounted for by the time of the diffusion through the unstirred layers. The thickness of the unstirred BLM layers was determined in the experiment.

As it was shown in [1–9] the hydrogen ion flux through the bilayer lipid membrane (BLM) induces pH shifts in the unstirred layers of the BLM. The magnitude of these shifts depends on the buffer capacity of the solutions bathing the membrane. The existence of pH shifts near the BLM was derived from the effect of buffer capacity on the flux of weak acids and bases through BLM [1] and from the generation of a electrical potential on the membrane in the presence of a protonophore [5–8]. Recently a direct method of measurement of pH shifts near the BLM with the help of pH microelectrode was introduced in our laboratory [10]. A qualitative agreement was demonstrated between the two methods. The fluxes in these experiments were induced by the ionophores nigericin and tributyltin.

In the present work a comparison of the results obtained by these two methods for a wide variety of systems inducing hydrogen ion flux through BLM was carried out. A quantitative agreement was demonstrated. The advantages of each method were discussed.

BLM was formed on a Teflon partition 0.4 mm in diameter, by a conventional method [11]. A membrane-forming solution contained 20 mg phosphatidylcholine from soybeans (Sigma) and 10 mg cholesterol (Serva) in 1 ml of *n*-decane. The thinning of the BLM was observed both visually and by measuring its capacity. The experiments were carried out at room temperature (21–23 °C). A protonophore tetrachlorotrifluoromethylbenzimidazole (TTFB) was added at both sides of the BLM.

The measurements of pH shifts near the BLM were carried out by means of a pH microelectrode prepared according to Ref. 12. The system was described in details in our previous work [10]. Briefly a grass-insulated tip-sensitive antimony pH microelectrode was driven perpendicular to the surface of the BLM through the hole in the rear part of the cell. Typically the electrode tip was about 10 μm. The process of BLM formation and pH microelectrode movements were observed through the transparent window in the front side of the cell. Smooth approach of microelectrode to the membrane was carried out using a hydraulic system attached to the reversible step-wise drive (step size about 1 μm). pH shifts were induced by the addition of permeating weak base $(NH_4)_2SO_4$ or ions in the presence of ionophores at the front or rear part of the BLM.

Abbreviations: TTFB, tetrachlorotrifluoromethylbenzimidazole, a protonophore; Mes, 2-(*N*-morpholino)ethanesulfonic acid; BLM, bilayer lipid membrane.

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We used a system of three electrodes for the simultaneous measurements of pH shifts and the BLM potential. The pH microelectrode and a common reference electrode were in the rear part of the cell whereas the second reference electrode was in the front part. Two electrometers were used as amplifiers. Control experiments showed that each channel of measurements worked independently.

Fig. 1 shows pH profiles near the BLM induced by the formation of the concentration gradient of $(NH_4)_2SO_4$ under the conditions of stirring the solutions. The superposition of pH profiles was carried out according to the time of the electrode movement under the conditions of constant speed (5 μm/s). Fig. 1 shows that the pH started to increase as the electrode approached the BLM from the side where the $(NH_4)_2SO_4$ concentration was lower in agreement with the scheme of proton-transfer reactions. However, at a definite position the steep change of the pH electrode potential took place. In the case of further movement of the electrode the BLM breakdown occurred. This phase of apparent pH decrease took place in the absence of the gradient of $(NH_4)_2SO_4$ on the BLM and was at the same distance from the surface of the membrane (curve 4). At the point marked by the arrow in Fig. 1 the speed was switched to the opposite direction. The symmetry of pH profiles indicated that the movement of the microelectrode had a negligible effect on the unstirred layer. The size of the unstirred layer was substantially larger if the solutions were not stirred (data not shown). The addition of 10 μM TTFB protonophore did not affect the pH distribution (data not shown).

The simultaneous recording of the BLM voltage in the presence of a protonophore (curve 1) and pH near the BLM (curve 2) measured by a pH microelectrode

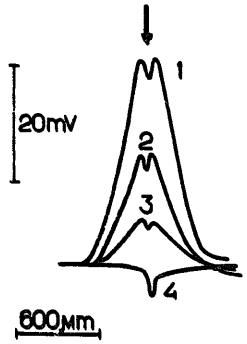

Fig. 1. The pH profile near the BLM measured upon bringing the electrode close to it (left side before the arrow) and taking the electrode away of it (after the arrow) after the addition of 1.75 mM of ammonium sulfate cis (curve 1) and 1.0, 1.5 and 1.75 mM trans side of the BLM (curves 2, 3 and 4 correspondingly) under the condition of stirring the solutions. The trans side is the side where the pH microelectrode was located. The solution comprised 1 mM Tris, 1 mM Mes, 100 mM choline chloride (pH 6.8), 10 μM TTFB. The electrode movement speed was 5 μm/s.

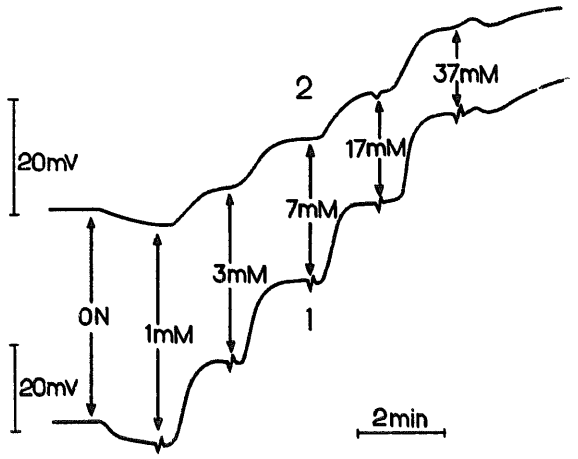

Fig. 2. Comparison of the kinetics of the potential generation on the BLM in the presence of a protonophore TTFB (curve 1) and the pH near BLM measured by pH microelectrode (curve 2, 93% of the maximum pH shift preceding the phase of steep pH change) after additions of ammonium sulfate and the start of stirring the solutions. The conditions were the same as in the legend to Fig. 1. ON, - the start of stirring the solutions.

in the vicinity of the BLM surface is presented in Fig. 2. The coincidence of the kinetics of both responses to the start of stirring the solutions as well as to new additions of ammonium sulfate is seen. It is worth noting that the response of the BLM voltage to the last addition of $(NH_4)_2SO_4$ was complex. Such a voltage behavior takes place frequently under high potential conditions on BLM (more than 100 mV). The pH response measured by the microelectrode was similar. These results show that the BLM voltage in the presence of TTFB reflects pH changes near the BLM. Control experiments showed that there were no pH changes measured by the pH microelectrode under conditions similar to Fig. 2 if the electrode was about 1 mm away from the surface of the membrane.

A plot of pH shifts measured by the pH microelectrode versus BLM voltage in the presence of TTFB is presented in Fig. 3. Lines 1 and 2 show the results of two experiments where pH shifts at the opposite sides of the BLM were measured, the pH shifts were opposite in sign. Actually we did not change the position of the microelectrode physically but added $(NH_4)_2SO_4$ at the opposite side of the BLM. Curve 3 is a sum of curves 1 and 2 and is close to the ideal dashed curve (curve 5) with slope unity. The agreement between these two methods would be actually better if one took into account the different responses of two channels to the change of pH, 50 mV for one pH unit by microelectrode and 58 mV by BLM voltage (curve 4). The difference between curve 4 and the ideal curve is less than 5%.

We checked with the pH microelectrode the systems described previously as generators of the BLM voltages in the presence of a protonophore [5–8] – the gradient of sodium acetate concentration, ionophores nigericin

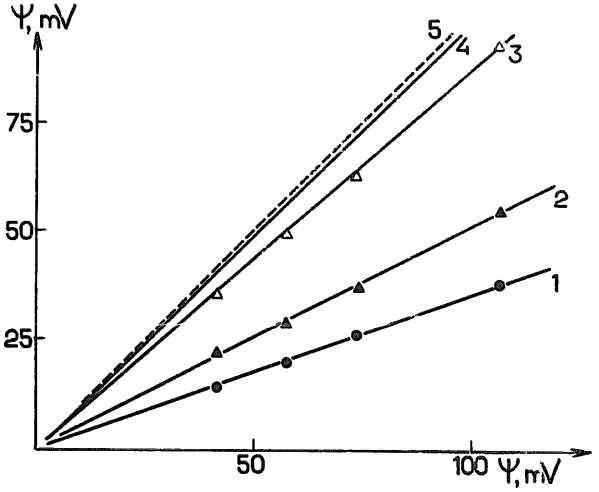

Fig. 3. pH change near the BLM measured by pH microelectrode (in millivolts, Y axis) versus the BLM voltage in the presence of TTFB (X-axis) under the condition of addition of ammonioum sulfate at one side of the membrane. Curve 1, measurements of surface pH changes on the opposite side of the BLM to the side where the $(NH_4)_2SO_4$ was added (93% of the maximum pH shift preceding the phase of steep pH change); curve 2, measurements of pH change at the same side as the addition of $(NH_4)_2SO_4$ (92% of the maximum pH shift preceding the phase of steep pH change); curve 3, the sum of curves 1 and 2; curve 4, the corrected curve for the different responses of these two channels of measurement to the pH change. Conditions were the same as in the legend to Fig. 1.

(potassium ions gradient), tributyltin (chloride ions), calcimycin (calcium ions). We detected pH shifts by the pH microelectrode parallel with voltage generation in all these systems.

Fig. 4 shows the scheme of pH profiles near BLM designed from pH microelectrode experiments. In agreement with the scheme of proton-transfer reactions (bottom of Fig. 4) the pH decreased on the side of BLM where $(NH_4)_2SO_4$ was added and increased on the opposite side. pH profiles at each side can be divided into three parts – a diffuse part of nonlinear

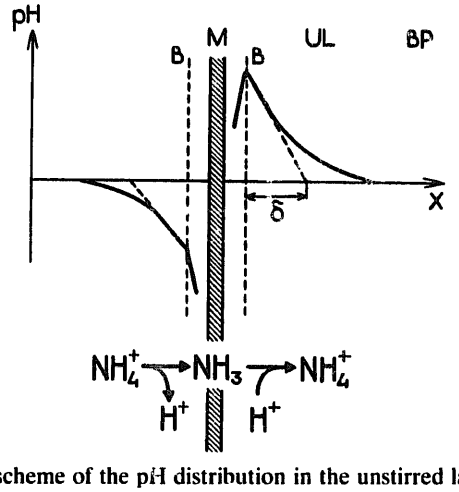

Fig. 4. The scheme of the pH distribution in the unstirred layer near the membrane (top) and proton-transfer reactions (bottom) under the conditions of ammonia permeation through the membrane (M). UL, unstirred layer; BP, bulk phase; B, break point.

pH change, a part of approximately linear pH change and a part of steep pH change near the surface of the BLM. In the last part (from point B to M and from M to B, Fig. 4) pH reduces at both sides of the BLM, the phenomenon being independent of the gradient of $(NH_4)_2SO_4$ on the membrane (Fig. 1). The most simple suggestion is that this part is the result of the reversible interaction of the pH electrode with the BLM and can not be interpreted as a pH change. On the other hand the described pH decrease near the surface of the membrane may be the result of the presence of pH titratable groups of phospholipids on the surface of the BLM.

We estimated the thickness of the unstirred layer (δ) by linear approximation [2,3] of a pH profile as presented in Fig. 4. The thickness of the unstirred layer in the experiment presented in Fig. 1 was 290 μm which was somewhat higher than the value derived from the permeability experiments [4]. However, in our experiments the unstirred layer thickness varied from 120 μm to 300 μm. This variation could be the function of the stirring bar position along the axis of the cell or some differences in hydraulic pressure on the BLM.

There is a relationship between the thickness of the unstirred layer (δ), diffusion coefficient (D) and a half-time response ($t_{1/2}$) associated with the delay for the diffusion through the unstirred layer [3]

$$t_{1/2} = 0.38\,\delta^2/D \qquad (1)$$

In the experiment of Fig. 1 the half-time of the voltage response to the addition of $(NH_4)_2SO_4$ was 50 s. According to Eqn. 1 we can estimate the effective diffusion coefficient of hydrogen ions as it was defined in Ref. 13. At $\delta = 290$ μm and $t_{1/2} = 50$ s one can obtain $D = 6.4 \cdot 10^{-6}$ cm^2/s. This value corresponds to the diffusion coefficient of molecules of the size of Tris and Mes and is substantially lower than the diffusion coefficient of hydrogen ions (about 10^{-4} cm^2/s). Therefore the main contribution to the diffusion of acid-base equivalents was made by the diffusion of buffer molecules as it can be expected from the ratio of free hydrogen ion (10^{-7} M) and buffer (10^{-3} M) concentrations [13,14].

The third diffuse part of pH profile (Fig. 4) was more expressed in the absence of the solution stirring [10] which indicates that it can be accounted for by the incomplete stirring of the solution on the border of the unstirred layer and the bulk phase. On the other hand the nonlinear pH distribution in the unstirred layer may take place even in the absence of chemical reactions in the unstirred layer [2,3].

Fig. 3 shows that the pH shifts were about 40% higher at the side of the BLM opposite to the side of $(NH_4)_2SO_4$ addition. This result can not be accounted

for by the different response of the Tris-Mes buffer mixture to the flux of acid and to the flux of base equivalents since experiments with sodium acetate showed that pH shifts were higher at the side of BLM opposite to the side of CH_3COONa addition as well. The difference in pH shifts may partially be a result of the increase of the buffer capacity of the solution where weak acids or base were added.

Both presented methods have advantages and shortcomings. The measurement of pH shifts by a pH microelectrode gives undoubtfully more information since it allows one to measure the pH profile in the unstirred layers. This method potentially enables one to estimate the flux through the membrane $J = D \cdot dC/dX$ from the measurements of dC/dX from pH profiles and the estimation of D from Eqn. 1. The disadvantage of this method is its considerable complexity and some uncertainty in the position of the electrode with respect to the BLM. The main advantage of the measurement of the BLM voltage in the presence of a protonophore is its simplicity, besides one can measure the sum of pH shifts at both sides of the membrane in one experiment. The disadvantage is the presence of the protonophore in the membrane which in general can affect the proporties of the membrane and the carrier under study.

So both techniques complement each other and give consistent results of pH shifts in the unstirred layers near the BLM under the conditions of hydrogen ion flux through the membrane.

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