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Effects of ultrasound on the steady-state transmembrane pH gradient and the permeability of acetic acid through bilayer lipid membranes

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The irradiation of bilayer lipid membranes with continuous ultrasound of a frequency of 8.2 MHz and a spacial peak time average (SPTA) intensity of 0.4 W/cm^2 reduces the thickness of the unstirred layer up to 40% of its initial value under our conditions. This result was obtained from measurements of the transmembrane potential which appears after the addition of a protonophore in the presence of a gradient of acetic acid. Ultrasound exposure decreases this potential when the pH of the buffer solutions is much higher than the pK of CH_3COOH and has no effect at low pH values. The latter can be explained by a simultaneous increase of the permeability of acetic acid and the buffer substances, respectively, due to ultrasound irradiation.

Introduction

Unstirred layers (UL) play an essential role in the transport processes through biological membranes since they generally cause a difference of the solute concentration adjacent to the membrane and the bulk solution [1].

The UL thickness of a biological membrane is determined by various factors such as shear stress and hydraulic pressure. There is some evidence that ultrasonic waves are able to reduce the thickness of the UL [2]. Such changes could be of importance when ultrasound is used in clinical therapy or diagnostics as well as in biotechnological applications. Cellular changes due to ultrasound irradiation, such as alteration in cell motility, the stimulation of cell synthesis and secretion appear to be associated with changes in the permeability of the cell (plasma) membrane and in the transport of ions and molecules across it [3].

Julian and Zentner [4] studied the ultrasonically mediated solute permeation through polymer barriers and came to the conclusion that effects on the stagnant aqueous diffusion layers were not responsible for the observed increases in permeability. At the same time an ultrasound stimulation of gramicidin incorporation into a bilayer lipid membrane was found [5]. An increase in the channel opening frequency was explained by a diminishing of near membrane non-stirred layers. However, the mechanism of this effect is not yet clear and it can also be discussed in terms of the dimerization process of the channel formation [6].

Furthermore, a planar bilayer lipid membrane (BLM) can be regarded as an appropriate model for studying the influence of ultrasound on the thickness of the UL of biological membranes. In the experiments described below sodium acetate was used as a model substance to study the mechanisms of the ultrasonically stimulated diffusion of weak electrolytes through a BLM. The diffusion of this type of substances through BLM was thoroughly investigated under normal conditions [7–10].

Materials and Methods

Formation of the membrane

BLM were formed on a teflon partition 1.2 mm in diameter by means of a conventional method [11]. The membrane forming solution contained 20 mg phosphatidylcholine from soybeans (Sigma) and 10 mg cholesterol (Serva) in 1 ml of n-decane (Merck). The buffer mixture consisted of 1 mM Tris (Fluka), 1 mM Mes (Boehringer Mannheim), 1 mM β-alanine (Merck) and 100 mM choline chloride (Fluka). The medium was

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stirred by magnetic bars to minimize the thickness of the UL. The agitation rates were the same in all runs. Ethanol solutions of the protonophores pentachlorophenol (PCP) at pH < 6.7 or carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at pH > 6.7 were added at both sides of the BLM. Their final concentration was $4 \cdot 10^{-5}$ M. PCP and CCCP were purchased from Fluka.

Measurement of the electrical properties of the BLM

All electrical parameters were monitored by calomel reference electrodes on both sides of the membrane. The BLM capacitance was measured by an ac method. A function generator set at 100 Hz transmitted a triangle wave with an amplitude of 50 mV to one electrode and the *x*-axis of an oscilloscope. The other electrode was connected with a Keithly 428 current amplifier passing the signal to the *y*-axis of the oscilloscope.

Conductance measurements were undertaken by a Keithly 617 electrometer applying a dc signal of 50 mV.

Transmembrane potential measurements were carried out by the electrometer too. The potential rises in the presence of a protonophore and a concentration gradient of sodium acetate. The latter generates a flux J through the BLM. Under buffered conditions, when the pH shift along the UL is small and assuming that the permeabilities of the UL for the acid and the acid anion are equal we can express the flux as [8]:

$$\frac{1}{J} = \frac{1}{P_{UL}[T^-] + P_{UL}[TH]} + \frac{1}{P_M[TH]} \tag{1}$$

where P_M is the membrane and P_{UL} the UL permeability coefficient, respectively. [TH] is the acid concentration, $[T^-]$ the concentration of the acid anion at that side of the BLM where the acid is added. J can be considered as the sum of two fluxes [9].

(1) The flux J_{TH} of the neutral acid through the membrane and the ULs. It is described by the equation:

$$\frac{1}{J_{TH}} = \frac{1}{P_M[TH]} + \frac{1}{P_{UL}[TH]} \tag{2}$$

(2) The flux J_{T^-} occurring in three steps: (a) the flux of the acid anions through the first UL which is accompanied by the association of protons, (b) the diffusion of the acid in its neutral form through the membrane and (c) the dissociation and the diffusion of the acid anions through the second UL. Unlike J_{TH} the flux J_{T^-} produces a pH shift in the ULs, whereas the pH of the bulk solutions does not change.

The pH difference between the ULs adjacent to the membrane can easily be measured by means of a transmembrane potential ϕ which occurs after the addition of a protonophore. Following the Nernst equation

$$\phi = \frac{RT}{F} \ln \frac{[H^+]_1}{[H^+]_2} \tag{3}$$

a pH difference of one produces a potential difference of 58 mV at room temperature. $[H^+]_1$ and $[H^+]_2$ are the proton concentrations at the different sides of the BLM, R, T and F have their usual meanings. The measurement of ϕ allows the estimation of J_{T^-}, which has been proposed by Antonenko and Yaguzhinsky [9]. Later the method was successfully used by others [12,13].

The pH gradient induces a buffer flux J_b described as the sum of the buffer fluxes on both sides of the BLM by the following equation [10]:

$$J_b = P_b B \log \frac{[H^+]_1}{[H^+]_2} \tag{4}$$

where P_b is the permeability of the ULs for the buffer, B is the buffer capacity. Eqn. 4 is based on the constance of the buffer capacity in the whole range of pH changes and the invariability of P_b for all buffer substances at a given UL thickness [14]. P_b can be substituted by the quotient of the diffusion coefficient D of acetate and buffer components and the thickness of the unstirred layer δ. In the steady state, the flux J_{T^-} must be equal to the buffer flux J_b. Eqns. 3 and 4 can be combined:

$$J_b = |J_{T^-}| = \frac{\phi}{\delta} AD \tag{5}$$

where $A = BF/(2.3\ RT)$.

Ultrasound exposure

The sound frequency (8.2 MHz) was chosen so that the wavelength was small compared to the diameter of the membrane. A plane circular transducer 8 mm in diameter was used. It was calibrated by radiation force measurements using suspended stainless steel spheres [15]. Continuous ultrasound waves were transmitted through a water tank onto the teflon chamber which had two sound windows on both ends made of a thin polyethylene film and a parafilm (Fig. 1). All measurements were performed in the far field of the transducer. A sound absorber consisting of castor oil prevented the formation of standing waves in the water tank.

A hydrophone was placed in the water tank to search for harmonic ultrasound signals emitted by cavitation bubbles. The experimental conditions where chosen so that cavitation did not occur. Before and after the sonication the temperature of the buffer solutions was measured.

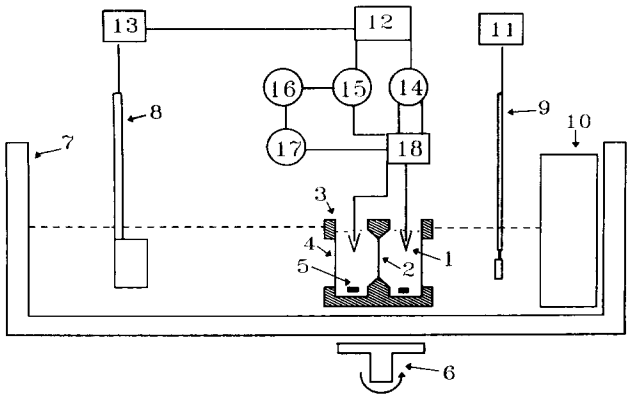

Fig. 1. Scheme of the experimental arrangement. 1, Reference electrodes; 2, bilayer lipid membrane; 3, teflon support; 4, acoustic window; 5, magnetic stirrer bar; 6, stirrer; 7, water bath; 8, ultrasound transducer; 9, ceramic hydrophone; 10, absorber; 11, selective nanovoltmeter; 12, personal computer; 13, rf generator; 14, electrometer; 15, current amplifier; 16, oscilloscope; 17, triangle wave generator; 18, switch: connects the electrodes either to 14 or to 15 and 17.

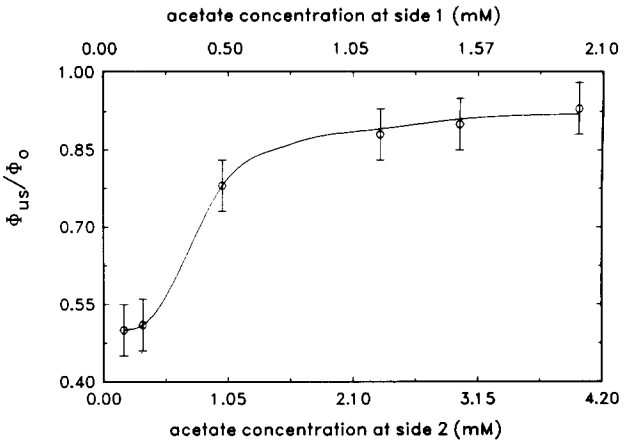

Fig. 3. Effect of the ultrasound on the transmembrane potential as a function of the acetate concentration. ϕ_o and ϕ_{us} are the transmembrane potentials without and with ultrasound irradiation (spacial peak time average intensity 0.4 W/cm^2) at the given concentration of sodium acetate. pH = 8.0. The quotient of the acetate concentrations at the both sides of the BLM c_1/c_2 was kept constant. The line was drawn by eye.

Results

For our bilayer lipid membranes containing a protonophore a conductance of $(3.3 \pm 0.9) \cdot 10^{-5}$ Ω^{-1} cm^{-2} and a capacitance of 0.54 ± 0.07 μF cm^{-2} was obtained. Ultrasound at all intensities applied in our experiments did not have any effect on the conductance and on the capacitance of a BLM.

The transmembrane potential measured as described in the previous section can be reduced both by the means of a stirrer and by ultrasound irradiation. Fig. 2 shows an example of the temporal course of an experiment.

The sound effect is not caused by the rise of temperature or by cavitational events. Neither the first nor the second were measurable up to an intensity of 0.5 W/cm^2.

The decrease of the transmembrane potential depends on the total amount of acetate in the solutions. Keeping the ratio of the salt concentrations c_1 and c_2 on both sides of the BLM constant it was diminished by the addition of acetate (Fig. 3).

Further experiments were carried out at low acetate concentrations, adding the salt at one side of the membrane only. The ultrasound effect on the transmembrane potential was measured as changes of the ratio ϕ_{us}/ϕ_o, where ϕ_o and ϕ_{us} are the transmembrane potentials under reference conditions and during sonification, respectively. The ratio decreases if the pH value increases in the range from 5.5 to 7.2. There was no influence of ultrasound and the stirrer at pH lower than 5.0. At pH higher than 7.3 the effect of ultrasound was maximum (Fig. 4).

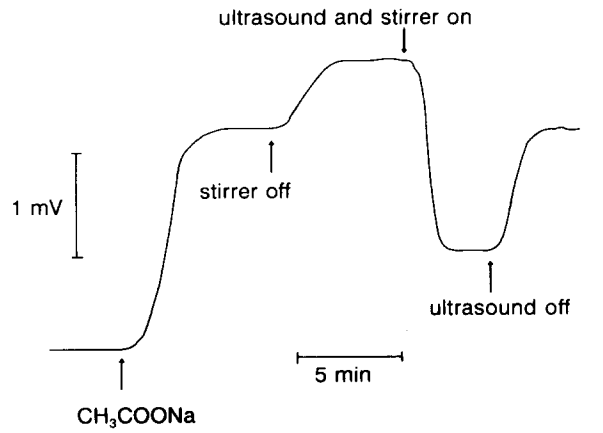

Fig. 2. Transmembrane potential of the BLM as a function of time. Sodium acetate was added at one side of the BLM (final concentration 0.9 mM) in the presence of a protonophore. pH of the solutions 8.0. Ultrasound intensity of 0.4 W cm^{-2} (spacial peak time average).

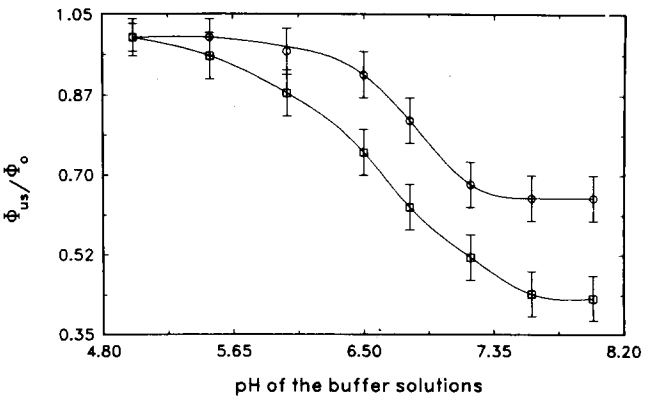

Fig. 4. Dependence of the ratio ϕ_{us}/ϕ_o on the pH value of the buffer solutions. The spacial peak time average intensity of ultrasound was 0.2 W/cm^2 (○) or 0.4 W/cm^2 (□).

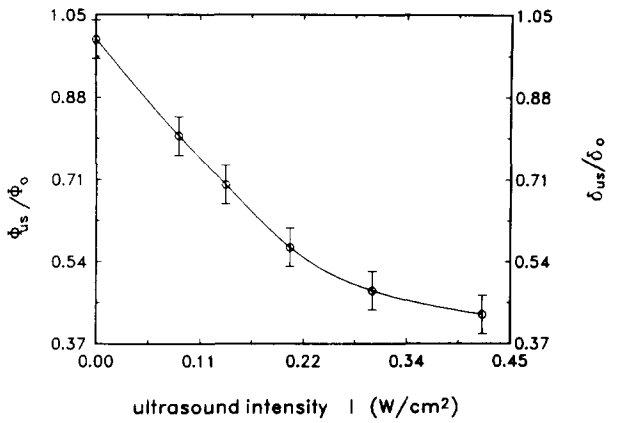

Fig. 5. Dependence of the transmembrane potential and the thickness of the unstirred layers on the ultrasound intensity. ϕ_o and ϕ_{us} indicate the potential, δ_o and δ_{us} the thickness of the unstirred layers under reference and irradiation conditions, respectively. pH of the solutions 8.0.

The ultrasound effect increases linearly with increasing intensity up to 0.3 W/cm^2 (Fig. 5), but the course of the curve indicates a saturation at intensities higher than 0.5 W/cm^2.

Discussion

The values for conductance and capacity correspond to those found in literature [16–18]. The lack of an effect of ultrasound on the membrane conductance and capacity (and therefore on the area of the BLM) agrees with the results of Rohr and Rooney [19].

As mentioned earlier, ultrasound is expected to decrease the thickness of the ULs δ_o to a certain thickness δ_{us}. Furthermore, the transmembrane potential is effected by stirring and ultrasound in the same direction at high pH values (Fig. 2). Both had no effect at low pH values. These results support the suggestion that the ultrasonic effect is based on the agitation of the liquid in a layer very close to the BLM.

Obviously, the behavior of the transmembrane potential depends on the rate limiting step of the transport process. Two extreme cases have to be distinguished.

(1) The transport of acetic acid through the membrane is determined by the diffusion rate through the membrane itself which is the case at high pH values [7]. The Eqns. 1 and 2 can be transformed substituting [TH] and [T$^-$] by the concentration of the acid [T$_0$] added at one side of the membrane [9]:

$$[T_0] = [T^-] + [TH] \text{ and } [TH] = [T_0]/(1+\alpha)$$

where $\alpha = 10^{pH-pK}$. The pK value of acetic acid is 4.75. Because of $\alpha >> 1$ we get:

$$J_{T^-} = J - J_{TH} = \frac{[T_0]P_M}{\alpha} - \frac{P_M P_{UL}[T_0]}{\alpha(P_M + P_{UL})} \tag{6}$$

The value of $\delta/2$ determined at one side of a BLM by a microelectrode technique amounts to 120 to 300 μm [20]. If the size of the UL regarding both sides of the BLM is not less than 400 μm and if we assume D to be 10^{-5} cm^2 s^{-1} then $P_{UL} = D/\delta$ will be larger than $2.5 \cdot 10^{-4}$ cm/s. Since P_M is about $6.9 \cdot 10^{-3}$ cm/s [21] we get the following equation:

$$\frac{\alpha J_{T^-}}{[T_0]P_M} = 1 - \frac{P_{UL}}{(P_M + P_{UL})} \approx 1 \tag{7}$$

This is an acceptable approximation even if the ultrasonic field reduces the size of the UL to one half of its initial value.

From Eqns. 5 and 7 we obtain:

$$\phi = \delta \frac{[T_0]P_M}{AD\alpha} \tag{8}$$

Consequently, ϕ can be used to estimate the sum of the thicknesses of the UL on both sides of the UL. However, the error of such a calculation will be very large because precise values of D and P_M for the system used are unknown. At pH 8 and for $[T_0] = 0.9$ mM and $B = 0.65$ mM, respectively, we find for δ a value of 1.2 ± 0.7 mm.

Therefore, in order to minimize the error we calculated the ratio ϕ_{us}/ϕ_o wich is equal to δ_{us}/δ_o rather than to use the absolute values of δ. In this way the error should be less than 10%.

(2) The limiting step of the acetate transport is the diffusion through the ULs. This occurs at low pH values [7]. If we assume that α is about unity Eqns. 1 and 2 can be transformed into:

$$J_{T^-} = J - J_{TH} = \frac{[T_0]P_{UL}P_M}{2P_{UL} + P_M} - \frac{[T_0]P_{UL}P_M}{2(P_{UL} + P_M)} \tag{9}$$

$$\frac{2J_{T^-}}{[T_0]P_{UL}} = \frac{P_M^2}{P_M^2 + 3P_M P_{UL} + 2P_{UL}^2} \tag{10}$$

Inserting the values assumed for P_M and P_{UL} at the right side of Eqn. 10 it can easily be shown that it is nearly unity. For smaller α this simplification is still valid even for a δ that is much smaller than assumed in our estimation. Considering Eqn. 5 we get for the potential difference:

$$\phi = \frac{[T_0]}{2A} \tag{11}$$

As seen from Eqn. 11 the transmembrane potential does not depend on the size of the ULs. Although an increase of the permeability of the UL for the acid should be caused by the ultrasound exposure this alteration will not be effective. It will be accompanied by a higher permeability of the buffer substances and consequently, no potential shift will be detectable.

Between both extreme cases the ultrasound effect on the potential increases with pH (see Fig. 4). For higher intensities it is observed at smaller α which can be explained by the limitations of the preconditions used in Eqn. 11.

Although a concentration dependence of the effect of ultrasound was recorded at high pH values Eqn. 8 can not be used to interpret the results because the pH shift in the UL at high acetate concentrations is not negligible. Previously it was found that at low concentrations of a weak base or acid the potential depends linearly on the *difference* between the salt concentrations on both sides of the BLM whereas at higher concentrations the potential is a function of the *ratio* of the salt concentrations [10]. The reason for this behavior is that in the first case the transport through the membrane and in the second case the diffusion through the ULs is the rate-limiting step. Consequently, we found in our experiments a decreasing effect of ultrasound on the transmembrane potential at higher acetate concentrations (Fig. 3).

Although the solutions in the chambers on both sides of the membrane are well stirred by two magnetic bars an additional effect on the solute transportation near the membrane due to acoustic streaming is likely. Unidirectional fluid circulation (quartz wind) is established in front of the sound transmitter. Its magnitude depends on the free propagation length of the sound waves in the liquid and on the sound intensity [22]. The effect of the latter on the thickness of the UL δ_{us}/δ_o measured in terms of the ratio ϕ_{us}/ϕ_o is shown in Fig. 5. It supports the streaming model for a wide range of the sound intensities used in our experiments. Furthermore, this suggestion would lead to the conclusion that the ULs on both sides of the sonified BLM are unsymmetrical in their dimensions.

The thickness of the unstirred layer along the BLM is in a complicated way dependent on the type of agitation [23]. Although, qualitatively estimated the quartz wind streaming appears to be small compared with the motions caused by the stirrers the former could be more effective due to its direction perpendicular to the BLM. On the other hand, other mechanical effects occurring within the UL like the relative movement between BLM and the liquid may also contribute to the reduction of the UL. This kind of effects should be observed on both sides of the BLM. Further experiments will be undertaken to investigate the properties of the UL in more detail.

Summarizing our results we conclude that the effect of ultrasound on the transport of weak acids through BLM can be explained by an increase of the permeability through the unstirred layers adjacent to the membrane rather than by changes of the permeability of the membrane itself. Under our conditions the decrease of the thickness of the ULs found at high ultrasound intensities was about 60%. If the transport through the UL is the rate limiting step in the transport process, i.e., at low acid concentrations and pH $\approx$ pK the transmembrane flux increased in the same way as the flux though the UL.

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