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The peculiarities of reactions catalyzed by alcohol dehydrogenase in unstirred layers adjacent to the bilayer lipid membrane

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The peculiarities of the kinetics of the enzymatic reaction taking place in the unstirred layer adjacent to the bilayer lipid membrane were studied for the case of the ethanol oxidation reaction catalyzed by alcohol dehydrogenase. A well-permeating substrate (ethanol, acetaldehyde) was added to one side of the bilayer lipid membrane and alcohol dehydrogenase to the other side. Hydrogen ions were produced in the unstirred layer in the course of the reaction $C_2H_5OH + NAD^+ \rightleftharpoons C_2H_4O + NADH + H^+$, which made it possible to measure the reaction rate by determining generation of the electrical potential on the bilayer lipid membrane in the presence of a protonophore due to local pH changes in the unstirred layer. It was shown that the kinetics of the reaction studied under these conditions are similar to those of reactions catalyzed by immobilized enzymes. At a high alcohol dehydrogenase concentration, the steady-state concentrations of substrates and products of the reaction near the membrane reach equilibrium values. The substrate oxidation rate is determined by the rate of product diffusion through the unstirred layer under these conditions. The system described presents a model of cell enzyme functioning when enzymes and substrates are in separate compartments and shows the peculiarity of soluble cell enzyme activity in the presence of unstirred layers.

Introduction

It was shown that the additional diffusion resistance due to the presence of unstirred layers near the surface of a catalyst make a significant contribution to the kinetics of the reactions catalyzed by immobilized enzymes [1]. It is well known that unstirred layers are present near model and natural membranes [2,3], but until recently their effect on enzymatic kinetics was studied only for the case of substrate-transporting membrane-bound enzymes [4–7]. In this paper the effect of unstirred layers near the bilayer lipid membrane on the activity of soluble enzyme, alcohol dehydrogenase (EC 1.1.1.1), is studied. The enzyme catalyzes the reaction [8,9]:

$$C_2H_5OH + NAD^+ \rightleftharpoons C_2H_4O + NADH^+ + H^+ \quad (1)$$

A well-permeating substrate (ethanol, acetaldehyde) is added to one side of the bilayer lipid membrane and alcohol dehydrogenase to the other side. The reaction 1 proceeds mainly in the unstirred layer adjacent to the surface of bilayer lipid membrane. The rate of the reaction is measured by determining the local pH shift in the unstirred layer. To determine the pH shift we apply our method of measuring pH changes in the unstirred layer, which consists of monitoring the bilayer lipid membrane potential in the presence of a

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Abbrviations: Hepes, *N*-1-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TTFB, tetrachlorotrifluoromethylbenzimidazole.

protonophore [10–13]. Local pH changes in unstirred layer in the course of enzyme-catalyzed reactions, viz. a carboanhydride reaction, were found first by Gutknecht et al. [14].

It is shown that at a high alcohol dehydrogenase concentration, the steady-state concentrations of substrates and products near the bilayer lipid membrane reach equilibrium values and the rate of the reaction is determined by the rate of product diffusion through the unstirred layer under these conditions.

Materials and Methods

Lipid bilayer membranes were formed on a hole in a Teflon partition, 0.4 mm in diameter, by the conventional technique [15]. A membrane-forming solution contained 20 mg of phosphatidylcholine (from soy beans, Sigma) and 20 mg cholesterol (Boehringer) in 1 ml of *n*-decane. The thinning of the bilayer lipid membrane was monitored both visually and by measuring its capacitance [10]. Solutions were stirred with a magnetic mixer, the agitation rates being the same in all runs. The potential difference was measured by two calomel reference electrodes connected with the medium by agar bridges filled with 1 M KCl on one side and having an input of a Keithley 301 amplifier and a recorder on the other side. The stock water solution of the protonophore tetrachlorotrifluoromethylbenzimidazole (TTFB, a gift of Prof. E.A. Liberman) was added to both sides of the bilayer lipid membrane. Yeast lyophilized alcohol dehydrogenase (Reanal, Hungary) was dissolved in 10 mM Tris-buffer (pH 7.5) before the start of the experiment. The activity of ADH was 2 μmol ethanol/min per mg protein and 10 μmol acetaldehyde/min per mg protein under the same conditions as in Fig. 1. 10 μM TTFB did not alter the activity of alcohol dehydrogenase. NAD and NADH were from Reanal, Tris and Hepes were from Serva. Other chemicals were from Reachim (U.S.S.R.). The experiments were carried out at room temperature.

Results

The addition of ethanol on one side of the bilayer lipid membrane and alcohol dehydrogenase on the other induces an electric potential on the bilayer lipid membrane in the presence of NAD and the TTFB protonophore (Fig. 1, curve 1). The potential has plus on the side of the membrane where ethanol is added. If alcohol dehydrogenase is absent, no potential is generated (data not shown). A direct measurement of the pH of the aqueous bathing solutions of the bilayer lipid membrane after the end of the experiment showed that it remained unchanged. As the buffer concentration increases, the magnitude of the bilayer lipid membrane potential decreases considerably under these conditions (Fig. 1, curve 1). This is not the effect of an increase in the ionic strength of the solutions, since the addition of 10 mM potassium chloride does not alter the bilayer lipid membrane potential (data not shown). Control experiments show that neither alcohol dehydrogenase nor ethanol changes the conductance of the bilayer lipid membrane either in the presence or in the absence of TTFB under these conditions.

Fig. 1, curve 2, shows the induction of the bilayer lipid membrane potential in the presence of TTFB upon the addition of acetaldehyde on one side of the membrane and alcohol dehydrogenase on the other. The potential has plus on the side of the bilayer lipid membrane where the

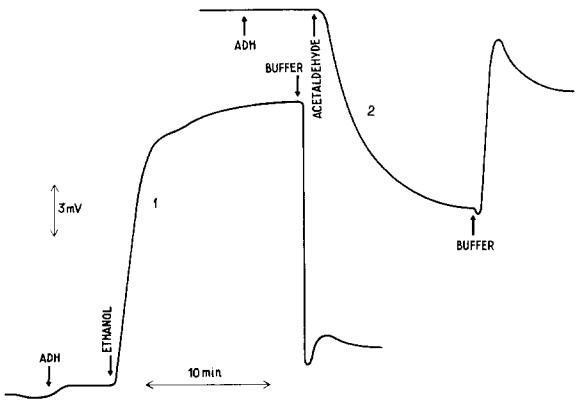

Fig. 1. Potential generation on the bilayer lipid membrane upon addition of ethanol (170 mM, curve 1) or acetaldehyde (1.1 mM, curve 2) on one side of the membrane and alcohol dehydrogenase (ADH) (curve 1 0.24 mg/ml, curve 2 0.016 mg/ml) on the other in the presence of TTFB. Curve 1. The solution was 1 mM Tris/3 mM NAD/100 mM KCl/10 μM TTFB (pH 7.5). Curve 2. The solution was 1 mM Tris/1 mM NADH/100 mM KCl/10 μM TTFB (pH 7.5). Buffer – the addition of 10 mM Hepes-Tris (pH 7.5) on both sides of the bilayer lipid membrane.

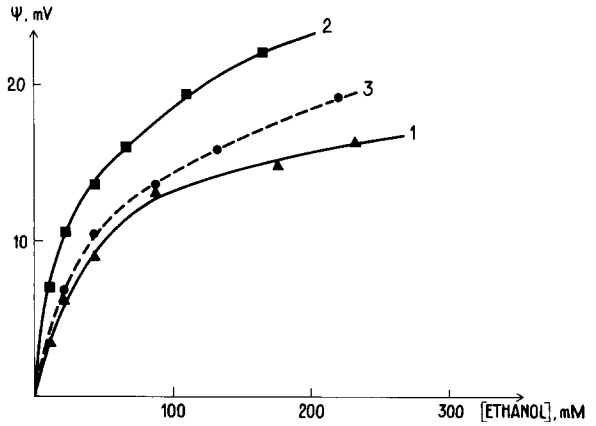

Fig. 2. The dependence of the steady-state value of the bilayer lipid membrane potential (curve 1) and the equilibrium value of pH electrode potential (curve 2) on the concentration of added ethanol. pH measurements were carried out in a separate cell, not in the bilayer lipid membrane one (zero potential corresponds to pH 7.5). The solution was as in Fig. 1, curve 1. The concentration of alcohol dehydrogenase was 0.24 mg/ml. The curve 3 was constructed from curve 2 by calculation so that each point was transferred along the abscissa axis with doubled argument.

enzyme is present. If alcohol dehydrogenase is absent, no potential is generated (data not shown). Both solutions contained 1 mM NADH in these experiments. As the buffer concentration increases, the bilayer lipid membrane potential decreases considerably. Acetaldehyde does not change the conductance of the bilayer lipid membrane, either.

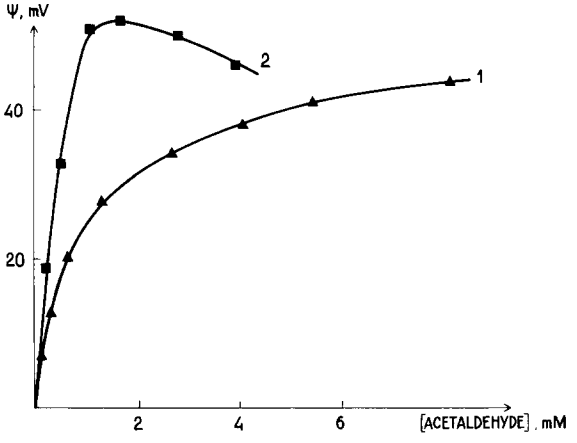

Fig. 3. The dependence of the steady-state bilayer lipid membrane potential value (curve 1) and of the pH electrode potential equilibrium value (curve 2) on the concentration of added acetaldehyde. pH measurements were carried out in a separate cell, not in the bilayer lipid membrane one (zero potential corresponds to pH 7.5). The solution was as in Fig. 1, curve 2. The alcohol dehydrogenase concentration was 0.08 mg/ml.

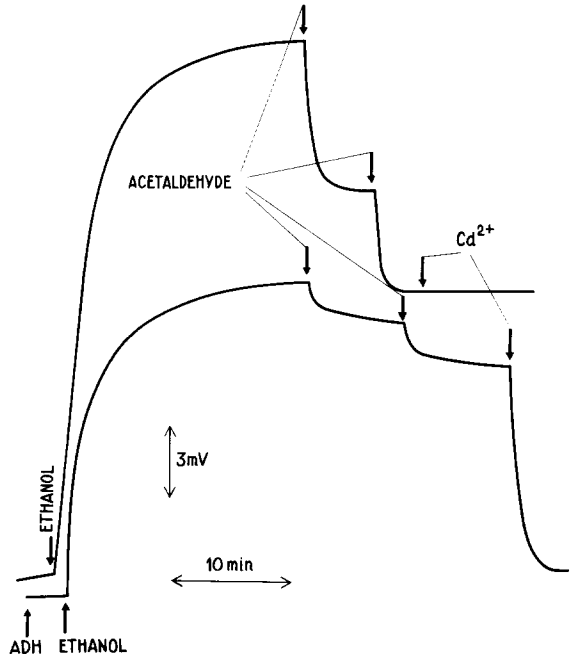

Fig. 4. The decrease in potential values on the bilayer lipid membrane (curve 1) and on pH electrode upon acetaldehyde addition (both additions were 0.3 mM). The solution was as in Fig. 1, curve 1. Alcohol dehydrogenase (ADH), 0.24 mg/ml; ethanol, 170 mM; Cd^{2+}, 50 μM.

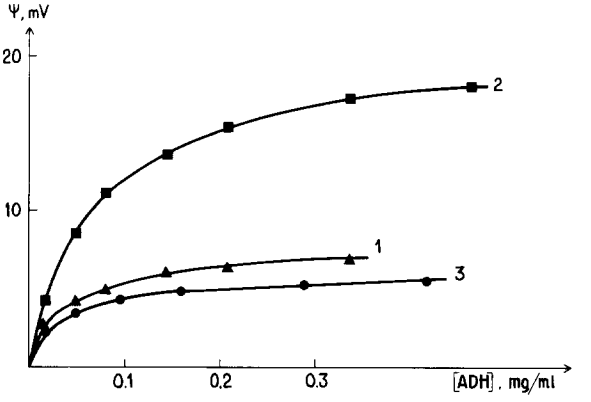

Fig. 5. The dependence of the bilayer lipid membrane potential on the alcohol dehydrogenase (ADH) concentration. Curve 1. The concentration of ethanol was 17 mM, the solution was as in Fig. 1, curve 1. Curve 2, 170 mM ethanol; the solution was as in Fig. 1, curve 1. Curve 3, 170 mM ethanol; the solution was 1 mM Tris/1 mM NAD/100 mM KCl/10 μM TTFB (pH 7.5).

Figs. 2 and 3 (curves 1) show the dependence of the magnitude of the bilayer potential in the presence of NAD and NADH on the concentration of ethanol and acetaldehyde, respectively. Figs. 2 and 3 (curves 2) present the data of experiments in a homogeneous system in which the equilibrium pH values of a solution with NAD and NADH versus the concentration of added ethanol and acetaldehyde, respectively, are given. The pH values are shown in millivolts according to the equation $\psi = 58\ \text{mV} \cdot \Delta\text{pH}$ (zero potential corresponds to pH = 7.5) for easy comparison with curves 1 of these figures.

As seen from Fig. 4, the magnitude of the ethanol-induced pH shift decreases considerably upon the addition of acetaldehyde both in the homogeneous (curve 2) and in the heterogeneous (curve 1) system. This decrease cannot be accounted for by contamination of NADH in the NAD sample, since no shift of the bilayer lipid membrane potential takes place upon the addition of acetaldehyde in the absence of ethanol. It is noteworthy that, according to kinetic pH measurements, the acetaldehyde at the concentration used does not diminish the initial rate of ethanol oxidation under these conditions. The difference between these two systems manifests itself in relation to enzyme inhibition. Cadmium ions, which inhibit enzyme activity by more than 95% under these conditions, do not affect the pH shift in the homogeneous system after an equilibrium has been reached (Fig. 4, curve 2), but decrease significantly the magnitude of the potential on the bilayer lipid membrane (Fig. 4, curve 1).

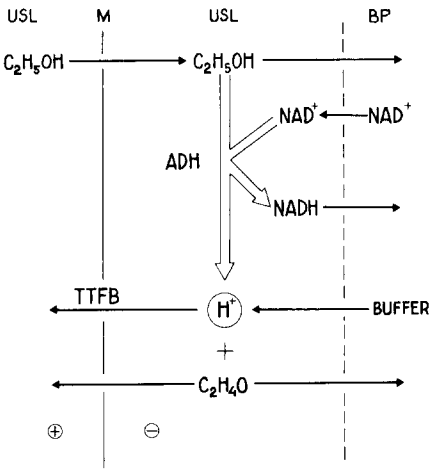

Fig. 7. The model of potential generation on the membrane (M) upon ethanol oxidation by alcohol dehydrogenase (ADH) in the presence of NAD. USL, unstirred layer; BP, bulk phase. Single arrow: the transfer of a substance. Double arrow: the chemical reaction.

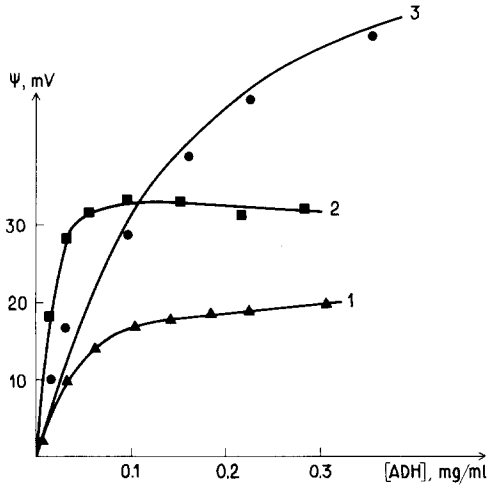

Fig. 6. The dependence of the bilayer lipid membrane potential on the concentration of alcohol dehydrogenase (ADH). Curve 1. The concentration of acetaldehyde was 0.3 mM. The solution was as in Fig. 1, curve 2. Curve 2, 6 mM, acetaldehyde; the solution was as in Fig. 1, curve 2. Curve 3, 6 mM acetaldehyde; the solution was 1 mM Tris/10 mM NADH/100 mM KCl/10 μM TTFB (pH 7.5).

Figs. 5 and 6 show a typical dependence of the bilayer lipid membrane potential on the enzyme concentration at different concentrations of ethanol and NAD (Fig. 5) or acetaldehyde and NADH (Fig. 6) in the solution.

Discussion

The induction of a bilayer lipid membrane potential in the presence of a protonophore is due to the formation of a pH gradient on the bilayer lipid membrane. Since in the course of the experiment the pH of the bathing solutions does not change, the pH gradient can form only in the unstirred layers adjacent to bilayer lipid membrane. This conclusion is supported by the fact that the value of potential decreases as the buffer capacity of the medium increases (Fig. 1). The phenomenon of the pH gradient formation in the unstirred layer near the bilayer lipid membrane

was studied thoroughly for the case of permeation of weak acids and bases through the bilayer lipid membrane [16–24].

Fig. 7 shows a scheme for the ethanol oxidation reaction in the system. Ethanol, permeating through the membrane, is oxidized by alcohol dehydrogenase in the presence of coenzyme NAD. The reaction is carried out in an unstirred layer of the bilayer lipid membrane and results in hydrogen ion production and unstirred layer acidification. TTFB, the protonophore, transports hydrogen cations through the membrane electrogenically. It induces an electric potential on the bilayer lipid membrane under open circuit conditions and the potential has plus on the side of the bilayer lipid membrane where ethanol is added. Thus, the scheme explains the sign of potential observed in the experiment. Hydrogen ions are consumed in the course of the back-reaction of acetaldehyde reduction accompanied by NADH oxidation. This results in alkalinization of the unstirred layer in our system and the potential has the opposite sign in the presence of TTFB.

After this preliminary discussion, let us consider some quantitative dependences. According to Fig. 7, the magnitude of the pH shift in the unstirred layer is determined by the ratio of the reaction rate and the flux of the buffer from the bulk phase to the membrane. If the buffer concentration is constant, the pH shift and the bilayer lipid membrane potential are proportional to the reaction rate which is determined by substrate and coenzyme concentrations as well as by concentrations of the reaction products. In general, the concentrations of products and substrates near the bilayer lipid membrane in the system can differ substantially from those in the bulk phase due to the reaction processing in the unstirred layer. One can visualize three limiting cases depending on the reaction rate:

(1) If the reaction rate is low, the substrate concentration in the unstirred layer does not decrease and the product concentration is less than the Michaelis constant of the back-reaction (kinetic regime).

(2) At a higher rate of the reaction a large gradient of substrate is formed which determines the kinetics of the whole process (diffusion regime).

(3) In the case of a reversible reaction the diffusion regime can transform into a 'thermodynamic' one which is characterized by nearly equal rates of the forward and backward reactions due to the high concentration of the reaction product in the unstirred layer. In the latter case pH near the bilayer lipid membrane is established in accordance with equilibrium Eqn. 1. The buffer flux can reduce the local pH shift in the system, and the steady-state pH in the unstirred layer is determined not only by equilibrium Eqn. 1, but also by the ratio of the transmembranous flux of the substrate and the flux of the buffer through the unstirred layer. The local pH shift is maintained by continuous substrate permeation into a reaction zone.

Let us consider which of these three limiting cases applies to our experiments. To discriminate between the kinetic and the diffusion regimes the estimation of diffusion parameter, ϕ, which is similar to the Thiele factor, is used [25]:

$$\phi = l\sqrt{\frac{v}{D \cdot S}}$$

where l is the thickness of the reaction zone; D, the substrate diffusion coefficient; S, the substrate concentration; v, the reaction rate. If $\phi \ll 1$ the system is in the kinetic regime, and if $\phi \gg 1$, the system is in the diffusion regime; $\phi \simeq 1$ corresponds to a transient regime [25]. It is essential that this criterion does not take into account the reversibility of the reaction. Let us estimate the magnitude of the diffusion parameter for the forward and the backward reaction in typical experimental conditions (Figs. 1–4):

(1) For ethanol oxidation $S_{NAD} = 3$ mM, $C_{ADH} = 0.24$ mg/ml, $v = 0.48$ mmol/min (see Materials and Methods).

(2) For the back reaction, $S_{NADH} = 1$ mM, $C_{ADH} = 0.08$ mg/ml, $v = 0.8$ mmol/min. By substituting $l = 2 \cdot 10^{-2}$ cm [18] and $D = 5 \cdot 10^{-6}$ Sm^2/s in Eqn. 2 one can obtain $\phi = 0.48$ and $\phi = 1.03$ for the forward and the backward reaction, respectively. Thus, this estimation shows that the gradients of substrate concentration in the unstirred layer can affect the kinetics of reaction 1 under our experimental conditions.

Let us analyse the experimental dependences of the bilayer lipid membrane potential on the concentration of the substrates (Figs. 2, 3), the coen-

zymes and the enzyme (Figs. 5, 6) by assuming that the system is in the kinetic regime. At the stady state, the flux of the hydrogen ions produced or consumed in the course of the reaction 1 is equal to the flux of the buffer from the bulk phase to the membrane which is proportional to the pH difference between the bulk phase and the bilayer lipid membrane (ΔpH) [24]

$$J = P_B \cdot B \cdot \Delta\text{pH} \qquad (3)$$

where P_B is the unstirred layer permeability for the buffer, B the buffer capacity. Therefore the bilayer lipid membrane potential in the presence of a protonophore must be proportional to the rate of enzymatic reaction. The dependence of the bilayer lipid membrane potential on substrate concentration must be of Michaelis-Menten type in this limiting case. Besides, the potential must be proportional to enzyme concentration.

However, the bilayer lipid membrane potential ceases to increase when the enzyme concentration exceeds the value of about 0.2 mg/ml (Figs. 5, 6). It may be assumed that the rate of the process is limited by the rate of NAD (NADH) diffusion. The rise in the pyridine nucleotide concentration does indeed cause an increase in the bilayer lipid membrane potential (Figs. 5, 6). Since the NAD (NADH) concentrations used are much higher than the Michaelis constants of these reactions [8,9], it may be concluded that the system is in the diffusion regime.

On the other hand, the idea of the system being in the diffusion regime contradicts the fact that the potential depends on substrate concentration at high enzyme and constant coenzyme concentrations (Figs. 5, 6). Thus, it should be suggested that reaction products accumulate in the unstirred layer of the bilayer lipid membrane at a high concentration and the back-reaction rate increases greatly. A decrease in the membrane potential upon acetaldehyde addition confirms this assumption, since it indicates the presence of NADH in the unstirred layer (Fig. 4). It may be inferred that at high enzyme concentration, the concentrations of substrates and products are near equilibrium, i.e., in the 'thermodynamic' regime. In this case the bilayer lipid membrane potential corresponds to the pH shift near the membrane which is established in accordance with Eqn. 1. An equilibrium position and a respective pH shift are determined by the ratio of steady-state substrate and product concentrations in the unstirred layer. The pH shift is equal to a pH gradient on the bilayer lipid membrane since the pH of the opposite unstirred layer remains constant and equals the bulk phase pH. Different levels of the potential at different substrate concentrations in the case of high enzyme concentration (Figs. 5, 6) are due to different equilibria established at different substrate concentrations according to Eqn. 1.

If the concentration of ethanol and/or acetaldehyde in the reaction zone near the bilayer lipid membrane differs insignificantly from that in the opposite bulk phase, the equilibrium position of reaction 1 in the unstirred layer is expected to be approximately the same as in the homogeneous system, all other conditions being the same. The pH shift in the unstirred layer must be the same as in the homogeneous system. Figs. 2 and 3 show that the pH shifts in these two systems are actually similar. This confirms the assumption that the system is in the 'thermodynamic' regime. The substrate concentration near the bilayer lipid membrane, regardless of its decrease in the course of the reaction, must be lower than in the opposite bulk phase by a factor of 2 in the case of permeation rate limitation by unstirred layers and by a factor of more than 2 in the case of the permeation rate limitation by the bilayer lipid membrane. We have not found the values of bilayer lipid membrane permeability for ethanol and acetaldehyde in the literature. Since the equilibrium of reaction 1 is shifted to the left [8,9], the degree of ethanol conversion in the unstirred layer cannot be high. If it is the unstirred layer that limits the ethanol permeation, the concentration of this substrate near the bilayer lipid membrane must be 2-times lower than that in the opposite bulk phase and respectively, 2-times lower than the concentration of ethanol used in experiments in the homogeneous system. After a correction for the above difference in the ethanol concentration, a coincidence of pH shifts in the unstirred layer and in the homogeneous system is evident (Fig. 2). Thus, the high reaction rate leads to an equilbrium ratio of the substrates and products of reaction 1 in the unstirred layer near the bilayer lipid mem-

brane under our experimental conditions.

The effect of the unstirred layer on the kinetics of reactions catalyzed by immobilized enzymes is well docoumented now [1]. In particular, a theoretical analysis of the process complicated by an equilibrium reached between the substrates and the products in the reaction zone, i.e., with the system being in a thermodynamic regime, was carried out [26]. We could not find any paper in the literature in which this situation was examined experimentally with immobilized enzymes. On the other hand, this case was observed for the reaction catalyzed by an inorganic catalyst [27]. We have shown in this paper that the 'thermodynamic regime' can be attained in a system where a substrate permeates through the membrane to the compartment containing a soluble enzyme.

Engasser and Horvath studied theoretically the kinetics of an enzymatic reaction under the conditions of substrate permeation through the membrane [28]. They proposed that only two compartments should be considered in this system, namely two bathing solutions on the opposite sides of the membrane. Two steady-state regimes of substrate transformation can exist in this case, i.e., diffusion and kinetic regimes [28]. The 'thermodynamic' regime establishing in this system means the cessation of the reaction proceeding. The presence of the third compartment, i.e., the unstirred layer, creates a possibility of existence of a third steady-state – a 'thermodynamic' regime.

The results obtained show that significant changes in substrate and product concentrations can occur in zones adjacent to the membranes due to enzyme activity. The system described presents a model of cell enzyme functioning when enzymes and substrates are separated by a membrane and substrates are transported to enzymes through the membrane.

It should be noted in conclusion that a large amount of hydrophilic enzymes are immobilized on the surface of biological membranes. For example, the enzymes of fatty acid oxidation are immobilized on the inner membrane of mitochondria [29,30]. The technique developed in this paper can be used for studying the peculiarities of the enzyme activity in the membrane-bound state.

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