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Enzyme Activity and Membrane Potential Interaction in an Artificial Enzyme Membrane

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Summary. The aim of this paper is to study the interaction between an enzyme reaction and membrane potentials or permeabilities. The study was performed with a proteic artificial membrane bearing cross-linked urease. The influence of the charge density in the amphoteric membrane on K^+ and Na^+ ion permeability was studied at different pH's. The urea and inhibitor effects on the enzyme membrane potential were experimentally observed. The potential variation resulting from the substrate introduction in the medium was recorded as a function of time. This potential difference vanishes in the presence of an inhibitor. The potential difference value was studied with different K^+ and Na^+ concentration gradients. The relationship between the potential difference and the substrate concentration is a sigmoid function.

The movement of ions across membranes has been described by numerous models. In particular, membrane charges were taken into account, either fixed as in Teorell's (1953), or mobile as in Rosenberg and Wilbrant's model (1955). The study of membranes considered as ion exchangers was put forward by Teorell, Helfferich and others. Their physico-chemical behavior was analyzed on a computer by Coster, George and Simons (1969) and by Conti and Eisenman (1965*a*, *b*).

The presence of enzyme molecules in the model membrane structure results in new effects and controls exerted on ion movements, which have been scarcely studied. Blumenthal, Caplan and Kedem (1967) used papain sandwiched between two ion exchange sheets for this purpose. In such a case, the enzyme is not actually included in the ion exchanger phase, so that a direct reciprocal effect between ion exchange and reaction could not be obtained in the membrane. The binding of the enzyme in the matrix of the

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membrane itself comes closer to this aim. Several techniques are available, which were recently reviewed by Thomas and Caplan (*in press*). The present work applies the techniques already described by Broun, Thomas, Gellf, Domurado, Berjonneau and Guillon (1973).

A systematic analysis of the factors affecting ion movement across an enzyme membrane necessitates the integration of both fixed charges and ionic species resulting from the enzyme reaction. The effect of the reaction can result in the appearance of ions, disappearance of ions, or transfer of charges from one molecule to another.

The effect of permanent membrane charges can be studied by the movement of phosphate, sodium and potassium across an artificial proteic membrane. By modifying the pH of the solution, which modifies the fixed-charge density of this amphoteric membrane, their effect on the ionic flux can be determined. This was done by using radioactive Na^+ and K^+.

The effect of the co-ions and counter-ions produced by the enzyme reaction on the movement of the above-mentioned ions was studied on a urea-urease system. Urease hydrolyzes urea into the carbonate anion and ammonium cation. The enzyme action results in the appearance of new co-ions and counter-ions inside the membrane, and in a modification of local pH.

This phenomenon was followed by the measurement of potential difference between both membrane compartments. The modification of ion diffusion resulting from the urease reaction in the presence of urea was immediately apparent from potential measurements. Thus, the instantaneous effect of the enzymic reaction on ionic flux across the membrane could be easily visualized. From these observations, some conclusions were derived concerning the possible effects of fixed membrane charges on movement of ions across the membrane.

Materials and Methods

Preparation of Artificial Enzyme Membranes

Active proteic films were produced by the previously described cross-linking method (Thomas, Broun & Selegny, 1972; Broun *et al.*, 1973). A bifunctional agent, glutaraldehyde, and a bulk protein, serumalbumin, were mixed together with one or several enzymes. This solution was spread on a planar glass surface. A complete insolubilization occurred as a result of the cross-linking reaction and active enzyme membranes were obtained. This process gave a homogeneous distribution of the active sites within the membrane. The resulting films were transparent and showed a good mechanical resistance. Mean diffusion coefficients were of the same magnitude as in collodion films (Broun *et al.*, 1973).

Various amounts of enzyme activity per unit volume of membrane (varying from 10 to 80 IU/ml) were obtained by introducing various enzyme concentrations in the initial mixture. The membrane used in this work contained serumalbumin at a final concentration of 40 mg/ml and glutaraldehyde at a concentration of 4 mg/ml. The urease activity (jackbean) introduced in the solution was 80 IU/ml.

Measurement of Enzyme Activity

Urease activity was determined by measuring the rate of ammonium ion formation. The appearance of ammonium ions was determined by using Berthelot's method (1859), or with a cation electrode (Beckman No. 39137). Alternatively, the formation of ammonium carbonate was determined with a pH-stat. When using Berthelot's and pH-stat methods, activity was measured using a 0.15 M urea solution in a 0.05 M sodium phosphate buffer. For the cation electrode measurements, a 0.15 M urea solution in a 0.05 M Tris-HCl buffer was used instead.

V_m values are given for each membrane in the experimental results section.

Experimental Determination of Diffusion Coefficients

Diffusion coefficients were measured in diffusion cells where the membrane separates two compartments containing metabolic solutions. The cells were made of "altuglass" with a diffusion area of 8 cm^2; both compartments had a volume of 90 cm^3. Stirring was performed by bubbling water-saturated air through both compartments.

Fluxes were measured by following the evolution of K^+ and Na^+ concentrations as a function of time. Effective experimental diffusion coefficients were obtained according to Fick's first law:

$$J = \bar{D}^* \frac{\Delta C}{e}$$

where J = instantaneous flux, ΔC = concentration difference across the membrane, e = thickness of the membrane and $\bar{D}^*$ = effective experimental diffusion coefficient.

Before each diffusion experiment, the membrane was equilibrated for 24 hr with the less concentrated ionic solution. Ion concentration was followed by using labeled Na^+ or K^+.

To measure the ion concentrations, a double-labeling method was used; i.e., $^{32}P - ^{24}Na$ and $^{32}P - ^{42}K$ systems.

The total activity of the sample (P + K or Na) was measured first. The half lives of ^{24}Na, ^{42}K and ^{32}P are, respectively, of 15 hr, 12.4 hr and 14.3 days. A second measurement after 5 days gave the phosphorus activity. The activities were never higher than 0.1 mC/liter.

Measurement of Potentials

If there is a salt concentration difference between both sides of an ion exchange membrane, a potential gradient between the solutions occurs without any external electrical stimulation. These potentials were measured using a UVA-type microvoltmeter (AOIP, Paris) with a high impedance for all scales, between two reference saturated KCl electrodes (Radiometer K 401, Copenhagen). The membrane area in the diffusion cells used for these experiments was only 0.5 cm^2 in order to avoid too rapid variations of ionic concentrations during, and between, measurements. The determination of the

effect of urease activity was obtained by using the same membrane both with and without urea and by comparing the resulting potential differences. In these experiments, urea concentration was 0.25 M in both compartments.

During the measurements, a stirring influence on the potential was not observed. In any case the unstirred layers are similar for each measurement.

Results and Discussion

It was previously shown that the binding of urease was obtainable with an enzyme activity yield of 50% (Broun *et al.*, 1973). This yield is the ratio between the actually immobilized enzyme activity and the initially introduced activity.

Binding conferred a better stability against thermodenaturation. The decrease of enzyme activity was less than 1% after 15 days at 4 °C. Hence, the stability of the enzyme membrane activity allowed reproducible physicochemical studies.

Effects of Membrane Charges on Permeability

The fluxes of Na^+ and K^+ and of their ionic counterparts were determined at several pH's. The system was defined by: the membrane thickness, $e = 5 \times 10^{-3}$ cm; the volume of each compartment, $V = 90\ cm^3$; the diffusion area, $S = 8\ cm^2$; the concentration in the first compartment, $C_1 = 10^{-2}$ M (at time zero); the concentration in the second compartment, $C_2 = 10^{-3}$ M (at time zero).

The permeability as a function of pH value inside the bulk solution is given in Fig. 1 for potassium phosphate buffer and sodium phosphate buffer. Effective experimental diffusion coefficients decreased as pH increased. Potassium phosphate diffusion coefficient was slightly higher than sodium phosphate diffusion coefficient. This relative difference increased with pH and became constant for pH's higher than 7.2. As the proteic membrane is amphoteric, the negative charge density increased together with the pH value. The shape of the diffusion coefficient-pH curves can be explained by this charge modification.

The albumin membrane offers amphoteric sites with weakly ionizable groups which give rise to cation exchange properties for pH values higher than 5, and anion exchange properties for pH values lower than 5. For low pH's, phosphate groups play the part of a counter-ion and Na^+ or K^+ of a co-ion. It is the opposite at high pH's. The permeability of this membrane to electrolytes is variable according to the ionization and the fixed-charge density. The increase of Donnan exclusion for pH's rising from 5

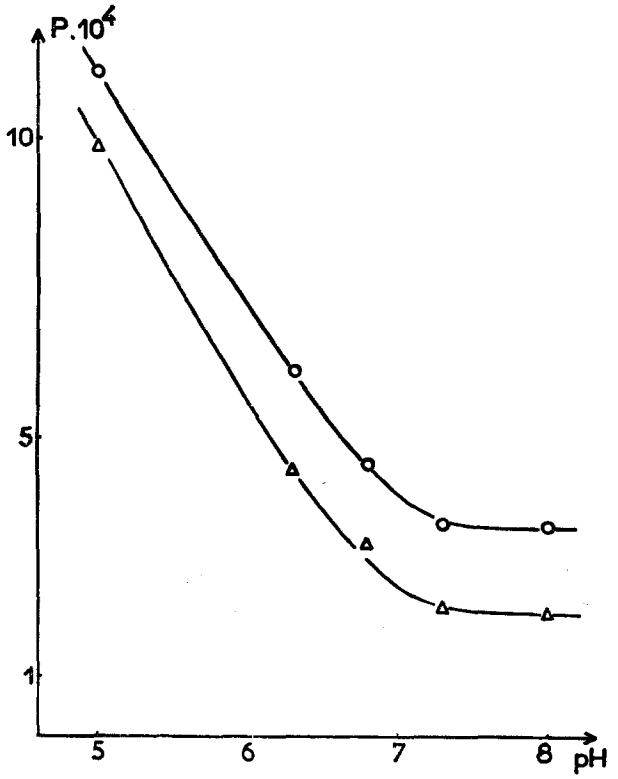


Fig. 1. Artificial protein membrane: Permeability coefficient P or $\bar{D}^*$ ($cm^2\,hr^{-1}$) of K^+ ($-\circ-\circ-$) and Na^+ ($-\triangle-\triangle-$) phosphate as a function of pH

to 8 can explain the permeability decrease (Helfferich, 1962). For the same ionization, the decrease of permeability to Na salts was more significant as compared to K salts, which can be interpreted as the consequence of an exclusion more efficient for Na^+ than for K^+ salts.

Effect of an Enzyme Reaction on the Membrane Potential Difference

Measurements were performed with sodium and potassium phosphate, both with and without urea. Membrane thickness, 5×10^{-3} cm; compartments volume, $V = 40\ cm^3$; diffusion area, $S = 0.5\ cm^2$; concentration in the first compartment, $C_1 = 10^{-2}$ M; concentration in the second compartment varied from $C_2 = 10^{-3}$ M to 10^{-2} M.

Potential measurements were performed at first without urea. Urea was then introduced into both compartments and the potential difference was recorded as a function of time (Fig. 2). Stable potential values were obtained 5 min after introducing urea. The introduction of the substrate into

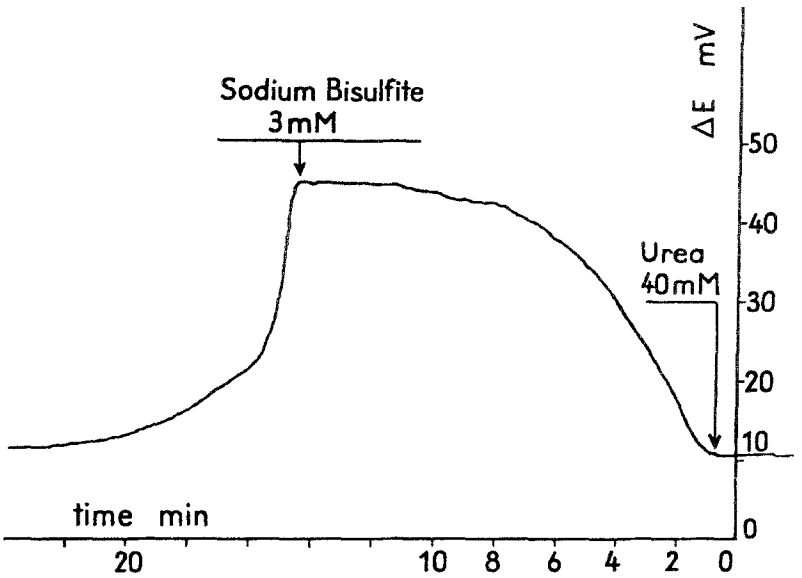


Fig. 2. Artificial urease membrane between two compartments. Membrane potential differences recorded as a function of time, when adding the substrate and an inhibitor into both compartments. NaH_2PO_4 concentrations in the first compartment 10^{-2} M and in the second one 10^{-3} M

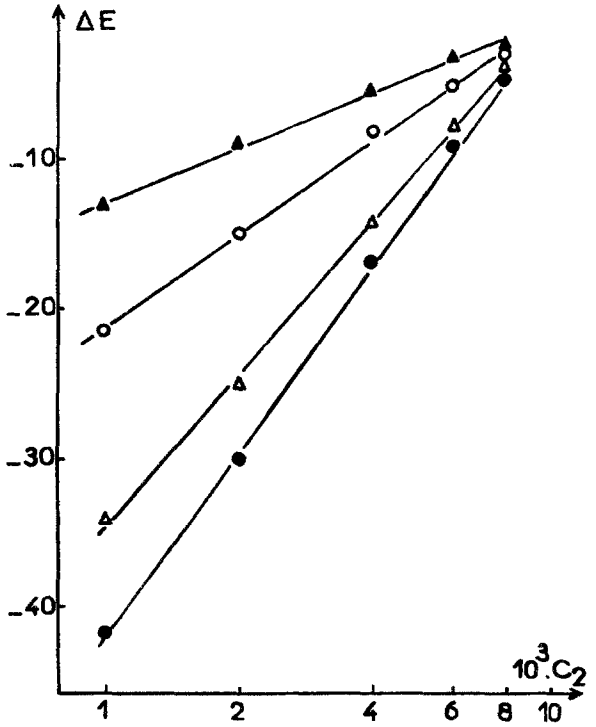


Fig. 3. Proteic membrane without enzyme activity. Membrane potential (ΔE in mV) as a function of salt concentration in compartment 2. The concentration in compartment 1 is constant and equal to 10^{-2} M: NaH_2PO_4 (▲ — ▲); Na_2HPO_4 (△ — △); KH_2PO_4 (○ — ○); K_2HPO_4 (● — ●)

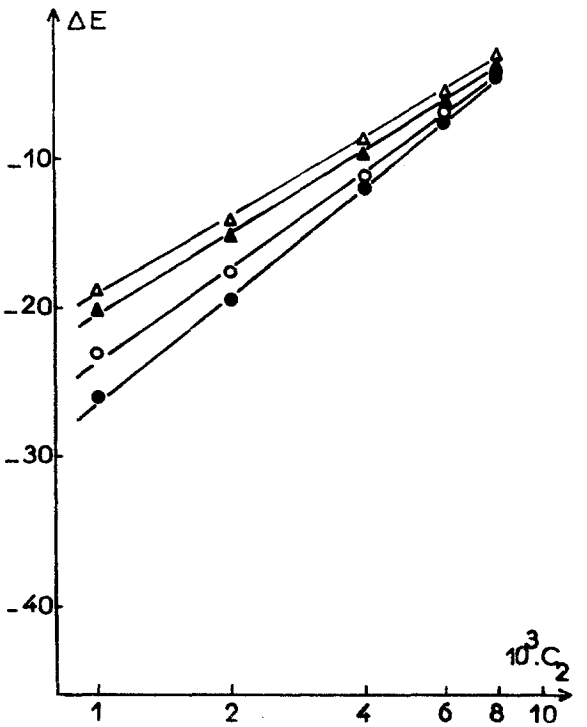


Fig. 4. Proteic membrane with urease activity. Membrane potential (ΔE in mV) as a function of the salt concentration in compartment 2. The concentration in compartment 1 is constant and equal to 10^{-2} M: NaH_2PO_4 (▲ – ▲); Na_2HPO_4 (△ – △); KH_2PO_4 (○ – ○); K_2HPO_4 (● – ●)

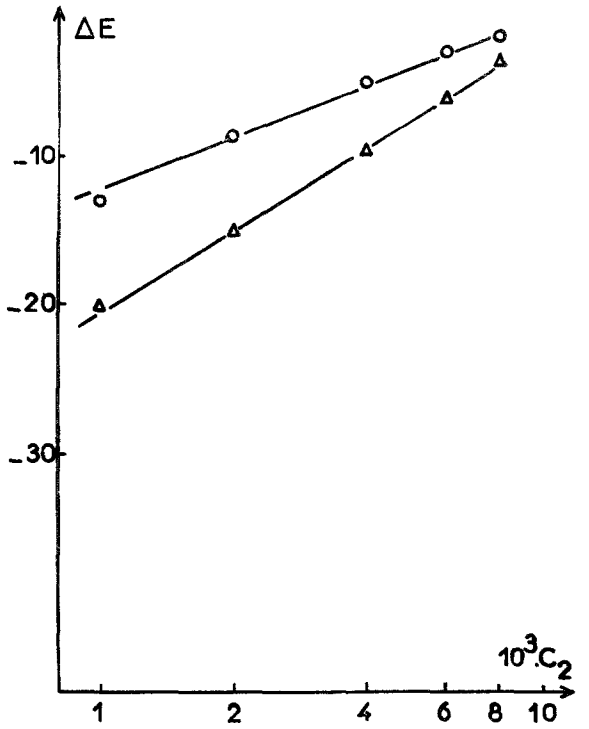
the system modified the membrane potential value, but the potential value of an albumin membrane without enzyme was not modified by introducing urea under the same conditions: By introducing an inhibitor, 3 mM bisulfite, into the active system, the potential difference returned to its initial value (Fig. 2).

The above data can be analyzed by separating the major factors. Two different conditions are to be observed:

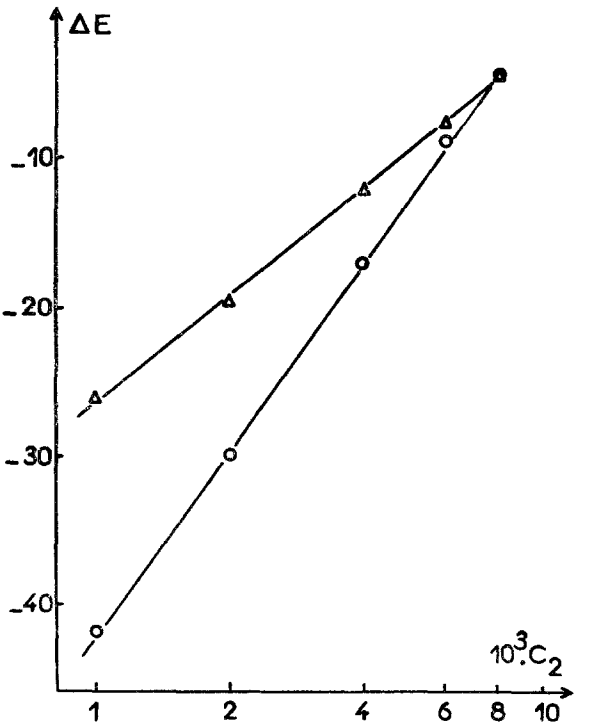
(*i*) The variation of ΔE as a function of the salt concentration gradient for a constant substrate concentration.

(*ii*) The variation of ΔE as a function of variable substrate concentration for a constant salt concentration gradient.

(*i*) Figs. 3 and 4 show the variation of membrane potential as a function of C_2 for an albumin membrane with (Fig. 3) and without (Fig. 4) enzyme activity by using different anions ($H_2PO_4^-$, HPO_4^{2-}) and different cations (Na^+, K^+). It is thus possible to compare ΔE curves for various salts with and without enzyme activity (Fig. 5*a–d*).



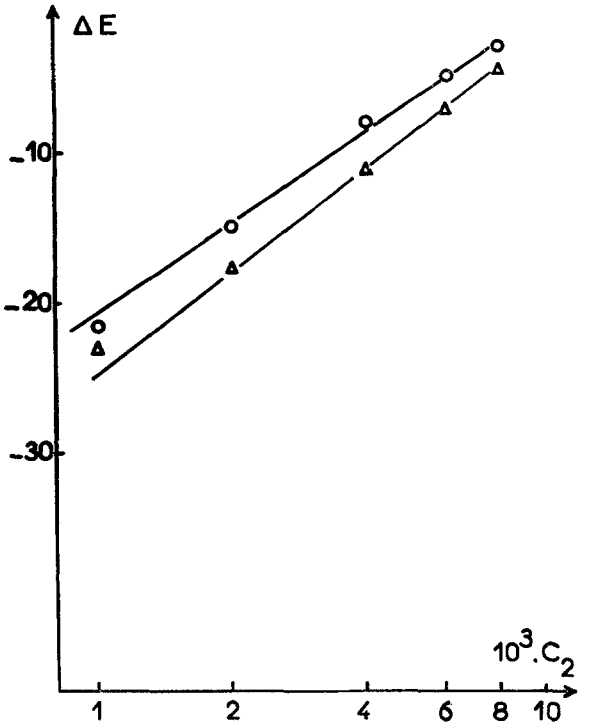
a



b

Fig. 5. Comparison of membrane potentials versus salt concentration gradient with and without enzyme activity: (*a*) NaH_2PO_4 with (△ — △) and without (○ — ○) activity;

c



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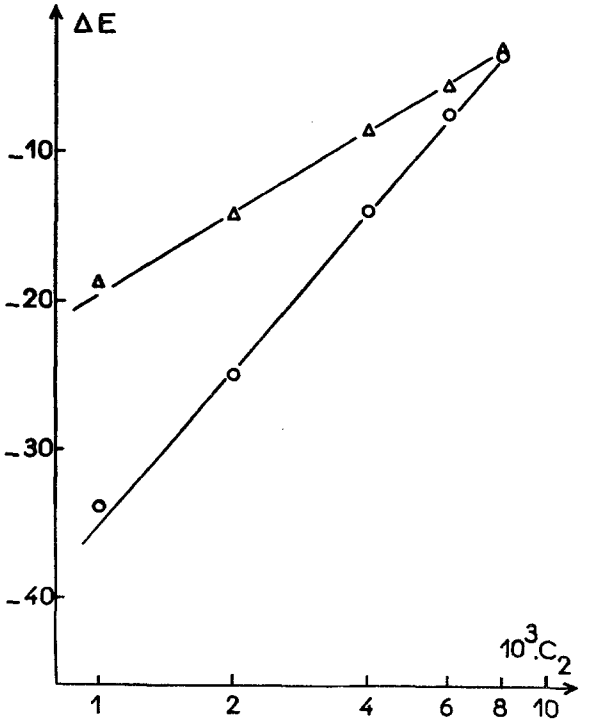

(*b*) Na_2HPO_4 with (△ — △) and without (○ — ○) activity; (*c*) KH_2PO_4 with (△ — △) and without (○ — ○) activity; (*d*) K_2HPO_4 with (△ — △) and without (○ — ○) activity

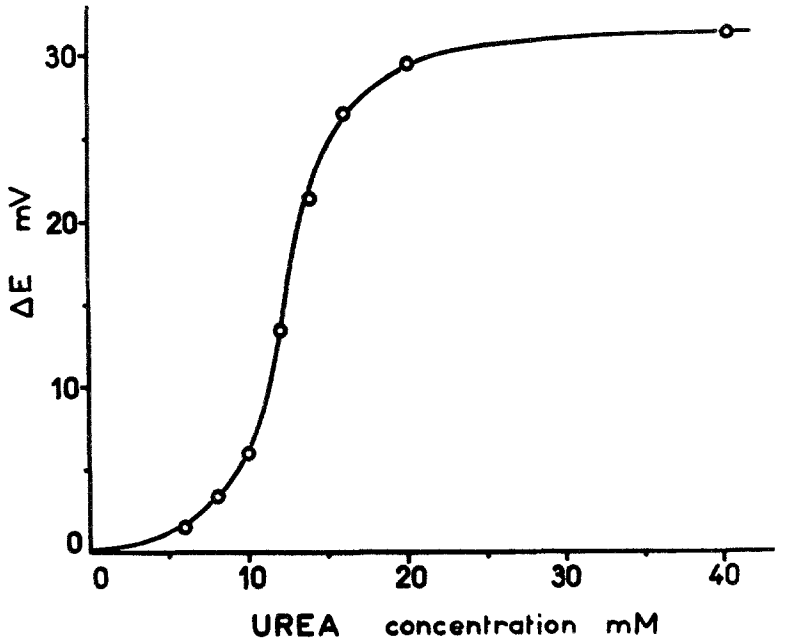

Fig. 6. Artificial urease membrane. Membrane potential difference as a function of substrate concentration in both compartments plotted with a linear scale. NaH_2PO_4 concentrations in the first compartment 10^{-2} M and in the second one 10^{-3} M

These results can be easily interpreted as the effect of urease activity producing ammonium carbonate from urea, which gives rise to a strong increase of the local pH value. With $H_2PO_4^-$ ions the pH both in the bulk solution and in the membrane without urea is around 5, but with urea the pH in the amphoteric membrane increases. The concentration of bound negative charges increases also, so that the transport number of phosphate ion decreases and the potential difference increases. With HPO_4^{2-} ions, the pH value outside is around 8 and the pH variation effect inside the membrane is quite negligible. On the other hand, the salt concentration inside is higher than outside as a result of the concentration profile of the enzyme product inside the membrane. When the salt concentration is higher, the difference between cation and anion transport numbers is smaller and the potential differences are reduced.

When the enzyme acts on urea, the above-described phenomena occur and the local concentrations in the intramembrane medium seem similar for any outside salt. The potential gradient curves are very closed in Fig. 4.

(*ii*) Fig. 6 shows the variation of the potential difference across an enzyme membrane as a function of the substrate concentration in both

compartments under steady-state conditions. The shape of this curve is sigmoidal. This effect is especially sharp in this figure because the substrate concentration is given using a linear scale and not a logarithmic one. At the outside, pH is nearly 5 and the enzyme activity is low. Up to the maximum pH 7.3, there is an autocatalytic effect inside the membrane (Thomas, 1973) due to the enzyme production of OH^-. A cooperative variation of the inner pH as a function of the substrate concentration outside occurs. The same effect is observed for the potential difference.

Conclusion

This paper deals with aspects of the reciprocal interaction between enzyme activity and ion movements in an artificial membrane. The influence of the membrane amphoteric properties on the ion permeabilities was shown. The study of an active artificial membrane gives a strong experimental evidence for a membrane potential modulation by an intramembrane enzyme activity. Due to obvious reasons this study is not possible at all in the classical context of free enzymes in solution. Nevertheless, the study of the reciprocal effects between an enzyme activity and a potential difference, in a well-defined context, can play a relevent part in the explanation of some results dealing with metabolism potential interaction in membrane physiology. In this way, artificial enzyme membranes are useful for studying these problems.

The next step of this work could be the reintroduction, in an artificial membrane, of enzymes playing an important part in the properties of structured biological systems, such as excitable membranes.

As already shown above, the membrane potential versus substrate concentration is a sigmoid function recalling the behavior of some biological systems (Changeux, Thiery, Tung & Kittel, 1967). The nonlinearity of the enzyme reaction coupled with the diffusion constraints could also cause some instabilities or periodic behavior of the membrane potentials and permeabilities (Thomas, *in press*).

References

Berthelot, M. 1859. Rapport de Chimie Appliquée. Paris, p. 254

Blumenthal, R., Caplan, S. R., Kedem, O. 1967. The coupling of an enzymatic reaction to transmembrane flow of electric current in a synthetic "active transport" system. *Biophys. J.* **7**:735

Broun, G., Thomas, D., Gellf, G., Domurado, D., Berjonneau, A. M., Guillon, C. 1973. New methods for binding enzyme molecules into a water-insoluble matrix: Properties after insolubilization. *Biotechnol. Bioeng.* **15**:359

Changeux, J. P., Thiery, J., Tung, Y., Kittel, C. 1967. On the cooperativity of biological membranes. *Proc. Nat. Acad. Sci.* **57**:335

Conti, F., Eisenman, G. 1965*a*. The steady state properties of ion exchange membranes with fixed charges. *Biophys. J.* **5**:247

Conti, F., Eisenman, G. 1965*b*. The steady state properties of ion exchange membranes with mobile sites. *Biophys. J.* **5**:211

Coster, H. G. L., George, E. P., Simons, R. 1969. The electrical characteristics of fixed membranes: Solution of the field equations. *Biophys. J.* **9**:666

Helfferich, F. 1962. Ion Exchange. McGraw-Hill Book Co., Inc., New York

Rosenberg, T., Wilbrant, W. 1955. The kinetics of membrane transports involving chemical reactions. *Exp. Cell Res.* **9**:49

Teorell, T. 1953. Transport processes and electrical phenomena in ionic membranes. *In:* Progress in Biophysics and Biophysical Chemistry. J. A. V. Boter and J. T. Randall, editors. Vol. 3, p. 305. Pergamon Press Inc., New York

Thomas, D. 1974. Functional organization in artificial enzyme membranes. Accomplishments and prospects. *In:* Membranes, Dissipative: Structures and Evolution. I. Prigogine, editor. Wiley Press, New York (*In press*)

Thomas, D., Broun, G., Selegny, E. 1972. Monoenzymatic model membranes, diffusion reaction kinetics and phenomena. *Biochimie* **54**:229

Thomas, D., Caplan, S. R. 1974. Artificial enzyme membrane. *In:* Membrane Separation Processes. P. Meares, editor. Elsevier, Amsterdam (*In press*)