MAGNETIC ENZYME MEMBRANES AS ACTIVE ELEMENTS OF ELECTROCHEMICAL SENSORS. SPECIFIC AMINO ACID ENZYME ELECTRODES*

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1. Introduction

Numerous reviews have been devoted to the subject of immobilized enzymes [1–3]. One of the applications of immobilized enzymes is its introduction as the active element of an electrochemical probe or sensor. Such enzyme electrodes possess the important enzyme characteristics of specificity and sensitivity, and are generally adaptable for continuous measurements. Electrochemical monitoring by means of enzyme electrodes can be carried out on whole blood or other biological media, thus eliminating preparation of the sample.

The use of an enzyme as a functional element of an electrochemical device was first reported by Clark and Lyons [4]. The earliest electrode incorporating and immobilized enzyme, however, was described by Updike and Hicks [5]. The enzyme electrodes recently reviewed [3–6] are generally done with enzyme entrapped into gel films. With the inclusion method strong drawbacks are observed : the enzyme activity is not stabilized and the mechanical properties of the active film are poor. The present paper deals with the use of a magnetic enzyme membrane bearing decarboxylases as the active part of specific sensors for tyrosine, phenylalanine, lysine measurement. Enzyme molecules are cross-linked together with an inert protein, such as albumin, by a bifunctional agent, such as glutaraldehyde, and magnetic ferrite particles are entrapped in the membrane structure. The membrane mechanical properties are similar to those of cellophane and the enzyme activity is stabilized for weeks. Robinson et al. [7], and Gellf and Boudrant [8] described immobilization of enzymes into magnetic particles but the present paper is the first item dealing with magnetic enzyme membranes. The method allows a direct fastening of the film on a pCO_2 electrode bearing a cylinder magnet, without O-ring. The active decarboxylase membrane is situated on the external surface of the gas permeable membrane When the electrode is in contact with solutions containing the appropriate amino acid, the local concentration of CO_2 generated within the membrane is monitored.

A well defined relationship exits between local pCO_2 level and external amino acid concentration. As far as selectivity is concerned, the gas electrodes are undoubtedly far superior to ion electrodes. Hence the described electrodes are solely dependent on the specificity of the enzyme system. It is noteworthy that for many amino acids the decarboxylases are far more specific than the deaminase or the oxidases. Guilbaut and Shu [9] and our group [10] have reported preliminary results dealing with amino acid electrodes based on the use of CO_2 sensors.

2. Materials and methods

2.1. *Membrane production*

The procedure for the manufacture of a membrane bearing lysine decarboxylase is given here in the following example: 5 mg of lysine decarboxylase (Sigma from *B. cadaveris*) were added to 2.5 ml of a solution containing 6% plasma albumin and 0.7% glutaraldehyde

* Part I.
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in a 0.02 M phosphate buffer, pH = 6.8, 3 g of magnetic iron oxide particles (100 μm in a diameter, purchased from La Radiotechnique, Evreux, France) were added as an inert charge before polymerisation occurred. The suspension was spread perfectly flat on a glass plate in order to obtain a membrane of homogeneous thickness. Cross-linking was effected at room temperature for 2 h. The plate with the magnetic protein layer was dipped in distilled water bath. The film easily separated from the glass plate, it was rinsed until the water no longer absorbed at 280 nm. No enzyme activity and no magnetic particles were observed in the rinse water.

2.2. *Enzyme activity measurements*

The decarboxylase kinetics were measured according to a method previously described [10] in a closed thermostated cell (Metrohm EA-8 76 1) with a pCO_2 electrode (E 5036 Radiometer) connected with a Radiometer 71 MK 2 pH-meter.

2.3. *Fixation of the magnetic enzyme membrane on the pCO_2 electrode*

The magnetic enzyme membrane is maintained by the magnetic field on a pCO_2 electrode (E 5036 Radiometer) modified according to the scheme given fig.1. The cylinder magnet added to the electrode was produced by 'La Radiotechnique'. No leak was observed between the magnet and the magnetic membrane.

2.4. *Calibration of the electrode and measurements*

The electrode is calibrated by bubbling two reference gases (0.5 and 1% CO_2 – Air Liquide). The measurements are preformed with samples bearing amino acids but no CO_2.

3. Theoretical section

Let us consider a coating (thickness e) between a bulk solution and the electrode surface. The reference axis is perpendicular to the membrane surface and the continuity equations for S and P inside the active membrane can be written as:

$$\frac{\partial S}{\partial t} = D_s \frac{\partial^2 S}{\partial x^2} - V_M \frac{S}{S + K_M} \qquad (1)$$

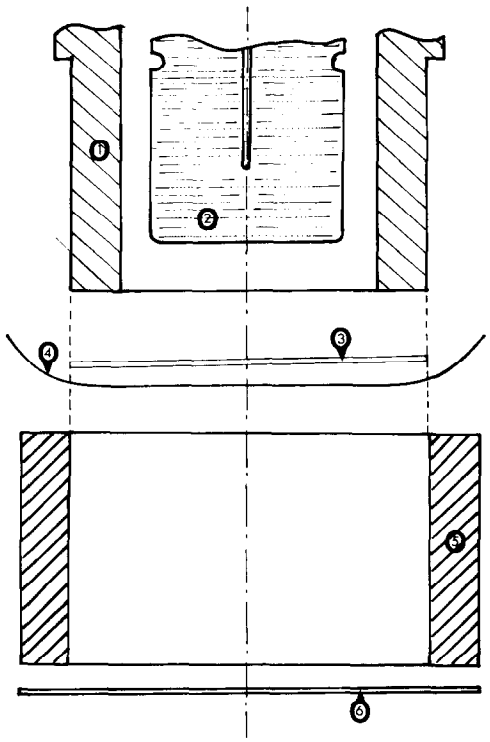

Fig.1. Scheme of a magnetic enzyme electrode. (1) Electrode jacket, (2) pH glass electrode, (3) porous sheet, (4) gas permeable membrane, (5) cylinder magnet, (6) magnetic enzyme membrane.

$$\frac{\partial P}{\partial t} = D_p \frac{\partial^2 P}{\partial x^2} + V_M \frac{S}{S + K_M} \qquad (2)$$

with S and P substrate and product concentrations respectively, V_M maximum enzyme activity per unit volume of membrane, K_M Michaelis constant and D_s, D_p diffusion coefficients.

By using dimensionless parameters, with the thickness and K_M as space and concentration units, under stationnary state conditions, (1), (2), can be written as:

$$\frac{\partial^2 s}{\partial x^2} = \sigma \frac{s}{1 + s} \qquad (3)$$

$$\frac{\partial^2 p}{\partial x^2} = \sigma \frac{D_s}{D_p} \frac{s}{1 + s} \qquad (4)$$

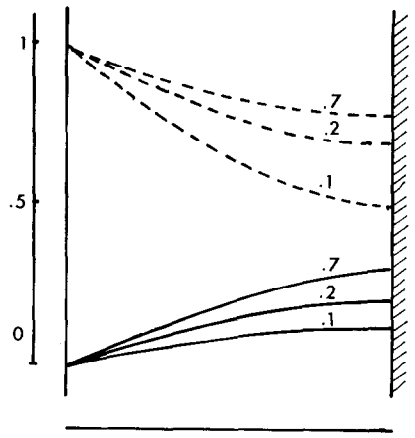

Fig.2. Computer calculation of the concentration profile evolution for the substrate (- - - -) and the product (——) within an active coating of an enzyme electrode. The parameter values used for calculation are: $V_M = 5 \times 10^{-5}$ mol cm^{-3} s^{-1}, $K_M = 2 \times 10^{-5}$ mol cm^{-3}, e = 5×10^{-3} cm, $D_p = D_s = 1.2 \times 10^{-5}$ cm^2 s^{-1}, time unit $\frac{e^2}{D_s}$

The only important parameter is a dimensionless one $\sigma = (\frac{V_M}{K_M} \frac{e^2}{D})$ similar to the Thiele modulus in Chemical Engineering. From (3) and (4):

$$D_s S + D_p P = D_s S_o + D_p P_o \qquad (5)$$

S_o and P_o are the concentration in the bulk solution. The definition of the system is completed by the boundary conditions [11].

The concentrations S_o and P_o are constant in the bulk solution, and along the electrode surface the fluxes are null $D_s (\frac{dS}{dx}) = 0$

These equations were solved numerically by computer according to previously described methods [11].

Examples of calculated concentration profiles of substrate and product inside the active coating are given in fig.2. The important point for applications is the relationship between the local product concentration measured by the sensor and the substrate concentration in the bulk solution. Calculated product concentrations along the electrode surface as a function of the substrate concentrations in the bulk solution are given (fig.3), for several values of σ parameter.

4. Experimental results

The activity yield after immobilization is 28%, 36% and 10% with lysine, tyrosine and phenylalanine decarboxylases respectively. The specificity after immobilization was checked. For example, in the case of lysine

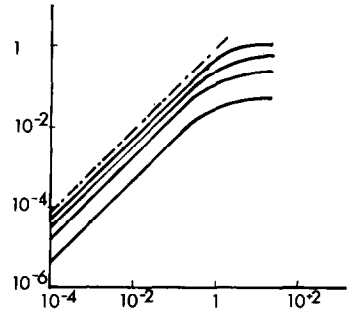

Fig.3. Calculated product concentration along the electrode as a function of the substrate concentration in the bulk solution. Results are given on a logarithmic scale.

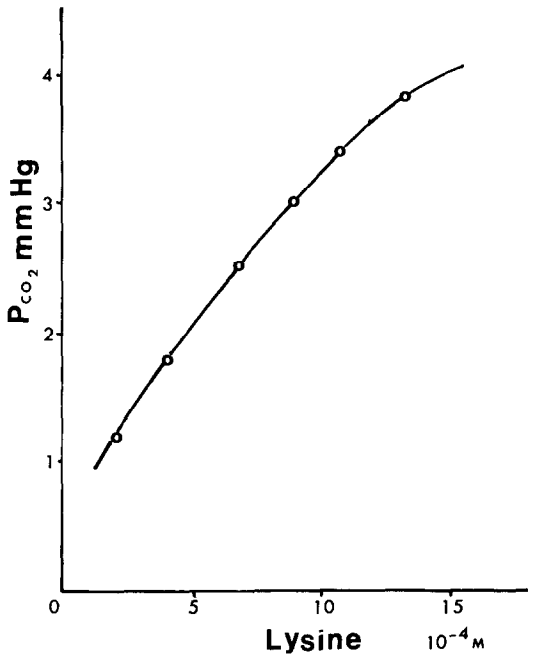

Fig.4. Calibration curve of a lysine decarboxylase electrode. Local pCO_2 measured as a function of lysine concentration. Measurements were done under stationary state conditions.

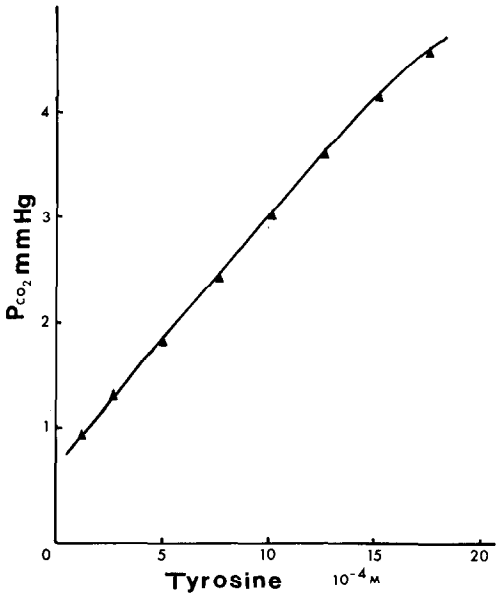

Fig.5. Calibration curve of a tyrosine decarboxylase electrode. Local pCO_2 measured as a function of tyrosine concentration. Measurements were done under stationary state conditions.

decarboxylase the effect of leucine, phenylalanine, tyrosine, arginine, methionine, histidine and cysteine is quite negligible.

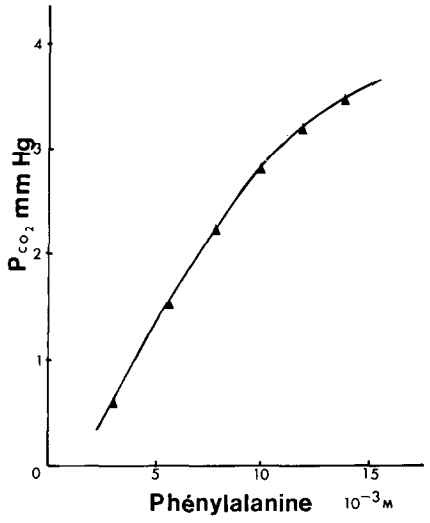

Fig.6. Calibration curve of a phenylalanine decarboxylase electrode-local pCO_2 measured as a function of phenylalanine concentration. Measurements were done under stationary state conditions.

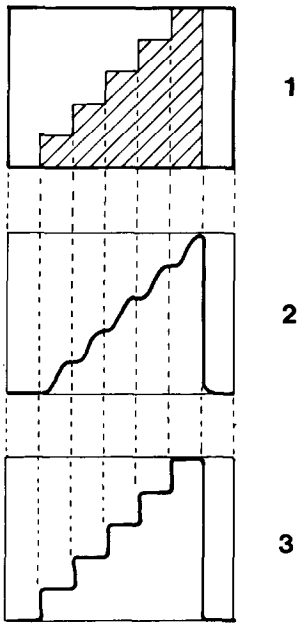

Fig.7. Lysine concentration generated (1) by the 'Ultrograd' pump and measured (2–3) with the enzyme electrode as a function of time. The solution was continuously flowing along the electrode. The curves were generated within one hour (2) and four hours respectively (3). The full scale deals with a concentration of lysine from 0 to 1 mM.

At 4°C, after ten days the proportion of the native and immobilized lysine decarboxylase activity is 32% and 100% respectively. At 22°C the relative activities are 67.5% and 100% after three days. Similar results were obtained with immobilized tyrosine and phenylalanine decarboxylase.

The lysine electrode proviously described was used in a buffer solution 0.02 M, pH 5.8. The local pCO_2 measured as a function of the lysine concentration is given in fig.4. There is a linear relation between measured pCO_2 and lysine concentration within a range from 10^{-4} to 1.5×10^{-3} M. No variation of the bulk solution pCO_2 was observed during the experiments. For series of measurements a reproductibility within 0.5% was observed. New series need a new calibration of the electrode.

Results obtained with tyrosine and phenylalanine electrodes are given in figs.5 and 6 respectively. Linearity was observed with the tyrosine between 2.5×10^{-4} M and 1.5×10^{-3} M and between 2.5 and 1.5×10^{-2} M with phenylalanine.

One of the interests of enzyme electrodes is in continuous measurements allowing a direct recording of an amino acid concentration in a biological fluid. The capacity of the electrode in this field was checked by generating a known function of time of lysine concentration with a pump (L. K. B. Ultrograd) in a solution flowing along the enzyme electrode. The signal given by the electrode and the known function generated are given in fig.7.

5. Conclusion

The basic principle of the described magnetic enzyme electrodes is a kinetic accumulation of CO_2 at the active layer electrode interface. The local pCO_2 level is linked to three simultaneous phenomena : substrate diffusion in, enzyme reaction CO_2 diffusion out. After a transient state there is a stationary state between the quantity of CO_2 produced by the enzyme reaction and the CO_2 diffusing from the active membrane to the bulk solution.

Continuous determination of free amino acids in biological media is useful in biological processing, fermentation, medicine, pharmaceutical industries and biological research.

No methods are presently available for any specific continuous measurement of lysine which is of nutritional importance in protein industrial syntheses; of phenylalanine and tyrosine which have to be monitored in several inborn diseases (phenylketonuria being the most important of them); of arginine and histidine which play a still imperfectly understood part in neurochemistry. The use of decarboxylase bearing membranes as sensors in such measurements could offer several novel advantages: (a) a simple device made of a currently manufactured electrode slightly modified by the use of an enzyme membrane; (b) The absence of any enzymic consumption due to the immobilization and the negligible consumption of substrate during the measurements; (c) The sensitivity which can be sharpened by a systematic study of the membrane parameters; (d) the continuous response of the electrode as long as it is in contact with the substrate solution; (e) the further feasibility as a miniature sensor.

The magnetic device introduced allows obviously a convenient use of the enzyme electrode, the active part can be removed and replaced without disturbance for the pCO_2 electrode itself. The enzyme electrodes are not only useful at the applied point of view but also at the fundamental point of view by allowing a direct measurement of an intra membrane concentration. The influence of simple structures on enzyme kinetics was studied with enzyme electrodes by our group, in the case of memory and oscillations obtained with enzyme systems [12,13].

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