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ELECTROCHEMICAL STUDY OF REACTIONS AT INTERFACES OF GLUCOSE OXIDASE COLLAGEN MEMBRANES

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Key words: Electrochemistry; Interface; Glucose oxidase; Collagen membrane

Summary

The operational behaviour of enzyme collagen membranes with surface-bound glucose oxidase has been studied by simultaneously recording the current outputs of two platinum anodes: whereas the first one was close to the enzymatic membrane, the second was placed into the bulk solution. Steady-state responses of both electrodes were measured when either glucose or hydrogen peroxide were added to the stirred buffer solution. They were used to determine the hydrogen peroxide fluxes, v_1 (towards the first electrode) and v_2 (towards the bulk phase). The glucose concentration and temperature dependence of v_1 and v_2 have been studied and the importance of diffusional limitations on the overall reaction rates were determined. Comparison of freely stirred and mounted enzymatic membrane enabled us to determine an equivalent working area at high glucose concentration.

Introduction

Collagen membranes having undergone an acyl-azide activation process are specially suitable for enzyme coupling [1] and the enzymic membranes obtained can be used either in enzymatic catalysis studies [2,3] or in analytical devices [4]. As shown with a large variety of enzyme-support conjugates, diffusional limitations appear to be one of the main results of immobilization

Abbreviations: ABTS, 2,2′-azino-di-(3-ethyl-benzthiazoline)sulfonate; E_1, electrode close to membrane; E_2, electrode in bulk solution.

[5,6]. Previously reported data obtained with collagen-bound aspartate aminotransferase have shown that kinetics can be modified after coupling: the enzyme exhibits a greater affinity for glutamate but a lower affinity for oxaloacetate [2] and the modified behaviour could be explained by differential diffusion limitations for the two substrates [7]. In this case, the measurements were performed with enzymic membranes freely immersed in the reaction vessel under constant stirring. With a more sophisticated approach, using a rotating membrane reactor and sorbitol dehydrogenase [8] the direct visualization of diffusional effects on the enzyme behaviour could be obtained. This paper presents a more complex situation where a glucose oxidase collagen membrane is pressed on a platinum anode. In our case, enzyme molecules are not randomly distributed inside the membrane but form two distinct layers [9]. One of these layers is in close contact with the platinum anode whereas the other is exposed to the stirred substrate solution. Hydrogen peroxide, one of the reaction products of the reduction of oxygen with glucose in the presence of glucose oxidase can be amperometrically detected in the immediate vicinity of the inner enzyme layer, thus providing a measurement of the membrane activity under these conditions. On the other hand, hydrogen peroxide diffusing towards the bulk solution can also be monitored amperometrically with another sensor, thus providing a second measurement of the membrane activity. This paper presents the relation between these two simultaneous monitorings of the same enzyme activity and their dependence on glucose concentration and temperature, taking into account diffusional effects.

Materials and Methods

1. Solutions and reagents

Insoluble films of highly polymerized reconstituted collagen, F70 type, were a gift of the Centre Technique du Cuir, Lyon, France. They are 20 cm wide and their thickness is about 0.1 mm in a dry state and 0.3–0.5 mm when swollen. Lyophilized β-D-glucose oxidase of fungal origin (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4, grade I, 210 U/mg), peroxidase from horseradish (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7, grade I, 250 U/mg), bovine liver catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6, 65 000 U/mg) and 2,2′-azino-di-(3-ethyl-benzthiazoline)sulfonate (ABTS) were supplied by Boehringer Mannheim.

The stock solution of 0.1 M glucose was allowed to mutarotate at room temperature at least 3 h before use and stored at 4°C. The stock solution of 0.1 M H_2O_2 was frequently assayed by titration with 0.1 N $Ce(SO_4)_2$ using ferrous *o*-phenanthroline as indicator.

2. Enzyme-collagen membranes

(a) Preparation. The previously described acyl-azide procedure for activation of collagen followed by coupling of β-D-glucose oxidase was used [1,4]. The available carboxyl groups on collagen were first esterified by immersion of the crude membranes into a methanol/0.2 M hydrochloric acid solution for at least 72 h at room temperature; then these membranes were treated overnight with 1% aqueous hydrazine solution and for 3 min in a 0.5 M sodium nitrite/0.3 M

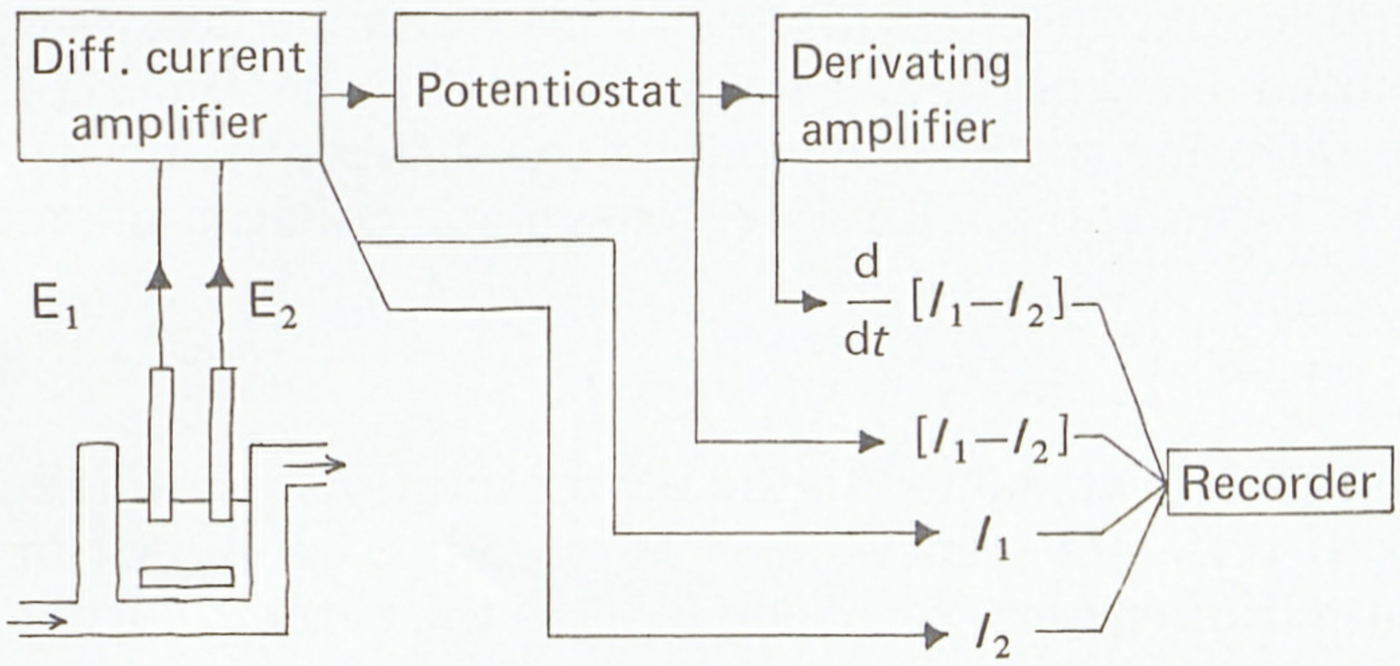


Fig. 1. Block diagram of electrodes and instruments. Potentiostat, constant voltage circuit.

hydrochloric acid mixture at 0—4°C prior to coupling. Thorough washings were performed at each step and at the end of the process. The enzyme coupling was obtained by 2—3 h immersion of activated membranes (2.5 × 1.5 cm) in 1.5 ml of 0.2 M borate buffer or 0.05 M glycine-NaOH buffer pH 9, containing 2.5 mg of β-D-glucose oxidase. After washing in 1 M KCl solution for 15—30 min, the enzymatic membranes were stored in 50 mM acetate buffer pH 4.5 at 0—4°C.

(b) Measurement of activity. The enzymatic membranes were dipped into 9 ml 0.2 M acetate buffer (pH 5.6) containing 0.1 M glucose at 30 ± 0.1°C under constant magnetic stirring. 0.2 ml of the reaction mixture were withdrawn every minute for 4 min and the hydrogen peroxide corresponding to the glucose oxidation, determined by adding 0.8 ml of the peroxidase-ABTS reagent and measurement of the absorbance at 420 nm after 30 min [10].

3. Instrumentation and glucose sensor

The glucose sensor consisted of two electrodes; both electrodes contained a platinum disk, an Ag/AgCl reference and a collagen membrane maintained in close contact by a screwed cap [4]. The electrodes E_1 and E_2 were filled with 0.2 M acetate buffer, 0.1 M KCl, pH 5.6 and mounted with a β-D-glucose oxidase collagen membrane and a non-enzymatic one, respectively. Previously described electronics were adapted to the present study (Fig. 1).

Prior to an experiment, electrodes E_1 and E_2 were allowed to equilibrate in 20 ml 50 mM acetate buffer (pH 5.6) for 15—30 min after stepping the potential of the working electrodes to +650 mV vs Ag/AgCl references. Unless otherwise stated, the temperature was carefully controlled at 30.0 ± 0.1°C using a Colora cryothermostat WK 5 DS.

Results

Glucose is oxidized in the presence of glucose oxidase according to the reaction:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

When associating a glucose oxidase collagen membrane in close contact with the platinum anode of electrode E_1, it is possible to detect amperometrically

H_2O_2, a product of the reaction, and thus to study the kinetics and the modified behaviour of the immobilized enzyme under these conditions. In order to detect hydrogen peroxide diffusing in the bulk phase, the differential device includes a second electrode E_2 mounted with a non-enzymatic collagen membrane as seen in Fig. 1.

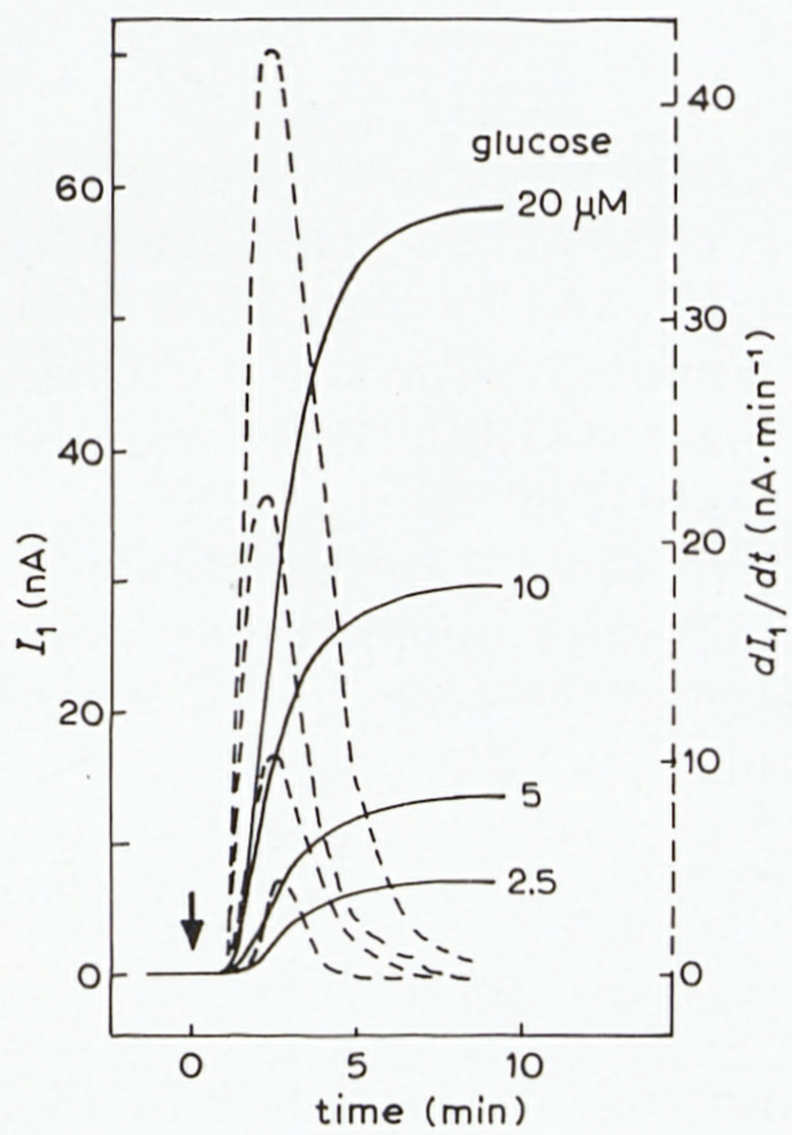
1. Glucose sensor responses

When samples containing glucose are added to the buffer solution into which both electrodes are dipped, different signals may be recorded as shown in Fig. 1: I_1 is the current output of enzyme electrode E_1, I_2 is the current output of the non-enzymatic electrode E_2.

As shown in Fig. 2, addition of glucose standards increases I_1 which reaches a steady-state value after 3—5 min; this is not an endpoint response since glucose is not completely converted within the reaction mixture, the very near vicinity of the membrane being only partially depleted. The inflexion of the I_1 vs time curve corresponding to the peak of its derivative, i.e. the dynamic response, is obtained after 30—90 s. When this device is used as a glucose sensor, I_2 compensates for electrochemical interferences such as ascorbate, uric acid and tyrosine: (I_1-I_2) and its derivative $d(I_1-I_2)/dt$ allow an accurate determination of glucose in the range $1 \cdot 10^{-7}$—$2 \cdot 10^{-3}$ M [4].

2. Hydrogen peroxide fluxes: electrochemical determination

Unlike many other enzymic membranes, enzyme molecules are not randomly distributed inside the collage matrix but form active layers, on both faces [9].



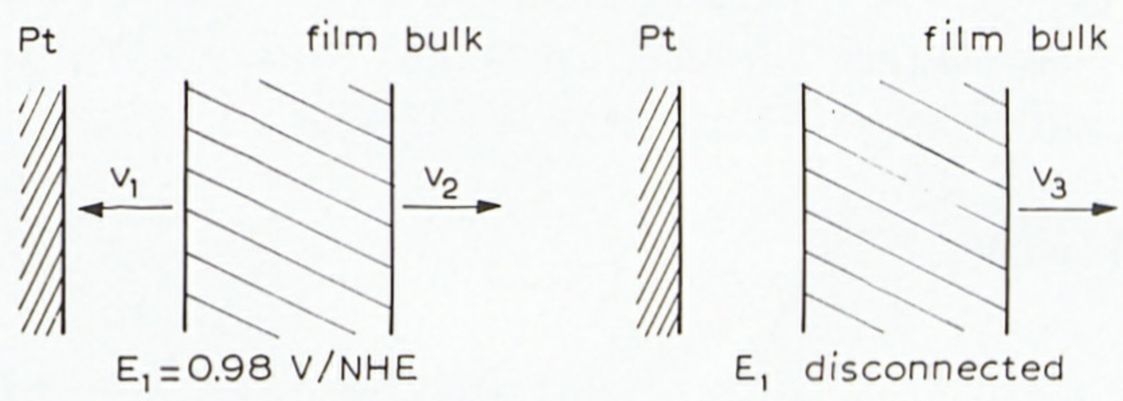


Fig. 2. Response of electrode E_1 to different glucose pulses. The dynamic responses are indicated by the dashed lines.

Fig. 3. Scheme of hydrogen peroxide fluxes on electrode E_1 in contact with glucose solution. Platinum (Pt) disk anodically polarized (left) or disconnected (right). Film, glucose oxidase collagen membrane; bulk, bulk glucose solution.

As the collagen membranes are permeable to small molecules, glucose may be oxidized either on the outer or on the inner face. In the first case, hydrogen peroxide crosses the membrane before reaching the platinum anode. In the latter, hydrogen peroxide is directly generated at the membrane-electrode interface. Two situations occur depending on the electrode E_1 potential (Fig. 3): when anodically polarized, part of hydrogen peroxide flux, v_1, is oxidized on platinum, the rest, v_2, diffusing towards the bulk solution; when E_1 is disconnected, v_3 is the flux towards the bulk solution.

In a first set of experiments, where glucose concentration and temperature were kept constant, the three fluxes v_1, v_2, v_3 have been determined (Fig. 4). Initially both electrodes are dipped in the buffer solution and anodically polarized. An H_2O_2 pulse yields steady-state currents on E_1 and E_2 proportional to the H_2O_2 concentration:

$$I_1 = I_2 = k\ [H_2O_2]$$

Then glucose is added, I_1 increases and reaches a steady-state value proportional to v_1 expressed in mol per min

$$v_1 = \frac{60 I_1}{2F}$$

where I_1 is expressed in ampere, F is the Faraday constant and 2 the number of electrons in the anodic oxidation of hydrogen peroxide

$$H_2O_2 \rightarrow O_2 + 2\ H^+ + 2e^-$$

Simultaneously, I_2 increases showing that the hydrogen peroxide is building up

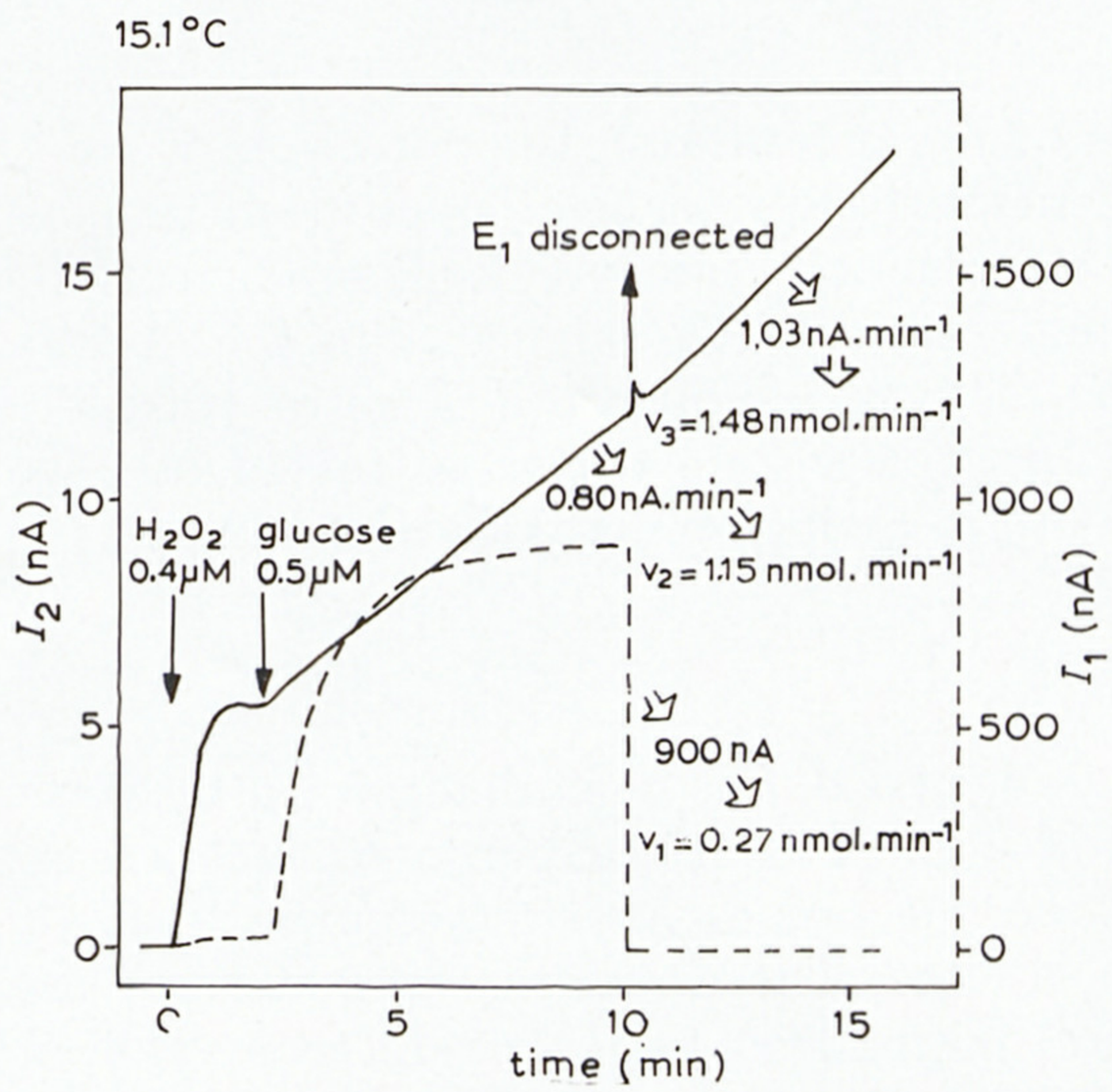


Fig. 4. Determination of hydrogen peroxide fluxes. Successive addition of 50 μl 0.16 mM hydrogen peroxide and 100 μl 0.1 M glucose solution into 20 ml acetate buffer. Electrode E_1, initially anodically polarized as electrode E_2, is disconnected at the end of the experiment. Note the different ordinates used for each electrode.

in the bulk solution. v_2 expressed in mol per min is given by

$$v_2 = \frac{dI_2}{dt} \cdot V \cdot \frac{1}{k}$$

where dI_2/dt is in A/min and V is the volume of the reaction mixture. When E_1 is disconnected, an increase of the slope of I_2 vs. time curve is observed, related to a faster building up of hydrogen peroxide in the soluton: v_3 is given by the same expression as v_2. Fig. 4 presents a typical experiment performed at 30°C in 0.5 mM glucose. Values corresponding to the above described steps are indicated on the curves, k being equal to 14 mA · M^{-1}. Further experiments were performed with various glucose concentrations and temperatures. The overall reaction rate of immobilized glucode oxidase, which takes into account the intrinsic enzymatic activity and diffusional limitations in the enzyme microenvironment, has been determined on both electrodes under these conditions.

3. Glucose concentration dependence

When plotting the values of v_1 and v_2 obtained for different concentrations of glucose in the range 100 nM—10 mM (Fig. 5), a Michaelis-Menten like-curve is obtained but the double reciprocal plot $v^{-1} = f[S]^{-1}$ does not show a straight line suggesting the presence of diffusional limitations [11]. However the maximum values of v_1 and v_2 fluxes for high concentrations of glucose can be tentatively determined as shown in Fig. 5B. As the curves obtained under such conditions were not linear, V_1 and V_2 were estimated by the ordinate of the intercept of their tangents for the highest concentrations of glucose. Returning to the direct plot of Fig. 5A, the glucose concentrations $S_{0.5}$ leading to the half value of V_1 and V_2 were estimated to be 3.7 and 1.8 mM, respectively.

4. Temperature dependence

We have compared the influence of temperature on the detection of H_2O_2 either exogenous or enzymatically generated in presence of glucose and glucose oxidase. The addition of H_2O_2 into the reaction vessel yields an anodic current in the absence of glucose which reaches a steady-state value withing 0.5–1 min. This response is proportional to H_2O_2 concentration in the range of 100 nM—10 mM. As previously mentionned for steady-state responses to glucose, we have expressed the steady-state response to hydrogen peroxide as a flux v_0:

$$v_0 = 60 \frac{I_2}{2F}$$

The temperature dependence of v_0, v_1 and v_2 has been determined between 15 and 40°C. As seen on Fig. 6A and B for a typical experiment, an increase of temperature has a smaller effect on the non-enzymatic response to H_2O_2 than on the enzymatic response to glucose. The direct representation of the H_2O_2 fluxes for a given temperature, normalized to their reference value at 15°C, exhibits a quasi linear relation to temperature (Fig. 6A). The slopes for temperatures lower than 30°C are approximatively equal to 0.04, 0.10 and 0.09 per °C for v_0, v_1 and v_2 respectively. When plotting the experimental data of Fig. 6A according to the Arrhenius representation, a non-linear pattern is

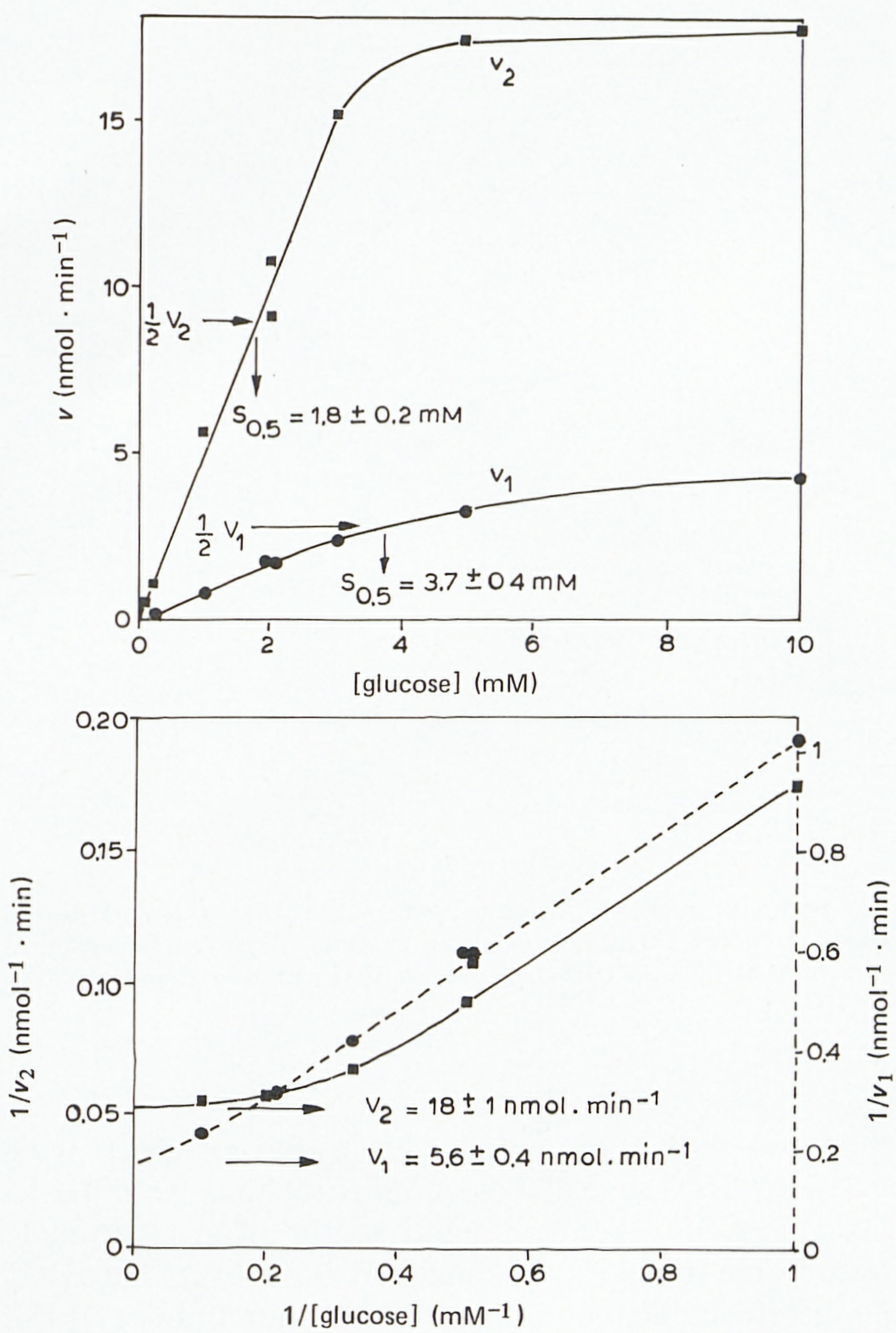


Fig. 5. Glucose concentration dependence of hydrogen peroxide fluxes v_1 (●) and v_2 (■). A (top), direct representation. B (bottom), Lineweaver-Burk plots. Note the different ordinates used for each electrode.

obtained (Fig. 6B). This is especially noticeable for v_1 and v_2. As previously mentioned for Lineweaver-Burk representations, such non-linear behaviour suggests diffusional limitations of enzymatic reactions. In this case, two activation energies were estimated around 15 and 40°C. At these two temperatures, the values for E_a were 13 and 12 kcal · mol^{-1} and 3.7 and 5.5 kcal · mol^{-1} for v_1 and v_2, respectively. The comparison of these figures illustrates that mass transfer phenomena play a more prominent role in the overall reaction rate at higher temperatures.

5. Hydrogen peroxide balance

Comparison of v_1, v_2 and v_3. In order to study the balance of the different steady-state H_2O_2 fluxes occurring at the enzyme membrane level, on electrode

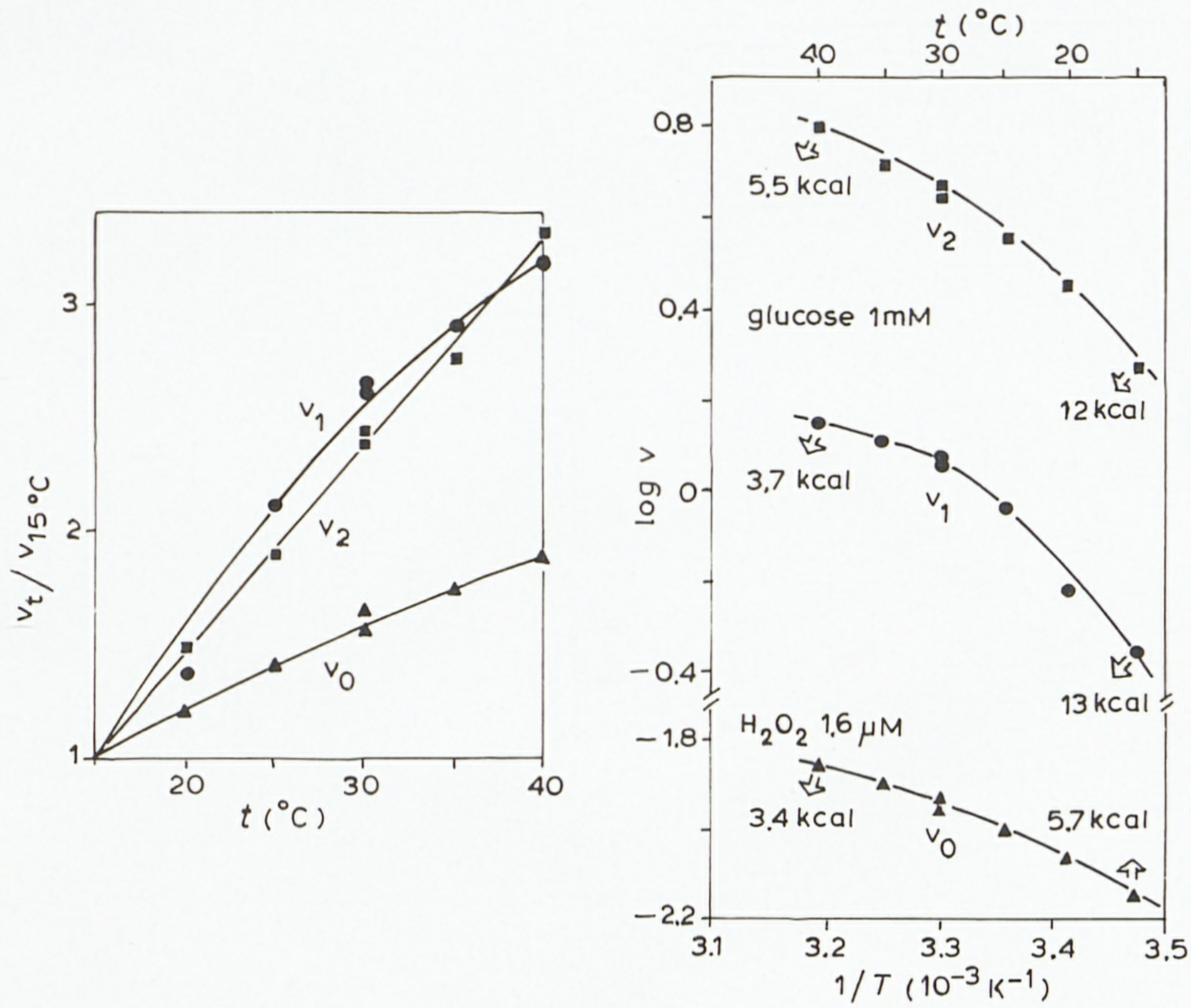


Fig. 6. Temperature dependence of hydrogen peroxide fluxes v_0 (▲), v_1 (●) and v_2 (■). Hydrogen peroxide and glucose solutions were 1.6 μM and 1.0 mM, respectively. A (left), direct representation of fluxes normalized to their respective value at 15°C. B (right), Arrhenius plots with corresponding activation energies values around 15 and 40°C.

E_1, experiments similar to those shown on Fig. 4 have been performed with temperatures ranging from 15 to 25°C and glucose concentrations from 0.1 to 1 mM (Fig. 7). As the ratios $(v_1 + v_2)/v_3$ were not significantly different from 1, in these glucose concentration and temperature ranges, it is obvious that H_2O_2 does not accumulate at the interface between the enzymatic membrane and the platinum disk. Furthermore there is no evidence of any effect of polarization of the platinum disk on the activity of the membrane. On the other hand, as shown by v_1/v_3 ratios, 17% of hydrogen peroxide enzymatically generated is electrochemically oxidized on the platinum anode of E_1. This percentage is independent of glucose concentration and temperature.

One side enzyme-bound collagen membrane. Glucose oxidase collagen membranes were specially prepared with enzyme bound to only one side. These membranes yield a larger response to glucose when the enzyme layer is pressed against the platinum disk: an increase of 160—260% of steady-state response v_1 was obtained showing that, although glucose has to diffuse through the membrane, the electrochemical detection of hydrogen peroxide is made easier when its enzymatic generation site is nearer to the electrode surface.

Collagen membranes freely stirred or mounted on electrode E_1. Maximum glucose oxidase activity was determined in 0.1 M glucose solutions corresponding to saturating substrate concentration. This determination was performed with membranes either freely stirred or mounted on disconnected electrode E_1,

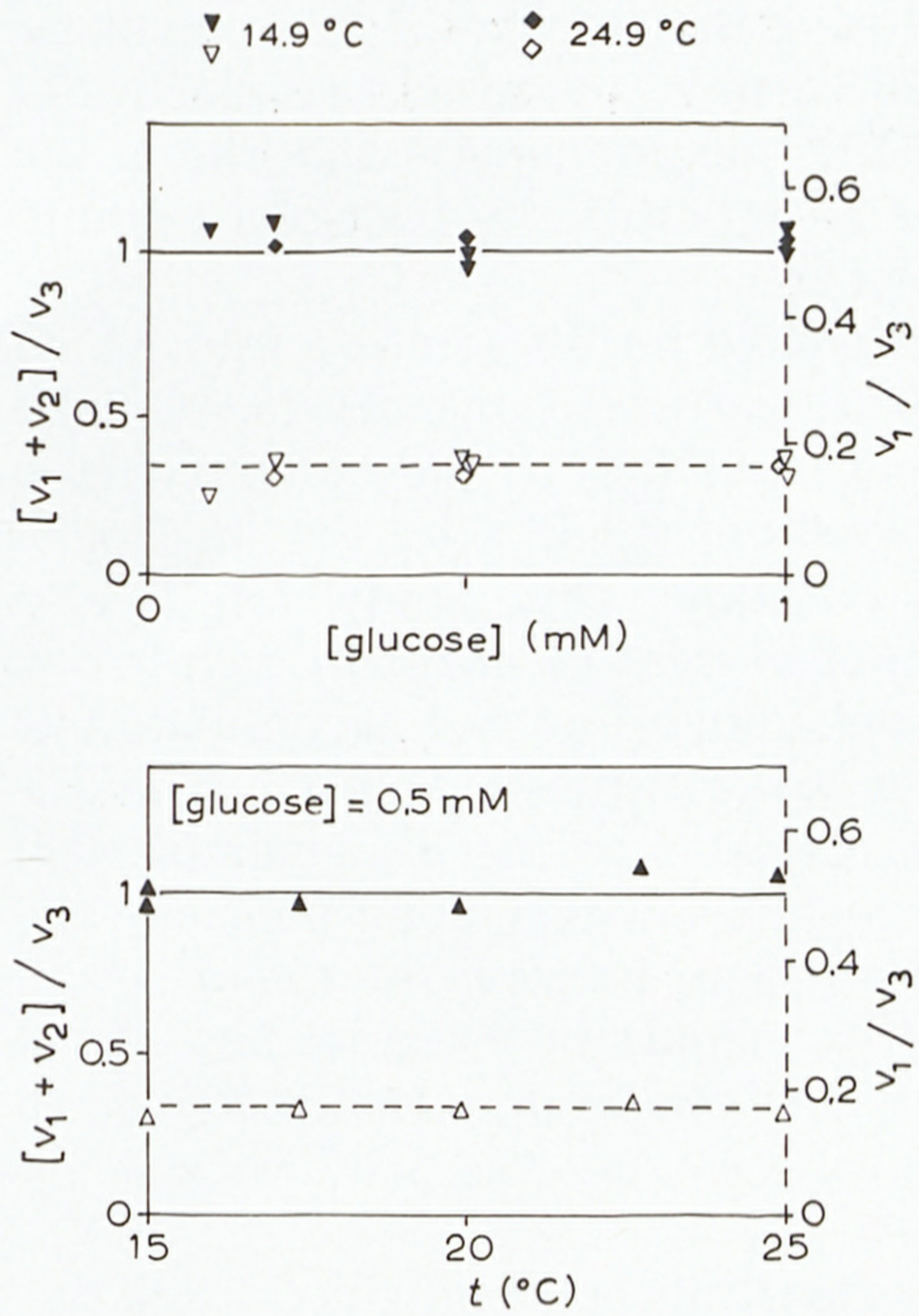


Fig. 7. Hydrogen peroxide balance. ———, $(v_1 + v_2)/v_3$, and - - - - - -, v_1/v_3 ratios. A (top), glucose concentration dependence. B (bottom), temperature dependence.

using the non-enzymatic electrode E_2. After calibration of the latter with hydrogen peroxide pulses, the rate of I_2 increase allowed the measurement of the corresponding values V_4 and V_3. The V_3/V_4 ratio was close to 0.26. Enzymatic surfaces in contact with the bulk solution are 0.4 cm^2, i.e. the aperture of the electrode cap when the membrane is mounted and 1.6 cm^2 for both faces of the freely stirred membrane. Their ratio, 0.25, is very close to the above mentioned one. This shows that the working area of the membrane mounted on the electrode is equivalent to its surface in contact with the bulk solution.

Discussion

The association of electrochemical sensors to enzymatic membranes, leading to the so-called enzyme electrodes [12] offers an opportunity for an experimental approach of heterogenous enzyme kinetics. Using a very sensitive electrochemical device we have been able to study new situations encountered when enzymatic membranes present only one side in contact with the stirred substrate solution.

Whereas previous electrochemical studies of such situations were generally performed with enzymes randomly distributed in an immobilized solution layer [4,13], in gels [15] or in cross-linked polymer matrixes [16,17], we have used reconstituted collagen membranes with surface-bound glucose oxidase on

both sides [9]. Moreover, instead of monitoring the activity of the enzymatic membrane by a sole electrode placed in its vicinity, we used two electrodes simultaneously, one in close contact with the membrane (E_1) and one in the bulk solution (E_2). This device enabled us to monitor the membrane-bound glucose oxidase activity from two different points.

When a pulse of glucose is added to the stirred buffer solution into which both electrodes are dipped, hydrogen peroxide is formed on both faces of the membrane. The linear increase of the output current I_2 of the non-enzymatic electrode E_2 demonstrates that, in steady-state conditions, the hydrogen peroxide flux towards to bulk solution is constant. This steady-state is also visualized by the constant level of the output current of enzymatic electrode E_1. The hydrogen peroxide enzymatically generated does not accumulate but diffuses towards the platinum disk of E_1 or towards the bulk phase. The difference between E_1 and E_2 output signals, is related to the influence of each electrodes on its environment: whereas E_2 presents a negligible influence on the hydrogen peroxide building-up in the bulk solution, E_1 plays the role of a well attracting hydrogen peroxide in the immediate vicinity of the inner face of the membrane. Thus, when E_1 is disconnected, this hydrogen peroxide depletion stops and its building up in the bulk phase is accelerated, as demonstrated by the increase of dI_2/dt.

The apparent equilibrium constant of the reaction between glucose and dissolved oxygen, as determined by the respective oxidoreduction potentials of the oxygen/hydrogen peroxide and gluconate/glucose systems, reaches $9.3 \cdot 10^{22}$ at pH 5.7. Thus, the occurrence of a hydrogen peroxide well in the vicinity of the enzymatic membrane has no influence on its activity. This was experimentally demonstrated by the balance of hydrogen peroxide generation when electrode E_1 is polarized or disconnected.

The simultaneous monitoring of hydrogen peroxide fluxes v_1 and v_2 towards E_1 and E_2 respectively, offers interesting possibilities for the determination of the rate limiting steps in the activity of the enzymatic membrane. The importance of diffusional limitations seems to be higher for v_1 as shown by its larger $S_{0.5}$ value (3.7 instead of 1.8 mM). We propose the following interpretation of these results. Glucose, if not completely depleted at the outer face level, has to diffuse through the membrane before being oxidized at the inner face level. Experiments done with glucose oxidase bound to only one side of the collagen membrane have indeed demonstrated that hydrogen peroxide molecules generated on the inner face of the membrane are more easily detected by the E_1 platinum anode than those produced on the outer face. However, as the outer face of the enzymatic collagen membrane is better exposed to glucose than the inner one, hydrogen peroxide is probably mainly generated on the outer face and has to diffuse through the membrane before reaching the E_1 platinum anode. Thus, whatever the contributions of both faces of the membrane in the production of the hydrogen peroxide flux v_1 detected on E_1, the necessity for both glucose and hydrogen peroxide to cross the membrane involves the occurrence of diffusion limitations more apparent on v_1 than on v_2.

Furthermore, we were able to define a working area by comparing the activity of an enzyme membrane either when freely stirred or when mounted

on E_1: the working area was equivalent to the membrane external surface in contact with the solution but the contribution of each face of the membrane has not yet been elucidated.

Finally, we think that the use of very sensitive electrochemical sensors placed both in the immediate vicinity of the membrane and in the bulk phase, offers a simple and promising experimental approach of heterogenous enzyme kinetics and a better understanding of their rate limiting steps.

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