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Covalent Linkage of Glucose Oxidase on Modified Glassy Carbon Electrodes. Kinetic Phenomena

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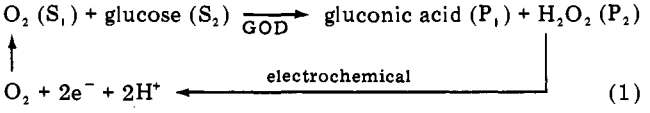
Received November 13, 1979

Abstract: Direct grafting of glucose oxidase molecules onto glassy carbon electrodes was done to optimize molecular proximity between enzymatic and electrochemical sites. Peroxide molecules produced by the enzyme reaction are measured and transformed into O_2 by the use of the electrode. Kinetic phenomena arising from the coupling between enzyme and electrochemical reactions were experimentally and theoretically studied as a function of the working potential values.

Recent works dealing with chemically modified electrodes have made possible the permanent chemical modification of the surface of various electrode materials.[1,2] Several chemical functions, electroactive or not, have been fixed onto electrode surfaces, generally through covalent linkages. Simultaneously, the analytical possibilities of an association between electrochemical sensors and enzyme membranes have been developed by numerous authors.[3]

The covalent binding of enzyme molecules as a quasi-monomolecular layer on the surface of a carbon electrode will be described in the present paper. The method of immobilization gives optimum molecular proximity between enzyme active sites and the electrochemical surface.

Owing to the possible amperometric measurement of one of the products (H_2O_2), glucose oxidase (GOD) was chosen as a model system. One of the substrates (O_2) can be electrochemically regenerated according to eq 1. In such a system, the enzyme activity can be controlled by the local electrochemical oxygen regeneration. The overall behavior is ruled by mass transfer phenomena near the electrode. In order to work under well-defined hydrodynamic conditions, a rotating cylinder electrode was used. The rotation speed can be varied from 100 to 1400 rpm.



(1)

Carbon is not a very attractive material for enzyme immobilization. Its chemical inertia toward coupling reagents is high, and mechanical strength decreases when the specific area increases. Enzyme adsorptions on graphite or activated carbon followed by glutaraldehyde[4,5] or soluble carbodiimide[6] cross-linking were described. Quite recently this last method was used after superficially oxidizing the carbon.[7,8] Glucose oxidase was immobilized with glutaraldehyde and bovine serum albumin on a carbon paste electrode.[9]

In order to get a direct covalent binding of the enzyme molecules, electrochemical oxidation and carbodiimide activation of the carbon were performed. Carbon oxidation gives

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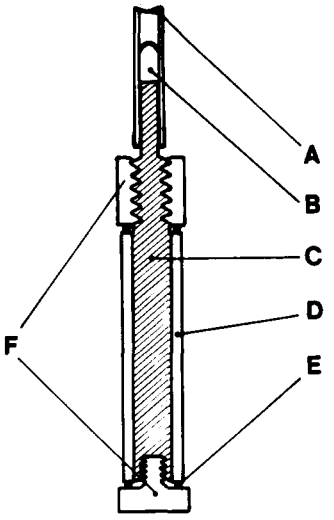
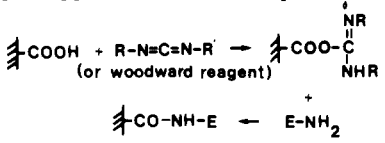


Figure 1. Rotating cylinder electrode of glassy carbon: (A) glass tube; (B) mercury electrical connection; (C) main part of the electrode in stainless steel adjusted to fit the internal diameter of D; (D) glassy carbon tube 1.2 cm in diameter, 5 cm long; (E) O rings; (F) Altuglass devices providing mechanical support and carbon isolation.

rise to numerous superficial oxygenated functions including COOH.[10,11] The immobilization process then occurs in two separate steps: support activation and protein binding.



The activation step was followed by an extensive rinse before protein binding. This procedure must lead to a mono- or a quasi-monomolecular layer since carboxylic functions of protein cannot be reactivated by free carbodiimide. Under these conditions, the resulting enzyme activity was necessarily low, especially when dealing with a nonporous support, which provided a good model of mass transfer effects.

Experimental Section

Production of Glucose Oxidase Electrodes. A tube of glassy carbon[12] was selected as a support. The external surface was used as a rotating cylinder (17.5 cm^2) (Figure 1). Owing to the difficulty in tooling glassy carbon, a maximum eccentricity of 0.5 mm was admitted.

The surface was carefully polished with abrasive paper and with alumina powders exhibiting decreasing grain size. The resulting surface was observed by scanning electron microscopy. The maximal roughness found was around 0.05 μm (strips were especially observed).

Each sample was submitted to the following steps: (a) Samples were treated with an alcohol-KOH solution and then ultrasonically cleaned. (b) Samples were electrochemically oxidized in 10% HNO_3 + 2.5% $K_2Cr_2O_7$ at 2.2 V/SCE during 10 s with a standard potentiostatic device. It was shown by scanning electron microscopy that a longer oxidation time gives rise to surface modifications higher than 0.05 μm. (c) COOH functions were activated with one of the following reagents: Woodward K reagent; morpholinocarbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; aminopropylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HCl. Activation was done during slow rotation of the electrode in a 0.05 M solution of the used carbodiimide-acetate buffer (0.05 M, pH 4.6). (d) Immobilization was then performed with 1 mg mL^{-1} GOD[13] in the above buffer at 4 °C for 2 h.

Each step was followed by extensive rinsing while the electrode was rapidly rotated.

Instrumentation and Procedures. The rotating electrode was placed into a 17-mL minireactor connected to the reference and counter electrodes by ceramic, porous plugs. A potentiostatic device was used. All experiments were carried out under continuous bubbling of pure oxygen. The used reactor may be fed with a pump in order to quickly renew the solution without any modification of the potential.

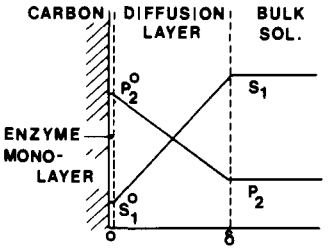


Figure 2. Geometry of the proposed scheme of the electrode-diffusion layer system and examples of concentration profiles: δ, diffusion layer thickness; the enzyme layer thickness is supposedly negligible with respect to δ.

For a given potential, measurements were performed as follows: After stabilization of the background current in the used buffer, a small sample of a stock solution of concentrated glucose or H_2O_2 was injected into the batch. Recorded current variations deal with the Δi definition given in the theoretical section. A control of the results dealing with electrode activities under the same experimental conditions was done with an autoanalyzer (Technicon) according to a bioenzymatic method.[14] Rotation speeds were stroboscopically measured.

Theoretical Model

Enzyme Layer (Figure 2). If a size of 8 nm for glucose oxidase molecules is assumed,[15] the order of magnitude of the expected coating activity can be calculated. The two assumptions are the existence of a monomolecular layer and a maximum filling coefficient of 60%.[16] Calculations give 10^{12} molecules of glucose oxidase fixed per cm^2 of electrode surface, i.e. 5×10^{-2} IU cm^{-2} on the basis of an activity of 200 IU mg^{-1} at 25 °C under pure oxygen with the enzyme used.

It is important to note that ten COOH functions per enzyme molecule are sufficient for good enzyme immobilization (10^{13} functions cm^{-2}). These levels of functions (1.6×10^{-11} mol cm^{-2}) are not measurable with glassy carbon exhibiting low specific area. In order to experimentally check the presence of COOH functions, a porous graphite submitted to the same oxidation treatments was used as a model. We have measured COOH functions largely in excess, as already demonstrated by many authors.[10,11]

Owing to the thinness of the enzyme layer, internal mass transfer can be neglected when compared with external transfer across the diffusion layer. In this way, concentrations of species are unique inside the thin active layer and near the electrode surface.

The glucose oxidase catalytic mechanism is know to be of the "ping-pong" type.[17]

From Figure 2 and with eq 1 conventions:

$$J = \frac{J_M}{1 + \frac{K_1}{S_1^0} + \frac{K_2}{S_2^0}} \qquad (2)$$

where J = product flux at $x = 0$; J_M = maximum chemical flux when the enzyme is working under zero-order kinetics for both substrates (mol cm^{-2} s^{-1}); K_1, K_2 = Michaelis constants for oxygen and glucose, respectively, with zero-order kinetic conditions for the second substrate (mol cm^{-3}); S_1^0, S_2^0 = substrate concentrations at $x = 0$. It is important to note that in this case we are dealing with a monomolecular layer, so that the classical V_M (mol cm^{-3} s^{-1}) cannot be used.

The external diffusion layer will be considered as a Nernst layer with a thickness depending on electrode rotation speed. Hydrodynamic studies of rotating cylinder electrodes have shown that turbulent convection is occurring even at low rotation speeds. With the present experiments, where rotation speeds surpass 25 rpm,[18,19]

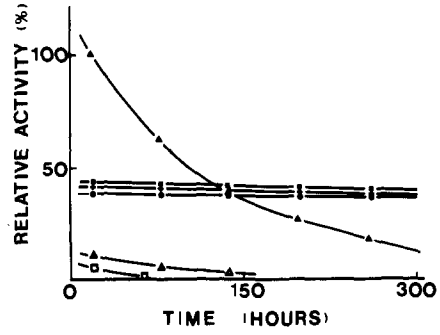


Figure 3. Glucose oxidase activity when immobilized onto carbon electrodes as a function of storage time: (△) simple adsorption without carbon oxidation; (▲) simple adsorption with oxidation; (□) morpholinocarbodiimide treatment without oxidation; (■) morpholinocarbodiimide activation after oxidation; (●) aminopropylcarbodiimide activation after oxidation; (+) Woodward "K" reagent activation after oxidation. The activity was obtained with Δi measurements at high rotating speed (see text for details). Diffusion limitation effects are negligible; glucose, 10^{-1} M; acetate buffer, 0.1 M, pH 5.6.

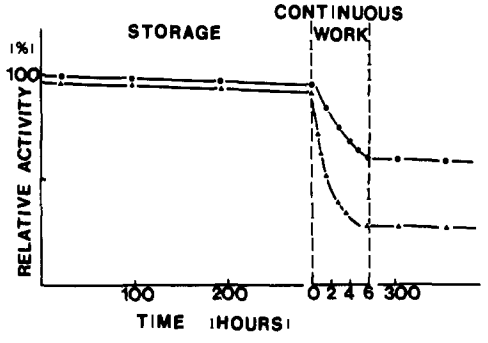


Figure 4. Glucose oxidase activity as a function of time when working or not. Time scale dealing with the continuous working period is expanding. Continuous work experiments were performed in the presence of 5 g L^{-1} (●) and 20 g L^{-1} (▲) glucose solutions, respectively. Other experimental conditions are those given in Figure 3.

$$\delta_i = 17.46 D_i^{1/3} (\text{rpm})^{-2/3} \quad (3)$$

with δ_i the thickness (cm) of the diffusion layer for species exhibiting a diffusion coefficient equal to D_i. Corresponding experimental thicknesses range from 10^{-3} to 2×10^{-2} cm.

Substrate feeding or product departure is given at $x = 0$ by:

$$J_i = \frac{D_i}{\delta_i}(C_i - C_i^0) = h_i(C_i - C_i^0) \quad (4)$$

with h_i as transport coefficient.

H_2O_2 Electrochemical Oxidation. Under the described experimental conditions (pH 5.6 with glassy carbon), H_2O_2 is electroactive from 0.6 to 0.7 V/SCE. Unfortunately, water oxidation occurs at 1 V/SCE. Measurements were made only between 0.7 and 1 V.

Δi was defined as current density increase obtained at a given potential, arising from either direct injection of H_2O_2 or injection of the enzyme substrates:

$$\Delta i = 2FK_V P_2^0 \quad (5)$$

K_V is the electrochemical reaction rate constant at a given potential V and P_2^0 is the H_2O_2 concentration at the electrode interface.

In the absence of enzyme activity with a concentration P_2 in the bulk solution, the continuity equation gives:

$$\Delta i = \frac{2FP_2}{\frac{1}{K_V} + \frac{1}{h_p}} \quad (6)$$

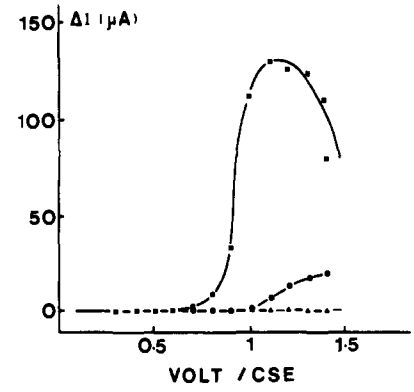


Figure 5. Current increase Δi dealing with the interfacial H_2O_2 molecules produced by the enzyme reaction as a function of working potential: (■) 0.1 M glucose, 1.26×10^{-3} M oxygen; (●) 0.1 M glucose under nitrogen bubbling (presence of a small signal with high potential values is linked to oxygen produced by water oxidation); (▲) blank test, electrode without enzymes in the presence of O_2 and glucose. Rotation speed, 1440 rpm; pH 5.6; 0.1 M acetate buffer; electrode area, 17.5 cm^2.

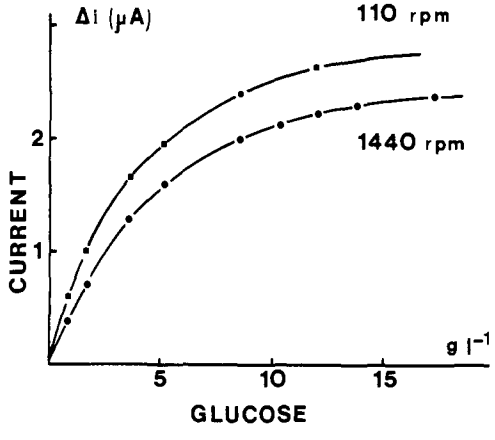


Figure 6. Electrode response as a function of glucose concentration with a working potential of 0.7 V/SCE: (■) rotating speed 110 rpm; (●) rotating speed 1440 rpm; O_2 bubbling; electrode area, 17.5 cm^2; 0.1 M acetate buffer, pH 5.6. Owing to the relatively low value of the electrochemical speed constant at 0.7 V, measured currents are small (see Table II).

This equation may be used in measuring K_V as a function of the potential, as well as h_p as a function of rotating speed in preliminary experiments without glucose and thus without enzymatic H_2O_2 production.

Enzyme Electrode. Owing to the low oxygen solubility (1.26×10^{-6} mol cm^{-3} under normal pressure with pure O_2 bubbling), glucose diffusion limitations can be neglected when compared to oxygen mass transfer (S_2 is always close to S_2^0).

Local concentrations of O_2 and H_2O_2 in the enzyme layer are dependant on three phenomena: the diffusion convection (hydrodynamic aspect); the electrode potential (electrochemical kinetic); the enzymatic rate (enzymatic kinetic).

In our case, the solution of this system is very simple because diffusion and reactions are spatially separated.

The continuity equation at $x = 0$ can be written as:

$$J_V = \frac{J_M}{1 + \frac{K_1}{S_1^0} + \frac{K_2}{S_2^0}} = h_s(S_1 - S_1^0) + K_V P_2^0$$

$$= h_p(P_2^0 - P_2) + K_V P_2^0 \quad (7)$$

and

$$\Delta i = 2FJ_V[K_V/(h_p + K_V)]$$

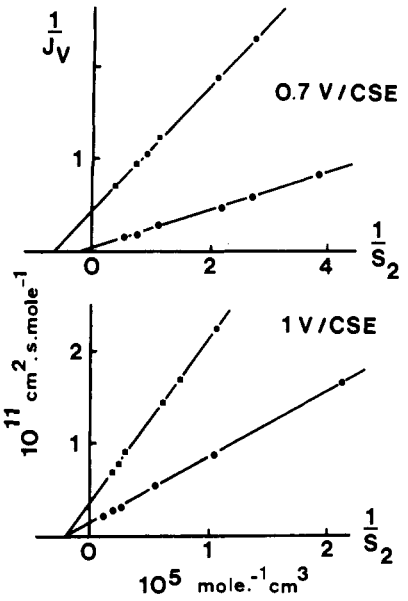


Figure 7. Typical Lineweaver-Burk plots dealing with two working potentials and two rotating speeds [(■) 110 rpm; (●) 1440 rpm]. J_V was calculated from measured Δi values. (Give attention only to the apparent Michaelis constant, since J_M was not exactly the same in both cases).

Table I

working voltage, V/SCE	K_2^M [a]	
	110 rpm	1440 rpm
0.7	1.2×10^{-2}	5.2×10^{-2}
0.8	1.6×10^{-2}	5.8×10^{-2}
0.9	2.7×10^{-2}	5.6×10^{-2}
1	5.8×10^{-2}	5.8×10^{-2}

[a] Apparent Michaelis constants (K_2^M) obtained from Lineweaver-Burk plots, pH 5.6, $O_2 = 1.26 \times 10^{-3}$ M (in solution $K_2^M = 5.7 \times 10^{-2}$ M).

with J_V the chemical glucose oxidase flux at a given potential.

Equation 7 is easily rewritten as:

$$a(S_1^0)^2 + b(S_1^0) + c = 0 \qquad (8)$$

with $a = (S_2 + K_2)(h_p h_s + K_V h_s)$, $b = J_M S_2^0 h_p + K_1 S_2^0 h_s (h_p + K_V) - (S_2^0 + K_2)(h_p h_s S_1 + K_V h_s S_1 + K_V h_p P_2)$, and $c = K_1 S_2^0 (h_p h_s S_1 + K_V h_s S_1 + K_V h_p P_2)$. Knowing S_1^0 allows simulation of J_V and P_2^0 by eq 7 and Δi by eq 5. Reciprocally, the experimental Δi value can successively give J_V, P_2^0, and S_1^0. The influence of rotation speed is introduced easily on h_i by eq 3.

Results and Discussion

Immobilization Optimization. The stability was tested each day during 2 weeks. The results (Figure 3) show the following.

Adsorption and immobilization were increased by superficial oxidation of glassy carbon electrodes. The presence of oxygenated functions increases surface hydrophilicity and makes carbodiimide activation possible.

The enzyme electrode activity did not depend on the nature of the carbodiimide used. Results with morpholino activation are discussed below.

Electrode response was stable under storage conditions when dealing with samples submitted to the overall immobilization process. This point is strong evidence for covalent binding of the enzyme molecules.

It is important to note that the results were not performed under continuous use of the enzyme electrode, but activities were discontinuously measured during storage.

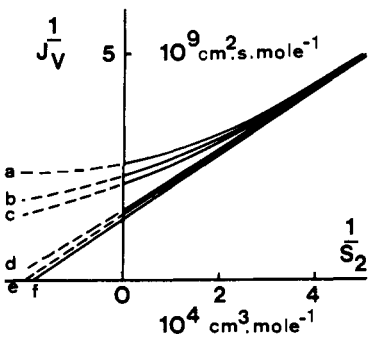


Figure 8. Simulated reciprocal plots with constant diffusion limitations ($\delta = 5 \times 10^{-2}$ cm) but with different K_V values: (curve a) $K_V = 0$; (b) 8.6×10^{-6}; (c) 3.8×10^{-5}; (d) 2.5×10^{-4}; (e) 1.1×10^{-3}; and (f) ∞ s^{-1}.

Table II. Numerical Values Used in the Theoretical Model

parameter	value	remarks
P_2	0 mol cm^{-3} at $t = 0$	calculations deal with initial velocity
S_1	1.26×10^{-6} mol cm^{-3}	saturating concn of O_2 in water under normal pressure at 25 °C
J_M	10^{-9} mol cm^{-2} s^{-1}	value corresponding to a mean J_V value of 0.3 IU per electrode
K_1	4.5×10^{-7} mol cm^{-3}	Michaelis constants of GOD measured in solution
K_2	6.5×10^{-5} mol cm^{-3}	
D_{P_2}	10^{-5} cm^2 s^{-1}	Stern[24]
D_{S_1}	2×10^{-5} cm^2 s^{-1}	Levich[25]
K_v		experimentally measured from eq 6 with a potential of:
	8.6×10^{-6} cm s^{-1}	0.7 V/SCE
	3.8×10^{-5}	0.8 V/SCE
	2.5×10^{-4}	0.9 V/SCE
	1.1×10^{-3}	1 V/SCE
δ_i	variation from 0 to 5 $\times 10^{-2}$ cm	

Glucose oxidase is quite stable when dealing with classical thermal denaturation, but a quick denaturation occurs under working conditions. The phenomenon needs some research to be well understood.[20] With an immobilized glucose-oxidase system, exhibiting high diffusion limitations, a large percentage of sites is not "working" at the beginning of the process and a kind of "reserve" exists. The present system is a monomolecular layer and resulting working denaturation is relatively higher (Figure 4). Direct amperometric measurement of glucose activity was very useful in overcoming reproducibility problems linked with measurement delays.

Owing to the imprecision of the physical size of the active layer, it was difficult to evaluate an activity yield after immobilization (yield = number of active enzyme molecules per total number of fixed protein molecules). The hypothesis of a monomolecular layer is based on the immobilization procedure and on the absence of surface modification after enzyme immobilization. No aggregates were observed by scanning electron microscopy within the resolution range (0.05 μm). The order of magnitude of measured activities was 0.02 IU cm^{-2} (under pure oxygen conditions). When compared with the evaluated activity by calculation (0.05 IU cm^{-2}), the yield could be about 40%. The value is similar to the results obtained with artificial enzyme membranes.[21]

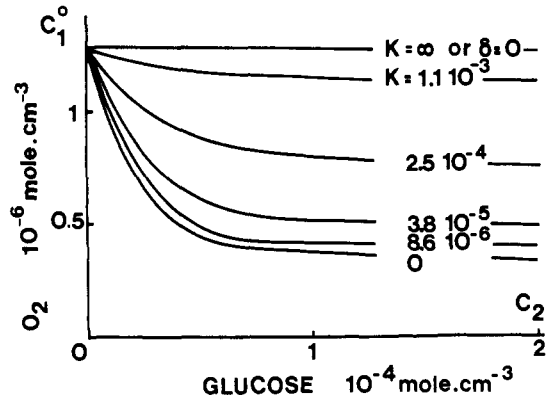


Figure 9. Simulated interfacial concentrations of oxygen under the conditions described in Figure 8 and Table II.

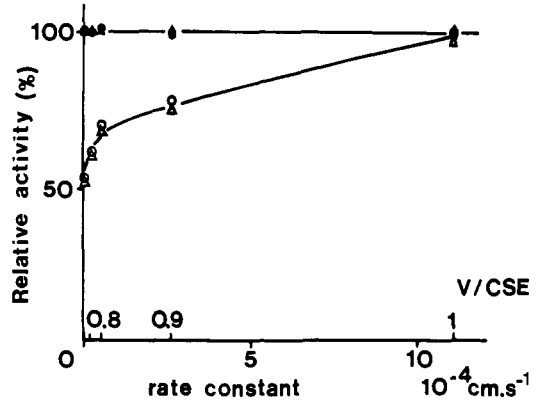


Figure 10. Experimental study of the glucose oxidase activity as a function of the electrochemical rate constant with two rotating speeds: (▲ and ●) rotating speed 1440 rpm; (△ and ○) rotating speed 110 rpm; (△ and ▲) electrochemically measured activity by Δ_i; (○ and ●) activity obtained by measurement of gluconic acid production. (a correction is given for the working denaturation in the last case); 0.10 M acetate buffer, pH 5.6, 20 g L^{-1} glucose, O_2 bubbling.

Kinetics Results. A typical variation of measured Δi as a fuunction of potential values is given in Figure 5. With a potential higher than 1.1 V, electrode response became irreversible and a quick enzyme denaturation was observed. Even in the presence of 0.1 M buffer, an important modification of the local pH could be responsible for the denaturation. Owing to previously introduced reasons, e.g., water oxidation, measurements were restricted to the range of potential 0.7-1 V/SCE.

Scheller et al.,[22] using a mercury electrode, observed electron transfer from the electrode to the prosthetic group of GOD from *Penicillium notatum*. In our case such a phenomenon was not observed even after numerous assays performed under different conditions.

Electrode responses as a function of glucose concentration exhibit a Michaelian behavior (Figure 6). Note that Δi values decrease when rpm increase. This does not imply that J_V necessarily decreases. In fact, calculations show that J_V increases in this case. Owing to the diffusion limitations giving a modulation of local O_2 and H_2O_2 concentrations, electrode responses are not directly proportional to enzyme activities. It is easier to discuss the results dealing with J_V evaluated from Δi through relations 5 and 7: the lower the rotation speed, the higher the O_2 diffusion limitation and the lower the apparent K_2^M (Figure 7); owing to O_2 electrochemical regeneration, apparent K_2^M increases with high electrode potential values (Figure 7).

It is of interest to note (Table I) that with the highest rotating speed, mass transfer effects appear to be negligible. Reciprocal plots were used to evaluate K_M values, but it is important to be aware of the limitations of this method when dealing with heterogeneous systems. These limitations on the linearity shown by our theoretical model (Figure 8) are now well known.[23]

A simulated reciprocal plot and local O_2 concentrations show the electrochemical regeneration effect when dealing with diffusion limitations or different regeneration conditions (Figures 8 and 9). Parameters used for the calculation are given in Table II. With a polarization layer of 5×10^{-2} cm and a potential of 1 V/SCE, electrochemical regeneration can provide up to 60% of the enzyme oxygen feeding.

Glucose oxidase activity as a function of electrochemical regeneration conditions was studied by measuring the activity with both the electrochemical Δi method previously discussed and gluconic acid measurement. Data obtained (Figure 10) show that both analytical methods give similar results and show once again the effect of O_2 regeneration in the system.

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