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Mass Transport and Reaction Kinetic Parameters Determined Electrochemically for Immobilized Glucose Oxidase†

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ABSTRACT: Mass-transfer resistances often have pronounced effects on the overall reaction rates of enzymes immobilized at interfaces or in polymeric matrices. In the present work glucose oxidase was immobilized on the surface of a platinum disk electrode by one of three attachment techniques: silane–glutaraldehyde, allylamine–glutaraldehyde, and albumin–glutaraldehyde. In one group of studies the electrodes were rotated, and methods were employed to determine the diffusion and shielding coefficients for transport of a model electroactive compound, i.e., potassium ferrocyanide, through the enzyme matrix. A model electrochemically active compound was used because glucose exhibits a very slow rate of electron transfer at a platinum surface. The diffusion coefficient for ferrocyanide was reduced 7% by the silane–enzyme and 25% by the allylamine–enzyme matrices. In a second group of studies the electrodes were held stationary. Marked internal diffusional resistance was noted for the albumin–glutaraldehyde–enzyme matrix. The calculated flux of ferrocyanide was decreased by a factor of 2000–8500 for transport through albumin–enzyme matrices 0.21–0.063 cm thick, as compared to transport through free solution. In a third group of studies the rotating enzyme-matrix electrode was utilized in determining apparent values of the Michaelis constant for glucose. The velocity of the reaction was determined by amperometric measurement of the concentration of hydrogen peroxide reaching the ring electrode. The results, determined from Eadie–Hofstee type plots of reaction current and substrate concentration, gave values between 12 and 36 mM for the three methods of immobilization. The results show that the mass-transfer resistance for diffusion of small molecules through immobilized glucose oxidase varied with the method of enzyme coupling. This study also shows that electrochemical techniques are useful in defining the relative rates of mass transfer and enzymatic reaction.

Many of the enzymatic reactions that take place in cells and tissues occur at interfaces or within some type of membrane, rather than in homogeneous solution (McLaren & Packer, 1970). In such heterogeneous sytems the kinetics of enzyme-catalyzed reactions may be quite different than in homogeneous solution (Engasser & Horvath, 1974). In particular, the rates at which substrates or products diffuse through the enzyme matrix may become slower than the rate of the enzyme-catalyzed reaction. This diffusional resistance is an important consideration in the study and application of enzymes immobilized on artificial matrices or supports (Kasche, 1983). Diffusional resistances can arise from transport limitations at the interface between the bulk solution and the enzyme support (i.e., external resistance) or from transport limitations within the support matrix (i.e., internal resistance) (Engasser & Horvath, 1976). One of the perplexing problems in the study of enzyme-catalyzed reactions in heterogeneous systems is the experimental determination of the degree of diffusional resistance. Highly sensitive methods are required for measuring the rate of transport of material through the enzyme-support matrix without causing undue perturbation of the system.

Several electrochemical methods, based on rotating and stationary electrodes, fulfill the requirements for high sensitivity and lack of a need to take samples. Rotating ring disk electrodes (RRDE)[1] are especially well suited for characterizing the rates of transport of material through an enzyme-support matrix (Bard & Faulkner, 1980); yet this technique has received surprisingly little application to date. A typical RRDE is shown in Figure 1. The disk and ring are insulated from each other and connected by lead wires to external circuitry. With suitable auxiliary and reference electrodes, the potential or current at the disk or ring can be controlled or measured. An enzyme matrix can be attached to the disk, and the RRDE can be rotated while immersed in a solution of substrate or other diffusate. When the RRDE is operated at different rotation speeds, the external diffusional resistance for the substrate can be selectively controlled, thus enabling the measurement of internal diffusional limitations. For characterization of external or internal mass-transfer resistances, the diffusate must undergo very rapid oxidation or reduction at the electrode in order not to influence the results of the measurements.

The RRDE was used to study the mass-transfer/reaction kinetics of glucose oxidase attached via octadecylamine, glutaraldehyde, and albumin to a carbon paste disk (Shu &

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[1] Abbreviation: RRDE, rotating ring disk electrode.

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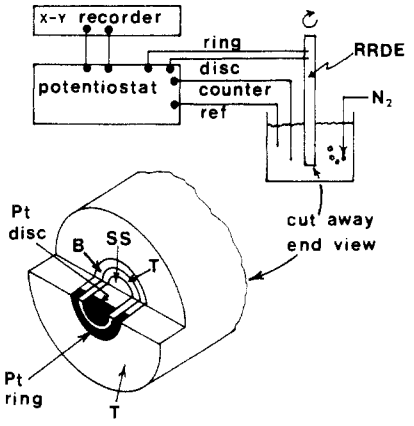

FIGURE 1: Description of RRDE. T is Teflon insulation, SS is stainless steel core for disk circuit, and B is brass core for ring circuit. A four-electrode arrangement was used (ring, disk, platinum counter, and Ag/AgCl reference electrodes). Disk 0.50-cm diameter; ring 0.75-cm inner diameter and 0.85-cm outer diameter.

Wilson, 1976) and later via 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide and allylamine to graphitic oxide and platinum disks (Kamin & Wilson 1980). Shu & Wilson (1976) used I^-/I_2 as an electrochemical mediator since this redox couple underwent rapid interfacial electron transfer while glucose exhibited much slower electron transfer and oxygen demonstrated irreversible electron transfer at these electrodes. Hydrogen peroxide from the glucose oxidase reaction oxidized I^- to I_2, which in turn was measured amperometrically at the ring or disk. Kamin & Wilson (1980) employed direct amperometric measurement of hydrogen peroxide at the ring. Glucose oxidase (Bourdillon et al., 1980) also was attached, by using several different carbodiimide reagents, to the surface of a cylinder of glassy carbon. This rotating electrode was used to study the combined rates of diffusion and reaction by amperometric measurement of the hydrogen peroxide produced.

The present paper describes the electrochemical measurement of diffusional resistances for transport of a model electroactive solute, i.e., potassium ferrocyanide, through matrices of glucose oxidase attached to platinum via three different coupling techniques. Results with both RRDE and stationary electrode techniques are included. This paper also reports the determination of the apparent kinetic constants for glucose oxidase (1) immobilized by different methods than described by previous authors, (2) for a wider range of glucose concentrations than reported previously, and (3) by using the Eadie–Hofstee rather than the Lineweaver–Burk approach to plot the enzyme electrode reaction variables of current and substrate concentration.

Materials and Methods

Covalent Immobilization of Glucose Oxidase to Platinum Disk of RRDE. Covalent attachment of glucose oxidase (EC 1.1.3.4) to the platinum disk electrode was carried out with either an alkylamine silane or allylamine as the coupling reagent. A Pine Instrument Model DT136 rotating electrode assembly with a removable 0.50-cm diameter platinum disk and a fixed platinum ring of 0.75-cm inner and 0.85-cm outer diameter was used (Figure 1). The assembled disk and ring were polished with 0.1-μm Gamal grade B alumina and rinsed with Milli Q treated water and then with 0.5 M sulfuric acid. Prior to attachment of the coupling agent, the disk was pretreated electrochemically in 0.5 M sulfuric acid under nitrogen to control the state of oxidation of the platinum surface. Before alkylsilane attachment, the potential of the disk was scanned in both directions at a rate of 3 V/min between 1900 and −450 mV, with respect to Ag/AgCl, until a reproducible cyclic voltammogram was obtained. Then the potential applied to the disk was maintained at 1900 mV for 5 min. Following this treatment the disk was removed from the RRDE assembly, rinsed, vacuum dried, and held for enzyme attachment. Glucose oxidase attachment by the silane method was based on a method described previously (Wingard et al., 1979). Briefly, the detached disk electrode was immersed 30 min in 10% (v/v) (γ-aminopropyl)triethoxysilane in dry benzene. After being rinsed with benzene and vacuum dried, the disk was held 1 h at ca. 4 °C in 2.5% glutaraldehyde in 50 mM, pH 7.0, sodium phosphate buffer. Enzyme coupling was carried out with the platinum–silane–glutaraldehyde disk in 2.5 mg/mL glucose oxidase (Sigma, type VII, about 125 units/mg) in the same buffer for 4 h at 4 °C. Just prior to use the disk was rinsed, brought to room temperature, and assembled in the RRDE.

Glucose oxidase attachment to platinum by allylamine was adapted from a published method (Kaman & Wilson, 1980). Electrochemical pretreatment of the disk consisted of scanning at 0.5 V/min from 400 to 1300 mV, then to −300 mV, and back to 400 mV, where the potential was held for 2 min. The electrochemically treated disk was immersed 30 min in 4% (w/v) aqueous allylamine. Then the disk was rinsed with the above buffer and placed in a solution prepared by mixing 20 mL of 1% (w/v) glucose oxidase in buffer and 1 mL of 25% glutaraldehyde.

^{125}I-Labeled glucose oxidase was prepared with lactoperoxidase, hydrogen peroxide, and Na^{125}I to give labeled material of specific activity of 2.6 μCi/μg and concentration of 39 μg/mL (4.2 units of enzyme activity/mL). The details of the ^{125}I-labeled enzyme preparation are described elsewhere (Wingard et al., 1983).

Cross-Linking of Glucose Oxidase on Platinum. Glucose oxidase also was immobilized by cross-linking in a matrix containing glucose oxidase (Sigma, type II), catalase, bovine serum albumin (Sigma, fraction V), and glutaraldehyde in 0.1 M, pH 7.4, phosphate buffer. The detailed procedure plus the spectrophotometric criteria used to ensure a sufficiently high content of monomeric glutaraldehyde are described elsewhere (Wingard et al., 1983). For the RRDE measurements, a 100-μL drop of the above mixture was placed on the platinum disk; cross-linking occurred over a 45-min period at room temperature. The enzyme-coated RRDE was rotated for 30 min at 1000 rpm in 0.1 M, pH 7.4, phosphate buffer to remove loosely bound protein. The estimated thickness of the matrix was 0.3 cm. The RRDE could not be refrigerated because of thermal contraction and subsequent leakage at the epoxy–platinum joints around the ring and disk; so the enzyme coated RRDE was used within 24 h of preparation.

For stationary electrode measurements, the enzyme–albumin–glutaraldehyde solution was poured into a 14.3 cm diameter by 0.063 cm deep well, having a 1.30 cm diameter by 0.05 mm thick piece of platinum foil in the bottom of the well. The cross-linked matrix was washed with 1.0 M potassium chloride for 2 h and then set overnight in 0.1 M phosphate buffer prior to use. The cross-linked matrix remained in the well during subsequent mass-transfer experiments.

Basis for Diffusion Measurements with RRDE. The resistance to diffusional transport of potassium ferrocyanide through the enzyme-support matrix was characterized with the RRDE under potentiostatic control, using a Pine Instrument Model RDE-3 unit (Figure 1). Measurements of the diffusion and shielding coefficients were carried out for this characterization. The measurements were made by operating

the RRDE at a constant rate of rotation. Viscous drag at the disk surface causes the fluid adjacent to the surface to rotate. The resulting centrifugal force sweeps the fluid across the face of the disk, in a well-defined manner (Bard & Faulkner, 1980). Under mass-transfer rate-limiting conditions, the maximum rate of solute diffusion to the disk occurs when the difference in concentration of solute between the bulk solution and the disk surface is a maximum. This occurs when the concentration at the disk surface is essentially zero. Under these diffusion-controlled conditions the "limiting" current due to oxidation of ferrocyanide at the disk is defined by the following expression:

$$i_l = 0.62nFAD^{2/3}w^{1/2}\nu^{-1/6}C_r^* \quad (1)$$

where i_l is the limiting current, n is the equivalents per mole, F is 96 485 C/equivalent, A is the disk area (cm^2), D is the diffusion coefficient (cm^2/s), w is the rotation speed (rad/s), ν is the kinematic viscosity (cm^2/s), and C_r^* is the concentration of ferrocyanide in the bulk solution (mol/cm^3) (Bard & Faulkner, 1980). The diffusion coefficient can be calculated from eq 1 by obtaining the slope of the linear plot of i_l vs. $\omega^{1/2}$ at constant C_r^*.

The shielding coefficient is based on an amperometric measurement of the ferrocyanide concentration that reaches the ring. If the matrix on the disk presents a large diffusional resistance, then little of the ferrocyanide gets through the matrix to be oxidized at the disk; hence, most of the ferrocyanide reaches the ring. The diffusional matrix on the disk thus "shields" the ring. The calculation is described more fully under Results.

Rates of Mass Transfer Measured with Stationary Electrode. The rates of mass transfer of potassium ferrocyanide through different thicknesses of cross-linked albumin–glutaraldehyde–enzyme matrices were determined with the enzyme matrix and platinum foil in a well, machined into a piece of poly(methyl methacrylate) resin. For the influx measurements, ferrocyanide was added to the bulk solution side of the well at time zero. The concentration of ferrocyanide on the platinum foil side of the well, i.e., on the opposite side of the enzyme matrix from the bulk solution, was measured by using linear sweep cyclic voltammetry. Similar efflux measurements were made by preloading the matrix with potassium ferrocyanide and following the change in concentration at the platinum foil as the ferrocyanide diffused through the matrix into bulk solution initially containing no ferrocyanide. Calculation of the rates of mass transfer of ferrocyanide through the enzyme matrix is described under Results. In the stationary electrode experiments the solution was stirred to exclude external mass-transfer effects.

Estimation of Apparent Kinetic Constants for Glucose Oxidase Immobilized on the Disk of the RRDE. The technique used was a modification of the simulation model originally developed by Mell & Maloy (1975) for an enzyme immobilized within a membrane matrix and placed on the end of a stationary electrode. Their electrode was placed in a solution of substrate, and the solution was stirred vigorously to eliminate external convection–diffusion mass-transfer resistances. This enabled the authors to simulate mathematically the relative rates of internal diffusion and enzyme-catalyzed reaction within the membrane under either mass-transfer or reaction-controlled conditions. Wilson & Shu (1976) adapted the simulation method to study the relative rates of external mass transfer and enzyme-catalyzed reaction with a rotating electrode. The use of these methods in the present work in calculating the kinetic parameters and in showing whether external mass transfer, internal mass transfer, or the enzyme-catalyzed reaction was rate controlling is described under Results. In the present work glucose oxidase was immobilized on the disk. The RRDE was rotated in air-saturated glucose solutions, containing 0.1 M sodium phosphate buffer, pH 6.0, at 25 °C. The hydrogen peroxide produced by the enzyme reaction diffused to the ring where the steady-state peroxide concentration was measured by determining the limiting ring current 1–2 min after the ring potential was stepped from −200 to 800 mV. The delay was to allow time for the potential step charging current to decay, while the magnitude of the step change was selected to produce rapid oxidation of the hydrogen peroxide. In these experiments the disk was open circuited. The steady-state ring current represented the rate at which hydrogen peroxide was reaching the ring. This was assumed to be the same as the rate of hydrogen peroxide production by the enzymatic reaction. The steady-state currents were obtained at different bulk solution concentrations of glucose and at different rates of rotation of the RRDE. These data were used for estimation of the apparent Michaelis constant and the apparent maximum current for the reaction catalyzed by the immobilized glucose oxidase.

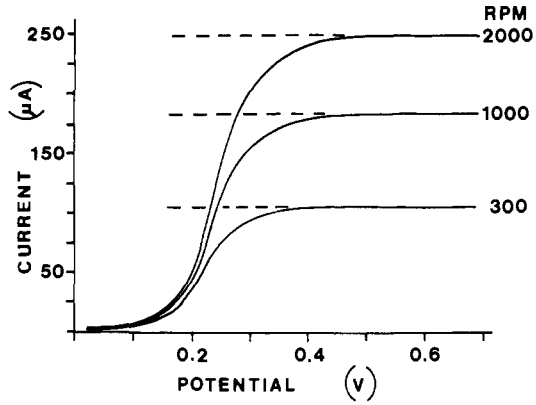

FIGURE 2: Anodic current vs. potential applied to the disk for selected rotational speeds with silane–glutaraldehyde–glucose oxidase coupled to the disk electrode surface. Bulk solution contained 1.95 M potassium ferrocyanide and 1 M KCl at 25 °C. Measurements were made in deoxygenated solution. The potential was scanned at 2 V/min from −100 to +700 mV. Dashed lines represent limiting currents.

Results

Diffusion and Shielding Coefficients. The diffusion coefficient was measured for the transport of potassium ferrocyanide from the bulk solution to the surface of the disk of the RRDE. Glucose oxidase was attached to the disk by the silane and allylamine techniques. The potential applied to the disk was varied at a fixed rate, and the current due to oxidation of ferrocyanide at the disk was measured. Typical results are shown in Figure 2 for glucose oxidase attached by the silane method. The mass-transfer limiting current was obtained at each rotation speed. A subsequent plot of limiting current (μA) vs. the square root of the rotation speed (rad/s) showed excellent linearity, as required from eq 1 (correlation coefficient 0.999), with a slope of 15.9 and an intercept of 17.2. The diffusion coefficient was calculated from eq 1 by using the following values: n = 1 electron-transfer equiv/mol for ferrocyanide, A = 0.196 cm^2 for the disk, C_r^* = 1.95 mmol/mL for bulk ferrocyanide concentration, ν = 0.01 cm^2/s for water. The diffusion coefficient results are summarized in Table I.

The same procedure, employed for measurement of diffusion coefficients through the silane– and allylamine–enzyme matrices, was also used with the cross-linked albumin–glutaraldehyde–glucose oxidase matrix. Instead of obtaining a maximum limiting current, as in Figure 2, the current in the

Table I: Diffusion and Shielding Coefficients for Potassium Ferrocyanide Transport through Immobilized Glucose Oxidase Matrix Attached to Disk of RRDE

attached to disk of RRDE	diffusion coefficient $\times 10^5$ (cm^2/s)	shielding coefficient
aqueous	0.61 ± 0.03	0.85
(aminopropyl)-triethoxysilane	0.66 ± 0.02	0.87
silane-glutaraldehyde-glucose oxidase	0.57 ± 0.05	0.87
allylamine-glutaraldehyde-glucose oxidase	0.46 ± 0.08	0.87
albumin-glutaraldehyde-glucose oxidase		0.99
literature (aqueous)	0.63[a]	
calculated[b]		0.84[b]

[a] Adams (1969). [b] Bard & Faulkner (1980). Theoretical value based on geometric considerations only. Experiments in present study done at 25 °C.

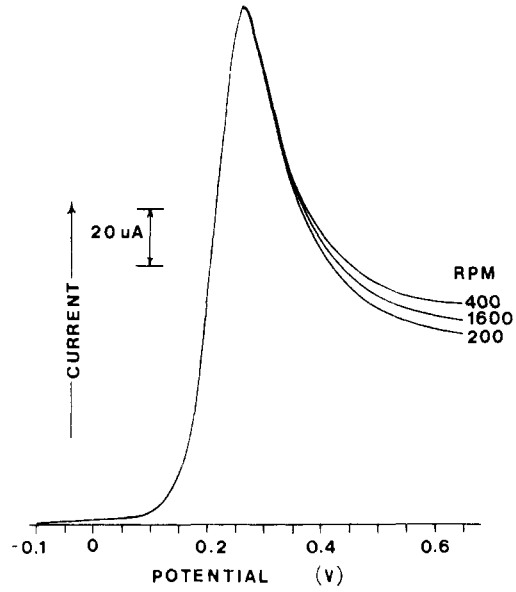

FIGURE 3: Measured anodic current at different applied disk potentials for a cross-linked albumin–glutaraldehyde–glucose oxidase matrix attached to the disk electrode of the RRDE. Solution contained 6 mM potassium ferrocyanide plus 1 M KCl. Disk potential scanned at 2 V/min −100 to +700 mV. Measurements done at 25 °C in deoxygenated solution.

albumin case decreased markedly at higher potentials (Figure 3). The maximum current was influenced only slightly by the rotation speed and thus did not follow the current vs. rotation relationship of eq 1. Determination of diffusion coefficients for ferrocyanide transport through the albumin–enzyme matrix could not be done under the experimental conditions that were used in Figure 3.

Shielding coefficients were determined by using the RRDE with glucose oxidase attached to the disk by the silane, allylamine, and cross-linked methods. To measure the shielding coefficient, a fixed potential of 800 mV was applied to the ring electrode. At this potential, any ferrocyanide reaching the ring was oxidized immediately to ferricyanide, so that the resulting mass-transfer limited ring current was taken as a direct measure of the concentration of ferrocyanide in the vicinity of the ring. At the same time that the ring current was being measured, the potential applied to the disk was changed at a fixed rate of 2 V/min from an initial value of −100 mV to a final value of +800 mV. At high disk potentials any ferrocyanide reaching the disk would be oxidized, thus reducing the concentration of ferrocyanide reaching the ring. The

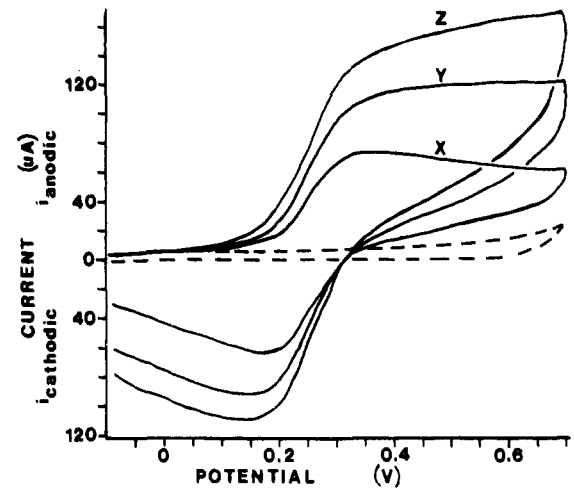

FIGURE 4: Tracings of cyclic voltammograms at a stationary platinum electrode, having a 0.063 cm thick albumin–glutaraldehyde–glucose oxidase matrix attached to the platinum surface. Voltammograms were obtained in 1 M KCl under nitrogen atmosphere at 25 °C and at a sweep rate of 3 V/min with 5–20 min between sweeps. At time equals zero the bulk solution concentration of potassium ferrocyanide was zero, shown by the dashed line. Voltammograms X, Y, and Z were taken at increasingly longer times after addition of ferrocyanide to give an initial bulk concentration of 1.2 mM.

shielding coefficient is defined as unity less the fractional reduction in limiting ring current due to ferrocyanide oxidation at the disk. Values of the shielding coefficients are listed in Table I for the different enzyme immobilization methods.

The concentrations of enzyme attached to the platinum disk via the silane and allylamine methods were determined by using ^{125}I-labeled glucose oxidase. The immobilized enzyme preparations were washed for 24 h to remove loosely attached enzyme. The results showed that 2.4×10^{-11} mol of enzyme protein/cm^2 of platinum were attached by the silane method and 9.2×10^{-11} mol/cm^2 by the allylamine technique. Continued washing with buffer over the next 13 days showed a total loss of only 6% from the silane–enzyme and 3% from the allylamine–enzyme electrodes. This indicated that the attachment was quite tight. Enzyme loading of the albumin–glutaraldehyde matrix was 1.9×10^{-11} mol of enzyme protein/cm^2 of 0.32 cm thick matrix or 4.9×10^{-11} mol/cm^3 of matrix (Wingard et al., 1983).

Mass Transfer at Stationary Electrode. Transport of potassium ferrocyanide through the 0.063 cm thick cross-linked albumin–glucose oxidase matrix was monitored by repetitive cyclic voltammograms. Figure 4 shows a scan before and several scans after the addition of ferrocyanide to the bulk solution side of the matrix. The peak anodic (oxidation) current occurred at about 350 mV and the peak cathodic (reductive) current at about 150 mV. The peak current for a reversible electrochemically active material such as ferrocyanide is related to the voltammetry variables as follows:

$$i_p = kn^{3/2}AD^{1/2}v^{1/2}C_r \quad (2)$$

where i_p is the peak current, k is a constant, v is the sweep rate of the applied potential (V/s), and C_r is the concentration of reduced solute in the enzyme matrix (mol/cm^3) (Bard & Faulkner, 1980). The other symbols are the same as for eq 1. Both influx and efflux measurements were made. Under influx conditions the peak current reached a maximum value, $i_{p,max}$, after 1000–1900 min. At this time C_r would have equaled C_r^*, the concentration of ferrocyanide in the bulk solution. At the start of the efflux experiments, the concentration at the electrode surface also was assumed to be the same as C_r^*. Extensive partitioning of ferrocyanide between

Table II: Initial Rate of Change of Ferrocyanide Concentration at Stationary Platinum Electrode for Ferrocyanide Diffusion through Albumin-Glutaraldehyde-Glucose Oxidase Matrix

thickness of matrix (cm)	C_r^* (mM)	initial rate of change of concn[a] (μM/min)	calculated flux (μmol cm^{-2} min^{-1}) with matrix[b]	calculated flux (μmol cm^{-2} min^{-1}) without matrix[c]
0.063	2.9	24 ± 3.0	0.002	17
0.16	5.5	40 ± 7.3	0.006	13
0.21	1.2	5 ± 0.1	0.001	2.1

[a] Mean of anodic, cathodic, influx, and efflux, ±SD, at 25 °C. [b] Calculated by using eq. 5; 25 °C. [c] Calculated by using eq 6; 25 °C.

the matrix and the solution was ruled out since spectrophotometric measurements (Winograd et al., 1969) showed that the bulk solution concentration of ferrocyanide did not change during the course of these experiments. Equation 2 can be used to set up the following ratio:

$$\frac{i_p}{i_{p,max}} = \frac{C_r}{C_r^*} \tag{3}$$

Upon differentiation and rearrangement, eq 3 gives

$$\frac{dC_r}{dt} = \frac{C_r^*}{i_{p,max}} \frac{di_p}{dt} \tag{4}$$

The initial values of di_p/dt were estimated for the peak anodic and cathodic currents by obtaining the initial slopes from the plots of i_p vs. time (Figure 5). The values of dC_r/dt, calculated from eq 4, are shown in Table II.

The initial values of dC_r/dt in Table II can be converted to the corresponding fluxes of ferrocyanide by using eq 5,

$$J_{init} = G\frac{dC_r}{dt} \tag{5}$$

where J_{init} is the initial flux in mol cm^{-2} min^{-1} and G is the surface-to-volume ratio in cm^3 cm^{-2}. G represents the volume of solution (per cm^2 of platinum surface) in which the change of concentration took place. Initially, G can be approximated as the volume of liquid that constituted the entire thickness of the matrix. The extent of diffusional resistance caused by the matrix can be estimated by comparing the flux through the matrix (calculated from eq 5) with the flux in the absence of the protein matrix. The flux without the matrix is calculated for diffusion of ferrocyanide through a quiescent solution of the same thickness as the matrix but without the cross-linked protein being present. Initially, the concentration of ferrocyanide at the platinum surface was zero. Therefore, the concentration gradient across the same thickness of quiescent solution would be C_r^*/d, where d represents the thickness of the matrix. When 0.61×10^{-5} cm^2/s is used as the diffusion coefficient for potassium ferrocyanide through aqueous solution (Table I), the initial flux of ferrocyanide can be calculated from eq 6 where J_{init} is in μmol cm^{-2} min^{-1}. The calculated

$$J_{init} = (0.61 \times 10^{-5})(60)\frac{C_r^*}{d} \tag{6}$$

fluxes for diffusion with and without the matrix are shown in Table II. Comparison of the flux values shows that a high degree of diffusional resistance was imparted by the cross-linked albumin–glucose oxidase matrix. This conclusion is in agreement with results reported elsewhere for enzyme activity measurements with cross-linked albumin–glucose oxidase matrices (Wingard et al., 1983).

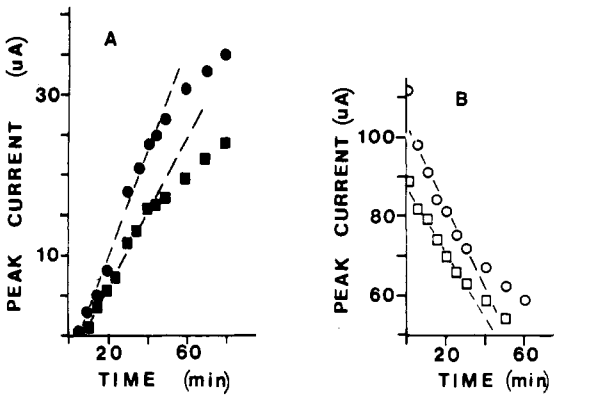

FIGURE 5: Peak anodic currents at 350 mV and cathodic currents at 150 mV from cyclic voltammograms for oxidation and reduction of ferrocyanide–ferricyanide, respectively, at stationary platinum foil electrodes. A matrix of albumin–glutaraldehyde–glucose oxidase was attached to the platinum surface, and the analyte had to pass through the matrix in moving between the bulk solution and the platinum surface. The currents are plotted against the time after addition of ferrocyanide to the bulk solution (influx) (A) or after saturation of the matrix with ferricyanide (efflux) (B). Circles represent anodic and squares cathodic currents. Solid symbols are for influx and open symbols for efflux. Slopes of dashed lines were taken as the initial rates of change of current.

Estimation of Kinetic Constants for Immobilized Glucose Oxidase Using RRDE. In determining the kinetic constants it is important to establish whether mass transfer or the enzymatic reaction is rate controlling under the conditions of the measurements. Since the mass-transfer and enzymatic reaction processes occur in series, the rate of mass transfer must equal the rate of the enzymatic reaction when the overall system is operating at steady state. These equalities can be expressed by eq 7 and 8 for enzymatic reaction plus external

$$(\beta_g^* - \beta) = H_E\left(\frac{\beta}{1+\beta}\right) \tag{7}$$

$$\frac{d^2\beta}{dz^2} = H_I^2\left(\frac{\beta}{1+\beta}\right) \tag{8}$$

and internal mass transfer processes, respectively (Engasser & Horvath, 1976), where β and z are the dimensionless glucose concentration and distance from the enzyme-matrix surface, respectively, β_g^* is the dimensionless glucose concentration in the bulk solution, and H_E and H_I are the dimensionless external and internal substrate moduli, respectively. The moduli represent the ratio of the rate of enzyme-catalyzed reaction to the rate of mass transfer. Thus, a large value (>10) of H_E or H_I indicates mass transfer as the rate-limiting step, while a small value (<0.1) is indicative of the reaction as rate controlling. The rate-controlling step for a given set of experimental conditions can be established by calculating values for H_E or H_I.

H_E is defined as follows:

$$H_E = \frac{k_3 C_E d}{h_s K_m'} = \frac{k_3 C_E t_K}{K_m'} \tag{9}$$

This definition is similar to that of Engasser & Howath (1976) with k_3C_E representing the maximum velocity of the enzyme reaction, C_E the enzyme concentration per volume, d the thickness of the enzyme matrix, K_m' the apparent Michaelis constant, and h_s the mass transfer coefficient for convection–diffusion movement of glucose through the external boundary layer. The modification, replacing d/h_s by t_K, gives the definition of H_E employed by Shu & Wilson (1976) for a convection-reaction system. In eq 9 t_K represents the time constant

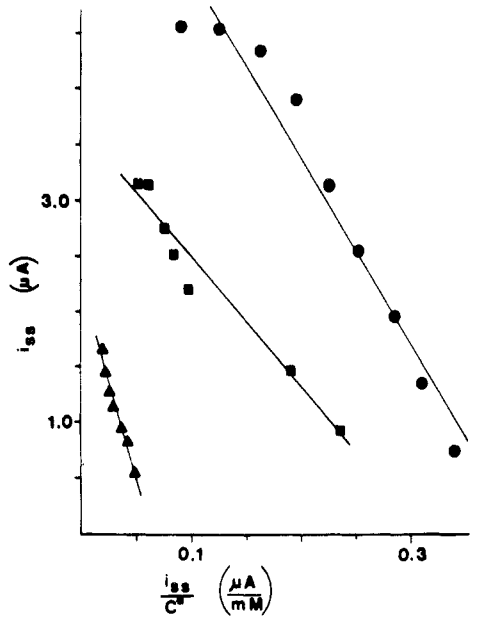

FIGURE 6: Eadie–Hofstee type plots of steady-state ring current and bulk solution concentration of glucose obtained with glucose oxidase immobilized on the disk of the RRDE. Steady-state current represents rate of arrival of hydrogen peroxide at ring and presumably also rate of glucose oxidase catalyzed production of hydrogen peroxide. Lines fit to eq 12 by linear least squares. Symbols represent glucose oxidase immobilization by allylamine (●), silane (▲), and albumin cross-linking (■) methods. Data obtained at 600 rpm, 25 °C, and aerobic conditions.

for the rate of convective flow past the ring–disk of the RRDE. At 600 rpm t_K equals 0.29 s on the basis of a diffusion coefficient for glucose in free solution of 0.67×10^{-5} cm^2/s at 25 °C (Weast, 1968) as calculated per the method of Prater & Bard (1970).

H_I is defined as follows:

$$H_I^2 = \frac{d^2 k_3 C_E}{D K_m'} \tag{10}$$

Values of H_E and H_I were calculated after first estimating K_m' and the maximum current, i_{max}, from the RRDE data.

Mell & Maloy (1975) used the following relationship to simulate the currrent–concentration interaction for an enzyme membrane operating under conditions where the enzymatic reaction was rate controlling:

$$\frac{i_{max}}{i_{ss}} = K_m' \frac{1}{C_g^*} + 1.0 \tag{11}$$

where i_{max} is defined as the ring current obtained when the enzymatic reaction was operating at maximum velocity. Equation 11 can be rearranged to the familiar Eadie–Hofstee form (eq 12):

$$i_{ss} = i_{max} - K_m' \left(\frac{i_{ss}}{C_g^*} \right) \tag{12}$$

where i_{ss} is the steady-state ring current and C_g^* is the concentration of glucose in the air saturated bulk solution. Equation 12 (i_{ss} vs. i_{ss}/C_g^*), rather than eq 11 ($1/i_{ss}$ vs. $1/C_g^*$), was used in the analysis of the data because eq 12 should give a clearer indication of the presence of external diffusional resistance to substrate transport (Engasser & Horvath, 1976). Plots of i_{ss} vs. i_{ss}/C_g^* are shown in Figure 6 for glucose oxidase immobilized on the platinum disk by the allylamine, silane, and albumin matrix techniques. The i_{max} and K_m' values obtained from these Eadie–Hofstee type plots are shown in Table III at three rotation speeds. The rotation speed should influence the values of K_m' only if external diffusional resistance was the rate-controlling process; the apparent Michaelis constant then would be expected to increase with greater diffusional resistance, i.e., lower rotation speed. The results in Table III show little change in K_m' with rotation rate. The values of K_m' in Table III are consistent with literature results. Weetall & Hersh (1970) obtained Michaelis values of 9.2 mM for soluble glucose oxidase and 7.1 mM for the enzyme immobilized on nickel oxide via silane–thiophosgene linkage. Their assays were carried out spectrophotometrically at pH 6.0. Kamin & Wilson (1980) reported spectrophotometrically measured values of K_m' of 3.1–19.1 mM for glucose oxidase immobilized in three different systems: (1) graphitic oxide and Teflon, (2) allylamine–platinum, and (3) carbon paste–octadecylamine. Similar electrodes measured electrochemically by these authors gave slightly higher values of K_m' of 5.2–29.0 mM. The K_m' values determined by Bourdillon et al. (1980) for glucose oxidase coupled by carbodiimides to a glass carbon cylinder ranged from 12 to 58 mM.

Table III: Kinetic Constants for Glucose Oxidase Immobilized on the Disk of the RRDE[a]

method of glucose oxidase immobilization	electrode (rpm)	i_{max} (μA)	K_m' (mM)	enzyme loading on disk (mol/cm^2)
allylamine	200	6.2	13	9.2×10^{-11}
	400	6.4	14	
	600	6.6	16	
silane	200	2.2	42	2.4×10^{-11}
	400	2.2	36	
	600	2.2	36	
albumin	200	3.9	5	1.8×10^{-11}
	400	3.4	6	
	600	3.6	12	

[a] Values obtained by fitting experimental data to eq 12.

Table IV: Calculated Values of H_E and H_I

coupling	d (cm)	$k_3 C_E$ (mol cm^{-3} s^{-1})	H_E[a]	H_I[a]
silane	0.0018	6.5×10^{-8}	5×10^{-4}	0.03
allylamine	0.0018	1.9×10^{-7}	3×10^{-3}	0.07
albumin	0.3	6.5×10^{-10}	2×10^{-5}	0.8

[a] Calculated by using values of i_{max} and K_m' at 600 rpm and $t_K = 0.29$ s.

Values for H_E and H_I were calculated by using eq 9 and 10 plus the Table III data for i_{max} and K_m' at 600 rpm. The maximum current was used to calculate dk_3C_E since by definition

$$i_{max} = nFAdk_3C_E \tag{13}$$

The values of d for the silane and allylamine methods of attachment were estimated to be the same as the boundary layer thickness, δ, at a rotation rate of 600 rpm. This thickness was calculated, as described in Bard & Faulkner (1980), by using eq 14. The calculated H_E and H_I values (Table IV)

$$\delta = \frac{1.61 D^{1/3} \nu^{1/6}}{w^{1/2}} \tag{14}$$

indicate that diffusional resistances were negligible and that the enzyme-catalyzed reaction was the rate-controlling step, with the exception of the albumin matrix. The calculated H_I value of 0.8 for albumin represented a minimum, since the diffusion coefficient for glucose in free solution, 6.7×10^{-6} cm^2/s at 25 °C (Weast, 1968), was used in the calculation.

If significant internal diffusional resistance was present, then the actual value of D would have been smaller. The albumin H_1 value would have been 2.7 or 8.5 if the glucose diffusion coefficient had been decreased by a factor of 10 or 100, respectively. Thus, for the albumin matrix the values of i_{max} and K_m' were influenced to some extent by internal diffusional resistance to glucose transport.

Discussion

The three electrochemical procedures used in this study all gave consistent results. The diffusional resistance of small molecules (potassium ferrocyanide or glucose) was markedly greater for the thick cross-linked albumin–enzyme matrix than for the thin allylamine–enzyme or silane–enzyme matrices.

The use of the Levich equation (eq 1) to measure diffusion coefficients was based on maintaining laminar flow of fluid across the face of the disk and on having essentially a homogeneous medium. The silane– or allylamine–glutaraldehyde–enzyme sequence attached to the surface of the disk most likely did not protrude beyond the thickness of the stagnant fluid boundary layer. The silane– and allylamine–glutaraldehyde chains were about 20 Å long on the basis of an average bond length of 1.6 Å (Gould, 1959), and the spin diameter of glucose oxidase was estimated as 170 Å (Messing, 1974). The total length of 190 Å was much less than the estimated 1.8×10^{-3} cm for the thickness of the hydrodynamic boundary layer. Since the experimentally determined limiting current varied linearly with the square root of the rotation speed (eq 1), it was concluded that the silane and allylamine systems behaved as quasi-homogenous media.

The albumin–enzyme matrix did not show similar homogeneity with the bulk solution. The ferrocyanide initially present in the vicinity of the disk surface underwent rapid oxidation, and the rate of ferrocyanide diffusion through the albumin–enzyme matrix was too slow to replenish the oxidized material (Figure 3). The current thus mirrored this depletion of ferrocyanide in the vicinity of the electrode. Use of a different rate for varying the potential applied to the electrode might have influenced the shape of the curve in Figure 3, but once the redox potential for ferrocyanide was exceeded (about 0.25 V), then the rate of sweep would not have been a major factor. The lack of effect of rotation speed on the current was further evidence that the mass-transfer resistance was located within the albumin–enzyme matrix and not in the fluid boundary layer.

The values of the diffusion coefficients obtained with eq 1 may have been influenced by the degree to which the silane and allylamine blocked part of the platinum surface. Murray (1980) calculated that a silane coating of $(0.8–4.0) \times 10^{-10}$ mol/cm^2 was roughly equivalent to a noncompacted monolayer of attached material. He pointed out that in general, the data in the literature suggest loosely organized surface structures with considerable opportunity for movement of the appendages and interfacial fluidity. Since the loadings in this study were $(2–9) \times 10^{-11}$ mol/cm^2, the surface probably was not covered with a monolayer of silane, although monolayer coverage by the attached glucose oxidase could not be rejected.

The shielding coefficient measurements were included in this study to provide additional data for the use of electrochemical techniques in the determination of immobilized enzyme mass-transfer parameters. Although the shielding coefficient is not a directly usable parameter in enzymology, it has application for describing mass-transfer events for enzymes in membranes.

In the stationary electrode cyclic voltammetry studies the bulk solution concentration was slightly different for each of the three matrix thicknesses (Table II). Since the 0.16 cm thick matrix had the highest initial bulk solution concentration of ferrocyanide, then the same matrix also would be expected to have the largest initial rate of change of concentration at the platinum side of the matrix. This is in accord with the results in Table II. The conclusion from Table II that the matrix reduced the rate of diffusion of ferrocyanide by a factor of 1000 or more is in agreement with potentiometric measurements made with a series of different thickness glucose oxidase–albumin–glutaraldehyde matrices (Wingard et al., 1983). A series of glucose oxidase albumin platinum matrices of 0.05, 0.16, 0.24, and 0.32 cm thick was used to determine the potentiometric response to air-saturated solutions having different concentrations of glucose. The potentiometric reesponse showed the presence of minor diffusional resistance for the 0.05 cm thick matrix. The resistance increased markedly with matrix thickness, until essentially no response was seen with the 0.32 cm thick matrix. Although different diffusional resistances would be expected for glucose and potassium ferrocyanide, due to molecular weight and ionic charge variances between the two molecules, still the marked influence of diffusional effects with the two solutes in quite similar enzyme matrices was strong support for the validity of the Table II results.

In the determination of enzyme kinetic parameters the concentration of immobilized enzyme is an important factor that can influence the degree of diffusional control. For example, a high concentration of enzyme loading in the immobilization matrix requires rapid transfer of substrate into the matrix to maintain the enzyme-catalyzed reaction rate at 50% or more of the maximum rate. Thus, a high enzyme loading often results in operation with internal mass transfer as the rate-limiting step. In the present study the enzyme loadings were rather low (Table III), thus favoring reaction kinetic control. The increase in i_{max} with the allylamine linkage was roughly proportional to the greater enzyme loading of the allylamine, as compared to the silane, linkage. However, comparison of the i_{max} and enzyme-loading results for the silane and albumin systems shows that the albumin electrode gave a larger i_{max} but with slightly less enzyme. This discrepancy may have resulted from differences in the percentage of enzyme that remained active on immobilization. This percentage was estimated by comparing k_3C_E, calculated from the electrochemical data (Table IV), with values of k_3C_E determined by independent means. Weibel & Bright (1971a) determined a value of 0.83×10^3 s^{-1} for k_3 (k_{cat} in referenced paper) at pH 7.4 and 25 °C and showed that this value did not change on immobilization of glucose oxidase (Weibel & Bright, 1971b). The enzyme-loading values in Table III constitute the total active plus inactive immobilized enzyme. These loadings can be converted from mol/cm^2 to mol/cm^3 through dividing by d from Table IV. The resulting values for the independently determined k_3C_E were 4.2×10^{-5} mol cm^{-3} s^{-1} for the allylamine coupling, 1.1×10^{-5} for the silane, and 5.0×10^{-8} for the albumin. Taking the ratio of the electrochemically determined values of k_3C_E (Table IV) to the ones calculated above shows that only 0.5%, 5.9%, and 13.0% of the allylamine-, silane-, and albumin-coupled enzymes, respectively, were active according to the electrochemical measurements. Thus, the higher value of i_{max} for the albumin, as compared to that of the silane, coupling could be explained by assuming that the higher retention of activity more than offset the lower loading of enzyme.

The principal reason for the larger values of K_m' obtained with the silane coupling, as compared to those of the allylamine

and albumin couplings, is not clear. Two possible explanations are that a smaller diffusion coefficient or less of a concentration gradient was present for glucose with the silane coupling. The data in Table I show that the diffusion coefficient for potassium ferrocyanide was reduced 14% by the silane–enzyme matrix and 30% by the allylamine–enzyme matrix, as compared to diffusion in aqueous solution. The smaller diffusion coefficient with allylamine may be attributed to the combination of greater loading plus a more tightly organized surface structure with less freedom of movement of the appendages. Carbon–carbon double bonds adsorb strongly to platinum (Land & Hubbard, 1973), so that all parts of the short length allylamine molecule, $CH_2{=}CH{-}CH_2{-}NH_2$, would be close to the platinum surface. The large glucose oxidase molecules would be displaced from the surface only by the length of the glutaraldehyde molecule. With the silane linkage one can envision the silicon binding to the platinum oxide to give a more loosely organized surface structure with the glucose oxidase molecules spaced 6–8 Å further from the platinum surface than with the allylamine.

No mention has been made of the role that the dissolved oxygen concentration might play. This study was carried out with air-saturated solutions, which could have become depleted of oxygen at the enzyme-electrode interface under high enzyme loadings or with prolonged operation. However, with the relatively low enzyme loadings used in this work, it is doubtful that the transport of oxygen was a major factor.

In summary, it is apparent that the resistance to transport of small molecules through the immobilized enzyme matrix was dependent on the method of glucose oxidase immobilization. However, by use of a variety of highly sensitive electrochemical techniques it was possible to estimate and in some cases quantify the degree of mass-transfer resistance. The study also demonstrated the combination of electrochemical measurements and Eadie–Hofstee type data analysis to obtain apparent values of the Michaelis constant for immobilized glucose oxidase.

Registry No. Glucose, 50-99-7; glucose oxidase, 9001-37-0; potassium ferrocyanide, 13943-58-3.

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