

Comparison of Spectrophotometric and Amperometric Rate Parameters of Enzymatic Reactions

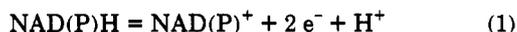
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In the context of current interest in electroanalytical sensors for biomedical applications, enzyme kinetic parameters obtained amperometrically were compared with those determined spectrophotometrically for ethanol oxidation catalyzed by alcohol dehydrogenase. The amperometry was conducted at a platinum rotated disk electrode, utilizing hexacyanoferrate(III/II) as an electron shuttle between the enzyme-catalyzed redox reaction and the electrode. Overall good agreement was seen between results obtained by the two methods except for V_{\max} . The amperometric Michaelis constant, K_M , for ethanol was 3.2 mM, comparable to a reported value of 3 under similar experimental conditions. The amperometric turnover rate, k_{cat} , was 448 s⁻¹, while the spectrophotometric literature assignment is 450 s⁻¹. The amperometric K_M for cosubstrate NAD was 0.80 mM versus spectrophotometric literature values ranging from 0.1 to 1 mM. On the other hand, the amperometric maximum initial reaction velocity, V_{\max} , was 15% lower than its spectrophotometric counterpart.

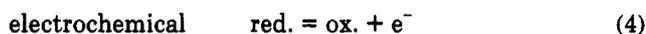
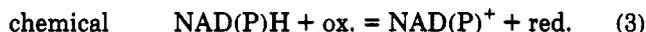
INTRODUCTION

Amperometric sensors have seen extensive biomedical application in recent years due to the ease with which they can be coupled to biologically important enzymatic redox reactions. Such oxidoreductase-catalyzed reactions invariably involve an electron-transfer step which can be transduced, by a suitably designed amperometric sensor, into a current which may serve to monitor substrate and/or product concentrations. X-ray crystallographic studies¹ and indirect evidence from mediator-modified enzymes^{2,3} suggest that catalytic sites of redox enzymes are often deeply embedded, minimizing the possibility of direct charge transfer from the site of the reaction to an electrode surface. In the large group of redox enzymes which depends on the soluble pyridine nucleotide cofactor, NAD(P)⁺/NAD(P)H, this limitation could, in principle, be overcome because the cofactor serves as a soluble charge carrier and is capable of electron transfer at an electrode. However the process



entails a large anodic overpotential at all known electrode materials and may result in decreased selectivity when other electrooxidizable species are present in solution. Moreover, NADH adsorbs strongly at platinum electrodes and proceeds through a complicated radical mechanism.⁴ These difficulties can be circumvented through the use of an amperometric

couple capable of homogeneous redox reaction, in aqueous solution, with the pyridine nucleotide moiety and having facile heterogeneous electron-transfer kinetics at the electrode surface. A generalized three-step sequence may consist, for instance, of the reactions



where *s-H* and *s* represent the reduced and oxidized forms of the substrate, respectively, and *ox.* and *red.* represent the oxidized and reduced forms of the amperometric couple.

The ADH-catalyzed oxidation of alcohols is a bireactant, biproduct reaction involving a ternary alcohol-NAD-ADH complex.⁵ This is similar to the enzyme mechanism proposed for alcohol oxidase-catalyzed oxidation of ethanol we have discussed in an earlier publication.⁶ Conventional single-substrate enzyme kinetics rely on the assumption that the overall process



proceeds from the substrate (S) to the product (P) in the presence of the enzyme (E) via the sequence



where ES is a binary substrate-enzyme complex (the "Michaelis-Menten" intermediate). This model leads to the well-known relationship

$$V_0 = \frac{V_{\max}[\text{S}]}{K_M + [\text{S}]} \quad (8)$$

where V_0 is the initial velocity of reaction 7 whose rate-determining step is reaction 9, V_{\max} denotes the limiting maximal velocity that would be observed if all the enzyme is present as the complexed ES form, [S] is the substrate concentration, and K_M is the so-called Michaelis-Menten constant. The parameter K_M expresses the relationship between the steady-state concentrations of reactants and intermediates,⁷ and it can be shown that

$$K_M = \frac{k_2 + k_{-1}}{k_1} = \frac{[\text{E}][\text{S}]}{[\text{ES}]} \quad (9)$$

When the substrate concentration equals the value of K_M ,

(5) Brändén, C.; Jörnvall, H.; Eklund, H.; Furugren, B. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XI, pp 103-190.

(6) Jordan, J.; Ciolkosz, M. *J. Solution Chem.* 1991, 20, 995-1000.

(7) Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, 1975; Chapter 6.

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† Deceased.

(1) Eklund, H.; Plapp, B. V.; Samama, J.; Brändén, C. *J. Biol. Chem.* 1982, 25, 14349-14358.

(2) Degani, Y.; Heller, A. *J. Phys. Chem.* 1987, 91, 1285-1289.

(3) Bartlett, P. N.; Whitaker, R. G.; Green, M. J.; Frew, J. *J. Chem. Soc., Chem. Commun.* 1987, 1603-1604.

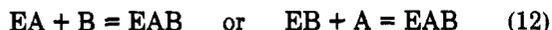
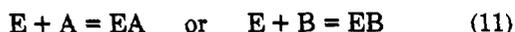
(4) Samec, Z.; Elving, P. *J. Electroanal. Chem. Interfacial Electrochem.* 1983, 144, 217-234.

the initial velocity, V_0 , is half of the saturation velocity, or $1/2 V_{\max}$.

Multisubstrate systems such as reaction 2 of the type



may proceed via ternary intermediate complexes through a sequential mechanism such as



Reaction sequence 11–13 is characterized by the feature that both the substrate (A) and the cosubstrate (B) first bind with the enzyme (E) in a ternary complex (EAB) before any products are released. If the transformation of the ternary complex into products is relatively slow and that of complex formation is rapid, the following simplified velocity equation may be obtained for such a system:⁸

$$V_0 = \frac{V_{\max}}{1 + \frac{(K_M)_A}{[A]} + \frac{(K_M)_B}{[B]} + \frac{(K_M)_{AB}}{[A][B]}} \quad (14)$$

where two Michaelis–Menten constants, $(K_M)_A$ and $(K_M)_B$, and corresponding substrate (A) and cosubstrate (B) concentrations are implicated, and the last term of the denominator relates to the mutual interaction of A and B at the binding site. Under conditions where the concentration of substrate A is much greater than its Michaelis constant, $[A] \gg (K_M)_A$, velocity eq 14 can be reduced to

$$V_0 = \frac{V_{\max}}{1 + (K_M)_B/[B]} = \frac{V_{\max}[B]}{(K_M)_B + [B]} \quad (15)$$

A similar approach with $[B] \gg (K_M)_B$ will result in a velocity equation in terms of substrate A and $(K_M)_A$. Such equations correspond to the velocity equation of a single-substrate reaction and allow simplified Michaelis constant evaluation.

As the reaction proceeds, substrate concentrations gradually decrease and, as the product concentration builds up, the unidirectionality of the reaction no longer exists. This invalidates the assumptions used to derive the V_0 equations. Hence, it is essential to make measurements within the time interval during which reaction conditions do not deviate significantly from those required for the velocity equations.

A variety of approaches have been used to monitor the progress of pyridine nucleotide-dependent enzymatic reactions. Spectrophotometric absorption of ultraviolet radiation by the reduced form of the cofactor, NAD(P)H, is the most commonly accepted method. However, few published studies have intercompared amperometrically and spectrophotometrically obtained enzyme kinetic parameters. Talbott⁹ found good agreement between spectrophotometrically determined values reported in the literature and his electrochemically determined Michaelis constant, K_m , for the substrate β -D-glucose but not for the cosubstrate of glucose oxidase, a bisubstrate, biproduct enzyme which does not involve a ternary complex. Other researchers have noted discrepancies as well. It is of general interest to compare results obtained spectrophotometrically and amperometrically, both for analytical purposes and as a way of probing

the possibility that mediators effect changes in the kinetic mechanism of the “natural” enzymatic reaction.

EXPERIMENTAL SECTION

Apparatus. Electrochemical experiments were performed in a water-jacketed, thermostated cell at a platinum rotated disk electrode (Pt-RDE, area = $3.2 \times 10^{-6} \text{ m}^2$) with a platinum wire auxiliary electrode and saturated calomel reference electrode (SCE). Constant-potential chronoamperometric experiments were carried out with an IBM EC/225 voltametric analyzer set at the desired potential. Data were acquired with a Rapid Systems 4 × 4 digital oscilloscope and/or a Yokogawa XYT recorder.

Reagents. All solutions were prepared with distilled water in which the conductivity was determined to be less than $11 \mu\Omega^{-1}$. Potassium hexacyanoferrate(III) (Baker), ethanol (Pharmco), β -nicotinamide adenine dinucleotides (NAD⁺ and NADH, Sigma), and yeast alcohol dehydrogenase, EC 1.1.1.1 (Sigma), were used without further purification. The supporting electrolyte was 0.2 M KCl in pH 8.8, 0.05 M pyrophosphate buffer.

Procedure. Unless otherwise stated, experiments were performed at 298 K under anaerobic conditions. Yeast alcohol dehydrogenase (Y-ADH) in concentrations appropriate to the experimental design (100 μM for initial velocity experiments, 400 nM for ethanol concentration determinations) was added to the electrochemical cell which held 10 mL of a buffered solution of ethanol and NAD⁺. Ethanol concentrations of 0.01–300 mM ($100K_M$) and NAD concentrations of 0.02–8 mM ($10K_M$), were used. Except as noted, the $K_3\text{Fe}(\text{CN})_6$ concentration was kept at 50 mM. Prior to each recording the working electrode was polished 3 min with 0.05- μm γ alumina (Buehler), rinsed in distilled water, and sonicated 10 s in distilled water. The cell was covered, and its contents were sparged prior to and blanketed during each experiment with N₂. The Pt-RDE was rotated at 900 or 1600 rpm with a Pine Instrument Co. analytical rotator. The potentiostat was set to maintain a potential difference of +500 mV vs SCE (a potential at which the anodic current was limited by mass transport of hexacyanoferrate from bulk solution to the electrode surface), and the anodic current was recorded as a function of time. Experiments to determine enzyme kinetic parameters were patterned after the initial velocity studies described by Segel.⁷ Slope measurements were made on the steepest part of the current–time response, representing the first 10–30 s of the reaction.

RESULTS AND DISCUSSION

Relationship of Current–Time Behavior to Hexacyanoferrate(II) Concentration. The ternary reaction sequence for the ADH-catalyzed oxidation of ethanol was

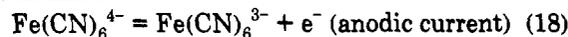
enzymatic



chemical



electrochemical



Reactions 16 and 17 are homogeneous reactions occurring in aqueous solution, while reaction 18 is heterogeneous, with electron transfer across the electrode–solution interface. Oxidation of ethanol, indicated by the forward direction of reaction 16, proceeds to virtual completion at pH 8.8. Reaction 18 is fast compared to reactions 16 and 17 at the applied potential (500 mV vs SCE) where the rate is mass transport limited. Figure 1 shows the relative concentration profile of the reactants and products in this sequence. Note that the $\text{Fe}(\text{CN})_6^{4-}$ concentration increases at twice the rate of ethanol depletion and that, after an initial decrease, the

(8) Michal, G. In *Principles of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie: New York, 1978; pp 29–34.

(9) Talbott, J. L. *Enzymatic Amperometry of Glucose*. Ph.D. Thesis, The Pennsylvania State University, 1987.

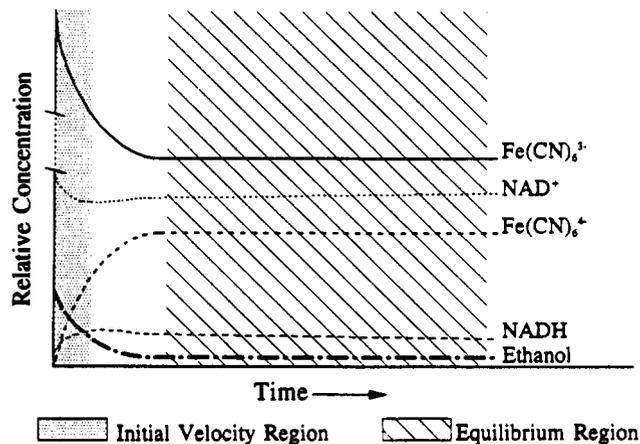


Figure 1. Relative concentration profile of reactants and products in ADH-catalyzed ethanol oxidation coupled to the electrode by hexacyanoferrate(II/III) (not to scale).

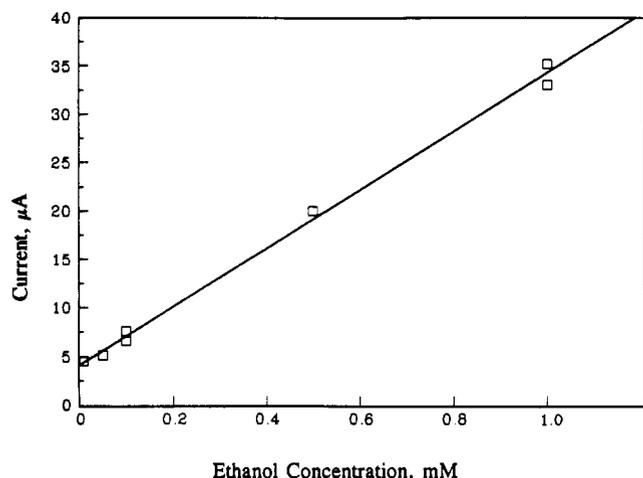


Figure 2. Linear range for amperometric ethanol concentration determination. Plot of i for hexacyanoferrate(II) oxidation at times corresponding to the equilibrium region of Figure 1.

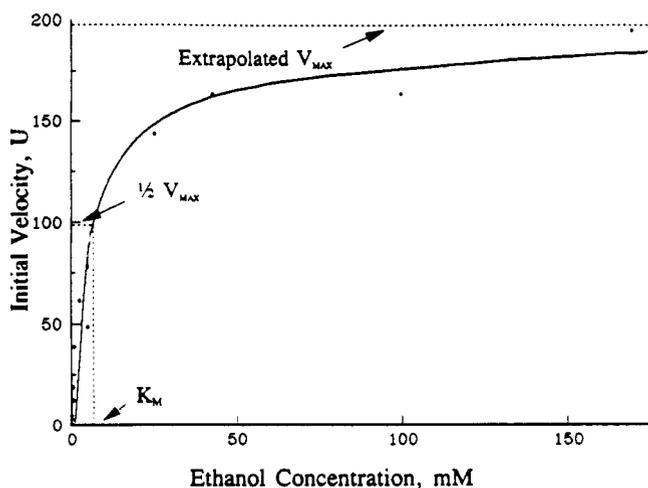


Figure 3. Michaelis plot showing initial reaction velocity in enzyme units. 1.0 unit of enzyme activity transforms 1.0 μmol of substrate/min at 298 K.

NAD⁺ concentration remains constant since virtually all NADH produced is immediately reoxidized by Fe(CN)₆³⁻ via reaction 17.

Hexacyanoferrate(II/III) meets many of the criteria for desirable redox couples, as listed by Coury et al.¹⁰ well-defined, reversible voltammetry, adequate aqueous solubility, availability in high purity, sufficient chemical stability, lack of

cross-reactivity with the enzymatic substrate, and ability to accept electrons from the reduced enzyme or the reduced cofactor. In addition, at moderate concentrations Fe(CN)₆³⁻ does not significantly affect the activity of the enzyme itself. The two-reaction sequence (reactions 17 and 18) has been studied at glassy carbon and carbon paste electrodes and applied to lactate dehydrogenase assays in serum.¹¹ Hexacyanoferrate(III) has been employed as a mediator or amperometric couple by other researchers with flavin enzymes.^{12,13} At a platinum electrode, hexacyanoferrate(II) ($E_{1/2} = 220$ mV vs SCE) is oxidized at a potential several hundred millivolts less anodic than NADH, significantly reducing the probability that side reactions (electrooxidation of other biological components which may be present in the sample) will occur. Nevertheless, some side reactions do occur. Analysis of the steady-state current obtained for these experiments which were allowed to proceed to virtual completion (equilibrium region of Figure 1) revealed an anodic current 2–9% in excess of the current predicted for 100% efficiency according to the Levich equation. The percent current excess is greatest for experiments using high enzyme concentrations and amounts to 0.6 $\mu\text{A}/\text{mg}$ of ADH. It is possible that impurities in the crystalline Y-ADH preparation, such as residual ethanol and NADH, are partly responsible for the observed current excess. On the other hand, some oxidation of ADH by hexacyanoferrate(III) may account for the excess current, which is proportional to the amount of enzyme present. We have encountered high oxidation currents and loss of enzyme activity with another enzyme, alcohol oxidase, in the presence of hexacyanoferrate(III) (unpublished data), and Durliat et al.¹³ have reported loss of enzyme activity with hexacyanoferrate(III) concentrations greater than 10 mM with lactate dehydrogenase. However, this effect with ADH is small and does not invalidate determination of ethanol concentration or kinetic parameters discussed below. Initial reaction rates were determined from the slope of the current–time graph during the first 30 s of the reaction (initial velocity region of Figure 1), using the Levich equation:

$$i_1 = 0.620nFAD^{2/3}\omega^{1/2}\nu^{-1/6}C^*$$

in which D , the diffusion coefficient of Fe(CN)₆⁴⁻, is 6.5×10^{-10} m²/s at 298 K (Adams¹⁴), ν , kinematic viscosity, is 1.0×10^{-6} m²/s, A is the area of the Pt-RDE, C^* is the bulk concentration of Fe(CN)₆⁴⁻, $n = 1$ is the number of electrons transferred in the electrooxidation of Fe(CN)₆⁴⁻, and F is the Faraday constant. Since the amount of Fe(CN)₆⁴⁻ generated in reaction 17 is stoichiometrically related to the amount of ethanol reacted

$$\Delta C^*_{\text{Fe(CN)}_6^{4-}} = -2\Delta C^*_{\text{ethanol}}$$

i_1 represents a measure of the amount of ethanol that has been oxidized. The rate of change of the limiting current then gives the rate of change of concentration, i.e., the reaction rate:

$$\frac{\Delta i_1}{\Delta t} = 0.620nFAD^{2/3}\omega^{1/2}\nu^{-1/6}\frac{\Delta C^*}{\Delta t}$$

Initial rate analysis of experiments in which concentrations of Fe(CN)₆³⁻ or NADH were varied while the other was held

(10) Coury, L. A., Jr.; Oliver, B. N.; Egekeze, J. O.; Sosnoff, C. S.; Brumfield, J. C.; Buck, R. P.; Murray, R. W. *Anal. Chem.* 1990, 62, 452–458.

(11) Thomas, L. C.; Christian, G. D. *Anal. Chim. Acta* 1976, 82, 265–272.

(12) Talbott, J.; Jordan, J. *Microchem. J.* 1988, 37, 5–12.

(13) Durliat, H.; Comtat, M.; Mahenc, J. *Anal. Chim. Acta* 1976, 85, 31–40.

(14) Adams, R. N. *Electrochemistry at Solid Electrodes*; Marcel Dekker, Inc.: New York, 1969.

Table I. Comparison of Spectrophotometric and Amperometric Kinetic Parameters

kinetic param	spectrophotometric value	electrochemical value	method of data analysis ⁷
V_{\max}	100% of specified ADH assay value ^a	85% of specified ADH assay value	Eadie-Hofstee
$K_M(\text{ethanol})$, mM	3	3.2 ^b	Eadie-Hofstee
$K_M(\text{NAD}^+)$, mM	0.1-1	0.80 ^c	Eadie-Hofstee
k_{cat} , s ⁻¹	450	448 ^d	$\Delta[\text{NADH}, \mu\text{M}]/\Delta t$ per μM enzyme

^a Information supplied by manufacturer. ^b Sample standard deviation, $s_x = \sqrt{[n\sum x^2 - (\sum x)^2]/n(n-1)} = 0.5$, $n = 16$. ^c $s = 0.08$, $n = 10$. ^d $s = 42$, $n = 14$.

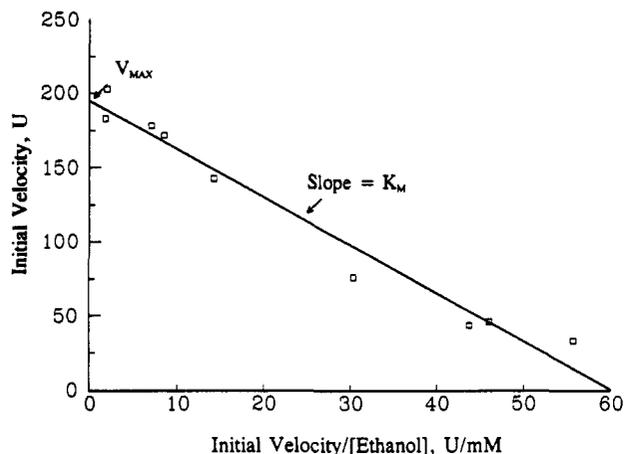


Figure 4. Eadie-Hofstee plot of initial reaction velocity versus initial velocity to ethanol concentration ratio. The Michaelis constant, K_M , for ethanol here is seen to be 3.2 mM.

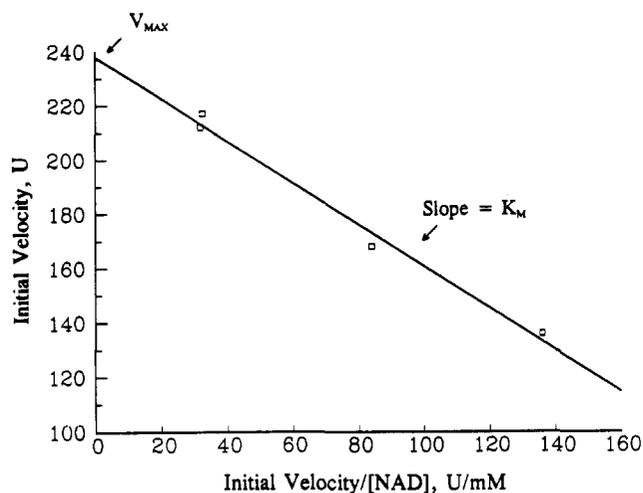


Figure 5. Eadie-Hofstee plot of initial reaction velocity versus velocity to NAD concentration ratio. The Michaelis constant, K_M , for NAD here is seen to be 0.8 mM.

constant clearly indicated that reaction 17 was first order with respect to both NADH and $\text{Fe}(\text{CN})_6^{3-}$. This is in agreement with the work of Carlson et al.¹⁵ who used ferrocenium salts as their 1-electron amperometric couple and found the reaction to be first order in both NADH and the amperometric couple. The rate constants for reaction 17 were determined from initial reaction rates to be 1.0 and 0.234 $\text{M}^{-1} \text{s}^{-1}$ at 298 and 279 K, respectively. An Arrhenius plot of these values, under the assumption that linearity prevails within this temperature range (not documented), indicates an activation energy barrier on the order of 50 kJ mol^{-1} .

The initial velocity of the ternary reaction sequence 16-18 was directly proportional to $\text{Fe}(\text{CN})_6^{3-}$ concentrations up to approximately 25 mM. At $\text{Fe}(\text{CN})_6^{3-}$ concentrations above 25 mM the velocity approached a maximum determined

primarily by the rate at which NADH was produced in reaction 16. Thus the rate-determining reaction in the sequence is reaction 16 and $\Delta i/\Delta t$ is a valid measure of the rate of enzyme-catalyzed ethanol oxidation under the prevailing experimental conditions. Concentration-dependent enzyme activity inhibition could not be discerned up to 150 mM $\text{Fe}(\text{CN})_6^{3-}$.

Enzyme Kinetic Parameters. Ethanol concentrations between 0.01 and 1 mM show an excellent linear current response ($R = 0.9982$), as can be seen from Figure 2. The current was measured 30 min after enzyme was added to the reaction mixture, at which time the reaction had nearly gone to completion (the so-called "equilibrium region" of Figure 1). This linearity is further documented in the Michaelis plot of initial reaction velocities (Figure 3). The maximum initial reaction velocity, V_{\max} , seen in Figure 3 was lower than the value determined spectrophotometrically with comparable NAD, ADH, and ethanol concentrations. The primary experimental difference was the presence of various concentrations of $\text{Fe}(\text{CN})_6^{3-}$ in the reaction mixture of the electrochemical experiments. Possibly, hexacyanoferrate(III) interacts with the enzyme in a concentration-independent manner and limits the maximum reaction velocity. The enzyme activity (V_{\max}) specified on the assay data sheet accompanying the Y-ADH purchased from Sigma was confirmed by spectrophotometric analysis (by the method of Hoch and Vallee as described in ref 21) in our laboratory. Electrochemical determination, however, consistently gave V_{\max} values approximately 15% lower than spectrophotometric determination.

Kinetic parameters determined amperometrically in our laboratory are summarized in Table I, together with spectrophotometric literature values. Michaelis constants, K_M , for ethanol and NAD were determined from initial reaction rates at the Pt-RDE. Analysis by Eadie-Hofstee plots and Lineweaver-Burk plots yielded similar values. A survey of the literature shows that these have been extensively studied spectrophotometrically. Dickinson and Monger,¹⁶ working at pH 7.05, obtained $K_M(\text{NAD}^+) = 0.109$ mM and $K_M(\text{ethanol}) = 21.7$ mM. Hayes and Velick¹⁷ determined the Michaelis constants, K_M , at pH 7.9 to be 0.17 mM (NAD^+) and 18 mM (ethanol). Burstein et al.¹⁸ reported K_M values of 1 and 3 mM for NAD^+ and ethanol, respectively, for ADH in pH 9.2 buffer solution. The considerable variability of the Michaelis constants reported above appears to be at least partially related to pH. A comparison of kinetic parameters determined spectrophotometrically with those determined electrochemically thus must take this factor into consideration. Albery¹⁹ worked out the steady-state kinetic equations for a conducting organic salt-membrane electrode and applied it to ADH-catalyzed alcohol oxidation.²⁰ He lists an "electrochemical Michaelis constant", K_{ME} , for ethanol of 2.7 mM at

(16) Dickinson, F. M.; Monger, G. P. *Biochem. J.* 1973, 131, 261-270.

(17) Hayes, J. E.; Velick, S. F. *J. Biol. Chem.* 1954, 207, 225-244.

(18) Burstein, C.; Ounissi, H.; Legoy, M. D.; Gellf, G.; Thomas, D. *Appl. Biochem. Biotechnol.* 1981, 329-338.

(19) Albery, W. John; Bartlett, P. N. *J. Electroanal. Chem. Interfacial Electrochem.* 1985, 194, 211-222.

(20) Albery, W. J.; Bartlett, P. N.; Cass, A. E. G.; Sim, K. W. *J. Electroanal. Chem. Interfacial Electrochem.* 1987, 218, 127-134.

(15) Carlson, B. W.; Miller, L. L.; Neta, P.; Grodkowski, J. *J. Am. Chem. Soc.* 1984, 106, 7233-7239.

pH 9.0. This is in good agreement with our results (K_M (ethanol) = 3.2 mM at pH 8.8) as determined from Eadie-Hofstee plots (Figure 4), in spite of experimental differences. The research reported here differs from the Albery experiment in that neither the enzyme nor the mediator were physically immobilized near the electrode surface. Thus, our system is dependent only on the well-documented diffusion coefficient of $\text{Fe}(\text{CN})_6^{4-}$ in water and is not affected by diffusion through membrane material. It is apparent that at pH closer to 7, the Michaelis constant for ethanol is much greater than in basic solution. This suggests that the enzyme has greater affinity for ethanol in basic solution, as anticipated from the fact that ethanol oxidation is optimal at pH 8.8 while equilibrium is strongly shifted in favor of aldehyde reduction²¹ at pH 7. The reverse trend for NAD^+ is difficult to explain but may in fact reflect variation due to experimental design. For many dehydrogenases the K_M for NAD^+ falls in the range 0.1–1 mM.²² As pH increased from 7.05 to 9.2, the reported K_M (NAD) increased from 0.109 to 1 mM. The K_M (NAD) = 0.80 mM value obtained amperometrically at pH 8.8 in our laboratory (Figure 5) is in general agreement with this pattern of pH dependency. Our amperometrically determined catalytic constant, k_{cat} , also known as the turnover number, was in excellent agreement with spectrophotometrically determined values.^{16,17}

(21) *Worthington Enzyme Manual*; Worthington, C. C., Ed.; Worthington Biochemical Corp.: Freehold, NJ, 1988.

(22) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman and Co.: San Francisco, 1977.

The averaged kinetic results of more than a dozen experiments are presented in Table I alongside spectrophotometrically determined parameters. Literature values used in this table are referenced in the discussion and were selected from research conducted at $\text{pH } 9 \pm 0.2$ whenever available.

CONCLUSIONS

A comparison of amperometrically (in the presence of an artificial mediator) and spectrophotometrically determined enzyme kinetic parameters shows overall good agreement between the two methods. We feel that the concordance between available spectrophotometric measurements in the literature and electrochemical measurements is generally so satisfactory that it indicates that the relevant enzymatic mechanisms of the natural enzymatic reaction and the one occurring in the presence of the mediator are the same. Only the maximum reaction velocity, V_{max} , determinations showed differences in excess of 10% which warrant further investigation.

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