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Studies towards the development of an ethanol sensor

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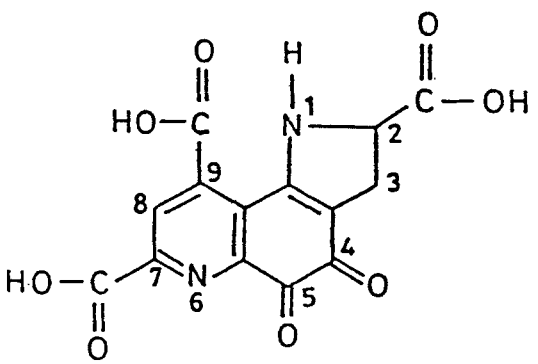
Abstract. An amperometric biosensor for ethanol is described. The sensor uses benzoquinone and ferrocene carboxylic acid as mediators for electron transfer between a quinoprotein, alcohol dehydrogenase and an edge plane pyrolytic graphite electrode. A linear current response proportional to ethanol concentration is observed in the range 1–10 mM.

Keywords. Benzoquinone; sensor; alcohol dehydrogenase; cyclic voltammetry.

1. Introduction

Biosensors based on NAD^+ and $NADP^+$ dehydogenases have attracted considerable attention because of their ubiquitous nature and large number[1]. While the cofactors, NAD^+ and $NADP^+$ aid in the enzymatic oxidation of the substrate, the direct oxidation of reduced NADH cannot be accomplished at low potentials without the help of mediators. In addition to those that use $NAD(P)^+$ as cosubstrates, there are a few dehydrogenases, termed quinoproteins[2], which contain the prosthetic group 2,7,9-tricarboxy-1H-pyrrolo (2,3-f)-quinoline-4,5 dione (PQQ).



An important property of these enzymes is that they do not require NAD^+ participation but use artificial electron acceptors[2,3]. Davis *et al*[4] have studied the reaction of the

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quinoprotein, alcohol dehydrogenase (ADH) using N,N,N',N'-tetramethyl-4-phenylenediamine (TMPD) as an electron acceptor in a biofuel cell with methanol as substrate and this has provided a sensitive method for the detection of primary alcohols. Cass *et al*[5] have suggested that ferricinium ion functions as a good electron acceptor at *p*H 10·5.

This paper reports cyclic voltammetric investigations of mediators such as ferrocene carboxylic acid (FCA) and *p*-benzoquinone (BQ) in the presence of alcohol dehydrogenase (ADH) and a typical substrate ethanol at an edge plane pyrolytic graphite (EPG) electrode. Murthy and Sharma[6] have recently shown BQ to be an efficient mediator, comparable to FCA in the development of sensors for a variety of substrates. The second order rate constant, for the reaction between the enzyme and the mediator at the optimum *p*H 10·5 for the oxidation of ethanol has been estimated. Sensor characteristics for ethanol have been evaluated in solution and at an EPG electrode on which ADH and BA were immobilized in polypyrrole (PPy) matrix.

2. Materials and methods

ADH (ADH from horse liver, 1 ea = 400 units, no EC number[7], and FCA were procured from Aldrich and used as received. Ethanol (HPLC grade, E. Merck, Germany) and pyrrole (Sisco Research Laboratories, Mumbai, India) were distilled before use. Benzoquinone (Aldrich) was recrystallized twice from hot solutions of *n*-hexane. Double distilled water was used for making a 0·2 M phosphate buffer. The enzyme solutions in buffer were deoxygenated by purging with N_2 for atleast 30 min prior to use to prevent any competitive oxidation by dissolved oxygen.

Cyclic voltammetry experiments using BAS voltammograph (CV-27) and an X-Y-t recorder were performed in a conventional three electrode electrochemical cell having a working volume of 5 ml. An EPG electrode (area 0·122 cm^2) supplied by LeCarbone Lorraine, France and polished with 600 grit SiC paper and 1 μm diamond paste served as the working electrode. The counter electrode was a Pt wire. All potentials were referred to the Ag/AgCl reference electrode.

2.1 *Electrochemical preparation of PPy membrane containing ADH and BQ*

A PPy membrane was prepared in the presence of ADH and BQ by applying a potential of 1·0 V (versus Ag/AgCl reference electrode) to an EPG electrode in a solution containing pyrrole (0·1 M), BQ (0·1 mM) and ADH (15 mg ml^{-1}). The electrolyte solution was 0·1 M potassium chloride solution deoxygenated before electropolymerization. The polymerization was continued until a charge of 1 C cm^{-2} was accumulated. This was followed by thorough washing of the electrode with phosphate buffer[8].

3. Results and discussion

The cyclic voltammogram of BQ at the EPG electrode in the presence of ADH is shown in figure 1a. BQ is known to adsorb on to EPG electrode. The electrochemistry of BQ so adsorbed, however, is stable for hours and is the same as in solution. Sensor configurations with adsorbed mediators and immobilized enzymes are known in the literature (for example, the archetypal glucose sensor with ferrocene mediator[1]). Upon the addition of ethanol, a large catalytic current flows (figure 1b) which is indicative of the

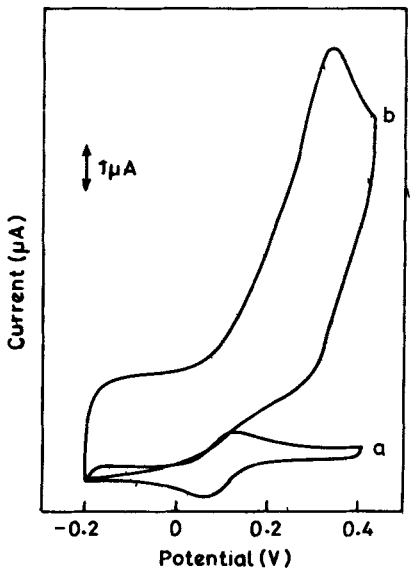

Figure 1. (a) Cyclic voltammogram of 1 mM BQ at an EPG electrode in the presence of 1 μM ADH. (b) As in (a) with 6 mM ethanol in 0·1 M NaOH–borax buffer *p*H 10·5, scan speed 5 mVs^{-1}.

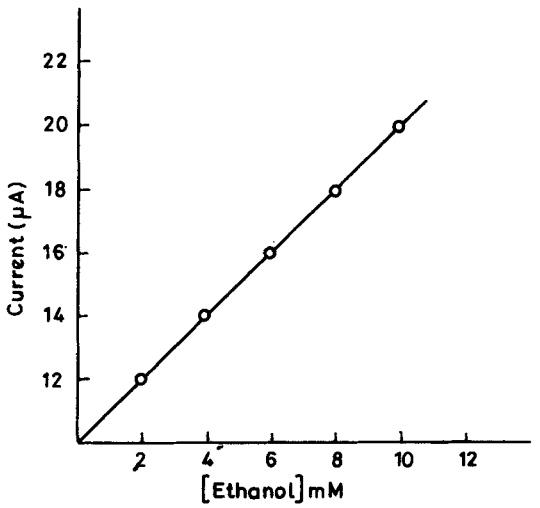

Figure 2. Variation of catalytic current with concentration of ethanol.

enzyme catalyzed oxidation of ethanol electrochemically coupled to BQ. The overall reaction catalyzed by the enzyme is as given below.

$$C_2H_5OH + \text{benzoquinone} \xrightarrow{\text{ADH}} CH_3CHO + \text{hydroquinone}$$

This behaviour is particularly apparent at slow scan rates (5 mVs^{-1})

The measured amperometric current for different concentrations of ethanol using BQ as mediator varied in the range 1–10 mM (figure 2). A straight line was obtained by linear regression analysis and the correlation coefficient (r) was found to be 0·982 ($y = 0{\cdot}20x + 0{\cdot}02$). A comparison with literature data will be in order. With N,N,N^1,N^1-tetramethyl-4 phenylenediamine (TMPD) as a mediator[5], the linear response was up to 10 nM only. Schumann *et al*[9] with their chloranil-pyrrole/pyrrole copolymer report a linear variation with current up to 2 mM. At N-methyl phenazinium-tetracyanoquinodimethane (NMP.TCNQ) electrode the linear response could not be stretched beyond 160 μM with complete saturation at 8 mM[10]. Thus, the observed response in our system is superior to that reported in the literature.The apparent Michaelis–Menten constant, K_m' was found to be 12 mM. The value is in fair agreement with that calculated from Eadie–Hofstee form, (1), of the Michaelis–Menten equation[11,12].

$$j_s = j_{max} - k_m'(j_s / C), \tag{1}$$

where j_s is the steady state current density, j_{max} is the maximum current density under saturating substrate conditions and C is the concentration of ethanol in solution. K'_m was found to be 13·5 mM. It may be compared with the K_m' value of 15·4 mM reported using NMPTCNQ electrode[10].

3.1 *Kinetic aspects*

Quantitative kinetic data for the reaction between the enzyme (ADH) and the mediator (BQ) was obtained according to the methodology of Nicholson and Shain[13]. The theory is suitable for obtaining kinetic information on reactions of the type:

$$Z + O \xrightarrow{k} R, \tag{2}$$

$$R - ne^- \Leftrightarrow O, \tag{3}$$

where O and R are the respective forms of the mediator and Z is the reduced enzyme.

To study the kinetic aspects, the current function, $i_p/v^{1/2}$ value (table 1) was plotted against log v (figure 3). The straight line observed for the BQ/H_2Q couple is typical for a simple reversible charge transfer process (figure 3A). On addition of ethanol, the variation of current function with logv was as expected for a catalytically coupled reaction (figure 3B–E). The data were analysed by making use of the working curve (figure 14 of ref. 13) which equates i_k/i_d (the ratio of kinetic to diffusion controlled current) to the kinetic parameter $(k_f/a)^{1/2}$ where k_f is the pseudo first order rate constant for the reaction between ADH and BQ and $a = nFv/RT$. The i_k/i_d values were obtained experimentally. The data (table 2) were then plotted for a series of ethanol concentrations between k_f/a and $1/v$. Under the pseudo first order conditions, the curves should be linear (figure 4). From the slope of each curve which equals k_fRT/nF, scan rate independent pseudo first order rate constant was obtained with BQ at each of the ethanol concentrations (table 3). The k_f values were obtained by carrying out the kinetic runs thrice and the uncertainty in these values was estimated to be ~2–3%. They plot of first order rate constant as a function of ethanol concentration was linear (figure 5). From the

Table 1. Cyclic volumetric data for BQ mediated ethanol/ADH reaction.

Scan rate ν (mVs^{-1})	$1/\nu$	$\nu^{1/2}$	$\log \nu$	Diffusion peak current i_d (μA)	Anodic peak current i_k (μA)				$i_d/\nu^{1/2}$	$I_k/\nu^{1/2}$			
					5 μM	10 μM	20 μM	30 μM		5 μM	10 μM	20 μM	30 μM
2	0·50	1·414	0·301	7	13	15	16	18	4·95	9·19	10·60	11·32	12·73
4	0·25	2·000	0·602	9	16	19	20	21	4·50	8·00	09·50	10·00	10·50
6	0·16	2·450	0·778	11	18	21	23	24	4·49	7·34	08·57	09·38	09·80
8	0·12	2·828	0·903	13	22	23	25	27	4·59	7·77	08·13	08·84	09·55
10	0·10	3·162	1·000	15	24	25	29	31	4·74	7·59	07·90	09·17	09·80
20	0·05	4·472	1·301	17	30	33	36	39	3·80	6·70	07·37	08·05	08·72

The concentrations of ethanol and BQ were 10 mM and 1 mM respectively

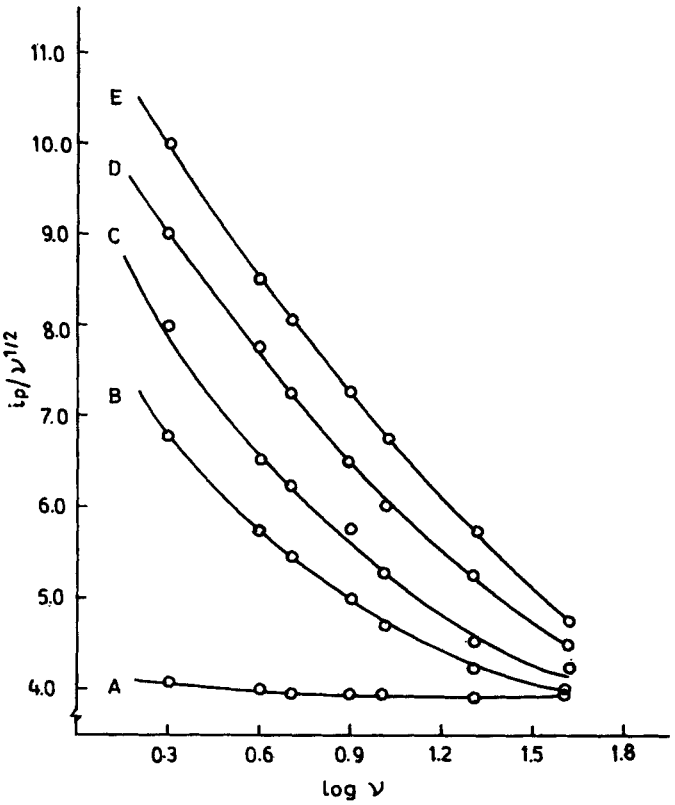

Figure 3. Variation of current function $i_p/\nu^{1/2}$ with $\log \nu$ for (A) diffusion controlled BQ/H_2Q redox reaction and for catalytically coupled reaction between BQ and ADH. The concentrations of ADD are (B) 5 μM, (C) 10 μM, (D) 20 μM, (E) 30 μM.

slope, k_f/(ethanol), second order rate constant, for the reaction between ADH and BQ was determined to be $3.8 \times 10^6\ M^{-1}s^{-1}$. In view of the fact that BQ is adsorbed on EPG electrode, the kinetic data should be viewed with caution. This value may be compared with the value $0.6 \times 10^5\ M^{-1}s^{-1}$ obtained for the substrates methanol and other primary alcohols employing ferrcenecarboxylic acid as mediator and ADH as the enzyme [5].

Similar observations have been made with FCA as mediator. The measured amperometric current for different concentrations of ethanol using FCA as mediator varied in the range 1–10 mM with a correlation coefficient (r) of 0·989 ($y = 0{\cdot}25x + 0{\cdot}03$). The apparent Michaelis–Menten constant, K'_m was found to be 12 mM. The second order rate constant for the reaction between ADH and FCA was calculated to be $1{\cdot}2 \times 10^5\ M^{-1}s^{-1}$. It can be seen that benzoquinone is an effective mediator for the enzymatic oxidation of ethanol.

The next set of experiments was carried out by immobilizing both ADH and BQ in a PPy matrix.

The cyclic voltammogram of the PPy electrode (containing BQ and ADH) in phosphate buffer displayed the characteristic electrochemistry of BQ as well as catalytic currents upon the addition of ethanol.

Table 2. Catalytic current data for the BQ mediated ethanol/ADH reaction.

Conc. of ADH (μM)	Scan rate ν (mVs^{-1})	Diffusion peak current i_d(μA)	An anodic peak current i_k(μA)	i_k/i_d	k_f/a
5	2	7	13	1·85	1·21
	4	9	16	1·77	1·06
	6	11	18	1·63	1·01
	8	13	22	1·69	0·95
	10	15	24	1·60	0·81
	20	17	30	1·76	0·45
10	2	7	15	2·14	1·50
	4	9	19	2·11	1·34
	6	11	21	1·90	1·23
	8	13	23	1·76	1·17
	10	15	25	1·66	1·03
	20	17	33	1·94	0·85
20	2	7	16	2·28	1·52
	4	9	20	2·11	1·36
	6	11	23	2·09	1·27
	8	13	25	1·92	1·24
	10	15	29	1·93	1·09
	20	17	36	2·11	0·95
30	2	7	18	2·57	1·84
	4	9	21	2·33	1·68
	6	11	24	2·18	1·55
	8	13	27	2·07	1·49
	10	15	31	2·06	1·37
	20	17	39	2·29	1·18

The concentrations of ethanol and BQ were 10 mM and 1 mM respectively

Table 3. Kinetic data for the reaction between reduced ADH and BQ

Conc. of ADH (μM)	k_f (s^{-1})	k (M^{-1}s^{-1})
5	0·662	
10	1·450	$3{\cdot}8 \times 10^6$
20	2·750	
30	3·520	

The measured amperometric current for different concentrations of ethanol varied in the range 1–8 mM. A straight line was obtained by linear regression analysis and the correlation coefficient (r) was found to be 0·988 [$y = 0{\cdot}4x + 0{\cdot}045$]. The apparent Michaelis–Menten constant, K'_m was found to be 9 mM. The immobilized configuration will have restrictions of diffusion of the analyte within the polymer film and this should increase the linear range compared to what was observed in the immobilized state. It is difficult to rationalize this observation in view of the uncertainty in the pore size of the membrane *vis-a-vis* the analyte size.

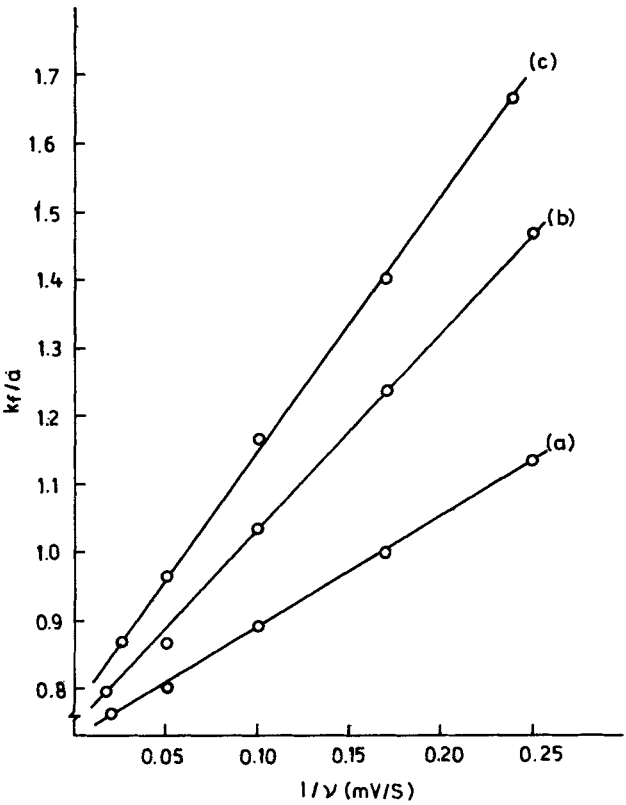

Figure 4. The kinetic parameter, k_f/a, as a function of $1/v$ for various ADH concentrations: (a) 10 μM, (b) 20 μM, (c) 30 μM.

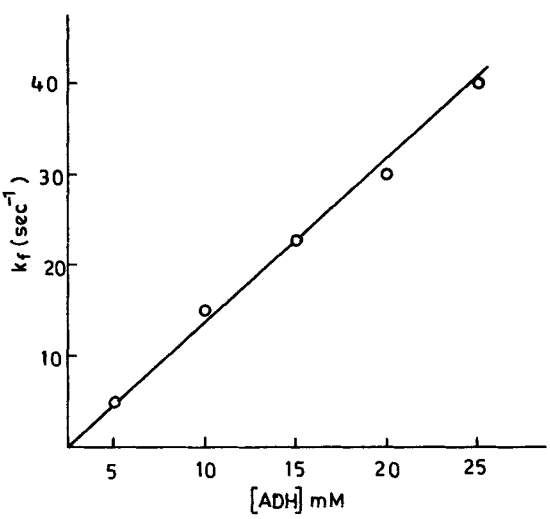

Figure 5. Plot of first order rate constant as a function of ADH concentration.

4. Conclusions

It is interesting that BQ and FCA act as effective mediators in the electrooxidation of ethanol using a quinoprotein ADH. The variation of the amperometric current along with the kinetic data demonstrate that this enzyme-catalysed reaction could be effectively employed for the development of a sensor for ethanol. The immobilization methodology described using PPy, holds a promise for the commercial development of an ethanol sensor.

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