

244 - Oxidation of 1,4-NADH at a Glassy Carbon Electrode: Effects of pH, Lewis Acids and Adsorption *

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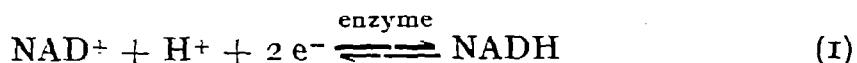
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Summary

The effects of pH, LEWIS acids [Ca(II) ; Zn(II)] and adsorption on the electrochemical oxidation of dihydronicotinamide adenine dinucleotide (1,4-NADH) at a glassy carbon electrode have been investigated in aqueous media using cyclic voltammetry. The oxidation of NADH to NAD⁺ (nicotinamide adenine dinucleotide) is highly irreversible and is only marginally dependent on pH ; the initial charge transfer is indicated to be rate-controlling step. Oxidation of at least some of the NADH as an adsorbed species makes the oxidation more difficult, as does the presence of Ca(II) and Zn(II) ; the possible causes for this behavior are examined.

Introduction

Reduced nicotinamide adenine dinucleotide (1,4-NADH ; dihydronicotinamide adenine dinucleotide) is formed on the reduction of nicotinamide adenine dinucleotide (NAD⁺, DPN⁺ ; coenzyme I) in a one-step process enzymatically (eq. 1) and in a two-step process electrochemically (eq. 2) :



Enzymatically, NADH can be oxidized back to NAD⁺ in a single step. Electrochemically, NADH can be oxidized back to NAD⁺ in a single step at a potential of ca. 0.4 V. The electrochemical reduction of NAD⁺

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also produces some 1,6-NADH, which is also oxidizable back to NAD⁺. The electrochemistry involved has been reviewed.^{1,2} (In the subsequent discussion, unless otherwise indicated, NADH refers to 1,4-NADH.)

In recent years, several papers have examined the electrochemical oxidation of NADH.³⁻⁸ The realization of conditions, under which reproducible current-potential curves could be obtained,^{5,7} has made possible the use of the electrochemical oxidation of NADH to NAD⁺ as a component of analytical methods based on the enzymatic reduction of NAD⁺,⁸ *i.e.*, chemical species, which undergo enzymatically catalyzed redox reactions with NAD⁺, can be determined by coulometric measurement of the NADH produced in the oxidation.

However, there is still considerable uncertainty about the detailed nature of the electrochemical oxidation of NADH. The present paper is concerned with the effects of BRØNSTED acids, as reflected in pH, and of LEWIS acids on the oxidation, and with the role played by adsorption of the NADH on a glassy carbon electrode, which is the type of electrode most often used in connection with the NADH oxidation.

Experimental

Chemicals

The purity of reduced nicotinamide adenine dinucleotide disodium salt (1,4-NADH) (P-L BIOCHEMICALS) was determined by enzymatic assay.⁹ Zinc perchlorate was obtained from G. F. SMITH and calcium perchlorate from J. T. BAKER. Background electrolyte and buffer solutions were prepared from reagent grade chemicals; TEAP refers to tetraethylammonium perchlorate and Tris to 2-amino-2-hydroxymethyl-1,3-propanediol.

Instrumentation

The jacketed electrochemical cell used was thermostatted at 25 °C. Potentials were measured against a saturated calomel electrode. The counter electrode was a platinum coil. The stationary glassy carbon electrode (G.C.E.) was a 0.3-cm (length) × 0.5-cm (diameter) glassy carbon disk (TOKAI ELECTRODE MANUFACTURING COMPANY of Tokyo), cemented into 5-mm i.d. glass tubing with Techkits E-7 epoxy adhesive; electrical connection was made by dipping a nickel wire into a mercury pool contained above the carbon. The glass tubing and carbon were ground flush to each other on a rotating 600-mesh SiC paper disk and then polished. Pretreatment of the electrode is subsequently described.

For cyclic voltammetry, a PAR Model 174 polarographic unit, an in-house built potentiostat,²² a HOUSTON INSTRUMENT Series 2000 X-Y recorder, and a TEKTRONIX 5103N cathode ray oscilloscope with suitable modules and cameras were used.

Procedures

Standard voltammetric procedures were used with the modifications subsequently noted.

Pretreatment of glassy carbon electrode. – The electrochemical characteristics of the oxidation of NADH on a G.C.E., are strongly dependent on the method used to prepare the electrode. In the cyclic voltammetric studies to be discussed, use of the following procedure to prepare the electrode surface prior to the running of each voltammetric sweep, resulted in a reproducible electrochemical response :

1. The electrode is vigorously sanded with 600-grade silicon carbide paper.
2. The electrode is then sprayed with distilled water under pressure to remove loose particulate carbon.
3. The excess water is daubed off the electrode surface with lens paper.
4. Electrode surface is allowed to air dry.

It was necessary to repeat this procedure before each scan because prolonged exposure of the electrode to NADH had the effect of passivating its surface. Within 2 to 3 min of being immersed in a solution of 2.6 mM NADH in 0.1 M TEAP and 0.01 M Tris (pH 7.2 to 9.6), the peak current, I_p , for the voltammetric NADH oxidation peak was noticeable attenuated and the peak potential, U_p , shifted positively. Exposure of the G.C.E. to a solution of 0.1 M TEAP and 0.01 M Tris alone produced no observable change in electrode behavior. Since rinsing the electrode surface with distilled water after exposure to NADH, did not regenerate the original characteristics of the electrode, a relatively strong adsorption of NADH on the glassy carbon surface is indicated.

Merely polishing the electrode surface with lens paper prior to each voltammetric sweep did not result in a reproducible electrochemical response for the NADH oxidation but rather had the effect of passivating the electrode surface. Fig. 1 shows the difference observed in the oxidation of NADH (A) at a G.C.E. which was sanded, rinsed, and allowed to air dry, and (B) at a G.C.E. which was sanded, rinsed, and polished with lens paper. Voltammogram (B) is significantly more irreversible than that of (A); U_p is ca. 320 mV positive and I_p is ca. 30 % less. In general, the magnitudes of the positive U_p shift and the I_p attenuation are dependent on the duration and vigor of the surface polishing.

Effect of pH on oxidation of NADH.

The effect of pH on the electrochemical oxidation of NADH at the G.C.E. was examined by cyclic voltammetry over the range of pH 7.2 to 9.6 in 0.1 M TEAP and 0.01 M Tris (the pH was adjusted by the addition of HClO₄) (Table 1). Under these conditions, the oxidation was

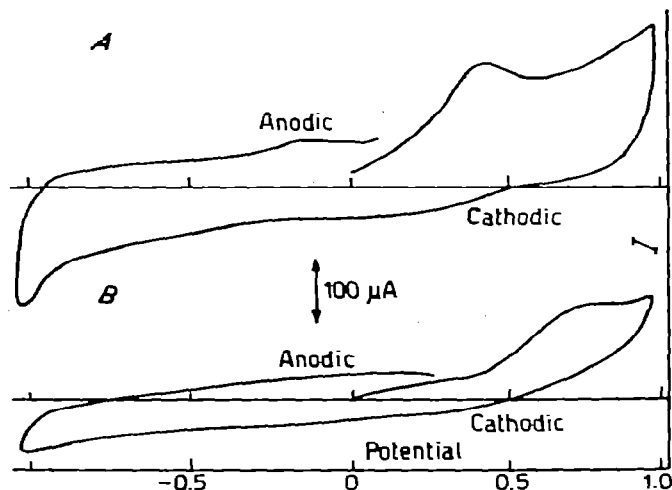


Fig. 1. Cyclic voltammograms of 2.6 mM NADH in 0.1 *M* TEAP and 0.01 *M* Tris showing the effects of electrode pretreatment. Pretreatment procedure: (A) sand with 600-grade silicon carbide paper, rinse with distilled water, and air dry; (B) sand with 600-grade silicon carbide paper, rinse with distilled water, and polish with lens paper. Scan rate = 40 mV/s. Scan starts at potential 0 in the anodic direction

highly irreversible. No complementary cathodic peak was observed within the scan rate, v , range of 0.04 to 20 V/s. At v in excess of 20 V/s, the anodic peak was quite broad and extended into the potential region where the oxidation of water to evolve oxygen occurred. Hence, peak potentials and currents could not be meaningfully determined at v greater than 20 V/s.

The effect of pH on the voltammetric peak potential, U_p , for the oxidation of NADH was dependent on v (Table I); the variation of U_p with pH for $v = 0.2$ V/s is given by

$$U_p = 0.53 - 0.012 \text{ pH} \quad (3)$$

At a rotating glassy carbon electrode (214 r.p.m.), BLAEDEL and JENKINS⁵ reported that the oxidation potential *vs.* pH relationship for 10 μM NADH in 0.1 *M* NaCl and 0.005 *M* phosphate buffer is given by

$$U_{1/2} = 0.512 - 0.017 \text{ pH} \quad (4)$$

The agreement in potential and pH-dependence is reasonable considering the difference in electroanalytical techniques.

If the oxidation of NADH proceeded reversibly with the loss of two electrons and a proton, U_p would be expected to shift 30 mV positive for each unit decrease in pH at 25 °C. The relationship of U_p to pH and the irreversible character of the cyclic voltammograms indicate that the oxidation does not occur reversibly.

Table I. Cyclic voltammetric data for the oxidation of 2.6 mM 1,4-NADH on a glassy carbon electrode.

pH ^a	ν mV/s	U_p V	$U_p - U_{p/2}$ mV	I_p μA	$(1 - \alpha)n_a^b$
Background : 0.1 M TEAP and 0.01 M Tris					
7.2	40	0.40	175	95	0.27
7.2	90	0.425	165	155	0.29
7.2	200	0.45	165	270	0.29
8.3	40	0.40	175	105	0.27
8.3	90	0.41	165	155	0.29
8.3	200	0.43	155	230	0.31
9.6	40	0.39	185	95	0.26
9.6	90	0.41	180	160	0.26
9.6	200	0.42	155	245	0.31
Background ; 0.1 M TEAP, 0.01 Tris and 50 mM Ca(II)					
7.2	40	0.41	145	115	0.33
7.2	90	0.425	155	210	0.31
7.2	200	0.46	135	370	0.35
9.6	40	0.41	145	135	0.33
9.6	90	0.425	135	210	0.35
9.6	200	0.455	130	375	0.37
Background ; 0.1 M TEAP, 0.01 M Tris and 21 mM Zn(II)					
7.2	40	0.425	120	130	0.40
7.2	90	0.455	115	220	0.42
7.2	200	0.485	130	355	0.37

^a The solution pH was adjusted by the addition of HClO₄.

^b The product $(1 - \alpha)n_a$ was calculated from the slope of the voltammogram and is equivalent to $1.857 RT/[F(U_p - U_{p/2})]$.¹²

Charge transfer characteristics

The slopes of 155 to 185 mV for the cyclic voltammetric peaks, $U_p - U_{p/2}$ (Table I), indicate that the rate-controlling step in the oxidation is the charge transfer.¹⁰ In order to determine a charge transfer rate constant, $k_{s,h}$, it would be necessary either for the rate to be sufficiently rapid that a cathodic peak could be observed or for a thermodynamic U^0 to be known. Since neither of these conditions could be met, $k_{s,h}$ could

not be determined. However, the product, $(1 - \alpha)n_a$, could be calculated from the slopes of the voltammetric peaks; α is the transfer coefficient for the charge transfer process,¹¹ and n_a is the number of electrons involved in the process (generally taken to be one) and $(1 - \alpha)$ represents the fraction of the applied overpotential which promotes the oxidation step. The relationship of the cyclic voltammetric peak slope to $(1 - \alpha)n_a$ is given by¹²

$$U_p - U_{p/2} = \frac{1.857 RT}{F(1 - \alpha)n_a} \quad (5)$$

Determinations of $(1 - \alpha)n_a$ based on nine cyclic voltammograms run at v of 0.04, 0.09 and 0.20 V/s were independent of solution pH with an average value of 0.28 and a standard deviation of 0.02. For 10 μ M NADH in 0.1 M NaCl and 0.005 M phosphate buffer at a rotating glassy carbon electrode (214 r.p.m.), BLAEDL and JENKINS⁵ reported a slope for a plot of U vs. $\log [(I_d - I)/I]$, which corresponds to an $(1 - \alpha)n_a$ of 0.17.

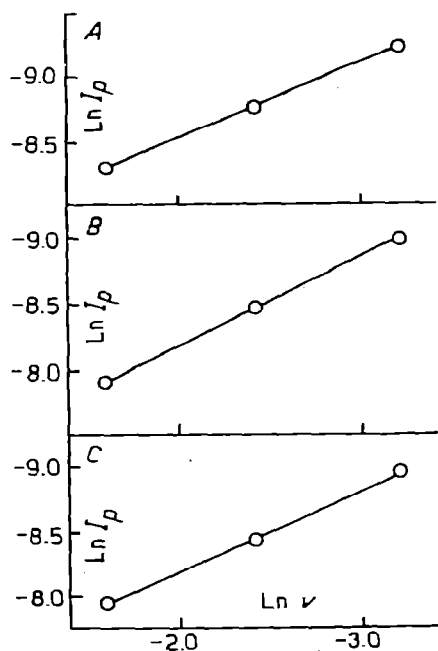


Fig. 2.

Variation of cyclic voltammetric peak current (I_p) with scan rate (v) for the oxidation of 2.6 mM NADH on a glassy carbon electrode. Solution conditions: (A) 0.1 M TEAP and 0.01 M Tris ($\ln I_p$ values were calculated from the average of I_p at pH 7.2, 8.3 and 9.6); (B) 0.1 M TEAP, 0.01 M Tris and 50 mM Ca(II) ($\ln I_p$ values were calculated from the average of I_p at pH 7.2 and 9.6); (C) 0.1 M TEAP, 0.01 M Tris and 21 mM Zn(II) at pH 7.2.

Effect of NADH adsorption.

In the limiting case, where all of the electroactive species undergoing a charge transfer is adsorbed at the solution-electrode interphase, the peak current, I_p , will be proportional to the scan rate, v , and a plot of $\ln I_p$ vs. $\ln v$ will have a slope of 1.0.¹³ If a non-adsorbed electroactive species undergoes charge transfer either reversibly or irreversibly, a slope

for $\ln I_p$ vs. $\ln v$ of 0.50 is predicted.¹⁰ Hence, a slope in excess of 0.50 indicates that some of the species undergoing charge transfer is adsorbed. Since a plot of $\ln I_p$ vs. $\ln v$ for 2.6 mM NADH in 0.1 M TEAP and 0.01 M Tris over the range of pH 7.2 to 9.6 has a slope of 0.58 (Fig. 2), it is apparent that some reduction of adsorbed NADH is taking place. In view of the passivation of the G.C.E. brought about by its exposure to NADH, it is not surprising that the relationship of I_p to v indicates the occurrence of adsorption.

The NADH adsorbed at the electrode-solution interphase is more difficult to oxidize than the NADH, which is in solution. Since adsorbed NADH occupies a lower free energy state, the electrode potential needed to oxidize it must overcome the energy of adsorption. Hence, oxidation of adsorbed NADH requires a more positive potential. Since the I_p vs. v relation indicates that some NADH is adsorbed, the true peak potentials for the oxidation of non-adsorbed NADH may be expected to be somewhat less positive than was experimentally observed. However, since the oxidation only shows one voltammetric peak, the oxidation potentials for adsorbed and non-adsorbed NADH must be sufficiently close that the experimentally observed voltammetric peak is a composite of the oxidation peaks for adsorbed and non-adsorbed NADH.

Effect of LEWIS acids:Ca(II).

The electrochemical oxidation of NADH at a G.C.E. in the presence of 50 mM Ca(II) in 0.1 M TEAP and 0.01 M Tris was studied at pH 7.2 and 9.6 in order to ascertain whether the addition of such a LEWIS acid, which is common in biological systems, would modify the oxidation.

In the presence of Ca(II), the oxidation of NADH is relatively insensitive to pH, *e.g.*, cyclic voltammograms at pH 7.2 and 9.6 were quite similar (Table 1). Addition of Ca(II) slightly shifts U_p positively (0–10 mV at pH 7.2 and 20–35 mV at pH 9.6) and increases I_p (from 25 % at 0.04 V/s to 40 % at 0.20 V/s). The slope of $\ln I_p$ vs. $\ln v$ is 0.73 (Fig. 2) as compared to one of 0.58 in the absence of Ca(II). This increase in slope indicates that addition of Ca(II) increased the adsorption of NADH, since the slope in the presence of Ca(II) more closely approximates that predicted for oxidation of an adsorbed species.

Since increased adsorption of NADH would produce a greater surface concentration of NADH, the increase in I_p brought about by Ca(II) addition is probably a consequence of the increased NADH adsorption. The slight positive shift in U_p may also result from an increase in the amount of adsorbed NADH, since a larger fraction of the species undergoing charge transfer will be in the lower energy adsorbed state and will be more difficult to oxidize.

In the potential region where the NADH oxidation occurs, the electrode-solution interphase and NADH are of opposite charge, *i.e.*, the former is positively charged due to the applied potential and the NADH is negatively charged due to phosphate dissociation in the pH region

involved. Since addition of 50 mM Ca(II) increases the ionic strength from 0.1 *M* to 0.25 *M*, a decrease in the activity coefficient of NADH and a corresponding decrease in adsorption would be expected. However, since adsorption of NADH is increased in the presence of Ca(II), it is likely that Ca(II) serves to further stabilize NADH adsorbed at the interphase.

Studies of the complexation of Ca(II) by adenine nucleotides have shown that Ca(II) is bound at the phosphate groups.^{14,15} If Ca(II) forms a 1:1 complex with NADH, the Ca(II) probably binds at the pyrophosphate linkage. The resulting complexed would be electrically neutral. Although the lack of a negative charge on the complexed NADH would result in decreased electrostatic attraction towards the positively charged electrode-solution interphase, the electrostatic repulsion between molecules of adsorbed complexed NADH would be less than that between molecules of adsorbed uncomplexed NADH which have a charge of -2. With less electrostatic repulsion between adsorbed species, a greater surface excess will occur at the interphase. In the presence of 50 mM Ca(II), the diminished repulsion between adsorbed molecules of complexed NADH is probably responsible for the increased NADH adsorption.

Effect of LEWIS acids : Zn(II).

The oxidation of 2.6 mM NADH in the presence of 21 mM Zn(II) at pH 7.2 (0.1 *M* TEAP and 0.01 *M* Tris buffer) was examined; U_p for the NADH oxidation shifted 20-35 mV positive and I_p increased by 35%. A plot of $\ln I_p$ vs. $\ln v$ gave a slope of 0.63 (Fig. 2), indicating that the adsorption of NADH was increased slightly by the Zn(II) addition.

Although much of the increase in I_p can be attributed to the increase in adsorbed NADH, the shift in U_p can not. Adsorption of NADH occurs to a lesser extent from a solution 21 mM in Zn(II) than from one 50 mM in Ca(II). However, U_p in the presence of Zn(II) was 10-25 mV positive of U_p in the presence of Ca(II) at the same pH. Since the positive shift is not the result of increased NADH adsorption, it may be indicative of an interaction between Zn(II) and NADH.

If NADH complexes with Zn(II), the LEWIS acid character of the Zn(II) ion could serve to lower the electron density in the dihydronicotinamide moiety, which would make NADH more difficult to oxidize. Nuclear magnetic resonance¹⁶ and infrared¹⁷ spectroscopic studies have indicated that Zn(II) binds adenosine-5'-triphosphate at both the adenine and phosphate moieties. It is highly probable that Zn(II) will bind these sites in NADH as well. It is not clear whether there is any significant interaction between Zn(II) and the dihydronicotinamide moiety. However, in view of the rather small shift in U_p at a Zn(II) : NADH ratio of 8, any interaction of this type would have to be quite weak.

Acknowledgement

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Note added in proof: Studies currently in progress indicate that the adsorption of NAD^+ generated by the oxidation of NADH may be an important factor in the adsorption behavior observed.