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616—COMPARATIVE ADSORPTION OF ADENINE AND NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) AT AN AQUEOUS SOLUTION | MERCURY INTERFACE

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SUMMARY

The relative extent of adsorption of adenine and NAD^+ at the 10-μM level from a solution of pH 4.8 and 0.5 *M* ionic strength (McIlvaine buffer) has been measured as a function of potential by an inverse normal pulse polarographic technic at a dropping mercury electrode (d.m.e.). Normalization of the pulse polarographic current resulting from pre-concentration of the electroactive species through adsorption compared to the current expected for a simple diffusion-controlled electron-transfer indicates that NAD^+ is strongly adsorbed over a broader potential range than adenine, and that its surface concentration is slightly greater; causes for such behavior are considered. Other normalization approaches indicate NAD^+ to be strongly adsorbed but comparably to adenine when account is taken of the relative effective areas occupied on adsorption.

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+; diphosphopyridine nucleotide, DPN^+; coenzyme I) (Fig. 1), is a coenzyme for the dehydrogenase enzymes which catalyze redox processes involving the transfer of hydrogen between substrate and coenzyme in virtually all biological systems, e.g., alcohol dehydrogenase catalyzes the simultaneous oxidation of ethanol to acetaldehyde and the reduction of NAD^+ to 1,4-NADH *via* effective net transfer of a hydride ion from ethanol to C(4) of the pyridine ring.

The gross reversibility of the NAD^+/NADH redox couple under physiological conditions has prompted extensive study by polarographic technics [1]. While the main features of the reduction pathway of NAD^+ have been reasonably well established, certain aspects of its electrochemical and related chemical behavior have been only partially explained. The present investigation was designed to establish more clearly the influence of its components on the adsorption of NAD^+.

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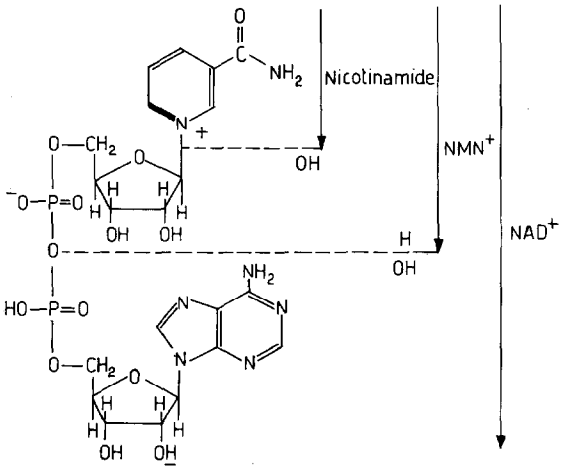

Fig. 1. Structure of nicotinamide adenine dinucleotide (NAD^+). Other names for this compound include diphosphopyridine nucleotide (DPN^+), coenzyme I, codehydrogenase and cozymase. Nicotinamide mononucleotide is designated by NMN^+.

Polarographic studies of nicotinamide mononucleotide (NMN^+) have shown that the presence of nicotinamide, ribose and phosphate moieties do not by themselves result in a high degree of surface-activity [2]. NAD^+, which contains two additional hydrophilic moieties (sugar and phosphate) plus a hydrophobic moiety (adenine) shows much greater surface-activity. In 0.4 *M* KCl and 0.1 *M* carbonate buffer (pH 9.6), NAD^+ considerably depresses the differential double-layer capacitance in the potential region from the mercury oxidation to the first reduction of NAD^+ at *ca.* -0.9 V, whereas NMN^+ only slightly decreases the capacitance prior to its first reduction peak [3]. Although desorption of NAD^+ and the dimer, which results from the initial one-electron reduction of NAD^+ to the free radical, does not occur until -1.4 V, it is difficult to evaluate the differential double-layer capacitance in the presence of a faradaic process, i.e., from -0.9 to -1.4 V. Since the addition of such hydrophilic groups as ribose and phosphate would not be expected to increase substantially adsorption from aqueous solution, the adenine moiety has been suggested as the most likely site for the adsorption of NAD^+ [4].

In order to clarify the role of adenine, the potential-dependent adsorption of adenine and NAD^+ was examined by a modified pulse polarographic technic [5] in McIlvaine buffer of pH 4.8 and 0.5 *M* ionic strength. The technic used is described in the Experimental section.

EXPERIMENTAL

In the variation on normal pulse polarography (n.p.p.) used to study potential-dependent adsorption, which has been called inverse normal pulse polarography

(i.n.p.p.), the initial polarization potential, U_i, is varied but the final potential, U_f, in each cycle remains constant [5–7]. The latter potential is selected to be on the limiting current portion of a polarographic wave, e.g., on the plateau of the one adenine wave or on that of the first diffusion-controlled NAD^+ wave. Specifically, for a controlled d.m.e., 5-s drop-time, an average current and standard deviation for each initial polarization potential, U_i, were calculated from the sampled current for 5–10 drops. The currents were obtained by stepping the potential from each U_i to a final polarization potential, U_f, of -1.48 V for adenine and -1.30 V for NAD^+, and then by sampling the current 2.9 ms after application of the pulse.

Chemicals, apparatus and procedures, unless otherwise indicated, were as previously described [2,3,5,7,8]. Potentials are reported with respect to the aqueous saturated calomel electrode (s.c.e.). Measurements were taken at 25 °C.

Chemicals

The reported analytical data and spectrophotometric assay indicated sufficient purity for electrochemical examination of the compounds used: nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide disodium salt (1,4-NADH), nicotinamide mononucleotide (NMN^+), and yeast alcohol dehydrogenase from P-L Biochemicals; adenine from Calbiochem. The purity of NAD^+ was confirmed by enzymatic assay [9], using 6200 for the 340-nm molar absorptivity of the reduced coenzyme.

Background electrolyte and buffer solutions were prepared from reagent grade chemicals [10].

Apparatus

A one-compartment cell with a water jacket and Luggin capillary (Fig. 4 of Ref. 11) was used with an in-house constructed potentiostat, which had a minimal response time [11]. Data were recorded on Houston 2000 x–y recorder and on a Tektronix 5103N power supplier/amplifier equipped with suitable modules and camera as a cathode ray oscilloscope system.

Procedure

The timing system, electrodes used and experimental methodology were the same as those described in Ref. 5.

RESULTS AND DISCUSSION

Reduction of adenine involves four electrons with subsequent deamination and an additional 2 e^- reduction occurring relatively slowly on the time scale of d.m.e. polarography. Since the adenine wave occurs on the shoulder of the background discharge due, in part, to the shift of that discharge to a more positive potential by

the presence of adenine, some catalytic reduction of hydrogen by adenine or its reduction products, superimposed on the adenine reduction, is likely [5,12,13]. However, a plot of the *d.c* polarographic I_l *versus* $h^{1/2}$, where h is the mercury height above the capillary tip, was linear, indicating diffusion control; consequently, the rate of hydrogen discharge must depend on the diffusion of adenine to the interface.

The first NAD^+ reduction wave, which is diffusion-controlled, [2] involves a one-electron process.

Adsorption as a function of potential

The variations of the currents measured, which are related to the extent of adsorption at U_i, are shown in Fig. 2 for adenine and for NAD^+ at the 10-μM concentration level.

Adenine shows maximum adsorption between -0.25 and -0.45 V, within which U_i range the reduction current is relatively constant. Using a similar pulse technic but with current sampling delayed until 6.2 ms after pulse application, Flemming [6] reported maximum adsorption at -0.43 V for 6.25 μM adenine in 0.5 M McIlvaine buffer of pH 5.

NAD^+ shows maximum adsorption from -0.15 to -0.55 V. The substantially lower currents observed, compared to those for adenine, result from the fact that NAD^+ undergoes only a one-electron reduction at the U_f for the NAD^+ study whereas adenine undergoes a multiple electron reduction at its U_f [5].

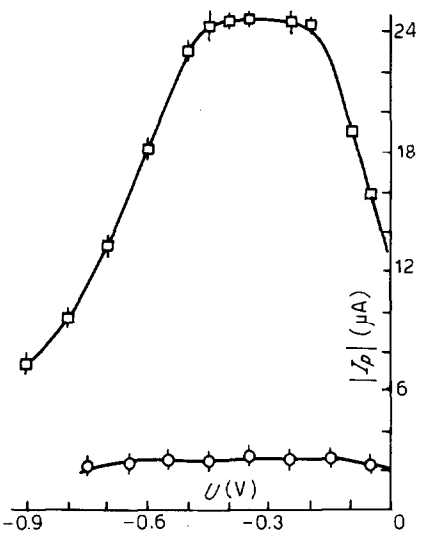

Fig. 2. Currents, I_p, obtained for 10 μM adenine (□) and 10 μM NAD^+ (○) in pH 4.8 McIlvaine buffer on modified (inverse) pulse polarography, on stepping the potential from the initial polarization potential, U_i, shown in the figure to a final polarization potential, U_f, of -1.48 V for adenine and -1.30 V for NAD^+. Controlled drop-time = 5.0 s; discharge period = 3 ms; current measurement period = 25 μs.

Relative adsorption of adenine and NAD$^+$

In order to normalize the data and thus to determine the relative increase in the pulse polarographic current, I_p, resulting from pre-concentration of electroactive species through adsorption, compared to the current, I_t, predicted for a simple diffusion-controlled electron-transfer at time t, I_p was divided by I_t, where

$$I_t = \frac{c_0 n \mathcal{F} A D_0^{1/2}}{\pi t^{1/2}} \tag{1}$$

A is the electrode area at 5 s, and t is the time between application of the pulse and cessation of current sampling. Values for $nD_0^{1/2}$ of 0.022 for adenine and 0.0020 for NAD$^+$ were calculated from reported diffusion current constants, I_d, [1,2,5], where $I_d = 607\ nD_0^{1/2}$. Plots of I_p/I_t *versus* U_i are given in Fig. 3.

In the potential region of maximum adsorption, the I_p/I_t ratio reaches a value of 6 for NAD$^+$ and 5.1 for adenine. The uncertainty in the I_p measurements corresponding to one standard deviation was about 1 μA. However, since the NAD$^+$ currents were smaller than those for adenine, the relative uncertainty in I_p is much larger for NAD$^+$ and the corresponding error bars (equivalent to one standard deviation for I_p/I_t) for the I_p/I_t ratio area are substantially greater. The overlap of the error bars in the potential region between -0.25 and -0.45 V indicates that the relative surface excess, Γ, i.e., the number of molecules adsorbed per unit interfacial area, is comparable for adenine and NAD$^+$.

Inasmuch as NAD$^+$ contains more hydrophilic groups than adenine, it may seem somewhat surprising that NAD$^+$ is strongly adsorbed over a broader potential region and that its surface concentration is slightly greater. In view of the effect of

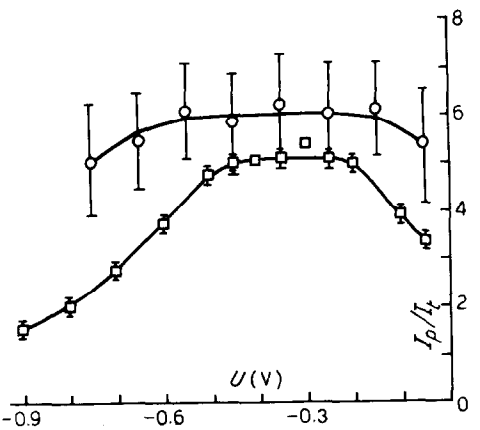

Fig. 3. Variation of normalized pulse polarographic current, I_p/I_t, with initial polarization potential, U_i, for 10 μM adenine (squares) and 10 μM NAD$^+$ (circles) in pH 4.8 McIlvaine buffer (0.5 M ionic strength). The procedure and conditions are given in Fig. 2. The predicted diffusion-controlled current, I_t, was calculated by equation (1).

ribose-phosphate on the potential-dependent adsorption of adenine nucleosides and nucleotides [13–16], the presence of ribose-phosphate groups in NAD^+ may be responsible for the greater NAD^+ adsorption, e.g., adenosine monophosphate is adsorbed over a broader potential region than adenine itself, with the extension of adsorption to the potential region positive of the potential of zero-charge. Since the interface has a net positive charge in this potential range, the increased adsorption of NAD^+ may result from electrostatic attraction between the negatively charged phosphate group and the interface, even though the primary adsorption site in both adenine and NAD^+ is the adenine ring.

Another approach to comparing the adsorption of adenine and NAD^+ would be to follow Anson's [17] treatment for the specific adsorption of a reactant, for which the total charge, Q, passed in a potential-step experiment is given by

$$Q = \frac{2n\mathcal{F}AcD^{1/2}t^{1/2}}{\pi^{1/2}} + Q_{dl} + n\mathcal{F}A\Gamma \tag{2}$$

where the first right-hand term is the integrated Cottrell equation for a diffusing reactant, the second term represents the double-layer charge, and the last term represents the charge due to reduction of the adsorbed reactant. If, under the temporal scale of the experiment, the last term can be assumed to be much greater than the other two, which should be true for short pulses, e.g., 2.9 ms, the current measured at t_d is given by

$$I_p = n\mathcal{F}A\Gamma f(t) \tag{3}$$

where $f(t)$ is some function of time relating the total charge to the current, i.e., $f(t)$ is the current decay curve for reduction of an adsorbed layer(s) of reactant.

Calculation of the I_p/I_t ratio gives

$$\frac{I_p}{I_t} = \frac{n\mathcal{F}A\Gamma f(t)}{n\mathcal{F}Ac_0D_0^{1/2}/(\pi^{1/2}t^{1/2})} = \frac{\Gamma f(t)}{c_0D_0^{1/2}/(\pi^{1/2}t^{1/2})} \tag{4}$$

If $f(t) = c_0D_0^{1/2}/(\pi^{1/2}t^{1/2})$, the current ratio would provide a measure of Γ; however, the current decay for reduction of an adsorbed layer, i.e., $f(t)$, would presumably not contain c_0 and $D_0^{1/2}$ terms. Consequently, the current ratio would be a measure of $[\Gamma/(c_0D_0^{1/2})] \times f'(t)$ *. Values of $D^{1/2}$, calculated from the previously cited $nD_0^{1/2}$ magnitudes, are 0.0055 for adenine and 0.0020 for NAD^+. Such normalization, when applied to the data of Fig. 3, results in Γ for adenine being greater by a factor of two or somewhat more than Γ for NAD^+; this is in accord with the relative magnitudes of the effective areas occupied by adenine and NAD^+ on adsorption with the principal plane of the adsorbate being parallel to the solution mercury interface, i.e., 55 $Å^2$ for adenine [18] and 125 $Å^2$ for NAD^+ in a folded configuration with parallel adenine and pyridinium rings [19].

Alternatively, division of equation 3 by $n\mathcal{F}A$ may allow a relative measure of Γ,

* Here, $f'(t) = (\pi t)^{1/2}f(t)$.

i.e.,

$$\frac{I_p}{n\mathcal{F}A} = \Gamma f(t) \tag{5}$$

If the current decay functions for adenine and NAD^+ are comparable, equation 5 provides a means of comparing surface excesses, e.g., by dividing the curves of Fig. 2 by the faradaic n values of 4 for adenine and 1 for NAD^+. When such a calculation is carried out, the corrected value, I_p/n, for adenine is approximately 2.5 times greater than the corresponding value for NAD^+. This suggests that the surface area occupied by one adsorbed molecule of NAD^+ is 2.5 times the area occupied by one adsorbed adenine molecule.

Using the previously reported effective coverage areas of 55 and 125 $Å^2$ for adenine and NAD^+, respectively, the ratio of effective coverage areas for NAD^+ *versus* adenine is 2.3. Hence, the relative magnitude of the effective occupied surface areas, which was calculated from the I_p values in Fig. 2 normalized only for the difference in the faradaic n values, is in good agreement with previously reported surface areas.

In two recent papers [20,21], Van Leeuwen discussed the influence of adsorption on limiting currents observed in pulse polarography. While the factors considered by Van Leeuwen can be significant, the effects in the present study are minimal or zero due to the difference in time scales. Thus, at high adsorbate concentration, the pulse duration (t_p) is 40 ms in the Van Leeuwen calculations [21] compared to 3 ms in the present study in which the current measurement period was 25 μs. The times before pulse application (t_0) also differ markedly: 1 s in the Van Leeuwen examples and 5 s in the present study. Consequently, the Van Leeuwen equations describe diffusion-controlled current at a point in time when all of the adsorbed material has already undergone charge transfer; in the present case, reduction of the adsorbed layer is the principal source of current.

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