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254 - Effect of the Adenine Moiety on the Electrochemical Behavior of Nicotinamide Adenine Dinucleotide; Possible Reduction of the Adenine

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Summary

The presence of the adenine moiety very markedly affects the adsorption phenomena at the solution|electrode interface associated with nicotinamide adenine dinucleotide (NAD^+) in aqueous media. Furthermore, a number of effects seen on the electrochemical reduction of NAD^+ may be associated with faradaic reduction of the adenine. Such a reduction, which has been postulated as an initial step in the pulse radiolysis of NAD^-, helps to rationalize the reversible reduction of adsorbed NAD^-, where the ultimate reduction site, *i.e.*, the pyridine ring, is separated from the solution|electrode interface by the adenine ring. The adenine moiety also participates in a redox couple with mercury.

Introduction

The pyridine nucleotide, nicotinamide adenine dinucleotide [NAD^+; also known as diphosphopyridine nucleotide (DPN^+) and coenzyme I] (Fig. 1), is a major coenzyme for the class of enzymes known as dehydrogenases, which catalyze reversible redox processes in virtually all biological systems, involving transfer of hydrogen between substrate and coenzyme, *e.g.*, yeast alcohol dehydrogenase (ADH) catalyzes oxidation of ethanol to acetaldehyde and the simultaneous reduction of NAD^+ to 1,4–NADH via the effective net transfer of a hydride ion from the alcohol to the 4–position of the pyridine ring.[1,2] (In subsequent discussion, NADH is taken to mean 1,4–NADH).

The gross reversible redox behavior of the NAD^+–NADH couple under physiological conditions has prompted study of the couple by polarographic technics.[3-6] While many aspects of the electrochemical behavior of NAD^+, NADH, and model compounds have been thoroughly examined and explained, others have been only partially clarified or have not been considered. One such item is that of the possible reduction of the adenine moiety in the NAD^-.

It is still not entirely clear what part the adenine moiety plays in the NAD redox pattern. Nuclear magnetic resonance, spectrophoto-

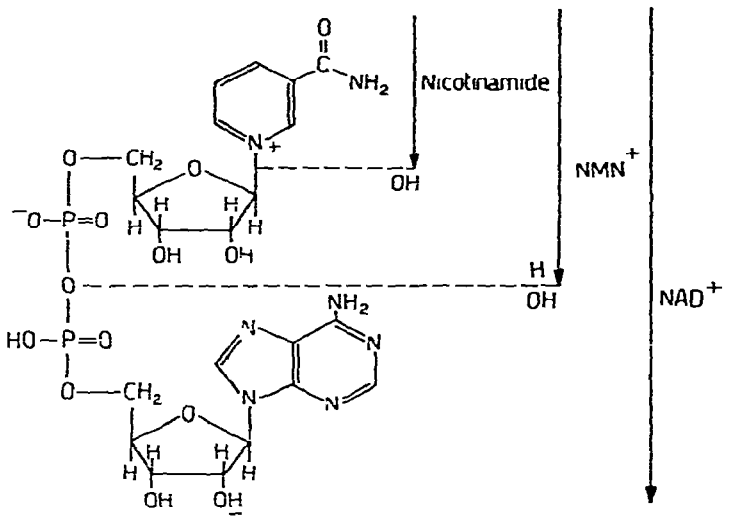

Fig. 1.
Formula for enzymatically active NAD^-, *i.e.*, β-nicotinamide adenine dinucleotide. The isomer having an α-glycosidic nicotinamide–ribose linkage does not exhibit enzymatic activity with yeast ADH. A related coenzyme, nicotinamide adenine dinucleotide phosphate ($NADP^+$), is formed when the underlined H is replaced by a $PO(OH)_2$ group. In deamino-NAD^- (DNAD), the adenine moiety is replaced by hypoxanthine.

metric and fluorescence studies show considerable interaction between the nicotinamide and adenine moieties in solution with a large fraction of the dinucleotide being present in a folded conformation.[7,8] A question is immediately raised as to whether the reducible species at the solution|electrode interface is folded or unfolded. Adenine could participate in the reduction with electrons received by it being transferred to the nicotinamide. The orientation of NAD^- at the electrode surface is obviously of importance in this respect. In adenine–cytosine dinucleotides, both bases can be electrochemically reduced in the pH range of 2 to 5.[9]

The present paper summarizes experimental observations on the electrochemical patterns seen with NAD^- and reasonable deductions from such data, which indicate the distinct possibility that the adenine moiety may be reduced even though this may only result in a transitory reduced state due to, for example, transfer of the added electron or electrons from the adenine ring to the pyridine ring. It is hoped that the present account will stimulate more detailed investigation of the role played by adenine in the electrochemical reduction of NAD^+ and the resulting implications for the behavior of the NAD^-–NADH couple in biological systems.

The phenomena subsequently discussed were seen in aqueous media unless otherwise specified.

Experimental

Chemicals, apparatus and procedures, which are not subsequently discussed, have been described.[10-15]

The buffer solutions used, prepared from reagent grade chemicals, are listed in Table 1.

Table 1. Buffer and background electrolyte solutions [a].

pH Range	Composition
0.0–1.8	HCl + KCl
2.0–8.0	$Na_2HPO_4 . 7\ H_2O$ + citric acid monohydrate + KCl
3.9–5.9	HOAc + NaOAc
5 4	HOAc + NaOHc + Et_4NCl [b]
7.8	H_3BO_3 + KCl + NaOH
9 0–10.0	K_2CO_3 + $KHCO_3$
9.0–10.0	K_2CO_3 + $KHCO_3$ + KCl [c]
9 0–10.0	K_2CO_3 + $KHCO_3$ + Et_4NCl [b]
9.0–10.0	NH_3 + NH_4Cl
11.0–12.0	KOH + KCl

[a] The final ionic strength of buffer solutions in all experiments was 0.5 *M*.
[b] Et_4NCl concentration : 0.4 *M*.
[c] KCl concentration : 0.4 *M*.

All potentials reported were measured with respect to the aqueous saturated calomel electrode (S.C.E.).

NAD^+, unless otherwise indicated, refers to the normal biologically active β form. Acronyms used to identify other compounds are as indicated : α–NAD^+ (α–nicotinamide adenine dinucleotide), $NADP^+$ (nicotinamide adenine dinucleotide phosphate ; triphosphopyridine nucleotide, TPN^+ ; coenzyme II), $DNAD^+$ (deamino–NAD^+ ; nicotinamide hypoxanthine dinucleotide), $DNADP^+$ (deamino–$NADP^+$; nicotinamide hypoxanthine dinucleotide phosphate), AMP (adenosine–5′–monophosphate), ADP (adenosine–5′–diphosphate), ATP (adenosine–5′–triphosphate), NMN^- (nicotinamide mononucleotide), NMNH (reduced nicotinamide mononucleotide), ADPR (adenosine diphosphate ribose).

General polarographic patterns

Adenine. – Adenine (6–aminopurine) is initially reduced electrolytically at a potential sufficient to reduce both the 1,6 and 3,2 N =C double bonds. On controlled potential electrolysis, it undergoes a six–electron (6 e^-) reduction, which probably involves

(*a*) 2 e⁻ reduction of the 1,6 double bond to produce a *gem*–diamine centered on C(6),
(*b*) immediate 2 e⁻ reduction of this product to 1,2,3,6–tetrahydroadenine,
(*c*) relatively slow deamination at the 6–position to form 2,3–dihydropurine,
(*d*) further 2 e⁻ reduction of the regenerated 1,6 double bond form 1,2,3,6–tetrahydropurine, and, finally,
(*e*) hydrolytic cleavage at the 2,3–position to form the same diazotizable amine as does purine in its overall 4 e⁻ reduction.[9,16-18]

Under polarographic conditions at the dropping mercury electrode (D.M.E.), adenine exhibits one cathodic wave of the current magnitude expected for a 4 e⁻ wave, *i.e.*, deamination does not proceed appreciably during the time scale involved in D.M.E. polarography. The pH–dependence of the wave is given by

$$E_{\frac{1}{2}} = -0.975 - 0.084\ \mathrm{pH} \qquad (1)$$

On cyclic voltammetry at the hanging mercury drop electrode (H.M.D.E.), adenine shows a single pH–dependent cathodic peak; no complementary anodic peak is observed on the return scan even at fast scan rate.[18] The peak current, I_p, is constant up to pH 5.5 and then, similar to the D.M.E. wave, decreases markedly with pH to vanish between pH 6 and 7; the wave is kinetically controlled in the pH region where it decreases. The latter effect is associated with the reducible form of adenine being the protonated species. In ammonia buffer, adenine gives an inflection close to the background discharge; point–by–point subtraction of the residual current yields a more or less well defined wave.[17] Background discharge occurs at less negative potentials in the presence of adenine.

In aprotic media, adenine undergoes a 1 e⁻ reduction of $E_{\frac{1}{2}}$ at —2.41 V in dimethyl–formamide and dimethyl–sulfoxide, and at —2.50 V in acetonitrile.[19]

$NAD_{ox} - NAD^-$, similar to other 1–substituted nicotinamides, shows two well–separated cathodic waves at the D.M.E.[13] Interpretation of the behavior patterns is complicated by

(*a*) the presence of adsorption phenomena,
(*b*) potential shifts for wave I*c* due to an irreversible dimerization following the initial reversible 1 e⁻ reduction,
(*c*) catalytic hydrogen evolution at lower pH, and
(*d*) decomposition above pH 10, producing reducible nicotinamide.

Consequently, the characteristics of waves I*c* and II*c* can be studied thoroughly and simultaneously only between pH 9 and 10. Over the pH range of 2 to 12, $E_{\frac{1}{2}}$ for wave I*c* is —0.89 ± 0.01 V in the presence of background and buffer cations which are not more strongly adsorbed than NAD^-. Between pH 9 and 10, $E_{\frac{1}{2}}$ for wave II*c* is —1.64 ± 0.03 V;

between pH 6 and 8, the wave does not appear to have a limiting plateau but gradually merges with solution discharge.

A small anodic wave is seen at times close to the potential of the mercury discharge.

Cyclic voltammetry indicates non-reduction of the wave I product in the wave II process and supports the notions that most of the $NAD^{\cdot}$ in the vicinity of the electrode forms the 1 e^- product before the potential for formation of the 2 e^- product is reached and that the peak IIc current is due to reduction of the $NAD^{\cdot}$, which diffuses through the depleted layer. At scan rates greater than 10 V/s and pH below 7, a small cathodic peak ($U_p = -1.35$ V) appears, which seems to grow with increasing scan rate; this peak may be due to adenine reduction and/or dimer desorption (*cf.* subsequent discussion).

In aprotic media, NAD^- gives a 1 e^- wave of $U_{1/2}$ equal to —0.98 V; the second wave is not seen before background discharge at —2.7 V.[20]

Effects of the adenine moiety in $NAD^{\cdot}$

The faradaic behavior of $NAD^{\cdot}$ largely reflects the presence of the pyridine moiety and is, therefore, much the same as that, for example, of $NMN^{\cdot}$ [11] (Fig. 1); however, under certain conditions, the voltammetric patterns are more complicated as a result of adsorption and apparent faradaic phenomena due to the adenine moiety.

Adsorption phenomena. – The steps or waves that appear in the *d.c.* polarographic patterns of NAD^- at the D.M.E. in addition to the two 1 e^- reduction steps can be explained — largely on the basis of *a.c.* polarography — in terms of adsorption phenomena, *e.g.*, adsorption of NAD^+ at the interface, replacing solvent molecules and background electrolyte and buffer species. In Et_4NCl–carbonate buffer, a wave appears at approximately —0.65 V; the *d.c.* capacitive current is depressed before this wave but is essentially identical to that for the background electrolyte alone after the wave. An *a.c.* tensammetric peak at about the same potential is primarily capacitive in nature; the double layer capacitance is depressed before the peak, but, afterwards, is essentially identical to that for the background alone. The *d.c.* wave is, therefore, a capacitive step; before the step, NAD^- is adsorbed; after the step, $Et_4N^{\cdot}$ is preferentially adsorbed. The strong adsorption of the cationic NAD^+ on the positive side of the electrocapillary maximum (e.c.m.) results from the large tendency for adenine to be adsorbed even in its protonated form.[9,21] On the negative potential side of the e.c.m., the surface-activity of the tetra-alkylammonium ion predominates.

In KCl–carbonate buffer, the *d.c.* polarographic pattern shows the capacitive current to be depressed over the entire potential region. A capacitive wave or maximum and an *a.c.* tensammetric peak, primarly capacitive in nature, which appear at a potential intermediate between waves I and II (*ca.* —1.3 V), has been ascribed to desorption of dimer; as the potential becomes more negative, the background electrolyte

cation (K^+) displaces the dimer because of increasing coulombic attraction of K^+ to the electrode.

The fact that the adenine moiety plays a major role in the adsorption process can be seen by comparing the adsorptive behavior of NAD^+ with NMN^+, which represents the nicotinamide portion of the NAD^+. NMN^+ and its reduction products are negligibly adsorbed.[11] NAD^+ behaves similarly to nearly all adenine nucleosides and nucleotides in being strongly adsorbed in the potential region prior to and in the vicinity of the e.c.m., and in giving rise to desorption peaks at more negative potential.[13,21] Similarly, the adenine or purine–related moiety of such NAD–related compounds as $NADP^+$, $DNAD^+$, $DNADP^+$, and α–NAD^+ causes adsorption behavior characteristic for each individual compound. On the addition of Et_4NCl, adsorption of the dinucleotides beyond the e.c.m. is minimized due to the preferential adsorption ot Et_4N^+; the *d.c.* polarographic patterns of all of the dinucleotides are then very similar to each other and to NMN^+.

Mercury–adenine compound formation. – D.M.E. polarograms of NAD^+ above pH 9 often exhibit, as mentioned, close to mercury discharge a small anodic wave, which also appears on *a.c.* polarography, *e.g.*, the in–phase and quadrature current components are identical at low frequency (15 Hz) but only the in–phase component appears at higher frequency (100 Hz).

The electrode reaction can best be studied by cyclic voltammetry. Above pH 8, a redox couple appears close to mercury discharge. In pH 9.3 carbonate buffer, the anodic and cathodic peaks of the couple are 20 mV or less apart and of identical height after repetitive cycling at 0.1 V/s in a potential region which includes both peaks. With increasing pH, both peaks become more negative, *i.e.*, the potential midway between them varies as follows :

$$U = 0.500 - 0.038\ \mathrm{pH} \qquad (2)$$

The peaks increase in height with concentration but become independent of concentration above 0.04 mM ; even 0.001 mM NAD^+ solutions yield peaks of constant height if the H.M.D.E. is placed in contact with the solution for a sufficient period of time before initiation of voltammetry. The constant peak heights indicate a process mainly dependent on electrode area. Similar results were found for AMP, ADP, ATP, and NADH, but not for nicotinamide or NMN^+. NAD^+ did not give the redox couple at the pyrolytic graphite electrode.

These results indicate reaction of the adenine moiety with oxidized mercury to form a compound which coats the electrode surface ; the redox couple represents oxidation and reduction of mercury. Similar results have been reported for adenine itself.[18] In the present study, a pH 4.8 solution of 10 μM adenine showed this peak pair.

Possible adenine reduction. – The adenine moiety of NAD^+ and

related compounds might be expected to behave similarly to such compounds as adenosine, AMP, ADP and ATP, whose electrochemical behavior resembles that of the parent adenine.[16,21] Up to about pH 5, these adenine-derived compounds generally exhibit a diffusion controlled 4 e^- wave due to reduction of the protonated species ; the wave is kinetically controlled at higher pH where it begins to disappear. $U_{\frac{1}{2}}$ becomes more negative with increasing pH. Solution discharge is more positive in their presence since adenine lowers the overpotential for hydrogen ion reduction even in slightly alkaline solution, where direct reduction is not seen, *e.g.*, ADP in pH 8 McIlvaine buffer shifts the background discharge 100 mV positive.

Some evidence supporting electrolytic reduction of the adenine moiety in NAD^+ has been reported.[22,23] Macroscale reduction of NAD^+ in acidic medium at a large mercury pool cathode (at an uncontrolled potential of —12 V *vs.* an isolated platinum anode) gave appreciable reduction of adenine, accompanied by an almost equivalent amount of deamination (under macroscale conditions, as previously noted, the initial 4 e^- reduction product of adenine can be further reduced as a result of slow deamination at the 6-position regenerating the 1,6 double bond). At pH 8, reduction of the adenine ring was sharply diminished and no deamination (as measured by ammonia evolution) was found.

On the basis of pulse radiolysis experiments, it was postulated that, when an electron reacts with the adenine moiety of NAD^+, it transfers rapidly to the nicotinamide ring.[24] Reduction by pulse radiolysis generally involves only a single electron, *e.g.*, NAD^+ forms a dimer. On electrolysis of NAD^+ in acidic solution at the H.M.D.E., virtually all of the NAD^+ in the vicinity of the electrode is converted to the dimer at a potential considerably more positive than the reduction potential of the adenine moiety ; thus, no transfer of electrons from the latter to the oxidized pyridine moiety would be expected.

In the present study, following well-formed NAD^+ D.M.E. wave Ic ($U_{\frac{1}{2}} = -0.89$ V), an ill-defined wave ($U_{\frac{1}{2}} = -1.3$ V) appears on the rising portion of solution discharge in pH 5 acetate buffer (discharge is 250 mV more positive in the presence of NAD^+) ; under the same conditions, NMN^+ does not exhibit the latter wave, which is best studied at low temperature (0.1 °C) in low capacity acetate buffers between pH 5 and 6. At pH 5.6, the limiting portion is virtually independent of mercury column height, but is strongly dependent on buffer capacity and NAD^+ concentration. In general, it is difficult to study the wave because of its sensitivity to experimental conditions.

In 0.5 *M* McIlvaine buffer of pH 4.8, 0.1 m*M* NAD^+ shows the same D.M.E. polarographic wave at —1.37 V that 10 μM adenine shows in the same potential region (—1.35 to —1.37 V) except that the wave height is approximately ten times that of the adenine wave.

On cyclic voltammetry of NAD^+ at pH 5.5, an oddly sharp peak appears at —1.36 V on the rising background discharge ; NMN^+ does not exhibit a similar peak (Fig. 2). Under similar conditions, only the peak corresponding to the first 1 e^- addition to NAD^+ has been reported.[25]

The potential and current of the peak seen at —1.36 V in acetate buffer depend on pH (Fig. 3):

$$U_p = -1.074 - 0.057\ \mathrm{pH} \qquad (3)$$

The current, which decreases from five times that od NAD^- peak I_c at pH 2 to zero at pH 6.5 and above, may involve a multiplicity of processes: direct adenine reduction, reduction of the pyridine moiety, catalytic hydrogen reduction promoted by adenine and the adenine and pyridine moiety reduction products, and sudden desorption of dimer, which might account for the sharpness of the peak. For example, the peak may be due to catalytic hydrogen evolution resulting from a rearrangement of the electrode double-layer during desorption, and/or reduction of unreduced NAD^- in the vicinity of the electrode as the dimer film on the latter breaks. At high scan rates (60 V/s), the NAD^- reduction appears as a broad peak due to overlapping of the two peaks at —1.1 and —1.3 V.

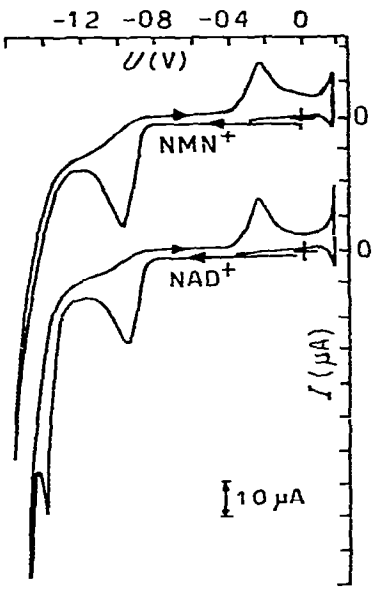

Fig. 2

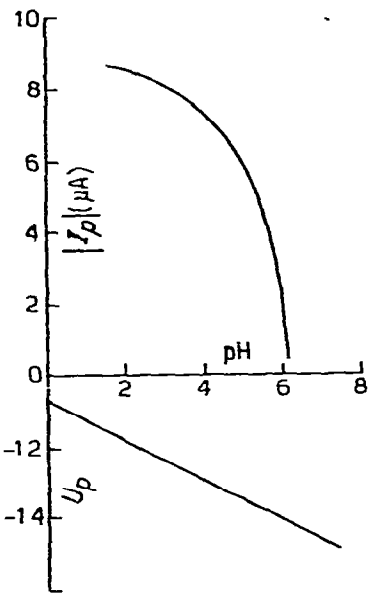

Fig. 3

Fig. 2.
Cyclic voltammograms at the H M D E. of NAD^+ (0.49 *M*) and NMN^+ (0.58 m*M*) in pH 5.5 acetate (0.05 *M*) buffer. Scan rate = 0.1 V/s. Electrode area = 0.029 cm^2

Fig. 3
Variation in the voltammetric behavior at the H M D E with pH of the cathodic peak due to possible reduction of the NAD^- adenine moiety. McIlvaine and acetate buffers, NAD^+ concentration = 0.32 m*M*; electrode area = 0.031 cm^2.

$NADP^+$ and α–NAD^+ behave similarly, except that the —1.3 V peak disappears at pH 5 for $NADP^+$ and at pH 7 for α–NAD^+. The peak is absent with NMN^-, $DNAD^+$ and $DNADP^+$, which do not contain the adenine moiety; the latter two compounds are more strongly adsorbed than NAD^+. In $DNAD^+$ and $DNAP^-$, reduction of the hypoxanthine moiety would be expected to be a 2 e^- process similar to that of hypoxanthine itself, which appears as an ill–defined inflection on background discharge.[16]

At higher frequencies (500–1000 Hz) on *a.c.* polarography of NAD^+, a faradaic peak (U_s = —1.32 V) is observed below pH 6 (height is greater at the higher frequency), which corresponds to the cyclic voltammetric peak at —1.36 V. $NADP^+$ and α–NAD^+ exhibit similar *a.c.* peaks; that for $NADP^+$ is more positive than the NAD^- peak while that for α–NAD^+ is more negative. In the case of the second harmonic *a.c.* curves, in addition to those corresponding to peaks I and II, at high enough frequency (500 Hz), an additional derivative pattern is noticeable, whose potential (—1.32 V) agrees with the fundamental *a.c.* and cyclic voltammetric patterns.

Exhaustive controlled–potential electrolysis of NAD^+ (wave I potential) shows the absence of adenine reduction, as indicated by absorption spectra and the subsequent regeneration of NAD^+ during oxidation of the dimeric product. This result is in contrast to the observation of adenine reduction during uncontrolled–potential electrolysis.[22] It is possible that in the latter case a specific chemical reductant for adenine was produced during the electrolysis.

Although all of the experimental evidence is consistent with reduction of the adenine moiety in NAD^+, conclusive evidence is not available. The facts that NAD^+ largely exists in aqueous solution in a folded configuration, that at mercury electrodes the adsorption site in NAD^+ is the adenine moiety, and that NAD^+ undergoes reversible reduction in the adsorbed state, can be rationalized by a situation, in which folded NAD^+ is the adsorbed species at the solution|electrode interface with the more hydrophobic adenine ring system facing the electrode and the more hydrophilic nicotinamide facing the solution with the electron–transfer process involving the passage of electrons from the electrode *via* the adenine to the pyridine ring.

Since adsorbed NAD^+ is initially reduced to a free radical, which dimerizes to an adsorbed dimer, it is difficult to use as a mechanism–evaluative test the fact that adsorbed NAD^+ is reduced about 0.1 V more easily than unadsorbed NAD^+.[13]

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