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Electrochemical Oxidation in Aqueous and Nonaqueous Media of Dihydropyridine Nucleotides NMNH, NADH, and NADPH

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Abstract: The electrochemical oxidation of dihydronicotinamide mononucleotide (NMNH), dihydronicotinamide adenine dinucleotide (NADH), and dihydronicotinamide adenine dinucleotide phosphate (NADPH) has been studied in aqueous media at carbon electrodes and in dimethyl sulfoxide at platinum electrodes. These 1,4-dihydropyridines undergo two-electron oxidation to generate the parent pyridine nucleotides, which, in turn, are reduced at considerably more negative potential in one-electron processes to free radicals which dimerize. The dimers are oxidized at considerably less positive potential than the dihydropyridines to regenerate the parent nucleotides. Protonation of the dihydropyridines by protons liberated at the electrode–solution interface during oxidation of the dihydropyridines or protons available from other solution species leads to their decomposition, producing species which further decompose to give products absorbing at 280–290 nm; the pseudo-first-order rate constants for the acid-catalyzed decomposition in aqueous solution are about 10^{-3} sec^{-1} at pH 4.1 and 10^{-5} sec^{-1} at pH 7.1 with NADH decomposing somewhat slower than the other two dihydropyridines. Bases such as pyridine and tributylamine and dissolved oxygen have no appreciable effect on the oxidation of the dihydropyridines in DMSO.

The importance of the pyridine nucleotides and their dihydro reduction products in the series of oxidation–reduction reactions termed the biological electron transport chain[2,3] emphasizes the desirability of thorough electrochemical investigation of their redox half-reactions. Although the electrochemical reduction of the pyridine nucleotides has been moderately extensively investigated,[3-5] there are only a few systematic studies of the electrochemical oxidation of relevant dihydropyridine derivatives.[6-8] Blaedel and Haas[6,9] investigated the oxidation of 1-alkyldihydropyridines (1-(2,6-dichlorobenzyl)-, 1-methyl-, 1-benzyl-, and 1-(*n*-propyl)-1,4-dihydronicotinamides) in acetonitrile at platinum and carbon electrodes. In the presence of bases such as pyridine or tributylamine, these RNH-type compounds underwent a 2 e oxidation to yield pyridinium salts (RN^+). In the absence of base, the RNH compounds were oxidized by a 1 e process to RN^+ and several unidentified species. These results suggest that the dihydropyridines might be oxidized in a 1 e process at acidic pH and in a 2 e process in basic pH. Such studies in media more acidic than pH 6 or 7 are hampered by the instability of the dihydro compounds in these media.[10] Leduc and Thevenot[7] concluded that 1-propyl- and 1-benzyl-1,4-dihydronicotinamides and NADH underwent a stepwise 2 e oxidation in aqueous solution to yield the corresponding nicotinamides (RN^+).

Oxidizing agents such as 2,6-dichlorophenol, indophenol, and malachite green have been successfully employed in the oxidation of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide and 1-(2,6-dichlorobenzyl)-1,6-dihydronicotinamide to the corresponding parent nicotinamides; most of the chemical oxidations have been explained in terms of the direct transfer of a hydride ion from the dihydropyridine to the oxidizing agents.[11] Enzymatic oxidations of NADH and NADPH to NAD^+ and $NADP^+$ are well known and have been similarly explained.[12]

As part of a systematic study by electrochemical techniques of the redox patterns and allied chemical behavior for the sequence of compounds from 3-nicotinamide itself through the NAD species, we have examined the electrochemical oxidation patterns for the following biologically active 1,4-dihydropyridine nucleotides in both aqueous and nonaqueous media: dihydronicotinamide mononucleotide (NMNH), dihydronicotinamide adenine dinucleotide (NADH), and dihydronicotinamide adenine dinucleotide phosphate (NADPH).

Experimental Section

Materials. 1,4-Dihydronicotinamide mononucleotide (NMNH, purity 90%), 1,4-dihydronicotinamide adenine dinucleotide (NADH, purity 98%), and 1,4-dihydronicotinamide adenine dinucleotide phosphate (NADPH, purity 96%) (Sigma Chemical) were

used as received since polarography and spectrophotometry, and the nature of the probable impurities, indicated the absence of significant interferences. Tetra-*n*-butylammonium perchlorate (Fisher Scientific; ACS Grade; dried under vacuum at 100° for 6 hr) was used as background electrolyte (0.1 *M*) in DMSO. Pyridine (Matheson; Spectral Grade) and tri-*n*-butylamine (Aldrich) were used. DMSO was purified by distillation; the middle fraction was used.

Buffer solutions (0.1 *M* in their minor constituent) were prepared from reagent grade chemicals; their ionic strengths were adjusted to 0.5 *M* with KCl, except for the pH 7.1 Tris buffer which had an ionic strength of 0.8 *M*. The solution components were HOAc and NaOAc for pH 3.8, 4.1, and 4.9, potassium hydrogen phthalate and NaOH for pH 5.6, and $KHCO_3$ and K_2CO_3 for pH 10.2; the pH 7.1, 8.1, 9.2 and 9.4 solutions were prepared from tris(hydroxymethyl)aminomethane (Tris) and HCl.

Electrodes. The working electrode for voltammetry in aqueous media was a 1-cm (length) × 0.3-cm (diameter) glassy carbon disk (Tokai Electrode Manufacturing Company of Tokyo), cemented into 5-mm o.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 by hand on a hospital tissue. Between runs, the electrode was rinsed with distilled water and wiped with a paper tissue; no further grinding with the SiC paper was required. The effective electrode area was found to be 0.0410 cm^2 by measurement of the anodic potassium ferrocyanide cyclic voltammetric peak ($n = 1$; $D = 6.32 \times 10^{-6}$ cm^2/sec).[13]

Large-scale electrolyses in aqueous media were made on a 1-cm (diameter) pyrolytic graphite (Pyroid; Space Age Materials Corp.) electrode, prepared similarly to the glassy carbon electrode. It was necessary to resurface this electrode between electrolyses on the SiC disk.

The reference electrodes used in both aqueous and nonaqueous studies were aqueous saturated calomel electrodes.

In DMSO, a platinum wire working electrode of 0.057 cm^2 area was employed in cyclic voltammetry. The counter electrode was a platinum coil.

Electrochemical Cells. A three-compartment, water-jacketed cell[14] was used in the aqueous studies. Nonaqueous experiments were performed in a previously described cell attached to a vacuum line.[15]

Spectrophotometry. Spectral studies were carried out in 1-cm quartz cells with a Beckman DB spectrophotometer connected to a Heathkit Model EUW-20 recorder.

Procedures. Aqueous polarographic dme and rotating glassy carbon electrode (rgce) studies were made at a scan rate, v, of about 3 mV/sec. A variable speed Caframo motor was used to rotate the rgce at 4 rps, except where otherwise specified. Cyclic voltammetric studies were made at v between 0.05 and 30 V/sec. A multipurpose instrument[16] was used for all electrochemical measurements.

In DMSO, the solution was degassed by a freeze–pump–thaw cycle and the vacuum was released by introduction of a nitrogen atmosphere. In the cyclic voltammetric studies, v ranged from 0.04 to 10 V/sec; at higher v, the peak current was not well defined as a result of the large charging current. The peak currents were measured, using the base line obtained by extrapolating the background current seen before the oxidation of the electroactive species. In this connection, the cyclic voltammetric curves obtained for background electrolytes alone were useful in checking the background currents observed at the potentials where peaks appear. The probable precision of peak current measurements by this procedure is about ±2%. Coulometric experiments were conducted with a coiled platinum working electrode with the number of coulombs passed being determined by using a voltage-to-frequency converter and counter combination.[17] Other electrochemical measurements were made with a multipurpose instrument, based on solid state operational amplifiers.[16,18]

In the hydrolytic stability studies, 10 ml of pH 4.1 buffer solution was added to the cell at 25° and deaerated by bubbling nitrogen through it for at least 15 min. A weighed portion of a dihydropyridine nucleotide was added (time of addition recorded). A voltammogram at the rgce was immediately recorded from 0 to 1.5 V, followed by a dme voltammogram (0 to −1.3 V); times at start and finish of each voltammogram were noted. This procedure was repeated at varying time intervals over a period of 4.5 hr for NADH and NADPH, and 10 hr for NMNH. The pH was then measured (it was unchanged at pH 4.1 in all cases) and adjusted to between pH 7 and 8 by addition of about 0.2 ml of saturated sodium hydroxide solution (resulting dilution was only about 2%). Final voltammograms were then recorded at the rgce and dme.

In the pH 7.1 stability studies, voltammetric scans from 0 to 1.4 V at the rgce were made on similarly prepared solutions at varying time intervals throughout a total period of about 24 hr. The rate constants were calculated from the decrease in i_l of the dihydropyridine anodic wave.

Results and Discussion

The electrochemical oxidation of the dihydropyridine nucleotides was studied by cyclic voltammetry, potential step electrolysis, rotating disk voltammetry, and controlled electrode potential electrolysis and coulometry in buffered aqueous media (pH 3.8 to 10.2) at glassy carbon electrodes (pyrolytic graphite electrodes were used for large scale electrolyses) and in dimethyl sulfoxide (DMSO; tetra-*n*-butylammonium perchlorate as background electrolyte) at platinum electrodes. All potentials are referred to the aqueous saturated calomel electrode unless otherwise specified.

In pH 7.1 aqueous medium, each dihydropyridine shows a single, well-defined, anodic peak at about 0.7 V on cyclic voltammetry (Table I; Figure 1). Reversal of the scan at a potential positive to this peak produces a cathodic peak at about −1.15 V, which is due to reduction of the product of the anodic peak process. Initial scan toward negative potentials did not generate this cathodic peak; it appeared only after an initial oxidation and was identified as due to reduction of the pyridine nucleotide itself; all of the latter show reduction peaks at −1.15 ± 0.03 V. A cathodic peak complementary to the anodic peak, corresponding to a reversible redox couple, was not seen in the 0.5 to 0.7 V region even at scan rate (v) of 30 V/sec.

The anodic peak height varied linearly with $v^{1/2}$ as expected from theory[19] (Figure 2). The ratio of the cathodic and anodic peak heights (i_{pc}/i_{pa}) increased with increasing v (Figure 3) to a limiting value between 0.3 and 0.4. For scan rates greater than those shown in Figure 3, the background charging current became large with respect to the peak currents and meaningful measurements could not be made.

Based on the difference in $E_{1/2}$ between pH 4.1 and 7.1, the anodic wave seen at the rotating glassy carbon electrode (rgce) shows a slight pH dependence of −0.011 V/pH for NADH and of −0.017 V/pH for NMNH and NADPH. It is possible that the observed shifts are due not to pH but to differences in buffer composition and/or ionic strength.

In DMSO, each dihydropyridine nucleotide also shows a single, well-defined anodic peak (Table I; Figure 4), whose height varies linearly with $v^{1/2}$; at v exceeding 10 V/sec, the peak merges with background discharge. Scan reversal positive to the oxidation peak generates a cathodic peak at about −1.0 V (Table I), which can be identified with the peaks shown by the pyridine nucleotides themselves, e.g., E_{pc} at a hmde of −0.99 V for NMN^+, −0.98 for NAD^+, and −1.06 for $NADP^+$. No complementary peaks were seen for the anodic and cathodic peaks even at v of 5 to 10 V/sec.

On the basis of the anodic processes in both water and DMSO being 2 e in nature with inconsequential loss due to acid-catalyzed reactions under cyclic voltammetric conditions (cf. subsequent discussion), the viscosities at 25° of 1.996 for DMSO and 0.894 for water lead to an expected current function (i_{pa}/AC) ratio for H_2O/DMSO of about 1.49. The ratios for the Table I data are 1.75 for NMNH, 1.50 for NADH, and 1.52 for NADPH. It is difficult to ac-

Table I. Voltammetric Behavior of Dihydropyridine Nucleotides[a]

Compd	Medium[b]	Anodic peak			Cathodic peak			
		E_{pa}, V	i_{pa}, μA	i_{pa}/AC, μA/(cm² mM)	$-E_{pc}$, V	i_{pc}, μA	i_{pc}/AC, μA/(cm² mM)	i_{pc}/i_{pa}
NMNH	H_2O	0.67	13.1	210	1.17	4.0	65	0.31
	DMSO	0.76	9.1	120	0.97	2.2	29	0.24
NADH	H_2O	0.67	11.8	204	1.13	3.0	52	0.25
	DMSO	0.89	16.0	136	0.95	4.2	36	0.26
NADPH	H_2O	0.72	10.7	240	1.18	2.5	55	0.23
	DMSO	0.84	3.6	158	1.00	1.1	48	0.31

[a] Scan rate was 0.10 V/sec; electrode types and areas were carbon (0.041 cm²) for H_2O and platinum (0.057 cm²) for DMSO solutions. Estimated error in current measurement is ±2%. [b] Aqueous solutions were pH 7.1.

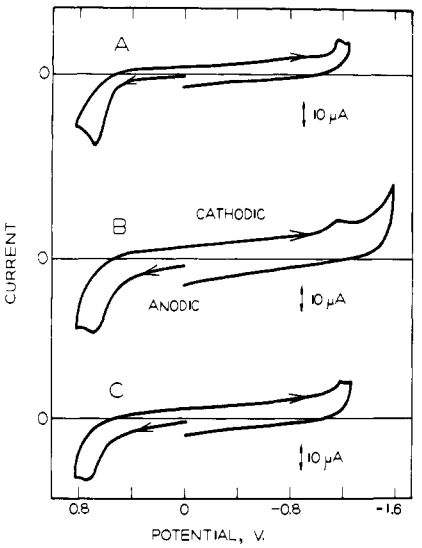


Figure 1. Cyclic voltammograms at a glassy carbon disk electrode in pH 7.1 aqueous Tris buffer of (A) NMNH (dihydronicotinamide mononucleotide) (1.5 m*M*), (B) NADH (dihydronicotinamide adenine dinucleotide) (1.4 m*M*), and (C) NADPH (dihydronicotinamide adenine dinucleotide phosphate) (1.1 m*M*). Scan rate: 0.2 V/sec. Arrowhead indicates direction of scan.

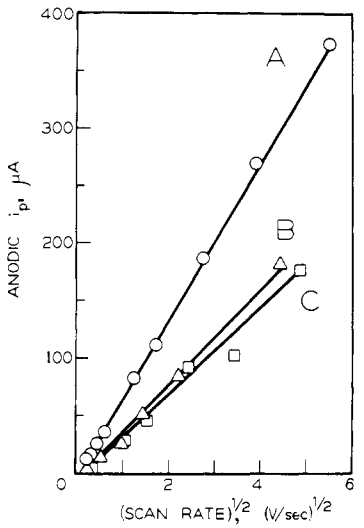


Figure 2. Effect of cyclic voltammetric scan rate, *v*, on the anodic peak height at a glassy carbon disk electrode in pH 7.1 aqueous Tris buffer for (A) NMNH (1.5 m*M*), (B) NADH (1.4 m*M*), and (C) NADPH (0.7 m*M*).

count for the ratio for NMNH being 17% higher than expected.

The overall irreversibility of the anodic electrode processes is demonstrated by the lack of complementary cathodic peaks, ($E_p - E_{p/2}$) values of 80 to 110 mV (Figure 1), and an ($E_p - E_{1/2}$) value of 70 mV for NADH at pH 7.1.

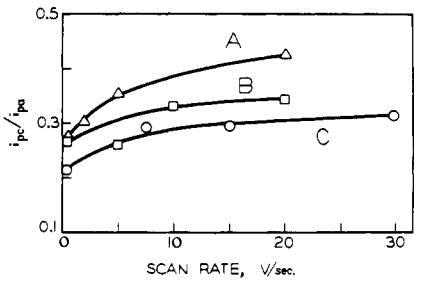


Figure 3. Effect of cyclic voltammetric scan rate, *v*, on the ratio of cathodic peak current (i_{pc}) to anodic peak current (i_{pa}) at a glassy carbon disk electrode in pH 7.1 aqueous Tris buffer for (A) NADH (1.1 m*M*), (B) NADPH (1.1 m*M*), and (C) NMNH (1.5 m*M*).

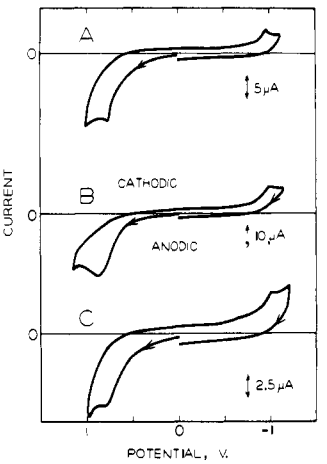


Figure 4. Cyclic voltammograms at a platinum electrode in dimethyl sulfoxide (Bu_4NClO_4 background) of (A) NMNH (1.3 m*M*), (B) NADH (2.1 m*M*), and (C) NADPH (0.4 m*M*). Scan rate: 0.2 V.sec. Arrowhead indicates direction of scan.

Irreversible cyclic voltammetric peak currents are defined by the expression[20]

$$i_p = [2.98 \times 10^5 n(\alpha n_a)^{1/2} A D^{1/2} C] v^{1/2} \quad (1)$$

where i_p is the peak current (μA) at scan rate *v* (V/sec), *A* is the electrode area (cm²), *D* is the diffusion current (cm²/sec), *C* is the bulk solution concentration (m*M*), α is the transfer coefficient, and n_a is the number of electrons in the rate-determining step. The number of electrons, *n*, transferred in the faradaic process was estimated by setting the slope of the $i_p - v^{1/2}$ plot for each dihydropyridine equal to the bracketed term in eq 1 and by estimating the product αn_a from the slope of the $E_{p/2}$-log *v* plot for each compound[20]

$$\alpha n_a = 0.0294/\text{slope} \quad (2)$$

The estimated αn_a values in pH 7.1 solution were 0.81, 0.46, and 1.01, for NMNH, NADH, and NADPH, respec-

Table II. Faradaic n Values for Dihydropyridine Nucleotides

Medium[a]	Compd	n^b	n^c	n^d
H_2O (pH 7.1)	NMNH	1.9	1.4	1.9
	NADH	2.0	1.3	2.0
	NADPH	2.0	1.5	2.4
DMSO	NMNH	2.0	1.6	2.0
	NADH	1.9	1.6	2.0
	NADPH	2.1	1.6	2.2

[a] Supporting electrolyte in DMSO was $(C_4H_9)_4NClO_4$. Tris buffer was used for the aqueous medium of pH 7.1. [b] Calculated from cyclic voltammetric data. [c] Calculated from controlled electrode potential coulometry. [d] Calculated from chronoamperometric data.

tively. On using D for the oxidized pyridine nucleotides,[21] the n values listed in Table II were calculated.

NMNH (1.32 mM in DMSO) shows an anodic peak at 0.84 V (v = 40 to 200 mV/sec) at a graphite disk electrode. Reversal of the scan at a potential positive to this peak results in a cathodic peak at −0.97 V. Since the high background current at the graphite electrode in DMSO made current measurements difficult, no further studies were done with graphite in DMSO.

Effect of Added Bases. Earlier studies[6] of NADH analogs in acetonitrile ascribed the increase in peak current to almost twice its original magnitude on pyridine and tri-*n*-butylamine addition to a 2 e oxidation process in the basic medium. The effects of these bases on the oxidation of 0.8 mM NADH in DMSO were examined. Pyridine in concentrations up to 129 mM did not significantly affect the anodic or cathodic peak currents.

Studies involving tributylamine were complicated by the closeness of its oxidation peak to that of NADH; the tributylamine peak shifts to more positive potential with increasing amine concentration. Thus, the 0.89-V peak grew with increasing amine concentration but, above 1.3 mM amine, a second peak separated from the 0.89-V peak, which decreased to its original magnitude, *i.e.*, the amine and NADH peaks are superimposed until the amine concentration is sufficiently large to shift the amine peak to a potential positive enough to resolve the two peaks. At high amine concentrations (50 mM), the NADH peak appeared on the rising portion of the amine peak, but the magnitude of its current was apparently unaffected.

In the presence of either tributylamine or pyridine, the two more positive peaks of the three anodic peaks observed for 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide in acetonitrile disappeared, and the least positive peak approximately doubled in height;[6] a similar effect was not observed in the present studies.

Effect of Dissolved Oxygen. Since oxygen has been found to have a considerable effect on the voltammetry of NADH analogs in acetonitrile,[6] the behavior of NADH in the presence of oxygen in DMSO was examined. The anodic NADH peak is unaffected by the presence of oxygen. On removal of oxygen by nitrogen purging, the anodic-cathodic pattern is identical to that of the original solution.

In the presence of oxygen, a large cathodic peak with E_p of −0.75 V appeared; this is in agreement with the reported $E_{1/2}$ of oxygen in DMSO ranging from −0.72 to −0.85 V with −0.73 and −0.77 V being favored.[22] It should be noted that in some experiments, apparently at random, a small peak of E_p = −0.75 V was observed, which may have been due to residual oxygen and/or to oxygen produced at the very positive potentials to which the original scan was carried in DMSO, perhaps by electrolysis of residual water.

Chronoamperometry. Chronoamperometry was used to estimate the number of electrons involved in the dihydropyridine oxidation. In DMSO, the working platinum electrode potential was stepped from 0 to well within the limiting current region: 0.90 V for NMNH, 1.06 V for NADH, and 1.00 V for NADPH. During electrolysis, the current decayed smoothly to a steady state value; typical decay curves are shown in Figure 5. The decay current may be fitted to the expression

$$i_t = nFAD^{1/2}C/\pi^{1/2}t^{1/2} \quad (3)$$

where i_t is the current at time t during electrolysis. On using D for the oxidized form of the pyridine nucleotides,[21] n values of 2 were calculated (Table II).

In pH 7.1 aqueous medium, the carbon electrode potential was stepped from 0 to 0.80 V and n was similarly calculated (Table II).

Controlled Potential Coulometry. Large-scale controlled electrode potential electrolysis at 0.80 V of pH 7.1 solutions of NMNH, NADH, and NADPH (three or four electrolyses of each) gave faradaic n values ranging between 1.2 and 1.7 (averages given in Table II). The solutions (originally 0.3 to 3.5 mM) were colorless throughout electrolysis, whose duration varied from 2 to 7 hr.[23] Oxidation of NADH at pH 10.2 gave an n of 1.5. Due to the relatively rapid decomposition of the dihydropyridines in acidic media, it was difficult to determine n accurately in such media.

Coulometric oxidation of the dihydropyridines in DMSO yielded an n of 1.6 (Table II). The electrolysis current decayed steadily with time and reached background value.

Product Identification. Prior to electrolytic oxidation in aqueous media (pH 7.1), NMNH had one ultraviolet absorption maximum at 338 nm (molar absorptivity (ϵ) = 3.6 × 10^3). After oxidation, which was not complete, the 338-nm peak decreased considerably (apparent ϵ = 0.5 × 10^3) and a new peak appeared at 265 nm (apparent ϵ = 3.2 × 10^3). After two of the four oxidations, a third peak attributable to decomposition reaction products appeared at about 275 nm with an absorbance of about 0.8 that of the 265-nm peak. Since NMN^+ shows a single peak at 265 nm (ϵ = 3.6 × 10^3), the major oxidation product is likely to be NMN^+.

NADH and NADPH themselves have two peaks each (Figure 6) at pH 7.1 at about 338 and 260 nm (ϵ = 5.0 × 10^3 and 15 × 10^3, respectively, for NADH, and 5.3 × 10^3 and 14 × 10^3, respectively, for NADPH). The 338-nm peak is attributable to the dihydronicotinamide moiety and the 260-nm peak to the adenine moiety (p 626 of ref 11). After electrolysis, the 338-nm peaks decreased to apparent ϵ values of 1.6 × 10^3 for NADH and 0.3 × 10^3 for NADPH, while the 260-nm peaks increased slightly with either no noticeable change in shape or a slight broadening on the long wavelength side of the peak, probably due to the absorption by the decomposition products, to apparent ϵ values of 16 × 10^3 for NADH and 15 × 10^3 for NADPH. NAD^+ and $NADP^+$ each show only one peak at about 260 nm (Figure 6) due to the combined absorption of the adenine and nicotinamide moieties with ϵ values of 18 × 10^3 for NAD^+ and 15 × 10^3 for $NADP^+$.

Prior to electrolytic oxidation of NNMH, NADH, or NADPH in pH 7.1 aqueous solution, no cathodic dme wave was visible between 0 and −1.6 V. After oxidation of NMNH (four runs), a wave of $E_{1/2}$ = −0.93 V appeared; in two runs, a second wave appeared ($E_{1/2}$ = −1.07 V), whose height was about 0.2 that of the first wave in one case and about equal in the other. Oxidation of NADH (three runs) produced a single wave ($E_{1/2}$ = −0.89 V); in a fourth run, a wave was also seen at −1.06 V (height was about 0.2 that of the first wave). After oxidation (three runs), NADPH solutions gave waves at $E_{1/2}$ of −0.90 V in all cases and of −1.07 in two cases; the latter wave was about 1.4 times the height of the −0.90-V wave in one case

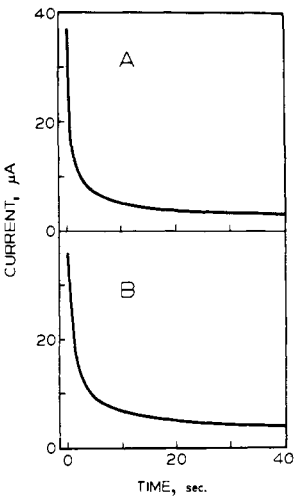


Figure 5. Typical chronoamperometric decay curves obtained at a platinum electrode in dimethyl sulfoxide (Bu_4NClO_4 background) for (A) NMNH (1.3 mM), stepped from 0 to 0.90 V, and (B) NADH (2.1 mM), stepped from 0 to 1.06 V.

and about 0.23 times its height in the other. Samples of pure NMN^+, NAD^+, and $NADP^+$ gave single dme waves at $E_{1/2}$ of −0.93, −0.89, and −0.90 V, respectively. The possible origin of the waves at ca. −1.06 V is subsequently discussed (cf. section on stability).

Large-scale reduction at −1.00 V at a mercury pool electrode of the pH 7.1 solutions obtained on large-scale electrolytic oxidation gave solutions which showed an anodic polarographic wave ($E_{1/2}$ = −0.24 V for the NMNH derivative and about −0.30 V for the NADH and NADPH derivatives). Identical anodic waves, observed[16,21,24] after reduction at −1.0 V of NMN^+, NAD^+, and $NADP^+$, have been attributed to oxidation of the dimers produced on 1 e reduction of the nicotinamides to the corresponding free radicals.

Addition of alcohol dehydrogenase (ADH) and ethanol to a pH 7.1 solution of the NADH oxidation product results in disappearance of the cathodic dme wave at −0.89 V and growth of a rgce anodic wave at 0.59 V. Since the NADH wave prior to the electrolysis was at 0.60 V, the enzymatic reaction probably regenerated the original dihydronicotinamide. ADH and ethanol react specifically with NAD^+ to yield NADH.[25]

The results of large-scale electrolyses in DMSO are in general agreement with these obtained in aqueous media. The dihydronicotinamides have absorption maxima at 263 and 335 nm. After controlled potential oxidation, absorption maxima are present at 263 and 280 nm. The 263-nm peak is due to the nicotinamide and adenine chromophores in the pyridine nucleotides;[11,26,27] the 280-nm peak is due to decomposition products.

Prior to electrolysis of the dihydropyridines in DMSO, an initial voltammetric scan toward negative potential shows the absence of any reducible material. After electrolysis, an initial scan toward negative potential produces a cathodic peak at ca. −1.0 V.

Stability of Dihydropyridine Nucleotides. After large-scale electrolytic oxidation of the dihydropyridines at pH 7.1, a second cathodic dme wave was sometimes observed at −1.06 or −1.07 V in addition to the wave always present at −0.89 to −0.93 V and due to the parent pyridine nucleotide. Examination of adenine, adenosine, and adenosine monophosphate at the rgce and dme in pH 7.1 solution revealed no oxidation or reduction waves between 1.2 and −1.6 V; consequently, none of the voltammetric waves observed prior to or after oxidation of the dihydropyridine nucleotides can be attributed to adenine species. Since a possible source of the −1.06-V wave was a compound produced on acid-catalyzed decomposition of the dihydropyridines, the latter was examined.

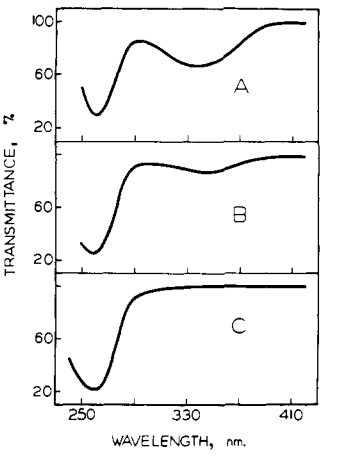


Figure 6. Ultraviolet spectra of 3.6×10^{-5} M aqueous solutions (pH 7.1 Tris buffer) of (A) NADH, (B) NADH after oxidation at 0.80 V at a pyrolytic graphite electrode for 6.6 hr, and (C) NAD^+.

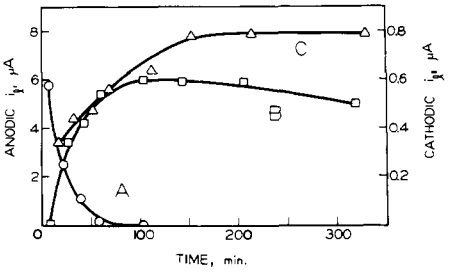


Figure 7. Variation in electroactivity (limiting current, i_l) with time of a pH 4.1 solution of NMNH (0.76 mM): (A) anodic wave (rgce) at ca. 0.68 V; (B) anodic wave (rgce) at ca. 1.08 V; (C) cathodic wave (dme) at ca. −0.96 V. At 575 min, B was 3.3 μA; at 590 min, C was 0.79 μA.

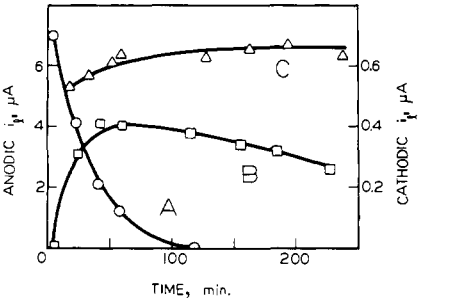


Figure 8. Variation in electroactivity (limiting current, i_l) with time of a pH 4.1 solution of NADH (0.62 mM): (A) anodic wave (rgce) at ca. 0.65 V; (B) anodic wave (rgce) at ca. 1.10 V; (C) cathodic wave (dme) at ca. −0.96 V.

Figures 7 to 9 summarize the changes in voltammetric patterns of pH 4.1 dihydropyridine solutions with time. In each figure, curve A corresponds to the original rgce anodic dihydronicotinamide wave (Ia) ($E_{1/2}$ between 0.64 and 0.71 V), and curves B to a rgce anodic wave (IIa) at about 1.1 V and C to a dme cathodic wave (Ic) ($E_{1/2}$ = −0.94 to −0.98 V), which appear with time. Neither wave IIa nor Ic was observed on repeated voltammetric scans of pH 7.1 solutions of the dihydropyridines, although wave Ia slowly diminished with time.[28] Wave Ic cannot be attributed to the parent pyridine nucleotides, since these give highly reproducible pH-independent dme waves at −0.93, −0.89, and −0.90 V for NMN^+, NAD^+, and $NADP^+$, respectively.

For each dihydropyridine, the wave Ia limiting current

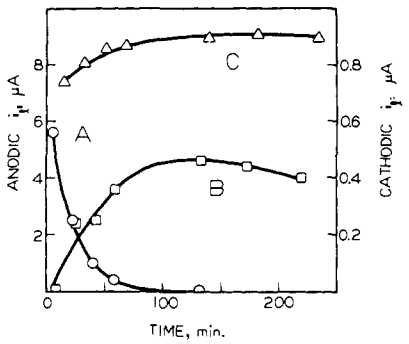


Figure 9. Variation in electroactivity (limiting current, i_l) with time of a pH 4.1 solution of NADPH (0.59 m*M*): (A) anodic wave (rgce) at ca. 0.67 V; (B) anodic wave (rgce) at ca. 1.08 V; (C) cathodic wave (dme) at ca. −0.98 V.

(i_l) at pH 4.1 decreased with time and disappeared completely after about 100 min; wave IIa increased from zero to a maximum value at about 100 min and then decreased; wave Ic increased and approached a limiting value after about 200 min. A plot of log [(i_l for wave Ia)/C] vs. time gave a straight line which extrapolated to a value at time zero, which was that expected for the compound involved, and from which line a rate constant for the decomposition could be calculated.[29]

The pseudo-first-order rate constants for the acid-catalyzed decomposition are estimated to be 8.2×10^{-4} sec^{-1} for NMNH, 5.9×10^{-4} sec^{-1} for NADH, and 8.5×10^{-4} for NADPH at pH 4.1, and 1.0×10^{-5} sec^{-1} for NMNH, 0.7×10^{-5} sec^{-1} for NADH, and 1.1×10^{-5} sec^{-1} for NADPH at pH 7.1.

The constancy with time of absorption spectra and voltammetric curves of DMSO solutions of the dihydropyridine nucleotides indicate their normal stability in such media.

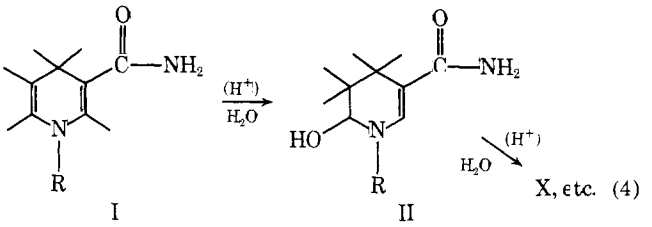
Identity of Acid-Hydrolysis Products. Upon adjustment of the solution pH, after standing, from 4.1 to 7 to 8 by NaOH addition, waves IIa and Ic disappeared, a small anodic wave appeared at about the same potential as that of the original dihydropyridine but with only 0.1–0.2 of its height, and a new dme cathodic wave (IIc) was observed at −1.06, −1.08, and −1.04 V respectively for original NMNH, NADH, and NADPH solutions. This was accompanied by a new cathodic wave for NMNH solution near background discharge at 1.23 V, whose large height would tend to indicate that it was due to discharge of a solution constituent such as oxygen evolution, and by a small cathodic wave for NADH solution at −0.91 V.

The dme wave ($E_{1/2}$ = −1.06 V; i_l = 0.23 μA) observed on adjustment of a 0.76-m*M* NMNH solution to pH 8.0 after 5 hr at pH 4.1 compares with waves of −1.07 V and 0.26 μA observed after 3-hr electrolysis in pH 7.1 buffer of 1.4 m*M* NMNH and 2-hr electrolysis of 0.32 m*M* NMNH. The two cathodic waves (−0.91 V and 0.14 μA; −1.08 V and 0.06 μA) seen on similar adjustment of a 0.62 m*M* NADH solution after 4 hr to pH 7.1 compare with an i_l of 0.09 μA at −1.06 V after 2.5-hr electrolysis of the one 0.37-m*M* NADH solution which showed a second wave (cf. Product Identification). After adjustment of a 0.59 m*M* NADPH solution (5 hr electrolysis) to pH 7.8, the single wave (−1.04 V; 0.17 μA) observed compared with one of −1.07 V and 0.17 μA seen after 7-hr electrolysis of 0.78 m*M* NADPH, and of −1.07 V and 0.78 μA seen after 4-hr electrolysis of 0.45 m*M* NADPH.

A pH 7.1 solution of NADH did not show any cathodic dme wave even after standing for 7 hr, which was the longest period used for an electrolysis.

Although it is not immediately obvious what species produce waves IIa and Ic, it is apparent that these result from the acidity of the solution (pH 4.1). The change to pH 7.1–8.0 results in the formation of wave IIc between −1.04 and −1.08 V. Although this is not proof that the waves sometimes observed at −1.06 or −1.07 V after electrolytic dihydropyridine oxidation were formed in a manner analogous to that by which wave IIc was formed, the closeness of the half-wave potentials does make it seem likely

There is general agreement in the literature[30,31] that the primary acid-catalyzed decomposition product of a compound with the general structure of I has structure II (eq 4) (compounds studied have included NMNH, NADH, and NADPH). Although there is some disagreement as to how compound II reacts further and as to the products of these reactions, there is unanimity that II does react to give secondary acid-reaction product(s).



(4)

However, since the following sequence of reactions is postulated to occur with a rapid first step and a slow second step[30]

$$\underset{\text{I}}{\text{RNH}} \xrightarrow{H^+} \text{H(RNH)}^+ \xrightarrow{OH^-} \underset{\text{II}}{\text{H(RNH)OH}} \qquad (5)$$

$H(RNH)^+$ is a likely intermediate species (cf. Mechanistic Pattern).

On the basis of the relative variation in magnitude of the waves in Figures 7 to 9 and previously reported data, it would be reasonable to assign the anodic wave at 0.6 to 0.7 V to oxidation of I (RNH; the dihydropyridine nucleotide), the anodic wave at *ca.* 1.1 V to oxidation of $H(RNH)^+$, and the cathodic wave at −0.96 to reduction of compound II and, perhaps, also of the secondary hydrolysis product(s). Subsequent change in pH from 4.1 to 7 to 8 could then alter the ease of reduction of the secondary hydrolysis products and/or the chemical nature of the latter to produce at times the dme wave at −1.06 or −1.07 V. An anodic wave at 1.1 V (vs. NHE) observed at a rotating platinum electrode for pH 4.6 to 7.0 solutions of 1 benzyl-1,4-dihydronicotinamide, which was about 0.4 V more positive than the expected RNH wave, was attributed[7] to the oxidation of compound II (eq 4), where R is benzyl.

Mechanistic Pattern

The following mechanism for the electrochemical oxidation of the 1,4-dihydropyridine nucleotides in both aqueous and nonaqueous media is considered to fit best the results obtained in the present study

$$\text{RNH} \longrightarrow \text{RN}^+ + \text{H}^+ + 2e \qquad (6)$$

$$\text{RNH} + \text{H}^+ \rightleftharpoons \text{H(RNH)}^+ \qquad (7)$$

$$\text{H(RNH)}^+ \longrightarrow \text{decomposition products absorbing at 280–290 nm} \qquad (8)$$

where RNH represents the dihydropyridine nucleotide, $H(RNH)^+$ is its protonated form, and RN^+ is the pyridine nucleotide itself (oxidized form). (Reaction 8 may well involve formation of H(RNH)OH as indicated in eq 4 and 5.) During the short time scales of cyclic voltammetric and rotating electrode experiments, reaction 8 proceeds to a negli-

gible extent and, consequently, the number of electrons involved in the electrode process approaches two per molecule of RNH (Table II). Under large-scale electrolysis conditions, reaction 8 removes enough RNH to give the observed coulometric n values of 1.3 to 1.6. $H(RNH)^+$ would not be electrolyzed at the applied potential of 0.80 V, since it would be expected to be considerably more difficult to oxidize than RNH ($E_{1/2}$ = 0.6 to 0.7 V) (cf. previous assignment of the anodic wave at ca. 1.1 V to oxidation of $H(RNH)^+$).

Results obtained in the present study are compatible with acid catalyzed decomposition, i.e., the RNH limiting current decreases with time on rotating disk voltammetry in acidic medium (pH 4.1) and the rate of decomposition decreases with increasing pH. For a rate constant of 1×10^{-5} sec^{-1}, the RNH will be 7, 14, and 19% decomposed after periods of 2, 4, and 6 hr (typical electrolysis times); during an actual electrolysis, the loss would be less due to constant removal of RNH by the electrochemical reaction (eq 6). This must be balanced against the possibility of decomposition occurring more rapidly during electrolysis due to a decrease in pH near the interface as a result of the generation of H^+ (eq 6) at the electrode surface. An appreciable decrease in pH from 7.1 would cause the dihydropyridines to decompose much more rapidly (compare rates at pH 7.1 and 4.1). The release of H^+ at the interface during electrolysis could sufficiently alter the pH in the vicinity of the electrode, in spite of the presence of a buffering system, to allow formation of acid-catalyzed, decomposition product, which would then be altered by the pH change upon diffusion into the bulk solution to yield a product corresponding to the second dme wave (cf. stability discussion). A check of references,[32] in which Tris was suggested for biochemical studies, failed to reveal any mention of the rate of equilibration of the buffer. The mean coulometric n value of 1.4 (Table II) for the dihydropyridines in aqueous solution would result if 30% of the dihydropyridine decomposed during electrolysis.

Acid-catalyzed decomposition reactions such as reactions 7 and 8 have been found[10] to give decomposition product(s) with an absorption maximum around 280–290 nm (cf. ref 30). Spectra of pH 3.8 and 4.1 solutions of the dihydropyridines for time periods about the same as those used for electrolyses, but at 1/20 of the concentration, show absorption maxima in the 280–290-nm region; the characteristic dihydronicotinamide moiety peak at 338 nm disappears during the same time period at pH 3.8 and 4.1 (the 338-nm peak only decreases slightly at pH 7.1, e.g., 1 to 2%).

Similar formation of protonated dihydropyridine nucleotides could occur during electrolysis in DMSO (eq 6 and 7); the rate of decomposition would be expected to be less due to the lower dielectric constant and higher viscosity, and the low residual water content. The coulometric n of 1.6 (Table II) corresponds to 20% dihydropyridine decomposition (eq 7).

Leduc and Thevenot[7] proposed the following two stepwise mechanisms (generalized from their equations for NADH)

$$RNH \xrightarrow[\text{slow}]{-e^-} \dot{R}HN^+ \underset{\text{rapid}}{\overset{-e^-}{\rightleftharpoons}} RNH^{2+} \underset{\text{rapid}}{\overset{-H^+}{\rightleftharpoons}} RN^+ \quad (9)$$

$$RNH \xrightarrow[\text{slow}]{-e^-} \dot{R}NH^+ \underset{\text{rapid}}{\overset{-H^+}{\rightleftharpoons}} \dot{R}N \overset{-e^-}{\rightleftharpoons} RN^+ \quad (10)$$

They were undecided as to which of these two reaction paths was actually followed. We have considered these mechanisms as well as other similar mechanisms, but have found no evidence which indicates a stepwise reaction within the time scales of even our fastest cyclic voltammetric studies.

Comparison with 1-Alkyl Analogs. It has been common in the study of the pyridine nucleotides to use 1-alkyl nicotinamides as model compounds, e.g., the early elucidation[33] of the nonenzymatic reduction of NAD^+. Such model compounds have also been moderately extensively used in electrochemical studies.[3,5] The caution that needs to be exercised in such situations is emphasized by the differences observed between NADH in DMSO in the present study and the 1-alkyl analogs in acetonitrile (AN) in the Blaedel and Haas[6] study. The number of electrons involved in oxidation of the analogs varied between 0.8 and 0.9, and was considered, together with other evidence, to be suggestive of the following process[6]

$$RNH \longrightarrow \dot{R}NH^+ + e \quad (11)$$

$$2\dot{R}NH^+ \longrightarrow RN^+ + H(RNH)^+ \quad (12)$$

$$RNH + H^+ \longrightarrow H(RNH)^+ \quad (13)$$

$$H(RNH)^+ \begin{cases} \nearrow RN^+ \\ \searrow \text{290-nm absorbing products} + \text{nonabsorbing products} \end{cases} \quad (14)$$

where H^+ in reaction 13 could be due to trace amounts of acidic impurities in the AN. The increase in the cyclic voltammetric i_p to a maximum of twice the original peak current on addition of pyridine or tri-*n*-butylamine was explained as due to the process

$$RNH \longrightarrow \dot{R}NH^+ + e \quad (15)$$

$$\dot{R}NH^+ + B \longrightarrow \dot{R}N + HB^+ \quad (16)$$

$$\dot{R}N \rightleftharpoons RN^+ + e \quad (17)$$

where B is sufficiently more basic than the electroactive species RNH and $\dot{R}N$ to ensure only minimum protonation of the latter, and the neutral pyridinyl radical $\dot{R}N$ is more readily oxidized than RNH and, consequently, is oxidized as it is formed.

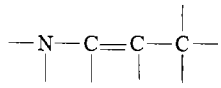
The mechanism of eq 15 to 17 does not seem to apply to DMSO solutions even on the basis of the assumption that DMSO is a sufficiently strong base to drive reaction 16 well to the right. It is difficult to evaluate the validity of this assumption. DMSO is a more basic solvent than AN; e.g., Huber[34a] classifies AN as an aprotic neutral solvent and DMSO as an aprotic basis solvent; for autodissociation of the pure solvent, $pK_a = -\log [CH_3CNH^+][CH_2CN^-] = 19.5$ for AN, and $-\log [C_2H_6SOH^+][C_2H_5SO^-] = 33.3$ for DMSO.[34b] (Dielectric constants are 36 for AN and 49 for DMSO.) However, if DMSO were a strong enough base to drive reaction 16 rapidly and completely to the right, faradaic n values of appreciably less than 2 would not be expected. On the other hand, if the difference in basicity of AN and DMSO is assumed not to be significant in respect to the oxidation of dihydropyridines, the fact that addition of base has a pronounced effect in AN but none in DMSO indicates either that the mechanisms in the two solvents must be different or that the compounds investigated in the two solvents must react differently.

The mechanistic path of eq 11 to 14 is excluded for DMSO by the lack of effect on pyridine and tributylamine addition.

Unfortunately, the poor solubility of NADH, NMNH, and NADPH in AN prevented a comparable study of these compounds in that solvent. On the other hand, the results obtained in aqueous medium indicate that the reaction scheme proposed in eq 6 to 8 is applicable throughout the pH range of 4 to 10.

For at least the present, differences in the behavioral patterns of the alkyl analogs and nucleotides are attributable to

differences in the basicity of the solvents employed (AN vs. DMSO) and to the presence of ribose and phosphate groups in the nucleotides. The ultimate product(s) of decomposition of $H(RNH)^+$ obtained in both studies has an absorption maxima at 280–290 nm, which indicates[35] the presence of a chomophore of the type



It is quite possible that the electroactive function producing the cathodic dme wave at ca. −1.06 V is not the same as the chromophore producing the 280-nm absorption, but that the compound containing the electroactive function also contains the chromophore, i.e., the chromophore may be in all of the acid-catalyzed decomposition products including $H(RNH)^+$.

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(29) In connection with the interpretation of Figures 7 to 9, it should be kept in mind that the i_l/C ratio (μA/mM) for the 2 e oxidation at the same rgce in pH 7.1 solution is 11 for NMNH and 12 for NADH and NADPH, and that the i_l/C ratio for 1 e reduction of the nicotinamides at the dme used for pH 7.1 solutions is 1.9 for NMN^+ and $NADP^+$, and 2.0 for NAD^+. Extrapolation of the log (i_l/C)–time plots derived for the dihydronicotinamide oxidation wave of Figures 7 to 9 (curve A) gives i_l/C values at time zero of 10 for NMNH, 13 for NADH, and 12 for NADPH; these values are in satisfactory agreement with the data at pH 7.1 where hydrolysis is very slow.
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