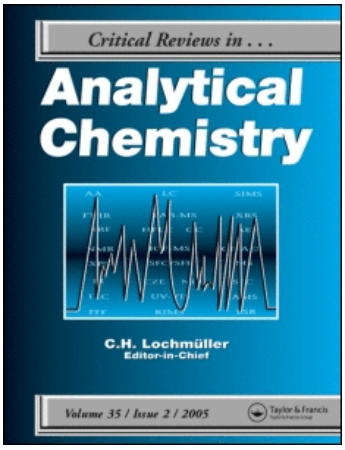

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NICOTINAMIDE-NAD SEQUENCE: REDOX PROCESSES AND RELATED BEHAVIOR: BEHAVIOR AND PROPERTIES OF INTERMEDIATE AND FINAL PRODUCTS

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I. INTRODUCTION

The senior author and his collaborators have long been concerned with the use of analytical chemical techniques – more specifically, experimental approaches and methodologies primarily based on polarography – to study problems of general chemical interest. Currently, electroanalytical approaches are being used in a systematic investigation of chemical phenomena involving biologically significant compounds, where such approaches seem to offer distinct advantages. Attention has been focused on the behavior of nucleic acid components, pyridine coenzymes, and relevant model compounds in solution as well as the electron-transfer interface. Behavior at the interface involves (a) adsorption of original, intermediate, and product species, (b) association in the adsorbed state, (c) mechanisms and kinetics of electron-transfer (redox) processes, and (d) chemical reactions (kinetics and mechanisms) involving reactant, intermediate (free radical, carbanion), and product species preceding, accompanying and following electron-transfer. Solution behavior includes (a) the "shape" of the compounds as reflected in structure, conformation, and association including stacking, (b) intra- and intermolecular association, and (c) orientation of the compound as it approaches the interface.

The compounds examined have been selected on the dual basis of (a) investigation in detail of the basic processes in the parent heterocycle, which is the site of oxidation and reduction; and (b) investigation of sequences of compounds in order to determine the basis for transferring information from simple to more complicated structure, thereby also isolating the characteristic behavioral features of the latter. Although the

compounds have been investigated in aqueous solution because biological processes occur in such media, many have also been examined in nonaqueous media in order better (a) to characterize the roles in the redox process of protons and of the free radical species formed in the postulated initial energy-controlling one-electron (*1e*) process, and (b) to simulate the varying environments encountered in biological systems. The results have been examined and correlated on the basis of structural, electrostatic, solvation, molecular orbital, and other pertinent characteristics and parameters, and evaluated with respect to the behavior of the compounds in biological systems, in particular to the extent to which electrochemical approaches can yield information pertinent to biological behavior.

In the present article, the application of polarographic techniques and methodology, and, to a lesser extent, spectrophotometry, in investigating the solution behavior, adsorption, redox processes including coupled chemical reactions, and allied aspects of biologically significant compounds and their redox products, has been exemplified by a review of the behavior in aqueous and nonaqueous media of the compound sequence from nicotinamide (3-carbamoylpyridine) to NAD^+ (nicotinamide adenine dinucleotide).

A. Nicotinamide Compound Sequence

The nicotinamide sequence of compounds is of considerable biological importance; nicotinamide (Figure 1) itself is widely distributed in plant and animal tissues and is an essential dietary factor for nearly all mammalian systems, probably because of its essential role in the production of certain pyridine nucleotides, e.g., NAD^+ and $NADP^+$, under physiological conditions. The latter compounds function as coenzymes for the pyridinoproteins, which are among the principal components in the Krebs citric acid cycle and in the electron transport chain in biological oxidation-reduction systems. In these reactions, they are reduced in two-electron (*2e*) processes to NADH and NADPH; thus, NAD^+ is a hydrogen-transferring coenzyme for the dehydrogenases. For example, yeast alcohol dehydrogenase (ADH) catalyzes the 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]

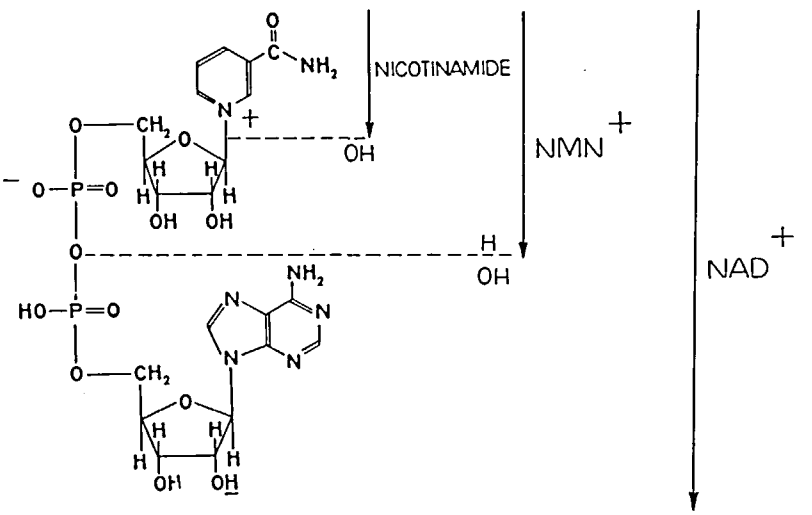


FIGURE 1. Formula for enzymatically active NAD^+, i.e., β-nicotinamide-adenine dinucleotide. The isomer having an α-glycosidic nicotinamide-ribose linkage does not exhibit enzymatic activity with yeast ADH. A related coenzyme, nicotinamide-adenine dinucleotide phosphate ($NADP^+$), is formed when the underlined H is replaced by a $PO(OH)_2$ group. In deamino-NAD^+ ($DNAD^+$), the adenine moiety is replaced by hypoxanthine. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+ \quad (1)$$

The gross reversible redox behavior exhibited by the NAD^+-NADH couple under physiological conditions,

$$NAD^+ + H^+ + 2e \rightleftharpoons NADH \quad (2)$$

has prompted extensive electrochemical study of the reaction. Typical of the potentiometric studies of the NAD^+-NADH redox couple are those by Rodkey and Donovan;[3,4] such studies require the use of a potential mediator or equivalent system since the NAD^+-NADH couple is not macroscopically reversible.

A basic understanding of the electrochemical redox behavior of the sequence from nicotinamide through the pyridine nucleotides should contribute to a better understanding of the biological electron transfer mechanisms, since the site of both biological and electrochemical redox activity in the pyridine nucleotides is the pyridine ring.

The literature on polarographic studies of the nicotinamide sequence in aqueous media has been reviewed.[5-10] While certain aspects of the electrochemical behavior have been thoroughly treated, other aspects have been only partially explained or not considered; the latter includes (a) the interpretation of the redox patterns of nicotinamide and nicotinamide mononucleotide (NMN^+), (b) the postulated electroactivity of the dimer produced on the initial 1*e* reduction, (c) the origin or origins of the second reduction step observed, and (d) the roles of adsorption at the interface and of kinetic (rate-controlled) processes, e.g., dimerization of the initially produced free radicals. Thus, the second reduction wave, observed at more negative potential, has been only fragmentarily studied, and has been assigned, *inter alia,* to reduction of the initially produced free radical, to reduction (site merely postulated) of the dimer produced by the free radical, and to a combination of both processes.

Although the polarographic behavior of many 1-substituted-3-carbamoylpyridinium ions has been investigated on the basis of their being model compounds for understanding the polarographic behavior of NAD^+, only in the case of the 1-methyl-3-carbamoylpyridinium ion (1-methylnicotinamide; MCP^+) have extensive data been collected. However, in many respects, NMN^+, which is structurally the simplest nicotinamide nucleotide, is a more realistic model compound than MCP^+ for understanding the behavior of the larger pyridine nucleotides such as NAD^+ and $NADP^+$. The electronic structure of the pyridine ring in NMN^+ would be expected to be more closely related to that of the coenzymes because of the similar inductive effects of N-substituents, e.g., the action of dilute base on MCP^+ is different from that on NMN^+ and NAD^+. The amide group of MCP^+ is readily hydrolyzed, but the nucleotides cleave at the nicotinamide-ribose linkage.[11] In addition, NMN^+, like the coenzymes, has a bulky N-substituent which, presumably, is effective in determining the isomeric form of the final wave Ic electrochemical reduction product, e.g., a 4,4′ as opposed to a 6,6′ dimer. The absence of the adenine moiety in both NMN^+ and MCP^+ simplifies interpretation of ultraviolet spectra (here characteristic only of the pyridine ring) and eliminates interfacial adsorption phenomena due to the presence of adenine.[12] In addition, an alkyl or aromatic substituent on N(1) contributes to hydrophobicity in that portion of the molecule while a sugar substituent is hydrophilic.

The two electrochemical studies[6,13] of NMN^+ reported previous to that of the present authors[14] are fragmentary and some of the results seem questionable; interesting results obtained for NMN^+ by Berg and Hanschmann[15,16] have, unfortunately, not yet been published.

Furthermore, although there is general agreement concerning the overall process for MCP^+, there is considerable disagreement on specifics, e.g., under similar conditions $E_{1/2}$ for the first of the two polarographic waves seen has been reported[17-20] as varying from −0.94 to −1.11 V. Two recent papers[6,8] report polarographic patterns for MCP^+ which are more complex than those seen by other investigators. Wave I is reported[17,20-22] to be due to a reversible 1*e* process, but the overall electrochemical process is irreversible due to subsequent dimerization;[17,20-22] however, the dimerization rate constant first obtained by cyclic voltammetry[22] differs by many orders of magnitude from that obtained by pulse radiolysis.[23] Other inconsistencies in the literature on MCP^+ involve potential data, presence or absence of catalytic hydrogen evolution, chemical and electrochemical properties of the reduction products, correlation of ultraviolet spectral properties of the latter with structure, and the role of adsorption phenomena.

In general, the polarography of nicotinamide itself has been primarily investigated[24-29] from the viewpoint of the catalytic hydrogen current (see section III.C.) which it produces; its fundamental behavior has been only meagerly interpreted and the reduction mechanism has not been fully understood.

Aside from a fragmentary study of NAD^+ in 50% dioxane,[30] the only study of the nicotinamide sequence in nonaqueous media is one by the present authors employing acetonitrile (AN) and dimethylsulfoxide (DMSO).[31] One need for studying the nicotinamide series of compounds in nonaqueous aprotic solvents arises from the poorer ability of these media, compared to water, to donate a proton; it should thus be possible to clarify the reduction mechanisms, particularly with respect to the observed second reduction step.

B. Scope of Present Paper

As a result of the considerations just outlined, as well as of others subsequently indicated, the authors have undertaken a systematic investigation in aqueous and nonaqueous media of the electrochemical and related chemical behavior of a sequence of nicotinamides. The techniques utilized included (a) normal DC polarography and phase-selective AC polarography at the dme (dropping mercury electrode), (b) cyclic voltammetry at the hmde (hanging mercury drop electrode), platinum electrode, pge (pyrolytic graphite electrode), and gce (glassy carbon electrode), (c) rotating disk electrode voltammetry, (d) potential step electrolysis (chronoamperometry), and (e) controlled electrode potential coulometry and preparative electrolysis at massive mercury, carbon and platinum electrodes. Partially and completely electrolyzed solutions prepared by the latter technique were used for spectrophotometric, chemical reactivity, enzymatic, electrochemical, and other examination.

The results of these studies furnish the primary framework in the present article for the experimental evidence upon which the behavior of the nicotinamide sequence of compounds is discussed. This approach is based on the facts that in these studies (a) the whole sequence was examined in some detail under compatible conditions, (b) a diversity of techniques was used, including perturbation techniques such as cyclic voltammetry and AC polarography, which had not been thoroughly utilized by previous investigators, and (c) the chemical and spectrophotometric behavior of reactant, intermediate, and product species was emphasized.

Stress has been placed on the results for the following series of compounds: nicotinamide, 3-(N-methylcarbamoyl)pyridine (N′-methylnicotinamide), 1-methyl-3-carbamoylpyridinium ion (MCP^+; frequently referred to as 1-methylnicotinamide), nicotinamide mononucleotide (NMN^+), nicotinamide adenine dinucleotide (NAD^+; also called coenzyme I, cozymase I, codehydrogenase I [Co I], and diphosphopyridine nucleotide [DPN^+]), nicotinamide adenine dinucleotide phosphate ($NADP^+$; triphosphopyridine nucleotide [TPN^+]; coenzyme II), deamino nicotinamide adenine dinucleotide (nicotinamide hypoxanthine dinucleotide, deamino-NAD^+ or $DNAD^+$), and nicotinamide hypoxanthine dinucleotide phosphate (deamino-$NADP^+$; $DNADP^+$). In addition to the normal β form of NAD^+, α-NAD^+ was also investigated. The structures of these compounds are evident from the schematic of Figure 1. Thus, NAD^+ is the condensation product of nicotinamide, adenine, two molecules of D-ribose, and two molecules of phosphoric acid; the positive charge on the quaternary nitrogen can be neutralized by an additional anion or by a dissociated phosphate anion; some of the acidic phosphate hydrogens can be replaced by other cations.

Two basically different structures are present in the nicotinamide series studies. Nicotinamide itself is a neutral species with the pyridine nitrogen lone pair of electrons occupying an sp^2 hybrid orbital localized in the molecular plane, which causes the compound to function as a base. The 1-substituted 3-nicotinamides (MCP^+, NMN^+, NAD^+, $NADP^+$, $DNAD^+$, etc.) contain a positively charged pyridine ring nitrogen (pyridinium species) due to loss of an electron on salt formation. Consequently, the redox behavior of nicotinamide would be expected to show a pH-dependence differing from those of the nucleotides except in the pH region where the ring nitrogen is protonated. The discussion has been presented with this difference in mind.

The inclusion of N′-methylnicotinamide was based partially on the expectation that its basic pK_a would be similar to that of nicotinamide and partially on the reported behavior of NAD^+ and $NADP^+$.[5,10] If a nicotinamide free radical produced in the initial one-electron (*1e*) process dimerizes in a manner similar to NAD^+, the site for

dimerization would be expected to be at the 4 or 6 position. Molecular models of the free radicals indicate dimerization at the 4 position in N'-methylnicotinamide to be sterically hindered due to free rotation of the methyl group.

Also investigated by the present authors and their colleagues was the oxidation at mercury, platinum, and carbon electrodes of the reduced forms of various nicotinamide species, in particular, the oxidation of NMNH, NADH and NADPH. [The so-called dihydropyridine nucleotides such as NADH are normally the 1,4-dihydropyridine species, where the one additional hydrogen is added *para* to the pyridine N(1).] Investigated in less detail were the redox patterns for hydrolytic and other decomposition products of the various compounds; previous studies from the authors' laboratory have included detailed examination of relevant purines such as adenine and hypoxanthine in aqueous and nonaqueous media.[10,12,32-34]

Emphasis has been placed by the authors on the use of rapid perturbation techniques in studying intermediate species and adsorption, and on the detailed examination of final products prepared by macroscale electrolysis. For example, in order to use electrochemical information for an understanding of biological systems, it is necessary to understand qualitatively and, where possible, quantitatively specific electrode-substrate phenomena which may not be present in biological systems, and which could, unless taken into account, invalidate correlations of biological and electrochemical behavior. Consequently, such phenomena as adsorption-desorption at the interface and substrate-electrode reaction must be investigated and, hopefully, understood. On the other hand, information obtained on adsorbate-adsorbent interactions may be directly applicable to biological systems.

It is recommended that readers of the present article also read the reviews by Underwood and Burnett,[9] and by Thevenot and Buvet,[6-8] whose views differ in many respects from those presented here. However, it should be mentioned that none of the present authors' papers dealing with the nicotinamide sequence had been published when the other reviews were prepared; the present authors expressed their earlier views in a brief review.[35]

C. Polarographic Techniques and Methodology

The theoretical and practical fundamentals of the polarographic techniques used to study the nicotinamides and the application of these techniques to the study of organic compounds have been well reviewed. (See References 36 to 45 on the techniques used, and References 9, 10, 41, and 46 to 54 on the polarographic and related electrochemical examination of biological molecules of the nicotinamide type.) The techniques used in determining the kinetics of reactions involving nicotinamides are briefly described in Section IV. B. 1.

II. INTERPRETATION OF ELECTROCHEMICAL BEHAVIOR

The present section is an attempt to summarize the behavior of the individual compounds of the nicotinamide-NAD^+ sequence and of the intermediates and products involved. Emphasis is placed on the factual data which provide the basis for the mechanistic and allied deductions and the correlations considered in the following sections. Due to lack of space, only essential features of the electrochemical behavior of the various compounds are given, as in Table 1. Details and data can be found in the papers summarized in the previously listed reviews,[5-10] e.g., papers by the present authors on behavior in aqueous media[14,35,55-57] and in nonaqueous media.[31,35,56] Behavior, unless specifically otherwise noted, is in aqueous media; all potentials cited are with respect to the aqueous saturated calomel electrode (sce), unless otherwise noted. The letters a and c, when added to Roman numerals designating waves and peaks, indicate that the waves and peaks are due, respectively, to anodic (oxidation) and cathodic (reduction) reactions.

A. Polarographic Patterns of the Nicotinamides

1. Aqueous Media

a. Nicotinamides

In acidic media, nicotinamide shows two closely adjacent cathodic waves with a closely following catalytic hydrogen discharge wave. $E_{1/2}$ for wave Ic, which is nearly pH-independent below pH 4 and above pH 9, varies linearly with pH between pH 4 and 9 (Figure 2):

$$E_{1/2} = -0.73 - 0.079\ \text{pH} \qquad (3)$$

N'-methylnicotinamide shows identical behavior (Figure 2). The catalytic wave disappears between

TABLE 1

Electrochemical Characteristics of Nicotinamides in Aqueous and Nonaqueous Media[a]

Compound	Solvent[b]	$-E_{1/2}$[c] V	I[d]	D[e] × 10^5 cm²/sec	$-E_p$[c] V
Nicotinamide	H_2O	I 1.60[f]	3.8	0.97	1.6–1.7
		II			
	AN	I 2.00			
		II 2.45			
	DMSO	I 2.01	1.23	0.30	2.10
		II 2.50			
N'-Methylnicotinamide	H_2O	I 1.60	2.1	1.19	1.6–1.7
MCP^+	H_2O	I 1.03	1.9	0.97	1.06–1.13
		II 1.59			1.68
	AN	1.04	3.3	2.2	
	DMSO	1.01	1.4	0.39	1.09
NMN^+	H_2O	I 0.98	1.37	0.50	1.05–1.22
		II 1.63			1.69
	DMSO	0.99	1.25	0.31	
NAD^+	H_2O	I 0.89–0.92	1.26	0.43	0.93
		II 1.67			1.6–1.7
	H_2O[g]	I 1.03	1.12	0.34	
		II 1.61			
	DMSO	0.98	1.20	0.28	1.03
α-NAD^+	H_2O	I 1.00	1.24	0.41	
		II 1.63			
$NADP^+$	H_2O	I 0.92	1.20	0.39	
		II 1.69			
	DMSO	1.06	0.91	0.16	1.16
$DNAD^+$	H_2O	I 0.91	1.13	0.34	
		II 1.64			
	DMSO	1.00	0.65	0.08	1.10
$DNADP^+$	H_2O	I 0.93	1.15	0.35	
		II 1.68			

[a]Data summarized from References 14, 31, 35, 55, 57 and 119. Except where otherwise noted, aqueous data were obtained in pH 9 to 10 carbonate buffer (0.5 *M*); nonaqueous data were obtained in designated solvent (0.1 *M* Et_4NClO_4).

[b]Viscosities in centipoises and dielectric constants in debyes at 25° are 0.894 and 78.3 for water (H_2O), 0.342 and 37.5 for acetonitrile (AN), and 1.996 and 46.7 for dimethylsulfoxide (DMSO).

[c]Potentials are referred to aqueous sce and are corrected for liquid junction potentials by using Rb(I) as a standard. Roman numbers refer to wave sequence; non-numbered data are for the first or only wave seen.

[d]Diffusion current constant, $I = i_d/cm^{2/3}t^{1/6}$. For aqueous media, i_d corresponds to the average current; for nonaqueous media, i_d corresponds to the maximum current at the instant the drop falls.

[e]The diffusion coefficient, D, has been calculated from the diffusion current, using the simple form of the Ilkovic equation and the appropriate numerical constant.

[f]$E_{1/2}$ is pH dependent; the value given is for pH 9 where the two waves seen in acid solution have coalesced.

[g]Background medium is 0.1 *M* pH 9.6 carbonate buffer, which is 0.4 *M* in Et_4NClO_4.

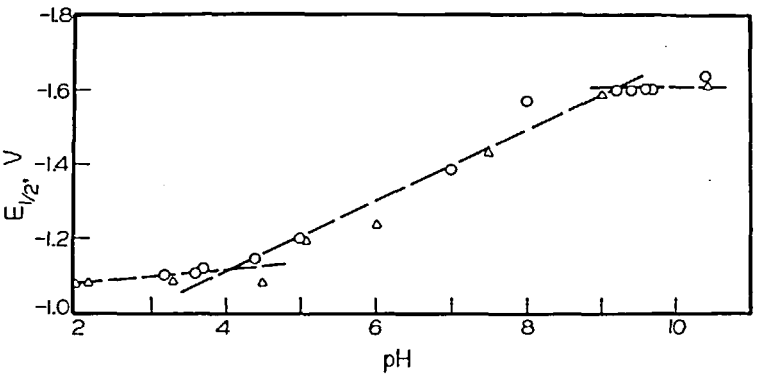


FIGURE 2. Variation with pH of the half-wave potential, $E_{1/2}$, for the first cathodic polarographic wave of nicotinamide (circles) and N'-methylnicotinamide (triangles). (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 345 (1974). With permission.)

pH 7 and 8. Between pH 3 and 7, the two nicotinamide waves are of equal height, result from 1*e* faradaic processes, and shift negatively with a constant 0.11-V separation in $E_{1/2}$. The nearly merged 2*e* wave seen above pH 8 shows an inflection near its midpoint, which disappears with increasing pH (see Table 1); addition of Et_4NCl to pH 9.4 carbonate buffer resolves the pattern into two apparently reversible 1*e* waves ($E_{1/2}$: -1.55, -1.68 V), based on wave slopes.

Cyclic voltammetry indicates that the wave Ic product is not further reduced in the wave IIc process, and that the product of the single cathodic 2*e* peak (E_{pc} = -1.72 V) at pH 12 produces an anodic peak at -0.21 V. At intermediate pH (9.4), sweep reversal halfway up the apparently single cathodic peak produces only anodic peak Ia; however, reversal after the cathodic peak produces two anodic peaks, which correspond to oxidation of the 1*e* and 2*e* reduction products (Figure 3). Repetitive scanning (12 V/sec) around the single cathodic peak and either peak Ia at pH 9.4 or peak IIa at pH 12 establishes a relatively high steady-state nicotinamide concentration at the interface; however, the cathodic peak decreases if the anodic peak is excluded from the repetitive cycle.

At higher scan rates, e.g., 14 to 32 V/sec for nicotinamide, complementary cathodic-anodic peak pairs are seen, whose 60-mV separation in peak potential is characteristic of a 1*e* reversible redox couple (Figure 4). The latter presence allows calculation of the dimerization rate constant of the free radical produced in the initial 1*e* process (see Section IV. B. 2).

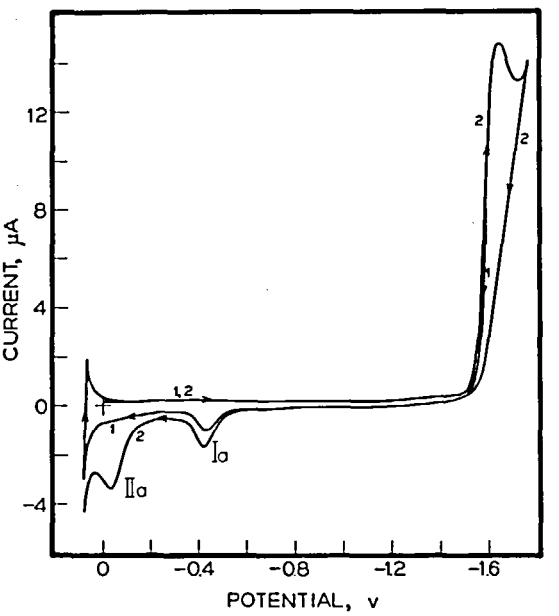


FIGURE 3. Cyclic voltammogram of nicotinamide (1.27 m*M*) in pH 9.4 carbonate buffer at the hmde. Roman numerals refer to waves, Arabic numerals to sweep. Scan rate = 100 mV/sec. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 345 (1974). With permission.)

b. 1-Substituted Nicotinamides

The 1-substituted nicotinamides generally show two well-separated 1*e* waves (Table 1). The electrochemical behavior patterns are obscured in some respects by the presence of adsorption phenomena involving the (ultimate) reduction product of the wave Ic process, and by potential shifts indicative of an irreversible dimerization

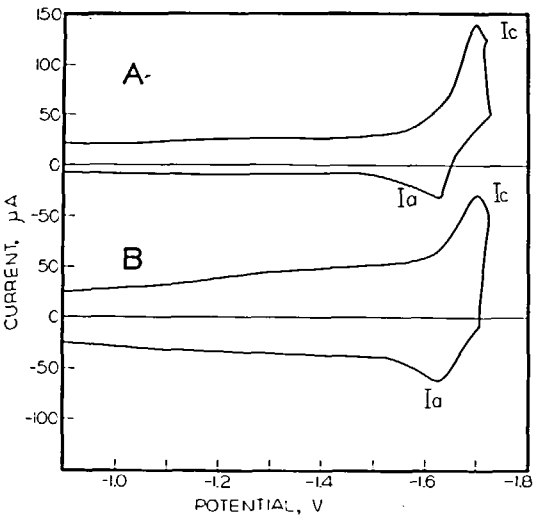


FIGURE 4. Cyclic voltammograms of (A) nicotinamide (scan rate = 23 V/sec) and (B) N'-methylnicotinamide (scan rate = 18 V/sec) in pH 9.0 carbonate buffer, showing the reversible cathodic-anodic peak couple for the first electron-transfer process present at high sweep rates. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 345 (1974). With permission.)

subsequent to a reversible 1*e* charge transfer for the wave Ic process (see subsequent discussion). Furthermore, below pH 6, wave IIc contains a considerable current component due to catalytic hydrogen evolution. For example, NMN^+ wave IIc decreases in height above pH 6, reaching at pH 9 a height identical to that of wave Ic; above pH 10, it is obscured by reduction of nicotinamide formed by NMN^+ hydrolysis.

Consequently, due to hydrogen evolution at lower pH and decomposition at higher pH, the characteristics of NMN^+ waves Ic and IIc can be studied thoroughly and simultaneously only between pH 9 and 10. The polarographic patterns obtained in this pH region depend on ionic strength and surfactant presence, e.g., Et_4N^+. The latter is adsorbed at the interface in the potential region where faradaic processes occur. Wave IIc is also usually better defined in the presence of KCl. In the pH 9 to 10 region, both waves are diffusion controlled (*h* and temperature variation), of equal height (diffusion current constant, *I*, magnitudes correspond to 1*e* faradaic processes), and proportional to concentration.

Cyclic voltammetry clearly indicates nonreduction of the wave I product in the wave II process. The fact that i_p for peak IIc is less than 10% that of Ic supports the notion that most of the 1-substituted nicotinamide 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 direct reduction of the 1-substituted nicotinamide which diffuses through the depleted layer. Peak IIc may also be due in part to reduction of nicotinamide which is present as a minor impurity.[57]

$E_{1/2}$ for wave I is generally pH-independent, except that $E_{1/2}$ for NMN^+ wave I shifts negatively between pH 5.0 and 7.5 due to secondary phosphate dissociation. $E_{1/2}$ for wave II may show slight pH-dependence. The confusion in the literature is shown by the fact that, in slightly alkaline solution, $E_{1/2}$ values for both MCP^+ waves are in good agreement for two reports,[14,17] 80 mV more positive than those in a third,[20] and 90 mV more negative than those in a fourth.[19] Since $\Delta E_{1/2}$ between the two waves is the same in all four studies, it is likely that the reference electrodes used differed in potential although all potentials are reported to be vs. sce; liquid junction potential effects should be small. The potential scale used in the present authors' investigations was frequently checked by examining a 0.5 m*M* $CdCl_2$ solution in 1.0 *M* HCl; at all times, the latter exhibited an $E_{1/2}$ of -0.642 ± 0.002 V, which is identical to the accepted value of -0.642 V.[58]

Typical of the wave patterns is that of MCP^+ (Figure 5A). After exhaustive electrolysis at a potential corresponding to the wave I plateau, both cathodic waves vanish and an anodic wave corresponding to the wave I product (dimer) appears (Figure 5B). After electrolysis at a wave II plateau potential, no waves are seen at mercury (Figure 5C), because oxidation of the postulated dihydropyridine product occurs at more positive potential than that for the oxidation of mercury; however, the oxidation is readily observed at the pge (Figure 6C). Sweep 1 to negative potential, initiated at -0.75 V, shows only background cathodic behavior; on the return sweep, a large anodic peak appears at -0.04 V due to oxidation of the 2*e* product; subsequent reversal toward more negative potential (Sweep 2) produces a cathodic peak at -1.18 V due to reduction of MCP^+ formed during the oxidation step. Finally, when the sweep is once again reversed toward more positive potential, an anodic peak appears at

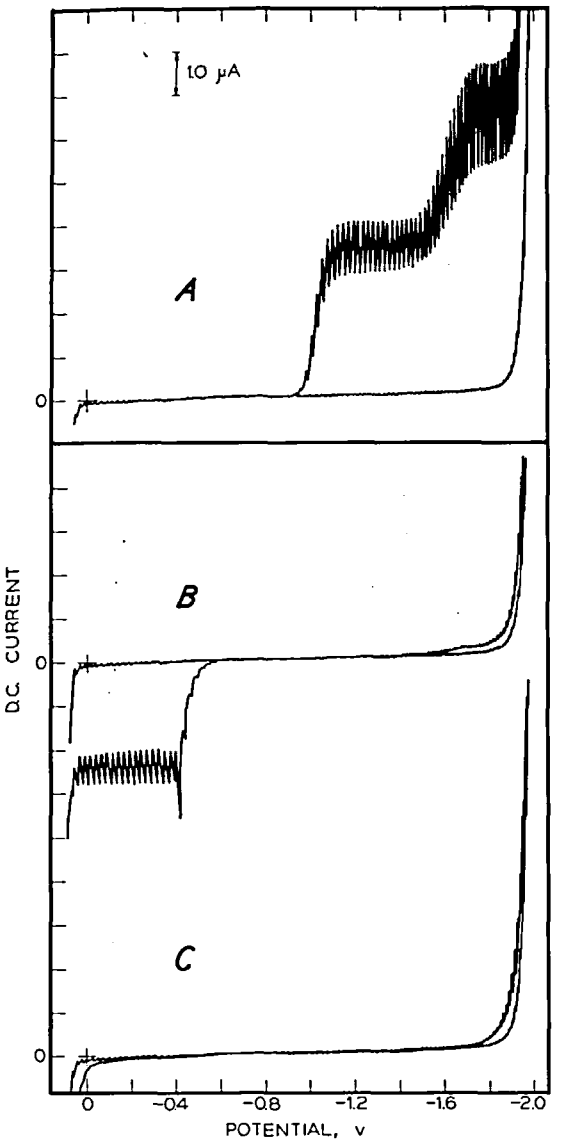


FIGURE 5. Electrolysis of 1-methyl-3-carbamoylpyridinium ion (MCP^+) (1.20 m*M*) in pH 10 KCl/carbonate buffer. DC polarograms: A, before electrolysis; B, after electrolysis at -1.25 V; C, after electrolysis at -1.80 V. Background polarogram shown for each case. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 1033 (1974). With permission.)

-0.34 V (dimer oxidation) in addition to the one at -0.04 V.

It should be emphasized that, in the case of all of the compounds investigated, polarographic, spectrophotometric, and enzymatic examinations of fully reduced solutions showed that the reduction product formed under lengthy macroscale conditions is the same as that formed under the more temporal conditions of cyclic voltammetry.

In the case of NAD^+, a very small anodic wave sometimes seen close to mercury discharge at pH 9 to 12 corresponds to formation of a mercury-adenine complex.[57] A similar wave is seen for ADP (adenosine diphosphate) but not for NMN^+.

i. Adsorption Phenomena

The presence of adsorption, as mentioned, is a complicating factor, especially in the case of compounds containing the adenine moiety. In general, adsorbability can be correlated – as expected – with the surface activity of component portions of the molecule.

Both the in-phase and quadrature components of the total alternating current are shown in figures such as Figure 7 along with the corresponding curves for the background electrolyte alone. In a potential region where a faradaic step does not occur, the quadrature (out-of-phase) current component is proportional to the differential double layer (DL) capacity at the solution-electrode interface and thus provides a convenient index to adsorption at the interface of the electroactive species and its products.

Adsorption of nicotinamide, N′-methylnicotinamide, and their reduction products at the solution-mercury interface is negligible. While MCP^+ is also negligibly adsorbed, its dimeric and dihyropyridine products are strongly and moderately adsorbed, respectively; NMN^+ and its reduction products are negligibly adsorbed. This behavior reflects the presence of a hydrophobic substituent on N(1) of MCP^+ and of a hydrophilic substituent on N(1) of NMN^+; the difference in adsorption of the MCP^+ dimeric product (strongly adsorbed) and the MCP^+ dihydropyridine product (moderately adsorbed) also reflects the difference in hydrophobic nature of the two compounds.

The manner in which adsorption can affect observed electrochemical patterns is well illustrated by the behavior of NAD^+ at pH 9 to 10. The waves that appear in the DC polarographic patterns in addition to the two 1*e* reduction steps can be explained, largely on the basis of AC polarography, in terms of adsorption phenomena.

At low ionic strength (μ), a slight maximum of the first kind appears on the rising portion of NAD^+ wave Ic and a small maximum of the second kind or capacity wave is seen on the limiting portion at -1.3 V; wave IIc appears as an ill-defined shoulder or inflection on solution

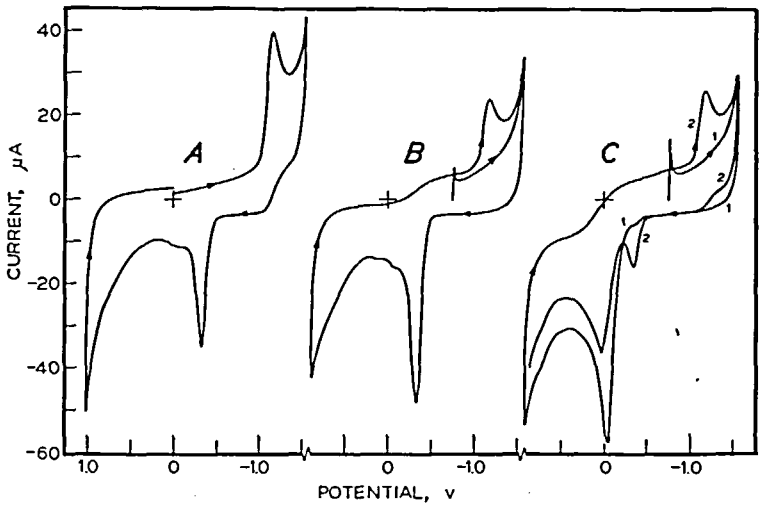


FIGURE 6. Electrolysis of 1.20 m*M* 1-methyl-3-carbamoylpyridinium ion (MCP^+) in pH 10 Et_4NCl/carbonate buffer. Cyclic voltammograms at the pge: A, before electrolysis; B, after electrolysis at −1.40 V; C, after electrolysis at −1.80 V. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 1033 (1974). With permission.)

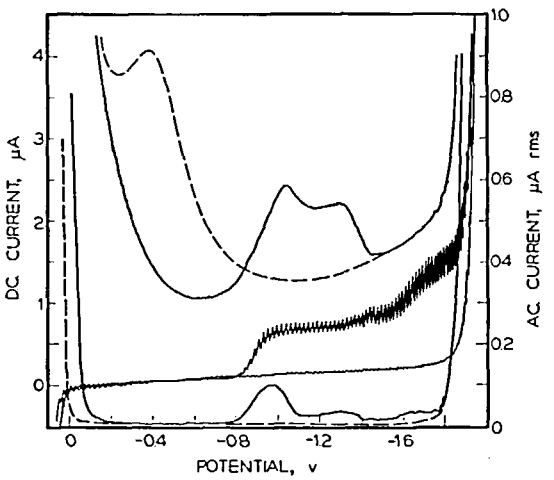


FIGURE 7. DC and AC polarograms of NAD^+ (0.31 m*M*) in pH 9.3 KCl/carbonate buffer. DC polarograms shown with and without electroactive species present. AC polarograms: solid lines represent in-phase (lower set) and quadrature (upper set) components of total AC current; dashed lines represent corresponding background currents. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

discharge. (On addition of 0.0008% Triton X-100 or 5m*M* Et_4NCl, both maxima disappear and wave Ic is well defined.) With increasing μ, the maximum of the first kind disappears, but the capacity wave grows and shifts to more negative potential; at the same time, wave IIc appears and grows in height (Figure 7). Characterization of wave IIc is difficult because of its proximity to the capacity wave.

The capacity wave is associated with desorption of the wave Ic dimeric product from the electrode surface. Any factor which would be expected to increase the surface activity of the dimer, e.g., by lowering its solubility and thus favoring its adsorption, shifts the wave to more negative potential; these include increased μ, decreased temperature, and increased NAD^+ concentration. A DC wave would not be expected to accompany a desorption process since a faradaic (reduction) current does not flow. However, such a DC wave might result, for example, from the desorption causing stirring, which would increase the limiting wave Ic current.

On AC polarography (Figure 7), NAD^+ shows a faradaic peak (in-phase component) at −0.97 V (equivalent to DC wave I process; corresponding quadrature component is at −1.04 V) and a barely discernible peak at −1.65 V (equivalent to DC wave II process; no detectable quadrature component). A faradaic peak at −0.01 V (not entirely seen in Figure 7) results from oxidation of mercury to form a compound with the adenine moiety. In the potential region prior to wave Ic, adsorption of NAD^+ depresses the DL capacity

with a minimum at -0.65 V. A quadrature peak at -1.29 V with only a small in-phase component and, therefore, almost entirely capacitive in nature, is a tensammetric peak resulting from desorption of the wave Ic product. Beyond -1.5 to -1.6 V, the DL capacity is the same as for the background alone, indicating that the wave IIc product is not adsorbed over the potential range of its formation.

The moderate sensitivity of the NAD^+ AC polarographic patterns to experimental variables is confined to adsorption-desorption phenomena, with faradaic processes remaining about the same. Factors expected to increase the dimer surface activity shift the tensammetric peak to more negative potential; these shifts are identical in sign and magnitude to those of the DC capacity wave.

Significant changes occur in the NAD^+ DC polarographic pattern at pH 9 to 10 on Et_4NCl addition, e.g., on increasing the Et_4NCl concentration at constant μ wave Ic shifts to more negative potential and wave IIc becomes distinct and grows in height (Figure 8); the solution discharge potential remains constant. At sufficiently high Et_4NCl concentration (Figures 8 and 9), two well-defined, diffusion-controlled waves of about equal height are seen; with increasing NAD^+ concentration (0.013 to 1.32 m*M* at pH 9.6), these remain of equal height, but $E_{1/2}$ of wave Ic becomes more positive (-1.09 to -1.00 V) and $E_{1/2}$ of wave IIc more negative (-1.58 to -1.65 V).

A small apparent wave of constant height at about -0.65 V (Figure 9), which becomes about 0.1 V more negative between 0.13 and 1.20 m*M* NAD^+, is due to the depression of the DC capacity current before this potential and, as subsequently discussed, is caused by a desorption-adsorption process.

Substitution of Et_4NCl for KCl in the background electrolyte, as expected, also significantly alters the AC polarographic pattern (Figures 7 and 9). NAD^+ now exhibits faradaic peaks at -1.09 and -1.64 V, a peak at zero volt corresponding to mercury oxidation, and a tensammetric peak at -0.67 V.

The depressed capacity at potentials prior to the tensammetric peak indicates that NAD^+ is absorbed at the interface, replacing solvent molecules and background ions. In the potential region of the peak, which is more negative than the ecm, NAD^+ is desorbed due to preferential

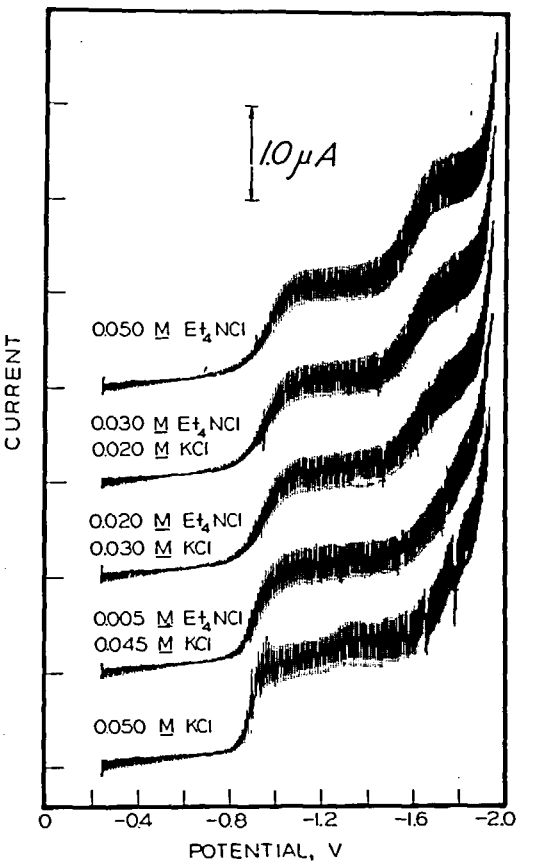


FIGURE 8. Variation in the DC polarographic behavior of NAD^+ (0.32 m*M*) with increasing Et_4NCl concentration. All solutions were maintained at 0.10 *M* ionic strength (0.05 *M* carbonate buffer plus Et_4NCl and KCl as shown; final pH 9.9). (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

adsorption of Et_4N^+. With increasing NAD^+ concentration (0.13 to 1.26 m*M*), E_p shifts from -0.64 to -0.73 V and the current increases by a factor of 2.5. With increasing Et_4N^+ concentration, E_p becomes more positive and the current increases. The small wave or kink in the DC pattern at -0.65 V is due to this NAD^+-desorption, Et_4N^+-adsorption process.

In the potential region following the tensammetric peak at -0.67 V, the DL capacity is nearly the same as that of background alone, indicating that NAD^+ and the two reduction products are not adsorbed, i.e., Et_4N^+ is preferentially adsorbed. Results are similar for NAD^+ concentration as high as 1.26 m*M*.

The DC and AC polarographic behaviors of

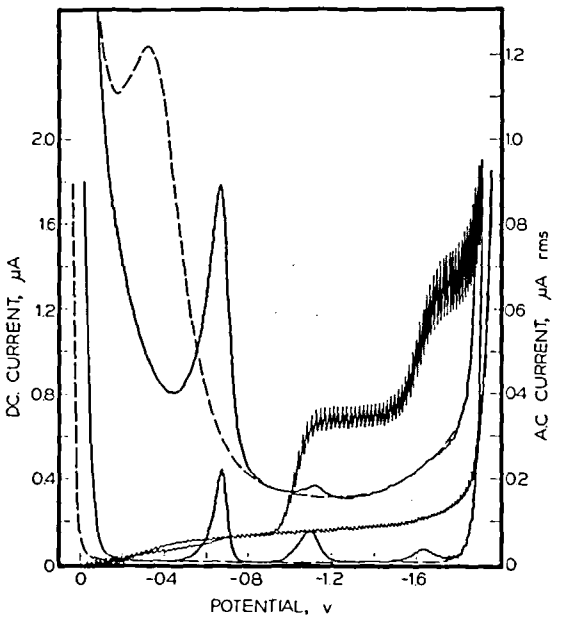


FIGURE 9. DC and AC polarograms of NAD^+ (0.31 *M*) in pH 9.6 Et_4NCl/carbonate buffer. DC polarograms shown with and without electroactive species present. AC polarograms: solid lines represent in-phase (lower set) and quadrature (upper set) components of total AC current; dashed lines represent corresponding background currents. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

α-NAD^+, $NADP^+$, $DNAD^+$ and $DNADP^+$ are generally similar to that of NAD^+. The principal difference in the absence of Et_4NCl is in the relative tendencies to adsorb at the interface. In every case, the oxidized species is adsorbed, causing a quadrature component depression prior to wave Ic. $DNAD^+$ is most strongly adsorbed; it exhibits in both acidic and basic solution a deep "adsorption well" or depression prior to wave Ic, as well as a depression in the DC charging current.

In the absence of Et_4N^+ ions (as in the case of NAD^+), the dimer formed in the 1*e* reduction of these compounds (corresponding to wave Ic) is adsorbed at the potential of its formation, but is desorbed at more negative potential. This results in the appearance of an AC peak between the peaks corresponding to waves Ic and IIc and, in some cases, of a small maximum of the second kind or capacity wave in the potential region corresponding to the limiting current of DC wave Ic. The most strongly adsorbed dimer appears to be that of α-NAD^+. On Et_4NCl addition, dimer adsorption disappears.

Exhaustive macroscale electrolysis of NAD^+ at pH 10 in KCl-containing buffer at a potential, at which adsorption of the reduction product would be expected, and in Et_4NCl-containing buffer at a potential at which the DL capacity is identical to that for background alone, gave faradaic *n* values of 0.96 ± 0.01, and solutions with nearly identical faradaic and spectral properties, thus showing little effect due to adsorption on the nature of the electrolysis products. Shift of the applied potential to −1.80 V, when electrolysis was complete, gave no additional current flow, confirming that the final wave I product is neither an intermediate in the formation of wave II ($E_{1/2}$ = −1.60 V) nor further reduced to a different product. In some experiments, a small wave was observed at about $E_{1/2}$ of −1.60 V, which was due to nicotinamide formed by alkaline decomposition of NAD^+.

The replacement of cationic NAD^+ by cationic Et_4N^+ as an adsorbed species at the interface at potentials more than slightly negative to the ecm, as well as the adsorption of NAD^+ on the positive side of the ecm, supports the assumption of a site other than the pyridinium ring being involved in adsorption of NAD^+; this is in agreement with the absence of adsorption of NMN^+. The probable site is that of the adenine moiety, which is known to be strongly adsorbed at mercury-water interfaces.[12,33]

ii. Free Radical Formation

Formation of a free radical in the initial 1*e* step and its subsequent dimerization in both aqueous and nonaqueous media for all of the compounds studied is clearly evident from the complementary cathodic-anodic peak pairs at high polarization (scan) rates on cyclic voltammetry and from the products on controlled potential electrolysis. Other characteristics also support the initial step as being a reversible 1*e* process producing a species which rapidly dimerizes (see Section II. A. 3).

2. Nonaqueous Media

Only nicotinamide and MCP^+ are sufficiently soluble in both of the aprotic solvents used (acetonitrile, AN, and dimethylsulfoxide, DMSO; dielectric constants at 25° are 37.5 and 46.7, respectively; viscosities are 0.342 and 1.996 cp, respectively) for their electrochemical behavior to be investigated in both solvents; the remaining nicotinamides were investigated only in DMSO (background electrolyte was generally 0.1 *M* Et_4NClO_4). Measured potentials, which are vs. the

aqueous sce, were corrected for liquid junction effects (E_j) on the basis of Rb(I), whose $E_{1/2}$ is -1.94 V in AN and -1.95 V in DMSO; the need for such corrections is well documented.[59,60]

Nicotinamide itself gives two well-formed, diffusion-controlled cathodic waves of equal height. Other compounds in the series (Figure 10A; Table 1) show only one wave; NMN^+ also shows a small prewave ($E_{1/2}$ = -0.38 V) due to adsorption (i_l is linearly proportional to h). The diffusion current constants, I, and wave slopes ($E_{1/4}$ - $E_{3/4}$) suggest reversible 1e faradaic processes followed by irreversible dimerization.[61]

[Some of the compounds used gave a cathodic wave at -1.96 V in DMSO, due to Na(I) introduced by use of sodium salts, and/or an anodic wave ($E_{1/2}$ = -0.20 V in AN and -0.18 V in DMSO), e.g., Figure 10A, where it is due to oxidation of mercury in the presence of chloride ion similarly introduced.]

Values of I for MCP^+ in AN are about 2.4 times those in DMSO, reflecting the difference in solvent viscosity, which affects the diffusion coefficient, D. The reciprocal of the ratio of the square roots of the viscosities, η, is 2.4, which is in good agreement with the experimental I and, consequently, $D^{1/2}$ ratios, i.e., the ηD products ($\times 10^5$) are 0.75 in AN and 0.78 in DMSO.

Values of D, calculated from polarographic I values (Table 1), decrease with increasing size of the molecule except for $DNAD^+$ whose D is much smaller in DMSO than would be expected on the basis of the Stokes-Einstein model. This anomaly may be due to greater solvation and/or association. $DNAD^+$ differs from the other NAD species in having a hypoxanthine moiety in place of the adenine moiety; the presence of the hydroxyl group on the hypoxanthine may favor association.

The product of controlled potential electrolysis of the nicotinamides carried out at a potential corresponding to the limiting current of wave Ic gives an anodic DC polarographic wave at -0.4 to -0.7 V (Figure 10B). This wave is attributed to dimer oxidation. This interpretation is confirmed by cyclic voltammetric data. The appearance and characteristics of a complementary cathodic-anodic peak couple, observed for each of the compounds at scan rates of 6 to 80 V/sec, supports wave Ic being due to a reversible 1e reduction followed by rapid dimerization. The reversible transfer of one electron in peak Ic is further corroborated by AC polarography.

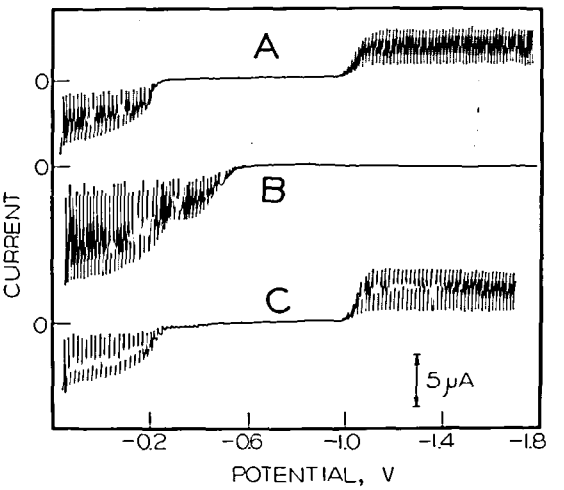


FIGURE 10. DC polarograms of 1-methyl-3-carbamoylpyridinium ion (MCP^+) (0.39 mM) in acetonitrile (0.1 M in Et_4NClO_4): A, before controlled potential electrolysis; B, after electrolysis at -1.30 V; C, after electrolysis at -0.19 V of the solution which gave polarogram B. (From Santhanam, K. S. V. and Elving, P. J., *J. Am. Chem. Soc.*, 95, 5482 (1973). Copyright by the American Chemical Society. With permission.)

Coulometric measurement at a potential corresponding to the limiting current of wave Ic gave for the overall number of electrons transferred in this process a faradaic n of one. Electrolytic oxidation of the resulting solution consumed the same number of coulombs as required for the reduction and regenerated all of the original compound (Figure 10C).

The follow-up chemical reaction connected with the wave of peak Ic process can be identified as dimerization from analysis of cyclic voltammetric curves, polarographic waves and spectrophotometry, e.g., examination of the solution obtained after controlled potential electroanalysis (see Section II. A. 3).

a. Effect of Proton Donor

The two 1e waves of nicotinamide and the one 1e wave observed for the 1-substituted nicotinamides in nonaqueous media, as in aqueous media, arise from reduction of the pyridine ring. Nicotinamide is initially reduced in nonaqueous media (wave Ic) to a free radical anion and the 1-substituted compounds to neutral free radicals;

this difference is evident in the reactivities to added proton donor. Thus, nicotinamide wave IIc decreases in height on adding benzoic acid or hydroquinone, and a new wave appears very close to original wave Ic, whose $E_{1/2}$ is shifted positively. With increasing proton donor concentration and accompanying growth of new wave IIc, the latter begins to merge with wave Ic; the combined wave reaches a limiting height of about twice that of original wave Ic at molar ratios exceeding 2 proton donor to 1 nicotinamide. The pattern observed in organic solvent containing proton donor is thus similar to that seen in aqueous medium of two almost merging 1*e* cathodic waves. On the basis of a benzoic acid pK_a of 22.0 in AN,[62] the $[H^+]$ required for appearance of a fully developed new nicotinamide wave II is 5×10^{-13} *M*.

In the presence of proton donor, the 1-substituted nicotinamides show markedly different behavior. The wave Ic height is almost unchanged but a new wave appears ($E_{1/2}$ = -1.79 V for MCP^+; -1.99 V for NAD^+), whose height increases with increasing proton donor concentration to a limiting value equal to the wave I height at a molar ratio of acid to 1-substituted nicotinamide of about 8. The $[H^+]$ required for appearance of a fully developed MCP^+ wave IIc is 6×10^{-13} *M*. This behavior is quite different from that of aromatic hydrocarbons in AN on proton addition (see subsequent mechanistic discussion).

b. Dimer Oxidation

The dimer oxidation peak (Ia) seen for nicotinamide on cyclic voltammetry is shifted about 0.4 V more positive in the presence of 2 m*M* hydroquinone as proton donor. However, the dimer oxidation peak potentials for the 1-substituted nicotinamides are almost unchanged by the presence of proton donor. This difference can be associated with the negatively charged nature of the nicotinamide 1*e* product and the uncharged nature of the other 1*e* products.

3. Evidence for Dimerization

The reversibility of the primary wave Ic process and the presence of a coupled following chemical reaction, which can be identified as a dimerization, can be established on the basis of the dme wave slopes, cyclic voltammetric ($E_p - E_{p/2}$) differences, AC faradaic peak currents and second harmonic curve shapes, and the appearance of complementary cathodic-anodic peak pairs on rapid cyclic voltammetry.

Dimerization reactions involving the primary product of electrochemical reduction can be differentiated from first order follow-up chemical reactions by the cyclic voltammetric potential difference, $E_p - E_{p/2}$, which at 25° should theoretically be $39/n$ mV for the second order reaction and $48/n$ mV for the first order, compared to $57/n$ in absence of a coupled chemical reaction.[63] Measurement of $E_p - E_{p/2}$ for peak Ic over a large number of curves ($v < 0.2$ V/sec) gave an average value of ca. 42 mV for each compound, suggesting dimerization. Similarly, polarographic wave Ic had slopes ($E_{1/4} - E_{3/4}$) close to the theoretically expected value of 46 mV for reversible 1*e* transfer followed by dimerization.[61]

The failure to see the anodic peak for the free radical oxidation at slow scan rates is due to the rate of the chemical reaction being faster than the sweep rate with consequent loss of the radical by the chemical reaction.

The fact that, at any pH, nicotinamide wave Ic becomes increasingly more evident with increasing concentration is in agreement with theory for an irreversible chemical reaction following a reversible charge transfer,[64] i.e., as the nicotinamide concentration is increased, the rate of the dimerization reaction increases, altering the ratio of concentrations of oxidized and reduced species at the electrode surface and thus causing the wave to shift to more positive potential. The significance of the positive shifts in wave Ic $E_{1/2}$ with increasing MCP^+ and NMN^+ concentration, and with drop-time, is somewhat obscured in the case of MCP^+ by the presence of adsorption phenomena involving the reduction product. The shifts in $E_{1/2}$ for MCP^+ wave IIc with concentration and ionic strength are explicable in terms of the rates of two competing reactions: dimerization of the initially produced radicals, and their reduction to produce wave II. For example, an increase in depolarizer concentration will increase the dimerization rate more rapidly than the rate of the electron-transfer process; thus, in order to obtain wave IIc, it is necessary to increase the rate of electrochemical reduction of the radicals, i.e., to make the electrode potential more negative.[65]

The effects of ionic strength (μ) and surfactants on the NMN^+ polarographic pattern appear to be related to changes in DL structure and the two charge centers of the electroactive species. The μ

effect suggests existence at low μ of an intramolecular complex of the ion-pair type between the negatively charged phosphate group and the positively charged pyridinium group; such complexation could increase the electron density in the nicotinamide nucleus. With increasing μ, the complex dissociates, causing wave Ic to become more positive and wave IIc to appear and grow; in the absence of tetralkylammonium salts, wave IIc exhibits partial kinetic control even at 0.5 *M* ionic strength. Wave IIc of MCP^+, which has no phosphate moiety, is well defined, even at low μ. The occurrence of MCP^+ wave Ic at a more negative potential than wave Ic of the dissociated form of NMN^+ may be due to the dimerization reaction as well as to structural features (see Section IV.A.2.).

The slope of NAD^+ wave Ic is generally greater than that for a reversible 1*e* transfer. $E_{1/2}$ becomes more positive with increase in concentration or drop-time; for a reversible electrode process in the absence of adsorption, such shifts, as mentioned, are indicative of irreversible dimerization subsequent to charge transfer.[61,64,66]

The latter is supported by the difference in the cyclic voltammetric patterns at slow and rapid scan rates, and the increase in the i_{pa}/i_{pc} ratio with increasing ν. $NADP^+$ and $DNAD^+$ behave similarly.

B. Reduction Products

The dimeric products of the initial 1*e* reduction and the dihydropyridine products of the 2*e* reduction were characterized by spectrophotometric, electrochemical, enzymatic, and chemical examination of the solutions obtained after controlled potential electrolysis. For example, complete reduction of the original compound could be verified by the absence of the original cathodic waves; the identities and amounts of the reduction products could be determined from ultraviolet spectra and the anodic waves observed for the electrolyzed solution, and from reverse controlled potential anodic coulometry (Tables 2 and 3).

It would perhaps have been useful to have isolated the individual dimeric and dihydropyridine reduction products and to have determined their exact composition and structure. However, it seemed questionable as to whether such a detailed study would have yielded information of an amount and importance which would be commensurate with the effort required. A chromatographic study to determine the homogeneity of the electrochemical reduction products, for example, would not be expected to yield definitive results due to the moderate instability of these substances (see subsequent discussion).

1. Spectra and Product Structure

Above 240 nm, 1-substituted 3-carbamoylpyridinium ions generally show a single absorption band at about 260 to 265 nm; the dihydropyridine reduction products show single bands at about 400 to 410 nm for the 1,2 species and at about 340 to 350 nm for the 1,4 species, and two bands at about 265 to 270 and 350 to 360 nm for the 1,6 species[67-73] These patterns have also been theoretically predicted.[73]

Wallenfels[74] was apparently the first to indicate that the dimeric reduction products of the 1-substituted 3-carbamoylpyridinium ions contain chromophores similar to those of the corresponding monomeric dihydropyridines, i.e., the corresponding dimer and dihydropyridine ultraviolet spectra do not differ with respect to the position of the bands although there is a difference in intensity. Thus, the 4,4′ dimers absorb at about 340 nm and the 6,6′ dimers at about 260 and 345 nm.

However, the presence of the adenine moiety introduces a complication. For example, the ultraviolet spectrum of NAD^+ exhibits a single band at 259 nm (ϵ = 17,800)[75,76] due to absorption by both the adenine and pyridine moieties; with the formation of 1,4-NADH, absorption due to the pyridine moiety shifts to a longer wavelength so that two bands are seen; λ_{max} = 259 nm (ϵ = 14,400)[75] and 338 nm (ϵ = 6,220).[75,76]

The same spectral considerations seem also to apply to nicotinamides containing a tertiary rather than a quaternary N(1). Nicotinamide itself (3-carbamoylpyridine) has an absorption band at about 261 nm; its dimeric and dihydropyridine reduction products each absorb at about 265 and 345 nm. The dimeric reduction product of N′-methylnicotinamide absorbs at 270 and 350 nm.

Table 2 summarizes the spectral data for the 1*e* and 2*e* reduction products of nicotinamide and 1-substituted derivatives.

2. Free Radicals

A variety of evidence has been presented supporting the formation of a neutral free radical on 1*e* reduction of nicotinamide and 1-substituted nicotinamides in aqueous media, and of 1-substi-

TABLE 2

Summary of Spectral Data for Reduction Products of Nicotinamide and Some 1-Substituted Nicotinamide Ions[a]

	Reduced species						
	Dimeric product			Dihydropyridine product			
Oxidized species	λ_{max} nm	ϵ	Method of reduction	λ_{max} nm	ϵ	Method of reduction	Ref.
Nicotinamide	266	12,100	Electrolytic	265	9,600	Electrolytic	55, 119
	346	5,500		346	4,400		
1-Methylnicotinamide (MCP^+)	277	12,500	Electrolytic	270	11,400	Electrolytic	14, 119
	358	8,300		358	6,600		
	275	14,700	X-Ray				23[b]
	355	9,600					
				266	8,100	Borohydride	83[b,c]
				358	5,900		
				360	7,000	Dithionite	86
				355	6,200	Dithionite	83[b,c]
							74[b,c]
1-Propylnicotinamide	255	3,800					
	357	8,300					
				265	–	Borohydride	120[e]
				350	6,700		
				346	5,800	Dithionite	120[e]
1-(2,6-Dichlorobenzyl)-nicotinamide	272	9,200	Zinc	265	9,400	Borohydride	74[b,d]
	350	6,500		355	7,500		
NMN^+	263	7,800	Electrolytic	259	4,400	Electrolytic	14, 119
	339	7,500		339	6,500		
NAD^+	259	33,100	Electrolytic				81
	340	6,650					
	259	32,000	Electrolytic				57, 119
	340	6,900					
	250	37,000	X-Ray				23[b]
	340	7,300					
				259	14,400	Enzymatic	75
				340	6,220	or dithionite	
				259	–	Borohydride	69
				345	6,220		
NAD^+ (primary acid catalyzed decomposition product of reduced species)	280	18,000	Original dimer prepared electrolytically				57, 119
				278	15,400	Dithionite	98
NAD^+ (mercury adduct of reduced species)	282	30,700	Original dimer prepared electrolytically				
				268	28,800	Dithionite	98

[a]Data taken from the literature for compounds where λ_{max} and ϵ values could be found for both the dimer and dihydropyridine reduction products; all data pertain to aqueous solutions unless otherwise stated.
[b]Data estimated from a published spectrum.
[c]Spectrum taken in ethanol.
[d]Spectrum taken in methanol.
[e]Spectrum taken in ether.

TABLE 3

Sequential Electrolysis of a Solution of NAD^+ and the Electrolytic Wave I Reduction Product[a]

		NAD^+				
Electrolysis potential	*n* Value[b]	Enzymatic concentration m*M*	Polarographic current[f] μA	Absorbance at 340 nm[g]	Reduction product current[h] μA	Nicotinamide current[i] μA
–	–	1.10[c]	2.13	0.011	0.00	–
-1.20[d]	1.02	0.01	0.00	0.785	1.76	0.27[j]
-0.10[e]	0.94	1.06	2.06	0.018	0.00	–
-1.20[d]	0.96	0.01	0.00	0.759	1.70	0.34[j]

[a]Solution: 0.5 *M* carbonate buffer; pH 9.0.
[b]Values of *n* are based on the initial NAD^+ concentration of 1.10 m*M*.
[c]Original concentration of NAD^+, before electrolysis, as determined enzymatically.
[d]Potential on the limiting portion of the first cathodic polarographic wave.
[e]Potential on the limiting portion of the anodic wave.
[f]Current for first cathodic wave.
[g]Absorbance of an aliquot of the electrolysis solution after a 1/5 dilution with the electrolysis buffer.
[h]Current for anodic wave produced in reduced solution.
[i]Current for small cathodic wave observed in reduced solution.
[j]Limiting currents correspond to nicotinamide concentrations of 0.051 and 0.064 m*M*.

tuted nicotinamides in nonaqueous media. In aprotic solvents, nicotinamide forms a free radical anion.

The free radicals derived from nicotinamide and 1-substituted nicotinamides are unstable in both aqueous and nonaqueous media; their half-lives are usually less than 1 msec. The stability of the radicals formed from 1-substituted isonicotinamides is greater, with lifetimes usually in the range of seconds,[77] which has allowed use of the esr technique to obtain information on their electronic distributions.

The formation of free radicals from nicotinamides by chemical reduction has also been reported. Thus, at pH 5 to 11, nicotinamide gives pyridinyl radicals on reaction with solvated electrons[78] with absorption maxima at 405 and ca. 280 nm (molar absorptivities of 3.0×10^3 and ca. $8.6 \times 10^3\ M^{-1}\ cm^{-1}$). The red shift at pH 14.2 of the free radical absorption maxima (molar absorptivities of 3.6×10^3 and $7 \times 10^3\ M^{-1}\ cm^{-1}$) is attributed to the formation of the ionized anionic radical.

A free radical has been identified by esr in the enzymatic reduction of NAD^+ in EtOH;[79] the radical lifetime was too brief to obtain a well-resolved esr spectrum. Other investigators[80] could not confirm the presence of free radicals in pyridine nucleotide linked ADH reactions.

The rates at which the free radicals obtained from pyridine nucleotides and model compounds dimerize are discussed in Section IV.B.2.

3. Dimers

The spectra of solutions (pH 9 to 10) of the various compounds after electrolysis at a potential on the plateau of the first 1*e* wave, which would be expected to produce a free radical with the latter rapidly dimerizing, can be readily summarized.

A nicotinamide solution has a single absorption maximum at 260 nm (ϵ = 2,980); the completely electrolyzed solution has maxima at 266 and 346 nm (Figure 11).

Solutions of MCP^+ before electrolysis have the single characteristic 264-nm band (ϵ = 4,600) (literature:[13] λ_{max} = 264 nm; ϵ = 4,600) (Figure 12A). Spectra, taken after electrolysis, show maxima at 277 nm (ϵ = 12,500) and 358 nm (ϵ = 8,300) (Figure 12B).

Prior to electrolysis, NMN^+ solutions exhibit the single characteristic pyridinium ion band (ϵ = 4,900) (Figure 13A). On KCN addition, the 265-nm band is replaced by one at 327 nm (ϵ = 6,300) (Figure 13A) due to cyanide addition at the 4 position of the pyridine ring (literature:[11,75] λ_{max} = 327 nm; ϵ = 5,900). The 265-nm band also disappears during electrolysis,

and bands with maxima at 263 nm (ϵ = 7,800) and 339 nm (ϵ = 7,500) appear and grow (Figure 13B). Reduction of the pyridine ring during electrolysis is also indicated by the fact that dilution of the electrolyzed solution with KCN solution gave the same spectrum as dilution with carbonate buffer.

Prior to electrolysis, NAD^+ solutions show a single absorption maximum at 259 nm (ϵ = 17,800) due to absorption by both pyridine and adenine moieties. The electrolyzed solutions have maxima at 259 nm (ϵ = 32,000) and 340 nm (ϵ = 6,900); the wavelengths agree with those reported for the dimer.[81] Enzymatic analysis of the solutions before and after electrolysis showed that the NAD^+ concentration had decreased to less than 1% of its original value; enzymatic analysis for the fully reduced coenzyme NADH was negative.

a. Correlation with Spectra

The molar absorptivities at 260 and 340 nm of the NAD-derived dimer prepared by the present authors[57] are close to those of an isolated electrochemically prepared dimeric NAD product,[81] and similar to those of a dimeric product (not isolated) quantitatively prepared by X-ray irradiation.[23] The 260-nm dimer absorption is greater than that expected from its two adenine moieties;[81] this implies absorption at two wavelengths by the nicotinamide portion (260 and 340 nm), and, in turn, at least some dimerization at the 6 position.[81] Nevertheless, workers[81] who isolated the NAD dimeric product regarded the 4,4' structure as the more likely, based in part on nmr spectra.

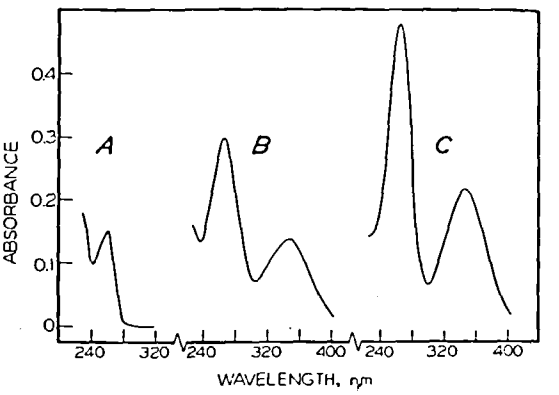


FIGURE 11. Absorption spectra obtained on electrolysis of nicotinamide (1.25 m*M*) in pH 10 Et_4NCl/carbonate buffer. Spectra obtained by a 1/25 dilution of electrolysis solutions with pH 10 carbonate buffer: A, before electrolysis; B, after electrolysis at −1.60 V; C, after electrolysis at −1.80 V. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 345 (1974). With permission.)

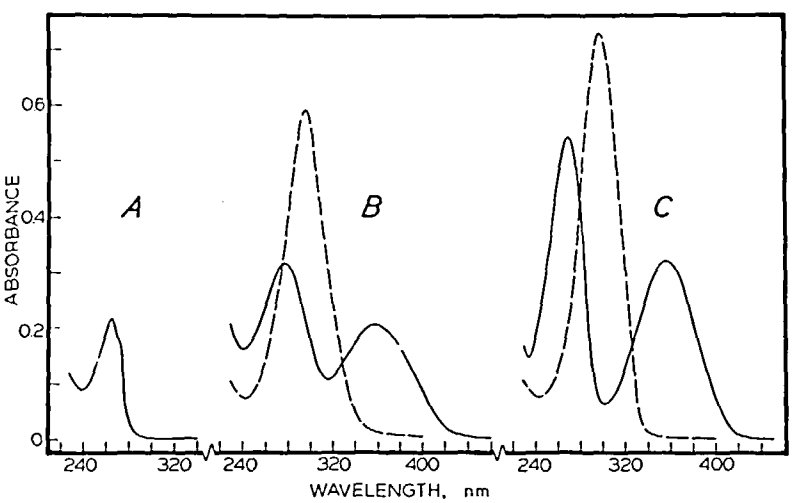


FIGURE 12. Electrolysis of 1-methyl-3-carbamoylpyridinium ion (MCP^+) (1.20 m*M*) in pH 10 Et_4NCl/carbonate buffer. Absorption spectra obtained by a 1/25 dilution of electrolysis solutions with buffers indicated. Solid line (pH 10 carbonate buffer): A, before electrolysis; B, after electrolysis at −1.40 V; C, after electrolysis at −1.80 V. Dashed line (McIlvaine buffer); B, acid catalyzed decomposition product of dimer (final pH = 7.4); C, acid catalyzed decomposition product of dihydropyridine species (final pH = 4.5). (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 1033 (1974). With permission.)

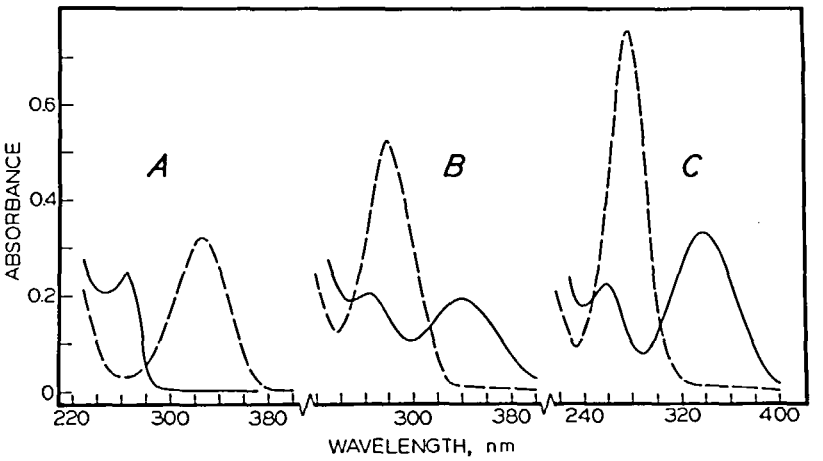


FIGURE 13. Electrolysis of NMN^+ (1.37 m*M*) in pH 9.6 Et_4NCl/carbonate buffer. Absorption spectra obtained by a 1/25 dilution of electrolysis solutions with buffer indicated. Solid line (pH 9.6 carbonate buffer): A, before electrolysis; B, after electrolysis at −1.3 V; C, after electrolysis at −1.8 V. Dashed line: A, cyanide addition product of NMN^+ (1.0 *M* KCN); B, acid catalyzed decomposition product of dimer (0.2 *M* phosphate buffer; pH 6.7); C, acid catalyzed decomposition product of dihydropyridine species (0.1 *M* phosphate buffer; pH 6.8). (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 1033 (1974). With permission.)

The 4,4′ structure is more acceptable than the 6,6′ with respect to steric interactions between the bulky N-substituents on the nicotinamide rings; however, the steric effect of the 3-carbamoyl group must also be considered since this group also protrudes from the reduced pyridine ring. Molecular models of the *dl* form of the 4,4′ dimer suggest that at least one amide function must be twisted out of the plane of the adjacent vinyl group for dimerization to occur; for the *meso* form, both amide groups must be twisted. For the 6,6′ dimer, however, very little deformation of the amide groups is necessary, although moderate interaction between N-substituents is seen. Amide deformation may be sufficient to hinder formation of the 4,4′ dimer which should exhibit a single absorption band at ca, 340 nm and, thus, could not be distinguished from other products containing a partially reduced 1,6 pyridine structure except in terms of the relative absorbances at 260 and 340 nm.

The NMN dimer, which also has bulky N-substituents, does appear to have largely a 6,6′ or a 4,6′ structure based on its spectrum,[14] which, unlike that of the NAD dimer, is due only to the nicotinamide moiety, i.e., the 260-nm region is not complicated by adenine absorption.

The spectra of electrolyzed MCP^+ solutions indicate the major – if not the exclusive – product to be the 6,6′ dimer.[14] The results of reducing MCP^+ by a variety of other than electrochemical methods are generally in good accord with those recorded by the present authors, e.g., X-ray irradiation results in free radical formation and a dimeric product[23,82] whose physical and chemical properties, including even molar absorptivities, are in remarkably close agreement with those of the electrolytically prepared dimer. The same is true of the dimeric product prepared by Zn reduction[68,74] and of the 2*e* reduction product prepared with borohydride.[82,83] Dithionite reduction, on the other hand, leads exclusively to a 1,4-dihydropyridine.[84-86]

Based on all available evidence, it is likely that solutions prepared by electrolysis at a potential on the plateau of wave Ic contain a mixture of isomers with the 6,6′ form predominating where the N-substituent is small, e.g., H or CH_3, and with the 4,4′ form being increasingly formed as the N-substituent increases in size.

Although formation of an isomer dimerized at the 2 position would be unlikely based on steric interactions evident from molecular models and the colorless electrolyzed solutions, such a possibility cannot be entirely ruled out; the transient greenish-yellow color seen during con-

trolled potential electrolysis[14,55,57] may be due to the formation of a small amount of such an isomer. Behavior analogous to that of the corresponding dihydropyridine might be expected; 1,2-NADH, for example, has an absorption maximum at 395 nm (compared to 340 and 345 nm for 1,4- and 1,6-NADH, respectively) and is much less stable than 1,4- or 1,6-NADH in slightly alkaline solution.[69,70] On hydrolysis, 1,2-NADH solutions lose their yellow color.

The ultimate product of the initial electrochemical reduction of the nicotinamide series in DMSO and AN, based on the spectral evidence, is the 6,6' dimer.[31]

b. Dimer Oxidation-Reduction

The ease of oxidation of the dimers is remarkable (Table 4). Electrochemical oxidation or reduction of carbon-carbon single bonds under polarographic conditions is generally limited to semiquinone type dimers, such as alloxantin, where the carbon atoms forming the single bond each carry a hydroxyl group.[87] Oxidation of the NAD dimer at pH 9.6 at the pge occurs at a potential about 0.6 V less positive than that for oxidation of NADH; simple inductive effects cannot account for the magnitude of the increased ease of oxidation of the dimer with respect to NADH. A reasonable explanation might be that molecular crowding, brought about by interactions between the substituents on the two rings, weakens the connecting bond.

The pH independence of dimer oxidation above pH 6 – for example, in the case of NAD – is consistent with the subsequently proposed redox scheme. Below pH 6, the potential becomes more positive with decreasing pH, indicating proton loss during oxidation, which may result from protonation of the dimer; this is consistent with promotion of catalytic hydrogen evolution at the dme by the dimer and its decomposition by acid catalyzed hydrolysis (see Section II.B.5.). With decreasing pH, the dimer anodic peak height at the hmde decreases because of the latter decomposition.

Previous investigators[88] have attributed a small cathodic wave, seen at a potential close to background discharge in a solution of the wave Ic electrolysis product, to reduction of dimer to 1,4-NADH, since 1,4-NADH activity slowly developed on further electrolysis at -1.84 V; the slow rate of reduction was ascribed to mercury surface adsorption phenomena. However, the cathodic wave was not examined. Under the same experimental conditions, NADP dimer solutions gave virtually identical results.[89]

A similar cathodic wave, seen in electrolyzed solutions of both NAD^+ and NMN^+, has been shown[14,57] to be due to free nicotinamide, present as an impurity and formed by decomposition of NMN^+ and NAD^+ during electrolysis under slightly alkaline conditions, e.g., as expected, the wave is not observed in electrolyzed solutions of MCP^+,[14] which decomposes under alkaline conditions via hydrolysis of the amide function. In addition, capacitance data indicate an absence of adsorption phenomena in the wave IIc potential region. The NAD dimer was not reduced to a dihydropyridine under controlled potential as negative as -1.8 V (-1.8 V corresponds to the foot of solution discharge; see Figure 9); however, when the potential was shifted into the background discharge region (-1.85 to -1.90 V; see Figure 9), the 340-nm absorption slowly increased, suggesting conversion of dimer to the more highly absorbing dihydropyridine. Nearly identical behavior was found for NMN^+.[14]

In view of the absence of adsorption, the slow formation of dihydropyridine from dimer suggests that a direct reduction is not involved (DC wave is absent), unless, coincidentally, the rising portion of the reduction wave occurs just beyond solution discharge; this seems unlikely, however, since the potential of solution discharge of final wave I electrolyzed solutions is identical to that for the background electrolyte alone. Indirect reduction may result via interaction of dimer with reduction products of background discharge, e.g., hydrogen radicals. Another possibility involves the fact that

TABLE 4

Half-Wave Potentials for Oxidation of Dimers Derived from Nicotinamides

Parent compound	$-E_{1/2}^{aq}$[a]	$-E_{1/2}^{DMSO}$[b]	$\Delta E_{1/2}$
Nicotinamide	0.45	0.65	0.20
MCP^+	0.45	0.36	-0.09
NMN^+	0.28	0.16	-0.12
NAD^+	0.28	0.31	0.03
$NADP^+$	0.28	0.33	0.05

[a] Values determined in aqueous solutions of pH 9 to 10.
[b] Values determined in DMSO containing $(C_2H_5)_4NClO_4$ as supporting electrolyte.

the NMN and NAD dimers decompose slowly under slightly alkaline conditions (oxygen absent) to NMN^+ and NAD^+ (Figure 14), which would be directly reduced (2*e* process) at a potential of -1.8 V to dihydropyridines. Further investigation of the matter of dimer reduction is obviously necessary.

Hanschmann's claim[16] that the dimers can be further reduced by continued irradiation to form dimers with 1,4,5,6-tetrahydropyridine components apparently contradicts the claim that the NAD dimer can be further reduced to a dihydropyridine. It would seem that the most susceptible bond for attack is in the ring and is not the dimeric connecting bond.

4. Dihydropyridines

The following summarizes the spectra and behavior observed for pH 9 to 10 solutions of the nicotinamides after exhaustive electrolysis at a potential on the second wave plateau, where a 2*e* reduction to the dihydropyridine species is expected.

A colorless electrolyzed solution of nicotinamide (n = 1.82) had absorption maxima at 265 and 346 nm (Figure 11) and exhibited two anodic

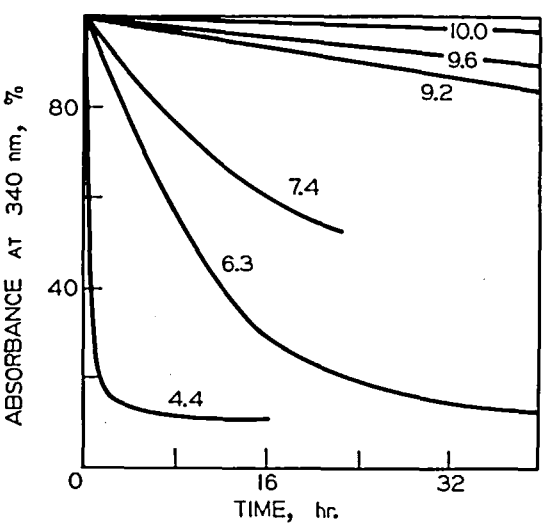


FIGURE 14. Effect of pH on stability of the NAD dimer (pH indicated on each curve). Buffer solutions and dimer concentration above pH 9: carbonate and 2.16 × 10^{-5} *M*; below pH 9: McIlvaine and 1.89 × 10^{-5} *M*. All solutions stored under air at room temperature. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

waves;[55] however, since the first wave height (dimer oxidation) was only about 6% that of the second (dihydropyridine oxidation), mostly the 2*e* reduction product was obtained. Oxidation of the freshly electrolyzed solution at 0.0 V, which corresponds to the 2*e* anodic wave limiting portion, gave a faradaic n of 1.69 based on the original nicotinamide content; the nicotinamide wave reappeared at 91% of its original height.

Electrolysis of MCP^+ in both KCl and Et_4NCl solutions gave a faradaic n of 1.95 ± 0.02, and colorless solutions of nearly identical spectral and faradaic properties, e.g., maxima at 270 nm (ϵ = 11,400) and 358 nm (ϵ = 6,600) (Figure 12), and the absence of both original cathodic waves (Figure 5); a small amount of the 1*e* product was formed (2.5% estimated from anodic wave height).[14] Oxidation of the 2*e* product is readily observed at the pge (Figure 6C; see previous discussion).

Completely electrolyzed solutions of NMN^+ (average n of 1.93), which were at most only a faint yellow, had maxima at 259 nm (ϵ = 4,400) and 339 nm (ϵ = 6,500) (Figure 13C).[14] Dilution with 1 *M* KCN did not alter the spectrum, indicating involvement of the pyridine ring in the reduction. Polarograms showed a small dimer anodic wave; based on its height, approximately 17% NMN^+ was converted to the 1*e* product. The pge voltammetric pattern was similar to that for 1,4-NMNH (Figure 15).

Electrolyzed solutions of NAD^+ had maxima at 259 and 341 nm.[57] Enzymatic analysis of a typical sample showed that the NAD^+ concentration decreased to less than 1% of its initial value and that an average of 54% had been converted to 1,4-NADH; the anodic wave indicated 11% dimer; the remaining 35%, based on coulometry and spectra, was considered to be 1,6-NADH. Other investigators found both 1,4-NADH and dimer after electrolysis at -1.85 V in $(n\text{-}Bu_4N)_2CO_3$ buffers; under optimal conditions, about 59% of the NAD^+ was converted to 1,4-NADH. However, only 80% of the initial NAD^+ could be accounted for as NAD^+, dimer, and 1,4-NADH; in view of the present discussion, the remaining material may have been 1,6-NADH.

a. Oxidation of Dihydropyridine Nucleotides

The importance of the dihydro reduction products of the pyridine nucleotides in the biological electron transport chain[9,11] emphasizes

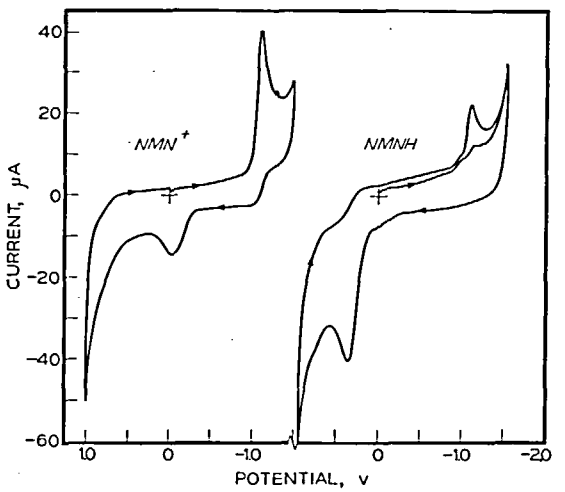


FIGURE 15. Cyclic voltammograms of NMN^+ (1.44 m*M*) and 1,4-NMNH (1.30 m*M*) at the pge in pH 9.6 Et_4NCl/carbonate buffer. Scan rate = 100 mV/sec. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 1033 (1974). With permission.)

the desirability of thorough electrochemical investigation of their redox half-reactions. Although there has been, as indicated, moderately extensive investigations,[5,9,10] of the electrochemical reduction of the pyridine nucleotides, there are only a few systematic studies of the electrochemical oxidation of relevant dihydropyridine derivatives.[15,16,90-92] Enzymatic oxidations of NADH and NADPH to NAD^+ and $NADP^+$ (2*e* process in each case) are well known.[93,94] Most of the chemical oxidations have been explained – similarly to the enzymatic oxidations – in terms of the direct transfer of a hydride ion from the dihydropyridine to the oxidizing agents.[72] In general, studies of the dihydronicotinamides are hampered by their acid-catalyzed hydrolytic instability, particularly at pH less than 6 or 7.

As part of the systematic study by electrochemical techniques of the redox patterns and allied chemical behavior for the nicotinamide sequence of compounds, commercially available and certified preparations of the following biologically active 1,4-dihydropyridine nucleotides were examined in both aqueous media (pH 4 to 10; carbon, mostly glassy carbon, electrodes) and nonaqueous media (DMSO; platinum electrodes): dihydronicotinamide mononucleotide (NMNH), dihydronicotinamide adenine dinucleotide (NADH), and dihydronicotinamide adenine dinucleotide phosphate (NADPH).[56]

i. Electrochemical Behavior

In pH 7.1 aqueous medium, each dihydropyridine nucleotide shows a single, well-defined, cyclic voltammetric anodic peak at about 0.7 V (Table 5; Figure 16), whose height varies linearly with $v^{1/2}$ as expected from theory for a diffusion-controlled process.[95] Reversal of the scan at a more positive potential produces a cathodic peak at about -1.15 V, which is due to reduction of the anodic peak process product. Initial scan towards negative potential did not generate this cathodic peak, which was identified as due to reduction of the parent pyridinium nucleotide itself. The ratio of the cathodic and anodic peak heights increased with increasing v to a limiting value between 0.3 and 0.4. A cathodic peak complementary to the anodic peak, corresponding to a reversible redox couple, was not seen even at a scan rate (v) of 30 V/sec.

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 these 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 5; Figure 17), whose height varies linearly with $v^{1/2}$ and which merges with background discharge at v exceeding 10 V/sec; no cathodic complementary peak was seen at v of 5 to 10 V/sec. Scan reversal positive to the oxidation peak generates a cathodic peak at about -1.0 V (Table 5), which can be identified with the peak shown by the pyridine nucleotide itself.

Faradaic n values, calculated from the cyclic voltammetric peak currents and potential step experiments (chronoamperometry), are given in Table 6.

On the basis of the anodic processes in both H_2O and DMSO being 2*e* in nature with inconsequential loss due to acid-catalyzed reactions under cyclic voltammetric conditions (see subsequent discussion), the viscosities at 25° of 1.996 for DMSO and 0.894 for H_2O lead to an expected current function (i_{pa}/AC) ratio for H_2O/DMSO of 1.49. The ratios for the Table 5 data are 1.75 for NMNH, 1.50 for NADH and 1.52 for NADPH. It is difficult to account for the ratio for NMNH being 17% higher than expected.

TABLE 5

Voltammetric Behavior of Dihydropyridine Nucleotides[a]

Compound	Medium[b]	Anodic peak E_{pa} V	Anodic peak i_{pa} μA	Anodic peak i_{pa}/AC^c μA/cm² mM	Cathodic peak $-E_{pc}$ V	Cathodic peak i_{pc} μA	Cathodic peak i_{pc}/AC^c 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.
[b]Aqueous solutions were pH 7.1.
[c]Normalized current: peak current divided by electrode area and depolarizer concentration.

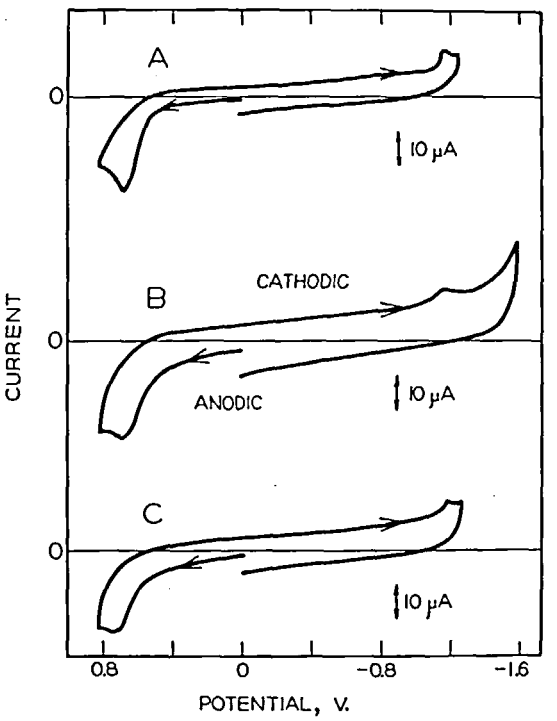


FIGURE 16. Cyclic voltammograms at a glassy carbon disk electrode in pH 7.1 aqueous Tris buffer of (A) NMNH (1.5 m*M*), (B) NADH (1.4 m*M*), and (C) NADPH (1.1 m*M*). Scan rate = 0.2 V/sec. Arrowhead indicates direction of scan. (From Braun, R. D., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 2591 (1975). Copyright by the American Chemical Society. With permission.)

Large-scale controlled electrode potential electrolysis at 0.80 V of pH 7.1 solutions of NMNH, NADH and NADPH (3 or 4 electrolyses of each) gave faradaic *n* values ranging between 1.2 and 1.7 (averages given in Table 6). The solutions (originally 0.3 to 3.5 m*M*) were colorless throughout electrolysis, whose duration varied from 2 to 7 hr. 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 6). The electrolysis current decayed steadily with time and reached background value.

ii. Product Identification and Behavior

Prior to electrolytic oxidation in aqueous media (pH 7.0), NMNH had one ultraviolet absorption maximum at 338 nm ($\epsilon = 3.6 \times 10^3$). After oxidation, this peak nearly vanished and a new peak appeared at 265 nm, whose ϵ matches that for the NMN^+ 265-nm peak. After some oxidations, a third peak attributable to decomposition reaction products appeared at about 275 nm.

NADH and NADPH have two peaks each (Figure 18) at pH 7.1 at about 338 and 260 nm ($\epsilon = 5.0 \times 10^3$ and 15×10^3, respectively, for NADH; 5.3×10^3 and 14×10^3, respectively, for NADPH); the 338-nm peak is attributable to the

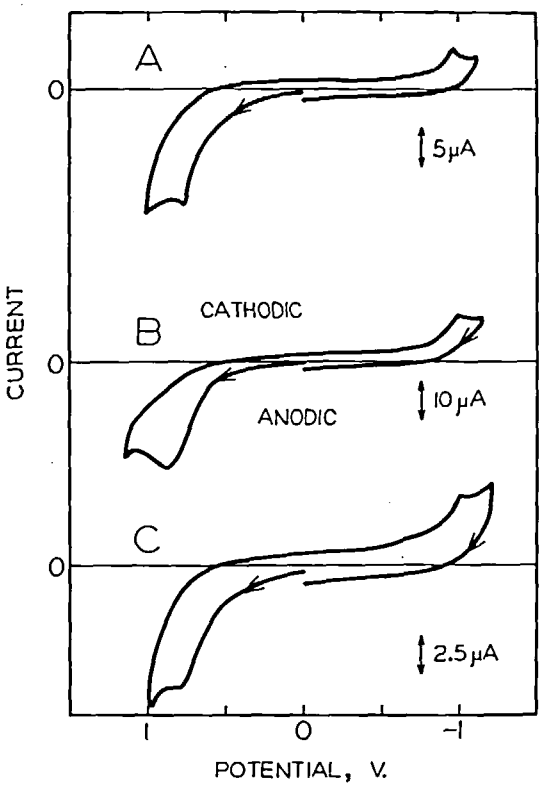


FIGURE 17. Cyclic voltammograms at a platinum electrode in dimethylsulfoxide (Bu_4NClO_4 background) of (A) NMNH (1.3 m*M*), (B) NADH (2.1 m*M*), and (C) NADPH (0.4 m*M*). Scan rate = 0.2 V/sec. Arrowhead indicates direction of scan. (From Braun, R. D., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 2591 (1975). Copyright by the American Chemical Society. With permission.)

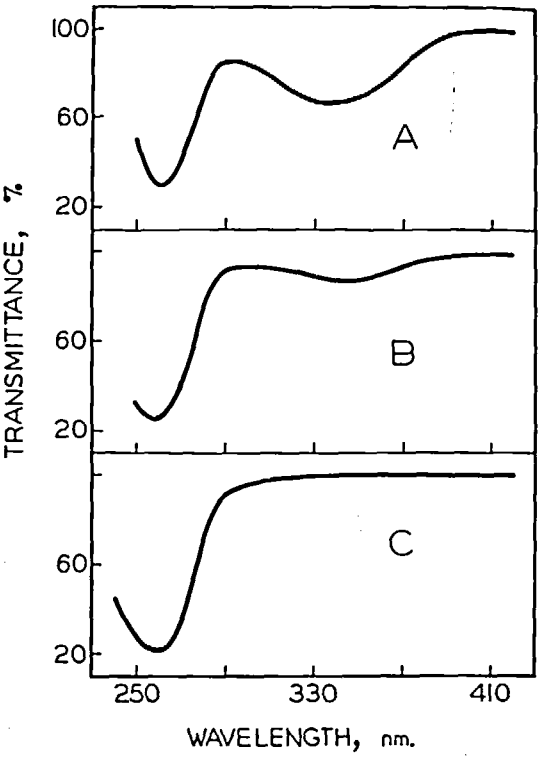


FIGURE 18. Ultraviolet spectra of 3.6×10^{-5} *M* aqueous solutions (pH 7.1 Tris buffer) of (A) NADH, (B) NADH after oxidation at 0.80 V at a pyrolytic graphite electrode for 6.6 hr, and (C) NAD^+. (From Braun, R. D., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 2591 (1975). Copyright by the American Chemical Society. With permission.)

TABLE 6

Faradaic *n* Values for Dihydropyridine Nucleotides

Medium[a]	Compound	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.

dihydronicotinamide moiety and the 260-nm peak to the adenine moiety (See Reference 72, p. 626). After electrolysis, the 338-nm peaks decreased considerably, while the 260-nm peaks increased slightly with either no noticeable change in shape or a slight broadening on the long wavelength side (probably due to absorption by decomposition products) to apparent ϵ values of 16×10^3 for the NADH product and 15×10^3 for the NADPH product. NAD^+ and $NADP^+$ each show only one peak at about 260 nm (Figure 18) 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 NMNH, NADH or NADP at pH 7.1, no cathodic dme wave was visible between 0 and -1.6 V. After exhaustive oxidation of NMNH, a wave of $E_{1/2} = -0.93$ V appeared and, at times, a second wave of $E_{1/2} = -1.07$ V. Exhaustive 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 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. Samples of pure NMN^+, NAD^+ and $NADP^+$ gave single dme waves at $E_{1/2}$ of -0.93 V, -0.89 V and -0.90 V, respectively. The possible origin of the waves at ca. -1.06 V is discussed in the next subsection.

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, in each case, showed an anodic polarographic wave identical to that attributed to oxidation of the dimers produced on 1*e* reduction of the parent nicotinamides.

Addition of alcohol dehydrogenase (ADH) and ethanol to a pH 7.1 solution of the NADH oxidation product resulted in regeneration of the original dihydronicotinamide. ADH and ethanol react specifically with NAD^+ to yield NADH.[96]

The results of large-scale electrolyses in DMSO generally agree with those for aqueous media. After controlled potential oxidation, the absorption maxima of the dihydronicotinamides at 263 and 335 nm are replaced by ones at 263 and 280 nm. The 263-nm peak is due to the nicotinamide and adenine chromophores in the pyridine nucleotides[71,72] (although the latter references are to aqueous solution, there is only a small solvent effect on the pyridine nucleotide absorption, as noted, for example, in Reference 35); the 280-nm peak is due to decomposition products. Prior to electrolysis, an initial voltammetric scan towards negative potential shows the absence of reducible material; after electrolysis, such a scan produces a cathodic peak at ca. -1.0 V.

iii. Stability of Dihydropyridine Nucleotides

The 1,4- and 1,6-dihydropyridine species are known to be rather acid-labile and to decompose at pH 8 and below.[86] Thus, the second cathodic dme wave sometimes observed at -1.06 or -1.07 V after large-scale electrolytic oxidation of the dihydropyridines at pH 7.1, was identified as due to a compound or compounds produced on acid-catalyzed decomposition of the dihydropyridines.

Figure 19 is typical of the changes in voltammetric patterns of pH 4.1 dihydropyridine solutions with time; 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 an 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; failure to see wave IIa may be due to occurrence of background discharge in the same potential region. 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.

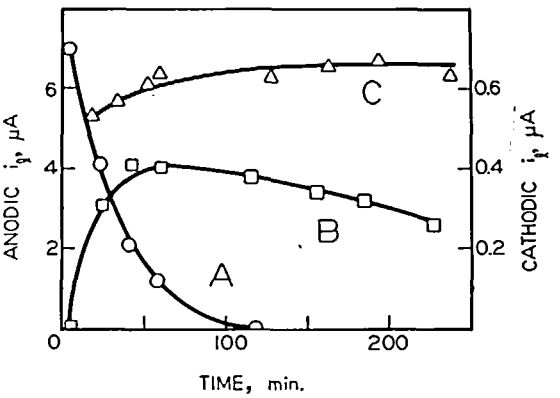


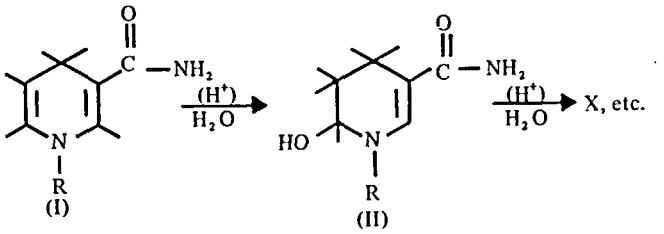
FIGURE 19. Variation in electroactivity (limiting current, i_l) with time of a pH 4.1 solution of NADH (0.62 m*M*): A, anodic wave (rgce) at ca. 0.65 V; B, anodic wave (rgce) at ca. 1.10 V; C, cathodic wave (dme) at ca. -0.96 V. (From Braun, R. D., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 2591 (1975). Copyright by the American Chemical Society. With permission.)

For each dihydropyridine, the wave Ia limiting current (i_l) at pH 4.1 decreased with time and disappeared completely after about 100 min; wave IIa increased from zero to a maximum height 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 (See Section IV.C.).

iv. Identity of Acid-Hydrolysis Products

There is general agreement in the literature[97-100] that the primary acid-catalyzed

decomposition product of a 1,4-dihydropyridine nucleotide (structure I) has structure II (Equation 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 reaction sequence is postulated to occur with a rapid first step and a slow second step,[97-99]

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

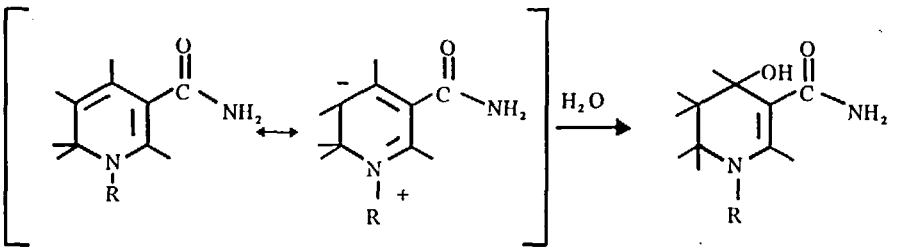
If the hydration occurs in two steps, as shown in Equation 5, and fast protonation is followed by a slow OH^- addition, the intermediate $H(RNH)^+$ will have measurable lifetime (see Section III on mechanistic patterns). [RNH represents the dihydropyridine nucleotide and $H(RNH)^+$ its protonated form.]

On the basis of the relative variation in magnitude of the observed waves, e.g., as in Figure 19, and previously reported data,[56] it is reasonable to assign the anodic wave at 0.6 to 0.7 V to oxidation of I (RNH), the anodic wave at ca. 1.1 V to oxidation of $H(RNH)^+$, and the cathodic wave at -0.96 V to reduction of II and, perhaps, also of the secondary hydrolysis product(s). Subsequent change in pH from 4.1 to 7 or 8 could then alter the ease of reduction of the secondary hydrolysis products and/or their chemical nature to produce at times the dme wave at -1.06 or -1.07 V.

Addition of H_2O to a 1,4- or 1,6-dihydronicotinamide would produce the equivalent of a 1,4,5,6-tetrahydro derivative, which would be expected to absorb in the 280 to 300 nm region;[97,101,102] addition to a 1,2 isomer would lead to a 1,2,5,6-tetrahydro compound, which would not absorb above 220 nm.[103] This same argument is applicable to the hydrolysis of the corresponding dimers.

5. Decomposition of the Reduction Products

The two reduction products of each nicotinamide (dimer and dihydropyridine) undergo acid-catalyzed decomposition even in alkaline solutions containing such proton donors as $H_2PO_4^-$, e.g., with time, the anodic voltammetric waves characteristic of each reduction product are lost and the product absorption peaks decrease and new peaks appear. With decreasing pH, the rate of decomposition increases; at any given pH, the dimer is less stable than the dihydropyridine species. The decomposition involves hydrolysis of the 4,5 double bond and can be regarded as an instance of enamine nucleophilicity:[104,105]



(6)

where R is H in the case of nicotinamide.

Thus, in the case of MCP^+ solutions electrolyzed at pH 10, the dimer concentration (measured by the 358-nm absorption) decreased at a rate of

6%/hr and that of the 2*e* product at 2%/hr. Since the time required for electrolysis was relatively short (40 min for the 1*e* product and 60 min for the 2*e* product), little product was lost and molar absorptivities were calculated assuming no loss. On dilution of aliquots of the electrolyzed solutions with buffer to obtain solutions of pH 9.3, 7.4, and 4.5, the dimer half-life in the latter was 112, 5.1 and 0.1 min, respectively, emphasizing the rapid increase in decomposition rate with decreasing pH. In all cases, as the two dimer absorption bands decreased in intensity, a new band developed at 296 nm (Figure 12B). At pH 7.4, an isosbestic point formed at 331 nm, suggesting that the dimer decomposed directly to its acid catalyzed product without going through an intermediate.

The effect of pH on stability of the dihydropyridine product is similar. At pH 9.3, the half-life of the MCP 2*e* product was 420 min; as the absorption band due to the acid-catalyzed product developed, an isosbestic point formed at 316 nm.

In alkaline solution, the two reduction products of nicotinamide and N′-methylnicotinamide are directly oxidized by molecular oxygen in air to the parent compound with the dimer being more easily oxidized than the dihydropyridine, which is consistent with their electrochemical oxidation potentials.[55] The MCP^+ products are also similarly oxidized.

Both reduction products of the pyridine nucleotides are much more stable over the pH range to acid-catalyzed hydrolysis and to oxidation by oxygen than the corresponding reduction products of nicotinamide and MCP^+, thus emphasizing the importance of the N(1) substituent on reactivity. For example, at pH 4.5, half-lives for hydrolytic decomposition are 0.1 min for MCP dimer and 16 min for NAD dimer; at pH 9.3 the half-lives are 112 min for MCP dimer and several days for NMN dimer.[14]

The problems in data interpretation, which may result from failure to take into account the acid instability of the reduction products, can be illustrated by a reexamination of the results reported in an earlier investigation of MCP^+.[20] The product isolated after electrolysis for several hours at the wave I potential in pH 7 to 8.1 phosphate buffers, had a molecular weight close to that expected for a dimeric product and a single absorption peak at 298 nm; it was postulated to be a 6,6′ dimer. The final wave I electrolysis solution gave a cathodic polarographic wave at about -1.65 V (depending on pH), which was attributed to reduction of the 4,5 double bonds of the dimer; no mention was made of an anodic wave due to dimer oxidation. Electrolysis on the -1.65 V wave plateau gave an n of 2.11 with no change in ultraviolet absorption spectrum; this was explained on the basis that, since the 4,5 double bond was only cross-conjugated with the main 298-nm chromophore, $-\overset{1}{N}-\overset{2}{C}=\overset{3}{C}-\overset{4}{C}=O$, removal of the 4,5 double bond by reduction would leave the basic chromophore intact.

In view of the results presented in the present paper, the compound isolated was actually the acid-catalyzed decomposition product of the dimer, which, in the pH range employed, has a rather short half-life compared to the electrolysis times used; this would account for presence of the 298-nm band and absence of the anodic wave. The origin of the -1.65-V cathodic wave is less clear, since its characteristics were not given. In pH 7.7 McIlvaine buffer,[14] the decomposition product of the dimer produces a catalytic hydrogen wave, (background discharge is about 0.25 V more positive), which, at 5 μA, had a potential of -1.67 V. Under the conditions previously used,[20] the catalytic wave may have exhibited a limiting portion, which might easily be taken to represent further reduction of dimer. Since electrolysis on this wave would not alter the catalyst (dimer decomposition product), no change in ultraviolet spectrum would be expected. During electrolysis, the limiting current of the wave might be expected to drop due to an increase in solution pH (the capacity of the 0.1 *M* phosphate buffer used would be low, around pH 8).

In the same study,[20] after electrolysis at potentials on MCP^+ wave II in pH 7.8 to 9.2 phosphate buffers, a product was isolated with a single band at 360 nm, which was identified as the 1,4-dihydropyridine. Under the pH conditions and time periods employed, a significant portion of the product should have been the acid-catalyzed decomposition product of the dihydropyridine species.

The stability of the dimers in AN and DMSO is indicated by the fact that, over a period of 24 hr, the spectrophotometric absorption peak and the anodic polarographic wave did not show any appreciable differences in magnitude.[31] The greater stability in nonaqueous media may obviously be explained as due to the low proton activity in

these media. On electrolysis of MCP^+ in AN at a potential on the wave Ic plateau in the presence of 50 m*M* benzoic acid, a 100% yield of the dimer (based on coulometric reversal oxidation) was obtained. The dimer yield is obviously not altered by the presence of a weak proton donor (pK_a of benzoic acid in AN = 22.0).

6. Summary on Reduction Product Identity

A feature of many of the dimers shown in Table 2 is the qualitative similarity of their ultraviolet absorption spectra to those of the corresponding dihydropyridines prepared by borohydride reduction. However, the long wavelength band and, to a lesser extent, the short wavelength band of a particular dimer have each a molar absorptivity only slightly greater than that for the corresponding band of the more fully reduced dihydro compound; this is true even though the dimer has twice as many chromophores per mole. The reason for such behavior is unclear. The two chromophores of the dimer are separated in all cases by two carbons and should, therefore, absorb independently of one another. In the case of the dimeric 1*e* products of pyrimidine and 2-aminopyrimidine,[32] where the dimer chromophores are also separated by two carbons, the molar absorptivity of the dimer is about twice that of the corresponding dihydro compound. However, these dimers are not substituted with bulky side chains as are the pyridine dimers.

Another question concerns the inability of the dimers to fluoresce. Dimeric products prepared from MCP^+,[82] 1-propyl-, 1-benzyl- and 1-dichlorobenzylnicotinamide,[74] and NAD^+[81,106] have all been reported as nonfluorescent. This contrasts with the fact that such dihydropyridines as 1-methyl-1,4-dihydronicotinamide[82] and 1,4-NADH[78] do fluoresce.

An electronic substituent effect does not readily account for either the anomalous ultraviolet absorption behavior or the absence of fluorescence. The dihydroxyacetone adduct of NAD^+, for example, which contains a carbon-carbon single bond at the 4 position of the pyridine ring, has about the same absorption and fluorescence properties as 1,4-NADH itself,[11] and undergoes similar acid-catalyzed decomposition. The lack of fluorescence from the dimeric structures may result from a steric effect such as molecular crowding of the two pyridine rings which very slightly interferes with the coplanarity of the chromophoric structure.[107]

Another problem involving the dimers is the fact that, although they can be quantitatively oxidized to the starting pyridinium compounds by electrolysis at a potential on the anodic wave, the oxidation process is slow (about three times as long as the corresponding 1*e* reduction) with only low levels of current flow.

Review of the literature, including work by the present authors, tends to leave one with the feeling that the various structures postulated for the electrolytic reduction products may not be entirely correct. The complicated nature of the problem is emphasized, for example, by the fact that, in the case of the ultimate 1*e* product, at least six different dimeric structures are possible but, because of the two asymmetric carbons at the point of dimerization, as many as 21 stereoisomers may exist (3 *meso* forms and 9 *dl*-pairs). Furthermore, the validity of using spectral arguments based on the dihydropyridines for assigning the position of dimerization in partially reduced pyridines has not been thoroughly tested, e.g., using these criteria structures have been assigned[68] to dimers of nicotinamide model compounds prepared by zinc reduction, but others have suggested caution.[81]

Based on all of the evidence, it is the authors' belief that macroscale electroreduction of the nicotinamides, model compounds, and pyridine nucleotides at potentials corresponding to both 1*e* and 2*e* processes, leads to mixtures of products. Thus, electrochemical reduction is nonspecific, unlike enzymatic reduction which, at least in the case of the coenzymes, is specific in that hydrogen addition occurs at the 4 position of the pyridine ring and stereospecific in that the hydrogen is added from only one side of the plane of the ring (see Section VI. B. 1). In formation of the dimers from the free radicals, mixtures of the 4,4′, 6,6′ and 4,6′ structures are possible. Because of the absence of a long wavelength (ca. 400 nm) absorption peak, a structure involving reduction at the 2 position would seem to be excluded, thus avoiding possible stereochemical problems. However, a pyridine ring partially reduced at the 2 position would be expected to be unstable (1,2-dihydropyridine is less stable than the 1,4 or 1,6 forms) and to decompose by hydrolysis during electrolysis to a product which does not absorb in the ultraviolet; a transient greenish-yellow color is

seen during electrolysis of NAD^+. However, only a minute amount could be involved since electrolysis at a potential on the anodic wave of an NAD dimer solution allows recovery of the original NAD^+. Underwood[9,81] and Hanschmann[15,16] state that conclusive evidence for the 4,4′ structure of the NAD dimer is not available; the adenine moiety at 260 nm masks the absorption of any structure reduced at the 6 position. The molar absorptivity of the NAD dimer at 260 nm is significantly greater than twice the molar absorptivity of adenosine (32,500 vs. 2 X 14,400).

III. MECHANISTIC PATTERNS

A. Redox Paths in Aqueous Media

1. Nicotinamides

The reaction scheme outlined in Figure 20 seems to fit best the electrochemical, chemical, and spectral data for nicotinamide (I) and N′-methylnicotinamide.[55]

At pH above the pK_a of nicotinamide (3.3 at 20°[108]), the initial reduction involves uptake of an electron and a proton to form a neutral free radical (II) (source of polarographic wave Ic); this is followed by irreversible dimerization to an apparent 6,6′ dimer (III). At the potential of wave IIc, nicotinamide is reduced to an apparent 1,6-dihydropyridine species (IV), which is equivalent to a further 1*e* reduction of the free radical; the dimer is not reduced at this potential. At a potential considerably more positive than that of wave Ic, the dimer is oxidized to nicotinamide. At still more positive potential, the dihydropyridine species is also oxidized to nicotinamide.

The pH dependencies of the two electrochemical reductions and the two electrochemical oxidations are compatible with the proposed mechanistic scheme. Below pH 4 (Figure 2), the wave Ic reduction process should show only slight pH dependence because most of the nicotinamide will be protonated at N(1) and any unprotonated compound will rapidly protonate as the protonated form is reduced. Under the conditions of cyclic voltammetry, peak potential Ic does become less pH dependent as the pH is lowered. The essential reactions in the wave Ic process are, consequently, as follows, where RN represents nicotinamide:

$$RN + H^+ \rightleftharpoons RNH^+ \quad (7)$$

$$RNH^+ + e \rightleftharpoons \dot{R}NH \quad (8)$$

$$2\,\dot{R}NH \longrightarrow (RNH)_2 \quad (9)$$

The assumption of essentially complete protonation before electron transfer is supported by the relatively slight pH dependence of $E_{1/2}$ and by the closeness of $E_{1/2}$ for nicotinamide (−1.11 V)

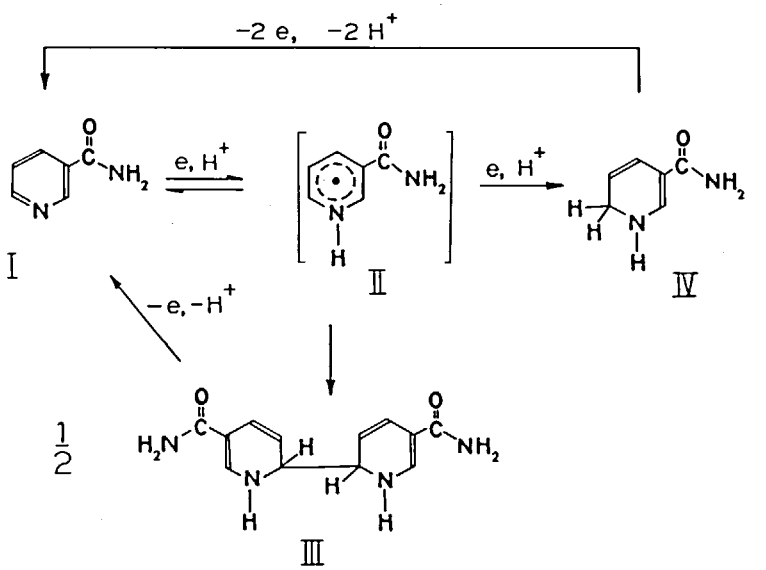


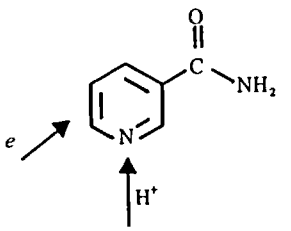
FIGURE 20. Reaction paths for the electrochemical behavior of nicotinamide and its reduction products. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Electrochem. Soc.*, 121, 345 (1974). With permission.)

and N′-methylnicotinamide (−1.08 V) to that for MCP^+ (−1.03 V),[14] where the nitrogen lone pair is not available and the electroactive species exists as a cation.

The wave II process involves Reactions 7 and 8 plus reduction of the free radical, which may proceed stepwise:

$$\dot{R}NH + H^+ + e \longrightarrow RNH_2 \quad (10)$$

Above pH 4, the energy-controlling step in the reduction involves essentially simultaneous addition of an electron entering the ring electronic system and of a proton localizing itself on N(1):



(11)

The electron density distribution in the nicotinamide ring based on resonance structures supports this model.[109] In the slightly acidic to slightly alkaline region (Figure 2), protonation is sufficiently rapid that it is not a limiting factor. The essential steps in the wave I process are, accordingly,

$$RN + H^+ + e \rightleftharpoons \dot{R}NH \quad (12)$$

$$2\,\dot{R}NH \longrightarrow (RNH)_2 \quad (13)$$

The pH dependence of 79 mV/pH unit for $E_{1/2}$ of wave I between pH 4 and 9 (60 mV expected for an uncoupled reversible 1*e* reaction) probably results from the rapid follow-up reaction as well as possible protonation subsequent to electron transfer.

Dimerization of the neutral free radical produced in acidic to slightly alkaline media must be exceedingly rapid since its oxidation is not seen even at high cyclic voltammetric scan rates. Oxidation of the free radical is seen at pH 9 and above (Figure 4).

At pH 12, two electrons add almost simultaneously to produce a polarographic wave, whose slope is near that expected for a reversible 2*e* wave. The following reactions are, accordingly, involved:

$$RN + e \rightleftharpoons \dot{R}N^- \quad (14)$$

$$2\,\dot{R}N^- \longrightarrow (RN)_2^{2-} \quad (15)$$

$$(RN)_2^{2-} + 2\,H^+ \longrightarrow (RNH)_2 \quad (16)$$

$$\dot{R}N^- + e \longrightarrow (:RN)^{2-} \quad (17)$$

$$(:RN)^{2-} + 2\,H^+ \longrightarrow RNH_2 \quad (18)$$

Where the two waves have coalesced, there may be a rapid protonation of the free radical anion to produce the neutral free radical, which would be reduced at the potential required for the first electron addition before it could dimerize, i.e.

$$\dot{R}N^- + H^+ \rightleftharpoons \dot{R}NH \quad (19)$$

$$\dot{R}NH + e \longrightarrow (:RNH)^- \quad (20)$$

$$(:RNH)^- + H^+ \longrightarrow RNH_2 \quad (21)$$

The proton source in the above reactions may be water or a buffer constituent such as HCO_3^-.

2. 1-Substituted Nicotinamides

The reaction scheme outlined in Figure 21 seems best to fit the available data (spectrophotometric, chemical, enzymatic, and electrochemical) for the 1-substituted nicotinamides and their reduction products.[14,57]

The electrochemical behavior of MCP^+ and NMN^+ is in some ways similar to that of nicotinamide itself. However, as might be expected for a positively charged pyridinium nucleus (V), the first reduction step is independent of pH and occurs at slightly more positive potential; thus, two well defined 1*e* waves appear in slightly alkaline solution rather than the merging waves seen for nicotinamide. The initial reversible uptake of an electron to form a neutral free radical (VI) (source of wave Ic) is followed by irreversible dimerization largely to an apparent 6,6′ dimer (VII). At the wave IIc potential, the cation is reduced in a 2*e* process to the dihydropyridine (VIII) (apparently the 1,6 species, although some 1,4 isomer may be produced in the case of NMN^+); the dimer is not reduced at this potential. The dimer can be oxidized to MCP^+ or NMN^+ at a potential considerably more positive than that of wave Ic; the dihydropyridine species is oxidizable at still more positive potential.

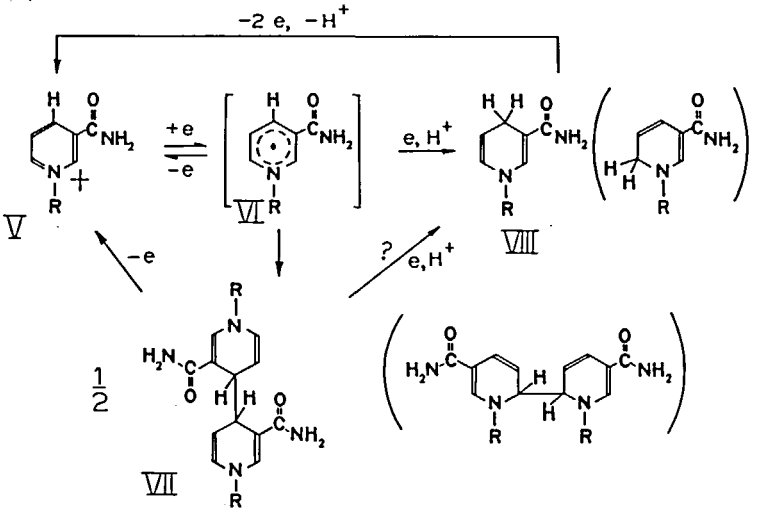


FIGURE 21. Reaction paths for the electrochemical behavior of nicotinamide-adenine dinucleotide, related compounds, and their corresponding reduction products. (From Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J., *J. Am. Chem. Soc.*, 97, 5083 (1975). Copyright by the American Chemical Society. With permission.)

The redox mechanism for NAD^+, which apparently is the same as those for $NADP^+$ and $DNAD^+$, differs only slightly. A reversible 1*e* addition to the pyridinium ion to produce a free radical (source of wave Ic) is followed by irreversible dimerization to VII, which is probably largely the 4,4′ isomer with some of the 6,6′ and 4,6′ forms, reflecting the transition from the 6,6′ isomer in the case of nicotinamide itself to the 4,4′ form as the substituent on the pyridinium nitrogen increases in size. At considerably more negative potential, the pyridinium ion is reduced to a dihydropyridine via direct 1*e* reduction of the free radical (source of wave IIc); reduction of the dimer is not involved. The wave IIc product is generally largely enzymatically active 1,4-NADH, but some 1,6-NADH also appears to be formed. At sufficiently positive potential, both the dimeric and dihydro products can be oxidized back to the original nucleotides. At potentials close to solution discharge, the dimer (at least for NAD^+) is very slowly reduced to a dihydropyridine species; although the mechanism for this reduction is unknown, it is unlikely that it involves a direct electron transfer.

Since the initial free radical products dimerize with a rate constant of about $10^6\ M^{-1}\ sec^{-1}$ (see Section IV.B.2.), dimerization would be expected to be essentially complete under dme polarographic conditions. The appearance of wave IIc at a potential where the dimer is stable to reduction, indicates that the second electron transfer is very rapid compared to the dimerization reaction or that dimerization occurs away from the interface, e.g., in the bulk solution. The data – cyclic voltammetric patterns in particular – support the concept of the second electron transfer being more rapid than the dimerization, i.e., NAD^+ is directly reduced to a dihydropyridine at the wave II potential in an overall 2*e* process.

The principal basis for postulating involvement of a proton in the overall electrode process for NAD^+ is the formation of enzymatically active NADH. The sequence of addition of electrons and protons could be (a) *e*, *e*, H^+, or (b) *e*, H^+, *e*. If protonation of the neutral free radical formed on addition of the first electron is very rapid as compared to its dimerization, either sequence could be competitive over the dimerization reaction in producing the dihydropyridine species; otherwise, sequence (a) would be more likely.

3. Dihydropyridine Nucleotides

The following mechanism for the electrochemical oxidation of the 1,4-dihydropyridine nucleotides in both aqueous and nonaqueous media is considered to best fit the results obtained by the present authors:[56]

$$RNH \longrightarrow RN^+ + H^+ + 2e \quad (22)$$

$$RNH + H^+ \rightleftharpoons H(RNH)^+ \quad (23)$$

$$H(RNH)^+ \longrightarrow \text{Decomposition products absorbing at 280 to 290 nm} \quad (24)$$

where RN^+ is the pyridine nucleotide itself (oxidized form). [Reaction 24 may involve formation of H(RNH)OH; see Equations 4 and 5.] During the short time-scales of cyclic voltammetric and rotating electrode experiments, Reaction 24 proceeds to a negligible extent and, consequently, the number of electrons involved in the electrode process approaches two per RNH molecule (Table 6). Under large-scale electrolysis conditions, Reaction 24 removes enough RNH to give the lower coulometric n values observed. $H(RNH)^+$ would not be electrolyzed at the applied potential (0.80 V), since it would be considerably more difficult to oxidize than RNH ($E_{1/2}$ = 0.6 to 0.7 V). Compare the previous assignment of the anodic wave at ca. 1.1 V to oxidation of $H(RNH)^+$.

The data are compatible with acid-catalyzed decomposition, i.e., the RNH limiting current decreases with time on rotating disk voltammetry at pH 4.1 and the rate of decomposition decreases with increasing pH. Thus, even on electrolysis at pH 7, a decrease in pH near the interface as a result of generation of H^+ (Equation 22) at the electrode surface might cause the decomposition to occur more rapidly. 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 produced upon diffusion into the bulk solution to yield a product corresponding to the second dme wave (see Section II.B.5.).

Acid-catalyzed decomposition reactions such as Reactions 23 and 24 give decomposition product(s) with an absorption maximum around 280 to 290 nm.[97-99,101,110] 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 to 290 nm region and the disappearance of the characteristic dihydronicotinamide moiety 338-nm peak; during the same time period, the 338-nm peak only decreases by 1 to 2% at pH 7.1.

Leduc and Thevenot[91,92] proposed the following stepwise mechanisms (generalized from their equations for NADH):

$$RNH \xrightarrow[\text{slow}]{-e} \dot{R}HN^+ \underset{\text{rapid}}{\overset{-e}{\rightleftharpoons}} RNH^{2+} \underset{\text{rapid}}{\overset{-H^+}{\rightleftharpoons}} RN^+ \quad (25)$$

$$RNH \xrightarrow[\text{slow}]{-e} \dot{R}NH^+ \underset{\text{rapid}}{\overset{-H^+}{\rightleftharpoons}} \dot{R}N \overset{-e}{\rightleftharpoons} RN^+ \quad (26)$$

They were undecided as to which path was actually followed. The present authors have considered these mechanisms as well as other similar mechanisms, but have found no evidence for a stepwise reaction within the time scales of even their fastest cyclic voltammetric studies. The postulation of the rapid protonation of NAD^+ or $\dot{NAD}$ in Equations 25 and 26 seems doubtful in view of the absence of an $E_{1/2}$ shift for NAD^+ with pH of the medium, and the absence of protonation of NAD^+ in controlled protonation experiments.[31]

B. Redox Paths in Nonaqueous Media

1. 1-Substituted Nicotinamides

The electrochemical redox pattern in nonaqueous media (AN and DMSO) for all of the cationic 1-substituted nicotinamides studied (MCP^+, NMN^+, NAD^+, $DNAD^+$, and $NADP^+$) involves an initial one-electron up-take (wave Ic), followed by rapid dimerization of the free-radical product:

$$RN^+ + e \rightleftharpoons \dot{R}N \quad (27)$$

$$2\,\dot{R}N \longrightarrow (RN)_2 \quad (28)$$

where RN^+ represents the 1-substituted nicotinamide. The dimer is oxidized at considerably more positive potential to the original nucleotide:

$$(RN)_2 \longrightarrow 2\,RN^+ + 2e \quad (29)$$

No reduction of dimer or of free radical occurs in nonaqueous media within the available potential range.

These results are in contrast with those in aqueous medium, in which the free radical $\dot{R}N$ is further reduced electrochemically to form the dihydronicotinamide, RNH. This difference in behavior is due to the low proton activity in AN

and DMSO. Two mechanisms may be formulated for the appearance of wave IIc in a nonaqueous medium on addition of proton donors. Either the protonated radical, $\dot{R}NH^+$, is being reduced or reduction of the neutral radical is facilitated by the proton presence. Since protonation of a radical generally makes its electrochemical reduction easier than that of the parent molecule,[111] it is unlikely that the protonated radical is involved in the wave IIc process, which occurs at more negative potential than that for reduction of RN^+. More likely, essentially simultaneous addition of a proton and an electron to $\dot{R}N$ is responsible for wave IIc (see Equation 11 for nicotinamide):

$$\dot{R}N + H^+ + e \longrightarrow RNH \qquad (30)$$

Wave IIc in aqueous medium has been claimed to be due to simultaneous reduction of dimer and neutral free radical;[88] however, the nonreducibility of the dimer in AN and DMSO in the available potential range (−2.6 V)[31] would seem to cast some doubt on the report of dimer reduction.

2. Nicotinamides

The electrochemical redox pattern for nicotinamide in nonaqueous media differs slightly from that for the 1-substituted nicotinamides; further reduction of the free radical (appearance of wave IIc) is observed even in the absence of proton donor. Based on analogy with the mechanisms postulated for the azabenzenes[112] and aromatic hydrocarbons[113-115] in AN, the mechanism for electrolytic reduction of nicotinamide in nonaqueous media can be summarized as follows:

$$RN + e \rightleftharpoons \dot{R}N^- \quad \text{(Wave I)} \qquad (31)$$

$$2\,\dot{R}N^- \longrightarrow (RN)_2^{2-} \qquad (32)$$

$$\dot{R}N^- + e \rightleftharpoons :RN^{2-} \quad \text{(Wave II)} \qquad (33)$$

$$:RN^{2-} + 2\,H^+ \longrightarrow RNH_2 \qquad (34)$$

where RN represents the neutral nicotinamide molecule. The negatively charged dimer is oxidized to the original molecule at less positive potential than the neutral dimers produced from the cationic nicotinamides ($E_{1/2}$ of −0.65 V for nicotinamide dimer vs. −0.16 to −0.33 for the others). This mechanism is consistent with that postulated for aqueous media, where the negatively charged dimer is protonated to give the neutral dimer.

On addition of proton donor, the negatively charged free radical is protonated to produce a species reducible at the potential of its formation and the wave Ic height increases to a maximum of twice its height in the absence of proton donor. Simultaneously, wave IIc diminishes.

3. Dihydropyridine Nucleotides

As has been indicated, the oxidation of the dihydropyridine nucleotides (NMNH, NADH, and NADPH) in DMSO proceeds by a mechanism similar to that in aqueous media. However, even though formation of protonated species, similar to those postulated for aqueous media, could occur during electrolysis in DMSO (Equations 22 and 23), the rate of decomposition would be expected to be less due to the lower dielectric constant, higher viscosity, and low residual water content. The coulometric n of 1.6 found for DMSO solutions somewhat exceeds that found for aqueous solutions (Table 6).

4. Comparison with Related Classes of Compounds
a. 1-Alkyl Nicotinamides

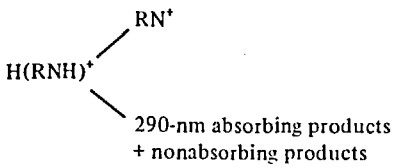
It has been common in the study of the pyridine nucleotides to use 1-alkyl nicotinamides as model compounds; such model compounds have also been frequently used in electrochemical studies.[9,10] The caution that needs to be exercised in such situations is emphasized by the differences observed between NADH in DMSO[56] and the 1-alkyl analogs[1-(2,6-dichlorobenzyl)-, 1-methyl-, 1-benzyl- and 1-(n-propyl)-1,4-dihydronicotinamides] in acetonitrile (AN) at platinum and carbon electrodes.[90]

The number of electrons involved in oxidation of the analogs varied between 0.8 and 0.9, and was considered,[90] together with other evidence, to be suggestive of the following process:

$$RNH \longrightarrow \dot{R}NH^+ + e \qquad (35)$$

$$2\,\dot{R}NH^+ \longrightarrow RN^+ + H(RNH)^+ \qquad (36)$$

$$RNH + H^+ \longrightarrow H(RNH)^+ \qquad (37)$$



(38)

where H^+ in Reaction 37 could be due to trace amounts of acidic impurities in the AN. The increase in cyclic voltammetric i_p to a maximum of twice the original i_p on addition of pyridine or tri-n-butylamine was explained as due to the process

$$RNH \longrightarrow \dot{R}NH^+ + e \quad (39)$$

$$\dot{R}NH^+ + B \longrightarrow \dot{R}N + HB^+ \quad (40)$$

$$\dot{R}N \rightleftharpoons RN^+ + e \quad (41)$$

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

The mechanism of Equations 39 to 41 does not seem to apply to DMSO solutions even if DMSO is assumed to be a sufficiently strong base to drive Reaction 40 well to the right. DMSO is a more basic solvent than AN; for autodissociation of the pure solvent, $-\log [CH_3CNH^+]\ [CH_2CN^-] = 19.5$ and $-\log [C_2H_6SOH^+]\ [C_2H_5SO^-] = 33.3$.[116] However, if DMSO were a strong enough base to drive Reaction 40 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 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 products investigated in the two solvents must react differently.

The mechanistic path of Equations 35 to 38 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 their study in that solvent. On the other hand, the results obtained in aqueous medium indicate that the reaction scheme proposed in Equations 22 to 24 is applicable throughout the pH range of 4 to 10.

For at least the present, differences in behavior of the alkyl analogs and the nucleotides can be attributed to differences in solvent basicity and the presence of ribose and phosphate groups in the nucleotides.

b. Aromatic Hydrocarbons

As was previously mentioned, the effect of proton addition on the 1-substituted nicotinamide reduction in nonaqueous media is quite different from that seen in the electrochemical reduction of aromatic hydrocarbons in nonaqueous media; that on nicotinamide is similar.

Reduction of aromatic hydrocarbons generally proceeds through two successive 1*e* additions with a potential separation not exceeding 0.6 V. Proton addition increases the first wave height with a corresponding decrease in the second. In the presence of a large excess of proton donor, most aromatic hydrocarbons show a single fully developed wave. The mechanism is considered to be

$$R + e \rightleftharpoons \dot{R}^- \quad (42)$$

$$\dot{R}^- + H^+ \longrightarrow \dot{R}H \quad (43)$$

$$\dot{R}H + e \longrightarrow RH^- \quad (44)$$

where R is the aromatic hydrocarbon. The protonated species $\dot{R}H$ has a higher electron affinity than R itself and, consequently, is reduced at the same potential as R.

In contrast, the 1-substituted nicotinamides form on reduction a neutral free radical, which, as opposed to a negatively charged free radical, has little tendency to react with the proton donor. Thus, wave Ic of the 1-substituted compounds is unaffected by added proton donor. The appearance of a new wave at more negative potential is due to reduction of the free radical in a process involving addition of both an electron and a proton.

The low proton activities (5 or 6×10^{-13} *M*) in nonaqueous media, at which wave IIc for the 1-substituted nicotinamides is fully developed (or wave IIc for nicotinamide has completely shifted to more positive potential), suggests either that the undissociated proton donor is involved in the protonation process or that rapid protonation of the 1-substituted nicotinamide radical is

accompanied by an equally rapid shift in the equilibrium:

$$HA \rightleftharpoons H^+ + A^- \quad (45)$$

Support for protonation involving the undissociated acid is indicated by the inappreciable dissociation of hydroquinone in DMSO, e.g., Kolthoff and Reddy[117] failed to see in DMSO the oxidation wave of hydroquinone, whose ionization is necessary for its oxidation.

C. Adsorption and Catalytic Hydrogen Evolution

In general, the height of wave Ic for nicotinamide and the 1-substituted nicotinamides is increased at low pH due to catalytic hydrogen evolution; at higher pH, where wave IIc is observed, it is usually accompanied by a similar hydrogen discharge.

A catalytic hydrogen evolution reaction occurs when a compound is present whose protonated adduct under the experimental conditions is more easily reducible, i.e., has a lower activation energy in respect to net addition of an electron to the proton, than the uncomplexed proton itself.

Adsorption of reduction products plays an important role in the catalytic evolution of hydrogen. In the case of MCP^+, for example, additional current in wave IIc region below pH 8 has been ascribed to catalytic hydrogen evolution because the observed limiting current increases rapidly with decreasing pH, increases with increasing buffer capacity at a given pH, and exhibits partial kinetic behavior.[14] At pH 7.4, the catalytic wave peak current occurs at −1.72 V, which is near the initial limiting portion of wave IIc. Little adsorption of either reduction product (dimer or dihydropyridine species) should occur at this potential since AC polarographic desorption peaks for both products occur at more positive potential. On the other hand, the mercury height-dependence of the catalytic wave suggests that an adsorbed species acts as catalyst, giving rise to a so-called surface catalytic wave.[118] This situation is explicable on the basis that weak adsorption corresponding to a very low surface coverage occurs even at potentials more negative than those of the desorption peaks.[65] Surface catalytic waves have been shown[65] to occur even wth surface coverages as low as 0.5%. With increasing negative potential, the surface coverage, although small, should decrease, which would account for the peaked shape of the wave, i.e., the rate of the electrode reaction increases with increasing negative potential but less catalyst is available due to decreasing surface coverage. The increase in catalytic wave height with decreasing h is due to the greater surface coverage at longer drop-time.[14]

D. Effects of the Reaction Medium

The present section emphasizes certain effects due to the solvent medium, which involve the electrochemical nicotinamide redox pattern, some aspects of which have already been discussed.

$E_{1/2}$ for wave Ic of the 1-substituted nicotinamides is not markedly affected by the medium (Table 1), indicating the absence of significant ion-pairing of the positively charged molecule with a counter ion in the solution. However, the mechanistic pathways are sensitive to the availability of protons in the medium. Thus, due to the low proton activity of DMSO and AN compared to H_2O, the second reduction wave is not seen in nonaqueous media. Addition of proton donors to the nonaqueous medium allows for further reduction of the initial wave Ic free radical product. These results are in contrast to the behavior of aromatic hydrocarbons[115] and azabenzenes,[114] where addition of proton donor enables further reduction of the free radical, thus increasing the height of the wave caused by the initial reduction of the parent molecule. It is evident that the proton affinity of the neutral radicals formed by reduction of the 1-substituted nicotinamides is much less than that of the negatively charged radicals formed by the hydrocarbons and azabenzenes. The reduction of nicotinamide itself in nonaqueous media is more akin to that of the aromatic hydrocarbons, as the product of the first wave reduction is a negatively charged radical.

The diffusion coefficient, D, of the nicotinamides in different solvents, calculated on the basis of the simple Ilkovic equation from polarographic diffusion current constant (I) values based on dme limiting currents, is presented in Table 1. With increasing viscosity, η, for example, the diffusion coefficients of nicotinamide and MCP^+ become smaller, but the product ηD remains fairly constant.

The effect of the medium on the rate of dimerization is illustrated by the dimerization of the nicotinamide free radical, which proceeds with k_d values of about 1.8×10^6 M^{-1} sec^{-1} in

aqueous alkaline medium, 1.2×10^5 in DMF, and 3.5×10^4 in DMSO. The activation energy for the dimerization decreases from 24 to 5 kcal $mole^{-1}$ on changing the solvent from H_2O to DMSO. The observed two orders of magnitude difference in k_d is attributable to differences in the nature of the free radical with solvent and in the dielectric constant of the medium ($\epsilon_{H_2O} = 78.3$; $\epsilon_{DMSO} = 46.7$; $\epsilon_{DMF} = 36.7$); a double sphere model for the dimerization reaction involving like charged species predicts variation of k_d with dielectric constant.

One report in the literature[77] suggests that free radicals derived from 1-substituted nicotinamides might possibly be more stable in nonaqueous than in aqueous media. Electrolysis at the wave Ic potential of 1-ethyl-4-carbamoylpyridinium species in AN produced a blue pyridinyl radical (1-ethyl-4-nicotinamide radical) suitable for electron spin resonance study; under aqueous conditions, this radical rapidly dimerizes.

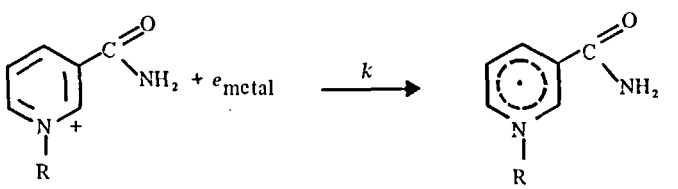
The half-wave potentials for oxidation of the dimers of the 1-substituted nicotinamides show very little variation with the medium (Table 4). The large difference for the nicotinamide dimer (0.2 V between H_2O and DMSO) may be attributed to the difference in nature of the dimer in the two media: neutral in H_2O and anionic in DMSO.

IV. KINETIC ASPECTS OF CHARGE-TRANSFER AND CHEMICAL REACTIONS

In general, kinetic steps in electrode processes may be resolved and measured by the use of suitably rapid relaxation methods, in which a perturbing impulse, e.g., a controlled potential increment, is applied to the system under investigation when it is at equilibrium, and the resulting transient relaxation or response of the system is measured, e.g., as a current flow. The techniques used to determine the kinetic parameters for charge-transfer and coupled chemical reactions have included cyclic voltammetry, chronoamperometry, chronocoulometry, faradaic rectification, phase-selective alternating current polarography, and pulse polarography, for instance, as in Reference 45.

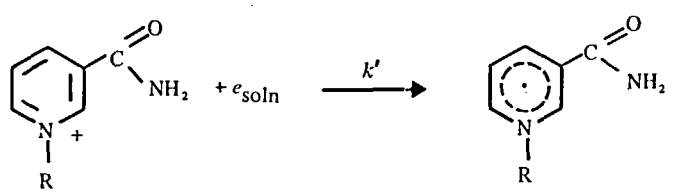
A. Heterogeneous (Charge-Transfer) Reaction Rates

The heterogeneous rate constant, k, for the reaction



(46)

has been related to the homogeneous electron-exchange constant, k', for the reaction



(47)

through Marcus's theory, who has shown[121] that, in general, the homogeneous and heterogeneous electron-transfer rate constants are related by the expression,

$$\frac{k}{Z_h} \leq \left(\frac{k'}{Z}\right)^{1/2} \quad (48)$$

where Z_h and Z are, respectively, the collisional frequencies for the heterogeneous and homogeneous electron transfers. The values of Z_h and Z are taken in the Marcus theory to be approximately 10^4 cm sec^{-1} and 10^{11} M^{-1} sec^{-1}.

The k' values have been determined in aqueous solution, using the technique of pulse radiolysis, to

be $4.1 \times 10^{10}\ M^{-1}\ sec^{-1}$ when R is CH_3 and $2.5 \times 10^{10}\ M^{-1}\ sec^{-1}$ when R is sugar-pyrophosphate-sugar-adenine (as in NAD^+).[23]

The heterogeneous rate constants for the first electron addition to MCP^+ and NAD^+ can then be estimated to be less than or equal to 6.4×10^3 and 5×10^3 cm sec^{-1}, respectively. Unfortunately, rate constants of such large magnitude cannot be determined using electrochemical techniques since the values are at or beyond the limit of these techniques. In this connection, it is appropriate to point out that k for anthracene has been experimentally determined to be 4.0 cm sec^{-1} while Marcus's theory gives a value of $k = 7.0 \times 10^2$ cm sec^{-1}.[113]

A major problem in applying such techniques as alternating current polarography to measure k for the initial electron addition to pyridinium species is the rapid dimerization of the free radical produced. Normally, this technique is limited to electron-transfer rates occurring with a k of 10^2 cm sec^{-1} or less due to interference from double layer charging currents; however, the presence of the dimerization process reduces the k value determined by the method due to the fact that the AC polarographic method measures the reversibility of the electron transfer process in terms of the current flow and, if the product of the electron transfer is lost by a chemical reaction, the faradaic currents are reduced (see the next section on reversibility).

1. Reversibility

Evaluation of the reversibility of the electron-transfer processes involved in the nicotinamide redox patterns is complicated by rapid follow-up chemical reactions, which result in the overall irreversibility of the electrode process, and, in some instances, by the adsorption of reactant and/or products. However, a strong claim of reversibility can be made for the first 1*e* addition in the case of all of the compounds investigated on the basis of the reversible oxidation of the primary radical product with supporting evidence being provided by the analysis of DC polarographic waves and cyclic voltammetric peaks, and the effects of concentration and drop-time (see, Section II.A.3. on evidence for dimerization).

Application of criteria such as those indicated support the generally irreversible nature of the second 1*e* addition process in the reduction of the nicotinamides and do not yield any definitive information in the case of the anodic processes involving the dimeric and dihydropyridine species.

The essentially irreversible faradaic AC polarographic response for wave Ic, i.e., observation for the first faradaic process of less than 3% of the alternating current theoretically expected for a reversible 1*e* process, results from the dimerization subsequent to electron transfer; under the conditions used, the reversibility of the overall electrochemical process is tested. The basic requirement for observation of chemical irreversibility on AC polarography is that the period of the applied alternating voltage sufficiently exceeds the half-life associated with the chemical step that reoxidation of the initial reduction product (free radical, in the present instance) cannot contribute significantly to the alternating current.[122] The greatest applied frequency (800 Hz), for which reliable data could be obtained, corresponds to a period of 1.8 msec; based on the dimerization rate constant found for the free radicals, a free radical half-life of at most a few tenths of a msec would be predicted; thus, most of the radicals dimerize before they can be reoxidized.

The AC polarographic behavior indicates a significant difference between the first and second electron-transfer processes for the nicotinamides. For example, in pH 9.4 Et_4NCl/carbonate buffer, the 1-substituted 3-carbamoylpyridinium ions exhibit an AC faradaic response for the first 1*e* step, which is always significantly greater than that for the second 1*e* step; in addition, the faradaic impedance for the first 1*e* step is always complex, having both resistive and capacitive components, while the impedance for the second 1*e* step is always entirely resistive in nature.

2. Formal Potentials

When a rapid chemical reaction follows a charge-transfer reaction, the resulting polarographic $E_{1/2}$ is not a simple criterion for the ease of reduction of the molecule. Different compounds can be validly compared only when the potentials are corrected for the effect of the chemical reactions. This can be done using cyclic voltammetric peak potentials and the relationship[123]

$$E_p = E_c^\circ - \frac{0.058}{3nF} \log \frac{4.78 \times 3\pi D_O}{2 D_R} \cdot \frac{RT k_d C^\circ}{nFv} \quad (49)$$

to calculate the formal potential, E_c°.

Using the k_d determined by the present authors,[14] E_c° for the initial electron addition process (wave Ic) in aqueous media,

$$MCP^+ + e \rightleftharpoons MCP\cdot \quad (50)$$

is calculated to be -1.18 V; the corresponding E_c° for NMN^+ is -1.24 V. However, in view of the uncertainties in k_d and in equating D_O and D_R, it would be best to round both values off to -1.2 V. Similarly corrected formal potentials for nicotinamide and 1-substituted nicotinamides in nonaqueous media are given in Table 7. It will be noted that, as a result of the irreversible dimerization reaction following charge-transfer, the polarographic $E_{1/2}$ values are about 0.1 to 0.2 V more positive than the calculated formal potentials.

In many instances, the significance of a chemical reaction which precedes or follows charge-transfer is either ignored or not recognized in considering the relation of potentials and accompanying chemical reactions. Bowie and Feldman,[124] for example, have reported what they regard as anomalous reductive dimerizations of alkylpyridinium ions by chromous perchlorate. They conclude that 1-benzyl-3-carbamoylpyridinium ion, which has a le reversible polarographic reduction potential of -1.06 V vs. sce,[17] should not accept an electron (be reduced) by the chromic/chromous couple which has an E° of -0.65 V vs. sce; yet, experimentally, the 1-benzyl-3-carbamoylpyridinium species is readily reduced by chromous ion to a dimeric product in good yield[74]. However, Bowie and Feldman[124] do not consider the possible effects of free radical formation and irreversible dimerization as component steps in the overall reaction.

The reaction in question, where RN^+ is 1-benzyl-3-carbamoylpyridinium ion,

$$RN^+ + Cr^{2+} \rightleftharpoons \dot{R}N + Cr^{3+} \quad (51)$$

has a cell potential, E_{cell}°, of -0.41 V, which is negative. Consequently, the redox reaction itself is not a spontaneous one. However, this does not exclude the possibility of at least trace amounts of free radicals being formed. The equilibrium con-

TABLE 7

Experimental Reduction Potentials and Hückel Molecular Orbital Data for Pyridines and Nicotinamides[a]

Molecule	$-E_{1/2}$ V	$-E_c^{\circ b}$ V	$-M_{m+1}$	$-\Delta(\Delta E_{solv})^c$ eV	$-\Delta(\Delta E_{solv})^d$ eV
Pyridine	2.78		0.841		
1-Methylpyridine	1.27		0.359	0.54	
2-Methylpyridine	2.80		0.837	-0.028	
4-Methylpyridine	2.86		0.832	-0.098	
Nicotinamide	2.00	2.16	0.762	0.622	0.46
1-Protonated nicotinamide	1.06	1.18	0.353	0.744	0.62
1-Methyl nicotinamide (MCP^+)	1.04	1.16	0.353	0.764	0.64
NMN^+	0.99	1.07	0.353	0.814	0.73
NAD^+	0.98	1.10	0.355	0.824	0.704
$NADP^+$	1.06	1.16	0.355	0.744	0.644

[a] MO calculations were done on an IBM 360 computer with standard coulomb and resonance integrals (see Reference 109, p. 106 to 110). Because of the positive charge on nitrogen in 1-substituted compounds, a small electronegativity was attributed to the *ortho* carbon atoms and their coulomb integrals have been modified to $\alpha_{c\ ortho} = \alpha_c + 0.3\ \beta$.

[b] Calculated from the cyclic voltammetric peak potentials in nonaqueous media after correction for the following chemical reaction.

[c] The values are relative to pyridine; $\Delta(\Delta E_{solv}) = \Delta E_{1/2} - (\Delta m)\ \beta$, where $\Delta E_{1/2}$ is the difference in half-wave potentials between pyridine and the compound of interest in the same solvent, and Δm is the difference in the HMO coefficients for the lowest unoccupied level of the same two molecules. β is taken as equal to -2.0.

[d] Calculated from the E_c° listed, using the approach of Footnote c.

stant, K, for the cell redox reaction can be calculated from the standard free energy change:

$$-\Delta G^\circ = RT \ln K = n F E^\circ_{cell} \quad (52)$$

$$\log K = \frac{n E_{cell}}{0.0591} = \frac{-0.41}{0.0591} = -6.94 \quad (53)$$

$$K = 1.1 \times 10^{-7} \quad (54)$$

When the redox reaction is coupled to a free radical irreversible dimerization, the overall reaction should be driven to the right, i.e.,

$$2\,\dot{R}N \longrightarrow (RN)_2 \quad (55)$$

B. Homogeneous (Chemical) Reaction Rates: Free Radical Dimerization

1. Methodology for Rate Constant Measurement

Free-radical dimerization reactions of the types encountered with nicotinamide and the 1-substituted nicotinamides, i.e.,

$$R + e \rightleftharpoons R^- \quad (56)$$

$$2R^- \longrightarrow (R_2)^{2-} \quad (57)$$

$$R^+ + e \rightleftharpoons \dot{R}^- \quad (58)$$

$$2\dot{R}^- \longrightarrow (R_2) \quad (59)$$

have been studied by a variety of techniques, e.g., pulse radiolysis,[23] electron spin resonance spectrometry,[77] cyclic voltammetry,[5;14,31,33-35,55] chronopotentiometry,[125] and dme polarography.[61] The essential aspects of the electrochemical techniques employed in the study of these dimerization reactions are subsequently described with emphasis on cyclic voltammetry which has found the most extensive use.

a. DME Polarography

Bonnaterre and Cauquis[61] have derived explicit expressions for the equation of the polarographic wave for reversible and irreversible dimerization reactions following electron transfer, from which the dimerization rate constant can be calculated. For a 1*e* reversible reaction followed by irreversible dimerization (the case pertinent to the nicotinamides),

$$E = E^\circ_c - \frac{RT}{F} \ln \frac{i^{2/3}}{i_d - i} - \frac{RT}{3F} \ln \frac{1.5\,F\,m}{\lambda_f^2\,C} \quad (60)$$

where $m = D/\delta$, $\lambda_f^2 = \delta^2\, k_d/D$, D = the diffusion coefficient, δ = thickness of the diffusion layer, and k_d = dimerization rate constant.

Since evaluation of the rate constant by this equation requires knowledge of the thickness of the diffusion layer and of the formal potential of the redox couple, only a few instances of the evaluation of k_d by this method have been reported. However, the equation has been useful in the nicotinamide studies as a diagnostic tool, since substitution of the appropriate relative current magnitudes leads to a value of 46 mV for the wave slope defined by $E_{1/4} - E_{3/4}$. Thus, the initial 1*e* wave for nicotinamide and the 1-substituted nicotinamides in nonaqueous media have a slope of 45 to 50 mV.[31]

b. Cyclic Voltammetry

Three approaches for calculating the dimerization rate constant, k_d, from cyclic voltammetric data have been used with the nicotinamides. One approach involves graphical correlation of the ratio of the anodic and cathodic peak heights (i_{pa}/i_{pc}) for the oxidation and the formation of the free radical with the kinetic parameter product, $k_d \tau C$, where τ is the time interval between E° and the potential at which direction of the potential sweep is reversed, and C is the bulk solution concentration of the original electroactive species; a second involves an explicit solution, relating k_d to the ratio of the observed anodic peak current, i_{pa}, to the normal diffusion-controlled anodic peak current, i_{pd}, at the same scan rate, ν; the third is based on the shift of peak potential, E_p, with ν. These approaches have been most extensively investigated from the theoretical and methodological viewpoints by Nicholson, Saveant, and their collaborators.[123,126-128]

i. Peak Current Ratio Method

For the case of a reversible electron transfer reaction followed irreversible dimerization,

$$O + n\,e \underset{i_{pa}}{\overset{i_{pc}}{\rightleftharpoons}} R \quad (61)$$

where n *e* electrons are added to a molecule O and R_2 is the resulting dimer, the cyclic voltammetric peak current for the cathodic process (reduction of O), i_{pc}, will exceed the peak current, i_{pa}, in the anodic process (oxidation of R) due to the loss of R by dimerization (Equation 62), i.e., the ratio of

i_{pa}/i_{pc} will be less than unity (Figure 22) and its magnitude will depend on k_d and ν.

Explicit expressions for the potential and the current for the situation represented by Equations 61 and 62 can be derived[123,126] by solving the dimensionless Fick's diffusional equations with the appropriate boundary conditions. The resulting equation for the current ratio, i_{pa}/i_{pc}, involves the terms k_d, C_o (the original bulk solution concentration) and τ (time taken to go from E° [generally assumed to be close to $E_{1/2}$] to E_λ, the switching potential); τ can be varied by control of ν and the switching potential. The variation of the kinetic parameter, $\log k_d C_o \tau$, is shown in Figure 23.

ii. Anodic Current Equation

In a slightly different approach, Saveant[127] obtained a closed form solution for the case of a reversible electron transfer reaction followed by irreversible dimerization. Current and potential functions were derived by applying the theory of chemical polarization with appropriate surface concentrations of R at the switching time θ. The anodic polarization curve under suitable conditions can be completely defined with the ratio of the observed anodic peak current, i_{pa}, to the theoretical normal diffusion anodic peak current, i_{pd} being

$$i_{pa}/i_{pd} = (0.67\,\pi\,k_d\,\theta\,C_o)^{-1/3} \qquad (62)$$

$$2\,R \xrightarrow{k_d} R_2 \qquad (63)$$

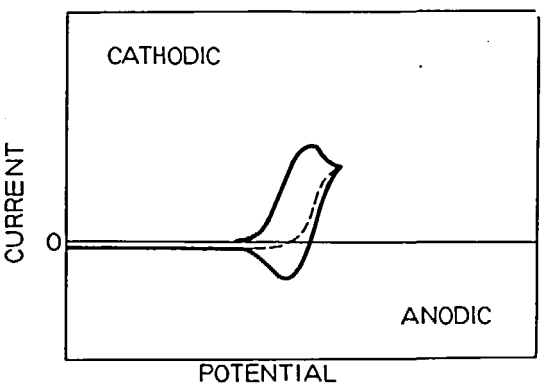


FIGURE 22. Cyclic voltammograms for a molecule undergoing reduction with no chemical complications following charge transfer (solid line) and with a dimerization reaction following charge transfer (dashed line).

A simple diagnostic criterion for the presence of a dimerization follow-up reaction is the linearity of a plot of the current ratio vs. $\theta^{-1/3}$; k_d can be calculated from the slope of the line.

iii. Peak Potential Method

Due to the loss of the primary electroreduction product by dimerization, the concentration of R at the electrode surface depends on k_d and ν.[126] Consequently, the peak potential for the reduction process, E_p, which is governed by the Nernst equation, can be related to k_d and ν:

$$E_p = E_c^\circ - \frac{RT}{3nF}\ln\frac{4.78 \times 3\pi D_O}{2D_R} - \frac{RT}{3nF}\ln\frac{a}{k_d C_o} \qquad (64)$$

where E_c° = formal potential of the system, $a = nF\nu/RT$, and D_O and D_R = diffusion coefficients of the oxidized and reduced forms. Evaluation and knowledge of many of the terms are avoided by measuring the shift of E_p with a, i.e., with scan rate, ν. Thus, the peak potential would shift $20/n$ mV for a tenfold change in the concentration or in the scan rate.

The accuracy of this method depends on accurate measurement of E_p, compensated for iR drop. The principal advantage of the method is that it can be employed to estimate k_d when no

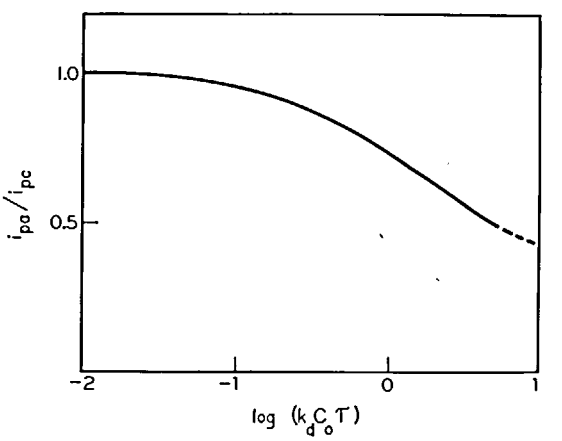


FIGURE 23. Variation of the cyclic voltammetric anodic-cathodic current ratio with log (k_d C_o τ), where k_d is the irreversible dimerization rate constant, C_o is the bulk concentration of the electroactive species, and τ is the switching time.

anodic peak corresponding to oxidation of the reduced species is seen, provided that E_c° is known.

iv. Numerical Method

A numerical method using digital simulation procedures described by Feldberg[129] was developed by Evans[130] for dimerization reactions. In this technique, Fick's equations for O and R, appropriately modified to include the reaction of Equation 62, were treated numerically to obtain the surface concentrations of O and R. From the latter, the current function values and the shift of peak potential were obtained as a function of the kinetic parameter. Typical curves are shown in Figure 24.

This approach has not yet been applied to the dimerization of free radicals derived from the nicotinamides.

c. Chronopotentiometry

In this technique, a constant current is applied to the polarographic cell for a short period, during which the potential of the working electrode is monitored as a function of time.[131,132] The result of the perturbation is a potential-time curve for the reduction or oxidation of a molecule with a characteristic transition time, τ, corresponding to the inflection at which suddenly the potential rapidly changes. Reversal of the direction of current flow produces another potential-time segment, corresponding to the complementary oxidation or reduction in the case of a reversible redox process. A typical chronopotentiogram for a reversible electron transfer process,

$$O + ne \rightleftharpoons R \qquad (65)$$

is shown in Figure 25.

For the case of dimerization of an electrolytically reduced product, the reversal (anodic) part of the chronopotentiogram would be absent if the rate of dimerization is faster than the transition time. However, by operating at asymmetric current densities (i.e., different forward and reverse current densities), the anodic transition time may be detected. The half-life of the initial product of the electrochemical reduction may be estimated by this procedure.

d. AC Polarography

The principles of AC polarography and its application for measuring the rates of chemical reactions coupled to the charge-transfer step are covered in References 38 and 43 to 45.

The expected AC polarographic response for a second order dimerization following charge transfer has only recently been theoretically analyzed and a suitable methodology for its application presented.[132a] When confronted by a second order kinetic step in the overall electrochemical

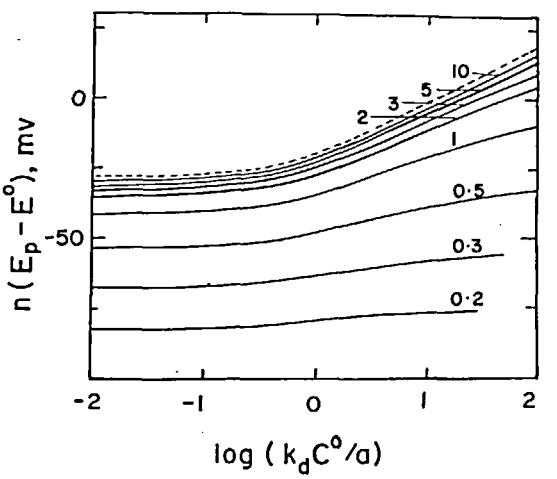


FIGURE 24. Cyclic voltammetric peak potential as a function of the dimensionless kinetic parameter for a dimerization reaction following charge transfer. The current function values are indicated on the curves. The dashed line corresponds to the curve for a reversible electron-transfer reaction. (From Evans, D. H., *J. Phys. Chem.*, 76, 1160 (1972). Copyright by the American Chemical Society. With permission.)

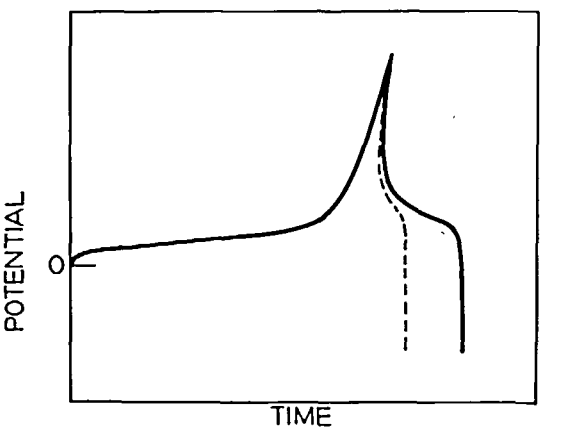


FIGURE 25. Chronopotentiograms for a molecule undergoing uncomplicated reversible electron transfer (solid line) and a dimerization reaction following charge transfer (dashed line).

process, the general tendency has been to treat the situation as one involving a pseudo first order reaction.[133,134] Since AC polarography has not yet been used to elucidate the dimerization reactions of pyridine nucleotide free radicals, the details of the approach are not being given.

2. *Dimerization Following Reduction Reactions*

Data for the free radical dimerization rate constant, k_d, for the nicotinamides and 1-substituted nicotinamides (pyridine nucleotides and related model compounds), determined electrochemically and by pulse radiolysis, are tabulated in Table 8.

a. Pulse Radiolysis Method

The first attempt at determining the dimerization rate constants for the neutral free radicals derived from NAD^+ and the model compound MCP^+ was made by Land and Swallow,[23] using a pulse radiolysis technique. The free radicals were

TABLE 8

Dimerization Rates of the Free Radicals Derived From Pyridine Nucleotides and Related Compounds[a]

Parent compound	Solvent[b]	Temperature °C	Method[c]	k_d[d] M^{-1} sec^{-1}	Ref.
Nicotinamide	H_2O, pH 9	30	CV-I	1.8×10^6	55
	DMSO	40	CV-I	3.5×10^4	31
N'-Methylnicotinamide	H_2O, pH 9	30	CV-I	4.9×10^6	55
MCP^+	H_2O, pH 5–9	30	CV-I	6.1×10^7	14
	pH 7	30	CV-E	2.24×10^{-2}	22
			PR	6.9×10^7	23
	AN	40	CV-I	1.0×10^6	31
NMN^+	H_2O, pH 5–9	30	CV-I	1.5×10^6	14
NAD^+	H_2O, pH 5	30	CV-I	2.2×10^6	57
	pH 7		CV-E	8.49×10^6	22
	pH 8		CCP	$>10^6$	125
	pH 9		CV-I	2.4×10^6	57
			PR	5.6×10^7	23
	DMSO	40	CV-I	9.0×10^5	31
$DNAD^+$	H_2O, pH 9	30	CV-I	1.7×10^6	57
	DMSO	40	CV-I	1.0×10^6	31
$NADP^+$	H_2O, pH 5	30	CV-I	4.3×10^6	57
	pH 7		CV-E	5.45×10^{10}	22
	pH 9	30	CV-I	1.6×10^6	57
	DMSO	40	CV-I	5×10^6	31

[a]The data for MCP^+ are included in the table, because of its frequent use as a model compound for NAD^+. Data for nicotinamide and N'-methylnicotinamide are included for comparative purposes.

[b]The pH is indicated for aqueous solutions; AN = acetonitrile; DMSO = dimethylsulfoxide.

[c]Methods used to produce the free radical and to measure the dimerization rate: CCP, cyclic chronopotentiometry; CV-I, cyclic voltammetry + peak current measurement; CV-E, cyclic voltammetry + peak potential measurement; PR, pulse radiolysis + spectrophotometric measurement.

[d]The dimerization rate constant, k_d, is presumably for the free radical anion formed on the initial 1*e* reduction in the case of nicotinamide in DMSO; in all other cases, it probably involves neutral free radicals.

generated by the reaction of NAD^+ or MCP^+ with the hydrated electron, as represented by Equation 47 (Section IV. A.). The hydrated electron was produced by the pulse radiolysis of aqueous solutions of MCP^+ or NAD^+. Land and Swallow were concerned with (a) determining the electron addition rate constant for the reaction, (b) determining the dimerization rate of the neutral free radical, and (c) identifying the dimer produced.

The dimerization of the radical was followed by monitoring the ultraviolet absorption of the dimer at 340 nm. By this method, the dimerization rate constants for the free radicals produced from MCP^+ and NAD^+ were determined as being $6.9 \times 10^7\ M^{-1}\ sec^{-1}$ and $5.6 \times 10^7\ M^{-1}\ sec^{-1}$. These results suggest the absence of any significant effect of the N(1) substituent on the dimerization rate constant; however, before such a conclusion is finally accepted, it would be desirable to have rate data for compounds with other substituents at the N(1) position of the pyridine ring. (Apparently, the dimerization rate constants for the free radicals derived from NMN^+, $NADP^+$ and deamino-NAD^+ have not been measured by pulse radiolysis.)

The electron addition rate constants were determined to be $4.1 \times 10^{10}\ M^{-1}\ sec^{-1}$ for MCP^+ and $2.5 \times 10^{10}\ M^{-1}\ sec^{-1}$ for NAD^+, i.e., electron addition is three orders of magnitude faster than the subsequent dimerization.

The identification of the dimers was based on the location of the maxima in their absorption spectra (see Section II. B. 3. a.).

b. Chronopotentiometric Method

Wilson and Epple[125] determined the half-life of the NAD neutral free radical, using cyclic chronopotentiometry. The cathodic transition, corresponding to a 1*e* reduction process, was well defined. However, the anodic transition in the chronopotentiogram was absent, suggesting loss of the radical by chemical reaction. Consequently, Wilson and Epple employed asymmetric current densities, where the cathodic and anodic current densitites differed by known factors, to obtain the reversal transition time for reoxidation of the radical. At an anodic current density, which was 0.1 of the cathodic current density, a barely detectably anodic transition time was obtained. From this, it was concluded that the half-life of the free radical is less than one millisecond; k_d can then be estimated to exceed $10^6\ M^{-1}\ sec^{-1}$.

c. Cyclic Voltammetric Methods

i. Nicotinamides

The dimerization rate constants for the free radicals derived from the initial 1*e* reduction of nicotinamide and N′-methylnicotinamide were determined by the cyclic voltammetric peak current ratio method in aqueous medium[55] and in nonaqueous media (AN and DMSO).[31] The precision of the experimental data is illustrated by Table 9; the temperature of 40° was selected as being close to biological temperature (36.8°). Some of the experiments in Table 9 were replicated; the best value for the nicotinamide free radical anion dimerization in DMSO is considered to be $3.5 \times 10^4\ M^{-1}\ sec^{-1}$.

At 30° in pH 9 aqueous solution, k_d is $(1.8 \pm 1.1) \times 10^6\ M^{-1}\ sec^{-1}$ (mean and standard deviation for 5 measurements) for nicotinamide, and $(4.9 \pm 1.2) \times 10^6\ M^{-1}\ sec^{-1}$ (6 measurements) for N′-methylnicotinamide.

ii. 1-Substituted Nicotinamides

Measurement by the cyclic voltammetric peak potential shift method of k_d for the neutral free radicals produced on reduction in aqueous solution of MCP^+, NAD^+ and $NADP^+$ gave values of 2.24×10^{-2}, 8.49×10^6 and $5.45 \times 10^{10}\ M^{-1}\ sec^{-1}$, respectively.[22]

In aqueous media, analysis of the i_{pa}/i_{pc} ratios

TABLE 9

Dimerization Rates for Nicotinamide Free Radical Anion in Nonaqueous Medium[a]

Scan rate V/sec	i_{pa}/i_{pc}	τ[b] msec	$k_d \times 10^{-4}$ $M^{-1}\ sec^{-1}$
6.25	0.35	64	4.1
6.25	0.35	64	4.1
7.50	0.37	66	3.5
7.50	0.38	66	3.5
8.33	0.40	48	3.6
10.71	0.45	37	3.5
13.63	0.47	29	3.8
15.00	0.45	33	3.9

[a]Measurements made by cyclic voltammetry in DMSO at 40°C, where the free radical exists as an anionic species. The dimerization rate constant, k_d, was calculated by Nicholson's graphical method.[126]
[b]Switching time.

for 15 measurements for τ ranging from 2 to 8 msec at 30° gave a k_d for the MCP free radical of $(6.1 \pm 1.1) \times 10^7\ M^{-1}\ sec^{-1}$ (mean and standard deviation);[14] similar determination of k_d for NMN^+ gave a value of $(1.5 \pm 0.1) \times 10^6\ M^{-1}\ sec^{-1}$ (3 measurements).[14]

Dimerization rate data for the NAD, NADP and DNAD free radicals in aqueous solution, based on the current ratio approach, are given in Tables 8 and 10.[57]

Values of k_d at 40° for the free radicals derived from the 1-substituted nicotinamides in nonaqueous media, as determined by the i_{pa}/i_{pc} ratio method (DMSO is solvent except where indicated), are $1 \times 10^6\ M^{-1}\ sec^{-1}$ for MCP^+ (AN), 9×10^5 for NAD^+, 1×10^6 for $DNAD^+$, and 5×10^6 for $NADP^+$ (see Table 8). The latter values may have to be regarded as approximate due to the large charging current contributions during fast cycling processes. The difference in magnitude between the rate constants in nonaqueous media for nicotinamide and the 1-substituted nicotinamides is in agreement with the charged and uncharged natures of the respective free radicals produced.

It is instructive to compare the values for the dimerization rate constant for the same compound as determined by different techniques, especially since the great difference between the values of k_d for the MCP free radical (Table 8) reported on the basis of potential shift measurement[22] and pulse radiolysis experiment,[23] raised the possibility that this difference might result from the influence of the electric field on the dimerization reaction, i.e., an electric field perturbation is present at the electrode at whose surface dimerization proceeds, whereas such fields are absent in the pulse radiolysis experiments.[9,10] However, the subsequently reported k_d, obtained by the current ratio method,[14] was in excellent agreement with the pulse radiolysis value.[23]

The latter agreement is of special significance since it would tend to indicate that the dimerization rate of the free radical, which is a neutral species, is not appreciably affected by the occurrence of the chemical reaction (dimerization) in the electrical double layer region. It is possible that the deviation of the earlier reported[22] electrochemically determined dimerization rate, which is stated in the original paper to be probably inaccurate due to complications caused by adsorption, resulted from an error in calculation.[14]

It is of interest to note in this connection that Puglisi and Bard[135] estimated the rate constant for dimerization of 1-benzylnicotinamide radical on the basis of rotating ring disk electrode experiments as exceeding $10^7\ M^{-1}\ sec^{-1}$.

Generally comparable values of k_d were found for the NMN^+, NAD^+, $NADP^+$ and $DNAD^+$ free radicals by the current ratio technique.[14,57] The

TABLE 10

Dimerization Rate Constants for Free Radicals Derived from Pyridine Nucleotides

Compound	pH[b]	Scan rate V/sec	Rate constant[a] at 30° k_d $M^{-1}\ sec^{-1}$	n	s $M^{-1}\ sec^{-1}$	E_a[c] kcal mol^{-1}
NAD^+	5.0	15–38	2.2×10^6	11	0.6	9
	9.0	13–26	2.4×10^6	3	2.1	
$NADP^+$	5.0	20–60	4.3×10^6	3	0.8	9
	9.0	20–60	1.6×10^6	3	2.6	
$DNAD^+$	9.0	20–36	1.7×10^6	3	2.3	

[a]Mean value of k_d is given together with standard deviation, s, for n measurements.
[b]Acetate buffer was used at pH 5 and carbonate buffer at pH 9; ionic strength was 0.5 M.
[c]Activation energies are based on Arrhenius plot of log k_d vs. T^{-1} between 7° and 50°.

value for NAD based on the potential shift approach[22] is also comparable. However, the NADP value obtained from the current ratio method is much less than that found by the potential shift method.[22]

The difference of an order of magnitude in k_d for the NAD free radical dimerization determined by pulse radiolysis and by cyclic voltammetry (current ratio method) is disturbing, expecially since the k_d values for the MCP^+ free radical determined by the two approaches agree quite well, as just indicated. Since the polarographic measurement is related to phenomena at an interface, adsorption and diffusion may affect the rate measurement. As a result of the presence of the adenine moiety, NAD^+ is strongly adsorbed as are also its dimeric and dihydropyridine reduction products. While MCP^+ is only slightly adsorbed, its dimeric and dihydropyridine reduction products are strongly and moderately adsorbed, respectively, due to their hydrophobic alkyl substituent. The relative values of k_d for NAD and MCP free radicals found in the electrochemical studies (2×10^6 and 6×10^7) are supported by the statement in another polarographic study[15,16] that NAD and NADP radicals have an appreciably longer lifetime than those of the N-alkyl models.

Since the k_d values determined by the current ratio method for $NADP^+$ and $DNAD^+$, which are also adsorbed, are comparable to k_d for NAD^+, these may also be low by an order of magnitude compared to those which would be determined by pulse radiolysis. It is also possible, of course, that k_d for the NAD free radical, determined by pulse radiolysis, is too high.

iii. Precision of Measurement

A general limitation in determining rate constants by the cyclic voltammetric peak current ratio method used by the present authors needs to be emphasized. The errors of precision in measurement of τ, the switching time, when it is in the range of 2 to 8 msec, and in the peak current ratio are 3 to 4% and 5%, respectively; this results in an overall error in k_d of ±15%.[14] There are also other factors which need consideration in the measurement of large rate constant values, e.g., adsorption, which was previously mentioned, and double layer charging effects, which would produce a capacity current linearly proportional to v. The latter factor would tend to limit the measurement of k_d by cyclic voltammetry to rates not exceeding the range of $10^7\ M^{-1}\ sec^{-1}$. Selection of the $E^\circ - E_\lambda$ may also be a source of error.

iv. Activation Energies and Frequency Factors

Activation energies, E_a, for the dimerization reactions in aqueous solution of the free radicals derived from MCP^+ and the pyridine nucleotides were calculated on the basis of the Arrhenius equation,

$$k_d = A \exp(-E_a/RT) \qquad (66)$$

by plotting log k_d vs. T^{-1} for the range of temperature from 7° to 50°. Frequency factors, A, were then calculated at a temperature of interest. The results are summarized in Table 11.

Activation energies are 4.1 kcal mol^{-1} for the MCP free radical and 3.6 kcal mol^{-1} for that of NMN^+;[14] the corresponding calculated frequency factors at 25° are 6×10^{10} and $8 \times 10^8\ M^{-1}\ sec^{-1}$, compared to that of about $10^{11}\ M^{-1}\ sec^{-1}$ expected for a reaction between neutral free radicals where there are no extensive solvation differences between reactants and products.[136] Consequently, the low experimental frequency factor for NMN^+ may indicate a large solvation difference between its free radical and the corresponding dimer, and/or the presence of small residual charges on the free radicals with the accompanying electrostatic repulsion. It is possible that the negative nature of the NMN free radical, as a result of the secondary phosphate dissociation

TABLE 11

Activation Energies and Frequency Factors Based on the Dimerization Rates for Pyridine Nucleotide Free Radicals[a]

Parent compound	k_d[b] $M^{-1}\ sec^{-1}$	E_a[c] kcal mol^{-1}	A[d] $M^{-1}\ sec^{-1}$
MCP^+	6×10^7	4.1	6.0×10^{10}
NMN^+	2×10^6	3.6	8.0×10^8
NAD^+	2×10^6	9.0	7.0×10^{12}
$NADP^+$	4×10^6	9.0	1×10^{13}

[a]Data for k_d and E_a are taken from References 14 and 57. Data for MCP^+ are included because of its importance as a model compound for NAD^+.
[b]Approximate values at 30° (see Tables 8 and 10 for more detail).
[c]Activation energies, generally calculated from Arrhenius plots for the range of 10° to 50°.
[d]Frequency factors at the temperature of interest (25° or 30°) calculated from the equation, $k_d = A \exp(-E_a/RT)$.

(pK_a of 6 to 7), is the cause (see discussion of the shift with pH of $E_{1/2}$ for NMN^+ wave I).[14]

The low activation energies of 9 kcal mol^{-1} for dimerization of the NAD and NADP free radicals are also in harmony with the rapid nature of the reaction and in general agreement with that expected for dimerization processes for neutral free radicals where electronic repulsions are not involved. The corresponding calculated frequency factors at 30° of 7×10^{12} M^{-1} sec^{-1} for NAD and 1×10^{13} for NADP are also in the range expected, as just noted.

It may be of interest to note for comparative purposes that nicotinamide and N'-methylnicotinamide in aqueous media gave activation energies (15° to 50° range) of 24 and 14 kcal mol^{-1}, respectively, from which frequency factors of 4×10^{23} and 1×10^8 M^{-1} sec^{-1}, respectively, are derived.[55] In DMSO, the nicotinamide free radical dimerization gave an activation energy (20° to 60° range) of about 5 kcal mol^{-1}.[31]

3. Dimerization Following Oxidation Reactions

Although the 1*e* oxidation of NADH (or other similarly reduced pyridine nucleotides) to the free radical NAD has not yet been observed electrochemically, e.g., Sections II.B.4. and V.C., it has been reported as resulting from chemical oxidation. Thus, the oxidation of NADH by chemical oxidizing agents such as $\dot{B}r_2^-$, produced during pulse radiolysis experiments, is assumed to proceed by the following sequence:[137]

$$\begin{array}{l} \mathrm{NADH} + \dot{\mathrm{B}}\mathrm{r}_2^- \xrightarrow{k_1} 2\,\mathrm{Br}^- + \mathrm{N\dot{A}DH}^+ \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow \\ 0.5\,(\mathrm{NAD})_2 \xleftarrow{k_2} \mathrm{N\dot{A}D} + \mathrm{H}^+ \end{array} \qquad (67)$$

The protonated $N\dot{A}DH^+$ decomposes to the neutral $N\dot{A}D$ radical, which subsequently dimerizes. The rate constant, k_2, for the dimerization is 9.0×10^8 M^{-1} sec^{-1}. The rate constants for Reaction 67 with $(\dot{C}NS)_2^-$ or $\dot{I}_2^-$ as oxidant are not significantly different: 4.7×10^8 M^{-1} sec^{-1} for $(\dot{C}NS)_2^-$ and 5×10^7 M^{-1} sec^{-1} for $\dot{I}_2^-$ In these experiments, the oxidation of NADH was followed by monitoring the decrease in the characteristic NADH absorption at 340 nm (ϵ_{max} = 6500). An intermediate produced in Reaction 67, which absorbs at 400 nm, has been identified as the $N\dot{A}D$ free radical.[137]

The instability of the $N\dot{A}D$ free radical in the presence of oxygen has been explained as due to the reaction:

$$\mathrm{N\dot{A}D} + \mathrm{O}_2 \xrightarrow{k_3} \mathrm{NAD}^+ + \mathrm{O}_2^- \qquad (68)$$

The rate constant, k_3, for Reaction 68 is 2.0×10^9 M^{-1} sec^{-1}.[137,138] NADH oxidation in the presence of oxygen gives 100% yield of NAD^+.

One-electron electrochemical oxidations of NADH model compounds [1-(2,6-dichlorobenzyl)-, 1-methyl-, 1-benzyl- and 1-(n-propyl)-1,4-dihydronicotinamides] in acetonitrile have been reported by Blaedel and Haas,[90] but the formation of $(NAD)_2$ was not observed. The products of these 1*e* oxidations have been identified to be the parent pyridinium derivatives from comparison of the cyclic voltammetric peak potentials of the latter with those of authentic samples. The original paper should be consulted for the mechanism suggested to explain these results.

C. Homogeneous (Chemical) Reaction Rates: Hydrolysis

The instability of the dihydropyridine nucleotides, RNH, in aqueous media as a result of acid-catalyzed decomposition has been reported[56] (see Section II.B.4.). The rate of decomposition of RNH can be monitored by following the decrease in limiting current (i_l) due to RNH oxidation (Figure 19). Plots of log i_l vs. time were linear, from which rate constants for the decomposition could be calculated.

The *pseudo* first order rate constants for the acid-catalyzed decomposition were estimated to be 8.2×10^{-4} sec^{-1} for NMNH, 5.9×10^{-4} sec^{-1} for NADH, and 8.5×10^{-4} sec^{-1} for NADPH at pH 4.1, and 1.0×10^{-5} sec^{-1} for NMNH, 0.8×10^{-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 indicated their normal stability in such media.

Rate data are presented in Section II.B.5. on the hydrolytic decomposition of the dimeric reduction products of MCP^+, NMN^+ and NAD^+, and of the dihydropyridine reduction product of MCP^+.

V. CORRELATION WITH THEORETICALLY CALCULATED PARAMETERS

A. Dimerization Site

1. Prediction by Theoretical Calculations

As early as 1959, Pullman and Pullman[139] predicted the sites of reactivity in the pyridine nucleotides on the basis of quantum mechanical calculations. Their approach assumed that a conjugated system will exert a certain maximum *pi* electron bonding power, and that the difference between the maximum *pi* bonding power and the actual bonding, as measured by a summation of the *pi* bond orders, could be taken as an indication of the surplus valence available to bind other groups at that position. These surplus valences, i.e., free valences, calculated for the model compound MCP^+ and the pyridine nucleotide NAD^+ using the above approach, yields the following results:

Position	MCP^+	NAD^+
2	0.658	0.658
4	0.580	0.575
5	0.394	0.394
6	0.666	0.658

Pullman and Pullman[139] postulated that reactions, which proceed through an ionic mechanism, would involve attack of the 1-substituted 3-nicotinamide at the *para* position of the pyridine ring and that reagents, which react to generate a nicotinamide free radical, would attack the *ortho* positions of the pyridine ring. They considered that, in general, the free valence index reflects the tendency to dimerization (radical attack). The greatest free valences correspond to the two *ortho* carbon atoms (positions 2 [0.658] and 6 [0.666] in the pyridine ring), which should, consequently, be preferentially attacked by free radicals.

2. Electrochemically Produced Free Radicals

The discussion of the structure of the dimer produced on the initial 1*e* reduction of the 1-substituted nicotinamides (Section II.B.3.a.) indicates that the 6,6′ dimer is produced in nonaqueous media but that, in aqueous media, the 6,6′ dimer predominates when the N-substituent is small with the 4,4′ form being increasingly formed as the N-substituent increases in size.

Negligible, if any, amounts of 2,2′ dimer are formed. The fact that the 6 position appears to be experimentally more favorable is explicable on the basis of the 2 position being less suitable for free radical attack, due to molecular crowding resulting from the amide group at position 3.

The stability of the free radicals of 1-substituted nicotinamides studied in the electrochemical investigations is strikingly different from that of the free radicals derived from 1-substituted isonicotinamides,[77,140] i.e., 1-substituted 4-carbamoylpyridines. The free radicals of 1-methylisonicotinamide[140] and 1-ethylisonicotinamide are more stable in AN than in aqueous medium, in which they rapidly dimerize,.e.g., stable esr signals have been obtained in AN. The instability of the free radical of 1-substituted nicotinamides in AN and DMSO can be correlated with the 4-position in the pyridine ring being the more reactive site; its availability, i.e., lack of substitution, would enable dimerization to occur readily. Thus, esr signals due to the free radicals of 4-substituted pyridines have been obtained[141] as a result of their stability, i.e., slowness of dimerization.

3. Chemically Produced Free Radicals

The reduction of pyridinium model compounds with $NaBH_4$ and $Na_2S_2O_4$ produces dihydropyridines without any evidence for free radical formation (dimers) (see Reference 142 and Section VI.C.1.). However, the reduction of such model compounds as 1-propyl-, 1-benzyl-, and 1-(2,6-dichlorobenzyl) pyridinium ions by Cr(II) does produce 6,6′ dimers (see Reference 74 and Section VI.C.2.).

B. Solvation Energy

At least two approaches have been used to estimate the solvation energy of an ion or molecule of the pyridine or pyridinium type: the Born approximation and molecular orbital (MO) calculations.

1. Born Relation

The free energy of solvation can be estimated from the Born relation,

$$-\Delta G_{solv} = N e (1 - D^{-1})/2 r \qquad (69)$$

where N is Avogadro's number, D is the dielectric constant of the solvent, and r is the radius of the

molecule. This approach or model assumes the molecule to be spherically symmetric in going from the gaseous phase to solution. From the free energy of solution, it has been possible to calculate the number of water molecules associated with an ion. This approach has been used by Kosower,[143] who estimated the solvation energy of pyridinium ion and its salts on the assumption that pyridinium ion has one half to one third of the free energy of solution of sodium ion (60 kcal mol^{-1}), based on not more than two water molecules being associated with the pyridinium ion. The solvation energy of 1-methylpyridinium iodide (MCP^+ I^-) was estimated to be 20 $kcal/mole^{-1}$.

2. MO Calculations

The half-wave potentials of several aromatic hydrocarbons and nitrogen-containing heterocyclic compounds have been successfully correlated with calculations of the energy levels of the molecules, as in Reference 144.

The relationship between $E_{1/2}$ for a reduction process,

$$R + e \rightleftharpoons R^- \quad (70)$$

and the electron affinity of the molecule (energy of the lowest unoccupied level) is expressed as

$$E_{1/2} = EA + \Delta E_{solv} - \gamma \quad (71)$$

where EA represents the electron affinity of the molecule, ΔE_{solv} is the difference in solvation energy of R^- and neutral molecule R, and γ is the appropriate correction term for the reference electrode used. For similarly structured molecules, ΔE_{solv} is usually assumed to be constant and, hence, a plot of $E_{1/2}$ vs. EA should give a straight line. As it is usually problematical to obtain reliable values of EA without making assumptions concerning the MO parameters, several authors have plotted the coefficients of the lowest unoccupied or empty molecular orbital level (LEMO) against the $E_{1/2}$. The two approaches are obviously related to one another.

The MO calculations for the nicotinamide series have been done by the Hückel method, using standard parameters;[145] for comparison, calculations were also made for a pyridine series. The half-wave potentials, MO data, and solvation energy differences are listed in Table 7. Introduction of an amide group, $-CONH_2$, in the 3-position of the pyridine ring reduces $E_{1/2}$ by 0.8 V due to increased contribution from the *pi* electrons of the amide group. Addition of the methyl group, $-CH_3$, at the 1-position of either pyridine or nicotinamide greatly facilitates reduction due to increased conjugation from the methyl group and the electropositive character of the nitrogen attracting the electrons towards the ring. Substitution of the methyl group elsewhere on the ring makes the reduction slightly more difficult than in the parent molecule.

The calculated differences in solvation energies for the two types of processes

$$R + e \rightleftharpoons R \quad (72)$$

and

$$R^+ + e \rightleftharpoons R \quad (73)$$

where R and R^+ represent the parent molecule, and R^- and $\dot{R}$ are negatively charged and neutral free radicals, given in Table 7, indicate that each type of process has a fairly characteristic value. The higher difference for the cationic species may result from the positively charged N(1) effectively ordering the solvent dipoles.

Since the ΔE_{solv} values are dependent on the value of β employed in the calculation of electron affinity (in the present calculations, the β value was taken as 2.0), it would be unreliable to place confidence in the absolute values of ΔE_{solv}. However, within a given series, one observes that ΔE_{solv} values are, as mentioned, nearly constant. Thus, pyridine, 2-methylpyridine, and 4-methylpyridine, which undergo electrochemical reduction through Reaction 72, have fairly constant ΔE_{solv} values as calculated from the measured $E_{1/2}$. Similarly, molecules with a substituent on the ring nitrogen (1-methylpyridine, 1-protonated-3-nicotinamide, 1-methyl-3-nicotinamide, NMN^+, NAD^+ and $NADP^+$), whose measured $E_{1/2}$ correspond to Reaction 73, also show a fair degree of constancy in the ΔE_{solv} values. More sophisticated MO approaches such as the extended Hückel theory (EHT) or self-consistent field (SCF) approaches in calculating EA should provide more reliable estimates of solvation energy. Song[146] has studied the flavin systems for intramolecular complexation, using semiempirical SCF procedures.

C. Oxidation of Dihydronicotinamides

In view of the two successive 1*e* reduction steps in the formation of NADH from NAD^+ under electrochemical conditions, it is worthwhile considering possible reasons for the failure to observe a symmetrical series of 1*e* oxidation steps for the electrochemical oxidation of NADH.

Postulation of a 1*e* oxidation scheme for dihydronicotinamides requires that the species $\dot{R}NH^+$ is oxidized at more positive potential than RNH, since further oxidation of $\dot{R}NH^+$ by $\dot{B}r_2^-$ does not occur (see Equation 67). Instead, $\dot{R}NH^+$ decomposes to yield the neutral radical, $\dot{R}N$, which subsequently dimerizes. The oxidation of $\dot{R}N$ to RN^+ does not seem to proceed under chemical oxidizing conditions in spite of the large difference in apparent formal potential of the two systems involved, i.e., E_c° of about -0.92 V for the $RN^+/\dot{R}N$ couple and exceeding 0.90 V for the $\dot{B}r_2^-/Br^-$ couple; the latter value is based on the fact that $\dot{B}r_2^-$ oxidizes RNH, whose oxidation occurs at 0.90 V. The absence of oxidation of $\dot{R}N$ may mean that the dimerization is faster than the chemical oxidation or that the activation energy for the chemical oxidation is very high.

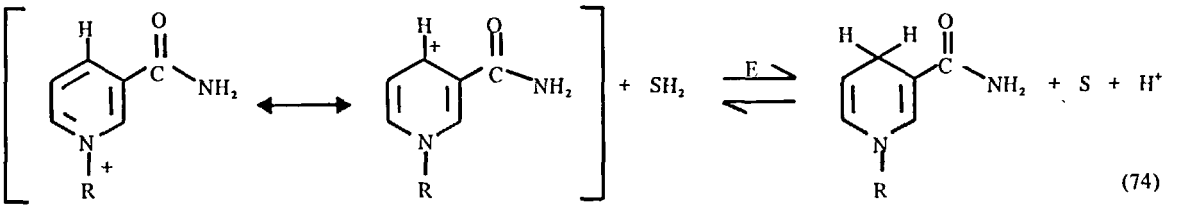
The 2*e* oxidation scheme is generally considered to involve the successive removal of electrons from the dihydronicotinamides with the assumption that $\dot{R}NH^+$ is easier to oxidize than RNH. Deprotonation of an oxidized product is believed to occur at some stage of the oxidation, i.e., after removal of either the first or second electron (see Reference 56 for a discussion of this topic). Based on analysis of cyclic voltammetric and chronoamperometric data, Braun et al.[56] conclude that deprotonation probably occurs after both electrons are removed. The protons liberated in this process protonate RNH, giving decomposition products which absorb in the 280 to 290 nm region.

It is apparent that the crucial factor in the preceding discussion is the ease of oxidizability of $\dot{R}NH^+$. Quantum chemical calculations[111] show that it should be easier to oxidize $\dot{R}NH^+$ than RNH. The explanation for this behavior may be as follows: Each of the *pi* bonding orbitals are doubly filled for RNH, which, on losing one electron, gives $\dot{R}NH^+$. Removal of the other electron from the depleted orbital should be easier than removal of the first electron since electron-electron attraction is removed in the first stage of oxidation. The magnitude of the difference in oxidizability would depend, however, on the molecular structure.

VI. MECHANISMS OF BIOLOGICAL OXIDATION-REDUCTION REACTIONS

The widespread involvement of the pyridine nucleotide coenzymes in biological redox reactions is well recognized. In combination with specific enzymes, the pyridine nucleotides catalyze virtually all degradative and biosynthetic processes requiring oxidation-reduction steps.

The function of the coenzyme NAD^+ in cellular metabolism, for example, is to act as the primary or initial oxidizing agent, which accepts electrons and hydrogen from metabolic intermediates. This biological oxidation of foodstuffs is catalyzed by a large number of different enzymes, appropriately called dehydrogenases.[147-149] A dehydrogenase catalyzed reaction is reversible and involves the net transfer of a hydride ion from substrate to C(4) of the pyridine ring and the release of a proton to the medium,



(74)

where S represents the oxidized substrate and SH_2 the reduced substrate. A specific example is the alcohol-dehydrogenase (ADH) catalyzed reversible oxidation of ethyl alcohol to acetaldehyde:

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+ \quad (75)$$

It has been generally recognized that the

coenzyme NAD^+ seems to be involved mainly in degradative (oxidative) biological pathways, whereas the coenzyme $NADP^+$ is involved mainly in biosynthetic pathways.[150,151]

Subsequent discussion of the role of the pyridine nucleotide coenzymes in biological redox reactions has been divided into four parts. The first part deals with the gross function of the pyridine nucleotides in intermediary metabolism. The second is concerned with the function of pyridine nucleotides at the molecular level, e.g., in regard to the chemistry and stereochemistry of the enzyme active site. Then, consideration is given to pyridine nucleotide chemical reactions and model chemical systems. Finally, the significance of electrochemical investigations in regard to the understanding of the biological redox mechanism is discussed.

A. Function of Pyridine Nucleotides in Intermediary Metabolism

The importance of the role, which the pyridine nucleotides play in biological redox processes, has been realized mainly through the elucidation of the detailed steps in intermediary metabolism. Thus, it is worthwhile to discuss briefly this subject; however, due to the complexity of the field, only certain points, important for the subsequent discussion, will be considered and the reader may wish to consult a more complete introductory discussion for additional information.[147,148]

The chief energy sources for most living organisms are carbohydrates, fats and proteins. In an animal cell, oxidation of these substances occurs by their passage through an integrated metabolic system which allows much of the released chemical energy to be ultimately trapped in a single energy-rich substance, adenosine triphosphate (ATP). ATP is then utilized by the cell as a general energy source for driving biosynthetic reactions and for the performance of other forms of work.

As an example of the utilization of a foodstuff by an aerobic cell, the metabolism of glucose will be considered. The direct combustion of a glucose by molecular oxygen has a high activation energy. One function of the cell is to provide suitable catalytic enzyme and coenzyme systems, which lower the activation energy for the reaction and, thus, allow it to take place rapidly.

The first phase of glucose metabolism (glycolysis) is thought to occur in the cell cytoplasm and involves formation of two moles of pyruvic acid, $CH_3COCOOH$, per mole of glucose; during the reaction sequence two moles of NAD^+ are converted to two moles of NADH. This breakdown of glucose requires a total of ten different, specific enzyme molecules, acting in sequence and in such a manner that the product of the first enzyme-catalyzed reaction becomes the substrate or reactant of the next. Thus, there are ten recognizable chemical reactions or stages in the overall breakdown of glucose to pyruvic acid.

In the second phase of aerobic energy production from glucose, the preliminary degradation product, pyruvic acid, is converted to acetyl-coenzyme A, which, in turn, is catalytically oxidized by molecular oxygen in a process called respiration. The enzyme systems, which catalyze respiration and the conservation of respiratory energy as ATP, are those of the Krebs citric acid cycle and of the electron transport chain. These enzyme systems are complex and do not occur singly in the free form in the soluble portion of the cell cytoplasm, but are fixed in geometrically specific arrays in mitochondria, which are subcellular structures often called the power plants of the cell.

The chemical reactions of the Krebs cycle and the electron transport chain allow for the complete oxidation of the acetic acid portion of acetyl-coenzyme A to CO_2 and water. In the Krebs cycle, four dehydrogenation steps occur, in which four pairs of electrons are enzymatically extracted from various intermediates of the cycle. Three pairs are accepted by three molecules of NAD^+ and the fourth pair is accepted by the flavoprotein, succinic dehydrogenase, which contains flavin adenine dinucleotide (FAD) as the electron carrier. These intermediate reduction products are reoxidized by donating their electrons to appropriate redox systems of the electron transport chain. Electrons entering the latter chain from NADH and $FADH_2$ travel through a series of redox couples of increasing positive potential until finally oxygen is reduced to water. Hydrogen ions, that are released to the medium through oxidation of NADH and $FADH_2$ and of intermediates in the Krebs cycle, are consumed in the final step of electron transport involving water formation.

The oxidation sequence and redox couples of the electron transport chain are reasonably well

understood, but the way in which the conversion of ADP to ATP is coupled to the sequence has not yet been satisfactorily elucidated. It is thought that the chain consists of eleven oxidation-reduction components arranged in four discrete enzyme complexes, three of which participate in the formation of ATP. A qualitative idea of the sequence of the various oxidation-reduction components[152] is shown in Figure 26, where f_D is the flavoprotein NADH dehydrogenase, f_s is the flavoprotein succinic dehydrogenase, a, a_3, b, c, and c_1 are five cytochromes, Fe is non-heme iron, CoQ is ubiquinone, and Cu is copper. In the sequence of reactions there shown, electrons travel from left to right and enter the chain of cytochromes through one or the other of the two flavoproteins. The cytochromes carry only one electron at a time, whereas NADH and the flavoproteins carry two at a time. It is thought that flavoprotein accepts 2 electrons from NADH, but, through the assumption of a semiquinone configuration, donates them in sequence as single electrons.

The magnitude of the free energy change as a pair of electrons moves the entire length of the chain, from NADH to oxygen, in the net reaction,

$$NADH + 0.5\ O_2 + H^+ \rightleftharpoons NAD^+ + H_2O \qquad (76)$$

is $-52{,}000$ cal mol^{-1}. In the electron transport chain, this overall free energy drop occurs in segments, three of which are relatively energetic and are coupled to ATP formation requiring +7000 cal mol^{-1}. Thus, for each mole of NADH formed in the initial stages of glucose metabolism (e.g., during glycolysis and Krebs cycle degradation), subsequent oxidation to NAD^+ by the electron transport chain yields three moles of ATP. Each mole of reduced succinic dehydrogenase ($FADH_2$) oxidized in the electron transport chain results in the formation of 2 moles of ATP.

Overall, the complete oxidation of glucose by an aerobic cell results in the formation of 38 moles of ATP per mole of glucose metabolized. Since the formation of ATP from ADP and inorganic phosphate requires a minimum input of +7000 cal mole^{-1}, the total free energy conserved corresponds to about 42% of the free energy available upon complete oxidation of glucose to carbon dioxide and water ($-686{,}000$ cal mole^{-1}).

In an anaerobic cell such as yeast, the situation is quite different. Because the Krebs cycle and the electron transport chain are not operative, pyruvic acid, the key compound produced in glycolysis, is not oxidized to acetyl-coenzyme A as in an aerobic cell, but, rather, is decarboxylated to acetaldehyde and carbon dioxide. In the final reaction of alcoholic fermentation, for example, acetaldehyde is reduced to ethanol by NADH in the presence of the enzyme, alcohol dehydrogenase. The fermentation of glucose by yeast results in the net production of only 2 moles of ATP per mole of glucose, which is obviously considerably less than that produced in an aerobic cell.

In both aerobic and anaerobic cells, NAD^+ is the primary oxidizing agent which accepts electrons from metabolic intermediates. However, since the amount of NAD^+ in any cell is limited, the reaction would soon cease if it were not for the existence of a mechanism for the reoxidation of the reduced pyridine nucleotide. In an aerobic cell, this is accomplished when NADH is reoxidized in the first step of the electron transport chain. In an anaerobic cell such as yeast, the reoxidation is accomplished when acetaldehyde is reduced to alcohol in the presence of alcohol dehydrogenase.

B. Function of Pyridine Nucleotides at the Molecular Level

Although a great deal is known about the gross

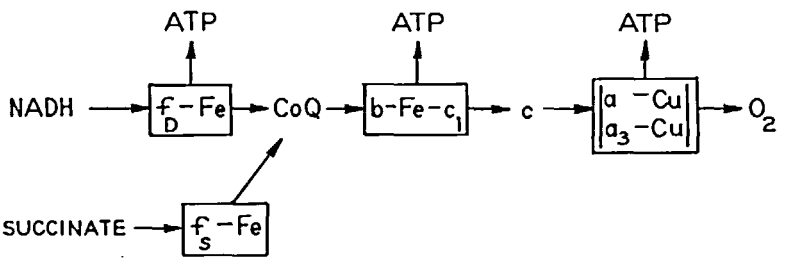


FIGURE 26. Essential sequence of redox components and steps in the electron transport chain. (Adapted from Green, D. E., *Compr. Biochem.*, 14, 309 (1966).

involvement of pyridine nucleotides in biological redox reactions, e.g., in intermediary metabolism, much less is understood regarding the detailed manner in which the coenzymes interact with dehydrogenases at the molecular level. Indeed, for any enzyme, there is not as yet a quantitative understanding of the mechanism and driving forces responsible for the enormous specific rate accelerations which are brought about by these substances.[153] Nevertheless, in the last 25 years considerable progress has been made in understanding the catalytic nature of pyridine nucleotide-dependent dehydrogenases, as reported, for example, in References 11, 72, 104, 149 and 154-159.

1. Stereospecificity of Hydrogen Transfer

A typical example of the general enzymatic hydrogen transfer reaction (Equation 74) is the yeast alcohol dehydrogenase (YADH) catalyzed reversible oxidation of ethanol to acetaldehyde (Equation 75). In this reaction, the transfer of hydrogen occurs in a stereospecific manner in both the forward and reverse directions. When NAD^+ is enzymatically reduced in D_2O (Equation 77), the reduced coenzyme does not contain deuterium.[1,160]

$$CH_3CH_2OD + NAD^+ \rightleftharpoons CH_3CHO + NADH + D^+ \quad (77)$$

$$CH_3CD_2OH + NAD^+ \rightleftharpoons CH_3CDO + NADD + H^+ \quad (78)$$

However, when NAD^+ is enzymatically reduced in ordinary water with 1,1-dideuteroethanol as substrate (Equation 78), the reduced coenzyme isolated from the reaction mixture contains one mole of deuterium per mole of reduced nucleotide, showing that hydrogen is directly transferred from the α-carbon of ethanol and that it does not exchange with the protons of the solvent medium. This result has given rise to the concept of "direct hydrogen transfer."[1,160]

A direct hydrogen transfer does not, however, necessarily rule out the occurrence of an intermediate reaction; a functional group on the enzyme itself, such as an amino acid side chain, could serve as a possible intermediate in the overall net transfer of hydrogen. The concept is recognized to mean only that there is no proton exchange with the solvent medium.[156]

That enzymatic reduction of coenzyme occurs at the C(4) *para* position rather than one of the *ortho* positions has been conclusively proven through various direct and indirect deuterium labeling experiments.[2,161] In addition, it has been shown that the two C(4) hydrogens of the reduced coenzyme are not equivalent. In the YADH-catalyzed oxidation of 1,1-dideuteroethanol, deuterium is transferred stereospecifically to only one side of the coenzyme pyridine ring, and, in the reverse reaction, the deuterium is removed from the same side of the ring.[1,160] Thus, the two sides of the pyridine ring of reduced coenzyme are distinguished from one another as side A and side B, with side A being defined as that side to and from which hydrogen is transferred by yeast alcohol dehydrogenase.[162] Some pyridine-nucleotide dependent dehydrogenases act on side A while others act on side B, but all are stereospecific for one side or the other. The absolute configuration with respect to A and B stereospecificity has been determined experimentally:[163] "When an enzyme of class A transfers hydrogen from a substrate to a pyridine nucleotide, the hydrogen is added to that side of the nicotinamide ring on which the ring atoms 1 to 6 appear in an anticlockwise order."

2. Chemistry of the Enzyme Active Site

The occurrence of direct stereospecific hydrogen transfer is the result of asymmetric binding of the coenzyme to the active site of the enzyme and suggests the formation of a highly structured ternary complex where substrate and coenzyme lie in close juxtaposition. The chemistry involved in these interactions has been extensively investigated with the hope that, eventually, such studies will allow elucidation of the mechanism of enzyme catalysis. A good example of this effort are the numerous investigations of the enzyme horse liver alcohol dehydrogenase (LADH).[149,154,154a,155]

Horse liver alcohol dehydrogenase exists in the form of isoenzymes with the ethanol-active component being predominant; the latter isoenzyme has a molecular weight of about 80,000 and occurs as a dimeric structure involving two identical polypeptide chains; the subunits are not enzymatically active in the monomeric state. Sequence studies show that the primary structure of each

chain contains 374 amino acid residues corresponding to a molecular weight of 39,847.[164-166]

X-Ray crystallographic investigation of LADH allows a three-dimensional view of this enzyme (secondary and tertiary structure) which is consistent with the primary structure work.[166a] Electron density distribution at 2.4 and 2.9 Å resolution shows LADH to be a dimeric molecule containing two identical subunits.[166b,166c] Each subunit is further divided into two parts separated by a deep active site cleft. The position of each of two zinc atoms per subunit is also established with one (the catalytic zinc atom) being bound at the bottom of the cleft by three protein ligands (two sulfur atoms from separate cysteine residues and one nitrogen atom from a histidine residue); depending on pH, a water molecule or hydroxyl ion is also bound to the catalytic zinc atom. The second zinc atom is liganded in a distorted tetrahedral arrangement by four sulfur atoms from cysteine residues and is thought to be essential for structural stability of the enzyme.[166a]

The overall chemistry of the LADH active site is only beginning to be understood. Dissociation constants for the binary complexes, enzyme-NAD^+ and enzyme-NADH, have been determined by both physical and kinetic methods;[149] for LADH and most other dehydrogenases, NADH binds more strongly than does NAD^+. The kinetic studies indicate a binding order in which free enzyme first combines with coenzyme and then with substrate.[167,168] Crystallographic evidence is consistent, suggesting that the binding of coenzyme to enzyme causes a conformational change in the LADH molecule whereby the actual binding site for alcohol is formed.[166a]

The binding of NADH to LADH shifts the 340-nm coenzyme absorption maximum to shorter wavelength[149] and also shifts NADH fluorescence toward shorter wavelength and increases its intensity.[72] Complexes of NAD^+ with anions such as cyanide (see subsequent discussion) are more stable with enzyme-bound NAD^+ than with the free coenzyme.[72,169] It is reported[149] that even the oxidation-reduction potential of the NAD^+-NADH couple is altered on binding to enzyme, e.g., in the case of LADH, the potential of the enzyme-coenzyme complex between pH 6 and 8 is 60 to 80 mV more positive than that of the free coenzyme system (see subsequent discussion). This is important due to the fact that rough calculations show the concentration of coenzyme binding sites within a cell to be of the same order of magnitude as the concentration of pyridine nucleotide;[72] under these conditions, most coenzyme would be bound. Such studies suggest that the binding of coenzyme to enzyme causes a change in the reactivity of coenzyme as well as creating favorable steric conditions for the enzyme reaction.[72]

The LADH X-ray crystallographic work, as mentioned, implicates a zinc atom at the active site, very close to the nicotinamide part of the coenzyme, e.g., it is thought that the binding of coenzyme and substrate to enzyme occurs in such a manner that the nicotinamide moiety and the alcohol group converge towards the region of the active site zinc atom; kinetic measurements support the hypothesis that substrate (alcohol) binds directly to the catalytic zinc atom with the latter acting as a Lewis catalyst.[149,166a,169a,169b] An assumed position for the coenzyme nicotinamide moiety in LADH places the C(4) carbon about 4.5 Å from the catalytic zinc atom with the A side facing zinc.[166a]

Inhibition by numerous organic and inorganic complex-forming agents supports the involvement of the enzyme-bound catalytic zinc atom in the reaction catalyzed by LADH;[149] 1,10-phenanthroline (o-phenanthroline), which binds to this zinc,[166a,170] is known to be an inhibitor of dehydrogenases, competitive with the coenzymes.[171] Based on X-ray studies,[166a] one of the sulfur (cysteine) ligands to the catalytic zinc atom can be selectively carboxymethylated with iodoacetate resulting in an inhibition of the enzyme; the enzyme is protected, however, by the presence of NADH.[172]

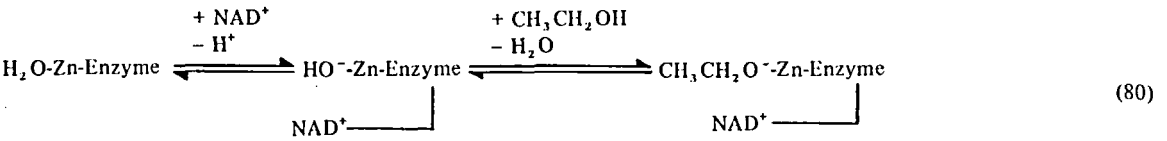
3. *Theoretical Models of the Enzyme Active Site*

Based on the results of crystallographic studies, kinetic studies, and primary structure work, Bränden and co-workers[166a,166b] have suggested a theoretical model for the active site of LADH. The proposed mechanism for the catalytic oxidation of alcohols by LADH is based on electrophilic catalysis mediated by the active site zinc atom; other investigators have also implicated zinc.[149,173,173a,173b,173c] The overall reaction for the oxidation of ethyl alcohol is given by Equation 79 where H_2O-Zn-Enzyme

(79)

refers to LADH thus emphasizing the importance of the hydrated active site zinc atom in the detailed mechanism.

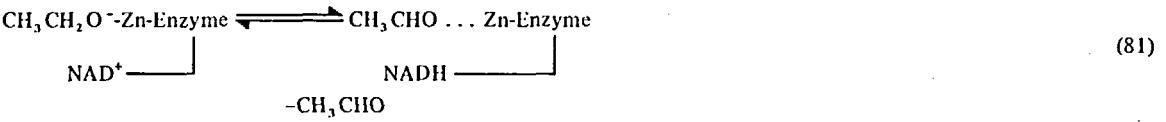
The first steps of the proposed[166a] multistep mechanism are given in Equation 80. Initially, NAD^+ binds rapidly to enzyme, but this is



(80)

followed by a slow isomerization step which induces a conformational change in the LADH molecule, thus altering some of the properties of the active site such as proton loss due to a lowering of the pKa of the zinc bound water molecule. It is thought that these changes cause a mutual stabilization in the binding of NAD^+ and alcohol. The binding of alcohol to zinc as a negatively charged alcoholate ion displaces the zinc bound hydroxyl ion, which forms a molecule of water by combining with the proton of the hydroxyl group of the alcohol.

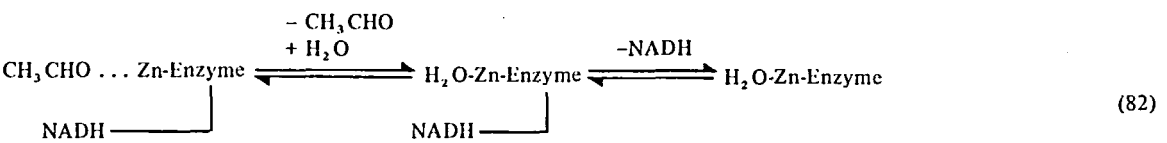
Equation 81 corresponds to the actual hydride transfer step where a hydrogen from the α-carbon atom of the alcoholate ion is transferred to



(81)

the C(4) carbon of the NAD^+ nicotinamide moiety. The intermediate stages of this reaction have not yet been elucidated (see subsequent discussion).

Equation 82 corresponds to loss of acetaldehyde and addition of water to the enzyme complex followed by slow isomerization and rapid loss of NADH.



(82)

The similarity between this proposed path for the alcohol dehydrogenase reaction[166a] and that of organic reactions involving the Meerwein-Pondorff-Oppenauer equilibrium is striking.[173b, 173d]

Based on crystallographic and kinetic

results,[166a] other mechanisms involving direct participation of cysteine[173] or reduction of enzyme[174-178] in the hydride transfer step are highly unlikely.

C. Pyridine Nucleotide Chemical Reactions and Model Chemical Systems

The conclusions drawn from studies of pyridine nucleotide chemical reactions and model chemical systems (enzyme absent) have often been used to support or reject various theoretical models of the biological redox reaction. The purpose of studying a model chemical system for an enzymatic reaction would be to try to characterize the reaction under isolated conditions so as to obtain clues regarding medium effects, and the nature and role of various functional groups present at the active site, as well as a description of the reaction transition state.[104] One question which is generally asked in the study of model dehydrogenase chemical systems is whether the electron transfer reaction per se occurs by a hydride ion mechanism (simultaneous transfer of a proton and two electrons) or by a free radical mechanism (two successive one-electron transfers with a free radical as an intermediate); *a priori*, both reaction mechanisms are possible for the biological reaction.

1. Hydride Ion Transfer

Support for a hydride ion mechanism is provided by the fact that the only chemical reducing agents capable of converting NAD^+ to enzymatically active NADH are dithionite and borohydride; reduction by both is thought to proceed by a polar reaction path and to involve hydride ion transfer.[2,179,180] The dithionate reduction yields entirely enzymatically active 1,4-NADH, but the product of borohydride reduction is only partially active, i.e., 1,2- and 1,6-NADH are also formed. The reduction of NAD^+ model compounds by these reducing agents occurs in a similar manner.[85,181]

Consistent with a hydride ion transfer is the fact that the 4-position of NAD^+ and various analogs is also subject to general attack by nucleophiles such as cyanide and enol anions. The cyanide addition reaction is a reversible localized interaction; transfer of a hydride ion from substrate to the 4-position of the coenzyme in an enzymatic process would be an analogous reaction. The ultraviolet absorption spectrum of the NAD-cyanide addition compound resembles that of 1,4-NADH in having a long wavelength absorption band at 327 nm rather than at 340 nm (see Figure 13A).

A model reaction, in which a simple transfer of a hydride ion may occur, is the reduction of thiobenzophenone (a thioketone) by dihydropyridines such as 1-benzyl-1,4-dihydronicotinamide and NADH[104,182,183] (Figure 27). The reaction occurs in the cold and proceeds more rapidly in polar than in nonpolar solvents; it is not sensitive to pH. The process involves direct hydrogen transfer with a deuterium isotope effect (rate

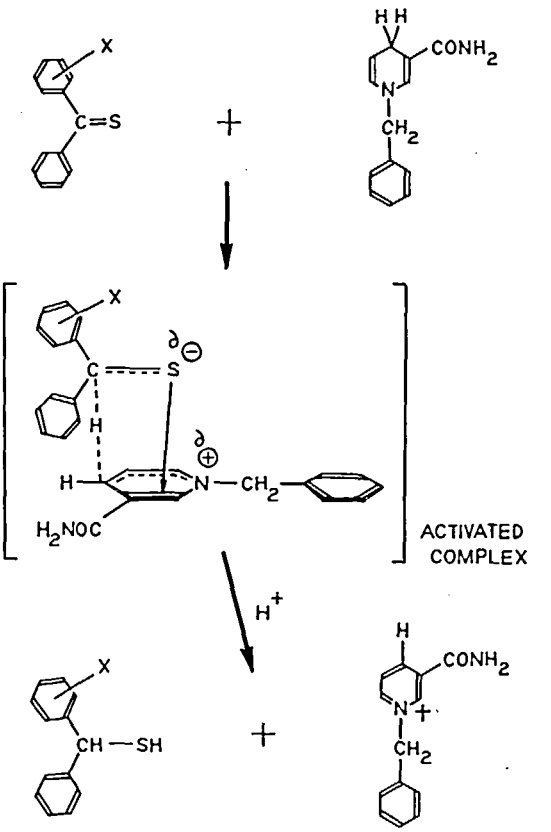
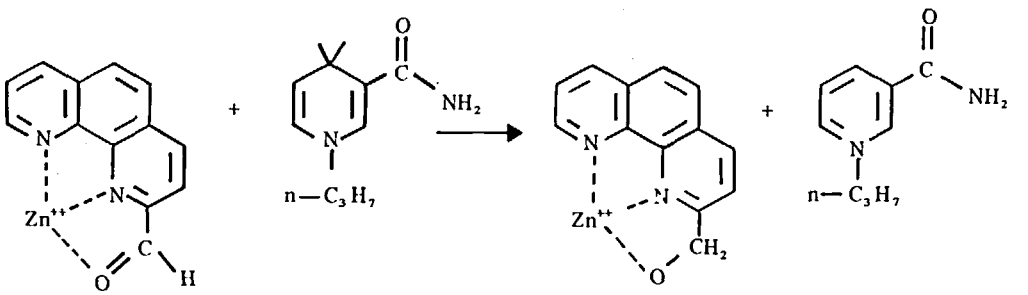


FIGURE 27. Proposed model reaction for simple transfer of a hydride ion: reduction of a thioketone by a dihydropyridine (Adapted from Kosower, E. M., *Molecular Biochemistry*, p. 166. Copyright 1962, McGraw-Hill. With permission.)

factor: k_H/k_D) of about 4. Free-radical chain inhibitors do not affect the reaction rate. A free hydride ion is not implied as an intermediate, but an activated complex with polar properties is thought to be formed; charge separation in the activated complex must be compensated internally, possibly by a charge transfer interaction between the benzhydryl mercaptide and the developing pyridinium ring.[104] Since electron-donating substituents slow the reaction whereas electron-withdrawing substituents accelerate it, the partial positive charge on the carbon in the thioketone group determines the rate of hydride ion transfer. In the case of an unactivated ketone or aldehyde (e.g., 3-octanone or acetaldehyde), the partial positive charge on the carbonyl carbon is apparently insufficient for reaction with a 1,4-dihydropyridine in the absence of enzyme; thus, the protein is essential for converting a poor chemical reaction into an effective enzymatic one, e.g., by inducing the necessary electron shift in the carbonyl group.[156,159,184] Such an electron shift is the basis for the enzymatic mechanism proposed by Bränden and coworkers,[166a,166b] in which the carbonyl group of an aldehyde is polarized by binding to the active site zinc atom (see Section VI.B.3.).

The example of a nonenzymatic reduction of an aldehyde by a dihydronicotinamide has been reported.[173c] In this model system, the zinc ion catalysis of the reaction between 1,10-phenanthroline-2-carboxaldehyde and N-propyl-1,4-dihydronicotinamide strongly suggests that either coordination or proximity to a metal ion is a feasible and efficient method for activation of a carbonyl group for reduction; no reaction is detected in the absence of the metal ion. Formation of the product, 1,10-phenanthroline-2-carbinol, proceeds by direct hydrogen transfer. The following equation is suggested for the reaction:



(82a)

2. Free Radical Transfer

Chemical evidence for the free radical reduction of pyridinium ions and free radical oxidation of dihydropyridines also appears in the literature.[72,157,158] A variety of chemical reducing reagents are capable of converting NAD^+ and model compounds such as MCP^+ to their corresponding free radicals, which then dimerize. Both the COO^- radical and hydrated electrons produced by pulse radiolysis are very potent in this regard. The $1e$ reduction of 1-alkyl nicotinamides with Zn/Cu, Mg or Cr(II) also results in dimeric products[74] which have physical and chemical properties close to those of the corresponding dimers produced by electrolysis.[14] There are very few, if any, studies reported where oxidized coenzymes or related model pyridinium ions are reduced chemically to dihydropyridines through free radical mechanisms.

The $1e$ oxidation of NADH has been carried out via chemical oxidizing agents such as $\dot{Br}_2^-$, $(\dot{CNS})_2^-$ and $\dot{I}_2^-$, which were generated by pulse radiolysis[137] (see Section IV. B. 3.). In these experiments, a neutral NAD radical was formed which subsequently dimerized; in the presence of oxygen, the neutral radical is further oxidized to NAD^+ with 100% yield.[137,138] The reactions involved are as follows:

$$\dot{Br}_2^- + NADH \rightleftharpoons 2\,Br^- + H^+ + NA\dot{D} \quad (83)$$

$$NA\dot{D} \rightleftharpoons 0.5\,(NAD)_2 \quad (84)$$

$$NA\dot{D} + O_2 \rightleftharpoons NAD^+ + O_2^- \quad (85)$$

NADH is also oxidized to NAD^+ by free radical reagents such as spirocyclohexylporphyrexide and prophyrindine as well as other molecules of suitable redox potential, which are capable of functioning as $1e$ acceptors such as ferricyanide; the porphyrexide analogue converts NADH to

NAD^+ almost quantitatively without extensive side reactions.[185] In contrast, NADH is not readily oxidized by certain reagents considered to be obligate 2-electron acceptors, e.g., o-iodosobenzoate, even in situations where these substances are considered to be powerful oxidizers. One conclusion from this study[185] was that the free radical oxidation of NADH may be important in relation to the functioning of the electron transport chain within an aerobic cell.

Other chemical evidence, which seems to implicate a free radical oxidation pathway, is the ready oxidation of 1-benzyl-1,4-dihydronicotinamide by diphenylpicryl hydrazyl derivatives.[181]

D. Significance of Electrochemical Studies

Electrochemical investigations of the pyridine coenzymes may be viewed as an extension of the study of coenzyme chemical reactions and model chemical systems, but with some important differences. Unlike the chemical studies where highly activated substrates or coenzyme derivatives are normally required for a net reaction, electrochemical studies utilize either the unaltered coenzymes or very closely related model compounds. A reaction is achieved in electrochemical work by making the potential at the solution-electrode interface sufficiently negative or positive (with respect to a given reference electrode) until, energetically, it is possible for a net reduction or oxidation to occur. Thus, one very important and immediate piece of information resulting from an electrochemical investigation, which may not be as easily obtained from chemical studies, is a better quantitative understanding of the energy requirements necessary for a net reaction to occur.

Electrochemical studies are also important from the point of view of being able to separate or differentiate the various steps of a multistep reaction mechanism, e.g., through the detection of radical intermediates and through the occurrence of non-ideal behavior such as irreversibility (see subsequent discussion). In regard to the NAD^+-NADH coenzyme system, the results of such studies allow speculation on the nature of the biological redox reaction.

1. Correlation of Results

The electrochemical results, previously discussed, show that the NAD^+-NADH system exhibits four distinct voltammetric steps at approximately the following half-wave potentials vs. sce (potentials depend on experimental conditions, e.g., the nature of the supporting electrolyte, solution pH and electrode type, as discussed in the references previously cited):

1. NAD^+ reduced to dihydropyridine, NADH: -1.6 to -1.8 V
2. NAD^+ reversibly reduced to free radical, $\dot{N}AD$, which further reacts to form dimer, $(NAD)_2$: -0.9 to -1.1 V
3. $(NAD)_2$ oxidized to NAD^+: -0.2 to -0.3 V
4. NADH oxidized to NAD^+: +0.4 to +0.8 V

The voltammetric waves in Figure 28 show approximately where each of the four processes occur on the potential scale; also shown is the formal potential, E_c°, for the NAD^+-NADH system, which falls about midway between the voltammetric reduction and oxidation steps.

At pH 7 and 25°, the formal potential for this couple is -0.315 V vs. nhe[186] (-0.557 V vs. sce). This value was calculated from thermal data and the equilibrium constants for the alcohol dehydrogenase catalyzed ethanol-acetaldehyde and 2-propanol-acetone reactions[186,187] and is in fair agreement with a potentiometric determination (-0.318 V vs. nhe at pH 7 and 30°),[3] in which milk xanthine oxidase and benzyl viologen were used, respectively, as catalyst and mediator; the slope of the E_c°-pH curve at 30° (-0.0303 V per pH unit) closely approaches the theoretical slope (-0.03007 V per pH unit) over the pH range of 6.5 to 10.5.[4,186] The potentiometric value of -0.318 V at pH 7.0 and 30° may be corrected by use of the temperature coefficient to -0.311 V at pH 7.0 and 25°[4,186] (see Reference 186 for an informative discussion of the E_c° value for the NAD^+-NADH couple and related systems).

Both wave I and wave II $E_{1/2}$ values for the electrochemical NAD^+ reduction are more negative than E_c° for the NAD^+-NADH couple (Figure 28). Such deviation of $E_{1/2}$ from E_c° is normally expressed as an overpotential, which represents the extra energy required to cause an electrochemical reaction to proceed at a reasonable rate; overpotential is a measure of departure from reversibility. This argument is not applicable, however, in the case of the electrochemical reduction of NAD^+ to a free radical (wave I); the NAD^+-$\dot{N}AD$ couple itself is considered reversible due to the fact that, on cyclic voltammetry, the corre-

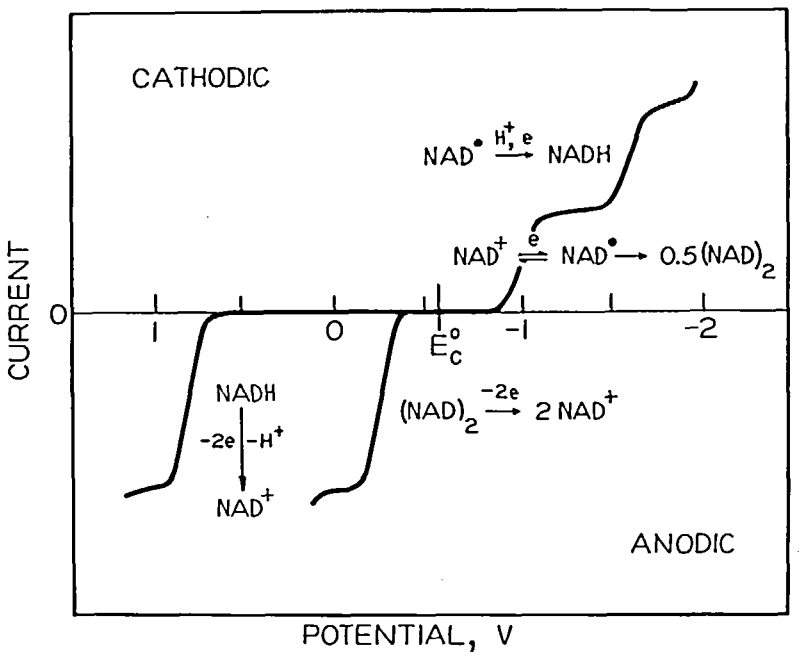


FIGURE 28. Composite representation of the voltammetric waves for the NAD^+-NADH system in relation to the calculated formal potential, E°_c, of -0.557 V for the half-reaction, $NAD^+ + 2e + H^+ \rightleftharpoons NADH$, at pH 7 and 25°. Potentials are vs. sce.

ponding cathodic and anodic peak potentials are separated by about the theoretically expected potential difference for a reversible couple. The E°_c value for the NAD^+-$NA\dot{D}$ couple in nonaqueous medium is about -1.10 vs. sce (Table 7). $E_{1/2}$ of NAD^+ wave I (-0.91 V vs. sce in the absence of tetraalkylammonium salts) occurs at considerably more positive potential than the formal potential for this couple; the positive potential shift may be due to irreversible dimer formation, as previously discussed, and strong adsorption of the dimeric reduction product at the interface, both of which phenomena would facilitate the electron transfer reaction.

The fact that the NAD^+-$NA\dot{D}$ couple is electrochemically reversible may be important in regard to understanding the biological electron transfer mechanism. A dehydrogenase-catalyzed oxidation of substrate (reduction of coenzyme) is, macroscopically, an apparent one-step process involving the net transfer of a hydride ion from substrate to C(4) of the pyridine ring. At the molecular level, however, it is possible that this reaction occurs in a stepwise fashion with the first step being reversible free radical formation:

$$NAD^+ \overset{e}{\rightleftharpoons} NA\dot{D} \overset{H\cdot \text{ or } H^+, e}{\rightleftharpoons} NADH \qquad (86)$$

The second step of the biological process would also be reversible, since, under equilibrium conditions, dehydrogenases give equal catalysis in both directions. Although the exact nature of this second step is unknown, it would presumably involve the transfer of either a hydrogen radical or a proton and electron in sequence or in concert. In most electrochemical studies, the second step (wave IIc) has been found to be irreversible; this is probably due to the fact that further electrochemical reduction of an initially formed coenzyme radical involves the rather complicated mechanism of transferring an electron across a solution-electrode interface with more or less simultaneous addition of a proton from the solvent.

If enzymatic reduction of the coenzyme is assumed to proceed through the stepwise free radical mechanism (Equation 86), then, based on the principle of microscopic reversibility,[188] which requires that the same transition state be rate-determining in both directions for a reaction

at equilibrium, the exact reverse of Reaction 86 would be expected for the corresponding biological oxidation process. Unfortunately, evidence for the stepwise electrochemical oxidation of NADH to NAD^+ is less clear-cut than for the stepwise electrochemical reduction of NAD^+ to NADH (see Section V. C.). Electrochemical oxidation of NADH in DMSO, as well as under aqueous conditions, is an apparent single 2*e* process; no evidence for a stepwise reaction within the time scales of even the most rapid cyclic voltammetric experiments can be detected.[56]

The nondetectability of a stepwise reaction during electrochemical NADH oxidation (+0.4 to +0.8 V) may be due to the fact that the first step of oxidation (conversion of NADH to NAD) is a slow reaction requiring a high activation energy whereas the second step (oxidation of NAD to NAD^+) is a very rapid reaction, i.e., NAD is already oxidizable at ca. -0.9 V. In such a situation, the second oxidation step would be expected to follow immediately after the first step and likely at such a rapid rate that a radical intermediate could not be detected.

It may be significant that, although NADH oxidation appears to be a single step 2*e* process, the electrochemical oxidation of a number of 1-alkyl NADH analogs in acetonitrile is best explained in terms of a stepwise mechanism where the oxidation is resolvable into at least two steps; the first involves loss of a single electron and is followed by one or more steps involving proton transfer and loss of the second electron to give the corresponding pyridinium ion.[90]

2. Analogy with the Biological Process

In attempting to transfer information and conclusions reached in electrochemical studies to the biological situation, account must be taken of the phenomena peculiar to heterogeneous electrode reactions such as adsorption, irreversible free radical dimerization, other aspects of irreversibility, and nonstereospecificity of reduction products. Under these conditions, reversibility in one step of a multistep reaction followed by irreversibility in another, as well as other non-ideal factors, might effect a separation of the overall reaction into steps (this situation is now under investigation). Thus, at an electrode surface, key electron-transfer steps requiring interconversion of NADH and NAD are irreversible (first step of NADH oxidation and second step of NAD^+ reduction).

As a working hypothesis, one may assume that the enzymatic NAD^+-NADH redox reaction proceeds by a stepwise free radical mechanism (Equation 86). The reversibility of the NAD^+-NADH couple under biological conditions, as compared to irreversible electrochemical behavior, must then obviously be related to enzyme activation of bound coenzyme and substrate species.

During biological reduction, the enzyme must in some fashion control the electron transfer process so that, when the first electron enters NAD^+, the energy required is sufficient to cause the second electron to enter rapidly, perhaps as a hydrogen radical or after a very rapid intervening chemical step such as protonation. With the rate of entry of the second electron sufficiently rapid, dimerization of an initially formed NAD free radical would not compete with dihydropyridine formation as in the case of electrochemical reduction. Free radical dimerization may also be prevented due to the binding of coenzyme (and the resulting free radical) as well as substrate species to specific enzyme sites. The binding of coenzyme and substrate in close juxtaposition on the surface of enzyme is no doubt a major reason why biological hydrogen transfer occurs in a direct stereospecific reduction. Electrochemical NADH formation is not a stereospecific reduction; it involves hydrogen transfer from the solvent medium rather than from a specific substrate.

The results of some chemical studies are more or less consistent with the electrochemical results in indicating that the biological reaction might follow a stepwise free radical mechanism (see Section VI. C. 2.). In one such study,[185] it was proposed that NADH dehydrogenase (the flavoprotein which links NADH to the cytochrome electron transport chain) may effect an activation of NADH by withdrawal of an electron or hydrogen radical to form the NAD free radical; this first step would be somewhat rate-limiting in comparison with the subsequent rapid loss of the second electron to flavoprotein with regeneration of NAD^+.[185]

Other chemical studies, however, favor a hydride ion mechanism (see Section VI. C. 1.). In addition, during the enzymatic action of alcohol dehydrogenases, no free radicals can be detected by electron spin resonance[80] or, at most, only a trace at the lower detection limit.[79] It is, conse-

quently, the opinion of many investigators and reviewers that the enzymatic reaction probably proceeds by a hydride ion mechanism rather than a free radical mechanism.[72,156,158,166a,184]

The question of whether enzymatic dehydrogenation proceeds by direct transfer of a hydride ion from substrate to coenzyme (i.e., simultaneous transfer of a proton and two electrons) or whether two successive 1*e* transfers are required, can be decided only if the various steps of the multistep mechanism can be separated; the term "simultaneous" obviously means only that no *detectable* time difference in terms of available approaches exists in the transfer of the two electrons. In the case of the NAD^+-NADH system, the electron spin resonance studies and, perhaps, some of the chemical studies may not be capable of distinguishing free radical steps, which occur in sufficiently rapid sequence, whereas at an electrode surface the different steps of the overall reaction are distinguishable.

VII. SUMMARY

The present article illustrates the application of analytical chemical techniques to the study of chemical phenomena, in particular, the use of electrochemical techniques and methodology – and, to a lesser extent, spectrophotometry – to investigate the solution behavior, adsorption, redox processes including coupled chemical reactions, and allied aspects of biologically significant compounds and of their intermediate and final redox products, e.g., the behavior of the free radicals produced by initial one-electron processes.

This approach is illustrated in the present instance by the consideration of the behavior in aqueous and nonaqueous media of a sequence of compounds ranging from nicotinamide (3-carbamoylpyridine) to NAD^+ and $NADP^+$; the latter compounds function as coenzymes for the pyridinoproteins which are principal components in the Krebs citric acid cycle and in the electron transport chain in biological redox reactions. An understanding of the various aspects of the electrochemical redox behavior of the sequence should aid in a more thorough understanding of biological electron transfer mechanisms, since the site of both biological and electrochemical redox activity in the pyridine nucleotides is the pyridine ring.

The desirability of studying the nicotinamide series of compounds in purely nonaqueous solvents like acetonitrile (AN) and dimethylsulfoxide (DMSO) is connected to the poorer proton-donating ability for these media, compared to water; consequently, it should be possible to clarify some uncertainties in the overall reduction mechanisms.

The compounds, upon whose behavior the discussion is predicated, include nicotinamide, N′-methylnicotinamide, 1-methylnicotinamide (1-methyl-3-carbamoylpyridinium ion; MCP^+), nicotinamide mononucleotide (NMN^+), nicotinamide adenine dinucleotide (NAD^+; DPN^+; coenzyme I), nicotinamide adenine dinucleotide phosphate ($NADP^+$; TPN^+; coenzyme II), deamino nicotinamide adenine dinucleotide (nicotinamide hypoxanthine dinucleotide; deamino-NAD^+; $DNAD^+$), and $DNADP^+$, as well as solutions of their dimeric and dihydropyridine reduction products, and three 1,4-dihydropyridine species [(dihydronicotinamide mononucleotide (NMNH), dihydronicotinamide adenine dinucleotide (NADH), and dihydronicotinamide adenine dinucleotide phosphate (NADPH)].

The detailed mechanism for the electrochemical reduction of the 1-substituted nicotinamides in aqueous media has been elucidated on the basis of the spectrophotometric, enzymatic, chemical and electrochemical properties of these compounds and their reduction products, and of similar studies on nicotinamides and adenines. An initial reversible one-electron (1*e*) addition to the pyridinium ring to produce a free radical is followed by irreversible dimerization; in the case of nicotinamide itself, the initial step involves simultaneous addition of an electron and a proton to form a free radical (only an electron in the pH range where the compound is protonated), which rapidly dimerizes to an apparent 6,6′ species; with increasing bulk of the N(1) substituent, formation of the 4,4′ dimer rather than the 6,6′ or 4,6′ species is favored. Oxidation of the free radical back to the parent compound can be observed at sufficiently high polarization rates on cyclic voltammetry, which allows calculation of the dimerization rate constants for the free radicals. At more negative potential, the free radical is reduced (1*e*, one-proton process) to a dihydropyridine (2*e* reduction product); a major fraction of the latter in the case of NAD^+ is enzymatically active 1,4-NADH, but 1,6-NADH is also formed under the experimental conditions used. Nico-

tinamide itself is reduced to an apparent 1,6-dihydropyridine species.

Neither the dimers nor the dihydropyridine species can be directly reduced electrochemically within the available potential range; however, at potentials close to background discharge, the NAD-derived dimer may be slowly converted to a dihydropyridine (probably by an indirect reduction process). Both dimer and dihydropyridine species are oxidized to the original nicotinamide species but at considerably more positive potentials than those necessary for their formation with the dimers being oxidized at considerably less positive potential than the dihydropyridines, which undergo apparently direct two-electron oxidation.

The rate constants for dimerization of the initially produced free radicals at 30° are about $2 \times 10^6\ M^{-1}\ sec^{-1}$ for nicotinamide, 6×10^7 for MCP^+, and 2×10^6 for the pyridine nucleotides; activation energies have been determined.

Both reduction products are susceptible to acid-catalyzed hydrolysis; the rate increases with decreasing pH; at any given pH, the dimer is less stable than the dihydropyridine species. Thus, protonation of the 1,4-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 absorb at 280 to 290 nm and which further decompose; the *pseudo* first order rate constants for the acid-catalyzed decomposition in aqueous solutions 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 NMNH or NADPH.

Adsorption of nicotinamide and N′-methylnicotinamide and their reduction products at the mercury-solution interface is negligible. While MCP^+ is negligibly adsorbed, its dimeric and dihydropyridine products are strongly and moderately adsorbed, respectively; NMN^+ and its reduction products are negligibly adsorbed, thus, reflecting the relative hydrophobic and hydrophilic nature of the N(1) substituent. Due to the presence of the adenine, which is strongly adsorbed, nucleotides containing this moiety and their dimeric products are normally strongly adsorbed.

In nonaqueous aprotic media, the 1-substituted nicotinamides show a redox pattern of initial single electron addition to the molecule to form a neutral free radical, which dimerizes at the 6 position. Further reduction of the radical requires proton participation, i.e., simultaneous addition of an electron and a proton to the ring is involved. The redox pattern for unsubstituted 3-nicotinamide consists of two successive one-electron additions (potentials separated by ca. 0.5 V) with the product of the initial one-electron reduction being a negatively charged free radical which dimerizes at the 6 position (rate constant for the dimerization is $3 \times 10^4\ M^{-1}\ sec^{-1}$). The rate constants for dimerization of the neutral free radicals derived from the 1-substituted nicotinamides are about $10^6\ M^{-1}\ sec^{-1}$. In general, in the presence of proton donors, the nicotinamides gave a wave pattern similar to that seen in aqueous media. The dimers show a greater stability in nonaqueous media than in aqueous media. However, the 1,4-dihydropyridine nucleotides do show proton-catalyzed decomposition.

Thermodynamic and kinetic properties for the compound sequence, such as corrected electrochemical potentials, chemical reaction rates, and adsorption at the solution-electron transfer interface, have been examined and correlated on the basis of structural, solvation, molecular orbital and other pertinent characteristics and parameters, and have been evaluated in respect to the behavior of the compounds when involved in biological systems.

The gross function of the pyridine nucleotides in intermediary metabolism as well as their function at the molecular level, e.g., in regard to the chemistry and stereochemistry of the enzyme active site, is discussed. Consideration is given to pyridine nucleotide chemical reactions and model chemical systems; the significance of electrochemical investigations in regard to the understanding of the biological redox mechanism is also considered.

Problems involved in correlation with biological behavior have been made explicit. For example, in attempting to transfer information and conclusions reached in electrochemical studies to the biological situation, account must be taken of the phenomena peculiar to heterogeneous electrode reactions such as adsorption, irreversible free radical dimerization, other aspects of irreversibility, and nonstereospecificity of reduction products. However, through the detection of radical intermediates and the occurrence of nonideal behavior such as irreversibility, electrochem-

ical studies of the NAD^+-NADH redox couple have been important from the point of view of being able to separate or differentiate the various steps of this multistep biological reaction mechanism.

As a working hypothesis, based on electrochemical work as well as some chemical studies, one may assume that the enzymatic NAD^+-NADH redox reaction proceeds by a stepwise free radical mechanism (two successive 1e transfers with a free radical as an intermediate) rather than by a hydride ion mechanism (simultaneous transfer of a proton and two electrons). In the case of the NAD^+-NADH system, a technique such as electron spin resonance may not be capable of distinguishing free radical steps, which occur in sufficiently rapid sequence, whereas at an electrode surface the different steps of the overall reaction are distinguishable.

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