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Structure of the Primary Acid Rearrangement Product of Reduced Nicotinamide Adenine Dinucleotide (NADH)†

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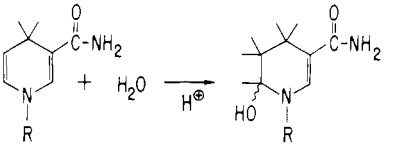
ABSTRACT: Evidence is presented for the α-$O^{2'}$-6B-cyclotetrahydronicotinamide adenine dinucleotide structure of the primary acid product of βNADH. This structure is based on the following results obtained by high frequency proton magnetic resonance at 220 MHz and by circular dichroism: (1) the acid product is a C-6 substituted tetrahydronicotinamide; (2) the reaction is stereospecific, generating only one diastereomeric form characterized by an axial C-4A proton and an equatorial substitution on the B side of the C-6 position; (3) both αNADH and βNADH yield the identical primary acid product; (4) the acid product has an α configuration and a static 3′-*endo*-ribose conformation; (5) only the 3′-ribose hydroxyl of the acid product is free to be acylated; and (6) blocking the 2′-hydroxyl of βNADH followed by incubation in dilute acid generates a compound with spectral properties of a 6-hydroxytetrahydronicotinamide. A reaction scheme is proposed to account for the observed acid-catalyzed chemistry of βNADH. Two pathways appear to be possible for the quantitative rearrangement of βNADH to the primary acid product: one through β-6-hydroxytetrahydronicotinamide adenine dinucleotide and the other through the acid-catalyzed anomerization of βNADH to αNADH. The causes of the stereospecificity of the cyclization reaction are discussed as are the possible implications of the acid-catalyzed chemistry on the nature of the transition state complex of βNAD$^+$ and βNADH in the active site of dehydrogenases.

The instability of the dihydronicotinamide moiety of βNADH[1] in dilute acid was first reported by Warburg *et al.* (1935) and Haas (1936). The reaction is characterized by a decrease in the ultraviolet (uv) absorption of the dihydronicotinamide ring at 340 nm with the concomitant appearance of a new chromophore at 280 nm. The resulting modified pyridine coenzyme has come to be referred to as the primary acid product. This reaction has been studied extensively with model compounds. Incubation of N-substituted dihydropyridines in acid results in the addition of water across the 5–6 double bond yielding a 6-hydroxytetrahydropyridine (Anderson and Berkelhammer, 1958) as is shown in eq 1. The acid-catalyzed hydration has been



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subsequently shown to be a general reaction of 1,4-dihydropyridine compounds (Stock *et al.*, 1961; Burton and Kaplan, 1963) and kinetic and structural investigations have also been reported (Johnston *et al.*, 1963; Alivisatos *et al.*, 1965; Choi and Alivisatos, 1968; Kim and Chaykin, 1968). X-Ray diffraction studies of the model compound 1-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide (Hope, 1969) have conclusively established the 6HTN structure and also the conformation of the 6HTN ring in the crystal.

A second modified reduced pyridine nucleotide is synthesized from βNADH by the enzyme glyceraldehyde-3-phosphate dehydrogenase at a pH below 7.5 (Rafter *et al.*, 1954). The enzyme-modified NADH also absorbs at 280 nm and has been called NADHX. This compound was

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[1] The NAD$^+$ nomenclature is used in order to abbreviate clearly the chemically modified pyridine coenzymes and analogs. The modifications are indicated by parentheses. Abbreviations used are: NMNH, dihydronicotinamide mononucleotide; (cTHN)MN and (cTHN)AD, $O^{2'}$-6B-cyclotetrahydronicotinamide nucleotide formed by the primary acid rearrangement of NMNH and NADH, respectively; cTHN, cyclotetrahydronicotinamide; (AcPy)ADH and (TN)ADH, reduced 3-acetylpyridine and 3-thionicotinamide analogs of NADH; (cTHAcPy)AD and (cTHTN)AD, the acid products of (AcPy)ADH and (TN)ADH, respectively; (6HTN)AD, β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide formed by glyceraldehyde-3-phosphate dehydrogenase (G3PD) from βNADH (originally called NADHX by Rafter *et al.*, 1954); 6HTN, 6-hydroxytetrahydronicotinamide; DCB-6HTN, 1-(2,6-dichlorobenzyl)-6HTN.

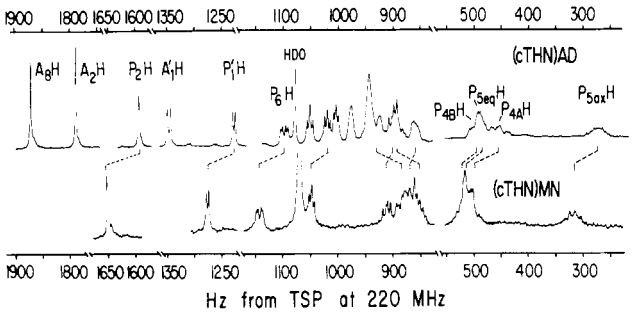
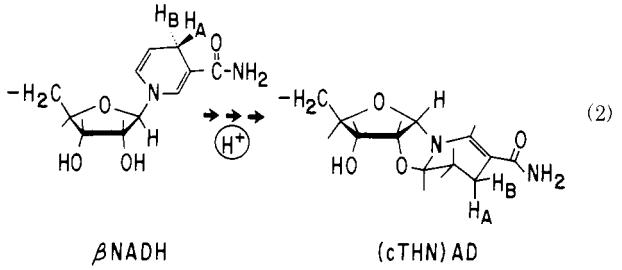


FIGURE 1: The pmr spectrum of (cTHN)AD and (cTHN)MN, 70 mM, 22°, D_2O, and pD 8.5.

thought to be structurally analogous to the primary acid product because the uv absorption spectra were nearly superimposable. Furthermore, NADHX is irreversibly converted to the acid product by incubation at pH 4 for a few minutes (Chaykin *et al.*, 1956; Meinhart *et al.*, 1956). However, the two modified coenzymes possess distinct enzymatic properties (Meinhart *et al.*, 1956; Stock *et al.*, 1961).

The complexity of the primary acid reactions of βNADH is evident from the circular dichroic (CD) investigation by Miles *et al.* (1968). The superimposability of the CD spectra for the acid products of αNADH and βNADH has led them to conclude that the acid products are identical, and further experiments indicated an α configuration for the acid product. Therefore, the primary acid reaction of βNADH involves an anomerization of the pyridine–ribose linkage. If the acid product is assumed to be an α6HTN nucleotide, then the question arises as to why the sterically more hindered α6HTN anomer would be favored over the β anomer, considering the preferential anomerization to βNADH of the less hindered dihydronicotinamide ring of αNADH (Woenckhaus and Zumpe, 1965; Oppenheimer *et al.*, 1971; Jacobson *et al.*, 1973).

In order to understand and clarify the chemistry of the reduced pyridine coenzymes in acid, we have investigated in detail the structure of the primary acid product of βNADH using proton magnetic resonance (pmr), CD, chemical modifications, and specific deuterium labeling. This paper presents the evidence for the acid-catalyzed rearrangement of βNADH to an α-$O^{2'}$-6B-cyclotetrahydronicotinamide nucleotide as shown in eq 2.



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Methods

Preparation of the Acid Product. The dinucleotides βNAD$^+$, αNAD$^+$, and βNADH were obtained from P-L Biochemicals and used without further purification. α-D-O^2,2′-Cyclouridine was purchased from Terra-marine Bioresearch. Reduced coenzyme specifically labeled on the B side, $NADD_B$, was prepared by pig heart lipoyl dehydrogenase (EC 1.6.4.3, Boehringer-Mannheim) reduction of βNAD$^+$ in D_2O (Oppenheimer *et al.*, 1971), while reduction with yeast alcohol dehydrogenase (EC 1.1.1.1, P-L Biochemicals) using [U-^{2}H]ethanol (C_2D_5OD, Merck, 99%) yielded $\beta NADD_A$. αNADH was prepared by dithionite reduction of αNAD$^+$ (Lehninger, 1957). (6HTN)AD was prepared according to the procedure outlined by Oppenheimer and Kaplan (1974a) and 1-(2,6-Dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide was synthesized according to the procedure of Kim and Chaykin (1968).

The primary acid product of NADH was prepared as described by Oppenheimer (1973), and (cTHAcPy)AD and (cTHTN)AD were prepared as described by Oppenheimer and Kaplan (1974b). The primary acid reaction is nearly quantitative with overall yields of ≥95% based on starting dihydronicotinamide. (cTHN)MN was prepared by both cleavage of (cTHN)AD with subsequent separation from 5′-AMP on a DEAE-cellulose column, and by the direct primary acid reaction of NMNH. The two sources gave identical pmr, uv, and CD spectra. (cTHN)MN($4D_B$) and (cTHN)MN($4D_A$) were prepared by cleavage of the respective specifically labeled dinucleotides, (cTHN)AD($4D_B$) and (cTHN)AD($4D_A$), with snake venom phosphodiesterase (EC 3.1.4.1, Boehringer-Mannheim).

The acylated nucleotides were prepared by adding the nucleotide, with vigorous stirring, to 100 ml of acetic anhydride–pyridine (50:50 by volume). The reaction mixture was maintained at room temperature by a water bath and within 1 hr the turbid solution cleared as the acylated nucleotide dissolved. The solution was evaporated to dryness and the glassy residue dissolved in methanol and then filtered to remove any insoluble nonacylated nucleotide. The methanol solution was then evaporated to dryness, dissolved in H_2O, and lyophilized to remove any volatile pyridine acetate. The acylated nucleotides were chromatographed on Dowex-1 and the complete acylation of the ribose hydroxyls was confirmed by pmr.

Spectral Determinations. The uv spectra were recorded on a Varian Associates Cary 14 and a Perkin-Elmer Colemann 124 scanning spectrophotometer. Circular dichroism spectra were recorded on a Varian Associates Cary 61 spectropolarimeter with a Xenon lamp. Spectra were obtained using a 1-cm pathlength cell and a thermostated block maintained at 25°.

Proton magnetic resonance spectra were recorded on a Varian Associates field sweep HR-220 proton magnetic resonance spectrometer under the same conditions as described in the following paper (Oppenheimer and Kaplan, 1974a).

Results

Primary Acid Product of NADH and NMNH. Proton Assignments. The complete pmr spectra of (cTHN)AD and (cTHN)MN are shown in Figure 1. The chemical shifts for the proton absorptions of the tetrahydropyridine rings of the acid products of NMNH, NADH, (AcPy)ADH, and (TN)ADH are listed in Table I. The proton assignments of the cTHN ring are made on the basis of deuterium labeling experiments and homonuclear spin decoupling (Oppenheimer, 1973). The upfield shifts of the cTHN of the dinucleotide relative to the mononucleotide indicate that there is a significant intramolecular interaction between the adenine and cTHN ring analogous to the folded interaction first discussed for NAD$^+$ and NADH by Jardetzky and Wade-Jardetzky (1966).

The spin–spin coupling constants of the tetrahydropyridine ring protons of DCB-6HTN, (cTHN)MN, (cTHN)AD,

TABLE I: Chemical Shifts of the Tetrahydropyridine Ring Protons.[a]

	PC_2H	PC_6H	PC_5H_{eq}	PC_5H_{ax}	PC_4H_A	PC_4H_B
(cTHN)MN	1648	1143	510	329	505	527
(cTHN)AD	1592	1098	485	278	450	492
(cTHAcPy)AD($4D_B$)	1683.5	1099.5	490.5	254	396	
(cTHTN)AD($4D_B$)	1747	1102.5	497.5	282	455	

[a] Chemical shifts are in hertz from sodium 3-trimethylsilylpropionate (tetradeuterio) at 220 MHz, 22°, 50 mM, and pD 8.5. The values are reported to within 0.5 Hz.

TABLE II: Spin–Spin Coupling Constants for the Tetrahydropyridine Ring Protons.

	J_{6-5ax}	J_{6-5eq}	J_{5ax-4A}	J_{5ax-4B}	$J_{5ax-5eq}$	J_{5eq-4A}	J_{5eq-4B}	J_{4A-4B}	J_{4A-2}
DCB-6HTN[a]	2.5	2.5	13.3	6.0	−13.1	5.6	2.0	−15.8	2.0
(cTHN)MN	9.5	4.2	13.0	5.1	−12.3	5.0	3.0	−16[b]	1.8
(cTHN)AD	9.5	4.2	13.0	5.3	−11.7	5.4	2.0	−16[b]	1.8
(cTHAcPy)AD($4D_B$)	9.6	4.1	13.5		−12.5	3.4			1.3
(cTHTN)AD($4D_B$)	9.7	4.2	13.0		−12.2	4.7			1.5

[a] The C-4 methylene protons cannot be absolutely assigned in DCB-6HTN; the 4A proton corresponds to the 4_{ax} proton, and the 4B proton to the 4_{eq} proton. Coupling constants are within 0.2 Hz. [b] Calculated from the computer simulation of the nonlabeled cTHN ring; the error limits are about ±0.4 Hz.

(cTHAcPy)AD, and (cTHN)AD are listed in Table II. The tetrahydropyridine proton coupling constants were determined from the acid product of the nucleotides specifically labeled with deuterium in the C-4A and C-4B positions. Computer simulation is necessary to confirm the values of the coupling constants in the unlabeled nucleotides and to determine the C-4 methylene geminal coupling constant because of the strong coupling between the protons even at 220 MHz.

Conformational Analysis. The correspondence of the coupling constants of the 6HTN ring C-4 and C-5 methylene protons with those for the cTHN protons (Table II) provides conclusive evidence that the conformation of the C-4 and C-5 methylenes in the primary acid product of NADH is the same as in the 6HTN ring. Thus, the cTHN ring likewise appears rigid without any indication of dynamic interconversions.[2] However, it is obvious from the values for the coupling constants of the C-6 proton to the C-5 methylene protons that a drastic alteration in the conformation of the C-6 substitution has occurred in the acid product of NADH. The large 9.5-Hz trans coupling constant between the C-6 and $C\text{-}5_{ax}$ proton and the small 4.2-Hz gauche coupling constant to the $C\text{-}5_{eq}$ proton are only consistent with a conformation in which the C-6 proton is axial and therefore the substitution at the C-6 position is equatorial. Thus, for the primary acid product of NADH and reduced analogs, the tetrahydropyridine ring assumes the same conformation as the 6HTN ring with the critical distinction that the substitution at the C-6 position is equatorial.

The absolute stereoconfiguration of an isolated asymmetric center cannot be unambiguously determined solely on the basis of the pmr spectroscopy. However, as in this case, if the geometry of a proton bound to an asymmetric carbon in a molecule can be related by spin–spin coupling to protons at another asymmetric carbon of known absolute configuration, then magnetic resonance provides a definitive method for determining the absolute molecular configuration. Such an analysis is possible for the protons of the primary acid product of NADH because specific deuterium labels of known absolute configuration can be generated enzymatically at the C-4 position. The absolute configuration of the enzymatic reduction of NAD^+ has been determined by Cornforth *et al.* (1962, 1965/1966). Subsequently the A and B protons of the C-4 methylene group have been assigned in the pmr spectrum of the enzymatically labeled NADH by Oppenheimer *et al.* (1971). Thus, the geometry of the C-6 substitution in the primary acid product can be determined absolutely by tracing the coupling constants, hence bond angles from the C-4A or C-4B protons to the C-5 protons (establishing the absolute configuration of the C-5 methylene proton) and from the C-5 protons to the C-6 proton. The conformational analysis of the C-4 labeled acid product establishes that the C-6 proton is on the A side of the ring and the C-6 substitution therefore is on the B side (Oppenheimer, 1973).[3]

An addition reaction at the C-6 of the tetrahydronicotinamide ring should generate an asymmetric center. The presence of the optically active D-ribose as well as other chiral centers in the dinucleotide precludes the existence of any plane of symmetry between the expected isomers; thus, they would be related as diastereomers. In principle the pmr spectra of diastereomers are nonsuperimposable; hence resonance doubling of the spectra would be expected (Mislow

[2] The data regarding the conformation of DCB-6HTN in solution are discussed in the following paper (Oppenheimer and Kaplan, 1974a).

[3] We have used the traditional A and B nomenclature for naming the substitutions of the acid reaction because of the simplicity in discussing substitutions onto either the A or B faces of the dihydronicotinamide ring. The *R* and *S* system of nomenclature (Cahn *et al.*, 1956, 1966) is less clear in this respect, *e.g.* in (cTHN)AD the configuration of the C-4A proton is *R*, while the C-6H proton which is on the same side of the cTHN ring as the C-4A proton has an *S* configuration.

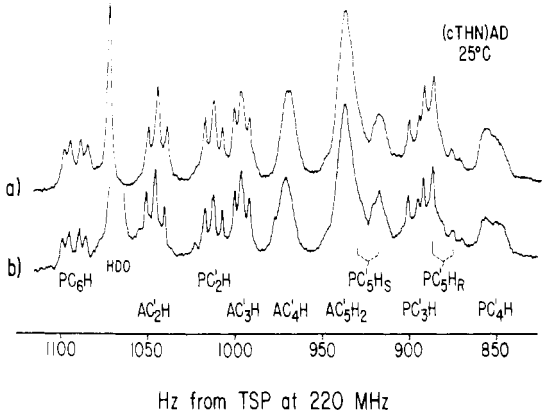


FIGURE 2: Ribose region of (cTHN)AD formed from βNADH (a) and αNADH (b), 70 mM, 25°, D_2O, and pD 8.5.

and Raban, 1966; Alworth, 1972). As can be seen in Figures 1 and 2 and in the spectra of the specifically labeled acid products which have been published elsewhere (Oppenheimer, 1973; Oppenheimer and Kaplan, 1974b), the pmr spectra of the acid product of NADH and NMNH consist of a single set of absorptions characterized by a unique and defined set of coupling constants. No evidence can be found for the presence of a mixture of diastereomers as in (6HTN)AD. Thus, we conclude that the primary acid reaction occurs stereospecifically, generating only one of the two possible diastereomeric forms attributable to a B-side C-6 substitution. There is no detectable concentration of the A side C-6 substitution in the acid product of NADH or any of the related dihydropyridine nucleotide analogs.

Ribose Conformation and Configuration. The configuration of the base–ribose linkage is important in understanding the chemistry involved in the acid rearrangement of NADH. Based on CD data, Miles *et al.* (1968) have proposed that both βNADH and αNADH yield the identical acid product. Furthermore, they have suggested that the modified coenzyme has an α linkage, thus requiring an acid-catalyzed anomerization of βNADH. High frequency pmr is uniquely suited to inyestigate the configuration of these nucleotides because of the detailed geometric information that can be obtained from the coupling constants of the ribose protons. The superimposability of the pmr spectra of the acid products generated from αNADH and βNADH shown in Figure 2 provides unequivocal proof for the identity of these compounds. The demonstration that these compounds are identical also confirms the occurrence of an anomerization reaction in the formation of the acid product.

The pyridine ribose protons are assigned by analysis of the coupling constants and by homonuclear and ^{31}P heteronuclear spin decoupling. The values of the chemical shifts and coupling constants for the pyridine and adenine ribose protons are listed in Tables III and IV. The PC-1′ proton appears as a doublet, the PC-2′ proton as a triplet, and the PC-3′ proton as a doublet of doublets. The PC-4′ and PC-5′ protons are characterized by additional ^1H–^{31}P coupling constants which can be decoupled by irradiation of the 5′-phosphate at 89 MHz. The PC-5′ protons are significantly nonequivalent (>0.1 ppm) in all the acid products and show the expected 11–12-Hz geminal coupling constant.

The chemical shifts of the pyridine ribose protons in the dinucleotide show differences in comparison with the values for the mononucleotides. The upfield shift of the C-1′, C-2′, and C-3′ proton absorptions in the dinucleotide suggests that the effects on these resonances are dominated by the anisotropic ring current shielding generated by the aromatic adenine. The downfield shift of the C-4′ and C-5′ protons indicates that the chemical shifts for these protons are dominated by the differences in charge between the 5′-phosphate of the mononucleotide and the 5′–5′-pyrophosphate of the dinucleotide.

The adenine ribose proton absorptions in the dinucleotide spectrum were assigned by a similar procedure of homonuclear and heteronuclear spin decoupling. The C-4′ and C-5′ protons are shifted downfield in comparison to AMP (pH 5.5) due to the differences in charge at the 5′ position. The adenine ribose protons in contrast to the pyridine ribose protons show very small effects due to intramolecular association with the cTHN ring. However, because it is nonaromatic, the cTHN ring would not be expected to generate a strong shielding field such as exists for the aromatic nicotinamide ring of NAD^+.

The pyridine ribose $J_{3'-4'}$ coupling constants of 8.8 Hz for (cTHN)AD and 8.6 Hz for (cTHN)MN are indicative of a large 150–160° dihedral angle for $\phi_{3'-4'}$ and correspond to a 3′ endo conformation[4] (Altona and Sundaralingam, 1973). The $J_{2'-3'}$ values of 5.4 Hz are also in close agreement with the 3′ endo conformation. If the cTHN nucleotide has a β configuration, the predicted value for $J_{1'-2'}$ in a 3′-endo conformation using the bond angles and coefficients for the Karplus relation listed by Altona and Sundaralingam (1973) would be about 0 Hz. For an α configuration, assuming that the αC-1′–C-2′ dihedral angle, $\alpha\phi_{1'-2'}$, can be represented by the βC-1′–C-2′ angle, $\beta\phi_{1'-2'} = -120°$, the values of $\alpha\phi_{1'-2'}$ for the 3′-endo conformation should be about −30°. This angle yields a calculated coupling constant for $J_{1'-2'}$ of between 6 and 6.5 Hz. The observed values of 4.5 Hz would therefore appear to correspond to the values for the α3′-endo configuration. The small discrepancy between the calculated and observed values probably reflects the inadequacy of assuming that the α anomer can be represented by interchanging the base and the C-1′ proton of the β anomer without any change occurring in the $\phi_{1'-2'}$ dihedral angle. On the other hand, if the acid product had a β configuration, the value of $J_{1'-2'}$ of 4.5 Hz would require a very large distortion of the ribose of greater than 40° without concomitant changes in the other coupling constants from the values for the 3′-endo conformer, a prospect which is highly unlikely. Finally, the similarity of the coupling pattern, especially the value of $J_{1'-2'}$ for the model compound α-D-O^2,2′-cyclouridine (Table IV), further supports the conclusion that the primary acid product is an α anomer.

Pmr data on the conformation of β nucleotides have been interpreted in terms of a dynamic system of interconverting conformers (Blackburn *et al.*, 1970; Hruska *et al.*, 1970; Grey *et al.*, 1971; Schleich *et al.*, 1972; Altona and Sundaralingam, 1973). For the cTHN nucleotides, interpretation of the data in terms of a static ribose conformation appears quite adequate. The observed J values correspond closely to the values for the pure α3′-endo conformer unlike the β nucleotides which tend to have coupling constants that are intermediate between the calculated values for the 2′-endo and 3′-endo conformers. Furthermore, any

[4] The nomenclature system contained in their analysis of the ribose conformations of β nucleotides will be used in the following discussion of cTHN nucleotides.

TABLE III: Tetrahydropyridine–Ribose Proton Chemical Shifts.[a]

	C-1′	C-2′	C-3′	C-4′	C-5′$_S$	C-5′$_R$
(cTHN)MN	1277	1048	908	867	877	844.5
(cTHN)MN-O$^{3'}$Ac	1295	1114	1199.5	951	876	847.5
Δ Chemical Shift	−22	−66	−191.5	−84	1	−3
(cTHN)AD	1218	1011	893.5	852	921.5	881.5
Adenine–ribose	1342	1044	995	970	936.5[b]	
(cTHAcPy)AD	1240	1029.5	908.5	867.5	930.5	899.5
(cTHTN)AD	1245.5	1025	901	863	926.5	884

[a] Chemical shifts in hertz relative to sodium trimethylsilylpropionate at 220 MHz; values are accurate to within 0.5 Hz. [b] The center of the C-5′ absorption. The nonequivalence is greater than 3 Hz but less than 6 Hz as determined by computer simulation.

TABLE IV: Tetrahydropyridine–Ribose Proton Coupling Constants.[a]

	$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$J_{4'-5'_S}$	$J_{4'-5'_R}$	$J_{5'_S-5'_R}$	$J_{5'_S-P}$[b]	$J_{5'_R-P}$[b]
(cTHN)MN	4.5	5.6	8.6	2.0	4.5	−11.2	4.5	4.5
(cTHN)MN-O$^{3'}$Ac	4.7	6.1	7.0	2.0	5.3	−11.7	4.5	4.5
(cTHN)AD	4.5	5.4	8.8	2.0	4.5	−11.5	4.5 ± 1	4.5 ± 1
Adenine–ribose	5.8	5.4	3.1	5.5[c]		−11.5 ± 0.5	4.5 ± 1	4.5 ± 1
(cTHAcPy)AD	4.5	5.5	8.5	2.0	4.3	−11.3	4.5 ± 1	4.5 ± 1
(cTHTN)AD	4.5	5.6	8.8	2.0	4.5	−11.4	4.5 ± 1	4.5 ± 1
α-D-O^{2},2′-Cyclouridine	5.4	5.6	9.0	2.1	4.4	−12.5		

[a] Coupling constants are accurate to within 0.2 Hz unless otherwise shown. [b] Measured by comparison with ^{31}P heteronuclear decoupled spectra. [c] Because of the small chemical-shift nonequivalence of the adenine C-5′ protons, only the sum of the couplings of the C-4′ proton to the C-5′ protons has significance.

significant dynamic interconversions of the ribose ring would be expected to cause accompanying alterations in the entire conformation of the cTHN ring due to the O$^{2'}$-6B linkage. We find no evidence for such dynamic effects in either the ribose or the cTHN ring.

Studies with molecular models indicate that the 3′-endo conformation in (cTHN)MN would be the least strained of any of the possible ribose conformations. However, we cannot tell at this time whether the O$^{2'}$-6B cyclization forces the ribose into the 3′-endo conformation or whether the 3′-endo conformation is intrinsic to this class of pyridine nucleotides.

Backbone Conformation; Rotamer Populations. Conformation around the C-4′–C-5′ Bond. The rotamer populations around the C-4′–C-5′ bond for both the pyridine ribose of (cTHN)AD and for the ribose of (cTHN)MN have been calculated by the method described by Blackburn *et al.* (1970). The 60° staggered rotational conformations and gauche coupling constants of 2 Hz and a trans coupling constant of 10.2 Hz are used as shown in Figure 3a. The percentage distributions of the rotamer populations are listed in Table V. As can be seen, the population of one of the trans-gauche rotamers is negligible.

Remin and Shugar (1972), citing evidence for a large number of 3′ and 5′ nucleotides, have proposed the assignment of the C-5′ exocyclic methylene protons. Using their assignment of the C-5′ methylene protons, we have assigned the downfield C-5′ absorption of the pyridine ribose to the C-5′$_S$ proton and the upfield C-5′ absorption to the C-5′$_R$ proton. The *R* and *S* pertain to the nomenclature for a prochiral center (Hanson, 1966). The assignment for the (cTHN)MN is reasonable since the C-5′$_S$ proton is in close proximity to the oxygen atom of the ribose ring in the gauche-gauche conformation and should be deshielded relative to the C-5′$_R$ proton. Furthermore, from studies with molecular models, the observed 3′-*endo*-ribose conformation in (cTHN)MN and (cTHN)AD should cause a strong interaction between the 3′-hydroxyl and the 5′-oxygen atom which should make the trans-gauche rotamer III energetically unfavorable compared to the gauche-trans rotamer. Finally, substitution of the 3′-ribose hydroxyl with an acyl

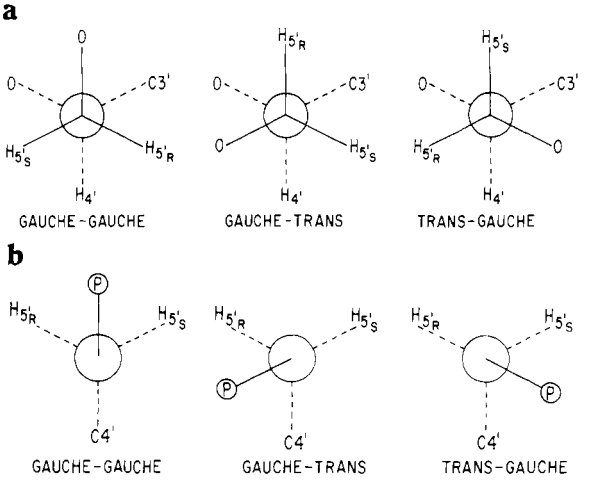


FIGURE 3: (a) The 60° staggered rotational conformations around the C-4′–C-5′ bond; (b) the 60° staggered rotational conformations around the C-5′–O bond.

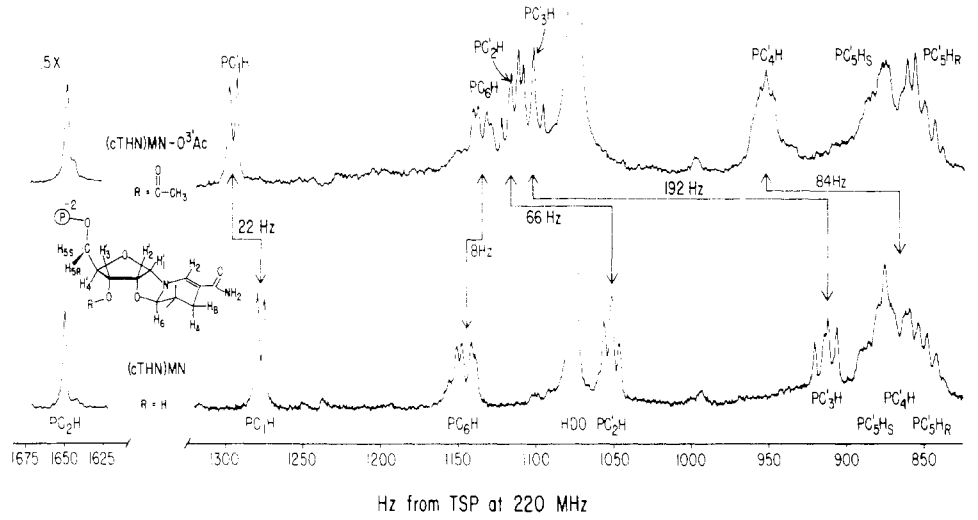


FIGURE 4: Comparison of the pmr spectrum of (cTHN)MN (bottom) with the exhaustively acylated (cTHN)MN (top), 50 mM, 22°, D_2O, and pD 8.5.

group does not significantly affect the values of $J_{4'-5'S}$ and $J_{4'-5'R}$. This would suggest that the trans–gauche population is already negligible in (cTHN)MN so that any additional steric hindrance caused by acylation has no further effect. Thus, for (cTHN)MN and (cTHN)AD, only the gauche–gauche and gauche–trans rotamers are present. This is also true for α-D-O^2,2′-cyclouridine.

Conformation around the C-5′–O–P Bond. The ^{31}P nucleus with a spin of ½ displays nuclear spin coupling to the hydrogen nucleus in a manner analogous to proton–proton spin coupling. The P–O–C–H vicinal coupling constants appear to follow a Karplus type dependence on the dihedral angle analogous to that for protons (Tsuboi *et al.*, 1968, 1969). The trans coupling constant is estimated to be 25 ± 3 Hz and the gauche coupling constant is 3 ± 2 Hz (Kainosho *et al.*, 1969; Hall and Malcolm, 1968). The method described by Blackburn *et al.* (1970) for the calculation of rotational isomers about the C-5′–C-4′ bond can be simply modified to calculate qualitatively the rotamer populations around the P–O–C-5′–H bond by using the values given above for the trans and gauche coupling constants and considering only the 60° staggered rotamers shown in Figure 3b. The rotamer populations for (cTHN)MN and (cTHN)AD are all exclusively gauche–gauche, *i.e.* >90%, and are in agreement with the results for NMN^+ and NMNH (Sarma and Mynott, 1973) and for other pyridine 5′ nucleotides (N.J. Oppenheimer and L.J. Arnold, unpublished data).

It is obvious from comparison of the calculated rotamer populations around the exocyclic methylene in the (THN)MN and (cTHN)AD that no significant changes in the rotamer populations have occurred. This is in spite of the fact that there is apparently significant intramolecular association in (cTHN)AD. The lack of change in rotamer populations does not necessarily argue against intramolecular association since it can be attributed to: (1) sufficient degrees of freedom around the O–P–O–P–O bonds to allow for the intramolecular associated conformations; any change in the angle around these bonds will not alter the coupling constants to the C-5′ protons and hence are unobservable; (2) the rotamer population may be so strongly governed by the local, steric interactions that the effects caused by the intramolecular association which is many bonds removed are insufficient to alter the population.

Acylation. The proposed α-$O^{2'}$-6B cyclic structure for the acid product of NADH implies that the 2′-ribose hydroxyl is critical to the reaction. Therefore, the determination of the number and position of free hydroxyls in the acid product and the blocking of the ribose hydroxyls prior to the acid reaction can provide evidence concerning participation of the 2′-hydroxyl in the acid-catalyzed reaction. Exhaustive acylation was chosen to determine the number and sites of the free hydroxyls because of the quantitative yield of the acylated nucleotides and the lack of complicating side reactions or decomposition.

Acylation of the free hydroxyl(s) in (cTHN)MN causes characteristic downfield shifts in the pmr spectrum of the ribose protons. The ribose proton of an acylated carbon is deshielded by 0.9–1.2 ppm while acylation of an adjacent carbon results in only 0.3–0.5-ppm deshielding. Thus, by comparing the chemical shifts of the corresponding ribose proton absorptions of (cTHN)MN with those of exhaustively acylated (cTHN)MN, the site or sites of acylation can be determined.

The chemical shifts and coupling constants of (cTHN)MN and the acylated (cTHN)MN are compared in Tables III and IV, and the ribose absorptions of the re-

TABLE V: Calculated Percentage Population Distributions of the Three 60° Staggered Rotamers around the C-4′–C-5′ Bond.

	Gauche–Gauche (I)	Gauche–Trans (II)	Trans–Gauche (III)
(cTHN)MN-O3′Ac	60	40	
(cTHN)MN	70	30	
(cTHN)AD	70	30	
Adenine–ribose	82	18[a]	
(cTHAcPy)AD	72	28	
(cTHTN)AD	70	30	
α-D-O^2,2′-Cyclouridine	66	29	5

[a] Because of the small chemical-shift nonequivalence of the adenine 5′ protons, only the sum of the gauche–trans and trans–gauche rotamer populations can be calculated.

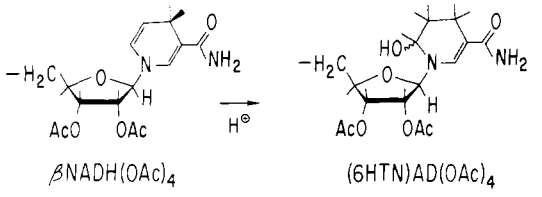
spective pmr spectra are shown in Figure 4. As can be seen, the C-3′ proton has shifted 191.5 Hz downfield, indicating the acylation of the 3′-hydroxyl. The C-2′ and C-4′ protons have shifted only 66 and 84 Hz downfield, respectively; thus, they are adjacent to the acylated 3′-hydroxyl but have not been acylated themselves.

The ribose proton assignments for (cTHN)MN-O$^{3'}$Ac are confirmed by homonuclear spin decoupling of the C-1′ proton from the C-2′ proton and the C-4′ proton from the C-3′ proton. The C-4′ and C-5′ protons are assigned by ^{31}P heteronuclear spin decoupling of the 5′-phosphate. The acylation of (cTHN)MN shows that only the C-3′ hydroxyl is acylated; the C-2′ oxygen is unavailable for acylation, as would be expected in the α-O$^{2'}$-6B cyclic structure.

Circular Dichroism. A strong, positive Cotton effect has been observed for the 280-nm absorption of the primary acid product of βNADH, with only a weak interaction occurring between the electronic transition dipoles of the adenine and cTHN chromophores (Miles *et al.*, 1968). Thus the CD spectrum of (cTHN)AD is not complicated by the strong reciprocal relationships that dominate the CD spectrum of βNAD$^+$ (Miles and Urry, 1968). The CD spectrum of (cTHN)AD is completely consistent with our proposed α-O$^{2'}$-6B cTHN structure. The large Cotton effect for the cTHN chromophore can be accounted for on the basis of the Kauzmann–Eyring rule (1941); chromophores with restricted rotation or fixed geometries within a molecule tend to have stronger Cotton effects than chromophores capable of free segmental motion. The cyclic structure has such a fixed geometry for the chromophore; also the acid product is a single pure optical isomer.

This is not the case for (6THN)AD,[5] where there is unhindered rotation around the ribosidic bond, a β anomeric configuration, and two diastereomeric forms. Thus, as would be expected, the CD spectrum of (6HTN)AD has essentially no Cotton effect associated with the 280-nm chromophore as is shown in Figure 5. The lack of a strong positive Cotton effect for the 6HTN nucleotide compared to the large positive Cotton effect for the cTHN nucleotide provides a criterion for distinguishing these two types of C-6 substituted tetrahydronicotinamide nucleotides.

The importance of a free hydroxyl at the ribose 2′ position for the formation of the cyclic structure can be tested by blocking the ribose hydroxyls of βNADH. Acylation of βNADH yields βNADH(OAc)$_4$ in which all four ribose hydroxyls have been acylated.[6] A decrease in absorption at 340 nm occurs when βNADH(OAc)$_4$ is incubated in dilute acid, analogous to the acid reaction of βNADH. The expected reaction is shown in eq 3. A comparison of the uv



(3)

spectra of the resulting acid product of the acylated derivative and (cTHN)AD is shown in Figure 5b. The primary acid product of βNADH(OAc)$_4$ has a significantly lower

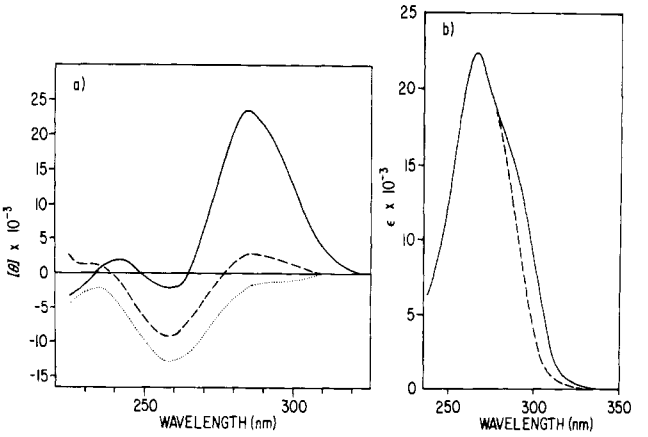


FIGURE 5: (a) Comparison of the CD spectra at 25° and pH 8.1 of (cTHN)AD (—); the acid product of exhaustively acylated βNADH, (6HTN)AD(OAc)$_4$ (- - -); and the enzymatically modified dinucleotide, (6HTN)AD (···). (b) Comparison of the uv spectra of (cTHN)AD (—) and (6HTN)AD(OAc)$_4$ (- - -).

extinction coefficient at 300 nm than (cTHN)AD. A similar phenomenon has been reported in the comparison of uv spectra of (6HTN)AD and the acid product of βNADH (Rafter *et al.*, 1954; Chaykin *et al.*, 1956; Meinhart *et al.*, 1956); however, the difference is small and not definitive.

The striking differences between the primary acid product of βNADH(OAc)$_4$ and (cTHN)AD are apparent from the CD spectra shown in Figure 5a. The acid product of the acylated dinucleotide, (6THN)AD(OAc)$_4$, where the 2′-hydroxyl is blocked shows almost no Cotton effect at 280 nm, analogous to the CD spectrum of (6THN)AD. This lack of a Cotton effect does not seem to be attributable to effects on the optical activity of the chromophore caused solely by acylation since the CD spectra of (cTHN)MN and (cTHN)MN-O$^{3'}$Ac have nearly identical positive Cotton effects as seen in Figure 6a. The shift to shorter wavelength of the Cotton effect in the CD spectrum of (cTHN)MN-O$^{3'}$Ac appears to be related to a corresponding shift in the uv spectrum shown in Figure 6b.

The blocking of the 2′-hydroxyl by acylation *before* the primary acid reaction yields a compound with spectral properties similar to that of (6HTN)AD and without the CD spectral characteristics of the cTHN ring. Acylation *after* the primary acid reaction yields only the 3′-acetate; the 2′-hydroxyl cannot be acylated and acylation of the 3′-hydroxyl does not significantly alter the CD spectrum.

The strong positive Cotton effect due to the locked chro-

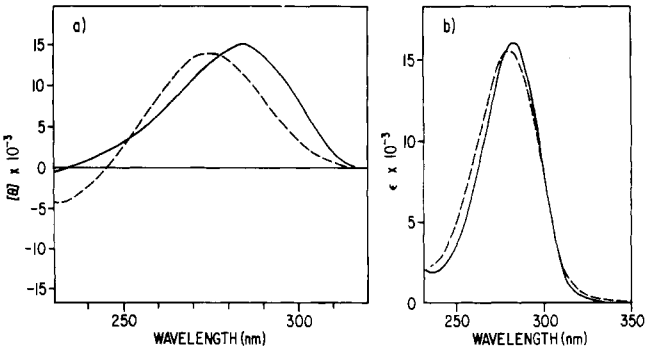


FIGURE 6: (a) Comparison of the CD spectrum of (cTHN)MN (—) with (cTHN)MN-O$^{3'}$Ac (- - -) at 25° and pH 8.1. (b) Comparison of the uv spectra of (cTHN)MN (—) and (cTHN)MN-O$^{3'}$Ac (- - -) at 25° and pH 8.1.

[5] The lack of a Cotton effect for the 280-nm chromophore of (6HTN)AD has also been recently reported by Zinner *et al.* (1973).

[6] A detailed analysis of the pmr spectrum of βNADH(OAc)$_4$ will be presented in a forthcoming paper on the conformation of βNAD$^+$ and βNADH in solution.

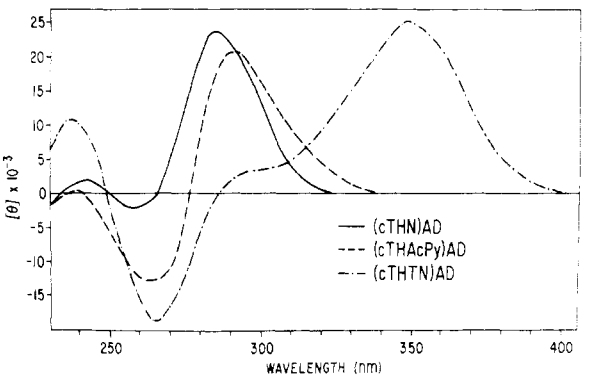


FIGURE 7: Comparison of the CD spectra of (cTHN)AD with the acid products of the reduced analogs (AcPy)ADH and (TN)ADH at 25° and pH 8.1.

mophore is also evident in the primary acid products of other NADH analogs. Figure 7 shows the CD spectra of (cTHAcPy)AD and (cTHTN)AD. For cTHAcPy the tetrahydropyridine chromophore absorption maximum is 303 nm and the CD maximum is at 292 nm, while for the two absorption bands of (cTHTN)AD, a minor band at ~280 nm and the major primary acid band at 345 nm, only the major band shows a strong CD absorption at 349 nm. These two uv absorptions in (cTHTN)AD are probably due to the more complex nature of the electronic transitions of the sulfur atom and probably correspond to the absorptions at ~300 and 395 nm for the dihydrothionicotinamide ring of (TN)ADH (Anderson *et al.*, 1959).

The similar Cotton effect for the acid products of βNADH, (TN)ADH and (AcPy)ADH indicates that the alignment of the chromophores of these three compounds is very similar. Thus we conclude that the specificity of the primary acid rearrangement is unaffected by the nature of the C-3 substitution of the dihydropyridine ring. Pmr studies of the acid product to these reduced analogs confirm an identical α-$O^{2'}$-6B cyclic structure (Oppenheimer and Kaplan, 1974b).

The evidence supporting the α-$O^{2'}$-6B-cyclotetrahydronicotinamide nucleotide structure for the primary acid rearrangement of βNADH can be summarized as follows: (1) the reaction is stereospecific; only one diastereomer is present; (2) both βNADH and αNADH give the identical compound; (3) the C-6 substitution of the tetrahydronicotinamide ring is equatorial and on the B side; (4) the base–ribose linkage has the α configuration; (5) the rearrangement reaction is unaffected by the C-3 substitution of the dihydropyridine ring; (6) acylation of (cTHN)MN yields only the 3′-acetate and does not affect the CD spectra; and (7) blocking the 2′-hydroxyl of βNADH yields an acid product without the strong positive Cotton effect at 280 nm and with a spectrum like that of (6HTN)AD.

Discussion

The formation of (cTHN)AD, the so-called "acid degradation product" of βNADH, requires that the following acid-catalyzed reactions occur quantitatively, though not necessarily in order: (1) an anomerization reaction from the β to the α anomer; (2) an irreversible protonation at C-5 of the dihydronicotinamide ring; and (3) an irreversible, stereospecific $O^{2'}$-6B cyclization reaction. The reaction scheme illustrated in Figure 8 is a summary of the proposed chemistry of βNADH in dilute acid. The scheme correlates previous reports on the acid-catalyzed reactions of βNADH and the structures of the modified pyridine coenzymes we have determined in our present investigation.

The mechanism of the acid-catalyzed anomerization is shown in steps 7 and 8 of Figure 8. Protonation of the ribose ring oxygen opens the ring with the concomitant formation

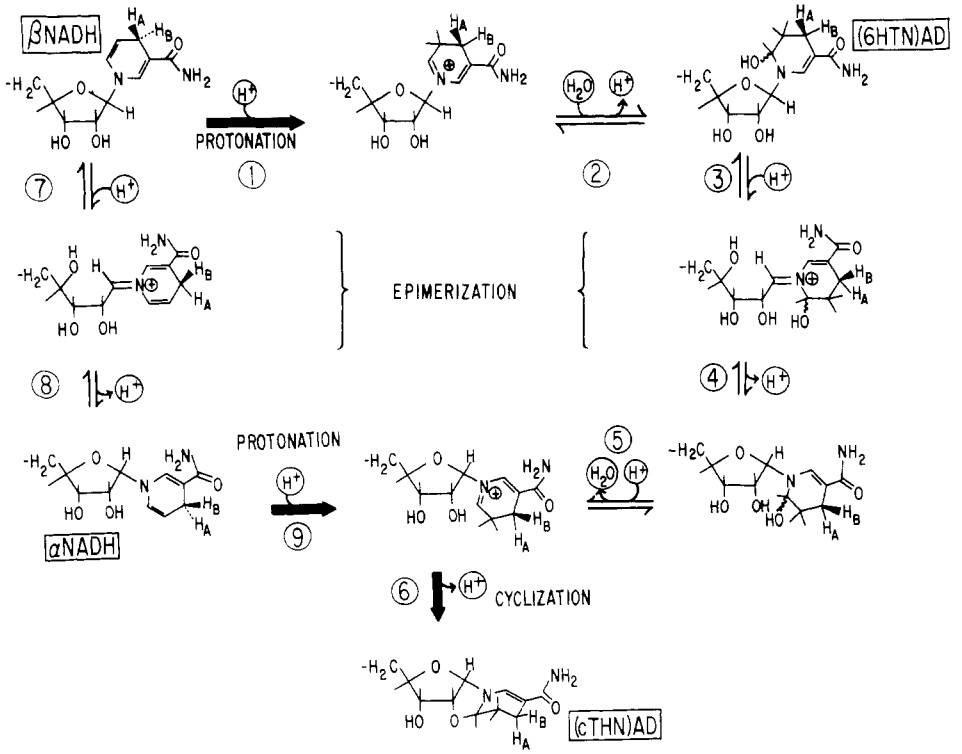


FIGURE 8: The proposed reaction scheme for the primary acid rearrangement showing the two possible pathways: (1) βNADH → αNADH → (cTHN)AD, steps 7, 8, 9, and 6; and (2) βNADH → (6HTN)AD → (cTHN)AD, steps 1–6.

of a double bond between the C-1′ and N-1 and generation of a positive charge on the nitrogen. This opened ring intermediate can then re-close to either the α or β anomer (Capon, 1969; Dekker and Goodman, 1970). The oxidized coenzymes αNAD$^+$ and βNAD$^+$ cannot anomerize by this mechanism because the N-1 nitrogen is already positively charged and thus is unable to donate further electrons to form the double bond to the C-1′ carbon.[7] Likewise, the tetrahydronicotinamide cationic intermediate formed by step 1 would not be expected to anomerize. On the other hand, the dihydronicotinamide–ribose linkage can anomerize as is shown in steps 7 and 8, as can the 6HTN–ribose linkage (steps 3 and 4). The occurrence of the later two anomerization reactions is supported in the literature. Anomerization of αNADH to βNADH has been reported by Woenckhaus and Zumpe (1965) and more recently we have found that this anomerization is an equilibration leading to a mixture of approximately 90% βNADH and 10% αNADH (Oppenheimer *et al.*, 1971). By inference the anomerization of 6THN–ribose has also been reported. NADHX incubated at pH 5.0 is readily converted into the primary acid product (Chaykin *et al.*, 1956; Meinhart *et al.*, 1956); thus β(6HTN)AD can anomerize to α-$O^{2'}$-6B(cTHN)AD. We have observed that this reaction occurs without exchange of the C-5 protons; thus the reaction of (6THN)AD to (cTHN)AD does not proceed *via* a NADH intermediate.

The primary acid rearrangement product of βNADH can arise by two pathways as is shown in Figure 8: (1) βNADH → αNADH → (cTHN)AD and (2) βNADH → (6HTN)AD → (cTHN)AD. The predominant sequence for this rearrangement reaction of βNADH to (cTHN)AD in dilute acid is currently under investigation. Evidence has been reported by Alivisatos *et al.* (1965) that the reaction of βNADH in concentrated phosphate solutions first forms (6HTN)AD which then subsequently rearranges to (cTHN)AD. Their result suggests that the polybasic anion-catalyzed reaction of βNADH favors protonation of the C-5 position to give (6HTN)AD followed by a slower conversion to the α anomer and the ultimate formation of (cTHN)AD.

The facile conversion of (6HTN)AD to (cTHN)AD by dilute acid raises the question as to how the nonspecific β-6-hydroxytetrahydronicotinamide nucleotide can rearrange to the stereospecific α-$O^{2'}$-6B-cyclotetrahydronicotinamide nucleotide. If the cycylization reaction occurs by nucleophilic displacement of the C-6 hydroxyl, then a mixture of the 6B and 6A cyclized nucleotides would be expected. Alternatively, if only one of the diastereomeric forms of (6HTN)AD could align itself to allow for displacement, then this favorably oriented isomer would cyclize while the unfavorably oriented isomer would remain as a 6-hydroxytetrahydronicotinamide. The exclusive presence of the single $O^{2'}$-6B diastereomer can be rationalized only if the hydroxylation step is reversible, generating an α-tetrahydronicotinamide (THN)$^+$ cation. Under these circumstances the addition of the 2′-hydroxyl would occur on the αTHN$^+$ ring as shown by steps 5 and 6.

Investigations into the mechanism of hydrolysis of enamines (Stamhuis and Maas, 1965; Maas *et al.*, 1967) indicate that the hydroxylation step can be reversible under mildly acidic conditions. Thus, it is quite plausible that an α-tetrahydronicotinamide cation can be generated following anomerization of the β(6HTN)AD to an α(6HTN)AD intermediate (steps 3–5). The generation of this α-tetrahydronicotinamide cation in close proximity to the 2′-ribose hydroxyl would explain the rapid and irreversible cyclization reaction that occurs. This cyclization reaction is similar to intramolecular cyclization reactions that have been reported for other tetrahydronicotinamide cations (Supple *et al.*, 1963).

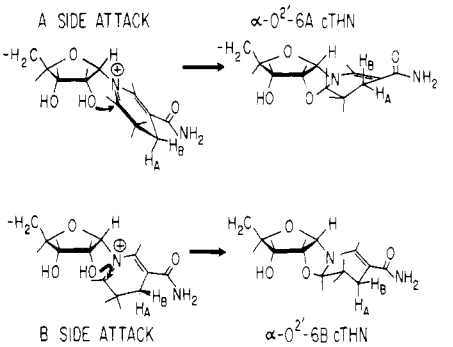


FIGURE 9: The two possible configurations that can be generated from the cyclization reaction between the 2′-hydroxyl and the tetrahydronicotinamide ring. The A-side attack requires the two rings to be coplanar and the B-side attack requires that the two rings be perpendicular.

The stereospecificity of the cyclization reaction can be accounted for by the steric considerations required in order for the 2′-ribose hydroxyl to attack the αTHN$^+$ intermediate. The αTHN$^+$ ring must be oriented with the 3-amido group away from the ribose in an anti conformation. The attack of the 2′-hydroxyl is limited to the rotational isomers in which the C-6 position is in close proximity; hence the syn conformations which would juxtapose the C-2 with the 2′-hydroxyl could not react. The reaction would then occur as is shown in Figure 9; in the B-side attack by the 2′-hydroxyl the plane of the αTHN$^+$ ring must be oriented at right angles to the plane of the ribose ring, while in an A-side attack the two rings must be coplanar. Thus, the stereospecificity of the 2′-hydroxyl attack would be governed by the difference in the energy of the two intermediates. Since the coplanar form resides at the energy maximum for rotation about the ribosidic bond while the perpendicular form is at the energy minimum (Coubeils *et al.*, 1971), the B-side attack would be favored by the difference in energy between the two intermediates. An energy difference of only 1.4 kcal at 25° (corresponding to a tenfold predominance) would be sufficient to make the A-side form practically unobservable by pmr spectroscopy in the presence of the predominant B form and from the calculations this energy difference could be expected to be even higher. The postulated dependence of the primary acid reaction on the energy barrier to rotation of the αTHN$^+$ ring around the ribosidic bond suggests that the specificity of the cyclization reaction would represent a general reaction of all dihydropyridine nucleotides in dilute acid, independent of the substituent at the C-3 position of the dihydropyridine ring. The strong, positive Cotton effects in the CD spectra for the acid products of NADH, (AcPy)ADH, and (TN)ADH support this interpretation.

Pmr studies of βNADH have shown that the dihydronicotinamide proton absorptions do not show significant changes in their chemical shifts down to pH 3 (Catterall *et*

[7] The anomerization of pseudouridine is a special case. Both the acid and base catalyze the opening of the ribose ring to generate a stable carbon-carbon double bond between the ribose C-1′ and the base, allowing for facile anomerization (Chambers, 1966).

al., 1969; Griffith *et al.*, 1970). These reports suggest that N-1 of the dihydronicotinamide ring apparently does not have a pK greater thn 2.5, otherwise some effect would be expected on the C-2 or C-6 protons. Likewise, the acid-catalyzed reactions of βNADH do not appear to involve direct protonation of N-1. Instead protonation occurs at either the C-5 or O-1′ position in the dihydronicotinamide nucleotide and is accompanied by a rearrangement of electrons or bonds to generate a positive charge on N-1. These protonated intermediates must either be in slow exchange on the pmr time scale with the unprotonated species (as would be the case for the irreversible protonation at C-5) or be present in concentrations of less than 5% at pH 3 in order to account for the apparent lack of titration effect.

The intriguing protonated intermediates in the acid-catalyzed reactions raise questions as to the role they might play in the activation of the coenzyme on a dehydrogenase. The inductive effects at C-5 or O-1′ caused by interactions with charged side chains of amino acid residues in the active side of the dehydrogenase could influence the redox potential of the bound coenzyme. By altering the electron density at N-1, these groups could activate the coenzyme in the transition of the aromatic nicotinamide ring with a full positive charge to or from the electron-rich, nonaromatic, uncharged dihydronicotinamide ring. These two inductive sites are alternative or complementary to the proposal of Kosower (1962) whereby the dehydrogenases might influence the microscopic redox potential of the coenzyme by inductive effects aimed directly at the ring nitrogen.

The determination of the structure of the primary acid product of βNADH as an α-$O^{2'}$-6B-cyclotetrahydronicotinamide adenine dinucleotide, that the reaction is stereospecific and involves an anomerization of the pyridine–ribose configuration, provides a new insight into the heretofore unsuspected complexity of the acid reaction of reduced pyridine nucleotides. The structure of (cTHN)AD takes into account the observations made over several decades by investigators using a variety of techniques and demonstrates the power and unique capabilities of high-frequency pmr spectroscopy in the determination of the structure of complex molecules of biological importance. Further studies into the kinetics and intermediates of these acid-catalyzed reactions of βNADH should yield information regarding the chemistry of the reduced pyridine coenzyme as it pertains to the active-site transition complexes of the dehydrogenases.

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Glyceraldehyde-3-phosphate Dehydrogenase Catalyzed Hydration of the 5–6 Double Bond of Reduced β-Nicotinamide Adenine Dinucleotide (βNADH). Formation of β-6-Hydroxy-1,4,5,6-tetrahydronicotinamide Adenine Dinucleotide†

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ABSTRACT: High-frequency proton magnetic resonance studies at 220 MHz of the modified nicotinamide coenzyme from βNADH by glyceraldehyde-3-phosphate dehydrogenase establish that the enzyme catalyzed the nonspecific addition of water across the 5–6 double bond of the dihydronicotinamide ring of βNADH to form a β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide, (6HTN)AD. Formation of (6HTN)AD in D_2O leads to a random incorporation of deuterium into the C-5 position with no evidence for exchange of the C-5 proton. Hydroxylation at the C-6 position occurs with the generation of unequal populations of the two diastereomers: 65% A-side hydroxyl and 35% B-side hydroxyl. The proton absorptions for each of the diastereomeric forms of the 6-hydroxytetrahydronicotinamide ring have been assigned and the coupling constants determined by computer analysis. Mechanisms for the formation of (6HTN)AD by glyceraldehyde-3-phosphate dehydrogenase are discussed involving catalysis by either the polybasic anion bound in the active site or by acidic amino acid residues in close proximity to the 5–6 double bond of βNADH.

Glyceraldehyde-3-phosphate dehydrogenase, G3PD,[1] in the pH range below 7.5 catalyzes the slow conversion of βNADH to a modified pyridine coenzyme absorbing at 280 nm (Rafter *et al.,* 1954). This compound has been referred to as NADHX and has been shown to have distinct enzymatic properties from those of the primary acid product of NADH (Meinhart *et al.,* 1956; Stock *et al.,* 1961). The structures of these two modified pyridine nucleotides are related since NADHX is rapidly and irreversibly converted to the primary acid product at pH 4 (Meinhart *et al.,* 1956). The isolation from yeast of an ATP-requiring enzyme which catalyzes the conversion of NADHX to NADH has led to speculation about possible NADH–phosphate intermediates in oxidative phosphorylation (Griffiths and Chaplain, 1962; Barltrop *et al.,* 1963; Bechara and Cilento, 1971, 1972). The presence of this enzyme which has NADHX as a substrate and the report that G3PD can be isolated with bound NADHX (Pfleiderer and Stock, 1962) indicate the probable synthesis and presence of NADHX *in vivo.* However, there is as yet no evidence for any physiological role for this modified pyridine coenzyme.

An enzymatic reaction which converts NADH to a form which is inactive with dehydrogenases, especially when catalyzed as a side reaction of the important enzyme G3PD, is of considerable interest. The formation of NADHX by G3PD whose normal reaction is to promote substrate level

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[1] The NAD^+ nomenclature is used in order to abbreviate more clearly these modified nicotinamide coenzymes. Abbreviations used are: (6HTN)AD, β-6-hydroxytetrahydronicotinamide adenine dinucleotide, formed by glyceraldehyde-3-phosphate dehydrogenase (G3PD) from βNADH [(6HTN)AD was originally called NADHX by Rafter *et al.* (1954)]; DCB-6HTN, dichlorobenzyl-6-hydroxytetrahydronicotinamide; (cTHN)AD, α-$O^{2'}$-6B-cyclotetrahydronicotinamide adenine dinucleotide, the primary acid product of βNADH.