Rafter, G.W., Chaykin, S., and Krebs, E.G. (1954), *J. Biol. Chem. 208,* 799.
Remin, M., and Shugar, D. (1972), *Biochem. Biophys. Res. Commun. 48,* 636.
Sarma, R.H., and Mynott, R.J. (1973), *J. Amer. Chem. Soc. 95,* 7470.
Schleich, T., Blackburn, B.J., Lapper, R.D., and Smith, I.C.P. (1972), *Biochemistry 11,* 137.
Stamhuis, E.J., and Maas, W. (1965), *J. Org. Chem. 30,* 2156.
Stock, A., Sann, E., and Pfleiderer, G. (1961), *Justus Liebigs Ann. Chem. 647,* 188.
Supple, J.H., Nelson, D.A., and Lyle, R.E. (1963), *Tetrahedron Lett. 24,* 1645.
Tsuboi, M., Kainosho, M., and Nakamura, A. (1968), *in* Recent Developments of Magnetic Resonance in Biological Systems, Furiwara, S., and Piette, I.H., Ed., Tokyo, Hirokawa Publishing Co., p 43.
Tsuboi, M., Takahashi, S., Kyogoku, Y., Hayatsu, H., and Kainosho, M. (1969), *Science 166,* 1504.
Warburg, O., Christian, W., and Griese, A. (1935), *Biochem. Z. 282,* 157.
Woenckhaus, C., and Zumpe, P. (1965), *Biochem. Z. 343,* 326.
Zinner, K., Faljoni, A., and Cilento, G. (1973), *Biochem. Biophys. Res. Commun. 51,* 181.

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†

Norman J. Oppenheimer‡ and Nathan O. Kaplan*

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.

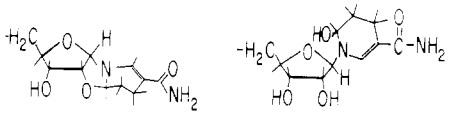
Glyceraldehyde-3-phosphate dehydrogenase, G3PD,[1] in the pH range below 7.5 catalyzes the slow conversion of βNADH to a modified pyridine coenzyme absorbing at 280 nm (Rafter *et al.,* 1954). This compound has been referred to as NADHX and has been shown to have distinct enzymatic properties from those 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

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92037. *Received March 20, 1974.* This work was supported in part by grants from the American Cancer Society (BC-60) and the National Institutes of Health (CA 11683).

‡ Present Address: Department of Chemistry, Indiana University, Bloomington, Ind. 47401.

[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.



(cTHN)AD (6HTN)AD

FIGURE 1: The structure of the tetrahydronicotinamide-ribose moieties of the primary acid product (cTHN)AD and the G3PD modified NADH, (6HTN)AD.

oxidative phosphorylation raises the possibility of a relationship between the reaction which forms a high-energy phosphoric acid-carboxylic acid anhydride bond and the formation of NADHX. Furthermore, in order to understand the chemistry of possible phosphate-dihydronicotinamide addition complexes, the structure of NADHX must be conclusively determined. This is especially important in view of the complex chemistry displayed by NADH in the overall primary acid reaction (Oppenheimer, 1973; Oppenheimer and Kaplan, 1974a).

We report here the results of proton magnetic resonance (pmr), circular dichroism (CD), and deuterium labeling experiments which conclusively establish that the modified coenzyme, NADHX, generated by G3PD is a β-6-hydroxytetrahydronicotinamide adenine dinucleotide, (6HTN)AD, in contrast to the primary acid product α-$O^{2'}$-6B-cyclotetrahydronicotinamide adenine dinucleotide, (cTHN)AD. The structure of both dinucleotides is shown in Figure 1. This investigation combined with the previous report (Oppenheimer and Kaplan, 1974a) on the primary acid reaction form a basis for a detailed understanding of the complex chemistry of NADH at low pH.

Methods

Preparation of Compounds. The dinucleotides, βNAD$^+$ and βNADH, were obtained from P-L Biochemicals and used without further purification. Reduced coenzyme specifically labeled on the B side, $NADD_B$, was prepared by reduction in D_2O with pig heart lipoyl dehydrogenase (EC 1.6.4.3, Boehringer-Mannheim) as described by Oppenheimer *et al.* (1971). The reduced coenzyme with both the A and B positions labeled with deuterium, $NADD_{AB}$, was prepared by oxidation of $NADD_B$ with the A specific enzyme yeast alcohol dehydrogenase (EC 1.1.1.1, P-L Biochemicals) to yield 4-deuterio-NAD$^+$ which was then reduced again by lipoyl dehydrogenase in D_2O. 1-(2,6-Dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide was prepared according to the procedure of Kim and Chaykin (1968).

Yeast G3PD (EC 1.2.1.12) was prepared from Fleishman's yeast according to the procedure of Butler and Jones (1970). The modified reduced coenzyme (6HTN)AD was prepared by incubation of 10 mM NADH with 15 mg/ml of yeast G3PD in 0.05 M pyrophosphate buffer at pH 6.0. When the 340-nm ultraviolet (uv) absorption of the dihydronicotinamide ring had decreased to less than 10% of the initial value the reaction was stopped by adding 2 M NH_4OH to pH 8.0. The (6HTN)AD was recovered from the G3PD by pressure dialysis through an Amicon PM-30 membrane. The (6HTN)AD solution was placed on a DEAE-11 cellulose column and eluted with a linear 5–500 mM ammonium bicarbonate gradient. The acid product, (cTHN)AD, was prepared by incubation of an unbuffered 10 mM NADH solution at pH 2.5 (Oppenheimer, 1973).

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

Proton magnetic resonance spectra were recorded on a Varian Associates field sweep HR-220 proton magnetic resonance spectrometer with the signal-to-noise ratio enhanced by a Nicolet 1074 computer. Homonuclear spin decoupling was performed using a Wavetek 131A voltage controlled oscillator. The samples were twice lyophilized from 99.8% D_2O and then dissolved in 100% D_2O. The sample volume was 0.2 ml and Teflon vortex plugs (Wilmad) were used. An internal standard, 3 mM sodium 3-trimethylsilylpropionate (tetradeuterio), was used and 1 mM EDTA was added to prevent line broadening from possible paramagnetic impurities. The pD of the samples was measured with a Radiometer Model 25 pH meter with a combination electrode using the standard electrode correction for D_2O, pD = meter reading + 0.4 (Glasoe and Long, 1960). Computer simulations of the pmr spectra were generated on a Nicolet 1080 computer using the Nicolet NMRCAL program, a six-spin program, and manipulated using subroutines of the Nicolet FTNMR program.

Results

DCB-6HTN. The values of the chemical shifts for the ring protons of DCB-6HTN are listed in Table I,[2] and the coupling constants are listed in Table II. The protons were assigned by appropriate deuterium labeling. A nonspecific deuterium label at the C-4 position generated by reduction of dichlorobenzylnicotinamide in D_2O followed by incubation in acid assigns the C-4 protons, while the formation of DCB-6HTN in D_2O–tetrahydrofuran assigns the C-5 protons. Homonuclear spin decoupling and analysis of the coupling constants were used to confirm the assignments (Oppenheimer, 1973).

The X-ray crystal structure for DCB-6HTN reported by Hope (1969) provides a basis for our pmr conformational analysis of the 6HTN ring in solution. In the crystal the substituents on the C-4, C-5, and C-6 atoms are aligned in a rigid, staggered conformation. We have calculated the dihedral angles between these substituents (Table III) using the atomic coordinates (Hope, 1969) and from these calculations have constructed the Newman projections shown in Figure 2. The important features of the conformation of the 6HTN ring in the crystal are: (1) the hydroxyl at the C-6 position is axial and (2) the protons on the C-4, C-5, and C-6 atoms are staggered with distinct axial and equatorial orientations.

Conformational analysis using pmr is possible because the vicinal coupling constants for protons on adjacent atoms are a function of the dihedral angle between the protons (Karplus, 1959, 1963). Therefore, if the conformation of the 6HTN ring in solution resembles the conformation in the crystal, the observed coupling constants in the pmr spectrum should correspond to the coupling constants that can be calculated using the observed dihedral angles in the crystal.

A comparison of the observed and calculated coupling

[2] The values for the chemical shifts of the C-4 and C-5 methylene protons of DCB-6HTN are corrected from the values listed in Table II (Oppenheimer, 1973) where the chemical shifts for these four protons are reported offset by 150 Hz.

TABLE I: Chemical Shifts of the Protons of DCB-6HTN,[a] (6HTN)AD,[b] and (cTHN)AD[b] at 220 MHz.[c]

	PC_2H	PC_6H	PC_4H_{ax}, A	PC_4H_{eq}, B	PC_5H_{eq}	PC_5H_{ax}	$PC_{1'}H$	AC_8H	AC_2H	$AC_{1'}H$
DCB-6HTN	1732	1142	644	590	500	395				
(6HTN)AD, A-OH	1592	1147.5	480	482.5	442	312	1084.5	1864	1799	1340.5
(6HTN)AD, B-OH	1594	1129.5	492.5	492.5	450	341	1084.5	1864	1799	1340.5
(cTHN)AD	1592	1098	450	492	485	278	1218	1868.5	1801.5	1342

[a] Hertz from tetramethylsilane in pyridine-d_5. [b] Hertz from sodium trimethylsilylpropionate in D_2O. [c] Chemical shifts are reported to within 0.5 Hz.

constants for the 6HTN protons listed in Table III reveals a negligible difference in the values for the coupling constants between the C-4 and C-5 methylene protons. The lack of an exact fit for the values of J_{5-6} does not necessarily mean that subtle changes in the conformation of the 6HTN ring occur when the molecule is in solution. The Karplus relationship is a semiempirical formula; thus, the values of the observed coupling constants reflect not only the dihedral angle between the protons, but to a lesser extent the electronegativity of the substituents, ring strain, altered electron density, etc. Therefore, the calculated value of the J_{5-6} of 4.0 Hz for 6HTN compared to the observed value of 2.5 Hz may reflect the electron withdrawing nature of the C-6 hydroxyl substitution and the inadequacy of using the same empirical constants for the calculation of J_{5-6} as were used for the calculation of the C-4 and C-5 methylene coupling constants. The qualitative fit of the data, however, makes it reasonable to conclude that the conformation of the 6HTN ring in solution differs little from that found in the crystal lattice and that the analysis of the coupling constants provides meaningful structural information. The small and equal values of J_{5-6} are consistent only with an equatorial proton, hence an axial substitution. The large trans and small gauche couplings between the C-4 and C-5 methylene protons are consistent with the staggered conformation observed in the crystal.

TABLE II: Coupling Constants of the 6HTN Protons of DCB-6HTN, (6HTN)AD, and (cTHN)AD.

Coupling Constants[a]	DCB-6HTN[b]	(6HTN)AD[c] A-OH[d]	(6HTN)AD[c] B-OH[e]	(cTHN)AD[d]
$J_{5_{ax}-5_{eq}}$	−13.1	−13.3	−13.3	−11.7
$J_{5_{ax}-4_{ax}}$	13.3	13.5	13.5[f]	13.0
$J_{5_{ax}-4_{eq}}$	6.0	5.3[f]	5.3	5.3
$J_{5_{ax}-6}$	2.5	2.5	2.5	9.5
$J_{5_{eq}-4_{ax}}$	5.6	5.0	5.0[f]	5.4
$J_{5_{eq}-4_{eq}}$	2.0	2.0[f]	2.0	2.0
$J_{5_{eq}-6}$	2.5	2.5	2.5	4.2
$J_{4_{ax}-4_{eq}}$	−15.8	−16[f]	−16[f]	−16[f]
$J_{4_{ax}-2}$	2.0	2.0	2.0[f]	1.8

[a] The coupling constant values are accurate to within 0.2 Hz except as noted in footnote *f*. [b] In pyridine-d_5. [c] Coupling constant values were obtained from (6HTN)AD($4D_B$) and (6HTN)AD($4D_{AB}$) except as noted in footnote *f*. [d] The C-4A proton is axial; the C-4B proton is equatorial. [e] The C-4B proton is axial; the C-4A proton is equatorial. [f] These coupling constants were obtained from the computer simulation of the spectrum. The values can be varied over a range of ±0.4 Hz before significantly affecting the fit of the computer-generated spectra.

TABLE III: Calculated Vicinal Coupling Constants for the 6HTN Protons.

	Dihedral Angle (deg) $\phi_{H_iH_j}$[a]	Coupling Constants (Hz) J_{calcd}[b]	J_{obsd}[c]
$H_{4_{ax}}H_{5_{ax}}$	173	13.4	13.3
$H_{4_{eq}}H_{5_{ax}}$	51	5.2	6.0
$H_{4_{ax}}H_{5_{eq}}$	46	6.0	5.6
$H_{4_{eq}}H_{5_{eq}}$	77	1.8	2.0
$H_{5_{ax}}H_6$	58	4.0	2.5
$H_{5_{eq}}H_6$	58	4.0	2.5

[a] ϕ, the dihedral angle between vicinal protons calculated from the positional parameters, after translation to orthogonal coordinates, for crystalline DCB-6HTN using the data contained in Table I of Hope (1969). [b] Calculated vicinal coupling constants for the dihedral angle ϕ using the equation $J_{vic} = A + B\cos 2\phi + C\cos 2\phi$. The empirical constants, $A = 7$ Hz, $B = -1$ Hz, and $C = 5.5$ Hz, were used (Bothner-By, 1965). [c] The experimentally observed values of the coupling constants.

The fit of the observed and calculated coupling constants also indicates that the 6HTN ring in solution is conformationally rigid. Rapid interconversion of the tetrahydronicotinamide ring would interchange the axial and equatorial protons, hence averaging the coupling constants to the population weighted, mean value of the trans and gauche coupling constants.

One final point should be made concerning the pmr spectrum of DCB-6HTN. The addition of hydroxyl at the C-6

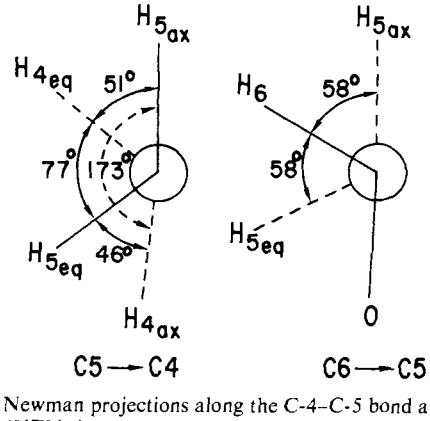


FIGURE 2: Newman projections along the C-4–C-5 bond and C-5–C-6 bond of the 6HTN ring.

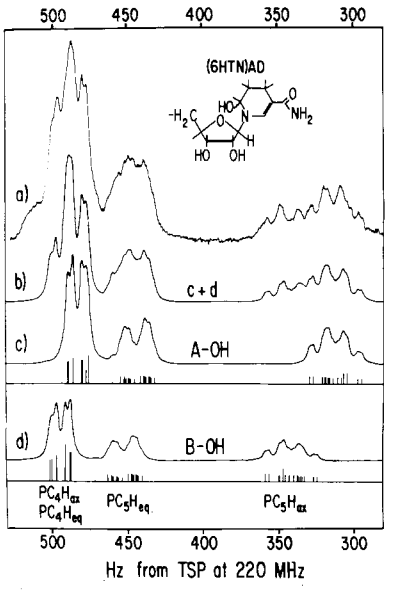


FIGURE 3: (a) The pmr spectrum of the C-4 and C-5 methylene protons of (6HTN)AD. (b) The computer-simulated spectrum of the methylene protons generated from the sum of the subspectra c and d. (c) The calculated subspectrum of the A side hydroxyl 6HTN; weighted amplitude, 65. (d) The calculated subspectrum of the B side hydroxyl 6HTN; weighted amplitude, 35. The values of chemical shifts and coupling constants used for these calculations are listed in Tables I and II.

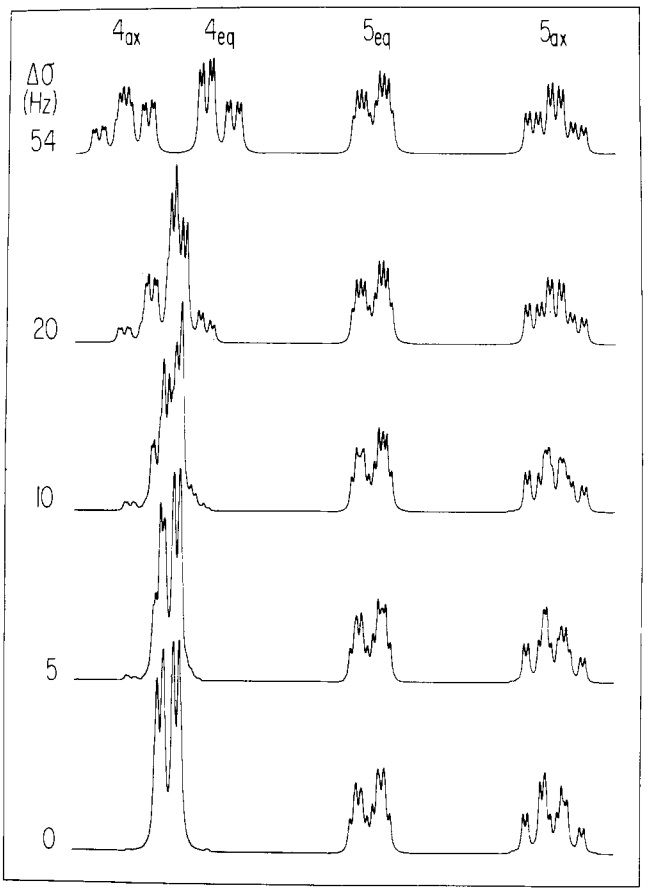


FIGURE 4: The effects of varying the chemical-shift nonequivalence of the C-4 methylene protons on the simulated spectral pattern of the methylene proton region. The top spectrum corresponds to the pattern found in DCB-6HTN; as the chemical-shift difference between the C-4 methylene protons decreases, distortions occur in the absorption pattern of the C-5 axial proton. The C-5 proton resembles the pattern for (6HTN)AD when the C-4 methylene protons are within 5 Hz of each other.

position generates an asymmetric center. However, since the two isomeric forms are related as mirror images, they are enantiomeric.[3] The pmr spectra of an enantiomeric pair are superimposable and indistinguishable in an isotropic solution (Mislow and Raban, 1966; Alworth, 1972); hence the spectrum of DCB-6HTN consists of a single set of absorptions.

(6HTN)AD. The chemical shifts for the 6HTN ring protons of (6HTN)AD are listed in Table I, and the coupling constants are listed in Table II. The pmr spectrum of the C-4 and C-5 methylene protons of (6HTN)AD is shown in Figure 3. The protons were assigned by deuterium labeling and by the similarity of the coupling constants to the 6HTN model compound. The chemical-shift values for the C-5 methylene protons were measured directly from the spectrum of (6HTN)AD($4D_{AB}$) formed from $NADD_{AB}$. The values for the chemical shifts and coupling constants of the C-4 methylene protons are based on the computer simulation of the spectrum shown in the botton of Figure 3. The large error limits for the C-4 chemical shifts and coupling constants reflect the range over which these parameters can be varied without altering the fit of the simulated spectrum. The computer analysis confirms the negative geminal coupling constants for the C-4 and C-5 methylene protons. The spectrum also indicates that the 6HTN rings of both diastereomeric forms have the same ring conformation (the presence of two diastereomeric forms of the 6HTN ring will be discussed below). The correspondence of the coupling constants of the 6HTN rings of the dinucleotides with DCB-(6HTN) establishes unequivocally the structure of the modified coenzyme as a 6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide. Furthermore, the observed conformation of the 6HTN ring based on pmr data is identical with the conformation of the DCB-6HTN molecule in the crystal (Hope, 1969).

The importance of computer simulation and deuterium labeling in the analysis of the pmr spectra of (6HTN)AD cannot be overly stressed. The top spectrum in Figure 4 is the computer simulation of the methylene protons of DCB-6HTN (the observed pmr spectrum is shown in Figure 5). The C-5_{ax} proton of DCB-6HTN has an overall appearance of a triplet with two large coupling constants of 13.3 and −13.1 Hz to the C-4_{ax} and C-5_{eq} protons, a smaller 6.0-Hz coupling constant to the C-4_{eq} proton, and a small 2.5-Hz coupling constant to the C-6 proton. In the pmr spectrum of (6HTN)AD, however, the C-5_{ax} protons have the overall appearance of a quartet (Figure 3). It would appear from the spectrum of (6HTN)AD that the two coupling constants from the C-5_{ax} proton to the C-4 methylene protons are nearly equal, about 9 Hz each. Furthermore, the C-4 methylene protons have the appearance of a two-proton doublet of doublets with a 9-Hz coupling constant to the C-5_{ax} proton and a 3.5-Hz coupling constant to the C-5_{eq} proton. This pattern for the C-4 methylene protons is in sharp contrast to the triplet pattern for the C-4_{ax} proton and the doublet pattern of the C-4_{eq} proton of DCB-6HTN model compound (Figure 5a).

The apparently obvious conclusion from these spectral differences would be that the conformation of the 6HTN ring of the dinucleotide was drastically different from that

[3] The term diastereomer will be used for compounds which are not related as enantiomers, *i.e.* stereoisomers which are not superimposable by any symmetry operation.

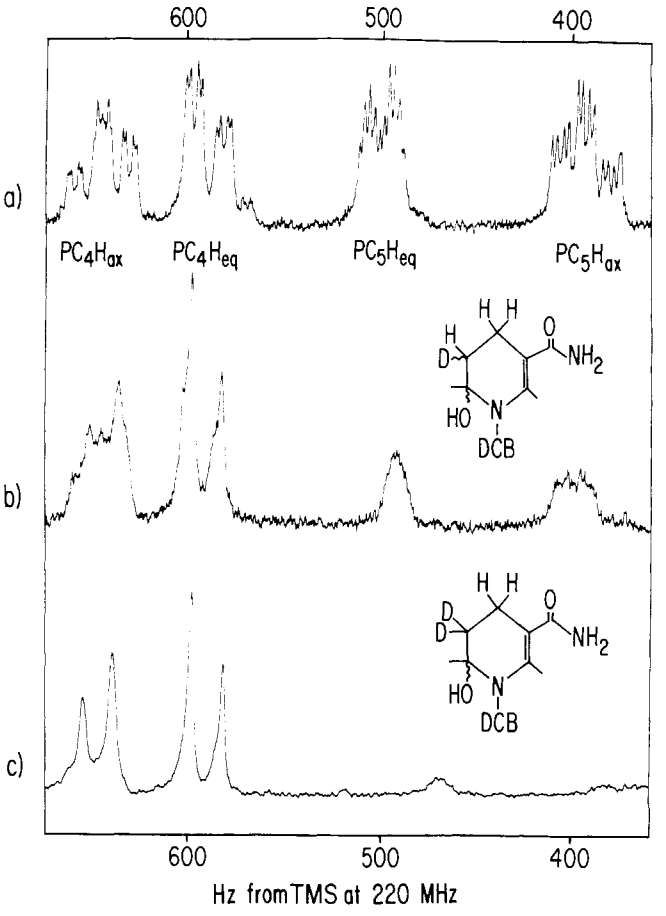


FIGURE 5: (a) The C-4 and C-5 methylene proton regions of the pmr spectrum of DCB-6HTN dissolved in pyridine-d_5. (b) DCB-6HTN formed in 40% D_2O–60% tetrahydrofuran; the C-5 methylene shows a nonspecific incorporation of deuterium. (c) DCB-6HTN formed in 1.5% D_2O–98.5% tetrahydrofuran; the C-5 methylene protons have completely exchanged with deuterium.

of DCB-6HTN. However, the observed equal values of $J_{5_{ax}-4_{ax}}$ and $J_{5_{ax}-4_{eq}}$ in the nonlabeled (6HTN)AD do not correspond to the values for these coupling constants measured in the specifically deuterium labeled dinucleotide, (6HTN)AD(4D_B), where $J_{5_{ax}-4_{ax}}$ = 13.5 Hz and $J_{5_{ax}-4_{eq}}$ = 5.3 Hz. The discrepancy between the coupling constants observed in the nonlabeled (6HTN)AD and DCB-6HTN or the deuterium labeled (6HTN)AD(4D_B) can be completely accounted for by the strong coupling[4] of the two C-4 protons as is shown by the simulated spectra in Figure 4.

The observed splitting pattern of the C-4 and C-5 protons of (6HTN)AD represents an example of a "deceptively simple" pmr spectrum (Becker, 1969). The chemical-shift equivalence of the C-4 protons in the B form and the 2.5-Hz nonequivalence in the A form of (6HTN)AD cause severe second-order effects in the coupling constants to the C-5 methylene protons. The ambiguity in the C-4–C-5 methylene proton coupling constants arises because the two strongly coupled C-4 protons are chemical-shift equivalent and hence are indistinguishable by pmr.[5] As a result of this

[4] A spin system is very strongly coupled if the ratio of the mutual coupling constants to the chemical-shift difference between the nuclei is less than 1.

[5] Chemical-shift equivalence is the fortuitous coincidence of the chemical shifts of nuclei. Chemical-shift equivalence is not to be confused with chemically or magnetically equivalent nuclei which are inherently indistinguishible because of symmetry and hence must have coincident chemical shifts.

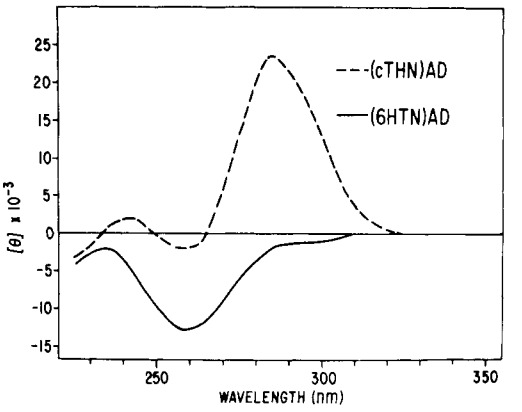


FIGURE 6: Comparison of the CD spectra at 25° and pH 8.1 of (cTHN)AD and the enzymatically modified NADH, (6HTN)AD.

fortuitous equivalence, the C-5_{ax} proton which should have a large 13.5-Hz trans coupling constant to the C-4_{eq} proton based on the expected conformation of the 6HTN ring instead shows an equal 9-Hz coupling constant to both the C-4 protons. The 9-Hz value corresponds to the arithmetic mean of the $J_{5_{ax}-4_{ax}}$ and $J_{5_{ax}-4_{eq}}$. The same is true for the C-5_{eq} proton; it is coupled to the C-4 protons with the values equal to the mean of $J_{5_{eq}-4_{ax}}$ and $J_{5_{eq}-4_{eq}}$.

The coupling constants observed for such a strongly coupled spin system are by themselves ambiguous; any values for the C-5 proton coupling constants can be used to generate the observed spectral pattern of the nonlabeled (6HTN)AD (Figure 3) subject only to the constraints that neither of the values are less than zero and that their sum equals 18.8 Hz for the C-5_{ax} proton and 7.0 Hz for the C-5_{eq} proton. The true coupling constants, that is the coupling constants that are solely a function of the conformation of the 6HTN ring *via* the Karplus relationship, can only be unambiguously determined from the coupling constants to the remaining proton of a specific deuterium label at the C-4 position. In the deuterium labeled compound the remaining C-4 proton is no longer strongly coupled to the geminal proton and thus has a first-order interaction with the other protons of the 6HTN ring. The unambiguous determination of the coupling constants for the specifically labeled (6HTN)AD and the fit of the C-5_{ax} proton's quartet shown in Figures 3 and 4 that can be obtained using these coupling constants provide unequivocal evidence that the conformation of the 6HTN ring in (6HTN)AD is identical with that for DCB-6HTN.

Circular Dichroism. As can be seen from Table II, the coupling constants of the C-4 and C-5 methylene protons in the 6HTN ring are quite similar to the corresponding couplings in the cTHN ring of the acid product. However, the values of the C-5 methylene–C-6 proton coupling constants are completely different and correspond to an equatorial C-6 substitution in the cTHN ring as opposed to the axial C-6 substitution in 6HTN. This large difference in structure is not manifest in their uv spectra, although subtle differences do exist; specifically (6HTN)AD has a lower extinction coefficient at 300 nm than (cTHN)AD (Meinhart *et al.*, 1956). As would be expected, however, a dramatic difference is observed in the CD spectra of (6HTN)AD *vs.* (cTHN)AD (Figure 6). The 280-nm chromophore of (cTHN)AD has a strong positive Cotton effect (Miles *et al.*, 1968) while the corresponding chromophore of (6HTN)AD has a negligible Cotton effect (Zinner *et al.*, 1973). The lack of a Cotton effect for (6HTN)AD can be

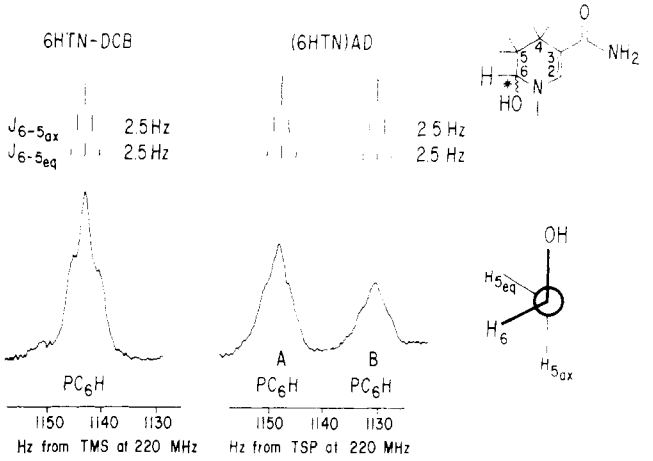


FIGURE 7: The C-6 proton absorption of DCB-6HTN, left, and (6HTN)AD, right. The small, equal coupling constant of 2.5 Hz to the C-5 methylene protons indicate a staggered conformation as shown in the Newman projection along the C-6–C-5 bond.

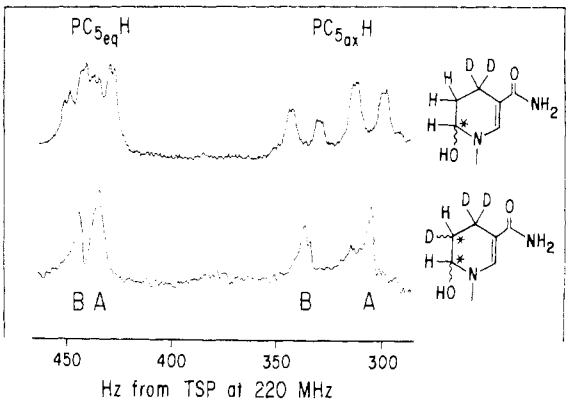


FIGURE 8: The pmr spectrum of the C-5 methylene protons of (6HTN)AD($4D_{AB}$) formed in H_2O (top spectrum) and in D_2O (bottom spectrum). The four peaks in the botton spectrum correspond to the four diastereomeric forms generated by the two asymmetric centers shown by asterisks. The absorptions correspond to the 6B-OH, 5_{ax}-D; 6A-OH, 5_{ax}-D; 6B-OH, 5_{eq}-D; and 6A-OH, 5_{eq}-D. The approximately equal areas under the axial and equatorial proton absorptions indicate a 50:50 random incorporation of deuterium at the C-5 position.

explained by the presence of two diastereomeric forms and free rotation around the β-ribosidic bond. The CD spectra are discussed in more detail in other papers on the primary acid product of NADH (Oppenheimer and Kaplan, 1974a,b).

Diastereomers. The addition of water across the 5–6 double bond of the dihydronicotinamide ring is expected to generate an asymmetric center at the C-6 position.[6] In DCB-6HTN the two stereoisomers are related as mirror images; hence they are enantiomeric and the corresponding protons of each isomer are magnetically equivalent under all isotropic conditions (Mislow and Raban, 1966). The faces of the dihydronicotinamide ring of NADH, however, are not enantiomeric. The A and B faces are not related as mirror images nor by any other symmetry operation because of the chirality of the attached D-ribose and the remainder of the dinucleotide. Thus the addition of hydroxyl to the C-6 position will lead to the generation of two nonsuperimposable stereoisomers, which by definition must be diastereomers. The presence of diastereomeric forms is expected to cause "resonance doubling" in the pmr spectrum, that is the superposition of two distinct spectra, one for each of the diastereomers, because in principle diastereomers are chemical-shift nonequivalent (Mislow and Raban, 1966). In other studies of the pyridine coenzymes, resonance doubling in the pmr spectrum due to the presence of diastereomeric forms has also been observed, *e.g.*, the NAD^+–cyanide adduct (Oppenheimer *et al.*, 1971) and the NAD^+–pyruvate adduct (Arnold and Kaplan, 1974). The C-6 proton absorptions of (6HTN)AD clearly show resonance doubling and therefore the presence of two diastereomeric forms (Figure 7). Resonance doubling is also clearly observable for the other proton absorptions of the 6HTN ring (Figures 3, 8, and 10).

The C-6 protons appear as a broadened triplet with equal and small 2.5-Hz coupling to the C-5 methylene protons as well as a small unresolved coupling of less than 1.0 Hz to the C-2 proton. The observed absorptions are identical with those for the C-6 proton of DCB-6HTN as is shown in Figure 7. The similarity in the coupling constants of both forms of (6HTN)AD establishes that the two diastereomers have the identical conformation, *i.e.* the C-6 hydroxyl is axial and differs only in the stereoconfiguration.

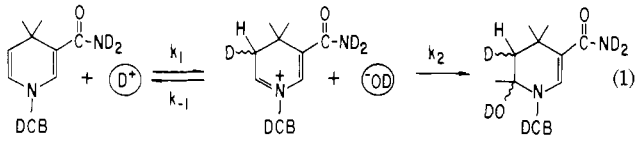
The C-6 proton absorptions of the two diastereomeric forms of (6HTN)AD are, however, of unequal population: a high population form, form A, and a low population form, form B, in a ratio of about 2:1 (65% to 35%). The presence of both diastereomeric forms demonstrates that the G3PD catalyzed reaction results in the nonspecific addition of hydroxyl at C-6 with a slight preference for hydroxylation of one side of the pyridine ring over the other. This lack of specificity is in sharp contrast to the generally assumed stereospecific nature of enzymatic reactions. The determination of the absolute configuration of the two forms by specific deuterium labeling will be discussed below.

Ribose–Base Configuration. The large value of the $J_{1'-2'}$ coupling constant of 7.1 Hz for the PC-1′ ribose proton is similar to the value reported for the PC-1′ ribose proton of βNADH (Oppenheimer *et al.*, 1971). This result is consistent with the 6HTN–ribose linkage having the same anomeric configuration as βNADH and a similar ribose conformation as well. Furthermore, the PC-1′ proton comes 135 Hz upfield from the αPC-1′ proton of the (cTHN)AD (Table I). The αPC-1′ ribose proton occurs about 0.2–0.5 ppm downfield from the βPC-1′ proton in NAD^+ (Lemieux and Lown, 1963) and in NADH (Oppenheimer and Kaplan, 1974c). The downfield position of the αPC-1′ ribose proton of (cTHN)AD relative to the βPC-1′ proton of (6HTN)AD is consistent with this general rule. The resemblance of the pmr spectrum of the ribose of (6HTN)AD to that of βNADH is also consistent with a similar ribose configuration and conformation. The PC-5′ ribose protons are chemical-shift equivalent as are the PC-5′ protons of βNADH and unlike the PC-5′ protons of (cTHN)AD and αNADH which show a large chemical-shift nonequivalence (unpublished data). The PC-5′ protons were assigned by ^{31}P heteronuclear decoupling. A more detailed analysis of the ribose proton absorptions was impossible because of the poor resolution due to the overlapping absorptions of the two diastereomeric forms.

Deuterium Incorporation. The acid reaction of model dihydronicotinamide compounds when performed with high concentrations of water shows a large kinetic deuterium or tritium isotope effect (Kim and Chaykin, 1968). However,

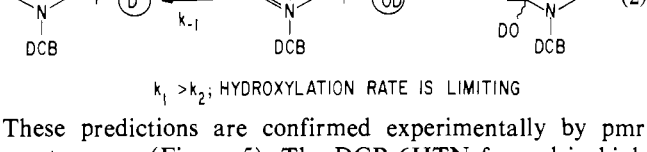
[6] Recently Zinner *et al.* (1973) have also commented upon the possibility of diastereomeric forms of the G3PD modified coenzyme based on the work of Meinhart *et al.* (1956).

as the reaction medium is made more anhydrous the kinetic isotope effects become negligible. This suggests that protonation is the rate limiting step of the hydration reaction under aqueous conditions and hydroxylation under anhydrous conditions. We have obtained conclusive evidence for this mechanism by the study of the incorporation of deuterium into the 6HTN ring. In 40% D_2O–60% tetrahydrofuran where protonation is rate limiting the reaction should lead to the random incorporation of only one deuterium atom at the C-5 position as shown in eq 1.[7] On the other hand in



$k_2 > k_1$; PROTONATION RATE IS LIMITING

1.5% D_2O–98.5% tetrahydrofuran where no deuterium isotope effect is observed the reaction should lead to the exchange of the C-5 proton with the incorporation of two atoms of deuterium at the C-5 position as is shown in eq 2.



$k_1 > k_2$; HYDROXYLATION RATE IS LIMITING

These predictions are confirmed experimentally by pmr spectroscopy (Figure 5). The DCB-6HTN formed in high D_2O concentration shows two broad peaks corresponding to the C-5_{eq} and C-5_{ax} protons. These two peaks have a combined area of one proton and are equal to half a proton each. The DCB-6HTN formed in low D_2O concentration, however, shows no absorptions due to either of the C-5 protons. The C-4 methylene protons now appear as an AB pattern with the C-4_{ax} proton broadened because of a long-range, 2.0-Hz coupling to the C-2 proton.

Kinetic studies were made of the D_2O solvent effect on the synthesis of (6HTN)AD by yeast G3PD. The rate of the reaction of 0.1 mM NADH at pH 6.2 and 6.6 in 0.1 M pyrophosphate buffer with 0.7 mg/ml of G3PD was compared with the rate in 0.1 M pyrophosphate buffer in D_2O at pD 6.2 and 6.6. The reaction has an apparent deuterium isotope effect of 1.7 ± 0.2 at pH 6.2/pD 6.2 and 2.1 ± 0.2 at pH 6.6/pD 6.6. The magnitude of the isotope effect is consistent with the isotope effects observed for the hydrolysis of enamines by Stamhuis and Maas (1965) and is also consistent with a mechanism involving protonation of the dihydronicotinamide ring as the rate limiting step in the formation of (6HTN)AD.

Deuterium incorporation experiments by Meinhart and Hines (1957) indicate that only one atom of deuterium is incorporated per mole of (6HTN)AD formed enzymatically in D_2O and therefore exchange at the C-5 position does not occur. The presence of only one atom of deuterium at the C-5 position is not sufficient evidence to support the hypothesis of a rate-limiting protonation step since enzymatic reactions are usually assumed to be stereospecific for the addition or removal of protons. Therefore a stereospecific enzyme should not exchange the C-5 proton even if the rate limiting step occurs after the protonation step.

[7] The nonspecific deuteration of the C-5 position of DCB-6HTN generates a diastereomeric pair, one isomer with deuterium in the 5_{ax} position and one with deuterium in the 5_{eq} position.

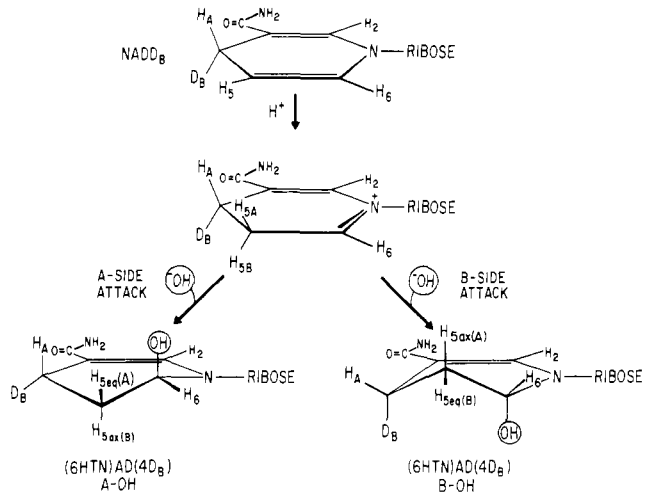


FIGURE 9: The determination of the absolute configuration of the C-6 hydroxyl by a specific C-4 deuterium label. The configuration of the C-4A proton–C-5_{ax} proton differs for the two diastereomers, A-hydroxyl ((6HTN)AD) and B-hydroxyl ((6HTN)AD). In the A-hydroxyl form the C-4A–C-5_{ax} conformation is trans; in the B-hydroxyl the C-4A–C-5_{ax} conformation is gauche. The measurement of the J_{4A-5ax} coupling constant for the two diastereomeric forms provides an unambiguous determination of the configuration of the C-6 hydroxyl relative to the known C-4A proton configuration.

The specificity of the enzymatic protonation reaction was determined by the preparation of (6HTN)AD($4D_{AB}$) in H_2O and comparing the spectrum of the C-5 methylene protons with those for the C-5 methylene protons of (6HTN)AD($4D_{AB}$) formed in D_2O (Figure 8). The unlabeled C-5 methylene groups of (6HTN)AD($4D_{AB}$) prepared in H_2O appear as two overlapping AX patterns; the large −13.3-Hz coupling constant is due to the geminal coupling between the C-5_{eq} and C-5_{ax} protons. The C-5 methylene protons of (6HTN)AD($4D_{AB}$) prepared in D_2O appear as singlets centered at the midpoints of each doublet. The area under the A form C-5_{eq} proton equals the area under the A form C-5_{ax} proton and the area under the B form C-5_{eq} proton equals the area under the B form C-5_{ax} proton. The presence of all four possible deuterium labels as well as an apparently equal distribution between the axial and equatorial protons of each form indicates that the protonation of the C-5 position occurs randomly (50:50), generating a nonspecific deuterium label. This result is consistent with protonation being rate limiting, otherwise the C-5 proton would have been expected to exchange, but leads to the inescapable conclusion that G3PD catalyzes a totally nonspecific protonation.

Configuration of the 6HTN Diastereomers. As discussed above the hydroxylation at the C-6 position generates two diastereomeric forms in unequal populations corresponding to an A side hydroxyl and a B side hydroxyl. The C-6 hydroxyl is axial in both forms; hence the configuration of the C-4 methylene protons will differ for the two diastereomers as shown in Figure 9. When the C-6 hydroxyl is on the A side, the C-4A proton is axial and the C-4B proton is equatorial. On the other hand when the C-6 hydroxyl is on the B side the C-4A proton is equatorial and the C-4B proton is axial. Thus, starting with a specific deuterium label at the C-4 position of the dihydronicotinamide ring of known absolute configuration (Cornforth *et al.*, 1962) the absolute configuration of the two diastereomeric forms of the 6HTN ring can be determined by the characteristic coupling constants between the specific C-4 proton and the C-5_{ax} proton

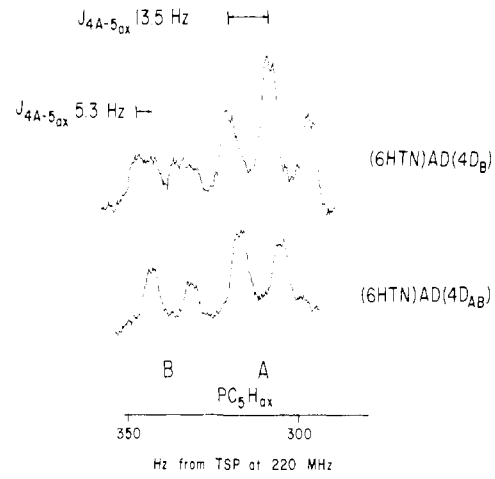


FIGURE 10: The pmr spectrum of the C-5 axial proton absorptions of the specifically labeled (6HTN)AD(4D$_B$). The large 13.5-Hz trans coupling constant to the 4A proton in the high population form establishes the absolute configuration of the C-6 hydroxyl as the A side. The small 5.3-Hz gauche coupling constant to the 4A proton in the low population form establishes the absolute configuration of the C-6 hydroxyl as the B side.

(Oppenheimer, 1973; Oppenheimer and Kaplan, 1974a,b). For (6HTN)AD(4D$_B$) prepared from specifically labeled NADD$_B$, the A side hydroxyl form would be expected to have a ~13-Hz $J_{4A\text{-}5_{ax}}$ trans coupling constant, while the B side hydroxyl form should have a 5-6-Hz $J_{4A\text{-}5_{ax}}$ gauche coupling constant. The general splitting pattern of the C-5$_{ax}$ protons would then consist of a triplet for the A side hydroxyl form and a doublet of doublets for the B side hydroxyl form. This is the pattern observed for the C-5$_{ax}$ protons of (6HTN)AD(4D$_B$) shown in Figure 10. The high population form has a value of 13.5 Hz for $J_{5_{ax}\text{-}4A}$ while the low population form has a value of 5.3 Hz for $J_{5_{ax}\text{-}4A}$. Thus, form A corresponds to a C-6 A hydroxyl and form B corresponds to a C-6 B hydroxyl.

The conformational analysis of the coupling constants in addition to the pmr studies of deuterium labeling and incorporation into the modified coenzyme formed by G3PD unambiguously establishes its structure to be a 6-hydroxytetrahydronicotinamide adenine dinucleotide.

Discussion

Substrate level oxidative phosphorylation catalyzed by G3PD occurs as two separate reactions catalyzed in the same active site: (1) the stereospecific reduction of the B side of the nicotinamide ring of NAD^+ with the concomitant oxidation of the aldehyde substrate to a thio ester (eq 3) and (2) the formation of a high-energy phosphoric anhydride bond by the nucleophilic attack of inorganic phosphate on the thio ester (eq 4). The reduction of NAD^+ (eq 3) is strongly pH dependent; below pH 7 the rate of this reaction decreases rapidly. The phosphorylation reaction (eq 4), however, is not severely affected since G3PD retains its phosphate transferase activity at pH below 7 (Rafter and Colowick, 1957).

$$NAD^+ + R\text{—}\overset{\overset{O}{\|}}{C}\text{—}H + HS\text{—}ENZ \rightleftharpoons R\text{—}\overset{\overset{O}{\|}}{C}\text{—}S\text{—}ENZ(NADH) + H^+ \quad (3)$$

$$P_i + R\text{—}\overset{\overset{O}{\|}}{C}\text{—}S\text{—}ENZ(NADH) \rightleftharpoons HS\text{—}ENZ + NADH + 1,3\text{—}DPG \quad (4)$$

Acetyl phosphate is a substrate for G3PD and above pH 8 it is reduced to acetaldehyde and inorganic phosphate with concomitant oxidation of NADH. As the pH is lowered, NADH is partitioned between oxidation to NAD^+ and formation of (6HTN)AD (Rafter and Colowick, 1957). The pH at which half the NADH is oxidized and half converted to (6HTN)AD is dependent on the polybasic anion present. The following anions are listed in order of their decreasing pH of 50% partition of NADH: pyrophosphate, 7.70; arsenate, 6.45; phosphate, 6.10; citrate, 6.05; acetate, 5.45 (Hilvers *et al.*, 1966). Previously Rafter *et al.* (1954) had observed that pyrophosphate increased the rate of formation of (6HTN)AD as did other polybasic anions. Furthermore, formation of the acyl enzyme by preincubation of G3PD with acetyl phosphate was also shown to increase markedly the rate of (6HTN)AD synthesis (Rafter and Colowick, 1957).

The observation that the rate of (6HTN)AD formation depends on the polybasic anion present and whether the enzyme is acylated suggested to us that the hydration of the 5-6 double bond of NADH to form (6HTN)AD might result from a reaction of a ternary complex of acyl-G3PD, polybasic anion, and NADH shown on the left side of eq 4. Since the hydration of NADH involves the generation of two asymmetric centers, a prochiral site at the C-5 position and a diastereomeric site at the C-6 position, the stereospecificity (or lack of it) of deuterium incorporation and the configuration of the C-6 hydroxyl should reflect the juxtaposition of the reacting groups in the active site.[8] The two steps of the hydration reaction of the dihydronicotinamide ring, the initial protonation and the subsequent hydroxylation, will be discussed below as they pertain to the possible participation of the polybasic anions or amino acid residues of the G3PD active site in the formation of (6HTN)AD.

Protonation. Stock *et al.* (1961) and Lowry *et al.* (1961) report that the dihydronicotinamide ring is unstable in concentrated neutral solutions of polybasic anions such as phosphate and that prolonged incubation presumably yielded a 6HTN structure. Alivisatos *et al.* (1965) report that in concentrated neutral phosphate the initial 280-nm absorbing product of the reaction of NADH is NADHX which is subsequently converted to the primary acid product.

The observed polybasic anion catalysis of the hydration of 5-6 double bond of NADH suggests that the formation of (6HTN)AD by G3PD might be a consequence of the polybasic anion bound in the active site. In order for G3PD to perform the reaction sequence outlined in eq 3 and 4 it seems reasonable to assume that the thio ester, dihydronicotinamide ring, and phosphate group all must be in close proximity to each other in the active site. Furthermore, since the normal dehydrogenase reaction for G3PD in the pH range below 7 is no longer rapidly turning over, this complex might be expected to have a considerable lifetime. Thus, the formation of (6HTN)AD by G3PD might represent catalysis by the high local concentration of phosphate

[8] Recent work by Arnold and Kaplan (1974) has demonstrated the power of this approach for the analysis of the active site of an enzyme by their study of the stereoconfiguration of the binary adduct of NAD^+ and pyruvate generated by heart type, chicken lactate dehydrogenase.

and NADH in the active site and by the increased time in which they would be in close proximity. Furthermore, the reaction might also reflect activation of the phosphate group, an activation which is normally manifested in the nucleophilic attack of the phosphate on the thio ester.

The proposed mechanism of polybasic anion catalysis of (6HTN)AD formation is attractive since it explains why G3PD is unique among all the dehydrogenases in catalyzing the formation of (6HTH)AD. However, catalysis by an acidic amino acid residue on the enzyme cannot be ruled out. The extremely conservative nature of the active-site amino acid sequence for G3PD (Jones and Harris, 1972) could account for an amino acid residue catalyzed formation of (6HTN)AD in G3PD from such diverse sources as yeast and rabbit muscle (Chaykin *et al.*, 1956; Rafter and Colowick, 1957). At present no choice can be made between these possibilities.

The nonspecific incorporation of deuterium into the C-5 position of (6HTN)AD formed in D_2O, however, strongly suggests that whatever group is catalyzing protonation must be symmetrically juxtaposed to the plane of the dihydronicotinamide ring and equally capable of protonating the A or B faces.

Hydroxylation. Any discussion of the hydroxylation reaction reflects upon the more basic question as to what is the specific reaction or reactions catalyzed by G3PD in the ultimate formation of (6HTN)AD. The asymmetry of the hydroxylation reaction could result from any of the following mechanisms: (1) a preferential attack of water on the A side of the tetrahydronicotinamide cation intermediate bound in the active site. This might be expected since G3PD is a B specific enzyme, hence the substrate binding site could partially block the B face of the dihydronicotinamide ring. (2) G3PD catalyzes only the formation of the protonated tetrahydronicotinamide cation. This intermediate is released from the enzyme into solution where it then subsequently adds the hydroxyl group nonenzymatically. The asymmetry of the hydroxyl addition would then be a function of the solution conformation of the tetrahydronicotinamide cationic intermediate much in the same manner that unequal diastereomeric populations of the NAD^+–cyanide adduct are generated (Oppenheimer *et al.*, 1971) or NAD^+–pyruvate adducts (Arnold and Kaplan, 1974). (3) The polybasic anion adds at the C-6 position as has been previously suggested (Chaykin *et al.*, 1956; Alivisatos *et al.*, 1964) and it is the tetrahydronicotinamide–anion complex that is released into solution where it subsequently hydrolyzes to the tetrahydronicotinamide cation which then adds hydroxyl as in case 2.

Such arguments on the mechanism of the formation of (6HTN)AD are not meant to be definitive but are an attempt to rationalize the unusual lack of stereospecificity shown by G3PD. The small turnover rate of this reaction as well as the possible involvement of phosphate or pyrophosphate makes this an ideal problem for a multinuclei nmr investigation. The determination of the binding characteristics of NADH at lower pH could provide information about the nature of the hydration reaction.

In conclusion the reactions of NADH with G3PD or dilute acid which were thought to be similar yield the very dissimilar compounds (6HTN)AD and (cTHN)AD. Furthermore, it is the enzymatic reaction which shows no stereoselectivity for the protonation at the C-5 position and only limited preference for the A side addition of hydroxyl; the nonenzymatic acid-catalyzed reaction is a quantitative, stereospecific rearrangement of NADH. Through further detailed kinetic and nmr studies of G3PD, especially in regard to the formation of (6HTN)AD, it is hoped that a more complete understanding of the binding and activation of phosphate and the coenzyme in the active site of G3PD will be obtained.

Acknowledgment

We wish to thank Drs. W.S. Allison and E.H. Cordes for valuable discussions and advice on this manuscript.

References

Alivisatos, S.G.A., Ungar, F., and Abraham, G.J. (1964), *Nature (London) 203,* 973.

Alivisatos, S.G.A., Ungar, F., and Abraham, G.J. (1965), *Biochemistry 4,* 2616.

Alworth, W.L. (1972), Stereochemistry and Its Application in Biochemistry, New York, N.Y., Wiley-Interscience, Chapter 3.

Arnold, L.J., Jr., and Kaplan, N.O. (1974), *J. Biol. Chem. 249,* 652.

Barltrop, J.A., Grubb, P.W., and Hesp, B. (1963), *Nature (London) 199,* 759.

Bechara, E.J.H., and Cilento, G. (1971), *Biochemistry 10,* 1837.

Bechara, E.J.H., and Cilento, G. (1972), *Biochemistry 11,* 2606.

Becker, E.D. (1969), High Resolution NMR, New York, N.Y., Academic Press, Chapter 7.

Bothner-By, A. A. (1965), *Advan. Magn. Resonance 1,* 195.

Butler, R.J.G., and Jones, G.M.T. (1970), *Biochem. J. 118,* 375.

Chaykin, S., Meinhart, J.O., and Krebs, E.G. (1956), *J. Biol. Chem. 220,* 811.

Cornforth, J.W., Ryback, G., Popjak, G., Donninger, C., and Schroepfer, G.J. (1962), *Biochem. Biophys. Res. Commun. 9,* 371.

Glasoe, P.K., and Long, F.A. (1960), *J. Phys. Chem. 64,* 188.

Griffiths, D.E., and Chaplain, R.A. (1962), *Biochem. Biophys. Res. Commun. 8,* 501.

Hilvers, A.G., Weenen, J.H.M., and van Dam, K. (1966), *Biochim. Biophys. Acta 128,* 74.

Hope, H. (1969), *Acta Crystallogr., Sect. B 25,* 78.

Jones, G.M.T., and Harris, J.I. (1972), *FEBS (Fed. Eur. Biochem. Soc.)* Lett. *22,* 185.

Karplus, M. (1959), *J. Chem. Phys. 30,* 11.

Karplus, M. (1963), *J. Amer. Chem. Soc. 85,* 2870.

Kim, C.S.Y., and Chaykin, S. (1968), *Biochemistry 7,* 2339.

Lemieux, R.U., and Lown, J.W. (1963), *Can. J. Chem. 41,* 889.

Lowry, O.H., Passoneau, J.V., Schulz, D.W., and Rock, M.K. (1961), *J. Biol. Chem. 236,* 2746.

Meinhart, J.O., Chaykin, S., and Krebs, E.G. (1956), *J. Biol. Chem. 220,* 821.

Meinhart, J.O., and Hines, M.C. (1957), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 16,* 425.

Miles, D.W., Urry, D.W., and Eyring, H. (1968), *Biochemistry 7,* 2333.

Mislow, K., and Raban, M. (1966), *Top. Stereochem. 1,* 1.

Oppenheimer, N.J. (1973), *Biochem. Biophys. Res. Commun. 50,* 683.

Oppenheimer, N.J., Arnold, L.J., Jr., and Kaplan, N.O.

(1971), *Proc. Nat. Acad. Sci. U.S. 68,* 3200.
Oppenheimer, N.J., and Kaplan, N.O. (1974a), *Biochemistry, 13,* 4675.
Oppenheimer, N.J., and Kaplan, N.O. (1974b), *Bioorg. Chem. 3,* 141.
Oppenheimer, N.J., and Kaplan, N.O. (1974c), *Arch. Biochem. Biophys.* (in press).
Pfleiderer, G., and Stock, A. (1962), *Biochem. Z. 336,* 56.
Rafter, G.W., Chaykin, S., and Krebs, E.G. (1954), *J. Biol. Chem. 208,* 799.
Rafter, G.W., and Colowick, S.P. (1957), *J. Biol. Chem. 244,* 373.
Stamhuis, E.J., and Maas, W. (1965), *J. Org. Chem. 30,* 2156.
Stock, A., Sann, E., and Pfleiderer, G. (1961), *Justus Liebigs Ann. Chem. 647,* 188.
Zinner, K., Faljoni, A., and Cilento, G. (1973), *Biochem. Biophys. Res. Commun. 51,* 181.

Topography of 16S RNA in 30S Ribosomal Subunits. Nucleotide Sequences and Location of Sites of Reaction with Kethoxal†

Harry F. Noller

ABSTRACT: The sites of reaction of kethoxal with 16S RNA in intact 30S ribosomal subunits have been identified. By means of a new "diagonal" electrophoresis method, 53 T1 oligonucleotides from modified sites were isolated, their nucleotide sequences determined, and sites of attachment of kethoxal unambiguously identified. These sequences define 26 sites within the 16S RNA chain, 16 of which can be located in the known sequence. The modified sites appear to fall into clusters, interspersed by regions 200–400 nucleotides long that are known to be involved in binding of ribosomal proteins. In addition to providing a catalog of RNA sequences that includes sites involved in ribosomal function, these data provide new information about the sequence of 16S RNA and its topography within the ribosome.

Indications of the functional participation of ribosomal RNA in protein synthesis have emerged from recent work by several groups (Senior and Holland, 1971; Bowman *et al.,* 1971; Helser *et al.,* 1971, 1972; Noller and Chaires, 1972; Lai *et al.,* 1973a,b; Held *et al.,* 1974). Studies in this laboratory have shown that the guanine-specific reagent kethoxal reacts with 30S ribosomal units causing inactivation of the particle in poly(U) directed *in vitro* protein synthesis (Noller and Chaires, 1972). Inactivation is attributable to loss of tRNA binding ability, and, correspondingly, protection against inactivation is afforded by prior binding of tRNA. Reconstitution experiments indicate that the target of inactivation is RNA. During the course of inactivation about 10 mol of kethoxal become attached/mol of 30S subunits. These modified sites include candidates for possible functional sites in 16S RNA. Thus, it is of great interest to identify these regions in the 16S RNA molecule. In addition, such information will reveal details of the conformation of 16S RNA within the intact ribosome, since kethoxal is known to react only with single-stranded regions of RNA (Litt, 1969).

Identification of modified sites in rRNA and other large RNA molecules is technically difficult due to the large number of fragments obtained from nuclease digests of these molecules. In order to simplify the identification of kethoxal-modified sites, a "diagonal" paper electrophoresis procedure has been devised. By use of this procedure, about 50 oligonucleotides have been isolated and their nucleotide sequences determined. These sequences correspond to about 26 sites within the 16S RNA, 16 of which can be located unambiguously in regions of known or partially known sequence as reported by Ehresmann *et al.* (1974). The modified sites appear to fall into five main clusters, interspersed by regions 200–400 nucleotides long which are known to be involved in binding of ribosomal proteins (Zimmerman *et al.,* 1972; Schaup *et al.,* 1971, 1973; Székely *et al.,* 1973). Some of the kethoxal sites correspond to sites of attack by T1 nuclease on intact 30S ribosomal subunits (Ehresmann *et al.,* 1972; Santer and Santer, 1973).

Materials and Methods

Buffers. Buffer A, 0.1 M NH_4Cl–0.01 M $MgCl_2$–0.02 M Tris (pH 7.5)–0.5 mM EDTA–6 mM β-mercaptoethanol; buffer B, 0.5 M NH_4Cl–0.01 M $MgCl_2$–0.02 M Tris (pH 7.5)–0.5 mM EDTA–6 mM β-mercaptoethanol; TMA II,[1] 0.3 mM $MgCl_2$–30 mM NH_4Cl–10 mM Tris (pH 7.5)–6 mM β-mercaptoethanol; kethoxal reaction buffer, 0.1 M potassium cacodylate (pH 7.0)–20 mM $MgCl_2$; SCE, 0.15 M NaCl–15 mM sodium citrate–10 mM EDTA (pH 7.0).

Enzymes. Ribonuclease T1 (Sankyo) was obtained from Calbiochem, ribonuclease A and snake venom phosphodiesterase from Worthington, and bacterial alkaline phosphatase from Sigma. U2 nuclease was a gift from Dr. Howard

† From Thimann Laboratories, University of California, Santa Cruz, California 95064. *Received July 19, 1974.* Supported by U. S. Public Health Service Grant No. 17129-01 from the National Institute of General Medical Sciences.

[1] Abbreviations used are: TMA II, 0.3 mM $MgCl_2$–30 mM NH_4Cl–10 mM Tris (pH 7.5)–6 mM β-mercaptoethanol; SCE, 0.15 M NaCl–15 mM sodium citrate–10 mM EDTA (pH 7.0); CMCT, *N*-cyclohexyl-*N'*-morpholinocarbodiimide metho-*p*-toluenesulfonate.