Investigation of the Conformation of β-Diphosphopyridine Nucleotide (β-Nicotinamide–Adenine Dinucleotide) and Pyridine Dinucleotide Analogs by Proton Magnetic Resonance*

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ABSTRACT: The conformation of β-diphosphopyridine nucleotide in aqueous solution (deuterium oxide) was examined in a study of α-diphosphopyridine nucleotide, β-diphosphopyridine nucleotide, and a series of nicotinamide dinucleotide analogs by proton magnetic resonance spectroscopy. The chemical shift data from adenosine diphosphoribose, α-diphosphopyridine nucleotide, and β-diphosphopyridine nucleotide at pH 7.0 indicate that in both α- and β-diphosphopyridine nucleotide there is intramolecular interaction between the pyridine and adenine moieties, the interaction being less in α-diphosphopyridine nucleotide. The chemical shift–pH dependence data suggest that there is maximal interaction between the pyridine and adenine moieties of β-diphosphopyridine nucleotide in the biological (neutral) pH range. Chemical shift data for the various analogs at pH values of 2.0 and 7.0 also indicate increased interaction at pH 7.0 and that a change of pH value from 7.0 to 2.0 results in the downfield shift of the adenine C_8, C_2, and C'_1 protons indicating the dissociation of the intramolecular pyridine–adenine complex. The enhanced interaction between pyridine and adenine moieties and consequent upfield shifting of proton magnetic resonance signals at neutral pH have been interpreted to result from the diamagnetic shielding of π-electron interactions of rings stacked in parallel planes as envisaged in a folded molecule of diphosphopyridine nucleotide. The downfield shift of the proton magnetic resonance signals at acidic pH indicates partial unfolding of the dinucleotide caused probably by the protonation of the adenine N_1. Chemical shift data for β-diphosphopyridine nucleotide at different pH values as well as at different temperatures suggest that the pyrimidine part of the adenine is involved directly in the folding interaction. Magnetic resonance data from the different analogs, in addition to supporting the above conclusion, show that hydrogen bonding of the type postulated as occurring between base pairs in biological systems does not play any significant role in maintaining the conformational integrity of the dinucleotide at the biological pH and temperature. It was further found that at pH 7.0 the analog *N*,*N*-dimethylnicotinamide–diphosphopyridine nucleotide, unlike the *N*-monomethyl- and *N*-monoethylnicotinamide–diphosphopyridine nucleotide, exhibited a tendency toward unfolding. Study of the molecular model of the *N*,*N*-dimethylnicotinamide–diphosphopyridine nucleotide in folded conformation shows that the methyl group *trans* to the oxygen hinders the folding of the rings. The approximate distance between the pyridine and adenine rings computed from van der Waal's and bond-length parameters for *N*,*N*-dimethylnicotinamide–diphosphopyridine nucleotide is less than 3.9 Å. Finally, it has been suggested, in order to account for the magnetic equivalence of the two hydrogens at C_4 of the dihydronicotinamide ring in the reduced β-diphosphopyridine nucleotide, that there may be a rapid equilibrium between two folded conformations, one in which the pyridine ring lies above the plane of the adenine ring, designated as the right-handed folding, and the other in which the pyridine ring lies below the plane of the adenine ring, the left-handed folding.

Several pertinent suggestions have been made regarding the conformation of pyridine dinucleotides in aqueous solution. Evidence for the intramolecular interaction between the adenine and pyridine rings in DPNH was provided by Weber (1957) from fluorescence measurements. Fluorescence transfer data led Shifrin and Kaplan (1959–1961) to propose a hydrogen-bonded structure in which the pyridine and adenine rings lie coplanar for effective energy transfer (Figure 1). Crick and Watson (1953) have suggested hydrogen bonding of this type between bases in DNA in order to account for its helical structure. The presence of hydrogen bonds in base–base interactions in various nucleic acids has been investigated by various physicochemical techniques (*i.e.*, X-ray diffractions by Hoogsteen (1963), infrared spectroscopy by Hamlin *et al.* (1965), and nuclear magnetic resonance by Katz and Penman (1966)). Based on fluorescence studies Velick (1958, 1961) inferred a folded conformation for DPNH in which the pyridine and adenine rings are stacked in parallel planes. In Velick's (1961) representation of DPNH, the nicotinamide ring is shown to lie above the plane of the adenine ring. Construction of β-DPN with atomic models

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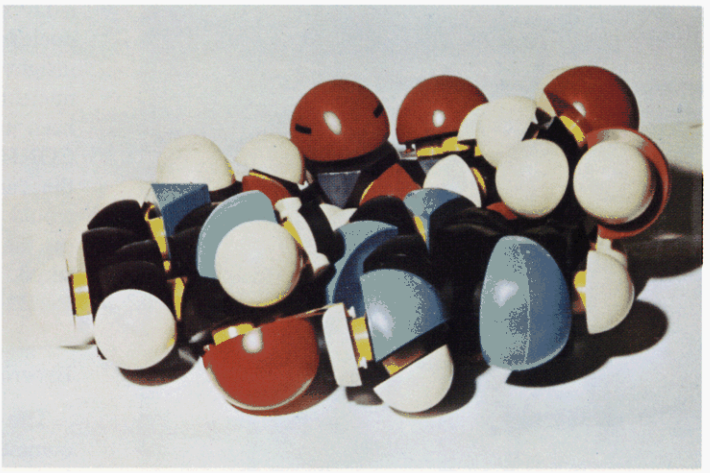

FIGURE 1: Hydrogen-bonded conformation for β-DPN. (The models have been constructed from Courtauld atoms.) The pyridine and adenine rings are coplanar and the $CONH_2$ group is out of the plane. The proposed two hydrogen bonds ($NH\cdots O{=}C$ and $N_1\cdots HN$) between the base pairs is limited by the length of hydrogen bonds. This model is facilitated if the $CONH_2$ group is not coplanar with the pyridine and adenine rings and considerable constraint is imposed on the systems. Hydrogen bonding between N_7 of adenine and the NH_2 group of $CONH_2$ side chain of nicotinamide also introduces considerable strain in the systems.

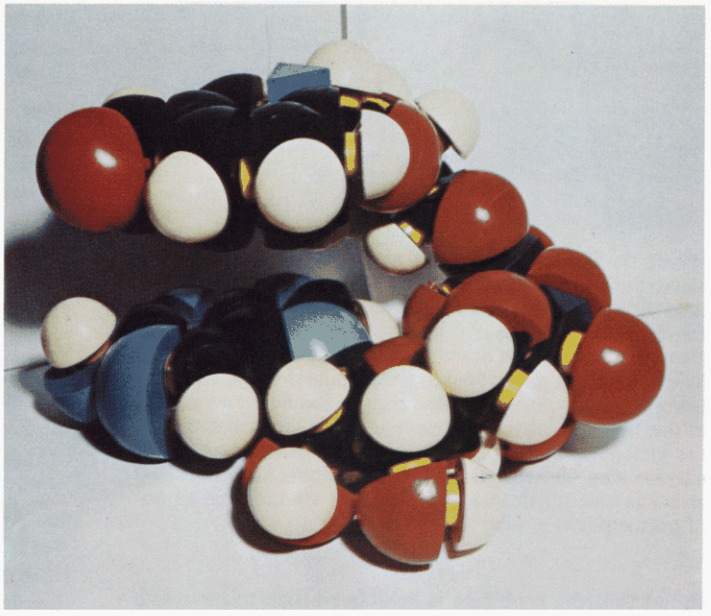

FIGURE 2: Folded conformation for β-DPN in which the pyridine ring lies above the plane of the adenine ring, arbitrarily chosen as the right-handed folding.

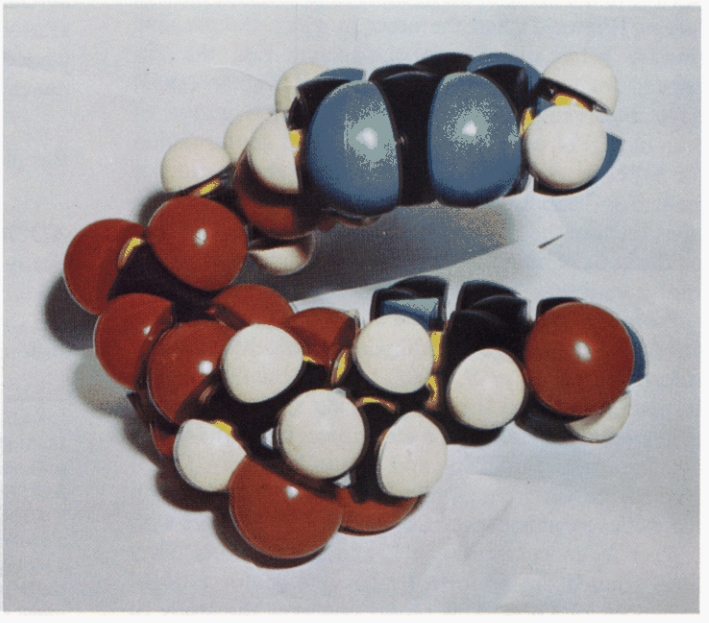

FIGURE 3: Folded conformation for β-DPN in which the pyridine ring lies below the plane of the adenine ring, the left-handed folding.

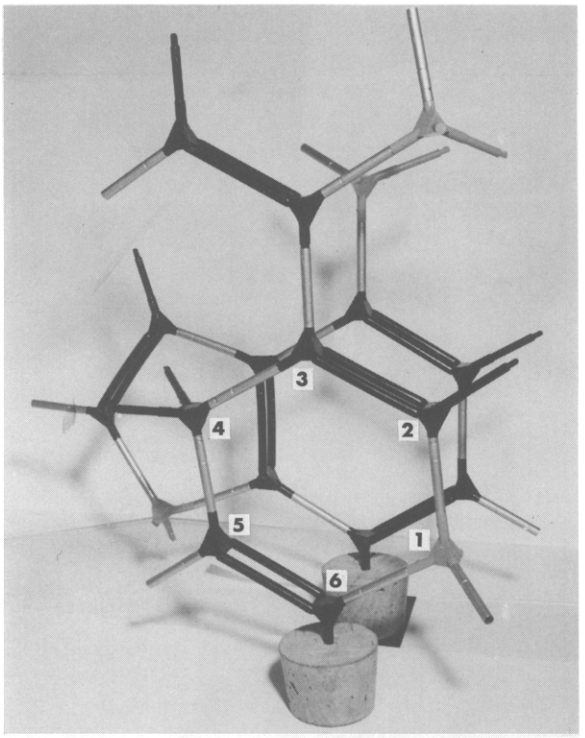

FIGURE 4: Skeletal models of the adenine and dihydronicotinamide ring to show the relative position of the two rings in one of the two folded conformations. The figure shows clearly that the two hydrogens at C_4 of the dihydronicotinamide ring are not chemically equivalent, one of the hydrogens being juxtaposed to the adenine ring and the other being remote from the adenine ring. It should be noted that in this figure no attempt is made to show the orientation or the juxtaposition of the $CONH_2$ group with respect to the adenine ring and that the N=C in adenine and the C=O group in the dihydronicotinamide ring are constructed out of geometrically similar C=C bonds.

shows that the molecule is flexible enough to have two folded conformations, one in which the pyridine ring lies above the plane of the adenine ring, the right-handed folding (Figure 2), and the other in which the pyridine ring lies below the plane of the adenine ring, the left-handed folding (Figure 3). Walter and Kaplan (1963) have interpreted the fluorescence titration curves of 5-amino-DPN on the basis of a folded conformation for DPN at neutral pH. Fluorescence studies do not lead to a distinct conformation for pyridine dinucleotides because the fluorescence data could be interpreted on the basis of both planar hydrogen-bonded and folded conformations.

Proton magnetic resonance study of DPNH in deuterium oxide and *N*-benzyldihydronicotinamide in chloroform led Meyer *et al.* (1962) to favor the folded conformation in which the pyridine ring spends considerable amount of time in the area above the plane of the adenine ring. Jardetzky and Wade-Jardetzky (1966) studied the variation of chemical shifts of the proton magnetic resonance spectra of pyridine nucleotides with respect to concentration, pH, and temperature. They have pointed out 64 formally possible conformational variations of DPN. This report concerns the study of α-DPN, β-DPN, and a series of β-DPN analogs using nuclear magnetic resonance in order to gain additional insight into the conformational features of pyridine dinucleotides in solution. Two classes of analogs have been used in this study, one involving the NH_2 of the $CONH_2$ group of nicotinamide and the other involving the carbonyl group of nicotinamide, both of which would tend to preclude hydrogen bonding. Their effects on the neighboring adenine protons have been measured. The effects of hypoxanthine in NHD[1] and AcPyHD on nicotinamide protons have been investigated.

Experimental Procedure

The coenzymes β-DPN, α-DPN, and DPNH were commercial preparations from Sigma or P. L. Biochemicals or California Biochemical Corp. The analogs 3-acetylpyridine–DPN, pyridine-3-aldehyde–DPN, NHD, and AcPyHD were products of P. L. Biochemicals. The analogs *N*-monomethylnicotinamide–DPN, *N*-monoethylnicotinamide–DPN, and *N*,*N*-dimethylnicotinamide–DPN were prepared by exchanging the nicotinamide of β-DPN for the respective pyridine bases with DPNase from pig brain according to the procedure of Kaplan and Ciotti (1956). The analogs 3-aminopyridine–DPN and 3-butyl pyridyl ketone–DPN were prepared in the same manner as described above.

The nuclear magnetic resonance spectra were obtained on high-resolution Varian A-60A, A-60, and HR-60 spectrometers. Shifts were measured with the use of DSS (E. Merck AG, Darmstadt, Germany) as an internal standard. An external reference, acetone, was used for the high-temperature work and the temperature calibrations were done by ethylene glycol. The precision of the measurements were found to be well within ± 0.5 cps. The nuclear magnetic resonance probe temperature was $38 \pm 1°$. All samples were lyophilized from deuterium oxide to remove exchangeable protons and then dissolved in deuterium oxide. Measurements were made on two different preparations and it was found that the agreement between the spectra from two different preparations was well within ± 0.5 cps. pH measurements were done before and after the nuclear magnetic resonance measurements.

Results and Discussion

The proton magnetic resonance spectra of β-DPN, ADPR, and α-DPN, taken consecutively under practically identical instrumental conditions on a 0.15 M solution at pH 7.0 with the same internal standard, DSS, are shown in Figure 5A–C, respectively. The chemical shift data[2] for ADPR, α-DPN, β-DPN, and a large

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; NHD, nicotinamide–hypoxanthine dinucleotide; AcPyHD, acetylpyridine–hypoxanthine dinucleotide.

[2] The chemical shifts are reported in cycles per second. The radiofrequency oscillator of the Varian Model A-60A nuclear magnetic resonance spectrometer is set at 60 Mcps. The chemishifts in cycles per second may be divided by the oscillator frequency and reported as parts per million as in Figures 6 and 8.

TABLE I: Chemical Shift of Adenine C_8H, C_2H, and C'_1 with Reference to DSS as an Internal Standard.[a]

Compound	Substituent in the 3 Position of Pyridine	Adenine Peaks (cps)		
		C_8H	C_2H	C'_1H
ADPR				
0.3 M, pH 7.0		512.0	492.0	370.5
0.075 M, pH 7.0		512.0	496.5	370.8
0.15 M, pH 7.0		512.0	495.0	370.8
pH 2.0		520.2	510.0	372.3
α-DPN				
pH 7.0	$CONH_2$	508.7	488.7	363.5
pH 2.0		518.8	508.5	368.8
β-DPN				
pH 7.0	$CONH_2$	504.0	486.0	363.0
pH 2.0		516.7	508.0	369.3
3-Acetylpyridine-DPN				
pH 7.0	$COCH_3$	504.2	486.0	363.0
pH 2.0		516.7	507.8	368.7
Pyridine-3-aldehyde-DPN				
pH 7.0	CHO	504.4	485.8	363.6
pH 2.0		517.0	508.0	369.3
3-Butylpyridyl ketone-DPN				
pH 7.0	$CO(CH_2)_3$-	503.5	486.0	362.5
pH 2.0	CH_3	516.6	508.0	368.8
3-Aminopyridine-DPN				
pH 7.0	NH_2	504.0	486.0	364.0
pH 2.0		517.0	508.0	368.8

[a] All measurements were made on a 0.15 M solution in D_2O at 38° in a high-resolution Varian Model A-60A spectrometer.

number of β-DPN analogs at pH 2.0 and 7.0 with reference to DSS as an internal standard are summarized in Tables I–III. In the case of NHD and AcPyHD the assignment of the pyridine C_5 proton and the adenine C_8 and C_2 protons was difficult because of the overlap of the pyridine C_5H resonance with the adenine C_8H and C_2H resonance. In all the analogs, except NHD and AcPyHD, to compare chemical shifts, the signals from the adenine C_8, C_2, and C'_1 are used rather than those from the pyridine protons because different substituents introduced on the pyridine ring tend to influence the chemical shifts of the pyridine protons.

Proton Magnetic Resonance Spectra of α-DPN, β-DPN, and ADPR at pH 7.0. The chemical shift data (Table I) for the adenine C_8H, C_2H, and C'_1H in α-DPN, β-DPN, and ADPR provide direct evidence for the intramolecular interaction between the pyridine and adenine rings in the dinucleotide. At pH 7.0, the adenine C_8H, C_2H, and C'_1H in both α- and β-DPN are shifted to higher fields compared with those in ADPR. The upfield of the proton magnetic resonance signals in the dinucleotide may be interpreted as resulting from diamagnetic shielding due to π-electron interactions of rings stacked in parallel planes as one would envisage for the DPN molecule in folded conformation. Ts'o *et al.* (1962) have discussed the diamagnetic shielding resulting from π-electron interactions of rings stacked in parallel. Thus it appears that both α- and β-DPN tend to occur in folded conformation at pH 7.0. Jardetzky and Wade-Jardetzky (1966) have come to the same conclusion on the basis of thermodynamic parameters derived from chemical shift–temperature dependence data for α- and β-DPN. However, it is worthwhile noting that while the adenine C_8H and C_2H in both α- and β-DPN are shifted upfield from those of ADPR, *the upfield shift is larger for the β isomer than for α-DPN.* This probably indicates less interaction between the pyridine and adenine moieties in the α isomer. It may also be noted that the chemical shifts of ADPR (Table I), like those of β-DPN (Jardetzky and Wade-Jardetzky, 1966), are independent of concentration. In the concentration range of 0.075–0.3 M the chemical shift of C_8H and C'_1H is constant, the C_2H shows small variation. This makes the comparison of the chemical

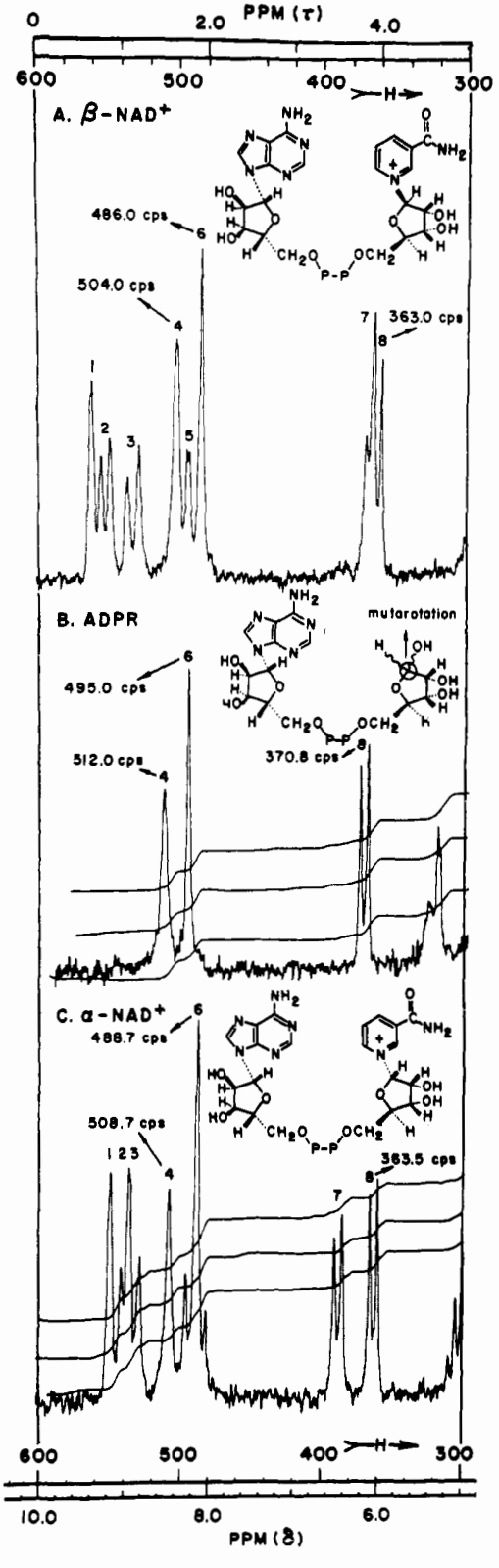

FIGURE 5: Proton magnetic resonance spectra of β-DPN (A), ADPR (B), and α-DPN (C) as reproduced directly from the nuclear magnetic resonance chart S60-C. The three spectra were taken consecutively under practically identical instrumental conditions in a high-resolution Varian Model A-60A spectrometer at a sweep width of 1000 cps and calibrated against DSS as an internal standard. The concentrations of the solutions were 0.15 M (pH 7.0) and the temperature was 38°. Peaks: (1) nicotinamide, C_2H; (2) nicotinamide, C_6H; (3) nicotinamide, C_4H; (4) adenine, C_8H; (5) nicotinamide, C_5H; (6) adenine, C_2H; (7) nicotinamide, C'_1H; and (8) adenine, C'_1H.

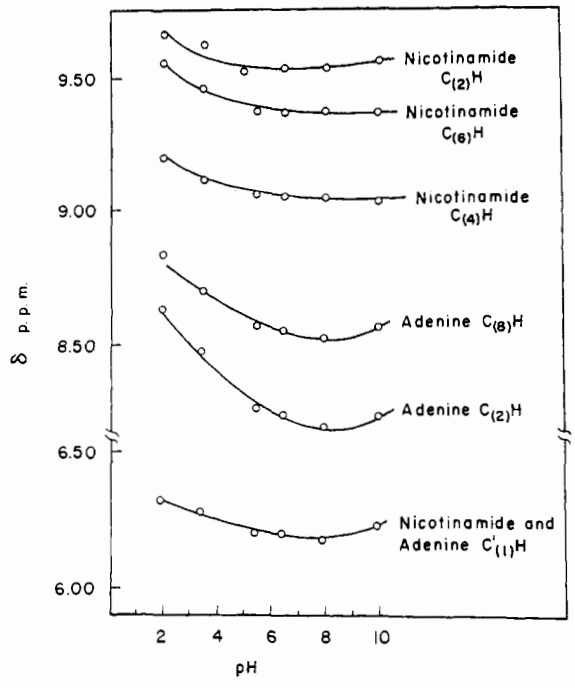

FIGURE 6: pH dependence of chemical shifts upon 0.1 M β-DPN at 38°.

shifts of ADPR with those of the dinucleotide a better one because of the absence of intermolecular complexing both in ADPR and the dinucleotide.

The comparison of the proton magnetic resonance spectra of α- and β-DPN clearly reveals the differences in the glycosidic linkage. In the β isomer the C'_1H is on the same side as the C'_2OH and C'_3OH of the D-ribofuranose ring. This hydrogen appears 19.7 cps upfield from the C'_1H of the α isomer in which the C'_1H resides on the same side as the C'_2H and C'_3H of the D-ribofuranose ring. In the α isomer the pyridine ring lies on the same side as the hydroxyl groups of the D-ribose and the pyridine C_2H and C_6H of the α isomer appears 12–13 cps upfield from the corresponding protons of the β isomer. It is instructive to note that neighboring protons and neighboring aromatic rings which reside on the same side as the hydroxyl groups of the D-ribofuranose ring are shielded, and this in turn reflects the difference in the geometry of the glycosidic linkage.

pH Dependence of Chemical Shifts in ADPR, α-DPN, β-DPN, and β-DPN Analogs. Change of pH from 7.0 to 2.0 results in the downfield shift of nuclear magnetic resonance chemical shifts in all the compounds, *including ADPR*, as can be seen from data in Table I–III and Figure 6. However, it can be seen that the effects of pH on the chemical shift of adenine C_8H, C_2H, and C'_1H are pronounced in the case of the dinucleotides compared with ADPR. The pH dependence of chemical shifts for β-DPN is shown in Figure 6. Any data for DPN in alkaline pH have to be interpreted with caution because DPN is known to decompose in that pH range. Minima of the curves or upfield shifting have been observed for the adenine C_2 and C_8 protons; the effect was slightly less for the nicotinamide C_2 and C_6 protons as well as the ribose C'_1 protons in the pH range 6–9. This

TABLE II: Chemical Shift of Adenine C_8H, C_2H, and C'_1H with Reference to DSS as an Internal Standard.[a]

Compound	Substituent in the 3 Position of Pyridine	Adenine Peaks (cps)		
		C_8H	C_2H	C'_1H
β-DPN				
pH 7.0	$CONH_2$	504.0	486.0	363.0
pH 2.0		516.7	508.0	369.3
N-Monomethylnicotinamide–DPN				
pH 7.0	$CONHCH_3$	504.0	486.2	363.2
pH 2.0		517.0	508.0	369.3
N,*N*-Dimethylnicotinamide–DPN				
pH 7.0	$CON(CH_3)_2$	506.7	488.4	363.5
pH 2.0		517.0	508.0	369.2
N-Monoethylnicotinamide–DPN				
pH 7.0	$CONHCH_2CH_3$	503.0	486.5	363.0
pH 2.0		516.5	508.0	369.3
N,*N*-Diethylnicotinamide–DPN	$CON(CH_2CH_3)_2$	Could not be prepared		

[a] All measurements were made on a 0.15 M solution in D_2O at 38° in a high-resolution Varian Model A-60A spectrometer.

suggests that the nicotinamide–adenine base interactions in β-DPN approach a maximum in the biological (neutral) pH range. Chemical shift data for the various analogs at pH 2.0 and 7.0 (Tables I–III) also indicate increased interaction at pH 7.0. The change of pH from 7.0 to 2.0 results in the downfield shift of the adenine C_8, C_2, and C'_1 protons, a further indication of the decreased interactions between base pairs at low pH.

The downfield shift observed in the case of ADPR with increasing pH is due to protonation of the adenine N_1. In the case of the dinucleotides the corresponding downfield shift is due to the combined effect of the same protonation and the diminution of the intramolecular interaction between base pairs. The partial unfolding of β-DPN and the DPN analogs at the lower pH is probably related to the protonation of the adenine N_1. The p*K* corresponding to the protonation of the N_1 of the adenine ring is approximately 4, indicating that below a pH of 4, the coenzyme and the analogs, in addition to the positive charge on the pyridine ring, have a second positive charge on the adenine ring. The decreased interaction observed at lower pH is probably caused by electrostatic repulsion by the two positive charges.

In Table IV the downfield shift, as a result of partial unfolding of the dinucleotide brought about by change of pH from 7.0 to 2.0, is calculated. In these calculations it has been assumed that the downfield shift observed in the case of ADPR with pH is due to protonation of the adenine ring and that in the case of the dinucleotides the combined effect of protonation and the resultant unfolding of the rings cause the downfield shift. Data in Table IV shows that in the folded conformation the adenine C_2H is shielded more than the C_8H by the magnetic anisotropy of the aromatic pyridine. This result implies that both in α- and β-DPN the pyridine ring is in close juxtaposition with the pyrimidine portion of the adenine ring rather than the imidazole part of it. It could also be seen that the effect of pH is less pronounced in the case of the α-DPN, indicating less interaction between the pyridine and adenine rings in the α isomer at pH 7.0.

At pH 2.0 the adenine C_8, C_2, and C'_1 protons have the same chemical shift in all the dinucleotide analogs (Tables I and II) implying that these analogs have dissociated to the same extent at pH 2.0. The same is true for NHD and AcPyHD, as can be seen from the constancy of chemical shift at pH 2.0 (Table III) for pyridine C_2, C_6, C_4, and C'_1 protons in β-DPN and NHD as well as 3-acetylpyridine–DPN and 3-acetylpyridine–hypoxanthine dinucleotide.

Data in Table I show that the adenine C_8H, C_2H, and C'_1H of β-DPN at pH 7.0 are shifted upfield from those of ADPR at pH 7.0 by 8.0, 9.0, and 7.8 cps, respectively. However, data on the right-hand side of Table IV show the downfield shift caused by partial unfolding of the dinucleotide brought about by pH decrease as 4.5, 7.0, and 4.8 cps, respectively, for the same protons. This indicates that *lowering the pH does not totally abolish the interaction between the two ring systems and that the dinucleotide does not assume a fully open conformation at pH 2.0.* This agrees with our model according to which at low pH protonation of adenine N_1 takes place and the resultant electrostatic repulsion between the ring systems diminishes the intramolecular interaction between

TABLE III: Chemical Shift of Pyridine C_2H, C_6H, C_4H, C_5H, and C'_1H with Reference to DSS As an Internal Standard.[a]

Compound	Pyridine Peaks (cps)				
	C_2H	C_6H	C_4H	C_5H	C'_1H
α-DPN					
pH 7.0	550.5	539.9	532.8		388.6
pH 2.0	558.5	547.7	540.2		396.6
β-DPN					
pH 7.0	562.7	553.4	534.0	495.8	368.9
pH 2.0	568.4	560.0	541.0	502.3	374.6
NHD (nicotinamide–hypoxanthine dinucleotide)					
pH 7.0	566.0	557.0	537.8		372.9
pH 2.0	568.2	559.9	540.5		374.9
3-Acetylpyridine–DPN					
pH 7.0	564.1	555.7	537.3	496.7	371.4
pH 2.0	570.4	562.7	546.8	504.3	377.4
3-Acetylpyridine–hypoxanthine dinucleotide					
pH 7.0	567.0	559.6	542.4		374.8
pH 2.0	569.5	561.0	546.8		377.3

[a] All measurements were made on a 0.15 M solutions in D_2O at 38° in a high-resolution Varian Model A-60A spectrometer.

TABLE IV: Separation of the Change in Chemical Shift Created by Protonation of Adenine N_1 and Consequent Unfolding of the Dinucleotide as a Result of pH Change from 7.0 to 2.0.

Compd (0.15 M)	Downfield Shift Caused by Protonation of Adenine N_1 and the Partial Unfolding of the Dinucleotide (cps)			Downfield Shift Caused by Partial Unfolding of the Dinucleotide (cps)		
	Adenine Peaks			Adenine Peaks		
	pH 7.0 pH 2.0 Δ Chemical Shifts[a]			pH 7.0 pH 2.0 $\Delta_{DPN} - \Delta_{ADPR}$ Chemical Shifts		
	C_8H	C_2H	C'_1H	C_8H	C_2H	C'_1H
ADPR	8.2	15.0	1.5	0	0	0
β-DPN	12.7	22.0	6.3	4.5	7.0	4.8
α-DPN	10.1	19.8	5.3	1.9	4.8	3.8

[a] Chemical shift at pH 2.0 − chemical shift at pH 7.0.

the base pairs. Recent nuclear magnetic resonance studies to be presented in a forthcoming paper on FAD with a doubly charged analog of DPN (nicotinamide$^+$–ribose–P–P–ribose–nicotinamide$^+$) further substantiate this point. Examination of Table I shows that at pH 2.0, the differences in the chemical shift between ADPR, α-DPN, and β-DPN still remain and this is because, as already outlined, the dinucleotide at pH 2.0 does not assume a fully open conformation and the interaction between the two ring systems is not totally annihilated.

At pH 8.0 the proton magnetic resonance signals are shifted downfield with increasing temperature except that adenine C_8H shows a small upward shift (Figure 7). This is in agreement with the observations of Jardetzky and Wade-Jardetzky (1966) and suggests that the molecule tends to unfold on heating at pH 8.0. It has not been possible to completely resolve the nicotinamide C_2 and C_6 peaks on heating. The average of these peaks is observed to change more rapidly than either the C_4 or ribose C'_1 protons. However, what is sig-

nificant is that the adenine C_2 protons exhibit far greater downfield shifting than C_8 protons, implying that the pyrimidine part of the adenine may be directly involved in the folding interaction.

Conformation of α-DPN. Data in Table I show that the adenine C_8H, C_2H, and C'_1H of α-DPN at pH 7.0 are shifted upfield from those of ADPR at pH 7.0 by 3.3, 6.3, and 7.3 cps, respectively. The upfield shift of the adenine protons in the dinucleotide indicates a folded conformation for the α isomer as has been suggested by Jardetzky and Wade-Jardetzky (1966). However, the adenine C_8H and C_2H in the α isomer has undergone an upfield shift of only 3.3 and 6.3 cps whereas the same protons in the β isomer undergo an upfield shift of 8 and 9 cps, respectively. This difference between the two isomers reflects the difference in the degree of intramolecular interaction between the base pairs in the two isomers. The pH data on the right-hand side of Table IV also show that the interaction between the two ring systems is less in α-DPN compared with that in β-DPN. In view of the fact that the proton magnetic resonance data strongly suggest a folded conformation for α-DPN, the earlier energy transfer data (Kaplan, 1960) have to be reinvestigated.

Proton Magnetic Resonance Spectra of β-DPN Analogs. The evidence presented so far is consistent with *a folded conformation for β-DPN* in which the pyridine ring is assumed to be in close juxtaposition with the pyrimidine portion of the adenine ring at biological pH and temperature. Magnetic resonance data, discussed below, for a variety of β-DPN analogs show that *hydrogen bonding of the type proposed to occur between base pairs* by Shifrin and Kaplan (1959–1961), Crick and Watson (1953), and others *does not appear to play any significant role in maintaining the conformational integrity of the dinucleotide at the biological pH and temperature.*

In all the analogs listed in Tables I and II except *N,N*-dimethylnicotinamide–DPN, the proton magnetic resonance signals from adenine C_8, C_2, and C'_1 protons appear at the same field at pH 7.0 and at a temperature of 38°. This indicates that the introduction of the substituents like $COCH_3$, CHO, $COCH_2(CH_2)_2CH_3$, NH_2, $CONHCH_3$, and $CONHCH_2CH_3$ in position 3 of the pyridine ring instead of the $CONH_2$ group does not affect the conformation of the dinucleotide and that the basic interaction between the pyridine and adenine rings remains the same. This would not be expected if the primary force of interaction between the two moieties were of a hydrogen-bonded nature[3] as in Figure 1. Thus in 3-acetylpyridine–DPN, in pyridine-3-aldehyde–DPN and in 3-butyl pyridyl ketone–DPN there is no NH_2 group available for hydrogen bonding as between the

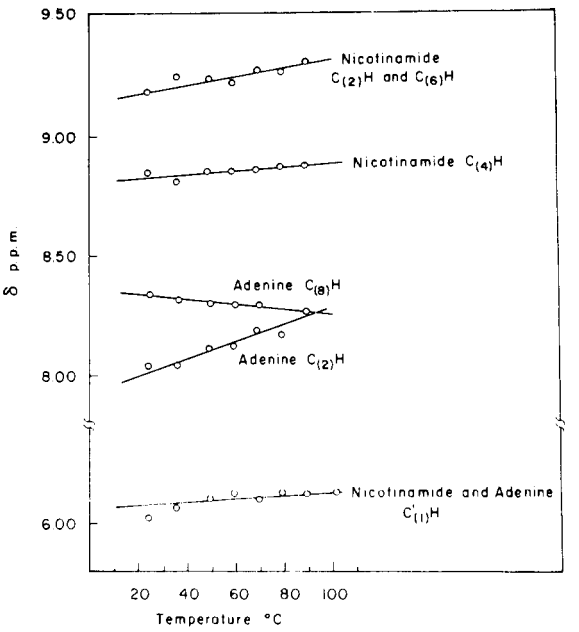

FIGURE 7: Temperature dependence of chemical shifts in 0.1 M β-DPN at pH 8.0.

N_1 of adenine and the NH_2 group of the nicotinamide moiety in DPN. It may be argued that this NH···N bond does not play a significant role in maintaining the integrity of the DPN conformation and that the hydrogen bonding between the C=O group of the pyridine ring and the NH_2 group at position 6 of adenine is the primary force of interaction. In the analogs NHD and 3-acetylpyridine–hypoxanthine dinucleotide, because of tautomerism, the only hydrogen bonding that could be present is of the NH···N type. Proton magnetic resonance and fluorescence data from NHD and proton magnetic resonance data from 3-acetylpyridine–hypoxanthine dinucleotide (*vide infra*) indicate that at pH 7.0 the purine and pyridine rings are dissociated from each other in these analogs, indicating that NH···N hydrogen bonding is insufficient to maintain the conformation of the dinucleotide. The chemical shift of adenine C_8, C_2, and C'_1 protons at pH 7.0 is the same for both β-DPN and 3-aminopyridine–DPN. This means that the basic interactions between the pyridine and adenine rings in the two compounds are the same, despite the absence of a C=O group in 3-aminopyridine–DPN to enter into hydrogen bonding of the type C=O···HN—.

The proton magnetic resonance spectra of the two hypoxanthine analogs, *i.e.*, NHD and 3-acetylpyridine–hypoxanthine dinucleotide, and that of *N,N*-dimethylnicotinamide–DPN deserve brief comments. The proton magnetic resonance spectra of NHD is shown in Figure 8. It could be seen from the spectra that the assignment of the pyridine C_5 proton and the adenine C_8 and C_2 protons was difficult because of the overlap of the pyridine C_5H resonance with the adenine C_8H and C_2H resonance peaks. In comparing the chemical shift of β-DPN and NHD as well as 3-acetylpyridine–DPN and the corresponding hypoxanthine analog, one has to confine one's attention to signals from the pyridine protons because replacement of NH_2 by OH itself could gen-

[3] Since the compounds were lyophilized from D_2O and the units which are supposed to hydrogen bond may carry deuterium instead of hydrogen, the term hydrogen bond used in this paper may imply deuterium bond. Substitution of deuterium for hydrogen does not change the ability of the units to hydrogen bond except that it will result in a slightly longer bond. The proton magnetic resonance measurements concern the nonexchangeable protons.

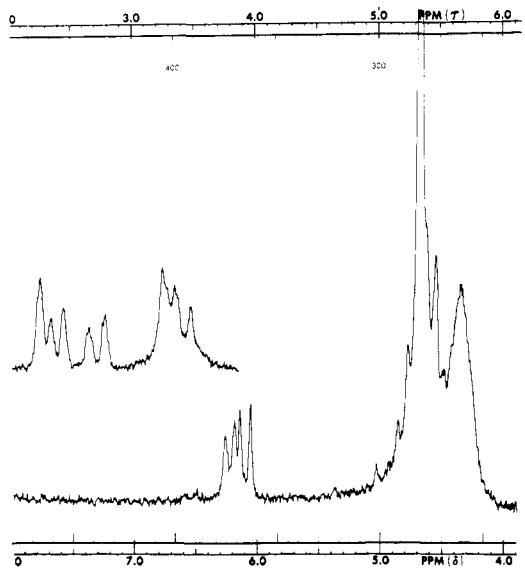

FIGURE 8: Proton magnetic resonance spectra of nicotinamide–hypoxanthine dinucleotide as reproduced directly from the nuclear magnetic resonance chart S60-C. Details are as in Figure 5.

erate differences in the chemical shift of adenine C_8, C_2, and C'_1 protons. At pH 2.0 the chemical shifts of pyridine C_2, C_6, C_4, and C'_1 are the same both in β-DPN and NHD (Table III). This means that both coenzymes are unfolded and dissociated to the same extent at pH 2.0. At pH 7.0 the proton magnetic resonance signals from pyridine C_2, C_6, C_4, and C'_1 protons are shifted to lower field[4] compared with those in β-DPN at pH 7.0, suggesting that in NHD the adenine and pyridine moieties are dissociated from each other. This also explains why Shifrin and Kaplan (1959) did not observe transfer of excitation energy in their energy transfer experiments with reduced NHD. The same lines of argument for the proton magnetic resonance chemical shift indicate that the analog 3-acetylpyridine–hypoxanthine dinucleotide is unfolded at pH 7.0 compared with the parent analog 3-acetylpyridine–DPN.

It is not quite clear why the hypoxanthine analogs are unfolded at pH 7.0. The tautomerism of the hypoxanthine moiety appears to be very significant. Mason (1957) has presented evidence using infrared spectroscopy that hydroxypurines themselves do not exist to any extent in the hydroxy form and that the most possible structure for 6-hydroxypurine is the one in which the tautomeric hydrogen atom derived from the hydroxy group is linked mainly to the N_1 rather than to the N_3 atom. So according to this, the hypoxanthine analogs have a C=O group at position 6 of the purine ring and the dissociation between the purine and pyrimidine moieties observed in the hypoxanthine analogs at pH 7.0 is most probably due to electrostatic repulsion between the rather negative oxygens of the carbonyl dipole of $CONH_2$ or $COCH_3$ groups and the carbonyl dipole of the tautomer of the hypoxanthine moiety. Electrostatic repulsions of this type are easily conceivable in a folded conformation rather than in a hydrogen-bonded one.

Steric Hindrance. The magnetic resonance data from the various analogs provide clues to the susceptibility of the dinucleotide to steric hindrance. In the folded conformation, the pyridine and adenine are stacked in parallel planes and as a result of this, substituents on the amide nitrogen, depending upon the orientation of the substituents, could hinder the folding of the rings. In the hydrogen-bonded conformation, the adenine and pyridine rings are coplanar and the two proposed hydrogen bonds (NH···O=C and N···HN) between the base pairs cannot take place if the $CONH_2$ group is in the same plane as the pyridine and purine. In an arrangement in which the pyridine, purine, and $CONH_2$ groups are coplanar, the C=O group will be too far distant from the adenine NH_2 if the hydrogen bonding between the adenine N_1 and the NH_2 of the amide takes place. In case the hydrogen bonding is between the amide C=O group and the adenine NH_2, the amide NH_2 will be too far removed from the adenine N_1. However such a difficulty could be overcome by twisting the $CONH_2$ group out of the plane of the pyridine ring and then the two hydrogen bonds could be formed as shown in Figure 1. In fact, the X-ray data of Wright and King (1954) indicate that the $CONH_2$ group of nicotinamide is not in the plane of the pyridine ring. If the pyridine and purine are coplanar and the $CONH_2$ group is out of the plane, as in a hydrogen-bonded conformation, one would not expect any steric hindrance from substituents on the amide nitrogen. This aspect of the problem was studied by proton magnetic resonance analysis of a series of amide nitrogen substituted analogs and this in turn unfolded some of the finer stereochemical features of this important biological molecule.

The analogs investigated were *N*-monomethylnicotinamide–DPN, *N*-monoethylnicotinamide–DPN, and *N,N*-dimethylnicotinamide–DPN. The analog *N,N*-diethylnicotinamide–DPN could not be prepared by the same method used for preparing other analogs. In the proton magnetic resonance spectra of *N*-monomethylnicotinamide–DPN and *N*-monoethylnicotinamide–DPN and β-DPN the adenine C_8H, C_2H, and C'_1H come at the same field. This indicates that the methyl or ethyl group on the nitrogen does not create any steric hindrance toward the adenine ring. In the case of *N,N*-dimethylnicotinamide–DPN,[5] the adenine C_8H and C_2H come at a field (506.7 and 488.4 cps, respectively) slightly lower than those in β-DPN (504.0 and 486.0 cps, respectively) suggesting that in the analog the two

[4] The hexagonal ring in hypoxanthine is not aromatic in character and its ring current anisotropy is small compared with the hexagonal ring of adenine, but its five-membered ring (the imidazole) has the same calculated ring current as that of the adenine (Griessner-Prettre and Pullman, 1965). This will contribute partly to the downfield shift of the pyridine resonance in NHD relative to DPN.

[5] *N,N*-Dimethylnicotinamide–DPN was prepared in two different batches. From each batch two different preparations were made to measure nuclear magnetic resonance spectra. The agreement among the final four spectra were well within ± 0.5 cps.

rings are less associated. In the proton magnetic resonance spectra of *N,N*-dimethylnicotinamide–DPN (Figure 9) the two methyl groups come at different fields, *i.e.*, 183.0 and 189.6 cps. This shows the double-bond character and restricted rotation about the C–N bond and that the two CH_3 groups reside in different electrical environments and as a result of this resonate at different fields. At 90° the two three-proton lines coalesced to a single six-proton line indicating free rotation of the C–N bond at high temperature. This is in accord with the observation of Phillips (1955) on the proton magnetic resonance spectra of *N,N*-dimethylacetamide and *N,N*-dimethylformamide. Observation of a single methyl proton resonance for *N*-monomethylnicotinamide–DPN and a clean triplet from CH_3 and a quartet from CH_2 in the case of *N*-monoethylnicotinamide–DPN points out that only one of the possible rotational isomers of these analogs is present. The fact that at pH 7.0 the dissociation between adenine and pyridine is observed only in *N,N*-dimethylnicotinamide–DPN and not in *N*-monomethyl- or even *N*-monoethylnicotinamide–DPN indicates that only one of the methyl groups in *N,N*-dimethylnicotinamide–DPN hinders the folding of the rings. Study of the molecular models of the folded conformation shows that the methyl group *trans* to the oxygen is the only methyl group which could hinder the folding. The slight dissociation caused by one of the methyl groups is probably steric in nature *via* van der Waal's repulsions. The fact that in *N,N*-dimethylnicotinamide–DPN the methyl group *trans* to the oxygen causes partial separation between the rings makes possible the calculation of the approximate distance between the adenine and pyridine rings in the folded conformation from the van der Waal's radius of a methyl group and the distance of the amide nitrogen from the plane of the pyridine ring. The van der Waal's radius of a methyl group found by a large number of workers approaches 2.0 Å. The displacement of the amide nitrogen in nicotinamide from the plane of the pyridine has been determined by Wright and King (1954) to be 0.46 Å. (In nicotinamide the amide group does not lie in the same plane as the pyridine ring.) This is most probably true in the case of the oxidized *N*-alkyl-substituted analogs of DPN. The C–N bond distance between the amide nitrogen and the carbon of the methyl group, from Wheland (1955), is 1.44 Å. Therefore the approximate distance from the plane of the pyridine ring to the outer edge of the van der Waal's sphere of the methyl group is calculated to be 0.46 + 1.44 + 2.0 = 3.90 Å. The distance between the adenine and pyridine rings in the folded conformation is slightly less than 3.90 Å because the plane which contains the amide group, as implied in this calculation, is not exactly perpendicular to the plane of the adenine ring.

β-DPNH. The earlier fluorescence transfer experiments by Shifrin and Kaplan (1960, 1961) and Velick (1961) as well as the proton magnetic resonance data by Meyer *et al.* (1962) and Jardetzky and Jardetzky (1966) support the idea that the conformation of β-DPNH in aqueous solutions is also a folded one. It has been found in this laboratory as well as shown by Meyer *et al.* (1962) and Jardetzky and Jardetzky (1966) that the two hydrogens at C_4 of the dihydronicotinamide moiety in β-DPNH are magnetically equivalent. This has been interpreted to suggest that the dihydronicotinamide ring is planar and that the $CONH_2$ group is in the same plane as the dihydropyridine ring. However, *according to a folded conformation, i.e.*, one in which the pyridine ring spends considerable time in the area above the plane of the adenine ring (Meyer *et al.*, 1962), one would expect the *two hydrogens at C_4 of the dihydropyridine ring in β-DPNH to be chemically different.* This is depicted in Figure 4 which is made from skeletal models and shows clearly that the two hydrogens at the C_4 of the dihydronicotinamide ring reside in different chemical environments, one of the hydrogens being juxtaposed to the adenine ring and the other being remote from the adenine ring. The chemical equivalence of the two pyridine C_4 hydrogens of β-DPNH in a folded conformation could be accounted for on the following grounds. (a) Free rotation of the N–C bond between the dihydropyridine ring and the ribose. Quite obviously, free rotation is impossible in the folded conformation because an adenine ring is juxtaposed to the pyridine. It is difficult to determine whether the dihydropyridine ring in an open conformation will undergo free rotation or not. Data available on analogous systems suggest that even in an open conformation the dihydropyridine ring would remain in some distinct conformation with respect to the ribose. The rotatory dispersion and circular dichroism data (Bender and Grisolia, 1968; Dr. C. Bender, private communication) show that in α-DPNH, β-DPNH, and β-NMNH the dihydropyridine ring is *not* free to rotate. Recent nuclear magnetic resonance studies (Schweizer *et al.*, 1968) clearly reveal that the adenine moiety in AMP is not free to rotate and that it is held in an *anti* conformation. Careful examination of the space-filling model of β-DPNH in open conformation shows that steric hindrance prevents free rotation of the dihydropyridine ring. Attempt at free rotation of the ring in

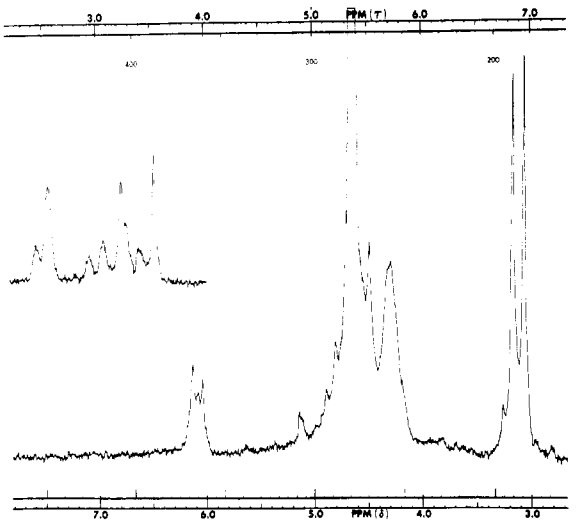

FIGURE 9: Proton magnetic resonance spectra of *N,N*-dimethylnicotinamide–DPN as reproduced directly from the nuclear magnetic resonance chart S60-C. Details are as in Figure 5. The two methyl groups on the nitrogen appear at 183.0 and 189.6 cps.

β-DPNH causes the pyridine hydrogens on C_2 and C_6 to approach the C'_2 hydrogen and the bridge oxygen of ribose beyond the desired van der Waal's distance. Such encroachment could result in steric hindrance and consequent preclusion of free rotation. We do not exclude the possibility that magnetic equivalence of the C_4 protons could result by minor oscillations or restricted rotation of the dihydropyridine ring. However, in view of all the above facts, we conclude that it is most unlikely that the dihydropyridine ring rotates freely in both the folded and open conformations of the dinucleotide. (b) Intermolecular complexing of pyridine and adenine rings, a situation in which the dihydropyridine ring is sandwiched between two adenine rings, one adenine ring from its own molecule and the other adenine ring from another molecule of β-DPNH. Jardetzky and Jardetzky (1966) have found that the chemical shifts in β-DPNH are independent of concentration and this argues against intermolecular complexing. (c) A situation in which there is a rapid equilibrium between two folded conformations, one in which the pyridine ring lies above the plane of the adenine ring (the right-handed folding, Figure 2) and the other in which the pyridine lies below the plane of the adenine ring (the left-handed folding, Figure 3).

Study of the molecular models shows that the dinucleotide is flexible enough to be folded either right handedly or left handedly and a rapid equilibrium between the two folded forms will average out the chemical shift of the two C_4 protons of the dihydronicotinamide ring in β-DPNH. This is because the C_4 proton juxtaposed to the adenine ring in the right-handed folding becomes remote from the adenine ring in the left-handed folding and *vice versa*. The fluorescence and magnetic resonance data so far available agree that pyridine dinucleotides exist in solution in a folded conformation: most probably there is a rapid equilibrium between two folded forms, one in which the pyridine ring lies above the plane of the adenine ring, the right-handed folding, and the other in which the pyridine ring lies below the plane of the adenine ring, the left-handed folding. The two orientations may help explain the existence of two classes of dehydrogenases with stereospecificity for the A and B side of the dihydropyridine ring of DPNH. Jardetzky and Wade-Jardetzky (1966) have estimated on the basis of thermodynamic parameters derived from chemical shift–temperature-dependent relations that only20–40% of the dinucleotide exists in folded conformation. A rapid exchange might occur between folded and unfolded as well as right- and left-handed forms. Our continuing nuclear magnetic resonance studies on flavin–adenine dinucleotide and the analog nicotinamide–nicotinamide dinucleotide, to be presented in a forthcoming paper, indicate that the nature of the interactions between the base pairs is of a hydrophobic nature and that the backbone of the dinucleotide is sufficiently flexible to permit such interactions.

Acknowledgments

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