High Frequency Nuclear Magnetic Resonance Study of the *M* and *P* Helices of Reduced Pyridine Dinucleotides*,†

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ABSTRACT: High frequency (220 MHz) nuclear magnetic resonance spectra of reduced triphosphopyridine nucleotide, reduced diphosphopyridine nucleotide, stereoselectively monodeuterated diphosphopyridine nucleotide, reduced nicotinamide–hypoxantine dinucleotide, reduced acetylpyridine diphosphopyridine nucleotide, reduced nicotinamide mononucleotide, reduced monodeuterated nicotinamide mononucleotide, and reduced acetylpyridine mononucleotide were investigated. The *geminal* C_4 protons of the dihydropyridine moiety of reduced triphosphopyridine nucleotide, reduced diphosphopyridine nucleotide, and reduced nicotinamide–hypoxanthine dinucleotide gave an AB quartet, indicating that the two C_4 geminal protons reside in different electronic and geometric environments. However, the geminal C_4 protons of the dihydropyridine moiety of reduced acetylpyridine diphosphopyridine nucleotide, reduced acetylpyridine mononucleotide, and reduced nicotinamide mononucleotide did not give an AB quartet. Comparison of the chemical shifts of the reduced dinucleotides to those of the corresponding reduced pyridine mononucleotides shows that the C_4H_2 resonance of the dinucleotides (except reduced nicotinamide–hypoxanthine dinucleotide) appears considerably upfield from those of the corresponding reduced pyridine mononucleotides, indicating that there is significant intramolecular interaction between the base pairs. At elevated temperatures, the AB quartet collapses to a singlet, moves downfield, and appears near the resonance of the mononucleotides, indicating that the dinucleotides unfold at elevated temperatures. A spectrum of the stereoselectively monodeuterated reduced diphosphopyridine nucleotide shows two signals from the remaining proton on the C_4 of the dihydropyridine moiety. On the other hand the same proton in the corresponding mononucleotide shows only one signal. This indicates significant population of the right- and left-handed folded dinucleotides. Specification of the molecular chirality of pyridine dinucleotides shows that the right- and left-handed folded dinucleotides are respectively *P* and *M* helices. The data further indicate that in aqueous solutions the reduced diphosphopyridine nucleotide exists as an equilibrium mixture of *P* and *M* helices undergoing slow exchange. Examination of the skeletal and space filling molecular models of *M* and *P* helices of dinucleotides reveals that the two helical forms are not equivalent, the nonequivalence being more pronounced in the reduced triphosphopyridine nucleotide, less pronounced in the diphosphopyridine nucleotide, and almost negligible in the acetylpyridine analog of the reduced coenzyme. In the *M* helix of triphosphopyridine nucleotide, the 2′-phosphate and the hydrogens of the $CONH_2$ side chain of the dihydropyridine moiety could hydrogen-bond, and this in turn would precipitate differences in the population densities of the two helical forms as well as in the chemical shifts of the geminal protons, which reside juxtaposed to the adenine ring in the two helical forms. In the case of diphosphopyridine nucleotide, the nonequivalence between the two helical forms may not be as prominent because of the absence of a large 2′ phosphate group.

In reduced acetylpyridine diphosphopyridine nucleotide, there is no chance for hydrogen bonding, and this may make the two helical forms almost equivalent. The upfield shifts in the dinucleotides from the corresponding mononucleotides indicate that the intramolecular interaction between the base pairs is strongest in reduced diphosphopyridine nucleotide and the corresponding acetylpyridine analog and is weaker in reduced triphosphopyridine nucleotide.

Recent investigations on the conformation of pyridine and flavin dinucleotides using 60 MHz nuclear magnetic resonance spectrometers by Jardetzky and Wade-Jardetzky (1966) as well as by Sarma *et al.* (1968b) have provided information about the molecular geometry and the nature of inter- and intramolecular interactions in the dinucleotides. However, the overlap of chemical shifts of environmentally nonequivalent and structurally distinct protons restricts the degree of knowledge one could obtain regarding the geometry of these molecules. Special mention should be made of the two geminal C_4 hydrogens of the dihydropyridine ring of the reduced coenzyme. In the 60 MHz spectrum, these two protons have the same chemical shift, an observation difficult to comprehend in view of the overwhelming evidence in favor of a folded conformation for the dinucleotide (Sarma *et al.*, 1968b). Recently, McDonald and Phillips (1967) as well as

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† This article contains two four-color figures. To avoid production difficulties these figures, as well as four-color figures from both accompanying articles, are printed together on pp 551, 552.

TABLE I: Chemical Shifts of the Adenine and Dihydropyridine Protons of Some Reduced Pyridine Mono- and Dinucleotides.[a]

Compounds	Adenine Peaks (Hz)		Dihydropyridine Peaks (Hz)					
						$PC_4H_AH_B$		
	AC_8H	AC_2H	PC_2H	PC_6H	PC_5H	H_A	H_B	$\nu_0\delta$[b]
23°								
NMNH[c]			1564.3	1076.5	1098.9	653.0	653.0	0
(AcPy)MNH			1648.5	1094.0	1139.0	635.0	635.0	0
TPNH	1865 ± 3	1806 ± 3	1522.5	*d*	*d*	602.7	620.7	18.0
DPNH	1857 ± 3	1789 ± 3	1522.5	1051.6	1046.5	584.3	598.9	14.6
NHDH	1855 ± 3	1800 ± 3	1535.3	*d*	*d*	627.4	634.2	6.8
(AcPy)DPNH	1860 ± 3	1785 ± 3	1600.8	*d*	*d*	575.7	575.7	0
5°								
NMNH			1555.0	1072.0	1084.0	642.7	642.7	0
(AcPy)MNH			1647.0	*d*	1134.3	628.0	628.0	0
TPNH	1863.5	1797.0	1516.0	1044.6	1084.3	575.6	601.4	25.8
DPNH	1853.5	1771.3	1510.5	1051.5	1041	560.5	581.9	21.4
(AcPy)DPNH	1854.3	1768.0	1595.0	1066.8	1070	548.7	548.7	0

[a] The nuclear magnetic resonance spectra were obtained on a high resolution Varian HRSC-IX superconducting solenoid 220 MHz nuclear magnetic resonance system. The measurements were made on 0.1 M (pH 7.5) D_2O solutions. Shifts were measured with DSS as an internal standard and precise calibrations were made with the use of an external oscillator. The letters P and A stand for pyridine and adenine; H_A and H_B are arbitrarily designated to be the two geminal protons at C_4 of the dihydropyridine ring of the reduced coenzyme and their chemical shifts are calculated theoretically from the AB quartet using the expression $C = {}^1/_2[(\nu_0\delta)^2 + J^2]^{1/2}$. [b] $\nu_0\delta$ is the difference in chemical shifts between H_A and H_B. [c] Equimolar mixture of NMNH + AMP or AcPyMNH + AMP, obtained by hydrolysis of the corresponding reduced pyridine dinucleotides by snake venom phosphodiesterase. [d] The chemical shifts could not be measured because the resonance was vitiated by the HDO peak.

Ferguson and Phillips (1967) have successfully employed high frequency nuclear magnetic resonance spectroscopy to investigate the conformational differences that exist between the native and denatured forms of ribonuclease, lysozyme, and cytochrome *c*. These considerations led us to study the proton magnetic resonance spectra of reduced pyridine nucleotides at a frequency of 220 MHz and as a result valuable information has been obtained about their molecular geometries.

Experimental Section

The nucleotides were obtained from P-L Biochemicals. Equimolar mixtures of NMNH[1] and AMP as well as (AcPy)-MNH and AMP were obtained by treating 0.1 M DPNH or 0.1 M (AcPy)DPNH with catalytic traces of snake venom phosphodiesterase (Worthington).

DPND was synthesized by reacting 1 g of C_2D_5OD with 200 mg (0.3 mM) of DPN and horse liver alcohol dehydrogenase in 25 ml (pH 10.0) of 0.1 M glycine–NaOH buffer. Progress of the reaction was followed by the increase in optical density at 340 mμ. When the reaction was complete, 75 ml of absolute ethanol was added and the solution centrifuged to get rid of any precipitated enzyme. The supernatant was vacuum evaporated to 25 ml, and 0.3 mM $BaCl_2$ was added to convert the sodium salt of DPNH into the less soluble barium salt. To the above solution 200 ml of absolute ethanol was added to precipitate the DPND. The solution was kept overnight in the cold room and the DPND was collected by decantation. The precipitate was dissolved in distilled water and converted into the sodium salt by double displacement with Na_2SO_4. The solution was centrifuged and the supernatant which contained the sodium salt of DPND was lyophilized. The excess salt and glycine were removed from DPND by passing it through a Sephadex G-10 column. NMND was synthesized from DPND using snake venom phosphodiesterase.

The 220 MHz spectra were obtained on a high resolution Varian HRSC-IX superconducting solenoid nuclear magnetic resonance system. Shifts were measured with DSS (E. Merck AG, Darmstadt, Germany) as an internal standard. Precise calibrations of the spectra were made with the use of an external audio oscillator and the precision of the measurements were within ±0.5 Hz. A Varian C-1024 computer of average transients was used to increase the signal to noise ratio in the case of 0.05 M DPNH solutions in 4 M NaCl.

Results and Discussion

The chemical shifts of the adenine and dihydropyridine protons of TPNH, DPNH, (AcPy)DPNH, and NHDH are summarized in Table I. Table I also contains the chemical

[1] Abbreviations used are: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; NHDH, reduced nicotinamide–hypoxanthine dinucleotide; (AcPy)DPNH, reduced acetylpyridine–adenine dinucleotide; NMNH, reduced nicotinamide mononucleotide; (AcPy)MNH, reduced acetylpyridine mononucleotide; DPND, stereoselectively C_4-monodueterated DPNH; NMND, stereoselectively C_4-monodeuterated NMNH.

shift data from the reduced mononucleotides, NMNH and (AcPy)MNH. The chemical shifts are reported in hertz. The radiofrequency oscillator of the Varian HRSC-IX superconducting nuclear magnetic resonance system is set at 220 MHz. The chemical shifts in hertz may be divided by the oscillator frequency and reported as parts per million.

The Geminal C_4 Protons of the Dihydropyridine Ring of Reduced Pyridine Dinucleotides. Nuclear magnetic resonance studies (Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968b) have strongly indicated that both the oxidized and reduced pyridine dinucleotides exist in a folded conformation, in which the pyridine and adenine rings are stacked in parallel planes. In a folded conformation, the two C_4 protons of the dihydropyridine ring of the reduced coenzyme are environmentally nonequivalent, and hence would be expected to appear at different fields in their nuclear magnetic resonance spectra (Figure 4, Sarma *et al.*, 1968b). The 60 MHz spectra of Meyer *et al.* (1962) as well as those of Jardetzky and Wade-Jardetzky (1966) showed both protons appearing at the same field. Our nuclear magnetic resonance investigations in D_2O at 0° as well as in 50% v/v D_2O–Me_2SO mixture at −20° showed them to be "magnetically equivalent" in the 60 MHz nuclear magnetic resonance system. Hollis (1967) has investigated the nuclear magnetic resonance spectra of DPNH in the 100 MHz system in the course of studies on the binding of coenzymes to enzymes; his spectrum did not distinguish any difference between the two geminal C_4 protons.

The spectra of the geminal C_4 protons of the dihydropyridine ring of TPNH, DPNH, (AcPy)DPNH, NMNH, and (AcPy)MNH taken in the 220 MHz system are shown in Figure 1. The nuclear magnetic resonance spectra of TPNH and DPNH at 5° are classic cases of AB spectra where the ratio of coupling constant (J) to the difference in chemical shift ($\nu_0\delta$) is large and indicates that the two protons are magnetically nonequivalent. Theoretically calculated geminal H–H spin–spin coupling constants as a function of the HCH bond angles vary from 32.3 Hz to 12.1 Hz as the HCH angle increases from 100 to 110° (Gutowsky *et al.*, 1959). The difference in chemical shifts between the two geminal protons should be small because the difference arises out of one proton being juxtaposed to the adenine ring and the second being remote from the adenine ring.[2] In the case of NHDH it is known that the interaction between the ring systems is significantly less as compared to DPNH and hence $\nu_0\delta$ would be still smaller.[3] If $J/\nu_0\delta$ were very small (0.02) for these two protons, we would have observed two doublets (*vide infra* for a discussion of the spin–spin splitting between the vicinal H_5 and the two geminal C_4 protons), each having components of equal intensity. As the ratio $J:\nu_0\delta$ increases, one should begin to observe an increase in the central components at the expense of the outer components. When there are very large values of this ratio, the central bands coalesce and the outer bands disappear.

In the 60 MHz nuclear magnetic resonance spectrum (Figure 1, see insert at bottom right) bands 1 and 4 could not be detected because they are overshadowed by the noise. This is due to the low signal to noise ratio of the 60 MHz nuclear magnetic resonance system and of the high gain and type of filter one has to use in order to record a spectrum of 0.1 M dinucleotide solutions. The 60 MHz spectrum does not detect bands 2 and 3 separately, because of the resolution limitations. Further, if the intrinsic line width were comparable with the separation in line width one would not be able to observe the AB quartet at 60 MHz. Analyses of the spectra (Emsley *et al.*, 1965; Pople *et al.*, 1959) show the chemical shift differences at 220 MHz between the two protons in TPNH to be 18 Hz, in DPNH to be 15 Hz, and in NHDH to be 6 Hz at room temperature. These differences are in agreement with our observations that in the hypoxanthine analog there is significantly less interaction between the base pairs[3] (Sarma *et al.*, 1968b).

What precipitates the magnetic nonequivalence of the C_4 geminal protons of the dihydropyridine ring of the reduced pyridine dinucleotide? It could be due to any of the following reasons, either in combination or alone.

(1) The dihydropyridine ring may exist in a puckered boat geometry in which one proton is quasi-axial and the other quasi-equatorial, the umbrella inversion of the trigonal nitrogen atom being sufficiently slow to make the C_4 axial and equatorial protons nonisochronous.

(2) The dihydropyridine ring is planar and the rate of rotation around the nitrogen–ribose glycosidic linkage is sufficiently slow compared to the nuclear magnetic resonance time scale. The orientation of the chiral centers of D-ribose with respect to the plane of the dihydropyridine ring would then induce a low order of asymmetry on the C_4 methylene of dihydropyridine.

(3) The amide side chain is not in the same plane as the dihydropyridine and the amide side chain would then shield the C_4 methylene proton to a different extent.

(4) The adenine ring of the dinucleotide is juxtaposed to the dihydropyridine ring so that the two protons at C_4 reside in different electronic and geometrical environments.

Assumption four appears as the most likely explanation for the reasons discussed below.[2] The difference in the chemical shifts at 23° between the geminal protons is 6 Hz in the case of NHDH, whereas in the case of DPNH and TPNH it is 15–18 Hz. Fluorescence transfer experiments (Shifrin and Kaplan, 1959) and nuclear magnetic resonance data[3] (Sarma *et al.*, 1968a,b) show that the intramolecular interaction between base pairs is significantly less in the hypoxanthine analog compared with the same in DPNH or TPNH. Reasons one, two, and three given above cannot account for the low $\nu_0\delta$ of NHDH compared with that of DPNH and TPNH. It is known that the dinucleotide unfolds at high temperatures (Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968b). Accordingly one would expect the geminal resonance to lose the AB pattern at elevated temperatures. Figure 1 shows the 220 MHz nuclear magnetic resonance spectrum of the geminal C_4 protons at 74°. At 5° (Figure 1) the center of the AB spectrum of DPNH was 571.2 Hz. At 74° the resonance has moved to a lower field of 637.5 Hz. The shift to the lower field is due to unfolding of the dinucleotide and consequent diminution of ring current shielding by the adenine magnetic anisotropy. In addition, at 74° the resonance

[2] In order to avoid confusion, it is sufficient at this point of this paper to explain the chemical nonequivalence of the two geminal C_4 protons of dihydropyridine, on the basis of a single folded conformation; *i.e.*, one proton is juxtaposed to the adenine ring and the other is remote from it. Later, we will present a discussion of the effect of chemical equilibria on the magnetic nonequivalence of the two geminal protons.

[3] Manuscript entitled "Geometry and Nature of the Folding Interactions between Base Pairs in Pyridine and Flavin Dinucleotides," in preparation.

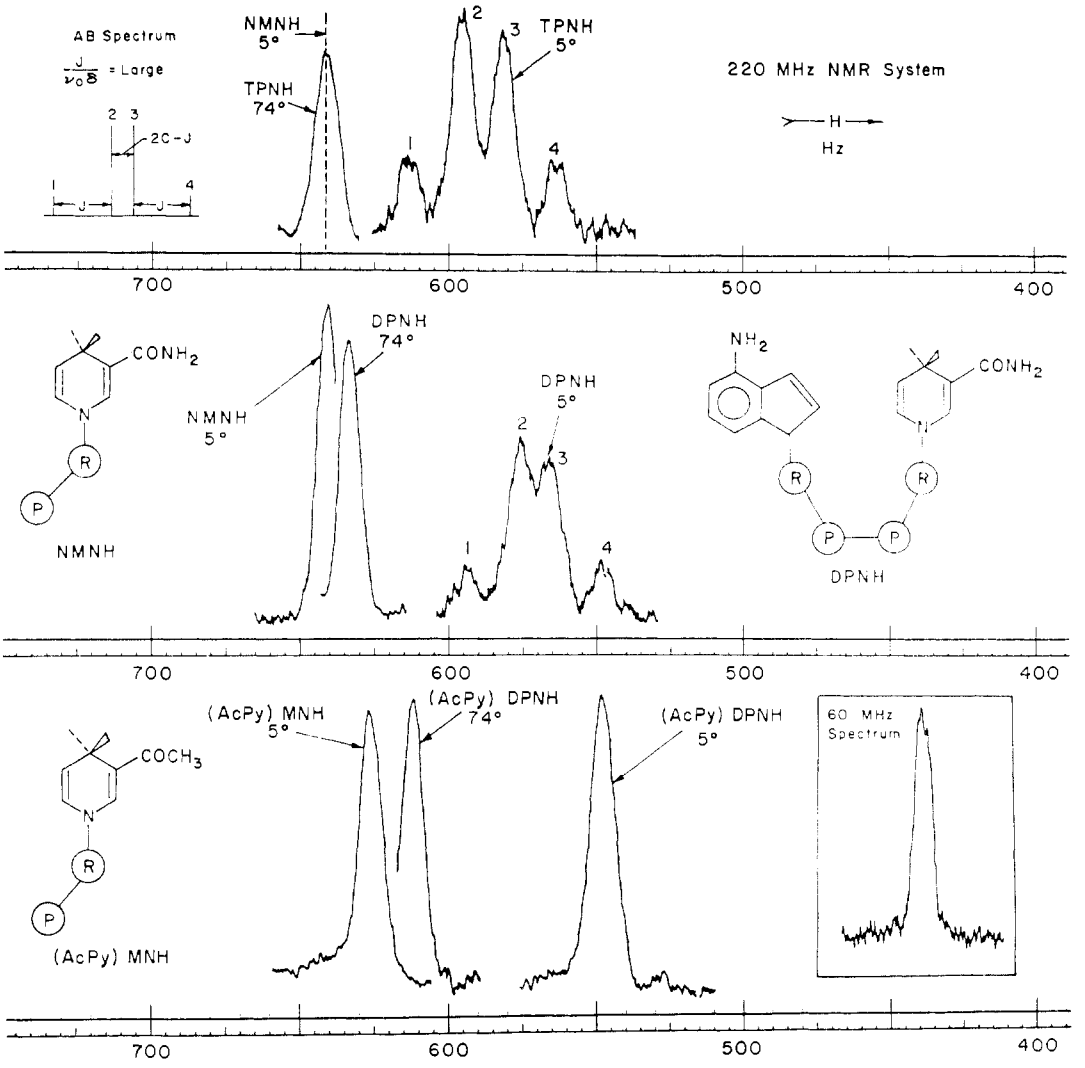


FIGURE 1: Nuclear magnetic resonance spectra of the two geminal C_4 protons of the dihydropyridine ring of TPNH, DPNH, (AcPy)DPNH, NMNH, and (AcPy)MNH taken in a 220 MHz nuclear magnetic resonance system at a sweep width of 500 Hz using DSS as an internal standard. The top of this figure shows the nuclear magnetic resonance signals from TPNH at 5 and 74° as well as the position where chemical shifts of NMNH at 5° appear. Top, left-hand corner, shows the theoretical pattern of an AB quartet where the ratio of coupling constant to the difference in chemical shift is large ($J:\nu_0\delta$). The middle of this figure shows the nuclear magnetic resonance signals from DPNH at 5 and 74° as well as the signal from NMNH at 5°. The bottom of this figure shows the nuclear magnetic resonance signals from (AcPy)DPNH at 5 and 74° as well as the signals from (AcPy)MNH at 5°. Bottom, right-hand corner, shows the 60 MHz nuclear magnetic resonance spectra of the two geminal C_4 protons of the dihydropyridine moiety of DPNH at 5°.

has become sharper and lost the AB pattern, indicating that the geminal protons are almost chemically equivalent. At 74° the inner bands 2 and 3 (Figure 1) are almost coalesced to a single band indicating that $\nu_0\delta$ is negligibly small between the geminal protons. When $\nu_0\delta$ is negligibly small, the outer bands 1 and 4 correspond to spectroscopically forbidden transitions and disappear completely. Figure 1 also shows the 220 MHz nuclear magnetic resonance spectrum of the geminal C_4 protons of the dihydropyridine in an equimolar mixture of AMP and NMNH. The equimolar mixture was obtained by breaking the diphosphate backbone with snake venom phosphodiesterase, The C_4 resonance appears at 642.7 Hz, *i.e.*, 71.5 Hz downfield from the center of the AB spectrum of DPNH (Figure 1). The downfield shift in the AMP + NMNH mixture indicates that in the dinucleotide the adenine is juxtaposed to the dihydropyridine ring and that hydrolysis of the diphosphate bridge annihilates the stacking of the base pairs. The C_4 methylene resonance from NMNH + AMP (Figure 1) is not an AB spectrum as in the case of DPNH; it is a singlet indicating that the two C_4 geminal protons are indeed magnetically equivalent. Discussions presented above show clearly that the magnetic nonequivalence of the two C_4 geminal protons of the dihydropyridine of the reduced pyridine dinucleotides arises out of its juxtaposition to the adenine ring. It is very important to point out at this time that the juxtaposition of the adenine to the dihydropyridine ring need not always generate an AB quartet for the geminal C_4 hydrogen. For example, the geminal C_4 hydrogens of (AcPy)DPNH do not produce an AB quartet. Discussion of the cause of the chemical nonequivalence of

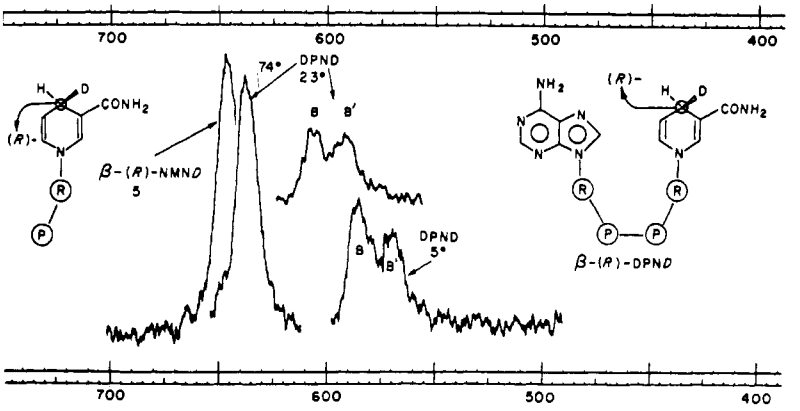


FIGURE 2: Nuclear magnetic resonance spectra (220 MHz) of the C_4H_B proton of the dihydropyridine of (*R*)-DPND at 5, 23, and 74° taken in the 220 MHz nuclear magnetic resonance system at a sweep width of 500 Hz. The figure also shows the spectrum of the C_4H_B proton of the dihydropyridine of (*R*)-NMND.

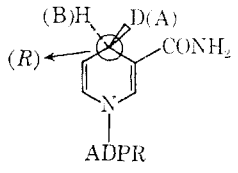
these geminal hydrogens in terms of the various possible equilibria is presented later. The absence of an AB pattern for the C_4 geminal protons in NMNH does not agree with a puckered boat geometry for the dihydropyridine ring. The same observation also discredits any explanation of magnetic nonequivalence of the C_4 geminal protons based on orientation of the chiral centers in D-ribose attached to the dihydropyridine or on the assumption that the amide side chain is not on the same plane as the dihydropyridine. The X-ray diffraction data of Karle (1961) on crystalline N-substituted 1,4-dihydronicotinamides has shown that the ring is planar. The 100 MHz nuclear magnetic resonance spectrum of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide in $CDCl_3$ shows *two symmetrical triplets* with the same $J_{4,5}$ = 3.5 Hz, $J_{5,6}$ being 8 Hz (Kim and Chaykin, 1968) for the C_5H resonance. The dihydropyridine C_5H resonance from DPNH is also a pair of symmetrical triplets. This indicates that the dihedral angles between C_5H and the two C_4 protons are the same as in a planar conformation of the ring as opposed to a puckered boat conformation.

The observation that the two geminal protons of the dihydropyridine ring reside in different electrical and geometric environments makes it possible to draw the following conclusions regarding the molecular geometry and mechanism of action of reduced pyridine dinucleotides.

Restriction of Rotation about the Dihydropyridine–Ribose Glycosidic Linkage. There is a certain degree of restriction of rotation about the dihydropyridine–ribose glycosidic linkage. It is possible to put an upper limit on the rate of reorientation of this bond to be something less than 10^2 sec^{-1}. However, with the present data, we cannot assign a lower limit on the rate of rotation about this bond. From the chemical shift difference at 220 MHz of 15 Hz, the chemical shift difference at 100 MHz is about 7 Hz. Since an AB spectrum is not observed at 100 MHz (Hollis, 1967) one *may not conclude* that the rate of rotation must be faster than 40 sec^{-1}. This is because it is very hard to tell whether the apparent fusion of AB pattern at 100 MHz is due to the fact that the rate of rotation is faster than 40 sec^{-1} or due to resolution limitations of the instrument. It could also be that the intrinsic line width is the same as the separation in line widths at 100 MHz.

Folded Conformation of the Dinucleotide. The reduced pyridine dinucleotides exist in a folded conformation. It could be folded in two different ways. In one folding arrangement, the dihydropyridine ring could spend a considerable amount of time above the plane of the adenine ring. This folding arrangement is arbitrarily designated as the right-handed folded conformer. In another folding arrangement, the dihydropyridine ring could spend a considerable amount of time below the plane of the adenine ring. This folding arrangement is arbitrarily designated as the left-handed folded conformer. It must be noted that the C_4 proton which is juxtaposed to the adenine ring in the right-handed folded conformer would reside remote from the adenine ring in the left-handed folded conformer. Recent high frequency nuclear magnetic resonance data (Sarma *et al.*, 1970) on *N*-methyl-*N*-ethylnicotinamide–adenine dinucleotide and on DPND (*vide infra*) indicate that there are significant populations of both types of folded conformers. Nuclear magnetic resonance data on *N*-methyl-*N*-ethylnicotinamide–adenine dinucleotide show clearly the existence of four nonequivalent N–CH_3 groups. This could happen only if there are significant populations of both right- and left-handed folded conformers. Each of the two possible geometric configurations of the DPN analog could then exist as a mixture of two conformers, giving rise to a total of four conformers and hence four nonequivalent N–CH_3 groups. The most desirable method to determine whether there are significant populations of both right- and left-handed folded conformers is to perform high frequency nuclear magnetic resonance studies on DPND. Dihydropyridine monodeuterated DPNH with the absolute configuration I (Cornforth *et al.*, 1962) was synthesized. The configuration of the new asymmetric center at C_4 in DPND is (*R*) according to the specification of asymmetric configurations in organic chemistry (Cahn *et al.*, 1956), and hence this DPND will hereafter be referred to as (*R*)-DPND. The nuclear magnetic resonance spectrum of (*R*)-DPND in the 220 MHz nuclear magnetic resonance system at 5, 23, and 74° is shown in Figure 2. The two individual resonances in Figure 2 designated as B and B′ with a difference in chemical shift of 14 Hz at 23° is from the same B proton in I. The two resonances cannot be the doublet from C_4H spin–spin splitting with C_5H because the coupling constants are independent of the frequency of the nuclear magnetic resonance system and the $J_{4,5}$ is only about 3 Hz (*vide infra*). Figure 2 also contains the 220 MHz nuclear magnetic resonance spectrum of the C_4HP proton of (*R*)-NMND and this is a singlet. From these observations we conclude that the

two individual resonances B and B′ of (*R*)-DPND are from the same B (I) proton which spends enough time in two different electrical and geometric environments.[4] In terms of the conformation of the dinucleotide this observation rules out the existence of a single folded conformation. One could



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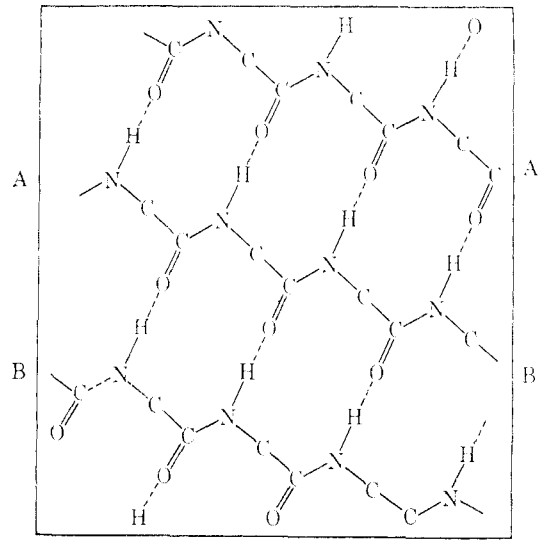
conjecture that there are significant populations of *syn* (dihydropyridine–ribose torsion angle ~150°) and *anti* (dihydropyridine–ribose torsion angle ~ −30°) conformations and that this gives the B and B′ signals in Figures 2. However, Sarma and Kaplan (1969b) have shown the orientation of the dihydropyridine with respect to the adjacent ribose in DPNH to be *syn*.

Hence, the appearance of B and B′ peaks in (*R*)-DPND indicates significant populations of both right-handed folded and left-handed folded dinucleotides. In this situation, the C_4B proton would spend considerable time juxtaposed to the adenine ring as in the left-handed folding and remote from the adenine ring as in the right-handed folding. At 74° as a result of unfolding of the dinucleotide, the difference in chemical shift between B and B′ vanishes and the signals B and B′ blend and move downfield. The chemical shift of the C_4B proton of (*R*)-DPND at 74° and that of the two C_4 geminal protons of DPNH at 73° is the same, *viz.* 638 Hz. It may be noted that the resonances B and B′ from (*R*)-DPND are broadened. This is because deuterium possesses an electric quadrupole moment of about 2.77×10^{-3} in multiples of $e \times 10^{-24}$ cm^2.

Specification of the Molecular Chirality of Pyridine Dinucleotides. In the above we have presented evidence for the existence of two distinct types of folded dinucleotides arbitrarily called right- and left-handed folded conformers. It is essential that we clarify the ambiguities associated with the arbitrary designations and *sharply* and *absolutely* define the geometry of the folded dinucleotide using the newer concepts in space relations, and also establish a symbiosis between the newer concepts in geometry and the arbitrary designations. Cahn *et al.* (1966) have provided a new concept of conformation based on the molecular chirality of the structure in question. This new concept of conformation has an agreed area of application and agreed area of nonapplication and penumbra. They define the term *chiral* as a model which has no element of symmetry except at most axes of rotation. *Asymmetry* is a sufficient, but not a necessary and sufficient condition for the existence of optical enantiomers. Chirality expresses the necessary and sufficient condition for the existence of optical enantiomers. Chirality means topological handedness. Cahn *et al.* (1966) propose a helicity rule to express the chiral nature of a helix. "*According as the identified helix is left- or right-handed, it is designated 'minus' and denoted by M or designated 'plus' and denoted by P.*" They illustrate the application of the above designations in the α-helical form of polypeptides which is shown in II,[5] opened out after making a cut parallel to the helical axis. If this projection is wrapped into a cylinder by joining the points AA, and the points BB, behind the paper, the peptide chain



II

will describe an *M* helix, whereas, if the junctions are made in front of the paper, the chain will describe a *P* helix.

In the case of the pyridine dinucleotides, the backbone of the dinucleotide could make a half turn of a right-handed helix, *i.e.*, the dinucleotide forms a *P* helix and thus the chiral description *P* as applied to the whole model is easily interpreted. It is this *P* helix of β-DPN that is shown in Figure 2 of the paper of Sarma *et al.* (1968b). The *P* helix of TPNH is shown in Figure 3.[6] In the *P* helix of DPN as well as in that of TPNH, the pyridine ring lies above the plane of the adenine ring, and to reverse the helix with respect to the observer makes no difference, *i.e.*, the *P* helix of the dinucleotide is a distinct structural species. The *P* helix of DPNH is shown schematically in Figure 5 (*vide infra*) and it should be noted that the A proton (Cornforth *et al.*, 1962) is juxtaposed to the adenine ring. The backbone of the dinucleotide could also make a half turn of a left-handed helix, *i.e.*, the dinucleotide forms an *M* helix. It is this *M* helix of β-DPN that is shown in Figure 3 of Sarma *et al.* (1968b). The *M* helix of TPNH is shown in Figure 4.[6] In the *M* helix shown in Figure 3 of the paper of Sarma *et al.* (1968b) as well as in Figure 4, the pyridine ring lies below the plane of the adenine ring, and to reverse the helix with respect to the observer makes no difference, *i.e.*, the *M* helix of the dinucleotide is a distinct structural species. The *M* helix of DPNH is shown schematically in Figure 5 and it should be noted that the A proton (Cornforth *et al.*, 1962) resides remote from the adenine ring. The high frequency nuclear magnetic resonance data from

[4] There may be conditions under which a fast exchange between the two helical forms occurs at biological temperature. This will cause B and B′ to coalesce to give a sharp single line (see below). These conditions are under investigation.

[5] Taken from Cahn *et al.*, 1966.

[6] Taken by Mr. John Galano, Camera Eye, 415 South Street, Waltham, Mass.

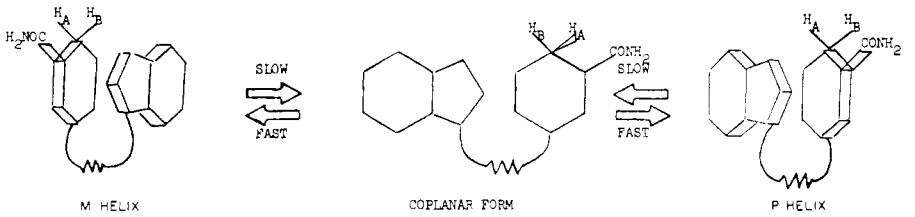


FIGURE 5: Schematic representation of the state of equilibrium in DPNH.

(*R*)-DPND and *N*-methyl-*N*-ethylnicotinamide–adenine dinucleotide (Sarma *et al.*, 1970) indicate that dinucleotide solutions contain significant populations of both *P* and *M* helices. We believe the specification of the molecular chirality of pyridine dinucleotides as *P* and *M* helices enables one to define sharply and absolutely the topological chirality of the folded dinucleotides and hence this nomenclature will be used throughout the rest of this paper and in forthcoming papers. Thus (*M*)-DPNH and (*P*)-DPNH respectively refer to the *M* helix of DPNH and the *P* helix of DPNH (Figure 5). After having defined the geometry of the folded dinucleotide in sharp and absolute terms of the newer concepts in space relation, it is essential that we establish a symbiosis between. the newer concepts in geometry and the arbitrary designations. Thus (*P*)-DPNH is the arbitrarily assigned right-handed folded conformer of DPNH, and (*M*)-DPNH is the arbitrarily assigned left-handed folded conformer of DPNH.

The Chemical Equivalence or Nonequivalence between the P and M Helices. Very careful examination of the skeletal and space filling molecular models suggests that very subtle differences exist between the *M* and *P* helices of TPNH as well as between those of DPNH. (i) In the *M* helix of TPNH (Figure 4) the C_4 proton nearer to the adenine ring (H_B) is exposed to the plane of the adenine as well as the 2′-phosphate; also one of the hydrogens of the $CONH_2$ group possibly could hydrogen bond to the oxygens of the phosphate. In the *M* helix the phosphate would also prevent the dihydropyridine ring from approaching the adenine ring beyond a certain distance. On the other hand, in the *P* helix (Figure 3) the dihydropyridine ring could approach the adenine much closer than it could in the *M* helix because the 2′-phosphate does not get in the way of folding interaction, *i.e.*, the C_4 proton juxtaposed to the adenine ring in the *P* helix (H_A) would undergo a larger ring current, shielding the C_4 proton juxtaposed to the adenine ring in the *M* helix. (ii) In the *M* helix of DPNH the C_4 proton juxtaposed to the adenine ring (H_B) resides in an environment which contains the plane of the adenine as well as the 2′- and 3′-hydroxyl groups of the ribose adjacent to the adenine. Furthermore, it is possible that the hydrogen of the $CONH_2$ group could hydrogen-bond to the 2′-OH group and stabilize the *M* helix. On the other hand, in the *P* helix, the C_4 proton juxtaposed to the adenine ring (H_A) resides in an environment which contains the plane of the adenine as well as the ether oxygen of the ribose adjacent to the adenine. In the case of DPNH, the difference between the *M* and *P* helical forms is not as pronounced as in the case of TPNH. (iii) The differences between the two helical forms of (AcPy)DPNH are negligible.

Population Difference between the P and M Helices. The nuclear magnetic resonance spectrum of (*R*)-DPND in Figure 2 very clearly shows that the areas of B and B′ are different, B being about 15% larger. This indicates that the two helical forms are populated to a different extent.

State of Equilibrium between the P and M Helices. One could envision the two limiting cases of rapid and slow exchange between the two helical forms. The inverse of the difference in chemical shifts between H_A and H_B in DPNH at 23° is 0.011 sec $[1/(2\pi \times \nu_0\delta)]$. For a slow exchange to occur, the mean lifetime of the two folded conformations should be larger than 0.011 sec, probably of the order of 0.1 or 0.333 sec. In the case of a fast exchange the lifetimes would be smaller than 0.011 sec. Normally, a molecule the size and shape of DPNH would be expected to undergo fast exchange. In fact, the observed AB quartet would agree with a rapid exchange between two nonequivalent helical forms populated to different extents. However, the following facts strongly argue against such a rapid exchange.[4]

(a) We have observed two peaks B and B′ from the same C_4H_B protons of (*R*)-DPND (Figure 2). A rapid exchange between two unequally populated nonequivalent helical forms of (*R*)-DPND should generate an average singlet.

(b) A rapid exchange between two unequally populated, nonequivalent helical forms of DPNH should generate a *symmetrical* AB quartet. Examination of bands 2 and 3 of the AB quartet of DPNH (Figure 1) show that the quartet is not symmetrical. This aspect has been further studied by recording the AB quartet from 0.05 M DPNH in 4 M NaCl in the temperature range of −7 to 23°. It has been very clearly observed that bands 1 and 2 possess higher intensities than bands 4 and 3. This indicates that the observed AB quartet is not a "pure" quartet but a "deceptively simple" AB quartet.

The observation of the peaks B and B′ (Figure 2) from (*R*)-DPND is consistent only with a slow exchange between the *P* and *M* helices. In the case of DPNH, a slow exchanging situation would be expected to result in a pair of AB quartets because the two helical forms are not equivalent. We believe that the observed asymmetric AB quartet results from the overlap of the two expected AB quartets from the two unequally populated, nonequivalent helical forms of DPNH. In Figure 5 we have schematically depicted this exchange. In addition to the two helical forms we have included a very low populated coplanar form as an intermediate. The coplanar form could rapidly go either to the *P* helix or to the *M* helix.

In the case of (AcPy)DPNH we did not observe an AB quartet (Figure 1). The observed singlet could result from two equally populated, equivalent helical forms undergoing fast exchange. It could also be that the chemical shifts of the H_A and H_B protons in (AcPy)DPNH become identical accidently. However, what is more important is the observation that the incorporation of a $COCH_3$ group instead of a $CONH_2$ group at C_3 of the dihydronicotinamide startlingly affects the asymmetry at the C_4-geminal protons.

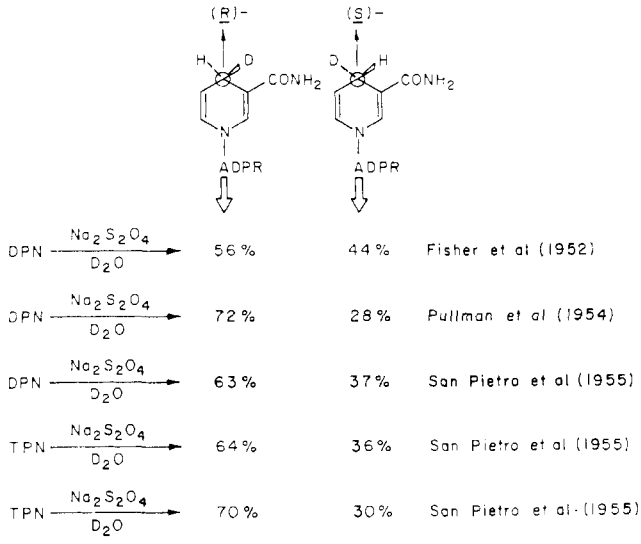


FIGURE 6: Percentages of the (*R*)- and (*S*)-DPND and TPND formed during the reduction of DPN and TPN by sodium hydrosulfite in deuterium oxide.

The Behavior of the Two Geminal C_4 Protons of the Dihydropyridine Moiety in Nonenzymatic Reactions. It has been observed by various people that the two geminal C_4 protons of the dihydropyridine moiety behave differentially in nonenzymatic oxidations and reductions. Figures 6 and 7 summarize the observations of Fisher *et al.* (1952), Pullman *et al.* (1954), and San Pietro *et al.* (1955). Sodium hydrosulfite reduction of DPN and TPN shows a preference for the formation of (*R*)-DPND and (*R*)-TPND (Figure 6). Ferricyanide oxidation of (*R*)-DPND leads to 65% retention of deuterium in the product, indicating a probable isotope effect. On the other hand, both enzymatic and ferricyanide oxidation

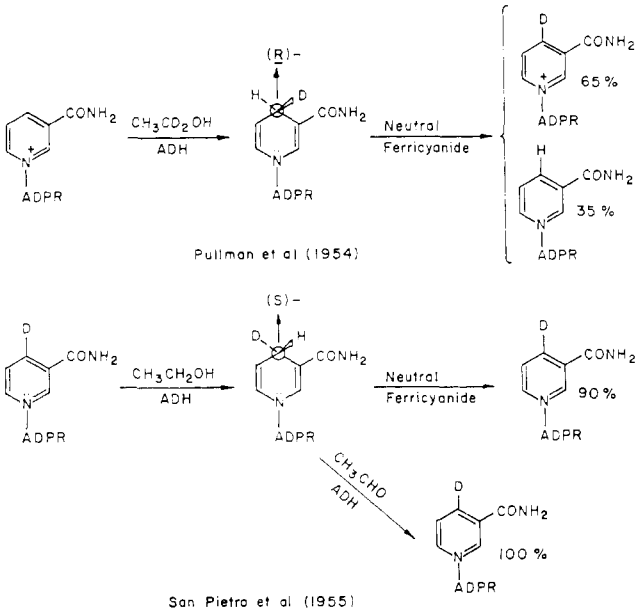


FIGURE 7: Neutral ferricyanide and enzymatic oxidation of (*R*)- and (*S*)-DPND.

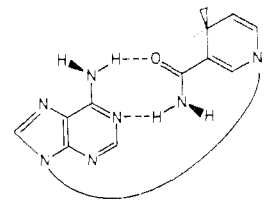


FIGURE 8: Hydrogen-bonded conformation of DPNH in which the adenine and dihydronicotinamide rings are *coplanar* for effective energy transfer.

of (*S*)-DPND leads to about 100% retention of deuterium in the product (Figure 7). The data in Figures 6 and 7 amply show that *the two C_4 geminal protons of the dihydropyridine ring of reduced pyridine dinucleotides behave differently toward chemical and enzymatic agents.* Such data are in excellent agreement with the present high frequency nuclear magnetic resonance observation that the two protons reside in different electronic and geometric environments. It is very difficult to escape the inference that the percentages of (*R*)-DPND and (*S*)-DPND shown in Figure 6 are related to the population of *M* and *P* helices of DPN. Proper integration of the nuclear magnetic resonance spectrum of DPND (Figure 2) is difficult. However, examination of the peaks B and B′ in Figure 2 reveals that their areas are not identical, which in turn indicate a difference in the population densities of the *M* and *P* helices of the dinucleotide. Also, as mentioned earlier, careful examination of the AB quartet of TPNH and DPNH in Figure 1 shows that the area of peaks 1 and 2 is not the same as that of peaks 3 and 4.

Enzyme Specificity. Sarma *et al.* (1968b) have conjectured the existence of pyridine dinucleotides as an equilibrium mixture of a right- and left-handed folded conformation and have wondered with circumspection whether the existence of two types of dehydrogenases specific to side A and side B of the ring is related to the two different folding arrangements of the dinucleotide. Nuclear magnetic resonance data from (*R*)-DPND and *N*-methyl-*N*-ethylnicotinamide–adenine dinucleotide (Sarma *et al.*, 1970) confirm the existence of both *M* or *P* helices. It is possible that the dehydrogenases specific to side A of the ring interact with only either the *M* or *P* helix (for example, the *M* helix of DPNH) and that as the reaction progresses the nonreactive conformer (for example, the *P* helix of DPNH) is converted into the reactive folded conformer. It is not possible to say with the present data which type of enzyme reacts with which helical conformation of the dinucleotide.

Hydrogen-Bonded Conformation. The magnetic nonequivalence of the two C_4-geminal protons of the dihydropyridine ring of the reduced coenzyme supports our original conclusion that (Sarma *et al.*, 1968b) hydrogen bonding of the type depicted in Figure 8 in which *adenine and pyridine are coplanar cannot* play any significant role in maintaining the conformational integrity of the pyridine dinucleotides. By the use of various DPN analogs Sarma *et al.* (1968b) showed hydrogen bonding *between base pairs* does not contribute anything toward determining the conformation of oxidized pyridine dinucleotides. Fluorescence transfer experiments (Shifrin and Kaplan, 1959; Weber, 1957) led to the postulation that the base pairs in the reduced coenzyme (*viz.*, DPNH) are

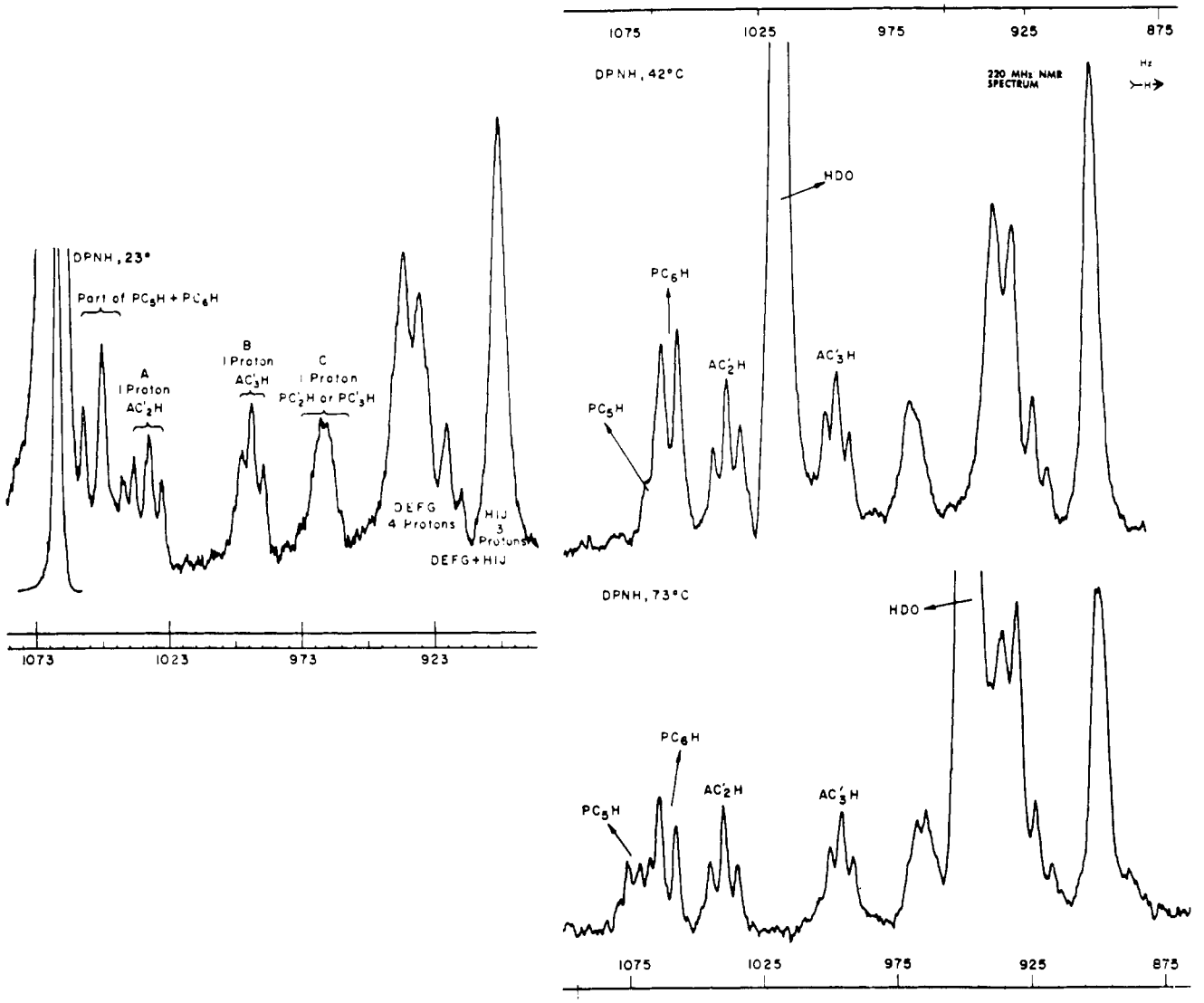


FIGURE 9: Nuclear magnetic resonance spectra (220 MHz) of DPNH near and upfield from the HDO peak at 23, 42, and 73°. The spectra were taken at a sweep width of 500 Hz using DSS as an internal standard. The letter P stands for the pyridine ring or the ribose adjacent to the pyridine ring. The letter A stands for the ribose adjacent to the adenine moiety.

hydrogen bonded, and the dihydropyridine and the adenine ring lie *coplanar* for effective energy transfer. In a conformation in which the adenine and dihydropyridine are *coplanar*, one would expect the two C_4 hydrogens to be environmentally equivalent as opposed to a conformation in which the base pairs are stacked in parallel planes.

The Vicinal Coupling between C_5H and the Two C_4 Protons of the Dihydropyridine Ring in Dihydronicotinamides and Reduced Pyridine Dinucleotides. The nuclear magnetic resonance spectra of N_1-substituted 1,4-dihydronicotinamides taken in the 100 MHz nuclear magnetic resonance system (Kim and Chaykin, 1968; Choi and Alivisatos, 1967) show that the C_5H is split into a pair of triplets by spin–spin splitting with the two C_4 protons and the one C_6 proton. Also it has been observed that the C_6H is split by the C_2H across the C–N–C bridge (Choi and Alivisatos, 1967). But what was not observed is the splitting of the two C_4 protons by the vicinal C_5H as well as the splitting of the C_2H by C_6H. In the 220 MHz nuclear magnetic resonance spectra of reduced pyridine dinucleotides at 23° one cannot locate either the C_6H or the C_5H because their resonances are vitiated by the large HDO peak. In the DPNH spectra at 23° part of their resonances could be seen immediately upfield from the HDO peak (Figure 9). By performing nuclear magnetic resonance measurements at high temperature, we were able to unearth the signals from the dihydropyridine C_5H and C_6H of DPNH for the first time. The top of Figure 9 is part of the 220 MHz nuclear magnetic resonance spectrum of DPNH at 42° and it would be noted that the HDO peak has moved considerably upfield revealing very clearly the C_6H and C_5H resonances. Still the dihydropyridine C_5H resonance is buried under the signal from C_6H. However, at 73° the C_5H resonance emerges out of the cluster of C_6H and C_5H resonances as an overlapping pair of symmetrical triplets (Figure 9, bottom). The $J_{5,6}$ is 7 Hz and $J_{4,5}$ is 3 Hz. The symmetry of the pair of triplets from dihydropyridine C_5H indicates (as pointed out

earlier) that the dihedral angles between C_5H and the two C_4 protons are the same (at least at 73°) in DPNH as in a planar conformation of the dihydropyridine moiety, as opposed to a puckered boat geometry. As is reported (Kim and Chaykin, 1968; Choi and Alivisatos, 1967) in the case of 1,4-dihydronicotinamides, we also could not observe the splitting of the C_4 proton by C_5 proton. This does not mean that the C_5H does not spin–spin split the two C_4 protons. On the contrary, the fact that a pair of triplets has been observed for the C_5H shows that there is mutual spin–spin splitting between C_5H and the two C_4 protons. However, we were not able to see the splitting of the C_4 proton resonances because the ratio of $J_{4,5}$ to the line width of the C_4 proton resonance is small. $J_{4,5}$ is 3 Hz and the line width at half-height of the resonance from the two C_4 protons is 16 Hz at 23°.

Acknowledgment

The authors gratefully acknowledge the enlightening advice as well as stimulating discussions of Dr. W. D. Phillips. The authors thank Dr. E. Grunwald, Dr. R. Lawler, and Dr. C. D. Jardetzky for very active discussions on the contents of this publication. They are particularly indebted to Dr. W. D. Phillips for arranging access to the 220 MHz nuclear magnetic resonance spectrometer. We also thank F. V. Ferrari for excellent technical assistance.

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