Conformation of Pyridine Nucleotides Studied by Phosphorus-31 and Hydrogen-1 Fast Fourier Transform Nuclear Magnetic Resonance Spectroscopy. III. Oxidized and Reduced Dinucleotides[1]

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Abstract: The proton nuclear magnetic resonance spectra of oxidized and reduced pyridine dinucleotides have been examined over a concentration range of 0.001–0.4 *M* in the Fourier transform mode. Assignments of the various protons were made from pH studies and homonuclear and ^{31}P decoupling experiments; certain earlier erroneous assignments were corrected. It has been shown that the adenine moiety prefers to exist in the anti conformation. The resonances from the adenine and the adjacent ribose moiety show strong concentration dependence, unlike the resonances from the nicotinamide nucleotide fragment. This observation is interpreted as indicating the formation of dimers involving adenine–adenine interaction at higher concentration. Analyses of the concentration data *vis-à-vis* the computed isoshielding lines for adenine suggest that each dinucleotide in the dimer exhibits a molecular chirality of (P)-helix. Comparison of the conformations of the individual mononucleotide components constituting the dinucleotide with that of the dinucleotide indicate that the monomers maintain, in general, their conformational integrity in the dinucleotide, as has been suggested for nucleic acids by Sundaralingam.

In paper I of this series,[2] the three-dimensional dynamic solution geometry of the oxidized and reduced pyridine mononucleotides was discussed. This is extended here to an examination of the parent dinucleotides. The conformation of the dinucleotides (especially β-DPN (I) and β-DPNH (II)) has been under intensive investigation by multinuclear nmr by several groups,[3–19] and the data have been rationalized on the ground that the dinucleotide, at biological pH and temperature, on a time-average basis, exists in a folded conformation in which the adenine and nicotinamide moieties are stacked in nearly parallel planes. This conclusion is in agreement with the solvent perturbation studies of Cross and Fisher,[20] fluorescence studies of Walter and Kaplan,[21] Velick,[22] and Weber,[23] circular dichroism studies of Miles and Urry,[24] and the temperature jump studies of Czerlinski and Hommes.[25] Jacobus[26] has indicated that the nmr data are also consistent with a conformation in which the dinucleotides may exist as a linear array. Jacobus[26] did not attempt to rationalize the ultraviolet,[20] fluorescence,[21–23] circular dichroism,[24] and temperature jump[25] data on the basis of a linear model. The essence of Jacobus'[26] argument was that the shifts which had been previously ascribed to ring current shifts mutually induced between the adenine and nicotinamide moieties in a folded molecule as well as the perturbation of chemical shifts by change in pH[4,6,14] can be accounted for by torsional variations of the backbone of a linear dinucleotide. However, theoretical and experimental evidence discussed elsewhere[27] shows that anisotropic shielding originating from torsional variations of covalent bonds is distant and angular dependent and that it should have no significant effect upon the chemical shifts of nuclei that are three or four bonds away in a linear molecule, and Sarma and Mynott[27] have reaffirmed that the existing data are best rationalized on the basis of an internally folded model.

Most of the ^1H nmr experiments previously reported[3–17] were carried out in continuous wave mode

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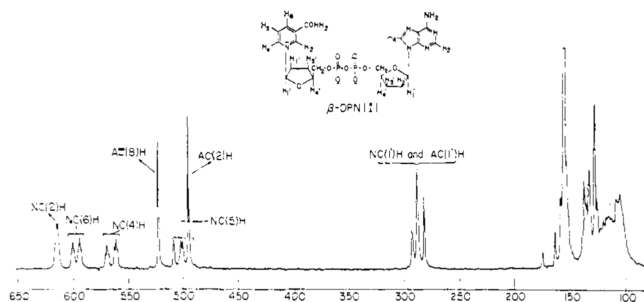


Figure 1. 4K fast Fourier transformed 100-MHz ^1H nmr spectrum of β-DPN (pH 7.5, 0.025 *M*) in "100%" D_2O. Number of pulses = 500; sampling frequency 2000 Hz; pulse width = 40 μsec; delay time, 0.5 sec; lock = ^{19}F of hexafluorobenzene. Chemical shifts are expressed in Hz downfield from TMA, 100-MHz system. Apodization mode = triangular. The letters A and N stand for the adenine and nicotinamide moieties, respectively.

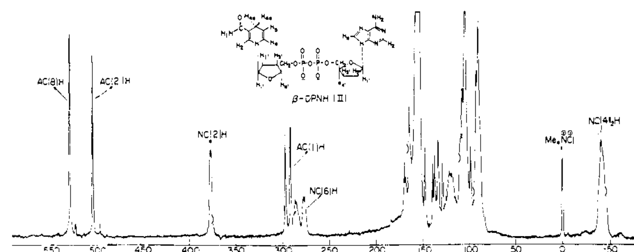


Figure 2. 4K fast Fourier transformed 100-MHz ^1H nmr spectrum of β-DPNH (pH 7.9, 0.025 *M*) in "100%" D_2O. Apodization mode = triangular. Remaining details are in Figure 1. Note that the fine couplings of nicotinamide C(2)H and C(6)H are discernible at the employed concentration of 0.025 *M*. Also, the outer transitions of the strongly coupled AB quartet of the dihydronicotinamide $C(4)H_2$ protons are detectable.

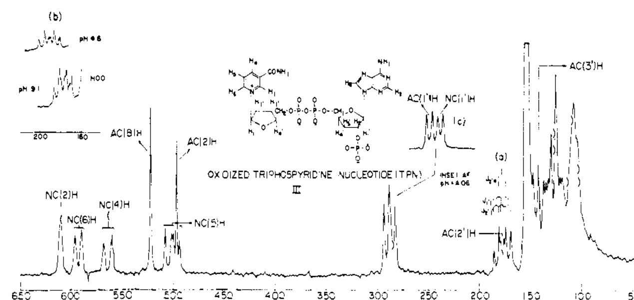
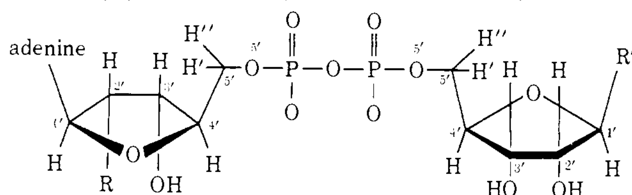


Figure 3. 2K fast Fourier transformed 100-MHz ^1H nmr spectrum of β-TPN (pH 7.9, 0.005 *M*) in "100%" D_2O. Rest of the details as in Figure 1. Inset (a), coupling pattern of adenine C(2')H; inset (b), effect of pH on the appearance of the multiplet from adenine C(2')H; inset (c), appearance of adenine C(1')H and nicotinamide C(1')H at pH ≃4.

at a concentration of approximately 0.1 *M*. Jardetzky and Wade-Jardetzky[6] reported that the chemical shifts of pyridine coenzymes are independent of concentration in the range of 1.0–0.005 *M*. A later investigation by Catterall, *et al.*,[4] at 0.05 and 0.005 *M* showed that there is a concentration dependence of the chemical shifts of certain protons in β-DPN and β-DPNH. The development of fast Fourier transform techniques in nmr spectroscopy now makes it possible to obtain high quality nmr spectra at dilutions not previously practicable. Accordingly, we have carried out a comprehensive examination of the effect of concentration upon the chemical shifts of the protons of β-DPN (I), β-DPNH (II), β-TPN (III), and β-TPNH (IV) and have



I = β-DPN, R = −OH; R′ = nicotinamide
II = β-DPNH, R = −OH; R′ = dihydronicotinamide
III = β-TPN, R = $-OPO_3^{2-}$; R′ = nicotinamide
IV = β-TPNH, R = $-OPO_3^{2-}$; R′ = dihydronicotinamide
V = ADPR, R = −OH; R′ = −OH

established that in reality the chemical shifts are highly dependent upon concentration and also that examination of the concentration profiles permits extension of one's knowledge of the molecular conformation of pyridine coenzymes. We report here the concentration profiles, together with chemical shifts at dilutions at which there is no longer a dependence, giving the most precise information yet obtained on the effects of intramolecular and intermolecular stacking upon chemical shifts.

In previous work, the main concern was to study the conformations of β-DPN and β-DPNH. In the present paper much more emphasis has been placed on the solution conformations of β-TPN and β-TPNH. During this work, we found that certain crucial assignments of chemical shifts of pyridine coenzymes in previous studies were wrong, and a full discussion of our assignments is reported here.

Experimental Section

The experiments were carried out on the same equipment as described in paper I[2] of this series. The coenzymes were commercial preparations. Other details are as given in paper I. Unless otherwise stated, the pH values reported for ^1H data are direct pH meter readings in D_2O solutions. Tetramethylammonium chloride (TMA) was used as an internal standard.

Results and Discussion

(a) Assignments of the Resonances in ADPR, β-DPN, and β-DPNH. The assignments of the adenine C(8)H, C(2)H, and C(1′)H of ADPR (V) has been made by Sarma, *et al.*,[14] and Kotowycz, *et al.*[28] (see Kotowycz, *et al.*,[28] for a 220-MHz ^1H nmr spectrum of ADPR). Figure 1 shows a 4K Fourier transformed 100-MHz ^1H spectrum of β-DPN (0.025 *M*, pH 7.5) taken with triangular apodization. The assignments indicated in Figure 1 are from previous investigations,[4,6,14] which we have confirmed by homonuclear decoupling experiments. Figure 2 shows the 100-MHz 4K Fourier transformed ^1H nmr spectrum of β-DPNH (pH 7.9, 0.025 *M*). This spectrum was taken under the same conditions as for β-DPN, except that square apodization was used to enhance the resolution. Except for assignments of nicotinamide C(1′)H and C(6)H, the assignments by Jardetzky and Wade-Jardetzky,[6] Meyer, *et al.*,[3] Catterall, *et al.*,[4] and Sarma and Kaplan[10] were found to be correct. The proper assignment for these resonances has been given by Oppenheimer, *et al.*,[15] and these are indicated in Figure 2.

(b) Assignments of the Resonances in β-TPN and β-TPNH. Figure 3 illustrates a 2K Fourier transformed 100-MHz ^1H spectrum of a 0.005 *M* solution of

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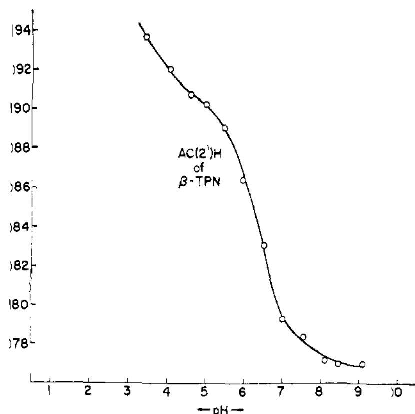
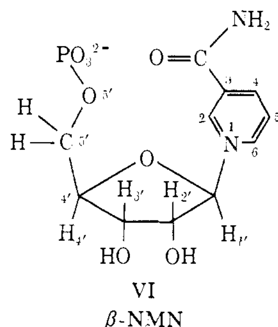


Figure 4. The pH profile of the ¹H chemical shift of adenine C(2′)H of β-TPN (0.005 *M*). The *y* axis shows the chemical shifts in Hz downfield from TMA.

β-TPN (pH 7.9). The assignments of the protons of the nicotinamide and adenine moieties are obvious from inspection and comparison with the spectra of the mononucleotides,[2,29] and are indicated in Figure 3. A comparison of the β-NMN (VI) spectrum (Figure 1,



Sarma and Mynott[2,29]) to that of β-TPN (Figure 3) shows that we have not been able to observe the fine long-range couplings of the nicotinamide moiety, because the β-TPN spectrum was a 2K Fourier transformed one whereas that of β-NMN was an 8K transformed one. The horizontal resolution of a 2K transform is 1 Hz in this case while that of an 8K transform is 0.25 Hz. A 2K transform was selected because spectra were to be recorded for the various dinucleotides over a range of concentration and pH. A typical 2K Fourier transformed spectrum of 2500 pulses required 0.7 hr whereas it would have required much longer to obtain an 8K Fourier transformed spectrum of comparable signal to noise ratio.

As will be seen from later discussion, the correct assignments of adenine C(1′)H, C(2′)H, and C(3′)H of β-TPN are crucial in arriving at the intramolecular conformation of β-TPN and hence we have attempted to assign them unambiguously.

At approximately 180 Hz downfield from TMA there is a multiplet with the appearance of a superimposed doublet of triplets (Figure 3) which is assigned to adenine C(2′)H because of the following evidence. (i) The coupling pattern shown as inset (a) in Figure 3, which is consistent with nearly equal coupling to C(1′)H and C(3′)H and larger coupling (6.7 Hz at pH 7.9) to the ^{31}P nucleus of the 2′-phosphate group. (ii) ^{31}P nmr experiments[17] have shown that the 2′-phosphate resonance is extremely sensitive to pH and the variation of the ^{31}P chemical shift of the 2′-phosphate group as a function of pH has a point of inflection at pH 6 (pH values are direct reading from pH meter, solution in H_2O) indicating that the p*K* for this phosphate group is 6.0. The pH profile of the ¹H chemical shift of the resonances assigned to adenine C(2′)H shows a variation in chemical shift of 13 Hz in the pH range of 7.5–4.5 (Figure 4) with a point of inflection at pH 6.4 (pH values are direct reading from pH meter, solution in D_2O). (iii) The same ^{31}P nmr investigations[17] showed that $^3J_{P-H(2')}$ is approximately 9.3 Hz at low pH ($\simeq$4) and 6.6 Hz at high pH ($\simeq$8), and the magnitude varies with the ionization of the 2′-phosphate group. In the *proton* spectrum, the large coupling of this multiplet, assigned to adenine C(2′)H, varies in the same manner, being 9.0 Hz at pH 4 and 6.7 at pH 9. (In Figure 3, we have illustrated the appearance of this multiplet at two pH values as inset (b).) (iv) This multiplet at $\simeq$180 Hz is absent in β-DPN which does not contain a 2′-phosphate group. (v) When the spectrum of β-TPN was taken under ^{31}P decoupling conditions the multiplet at $\simeq$180 Hz collapsed to a quartet. (vi) Homonuclear decoupling, discussed below.

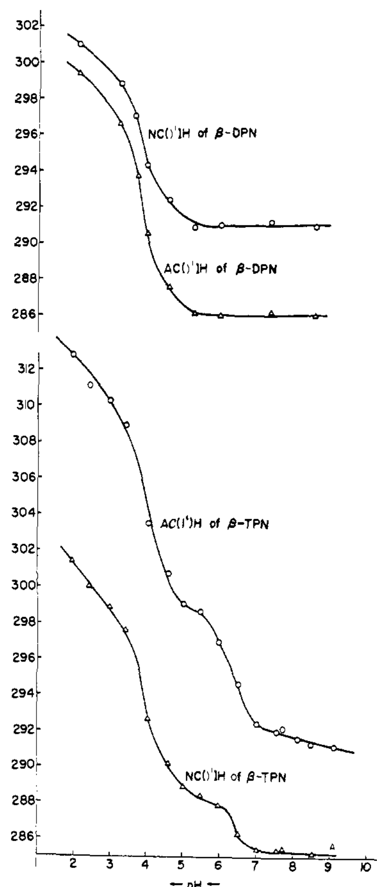


Figure 5. The pH profile of the ¹H chemical shifts of adenine C(1′)H and nicotinamide C(1′)H of β-DPN and β-TPN (0.005 *M*). The *y* axis shows the chemical shifts in Hz downfield from TMA.

The group of resonances at $\simeq$288 Hz downfield of TMA was assigned by Jardetzky and Wade-Jardetzky[6] to nicotinamide C(1′)H and adenine C(1′)H of β-TPN. At pH values higher than 7.0 these doublets (due to coupling between C(1′)H and C(2′)H) are partially

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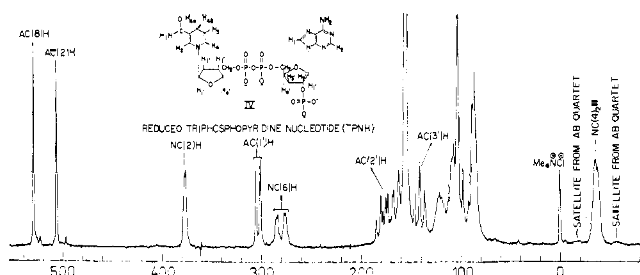

Figure 6. 4K fast Fourier transformed 100-MHz ^{1}H nmr spectrum of β-TPNH (pH 8.0, 0.025 M). Details as in Figure 1.

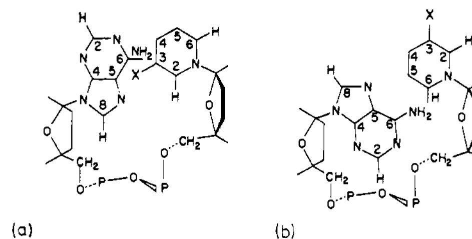

Figure 7. (a) and (b), respectively, show the anti and syn torsional diastereomers constrained to the adenine–ribose glycosidic linkage of β-DPN. The nicotinamide moiety in (a) is syn, and in (b) it is anti.

superimposed and appear as a triplet, but, as pH is lowered, they appear as a pair of doublets (see inset (c), Figure 3). According to Jardetzky and Wade-Jardetzky, the doublet to the lower field originates from nicotinamide C(1′)H and that at the higher field from adenine C(1′)H. Based on the following observations we are reversing these original assignments,[6] *i.e.*, we assign the low field doublet to adenine C(1′)H and the high field one to nicotinamide C(1′)H. (i) Irradiation of the center of the multiplet of adenine C(2′)H produces collapse of the presently assigned resonance due to adenine C(1′)H. (ii) The pH profiles of adenine C(1′)H and nicotinamide C(1′)H for β-DPN and β-TPN (0.005 M) as assigned in this paper are shown in Figure 5. The curves for β-DPN display no pH dependence at pH values above 5.0. On the other hand, the chemical shifts of adenine C(1′)H and nicotinamide C(1′)H of β-TPN show a pronounced dependence of pH in this range, unequivocally due to the ionization of the 2′-phosphate group at the adenine ribose. Important is the observation that the adenine C(1′)H of β-TPN, as assigned in this paper, shows a shift of 6.8 Hz in the pH range of 5.2–7.5, the midpoint of the shift being approximately pH 6.4, the pK for the 2′-phosphate group in D_2O.[17] The nicotinamide C(1′)H of β-TPN, as assigned in this paper, shows a shift of 2.8 Hz between pH 5.7 and 7.5, the midpoint being $\simeq$6.3. The chemical shifts of adenine C(1′)H and nicotinamide C(1′)H of β-TPN and β-DPN display shifts to lower fields at pH values below 5, the midpoint being pH $\simeq$4. The adenine C(1′)H shows a large shift in this range. These shifts are due to the protonation of the adenine N(1) and subsequent unfolding of the coenzyme, discussed elsewhere.[27] The shift of adenine C(1′)H of β-TPN, centered around pH $\simeq$6.3, is well over twice as large as in the case of nicotinamide C(1′)H. This large shift is due to the direct influence of the 2′-phosphate group upon the chemical shift of adenine C(1′)H which is juxtaposed to the phosphate. The smaller effect seen on the nicotinamide C(1′)H is due to pH-induced change in intramolecular conformation, discussed elsewhere.[27]

The triplet at $\simeq$140 Hz (Figure 3) was assigned to adenine C(3′)H from homonuclear decoupling experiments. The assignments of the resonances in β-TPNH are shown in Figure 6 and are based on homonuclear decoupling and ^{31}P decoupling experiments. The remaining resonances in the various dinucleotides could not be assigned due to extreme overlap of transitions.

(c) Conformational Nomenclature for Pyridine Dinucleotides. The nomenclature described in paper I of this series,[2] based on Sundaralingam[30] and Donohue and Trueblood,[31] will be used. As before[2] the term torsional diastereomer is used to describe all stereoisomers which are not enantioners.

(d) Sugar–Base Torsion Angle Constrained to the Adenine–Ribose Glycosidic Linkage. Figures 7a and 7b show the anti and syn conformations for the adenine moiety in pyridine coenzymes. Chan and Nelson[32] and Schweizer, *et al.*,[33] have shown that the adenine moiety in 5′-AMP prefers to exist in the anti conformation. Sarma and Kaplan[9] hypothesized that the adenine in pyridine coenzymes may therefore also exist in the anti conformation although there was no experimental evidence to support this. Chan and Nelson[32] have proposed a method to distinguish between anti and syn conformations in nucleotides which makes use of the following facts. (i) The adenine C(8)H is juxtaposed to the pyrophosphate backbone in the anti form whereas adenine C(2)H is juxtaposed in the syn form (Figure 7). (ii) The effectiveness of a paramagnetic center in broadening a nuclear resonance varies as the square of the paramagnetic moment and as the inverse sixth power of the separation between the nucleus and the center. Accordingly, if a paramagnetic ion like Mn(II), possessing a long electronic relaxation time, can bind to the pyrophosphate backbone of pyridine coenzymes, one would expect the paramagnetic center to broaden the adenine C(8)H or C(2)H depending upon whether the conformation is anti or syn. Should there be no preference for either anti or syn, both the adenine C(8)H and C(2)H will be broadened. Shulman, *et al.*,[34] and Walaas[35] have shown that Mn(II) ions bind primarily to the phosphate group of nucleotides. Accordingly, we have examined ^{31}P nmr spectra of β-DPNH in the presence of 0.001 M EDTA and in the presence of 175 μM $MnCl_2$ (ratio of DPNH/Mn(II) = 2300) and these are shown in Figure 8. Inspection of the ^{31}P and ^{31}P{^{1}H} spectra in Figure 8, in the presence and absence of Mn(II) ions, shows that the paramagnetic ions broaden the ^{31}P resonances beyond recognition. The observation is best rationalized on the basis of the binding of Mn(II) ions to the pyrophosphate backbone of coenzymes. The ^{1}H nmr spectra of β-DPNH and β-DPN at a concentration of 0.07 M were taken on a 220-MHz system in the presence and

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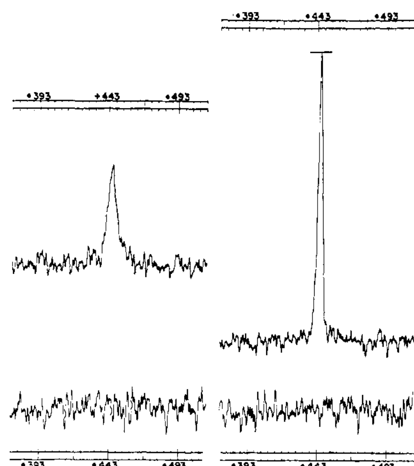

Figure 8. ^{31}P nmr spectrum of 0.4 M DPNH: top left, 0.4 M DPNH in 0.001 M EDTA; bottom left, 0.4 M DPNH in 175 μM $MnCl_2$; top right; 0.4 M DPNH in 0.001 M EDTA, proton decoupled ^{31}P spectrum; bottom right, 0.4 M DPNH in 175 μM $MnCl_2$, proton decoupled ^{31}P spectrum; sweep width = 500 Hz, temperature = 30.5°. Chemical shifts are reported in Hz (40.5 MHz, measuring channel) upfield from external 85% H_3PO_4 capillary.

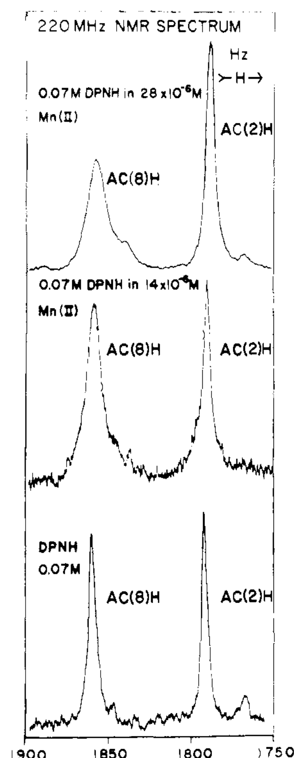

Figure 9. Effect of Mn(II) ions on the line widths of the adenine C(8)H and C(2)H resonances of the 220-MHz ^1H nmr spectrum of β-DPNH. Chemical shifts are expressed in Hz, downfield from 2,2-dimethyl-2-silapentane-5-sulfonate.

absence of Mn(II) ions (Figures 9 and 10). The 220-MHz system was used to separate the resonances as much as possible. Concentrations below[36] 0.07 M could not be examined because Fourier transform capabilities were not available with the 220-MHz system, and a conventional computer of average transients

(36) When the same ^1H nmr experiments were performed at a frequency of 100 MHz and a concentration of 0.005 M, similar results were obtained.

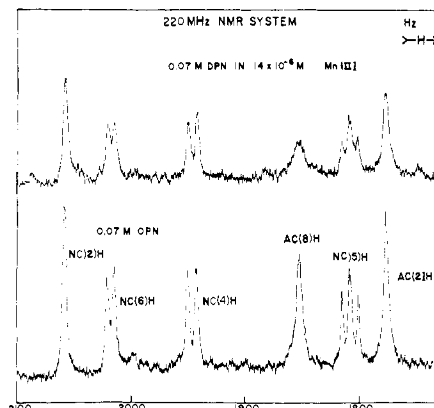

Figure 10. Effect of Mn(II) ions on the line widths of the low field region of the 220-MHz ^1H nmr spectrum of β-DPN.

was used. The ratio of the concentration of the coenzyme to that of Mn(II) ions was the same for experiments depicted in Figures 8–10. The observed broadening of the adenine C(8)H resonances in β-DPN and β-DPNH indicates that the adenine moiety tends to assume an anti conformation. Berthod and Pullman[37] have shown by calculations involving perturbation configuration interaction using localized orbitals that anti is the preferred conformer in cases similar to pyridine coenzymes. Examination of Figures 9 and 10 shows that in the presence of Mn(II) ions the adenine C(2)H has undergone little or no broadening, which can be rationalized if the adenine exists mostly or entirely in an anti arrangement. Such a conclusion may not agree with the theoretical calculations of Haschemeyer and Rich[38] or Lakshminarayanan and Sasisekharan,[39] who suggest that for purine nucleosides, both syn and anti conformations are allowed, but that the anti conformer is preferred for ^3E sugar pucker. There is no evidence that the ribose adjacent to the adenine moiety in pyridine coenzymes is a conformationally pure ^3E (*vide intra*) and probably it is not. Nevertheless, the Mn(II) ion binding studies are explained best if the adenine moiety is locked in the anti conformation. It is possible that the intramolecularly folded conformation (and the consequent juxtaposition of the nicotinamide system) of the dinucleotide prevents an otherwise energetically feasible[38,39] torsional interconversion between anti ⇄ syn diastereomers.

(e) Sugar–Base Torsion Angle Constrained to the Nicotinamide–Ribose Glycosidic Linkage. Paramagnetic ion binding studies are unreliable when used to distinguish whether the nicotinamide moiety in the dinucleotides exists in the syn or the anti conformation, because the hydrogen nuclei in the nicotinamide moiety are coupled to each other and shortening the relaxation time of any of the nuclei may cause "false" broadening by relaxation decoupling. In paper I of this series,[2] it was shown that the nicotinamide moiety prefers to exist in the syn and anti conformations in β-NMN and β-NMNH (VII), respectively. It is reasonable to believe that in the parent dinucleotides the same arrangement is preserved (*vide infra*).

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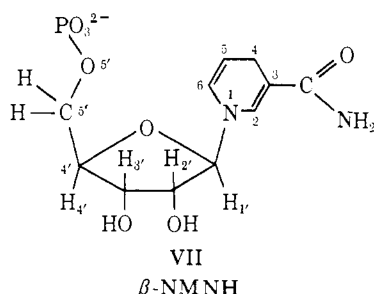
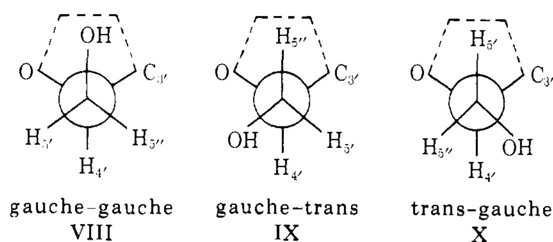
(38) A. E. V. Haschemeyer and A. Rich, *J. Mol. Biol.*, **27**, 369 (1967).
(39) A. V. Lakshminarayanan and V. Sasisekharan, *Biopolymers*, **8**, 475 (1969).



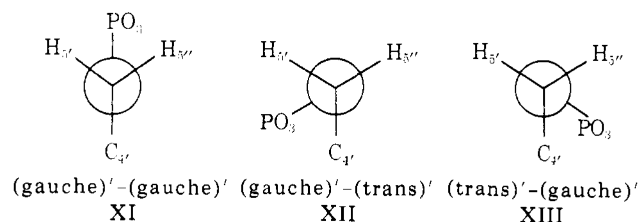
VII

β-NMNH

(f) Conformation of the Backbone and Its Relation to Sundaralingam Concept of Rigidity.[40] The backbone stereochemistry is defined by: (i) the conformation of the two ribofuranose rings, (ii) the torsional angles about the two C(4′)-C(5′) bonds (VIII–X), and (iii) the



torsional angles about the two C(5′)-O(5′) bonds (XI–XIII).



(i) It has been recently suggested[2] that ribose coupling constants are best rationalized on the basis of two conformations, ^3E and ^2E, undergoing interconversion by pseudorotation.[40–42] In Table I, we have summarized the coupling constants for the adenine ribose protons in the various dinucleotides, and for 5′-AMP and 5′-ADP. Although the small differences in a given coupling constant (*e.g.*, $J_{1'2'}$ of β-TPN and β-TPNH) between the various compounds are real, they do not represent any major differences in the time-average ribose pucker. One may conclude that the ribose adjacent to the adenine moiety in the molecules listed in Table I is undergoing pseudorotational interconversion between various puckered forms, which are probably mainly ^2E and ^3E (S and N forms, in the new Sundaralingam nomenclature[43]). It should be noted that very little difference exists in the time-average pucker of the ribose moiety adjacent to the adenine moiety between the mononucleotides and the dinucleotides. We have discussed elsewhere[2] that the ribose adjacent to the nicotinamide moiety of the mono- and dinucleotides also exists as a flexible ring system, preferring ^2E and ^3E modes in the pseudorotational itinerary.

(40) M. Sundaralingam in ref 27.

(41) J. E. Kilpatrick, K. S. Pitzer, and R. S. Pitzer, *J. Amer. Chem. Soc.*, **69**, 2483 (1947).

(42) L. D. Hall, P. R. Steiner, and C. Pedersen, *Can. J. Chem.*, **48**, 1155 (1970).

(43) C. Altona and M. Sundaralingam, *J. Amer. Chem. Soc.*, **95**, 2333 (1973).

Table I. Adenine-Ribose Coupling Constants (Hz), pH ≃8; Concentration 0.05 *M*, Unless Otherwise Stated

Compd	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$
β-DPN[a]	5.5	5.0	4.5
β-DPNH[a,b]	5.5	5.5	3.8
β-TPN[c]	5.2	5.1	4.9
β-TPNH[c]	4.6	4.9	5.0
5′-AMP[d]	5.7	5.1	3.7
5′-ADP[e]	5.6	5.1	3.8

[a] Values from Oppenheimer, *et al.*[15] [b] The corresponding *J* values determined earlier[11] are 5.1, 5.0, and 4.1. The values in this table are more accurate because these were determined in a 220-MHz system with a high degree of homogeneity. [c] Redetermined for the present work because the earlier values[11] were obtained from line-width measurements because the 220-MHz system employed in the earlier work could not resolve the individual components of the multiplet from adenine C(2′)H in β-TPN and β-TPNH. [d] Concentration, 0.1 *M*; it has been reported [ref 32; P. O. P. Ts'o, N. S. Kondon, M. P. Schweizer, and D. P. Hollis, *Biochemistry*, **8**, 997 (1969); S. S. Danyluk and F. E. Hruska, *ibid.*, **7**, 1038 (1968)] that the value of $J_{1'2'}$ for 5′-AMP increases slightly as the concentration is reduced. Sarma and Evans (unpublished results, 1973) have examined the ^1H-{^{31}P} spectra of 5′-AMP in the range 0.4–0.001 *M* and have also observed this increase in $J_{1'2'}$ with dilution; in addition $J_{3'4'}$ shows a corresponding decrease with dilution. These data are consistent with the view that in these concentration ranges 5′-AMP exists as a dynamic equilibrium of $^2E \rightleftarrows {}^3E$ with a small shift in population toward ^2E at lower concentration. [e] Concentration 0.1 *M* (pH 5.2).

(ii) To obtain the rotamer populations about the C(4′)-C(5′) bonds of dinucleotides by a Karplus treatment of vicinal H-H coupling constants, the four coupling constants $XJ_{4'5'}$, $XJ_{45''}$ (X = N for nicotinomide and A for adenine ends of the molecule, respectively) must be obtained. Experimentally, the overlap of transitions in the dinucleotide spectra prevents their determination. In the mononucleotides, these are all predominantly gauche-gauche (VIII).

(iii) It has been shown[44] by ^1H and ^{31}P Fourier transform nmr and computer line-shape simulation that the torsional diastereomers constrained to the C(5′)-O(5′) bonds of oxidized pyridine coenzymes are predominantly gauche-gauche (XI) at both the adenine and nicotinamide ends. It has further been shown[29,44] that this is also the preferred conformation in the mononucleotides β-NMN, β-NMNH, 5′-AMP, and 5′ADP.

As early as 1969, Sundaralingam[30] suggested that "the preferred conformation of the nucleotide unit in the nucleic acids and polynucleotides is the same as that found for the nucleotide itself." Recently, Sundaralingam[40] and coworkers[45] have elaborated this idea into what he calls the concept of conformational rigidity according to which di-, oligo-, and polynucleotides can be considered to be made up of single mononucleo*tide* units (not nucleo*side* units) in which the monomers, to a considerable extent, maintain their conformational integrity, and that di-, oligo-, and polynucleotides achieve their three-dimensional geometry mainly by flexibility about the P-O bond. The available information suggests that the Sundaralingam concept is applicable to the solution conformation of pyridine coenzymes.

The data from nmr investigation collected above

(44) R. H. Sarma, R. J. Mynott, D. J. Wood, and F. E. Hruska, *J. Chem. Soc., Chem. Commun.*, 140 (1973)

(45) J. Rubin, T. Brennan, and M. Sundaralingam, *Biochemistry*, 11, 3112 (1972).

Table II. Chemical shifts[a] of the Base Protons in Pyridine Mono- and Dinucleotides, 0.005 *M*, at 6 and 30°, Expressed in Hertz at 100-MHz Measuring Frequency

Nuclei	β-NMN, pH[b] 7.4			β-NMNH, pH 9.0			ADPR, pH 7.4			β-DPN, pH 7.7			β-TPN, pH 8.5			β-DPNH, pH 8.6			α-TPNH, pH 8.5		
	6°	30°	Δδ[c]	6°	30°	Δδ	6°	30°	Δδ	6°	30°	Δδ	6°	30°	Δδ	6°	30°	Δδ	6°	30°	Δδ
NC(2)H	641.8	640.9	−0.9	399.0	397.5	+1.5				614.7	615.5	+0.8	610.1	611.0	+0.9	376.0	377.0	+1.0	373.8	376.3	+2.5
NC(6)H	615.1	614.9	−0.2	307.7	305.8	−1.9				595.8	597.9	+2.1	592.3	593.5	+1.2	279.3	281.7	+2.4	277.8	279.5	+1.7
NC(4)H	581.4	581.4	0	11.3[d]	11.6[d]	+0.3				563.6	566.4	+2.8	562.9	564.7	+1.8	47.7[d]	41.6[d]	+6.1	41.0[d]	36.6[d]	+4.4
NC(5)H	512.1	512.4	+0.3	185.2	184.6	−0.6				499.4	501.5	+2.1	500.1	501.5	+1.4						
AC(2)H							506.7	508.8	+2.1	496.5	499.7	+3.2	495.0	497.0	+2.0	504.5	506.7	+2.2	505.1	506.3	+1.2
AC(8)H							533.1	533.0	−0.1	524.8	524.8	0	523.7	522.9	−0.8	530.1	530.2	+0.1	529.9	529.1	−0.8

[a] Chemical shifts are accurate within ±0.5 Hz and are, unless stated, downfield from TMA. [b] pH given is the direct reading from pH meter of solutions in D_2O. Chemical shifts of β-NMN, β-NMNH, ADPR, β-DPN, and β-DPNH are independent of pH at values above 7.5. Chemical shifts of β-TPN and β-TPNH are independent of pH at values above 7.5. [c] Δδ is the difference in chemical shifts at 6 and 30°. [d] Chemical shifts expressed in Hz at fields higher from TMA.

show that 5′-AMP and pyridine coenzymes have, at least in a *qualitative sense*, identical conformations with respect to the sugar–base glycosidic linkage and torsional angles constrained to the C(5′)–O(5′) bond, and that the ribofuranose ring in the monomer and dinucleotide have the same time-average pucker. Similarly, a comparison of the conformations of β-NMN and β-NMNH[2,29] with the respective fragments of the corresponding pyridine coenzymes indicates maintenance of the conformational integrity of the C(5′)–O(5′) torsional angle and the ribose pucker. Thus all the evidence available is in agreement with the idea that the mononucleotides maintain their conformational integrity in the dinucleotides. This further suggests that those features for which there is no *direct* evidence regarding their conformation in the dinucleotide may *also* be the same as in the mononucleotides, *i.e.*, the torsion about the C(4′)–C(5′) bonds and the nicotinamide or dihydronicotinamide base glycosidic linkage.

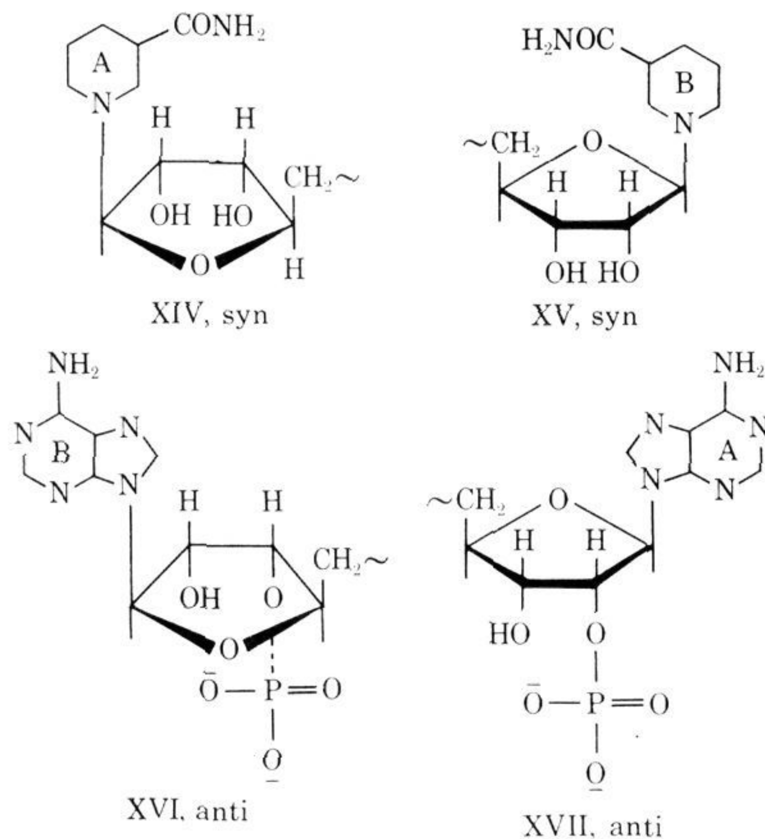
It is possible for the mononucleotides to retain their conformational integrity and at the same time for the base pairs of the dinucleotides to stack in nearly parallel planes by rotation about the P–O bonds. However, the Sundaralingam concept does not imply[40,45] that the monomer and dimer have absolutely identical conformations, and it is anticipated that perturbations of the various torsion angles may take place as a result of P–O rotation and stacking interactions.

(g) On the Intramolecular Conformation of Pyridine Coenzymes, Whether Folded, Linear, or Coplanar. Published proton nmr data[4–16] on pyridine coenzymes have been rationalized on the basis of a folded conformation in which the base pairs are stacked in nearly parallel planes on a time-average basis. These data were obtained in the concentration range of 0.05–0.1 *M* and the conclusions with regard to the intramolecular conformation were made on the assumption that the proton chemical shifts in this concentration range are independent of concentration and hence of intermolecular contributions. Further data reported by Jardetzky and Wade-Jardetzky[6] indicated that chemical shifts are independent of concentrations. Reinvestigations elsewhere[4] and in this laboratory showed (*vide infra*) that in fact pyridine dinucleotide chemical shifts exhibit a strong concentration dependence at concentration levels above 0.005 *M*. Criticisms have been leveled[26] that, in the past, neither a linear nor a coplanar model has been considered for the coenzymes and that all the interpretations were based on *a priori* folded model. In a recent article[27] from this laboratory it has been reaffirmed that the ^1H nmr data for the various mononucleotides and dinucleotides, obtained at *0.005 M concentration levels*, are best rationalized on the basis of a *time average* folded model for the coenzyme and that such data are totally inconsistent with the linear and the coplanar models.

The importance of carrying out investigations, whenever possible, at dilutions at which there is no longer a concentration dependence (*ca.* 0.005 *M* for pyridine coenzymes) to obtain information regarding intramolecular conformation, is illustrated by the data in Table II. Comparison of the data in Table II with those of previous investigators,[6,8,12,14,16] who conducted temperature studies at 0.1 *M* concentration level, reveals

that the temperature-induced changes in chemical shifts are considerably smaller at low concentration. This indicates the dangers of using chemical shifts at various temperatures at higher concentration to obtain information regarding intramolecular conformation and dynamics because such data monitor simultaneously inter- and intramolecular events.

(h) Distinctions between the Various Possible Folded Conformations. Examinations of the space filling and stereomodel of a dinucleotide such as β-TPN, in which adenine is anti and nicotinamide is syn, show that the molecule can exist in four different folded forms.[46] To describe these four different arrangements, we have labeled each side of the base pairs by the letters A and B (XIV–XVII). Inspections of XIV–XVII show that



the B sides of the bases are crowded in the syn form and A sides in the anti forms because of the presence of the hydroxyl and phosphate groups. The four possible folded forms are the following.

(i) (P)-B-anti-A-syn. The backbone of the coenzyme makes a turn of a right-handed helix so that the B side of adenine interacts with the A side of nicotinamide. In this arrangement, the bulky substituents on the ribose moieties lie on the outside and the two base pairs are free to stack over each other. This conformation is designated as (P)-B-anti-A-syn, in which the letter P describes the chirality,[47] the first letter B followed by anti stands for the side of adenine facing the nicotinamide and the conformation of the adenine with respect to its glycosidic linkage, and the second letter A followed by syn designates the side of nicotinamide facing the adenine and the conformation of nicotinamide with respect to its glycosidic linkage.

(ii) (P)-B-anti-B-syn. This designates a structure in which torsional variations of the backbone of (P)-B-anti-A-syn create a new folded form in which the B side of adenine faces the B side of nicotinamide.

(iii) (M′)-A-anti-B-syn.[48] The backbone of the dinucleotide makes a turn of a left-handed helix so that the A side of adenine stacks over the B side of nicotinamide. In this conformation the inside of the helix is highly crowded by substituents originating from the two ribose moieties. The B side of adenine and A side of nicotinamide are freely exposed to the environment.

(46) Jardetzky and Wade-Jardetzky[6] have proposed 64 possibilities.

(47) R. S. Cahn, C. Ingold, and V. Prelog, *Angew. Chem., Int. Ed. Engl.*, 5, 385 (1966).

(48) (M′)- is used rather than (M)- because the two helical forms discussed here are not mirror images and do not constitute an enantiomorphic pair.

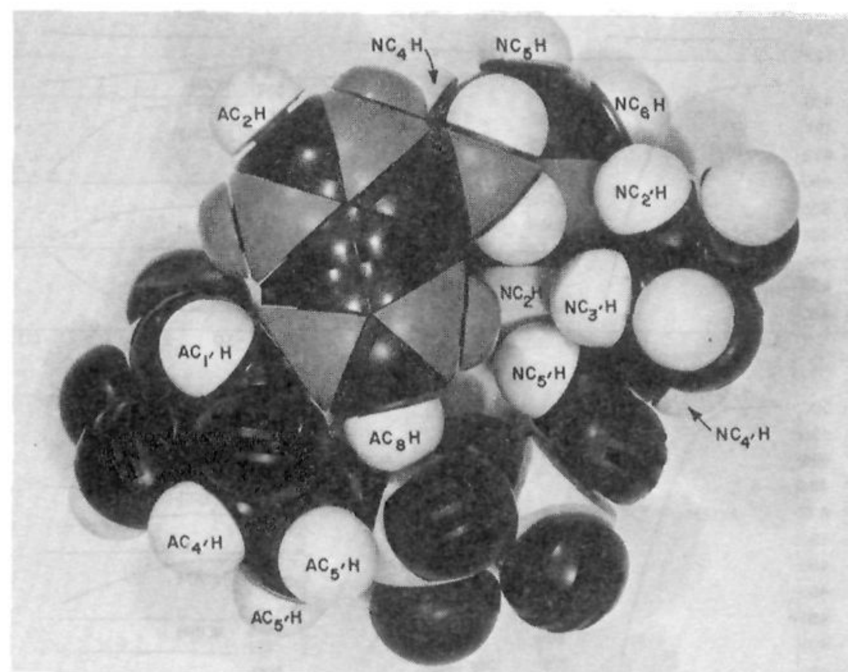


Figure 11. Space-filling model of (M′)-A-anti-B-syn conformation of β-TPN. Note that the top surface of adenine (B) is fully exposed to the environment and interaction with another adenine will be expected to shield adenine C(2)H, C(8)H, and C(1′)H.

(iv) (M′)-A-anti-A-syn. This conformation can be produced by torsional variations of the backbone of the (M′)-AB helix to create a folded form in which the A side of adenine stacks with the A side of nicotinamide.

One may attempt to distinguish among the above possibilities by taking into account the fact that in the (M′)-helical system, the B side of adenine resides outside the helix and the entire B surface and the nearby environment are free from substituents from the D-ribose moiety (Figure 11). Hence, one would expect the B surface of adenine in the (M′)-helix to freely intercalate with extraneous purine rings, if the experiments were conducted in purine solutions. However, in the (P)-helical system, it is the A side of adenine which lies outside and this side is highly crowded from substituents originating from the D-ribose, especially in the case of β-TPN and β-TPNH where the bulky 2′-phosphate group will strongly hinder a free close overlap of extraneous purine with the A side of adenine in the (P)-helical system. However, extraneous purine may disturb the native conformations. Instead, the weak intermolecular interactions of the coenzyme itself may be used. We have undertaken a detailed study of the concentration dependence of the chemical shifts of pyridine coenzymes. Concentration profiles for β-TPN, β-DPN, β-TPNH, and β-DPNH are shown in Figures 12 and 13. The differences in the behavior of the pair β-TPN and β-DPN on the one hand and the pair β-TPNH and β-DPNH on the other come into sharp focus in these experiments. Adenine C(2)H of β-TPN experiences an upfield shift of 16 Hz in going from infinite dilution to 0.4 *M* concentration; the same proton of β-DPN displays a shift of 26 Hz in the same range. The adenine C(8)H of β-TPN remains constant in the same range whereas adenine C(8)H of β-DPN shows a shift to higher fields by 12 Hz. Effects of

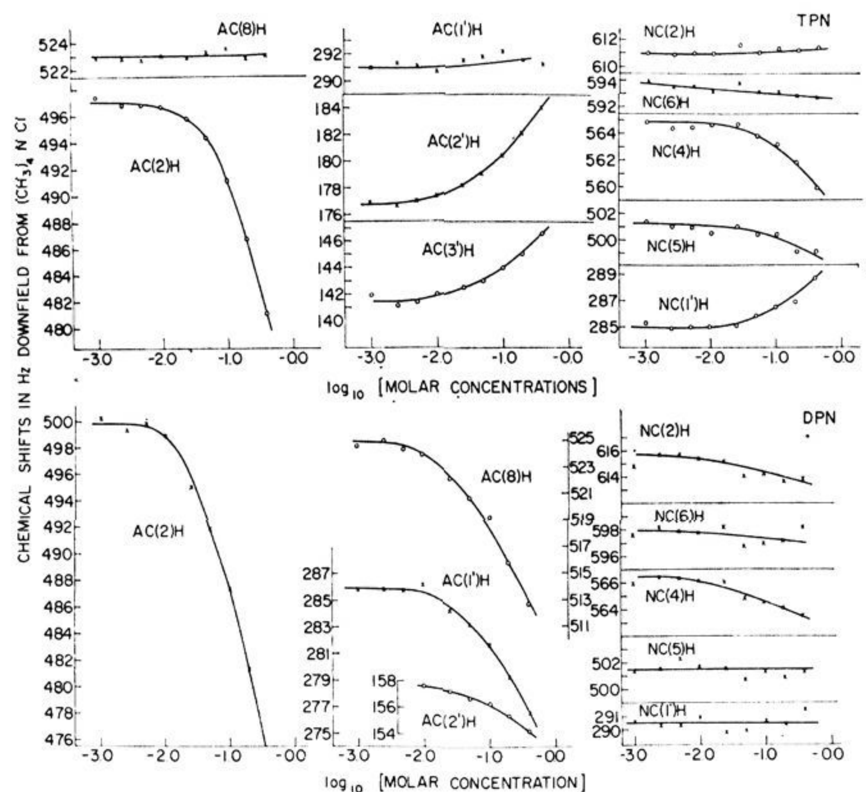


Figure 12. Concentration dependence of the chemical shifts of protons in β-TPN (top) and β-DPN (bottom). The letters A and N identify the protons in the adenine and nicotinamide fragments. Chemical shifts are expressed in Hz downfield from TMA.

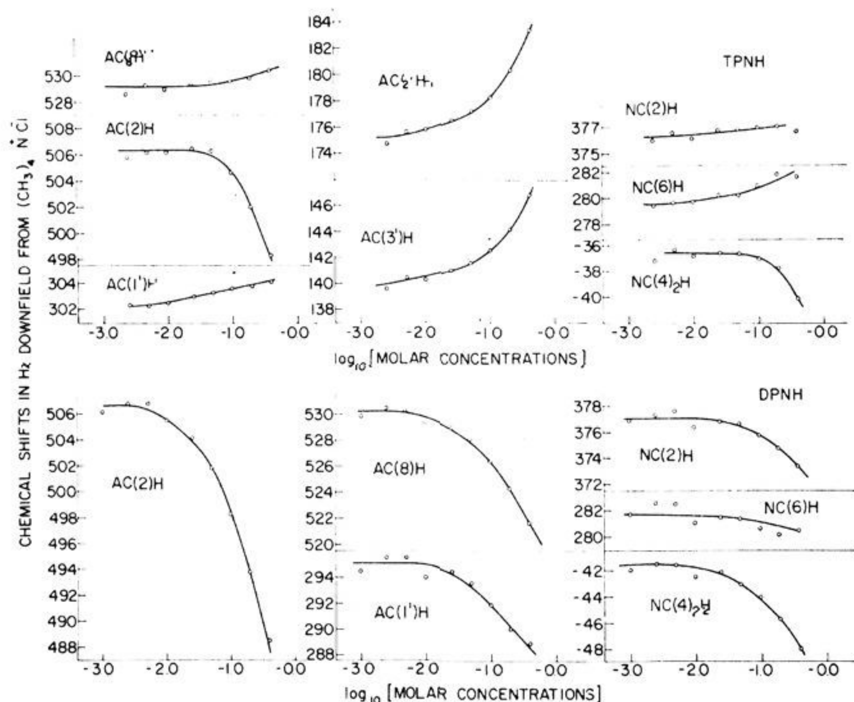


Figure 13. Concentration dependence of the chemical shifts of the protons in β-TPNH (top) and β-DPNH (bottom).

concentration on the ribose protons are very dramatic indeed. The adenine C(1′)H of β-TPN remains virtually constant, C(2′)H shows a shift of 7.5 Hz *to lower fields*, C(3′)H showing a shift of 5 Hz to *lower field*. On the other hand, in β-DPN, adenine C(1′)H and C(2′)H *show shifts of 9 and 4.5 Hz to higher fields*. The nicotinamide C(1′)H of β-DPN remains constant whereas the same proton in β-TPN moves to *lower fields* (4 Hz) *at higher concentration*. Similar differences in the concentration profiles of β-TPNH and β-DPNH are also observed (Figure 13).

To our knowledge, this is the first time one has experimentally observed shifts to lower fields at higher concentrations in the stacking interactions between aromatic systems. Shifts in this direction have been predicted by several theoretical calculations.[49–51]

(49) C. E. Johnson, Jr., and F. E. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958).

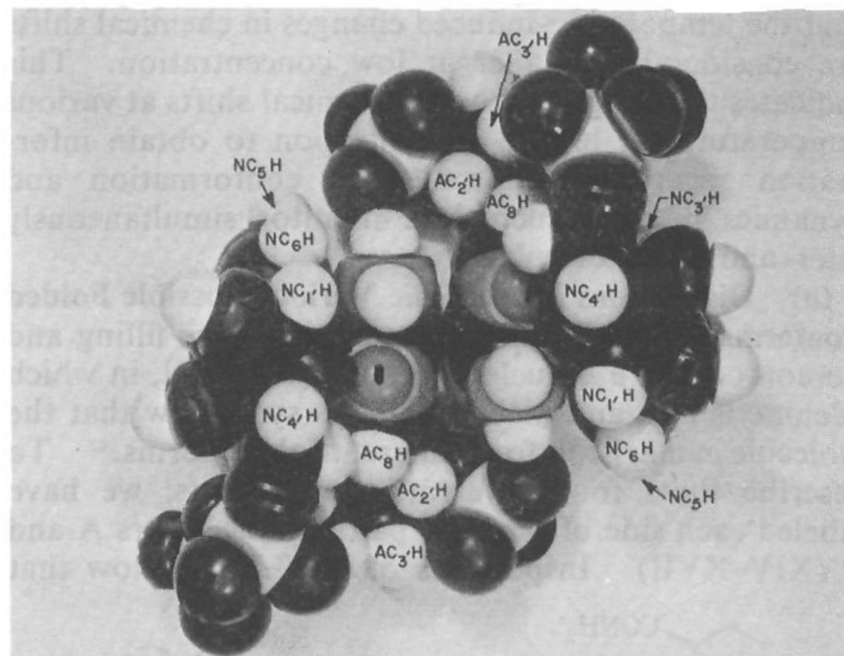


Figure 14. Space-filling model of the stacking between two (P)-B-anti-A-syn conformers of β-TPN.

It appears that the observed difference in the concentration profiles of β-TPN and β-DPN as well as that between β-TPNH and β-DPNH is caused by the presence of the bulky 2′-phosphate group, the geometric disposition of which in the isolated molecule interferes with the stacking interaction between the two adenine moieties in a possible dimer that may form at higher concentration levels. Since the nicotinamide resonances (Figure 12), relative to the adenine ones, have undergone considerably less perturbation, one may rule out any significant existence of dimers involving direct intermolecular stacking between adenine and nicotinamide moieties or between nicotinamide moieties only. Theoretically computed isoshielding contours for nicotinamide[52] indicate that nicotinamide–nicotinamide or nicotinamide–adenine interaction would cause the nicotinamide protons to move to higher fields, the magnitudes of the shifts being at least as large as those predicted for adenine–adenine interaction. Concentration-dependent perturbation experienced by the protons of the adenine region indicates the formation of dimers in which intermolecular stacking occurs between justaposed adenine moieties. For β-TPN, this stacking manifests itself in the shielding of adenine C(2)H (16 Hz), neither shielding nor deshielding of adenine C(8)H and C(1′)H, and deshielding of adenine C(2′)H (7.5 Hz) and adenine C(3′)H (5.0 Hz). If an isolated molecule of β-TPN existed as a (M′)-helix, the free B surface of adenine would be expected to "comfortably" stack over a neighboring adenine moiety with no hindrance from substituents from the D-ribose. This would be expected to result in considerable upfield shifts of adenine C(2)H, C(8)H, and C(1′)H and relatively small upfield shifts of adenine C(2′)H and C(3′)H, the protons which lie inside in the (M′)-helix (Figure 11). This is not what is observed.

The concentration data on β-TPN, we believe, are in agreement with stacking between two (P)-helical forms as shown in Figure 14. It can be seen from

(50) C. Griessner-Prette and B. Pullman, *J. Theor. Biol.*, **27**, 87 (1970).

(51) R. B. Mallion and C. W. Haigh, *Org. Magn. Resonance*, **4**, 203 (1972).

(52) C. Griessner-Prette and B. Pullman, personal communication, 1972.

Figure 14 that the bulky 2′-phosphate group partially precludes the close and complete overlap between faces "A" of two adenine moieties of the two molecules of β-TPN involved in dimerization. Such hindrance to complete overlap causes the adenine C(2)H to be shielded, C(8)H and C(1′)H to be unaffected, and adenine C(2′)H and C(3′)H to be deshielded.

It must be noted that the adenine C(2)H of β-DPN has undergone an upfield shift of 26 Hz whereas the same proton of β-TPN undergoes a shift of only 16 Hz. This may result from an internal compensation of the ring current shielding by the deshielding originating from the 2′-phosphate dianion from the adjacent β-TPN molecule, located as shown in Figure 14, in addition to other factors. Table III contains the spatial

Table III. Cyclindrical Coordinates p and z for the Protons in β-TPN Dimer Shown in Figure 14

Protons	p, Å[a]	z, Å[a]	Obsd Δδ, Hz[b]	Ring current zone,[c] Hz: Adenine[d]	Benzene[e]	Benzene[f]
AC(2)H	3.0	3.6	−16.0	−25	−20	−8.1
AC(8)H	5.0	3.6	0.0	0	n	n
AC(1′)H	4.8	3.3	0.0	−1	n	n
AC(2′)H	5.1	2.5	+7.5	+7	+6.2	+2.2
AC(3′)H	7.5	3.4	+5.0	+3	d	+1

[a] Measured values for p and z are accurate only to ±0.5 Å. [b] (−) refers to upfield shifts. (+) refers to downfield shifts. [c] n = neutral, d = deshielding. [d] From Giessner-Prettre and Pullman,[52] who provided the authors with isoshielding diagrams for adenine for z values ranging from 2 to 5 Å at intervals of 0.2 Å. Their method of calculation is described in ref 49. [e] From ref 48. [f] From ref 50.

positions and predicted shielding patterns for the observable protons in Figure 14: p and z are the cylindrical coordinates (±0.5 Å) of the protons relative to the center of the adenine moiety, p being the in-plane projection and z the vertical distance from the plane. Also given in the table are the upfield or downfield shifts predicted from Giessner-Prettre and Pullman[50,52] for stacking between adenine moieties. For the purpose of comparison, we also provide the stacking data for benzene from Mallion and Haigh[51] and Johnson and Bovey.[49] In Figure 15, we have shown the projection of one adenine over another adenosine moiety along with the isoshielding surface as the background. It should be emphasized that the isoshielding surface in Figure 15 lies at a z value of 3.4 Å, whereas the z value for the protons in Figure 14 range from 2.5 to 3.6 Å. One should also realize the fact that the experimentally observed shifts reported in Table III are the time-averaged values for these protons which exchange between their monomer and dimer environments. Unfortunately, we have not been able to obtain a plateau at the highest concentration employed, 0.4 M, because spectra recorded at concentrations higher than 0.4 M gave greatly broadened lines, which could not be used for measuring chemical shifts accurately.

Inspection of Table III shows that there is excellent agreement between the predicted and observed *direction* of the shifts. Given the inaccuracy involved in the measurement of z and p, the assumptions involved in the calculations[49-51] and other uncorrectable factors,[53] one may also conclude that the agreement be-

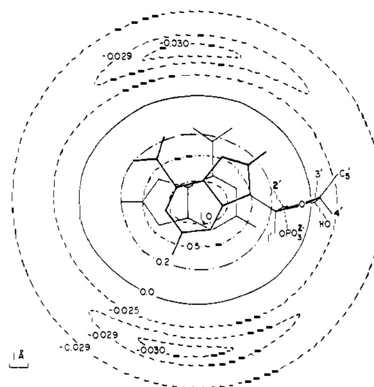


Figure 15. Geometric orientations between the two adenine fragments in the dimer of the (P)-B-anti-A-syn conformation of β-TPN. Isoshielding lines (z = 3.4 Å) are from ref 49. Ribose in the diagram appears puckered, but no puckering is intended other than to show the various atoms of the ribose.

tween the theoretically predicted magnitude of the shifts and the corresponding observed ones is reasonable. The proposed stacking of a (P)-helix with another (P)-helix (Figures 14 and 15) is also in agreement with the small shieldings that the nicotinamide C(4)H and C(5)H experience (z = 7 Å) because these protons project from the periphery of the intramolecularly stacked (P)-helix and hence are susceptible to shielding in the intermolecular complex. According to the model, nicotinamide C(1′)H would experience a small deshielding and in fact this proton experiences a deshielding of 4 Hz. The model presented in Figures 14 and 15 is only one of the several possibilities of the intermolecular stacking between the adenine moieties of two (P)-helices. We have selected this particular mode of stacking because of its following characteristics. (1) The two phosphate dianion groups, each at the 2′ positions of the adenine riboses, are as far apart as possible to minimize electrostatic repulsion. (2) The spatial orientation of the two adenine moieties is such that each affects the protons on the other in an identical manner. (3) All the hydrophilic groups such as the hydroxyls, the pyrophosphate backbone, and the 2′-phosphate groups lie outside, fully exposed to water, whereas the two hydrophobic adenine moieties lie inside.

It is our belief that the concentration data and their theoretical interpretation presented above are most consistent with the stacking between two (P)-helices. The data are inconsistent with dimerization between two (M′)-helices or for that matter between one (M′)-helix and another (P)-helix. From these data, we believe, that one can make the reasonable conclusion that a single molecule of β-TPN exists in deuterium oxide as a (P)-helix. It is not possible to say from the data whether the preferred conformation for β-TPN is (P)-B-anti-A-syn or (P)-B-anti-B-syn, although molecular models suggest that the (P)-B-anti-A-syn form is slightly less crowded, and the shielding data on the nicotinamide

(53) The experimentally observed shifts reported in Table III are the difference in chemical shifts between the isolated monomer (infinite dilution) and that of a dimer which exchanges with a monomer. Actually, one should use the difference in chemical shift between the isolated monomer and an isolated dimer. For reasons presented in the text, we cannot obtain accurate experimental data at concentrations above 0.4 M and hence no S-shaped concentration profile. Further, it is assumed that the intramolecular conformation is the same at all concentrations.

protons if anything fit the former than the latter. The concentration data on β-DPN do not permit a distinction to be made between the (M′)- and (P)-helical forms.

Theoretically computed isoshielding lines[52] for a dihydronicotinamide moiety indicate that this molecule possesses little or no ring current field and, hence, in the case of β-TPNH, one cannot rule out a dihydropyridine–dihydropyridine interaction. Nevertheless, the magnitude and direction of the shifts of the protons of the adenosine moiety (Figure 13) indicate that adenine–adenine interaction exists and these data are compatible with dimerization between two (P)-helices of β-TPNH with adenine–adenine interaction. β-TPNH may exist either as (P)-B-anti-B-anti or as (P)-B-anti-A-anti because the preferred torsional diastereomer constrained to the nicotinamide–ribose glycosidic linkage is anti (*vide supra*) and that in such a conformation the less crowded side is the B side. X-Ray data on lactate dehydrogenase reduced coenzyme binary complex indicate that the dihydronicotinamide is anti in the complex.[54] The data do not allow a distinction between the (M′)- and (P)-helices of β-DPNH.

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Mass Spectrometry of Nucleic Acid Components. *N*,*O*-Permethyl Derivatives of Nucleosides

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Abstract: Reaction between nucleosides and methylsulfinyl carbanion–CH_3I or–CD_3I produces permethyl derivatives which are O-methylated in the sugar, and methylated at the following positions in the base: adenosine, N^6,N^6; guanosine, N^2,N^2,O^6 and N^2,N^2,N-1 (~1:1); uridine, N-3; cytidine, N^4,N^4. The reaction can be carried out on a microgram scale; the products are chemically stable, exhibit relatively low molecular weights, and are sufficiently volatile for gas chromatography–mass spectrometry. Principal fragmentation pathways have been studied, based on D, ^{18}O, and substituent labeling, and measurement of exact mass, using 37 nucleoside and deoxynucleoside models. Major reactions are initiated by transfer of hydrogen from the sugar moiety to the charge-localized base. As an exception, 25% of rearranged hydrogen in the base + H ion is derived specifically from the *O*-2′-methyl group. The influential role of base–2′ interactions is also shown by the effect of methoxyl orientation at C-2′ (ribose *vs.* arabinose) on sugar − H ion abundance. Otherwise, steric features of the sugar have significant effects upon ion abundance, but hydroxyl orientation in the parent nucleoside cannot be assigned directly from the mass spectrum. Characteristic elimination of methylenimine from dimethylamino groups in the base proceeds by a complex mechanism which can include participation of other sterically accessible methyl groups, *e.g.*, the C-5 methyl function in the permethyl derivative of 5-methylcytidine.

Detailed studies of electron impact induced fragmentation of nucleosides and their analogs have led to a clearer understanding of the complex reaction paths which are involved and have provided a powerful tool for the determination of nucleoside structure. However, the dominant experimental problem, particularly for many modified nucleosides which occur in transfer RNA, continues to be low volatility resulting from the presence of multiple hydroxyl and amino groups. Substantial progress in this respect has been made using the field desorption technique in lieu of conventional vaporization,[1] but the question of sensitivity is still unclear and it is evident that the low degree of fragmentation may represent a net loss in structural information compared with electron ionization methods. Chemical derivatization to reduce polarity currently remains as the single most effective approach and in some cases permits the added advantage of gas chromatography. Acetyl,[2,3] phenylboronyl,[3] *O*-isopropylidene,[3] trimethylsilyl,[4] and trifluoroacetyl[5] blocking groups have been proposed for this purpose, but detailed studies have been reported only for the latter derivative.[6,7]

In an effort to explore new approaches to this prob-

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