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Proton Magnetic Resonance Study of the Intramolecular Association and Conformation of the α and β Pyridine Mononucleotides and Nucleosides[†]

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ABSTRACT: The chemical shifts and coupling constants are reported for the proton nuclear magnetic resonance (NMR) spectra of the α and β anomers of the oxidized and reduced pyridine mononucleotides and nucleosides. The pseudorotational conformation analyses of the ribose coupling constants indicate that the ribose conformation for β-nicotinamide mononucleotide, βNMN, can best be described by a 3:1 mixture of interconverting 3′-exo (S) and 2′-exo (N) conformers. Reduction of βNMN to βNMNH results in phase angles consistent with interconverting 2′-endo (S) and 3′-endo (N) conformers without changes in the conformer populations. Cleavage of the 5′-phosphate from βNMN has a significant effect on the phase angles (becoming more like those for βNMNH), conformer population (the N and S conformers become nearly equal), and the distribution of the rotational isomers around the ribose 4′-5′ bond to the exocyclic methylene (the gauche-gauche population decreases by about 25%). In contrast, for βNMNH these parameters are all insensitive to dephosphorylation. The pseudorotational analysis has been extended to define the conformational parameters of α nucleotides. Analysis of the coupling constants for the α anomers indicates that the phase angles, conformer populations, and rotational isomers are generally insensitive to dephosphorylation, whereas both the phase angle and conformer populations are strongly dependent on the redox state of the base, αNMN being predominantly 2′-endo and αNMNH exclusively 2′-exo. The rotational isomers around the 4′-5′ and 5′-O bonds are found to be insensitive to the large changes in ribose conformation in the absence of any interaction with the base. The results are discussed in terms of relative contributions from base-ribose, ribose-side chain, and base-side chain interactions to the general conformational restraints imposed by the *cis*-2′,3′-hydroxyl interaction in β nucleotides and the additional *cis*-2′-hydroxyl-base interaction in α nucleotides. The significance of these interactions with respect to the enzymatic and nonenzymatic properties of the pyridine nucleotides is also considered.

Previous proton NMR[1] investigations of the pyridine mononucleotides (Oppenheimer et al., 1971; Birdsall and Feeney, 1972; Birdsall et al., 1975; Blumenstein and Raftery, 1972; Sarma and Mynott, 1973; Egan et al., 1975; Zens et al., 1975) have dealt exclusively with the β anomers because of their significance as the active moiety in βNAD(P) and βNAD(P)H, the "natural" coenzymes for the pyridine nucleotide linked dehydrogenases.[1] In contrast, the α-pyridine nucleotides have not been investigated in detail despite their isolation from a number of tissue extracts and organisms (Kaplan et al., 1955; Ricci et al., 1965), the equilibration of α- and β-reduced

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[1] Abbreviations used are: NMN, nicotinamide mononucleotide; NMNH, reduced NMN; NR, nicotinamide ribonucleoside; NRN, reduced NR; TSP, 3-trimethylsilylpropionate-*2,2,3,3-*d_4, sodium salt; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance; α and β refer to the anomeric configuration of the pyridine base to the ribose; P is the pseudorotational phase angle and τ_m the degree of puckering for the ribose ring as defined by Altona and Sundaralingam (1972, 1973).

coenzymes (Oppenheimer et al., 1971), and the stereoselective oxidation of αNADH by common dehydrogenases (Oppenheimer and Kaplan, 1975). Thus, an understanding of the structure–function relationship for coenzyme binding to dehydrogenases requires detailed information concerning the conformational forces acting in both anomeric forms.

A comprehensive understanding of the magnitude and significance of specific intramolecular interactions requires a systematic investigation of the pyridine mononucleotides involving alterations of all three nucleotide moieties: the base, the ribose, and the 5′ substituent. The perturbations engendered by the oxidized or reduced base, the α or β anomers, and the presence or absence of the 5′-phosphate allow the pyridine nucleotides to form a set of compounds in which different intramolecular interactions predominate, thus enabling relative estimates of the significance of each in determining the observed solution conformation. Expansion of the Altona and Sundaralingam (1972, 1973) pseudorotational analysis of ribose conformation to include the α anomeric configuration allows a quantitative calculation of the redox and dephosphorylation-dependent conformational changes for both the α- and β-pyridine mononucleotides. Finally, the pyridine nucleotides differ from the purine and pyrimidine nucleotides only in the nature of the base, hence, information concerning relations between conformation and specific intramolecular interactions should be directly applicable to the broader subject of the conformational forces in nucleic acids.

Methods

Preparation of Mononucleotides. βNAD, βNADH, and αNAD were obtained from PL Biochemicals and used without further purification. βNMN, βNMNH, and αNMN were prepared from the respective dinucleotides by cleavage of the pyrophosphate linkage with snake venom phosphodiesterase (EC 3.1.4.1, Boehringer-Mannheim) according to the procedure of Kaplan and Stolzenbach, (1957) and separated from 5′-AMP on a DEAE-cellulose column eluted with a 0.005–0.5 M ammonium bicarbonate linear gradient. αNMNH and αNRH were prepared by dithionite reduction of αNMN and αNR, respectively (Lehninger, 1957). The nucleosides βNR, βNRH, and αNR were prepared from the respective mononucleotides by hydrolysis of the 5′-phosphate ester with alkaline phosphatase (EC 3.1.3.1, Sigma).

Spectral Determinations. Proton magnetic resonance spectra were recorded on a Varian Associates field sweep HR-220 spectrometer with the signal-to-noise ratio enhanced by a Nicolet 1074 computer. Homonuclear spin decoupling was performed using a Wavetek voltage-controlled oscillator. Heteronuclear ^{31}P spin decoupling was performed with an Accutronics, Inc. JM 201M voltage-controlled crystal oscillator centered at 89.066 MHz and an Amplifier Research Corp. Model 6L wide-band radiofrequency power amplifier connected to the decoupling coils by a tuned network. The resulting spectra were analyzed using the Nicolet iterative computer simulation program ITRCAL.

Samples were twice lyophilized from 99.8% D_2O and then dissolved in 100% D_2O to a final concentration of 40 mM. Sample temperature was 22 °C. The pD of the solutions is as noted in the tables and was measured with a Radiometer Model 25 pH meter with a combination electrode and reported using the electrode correction for D_2O, pD = meter reading + 0.4 (Glasoe and Long, 1960). The sample volume was 0.2 ml with vortexing suppressed by Teflon plugs (Wilmad). An internal standard, 3 mM TSP, was used with 1 mM EDTA added to prevent line broadening from possible paramagnetic ion impurities.

Results

Spectral Assignments. The chemical shifts of the ribose protons are listed in Table I and the coupling constants in Table II. The ribose protons of αNMN and αNMNH were assigned by homonuclear proton decoupling and heteronuclear phosphorous decoupling. The assignments of the ribose protons are consistent with the expected deshielding of the C1′ proton in the α anomer (Lemieux and Lown, 1963). The ribose absorptions of βNR, βNRH, αNR, and αNRH were assigned by homonuclear spin decoupling and are consistent with the upfield shifts expected upon removal of the 5′-phosphate. The ^{1}H NMR spectra of αNR and αNRH are shown in Figure 1. The assignments of the β-mononucleotide proton absorptions have been reported previously (Oppenheimer et al., 1971; Birdsall and Feeney, 1972; Sarma and Mynott, 1973). The C5′ protons for the α anomers are tentatively assigned by analogy to the C5′ protons of α-O$^{2'}$-6B cyclotetrahydronicotinamide mononucleotide, α(cTHN)MN (Oppenheimer and Kaplan, 1974). The same assignments are suggested for the β anomers and are consistent with the assignments proposed for the 5′ protons of other β nucleotides (Remin and Shugar, 1972). The discussions related to the 5′ protons, however, will not critically depend on the validity of these specific assignments.

The chemical shifts for the pyridine ring protons are listed in Table III. The nicotinamide protons of αNMN were assigned by comparison to the absorptions in αNAD where the nicotinamide proton absorptions have been unequivocally assigned (Oppenheimer and Kaplan, 1975). The distinctive absorptions of the dihydronicotinamide protons of αNMNH were assigned by comparison with the spectrum of αNADH (Oppenheimer, et al., 1971; Oppenheimer and Kaplan, 1975).

The coupling constants of the nicotinamide and dihydronicotinamide ring protons in the β nucleosides and α nucleotides/nucleosides do not differ by more than the accuracy limits, ±0.15 Hz from the values reported for βNMN and βNMNH (Birdsall and Feeney, 1972; Sarma and Mynott, 1973).

Chemical Shifts of the Pyridine Protons. Titration of the 5′-phosphate of βNMN leads to a substantial 0.15 ppm combined shift of both the N2 and N6 protons, whereas titration of the 5′ phosphate of αNMN causes only a small 0.03 ppm combined shift (Sarma and Kaplan, 1969). The shift of the N2 and N6 protons in βNMNH with titration is only about one-fourth the shift of βNMN (Sarma and Mynott, 1973). However, there is a larger 0.12 ppm shift of βNMNH with dephosphorylation to βNRH (Table III). These results are consistent with the expected closer proximity of the pyridine ring to the charged 5′-phosphate moiety in the β nucleotides as compared to the α anomers. There may also be an additional screening of the charge interaction by the intervening ribose moiety in the α anomers.

Ribose Conformational Analysis. The pseudorotational analysis of nucleotide conformation is based on the principle that any given ribose conformer can be uniquely defined by only two parameters, the pseudorotational phase angle, P, and the degree of puckering, τ_m (Altona and Sundaralingam, 1972). ^{1}H NMR studies summarized by Altona and Sundaralingam (1973) show that in solution the ribose moiety of purine and pyrimidine nucleotides/nucleosides can be accurately described as corresponding to the rapid interconversion of two distinct types of conformers. The phase angles for these two types of conformers are found to range between 0 and 36° for

TABLE I: Chemical Shifts of Ribose Protons.[a]

pD	Compound	C1′	C2′	C3′	C4′	C5′$_S$	C5′$_R$
8.5	αNMN$^+$	6.580	5.040	4.462	4.912	4.041	3.977
4.5	αNMN$^+$	6.573	4.972	4.449	4.914	4.181	4.101
8.5	αNR	6.557	4.907	4.382	4.777	3.927	3.805
8.5	βNMN$^+$	6.177	4.670	4.477	4.618	4.205	4.032
4.5	βNMN$^+$	6.218	4.591	4.464	4.659	4.314	4.164
8.5	βNR$^+$	6.275	4.533	4.370	4.475	4.060	3.917
8.5	αNMNH	5.218	4.402	4.336	4.241	3.918	3.836
8.5	αNRH	5.168	4.334	4.252	4.143	3.830	3.673
8.5	βNMNH	4.900	4.345	4.250	4.105	(3.870)[b]	(3.870)[b]
8.5	βNRH	4.893	4.218	4.145	3.982	3.759	3.705

[a] Chemical shifts are in ppm from TSP and are reported to within 0.002 ppm. [b] The chemical shift difference of the C5′ protons of βNMNH is less than 0.01 ppm, so only the value of the center of the absorption is given.

TABLE II: Ribose Proton Coupling Constants.[a]

pD		$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$J_{4'-5'_S}$	$J_{4'-5'_R}$	$J_{5'_S-5'_R}$	$J_{5'_S-P}$	$J_{5'_R-P}$	$^4J_{4'-P}$
8.5	αNMN	5.6	5.0	2.2	3.0	3.3	−11.9	4.9	5.8	1.8
4.5	αNMN	5.7	4.9	2.5	2.7	3.2	−11.8	5.6	5.6	2.0
8.5	αNR	5.4	4.7	3.4	3.2	4.7	−12.6			
8.5	βNMN	5.8	4.8	2.1	2.4	1.9	−11.8	4.0	4.2	2.3
4.5	βNMN	5.4	4.9	2.4	2.4	2.0	−12.0	4.2	4.8	2.1
8.5	βNR	4.4	5.0	4.7	2.8	3.5	−13.1			
8.5	αNMNH	4.7	5.2	5.7	2.9	3.9	−11.2	4.3	5.3	0.9
8.5	αNRH	4.4	4.9	7.0	2.6	4.7	−12.5			
8.5	βNMNH	7.5[b]	5.5	2.2	(3.9)[c]	(3.9)[c]		(4.8)[c]	(4.8)[c]	1.5
8.5	βNRH	7.4	5.1	2.8	3.6	4.7	−12.6			

[a] Coupling constants obtained at 220 MHz and are accurate to within ±0.15 Hz. [b] Results obtained at 100 MHz (Sarma and Mynott, 1973) report a smaller value (6.8 Hz) for $J_{1'-2'}$. This discrepancy may arise from second-order effects caused by the strong coupling of the N2′ and N3′ protons at the lower frequency. See Hruska et al., 1970 for a brief discussion of this problem. [c] Only the mean value of these coupling constants can be determined because of the chemical shift equivalence of the C5′ methylene protons.

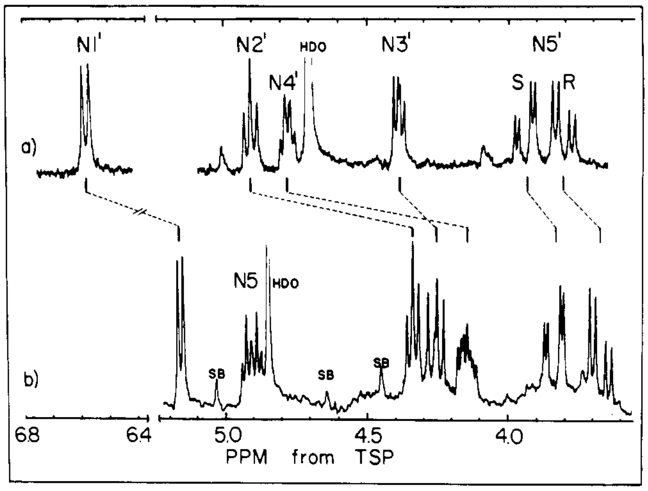

FIGURE 1: ^{1}H NMR spectra of the ribose proton absorptions of (a) αNR and (b) αNRH.

TABLE III: Chemical Shifts of the Pyridine Ring Protons.[a]

pD	Compound	N2	N6	N4	N5
		Nicotinamide Protons			
8.5	αNMN$^+$	9.335	9.118	8.961	8.223
4.5	αNMN$^+$	9.309	9.097	8.957	8.217
8.5	αNR$^+$	9.305	9.086	8.970	8.239
8.5	βNMN$^+$	9.589	9.332	8.989	8.302
4.5	βNMN$^+$	9.475	9.295	8.991	8.305
8.5	βNR$^+$	9.622	9.296	9.000	8.298
		Dihydronicotinamide Protons			
8.5	αNMNH	7.250	6.158	3.073	4.911
8.5	αNRH	7.230	6.141	3.075	4.911
8.5	βNMNH	7.166	6.239	3.075	5.036
8.5	βNRH	7.156	6.117	3.075	5.009

[a] Chemical shifts are in ppm from TSP and are reported to within 0.002 ppm.

the N-type conformer and 144–180° for the S-type conformer.

Interconversion of the S and N type conformers occurs by surmounting an energy barrier corresponding to the unfavorable ribose conformers with phase angles of either 90° (O′-endo) or 270° (O′-exo). In these transition states the four ring carbons are coplanar and have their substituents eclipsed. Thus, these states correspond to maxima of steric hindrance. In contrast, the conformers with phase angles equal to 0 and 180° correspond to minima in steric hindrance, since the 2′ and 3′ hydroxyls are staggered. The observed ranges of phase angles for β purine and pyrimidine nucleotides/nucleosides (Altona and Sundaralingam, 1972; 1973; Davies and Danyluk, 1974) are intuitively reasonable, since they are near the expected energy minima of P equal to 0 and 180°, although skewed towards the 90° half.

TABLE IV: Conformational Parameters of the α and β Pyridine Nucleotides

	pD	^{N}P	^{S}P	$^{N}\tau_m$	$^{S}\tau_m$	[N]	[S]	gg	g′g′
αNMN	8.5	352	166	35	39	26	74	67	74
αNMN	4.5	355	169	35	39	29	71	71	71
αNR	8.5	354	169	37	40	40	60	51	
βNMN	8.5	352	190	35	38	26	74	87	88
βNMN	4.5	350	190	35	38	30	70	86	83
βNR	8.5	10	174	35	38	49	51	67	
αNMNH	8.5	334	150	37	41	87	13	62	80
αNRH	8.5	335	152	39	43	~100		57	
βNMNH	8.5	24	159	35	37	19	81	52	80
βNRH	8.5	27	155	39	42	25	75	47	

A consequence of the observed range of phase angles is that the value of the ribose coupling constant $J_{2'-3'}$ should define the value of the time-averaged absolute value of the dihedral angle between the C2′ and C3′ protons, $\theta_{2'-3'}$, which in turn can be used as the absolute value of τ_m (Altona and Sundaralingam, 1973).[2] This proposal is supported experimentally by the analysis of the ^{1}H NMR spectra for deoxyribonucleotides (Davies and Danyluk, 1974). The extra coupling constant $J_{2''-3'}$ makes the pseudorotational analysis fully defined and indicates that the value of $J_{2'-3'}$ does correspond to the absolute value of the average dihedral angle. The skewed distribution of the values of ^{N}P and ^{S}P for β purine and pyrimidine nucleotides toward 90° will be discussed below.

β Anomers. The mean values of $J_{2'-3'}$, and the sum of $J_{1'-2'+3'-4'}$ for the pyridine nucleotides/nucleosides are smaller than those used by Altona and Sundaralingam (1973) to derive the parameters for the Karplus relations (Karplus, 1959, 1963) and has prompted our use of the parameters derived by Davies and Danyluk (1974), i.e., $A = 9.8$ Hz and $B = 0.9$ Hz. The values of $J_{1'-2'}$ and $J_{3'-4'}$ for the N- and S-type conformers were calculated for incremental changes in τ_2 corresponding to values of $J_{2'-3'}$ of 4.4–6.0 Hz using the equations given by Altona and Sundaralingam (1973) with the values of θ, the dihedral angle, calculated by the indirect method. The value of τ_m was calculated from the derived values of ^{N}P, ^{S}P and the experimentally determined $J_{2'-3'}$. As discussed by Davies and Danyluk (1974), the experimental error in calculating ^{N}P and ^{S}P is on the order of ±2°; however, the systematic errors introduced by the underlying assumptions of this method probably make the range of significance approximately ±5° for studies comparing dissimilar compounds.[3]

The values of P and τ_m derived by the full pseudorotational analysis for the β anomers are listed in Table IV. The results indicate that substantial differences occur in the phase angles with either oxidation–reduction of the base or dephosphorylation of the ribose without major change in the degree of puckering. The phase angles for βNMN are clearly outside the range found for purine and pyrimidines (Altona and Sundaralingam, 1973; Davies and Danyluk, 1974) and correspond to the conformers characterized as 2′- and 3′-exo. The coupling constants for βNMN are not indicative of a more highly puckered ring (increased τ_m), since the small value of $J_{1'-2'} + J_{3'-4'}$ is not accompanied by the abnormally high value of $J_{2'-3'}$ characteristic of such distortion.

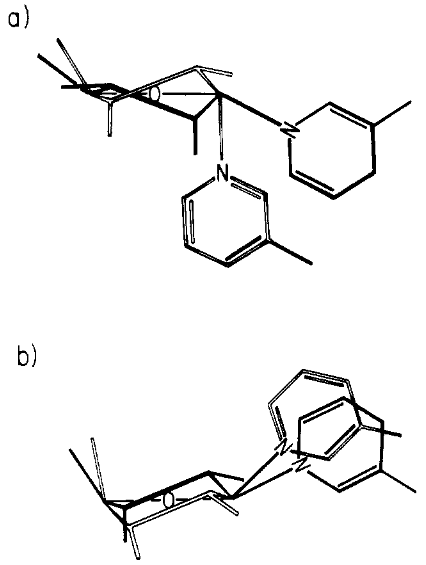

FIGURE 2: The redox-dependent conformational changes for the pyridine mononucleotides shown by superimposing the predominant conformers (a), 2′-exo for αNMNH (solid lines), 2′-endo for αNMN (open lines); (b), 2′-endo for βNMNH (solid lines), 2′-exo for βNMN (open lines).

The values of ^{N}P and ^{S}P for βNR, βNMNH, and βNRH indicate a predominance of the "more typical" 2′-/3′-endo types of conformers. Note that the large change in phase angle with oxidation–reduction of the mononucleo*tide* is not accompanied by a corresponding change in N and S type conformer populations. In contrast, dephosphorylation of βNMN to βNR results in both a shift in ^{N}P and ^{S}P towards 90° and a nearly equal distribution of S and N type conformers. The structures of the predominant conformers and the relative orientation of the pyridine ring for βNMN and βNMNH are compared in Figure 2.

α Nucleotides. The α nucleotides/nucleosides also undergo dynamic interconversion of conformers (Grey et al., 1971), thus, a quantitative pseudorotational analysis for the α anomers is possible based on the following assumptions: (1) the values of $\theta_{1'-2'}$ for α nucleotides can be derived from the values for β nucleotides by interchange of the 1′ proton and the base;

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[2] Any ensemble average model would be energetically less favorable, since it would require the observed value of $J_{2'-3'}$ to be the average of a large value of $J_{2'-3'}$, corresponding to a conformer in which the ribose substituents are eclipsed, and a small value of $J_{2'-3'}$, indicative of a highly puckered ribose conformation.

[3] The electronegativity of the substituent at C1′ can have an influence on the Karplus relation that defines the ribose coupling constants. However, since the chemical shift of the C1′ proton for purine, pyrimidine, and pyridinium nucleosides/nucleotides are similar, the effective electronegativities of the bases should also be comparable (to a first approximation). In contrast, the ca. 1.5 ppm upfield shift of the C1′ proton in dihydropyridine nucleosides/nucleotides indicates greater electron density at C1′ that may result in a contribution to the value of $J_{1'-2'}$ of as much as 0.5 Hz (Laszlo and Schleyer, 1963). As a consequence, the values of ^{N}P may be 5° less and ^{S}P 5° more. This would not alter the conclusion that the 2′-/3′-endo type conformers predominate for dihydropyridine nucleosides/nucleotides.

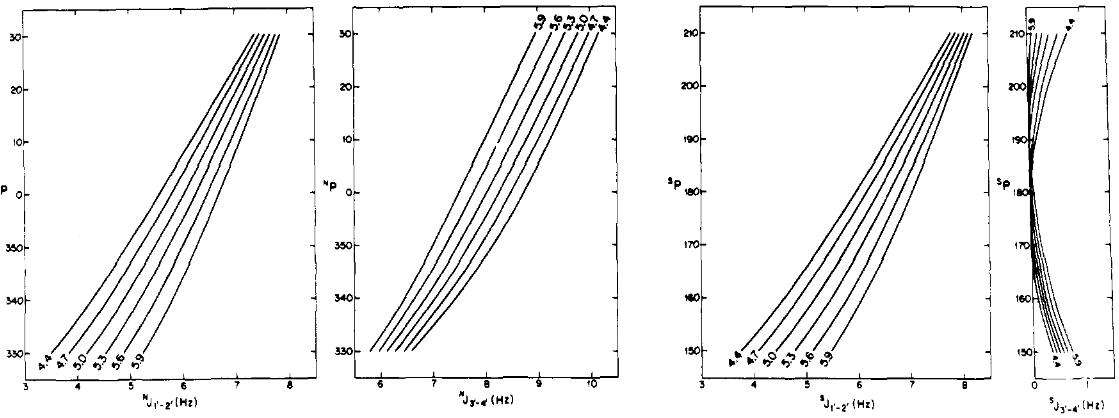

FIGURE 3: Computed values of the phase angle, P, and ribose proton coupling constants in α nucleotides for different values of $J_{2'-3'}$ using the Karplus relation ${}^{3}J_{H,H} = 9.8 \cos^2 \theta - 0.9 \cos \theta$ (Davies and Danyluk, 1974) according to the methods outlined by Altona and Sundaralingam (1973). (a), ${}^{N}P$ vs. ${}^{N}J_{1'-2'}$; (b), ${}^{N}P$ vs. ${}^{N}J_{3'-4'}$; (c) ${}^{s}P$ vs. ${}^{s}J_{1'-2'}$; (d) ${}^{s}P$ vs. ${}^{s}J_{3'-4'}$. The intersection of the observed value of $J_{1'-2'}$ with the diagonal line corresponding to the observed value of $J_{2'-3'}$ defines the phase angle ${}^{N}P$ using graph a and ${}^{S}P$ using graph c. The values of ${}^{N}P$ and ${}^{S}P$ can then be used to calculate values of ${}^{N}J_{3'-4'}$ and ${}^{S}J_{3'-4'}$ using graphs b and d, respectively. The fractional conformer populations then correspond to $[S] = ({}^{N}J_{3'-4'} - J_{3'-4'})/({}^{N}J_{3'-4'} - {}^{S}J_{3'-4'})$ and $[N] = 1 - [S]$ where $J_{3'-4'}$ is the observed value.

(2) the value of $J_{1'-2'}$ approximately defines the absolute value of $\theta_{1'-2'}$ rather than corresponding to an ensemble average of coupling constants for conformers with widely differing values of $J_{1'-2'}$; (3) the same Karplus relation can be used for analysis of both α and β anomers. This final assumption is not unwarranted. The uncertainty in the Karplus relation should be similar for both anomers, since the differences in juxtaposition of the substituent between anomers are comparable to differences in various conformers.

These assumptions, combined with the appropriately modified equations of Altona and Sundaralingam (1973) and the Karplus relation constants of Davies and Danyluk (1974), allow us to calculate $\alpha^{N}J_{1'-2'}$ and $\alpha^{S}J_{1'-2'}$ for values of $J_{2'-3'}$ varying from 4.4 to 6.0 Hz (Figure 3). The resulting pseudorotational phase angles of the contributing conformers in the α pyridine nucleotides/nucleosides, ${}^{N}P$, ${}^{S}P$, and τ_m are listed in Table IV.

As can be seen, the N-phase angles for the α anomers show a large difference with respect to the β anomers. The value of ${}^{N}P$ is skewed towards 270° and is indicative of the 2′-exo class of conformers, while the value of ${}^{S}P$ indicates a 2′-endo type conformer similar for both α and β anomers. X-ray crystallographic studies, summarized by Sundaralingam (1971), indicate a preference for 2′-exo or 2′-endo conformations in vitamin B_{12}, and 2′-exo in α-pseudouridine. Thus, the conformations calculated from ${}^{1}H$ NMR data are in qualitative agreement with the X-ray crystallographic results for α ribonucleotides/ribonucleosides.

The oxidized compounds αNMN and αNR show a 3:1 predominance of the S-type (2′-endo) conformer, whereas αNMNH and αNRH are close to being exclusively the N type (2′-exo), which is shown in Figure 2. The redox-mediated change in the value of the phase angles for the α anomers is, however, about two-thirds that observed for the β anomers. It is difficult to ascribe the large population changes in the α anomers to the changes in phase angles alone, since essentially no change in population is observed for the β anomers where larger changes in the phase angle values occur. Dephosphorylation of αNMN or αNMNH causes about a 10% change in conformer populations. In comparison, dephosphorylation of βNMN causes a 20% decrease in S-type conformer and dephosphorylation of βNMNH causes a 5% change. We believe that changes greater than 5% are significant.

Rotamer Populations. The populations of rotational isomers, rotamers, around the C5′ O–P bond can be calculated from the values of the heteronuclear proton–phosphate coupling constant, $J_{5'_S-P}$ and $J_{5'_R-P}$, using the limiting values of 21 Hz for the trans-coupling constant and 3 Hz for the gauche-coupling constants (Hall and Malcolm, 1968; Tsuboi et al., 1968; Kainosho et al., 1969; Donaldson et al., 1972) The calculated rotamer populations in the β mononucleotides are in agreement with the values reported by Sarma and Mynott (1973) and indicate a predominance of the gauche-gauche (g′g′) rotamer. The larger values of these coupling constants in the α nucleotides compared to the β nucleotides and βNMNH compared to βNMN reflect a greater contribution from the g′t′ and t′g′ rotamers in the former compounds.

The populations of all three rotamers around the C5′–C4′ bond can be calculated from the values of $J_{4'-5'_S}$ and $J_{4'-5'_R}$ (Blackburn et al., 1970) using the limiting values of $J_{\text{trans}} =$ 11.5 Hz and $J_{\text{gauche}} = 1.5$ Hz (Wood et al., 1973). For all the pyridine nucleotides and nucleosides except βNMNH this calculation is straightforward. For βNMNH, the C5′ proton absorptions are chemical shift equivalent within experimental limits even at 220 MHz, i.e., $\Delta\delta$ is less than 0.01 ppm, hence, the spectral pattern is deceptively simple, Thus, in the spectrum of βNMNH the individual values of $J_{4'-5'_R}$ are indeterminant; only their sum can be measured and, likewise, only the sum of the gt and tg populations has any significance. The strong coupling does not, however, affect the calculation of the gg populations.

The calculated rotamer populations of the nucleotides are listed in Table IV. For βNMN, the gg rotamer around the C4′–C5′ bond strongly predominates. Reduction of the β-nicotinamide ring tends to equalize the populations with the gg contribution decreasing to about 52% in βNMNH, in agreement with the data reported by Sarma and Mynott (1973). Likewise, dephosphorylation of βNMN decreases the gg population to 67%. No such decrease is observed for the dephosphorylation of βNMNH where the rotamer population remains nearly the same at 47% gg.

The oxidation state of the α-nicotinamide ring exerts a much weaker influence on the rotamer populations than found for the β anomers. Reduction of αNMN to αNMNH is accompanied by only a 5% decrease in the gg rotamer and for the nucleotides there is a 6% increase.

The values of long range, four bond, coupling constants such as ${}^{4}J_{4'-P}$ have strong stereoconfiguration dependencies, with

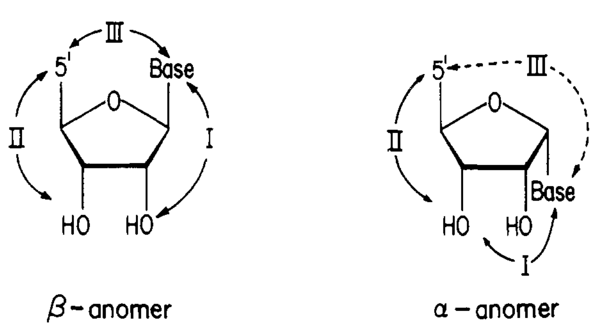

FIGURE 4: The three dominant intramolecular interactions in nucleotides and nucleosides; (I) base-ribose; (II) ribose-5'; (III) base-5'.

maximal coupling for the trans planar, "M", gg-g'g' orientation (Barfield, 1971; Hall and Malcolm, 1972 a,b; Sarma et al., 1973). The differences in the values of $^4J_{4'\text{-P}}$ observed in the spectrum of βNMN and βNMNH primarily reflect the smaller gg rotamer population in βNMNH that decreases the possible amount of gg-g'g' orientation. A series of nucleotides, including βNMN and βNMNH, have been shown to have a correlation between $^4J_{4'\text{-P}}$ and the populations of gg and g'g' rotamers (Sarma et al., 1973). Further analysis of the conformational restraints on the correlation of gg and g'g' rotamers suggests that the large decrease observed in $^4J_{4'\text{-P}}$ upon reduction of αNMN could be accounted for by a change in *correlation* of the gg and g'g' rotamers, since the changes in rotamer populations themselves are small. In αNMN the gt-g't' type orientations (including gt-t'g', tg-g't', and tg-t'g') may be stabilized by a coulombic attraction to the nicotinamide ring, since those orientations allow the closest approach of the two charged moieties. This stabilization could lead in turn to a maximization of the gg-g'g' orientation with the resulting large value of $^4J_{4'\text{-P}}$. The absence of any stabilizing interaction in αNMNH should result in minimization of the gt-g't' type orientations, hence, decreasing the gg-g'g' population along with the value of $^4J_{4'\text{-P}}$ without any correspondingly large changes in either gg or g'g' rotamer populations.[4]

Discussion

The important aspects of the solution conformation of any mononucleotide or mononucleoside are defined by three parameters: (1) the dihedral angle around the glycosidic bond, (2) the ribose conformation, and (3) the dihedral angles around the C4'-C5' and C5'-O bonds (Sundaralingam, 1969; Saenger, 1973). These conformational parameters, in turn, are governed by an intraribose interaction between the 2'- and 3'-hydroxyls and the intramolecular interactions between the three functional groups which compose a nucleotide/nucleoside; the base, the sugar, and the 5' side chain. The latter three interactions are schematically illustrated in Figure 4.

Intraribose Interaction. The cis diol of ribonucleotides imposes severe conformational constraints, as amply illustrated by the observed conformers (Altona and Sundaralingam, 1972) which occupy a narrow range of phase space corresponding to maximum staggering of the cis diol. It is apparent that other types of intramolecular interactions can at best cause only relatively small perturbations away from this conformational energy well.

I. Base-Ribose Interactions. Two general types predominate: the physical contact between the ribose ring and the adjacent substituents on the purine, pyridine, or pyrimidine rings, and through bond interactions reflecting the most favorable interaction of the electron orbitals of the base with those of the ribose oxygen, i.e., the aromatic and reverse-anomeric effects (Lemieux, 1971). In addition, there is possible hydrogen bonding (T'so et al., 1966) and dipolar interaction with the ribose 2'-hydroxyl.

II. Ribose-5' Hydroxyl-Phosphate Interactions. The dominant interactions are either coulombic repulsion or physical contact between the 5'-oxygen or -phosphate and the 3'-hydroxyl or the unshared electron pairs on the ribose ring oxygen.

III. Base-5' Hydroxyl-Phosphate Interactions. These interactions primarily involve coulombic attraction or repulsion, van der Waals (induced dipolar) attraction, hydrogen bonding, or physical contact of the moieties.

It is obvious from Figure 4 that all three interactions will be important in defining the conformation of β nucleotides because of the close proximity of the base to the ribose and 5'-phosphate. On the other hand, in the α mononucleotides the large distance and the intervening ribose between the 5'-phosphate and the base would be expected to minimize contributions from the Base-5' (III) interactions. This makes the study of the α nucleotides important, since contributions from base-ribose (I) and ribose-5' (II) interactions can be studied in the near absence of any base-5' (III) interaction. As examples, changes in the α-ribose conformation with dephosphorylation should reflect predominantly a Ribose-5' (II) interaction, while oxidation or reduction of the α-pyridine ring should cause changes primarily to a direct base-ribose (I) interaction. The magnitude of the type II interaction in the α-nucleotide → α-nucleoside pair can be used to estimate the contribution of type I interactions for the oxidized/reduced pair. These estimates can then be used to estimate the contributions of all three interactions in determining the conformation of the β nucleotides. The pertinent data are summarized in Table V.

Base-Ribose Interactions (I)

Anomeric and Reverse Effects. The concept of anomeric and reverse anomeric effects developed by Lemieux (1971) to predict the preferred orientation of C1' substituents of pyranosides does not appear directly applicable to either α- or β-pyridine ribofuranosides. The conformations of the ribofuranose ring that allow the staggered orientations of the base and the orbitals of the ring oxygen's unshared electron pairs, namely, the O'-exo-1'-endo and O'-endo-1'-exo conformers, are unfavorable because they eclipse the 2'- and 3'-hydroxyls. Instead, the observed ribose conformers tend to favor the base and the electron orbitals having a nearly eclipsed orientation,

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[4] An interesting analysis of the overall backbone conformation can be developed based upon the restrictions imposed by the values of the individual gg and g'g' rotamer populations that are calculated from the appropriate vicinal coupling constants. These restrictions lead to the following conclusions: for a defined gg and g'g' population, maximizing the gg-g'g' orientation has as a direct consequence the maximizing of the unfavorable gt-g't' type orientations, and, conversely, minimizing the gg-g'g' orientation should result in no gt-g't' type orientations at all. As an example, given the following rotamer populations, gg = 50% and g'g' = 75%; maximizing the gg-g'g' orientation results in the following distribution: gg-g'g' = 50%, gt-g'g' type = 25%, and gt-g't' type = 25%, whereas minimizing the gg-g'g' orientation gives: gg-g'g' = 25%, gg-g't' type = 25%, gt-g'g' type = 50%, and *no* gt-g't' type. The overall correlation of the individual rotamer populations depends upon the energies of the various orientations with respect to each other. Therefore, the populations of the overall orientations can change in response to alteration of the total interaction potential even in the absence of any significant change in the individual gg or g'g' rotamer populations. Since the value of $^4J_{4'\text{-P}}$ depends on the orientation and correlation of both the 4'-5' and 5'-O bonds, it should be a much more sensitive probe to changes in correlation than either set of vicinal coupling constants.

TABLE V: Summary of Results.

	Decrease in N-Type Conformer (%)	Decrease in gg Rotamer (%)	Predominant Interaction[a]
αNMN → αNMNH	61	5	I
αNR → αNRH	60	−6	I
αNMN → αNR	14	16	II
αNMNH → αNRH	~13	5	[b]
βNMN → βNMNH	−7	35	III
βNR → βNRH	−24	20	III
βNMN → βNR	24	20	III
βNMNH → βNRH	6	5	[b]

[a] The numerals refer to the three types of interactions illustrated in Figure 5. I, base-ribose; II, ribose-5′; III, base-5′. [b] None of the interactions appears to be affected by dephosphorylation.

TABLE VI: Predicted Ribose Conformers Based on Anomeric Interactions.

	Predicted Conformer	Predominant Conformer[a]
αNMN	2′-exo	2′-endo
βNMN	2′-endo	3′-exo
αNMNH	2′-endo	2′-exo
βNMNH	2′-exo	2′-endo

[a] Based on pseudorotational analysis summarized in Table IV.

hence, one that is much less distinct than those for the corresponding pyranoside.

If we were to assume that the anomeric interactions determine the preferred conformation we would then make the following predictions: (1) the nicotinamide ring should be equatorial, allowing maximal interaction with both orbitals of the ring oxygen, hence, 2′-endo (S) in βNMN and 2′-exo (N) in αNMN; (2) the dihydronicotinamide ring should be axial, minimizing the interaction between the electron-rich base and the orbitals of the ring oxygen, hence, 2′-exo (N) for βNMNH and 2′-endo (S) for αNMNH. As can be seen from Table VI, the predictions do not correspond to the observed conformers even though nicotinamide pyranosides do give the predicted conformations (Lemieux 1971). Therefore, it is apparent that the anomeric effects do not play a determining role in defining the pyridine ribofuranose conformation, i.e., the anomeric effects do not significantly influence the value of ^{N}P or ^{S}P. This is not to say that the anomeric effects are without any influence. Indeed, if one looks at the population distribution of N- and S-type conformers for the β-pyridine nucleotides/nucleosides a correlation can be made between the predominant conformer *population* and the expected anomeric effect; the favored 3′-exo permits greater overlap than 2′-exo in βNMN and the favored 2′-endo permits less overlap than 3′-endo in βNMNH.

Base-Glycosidic Torsional Conformation. Direct base-ribose interactions probably can be excluded from providing the driving force for either syn or anti rotamers because of the symmetrical nature of the N-2 and -6 positions. Therefore, any preference for the syn or anti orientations should arise primarily from interaction of the 3-carboxamido group with the 5′ substituent.

Proton relaxation studies (Zens et al., 1975), homonuclear nuclear Overhauser effect experiments (Egan et al., 1975), and Lanthanide shift reagents (Birdsall et al., 1975; Lee and Raszka, 1975) indicate the lack of a strong preference for either syn or anti orientations in βNMN or βNMNH.

Interactions with the 2′-Hydroxyl. The large change in the α-ribose conformation between αNMN and αNMNH and the predominance of the 2′-endo (S) conformer in αNMN can be accounted for by an intramolecular association of the positively charged nicotinamide ring with the 2′-hydroxyl of the ribose diol, $pK \sim 11.5$–12.5. As evidence we site the 0.64 ppm upfield shift of the 2′-proton of αNMN upon reduction compared to a 0.33 ppm shift of the 2′-proton in βNMN. In contrast, the shifts for the 1′ protons are comparable, 1.36 and 1.27 ppm for α and βNMN, respectively. The deshielding of the 2′-proton can be attributed to polarization of the C2′–H bond through electron withdrawal from the 2′-hydroxyl to the positively charged nicotinamide ring. Such an interaction would account for the increased stability (Lindquist and Cordes, 1968) of the α-anomer, since nucleophilic attack at C1′ could be blocked by the 2′-hydroxyl, and its lower redox potential (Kaplan, 1960), since the increase in electron density on the nicotinamide ring could decrease its electron affinity. This latter effect may also account for the upfield shift of the nicotinamide protons of the α-anomer relative to the β-anomer (Table III). Diamagnetic anisotropy of the 2′-hydroxyl can be excluded as the sole source of this shift, since the very large differences in chemical shifts observed for αNR/βNR of 0.318 ppm for N2 and 0.211 ppm for N6 are not seen in αNRH/βNRH where the shifts are only 0.073 and 0.023 ppm for the N2 and N6 protons, respectively.

Consideration of the interaction potential between the three adjacent cis substituents can account for the observed conformers of the α-pyridine nucleotides. Conformers with $^{N}P = 324°$ or $^{S}P = 136°$ have the base and the 2′-hydroxyl in a maximally staggered orientation, whereas the 2′- and 3′-hydroxyl are maximally staggered in conformers with $^{N}P = 0°$ or $^{S}P = 180°$. Thus, if we assume that the magnitude of the steric interaction between the 2′- and 3′-hydroxyl is comparable to that between the base and the 2′-hydroxyl then intermediate values of the phase angles, $^{N}P = 342°$ and $^{S}P = 154°$ would be expected in order to maximize the relative distances of all three cis substituents. The degree to which the base is attracted to or repelled by the 2′-hydroxyl should then be reflected in the observed value of the phase angles relative to these values. Attraction should allow closer approach of the 2′-hydroxyl and base, hence, shifting the phase angles towards 0 and 180°, whereas repulsion would favor a more staggered orientation of the 2′-hydroxyl and base, thus shifting the values of the phase angles towards 324 and 136°. As can be seen in Table IV, the values of P for αNMN and αNR fit the former case, while those for αNMNH and αNRH fit the latter.

The observed predominance of the S-type 2′-endo conformer in αNMN results from the closer approach of the 2′- and 3′-hydroxyls to the positively charged nicotinamide ring in the 2′-endo conformer compared to the N-type 2′-exo conformer. Reduction of αNMN, however, juxtaposes the electron-rich dihydronicotinamide ring with the 2′-hydroxyl, hence, favoring the N-type 2′-exo conformer which maximizes the distance from the 2′-hydroxyl, thus accounting for the large shift in conformer populations between αNMN and αNMNH. Other perturbations, such as cleavage of the 5′-phosphate, do not drastically alter this interaction in the α anomers, since the phosphate resides predominantly above the ribose ring, away from the pyridine ring.

Ribose-5′ Hydroxyl/Phosphate Interactions (*II*)

Effects on Rotamer Populations. It is clear from the results that no direct correlation can be drawn in the pyridine nucleotides between the ribose conformation and the rotamer populations. Comparison of αNMN with αNMNH or αNR with αNRH (Table V) shows that the rotamer populations differ by less than 10% for changes in ribose conformer populations of as large as 70%. Only the dephosphorylation of αNMN to αNR shows a percent change in rotamer population greater than the percent change in conformer population and this result can be attributed to a weak, Base-5′ (III) interaction. The large changes in rotamer populations in the β nucleotides can be explained by Base-5′ interactions, whereas the absence of change with dephosphorylation of βNMNH or in the α nucleotides strongly argues against the notion that the ribose conformation directly defines the rotamer population.

The ribose coupling constants for βNMN correspond to phase angles that are skewed towards the 270° half of phase space. This represents a shift to conformers that decrease the base-5′ distance as would be expected considering the favorable electrostatic interaction in βNMN. The fact that protonation of the phosphate dianion does not result in significant changes in the pseudorotational parameters suggests that the coulombic attraction to the monoanion is sufficient to maintain the 2′- and 3′-exo conformers.

Reduction of βNMN causes the unfavorable juxtaposition of the electron-rich dihydronicotinamide ring with the 5′-oxygen. The resulting electrostatic repulsion should then favor those conformers that increase the Base-5′ distance, i.e., the phase angle should shift towards 90° as is observed. The absence of change in either phase angle or conformer population with dephosphorylation of βNMNH suggests that the base and the 5′-oxygen interact and that additional repulsion due to the phosphate anion is negligible.

The intermediate values of P for the ribose ring of βNR relative to those for βNMN and βNMNH may indicate a small interaction with the 5′-hydroxyl via an induced dipolar attraction or a hydrogen bond. In the absence of the charged 5′-phosphate group, the base-5′ hydroxyl interaction apparently can neither maintain the 2′-/3′-exo type conformers nor the larger population of gg and g′g′ rotamers.

The gg rotamer around the C4′–C5′ bond and g′g′ around the C5′–O bond, in general, allows the closest approach of the 5′-phosphate to the base, whereas contributions from the other rotamer populations greatly increase that distance. In βNMN, any rotamers other than gg and g′g′ would be expected to be highly unfavorable, since work would have to be done against the electric field gradient of the nicotinamide ring. In contrast, the expected coulombic repulsion in βNMNH would make such a close approach of the 5′-phosphate energetically unfavorable.

The coulombic interaction in βNMN should also be reflected in its physical and enzymatic properties. Thus, the report by Blumenstein and Raftery (1972) that the pK of the 5′-phosphate in βNMN is some 0.4 pH units lower than for 5′-AMP or βNMNH is consistent with the presence of zwitterionic interactions that are known to alter the pK of the groups involved. Likewise, βNMN is dephosphorylated by prostatic phosphatase at one-tenth the rate as compared to βNMNH, αNMN, or αNMNH (Kaplan, 1960).

The significant differences in ribose phase angles for βNMN relative to βNMNH result in substantial changes in the intramolecular distances (Figure 2). The difference in angle between the 4′–5′ bond and the 1′–N bond in 2′-/3′-exo type conformers and 2′-/3′-endo type conformers is about 40°. This difference would result in a large displacement of the important N5 position of the pyridine ring with oxidation or reduction in the active site of a dehydrogenase.

A pattern emerges in regards to the β nucleotides. If we assume that in the absence of any base-5′ interaction the preferred conformers should correspond to phase angles of 0 or 180° (minimizing the interaction between the 2′- and 3′-*cis*-hydroxyls) then positive Base-5′ interactions (attractions) should shift the phase angles towards 270°, decreasing the base-5′ separation, whereas negative interactions (repulsion) should shift the phase angles towards 90°, increasing their separation. The nature of the Base-5′ interaction should also be reflected in the rotamer populations around the bonds to the exocyclic methylene attraction favoring gg and g′g′ rotamers, repulsion increasing the populations of gt and g′t′-type rotamers. Thus, the apparent correlation between rotamer and conformer populations in β-purine and β-pyrimidine nucleotides (Hruska et al., 1971; 1974) can be rationalized on the basis of Base-5′ interactions. The α nucleotides appear to be governed exclusively by the interaction between the base and the 2′-hydroxyl.

The results clearly question the applicability to the pyridine nucleotides/nucleosides of any direct correlation because of the ribose conformer population distributions and the rotamer populations, as have been calculated by Lakshminarayanan and Sasisekharan (1969) and Wilson and Rahman (1971). Instead, our results demonstrate the importance of the intramolecular interactions of the base with the sugar and 5′-hydroxyl or phosphate in accord with the recent detailed calculations of nucleotide conformations (Berthod and Pullman, 1973; Yathindra and Sundaralingam, 1973a,b,c).

Finally, the intramolecular coulombic interaction observed in the mononucleotides is also evident in the respective α and β dinucleotides, suggesting a conformational "rigidity", i.e., dominance of the shorter range interactions observed in the α and β mononucleotides/mononucleosides. The detailed analysis of the conformations of these dinucleotides will be discussed in forthcoming projects.

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