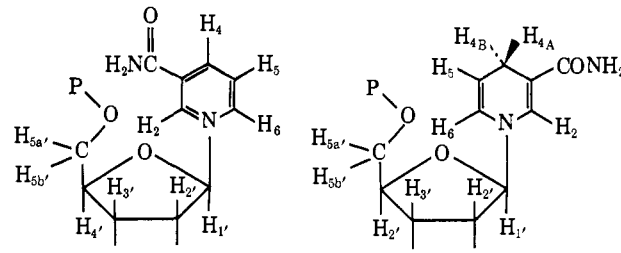
Conformation of Pyridine Nucleotides Studied by Phosphorus-31 and Hydrogen-1 Fast Fourier Transform Nuclear Magnetic Resonance Spectroscopy. I. Oxidized and Reduced Mononucleotides[1]

Ramaswamy H. Sarma* and Richard J. Mynott

Contribution from the Department of Chemistry, State University of New York at Albany, Albany, New York 12222. *Received July 3, 1972*

Abstract: The proton nuclear magnetic resonance spectra of aqueous (deuterium oxide) solutions of the oxidized and reduced β-nicotinamide mononucleotides (β-NMN and β-NMNH, respectively) have been examined over a range of pH and concentrations in the Fourier mode. The complete analysis of the spectra indicates that (a) the preferred torsional isomer constrained to the $C_{5'}$–$O_{5'}$ bond is gauche–gauche in both molecules, (b) the rotamers about the $C_{4'}$–$C_{5'}$ bond show a definite preference for the gauche–gauche form, this preference being most marked in the case of β-NMN, and (c) a difference exists between the time-averaged ribose backbone conformations of the oxidized and reduced nucleotides. ^1H and ^{31}P nuclear magnetic studies of the perturbation due to protonation of the phosphate group indicate that β-NMN probably exists mainly in the syn form and β-NMNH mainly in the anti form.

No nucleotides are known to be involved in so many important biochemical reactions as the pyridine coenzymes. Thus, their oxidation–reduction and subsequent transport of the electrons to molecular oxygen generate the energy by formation of ATP; over 150 pyridine nucleotide dependent dehydrogenases control various biological reactions; they are even involved in the synthesis and repair of DNA. There have been intensive investigations in the past using various forms of spectroscopy regarding their solution conformation in order to understand their structure–function relationship.[2-24] The availability of a large Fourier transform system in our laboratory enabled us to obtain magnetic resonance spectra of these coenzymes superior to any hitherto reported in the concentration range of 0.1–0.001 *M* and this paper, as well as a subsequent one, describes our recent studies on the conformations of the pyridine mono- and dinucleotides, respectively. The structures of the oxidized and reduced β-nicotinamide mononucleotides (β-NMN and β-NMNH, respectively) are shown in I and II.



I β-NMN (syn)

II β-NMNH (anti)

Experimental Section

The proton nmr spectra were recorded at 100 MHz on a Varian HA 100D spectrometer interfaced to a Digilab FTS-3 Fourier transform data system. The system has a total memory of 132K and is capable of performing a maximum of 64K, single precision (16 bits per word length), or a 32K, double precision (32 bits per word length) transform and possesses an adequate dynamic range. The frequencies for the proton (observing) channel and fluorine (lock) channel were derived from a Digilab 10-94 frequency synthesizer and a Digilab 400-2 pulser. Hexafluorobenzene in a 1-mm capillary served as an external reference as well as provided the signal for the ^{19}F lock. The internal reference was tetramethylammonium chloride (TMA).[25] The sample temperature was 30.5°. The 220-MHz nmr spectra were recorded using the Varian HRSC-1X 220-MHz superconducting system in the continuous wave mode.

The ^{31}P nmr spectra were examined on a Varian HA-100D spec-

(1) Presented in part: (a) R. H. Sarma and R. J. Mynott, International Symposium on the Conformation of Biological Molecules and Polymers, Jerusalem, Israel, April 1972; (b) R. H. Sarma and R. J. Mynott, *J. Chem. Soc., Chem. Commun.*, 975 (1972).
(2) W. L. Meyer, H. R. Mahler, and R. H. Baker, Jr., *Biochim. Biophys. Acta*, **64**, 353 (1962).
(3) W. A. Catterall, D. P. Hollis, and C. F. Walter, *Biochemistry*, **8**, 4032 (1969).
(4) D. G. Cross and H. F. Fisher, *ibid.*, **8**, 1147 (1969).
(5) G. Czerlinski and F. Hommes, *Biochim. Biophys. Acta*, **79**, 46 (1964).
(6) D. P. Hollis, *Org. Magn. Resonance*, **1**, 305 (1969).
(7) J. Jacobus, *Biochemistry*, **10**, 161 (1971).
(8) O. Jardetzky and N. G. Wade-Jardetzky, *J. Biol. Chem.*, **241**, 85 (1966).
(9) N. O. Kaplan and R. H. Sarma in "Pyridine Nucleotide-Dependent Dehydrogenanses," H. Sund, Ed., Springer-Verlag, West Berlin, 1970, pp 39–56.
(10) D. W. Miles and D. W. Urry, *Biochemistry*, **7**, 2797 (1968).
(11) D. J. Patel, *Nature (London)*, **221**, 1239 (1969).
(12) R. H. Sarma, P. Dannies, and N. O. Kaplan, *Biochemistry*, **7**, 4359 (1968).
(13) R. H. Sarma and N. O. Kaplan, *J. Biol. Chem.*, **244**, 771 (1969).
(14) R. H. Sarma and N. O. Kaplan, *Biochem. Biophys. Res. Commun.*, **36**, 780 (1969).
(15) R. H. Sarma and N. O. Kaplan, *Biochemistry*, **9**, 539 (1970).
(16) R. H. Sarma and N. O. Kaplan, *ibid.*, **9**, 557 (1970).
(17) R. H. Sarma and N. O. Kaplan, *Proc. Nat. Acad. Sci. U. S.*, **67**, 1375 (1970).
(18) R. H. Sarma, M. Moore, and N. O. Kaplan, *Biochemistry*, **9**, 549 (1970).
(19) R. H. Sarma, V. Ross, and N. O. Kaplan, *ibid.*, **7**, 3052 (1968).
(20) S. F. Velick, *J. Biol. Chem.*, **233**, 1455 (1958).
(21) S. F. Velick in "Light and Life," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1961, p 108.
(22) P. Walter and N. O. Kaplan, *J. Biol. Chem.*, **238**, 2323 (1963).
(23) G. Weber, *Nature (London)*, **180**, 1409 (1957).
(24) N. Oppenheimer, L. Arnold, and N. O. Kaplan, *Proc. Nat. Acad. Sci. U. S.*, **68**, 3200 (1972).

(25) Under conditions of 8K or 16K input point transforms, the resolution was so good that the TMA peak appeared as a triplet due to spin coupling between ^1H and ^{14}N (J = 0.56 Hz). In fact homogeniety controls of the magnetic field were adjusted until TMA appeared as a triplet under the above conditions.

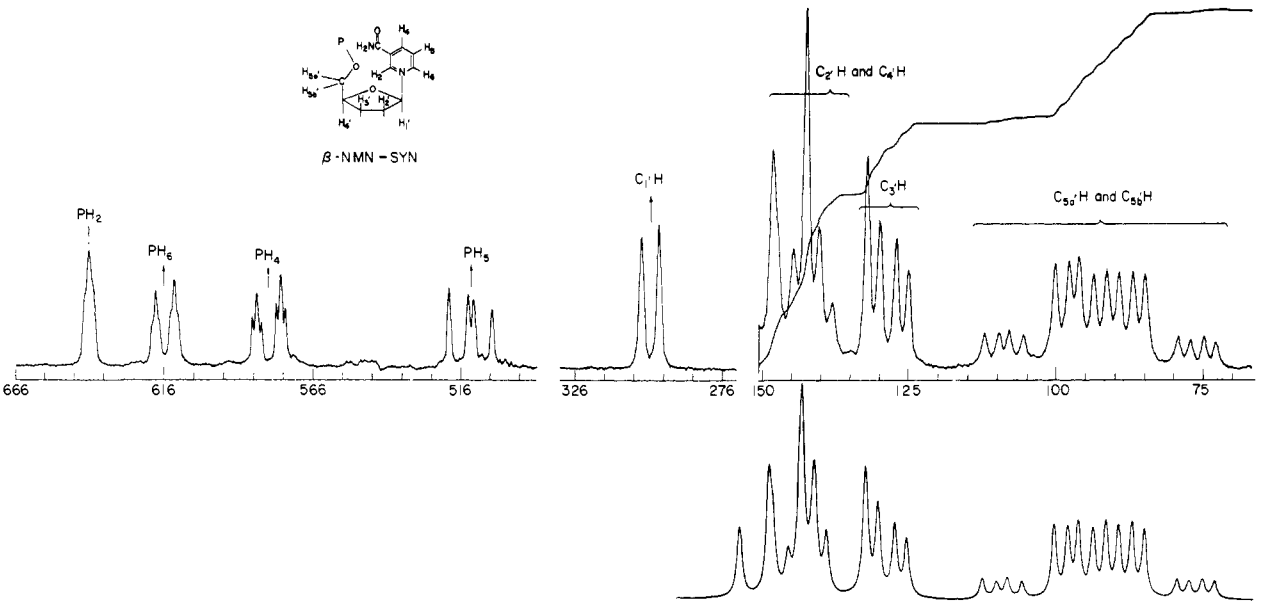


Figure 1. Fourier transformed ^1H nmr spectrum of a 0.1 M solution of β-NMN in 100% D_2O, pH 8.3; number of pulses = 500; pulse width = 40 μsec; sampling frequency = 2000 Hz per sec; number of input points = 8192; delay time = 0.0 sec; lock = ^{19}F of hexafluorobenzene in 1-mm capillary. The x scale was selected so that 1000 Hz will be displayed in at first 200 and then at 400 cm of chart paper. The letter P stands for the pyridine moiety. Chemical shifts are expressed in hertz downfield from $(CH_3)_4N^+Cl^-$ 100-MHz nmr system. The computer-simulated spectrum of the ribose region of β-NMN is shown in the bottom of Figure 1. One of the transitions in the simulation was obscured by the HDO resonance.

trometer at 40.48 MHz in continuous wave mode. The instrument has a double tuned probe capable of accepting ^{31}P and ^1H radiofrequencies. Decoupling of the protons of the ribose moieties was carried out by coherent irradiation in the proton range with an input derived from Hewlett-Packard Model 5105A frequency synthesizer and Hewlett-Packard Model 5110B synthesizer driver. The input was amplified to approximately 1 W by a Hewlett-Packard 230A signal generator power amplifier; the sample temperature was kept at 30.5° by cooling with a nitrogen stream. The spectrometer was operated in HA frequency sweep mode, locking onto a capillary containing the reference compound. This capillary was centered in the sample tube by a Wilmad Teflon microcell insert. Aqueous orthophosphoric acid (85%) was employed as the reference. In those cases where the resonance of the sample was close to phosphoric acid, P_4O_6 was used as the reference. The signal to noise ratios were enhanced on a Varian C-1024 computer of average transients (CAT). The internal sweep oscillator was replaced with an external voltage controlled generator, Hewlett-Packard 3310A, using a ramp voltage derived from the CAT.

The spectra were analyzed by the computer program LAOCN III which gave the line positions and intensities. The initial parameters for the simulation were derived from the 100- and 220-MHz spectra. Spectra were obtained in the concentration range of 0.0025–0.1 M in the Fourier mode. Even though at 0.1 M level concentrations one can obtain continuous wave spectra, we found the 8K and 16K fast Fourier transformed spectra to be very much superior, since the fine couplings were discernible. For a solution of 0.1 M using about 500 pulses (pulse width 40 μsec) and 8K transform, considerable increases in signal to noise ratios were obtained in very short time, greatly helping the spectrum analysis as the spectrum envelopes were then very clearly defined.

The mononucleotides were commercial preparations of adequate purity and were used without further purification. The samples were lyophilized two times from 99.8% D_2O and the spectra were taken in commercial "100%" D_2O. pH measurements were made before and after the run. The typical concentration of the internal standard TMA was 0.004 M for 0.1 M solution of nucleotide and 0.00004 M for a 0.001 M nucleotide. The pH reported are pH meter readings from a Leeds and Northrop or a Fisher Accument Model 320 pH meter. ^{31}P measurements were made in H_2O solutions.

Results and Discussion

(a) Assignments of Resonances in β-NMN (I). Assignments of the resonances of the nicotinamide moiety and of $C_1'H$ have been made in the past by Jardetzky and Wade-Jardetzky[8] as well as Sarma and Kaplan.[14] These assignments were used in the present work and verified by homonuclear decoupling in the continuous wave mode. They are indicated in Figure 1. However, the earlier investigators[8,14–16] have failed to observe the fine details which now we have been able to discern in 8K Fourier transformed spectra (Figure 1). Thus the resonance of the pyridine C_4H is split into a well-resolved doublet of triplets, due to coupling to pyridine C_5H and nearly equal weak coupling to pyridine C_6H and C_2H. The pyridine C_5H is a doublet of doublets due to coupling to pyridine C_4H and C_6H, but $^5J_{2-5}$ is too small to detect; pyridine C_6H is split into a doublet, J = 6.3 Hz, by C_5H. The signal is less well resolved than that of C_4H but appears to be split into triplets, due to coupling to C_2H and C_4H. The poorer resolution may arise from further weak splitting due to $C_1'H$ or to the adjacent nitrogen, or both. The signal due to pyridine C_2H has indications of fine structure but no well-defined features.

In the ribose region, $C_1'H$ is a doublet at 300.4 Hz to lower field than TMA with no indications of fine structure. The remaining ribose signals were assigned as follows. The 16 peaks at highest field (Figure 1) were assigned to the $C_{5a'}$ and $C_{5b'}$ protons of β-NMN. Evidence for this is that these transitions move as a group as the pH is reduced from 9 to 2 and that the shift of this group is *ca.* 12 Hz, by far the largest change found on varying the pH of any of the ribose protons. This would be expected if the phosphate group were adjacent. Furthermore, this group of lines appears to arise from two protons with a coupling constant of 12.1 Hz, consistent only with being geminal coupling. A satisfactory preliminary analysis of these lines can be made by considering them to arise from the AB region of an ABMX system (5a′ and 5b′ being A and B, $C_4'H$ = M, ^{31}P = X) which consist of four ab subspectra.

Table I. Chemical Shifts and Coupling Constants of β-NMN and β-NMNH, 0.1 *M* Solution in 100.0% D_2O.[a] Data from the 100-MHz Spectra

Nucleotides	Proton designation	Chemical shifts, δ, Hz			Nuclei coupled	Coupling constants, J, Hz	
		pH 8.3	pH 4.0	Δδ pH 8.3 → 4.0		pH 8.3	pH 4.0
β-NMN	H_2	640.9	628.7	12.2	J_{2-4}	1.5	1.5
	H_4	580.9	581.2	−0.3	J_{2-5}	n.d.	n.d.
	H_5	512.6	512.6	0.0	J_{2-6}	n.d.	n.d.
	H_6	615.6	610.9	4.7	J_{4-5}	8.2	8.0
	$H_{1'}$	300.4	304.4	−4.0	J_{4-6}	1.5	1.5
	$H_{2'}$	148.1	139.0	9.1	J_{5-6}	6.3	6.3
	$H_{3'}$	129.2	127.0	2.2	$J_{1'-2'}$	5.7	5.2
	$H_{4'}$	142.0	147.9	−5.9	$J_{2'-3'}$	5.0	5.0
	$H_{5a'}$	101.3	112.8	−11.4	$J_{3'-4'}$	2.1	2.6
	$H_{5b'}$	84.2	97.3	−13.1	$J_{4'-5a'}$	2.6	2.4
					$J_{4'-5b'}$	2.0	2.3
					$J_{5a'-^{31}P}$	4.2	4.4
					$J_{5b'-^{31}P}$	4.4	5.1
					$J_{5a'-5b'}$	12.1	12.1
β-NMNH[b]	H_2	397.5	396.1	1.4	J_{2-6}	1.7	1.6
	H_6	304.6	300.2	4.4	J_{5-6}	8.2	8.1
	H_5	184.3	183.7	0.6	J_{4-6}	1.6	1.8
	$(H_4)_2$	−11.7	−12.0	0.3	J_{4-5}	3.5	3.4
	$H_{1'}$	170.4	171.9	−1.5	J_{2-5}	Not resolved	
	$H_{2'}$	115.1	109.3	3.8	J_{2-4}	Not resolved	
	$H_{3'}$	106.4	104.7	1.7	$J_{1'-2'}$	6.8	6.8
	$H_{4'}$	90.0	92.2	−2.2	$J_{2'-3'}$	5.4	5.4
	$H_{5a'}$	67.5	79.5	−12.0	$J_{3'-4'}$	1.9[c]	1.8
	$H_{5b'}$[d]	67.5	79.5	−12.0	$J_{4'-5a'}$, $J_{4'-5b'}$[d]	4.0	3.0
					$J_{5a'-^{31}P}$[d], $J_{5b'-^{31}P}$[d]	4.8	5.1

[a] The chemical shifts are accurate to at least 0.2 Hz, the coupling constants to 0.1 Hz. For β-NMNH (low pH), chemical shifts are accurate to 0.3 Hz, coupling constants to 0.2 Hz. [b] pH values were 8.1 and 5.0. [c] From 220-MHz nmr spectrum. [d] No difference in chemical shifts observable between $C_{5a'}$ and $C_{5b'}$ protons even at 220 MHz.

This shows that $J_{4'-5a'}$ and $J_{4'-5b'}$ have the same sign and also that $J_{5a'-^{31}P}$ and $J_{5b'-^{31}P}$ have the same sign. No attempt was made to say which of the 5′ protons has the higher chemical shift. In completing the analysis, the value of $J_{1'-2'}$ was obtained from the doublet of $C_{1'}H$, while those of $J_{4'-5a'}$ and $J_{4'-5b'}$ were obtained from the 5′ spectra as described above. Trial calculations and computer simulations (Figure 1, bottom) then allowed assignment of the chemical shifts of $C_{2'}H$, $C_{3'}H$, and $C_{4'}H$ and values to $J_{2'-3'}$ and $J_{3'-4'}$. These were confirmed by comparison with the 220-MHz spectrum (Figure 2). The chemical shifts and coupling constants for β-NMN are compiled in Table I.

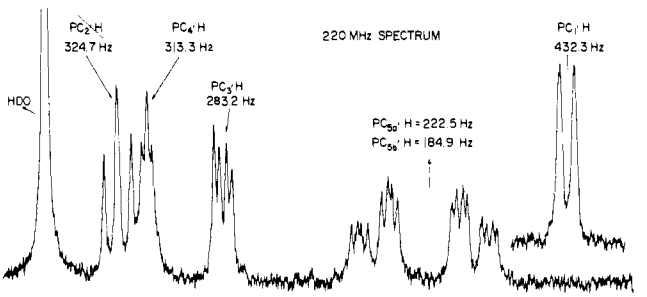


Figure 2. Continuous wave 220-MHz ^1H nmr spectrum of the ribose region of β-NMN.

(b) Assignment of the Resonances in β-NMNH (II). The 100-MHz proton 8K Fourier transformed spectrum of β-NMNH is illustrated in Figure 3; the assignments are also indicated. The pyridine C_4H_A and C_4H_B can be assigned by inspection and from the coupling pattern. There was no detectable inequivalence between C_4H_A and C_4H_B at 100 or at 220 MHz. The signal at 304.6 Hz to lower field than TMA was originally assigned to $C_{1'}H$ by Jardetzky and Wade-Jardetzky[8] and by Sarma and Kaplan.[16] Similar assignments in the reduced dinucleotides were made by various investigators.[2,3,8,14,16] Recent homonuclear decoupling experiments in this laboratory, as well as by Oppenheimer, *et al.*,[24] show that this is a wrong assignment. We assign now the resonance at 304.6 Hz to C_6H. Moreover, the fine coupling pattern of this resonance which is clear in the 8K Fourier transformed spectrum (Figure 3), a doublet of doublets of triplets, arising from coupling to C_5H, C_2H, and C_4H_A and C_4H_B, respectively, fits this assignment. The coupling pattern of C_2H and C_5H, including the long-range coupling, is consistent with the assignments given in Figure 3.

The 100-MHz spectrum does not permit assignments of the ribose protons except for the $C_{1'}H$. The 220-MHz spectrum (inset, Figure 3) was near enough to first order to make assignments much easier. In the 220-MHz spectrum, the peaks due to $C_{2'}H$ can be picked out since $J_{1'-2'}$ is known from the $C_{1'}H$ resonance. The peak at ≃198.6 Hz, 220 MHz, to lower field than TMA must be due to $C_{4'}H$, as the coupling to $C_{3'}$, $C_{5a'}$, and $C_{5b'}$ protons will produce a broad signal. The remaining signals, due to the $C_{3'}$ and the two $C_{5'}$ protons, can be assigned on the basis of relative intensities. The final results were obtained by refining the computer simulated spectra at 100 and at 220 MHz. The chemical shifts and coupling constants for β-

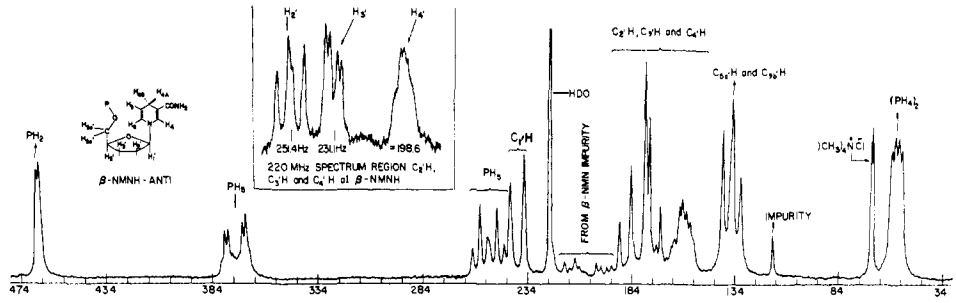


Figure 3. Fourier transformed ^1H nmr spectrum of a 0.1 M solution of β-NMNH, pH 8.1. Remaining details as in Figure 1. Note the position of $(CH_3)_4N^+Cl^-$ between 64 and 74 Hz. The zero point was selected by the computer in transforming the free induction decay to the frequency domain.

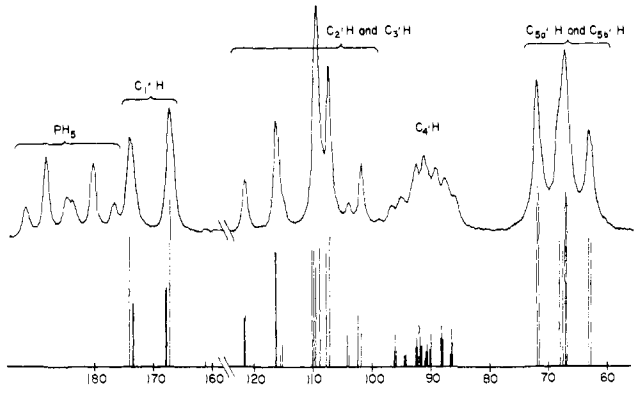


Figure 4. Top: 16K fast Fourier transformed ^1H nmr spectrum of β-NMNH. Only the ribose region and pyridine C_5H is shown. Bottom: the computer simulated spectrum of the ribose region of β-NMNH. Line shape simulation indicates a long range $J_{4'P} \simeq 1.8$ Hz.

NMNH are compiled in Table I. The computer simulated spectrum is shown in Figure 4.

(c) Concentration Dependence of the Chemical Shifts of β-NMN and β-NMNH. The Fourier transformed ^1H nmr spectra of β-NMN were recorded at the following concentrations: 0.4, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025 M. The concentration had no effect on either the coupling constants or the chemical shifts of the various protons at concentration levels below 0.1 M. In the range of 0.4–0.1 M, the pyridine C_2H showed a shift of 1.1 Hz to lower fields at low concentration and pyridine C_6H showed a shift of 1.9 Hz to higher fields. These shifts are very small for the range of concentrations examined and may have no significance. In the case of β-NMNH, examination of spectra at 0.1 and 0.01 M does not show any shifts larger than 1 Hz. The data enable one to conclude that β-NMN and β-NMNH do not undergo significant intermolecular association. In the case of β-NMNH, however, one should realize that even if intermolecular stacking occurs, the shifts would not be very large because the base does not possess as large a ring current as the pyridine moiety of β-NMN.

(d) Conformational Nomenclature for Pyridine Nucleotides. This is the first time an attempt is made to describe in *detail* the conformation of pyridine nucleotides and in order to make their descriptions compatible with the X-ray study of purine and pyrimidine nucleotides, the following nomenclature will be used.

(i) The Sundaralingam[26] definitions will be used to define the sense of torsion around $O_{5'}$–$C_{5'}$ (ϕ) and $C_{5'}$–$C_{4'}$ (ψ) bonds of the ribose.

(ii) The sugar conformation follows the proposal of Sundaralingam.[27,28] An envelope conformation is represented by E and the twist one by T. In the envelope description, four atoms of the ribose ring lie in a plane and the atom deviating from the plane is defined with respect to the $C_{5'}$. Thus, $C_{3'}$-endo = ^3E, *i.e.*, $C_{3'}$ lies on the same side as $C_{5'}$. In the same way $C_{3'}$-exo = $_3E$ means the $C_{3'}$ deviates from the plane containing $C_{1'}$, $C_{2'}$, $C_{4'}$, and $O_{1'}$ and on the opposite side of $C_{5'}$. The twist conformation $C_{2'}$-endo–$C_{3'}$-exo is represented as $^2T_3$ when the $C_{2'}$ atom shows a major puckering and the $C_{3'}$ a minor puckering from the plane of $C_{1'}$, $O_{1'}$, and $C_{4'}$. (See Figure 8a and 8b in Sundaralingam.[26])

(iii) The torsional diastereomers[29] constrained to the nicotinamide–ribose glycosidic linkage are described according to Donohue and Trueblood[30] nomenclature for purine and pyrimidine nucleotides. However, definitions of torsion angle (χ) follow the Sundaralingam description.[26]

(iv) Recourse to Newman projections will be made to describe the various torsional diastereomers.

(e) Torsional Isomers around the $O_{5'}$–$C_{5'}$ Bond. This rotation is denoted by ϕ. The value of $\phi = 0$ when the $C_{5'}$–$C_{4'}$ bond is cis planar to P–$O_{5'}$ bond with respect to rotation around the $O_{5'}$–$C_{5'}$ bond. The angle ϕ can assume values 0–360° for a right-handed rotation.[26] Lakshminarayanan and Sasisekharan[31] have calculated the allowed conformations around ϕ in ribose–phosphate units by plotting the variations of total potential energy (van der Waals nonbonded energy + electrostatic energy + torsional energy) as a function of ϕ and have concluded that there are three minima which correspond to the Sundaralingam sense of rotation[26] at 60, 180, and 300°, the deepest minima being at ϕ = 180°. These three possible torsional isomers constrained to $O_{5'}$–$C_{5'}$ can be represented by traditional Newman projections as in III, IV, and V. From the X-ray study of a large number of purine and pyrimidine nucleotides, Sundaralingam[26] concludes that the preferred conformation of 5′ nucleotides is gauche–gauche

(26) M. Sundaralingam, *Biopolymers*, 7, 821 (1969).

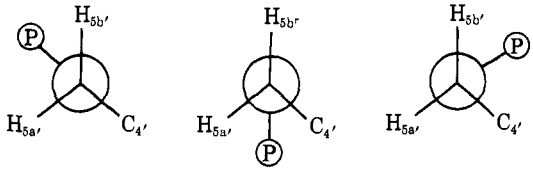
(27) M. Sundaralingam, *J. Amer. Chem. Soc.*, 87, 599 (1965).

(28) M. Sundaralingam, S. T. Rao, and J. Abola, *ibid.*, 93, 9055 (1971).

(29) K. Mislow, "Introduction to Stereochemistry," W. A. Benjamin, New York, N. Y., 1966. According to Mislow the term diastereomer is used to describe all stereoisomers which are non enantiomers.

(30) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960).

(31) A. V. Lakshminarayanan and V. Sasisekharan, *Biopolymers*, 8, 489 (1969).



gauche–gauche $\phi = 180°$ III | gauche–trans $\phi = 60°$ IV | trans–gauche $\phi = 300°$ V

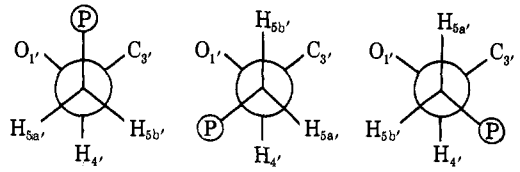
III which is in good agreement with the calculations of Lakshminarayanan and Sasisekharan.[31]

In order to determine whether this is also true of pyridine nucleotides, we have analyzed the coupling constants for coupling between the ^{31}P and the $C_{5'}$ protons in β-NMN and β-NMNH. Molecules containing the system H–C–O–P have been reported to show an angular dependence of spin–spin coupling similar to that found in H–C–C–H systems. The gauche coupling is in the range of 3 ± 2 Hz and the trans in the range of 25 ± 3 Hz (Kainosho, *et al.*,[32] Hall and Malcolm,[33] Tsuboi, *et al.*,[34,35] Schleich, *et al.*,[36] and Smith, *et al.*[37]). The observed values for β-NMN at pH 8 are $J_{5a'-P}$ = 4.2 and $J_{5b'-P}$ = 4.4 Hz. The corresponding values for β-NMNH are 4.0 and 4.8 Hz, respectively (Table I). Two features to be noted: (i) the coupling of the two 5′ protons to the phosphorus is nearly equal and (ii) the magnitude of these two couplings clearly lies in the gauche range. Thus, the data very strongly suggest that in β-NMN and β-NMNH, the torsional isomer constrained to the $O_{5'}$–$C_{5'}$ bond is gauche–gauche III ($\phi \simeq 180°$). Insofar as the differences in total potential energy between the forms III, IV, and V are less than 0.5 kcal,[31] one may expect a rapid exchange among these conformers. Should such an exchange exist, the population of the gauche–gauche form would be significantly high. The data in Table I show that the $J_{5'-P}$ have a slight pH dependence. This may originate from protonation of the phosphate group or small shift in conformer population or both.

(f) Torsional Diastereomers Constrained to the $C_{5'}$–$C_{4'}$ Bond. Sundaralingam[26] denotes this rotation angle by ψ. The value of $\psi = 0$ when the $C_{4'}$–$C_{3'}$ bond is cis planar to the $O_{5'}$–$C_{5'}$ bond with respect to rotation around the $C_{5'}$–$C_{4'}$ bond,[26] *i.e.*, fully eclipsed. The Sundaralingam[26] sense of rotation will be used in the following discussion. It has been recognized by Sundaralingam[26] as well as by Shefter and Trueblood[38] that there are three allowed ranges for ψ. These are 35–65, 160–190, and 295–325°. For purine and pyrimidine nucleotides the most preferred range is reported to be[26] 35–60°, the least preferred being the 295–325° range.

(32) M. Kainosho, A. Nakamura, and M. Tsuboi, *Bull. Chem. Soc. Jap.*, **42**, 1713 (1969).
(33) L. D. Hall and R. B. Malcolm, *Chem. Ind. (London)*, 92 (1968).
(34) M. Tsuboi, M. Kainosho, and A. Nakamura, in "Recent Developments of Magnetic Resonance in Biological Systems," S. Fujiwara and L. H. Piette, Ed., Hirokawa Publishing Co., Tokyo, 1969, p 43.
(35) M. Tsuboi, S. Takahashi, Y. Kyogoku, H. Hayatsu, T. Ukita, and M. Kainosho, *Science*, **166**, 1504 (1969).
(36) T. Schleich, B. J. Blackburn, R. D. Lapper, and I. C. P. Smith, *Biochemistry*, **11**, 137 (1972).
(37) I. C. P. Smith, H. H. Mantsch, R. D. Lapper, R. Deslauriers, and T. Schleich, Proceedings of the International Symposium on the Conformation of Biological Molecules and Polymers, Jerusalem, Israel, April 1972, in press.
(38) E. Shefter and K. N. Trueblood, *Acta Crystallogr.*, **18**, 1067 (1965).

The potential energy calculations[31] also show that there are three comparable minima occurring around ψ = 60, 180, and 300°. The Newman projections of these three minima are shown in VI, VII, and VIII.



gauche–gauche $\psi = 60°$ VI | gauche–trans $\psi = 180°$ VII | trans–gauche $\psi = 300°$ VIII

From extensive compilation of X-ray data, Sundaralingam[26] concludes that 5′ nucleotides have in general ψ values in the gauche–gauche VI range. In order to determine the torsional diastereomers constrained to the $C_{5'}$–$C_{4'}$ bond of pyridine mononucleotides, we have obtained the coupling constants for coupling between the $C_{4'}$ and $C_{5a'}$ protons as well as $C_{4'}$ and $C_{5b'}$ protons of β-NMN and β-NMNH (Table I). Hruska, *et al.*,[39] and Blackburn, *et al.*,[40] have developed equations to calculate the populations of conformers VI, VII, and VIII from the vicinal $J_{4'-5'}$ coupling constants. Among the assumptions made in these calculations it is considered that the rotamers interconvert rapidly, that the observed coupling constants are weighted averages, and that the Karplus equation is valid. The results of such a calculation for β-NMN and β-NMNH are shown in Table II. The results of the calculation reveal that in

Table II. Calculated Population of the Three Classical 60° Staggered Torsional Isomers around $C_{4'}$–$C_{5'}$ Bond in Pyridine Mononucleotides

		Percentages of—		
Nucleotide	pH	Gauche–gauche	Gauche–trans	Trans–gauche
β-NMN	8.3	93	0 or 7[a]	7 or 0[a]
	4.0	91	4–5	4–5
β-NMNH	8.1	50	25	25
	5.0	75	12–13	12–13

[a] No attempt was made to assign the 5a′ or 5b′ protons to either the higher or the lower chemical shift of the 5′ region.

β-NMN, the gauche–gauche VI torsional isomer significantly predominates (93%) over the other isomers, an observation compatible with the predictions of Sundaralingam.[26] The small difference one observes in population between the monoanion (pH 4.0) and dianion (pH 8.3) species of β-NMN may be due to an actual shift in population or due to a differential effect of charge on $J_{4'-5a'}$ and $J_{4'-5b'}$. Note that $H_{5a'}$ and $H_{5b'}$ chemical shifts are shifted to lower fields by different magnitudes (11.4 and 13.2 Hz, respectively, Table I) as the phosphate changes from dianion to monoanion.

Nuclear magnetic resonance data from β-NMNH clearly show that this reduced mononucleotide behaves quite differently from β-NMN. Thus, no differences in chemical shifts *were detected* between the geminal $C_{5'}$ hydrogens of β-NMNH whereas in β-NMN the cor-

(39) F. E. Hruska, A. A. Grey, and I. C. P. Smith, *J. Amer. Chem. Soc.*, **92**, 4088 (1970).
(40) B. J. Blackburn, A. A. Grey, I. C. P. Smith, and F. E. Hruska, *Can. J. Chem.*, **48**, 2866 (1970).

responding protons show a difference in chemical shifts of 17.1 Hz (Table I). This *does not* mean that no difference exists in chemical shifts between 5a′ and 5b′ protons of β-NMNH or that a rapid exchange among an equally populated gauche–gauche VI, gauche–trans VII, and trans–gauche VIII species takes place. In fact, calculations of rotamer population from the $J_{4'-5'}$ coupling constants (Table II) indicate that the gauche–gauche form is relatively predominant (50% at pH 8), but *the population is much less than that of the same torsional isomer in β-NMN.*

The theoretical calculations of Lakshminarayanan and Sasisekharan[31] have correlated the torsion around $C_{5'}$–$C_{4'}$ to various ribose puckerings. In the cases of a ^{3}E and $_2$E, the preferred torsional isomer around $C_{5'}$–$C_{4'}$ is gauche–gauche VI. The ^{2}E and $_3$E ribose tends to favor the trans–gauche VIII over the gauche–gauche VI. In the cases of β-NMN and β-NMNH, the predominant population is gauche–gauche, but there is a definite shift in the population of the various isomers as the mononucleotide become reduced. If one can accept the validity of the calculations[31] one may conclude that when β-NMN is reduced to β-NMNH, the time-averaged ribose conformation shifts from ^{3}E and $_2$E toward ^{2}E and $_3$E.

(g) Conformation of the D-Ribose Moiety. A partial search of literature[26–28,41–50] shows that the following conformations for the sugar ring have been encountered: ^{2}E, $_2$E, ^{3}E, $_3$E, ^{2}T$_3$, ^{3}T$_2$, ^{2}T$_1$, ^{0}T$_4$, and ^{3}T$_4$ in crystals (our list is not complete since no exhaustive search was made) indicating that the sugar ring indeed can assume various types of puckering. The following thoughts have been expressed by theoreticians, X-ray scientists, and nmr investigators on the conformation of ribose. (i) The five-membered ring of the ribose itself may be considered to be a rigid unit.[31,51] (ii) Conformational interconversions among the various sugar conformers (^{2}T$_3 \rightleftharpoons {}^3T_2$) are energetically equivalent to normal hydrogen bond energies or crystal packing forces.[26] (iii) In solution, the ribose moiety of nucleosides and nucleotides may exist as a rapidly exchanging mixture of ^{2}E, $_3$E, $_2$E, and ^{3}E.[36,39] (iv) Smith and Jardetzky[52] have predicted 20 possible bucklings for D-ribose and have provided a table of coupling constants theoretically relating them to each of the 20 individual puckerings.

The presently observed coupling constants $J_{1'-2'}$, $J_{2'-3'}$, and $J_{3'-4'}$ of the pyridine nucleotides (Table I) will be analyzed *vis-à-vis* the above comments. In the past, Sarma and Kaplan[16] have analyzed ribose conformation from one or two coupling constants which were available in 1969 using Smith and Jardetzky's table[52] and have concluded that the ribose adjacent to the pyridine moiety is $_3$T^4 (!) and ^{2}T$_3$, respectively, in the oxidized and reduced coenzymes. In the last three years a large number of coupling constants have become available and it is now possible to determine all the relevent coupling constants (not just $J_{1'-2'}$) and it is becoming increasingly clear that Karplus's equation may not be able to predict the exact puckering of the ribose. Hruska, *et al.*,[39] and Schleich, *et al.*,[36] have published tables relating coupling constants to ribose conformation. Table III contains the theoretically predicted coupling constants from Hruska, *et al.*,[39] Schleich, *et al.*,[36] and Smith and Jardetzky[52] for two conformations of D-ribose. An inspection of the table and observation of the discrepancies that exist among the J values clearly illustrate the difficulty of rationally using Karplus's equation to distinguish the *small differences that exist in dihedral angles* among the 20 possible conformations of ribose.[53] Theoretically, one could use a Karplus type equation for such purposes, provided one develops a new Karplus equation for D-ribose which takes into consideration the effect of the following factors on the relevent coupling constants: (i) the nature of the substituents on the 1′ (*e.g.*, the base), 2′ (*e.g.*, H, OH, or OPO_3), 3′ (*e.g.*, OPO_3 or OH), and 4′ (*e.g.*, CH_2OH or CH_2OPO_3), (ii) the torsion angles ψ and χ, (iii) state of hybridization of carbon bearing equatorial –OH,[27] and (iv) the dynamics of the five-membered ring. No such refined Karplus curve is available at present. We understand that Professor Pullman's group (personal communication to R. H. S. from Professor Pullman) is actively engaged in deriving a refined Karplus type relation from the wave equation. We do not believe that the Karplus equation as is used today could distinguish between differences that exist between conformations such as ^{2}E, $_3$E, ^{2}T$_3$, etc., because they involve small changes in dihedral angles which lie within the uncertainty of the equation. Recently Hruska[54] has suggested that the limitations inherent in Karplus's equation are such that it can be realistically used to distinguish between only two conformations, *viz.* ^{3}E and ^{2}E. We agree with Hruska's[54] approach and found the conformational map in

Table III. Ribose Conformation and Estimated Dihedral Angles and Spin–Spin Coupling Constants

Conformation	$\phi_{1'2'}$, deg	$J_{1',2'}$, Hz	$\phi_{2'3'}$, deg	$J_{2'3'}$, Hz	$\phi_{3'4'}$, deg	$J_{3'4'}$, Hz	Ref
$C_{2'}$-endo	155	8.3	35	5.9	101	0	36
	165	9.5	45	4.3	105	0.4	39
	165	8.6	45	3.9	105	0.4	52
$C_{3'}$-exo	140	5.8	28	7.0	95	0	36
	145	6.7	40	5.0	100	0	39
	135	4.6	45	3.9	75	0.2	52

(41) D. W. Young, P. Tollin, and H. R. Wilson, *Acta Crystallogr., Sect. B*, **25**, 1423 (1969).
(42) D. G. Watson, D. J. Sutor, and P. Tollin, *Acta Crystallogr.*, **19**, 111 (1965).
(43) J. Konnert, I. L. Karle, and J. Karle, *Acta Crystallogr., Sect. B*, **26**, 770 (1970).
(44) W. Saenger and K. H. Sheit, *J. Mol. Biol.*, **50**, 153 (1970).
(45) G. H. Y. Lin, M. Sundaralingam, and S. K. Arora, *J. Amer. Chem. Soc.*, **93**, 1235 (1971).
(46) E. Shefter and T. I. Kalman, *Biochem. Biophys. Res. Commun.* **32**, 878 (1968).
(47) M. Sundaralingam and S. K. Arora, *Proc. Nat. Acad. Sci. U. S.*, **64**, 1021 (1969).
(48) S. T. Rao and M. Sundaralingam, *J. Amer. Chem. Soc.*, **92**, 4963 (1970).
(49) W. Saenger, D. Suck, and K. H. Scheit, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **5**, 262 (1969).
(50) D. C. Fries and M. Sundaralingam, *Acta Crystallogr., Sect. B*, **27**, 401 (1971).
(51) V. Sasisekharan, A. V. Lakshminarayanan, and G. N. Ramachandran in "Conformation of Biopolymers," Vol. 2, G. N. Ramachandran, Ed., Academic Press, New York, N. Y., 1967, p 641.
(52) M. Smith and C. D. Jardetzky, *J. Mol. Spectrosc.*, **28**, 70 (1968).
(53) The Karplus equation is quite valid to determine the conformer populations such as gauche–gauche, etc., around the C_4'–C_5' bond because the difference in dihedral angle involved is quite large.
(54) F. Hruska, Proceedings of the International Symposium on the Conformation of Biological Molecules and Polymers, Jerusalem, Israel, April 1972.

Hruska to be useful in arriving at a *qualitative* idea regarding the time-averaged conformation of the ribose moiety in pyridine nucleotides.

The observed $J_{1'-2'}$, $J_{2'-3'}$, and $J_{3'-4'}$ for β-NMN and β-NMNH (Table I) clearly indicate the lack of conformational purity and that in both molecules the ribose exists as an equilibrium mixture of ^2E and ^3E conformations, the population of the ^2E being larger for β-NMNH. The $^2E \rightleftharpoons {}^3E$ interconversion can take place through the Sundaralingam pseudorotational itinerary of the ribofuranose ring.[55] Such a shift in population is compatible with the observation that when β-NMN is reduced to form β-NMNH, the gauche–gauche torsional diastereomer around $C_{5'}$–$C_{4'}$ depopulates.[31] This was discussed in section f above. In the case of the oxidized and reduced dinucleotides, β-DPN and β-DPNH, Oppenheimer, *et al.*,[24] have determined the J values for the ribose adjacent to the pyridine moiety. Their values for β-DPN are: $J_{1'-2'}$ = 5.4, $J_{2'-3'}$ = 6.1, and $J_{3'-4'}$ = 2.3 Hz, the corresponding values for β-DPNH being $J_{1'-2'}$ = 7.4 and $J_{2'-3'}$ = 4.0 Hz. Oppenheimer, *et al.*,[24] continue to use the Smith–Jardetzky table[52] to predict ribose conformation from J values as has been used previously by Sarma and Kaplan[16] and have concluded that the ribose adjacent to the pyrdine moiety exists in a $_3T^4$ "configuration" in *both* oxidized and reduced coenzymes. In this paper, we have expressed our doubts regarding the validity of Smith–Jardetzky table[52] which assumes the ribose to be a rigid moiety and further assumes that the Karplus equation can distinguish the small differences in dihedral angles. These assumptions are unjustifiable and the prediction of the existence of a $_3T^4$ ribose, never reported by crystallographers, amply illustrates the failures in this approach. It is our belief that the coupling constants for β-DPN and β-DPNH quoted above indicate that the ribose adjacent to the pyridine moiety of both β-DPN and β-DPNH exists as an equilibrium mixture of ^2E and ^3E conformations, the population of the ^2E being larger for β-DPNH. The energy barrier for a $^2E \rightleftharpoons {}^3E$ interconversion is probably of the order of 2–3 kcal and can easily be offset by interaction with the solvent.

In addition to the coupling constants, one could also use the chemical shifts of the ribose protons to determine whether a change in the time-averaged conformation of ribose takes place as β-NMN is reduced to β-NMNH. In Table I we provide the chemical shifts of 1′, 2′, 3′, 4′, 5a′, and 5b′ protons of β-NMN and β-NMNH at pH values when the phosphate is monoanion and dianion (*vide infra*). The coupling constants for both mononucleotides suggest that the ribose conformation is independent of the state of ionization of phosphate. Examination of the chemical shifts of the ribose protons for the monoanion and dianion shows some interesting correlations. In both β-NMN and β-NMNH, as the molecule goes from *dianion to monoanion state*, the following changes are noted: (i) the two 5′ protons are deshielded, (ii) the 4′ and 1′ protons are deshielded, and (iii) the 3′ and 2′ protons are shielded.

The effect on the two 5′ protons is expected because they are juxtaposed to the phosphate. What is remarkable is that the 4′ and 1′ protons which are "exo" are deshielded and the 3′ and 2′ protons which are "endo" are shielded. The terms "exo" and "endo" are used to indicate whether the protons are on the opposite or on the same side as $C_{5'}$. This observation clearly establishes the angular dependence of the anisotropic shielding or deshielding originating from the phosphate group experimentally even though very little theoretical detail of such a dependence is known. Should the ribose moieties of β-NMN and β-NMNH possess different conformations, one would expect the ribose protons to experience differential anisotropic effects from the phosphate group. Inspection of the data in Table I illustrates that this is what we are indeed observing. Thus, in the case of β-NMN, the exo protons, 4′ and 1′, shift to lower fields by 6.0 and 4.0 Hz, respectively, as the molecule goes from dianion to monoanion. In β-NMNH, the same protons show only a shift of 2.1 and 1.2 Hz. The endo protons, 2′ and 3′, of β-NMN show shifts to higher fields by 2.8 and 8.7 Hz, respectively, whereas the same protons of β-NMNH experience shifts only by 1.6 and 4.0 Hz. It is our belief that these differences in the behavior of the ribose proton chemical shifts of β-NMN and β-NMNH originate because these two molecules have a different time-averaged conformation for their ribose phosphate backbones.

The observed time-averaged difference in the ribose and phosphate conformation between β-NMN and β-NMNH is also evident in the chemical shifts of the individual ribose protons in these two mononucleotides (Table I). It can be seen that the 1′, 2′, 3′, 4′, 5a′, and 5b′ ribose protons of β-NMNH move to higher fields by 130, 33, 23, 51, 33, and 16 Hz, respectively, compared to the same protons of β-NMN. The following three factors mainly account for this shift: (i) nicotinamide moiety of β-NMN is more electronegative than the dihydronicotinamide ring of β-NMNH; (ii) the ring current fields of nicotinamide moiety is much stronger than that of dihydronicotinamide ring; (iii) insofar as the time-averaged ribose and phosphate conformations are different in β-NMN and β-NMNH, the local anisotropic shielding and deshielding originating from the phosphate group, the amide group, and the other covalent bonds and groups will affect the ribose proton chemical shifts differently in β-NMN and β-NMNH.

(h) Sugar–Base Torsion Angle. This angle is denoted by χ and has a value of zero[26] when the N_1–C_6 bond in the pyridine moiety is cis planar to the $O_{1'}$–$C_{1'}$ bond with respect to rotation about the $C_{1'}$–N bond. Clockwise rotation of the base[26] will give χ values from 0 to 360°. Donohue and Trueblood[30] have recognized two preferred ranges called anti and syn for the value of χ. In general, the region χ = 0–70° is the anti range and χ = 210–260° is the syn range. β-NMN in I is shown syn and β-NMNH in II is shown anti. The Newman projections of syn and anti conformations for β-NMN are shown in IX and X.

Haschemeyer and Rich,[56] Sundaralingam,[26] Lakshminarayanan and Sasisekharan,[57] and Prestegard and Chan[58] have attempted to correlate the sugar puckering

(55) M. Sundaralingam, Proceedings of the International symposium on the Conformation of Biological Molecules and Polymers, Jerusalem, Israel, April 1972.

(56) A. E. V. Haschemeyer and A. Rich, *J. Mol. Biol.*, 27, 369 (1967).

(57) A. V. Lakshminarayanan and V. Sasisekharan, *Biopolymers*, 8 475 (1969).

(58) J. H. Prestegard and S. I. Chan, *J. Amer. Chem. Soc.*, 91, 2843 (1969).

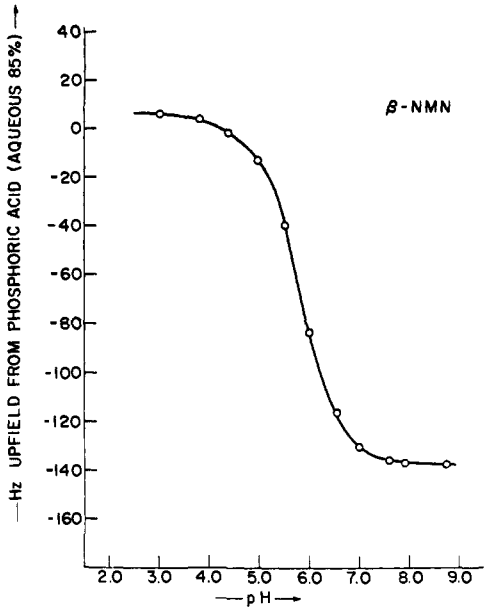
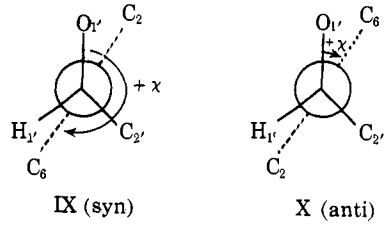


Figure 5. The variation of the ^{31}P chemical shifts of β-NMN as a function of pH. Concentration 0.1 *M* in 10^{-3} *M* EDTA. Chemical shifts are expressed in hertz downfield from external 85% H_3PO_4 capillary.



to the χ values. In view of the existence of some correlation between sugar pucker and the value of χ for purines and pyrimidines, we are interested in finding out whether any correlation exists for the oxidized and reduced pyridine mononucleotides which are shown in earlier discussion to possess different ribose–phosphate conformation on a time-averaged basis.

From stereomodels of β-NMN and β-NMNH (Metalo Glass, Inc, designed by Dr. Christine Jardetzky from X-ray data), we have measured the distance between the phosphorus atom and the protons of pyridine moiety in both the syn and anti forms. The data are given in Table IV. From these data it appears

Table IV. Through-Space Distance (Å) between the 5′-Phosphate Group (Center of Phosphorus) and the Various Protons in β-NMN[a]

Sugar–base torsion	Conformation of exocyclic CH_2OPO_3	Protons				
		H_2	H_6	H_4	H_5	$H_{1'}$
Syn	Gauche–gauche	2.8	6.8	6.0	7.3	6.0
	Gauche–trans	3.9	7.3	7.0	8.0	6.3
	Trans–gauche	4.1	8.0	8.0	9.1	6.6
Anti	Gauche–gauche	6.8	2.8	6.0	3.8	6.0
	Gauche–trans	7.3	3.9	7.0	5.1	6.1
	Trans–gauche	8.0	4.1	8.0	5.9	6.6

[a] In β-NMNH, the distances are the same except for the C_4H protons which are off by a few tenths of an Å.

that any perturbation of the phosphate group should affect significantly the pyridine C_2H resonance in the syn form; the pyridine C_6H should be affected in the anti form. If both forms are present, the relative extents

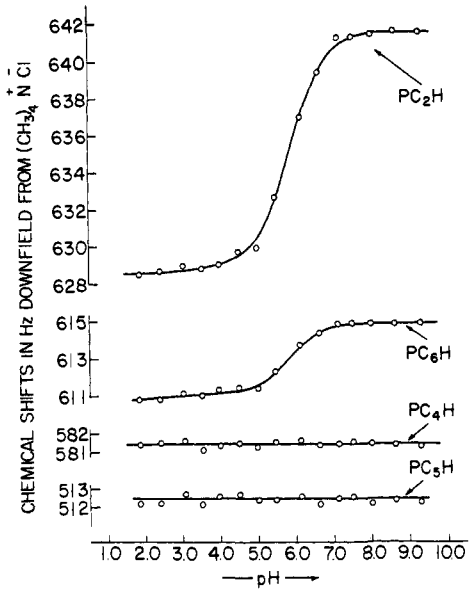


Figure 6. The variation of the ^1H chemical shifts of β-NMN as a function of pH; concentration 0.01 *M*. Spectra taken in the Fourier transform mode at 100 MHz. Chemical shifts are expressed in hertz downfield from $(CH_3)_4N^+Cl^-$.

of perturbation of C_2H and C_6H resonances will reflect their population differences, provided χ(anti) − χ(syn) = 180°.

Ionization of the 5′-phosphate group is a good perturbation.[59] The $^{31}P-\{^1H\}$ nmr chemical shift data for β-NMN as a function of pH is shown in Figure 5 and the p*K* for the phosphate group estimated from Figure 5 is 5.8. The effect of pH on the ^1H chemical shifts of β-NMN is shown in Figure 6 and the data clearly show that the pyridine C_2H chemical shift undergoes a significant perturbation as the phosphate group becomes protonated. This large perturbation of the pyridine C_2H chemical shift may be rationalized on the ground that β-NMN predominantly exists in the syn (I, IX) conformation. This conclusion is reasonable since the pyridine C_2H resonance would not otherwise be sensitive to perturbation in the vicinity of the phosphate group. Arguments similar to these have been used by Schweizer, *et al.*,[59] to conclude that 5′-AMP exists predominantly in the anti form, a conclusion verified by the Mn^{2+} ion perturbation studies of Chan and Nelson.[60] The data in Figure 6 also show that pyridine C_6H which resides approximately 6.8 Å from the phosphate group (Table IV) in the gauche–gauche–syn form has also been perturbed to a small extent, whereas pyridine C_4H and C_5H residing approximately at the same distance are not affected at all. From this one may conclude that β-NMN in deuterium oxide may not exist entirely in the syn form but as a fast equilibrium between the syn and anti forms (IX, X), syn being the predominant form. Examination of the pH data for β-NMNH[61] shows (Table I) that in the

(59) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Amer. Chem. Soc.*, 90,1042 (1968).

(60) S. I. Chan and J. H. Nelson, *ibid.*, 91, 168 (1969).

(61) We realize that β-NMNH slowly decomposes at pH values[62] below 7.0. In order to offset this problem, the experiments were done at 0.1 *M* and the spectra were taken immediately after adjusting the pH. During the course of the experiment we did not notice more than 2–3% decomposition. The use of Fourier transform techniques also allowed us to obtain a good spectrum very rapidly.

(62) R. M. Burton and N. O. Kaplan, *Arch. Biochem. Biophys.*, 101, 139 (1963).

reduced mononucleotide, the C_6H undergoes a larger perturbation (4.4 Hz) compared with C_2H (1.4 Hz). Following the arguments of Schweizer, *et al.*,[59] one may conclude that the preferred conformation around the glycosidic linkage may be anti (II) for β-NMNH. Such conclusion is compatible with the X-ray data on lactate dehydrogenase reduced coenzyme complex.[63]

The conclusion that in β-NMN and β-NMNH the preferred conformations may be syn and anti, respectively, does not imply that the same arrangement may exist in the parent oxidized and reduced dinucleotides, although there is no reason to believe that this may not be the case. Our continuing studies indicate that in β-NMN, β-DPN, and β-TPN, the preferred conformation of the nicotinamide moiety is likely to be syn and that in β-NMNH, β-DPNH, and β-TPNH to be anti. A preliminary communication on this subject has been published from this laboratory[64] and this will be discussed in detail in paper II of this series.

Acknowledgment. The authors are grateful for the support of this research to the following grants: (1) CA 12462-01 from the National Cancer Institute of the National Institutes of Health, (2) GB-28015 from the National Science Foundation, (3) GP-28061 from the National Science Foundation, and (4) 020-7212-A from the Research Foundation of the State University of New York. We thank Mr. Robert C. H. Lee for the line shape simulations and Dr. C. W. Haigh for providing the line shape programs.

(63) M. J. Adams, A. McPherson, Jr., M. G. Rossman, R. W. Schevitz, I. E. Smiley, and A. J. Wonacott in ref 9, pp 157–174.

(64) R. H. Sarma and R. J. Mynott, *J. Chem. Soc., Chem. Commun.*, 977 (1972).

Bromide Ion Probe Nuclear Magnetic Resonance Studies of Protein Conformation. Application to Methemoglobin

T. R. Collins, Z. Starčuk, A. H. Burr, and E. J. Wells*

Contribution from the Department of Biological Sciences and the Department of Chemistry, Simon Fraser University, Burnaby 2, British Columbia, Canada. *Received August 9, 1972*

Abstract: The application of the bromide ion as an nmr probe of protein conformation has been investigated both theoretically and experimentally. It is shown that the bromide ion has some advantages over the more commonly used chloride ion, the most important being its ability to yield values for the free-to-bound exchange rate constant of the probe itself, in addition to the correlation time for reorientation of the bound probe. It is shown experimentally that the exchange rates are fast, near the diffusion limit, and are sensitive to protein conformational effects near the binding site. Also, each of two equally abundant Br isotopes can be studied for an independent check of the conformational parameters. The bromide probe technique has been tested by measurements of signal-averaged ^{81}Br and ^{79}Br free induction decay constants. The reorientational correlation time for the bromide probe bound *via* mercury to the F9(93)β-sulfhydryl groups of horse methemoglobin decreased from $(1.48 \pm 0.04) \times 10^{-10}$ sec at pH 7.0 to $(0.8 \pm 0.2) \times 10^{-10}$ sec at pH 10, while the halide exchange rate decreased from $(3.27 \pm 0.06) \times 10^7$ to $(1.2 \pm 0.2) \times 10^7$ l. mol^{-1} sec^{-1}. These effects are interpreted in terms of conformational differences between acid and alkaline methemoglobin.

There has been considerable interest in the application of nuclear magnetic resonance (nmr) and other probes to the study of the conformation of proteins.[1] The nmr halide probe method[2] detects changes in the halide nuclear transverse relaxation time, T_2, as a result of halide exchange between symmetrically solvated free halide ion (T_2 long) and a probe site where the quadrupolar halide nucleus is bound tightly to a specific region within a protein (T_2 short). The resulting relaxation time is dependent on the relative concentrations of free and bound sites, and the physical environment of the bound halide. In its most common application, a solution of protein is prepared in aqueous sodium halide, and specific binding sites on the protein may be titrated with a metal such as mercury which binds only to these sites and which subsequently complexes halide from the solution. Stengle and Baldeschwieler have shown[2] that the method leads in principle to the determination of the number of metal binding sites on the protein and the correlation time for reorientation of the halogen–metal bond attached to the protein. A feature of the technique is that small concentrations of labeled protein (10^{-4}–10^{-6} *M*) can cause observable changes in the halogen nucleus T_2, due to a "chemical amplifier" effect originating in the very large difference in the field gradient present at the halogen nucleus in its free and bound sites.

In most halide-probe studies on proteins the number and availability of metal ion binding sites were determined by measurement of the ^{35}Cl steady-state nmr line width. Stengle and Baldeschwieler[3] titrated the sulfhydryl groups of equine and human hemoglobin with mercuric chloride, finding two sites in the native protein. A similar study by Ellis, *et al.*,[4] revealed one and

(1) G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, 1, 65 (1968).

(2) T. R. Stengle and J. D. Baldeschwieler, *Proc. Nat. Acad. Sci. U. S.*, 55, 1020 (1966).

(3) T. R. Stengle and J. D. Baldeschwieler, *J. Amer. Chem. Soc.*, 89, 3045 (1967).

(4) W. D. Ellis, H. B. Dunford, and J. S. Martin, *Can. J. Biochem.*, 47, 157 (1969).