# A Nuclear Magnetic Resonance Investigation of the Conformation of Nicotinamide Mononucleotide in Aqueous Solution

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**Abstract:** The conformation of a flexible molecule, nicotinamide mononucleotide (NMN), has been investigated by fitting lanthanide-induced pseudocontact shift and broadening data to a mixture of conformers. The lanthanide ion binds to the phosphate group of NMN to form a 1:1 complex and it is shown that the binding does not perturb the conformation of the molecule. The lanthanide-induced shifts (LIS) for the protons are pseudocontact in origin, whereas there is evidence for contact shifts for the $^{13}C$ nuclei in the nicotinamide ring. It is shown that it is not possible to define the conformation of NMN using *only* the lanthanide ion data. A conformational solution which fits the LIS and broadening data but which disagrees with spin–spin coupling constant data was obtained and rejected. By considering all the available conformational information it is found that the simplest solution which agrees with all the observed data has the molecules with "free rotation" about the O(5′)–P bond and a 2:1 mixture of syn C(2′)-endo and anti C(3′)-endo conformers.

For many years the most widespread NMR method of obtaining conformational information for small molecules in solution has relied on the relationships between three-bond coupling constants and dihedral angles. This approach gives detailed information only for the parts of the molecule which have the magnetic nuclei required for the appropriate three-bond coupling constants. Another method is to use nuclear Overhauser enhancement (NOE) measurements to obtain distance information between nuclei involved in a mutual dipolar relaxation process. Once again the conformational information is limited to fragments of the whole molecule because of the short-range nature of the interactions. Recently a method for conformational analysis based on lanthanide-induced shift (LIS) studies has been developed and this promises to provide a more general method for defining the full three-dimensional structure of a molecule in solution.[1] For a molecule of fixed conformation which binds a single lanthanide ion at a unique binding site the analysis of the observed pseudocontact shifts can define the conformational situation of magnetic nuclei within ~10 Å of the bound lanthanide. Thus the conformations of the nucleotides AMP and cAMP in aqueous solution have been deduced from LIS and broadening data assuming the presence of a unique conformation.[2,3]

These studies encouraged us to examine whether or not it is possible to use this method to study a compound known to exist in multiple conformations. We have chosen to study nicotinamide mononucleotide (NMN) for which there is substantial NMR evidence suggesting that the molecule does not exist as a single conformer in solution. For such a molecule it is not possible to use computer programs designed to search for a unique conformation which fits the observed lanthanide-induced pseudocontact shifts. Instead, the shift data are fitted to mixtures of conformations which are considered to be present from the results of other experimental NMR evidence such as coupling constants and nuclear Overhauser enhancement (NOE) experiments and from theoretical potential energy calculations. Using this approach we have attempted to obtain some quantitative estimation of the relative populations of the dominant conformers for NMN in solution.

## Experimental Section

NMN was obtained from Sigma Chemical Co. and used without further purification. The lanthanide ion solutions were made by dissolving the lanthanide oxide (obtained from Koch-Light Laboratories Ltd.) in $D_2O$ with a small excess of DCl and warming. The lanthanide ($Ln^{3+}$) concentration was determined by titration against ethylenediaminetetraacetic acid, EDTA.[4]

The $^1H$ and $^{13}C$ spectra were recorded on a Varian XL-100 spectrometer. The $^{13}C$ spectra were accumulated by the Fourier transform technique under conditions of complete proton decoupling. Solutions in deuterium oxide were examined and the deuterium in the solvent was used for field frequency locking the Varian XL-100 spectrometer.

The $^1H$ spectra were recorded at a constant pH and constant NMN concentration (pH 4.4, 4.0 m*M*) and the lanthanide ion concentration was varied. The $^{13}C$ experiments were carried out at 100 m*M* NMN (pH 4.4). It has been shown by dilution studies on the $^1H$ chemical shifts that there is no self-association of NMN at this concentration in $D_2O$.[5]

## Results

**A. [$^1$H]Lanthanide Shift Data.** The [$^1$H]lanthanide-induced shift (LIS) experiments were carried out at constant NMN (I) concentration (4.0 m*M*) and the lanthanide ion concentration was varied (0–10.0 m*M*). The H(5′) protons have by far the largest induced chemical shifts (2.57 ppm at pH 4.4 and 0.31 ppm at pH 1.7 at 10 m*M* $Eu^{3+}$ concentration) suggesting that the lanthanide ion is binding to the phosphate group. The pH dependence of the LIS for all the protons in NMN is consistent with the lanthanide binding strongly to the ionized phosphate group. Thus the shifts observed for the nicotinamide ring protons in the presence of $Eu^{3+}$ at pH 4.4 (where the NMN predominantly binds as the dianion) are all an order of magnitude greater than those observed at pH 1.7 (where the binding is to the monoanionic phosphate group) (see Figure 1).[6] These experiments were repeated using other lanthanide ions and in all cases the observed induced shifts indicated that the lanthanide ion binds strongly to the charged phosphate groups.

The titration curves at both pH values are monotonic and the ratios of the observed shifts are independent of the concentration of $Eu^{3+}$. The data can be fitted to binding curves for a 1:1 complex and the same "equilibrium constant"[7] is obtained for each proton (56 ± 5 l. $M^{-1}$). This shows that there is only one complexed form in solution.

The [$^1$H]lanthanide-induced shift data for all the protons in NMN are shown in Table I. The observed shifts have been corrected for diamagnetic shifts by subtracting the shifts due to $La^{3+}$ and are expressed relative to the shift observed for the H(5′) protons (taken as 100). The shift ratios are identical for $Eu^{3+}$, $Nd^{3+}$, and $Pr^{3+}$. It was not possible to follow all the ribose protons with $Nd^{3+}$ and $Pr^{3+}$ because

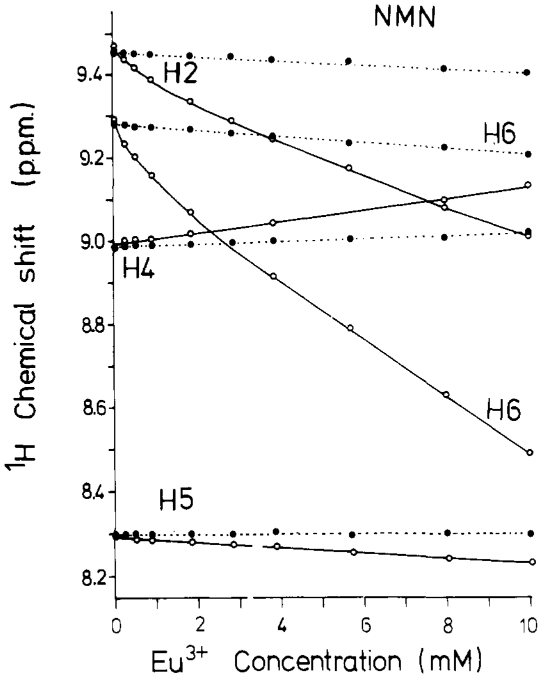


**Figure 1.** The $^1$H chemical shifts (ppm from DSS reference) of the nicotinamide ring protons in NMN as a function of Eu$^{3+}$ concentration: (●- - - -●) pH 1.7; (○——○) pH 4.4 (DSS is sodium 2,2-dimethyl-2-silapentane-5-sulfonate).

**Table I.** Ratios of the [$^1$H]Lanthanide-Induced Shifts Relative to the 5' Protons (NMN 4 m*M*, pH 4.4)[a]

| Proton | Eu$^{3+}$, 10 m*M* | Nd$^{3+}$, 10 m*M* | Pr$^{3+}$, 1 m*M* | Yb$^{3+}$, 2 m*M* |
|---|---|---|---|---|
| | | Nicotinamide Ring | | |
| 2 | 19 | 21 | 21 | 23 |
| 4 | −6 | −7 | −6 | −3 |
| 5 | 3 | 2 | 3 | 4 |
| 6 | 31 | 29 | 28 | 44[b] |
| | | Ribose Ring | | |
| 1' | 11 | 11 | 12 | 13 |
| 2' | 39 | | | 47 |
| 3' | 40 | | | 47 |
| 4' | 26 | | | 19 |
| 5' | 100 | 100 | 100 | 100 |

[a] The shift ratios are corrected for diamagnetic binding effects by subtraction of the shifts due to an equivalent concentration of La$^{3+}$. [b] In the presence of Yb$^{3+}$ the shift ratio of H(6) is slightly different from that observed with the other lanthanide ions. Yb$^{3+}$ is at the end of the lanthanide series and perhaps Lu$^{3+}$ should be used for correcting the observed shifts for this ion.

**Table II.** [$^{13}$C] Lanthanide Induced Shifts (ppm) of NMN[a]

| Carbon resonance[b] | Nd$^{3+}$, 25 m*M* | Eu$^{3+}$, 25 m*M* | Yb$^{3+}$, 5 m*M*[c] | La$^{3+}$, 25 m*M* |
|---|---|---|---|---|
| C(2) | −0.81 | 0.58 | 0.24 | 0.32 |
| C(3) | 1.07 | −1.47 | 0.49 | −0.18 |
| C(4) | −0.30 | 0.14 | 0.20 | −0.14 |
| C(5) | −0.05 | 0.11 | 0.13 | −0.03 |
| C(6) | −0.33 | 0.04 | −0.02 | 0.62 |
| C(1') | −0.26 | 0.05 | 0.0 | 0.14 |
| C(3') | 0.06 | −0.03 | −0.16 | −0.44 |
| C(2') | −0.02 | −0.06 | −0.13 | −0.09 |
| C(4') | −0.06 | −0.08 | −0.18 | −0.31 |
| C(5') | 0.56 | −0.69 | −0.88 | −0.12 |

[a] The paramagnetic ion shifts are corrected for the diamagnetic shifts produced by an equivalent concentration of La$^{3+}$. Positive shifts are downfield from an internal dioxane reference. The errors in the [$^{13}$C] lanthanide-induced shifts are large (±0.15 ppm) because of the severe line broadening (exchange broadening) resulting from the low dissociation rate constants for the tightly bound lanthanide–NMN dianion complexes. [b] Assignments of Birdsall and Feeney.[9] [c] At 25 m*M* Yb$^{3+}$ extensive line broadening was observed.

these lanthanide ions produce downfield shifts for the ribose protons placing the signals under the HOD peak. The good agreement of the proton shift ratios for several lanthanide ions shows that there is no contact contribution in the shifts.

**B. [$^{13}$C]Lanthanide Shift Data.** The lanthanide-induced shifts observed for the $^{13}$C signals of NMN (pH 4.4, 100 m*M*) are listed in Table II. The values have been corrected for the contribution to the shielding from binding diamagnetic lanthanide ions (La$^{3+}$). In contrast to the very small La$^{3+}$ induced shifts observed in the $^1$H spectra, large changes in the shielding of the $^{13}$C nuclei are obtained and these are sometimes larger than the effects of Nd$^{3+}$ or Eu$^{3+}$ [e.g., C(6), C(4'), and C(2')].

The proton spectra of these samples (with a ratio of NMN to Ln of 4:1) showed broadened signals but very small shifts (e.g., in the presence of Nd$^{3+}$ the nicotinamide ring protons were only shifted 0.1 ppm). Since the proton lanthanide shifts have been shown to be pseudocontact in nature one would expect the pseudocontact shifts of the $^{13}$C signals to be of the same order of magnitude. Some of the shifts measured for the nicotinamide ring $^{13}$C resonances are much too large to be pseudocontact [e.g., shifts due to Nd$^{3+}$, C(2) −0.81 and C(3) 1.07 ppm; shifts due to Eu$^{3+}$, C(2) 0.58 and C(3) −1.47 ppm] and must arise mainly from a contact mechanism.

If the nicotinamide ring $^{13}$C shifts are entirely contact in origin one would expect that there would be the same distribution of shifts around the ring for different lanthanide ions. Within the error of the measurements (±0.15 ppm on the broadened lines) there is reasonable agreement between Nd$^{3+}$ and Eu$^{3+}$ assuming the contact shifts are of opposite sign as has been shown in the recent paper of Dobson et al.[9] The shifts observed for C(2) and C(3) with Yb are smaller and in the same direction as the Eu shifts which also agree with previous results.[9]

Since the contact shift is a through-bond phenomenon, one can postulate that the contact shifts are introduced into the nicotinamide ring by way of an overlap of orbitals involving the lanthanide ion and the nicotinamide amide group. The largest effects are seen at the nicotinamide C(2) and C(3) atoms and the nicotinamide ring is too far from the phosphate group for the effect to have been transmitted through the ribose ring. Space-filling models show that an interaction involving the amide group and a lanthanide ion bound to the phosphate group can only occur if the nicotinamide ring is in the syn conformation. This interaction which gives rise to the contact shifts does not change the relative energies of the syn/anti conformers in the system (see section D). Thus the $^{13}$C LIS data show that there must be some syn conformer in solution but the shifts are not capable of quantitative interpretation.

**C. Gd$^{3+}$ Broadening Data.** Conformational information can also be obtained from considering the contributions to relaxation (as measured by line broadening) from bound Gd$^{3+}$ ions.[2] If it is assumed that the relaxation contribution from this paramagnetic ion is dominated by the dipolar interaction, then the contributions to the relaxation rates should be inversely proportional to $r_i^6$.

The broadening experiments were carried out at 4.00 m*M* NMN and 0–60 $\mu M$ Gd$^{3+}$. The qualitative order of broadening was 5' > 2 > 6 ~ 5 > 4 ~ 1'. The apparent line widths measured at high Gd$^{3+}$ concentrations relative to H(2) are given in Table IV. The chemical exchange spin decoupling of the multiplet proton signals, caused by Gd$^{3+}$, can give rise to errors in these measurements. However, by examining line broadening at high concentrations of $Gd^{3+}$, where the proton multiplet is completely decoupled, these errors can be minimized.

**D. The Binding of Lanthanide Ions to NMN Does Not Alter Its Conformation in Solution.** By monitoring conformationally dependent vicinal coupling constants in the presence of lanthanide ions one can detect changes in conformation. The proton coupling constants $J_{1'-2'}$, $J_{2'-3'}$, and $J_{4'-5'}$ do not vary (within 0.2 Hz) as the lanthanide concentration is changed over the range 0–10 m*M* at which concentration approximately 40% of the NMN molecules are complexed. This shows that the binding of lanthanide ions does not change the conformation of the ribose ring in NMN. In addition, the $J_{5'-P}$ coupling constants are unchanged in the presence of a 10 molar excess of $La^{3+}$ showing that the conformation of the P–O–C–H backbone is also unaffected by the presence of lanthanide ions.

From a consideration of the shift ratios at different lanthanide concentrations one can determine whether or not conformational changes accompany the binding of lanthanide ions. For example, if lanthanide ions changed the composition of the equilibrium mixture of syn and anti conformers (see ref 10) of the nicotinamide ring in NMN, this change would be reflected in the ratio of the shift observed for H(2) relative to that of H(6) at different lanthanide ion concentrations. This analysis assumes that the lanthanide ion binds to the phosphate group in *both* conformers under consideration. The ratio of lanthanide-induced shifts of H(2) to H(6) remains constant (within 5% over the total range of lanthanide ion concentration) thus showing that the binding of lanthanide ions has not changed the proportions of syn and anti conformers in solution.

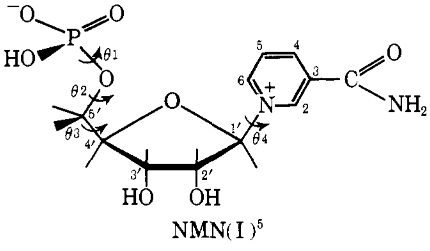
The constancy of the observed chemical shift ratios for the protons in NMN at different lanthanide ion concentrations indicates that the association constants for the binding of lanthanide ions to the different conformers are similar.

## Discussion

Our approach to the investigation of the conformation of NMN involves the collation of LIS and broadening data with conformational evidence obtained by other methods.

**A. Conformational Information Obtained from Coupling Constants and NOE Measurements.** NMN (I) is a flexible molecule with the possibility of rotation about four bonds ($\theta 1$–$\theta 4$);[10] in addition the ribose ring can exist in various puckered forms. NMR studies not involving paramagnetic probes can provide some conformational information for such molecules; there are well-established procedures relating three-bond coupling constants to dihedral angles in rigid molecules and to rotamer population in flexible molecules.

(i) **Torsion Angles $\theta 2$ and $\theta 3$.** From measurements of the $J_{5'a-P}$ and $J_{5'b-P}$ coupling constants it has been shown that about 90% of the molecules of NMN have the phosphorus atom gauche to both 5′ protons[5] ($\theta 2 = 360°$). Likewise, the coupling constants $J_{4'-5'a}$ and $J_{4'-5'b}$ have been measured[5,8] and indicate that more than 90% of the molecules have the 4′ proton gauche to both 5′ protons[5] ($\theta 3 = 240°$). Thus the phosphate backbone conformation of NMN is well defined.



NMN(I)[5]

(ii) **Torsion Angle $\theta 4$ (Syn and Anti).** The syn and anti conformations can exist over a range of glycosidic bond angles, $\theta 4$ varying by as much as 90° within a conformation. The chemical shift changes of the nicotinamide ring protons as a function of pH have been used to conclude that NMN exists entirely in the syn conformation[11] although these results are not inconsistent with a mixture of conformers. Semiempirical energy calculations indicate that only small energy differences exist between the syn and anti forms and one would therefore expect a mixture of conformers to exist.[12] Egan et al.[13] carried out nuclear Overhauser effect (NOE) experiments on NMN and concluded that the syn and anti conformers are nearly equally populated.

(iii) **Ribose Ring.** Although a five-membered ring is flexible, substitutions on the ribose ring make certain conformations more probable than others. Surveys of the nucleotide crystal structure literature showed that the commonly found ring pucker conformations fall into two classes, the S conformations (similar to C(2′)-endo) and the N conformations (similar to C(3′)-endo).[14] Recently Altona and Sundaralingam[15] have compiled a table of the ribose ring H–H coupling constants in nucleotides for different equilibrium mixtures of S and N sugar conformations. Their table was derived using data from nucleotides with uncharged bases (unlike NMN) and one would expect that the $J_{1'-2'}$ values in NMN to be smaller[16] than those considered by Altona and Sundaralingam. The coupling constants $J_{2'-3'}$ and $J_{3'-4'}$ will be almost unaffected by the charge on the base and we have used these to estimate the S and N equilibrium composition. The observed coupling constants are $J_{2'-3'} = 5.0$ Hz, $J_{3'-4'} = 2.6$ Hz and representative coupling constants predicted for an equilibrium mixture containing 70% S + 30% N are $J_{2'-3'} = 5.2$ Hz, $J_{3'-4'} = 3.0$ Hz.

Obviously, any acceptable conformational structures must be consistent with both the pseudocontact chemical shift data and the coupling constant information.

**B. Conformational Information from LIS and Broadening Data.** Before commencing the analysis of the data obtained using paramagnetic ion probes it is necessary to know (1) the stoichiometry of the complex, (2) that the binding of the lanthanide does not distort the conformation, and (3) that the lanthanide-induced shifts are of pseudocontact origin.

In the results section we provide evidence that only a 1:1 complex is formed and that neither the conformation of the ring nor the syn/anti ratio is altered when a lanthanide ion binds to NMN. Further, the LIS for each proton for different lanthanide ions have constant shift ratios, indicating the shifts are of pseudocontact origin. (In contrast, the $^{13}C$ shift ratios are not constant, suggesting a contact shift contribution to the LIS.)

The magnitude of the pseudocontact shift $\Delta\nu/\nu_0$ is given by the equation

$$\frac{\Delta\nu}{\nu_0} = D\left(\frac{3\cos^2\theta_i - 1}{r_i^3}\right) + D'\left(\frac{\sin 2\theta_i \cos^2\phi_i}{r_i^3}\right)$$

where $D$ and $D'$ are constants for each lanthanide complex at a given temperature, $\theta_i$ is the angle between the principal symmetry axis of the complexed ion and the distance vector $r_i$ between the ion and a specific nucleus and $\phi_i$ is the angle between the equatorial projection of $r_i$ and the vector from the lanthanide ion. If one has an axially symmetric complex the second term in the equation is zero. In such a case one can see that the ratios of the shifts observed for each nucleus in the molecule would be the same for all lanthanide ions. There is no evidence that the Ln–NMN complex is axially symmetric. It is not possible to have free rotation about an axis which goes through the lanthanide ion which would make a nonaxially symmetric complex behave as an effectively axially symmetric complex due to the averaging

Table III. The Four Families of Solutions from the BURLESK Program That Fit the $^1$H LIS Data within a Tolerance of ±10 [C(2')-Endo Sugar Conformation] [a]

| Family | $\theta 1$ | $\theta 2$ | $\theta 3$ | $\theta 4$ |
|---|---|---|---|---|
| A[b] | 90–100 | 10–20 | 110–120 | 210–240 |
| B[b] | 150 | 20 | 210–220 | 220–240 |
| C | 220–230 | 350–10 | 220–330 | 320–340 |
| D[b,c] | 220–230 | 340–10 | 320–330 | 160–190 |

[a] See ref 19. [b] These anti solutions may be ruled out on the basis of the $^{13}$C LIS results. [c] The solution obtained for the $^1$H LIS for AMP, pH 1, was $\theta 1 = 190°$, $\theta 2 = 354°$, $\theta 3 = 320°$, $\theta 4 = 194°$.[2]

Table IV. Observed and Calculated Shift and Broadening Ratios for NMN [Fixed Lanthanide Ion, C(2')-Endo, 50% Syn, 50% Anti] [a]

| | Shift and broadening ratios | | | |
|---|---|---|---|---|
| | Obsd | Calcd $\theta 1 = 260°$, $\theta 2 = 350°$, $\theta 3 = 280°$, C(2')-endo | | |
| Proton | | 50% syn, 50% anti | Syn, $\theta 4 = 15°$ | Anti, $\theta 4 = 245°$ |
| $1'$ | 11 (6) | 12 (5) | | |
| $2'$ | 39 | 45 | | |
| $3'$ | 40 | 35 | | |
| $4'$ | 26 | 25 | | |
| $5'$ | 100 (–)[b] | 100 (100) | | |
| 2 | 19 (30) | 23 (62) | 44 (120) | 1 (4) |
| 4 | –6 (4) | –7 (6) | –7 (6) | –7 (6) |
| 5 | 2 (15)[c] | 4 (13) | 1 (2) | 7 (24) |
| 6 | 31 (18) | 35 (22) | 8 (3) | 62 (41) |

[a] The broadening data are listed in parentheses adjacent to shift data, the observed broadening data being normalized to the broadening of H(2) (taken as 30). This resonance is chosen for normalization because it exhibits only small proton spin couplings and, except for the 5' protons, has the largest observable broadening. [b] The broadening of the 5' Protons is very difficult to measure because of the chemical nonequivalence of the 5' protons and the multiple spin–spin couplings on these resonances. The broadening ratio may be estimated to be in the range 50–200. [c] The H(5) proton has large spin–spin couplings to H(4) and H(6) and thus the broadening of this multiplet resonance is also difficult to measure.

effect.[17] However, the shift ratios in the presence of different ions are equal and, since this is true for all the protons in the molecule, one term in the equation must be predominant. In these calculations we have chosen the first term to be the important term.

The lanthanide ions bind to the ionized phosphate group in NMN as was found for AMP;[2] the lanthanide ion is located in the plane defined by the O–P–O angle and bound to two of the oxygen atoms not attached to the carbon. The principal axis of symmetry is taken to be through the lanthanide ion, bisecting the O–P–O angle.[18]

The BURLESK program (provided by the Oxford Enzyme Group) searches for conformations which agree with the LIS data by rotating bonds sequentially until a conformation is found which is acceptable on van der Waal's distance criteria and which also gives calculated shifts in agreement with the LIS data within a preset tolerance of the shift ratios. The success of this method depends on the existence of a unique conformation of the molecules in solution. It is interesting that application of the BURLESK program to the NMN LIS proton data gives solutions for unique conformations (see Table III) despite the evidence that NMN exists as a mixture of conformers in solution. However, none of these unique conformations agrees with the coupling constant and $Gd^{3+}$ line-broadening data.

(i) **Analysis of LIS Data for Mixtures of Conformers.** The procedure adopted was to attempt to first fit the sugar and backbone $^1$H LIS data before considering mixtures of the syn and anti conformations. Because the position of the H(4) proton relative to the lanthnide ion is independent of the syn/anti position of the nicotinamide ring, we can include the H(4) LIS data in the search for the best fit for the sugar and phosphate backbone conformation. Only one solution (within a tolerance of ±6) was obtained $\theta 1 = 260°$, $\theta 2 = 350°$, $\theta 3 = 280°$, C(2')-endo ribose ring, see Table IV).[19] For this solution a 1:1 mixture of syn and anti conformers is in good agreement with both the LIS and the broadening results for all the protons (Table IV). However, this solution which agrees with all the LIS and broadening data can be shown to be incorrect. The backbone conformation ($\theta 2 = 350°$, $\theta 3 = 280°$) differs from that predicted from coupling constant information ($\theta 2 = 360°$, $\theta 3 = 240°$) and implies almost an eclipsed conformation. In addition, the position of the glycosidic bond of the anti conformer ($\theta 4 = 245°$) is at one extreme end of the allowed anti range. *This indicates that the computer fitting of the LIS and broadening data alone is not a sufficient criterion for a conformational solution; solutions must also be compatible with the coupling constant information.*

The procedure we have adopted is to consider (1) the phosphate backbone conformation as determined from coupling constant information ($\theta 2 = 360°$, $\theta 3 = 240°$) and (2) the ribose ring as a mixture of C(2')-endo and C(3')-endo conformations. By rotating the 5'-O–P bond, $\theta 1$, the position of the lanthanide ion relative to the sugar is changed and the pseudocontact shifts of the ribose ring protons for the various $\theta 1$ values have been calculated (Figures 2 and 3). In certain conformations the two 5' protons have very different pseudocontact shifts (e.g., 30°, Figure 2) whereas in other regions the 5' protons exhibit the same shift. Experimentally we find the 5' protons shift to the same extent. This means either (a) the preferred conformation is in the range $\theta 1 = 150–210°$ or (b) there is rotation about the 5'-O–P bond resulting in the observed shifts being an averaged value which is the same for both protons.

In case a the LIS data of the sugar protons can be fitted to a 70:30 mixture of C(2')-endo and C(3')-endo conformations[20] assuming $\theta 1 = 250°$ for C(2')-endo and $\theta 1 = 115°$ for the C(3')-endo. This solution is consistent with the coupling constant data. However, no solutions which fit the nicotinamide $^1$H LIS data can be found for the syn, anti, or combinations of the syn and anti conformations of the nicotinamide ring. Thus in this model where the lanthanide ion is assumed to be in a fixed position relative to the ribose ring, we cannot obtain a solution which is consistent with the coupling constant data.

There is no a priori reason for a unique position of the lanthanide ion and, in fact, energy calculations predict essentially free rotation about the O(5')–P bond.[12] In addition, a lanthanide ion bound to two of the oxygen atoms on the phosphate group is not hindered sterically by the nicotinamide ring. For rotation about the O(5')–P bond there are at least six rotameric states; averaging over these rotamers will lead essentially to the same results as assuming free rotation about the O(5')–P bond. We have calculated the shifts for the C(2')-endo and C(3')-endo sugar conformations assuming such free rotation of the phosphate group (case b) (Table V). Excellent agreement between the observed and predicted shifts was obtained with a 2:1 ratio of the C(2')-endo to the C(3')-endo form (consistent with the ribose ring $^1$H–$^1$H coupling constant data). When the syn and anti ranges of both the C(2')-endo and C(3')-endo conformations were examined only one solution could be found that fitted the observed shift and broadening data. That solution, interestingly, was a 2:1 mixture of syn C(2')-endo and anti C(3')-endo (Table V). Such a solution would imply that the syn/anti conformation and the nature of the ribose

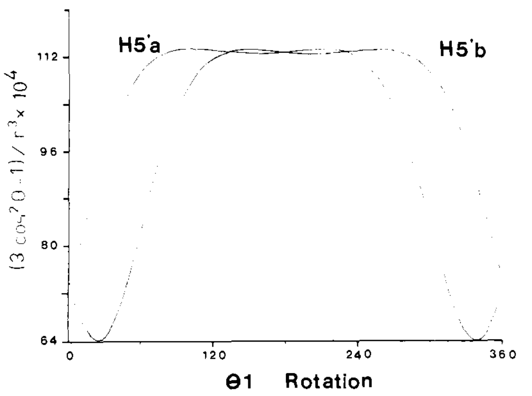


**Figure 2.** The calculated pseudocontact chemical shift parameter [(3 $\cos^2\theta - 1)/r^3$] for the H(5′) protons of NMN as a function of the 5′O-P bond rotation (torsion angle θ1) with θ2 = 360°.

**Table V.** Observed and Calculated Shift and Broadening Ratios for NMN [Averaged Position for Lanthanide Ion, 67% Syn C(2′)-endo, 33% Anti C(3′)-Endo] [a]

| | Shift and broadening ratios | | | |
|---|---|---|---|---|
| | Obsd | Calcd | | |
| | | θ1 rotated, θ2 = 360°, θ3 = 240° | | |
| Proton | | 67% syn C(2′)-endo, 33% anti C(3′)-endo | Syn C(2′)-endo, θ4 = 10° | Anti C(3′)-endo, θ4 = 190° |
| 1′ | 11 (6) | 15 (4) | 16 (4) | 13 (3) |
| 2′ | 39 | 40 | 53 | 13 |
| 3′ | 40 | 37 | 35 | 41 |
| 4′ | 26 | 27 | 27 | 29 |
| 5′ | 100 (50–200) | 100 (182) | 100 (182) | 100 (182) |
| 2 | 19 (30) | 23 (28) | 31 (41) | 7 (2) |
| 4 | −6 (4) | −4 (5) | −5 (5) | −3 (5) |
| 5 | 2 (15) | −1 (19) | 1 (2) | −6 (54) |
| 6 | 31 (18) | 26 (19) | 7 (3) | 65 (51) |

[a] The broadening data are listed in parentheses adjacent to the shift data.

ring pucker are correlated. Although this is the simplest solution found to agree with the shift, broadening, coupling constants, and NOE experimental evidence, it should be emphasized that more complex averaged solutions which fit the data could possibly be found.

## Conclusions

For flexible molecules which exist as a mixture of interconverting conformations, the use of pseudocontact chemical shifts is of only limited usefulness in defining the conformational state of the molecule. It is seen that if conformational information from other sources (such as spin-coupling constants) is ignored, then the use of pseudocontact shift and line broadening data alone can lead to incorrect conformations. However, by using a combination of all the data available a semiquantitative picture of the conformational state is achievable.

Probably the most useful applications of pseudocontact shift studies to flexible molecules will be in helping to confirm theoretical potential energy calculations. From a series of detailed potential energy maps for a molecule one can predict the populations of all the conformations contributing to the equilibrium and use this information to compute the expected coupling constants and pseudocontact shift and line-broadening ratios for the magnetic nuclei in the molecule. Agreement between the observed and calculated data for all the magnetic nuclei would then be a necessary (but not sufficient) criterion of the correctness of a calculation.

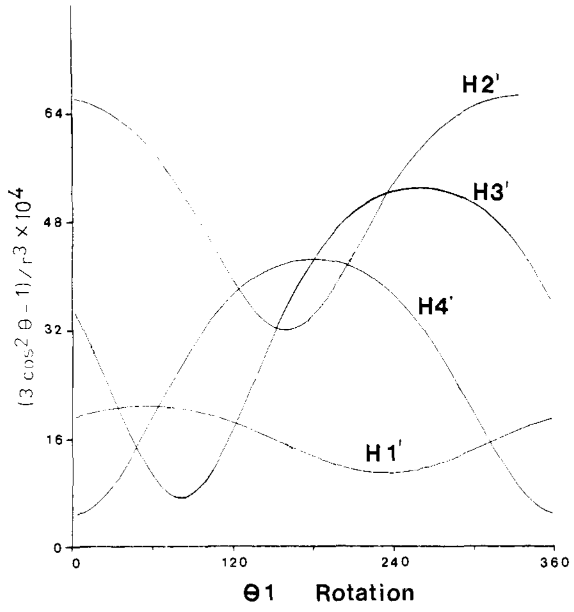


**Figure 3.** The calculated pseudocontact chemical shift parameter [(3 $\cos^2\theta - 1)/r^3$] for the ribose protons in NMN as a function of the 5′-O-P bond rotation (torsion angle θ1) with θ2 = 360°, θ3 = 240° and with C(2′)-endo conformation.

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## References and Notes

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(6) The shift ratios at pH 4.4 and 1.7 are similar for all the protons. This indicates that (1) the conformations of the complexed dianion and monoanion species are broadly similar, (2) the lanthanide ion binds in the same manner in these two complexes, and (3) small amounts of monoanion complexes coexisting with the dianionic complexes at pH 4.4 will not interfere with the analysis.
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(10) For bonds θ1, θ2, θ3, and θ4 in NMN (I), positive angles (0-360°) are defined as right-handed rotation. When one looks along any bond, the far atom rotates clockwise relative to the near atom. The zero angles for each bond are defined as follows: θ1 = 0° when the C(5′)-O(5′) bond is trans planar to the P-O (this O is that not bonded to Ln) bond, θ2 = 0° when the C(5′)-C(4′) bond is trans planar to the P-O(5′) bond, θ3 = 0° when the C(4′)-C(3′) bond is trans planar to the O(5′)-C(5′) bond, and θ4 = 0° when the N-C(2) bond is cis planar to the C(1′)-ring O bond. In the syn conformation of NMN, θ4 is in the range 320-340°, the carboxamide group being above the ribose ring. The anti conformation (θ4 = 140-250°) is depicted in I. Our definitions of the angles are related to those of M. Sundaralingam, *Biopolymers*, **7**, 821-860 (1969), as follows: θ1 = ω + 180°, θ2 = φ + 180°, θ3 = ψ + 180°, θ4 = χ − 180°.
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(18) Other positions for the lanthanide ion were tried but no solutions to the BURLESK program were found.

(19) No solutions using the C(3′)-endo ribose ring conformation could be found.

(20) When averaging conformations it is necessary to average absolute shifts and not shift ratios. For a mixture of *n* conformations of fractional populations, $\alpha_n$, the calculated chemical shifts for a proton $H_1$ is given by

$$\partial H_1 = K\sum_n \alpha_n f\{\theta_n(1), r_n(1)\}$$

where f $\{\theta_n(1), r_n(1)\}$ is the angle and distance term in the pseudocontact equation for proton 1 and for $H_2$

$$\partial H_2 = K\sum_n \alpha_n f\{\theta_n(2), r_n(2)\}$$

It is possible to compare calculated and experimental shift ratios using these values

$$\frac{\partial H_1}{\partial H_2} = \frac{\sum_n \alpha_n f\{\theta_n(1), r_n(1)\}}{\sum_n \alpha_n f\{\theta_n(2), r_n(2)\}}$$

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# Nuclear Magnetic Resonance Studies on Pyridine Dinucleotides. II. Solution Conformational Dynamics of Nicotinamide Adenine Dinucleotide and Nicotinamide Mononucleotide as Viewed by Proton $T_1$ Measurements[1]

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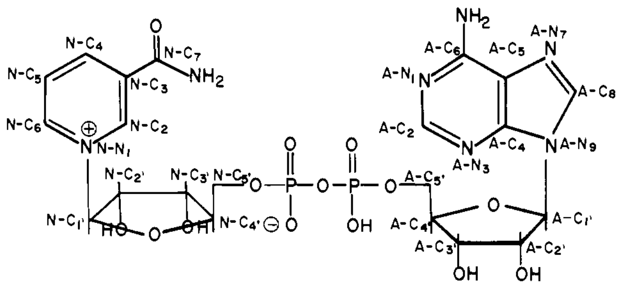
**Abstract:** The $^1$H $T_1$'s of NAD$^+$ and NMN$^+$ have been measured as a function of temperature. The measurements demonstrate that these $^1$H $T_1$'s are dominated by the dipole-dipole mechanism. The reorientational activation energy extracted from these measurements is 4.6 ± 0.4 kcal/mol for both NAD$^+$ and NMN$^+$. Proton and $^{13}$C $T_1$ measurements were used to establish that the pyridyl protons $T_1$'s can be described by a single correlation time, $\tau_c = 2.3 \times 10^{-10}$ sec (NAD$^+$). Proton $T_1$ measurements have also been made on NAD$^+$ deuterated at positions 2 or 6 or 2 and 6 of the pyridyl ring. Using these data and recent X-ray measurements, specific microdynamic models for the motion with NAD$^+$ can be made and tested. These and other measurements support the notion that the microdynamic motion of the pyridyl ring in NAD$^+$ can be described by a two-state model involving the rapid interconversion of syn and anti states with approximately equal populations of the syn and anti conformers. The pD dependence of the $T_1$'s for the pyridyl $^1$H's is presented. The implications of these data with regard to the "folded" structure for NAD$^+$ are discussed.

Since the initial $^1$H NMR study of NADH[2] reported in 1962 by Mahler and coworkers,[3] there have been many papers published that discuss various aspects of the conformational dynamics of pyridine dinucleotides in solution.[3-25] With the possible exception of two of these papers,[14,20] the current hypothesis describing the solution dynamics of nicotinamide adenine dinucleotide (NAD$^+$, I) may be summarized in the following manner. NAD$^+$ is thought to exist in predominately two forms, a folded form in which the two aromatic rings are approximately parallel to each other and an extended form. Furthermore, these species are envisioned to undergo a rapid (on the NMR chemical shift time scale) interconversion. The equilibrium constant describing this process is pH dependent. At pH values greater than 4, the equilibrium is shifted in such a way that the folded form becomes more prevalent. Various estimates of the percent folded species have been made. Jardetzky,[4] using thermodynamic parameters, concluded that 20-40% of NAD$^+$ was folded at neutral pH and room temperature. This conclusion has been further supported by chemical evidence put forth by Oppenheimer et al.[15] from the unequal addition of cyanide to the A and B side of the pyridine ring. However, a more recent thermodynamic study by Hollis and coworkers[16] has concluded that only 15% of the folded conformer exists at any one time. For pH values less than 4, the equilibrium is such that the extended form predominates in solution. The pH dependence of the $^1$H NMR of NADH and NADPH has been characterized.[4] However, because of the inherent instability of these compounds in acidic media, these systems have been primarily examined at pH levels of 7 or higher. At elevated values of pH these dinucleotides are believed to exist in the folded form.[10-13,15] The evidence used to support this conclusion is based on the appearance of an AB quartet (at 220 MHz) for the N-$H_4$ hydrogens of the nicotinamide portion of these dinucleotides.



I

The validity of the preceding conclusions rests on the implicit assumption that in the folded form the overall reorientational motion (tumbling) of the dinucleotide is fast compared to the internal rotation of the bases with respect to their contiguous sugars. Furthermore, it has been reported by Sarma and Kaplan[10-13] and more recently by Sarma and Mynott[22,24] that in nicotinamide mononucleotide (NMN$^+$, II) the nicotinamide base must be in the syn conformation with respect to its contiguous ribose. That is, the dihedral