The Conformation of Pyridine Dinucleotides in Solution*

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SUMMARY

The dependence of chemical shifts in the nuclear magnetic resonance spectra of pyridine nucleotides on concentration, pH, and temperature has been examined. The data indicate that all of the dinucleotides exist predominantly in a folded conformation, with the planes of the two rings stacked in parallel. The free energy of formation of the intramolecular complexes has been estimated to be of the order of -0.5 to -1.0 Kcal per mole. The present data do not allow a distinction between a single complex with a unique geometrical configuration and a multiplicity of complexes undergoing rapid exchange. However, the relative instability of the complexes, together with a detailed examination of the 64 alternative structures with the aid of molecular models, leads us to prefer the latter possibility.

The conformation of pyridine dinucleotides in aqueous solution is of some interest in the study of the transition complexes formed by them in the course of an enzymatic reaction. No conclusive evidence on the nature of this conformation has been advanced so far, but several relevant suggestions have been made. In the case of reduced nicotinamide adenine dinucleotide, Weber (1) postulated an intramolecular complex between the adenine and the dihydropyridine rings in order to account for the pyridine fluorescence at 4680 A following absorption by adenine at 2540 A. Extending the fluorescence studies, Velick (2, 3) proposed a folded conformation for NADH, with the two rings stacked in parallel. A folded conformation was also favored by Meyer, Mahler, and Baker (4) on the basis of chemical shifts in the nuclear magnetic resonance spectra of NADH in D$_2$O, as compared to those of N-benzyldihydronicotinamide in chloroform. On the other hand, Shifrin and Kaplan (5) have considered the possibility of a hydrogen-bonded structure, with the two rings lying in the same plane.

All conceivable stable conformations of a dinucleotide may be regarded as special cases of one of the three conformational prototypes shown in Fig. 1: open chain, planar hydrogen-bonded, and folded. It is not possible to distinguish between these three alternatives by studies of fluorescence transfer (1-3) or hypochromism. Fluorescence transfer is known to occur in systems in which the donor and the acceptor rings are neither in direct contact nor in a fixed orientation with respect to each other (6), and hypochromism could result from intermolecular aggregation frequently found in this class of compounds (7). Nuclear magnetic resonance measurements can provide adequate evidence for assigning the conformation, and the findings of Meyer et al. (4) are suggestive. However, the shifts on which their conclusion was based were not corrected for effects of substituent, solvent, and concentration. Since such corrections are of the same order of magnitude (8) as the shifts observed by Meyer et al., the validity of the conclusion remains uncertain.

We have been led to a systematic study of the conformation of pyridine nucleotides by our findings on the interaction of NAD with yeast alcohol dehydrogenase (9). The observed selective broadening of the pyridine peaks could not be explained if the bound coenzyme remained in the form of an intramolecular complex. Therefore, such a complex either did not exist, even in the free dinucleotide, or was dissociated upon combination with the enzyme.

The findings reported in this paper have substantiated the conclusion that pyridine dinucleotides can exist in a folded conformation, in equilibrium with the open chain form. They strongly suggest that it is indeed the exchange between the two forms which gives rise to the chemical relaxation observed by Czerlinski and Hommes (10) in temperature jump experiments on NADH and NADPH solutions. A complete unfolding of the coenzyme molecule apparently occurs in the course of some enzymatic reactions.

EXPERIMENTAL PROCEDURE

The NMR$^1$ spectra were obtained on a high resolution Varian model V3200B 60-nc spectrometer, as described previously (7, 8). At low concentrations (10$^{-3}$ M), an average response computer (Mnemotron CAT 400A) was used to improve the signal to noise ratio (9). Shifts were measured by direct side band superposition in the more concentrated and by interpolation in the more dilute solutions, with the use of hexamethyldisiloxane as an external and acetone as an internal reference, as described previously (7). The precision of the measurements is ±0.2 cps. $\beta$-NAD, $\alpha$-NAD, NADH, NADPH, NADP, NMN, and AMP were commercial preparations obtained from Sigma, purified whenever necessary by the procedure of Kornberg and Horecker (11). NMNH was prepared by reduction of NMN with dithionite (12). The purity of the commercial samples varied somewhat, judging from differences in the degree of discoloration. The more discolored samples gave poorly resolved NMR spectra, but no new peaks were observed. The chemical shifts of the

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$\beta$-

$\alpha$-

NAD-

NADH-

NADPH-

NADP-

NMN-

AMP-

NMNH-

$\beta$-

$\alpha$-

NAD-

NADH-

NADPH-

NADP-

NMN-

AMP-

NMNH-

$\beta$-

$\alpha$-

NAD-

NADH-

NADPH-

NADP-

NMN-

AMP-

NMNH-

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NAD-

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NADPH-

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NADPH-

NADP-

NMN-

AMP-

NMNH-

$\beta$-

$\alpha$-

NAD-

NADH-

NADPH-

NADP-

NMN-

AMP-

NMNH-

$\beta$-

$\alpha$-

NAD-

NADH-

NADPH-
main peaks were identical in the spectra of the unpurified commercial samples and in samples purified and found to be homogeneous by paper chromatography in a phosphate-ammonium sulfate-propanol-1 system. This fact is of some importance because some of the differences in chemical shifts which are here interpreted in terms of differences in conformation could conceivably have resulted from interactions with the impurity. All samples were lyophilized from D₂O to remove exchangeable hydrogens, dissolved in the appropriate solvent, and sealed under vacuum in NMR cells. Measurements were made on each sam-

![Diagram](image)

**Fig. 1.** Possible conformations of NAD

<table>
<thead>
<tr>
<th>Compound</th>
<th>Adenine peaks</th>
<th>Pyridine peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Η(α)</td>
<td>Η(β)</td>
</tr>
<tr>
<td>α-NAD</td>
<td>524.6</td>
<td>502.6</td>
</tr>
<tr>
<td>β-NAD</td>
<td>520.4</td>
<td>501.2</td>
</tr>
<tr>
<td>NADP</td>
<td>523.3</td>
<td>503.1</td>
</tr>
<tr>
<td>NMN + AMP</td>
<td>532.3</td>
<td>508.3</td>
</tr>
<tr>
<td>NMN</td>
<td>532.5</td>
<td>508.1</td>
</tr>
<tr>
<td>AMP</td>
<td>532.4</td>
<td>508.2</td>
</tr>
<tr>
<td>NADPH</td>
<td>524.3</td>
<td>504.7</td>
</tr>
</tbody>
</table>

**Table I**

*Chemical shift referred to hexamethyldisiloxane*

All measurements were made at room temperature on 0.1 M solutions at pH 7.3.

![Diagram](image)

**Fig. 2.** Proton magnetic resonance spectra of (a) β-NAD and (b) an equimolar mixture of NMN and AMP. Peaks: 1, pyridine H(α); 2, pyridine H(β); 3, pyridine H(γ); 4, adenine H(δ); 5, pyridine H(β); 6, adenine H(ε); 7, pyridine H(ζ); 8, adenine H(η); 9, solvent; 10, H(η'), H(η''), H(η'''), and H(η''''); 11, acetone marker. Sweep rates are comparable.
ple immediately after preparation. Measurements were also made at room temperature on each sample after it had been subjected to higher temperature. The pH was determined both at the time of preparation and after all NMR measurements were finished. Measurements made on solutions showing any change from the initial values were discarded. All points on pH, concentration, and temperature figures represent the average of measurements made on two different preparations.

RESULTS AND DISCUSSION

α- and β-NAD; β-NADP—The NMR spectra of β-NAD and an equimolar mixture of its constituent mononucleotides in D₂O at pH 6.8 are shown in Fig. 2. The assignment of peaks has been given previously (9) and is consistent with that made by Kowalewski and Kowalewski (13) and Lemieux and Lown (14). The spectrum of β-NADP is identical with that of β-NAD; that of α-NAD differs from it only in the magnitude of the chemical shifts, as noted in Table I.

It is apparent that in the dinucleotide the aromatic peaks and the H'(4) peaks of the ribose are shifted to higher fields, as compared to the mixture of the mononucleotides. These shifts are not very large (5 to 15 cps) and are not identical for different peaks in the spectrum. This fact, together with the observation that the position of the internal standard line is identical in the two solutions (at 152.1 cps), precludes any simple explanation in terms of differences in diamagnetic susceptibility. Rather, it indicates a specific structural difference, affecting different protons to a different extent.

It has been pointed out previously (7) that a finding such as that shown in Fig. 2 could result from the formation of either intramolecular complexes, or a combination of both. In the present case, complexes formed between either like or unlike rings would give similar results. However, the alternatives can be distinguished by the study of the dependence of the chemical shifts on pH and concentration.

The pH dependence of the chemical shifts for the mono- and dinucleotides is shown in Fig. 3. In the mononucleotides the shifts of the adenine peaks reflect the pK of N(9)H at 3.8; the shifts of the pyridine are independent of pH over the accessible range; and there is a slight indication of an upfield shift just before the mixture of the mononucleotides. These shifts are not very large (5 to 15 cps) and are not identical for different peaks in the spectrum. This fact, together with the observation that the position of the internal standard line is identical in the two solutions (at 152.1 cps), precludes any simple explanation in terms of differences in diamagnetic susceptibility. Rather, it indicates a specific structural difference, affecting different protons to a different extent.

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before precipitation, probably resulting from the formation of microcrystalline aggregates. In the dinucleotide, however, both the adenine and the pyridine shifts show a transition with a pK of approximately 4, near the pK of the adenine. It is unfortunate that the precipitation of NMN in the lower pH range precludes a direct comparison of the shifts. However, a change in the pyridine shifts to lower fields in this pH range is sufficiently unlikely (pK values for the protonation of a substituted pyridine are much lower) so that the observed transition constitutes reasonable evidence for an interaction between the pyridine and the adenine rings. This interpretation is strengthened by the fact that the ring shifts in the dinucleotides in the low pH range are the same as those observed for the mononucleotides, when the mononucleotide ring bears a positive charge (below pH 3 for AMP; in the entire pH range for NMN). Positively charged rings would not be expected to form complexes with each other, so that the transition at approximately pK 4 should represent a dissociation of the pyridine-adenine complexes. This is further borne out by the finding that the downfield shift of the AMP peaks with decreasing pH is greater in the dinucleotide than in the mononucleotide.

The intramolecular nature of the complex becomes apparent from the lack of a concentration dependence of the chemical shift in the dinucleotide, shown in Fig. 4. The pyridine moiety forms no intermolecular complexes in either the mono- or the dinucleotide form. This would be expected, since the ring is charged in either case. In the dinucleotide an intramolecular complex is revealed by the upfield shifts of all dinucleotide peaks compared to the shifts of mononucleotide peaks over the entire concentration range from 1 mM to 1 M. The contrast between the behavior of the mono- and the dinucleotide is more striking in the case of adenine. The difference in the shifts is greatest at infinite dilution and progressively decreases as the concentration is increased, permitting the formation of molecular aggregates of AMP. The absence of concentration dependence of adenine peaks in NAD suggests that adenine does not form larger stacks if complexed with the pyridine ring.

Some indication of the stability of the complexes can be obtained by assuming an equilibrium of the type

$$\text{(NAD)}_n \rightleftharpoons \text{(NAD)}_c$$

where \(n\) and \(c\) denote unfolded and complexed folded forms. The stability constant for the folded form,

$$K = \frac{[\text{(NAD)}_c]}{[\text{(NAD)}_n]} = \frac{p}{1 - p}$$

(1)

(where \(p\) is the fraction of molecules in the folded form), is easily expressed in terms of the observed chemical shifts \(\delta\) and the shifts in the folded form \(\delta_c\). Since we are dealing with a case of rapid exchange,

$$\delta = p\delta_c + (1 - p)\delta$$

(2)

or

$$\Delta\delta = \delta - \delta_c = p(\delta_c - \delta) = p\Delta\delta_c$$

we have

$$K = \frac{\Delta\delta}{\Delta\delta_c - \Delta\delta}$$

(3)
\[ \delta_n \] is given by the chemical shifts of the monomers at infinite dilution. There is no direct experimental measure of \[ \delta_n \]. Theoretical estimates, based on calculations of ring currents (15, 16), are not very satisfactory for heterocyclic rings and lead to widely divergent figures for \[ \Delta \delta_n \] (30 to 120 cps), depending on the assumptions made. In the present case an empirical procedure for estimating \[ \Delta \delta_n \] proved to be feasible when the temperature dependence of the chemical shifts was examined.

The plot of chemical shift with respect to temperature is shown in Fig. 5. It is apparent that (a) the variation of the shifts with temperature is small, indicating that the enthalpy of formation for the complex is small, and (b) the shifts are to lower fields, suggesting that the complex tends to dissociate with increasing temperature. The smallness of the downfield shifts reflects in part an upfield shift of the standard. This cannot be avoided since it is not possible to measure absolute shifts. However, the argument here rests not on the extent of the temperature variation, but on the convergence of the lines for dinucleotide and mononucleotide for each resonance peak.

On the reasonable assumption that \[ \delta_n \] is a constant characteristic of a given complex, the variation of the observed shift with respect to \[ K \] can be found by differentiating Equation 3. Defining for convenience the inverse of the chemical shifts \[ \rho_n = 1/\Delta \delta_n \] and \[ \rho_0 = 1/\Delta \delta_0 \], one obtains

\[ \frac{d\rho_n}{d \ln K} = \rho_n - \rho_1 \] (4)

which, combined with the usual expressions for the temperature variation of the equilibrium constants, yields

\[ \frac{d\rho_n}{dT} = \frac{(\rho_n - \rho_0) \Delta S}{R} \] (5)

\[ \frac{d\rho_n}{d(1/T)} = \frac{(\rho_n - \rho_0) \Delta H}{R} \] (6)

where \[ \Delta H \] and \[ \Delta S \] are the enthalpy and entropy of complex formation, \[ R \] is the gas constant, and \[ T \] the absolute temperature. Equations 5 and 6 can be solved numerically, with the use of experimental values of \[ \rho_n \] at different temperatures, without prior knowledge of \[ \rho_0 \], provided that the variation of either \[ \Delta S \] or \[ \Delta H \] with temperature can be neglected. This is certainly not justified in general, but is a reasonable approximation in the present case: over the narrow accessible temperature range, the plots of \[ \Delta \rho_n/\Delta T \] against \[ \rho_n \] and of \[ \Delta \rho_n/\Delta(1/T) \] against \[ \rho_n \] are almost linear.

The thermodynamic parameters calculated in this manner from the observed chemical shifts are rough approximations, indicating an order of magnitude, not an accurate value.\(^2\) Extremes of the values calculated for pyridine peaks \[ \Pi_{(2)} \] and \[ \Pi_{(3)} \], for which the temperature variation of chemical shifts is sufficiently large, are given in Table II. The value of \[ \Delta \delta_n \] used in these calculations is the difference in the chemical shifts between AMP and NAD protons, shown in Fig. 5. For the remaining peaks the shifts are small and their temperature variation over a narrow range is of the same order of magnitude as the accuracy of the shift measurement, so that a meaningful calculation is not presently possible.

\(^2\) To determine these values both accurately and precisely, appreciably higher resolution (300 me) and greater stability of the field at higher temperatures are necessary. Although it is likely that such measurements will be feasible in the future, they are beyond the capabilities of the existing spectrometers.

Table II

<table>
<thead>
<tr>
<th>Peak</th>
<th>[ \rho_n ]</th>
<th>[ \Delta \delta_n ]</th>
<th>[ \Delta \delta(1/2) ]</th>
<th>[ \Delta \delta(3/4) ]</th>
<th>[ \Delta \delta(5/4) ]</th>
<th>[ \Delta \delta(7/4) ]</th>
<th>[ \Delta \delta(9/4) ]</th>
</tr>
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<tbody>
<tr>
<td>[ H_{(2)} ]</td>
<td>-0.021</td>
<td>-5.0</td>
<td>-5.73</td>
<td>-0.68</td>
<td>-18.5</td>
<td>0.309</td>
<td>27</td>
</tr>
<tr>
<td>[ H_{(3)} ]</td>
<td>-0.040</td>
<td>-25</td>
<td>-5.43</td>
<td>-0.94</td>
<td>-16.4</td>
<td>0.612</td>
<td>38</td>
</tr>
</tbody>
</table>

The available results indicate that between 20 and 40% of the dinucleotide is present in the form of a folded conformation.

The same line of argument leads to closely similar values of thermodynamic parameters for the folded form of \[ \alpha \]-NAD. It should be noted that the conclusion reached in the present study, that \[ \alpha \]-NAD as well as \[ \beta \]-NAD exists in the folded conformation, is different from that drawn by Kaplan from fluorescence transfer data (17). The difference reflects the difference in methods. Many more uncertainties are inherent in the interpretation of fluorescence transfer data than in the interpretation of NMR chemical shifts in this case.

\[ \beta \]-NADH and \[ \beta \]-NADPH—The pH dependence of shifts in the NADH is shown in Fig. 6. A decrease in the areas of Peaks \[ H_{(2)} \] and \[ H_{(3)} \] corresponding to a decrease in the optical density at
Conformation of Pyridine Nucleotides

Fig. 6. pH dependence of chemical shifts in 0.1 M β-NADH and an equimolar mixture of NMNH and AMP

The data on the concentration dependence of shifts for NADH and its constituent monomers are given in Fig. 7, and those for NADPH are included in Table I. These data substantiate the conclusions that all forms of pyridine nucleotides form stacked intramolecular complexes. Fig. 7 shows that the shifts of the adenine peaks to higher fields in NADH solutions are apparent only at low concentrations. This is perhaps the most striking indication that no complexes involving more than 1 dinucleotide molecule are formed.

The temperature dependence of the spectra of the reduced pyridine nucleotides has been given only a cursory examination, as it was found to differ little from that of the oxidized form. The values of the thermodynamic parameters obtained therefore also appear to be of the same order of magnitude as those for the oxidized forms. This is somewhat surprising since one might expect the complexes of the reduced forms to be less stable because of the absence of the positive charge. It must be remembered, however, that the error in making this type of estimate from the present data is rather large because the estimate is based on relative rather than absolute chemical shifts (i.e., those determined with respect to a standard kept at room temperature). Differences in stability between the complexes of different dinucleotides may well become apparent when more refined measurements become possible.

Geometrical Considerations—The possibility of obtaining information on the geometry of the intramolecular complexes has been examined in detail and deserves a brief comment. For each of the dinucleotides there are 64 possible structures in which the two rings are stacked in parallel as indicated in Fig. 1, Part 2. Thirty-two of these correspond to one turn of a right-handed helix, and 32 to one turn of a left-handed helix. If only one type of complex were formed, all but four of these structures could be eliminated on the basis of the relative magnitude of the chemical shifts. In view of the instability of the complex, however, it is rather likely that there is a rapid exchange between several different forms. Prediction of an “average” conformation from the NMR shifts does not in this case lead to any unique features, such as the stacking of 6-membered rings in purine derivatives (7). It is nevertheless worth noting that the data unmistakably
reflect a difference in geometry between the complexes of the α and the β form, as might be expected from differences in the configuration about the C'(1) atom.

**REFERENCES**

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