Nuclear Magnetic Resonance Studies on Pyridine Dinucleotides. 7.[1] The Solution Conformational Dynamics of the Adenosine Portion of Nicotinamide Adenine Dinucleotide and Other Related Purine Containing Compounds

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Abstract: Proton spin-lattice relaxation times, T_1, have been determined for the following purine containing compounds: adenosine 5′-monophosphate (5′-AMP), adenosine 3′-monophosphate (3′-AMP), adenosine 2′-monophosphate (2′-AMP), inosine 5′-monophosphate (5′-IMP), adenosine, inosine, purine riboside, and nicotinamide adenine dinucleotide (NAD^+). The T_1 values of selected protons were determined for each of the above compounds in the presence and absence of a deuterium in the 8-position on the purine ring. These data reflect several important features of the solution conformational dynamics of these molecules. First, both 5′-AMP and inosine studied at 5 mM concentration and at 5 °C are shown to participate in a stereospecific (head-to-tail) intermolecular association. Further, it was demonstrated this intermolecular association is responsible, in part, for the preferential anti conformation of the purine base with respect to its contiguous ribose in these two systems. The T_1 data on the remaining compounds are consistent with the notion that there is a considerable amount of conformational mobility about the glycosidic bond in these systems. However, no definite conclusions can be drawn as to specific microdynamic models for this conformational mobility. Finally, the ^1H T_1's were also measured for a series of deuterium labeled NAD^+ molecules. From the lack of intra-ring dipole-dipole relaxation, it is demonstrated that the two aromatic rings cannot approach one another closer than 4.5 Å for a time long compared to the reorientational correlation time for NAD^+, 2.6×10^{-10} s. These data are discussed in terms of the "significance" of the "intramolecular stacked" model of NAD^+.

There have been several NMR studies reported which are concerned with the solution conformational dynamics of pyridine and purine bases with respect to their contiguous ribose moiety. The NMR parameters that have been employed in analysis of this problem are quite varied, i.e., ^1H and ^{13}C chemical shifts,[2-40] *intra-* and *inter*molecular nuclear Overhauser effects (NOE),[41-47] and ^1H and ^{13}C spin-lattice relaxation times, T_1.[48-52] The conclusions drawn from these data have not always been consistent with one another. Specific examples will be presented in the Discussion section of this paper.

Two conformational possibilities (of the potentially infinite set) of the purine ring with respect to its contiguous ribose are depicted in Figure 1; these are the so-called syn and anti conformers.[53] Relaxation and NOE results depend upon the conformationally averaged interatomic distance and the microdynamic processes which average these parameters,[54] whereas, ^1H and ^{13}C chemical shifts *do not* depend *explicitly* on specific interatomic distances.[55] Because ^1H spin-lattice relaxation times *are explicitly* dependent upon the conformationally averaged interatomic distance between pairs of protons, it represents the method of choice to study the dynamic stereochemistry in these systems. Furthermore, application of the so-called ^2H-difference method allows these distances and the necessary correlation times to be measured.[56]

Due to the relative ease in which a conformationally averaged interatomic distance can be extracted from the analysis of a ^2H-difference experiment, and to its recent introduction, a brief summary of the method is warranted. Reference to Figure 1 allows one to easily visualize the importance that hydrogen A-H_8[57] can have in determining the spin-lattice relaxation time T_1, for hydrogen A-$H_{1'}$. Equation 1 gives the expression for the dipole-dipole relaxation rate for hydrogen A-$H_{1'}$.

$$T_1^{DD-1}(\text{A-H}_{1'}) = \tfrac{3}{2}\gamma_H^4\hbar^4[\langle r^{-6}\rangle_{1'8}\tau_{c1'8} + \sum_{i\neq 1'}\langle r^{-6}\rangle_{1'i}\tau_{c1'i}] \quad (1)$$

Here γ_H is the magnetogyric ratio of hydrogen, $\hbar$ is Planck's constant divided by 2π, $\langle r^{-6}\rangle_{1'i}$ is the conformationally averaged value of the inverse sixth power of the distance between hydrogen A-$H_{1'}$ and proton i, and τ_c represents the average reorientational correlation time of the vector connecting proton i and A-$H_{1'}$. Deuteration of the 8-position in the purine ring effectively removes the contribution of that spin to the dipolar relaxation of A-$H_{1'}$. Hence, subtraction of the two T_1^{DD-1} (A-$H_{1'}$) values, with and without a ^2H in the 8-position, yields

$$\Delta[T_1^{DD-1}(\text{A-H}_{1'})] = \tfrac{3}{2}\gamma^4h^2\langle r^{-6}\rangle_{1'8}\tau_{c1'8} \quad (2)$$

Equation 2 demonstrates that *if* a value for $\tau_{c1'8}$ was available, the conformationally averaged interatomic distance between A-$H_{1'}$ and A-H_8 could be determined by *simple algebra!* By performing a relatively simple set of T_1 experiments (^2H and ^{13}C) a reasonably precise value of τ_c can be determined.[56] Hence, the conformationally averaged distance can be extracted from eq 2.

Utilizing the ^2H-difference method we report correlation times and conformationally averaged values of the A-$H_{1'}$ to A-H_8 distance in the following molecules:[57] 5′-AMP, 3′-AMP, 2′-AMP, 5′-IMP, adenosine, purine riboside, and NAD^+. With these data in hand, arguments are put forth that demonstrate (a) 5′-AMP and inosine undergo a stereospecific head-to-tail intermolecular association at 5 °C and 5 mM; (b) there is a considerable amount of conformational flexibility of the purine ring about its contiguous ribose in these systems; (c) "simple direct" ^1H chemical shift arguments used by some researchers to elucidate the relative syn and anti forms of the purine base are shown to be without merit; and (d) finally, by lack of intra-ring dipole-dipole relaxation in NAD^+, it is demonstrated that the two aromatic rings within NAD^+ on a time average do not approach one another closer than 4.5 Å for a time long compared to the overall reorientational correlation time for NAD^+.

Table I. Relaxation Data and Calculated Conformationally Averaged Distance of $\langle r \rangle_{1'8}$ and $\langle r \rangle_{2\ 8}$ for 5′-AMP

	T_1 (A-H_2)[a]	$\Delta[T_1^{-1}$ (A-H_2)][b]	$\langle r \rangle_{2-8}$[c]	T_1 (A-H_8)[a]	T_1 (A-$H_{1'}$)[a]	$\Delta[T_1^{-1}$ (A-$H_{1'}$)][b]	$\langle r \rangle_{1'8}$[c]	$T_1(^2H)$[d]	τ_c[e]
5′-AMP (5 °C)	1.86	0.26	3.5	1.73	1.30	0.09	3.7	7.7	2.9
5′-AMP-A-$^2H_8$(5 °C)	3.60				1.61				
5′-AMP (6 M Urea, 5 °C)	3.86	0.003		0.92	1.23	0.10			
5′-AMP-A-$^2H_8$ (6 M Urea, 5 °C)	3.91				1.40				
5′-AMP (30 °C)	9.72	0.002		1.86	2.75	0.07	3.3	21.2	1.1
5′-AMP-A-$^2H_8$ (30 °C)	9.91				3.36				

[a] The units for the T_1 values are in seconds. [b] The units for the relaxation rate are seconds^{-1}. [c] Conformationally averaged distance between hydrogens in angstroms. See text for details on how these values were calculated. [d] The units for these T_1 values are in milliseconds. [e] τ_c is given in 10^{-10} s and they were calculated by the procedure outlined in ref 56.

Experimental Section

NMR Measurements. The ^1H and ^2H spin–lattice relaxation time measurements were made on a Varian XL-100-15 NMR spectrometer operating at 100.1 and 15.4 MHz, respectively. The spectrometer is equipped with a 16K (1K = 1024 words) 620 I computer with a two million word disk (VDM-36). The deuterium T_1's were inferred directly from the line width of the deuterium resonance by assuming that $T_1 = T_2 = T_2^*$.[58] The proton T_1's were determined using the Freeman–Hill[59] inversion recovery pulse sequence. The pulse delay between pulses in both the deuterium and proton NMR experiments was in all cases at least $5T_1$'s. The T_1's were determined using standard least-squares techniques with peak heights being used for the A-H_2 and A-H_8 resonances and areas being utilized for the resonances showing fine structure (A-$H_{1'}$, A-$H_{2'}$, etc.). The area of these resonances was determined using a Geotec compensating polar planimeter. The range of τ values employed for a given T_1 experiment in all cases did not exceed $2T_1$'s in order to minimize the potential nonexponential relaxation due to multispin relaxation effects.[54] The operating temperature of the T_1 experiments was measured with a thermocouple both before and after each experiment. A given experiment was considered valid only if the temperature remained constant within ±0.5 °C during the duration of the experiment. The reproducibility of the ^1H T_1's measured was typically ±5%. The relative uncertainty of the ^2H T_1's was typically less than 10%.

Materials and Sample Preparation. The NAD^+, NADH, 3′-AMP, 2′-AMP, 5′-IMP, and purine riboside were purchased from P-L Biochemicals. The adenosine and 5′-AMP were purchased from Sigma Chemical Co. and Aldrich Chemical Co., respectively. The nucleotides and nucleosides purchased from commercial sources were used without further purification. The 99.8 and 100% D_2O, NaOD, and DCl were purchased from Merk, Sharp, and Dohme.

The glassware (NMR tubes, glass spatulas, storage containers, etc.) used in these experiments was immersed in concentrated nitric acid for at least 2 h and rinsed several times with glass distilled H_2O in order to minimize the possibility of paramagnetic impurities influencing the T_1 experiments.

The ^1H NMR samples were prepared as 0.005 M nucleotide or nucleoside solutions in 0.68 M potassium phosphate buffer. The samples also had 10^{-4} M EDTA added in order to quench the effect of any paramagnetic ions on the observed ^1H T_1's. The D_2O solution was then adjusted to a pD of 7 and the solution was lyophilized a minimum of two times from 99.8% D_2O and at least twice from 100% D_2O. The samples were then made up from 100% D_2O in 12-mm NMR tubes and were freeze–pump–thaw degassed five times on a vacuum line at 10^{-5} Torr before being sealed. The deuterium NMR samples were prepared in a manner analogous to the proton samples, except that they were made up in glass-distilled H_2O and were degassed oly twice due to the lack of any effect of dissolved paramagnetic oxygen on the T_1's of deuterium.

The preparation of the various ^2H-labeled compounds employed in this study has been described elsewhere.[56]

Results and Discussion

Temperature Dependence of $\langle r \rangle_{1'8}$ in 5′-AMP. The measured ^2H and ^1H spin–lattice relaxation data, correlation times, and calculated values for the conformationally averaged internuclear distances for 5′-AMP are presented in Table I. A word of caution is pertinent at this point with respect to the precise definition of a conformationally averaged internuclear distance. Relaxation data, via eq 2, provides a vehicle for the evaluation of the average value of an internuclear distance, r, raised to the *minus six power*. This scheme is *not* averaging r but rather r^{-6}, and hence, the method will only be applicable to interatomic distances which are less than approximately 5 Å, the value beyond which the differential relaxation rate becomes insignificant. With this point in mind, it is interesting that the value of $\langle r \rangle_{1'8}$ in 5′-AMP is temperature dependent. At 5 °C the calculated value of $\langle r \rangle_{1'8}$ is 3.7 Å, whereas, at 30 °C it is 3.3 Å. It is enlightening to note that the relative uncertainty in the calculated value of $\langle r \rangle$ is relatively insensitive to the combined uncertainty in both $\Delta(T_1^{-1})$ and τ_c. For example, with a combined relative uncertainty of 20% (a worse case) the relative uncertainty in $\langle r \rangle$ is less than 2%. Therefore, the 12% variation in $\langle r \rangle_{1'8}$ is experimentally meaningful.

Figure 1 shows two possible conformational extremes of a purine ring with respect to its contiguous ribose. In the so-called syn conformer the 1′–8 distance is approximately 2.4 Å, whereas, in the corresponding anti conformation this distance is approximately 3.7 Å.[60] It is apparent from these data that 5′-AMP at 5 °C is almost exclusively in the anti conformation. In contrast to this behavior, it is evident from the data presented in Table I that 5′-AMP undergoes a conformational change in raising the temperature from 5 to 30 °C.

In an attempt to visualize this conformational difference, several microdynamic models were developed which represent a variety of plausible conformational possibilities for 5′-AMP. It is clear from the data summarized in Table II that there is only one model (of those considered) that is consistent with the experimental data for 5′-AMP at 5 °C. That is, the model which only allows a small librational angle about a dihedral angle of 180°. Clearly, any model which was more restrictive with respect to the magnitude of the librational angle would also be consistent with the data.

Applying the results of these calculations to the experimental data for 5′-AMP at 30 °C leads to ambiguous results. That is, the experimental data are consistent with two of the models described in Table II. A simple two-state model, where the purine ring spends 92% of its time in the anti conformer and only 8% of the time in syn conformer is consistent with the experimental observations. In addition, a model which allows large amplitude rotations (~120°) about the pure anti position is also consistent with the data. The relaxation data with respect to the 1′–8 interaction can not distinguish between these models. Reference to molecular models does not allow an unambiguous discrimination between these alternatives. It should also be evident that certain linear combinations of the six models proposed in Table II could be made to be consistent with the experimental data. The only unambiguous conclusion that can be drawn from the calculations summarized in Table I is that the motion of the *purine ring about its glycosidic bond*

Table II. Microdynamic Models for the Conformationally Averaged Value of $\langle r \rangle_{1'8}$ in 5'-AMP

Model	Calcd $\langle r \rangle_{1'8}$
Two state[a]	2.62 (3.3)[a]
Free rotor [b]	2.82
Average 0 + 90 °[c]	2.58
Average 180 ± 120 °[c]	3.27
Average 180 ± 90 °[c]	3.46
Average 180 ± 20 °[c]	3.70

[a] This is the average of r^{-6}, assuming equal populations of pure syn and pure anti states. The value in parentheses was obtained by a simple average of r^{-6} for a system composed of 8% syn and 92% anti. [b] The free rotor model is the average of r^{-6} taken every 5 °C as shown in Figure 1, assuming all the intermediate states are equally probable. [c] This is the average of r^{-6} every 5° over the range indicated as shown in Figure 1, assuming that each intermediate value over this range is equally probable.

in 5'-AMP at 5 °C is conformationally more restrictive than at 30 °C.

The conclusion that 5'-AMP undergoes a conformation change in going from 5 to 30 °C is supported by yet another set of relaxation data. At 5 °C the ratio of the T_1(A-H_2) to that of T_1(A-H_8) is approximately one. At 30 °C this ratio is greater than five. Although it is possible to explain some of this change in terms of differences in intermolecular interactions (5'-AMP is known to be highly associated in solution),[2-4] it should be pointed out that the T_1 of the A-H_8 did not change within experimental error upon changing the temperature. The above information can best be rationalized in terms of an intramolecular conformation change, since in raising the temperature, one would expect that all of the measured T_1's would become longer for the same set of time averaged bond distances and angles. Examination of the T_1 for the A-H_2 proton at 30 and 5 °C indicates that it increased by a factor of 5.2, whereas the inverse ratio of the correlation times only increased by a factor of 2.6. This increase in the T_1 of A-H_2 over that predicted from the inverse ratio of the correlation times is most probably due to a diminished amount of *inter*molecular contributions to the relaxation of A-H_2 at elevated temperature (this facet of the investigation will be discussed below). From the above arguments it appears that the T_1 of A-H_8 should increase due to its decreased correlation time and lessened intermolecular relaxation pathways. It is thus evident that the only way that the T_1 of A-H_8 could be nearly the same over this temperature range is that there is a more efficient relaxation pathway available due to a conformational change about the glycosidic linkage. Examination of a Dreiding model of 5'-AMP shows that the $r_{2'8}$ distance is very sensitive to small changes in the dihedral angle subtended by hydrogens 1' and 8, whereas the distance of A-H_8 to other ribose protons, i.e., 3', 4', 5', and 5'' is not. If one moves the purine ring about its glycosidic bond in a manner which is consistent with the increased flexibility demonstrated by the calculated $\langle r \rangle_{1'8}$ value of 3.3 Å at 30 °C, it becomes evident that the value of $\langle r \rangle_{2'8}$ will be short enough to reduce the relaxation time of the A-H_8 proton in 5'-AMP considerably.

It should be noted that this conclusion also lends strong support to the limited flexibility of the purine ring about its glycosidic linkage in 5'-AMP at 5 °C. In considering the above arguments it is likely that the increased flexibility of 5'-AMP at 30 °C is due to a reduction of intermolecular association which was, in all probability, responsible for the two-fold increase in the T_1 of A-H_2 over that expected from the inverse of the correlation times in going from 5 to 30 °C. This hypothesis is examined in more detail in the next section.

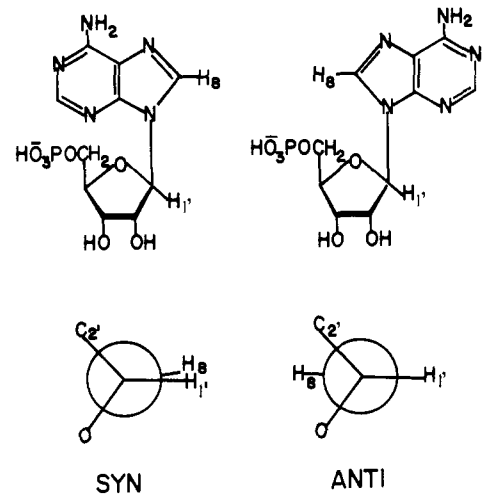

Figure 1. The syn and anti conformation of 5'-AMP. The syn conformation is defined here when the dihedral angle between A-H_8 and A-$H_{1'}$ is 0°. The anti position is defined when the dihedral angle subtended by A-H_8 and A-$H_{1'}$ is 180°. This convention has previously been used by Schirmer and co-workers.[42]

Microdynamic Model for the Intermolecular Association of 5-AMP in Solution. As previously mentioned, substitution of the A-H_8 position on the purine ring with deuterium at 5 °C causes an increase in the T_1 for the A-H_2 hydrogen by a factor of 2. This result thus accounts for the discrepancy between the ratio of A-H_2 T_1's and the inverse of their correlation times in going from 5 to 30 °C. Considering the magnitude of the increase in the T_1 for A-H_2 upon deuteration at the A-H_8 position two conclusions are clearly evident: (1) the relaxation of A-H_2 by A-H_8 is intermolecular in origin, since the intramolecular A-H_2 to A-H_8 distance of approximately 6.5 Å is too remote to affect the relaxation of either proton; (2) the time-averaged intermolecular distance of A-H_8 for A-H_2 must be relatively short. These phenomena were first noted by Gueron et al.[46] in solutions of 50 mM solutions of A-$^2H_8$-5'-AMP at 23 °C.

Before we proceed to calculate this intermolecular $\langle r \rangle_{2-8}$ distance using the ^{2}H-difference method, we feel that the exact nature of this intermolecular interaction should be discussed further. In order to test the hypothesis that this interaction is indeed due to an intermolecular association, we attempted to disrupt this interaction by adding a denaturing reagent such as urea. In Table I the result of such an experiment is presented. These data indicate that 5'-AMP in the presence of 6 M urea does not manifest an intermolecular dipolar interaction, since the T_1 of A-H_2 in the presence and absence of deuterium at the A-H_8 position is the same. It should also be noted that the ratio of the T_1's for these hydrogens has gone from approximately one in the absence of urea to greater than four in the presence of urea. That these ratios parallel those observed for the temperature change indicates the intermolecular association of 5'-AMP at 5 °C is the origin of the conformational "rigidity" of the purine ring with respect to its contiguous ribose.

In order to better formulate a model for this intermolecular association we calculated the time-averaged distance of $\langle r \rangle_{2-8}$ in 5'-AMP at 5 °C using the deuterium difference method. In Table I this result is listed as 3.5 Å. This corresponds to the time-averaged distance of two A-H_8 protons from different molecules arranged about an A-H_2 proton of yet another 5'-AMP molecule. The choice of three purines to be involved in the stack is arbitrary. The number of molecules in the stack could vary from two to n, where n is a relatively large number and time dependent. However, if the stack is parallel and further it has more than three molecules within the stack, it

Table III. Relaxation Data and Calculated Conformationally Averaged Distance of $\langle r \rangle_{1'8}$ for 3′-AMP, 2′-AMP, Adenosine, Purine Riboside, 5′-IMP, and Inosine[a]

	T_1 (A-H_2)	T_1 (A-H_8)	T_1 (A-$H_{1'}$)	$\Delta[T_1^{-1}$ (A-$H_{1'}$)][b]	$\langle r \rangle_{1'8}$[c]	T_1 (^{2}H)	τ_c[d]
3′-AMP	4.09	0.73	0.96	0.53	2.7	9.4	2.4
3′-AMP-A-$^2H_8$	4.27		1.76				
2′-AMP	4.57	0.73	0.91	0.56	2.7	9.6	2.3
2′-AMP-A-$^2H_8$	5.02		1.86				
Adenosine	2.39	0.75	0.98	0.46	2.6	12.9	1.7
Adenosine-A-$^2H_8$	2.57		1.77				
Purine riboside	3.36	0.86	1.46	0.27	2.7	17.1	1.3
Purine riboside-A-$^2H_8$	3.81		2.41				
5′-IMP	3.47	0.78	1.26	0.16	3.4	7.4	3.0
5′-IMP-A-$^2H_8$	3.60		1.59				
Inosine	4.01	1.23	1.42	0.055	3.8	11.4	2.0
Inosine-A-$^2H_8$	7.24		1.54				

[a] The units for the ^{1}H T_1's are in seconds, whereas, the units for the ^{2}H T_1's are in milliseconds. Furthermore, the temperature was 5 °C. [b] Relaxation rate in seconds^{-1}. [c] Conformationally averaged distance between hydrogens in angstroms. See text for the details of the calculation of this quantity. [d] Correlation time in 10^{-10} s and they were calculated from the procedure outlined in ref 55.

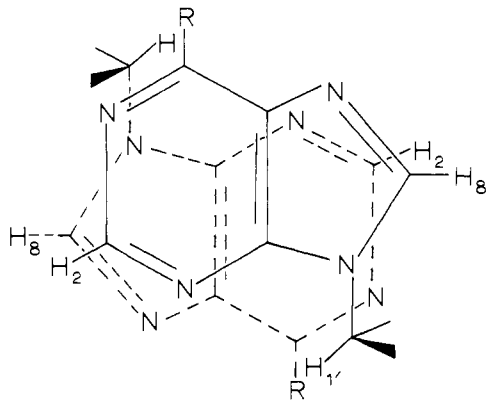

Figure 2. A schematic representation of a possible head-to-tail stack for the purine ring of 5′-AMP. R denotes a generalized substituent to the purine ring.

follows that only the contiguous molecules within the stack will contribute significantly to the intermolecular relaxation of hydrogen 2 by hydrogens 8. Figure 2 schematically shows a possible stacking arrangement for a pair of 5′-AMP molecules at 5 °C. This "head-to-tail" stacking model has previously been cited by Gueron and co-workers.[46] This geometrical description of the stack is different from the head-to-head stack suggested by Broom, Schweizer, and Ts'o in 1967.[3] Furthermore, it should be clear from their description of the head-to-head stack that hydrogen 2 *cannot* be relaxed by an inter- or intramolecular dipole–dipole relaxation process via hydrogen 8. Therefore, the head-to-tail stack proposed by Gueron and co-workers[46] is the only model which is consistent with both ^{1}H and ^{13}C[44b] chemical shift and relaxation data. In Figure 2 the A-H_2 proton lies over the imidazole ring in a manner such that it is in a region of maximum shielding. The A-H_8 proton, however, lies outside the region of maximum shielding by the pyrimidine ring as do the ribose protons.[46] This arrangement corresponds well with the chemical shift concentration and temperature dependence for 5′-AMP, which shows the A-H_2 proton as having the largest chemical shift perturbation upon changes of either the concentration or temperature.[28] It is not possible using the information from the A-H_2–A-H_8 dipole–dipole interaction to make any arguments as to the relative orientation of the various ribose units. It is clear, however, that any stacking arrangement must take into account the closeness of the interacting A-H_8 and A-H_2 protons. Recent empirical shielding calculations performed by Sarma and Mynott[26] show a model for the stacking of 5′-AMP. Unfortunately, the best fit for the experimental parameters employed came when the A-H_2 and A-H_8 protons were apparently distant from one another. This conclusion is clearly not consistent with the relaxation data presented here.

Finally, it should be noted that the A-H_2–A-H_8 intermolecular dipole–dipole interaction has been observed to some extent for all the nucleotides or nucleosides studied here. The exact nature and extent of influence of this interaction on the purine ring orientation about the glycosidic bond for these molecules is yet to be determined.

Conformationally Averaged $\langle r \rangle_{1'8}$ Distance for 2′-AMP, 3′-AMP, 5′-IMP, Adenosine, Purine Riboside, and Inosine. In Table III the measured deuterium and proton spin–lattice relaxation data, correlation times, and calculated conformationally averaged intermolecular distances for the above compounds are presented. From the tabulated values of $\langle r \rangle_{1'8}$ in Table III, it is apparent that significant amounts of both syn and anti character exist about the glycosidic linkage in the molecules 2′-AMP, 3′-AMP, adenosine, and purine riboside. For these molecules, the calculated values of $\langle r \rangle_{1'8}$ are essentially the same, 2.7 ± 0.1 Å. It should also be noted that these molecules have a smaller A-H_2/A-H_8 *intermolecular* interaction compared to 5′-AMP at the same temperature. As in the case of 5′-AMP at higher temperature, these data can be interpreted as a reflection that there is an increased flexibility of the purine base about its glycosidic linkage. That the intermolecular association at 5 °C in these systems is less than that of 5′-AMP is also apparent from their respective correlation times.

In comparison to the above mentioned compounds, the base-to-contiguous ribose conformation in 5-IMP has significantly more anti character to it, with the calculated value of $\langle r \rangle_{1'8}$ being similar to that of 5′-AMP at 30 °C. In addition, inosine exhibits a nearly exclusive anti character, with a $\langle r \rangle_{1'8}$ that is similar to 5′-AMP at 5 °C. Furthermore, the ratio of the T_1's for A-H_2 with and without a deuterium in the 8-position is 1.8. Therefore, one has to conclude that there is a stereospecific intermolecular association in inosine which is analogous to that manifested by 5′-AMP at 5 °C. It is interesting to note that at 25 °C Broom, Schweizer, and Ts'o demonstrated that inosine is considerably less stacked than adenine nucleotides. The difference noted here is, in all probability, due to the 20 °C lower temperature employed in the present study.

To what extent the intermolecular association influences the base to contiguous ribose conformational dynamics is uncertain at the present time. For those systems in which it appears to

Table IV. Relaxation Data and Calculated Conformationally Averaged Distance of $\langle r \rangle_{1'8}$ for NAD^+ [a]

	T_1 (A-H_2)	T_1 (A-H_8)	T_1 (A-$H_{1'}$)	$\Delta[T_1^{-1}$ (A-$H_{1'}$)]	$\langle r \rangle_{1'8}$	T_1 (^{2}H)	τ_c [b]
NAD^+	2.13	0.45	0.87	0.17	3.3		2.8
NAD^+-A-$^2H_8$	2.19		1.02			8.0	2.8
NAD^+-N-$^2H_{2,6}$	2.08	0.46					
NAD^+-N-$^2H_4$	2.03	0.48				7.8	2.6

[a] The units on the ^1H relaxation times and relaxation rates are seconds and seconds^{-1}, respectively. The ^2H relaxation times are in milliseconds. [b] The correlation times are in units of 10^{-10} s and they were determined by the procedure outlined in ref 55.

be stereospecific (5′-AMP and inosine) at 5 °C, it appears to lead to an anti conformation. Further research is needed, however, before this statement can be generalized.

It is of interest to compare the conclusions of our relaxation data to the "simple direct ^1H NMR" chemical shift arguments put forth by Sarma and co-workers.[28,29] They stated that because the ^1H chemical shift of A-H_2 was constant in adenine, adenosine, and 5′-AMP that it would be an indicator of an anti conformation in these systems. Furthermore, these authors utilized a 0.02–0.06 ppm chemical shift with respect to 5′-AMP to indicate a "considerable amount of time over the ribose moiety as in the syn conformation" for 8-Br-5′-AMP! These conclusions are not consistent with the relaxation data. That is, at 5 °C (see Tables I and III) the values of $\langle r \rangle_{1'8}$ for adenosine and 5′-AMP strongly support the notion that these molecules do *not* have the same conformational flexibility about the glycosidic linkage. Therefore, the above mentioned chemical shift approach to conformational dynamics has little, if any, utility at all!

Intra- and Intermolecular Association and Calculated $\langle r \rangle_{1'8}$ Distance in NAD^+. In Table IV the calculated $\langle r \rangle_{1'8}$ distance, correlation time, and relaxation data for NAD^+ are presented. From these data it is seen that the calculated value of $\langle r \rangle_{1'8}$ is 3.3 Å. This value may be compared to the 3.3 Å found for 5′-AMP in its nonassociated form and 3.4 Å for 5′-IMP which shows little evidence of stereospecific intermolecular association. In Table II it is seen that a value of 3.3 Å for $\langle r \rangle_{1'8}$ is most closely duplicated by the 180 ± 120 ° the 180 ± 90°, or the syn/anti two-state microdynamic models. As previously discussed these models cannot be unambiguously distinguished. However, all of these models represent a considerable amount of flexibility of the purine ring about the glycosidic bond. Furthermore, it is evident from the T_1 data that the purine ring in NAD^+ has the same conformational preference about its glycosidic linkage that its analogous monomer 5′-AMP has when it is not associated. This result is now compared with the pyridyl portion of NAD^+ and its analogous monomer, NMN^+. In our previous work[48] on the conformational preference of the pyridyl portion of NAD^+, we found that a two-state model best approximated the pyridyl portion of NAD^+. In this microdynamic model both the syn and anti conformations of the pyridyl ring about the glycosidic linkage are nearly equally populated with the possibility of a slight anti conformational preference. This model is also characterized by a significant amount of flexibility of the pyridyl ring about the glycosidic linkage. For NMN^+ a similar picture of the conformational dynamics of the pyridyl ring about its contiguous ribose has been formulated by the NOE studies.[43,45]

Jacobus and co-workers[43,45] found that both the syn and anti conformations of the pyridyl ring in NMN^+ about its glycosidic linkage are nearly equally populated. They further conclude that there is a slight anti preference (60/40) and that the pyridyl ring is on a time-averaged basis very mobile about its glycosidic linkage. From the above discussion it is now evident that the components within NAD^+ behave in a manner analogous to their counterparts 5′-AMP and NMN^+, with both the pyridyl and purine rings having a significant amount of flexibility about their glycosidic linkages. It is essential to point out that these motions about the respective glycosidic bonds are, in all probability, slow compared to the overall tumbling time of NAD^+.[42,48]

The question of whether NAD^+ exists in a stereospecific stacked (or folded) form in solution, and, if so, to what extent, has not been unambiguously answered. The data in Table IV are germane to this question. First, it is apparent that deuteration of A-H_8 causes little change in the T_1 of A-H_2. Hence, we can conclude that *if intermolecular* association is present in NAD^+ solutions at 5 mM, it can not be stereospecific in the same sense as 5′-AMP. Proton and ^{13}C NMR chemical shift data are consistent with the notion that NAD^+ is not associated at this concentration.[26,38] Furthermore, deuteration of either N-H_2, N-H_6, or N-H_4 in the pyridyl ring has no influence on the T_1's of the adenyl hydrogens in NAD^+. From the magnitude of adenyl ^1H T_1's and assuming an experimental error in the T_1's of 5%, we can stipulate that on a time-averaged basis the aromatic rings within NAD^+ do not approach one another closer than 4.5 Å. That is, for a time comparable to the reorientational correlation time of NAD^+, as a unit, the aromatic rings do not get closer than 4.5 Å. This, however, does not imply that the two aromatic rings cannot approach one another closer than 4.5 Å. It means that the lifetime of such a conformation is not comparable to the reorientational correlation time. *Therefore, if we further agree that significant stacking interactions are those which arise when the inter-ring distance is less than 4 Å,*[61] *we have to conclude that there is no significant intramolecular stacking interactions within NAD^+ for a time comparable to the reorientation correlation time for NAD^+, 2.0×10^{-10} s.*

In view of the above conclusions, it is appropriate at this point to discuss the relaxation data in light of previous work on conformational dynamics of NAD^+. It is apparent from ^1H and ^{13}C NMR chemical shift data there is, at the very least, a transient interaction between the two aromatic rings within NAD^+. However, it is essential to keep in mind that the so-called "ring current" or "neighboring group anisotropy" contribution to the shielding of a given nucleus is proportional to r^{-3}, whereas the relaxation data are a reflection of interactions which are proportional to r^{-6}. Herein lies a key difference between the two approaches. That is, the relaxation data are sensitive to "short range" interactions, whereas the shielding constant is more responsive to long range effects. More specifically, the shielding constant is experiencing an intramolecular interaction (the degree of specificity is still a matter of question), whereas the ^1H spin–lattice relaxation data are simply pointing out that such an interaction must be operating over distances in excess of 4.5 Å. Furthermore, even though it is difficult to imagine a specific interaction between the two rings at such a distance, the relaxation data and chemical shift data to date cannot be unambiguously interpreted with respect to this point.

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(57) Abbreviations used: 5′- or 3′- or 2′-AMP, adenosine 5′- or 3′- or 2′-monophosphate; 5′-IMP, inosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, 1,4-dihydronicotinamide adenine dinucleotide. Further, we use the atom numbering nomenclature defined in ref 47.
(58) T_2^* denotes an effective transverse relaxation time that includes the natural T_2 for the spin system plus other terms that can contribute to the line width, i.e., unhomogeneous magnetic fields, chemical exchange, etc.
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