Studies of the Interaction of Adenine and Nicotinamide Ring Systems in Aqueous Solution by High Resolution Nuclear Magnetic Resonance†

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ABSTRACT: A model system for NAD^+ has been investigated using a paramagnetic transition metal ion as a probe. The well-known complexation of Mn^{2+} by adenine nucleotides was utilized to "label" adenosine 5′-diphosphate. A broadening effect on the 100-MHz proton nuclear magnetic resonance spectrum of N^1-methylnicotinamide due to the adenine–metal ion complex was observed. It was found that the nicotinamide species showed no evidence for interaction with Mn^{2+} in the absence of the adenine nucleotide. These observations have led to the proposal that N^1-methylnicotinamide associates with the adenine moiety of the adenine nucleotide–metal complex. This suggests a tendency of adenine and nicotinamide rings to interact in aqueous solution implying some tendency of the coenzyme NAD^+ to occur in a folded or stacked conformation.

A continuing interest in this laboratory is the nature of the association of the coenzyme NAD^+/NADH with alcohol dehydrogenases (Hollis et al., 1966; Hollis, 1967; Czeisler and Hollis, 1973) and the conformation of the coenzyme in the bound and free states (Catterall et al., 1969; Hollis, 1969; McDonald et al., 1972). In investigating these questions we have been searching for model systems which would allow the evaluation of the contributions of the various sections of the coenzyme to its binding to enzyme sites. Significant results were obtained by a combination of X-ray and kinetic studies (McPherson, 1970) by studying the binding of adenosine 5′-monophosphate (AMP) and nicotinamide mononucleotide (NMN^+) to dogfish lactate dehydrogenase. We have been studying a combination of adenosine 5′-diphosphate (ADP) and N^1-methylnicotinamide ($NMeN^+$) as a model for NAD^+. This combination avoids the difficulty of the repulsion of negative charges located on the phosphate groups of two nucleotides such as AMP and NMN^+. The two molecules selected have a pyrophosphate group as does the coenzyme. This factor combined with the same total number of moieties as the coenzyme (the methyl group is considered as a single moiety replacing the ribose ring of the coenzyme) suggests this system as a good primary model. Here we report results obtained for this model relating to the conformation of the coenzyme in aqueous solution.

Considerable attention has been devoted in recent years to the conformation of NAD^+ in aqueous solution. In many studies nuclear magnetic resonance (NMR) has been the main technique used (Jardetzky and Wade-Jardetzky, 1966; Sarma et al., 1968, 1970, 1973; Sarma and Kaplan, 1969a,b, 1970a,b; Catterall et al., 1969; McDonald et al., 1972; Ellis et al., 1972; Blumenstein and Raftery, 1972; Sarma and Mynott, 1973a,b). The early work of Jardetzky and Wade-Jardetzky (1966) compared the proton chemical shifts of the coenzyme with those of a model system containing adenine and nicotinamide mononucleotides. Several groups have studied the concentration, temperature, and solvent effects on the chemical shifts, in terms of shielding effects due to ring–ring interactions. An analysis of the proton chemical shifts by McDonald et al. (1972) suggested a dynamic, multistate folding process, with about 15% of the oxidized dinucleotide in the folded form. An alternate interpretation was discussed by Ellis et al. (1972), who suggested that the chemical shift effects could be explained in terms of a nonspecific "intramolecular aromatic-induced solvent shift" (Laszlo, 1967). Ellis et al. (1972) also showed that the pH dependence of the ^{13}C chemical shifts of NAD^+ are consistent with long range substituent effects. Accordingly, unfolding due to electrostatic repulsion of the nicotinamide quaternary nitrogen and a protonated N-1 of the adenine ring would not be required to explain the data.

This alternate interpretation of the chemical shift data suggests the need for further studies of adenine ring nicotinamide ring interactions using a complementary approach. One approach is the use of relaxation effects as an indicator of molecular interactions. Nuclear magnetic relaxation is commonly dominated by magnetic dipole–dipole interactions and it varies as the inverse sixth power of the interdipole distance, providing a geometric measure of the interaction. In internuclear magnetic interactions each dipole is quite weak and relaxation effects, as reflected in resonance line widths, are small. By contrast, the large magnetic dipole of a paramagnetic transition metal ion produces a much larger relaxation effect on a nuclear dipole. Thus, other conditions being equal, the paramagnetic ion causes a much larger spectral effect. We have used this paramagnetic ion effect in the present work to probe the interaction of nicotinamide and adenine rings as a model system for NAD^+. A similar approach has been used previously by Evans and Sarma (1974).

Experimental Section

All materials were reagent grade. N^1-Methylnicotinamide chloride was obtained from K & K Laboratories, Inc. (Plainview, N.Y.). Nicotinamide mononucleotide and 5′-adenosine diphosphate were from Sigma Chemical Co. (St. Louis, Mo.). 5′-Adenosine monophosphate was from Schwarz/Mann (Orangeburg, N.Y.). Nicotinamide was from Eastman Organic Chemicals (Rochester, N.Y.). Manganous chloride tetrahydrate and sodium oxalate were from J. T. Baker Chemical Co. (Phillipsburg, N.J.). Deuterium

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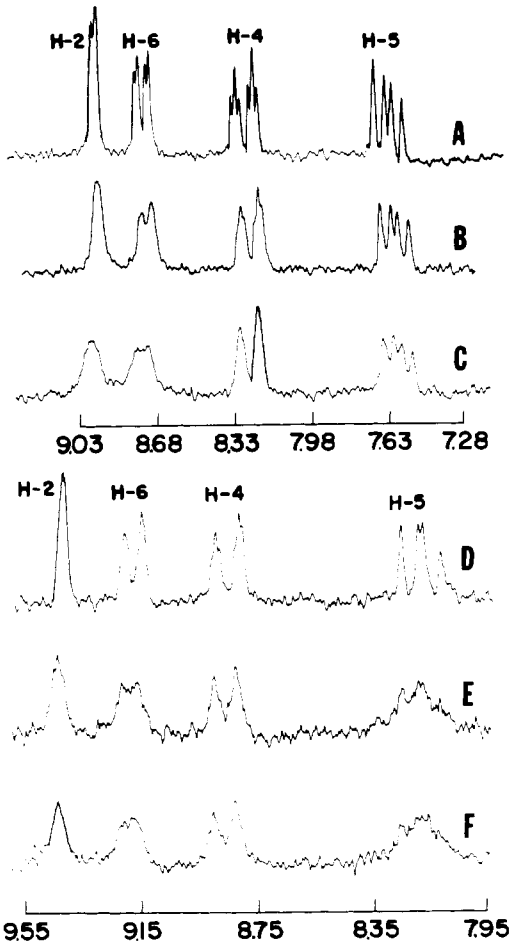

FIGURE 1: The 100-MHz spectra of 25 m*M* nicotinamide (A–C) and 25 m*M* NMN^+ (D–F) in D_2O at pD 7.6. All solutions contain 0.02 m*M* EDTA and 10 m*M* sulfonate. Sequence of traces is with increasing concentrations of $MnCl_2$ in each case. (A) No $MnCl_2$; (B) 0.08 m*M*; (C) 0.28 m*M*; (D) no $MnCl_2$; (E) 0.08 m*M*; (F) 0.012 m*M*. All spectra resulted from 9 or 16 passes at about 2.0 Hz/sec. Scale is in parts per million from the sulfonate reference.

oxide (99.7 atom %, Diaprep, Inc.) and EDTA (sodium salt, tetrahydrate) were from Aldrich Chemical Co. (Milwaukee, Wis.).

Proton NMR spectra at 100 MHz were obtained with the Varian HA-100 high-resolution spectrometer equipped with a 15-in. magnet. Large diameter (12 mm o.d.) sample tubes (Wilmad, Inc.; Buena, N.J.) were used. The sample temperature was 32°. Signal-to-noise enhancement was achieved with a Varian 1024 computer of average transients equipped with a voltage controlled oscillator for frequency sweep. An internal reference signal, for field-frequency locking, was obtained by the addition of a small quantity of sodium 3-(trimethylsilyl)propanesulfonate (hereafter referred to as the sulfonate), a product of E. Merck Ag. (Darmstadt, Germany) purchased from Brinkmann Instruments, Inc. (Westburg, N.J.). The amplitude of the reference signal was monitored with each spectrum.

All solutions were prepared in D_2O and adjusted to pD 7.6 with NaOD or DCl in D_2O.

Results and Discussion

The spectra in Figure 1A–C show the effect of the Mn^{2+} ion on the spectrum of nicotinamide. Because pyridine is known to be a ligand of manganous ion (Atkinson and Bauman, 1963) the broadening effects with increasing metal ion concentration are quite reasonable. This is all the more expected for a molecule such as nicotinamide with a second ligand site such as the amide group. The preferential broadening of H-2 and H-6 is consistent with Mn^{2+} binding to the ring nitrogen. No shifts were detected suggesting the importance of the dipolar contribution to the relaxation over the scalar term of the Solomon–Bloembergen equations (Solomon, 1955; Solomon and Bloembergen, 1955; Bloembergen, 1957).

Figure 1 also shows (D–F) the effect of the manganous ion on the nicotinamide ring proton signals of NMN^+. In this case the broadening occurs at a lower concentration of metal ion possibly indicating a larger association constant for the complex or a closer approach of the Mn^{2+} to the affected proton. The pyridine nitrogen is unavailable as a binding site due to its attachment to the ribose group. This suggests binding of the metal at the phosphate of the nucleotide with a possible secondary site at the amide. The preferential broadening of H-5 and H-6 indicates orientation of the phosphate bound Mn^{2+} toward the H-5, H-6 side of the nicotinamide ring.

Figure 2A and B show the effect of Mn^{2+} on the nicotinamide ring proton resonances of N^1-methylnicotinamide (chloride). That the spectrum is essentially unaffected by the addition of Mn^{2+} can be rationalized by two factors. Firstly, the primary ligand site, the ring nitrogen of the pyridine system, is occupied by the N^1-methyl group. Secondly, the pyridine nitrogen is quaternary giving the entire ring system a diffuse positive charge. The electrostatic repulsion between the positive ring and the positive metal ion almost certainly prevents significant complex formation at the secondary site as well. In the case of the effect of Mn^{2+} on the spectrum of NMN^+, charge neutralization of the metal ion by the phosphate probably alleviates the electrostatic interaction between ring and metal ion. Such a charge neutralization could allow a closer approach of the manganese toward the ring, a highly improbable arrangement for $NMeN^+$. Effective outer sphere dipolar relaxation of the $NMeN^+$ ring protons is probably also prevented by the unfavorable charge interaction of the two species; hence we find essentially no relaxation effects. It is interesting that in trace B of Figure 2 the low-field resonance is of lower relative intensity than in trace A. The sample used in trace B was from the same stock solution of $NMeN^+$ but recorded some time after trace A. This appears to be evidence for slow exchange of the nicotinamide C-2 proton with the deuterons of the D_2O solvent, which is quite reasonable in view of the rapid exchange of this proton above pD 10 (San Pietro, 1955).

Figure 2C–F show a sequence of spectra for $NMeN^+$ as in A and B. However, in this case the measurements were taken in the presence of a low concentration of ADP. The obvious effect is that at a concentration of 0.60 m*M* $MnCl_2$, broadening of the $NMeN^+$ spectrum by Mn^{2+} does occur in the presence of ADP but does not occur in its absence. The most direct interpretation of this observation is that the $NMeN^+$ species cannot bind to the metal ion alone whereas it can bind to a Mn–ADP complex (Izatt et al., 1971). To establish that the species causing the observed broadening is actually an ADP–Mn complex, spectra were recorded as a function of ADP concentration at constant Mn^{2+} concentration. The results in Figure 3 show that the broadening of the $NMeN^+$ spectrum depends on the concentration of the adenine nucleotide. Thus the concentration of the species causing the relaxation effects which are reflected in the

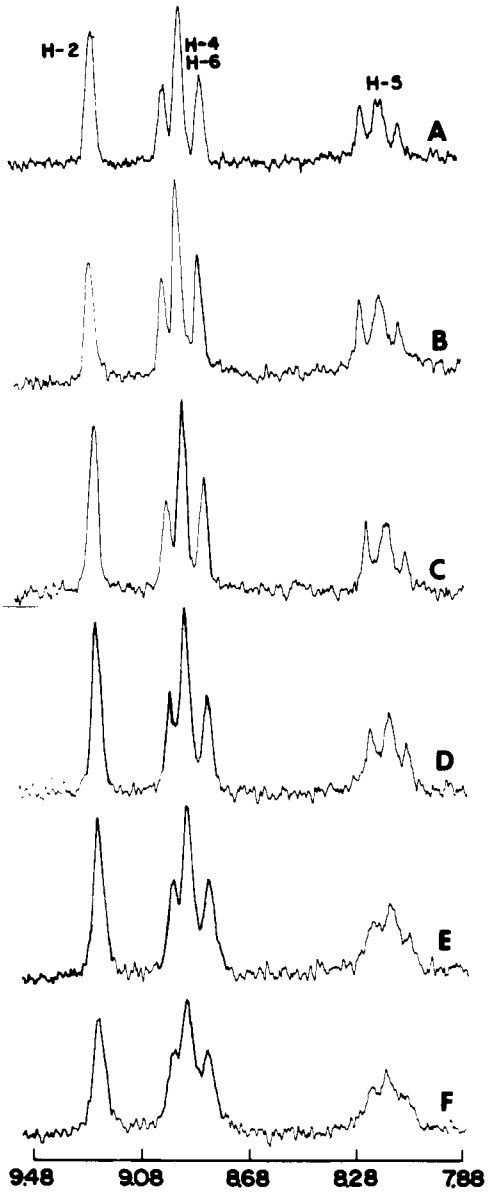

FIGURE 2: The 100-MHz spectra of 25 m*M* *N'*-methylnicotinamide in D_2O at pD 7.6. All solutions contain 0.02 m*M* EDTA and 10 m*M* sulfonate. In addition, C, D, and F contain 1.0 m*M* ADP. Sequence of traces is with increasing concentrations of $MnCl_2$ in each case. (A) No $MnCl_2$; (B) 0.60 m*M* $MnCl_2$; (C) no $MnCl_2$; (D) 0.20 m*M*; (E) 0.4 m*M*; (F) 0.60 m*M*. All traces resulted from 16 passes at 2.0 Hz/sec. Scale is in parts per million from the sulfonate reference.

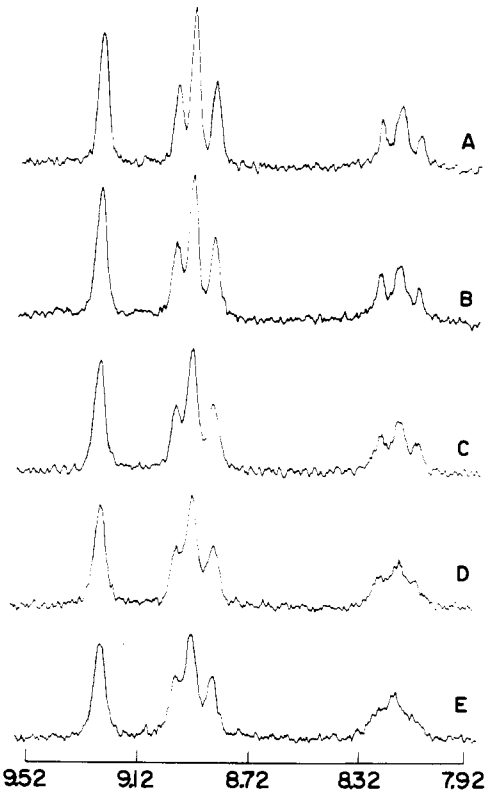

FIGURE 3: The 100-MHz spectra of 20 m*M* N^1-methylnicotinamide in D_2O at pD 7.6. All solutions contain 0.02 m*M* EDTA, 7.5 m*M* sulfonate, and 0.60 m*M* $MnCl_2$. Sequence of traces is with increasing ADP concentration. (A) No ADP; (B) 0.20 m*M*; (C) 0.60 m*M*; (D) 1.0 m*M*; (E) 1.2 m*M*. All spectra are the result of 25 passes at a sweep rate of 2.0 Hz/sec. Scale is in parts per million from the sulfonate reference.

$NMeN^+$ spectrum depends on both metal and ADP concentration. At the pH of these studies, the dissociation constant for the 1:1 ADP-Mn complex is about 10^{-4} *M* (Walaas, 1958; Taqui Khan and Martell, 1967). It is then easy to show that for $[Mn^{2+}] < [ADP]$ most of the Mn^{2+} ion is in the complexed form.

Two questions now arise. The first is whether simple complexation or chelation of the Mn^{2+} ion alters it so that the $NMeN^+$ can bind directly to it where such an interaction would not be possible with the uncomplexed ion. The spectra in Figure 4 show the effect of the addition of sodium oxalate to a solution containing $NMeN^+$ and Mn^{2+} ion. The dissociation constant for the 1:1 oxalate-Mn complex (McAuley and Nancollas, 1961) is very close to that for the ADP-Mn complex (Walaas, 1958; Taqui Khan and Martell, 1967). Hence, at comparable concentrations of ligand, ADP, or oxalate, the concentration of complexed Mn^{2+} should be the same. Figure 4 shows that the oxalate-Mn complex causes essentially no detectable nuclear magnetic relaxation of the protons of $NMeN^+$ in excess of that due to uncomplexed Mn^{2+}. Thus simple complexation or chelation of the Mn^{2+} ion does not seem to allow it to complex directly with the NMeN.

The effects shown in Figures 1-4 are quite different from those observed for the sulfonate internal reference signal. Changes in the sulfonate line width were detected as changes in the reference signal level which was recorded with each spectrum. With increasing Mn^{2+} concentration the signal level decreased, indicating a broadening of the reference signal. Subsequent addition of ADP caused the reference signal to increase again to the original level as measured in the absence of Mn^{2+}, indicating renarrowing of the sulfonate signal. Yet while this signal was narrowed the signals for $NMeN^+$ were broadened by the addition of ADP as shown in Figure 3. The addition of oxalate to a solution of Mn^{2+} and $NMeN^+$ also caused the reference signal level to increase indicating its narrowing. Yet the addition of oxalate to form the oxalate-Mn complex caused no change in the $NMeN^+$ spectrum. Thus the sulfonate molecules had negligible access to the "relaxation sphere" of the complexed manganous ion, while $NMeN^+$ was affected by the Mn^{2+}-ADP complex but not by free Mn^{2+}.

The second question concerns the nature of the electrostatic interaction between $NMeN^+$ and the ADP-Mn complex. Since at pH 7-8 ADP has a charge of -3 while the manganous ion has a charge of $+2$, the complex should

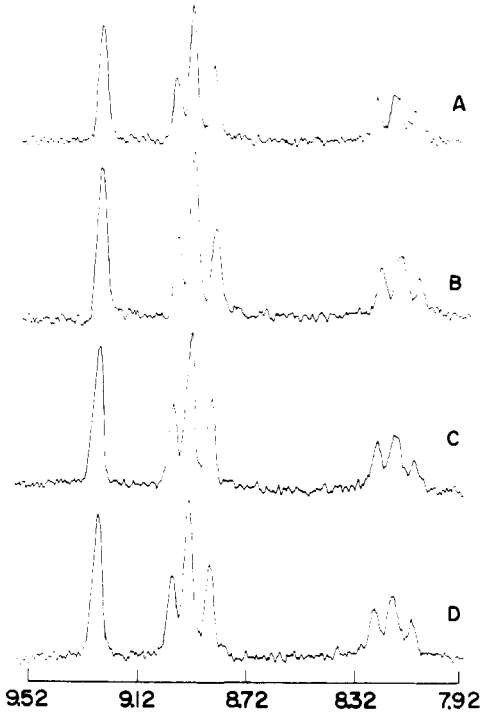

FIGURE 4: The 100-MHz spectra of 20 mM N^1-methylnicotinamide in D_2O at pD 7.6. All solutions contain 0.02 mM EDTA, 7.5 mM sulfonate, and 0.60 mM $MnCl_2$. Sequence of traces is with increasing sodium oxalate concentration. (A) No oxalate; (B) 0.60 mM; (C) 1.0 mM; (D) 1.20 mM. All spectra are the result of 25 passes at a sweep rate of 2.0 Hz/sec. Scale is in parts per million from the sulfonate reference.

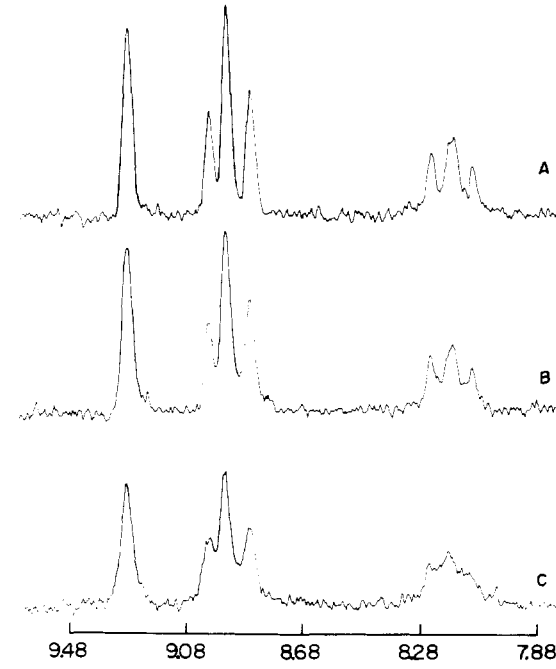

FIGURE 5: The 100-MHz spectra of 20 mM N^1-methylnicotinamide in D_2O at pD 7.6. All solutions contain 0.02 mM EDTA and 7.5 mM sulfonate. Trace A contains neither $MnCl_2$ or AMP. Trace B contains 1.0 mM $MnCl_2$. Trace C contains 1.0 mM $MnCl_2$ and 6.0 mM AMP. Spectra are the result of 25 passes at a sweep rate of 2.0 Hz/sec. Scale is in parts per million from the sulfonate reference.

have a net charge of -1. This net charge could cause an association interaction permitting nuclear magnetic relaxation of the $NMeN^+$ protons. To investigate this possibility the experiment was repeated in the presence and absence of AMP. The AMP-Mn complex should be neutral in charge due to the -2 charge of AMP. The difficulty in this case is that the dissociation constant of the 1:1 AMP-Mn complex (Walaas, 1958; Taqui Khan and Martell, 1967) is much greater than for the comparable complex of ADP. This has the effect of lowering the concentration of complex in solution, and thus reducing any possible relaxation effect on the $NMeN^+$. In order to maintain the ratio of $NMeN^+$ to adenine-metal complex, the concentrations of Mn^{2+} and adenine nucleotide were increased. Figure 5 shows the effect of the addition of AMP to a solution of $NMeN^+$. The effect is essentially the same as that observed upon addition of ADP. Thus the presence of the adenine ring and metal ion are the determining factors in the line broadening effects while the charge of the complex does not appear to be relevant. Note that at the concentration of 1.0 mM $MnCl_2$ a very slight direct effect on $NMeN^+$ is observed in trace B.

An additional concern is the position of the metal ion relative to the adenine ring. The manganous ion is probably bound to the phosphate groups of ADP and perhaps to the adenine ring itself at N-7 (Cohn and Hughes, 1962). Evidence from studies of AMP (Chan and Nelson, 1969; Kotowycz and Hayamizu, 1973; Kotowycz and Suzuki, 1973) and ATP (Cohn and Hughes, 1962; Sternlicht et al., 1965a,b; Glassman et al., 1971) generally suggested that the manganous ion is between the phosphate and the N-7 of the adenine ring. Evidence from optical rotatory dispersion (Brintzinger, 1961) suggests interaction of the metal ion with the 2′- and 3′-hydroxy groups of the ribose ring, consistent with its position near the phosphate and N-7 sites. There are at least three ways to rationalize the broadening which occurs in the presence but not in the absence of the adenine nucleotide. First, $NMeN^+$ and Mn^{2+} could associate resulting in a severe shortening of τ_e, the electron correlation time. The correlation time which determines the relaxation rate is the period of the fastest process which modulates the magnetic interaction. Thus, if τ_e were very short it would determine the relaxation rate which would be quite small, i.e., no broadening. A second explanation invokes the exchange of $NMeN^+$ between an NMeN-metal complex and the free solution state. If the residence time, τ_m, of $NMeN^+$ in such a complex is sufficiently long there may be no broadening in the spectrum of the ligand. Formation of a ternary complex with ADP could then lead to line broadening by increasing τ_e or decreasing τ_m, respectively. Both of these explanations may be eliminated since they require formation of a relatively stable complex from two positively charged species. The electrostatic repulsion between Mn^{2+} and $NMeN^+$ almost certainly precludes formation of such a complex. A third way to explain the results relies on the tendency of adenine nucleotides to chelate metal ions (Izatt et al., 1971). A complex of adenine nucleotide and Mn^{2+} would not be prevented by Coulombic repulsion from forming a complex with $NMeN^+$ causing nuclear magnetic relaxation of the latter. That charge neutralization alone of the Mn^{2+} is not sufficient to allow its interaction with $NMeN^+$ is shown by the lack of spectral broadening in the presence of the oxalate-Mn^{2+} complex. Thus the $NMeN^+$ does not attach itself to the Mn^{2+} ion directly even when the charge of the metal ion is neutralized.

We conclude that the association of $NMeN^+$ and the adenine nucleotide-manganese complex is due to an interaction between the adenine and nicotinamide rings. We cannot tell to what extent the manganous ion might polarize

the adenine ring or otherwise predispose it to associate with $NMeN^+$. Nevertheless, the present results together with chemical shift data (Jardetzky and Wade-Jardetzky, 1966; Sarma et al., 1968, 1970, 1973; Sarma and Kaplan, 1969a,b, 1970a,b; Catterall et al., 1969; McDonald et al., 1972; Blumenstein and Raftery, 1972; Sarma and Mynott, 1973a,b) indicate that weak forces between nicotinamide and adenine rings in aqueous solution produce a measurable fraction of NAD^+ molecules, in a folded or stacked arrangement.

In summary, we have observed that the 100-MHz proton NMR spectrum of N^1-methylnicotinamide in aqueous solution is not affected when manganous ion is added to a concentration level of 0.60 m*M*. This effect is explained in terms of charge repulsion of the two positive species. Addition of 5′-adenosine diphosphate to a solution of $NMeN^+$ and Mn^{2+} results in a broadening of the spectrum of $NMeN^+$. Addition of oxalate ion, in place of ADP, does not cause broadening. Our interpretation of the results is that molecular association occurs between nicotinamide and adenine rings. This supports the idea that the coenzyme NAD^+ has a measurable tendency to exist in a folded conformation in aqueous medium.

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