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Natural Abundance ^{13}C Nuclear Magnetic Resonance Spectra of Nicotinamide Adenine Dinucleotide and Related Nucleotides†

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ABSTRACT: The natural abundance carbon-13 spectra of the following nucleotides have been obtained and assigned: reduced and oxidized nicotinamide adenine dinucleotide, reduced and oxidized nicotinamide adenine dinucleotide phosphate, reduced and oxidized nicotinamide mononucleotide, α-nicotinamide adenine dinucleotide, and adenylic acid. Small shifts (2–6 Hz) are observed in the nicotinamide carbon resonances of nicotinamide adenine dinucleotide upon protonation of the adenine ring, indicating an intramolecular interaction between the two bases. Titration of the phosphate group in nicotinamide mononucleotide results in shifts of the nicotinamide resonances, an effect which is not seen in the titration of reduced nicotinamide mononucleotide. This effect is due to an interaction between the negatively charged phosphate and the positively charged nicotinamide ring. The spectra due to the nicotinamide portion of nicotinamide adenine dinucleotide and nicotinamide mononucleotide are virtually identical, while differences exist between the spectra due to the dihydronicotinamide moieties of reduced nicotinamide adenine dinucleotide and reduced nicotinamide mononucleotide. A likely explanation for the latter effect is a slightly different orientation of the dihydronicotinamide ring relative to the ribose ring in the reduced mononucleotide as compared to the reduced dinucleotide. The shifts due to the nicotinamide moiety of α-nicotinamide adenine dinucleotide differ by a considerable amount (25–40 Hz) from the corresponding resonances in the β-dinucleotide. The chemical shifts of the ribose carbons are highly dependent on the attached base, but are not greatly different between the corresponding mono- and dinucleotides. Very minor effects upon the carbon-13 spectra were seen in varying the concentration of nicotinamide adenine dinucleotide from 0.1 to 0.008 M, thus indicating that intermolecular base stacking does not have a large effect on the ^{13}C shifts observed with this compound. The T_1 of the carbonyl carbon in nicotinamide adenine dinucleotide-*carbonyl*-^{13}C was determined to be 14 ± 2 sec.

Nicotinamide adenine dinucleotide (NAD^+ [1]) has been widely studied by pmr methods (Jardetzky *et al.*, 1963; Jardetzky and Wade-Jardetzky, 1966; Catterall *et al.*, 1969; Hollis, 1969; Sarma *et al.*, 1968a,b, 1970; Sarma and Kaplan, 1969a,b, 1970a,b; Griffith *et al.*, 1970). One major conclusion from these studies is that at room temperature above pH 4, the pK_a value of N-1 of the adenine ring in aqueous medium, the dinucleotide exists in folded form,[2] with the nicotinamide and adenine rings lying in parallel planes. It has been shown, however, that raising the temperature (Sarma and Kaplan, 1970a), lowering the pH below a value of 4 (Catterall *et al.*, 1969), or going to a methanol–water solvent mixture (Catterall *et al.*, 1969) causes the dinucleotide to unfold. In a recent communication from this laboratory (Blumenstein and Raftery, 1972), utilizing ^{31}P nmr, we have suggested an electrostatic interaction between the negatively charged diphosphate backbone and the positively charged nitrogen of the nicotinamide ring of NAD^+. This interaction was pH independent, indicating that it did not depend on the folding of the dinucleotide.

The natural abundance cmr results presented here give additional information concerning the solution conformation of NAD^+ and related compounds. Also, an indication is given of what kind of problems are especially suited to study by

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[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide; $NMNH_2$, reduced nitotinamide mononucleotide; α-NAD^+, α-nicotinamide adenine dinucleotide; AMP, 5′-adenylic acid; pmr, proton magnetic resonance spectroscopy; nmr, nuclear magnetic resonance spectroscopy; cmr, ^{13}C nuclear magnetic resonance spectroscopy; UMP, uridylic acid.

[2] The terminology "folded form" as used in this paper refers to an equilibrium mixture of folded and unfolded dinucleotide.

TABLE I: ^{13}C Chemical Shifts of NAD^+ and Related Nucleotides.[a]

		Adenine Ring					Adenine Ribose				
	pH	A-2	A-4	A-5	A-6	A-8	A-1′	A-2′	A-3′	A-4′	A-5′
AMP	2.5	801	718	1468	674	868	2233	2569	2681	2331	2829
AMP	5.0	627	713	1476	558	931	2256	2576	2680	2338	2830
AMP	8.5	611	707	1473	544	926	2262	2576	2673	2320	2853
NAD^+	2.5	799	720	1471	677	865	2236	2275	2683	2333	2811
NAD^+	7.5	607	706	1471	545	933	2265	2587	2678	2340	2806
NAD^+ (0.008 M)	8.0	601				931	2264	2591	2678	2342	2810
NADH	8.0	608	708	1469	541	937	2255	2682	2575	2350	2810
α-NAD^+	2.5	795	719	1470	671	869	2237	2681	2568	2332	2805
α-NAD^+	7.5	609	710	1475	543	932	2267	2680	2574	2335	2802
$NADP^+$	2.5	799	718	1474	676	853	2264	2685	2510	2337	2806
$NADP^+$	5.1	631	710	1480	565	920	2287	2688	2527	2349	2806
$NADP^+$	8.0	610	706	1475	546	929	2281	2685	2537	2361	2803
NADPH	8.1	610	700	1464	539	931	2264	2684	2529	2370	2804
	pH	N-2	N-3	N-4	N-5	N-6	N-1′	N-2′	N-3′	N-4′	N-5′
		Nicotinamide Ring					Nicotinamide Ribose				
NMN	4.0	929	1078	774	1116	863	1933	2493	2663	2253	2837
NMN	8.0	932	1078	768	1116	846	1922	2492	2652	2228	2863
NAD^+	2.5	930	1081	776	1111	862	1934	2497	2668	2257	2811
NAD^+	7.5	931	1087	782	1113	867	1933	2497	2674	2249	2815
NAD^+ (0.008 M)	8.0	929		779	1111	865	1931	2497	2674	2242	2816
α-NAD^+	2.5	904	1127	795	1151	836	1914	2647	2669	2256	2804
α-NAD^+	7.5	914	1139	808	1156	844	1914	2467	2668	2254	2802
$NADP^+$	2.4	933	1083	776	1111	863	1936	2499	2671	2264	2815
$NADP^+$	5.1	933	1089	783	1114	870	1936	2500	2676	2265	2820
$NADP^+$	8.0	935	1094	788	1116	875	1936	2500	2679	2273	2817
		Dihydronicotinamide Ring					Dihydronicotinamide Ribose				
$NMNH_2$	5.2	974	1919		1798	1307	2061	2673		2372	2819
								2668			
$NMNH_2$	8.5	971	1918	3897	1797	1309	2059	2671	2671	2357	2841
NADH	8.0	971	1933	3899	1801	1330	2056	2676		2380	2791
								2668			
NADPH	8.2	974	1934		1804	1334	2058	2674		2378	2794
								2668			

[a] Shifts are in hertz at 25.1 MHz and are referred to a $CH_3{}^{13}CO_2H$ standard. All shifts are upfield relative to the standard.

cmr spectroscopy, as well as those which are better studied by pmr, or other methods.

Results

The positions of the ^{13}C resonances of the nucleotides used in this study are listed in Table I. The spectra of NAD^+ and NADH are shown in Figure 1. Spectra were taken at pH values above and below the pK_a values of ionizable groups in the molecules to monitor the effects of these ionizations on the ^{13}C spectra. The differing intensities of the peaks in each spectrum arise primarily from partial saturation of quaternary carbons. It is thus observed that peaks due to C-4, -5, and -6 of the adenine ring are less intense than any others. The decreased peak heights of the 4′ and 5′ peaks of the ribose rings are not due to decreased intensity of those peaks but rather to splitting caused by the phosphorus atoms of the diphosphate backbone.

Assignment of Resonances. Since the ^{13}C spectrum of AMP (at pH 7) has been previously reported (Dorman and Roberts, 1970) and since it was found that the adenine ring resonance positions are very similar in all compounds containing this moiety, these resonances could be assigned in the high pH spectra of NAD^+, α-NAD^+, NADH, $NADP^+$, and NADPH, in addition to AMP. Furthermore, the spectrum of ATP at pH 2.5 has been reported (Dorman and Roberts, 1970), and it was found that at low pH resonances of the nucleotides used in this study corresponded almost exactly to the ATP resonances. Similarly, from the AMP assignments for the ribose ring, the peaks due to C-1′, -4′, and -5′ of the corresponding ribose in NAD^+, α-NAD^+, and NADH could be assigned.

There has been a disagreement on the assignment of the 2′ and 3′ resonances in nucleotides, with one assignment (Dorman and Roberts, 1970) being based on a comparison of normal nucleotides with 2′-deoxynucleotides, and the other assignments (Mantsch and Smith, 1972) based on the effect of a phosphate group on the chemical shifts of the ribose ring carbons. In order to definitively assign C-2′ and -3′ of AMP, single frequency decoupling was used, and it was found that the resonance due to C-2′ is further downfield than that due to C-3′. This is in agreement with the newest assignment (Mantsch and Smith, 1972) made for C-2′ or -3′ of 5′-UMP.

C-1′, -4′ and -5′ of the riboses in NMN and $NMNH_2$ could be assigned as described previously (Dorman and

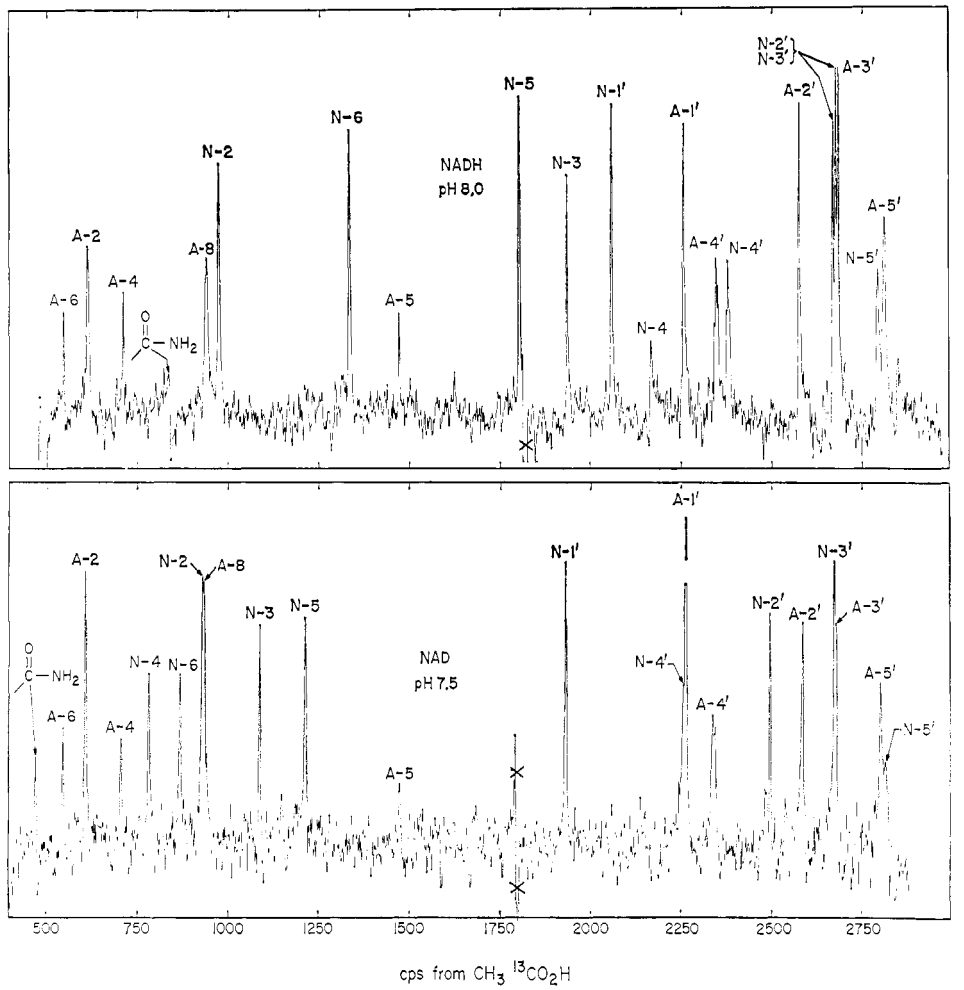

FIGURE 1: Natural abundance, proton noise decoupled ^{13}C spectra of 0.1 M NAD^+ (lower) and NADH (upper). Accumulation time for each spectrum was 2 hr. A and N refer to the adenine and nicotinamide (or dihydronicotinamide) rings, respectively. In each spectrum, the carbonyl resonance is folded in from the left side, while in the NADH spectrum the N-4 peak is folded in from the right side. In each spectrum, the peak marked with a cross at 1800 cps is probably due to the methyl group of acetone, which is an impurity in the nucleotide samples. This peak is folded in from the right side.

Roberts, 1970). The 2′ and 3′ resonances of NMN were assigned by single frequency decoupling, while the 2′ and 3′ resonances of $NMNH_2$ either exactly coincided or were too close to be individually assigned. Ribose assignments in the dinucleotides were made on the basis of the mononucleotide assignments, the only difficulties being encountered in the case of the 5′ carbons, which are very close to each other in the dinucleotides, and whose positions are somewhat shifted from the mononucleotides. Since the 5′ peak of AMP is downfield of the corresponding peak in NMN, the further downfield 5′ peak of NAD^+ was assigned to adenine ribose. Similarly, the 5′ peak of AMP is upfield of the corresponding peak in $NMNH_2$, so in NADH the adenine ribose was assumed to give rise to the 5′ peak to highest field.

The spectrum of the ribose adjacent to the adenine ring of NADPH (and $NADP^+$) is very complex, due to the additional phosphate which is expected to split the 1′, 2′, and 3′ resonances. This ribose portion of the NADPH spectrum is shown in Figure 2 and was assigned by comparing the ribose chemical shifts with those of AMP, in a manner described for the assignment of 2′-UMP (Mantsch and Smith, 1972). The $^{13}C-^{31}P$ coupling constants are also similar to those found in 2′-UMP, being 8 Hz for $J_{C\text{-}1'\text{-}P}$, 4 Hz for $J_{C\text{-}2'\text{-}P}$ and 2 Hz for $J_{C\text{-}3'\text{-}P}$ in NADPH, while the corresponding values in 2′-UMP are 9, 4.5, and 3 Hz (Mantsch and Smith, 1972). The spectrum due to the adenine ribose of $NADP^+$ was virtually identical with that of NADPH and was therefore assigned identically.

The assignment of C-2, -4, and -6 of the nicotinamide ring in the oxidized nucleotides was done by normal decoupling, C-3 was assigned by off-resonance decoupling, and the remaining peak due to this moiety was assigned to C-5. The decoupling experiments were performed on NAD^+, and due to the great similarity of the spectra, are almost certainly valid for NMN^+ and $NADP^+$. The spectrum of α-NAD^+ is somewhat different (see below) but, since the proton spectrum has not been definitively assigned, peaks were assigned by assuming that each peak in α-NAD^+ was due to the carbon which corresponded to the closest peak in the NAD^+ spectrum.

The peak to lowest field due to the dihydronicotinamide moiety of NADH could be decoupled by placing the decoupler at a frequency corresponding to N-2–H. Decreasing the decoupler frequency (*i.e.*, moving upfield) by 100 Hz caused decoupling of two resonances, one of which had previously been assigned to A-1′, and the other of which was now assigned to N-6. Moving the decoupler frequency 140 Hz further upfield caused decoupling of two more resonances, one of which was previously assigned to N-1′ and the second of which was now assigned to N-5. The peak due to N-3 could be assigned by off-resonance decoupling and C-4, being a sat-

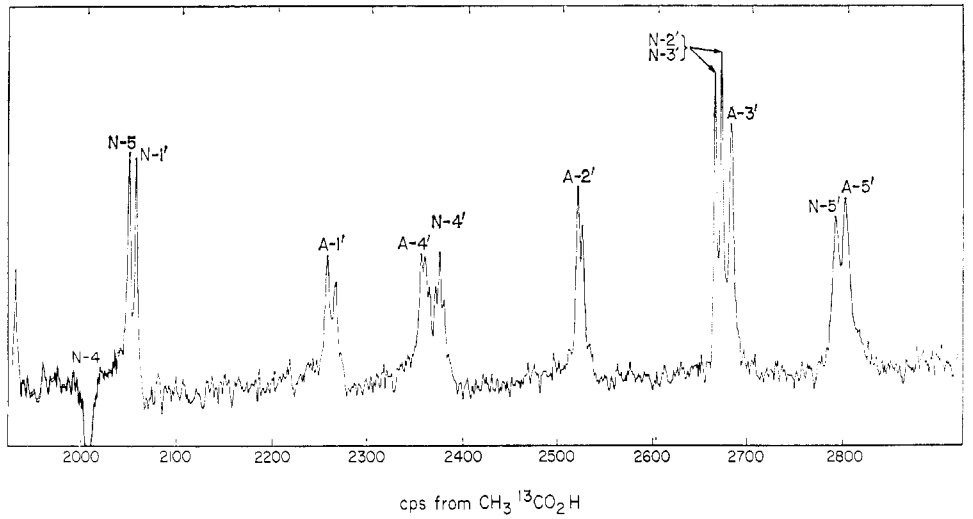

FIGURE 2: Ribose region of the ^{13}C spectrum of NADPH. The N-4 peak is folded in from the right side of the spectrum, while the N-5 peak (which is completely in phase) is folded in from the left side.

urated carbon, came in a totally different area of the spectrum and was in fact outside the spectral region which was observed. The peak was sometimes "folded back" into the spectrum, but it was often not seen when using a 2500-Hz sweep width. Its position was measured (in NADH and $NMNH_2$) by using a 5000-Hz sweep width.

It should be noted that the ^{13}C assignments are in agreement with the newest assignments for the proton spectrum of NADH (Oppenheimer *et al.*, 1971), and are in fact a strong confirmation of their accuracy. Using the old assignments, wherein the N-1′ and N-6 were reversed, the ^{13}C resonances of these carbons would exchange their positions, a shift of 30 ppm, and both resonances would then have phenomenally anomalous positions. The presently assigned positions of these resonances are quite reasonable.

The assignments for NAD^+ at low pH are in agreement with recently published assignments (Birdsall *et al.*, 1972) for this compound at pH 3.

The relaxation time (T_1) of the carbonyl carbon of NAD^+-*carbonyl*-^{13}C (Blumenstein and Raftery, 1972) was determined using the $[\ldots T\ldots 90°(S_\infty)\ldots T\ldots 180°\ldots t\ldots 90°(S_t)\ldots]_n$ pulse sequence described by Freeman and Hill (1971). The T_1 value was found to be 14 ± 2 sec and within experimental error was pH independent.

Discussion

pH Effects. The protonation of the adenine ring in NAD^+ leads to small downfield shifts (2–6 Hz) of all resonances due to the carbons on the nicotinamide ring. This is in agreement with proton results (Catterall *et al.*, 1969) and is indicative of the dinucleotide at neutral pH and an unfolding at low pH. The effect of folding of the dinucleotide is of similar magnitude (0.1–0.2 ppm) to that found in pmr spectra (Catterall *et al.*, 1969). This is not surprising since if the effect is due to the ring current of the adenine ring, it is magnetic rather than electric in nature, and should be the same for all nuclei.

As expected, the ^{13}C spectrum of the nicotinamide portion of NAD^+ at low pH, where the dinucleotide is unfolded, is virtually identical with that of NMN at pH 4, where the mononucleotide has a singly ionized phosphate group. The spectrum of NMN when it is doubly ionized (pH 8) is different, however, due to an interaction between the positively charged nicotinamide ring and the phosphate group. It is therefore not valid to determine the effect of the adenine ring on the nicotinamide spectrum by comparing NAD^+ and NMN when both are at pH 7, as was done with proton data by Sarma and Kaplan (1969a). On the basis of this comparison they reached the conclusion that the adenine ring shielded certain protons of the nicotinamide ring more than others. If, however, they had compared the shifts of NAD^+ at pH 7 with NMN at pH 4.5 they would have found (using data presented in that paper) that all of the protons were shielded virtually the same amount. Again, the differential shift which they claimed existed between acetylpyridine adenine dinucleotide and its corresponding mononucleotide vanishes when one uses the mononucleotide data at pH 4.5 rather than pH 8. The fact that the nicotinamide ring protons are shielded virtually equally by the adenine ring is expected, since this is what one would expect from a maximum interaction between the rings. Other workers (Catterall *et al.*, 1969) have determined the adenine shielding in NAD^+ by comparing high and low pH pmr spectra of the dinucleotide and have indeed found similar shieldings on all protons.

As already mentioned, the chemical shifts of certain nicotinamide carbons are affected by a change in ionization state of the phosphate group in NMN. These changes are not present in the $NMNH_2$ spectrum and confirm earlier results obtained in this laboratory from ^{31}P studies (Blumenstein and Raftery, 1972) that the presence of a positive charge in the nicotinamide ring causes an interaction with the phosphate group. Somewhat surprisingly, while in the pmr work (Sarma and Kaplan, 1969a) H-2 is most affected by a change in phosphate ionization state (0.1 ppm) and H-6 less affected (0.05 ppm) and other protons are unchanged, in the carbon spectrum C-6 is most shifted (0.7 ppm), C-4 affected somewhat (0.3 ppm), and other carbons very slightly changed. In both the carbon and proton spectra, shifts are to higher field at low pH. The proton results have been interpreted as indicating that the nucleotide exists in the syn conformation, and since recent theoretical studies (Berthod and Pullman, 1971) support this view one must wonder as to the origin of the carbon shifts, which at first glance appear to support an anti conformation. It can, however, be deduced from the direction and magnitude of the carbon shifts that they must arise from causes somewhat different than the proton shifts since neither of the reasons given (Schweizer *et al.*, 1968) for the specific shielding of aromatic ring protons by the phosphate group of

mononucleotides explain the shifts. Thus, if the shift were caused by magnetic anisotropy due to the negative charge, it would have to be of the same magnitude (in parts per million) in carbon and proton spectra, while in fact the observed carbon shifts are much larger. Alternatively, it is suggested (Schweizer *et al.*, 1968) that the negative charge pulls the proton away from the carbon, decreasing the charge density at the proton, and thereby shifting it downfield with increasing phosphate ionization. Such an effect, however, would increase the charge density at the carbon and cause the shift to be opposite to that of the proton, while in fact shifts in the same direction are observed. Also, the general effect of a negative charge would be to increase the charge density at both the proton and the carbon, thereby causing upfield shifts at high pH, while as just noted the observed shifts are upfield at low pH. A likely explanation for the ^{13}C shifts is that the phosphate–nitrogen interaction causes a redistribution of charge around the nicotinamide ring leading to shifts of certain carbons. Examination of the chemical-shift changes which occur in the adenine ring carbons of NAD^+ when the adenine N-1 is protonated illustrates the large charge effects seen in the ^{13}C spectra of aromatic systems. Thus, as expected, both C-2 and C-6 of the adenine ring of NAD^+ exhibit large chemical shifts when the ring is protonated, but while C-4 and C-5 display very slight shifts, C-8 exhibits a very large shift. It is clear, therefore, that the shifts induced in the nicotinamide ring of NMN by the phosphate group are not necessarily an indication of which carbons are spatially closest to this group. It should be pointed out that a redistribution of charge around the nicotinamide ring will also affect the proton spectrum, so the observation of a shift in H-2 upon ionization of the phosphate does not necessarily imply the syn conformation for NMN.

Dihydronicotinamide Conformation. As was mentioned previously, the ^{13}C resonances due to the nicotinamide ring in NMN^+ are virtually identical (at low pH) with those in NAD^+. This equivalence is not the case for the dihydropyridine moieties in $NMNH_2$ and NADH, since examination of Table I shows a 15-Hz shift difference for C-3 and a 22-Hz shift difference for C-6, in both cases the higher field shift being observed in NADH. One explanation for these results is the recent suggestion (Oppenheimer *et al.*, 1971) that the dihydropyridine ring is puckered at the C-4 in NADH, while it is flat in $NMNH_2$. Such a change, however, would be expected to have a large effect on the C-4 chemical shift, while no chemical-shift difference between NADH and $NMNH_2$ is seen at this position. It is possible that the chemical-shift difference observed at C-6 is due to a different orientation of the dihydropyridine ring relative to the attached ribose in $NMNH_2$ and NADH. Thus, if the C-6 experiences steric hindrance in NADH (Sarma and Kaplan, 1969a), it is possible that in $NMNH_2$, the dihydropyridine ring, no longer constrained by the parallel adenine ring, can twist around the glycoside bond to avoid this strain. The origin of the shifts of C-3 is unclear, though perhaps it involves the attached carboxamide side chain rather than a direct interaction involving C-3.

α-NAD+. The position of the nicotinamide resonances in NAD^+ differs by 1–1.5 ppm from the corresponding resonances in α-NAD^+. The shifts go in both directions but the largest shifts and the "total" shift (Perlin, 1971) are upfield in going from β-NAD^+ to α-NAD^+. This corresponds to greater steric interaction between the nicotinamide ring and the adjacent ribose when the latter is in the α configuration. It is of interest that the nicotinamide resonances of α-NAD^+ are more pH dependent than are the corresponding peaks in β-NAD^+. A possible explanation is that there is greater nicotinamide–adenine interaction in α-NAD^+ than in β-NAD^+. Another explanation is similar to that for the difference in the C-6 resonances of NADH and $NMNH_2$, *i.e.*, in α-NAD^+, when the dinucleotide unfolds at low pH, the nicotinamide ring twists to relieve some steric strain.

Ribose Conformation. The ^{13}C spectrum of ribose rings of the mono- and dinucleotides used in this study is much clearer than the 220-MHz proton spectrum, and it is observed that the shifts in the dinucleotides are virtually identical with those in the mononucleotide (except in the case of the 5′ carbons, where the phosphate present in the mononucleotides causes a somewhat different shift than does the dinucleotides' pyrophosphate). It would thus appear that the ribose conformations are identical in the mono- and dinucleotides and are solely a function of the base to which the ribose is attached.

It can be seen (Table I) that the ribose chemical shifts are very dependent on the base which is attached. It is observed that the largest chemical-shift differences are seen in C-1′ and C-2′, so these shifts can be partially explained in terms of different electron withdrawing abilities of the adenine, nicotinamide, and dihydronicotinamide rings. C-3′ and C-5′ are affected very slightly by a change in the moiety attached to the ribose but, surprisingly, C-4′ shows rather large shift differences, with the resonance in AMP occurring 90 Hz to higher field of that in NMN, and the $NMNH_2$ resonance occurring 40 Hz to higher field of the AMP resonance. It is not clear whether these shifts reflect different ribose conformations or are due to long-range electric effects caused by the bases attached to the riboses.

One effect common to the three mononucleotides, NMN, $NMNH_2$, and AMP, is that in going from a singly ionized phosphate to a doubly ionized phosphate the C-5′ shifts upfield by about 1 ppm, while the C-4′ resonance shifts downfield by about 0.8 ppm. Comparing the work of Grant (Jones *et al.*, 1970) on nucleotides with that of Roberts (Dorman and Roberts, 1970) on nucleotides, one sees that addition of a phosphate to nucleosides causes a downfield shift on the 5′ resonance of 2–2.5 ppm and an upfield shift of ~1 ppm on the 4′ resonance. The anomalous shifts often observed with ^{13}C have been discussed elsewhere (Horsley and Sternlicht, 1968) where it is noted that redistribution of charge between carbons and different nuclei or other carbons often leads to shifts which are opposite to those seen in pmr work and which are often surprisingly small.

It must be noted that far more work must be done on correlating cmr data with sugar conformation, but the large shift differences which are observed among various sugars indicate that cmr spectroscopy should be a very valuable tool in this area.

Many small shifts are seen in both ribose rings of NAD^+ when its adenine ring is titrated, the largest shift of course being observed in C-1 of the ribose bound to adenine. The shifts in the nicotinamide ribose are again indicative of an effect on the entire molecule which occurs when the adenine ring is protonated.

Base Stacking. Changing the concentration of NAD^+ from 0.1 to 0.008 M has a very small effect on the spectrum, with changes of less than 5 Hz being observed. A comparison of the spectral positions of the AMP resonances in this study, using 0.1 M nucleotide, with those of a previous study (Dorman and Roberts, 1970), using 1–2 M nucleotide, again reveal that very small changes (less than 5 Hz at 25.1 MHz) occur even with fairly large changes in concentration.

It is clear from the pH studies of NAD^+, as well as the con-

centration studies of NAD^+ and AMP, that the study of intermolecular and intramolecular base stacking, as well as aromatic ring current effects in general by cmr techniques, requires exceedingly great precision of measurement and would often better be studied by pmr, where the shifts in hertz will be larger due to the higher frequency of the proton absorptions. There are, however, many cases where the cmr study of such effects will no doubt prove profitable, such as in complex molecules where by dint of greater spectral resolution the ^{13}C peaks can be more easily resolved and identified than the corresponding proton peaks, or in cases where the carbon atom is not bonded to any protons, and is therefore the only probe one has at a given position.

Relaxation Times. The T_1 value of 14 ± 2 sec determined for the carbonyl carbon of NAD^+ indicates that the amide nitrogen does not provide an efficient mechanism for the relaxation of this carbon. Furthermore, the fact that the T_1 is not greatly altered in going from low to high pH indicates that folding of the molecule also has no large effect on the relaxation. It should be noted that the purity of the NAD^+ has a very large effect on the T_1, with values of from 1 to 5 sec being obtained when the dinucleotide was used without first being purified by ion exchange chromatography. It has been previously observed (Blumenstein and Raftery, 1972) that paramagnetic ion concentration has a very great effect on the ^{31}P spectra of NAD^+ and NADH, and also causes a substantial shortening of the T_1 values for the ^{31}P atoms of ATP (M. P. Klein, personal communication).

Experimental Section

Materials. Mono- and dinucleotides used in this study were purchased from Sigma.

Methods. Spectra were taken on a XL-100-15D nmr spectrometer operating at 25.1 MHz. Proton noise decoupling was used in all experiments, except those in which single frequency decoupling was employed to aid in peak assignments. The Fourier transform technique was used to acquire all spectra. Conditions were such (acquisition time 0.4 sec, pulse width 25 μsec ($\pi/2$ = 105 μsec)) that all peaks were observed, though those with fairly long relaxation times were partially saturated. Most spectra were run at a 2500-Hz spectral width, with peak positions being determined from the computer-generated printout, using the solvent, D_2O, as internal standard.

Most samples had a concentration of approximately 0.1 M nucleotide and also contained 0.1 M phosphate. Spectra were acquired in 2-hr. Certain samples (NMN, $NMNH_2$, α-NAD^+) contained 0.05 M nucleotide, and spectra of these samples took 8 hr to accumulate. The low concentration (0.008 M) NAD^+ sample was run under different conditions (acquisition time of 0.2 sec and pulse width of 100 μsec) and also took 8 hr. Under these conditions, quaternary carbons were not seen.

Most chemical shifts given are the average of two runs, performed on different days. Almost all values obtained in the two runs were within 3 Hz of each other.

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