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Raman Spectroscopy of Oxidized and Reduced Nicotinamide Adenine Dinucleotides†

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Received October 18, 1985; Revised Manuscript Received March 18, 1986

ABSTRACT: We have measured the Raman spectra of oxidized nicotinamide adenine dinucleotide, NAD^+, and its reduced form, NADH, as well as a series of fragments and analogues of NAD^+ and NADH. In addition, we have studied the effects of pH as well as deuteration of the exchangable protons on the Raman spectra of these molecules. In comparing the positions and intensities of the peaks in the fragment and analogue spectra with those of NADH and NAD^+, we find that it is useful to consider these large molecules as consisting of component parts, namely, adenine, two ribose groups, two phosphate groups, and nicotinamide, for the purpose of assigning their spectral features. The Raman bands of NADH and NAD^+ are found generally to arise from molecular motions in one or another of these molecular moieties, although some peaks are not quite so easily identified in this way. This type of assignment is the first step in a detailed understanding of the Raman spectra of NAD^+ and NADH. This is needed to understand the binding properties of NADH and NAD^+ acting as coenzymes with the NAD-linked dehydrogenases as deduced recently by using Raman spectroscopy.

It is well-known that nicotinamide adenine dinucleotide, NAD^+,[1] and its reduced form, NADH, are essential in many oxidation–reduction reactions. The NAD-linked dehydrogenases, a class of more than 300 enzymes, use these molecules as coenzymes. The enzymatic reactions of many NAD-linked dehydrogenases are strictly ordered, with the formation of the binary product of enzyme and coenzyme as the first and last steps (Theorell & Chance, 1951; Nygaard & Theorell, 1955; Wratten & Cleland, 1963). A clear understanding of how NAD^+/NADH binds at the active sites of these dehydrogenases is necessary to understanding their enzymatic behavior. Various techniques have been used to study these interactions, including circular polarization

† This work was supported in part by Grant PCM-8202840 from the National Science Foundation, Grant GM 35183 from the National Institutes of Health, Grant RR 08168 from the National Institutes of Health (MBRS program), and a grant of the City University PSC-BHE Faculty Award Program.
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[1] Abbreviations: NAD^+, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; 9EtAd, 9-ethyladenine; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ADPR, adenosine 5′-diphosphoribose; NMN^+, oxidized β-nicotinamide mononucleotide; NMNH, reduced β-nicotinamide mononucleotide; 1-MN^+, 1-methylnicotinamide; 3-$PAAD^+$, 3-pyridinealdehyde adenine dinucleotide (oxidized).

0006-2960/86/0425-4941$01.50/0

fluorescence spectroscopy (Schlessinger et al., 1975), affinity chromatography (Thompson et al., 1975), equilibrium and kinetics studies (Suhadolnik et al., 1977), calorimetry studies (Subramanian & Ross, 1977, 1978), and X-ray crystallography (Ohlsson et al., 1974; Wierenga et al., 1985). However, the molecule binding properties of NAD^+/NADH with the NAD-linked enzymes are poorly understood.

Recently, Yue et al. (1984) reported the classical Raman spectrum of NADH when bound to the enzyme liver alcohol dehydrogenase (EC 1.1.1.1), by employing sensitive difference techniques. It was shown that significant changes occur in the Raman spectrum of NADH upon binding to LADH. From these changes, it was deduced that both the adenine and the nicotinamide moieties of NADH are directly involved in binding, as is one or both of the $-NH_2$ moieties of NADH (see Figure 1). It is clear that such Raman studies are capable of yielding valuable information concerning the binding of NADH or NAD^+ to the NAD-linked enzymes. However, a more detailed understanding of the Raman spectral features is hindered by the very limited understanding of the Raman spectrum of NAD^+ and NADH.

Our goal here is to assign the Raman bands of NADH and NAD^+ to simpler molecular moieties by obtaining the Raman spectra of portions of these rather complicated molecules and comparing these results to those of NAD^+ and NADH. To do so, we have measured the Raman spectra of various fragments and analogues of NAD^+ and NADH. In particular, we report the Raman spectra of adenine, adenosine, AMP, ADP, ADPR, NMN^+, NMNH, 1-methylnicotinamide iodide, 9-ethyladenine, and 3-$PAAD^+$. In addition, the effects of pH and the deuteration of the exchangable protons on the Raman spectra of NADH, NAD^+, and their fragments have been obtained. By comparing the Raman spectra of the molecular fragments to that of NAD^+ and NADH, we can assign the peaks in the NAD^+ and NADH spectra to individual groups.

Several Raman studies on NAD^+ and/or NADH have been reported previously (Patrick et al., 1974; Forrest, 1976; Nishimura & Tsuboi, 1980a; Barrett, 1980). These studies are mostly concerned with the assignment of the C=O stretching mode with little or no discussion of the other spectral features. More recently, ultraviolet resonance Raman studies of NADH and its analogues (Nishimura & Tsuboi, 1980b; Bowman & Spiro, 1980; Rodgers & Peticolas, 1980) have presented a more complete examination of some of the spectral features of NADH. Other studies on molecules containing portions of NADH or NAD^+ are available (Lord & Thomas, 1967; Rimai et al., 1969; Lafleur et al., 1972). We compare our results to these studies and also discuss the assignment of particular bands in terms of specific nuclear motions where these previous studies have made such an assignment.

Materials and Methods

NADH (grade III), NAD^+ (grade III), 3-$PAAD^+$, NMN^+, NMNH, 1-methylnicotinamide iodide, 9-ethyladenine, adenine (Sigma grade), adenosine (Sigma grade), ADP (grade I), AMP (Sigma grade), and adenosine 5′-diphosphoribose were purchased from Sigma Chemical Co. (St. Louis, MO). All samples were dissolved in 0.1 M pyrophosphate buffer (pH 9.6) or phosphate buffer (pH 7.0 and pH 5.0) depending on the required experimental conditions. Samples at pH lower than 5.0 were prepared by adding dilute phosphoric acid to obtain the desired pH. Concentrations of all samples (ranging from 1.5 to 250 mM) were determined spectroscopically, if possible, by using accepted extinction coefficients. Most Raman measurements were carried out at room temperature except where mentioned otherwise. All samples of adenine and samples of adenosine at pH lower than 9.6 were measured at 60 °C because of their low solubilities at room temperature. The temperature was maintained by a thermal bath/circulator. Ultraviolet/visible spectra measurements were obtained before and after each Raman measurement. No obvious changes in the absorption spectra were observed, indicating that no appreciable degradation of samples occurred during the Raman measurement. The Raman spectra of all samples were obtained by one of these two systems: (1) a standard scanning system consisting of a Spex 1401 double spectrometer controlled by a Compudrive controller (CD2A, Spex Industries, Metuchen, NJ), a cooled RCA 31034 photomultiplier, and photon-counting electronics; (2) an OMA system that consists of a Triplemate spectrometer (Spex Industries, Metuchen, NJ) and a solid-state detector system of a Model 1420 water-cooled photodiode array and Model 1218 controller (EG&G, Princeton Applied Research, Princeton, NJ). Both spectrometers were interfaced to an LSI-11 microcomputer (Digital Equipment Corp., Marlboro, MA), which was also used for data analysis. All spectra lines measured with the OMA system were calibrated against known lines of toluene. Spectra measured with the scanning spectrometer are accurate to within ± 2 cm^{-1}. The OMA system has an accuracy of ± 1.5 cm^{-1}. Most spectra were measured by using either 488-nm or 514.5-nm lines from a Model 165 argon ion laser (Spectra Physics, Mountainview, CA). Blue-green lines from a Coherent (Palo Alto, CA) Model CR-2000 krypton ion laser were also used. The water/buffer background Raman spectrum has been subtracted from the presented data.

Results and Discussion

Figure 1 shows molecular schematics of NADH and NAD^+ and the different moieties of NADH/NAD^+ used in this study. The Raman spectra of NAD^+ and NADH are shown in Figures 2b and 3b, respectively. The Raman spectra of the two major fragments of NAD^+ and NADH (NMN^+ or NMNH and AMP) are also shown in Figures 2 and 3. It is clear that peaks in NAD^+ and NADH spectra can be identified as being from either NMN^+/NMNH or the AMP moiety. Measurements of equimolar concentrations of NMN^+/NMNH and AMP when added reproduce the spectra of NAD^+ and NADH very well. The small differences in the summed spectra when compared to the NAD^+ or NADH spectra are due to peaks that can be easily assigned to structural changes due to the joining of the two fragments. This shows that the two major fragments are basically independent of each other with regard to their Raman spectra. From measurement of known mixtures of NAD^+ and NADH, we found that the intensity of the 1338-cm^{-1} peak, due to the adenine moiety (as will be discussed), is the same in NADH and NAD^+ as expected.

Table I summarizes all the major band positions and relative band intensities in the fragments and analogues of NADH/NAD^+ at pH 7.0. The intensities were calculated relative to the 1338-cm^{-1} adenine band (arbitrarily set at 10), whenever it is present, and indirectly through the peak at 1688 cm^{-1} of NADH or the peak at 1032 cm^{-1} of NAD^+ for fragments not containing adenine.

Assignments of the major peaks of NADH and NAD^+ to a particular group is based mainly on the peak's presence in the corresponding fragment spectrum containing the moiety with about the same intensity. For example, the peak at 1308 cm^{-1} is assigned to adenine since it is observed in the spectra of all adenine-containing fragments and is not present in fragment spectra where adenine is absent. Likewise, the peak at 1032 cm^{-1} is assigned to molecular motions in the oxidized

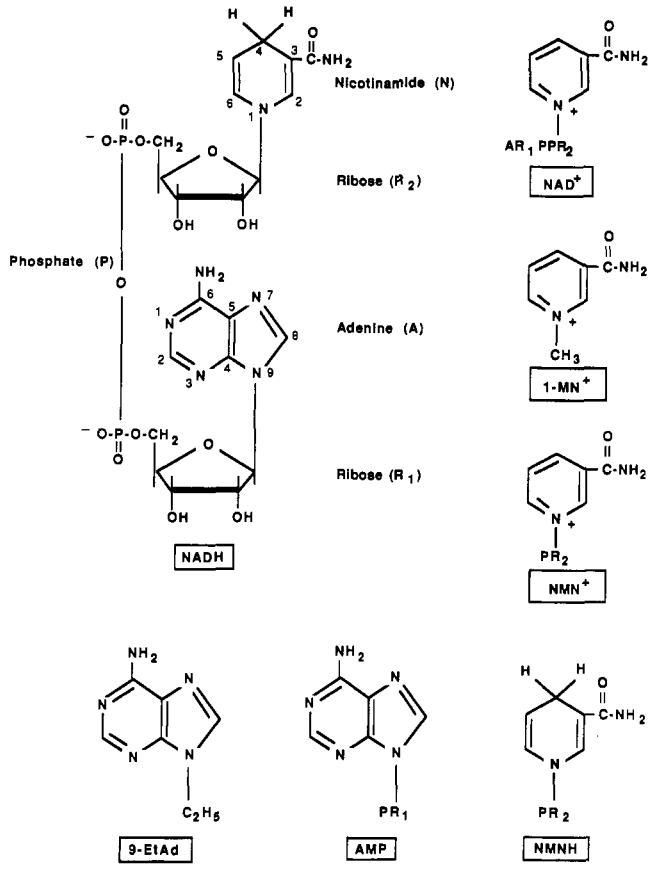

FIGURE 1: Schematic of NADH, NAD^+, NMNH, 1-MN^+, NMN^+, AMP, and 9EtAd. N, nicotinamide; R_2, ribose next to N; P, phosphate; R_1, ribose next to adenine; A, adenine.

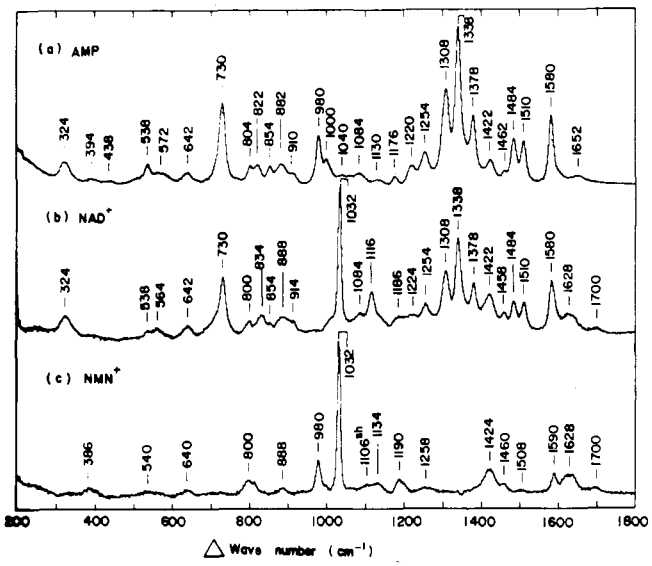

FIGURE 2: Raman spectrum of (a) AMP (110 mM), (b) NAD^+ (45 mM), and (c) NMN^+ (78 mM) in 0.1 M phosphate buffer (pH 7.0) at room temperature. Laser line was 488 nm with 100-mW incident power, and the resolution was 8 cm^{-1}. The spectrum of AMP was amplified by a factor of $^5/_3$ for clarity. As taken on the scanning appratus, each spectrum consists of 800 channels with an effective dwell time of 4 s/channel. Thus, each spectrum took about 1 h to take. Our reticon spectrometer (see Materials and Methods) is about 100 times faster.

nicotinamide moiety since this band is present in the spectra of all fragments containing oxidized nicotinamide. Further assistance in the assignments comes from the pH dependences and deuteration effects of the various bands. Table II summarizes the assignments of all the major peaks in NADH and

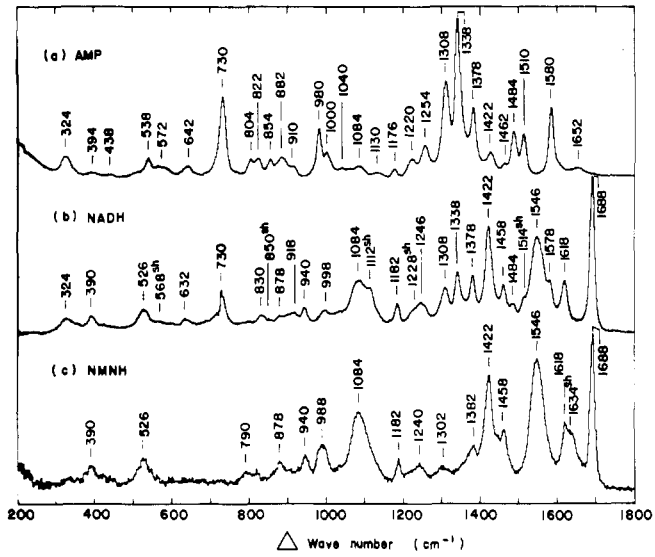

FIGURE 3: Raman spectrum of (a) AMP (110 mM), (b) NADH (67 mM), and (c) NMNH (16 mM). Conditions are as in Figure 2. The spectrum of AMP was amplified by a factor of 2.5 for clarity.

NAD^+ spectra obtained in this way. Rodgers and Peticolas (1980) previously assigned many of the bands in the classical Raman spectrum of NADH using resonance Raman techniques. In general, the agreement between their study and this is excellent.

Adenine-Related Peaks. The Raman spectrum of the adenine moiety (with and without the ribose and/or phosphate) has been studied extensively, mainly in conjunction with the other bases found in nucleic acids (Lord & Thomas, 1967; Rimai et al., 1969; Lafleur et al., 1972). More recently, pre-resonance and resonance Raman studies of adenine were reported (Nishimura et al., 1978; Chinsky et al., 1978; Bushaw et al., 1980; Kubasek et al., 1985; Fodor & Spiro, 1985). In general, our assignments agree with these results. The 1308-, 1338-, 1378-, 1422-, 1484-, 1510-, and 1580-cm^{-1} bands in NAD^+ are known as ring modes for adenine. Our results show that some of these modes, namely, the 1338-, 1378-, and 1422-cm^{-1} bands, are at slightly different frequencies in the spectrum of adenosine, indicating that these modes are affected by the ribose, R_1, in various degrees. The 1510- and 1580-cm^{-1} bands are not present in the adenine spectrum, but similar peaks to those found in adenosine are observed in the 9-ethyladenine spectrum. Peaks at 538, 642, and 730 cm^{-1} are also due to adenine, with the 730-cm^{-1} peak affected to a degree by the ribose.

All of these modes are also present in the spectrum of NADH. Some peaks appear to have different intensities because of the presence of additional bands due to the reduced nicotinamide moiety with very close, or identical, frequencies (see Table II). For example, the 538-cm^{-1} adenosine peak is apparently unresolved in the broad 526-cm^{-1} peak of NADH since the broad peak contains contribution of a peak at 526 cm^{-1} coming from the reduced nicotinamide moiety. Similarly, the 1378- and 1422-cm^{-1} peaks of NADH have significant Raman contributions from nicotinamide-related modes in addition to those from adenosine. The labeled peak at 1246 cm^{-1} in the NADH spectrum would appear to consist of the 1254-cm^{-1} adenine-related band the 1240-cm^{-1} nicotinamide-related band.

Nicotinamide-Related Peaks. The nicotinamide ring structures of NAD^+ and NADH are very different and thus yield very different Raman spectra (e.g., contrast NMN^+ and NMNH in Figures 2c and 3c). The Raman spectrum of

Table I: Raman Peak Frequencies (cm^{-1}) and Relative Intensities (in Parentheses) of Adenine, 9EtAd, Adenosine, AMP, ADP, ADPR, NMNH, NMN^+, and 1-MN^+ in pH 7.0 Buffer[a]

adenine	9EtAd	adenosine	AMP	ADP	ADPR	NMNH	NMN^+	1-MN^+
302 (1)	333 (3)	316 (1)	324 (1)	324 (2)	328 (3)			
								371 (1)
	391^{br} (1)		394^{br} (0)			390 (3)	386 (1)	
			438^{br} (0)	410 (1)				410^{br} (1)
				456 (2)				
								462^{br} (1)
						524^{br} (4)		530 (3)
539 (3)	531 (2)	538 (2)	538 (1)	538 (1)	538 (2)		540^{br} (1)	
				552 (4)	560^{sh}			551^{sh}
	582 (1)		572^{br} (1)					
622 (3)					632^{br} (1)			
		630^{br} (1)	642 (1)	640 (1)			640^{br} (1)	638^{br} (1)
	714 (4)							
722 (11)								
	732 (9)	730 (8)	730 (5)	732 (6)	732 (6)			735 (4)
				788 (7)		790 (2)		
	802 (2)		804 (1)		802 (6)		800 (2)	
			822 (1)	826 (1)	830^{sh}			
			854 (1)	850 (3)	850^{sh}			
872 (2)	876 (2)	868 (2)	882 (1)	880 (3)	880 (4)	878 (4)	888 (1)	
	912 (1)	910^{br} (1)	910 (1)	910^{sh}	910^{sh}			
948 (3)						944 (4)		
	961^{sh}							
			980 (3)				980 (4)	
990 (4)	991 (4)	990 (2)				988^{br} (4)		990 (1)
			1000 (2)					
		1008^{sh}						
			1040 (1)	1036 (5)			1032 (15)	1034 (15)
1079 (1)	1079 (2)		1084 (1)	1080 (3)	1078 (4)	1084^{br} (9)		
	1093 (2)							
				1110 (2)			1106^{sh}	
1121 (1)		1132 (1)	1130 (0)		1116 (3)			
							1134 (2)	
1148^{sh}								
		1178 (1)	1176 (1)	1182 (1)	1178 (1)			
						1186 (4)	1190 (2)	
	1196^{br} (2)							
								1217 (2)
		1214 (2)	1220 (1)	1228^{sh}	1230^{sh}			
						1240 (4)		
1249 (3)	1254 (2)	1254 (2)	1254 (2)	1266 (4)	1252 (3)		1258^{br} (1)	
	1279 (3)							
								1300^{br} (0)
1309 (6)	1311 (6)	1308 (7)	1308 (6)	1310 (6)	1308 (7)	1302 (3)		
1330 (10)	1335 (10)	1338 (10)	1338 (10)	1340 (10)	1340 (10)			
1363 (4)	1368 (6)	1378 (4)	1378 (4)	1380 (5)	1382 (6)	1382 (6)		
1396 (3)								
1419 (2)	1418 (2)	1428 (2)	1422 (2)	1426 (2)	1428 (4)	1422 (13)	1424 (3)	1418 (3)
	1451 (3)			1450 (4)				
	1463^{sh}		1462 (1)		1462 (3)	1458 (7)	1460 (2)	1471^{sh}
1485 (5)	1489 (1)	1486 (3)	1484 (3)	1486 (4)	1486 (5)			
1497^{sh}								
	1516 (3)	1512 (3)	1510 (3)	1512 (3)	1510 (5)		1508^{br} (0)	1511 (0)
						1546^{br} (15)		
1569^{sh}								
	1580 (3)	1582 (5)	1580 (4)	1584 (5)	1584 (7)			
							1590 (3)	1595 (2)
1596 (7)								
						1618 (8)		
	1640^{br} (2)		1652^{br} (1)	1656^{br} (1)	1646^{br} (1)	1634^{sh}	1630^{br} (3)	1632^{br} (2)
						1690 (25)	1700 (1)	1692 (1)

[a] sh, shoulder; br, broad.

NAD^+ is very similar to that of ADPR, indicating that the oxidized nicotinamide moiety does not contribute much to the spectrum of NAD^+. The major exception to this is a mode at 1032 cm^{-1}, a prominent peak in the NAD^+ (and the NMN^+) spectrum. This can clearly be assigned to an aromatic ring breathing mode of oxidized nicotinamide. There are also two smaller bands at 800 and 1186 cm^{-1} (shoulder) in the NAD^+ spectrum; these are due to molecular motions located on the nicotinamide moiety as well as the ribose, R_2. Peaks at 642, 1422, and 1580 cm^{-1} have substantial contributions from bands due to nicotinamide at similar positions in addition to adenosine. The broad peak at about 1628 cm^{-1} is also due mostly to nicotinamide; however, some of this mode's intensity arises from adenine since adenine also has a weak broad peak at that position. Many investigators have reported different values for the highest frequency mode observed in the Raman spectrum of NAD^+, which is attributed to the C=O stretch (Forrest, 1976; Barrett, 1980; Nishimura & Tsuboi, 1980a). We have consistently observed a small shoulder at 1700 cm^{-1} in the NAD^+ spectrum, which may have been neglected in

Table II: Assignment of Peaks in the Raman Spectrum of NADH and NAD+ (Relative Intensity in Parentheses)[a]

peak frequency (cm^{-1})		
NAD+	NADH	assignment[b]
324br (2)	324br (3)	P
	390 (3)	N
538 (1)	526br (4)	A for NAD+; 0.2A + 0.8N for NADH
564br (1)	568sh	P/R
642br (2)	632br (2)	$0.5A/R_1 + 0.5N$ for NAD+; A/R_1 for NADH
730 (7)	730 (7)	$A/\delta R_1$
800 (0)		R_2/N
834 (2)	830br (2)	P
854 (1)	850sh	R_1/P
888br (2)	878 (3)	P
914 (2)	918 (3)	P
	940 (4)	N
	998 (4)	N
1032 (15)		N
1084 (3)	1084 (9)	P for NAD+; $0.3P + 0.7N^*$ ($-NH_2$) for NADH
1116 (4)	1112sh	R_2/pyrophosphate
	1182 (5)	N
1186br (3)		R_2/N
1224 (3)	1228sh	P
1254 (4)	1246 (5)	A* for NAD+; $0.8N^* + 0.2A^*$ for NADH
1308 (7)	1308 (7)	A
1338 (10)	1338 (10)	$A/\delta R_1$
1378 (6)	1378 (9)	A/R_1 for NAD+; $0.7A/R_1 + 0.3N^*$ for NADH
1422 (5)	1422 (17)	$0.5A/\delta R_1^* + 0.5N^*$ for NAD+; $0.1A/\delta R_1^* + 0.9N^*$ for NADH
1458 (3)	1458 (8)	$R_2/\delta N$
1484 (4)	1484 (4)	A
1510 (4)	1514sh	$A/\delta R_1$
	1546 (16)	N*
1580 (6)	1578sh	$0.8A/\delta R_1 + 0.2N$ for NAD+; $A/\delta R_1$ for NADH
	1618 (9)	N
1628br (3)		N*
	1688 (25)	N
1700 (1)		N*

[a] br, broad; sh, shoulder; asterisk, deuteration effect. Symbols: P, phosphate; A, adenine; N, nicotinamide; R, ribose (R_1, near A; R_2, near N as in Figure 1); δ, small influence. Modes involving more than one moiety are indicated by symbols separated by a slash. Peaks containing two separate degenerate or nearly degenerate modes are indicated by plus sign separating the two modes with the relative contribution in intensity in front of the symbol.

some of these earlier studies. This band is clearly visible if the contribution due to water/buffer is subtracted, as in Figure 2b. Our results (discussed later) indicate a substantial deuteration effect in the 1700-cm^{-1} mode, making it unlikely to be a simple C=O stretching mode.

In contrast to NAD+, the reduced nicotinamide moiety yields many prominent peaks in the NADH spectrum. By comparison of the Raman spectrum of NADH and NMNH, it is easy to identify the bands at 390, 526, 940, 1084, 1182, 1378, 1422, 1546, 1618, and 1688 cm^{-1} as due to the nicotinamide moiety. The peak at 1084 cm^{-1} has been assigned to the rocking motion of $-NH_2$ of the amide (Bowman & Spiro, 1980; Yue et al., 1984). Upon deuteration, it shifts down to 908 cm^{-1} as previously reported. The strong 1688-cm^{-1} band has often been attributed to the C=O carbonyl stretch (Patrick et al., 1974; Forrest, 1976; Barrett, 1980; Nishimura & Tsuboi, 1980a; Bowman & Spiro, 1980). This assignment, however, was recently questioned by Rodgers and Peticolas (1980).

Peaks due to Phosphate. We have assigned peaks that appear in the AMP spectrum but not in adenosine's and that also double in intensity in ADP's spectrum to phosphate modes.

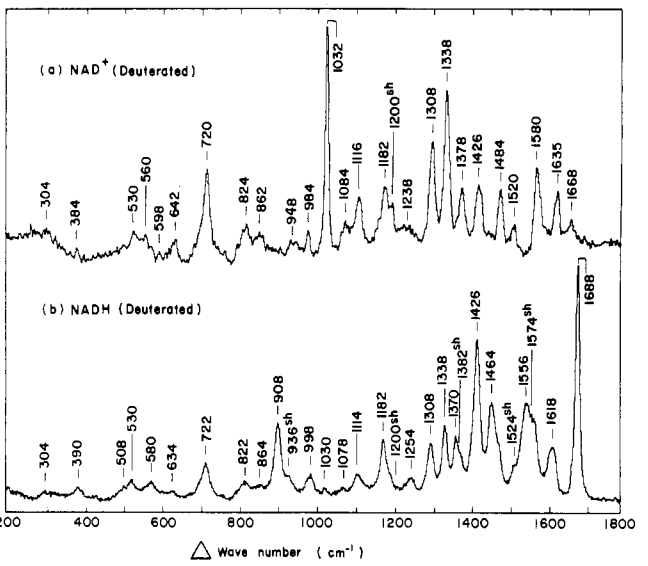

FIGURE 4: Raman spectrum of (a) NAD+ (62 mM) and (b) NADH (41 mM) in 0.1 M phosphate/D_2O buffer (pD 7.0). Other conditions are as in Figure 2.

Further support for these assignments comes from the pH dependencies of these bands. ADP has an apparent pK at 6.3 (Alberty et al., 1951; Bock et al., 1956) associated with the ionization of the phosphate's last proton, suggesting that NAD+ and NADH would show similar titration behavior. In the titrations of NADH or NAD+ through pH 6, we found those bands assigned to the phosphate moiety indeed show significant changes.

We give special mention to the phosphate band at 1084 cm^{-1} (Rimai et al., 1969) in the spectrum of NAD+. This band is not visible in NADH because of the much more dominant rocking mode of the amide's $-NH_2$ at 1084 cm^{-1}. Upon deuteration of NADH, however, the phosphate peak revealed as the peak due to $-NH_2$ is downshifted to 908 cm^{-1}.

Deuteration Effects. Figure 4 shows the Raman spectra of NAD+ and NADH in D_2O/buffer. At pD 7.0, only the two $-NH_2$ groups and the hydroxyl groups of the ribose rings are deuterated. Nevertheless, there are a number of significant changes in the NAD+ and NADH spectra, despite these apparently relatively minor changes. These indicate that many modes involve motions associated with these $-NH_2$ groups or the ribose moiety to varying degrees. We discuss in turn the changes observed upon deuteration for bands assigned to adenine, oxidized nicotinamide ring, reduced nicotinamide ring, and ribose/phosphate moiety. Spectra of deuterated fragments like AMP, NMN+, and NMNH were also measured to clarify the changes (data not shown). Modes that show a deuteration effect are indicated by an asterisk in Table II.

The 1422-cm^{-1} peak in both NAD+ and NADH contains an adenine ring mode which is upshifted slightly by 4 cm^{-1} in D_2O/buffer, showing that this mode involves the $-NH_2$ group to a degree. The broad peaks at 1246 cm^{-1} for NADH and 1254 cm^{-1} for NAD+ contain more than one mode. The adenine mode at 1254 cm^{-1} is common in both coenzymes. In D_2O/buffer, this band disappears. (For NADH, a weaker new peak due to the reduced nicotinamide ring appears at aboout the same frequency in D_2O/buffer; see later.) Upon deuteration, two new bands, a shoulder at 1200 cm^{-1} and a peak at 1182 cm^{-1}, appear. Both can be identified with the adenine moiety since similar peaks appear when AMP is deuterated. The 1182-cm^{-1} mode has been assigned to a $-ND_2$ scissor mode by Tsuboi et al. (1973) in their studies of adenine. Note that the peak at 1182 cm^{-1} observed in the deuterated NAD+

and NADH spectra is also partly due to a mode associated with the nicotinamide ring; this is discussed later.

A number of spectral changes can be associated with the effects of deuteration upon the oxidized nicotinamide moiety. The broad peak at 1628 cm^{-1} of NAD^+ in H_2O solution is replaced by a peak at 1635 cm^{-1} for deuterated NAD^+. Similar spectral changes occur for NMN^+. The weak peak at 1700 cm^{-1} in the protonated NAD^+ spectrum disappears upon deuteration and a new peak at 1668 cm^{-1} appears in D_2O/buffer, suggesting a 32 cm^{-1} downward shift of the 1700-cm^{-1} band upon deuteration. This is not the case. The peak at 1668 cm^{-1} is also present in deuterated NMN^+ but not in any of the adenine-containing deuterated fragments, which implies that both peaks are associated with the nicotinamide moiety. However, in the spectrum of 3-$PAAD^+$, where the carboxamide is replaced by an aldehyde, the 1700-cm^{-1} peak is present in H_2O, but the 1668-cm^{-1} peak is missing in D_2O/buffer. It is clear that both modes are related to motion of the $-NH_2$ moiety but are different modes. The weak broad peak at 1258 cm^{-1} in the protonated NMN^+ spectrum, which is part of the observed 1254-cm^{-1} peak in the spectrum of NAD^+, disappears upon deuteration. Correspondingly, a broad peak at 1238 cm^{-1} appears in the deuterated NMN^+ and NAD^+ spectra, suggesting that the oxidized nicotinamide 1258-cm^{-1} mode shifts to 1238 cm^{-1} upon deuteration. The nature of the new broad peak around 948 cm^{-1} in the deuterated spectrum is not clear. However, it is likely to be associated with the nicotinamide moiety since a similar new peak (shoulder) appears in the NMN^+ spectrum in D_2O while no corresponding peak is observed in the D_2O spectra of AMP, ADP, and ADPR.

The most prominent change in the Raman spectrum of NADH in D_2O is the downshift of the 1084-cm^{-1} mode to 908 cm^{-1}. As mentioned earlier, this mode is associated with the nicotinamide $-NH_2$ rock. The 1240-cm^{-1} NMNH mode, which is part of the observed 1246-cm^{-1} mode in the spectrum of NADH, disappears upon deuteration. A "new" peak due to the nicotinamide moiety appears at 1254 cm^{-1} in the deuterated spectrum. Note that the original 1254-cm^{-1} peak (seen as part of the 1246-cm^{-1} side of NADH) in the H_2O spectrum, which is due to adenine moiety, disappears upon deuteration (see earlier discussion). Similar behavior is observed with the spectrum of NMNH upon deuteration, confirming our assignments. The 1378-cm^{-1} peak, due in part mostly to a mode from the reduced nicotinamide moiety (see Table II), appears to downshift to 1370 cm^{-1}. A shoulder labeled at 1382 cm^{-1} in Figure 4b, is probably due to the 1378-cm^{-1} adenine band, which remains unaffected by deuteration. The corresponding but weaker mode in NAD^+ spectrum, which is due solely to adenosine, is unaffected. In addition, the 1546-cm^{-1} mode is upshifted to 1556 cm^{-1} in D_2O/buffer.

The regions, 300–600 and 700–900 cm^{-1}, show a number of changes; this is expected as there are many modes due to ribose/phosphate in this region.

pH Dependence. No changes in the Raman spectrum of NAD^+ between pH 8.0 and pH 5.0 and NADH between pH 9.6 and pH 7.0 are observed. NAD^+ is not stable at pH higher than pH 8, and its Raman signal rapidly disappears irreversibly. For NADH, irreversible changes in the absorption spectrum occur at a pH below approximately 6. At pH 5.0, for example the 340-nm absorption peak disappears completely in several hours. Significant changes are also observed in the Raman spectrum of NADH at a pH lower than 6. Between pH 4.0 and pH 6.0, all the changes are associated with nicotinamide-related peaks. Below pH 4.0, changes in the adenine-related peaks are also observed (as discussed later). For example, the intensities of the peaks at 998, 1422, and 1688 cm^{-1} due to nicotinamide are gradually reduced as the pH is lowered. The 1688-cm^{-1} peak disappears completely at pH 3. In the meantime, the intensities of the 1618-, 1084-, and 878-cm^{-1} bands increase, especially that of the 878-cm^{-1} band, which increases by over 10-fold from pH 7.0 to pH 5.0. Other new bands at 800, 495, and 433 cm^{-1} also start to appear at pH 5.0 with relative intensities of 4, 4, and 3, respectively. These changes are all probably related to acid denaturation of the reduced nicotinamide moiety.

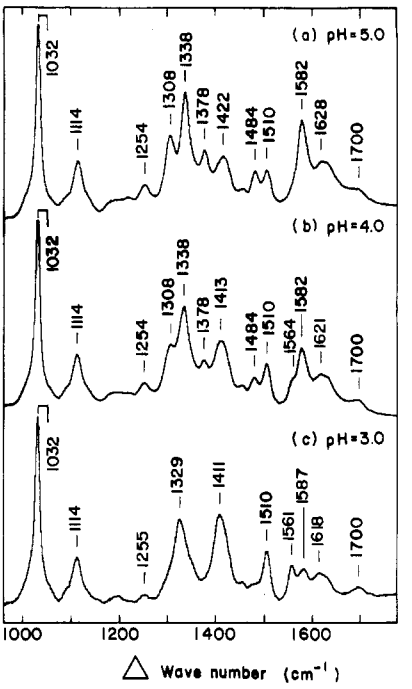

FIGURE 5: Raman spectrum of NAD^+ (66 mM) as a function of pH: (a) pH 5.0; (b) pH 4.0; (c) pH 3.0. Sample at pH 5.0 was in 0.1 M phosphate buffer. Phosphoric acid was added to the sample to lower the pH to the desired values. The resolution was 6 cm^{-1}; otherwise conditions are as in Figure 2.

Major spectral changes occur with the adenine-related peaks when the pH is lowered from pH 5.0 for both NAD^+ and NADH. Figure 5 shows the Raman spectrum of NAD^+ at pH 5.0, 4.0, and 3.0. At pH above 5.0, the four peaks associated with the adenine moiety at 1308, 1338, 1378, and 1422 cm^{-1} are easily distinguishable. However, as the pH is lowered, these bands are replaced by the two bands at 1329 and 1411 cm^{-1} at pH 3.0. There are other spectral changes also, which are evident from Figure 5. These spectral changes, which are reversible, are probably associated with the titration of a group with a p*K* around 3.9 (Moore & Underwood, 1969). It is likely to be due to a protonation of the adenine ring (Cochran, 1951; Broomhead, 1951; Zubay, 1958; Jardetzky & Jardetzky, 1960; Ellis et al., 1973). Protonation would clearly cause major changes in the ring modes of adenine as observed. Moreover, we observe an effect on the spectrum of NAD^+ at low pH when D_2O/deuterated buffer solutions are measured (data not shown). Similar changes are observed in the Raman spectrum of AMP and in the adenine-related peaks of NADH as pH is varied from 5 to 3.

Stacking. It is well-known that NADH and NAD^+ exist in predominantly folded forms in solution at room temperature, with the adenine and nicotinamide ring stacked together (Meyer et al., 1962; Cross & Fisher, 1969; Caterall et al., 1969; Oppenheimer et al., 1971; McDonald et al., 1972). We have measured the Raman spectrum of NADH at 75 °C and

with a strong destacking agent, 8 M urea. Under these conditions the open form predominantes (Cross & Fisher, 1969; Caterall et al., 1969; McDonald et al., 1972; Oppenheimer et al., 1978). We have observed that the Raman spectra of these two forms are essentially identical (Yue et al., 1984) with a small downward shift of the peak at 1546 cm^{-1} as the only change. NAD^+ is unstable at high temperatures. However, in 8 M urea, we observed no change in the Raman spectrum of NAD^+. We conclude, therefore, that stacking has no effect on the Raman spectrum of NADH and NAD^+.

In summary, we have found it possible to assign the major Raman peaks of NAD^+ and NADH to smaller components of these rather sizable molecules by studying the Raman spectra arising from a series of fragments and analogues. Major changes in the Raman spectrum associated with the adenine moiety are observed when adenine is protonated at pH 3.9. Stacking seems to have a very small or no effect on the Raman spectra of NAD^+ and NADH. More detailed assignments of NADH and NAD^+ Raman spectral features are in progress with isotopically labeled NAD^+ and NADH.

Registry No. NAD^+, 53-84-9; NADH, 58-68-4; 9EtAd, 2715-68-6; AMP, 61-19-8; ADP, 58-64-0; ADPR, 20762-30-5; NMN^+, 1094-61-7; NMNH, 4229-56-5; 1-MN^+, 3106-60-3; adenine, 73-24-5; adenosine, 58-61-7.

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