RAMAN AND INFRARED INVESTIGATION OF THE CARBONYL FREQUENCY OF NAD

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DAN MICHAEL PATRICK II
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ABSTRACT

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THE CARBONYL FREQUENCY OF NAD

By

Dan Michael Patrick II

The coenzyme NAD was studied by Raman and infrared spectroscopy. This spectroscopic investigation was confined to the carbonyl group of the nicotinamide moiety, which was monitored to seek evidence regarding the possibility of an intramolecular hydrogen bond existing between the amino group of the adenine moiety and the carbonyl group of the nicotinamide moiety of NAD.

The following compounds were chosen as models to provide a basis for interpreting the spectrum of NAD: nicotinamide, 3-acetyl pyridine, nicotinaldehyde, the methylated derivatives of these compounds, and nipecotamide.

It was concluded that, in hydrogen bonding solvents, NAD+ exists with a significant $\overset{O^-}{\underset{C = NH_2}{|}}{}^{+}$ resonance contribution. In nonhydrogen bonding solvents, this contribution is decreased, and the carbonyl frequency has a more classical value.

NAD+, NADH, and the aldehyde analog of NAD+ were examined at room temperature and at 70°C. It has been reported that at increased temperatures the intramolecular stacking is destroyed and NAD is found in an open configuration. The lack of a temperature dependence of the carbonyl frequency strongly suggests the absence of an intramolecular hydrogen bond between the amino group of the adenine moiety and the carbonyl of the nicotinamide group of NAD.

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INTRODUCTION

Cofactors are nonprotein structures which many enzymes require for activity. The cofactor can be a metal ion or a complex organic molecule, which is classified as a coenzyme.

NAD is the symbol for the oxidoreduction coenzyme, nicotinamide adenine dinucleotide, which is involved in the transfer of hydrogen atoms and electrons. Figure 1 is the NAD structure drawn with no consideration given to its conformation.

Interactions between the adenine and nicotinamide moieties in NAD have been detected by various spectroscopic techniques and evidence has been accumulated which indicates that in aqueous solutions and at room temperature NAD tends to exist in a folded or internally complexed form.[1-7] The suggested structure seems to resemble the stacked structures of polynucleotides and nucleic acids.

Miles and Urry[1] examined NAD using circular dichroism and absorption spectroscopy. The analysis dealt with the effects of interbase coupling on the electronic transitions. The presence of reciprocal relations in the circular dichroism spectra gave strong evidence that the folded

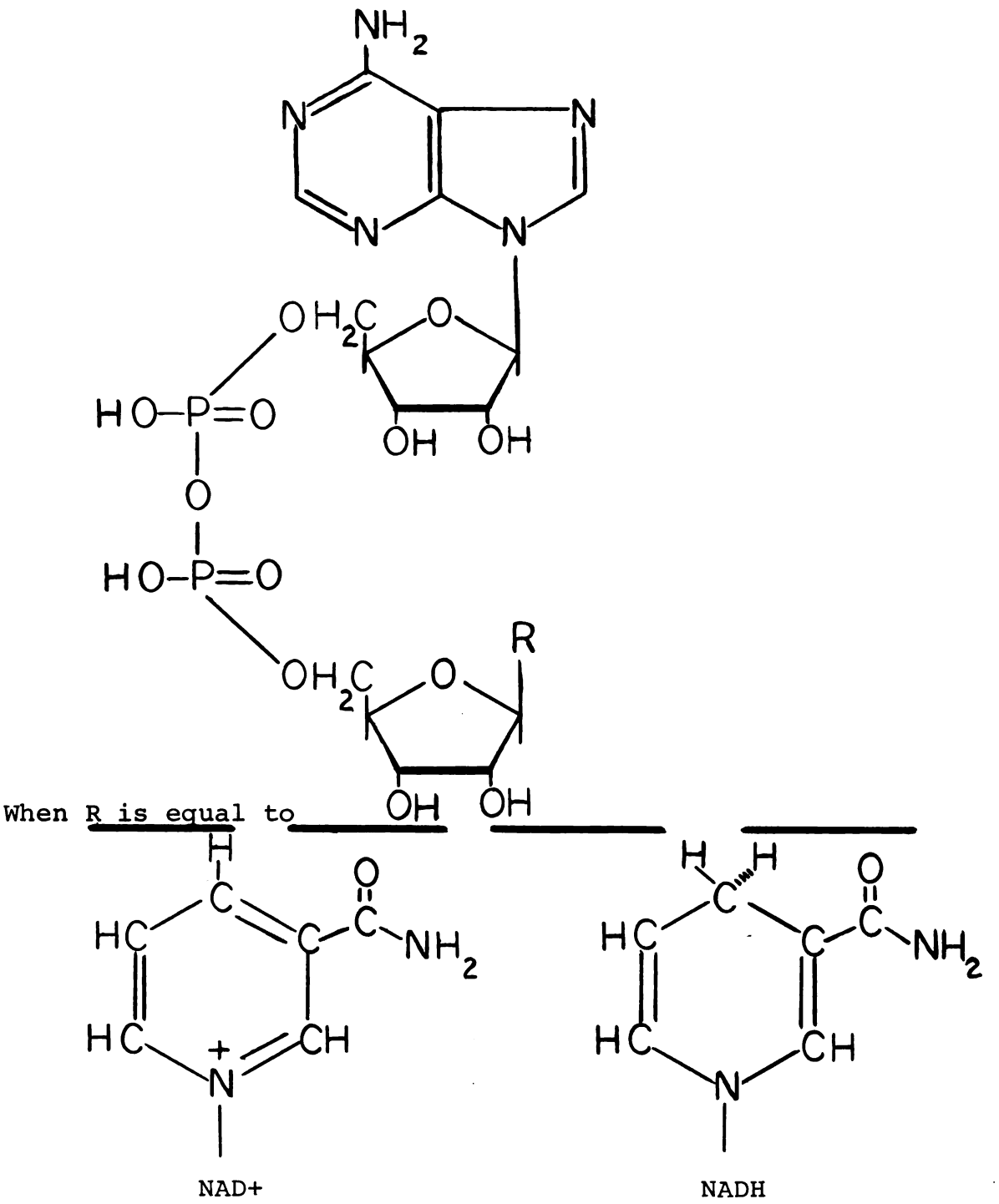


Figure 1. Structure of NAD.

state is substantially populated at low temperatures. This examination was performed on both alpha and beta NAD, which are the two isomers of the coenzyme corresponding to the alpha and beta conformation around the glycoside linkage.

As an experimental parameter is varied which brings two different chromophoric groups into juxtaposition, one might expect that close lying transitions, in particular, exhibit a coupling in which the circular dichroism peak due to a transition in the first chromophore becomes more positive while the circular dichroism peak of a close-lying electronic transition in the second chromophore becomes more negative in a reciprocal manner. This coupling has been called "reciprocal relations" in optical rotation.[8]

Scott et al.[2] examined the fluorescence properties of NADH and model compounds in which the linkage of the adenine and the dihydronicotinamide moieties of NADH was constructed with tri- and hexamethylene chains. They concluded that in water NADH behaves like a molecule in which the adenine and dihydronicotinamide moieties are proximate, as in the trimethylene model compound. In nonaqueous media NADH resembled the hexamethylene model compound, in which the terminal heterocyclic groups are remote.

Secrist and Leonard[3] constructed an "abbreviated" model system of NAD+ which contained the elements of the two heterocyclic rings and the sugar. These compounds were constructed to provide an improved model system which more

closely resembles NAD than those with variable length methylene groups between the adenine and the nicotinamide moieties for investigating the base interactions. The "abbreviated" model systems and NAD were studied by ultraviolet spectroscopy and circular dichroism. The ultraviolet hypochroism of the model systems indicated the presence of an inter-ring interaction which was greater than the one shown for NAD+. The spectroscopic techniques employed verified the existence of an intramolecular interaction in aqueous media between the quaternized nicotinamide ring and the adenine ring.

Nuclear magnetic resonance spectroscopy has been employed to study NAD and the following observations have been made: (1) There are differences in the chemical shifts of the adenine and the nicotinamide protons as a function of the pH.[4] (2) The C-2 and the C-6 protons of the nicotinamide moiety have a differential shielding and it is stated that they are symmetrically disposed with reference to the N-glycoside bond.[5] (3) NADH has an NMR spectrum which contains an AB quartet.[6] (4) The NMR spectrum of N-methyl-N-ethylnicotinamide adenine dinucleotide has four N-methyl resonances.[7]

From the above NMR data, Sarma and Kaplan have proposed a helical model for NAD. The identification of the specific conformation of the folded form by NMR spectroscopy and the proposed Sarma-Kaplan model are still undergoing critical analysis.[9]

Adams et al.[10] have analyzed the lactate dehydrogenase-NAD complex using an X-ray structural determination. The NAD associated with the enzyme was in an essentially linear array.

NAD has complete torsional freedom over the entire linkage from the adenine moiety to the pyridine moiety of the molecule. Thus, a question to be considered is: Does an intramolecular hydrogen bond exist between the amino group of the adenine moiety and the carbonyl of the nicotinamide moiety which would be responsible for the folded conformation and complement the base stacking?

An intramolecular hydrogen bond occurs only when a proton donor and a proton acceptor site on the same molecule are in a favorable spatial configuration; that is, the distance between the hydrogen of the donor group and the acceptor site is between 1.4 and 2.5 Å, and the angular orientation of the acceptor site does not deviate greatly from the bond axis of the donor group, A-H.[11]

The nature of hydrogen bonding can be examined by spectroscopic techniques. From them, one can obtain evidence relating to the formation of a weak bond, the involvement of a specific covalently bound hydrogen atom, and the participation of a specific acceptor group. With this background information, it was decided to employ laser-Raman techniques to monitor the carbonyl group in NAD as a possible acceptor site. The vibrational modes of the hydrogen

bond acceptor, in this case C=O, can be shifted by hydrogen bonding. These shifts are always to lower frequencies.[12]

The analysis of the carbonyl stretching frequencies in various types of carbonyl compounds suggests that the observed band position results from the interplay of several factors, among which the following are clearly important: (1) Physical state of the compound, (2) Inductive effects, (3) Electronic and mass effects of neighboring carbonyl groups, (4) Hydrogen bonding, (5) Enolization, and (6) Solvent effects.

Raman spectroscopy is based on the interaction of light with a molecule resulting in inelastic photon scattering. Vibrational Raman scattering occurs if there is a concomitant change in the polarizability of the molecule, that is when the interaction of the electromagnetic field with the vibrational motion produces an induced dipole moment. There is low interference of water compared to infrared spectroscopy. Therefore, aqueous solutions of biological systems can be examined precisely in the state in which they are found, although higher concentrations are normally necessary since the second order Raman effect is rather weak. Thus, as a check on positions and assignments, infrared spectroscopy can also be employed. Infrared is also employed because the carbonyl stretching absorption band in the region of 1870-1540 cm^{-1} has a relatively constant position, high intensity, and relative freedom from interfering

bands, making this one of the easiest bands to recognize in infrared spectra.[13]

This thesis deals with the Raman and infrared spectroscopic study of the carbonyl frequency of NAD, with the specific purpose being to seek evidence for or against the formation of an intramolecular hydrogen bond.

CHAPTER I

PRELIMINARY REVIEW OF THE CARBONYL FREQUENCY OF THE PYRIDINE MOIETY OF NAD+

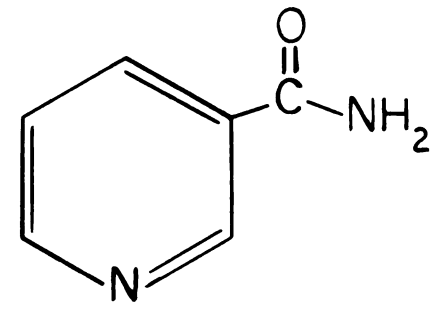
Introduction

In a vibrational spectroscopic study one must determine whether the selected methods and techniques are applicable to the problem of interest.

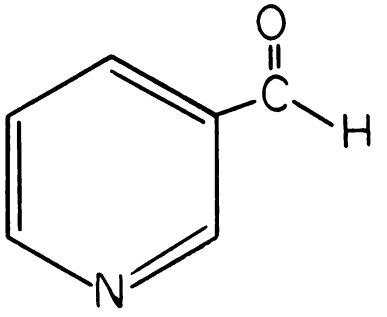
Initially, nicotinamide, nicotinaldehyde, 3-acetyl pyridine and nipecotamide were studied at room temperature and in various solvent systems as models for the pyridine moiety of NAD+. Figure 2 depicts the structures of the compounds that were explored in this preliminary study. The data obtained showed that Raman spectroscopy represented a potentially valuable method for studying the environment of the carbonyl group of NAD+. They also established a basis for interpreting the Raman spectra of the intact NAD+ molecule.

Experimental

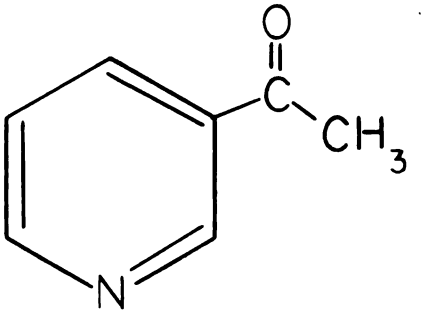
Nicotinamide was obtained from the Sigma Company and 3-acetyl pyridine, nicotinaldehyde and nipecotamide were obtained from the Aldrich Chemical Company and were



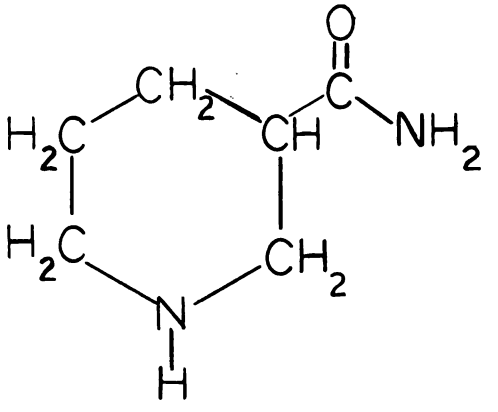
Nicotinamide



Nicotinaldehyde



3-Acetyl pyridine



Nipecotamide

Figure 2. Structures of compounds examined in Chapter I.

used without further purification. The solvents employed were: p-dioxane from the Matheson, Coleman and Bell Company, deuterium oxide from the Columbia Organic Chemicals Company, methyl alcohol-d_4 from the British Oxygen Company, and acetonitrile and dimethylsulfoxide from the J. T. Baker Company. The dimethylsulfoxide was further distilled over 4A molecular sieves to insure the absence of any water. The remaining solvents were used without further purification.

The instrument used was a laser-Raman spectrometer[14] consisting of: either a Spectra-Physics Model 164 Ar^+ laser (providing a maximum output of 1.6 watts of 5145 Å radiation) or a Spectra-Physics Model 165 Kr^+ laser (providing a maximum output of 0.76 watts of 6471 Å radiation); the Spectra-Physics 265 exciter; Spex 1400 double monochrometer; RCA C31034 photomultiplier tube; Victoreen VTE-1 D.C. amplifier; and a Hewlett-Packard Moseley 7100 B strip chart recorder.

Typical Raman instrument settings using the Ar^+ laser were: time constant of 1 or 3 seconds; detector temperature less than -20°C, and voltage between 1000-1300 volts; scan speed 10-2.5 Å/min.; slits 100-200-100 microns; sensitivity 10^{-10} amps; 5145 Å exciting line with power output 1.0-1.3 watts; chart speed of 0.5-0.2 in/min. When the Kr^+ laser was substituted the following changes were made: slits 200-200-200 microns; sensitivity 10^{-11} amps; 6471 Å

exciting line with power output 0.5-0.76 watts; photomultiplier voltage of 1000-1100 volts.

The cells employed for the Raman samples were four millimeter o.d. glass tubing, which was first sealed at one end, bent into an L-shape design, sample added, and then sealed at the other end. The laser beam was focused into the glass tubing by a lens (focal-length 10 cm.). The Raman scattering was collected at 90° from the laser's incident direction and focused on the monochromator entrance slit. See Figure 3 for a schematic representation of the Raman sample cell and the illumination and scattering optics.

A Perkin-Elmer 225 infrared spectrometer was also used. The IR spectra were all double beam. Aqueous solutions were run in 0.2 millimeter thick cells with IRTRAN windows, whereas the nonaqueous solutions were run in 0.103 millimeter thick cells with NaCl windows. The following IR instrument settings were employed: slit program 4; pen traverse time 3 seconds; gain between 100-500.

All solutions examined by Raman analysis were 0.25M concentration except the nicotinamide solutions in acetonitrile and p-dioxane and the aqueous nipecotamide. They were 0.15M, 0.20M, and 1.0M respectively. The solutions inspected using IR analysis were saturated solutions, except the D_2O solution of nicotinamide and the DMSO solutions of nicotinamide and nipecotamide which were 0.25M.

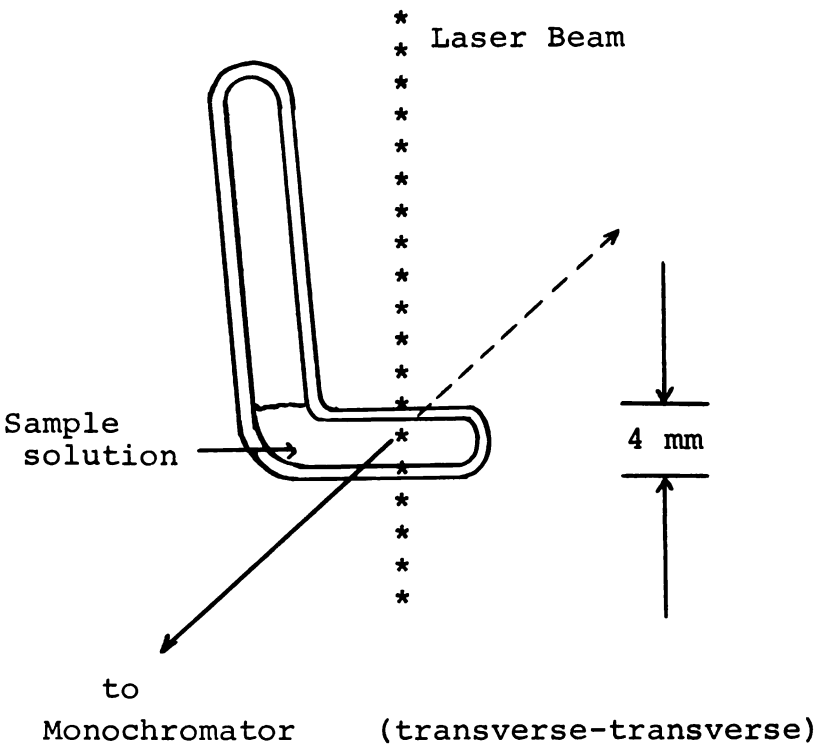


Figure 3. Raman cell.

Figures 4 through 9 are examples of the Raman and IR spectra that were obtained. Table 1 lists the values of the assigned carbonyl frequencies for nicotinamide, 3-acetyl pyridine, and nicotinaldehyde in the various solutions. Table 2 lists the assigned carbonyl frequencies for nipecotamide in D_2O and DMSO.

Solutions of 0.25M nicotinamide in varying percentages of D_2O and p-dioxane by volume were prepared and were examined by Raman analysis, and Table 3 lists the values for the assigned carbonyl frequency.

The error limit or reproducibility of all assignments is ± 1.0 cm^{-1} for both Raman and IR values.

Results and Conclusion

Nicotinamide, 3-acetyl pyridine and nicotinaldehyde are similar in that each contains a pyridine ring with a carbonyl in the 3 position. As a result, any resonance or inductive effects of the ring system on the carbonyl frequency should be identical for the three compounds.

The above mentioned three compounds differ in the R group substituted on the carbonyl group. Basically, none of the R groups, H, NH_2, and CH_3, present any major steric hindrance to solvent interactions at the carbonyl position. Consequently, in considering the electronic distribution of the carbonyl group, any changes brought about by hydrogen bonding of a solvent system should also be similar for the three compounds.

Figure 4. IR spectrum of nicotinamide in chloroform.

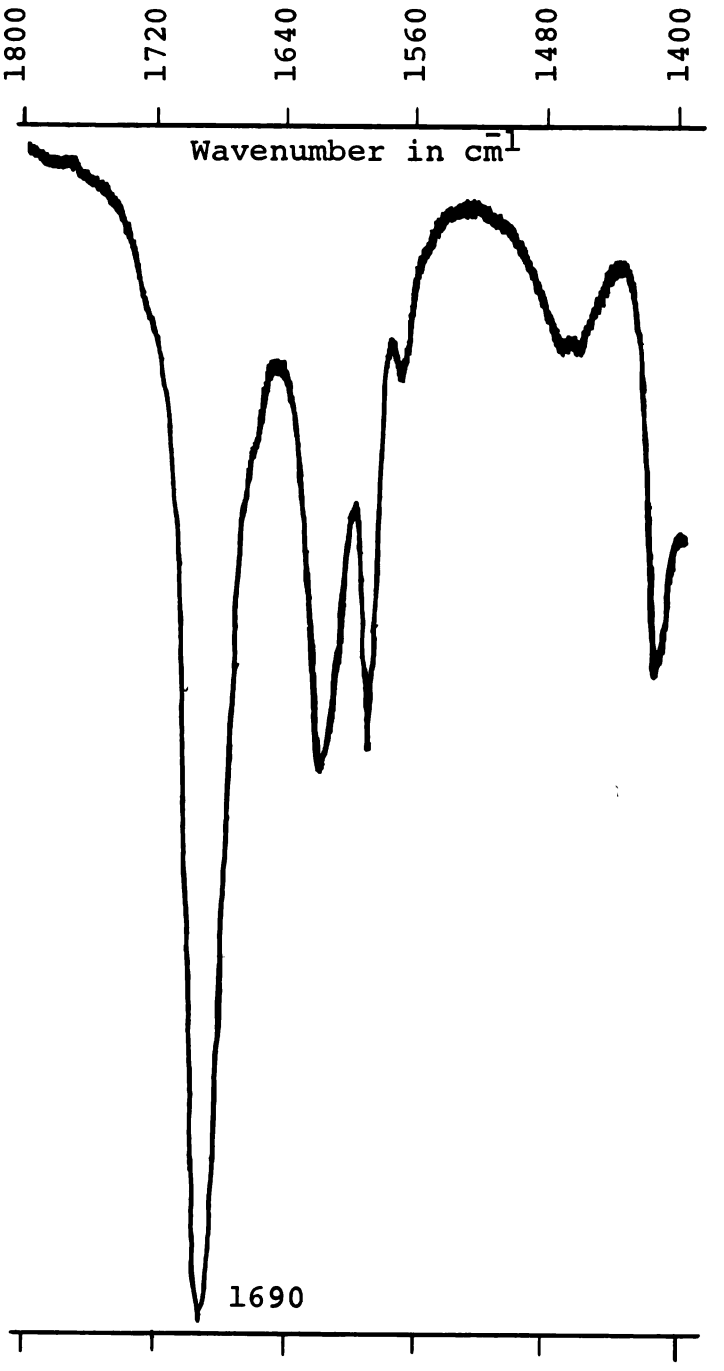


Figure 5. IR spectrum of nicotinamide in p-dioxane.

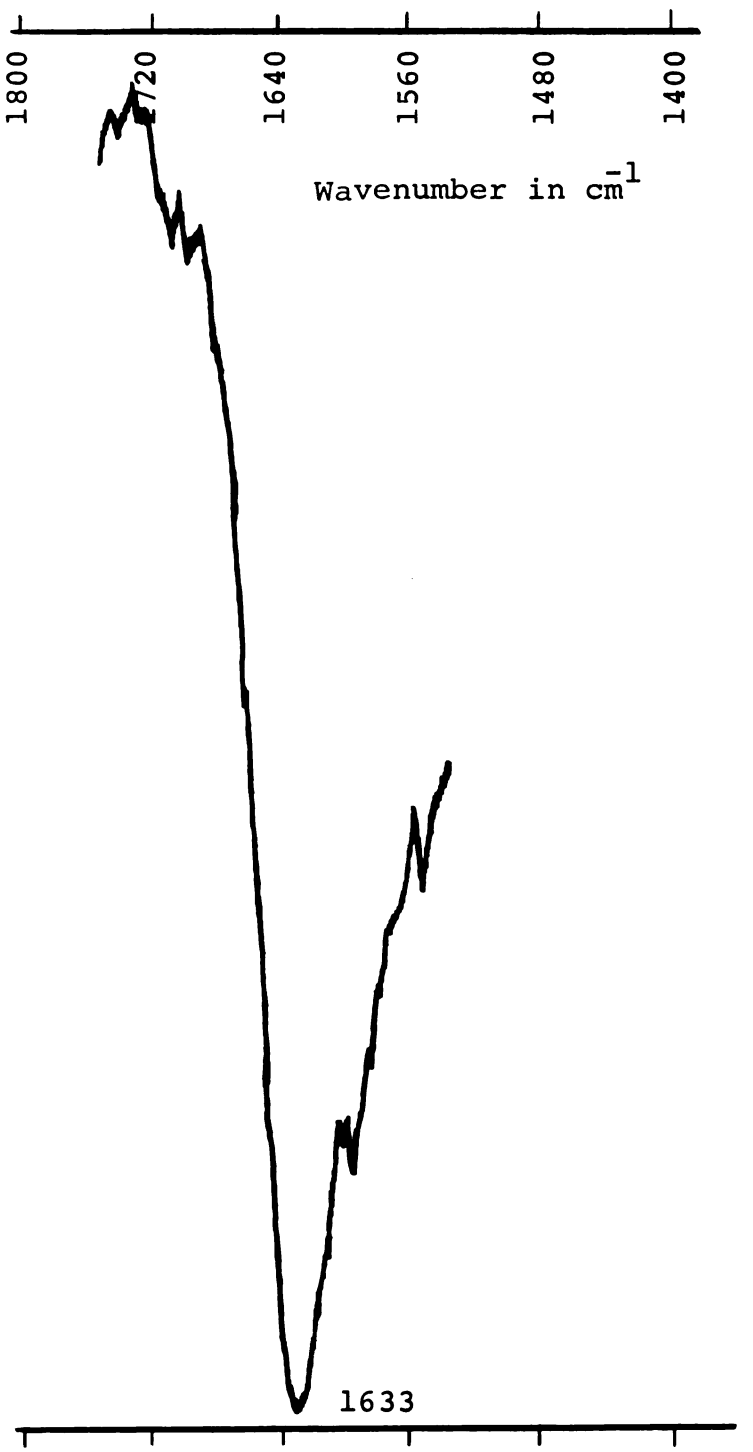


Figure 6. IR spectrum of nicotinamide in D_2O.

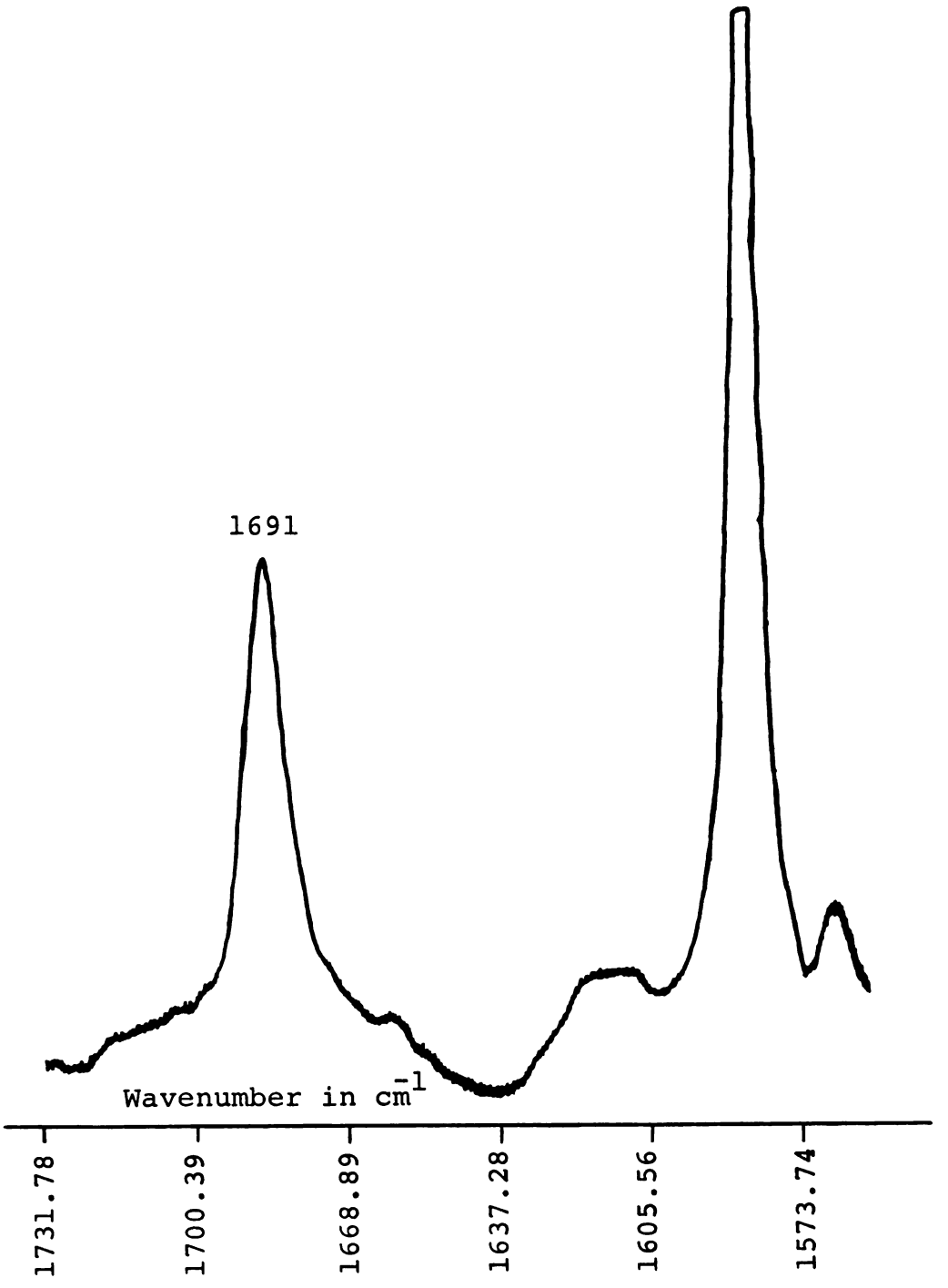


Figure 7. Raman spectrum of nicotinamide in p-dioxane.

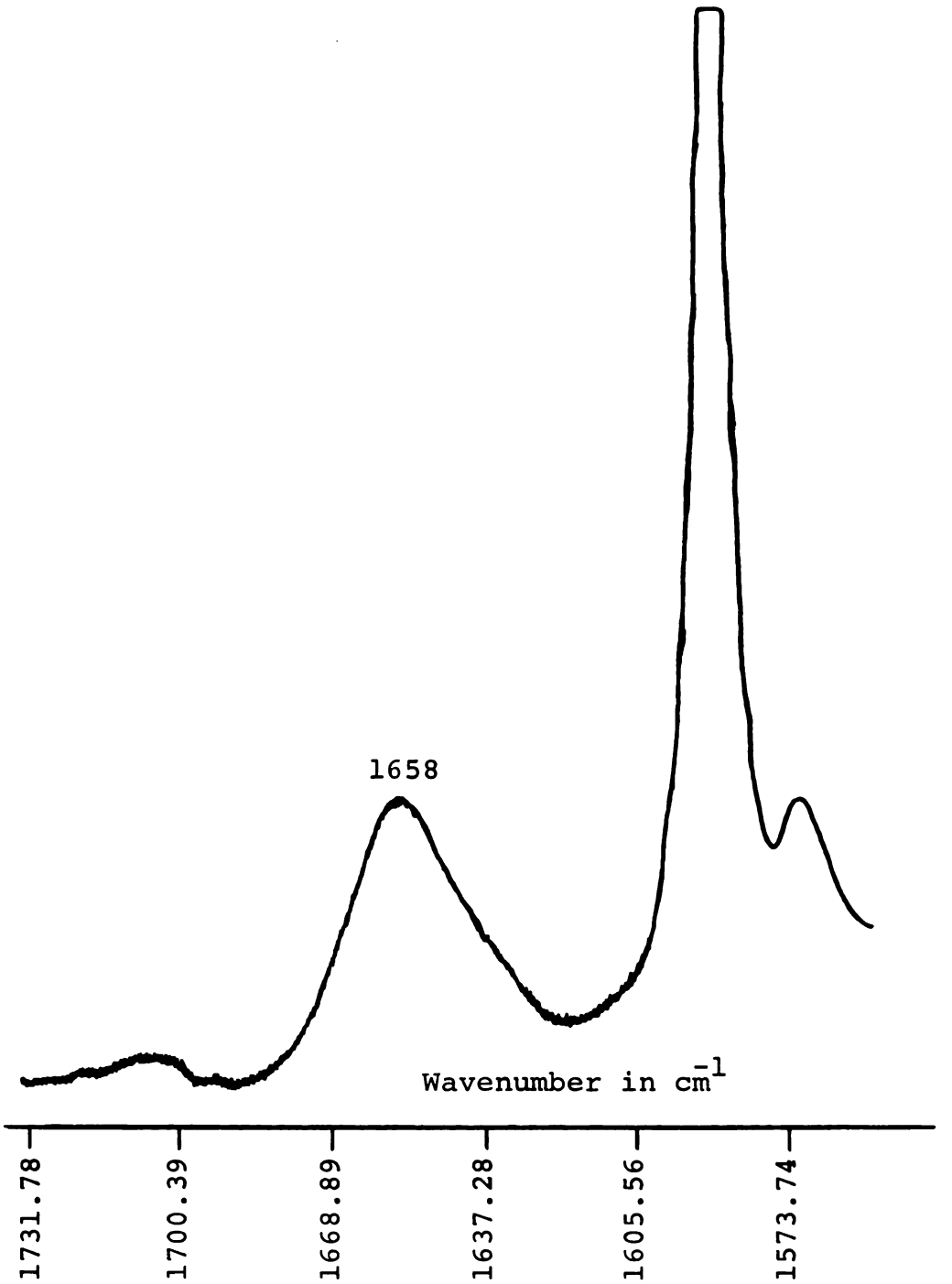


Figure 8. Raman spectrum of nicotinamide in 75 percent p-dioxane and 25 percent D_2O.

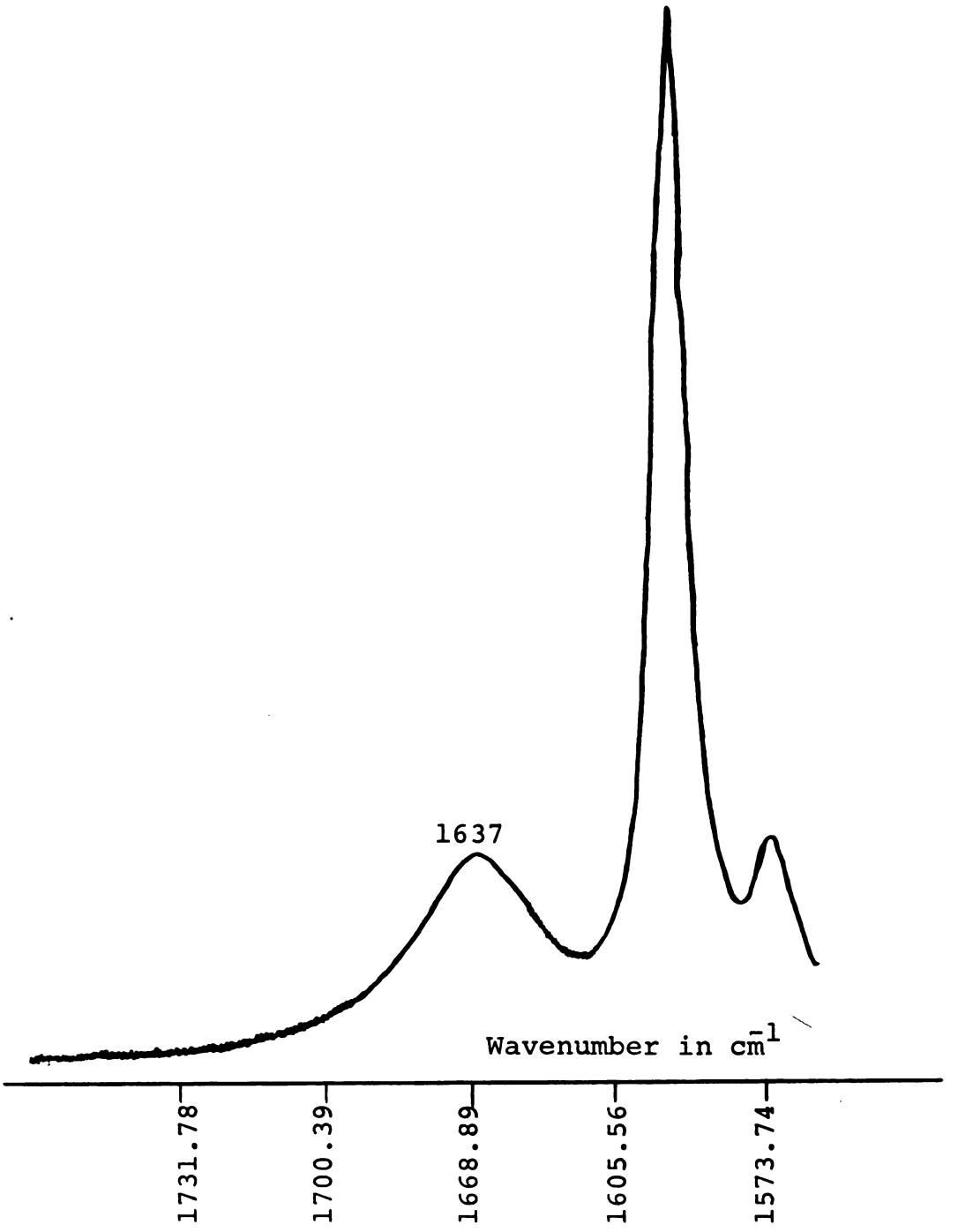


Figure 9. Raman spectrum of nicotinamide in D_2O.

Table 1. Positions of the assigned carbonyl frequencies for nicotinamide, 3-acetyl pyridine, and nicotinaldehyde.

Solvent	Carbonyl Position in cm^{-1}				Solvent Properties	
	R Group				Dipole moment[15]	Dielectric constant[16,17]
	NH_2		H	CH_3		
	Raman	IR	Raman			
D_2O	1637	1633	1705	1684	1.88 (D)	78.25
CD_3OD	1658	1654	1711	1693 1685	2.97*	32.63*
CH_3CN	1689	1690	1707	1692	3.39	38.8
DMSO	1686	1687	1702	1688	3.9 (B)	48.9
p-dioxane	1691	1690	1707	1692	0	2.2
H_2O	--	--	1703	1685	1.92	78.54
C_6H_6	--	1690	--	--	0	2.28
$CHCl_3$	--	1690	--	--	1.55	4.81
Pure Cmpd	1675s	--	1700n	1688n	-----	-----

s solid
n neat
* Values for methanol, CH_3OH
Error limits on all assignments, both Raman and IR are ± 1.0 cm^{-1}

(B) in a benzene solution
(D) in a dioxane solution

Basic Structure is =

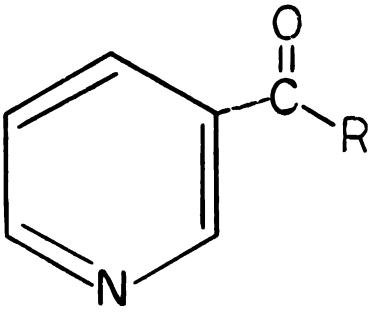


Table 2. Position of the carbonyl frequency for nipecotinamide.

Carbonyl Position in cm^{-1}			
Solvent	Raman	IR	Comments
D_2O	1623	1621	1.0 M
DMSO	1672	1674	0.25 M

Error limits for Position ± 1.0 cm^{-1}

Table 3. Effect of the solvent composition on the carbonyl frequency of nicotinamide.

Percent of p-dioxane	Percent of D_2O	Position in cm^{-1}	Width at Half Height in cm^{-1}
100	0	1691	11
94	6	1665	27
88	12	1659	27
75	25	1658	25
50	50	1648	37
25	75	1639	32
0	100	1637	32

Error limits:

Width at Half Height: ± 10% for each indicated value.

Position: ± 1.0 cm^{-1}

Upon inspection of the data in Table 1 for 3-acetyl pyridine and nicotinaldehyde, one observes that the carbonyl frequency varies only 9 cm^{-1} and 11 cm^{-1} for the respective compounds over the entire range of solvents employed. Thus, one can conclude that the solvent has only a minor effect upon the location of the carbonyl position.

When comparing the results of nicotinamide with those for nicotinaldehyde and 3-acetyl pyridine, one observes an anomalous effect in a D_2O or CD_3OD solution. All of the remaining solvents are within 5 cm^{-1} of each other and the agreement between the IR and Raman data is excellent. As Table 1 indicates, this anomalous effect is apparently not related to the dielectric constant or dipole moment properties of the solvents. D_2O and CD_3OD differ from the other solvents due to their hydrogen bonding properties.

In considering the question of an intermolecular hydrogen bond in nicotinamide, it must be pointed out that in all cases only one carbonyl frequency was observed. Often, such systems result in two carbonyl frequencies being observed. One is due to a free and the other due to a hydrogen bonded carbonyl group.

Intermolecular hydrogen bonding involves association of two or more molecules. The extent of intermolecular hydrogen bonding is temperature dependent. Also, the bands that result from intermolecular hydrogen bonding generally disappear at low concentrations, less than about 0.01M in nonpolar solvents.[18]

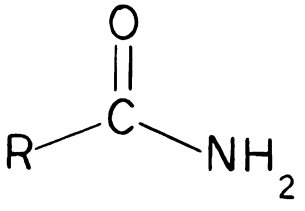
Figure 4 is an IR spectrum of a saturated CH_3Cl solution of nicotinamide, which is less than 0.01M. Figures 5 and 7 are the IR and Raman spectra of a 0.20M nicotinamide solution in p-dioxane. There is no change in the carbonyl position. Thus, there is no evidence of a hydrogen bonded carbonyl resulting from an intermolecular hydrogen bond. Therefore, intermolecular effects should present no problem when interpreting the other spectral data obtained in this study, typically using concentrations of approximately 0.25M.

When one considers Table 3, one observes that the carbonyl frequency is continually lowered as D_2O is added to a dioxane solution of nicotinamide. Dioxane is considered to be an inert solvent because it shows no spectral evidence of an interaction with the carbonyl group. Therefore, the lowering of the carbonyl frequency is related to the presence of D_2O, which is a hydrogen bonding solvent. This study confirms the assignment of 1637 cm^{-1} in an aqueous solvent as the carbonyl frequency.

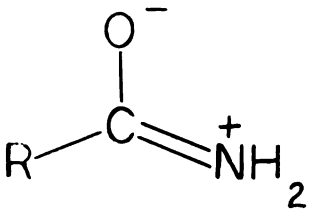
Nipecotamide was also examined. The only double bond is the carbonyl group in the 3 position. As a result, there is no possible interaction with the ring system. When Table 2 is examined and compared with the data for nicotinamide, one observes the same solvent effect. That is, the presence of a hydrogen bonding solvent results in a major lowering of the carbonyl frequency when an amino

group is adjacent to it. This solvent effect is not present with nicotinaldehyde and 3-acetyl pyridine. So, the lowering of the carbonyl frequency is not due to the solvent itself. It appears to be directly related to the presence of an adjacent amino group.

In terms of the adjacent amino group, one can write the following resonance structure:[13]



(I)



(II)

The resonance effect increases the carbonyl bond length and reduces the frequency of absorption.[13] As a result of increased interaction (via hydrogen bonding) with resonance form II, hydrogen bonding solvents could increase the contribution of such an effect; this could provide an explanation for the anomalous lowering of the carbonyl frequency in a hydrogen bonding solvent. In a nonhydrogen bonding solvent, form II would be expected to make a lesser contribution to the electronic structure of the carbonyl.

CHAPTER II

ANALYSIS OF THE CARBONYL FREQUENCY OF QUATERNARY PYRIDINE MODEL COMPOUNDS

Introduction

In the previous chapter it was noted that in an aqueous medium nicotinamide appears to exhibit a resonance effect between the carbonyl group and the adjacent amino group. NAD+ exists with a plus charge on the quaternary pyridine ring. What must be considered next is to what extent a positive charge on the pyridine ring alters the carbonyl frequency.

In these experiments, N-methyl nicotinamide iodide, N-methyl 3-acetyl pyridine iodide, and N-methyl nicotinaldehyde iodide were studied at room temperature and in different solvent systems for the above purpose. NAD+ has an N-glycoside bond to the quaternary pyridine ring. If quaternization of the nitrogen in the pyridine ring does result in delocalization of the electrons from the carbonyl group, it should make no drastic difference as to the group that provides the quaternary linkage to the pyridine ring. Therefore, the methylated compounds of nicotinamide, nicotinaldehyde, and 3-acetyl pyridine were selected as model

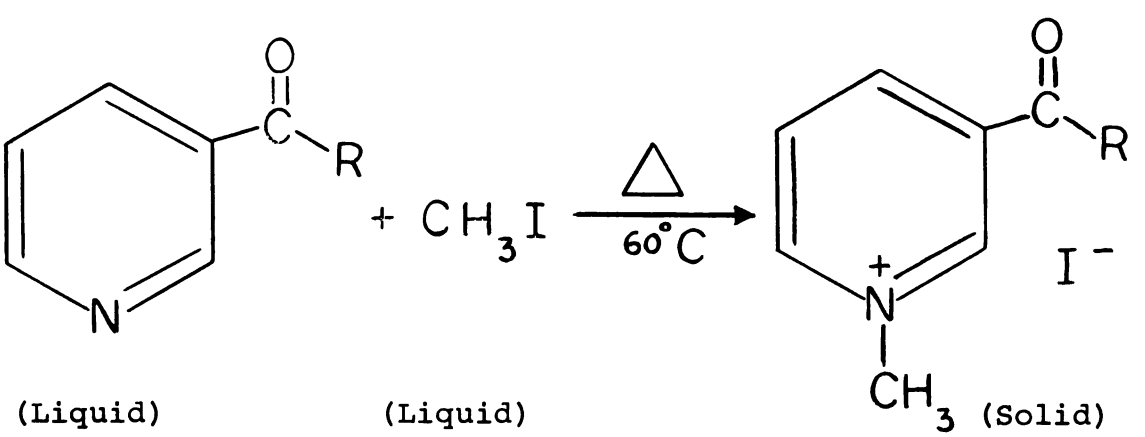
compounds. Figure 10 is the structure of the compounds inspected in this phase of the study.

Experimental

N-methyl nicotinamide iodide was obtained from the Sigma Company, methyl iodide was obtained from the J. T. Baker Company, nicotinaldehyde and 3-acetyl pyridine were obtained from the Aldrich Chemical Company and all were used without further purification. The solvents employed were deuterium oxide from the Columbia Organic Chemical Company and dimethylsulfoxide from the J. T. Baker Company. The dimethylsulfoxide was further distilled over 4A molecular sieves to insure the absence of any water.

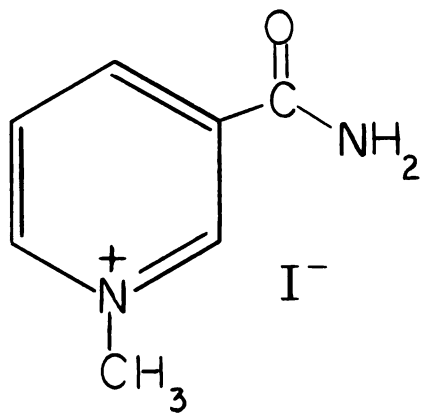
The Raman spectrometer and cells used were described in the previous chapter (see Chapter I, Experimental).

Nicotinaldehyde and 3-acetyl pyridine were methylated by the following reaction:[19]

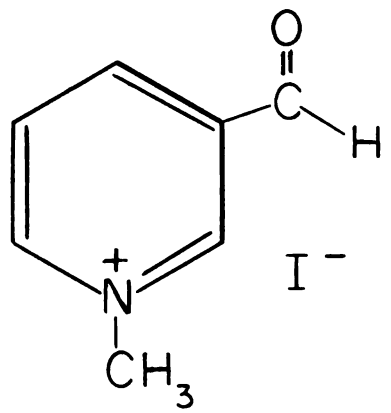


(Liquid) (Liquid) (Solid)

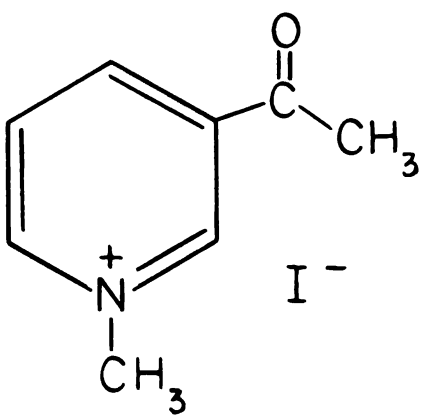
where R = CH_3 and H



N-methyl nicotinamide iodide



N-methyl nicotinaldehyde iodide



N-methyl 3-acetyl pyridine iodide

Figure 10. Structures of compounds examined in Chapter II.

The synthesis utilized a four-fold excess of methyl iodide. The reaction began almost immediately, with a yellow solid being formed in both cases. The reaction was kept at 60°C. to allow the excess CH_3I to evaporate. The products were dried and recrystallized twice from spectral-grade isopropyl alcohol (Matheson, Coleman, and Bell Company). The melting points were taken and are compared in Table 4 with the literature values. The agreement is excellent.

The Raman spectra of the solid methylated model compounds were taken using the Kr^+ laser and the 6471 Å exciting line. The crystals were placed in melting point capillary tubes and the open end was at 90° to the incident laser light. Spectra were obtained for 0.25M solutions of the three methylated compounds in D_2O and dimethylsulfoxide. The other solvents used in the earlier study (Table 1) dissolved inadequate amounts to permit obtaining of Raman spectra. The solutions were examined using the Kr^+ laser and the 6471 Å exciting line, and in addition, the D_2O solutions were examined using the Ar^+ laser and the 5145 Å exciting line. There was excellent agreement (± 1.0 cm^{-1}) between the spectra obtained using the two light sources. Figure 11 is the Raman spectrum of N-methyl nicotinamide iodide in DMSO using the Kr^+ laser and Figure 12 is the Raman spectrum of N-methyl nicotinamide iodide in D_2O using the Ar^+ laser. Table 5 lists the values obtained for the carbonyl frequencies observed for the solutions and the solids.

Table 4. Melting point values for N-methyl nicotinaldehyde iodide and N-methyl 3-acetyl pyridine iodide in °C.

Compound	This work	Literature values	
N-methyl nicotinaldehyde iodide	174-175	172.5-173.5	Boger, Black, San Pietro
		173	Ginsburg, Wilson
		173-175	Ellin, Kondritzer
		174	Pannizon
N-methyl 3-acetyl pyridine iodide	163-165	163.5	Boger, Black, San Pietro
		163-164	Ginsburg, Wilson
		162	Pfleiderer, Sann, Stock
		160-163	Akagi, Paretsky

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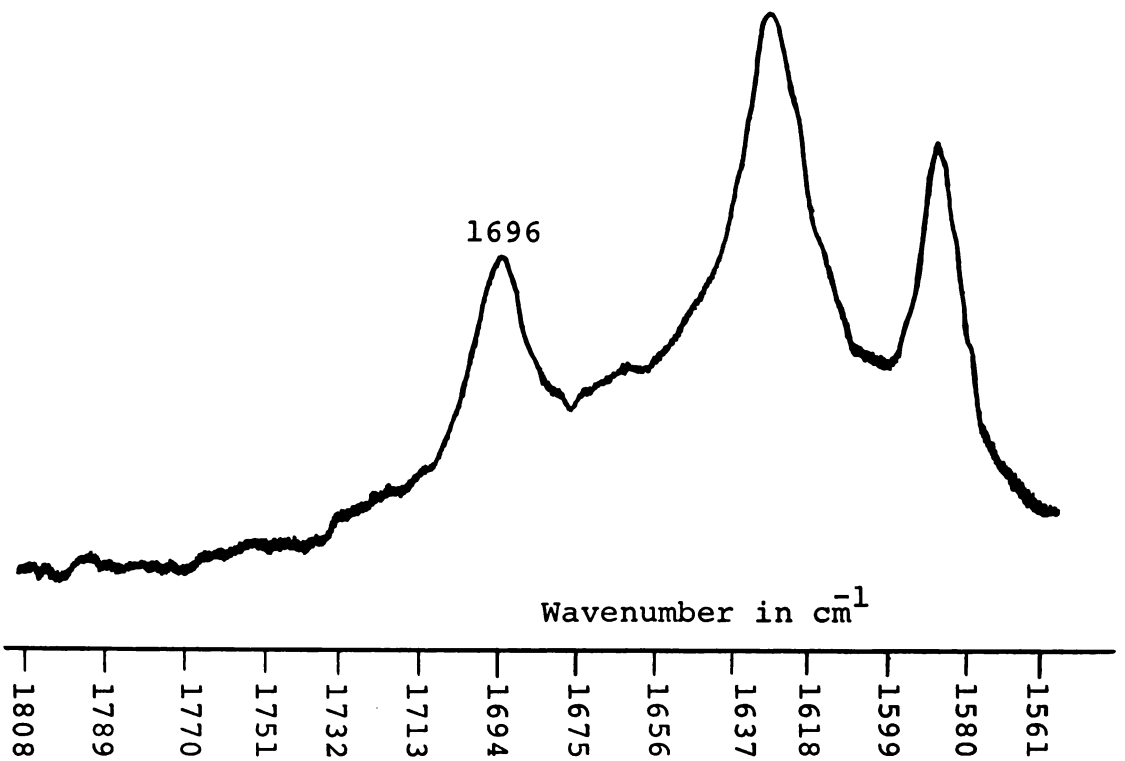


Figure 11. Raman spectrum of N-methyl nicotinamide iodide in dimethylsulfoxide.

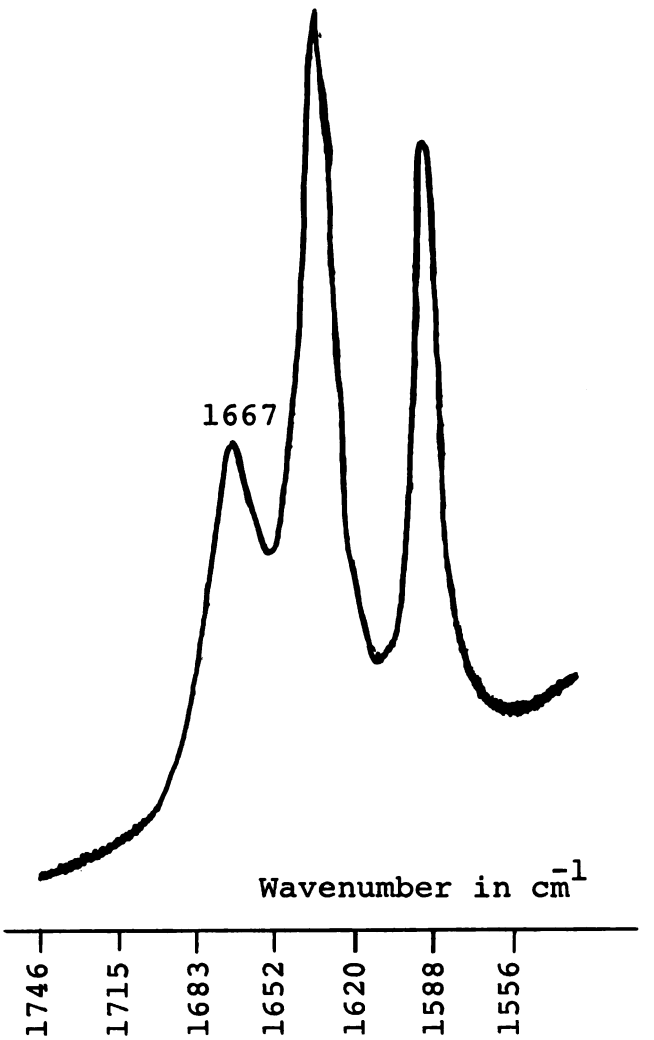


Figure 12. Raman spectrum of N-methyl nicotinamide iodide in D_2O.

Table 5. Position of the carbonyl frequency for N-methyl nicotinamide iodide, N-methyl 3-acetyl pyridine iodide, and N-methyl nicotinaldehyde iodide.

Solvent	IODIDE SALTS					
	N-methyl nicotinamide		N-methyl nicotinaldehyde		N-methyl 3-acetyl pyridine	
D_2O	1667	(1637)	1720	(1705)	1704	(1684)
DMSO	1696	(1686)	1715	(1702)	1702	(1688)
pure (solid)	1680		1699		1702	

The values in parenthesis are the values of the non-methylated compounds from Table 1.

Error limit ± 1.0 cm^{-1} for all carbonyl positions.

Results and Conclusion

Upon inspection of the data in Table 5, one finds there has been a slight increase in the carbonyl frequency (on the order of 10-20 cm^{-1}) when the values of the methylated compounds are compared with those for the nonmethylated derivatives. An increase in the carbonyl frequency strongly suggests that the electrons of the carbonyl group have become more localized, resulting in an increased double bond character. Thus, there is no indication that the presence of a plus charge on the pyridine ring system results in further delocalization of electrons from the carbonyl group into the ring.

Of the solvents used in the previous study (Table 1), only deuterium oxide and dimethylsulfoxide would dissolve a sufficient concentration for Raman spectra of reasonable intensity. Therefore, a solvent study as extensive as the one performed on the nonmethylated derivatives could not be completed.

As shown in Table 5, N-methyl nicotinaldehyde and N-methyl 3-acetyl pyridine iodide, like the nonmethylated compounds, shows only minor differences between the carbonyl frequencies in the different solvents. Thus, if an extensive solvent study could have been done, the results obtained would probably have been very similar to the data obtained for the nonmethylated derivatives.

As with nicotinamide, however, N-methyl nicotinamide iodide shows an anomalous effect on the carbonyl frequency with the presence of a hydrogen bonding solvent. The difference between the DMSO and the D_2O values is 29 cm^{-1} as compared with 49 cm^{-1} for nicotinamide. One can again extend the same arguments that were advanced for the anomaly of nicotinamide in solution to explain the anomaly of N-methyl nicotinamide in solution. The electronic distribution of the carbonyl group has been changed due to a resonance effect between the carbonyl group and the adjacent amino group and this effect is facilitated by the presence of a hydrogen bonding solvent.

CHAPTER III

ANALYSIS OF THE CARBONYL FREQUENCY OF NAD

Introduction

In their circular dichroism study, Miles and Urry[1] noted that a reciprocal behavior was observed for both beta NAD+ and NADH as the temperature was increased above room temperature. They concluded that at room temperature NAD exists in a stacked conformation and the reciprocal behavior that is observed as the temperature is increased to 65°C. is due to the molecule changing conformation and going to an open form, that is, no stacking between the adenine and the nicotinamide moieties.

With this observation in mind, studies were performed on beta NAD+, NADH, the 3-aldehyde analog of NAD+, NMNH, and N-methyl nicotinaldehyde iodide to determine the effects of temperature on the carbonyl frequency of these compounds. NMNH is the abbreviation for the reduced form of nicotinamide mononucleotide and Figure 13 shows the structure of this compound. In addition, the 3-acetyl analog of NADH was examined at room temperature. Ultraviolet spectra were also obtained in several cases in order to

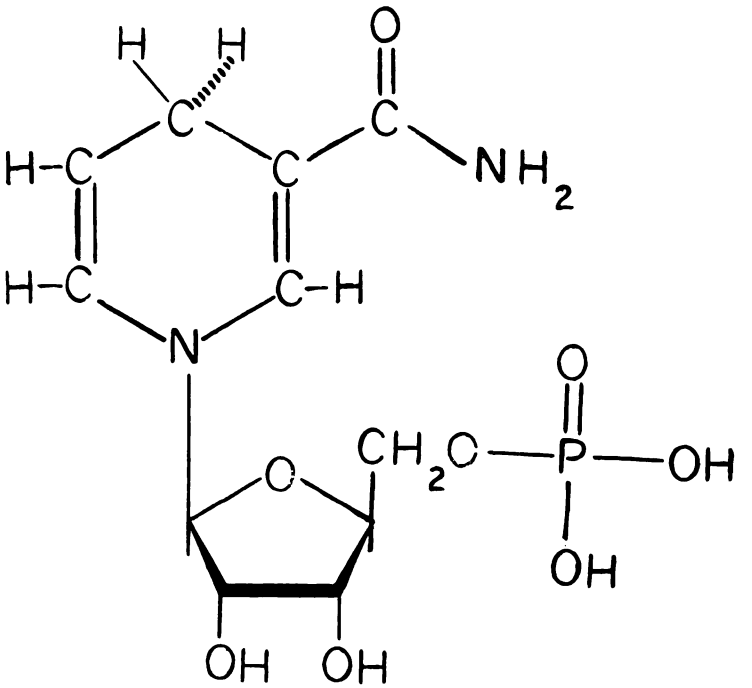


Figure 13. Structure of NMNH.

investigate possible correlation between the electronic and vibrational spectra.

Experimental

Beta NAD+, NADH, NMNH, and the 3-aldehyde analog of NAD+ were all obtained from the Sigma Company and the 3-acetyl analog of NADH was obtained from P-L Biochemicals, Inc.. All were used without further purification. The solvents used were deuterium oxide (Columbia Organic Chemicals) and dimethylsulfoxide (J. T. Baker). The dimethylsulfoxide was purified as described previously. The N-methyl nicotinaldehyde iodide was that prepared in the previous chapter (see Chapter II, Experimental).

A Coleman 124 double beam Spectrophotometer and quartz cells were used to take UV spectra. The following compounds were inspected by UV: 0.1mM NADH in DMSO and H_2O; 0.2mM N-methyl nicotinamide iodide, N-methyl nicotinaldehyde, and N-methyl 3-acetyl pyridine iodide in H_2O; 0.1mM NMNH in H_2O; and a 0.1mM solution of the 3-acetyl analog of NADH in H_2O and DMSO. The values obtained are listed in Table 6.

The Raman and IR spectrometers and cells used were those described in the first chapter (see Chapter I, Experimental).

Temperature studies were carried out by placing the sample capillary in an unsilvered Dewar cell, as depicted

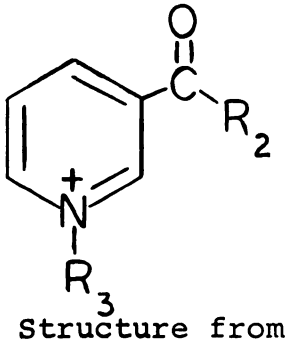
Table 6. Ultraviolet spectra of selected compounds.

Pyridine Derivatives		Dihydro Derivatives			$\lambda_{max.}$ in nm	
R_2	R_3	R_4	R_5	Solvent System	This Work	Literature[20,21]
NH_2	CH_3			H_2O	264	---
CH_3	CH_3			H_2O	265	---
H	CH_3			H_2O	262	---
NH_2	ribose 5 phosphate			H_2O	---	263
		NH_2	M	H_2O	338	338
		NH_2	M	DMSO	332	---
		CH_3	M	H_2O	362	363
		CH_3	M	DMSO	354	---
		H	M	H_2O	---	358
		NH_2	$CH_2C_6H_5$	CH_3OH	---	352
		CH_3	$CH_2C_6H_5$	CH_3OH	---	371
		NH_2	ribose 5 phosphate	H_2O	337	---

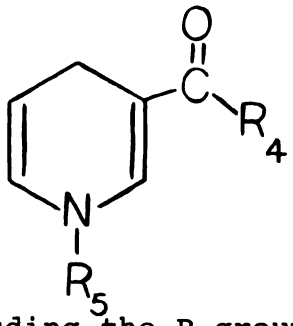
Error limit: ± 1.5 nm

Basic Structures:

Pyridine Derivatives



Dihydro Derivatives



M: Structure from Figure 1, excluding the R group

in Figure 14. Nitrogen gas was passed through coils wrapped in heating tape and placed in an auxillary Dewar. The flow rate of the nitrogen gas, which could be accurately controlled, determined the temperature. Using a Leeds and Northrup millivolt potentiometer, the temperature was measured in the sample chamber by a Copper-Constantan thermocouple placed in a mercury-filled tube adjacent to the sample.

Raman spectra were taken of NAD+, NADH, NMNH, the aldehyde analog of NAD+, and N-methyl nicotinaldehyde iodide at room temperature (19°C.), and at 70°C. The 3-acetyl analog of NADH was examined at room temperature (21°C.). The NADH, NMNH, and the 3-acetyl analog of NADH solutions were scanned using the Kr^+ laser and the 6471 Å exciting line and the others were inspected using the Ar^+ laser and the 5145 Å exciting line. Solutions of 0.25M were prepared in D_2O. NADH and the 3-acetyl analog of NADH were also scanned in DMSO solutions. The solubility properties of the compounds studied prevented any extensive examination of the solvent effect upon the carbonyl frequencies. Table 7 lists the carbonyl frequency values obtained in the temperature studies.

Figure 15 is the Raman spectrum of NMNH in D_2O at 19°C. and was obtained using the Kr^+ laser. Figure 16 is the IR spectrum of a 0.1M NAD+ solution in D_2O. Figure 17 is the Raman spectrum of NAD+ in D_2O at 19°C. using the Ar^+ laser.

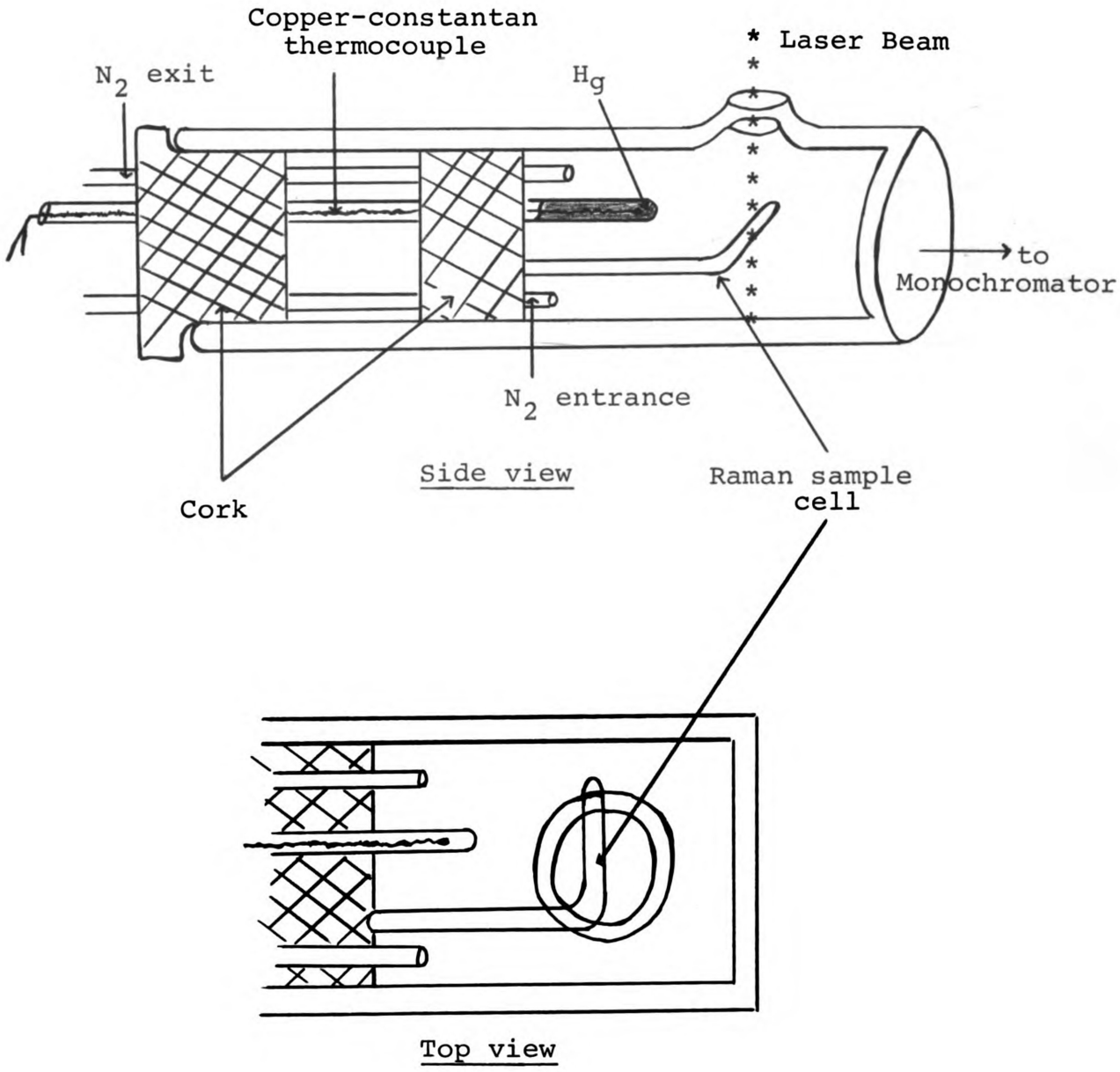


Figure 14. Raman temperature control unit[22].

Table 7. Effect of temperature on the carbonyl frequency in the Raman spectra of NAD and similar molecules.

Compound	Carbonyl Frequency (cm^{-1})	
	Temperature 19°C.	70°C.
NAD+ in D_2O	1667	1667
NAD+ in D_2O	1666 (IR)	
3-Aldehyde analog of NAD+ in D_2O	1717	1717
N-methyl nicotinaldehyde iodide in D_2O	1720	1720
NMNH in D_2O	1689	1689
NADH in H_2O	1689	1689
NADH in D_2O	1689	1689
NADH in DMSO	1689	1689
3-Acetyl analog of NADH in D_2O	1679*	
3-Acetyl analog of NADH in DMSO	1679*	

Error limit: ± 1.0 cm^{-1} for all carbonyl positions.

*Temperature was 21°C.

IR infrared value

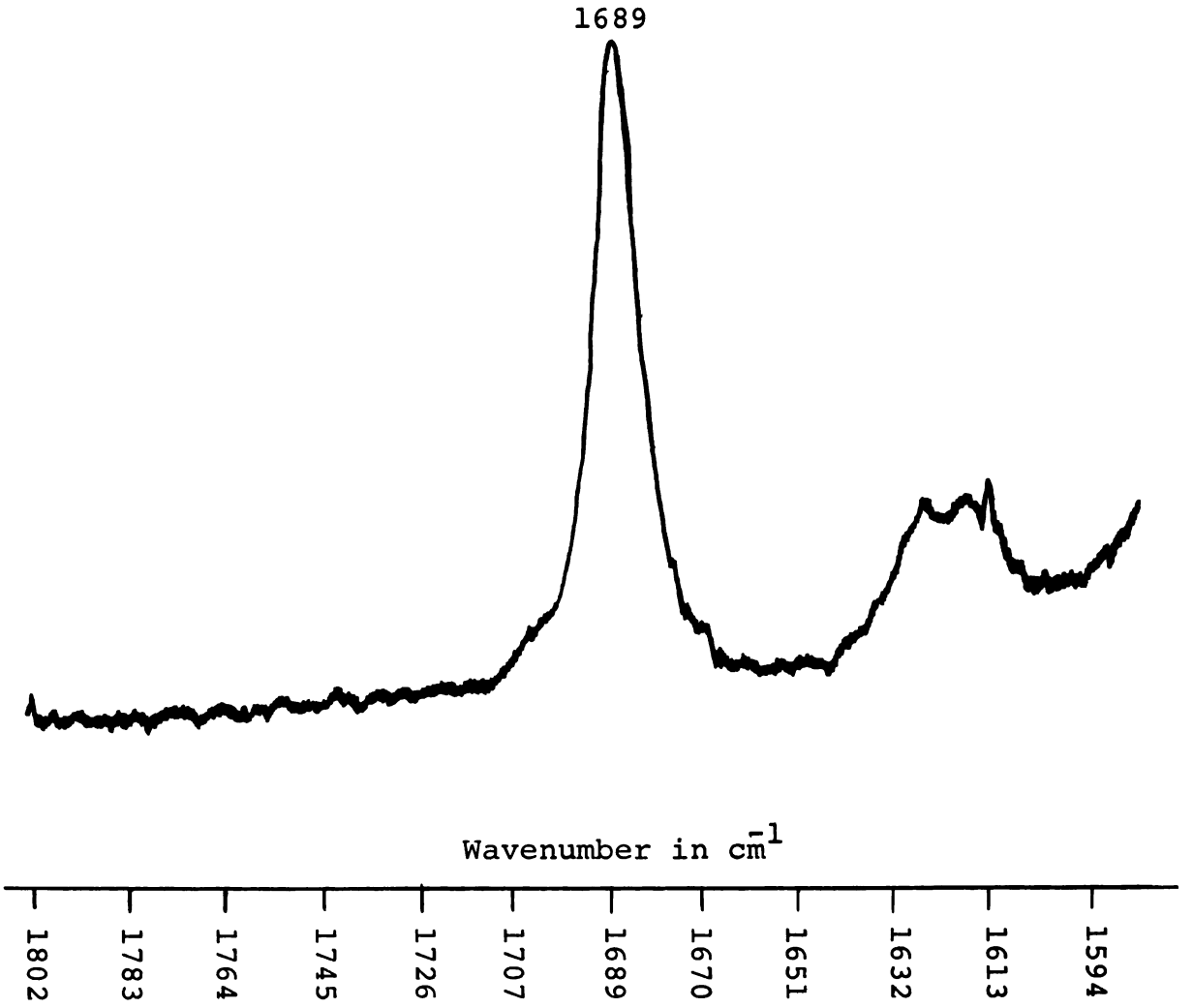


Figure 15. Raman spectrum of NMNH in D_2O.

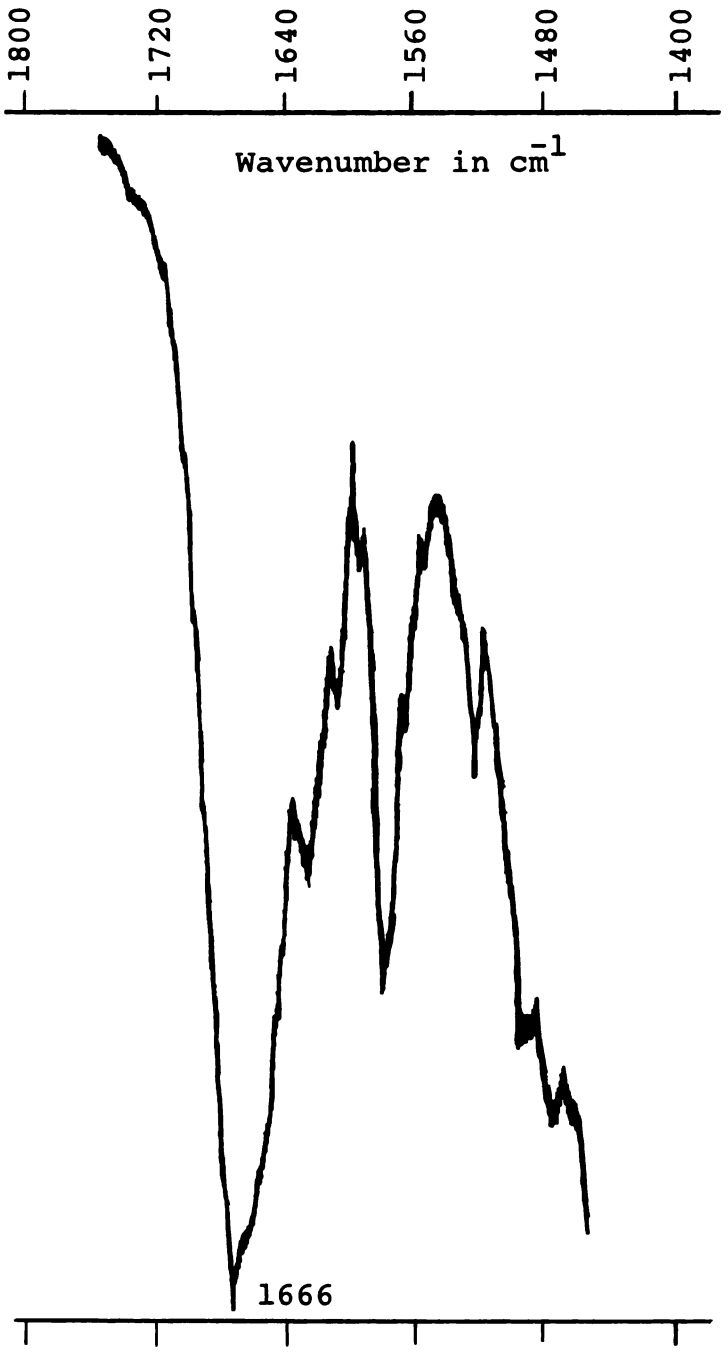


Figure 16. IR spectrum of NAD+ in D_2O.

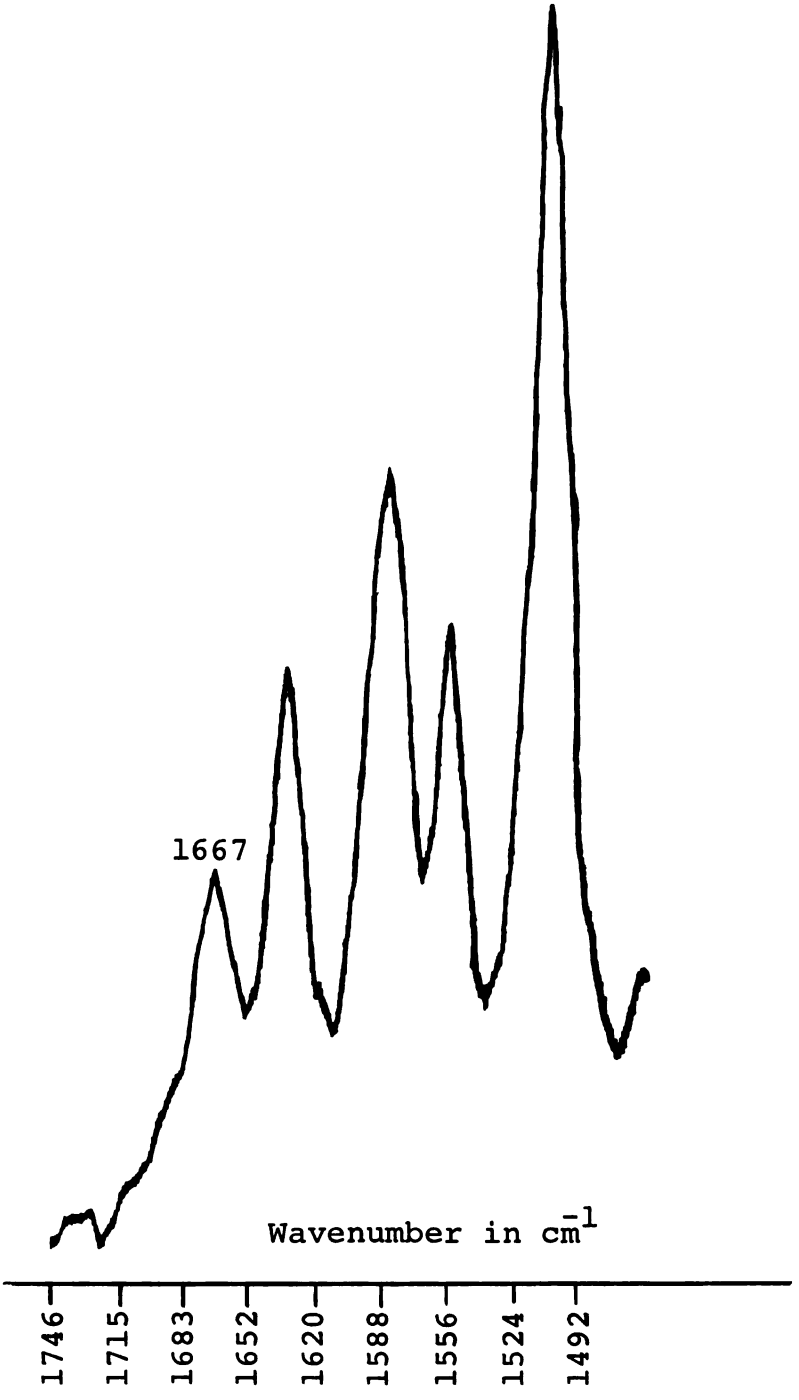


Figure 17. Raman spectrum of NAD+ in D_2O.

Results and Conclusion

Intramolecular hydrogen bonding is an internal effect and persists at very low concentrations, but the extent of intramolecular bonding would be expected to be temperature dependent.[13] If an intramolecular hydrogen bond existed between the adenine amino group and the carbonyl group of the nicotinamide, it should be reflected in a lowering of the carbonyl frequency.[12] With an increase in temperature, which has been shown[1] to disrupt the interactions between the rings, the perturbation in the carbonyl frequency would be removed. As shown in Table 7, there is no change in the carbonyl frequency of NAD+, NADH, and the 3-aldehyde analog of NAD+, with the change in temperature. This lack of a temperature dependence of the carbonyl frequency in the compounds considered may be interpreted as evidence against the existence of an intramolecular hydrogen bond between the amino adenine group and the nicotinamide carbonyl group of NAD.

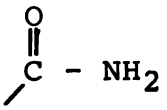
There is also excellent agreement between the carbonyl frequencies observed for NAD+ and the aldehyde analog of NAD+ (Table 7) and the values found for the corresponding N-methyl derivatives (Table 5); this provides further support for the view that the N-methyl compounds represent useful models for the more complex pyridine nucleotides. Furthermore, the agreement between the position and the effect of solvent on the carbonyl frequency of NAD+ and that

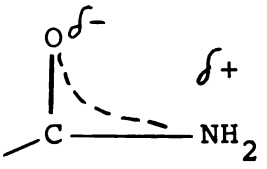
of the model compound, N-methyl nicotinamide iodide, indicates that the resonance interaction proposed to exist in the model compound (see Chapter II) also exists in NAD+.

When one scans Table 6 for the oxidized methylated compounds that were considered in Chapter II, one finds that the values obtained are very similar to NMN. Thus, the electronic transitions in the ring system of the oxidized compounds are insensitive to changes in the substituents on the ring nitrogen or the three position of the ring. This again indicates that the N-methyl derivatives serve as adequate model compounds for the NAD oxidized analogs. Also, the suggested resonance interaction between the carbonyl group and the adjacent amino group apparently does not affect the electronic transitions of the ring.

In contrast, the UV data indicate that the electronic transitions in the dihydro compounds <u>are</u> appreciably affected by the nature of the substituents on the nitrogen of the ring and in the three position. The data in Table 6 show that when the substituent in the three position is held constant, there is a difference in the absorption maximum dependent on the substituent in the one position of the dihydro ring. When one keeps the substituent in the one position constant, one finds that changing the substituent in the three position also results in major changes. Thus, it is clear that, in contrast to the oxidized compounds, there must be extensive electronic interactions

between the group at the three position (which includes the carbonyl of direct interest in the present study) of the dihydro pyridine ring and the rest of the molecule. The nature of these interactions in the dihydro compounds can only be elucidated by a more extensive study.

Sarma and Kaplan[23] stated at a symposium on "Pyridine Nucleotide-Dependent Dehydrogenases" that: "It is difficult to generalize why the different analogs substituted in the three position of the pyridine moiety react so differently with the various pyridine nucleotide dehydrogenases. The variation is certainly not attributable to difference in the stacking of the bases. The possibility exists that different enzymes can distinguish changes in the side chain at the three position." Thus, the electronic distribution of the amide group of NAD+ and NADH could be of major importance in determining interaction at the binding sites for the coenzymes. The present investigation provides considerable evidence in support of the view that, in aqueous solutions, the three position  group of NAD+ is not a "typical" amide, but rather is more adequately represented by the structure:



Furthermore, this structure does not appear to make a significant contribution to the electronic structure of NADH. Thus, changes in oxidation state of the ring affect not only the ring itself, but also the electronic properties of the three substituent. These differences are likely to be of great significance in determining enzyme-coenzyme interactions.

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