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Proton Nuclear Magnetic Resonance Study of the Conformation and Configuration of the Cyclized Pyridine Nucleotide Adducts†

L. J. Arnold, Jr., N. J. Oppenheimer,* C.-Y. Lee,‡ and N. O. Kaplan

ABSTRACT: We have closely examined by high-frequency ^{1}H nuclear magnetic resonance spectroscopy the structure of the adducts which form when various carbonyl compounds react with pyridine nucleotides at elevated pH. These studies show that the adducts of *N*-(2,6-dichlorobenzyl)nicotinamide–acetone, *N*-(2,6-dichlorobenzyl)nicotinamide–pyruvate, NMN–pyruvate, NAD–pyruvate, NAD–acetaldehyde, and NAD–oxaloacetate form with identical structural features as well as configuration. The following structural features are observed: (1) the adducts are pyridine N-4-substituted compounds; (2) a second six-membered ring forms by addition of the nicotinamide amido to the carbonyl group of the compound forming the addition complex; (3) cyclization occurs stereospecifically, indicating that the stereochemistry is predetermined by the initial attack at the N-4 position; (4) two diastereomeric forms are observed for each nucleotide adduct. Finally, the determination of configuration at all symmetric carbon atoms in these adducts will be discussed.

The nucleophilic addition of carbonyl compounds to NAD$^+$ has been extensively studied because of the important biochemical properties of the resulting adducts. These adducts are specific inhibitors of various dehydrogenases (Long & Kaplan, 1973; Everse et al., 1971, 1972), and they have been used as specific eluants for the purification of dehydrogenases by affinity chromatography (Lee et al., 1974; Kaplan et al., 1974). Furthermore, they are thought to be related to the abortive ternary complex of NAD$^+$, pyruvate, and heart type lactate dehydrogenase which serves a regulatory function in heart muscle (Everse et al., 1972; Arnold & Kaplan, 1974). Investigations into the chemical properties of these adducts by Burton & Kaplan (1954) have led to the proposal that the adducts originate from the nucleophilic attack by the α carbon of a carbonyl compound on the N-4 position of NAD$^+$. Subsequent studies of NAD$^+$ adducts (Burton et al., 1957; Dolin & Jacobson, 1964) and nicotinamide derivatives (Ludowieg et al., 1964) have substantiated this proposal.

In order to understand the origins of substrate specificity reflected in the specificity of inhibition by the NAD adducts, detailed knowledge of their chemistry and conformation is required. ^{1}H NMR provides a unique tool for such investigations since resonances of diastereomers are in principle nonequivalent (Mislow & Raban, 1966) and conformations can be determined from the angular dependence of vicinal coupling constants (Karplus, 1963; Gutowsky et al., 1959). Thus at high magnetic fields it is possible to resolve and assign the absorptions for specific diastereomeric forms of the adducts. From this information it is possible to determine the stereoselectivity of the reactions and the populations of the resulting forms, as well as the conformation and configuration of the adducts. In this study we provide ^{1}H NMR data for a number of biologically important NAD$^+$ adducts and related model compounds and determine their conformations as well as configurations.

Experimental Section

Materials

N-(2,6-Dichlorobenzyl)nicotinamide–Acetone Adduct. One gram of *N*-(2,6-dichlorobenzyl)nicotinamide (DCB-Nic[1]), prepared according to Krohnke & Ellegast (1956), was dissolved in 60 mL of acetone:water (1:1). To this solution was added 3 mL of a saturated solution of sodium carbonate. After 5 min, 1 volume of water was added and the reaction mixture was placed in the freezer at −20 °C, whereupon the DCB–Nic–acetone adduct crystallized to give a yield of 800 mg (66%).

N-(2,6-Dichlorobenzyl)nicotinamide–Pyruvate Adduct. Column purification of the DCB-Nic–pyruvate adduct was

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[1] Abbreviations used: NAD$^+$, nicotinamide adenine dinucleotide; NAD–pyruvate, NAD–acetaldehyde, and NAD–oxaloacetate are the pyruvate, acetaldehyde, and oxaloacetate adducts of NAD$^+$, respectively; Nic, nicotinamide, DCB, *N*-(2,6-dichlorobenzyl); DCB-nicotinamide–acetone and DCB-nicotinamide–pyruvate are the acetone and pyruvate adducts of DCB-nicotinamide, respectively; N-2, N-4, N-5, N-6, N-9, N-10ax, and N-10eq are the nicotinamide 2, 4, 5, 6, 9, 10 axial, and 10 equatorial protons, respectively, and A-2 and A-8 refer to the adenine 2 and 8 protons; TSP, sodium trimethylsilylpropionate-*2,2,3,3-d*$_4$; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; TMAC, tetramethylammonium chloride; Me_4Si, tetramethylsilane; EDTA, (ethylenedinitrilo)tetraacetic acid; forms *R* and *S* are the forms with an *R* and *S* configuration at N-4 of the adducts.

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found to be futile. Attempts to lyophilize "pure" material (based on the UV absorption of the column fraction) led to multiple degradation products reflecting both rearrangement and oxidation. This problem was circumvented by preparing the adduct in situ just prior to obtaining its ^1H NMR spectrum. A D_2O solution of 0.2 M DCB-Nic and 0.6 M pyruvate was carefully titrated with NaOD until the ^1H NMR spectrum revealed that all the DCB-Nic had reacted to form the reduced adduct. At this point additional NaOD was added to raise the pD to about 12 in order to exchange the protons of the unreacted pyruvate. By this procedure only the proton resonances of the DCB-Nic–pyruvate adduct remained.

NAD–Pyruvate, NAD–Acetaldehyde, and NAD–Oxaloacetate Adducts. The NAD–pyruvate, NAD–acetaldehyde, and NAD–oxaloacetate adducts were synthesized according to the procedure of Everse et al. (1971) with alterations in the DEAE-11 chromatography. Elution of these adducts from the DEAE-11 column were achieved with a linear 0–0.5 M ammonium bicarbonate gradient which was prepared in oxygen-free water. All these adducts were found to elute at approximately 0.35 M ammonium bicarbonate and were stable to lyophilization. The purity of the final products was greater than 90%.

NMN–Pyruvate Adduct. The NMN–pyruvate adduct was prepared by two different methods.

Method 1. The NAD–pyruvate adduct (133 μmol) and phosphodiesterase (0.2 mg) (Boehringer and Mannheim) were incubated for 3 h in 50 mL of dilute ammonium bicarbonate, pH 8. The resulting mixture of AMP and the NMN–pyruvate adduct was diluted to 200 mL and applied to a 100-mL DEAE-11 column in the bicarbonate form. A 0–0.4 M linear ammonium bicarbonate gradient eluted the AMP first followed by the NMN–pyruvate adduct at approximately 0.2 M ammonium bicarbonate. The fractions with A_{260}/A_{340} of less than 0.2 were pooled and lyophilized. The yield was 38 μmol (31%) of the NMN–pyruvate adduct.

Method 2. A solution of NMN^+ and AMP was prepared by adjusting a 6-mL solution of 300 μmol of NAD^+ to pH 7 with ammonium bicarbonate and adding 0.2 mg of phosphodiesterase (Boehringer and Mannheim). Upon completion of hydrolysis, pyruvate (2.7 mmol) was added and the pH raised to 11.5 by the addition of 1 N sodium hydroxide. The ensuing reaction was quenched by adjusting the pH to 9.0 with ammonium bicarbonate when the A_{340}/A_{260} reached 0.33. The material was then purified as described in method 1. The yield of the NMN–pyruvate adduct was 200 μmol (66%).

Methods

Proton Magnetic Resonance Measurements. Proton magnetic resonance spectra were obtained on a Varian HR 220 spectrometer. When necessary, the signal-to-noise ratio of the spectra was increased by signal averaging with a Nicolet 1074 computer or subsequently by a Nicolet Fourier transform system. Samples were lyophilized twice from 99.8% D_2O and then dissolved in 100% D_2O (Wilmad). Spectra were obtained at the concentrations indicated and, at 22 °C, the ambient temperature of the probe. Sample volumes were 0.25 mL and Wilmad vortex plugs were used. Internal standards of TMAC (tetramethylammonium chloride), DSS (4,4-dimethyl-4-silapentanesulfonate), Me_4Si (tetramethylsilane), or TSP (sodium trimethylsilylpropionate-*2,2,3,3-d*$_4$) were used, and 1 mM EDTA was added to suppress line broadening from possible paramagnetic impurities. The pD was measured on a Corning Model 12 pH meter and the standard electrode correction was made; pD = meter reading + 0.4 (Glasoe & Long, 1960).

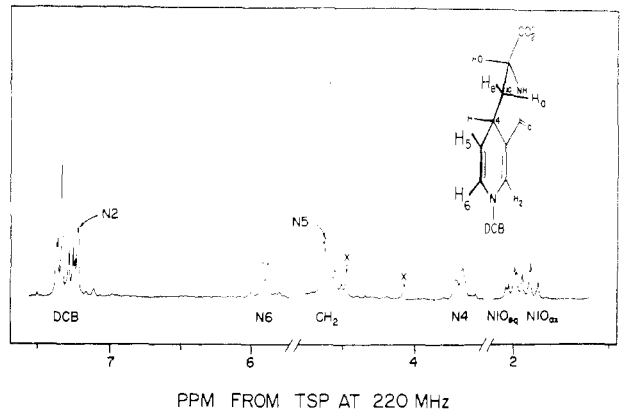

FIGURE 1: The ^1H NMR spectrum of the DCB-nicotinamide–pyruvate adduct.[2] The adduct is dissolved in D_2O:CD_3OD (60:40). The chemical shifts are in hertz from TSP at 22 °C.

Computer simulations of the ^1H NMR spectra were generated by using the Nicolet ITRCAL program. The chemical shifts and coupling constants of the observed spectra were fitted to within 0.1 Hz.

Ultraviolet Measurements. Ultraviolet spectra were obtained by using a Perkin-Elmer Coleman 124 double beam spectrophotometer and additional ultraviolet measurements were acquired with a Zeiss Model M4 QII.

Results

Cyclized N-(2,6-Dichlorobenzyl)-1,4-dihydronicotinamide Adducts. The possibility of four distinct diastereomeric forms and the expected complexity of the ^1H NMR spectrum of the NAD adducts required the initial examination of simple model nicotinamide compounds. The DCB-nicotinamide system was chosen for the following reasons. (1) The protons of the DCB group do not overlap with the resonances of the dihydronicotinamide adduct, unlike *N*-alkyl substituents. (2) The adducts are readily synthesized and are relatively stable. (3) The adduct possesses a plane of symmetry; thus, there can be at most only two diastereomeric forms. Consequently, the model compounds can provide information about both the intrinsic stereoselectivity of the initial addition reaction and the subsequent cyclization reaction. (4) The lower molecular weight of the model compounds, hence, faster molecular reorientation, compared with the dinucleotide adducts, generally provides better resolution and allows measurement of the small, long-range scalar coupling constants.

Assignments. The ^1H NMR spectrum of the DCB-nicotinamide–pyruvate adduct is shown in Figure 1[2] and that of the DCB-nicotinamide–acetone adduct in Figure 2. Although the substituent at N-9 differs (carboxyl for pyruvate and methyl for acetone), the overall similarity of the spectra indicates that the N-9 substituent has little effect on the conformation of the adduct. The results of the DCB-nicotinamide–acetone adduct are in accord with previous studies by Ludowieg et al. (1964) on the *n*-propylnicotin-

[2] It should be pointed out that in this spectrum the N-4 proton resonance is asymmetric. In reality it should be symmetrical. Due to the instability of this adduct, it was prepared immediately before obtaining the spectrum by adding NaOD to a solution of pyruvate and DCB-nicotinamide. Under these conditions, pyruvate undergoes approximately 10% deuterium exchange before adduct formation. Deuterium at the N-10 position collapses the N-4 resonance to a narrow doublet and at the same time shifts the resonance slightly upfield because deuterium is more electron donating than hydrogen. The superposition of this deuterium-labeled material on the unexchanged spectrum produces the asymmetric N-4 resonance. When the DCB-nicotinamide–pyruvate adduct is formed in H_2O/CH_3OH, the N-4 is indeed symmetric.

Table I: Coupling Constants of the Cyclized Adducts[a]

	$^4J_{2-4}$	$^4J_{2-6}$	$^3J_{4-5}$	$^4J_{4-6}$	$^3J_{4-10eq}$	$^3J_{4-10ax}$	$^3J_{5-6}$	$^3J_{9-10eq}$	$^3J_{9-10ax}$	$^2J_{10gem}$
DCB-Nic-pyruvate	<1.0	1.2	2.0	2.2	3.6	13.0	7.8	*	*	−13.0
DCB-Nic-acetone	<1.0	1.5	2.1	2.1	3.6	12.8	8.0	*	*	−13.0
NMN-pyruvate	-	1.2	2.0	2.1	3.6	13.2	7.8	*	*	−13.0
NAD-pyruvate	-	-	1.9	-	3.6	13.2	7.9	*	*	−13.2
NAD-acetaldehyde	-	-	2.1	-	3.6	13.6	8.2	2.2	3.3	−13.2
NAD-oxaloacetate	-	-	~2.0	-	*	12.1	8.1	*	*	*

[a] The concentrations are 80 mM and the temperature is 22 °C. The pD is 8.0. An asterisk indicates that the coupling constant is not present. A dash indicates that the coupling constant is unresolved.

Table II: Chemical Shifts of the Cyclized Adducts[a]

	form	N-2	N-4	N-5	N-6	N-10 ax	N-10 eq	N-1′	A-2	A-8
NMN-pyruvate	*R*	7.265	3.745	4.922	6.305	2.036	2.138	5.436		
	S	7.265	3.745	4.977	6.418	1.975	2.145	5.474		
NAD-pyruvate	*R*	7.089	3.480	4.670	6.061	1.850	1.993	4.875	8.105	8.432
	S	7.120	3.480	4.690	6.139	1.770	1.970	4.934	8.105	3.433
$\Delta\delta$[b]	*R*	0.176	0.265	0.252	0.244	0.186	0.145	0.561		
	S	0.145	0.265	0.287	0.279	0.255	0.175	0.540		
NAD-acetaldehyde	*R*	7.069	3.458	4.642	6.098	1.575	1.828	4.895	8.093	8.440
	S	7.069	3.458	4.711	6.193	1.480	1.861	4.945	8.093	8.440
NAD-oxaloacetate	*R*	7.183	3.609	-	6.250	2.824	*	-	8.183	8.478
	S	7.142	3.690	-	6.162	2.835	*	-	8.183	8.465
DCB-Nic-pyruvate[c]		7.244	3.652	4.606	5.904	1.910	2.049			
DCB-Nic-acetone[d]		7.230	3.768	4.511	5.870	1.745	2.005			

[a] The concentration is 80 mM and the temperature is 22 °C. The chemical shifts are in ppm downfield from TSP at 220 MHz and the pD is 8.0. [b] Differential chemical shifts of the NAD-pyruvate adduct and the NMN-pyruvate adduct. [c] In D_2O-CD_3OD (60:40). [d] In $CDCl_3$ chemical shifts are from Me_4Si. An asterisk indicates that the proton is not present. A dash indicates that the resonance is unresolved.

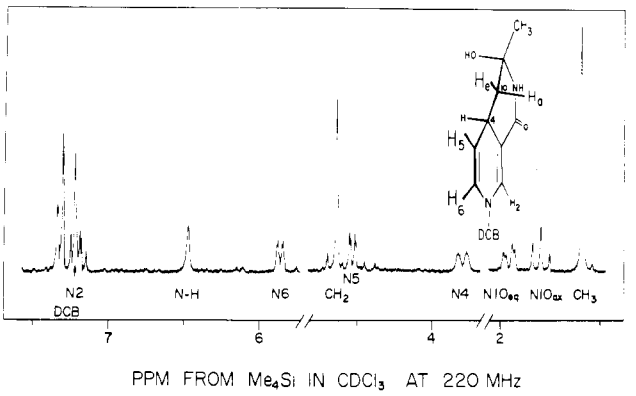

FIGURE 2: The ^{1}H NMR spectrum of the DCB-nicotinamide–acetone adduct. The chemical shifts are in Hertz from Me_4Si (tetramethylsilane) and the solvent is CD_3Cl at 22 °C.

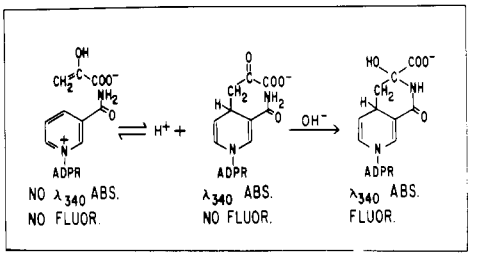

FIGURE 3: The proposed mechanism for the base catalyzed adduct formation of the chemically produced NAD–pyruvate adduct.

amide–acetone adduct. However, with the use of the DCB substituent, we have been able to resolve the spectral parameters. The assignment of the proton resonances for these compounds is based on homonuclear spin decoupling and analogy to the chemical shifts and coupling constants of other model nicotinamide adducts and to 1,4-dihydronicotinamide compounds. The values of the coupling constants are listed in Table I and the chemical shifts are listed in Table II.

Structure of Cyclized Adducts. The formation of the cyclized adducts as proposed by Ozols & Marinetti (1969) and Everse et al. (1971) involves two discrete addition reactions: (1) the addition of the nucleophilic α carbon to the N-4 position; and (2) the attack on the carbonyl by the amido nitrogen to form a six-membered ring as shown in Figure 3. Our ^{1}H NMR data for the model compounds shown in Figures 1 and 2 confirm this structure by the following evidence: (1) the spectra correspond to a 1,4-dihydropyridine compound; (2) decoupling experiments show that a methylene group is directly attached to the N-4 position; (3) the N-10 methylene group is approximately 1.4 ppm upfield from the chemical shift that would be expected if it were adjacent to a carbonyl group (the observed chemical shift is consistent with its being adjacent to a carbinolamine); and (4) the broad, single proton NH resonance at 6.45 ppm (see Figure 2) is consistent with an N-substituted amido and is further evidence for cyclization. The observation of the NH resonance in organic solvents also precludes the possibility that dehydration across the N-9–N-10 bond has occurred to any significant degree, as has been previously suggested (Ozols & Marinetti, 1969).

Magnetic resonance spectra of compounds in an isotropic environment in principle can resolve diastereomers but not enantiomers (Mislow & Raban, 1966). Since the nicotinamide ring is prochiral, addition to the N-4 position in an achiral environment cannot be stereoselective. This requires that a pair of enantiomers be present, which are not discernible. At the same time, the fact that the model adducts consist of a single set of unique resonances (see Figures 1 and 2) is *inconsistent* with the presence of diastereomers. Together these observations require that the cyclization reaction is stereospecific, the stereochemistry of ring closure depending solely on the configuration of the initial attack regard N-4. The precise geometry of the adduct will be discussed below.

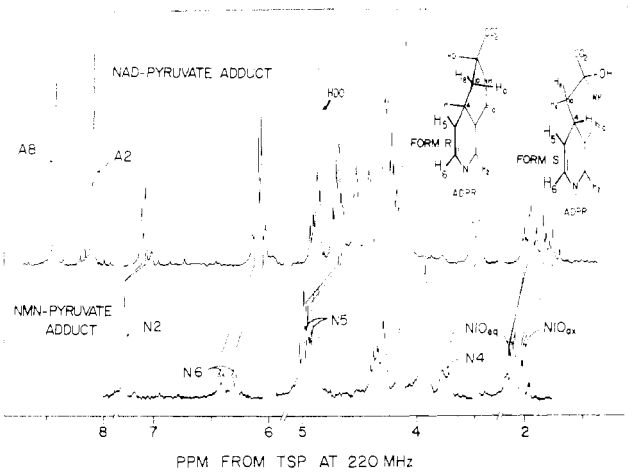

FIGURE 4: The NMN–pyruvate and NAD–pyruvate adducts at 22 °C. Samples are in D_2O at 80 mM using TSP as an internal standard. Note that both of these spectra show the presence of two forms. These spectra also indicate the chemical shift changes which occur in the two forms in going from the mononucleotide to the dinucleotide. In the case of the dinucleotide, the forms are present as a high (form *R*) and low (form *S*) population species. Note that form *R* has an *R* configuration at N-4 and form *S* has an *S* configuration at N4.

^1H NMR Spectra of the Cyclized NMN^+ and NAD^+ Adducts. The ^1H NMR spectra of the NMN–pyruvate and NAD–pyruvate adducts are shown in Figure 4. These spectra reveal the added complexity induced by the chiral ribose moiety which precludes enantiomeric forms. Now the two forms of the adduct are related as diastereomers since they are magnetically nonequivalent. This complexity should not obscure the fact that the spectrum of each form is very similar to those of the model compounds, especially in regard to coupling constants. Therefore, the observation of only two forms indicates that the stereoselectivity of cyclization found in the model compounds is retained in the nucleotide adducts.

As can be seen in Figure 4, the proportions of the two forms of the dinucleotide adduct are about 1/0.7, whereas in the mononucleotide the populations are equal. These results are analogous to those found for the NAD cyanide adducts (Oppenheimer et al., 1971), and on this basis we assign the higher population as a nicotinamide A-side addition product (*R* form), and the smaller population as a B-side addition product (S form). This assignment has been further substantiated by enzymic preparation of the cyclized adduct which yields only the *R* form (Arnold & Kaplan, 1974).

Assignments of the Pyruvate Adduct Resonances. The resonances for each of the two forms can be readily assigned by homonuclear spin decoupling together with computer simulation (data not shown). The specific correspondence of the resonances for each form of NMN–pyruvate with those in the dinucleotide adduct is more difficult since the population of the two forms is equal when NMN–pyruvate is prepared by attack of pyruvate on NMN^+. Nevertheless, these assignments can be made unambiguously by preparing the NMN–pyruvate adduct from the NAD–pyruvate adduct where the populations of the two forms are different (1/0.7). Snake venom phosphodiesterase cleavage of the NAD–pyruvate adduct and subsequent separation of the nucleotides by DEAE-11 chromatography yielded the NMN–pyruvate adduct with the same population ratio of diasteromers as found in the dinucleotide, thus establishing the correspondence between the resonances of the two forms. This observation also verifies the model that the intact backbone of NAD^+ is responsible for the unequal population ratios (Oppenheimer et al., 1971).

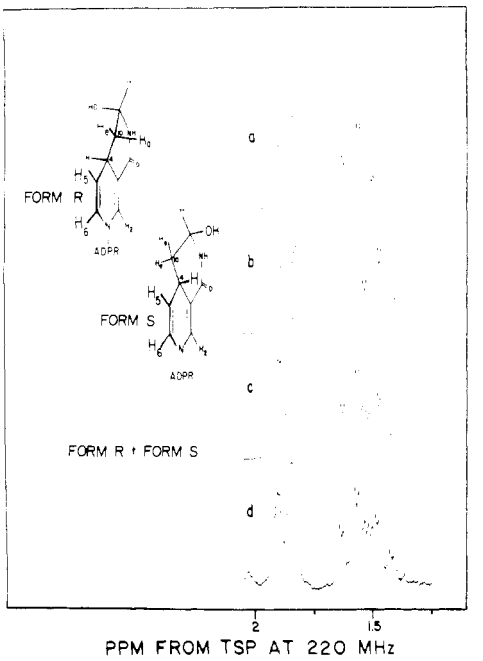

FIGURE 5: The N-10 region of the NAD–acetaldehyde adduct. a and b are the computer simulations of forms *R* and *S*; c is a and b summed in the ratio of 1:0.7; and d is the spectrum obtained empirically. The simulations were obtained by using the coupling constants given for the NAD–acetaldehyde adduct in Table I and the chemical shifts in Table II. This decoupling experiment reveals that N-9 has small coupling constants to the N-10ax (3.3 Hz) and the N-10eq (2.2 Hz); thus, the N-9 proton is gauche–gauche to the N-10 methylene protons. Concentrations are 80 mM and chemical shifts are from TSP at 22 °C.

The assignment of the absolute configuration of the two forms will be discussed below.

Assignments of the Acetaldehyde Adduct Resonances. The coupling constants and chemical shifts of the NAD–acetaldehyde adduct[3] are very similar to the corresponding values for the NAD–pyruvate adduct. In addition, the substitution of a proton for the N-9 carbonyl group results in a new resonance. This proton has been assigned by homonuclear spin decoupling of the N-10 protons and is found to be superimposed on the N-1′ proton resonance. Computer analysis of the N-10 methylene region was used to determine the vicinal coupling constants and the simulated spectra are compared with the observed spectrum in Figure 5. This analysis establishes the vicinal coupling constants of the N-9 methine and the N-10 methylene for both forms to be 2.2 Hz and 3.3 Hz (see Table I).

Coupling Constant Analysis of the Cyclized Adducts. The coupling constants we have determined for both the model DCB-nicotinamide and corresponding resonances of the pyridine nucleotide cyclized adducts are listed in Table I. Some of the smaller coupling constants of the nucleotide adducts were not resolved but could be estimated from the values determined for corresponding model compounds. As can be seen, the coupling constants of the model compound adducts are virtually identical with those of the nucleotide adducts. Figure 6 shows typical coupling constant values of the cyclized nicotinamide adducts superimposed on the general structure of these adducts. Note that the NAD–acetaldehyde adduct possesses two additional coupling constants due to the N-9 proton.

[3] Attempts to prepare the DCB-nicotinamide–acetaldehyde adduct were unsuccessful due to its instability.

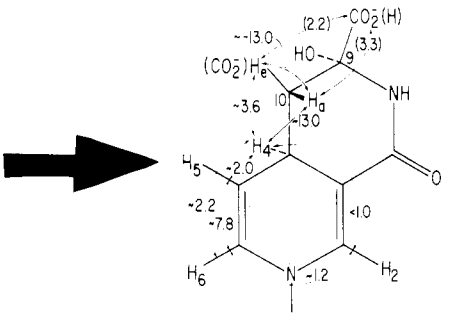

FIGURE 6: The structure and approximate coupling constants of the cyclized adducts. Note that as determined all of the adducts are closely related in structure. Furthermore, the adducts exist predominately in two forms, the form shown here and its mirror image, in a ratio of 1:0.7.

These cyclized adducts show a distinct decrease in the value of $^3J_{4-5}$ compared with other 1,4-dihydropyridine derivatives such as NMNH, 3.4 Hz (Oppenheimer et al., 1971), and NMN–CN, 4.5 Hz (Arnold, 1975). The value of 2.0–2.2 Hz in these cyclized adducts is consistent with an axial orientation of the N-4 proton. The orientation of the N-4 proton can be independently determined by analysis of $^4J_{4-6}$ since four bond coupling through a π bond is maximal for a dihedral angle of 90°. The observed value of $^4J_{4-6}$ in the cyclized adduct is 2.2 Hz, whereas in NMNH it is 1.6 Hz (Sarma & Mynott, 1972) and in the cyanide adduct it is only 0.8 Hz (Arnold, 1975).

Conformation about Carbons N-4 and N-10 in the Cyclized Adducts. The value of the vicinal coupling constants between the N-10 methylene protons and the N-4 proton can be correlated to the geometry of the molecule via the appropriate Karplus relationships (Gutowsky et al., 1959; Karplus, 1963). In the absence of dynamic interconversion between significant populations of conformers, the coupling constants for six-membered rings can be used to unambiguously establish relative orientations. Vicinal coupling constants greater than 9 Hz indicate the predominance of a trans orientation and values less than 6 Hz a gauche orientation, either as a gauche–trans or gauche–gauche conformation. Thus the N-10 proton at 1.911 ppm for the DCB-nicotinamide–pyruvate adduct must be axial since it shows a large, 13-Hz trans coupling to the N-4, whereas the other N-10 proton at 2.049 ppm shows the small gauche coupling constant of 3.6 Hz expected for an equatorial orientation. Furthermore, these values indicate the overwhelming predominance of a single conformer and that other conformers, if present, have only a minor effect on the coupling constants.

Conformation at N-9 in the NAD–Acetaldehyde Adducts. The steric constraints of the six-membered cyclized ring permit only two possible staggered geometries for the N-9 substituents with respect to the N-10 methylene protons. These two configurations are shown in Figure 7. In the case of the NAD–acetaldehyde adduct, the configuration at this position can be obtained from the N-9 vicinal coupling constants to the N-10 methylene protons. The two small coupling constants of 2.2 Hz and 3.3 Hz which are observed for the N-9 proton unambiguously define it as gauche to both N-10 methylene protons. Finally, knowing the conformation of the N-9–N-10 and N-10–N-4 bonds also establishes the configuration at N-9. Thus the high population form of the NAD–acetaldehyde adduct has an *R*-(N-4), *S*-(N-9) configuration and the low population form is *S*-(N-4), *R*-(N-9). See Figures 5 and 6.

Conformation at N-9 in the NAD–Pyruvate Adduct. Like the NAD–acetaldehyde adduct, the NAD–pyruvate adduct can have two possible configurations at N-10, but, unlike the acetaldehyde adduct, the pyruvate adduct has no N-9 proton. Consequently, coupling constant analysis alone cannot be used to determine the configuration of this position. The stereochemistry at N-9 can be easily distinguished by using the paramagnetic broadening agent, gadolinium. This ion broadens proton resonances with a $1/r^6$ distance dependence and shows no angular contribution. Furthermore, it complexes primarily with anionic functional groups. As shown in Figure 7, the axial, gauche–trans orientation of the carboxylate will juxtapose the gadolinium ion to the N-10eq proton but not the N-10ax proton, whereas a gauche–gauche orientation of the carboxylate places the gadolinium approximately equidistant from both the N-10ax and N-10 eq protons. We have chosen to examine the DCB-nicotinamide–pyruvate adduct since this model adduct has a relatively simple ^{1}H NMR spectrum and only one anionic binding site, the carboxylate at N-10.

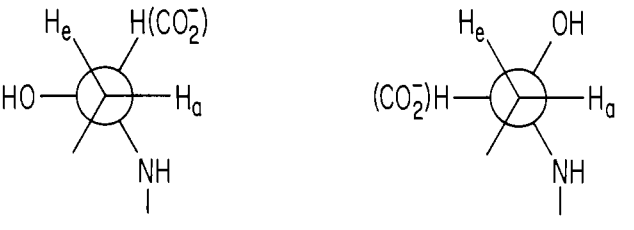

FIGURE 7: The two possible orientations about the N-9–N-10 bond in the pyruvate and acetaldehyde adducts. Coupling constants and line-broadening studies indicate that these adducts exist in a gauche–gauche orientation at the N-9–N-10 bond.

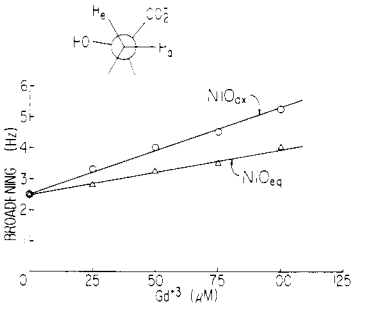

FIGURE 8: Broadening of the N-10 protons of the DCB-nicotinamide–pyruvate adduct by gadolinium. These results indicate that the N-10ax proton is slightly closer to the N-9 carboxyl group than is the N-10eq proton.

The effect of increasing gadolinium concentration from 0 to 100 μM on the line widths of a 0.1 M solution of the DCB-nicotinamide–pyruvate adduct is shown in Figure 8. The line widths were measured by first computer simulating the coupling pattern and then fitting it to the experimentally observed line widths. The results show that the broadening of the N-10ax proton is twice that of the N-10eq proton. Based on the $1/r^6$ distance dependence, this result indicates that the carboxylate is approximately 10% closer to the N-10ax proton than it is to the N-10eq proton. Thus the carboxylate must be gauche with respect to both the N-10 methylene protons. A gauche–trans orientation of the carboxylate can be excluded since such a conformation should cause a nearly sixfold greater broadening of the N-10eq proton relative to the N-10ax. These results establish the configuration at N-9. Therefore, the pyruvate adduct consists of a high population, *R*-(N-4), *R*-(N-9), and a low population, *S*-(N-4), *S*-(N-9) pair. See Figures 4 and 6.

This result establishes two important points about the

stereochemistry of this adduct. First, the stereospecificity of the attack by the amido nitrogen on the carbonyl is independent of the substituent on N-9. Second, the configuration of the cyclized ring (the orientation of the N-9 substituent) is independent of the substituent at N-9.

Assignments of the Oxaloacetate Adduct Resonances. The assignments of the NAD–oxaloacetate adducts were made by analogy to the NAD–pyruvate adduct and by homonuclear decoupling. Assignments were also greatly simplified by the fact that only a single proton is present at N-10 of this adduct (see Figure 6). As a result, the resonances corresponding to N-10 could be easily interpreted without computer simulation.

In principle, as many as eight magnetically and optically distinguishable forms of the NAD–oxaloacetate adduct are possible since asymmetric centers are generated at N-4, N-9, and N-10. The observation of only two diastereomeric forms requires that the carboxyl group α to the carbonyl in oxaloacetate must be oriented stereospecifically when oxaloacetate adds to the N-4 position of NAD^+. Based on the observation of a large trans $^3J_{4-10ax}$ coupling constant and the results for the acetaldehyde and pyruvate adducts, we assign the configurations of the two forms as: high population, *R*-(N-4), *R*-(N-10), *R*-(N-9), and low population, *S*-(N-4), *S*-(N-10), *S*-(N-9). See Figure 6.

Chemical Shifts of the Cyclized Adducts. The chemical shifts of the cyclized nicotinamide adducts are listed in Table II. The data show the general similarity of the magnetic environments for the corresponding protons of the various adducts which include those of the model compounds as well as the dinucleotides. Note, however, that the protons of the NAD–pyruvate adduct are shielded relative to the corresponding absorptions in NMN–pyruvate, and that the N-2, N-4, N-5, and N-6 resonances are all shielded between 30 Hz (0.136 ppm) and 65 Hz (0.295 ppm). Similar shielding of the pyridine moieties in dinucleotides relative to mononucleotides is observed in βNAD^+ and $\beta NADH$ (Jardetzky & Wade-Jardetzky, 1966; Sarma & Kaplan, 1969; Patel, 1969; Oppenheimer et al., 1971). These results have been interpreted in terms of a fast exchange between a specific folded form and an open, unassociated form in which the B face of the pyridine ring stacks againt the adenine ring (Oppenheimer et al., 1978).

A major difference is observed in the spectra of the NAD-cyclized adducts compared with other dihydropyridine dinucleotides, e.g., NADH analogues and NAD–CN. The N-4 protons of the diastereomeric forms of some of the adducts show little or no magnetic nonequivalence. This is particularly evident for the NAD–acetaldehyde and NAD–pyruvate adducts (see Table II). In these two adducts, the N-4 resonances of the diastereomers are virtually identical in comparison with NADH and NAD–CN in which the N-4B proton is preferentially shifted about 0.05–0.15 ppm more than the N-4A proton. The equivalence of the N-4 resonances, however, cannot be generalized to all adducts since the NAD–oxaloacetate adduct shows a substantial 0.08-ppm nonequivalence for the N-4 proton resonances.

Discussion

These results confirm the general structure of the adducts as proposed by Everse and co-workers (1971, 1972) and provide important new information regarding their stereochemistry and conformation. The properties of the cyclized adducts of NAD^+ can be summarized as follows. (1) They are N-4-substituted 1,4-dihydropyridine adducts of NAD^+. (2) In alkali, a second six-membered ring forms by an attack of the 3-carboxamido nitrogen on the N-10 carbonyl. (3) The attack which forms the second ring is stereospecific with the configuration of the product being determined by the conformation of the acyclic adduct (see Figure 6). (4) The observed stereospecificity of cyclization is not influenced by the substituent at N-9 for the compounds we have studied. (5) Only the two diastereomeric forms generated by the initial attack at N-4 are detected; the subsequent cyclization occurs with high stereoselectivity. For example, the NAD–oxaloacetate adduct shows only the *RRR* and *SSS* forms even though there are potentially eight different forms. (6) The proton resonances of the NAD^+ adducts show considerable upfield shifts relative to the corresponding NMN^+ adducts. This result indicates a significant contribution to the solution conformation from intramolecular association with the adenine moiety, in analogy to NAD^+ and NADH. (7) Although strongly shifted upfield, the N-4 proton resonances of the *R* and *S* forms show little chemical shift nonequivalence for the NAD–pyruvate and NAD–acetaldehyde adducts in contrast to either NADH or the NAD–cyanide adduct.

The overwhelming feature of these adducts is that they form stereospecifically with no special dependence on the substituents at N-9 and N-10. See Figure 6. An analysis of the preferred conformations of the N-9–N-10 bond and N-4–N-10 bond and those conformations which can lead to ring closure shows that this is expected. Figure 9 shows the basis for this selectivity. Of the rotamer populations shown, I and II are the only ones which can lead to ring closure. However, of these two rotamers, II is preferred for all the adducts since I would eclipse the carbonyl substituent with the dihydropyridine ring. At the same time, the N-9–N-10 bond should have the *R* substituent trans to the N-4–N-10 bound as is typical for aldehydes and ketones (Karabatsos & Fenoglio, 1969). This places the carbonyl in a position for attack by the amido nitrogen. Upon ring closure, the product shows the configurations we observe.

It must be emphasized that, until cyclization occurs, the addition at N-4 is rapidly reversible. Thus, even though the oxaloacetate may have formed the somewhat stable rotamer II, but with the R_1 and R_2 ligands interchanged, it could not lead to facile ring closure due to steric effects. As a result, it would be expected to dissociate and re-form to the structure shown for rotamer II which does lead to unrestricted ring closure.

Knowledge of the structures of these adducts provides us possible insight into their interactions with dehydrogenases. Since dehydrogenases catalyze the stereospecific transfer of hydride (for a review, see You et al., 1978), these adducts would be expected to serve as probes for the stereochemical factors governing binding. Previous studies have shown that the cyclized adducts formed by the reaction of oxidized substrates with NAD^+ are selective inhibitors of the corresponding dehydrogenases (Everse et al., 1972). For example, the NAD–pyruvate adduct inhibits lactate dehydrogenases (LDH), whereas LDH is not inhibited by either the oxaloacetate or acetaldehyde adducts of NAD^+. Surprisingly, LDH shows the same affinity for both forms (*R* and *S*) of the cyclized pyruvate adduct (Everse et al., 1971) and has a K_D of 2.5×10^7 M as determined by fluorometric titration measurements (McKay & Kaplan, 1964).

Therefore, it is apparent that LDH "recognizes" only discrete regions of the dihydropyridine moiety of the NAD–pyruvate adducts. The absence of selective binding of the diastereomeric forms suggests that the regions of the dihydropyridine moiety of the NAD–pyruvate responsible for binding to LDH occur in or near the plane of the dihydropyridine ring, since it is about this plane that both forms of

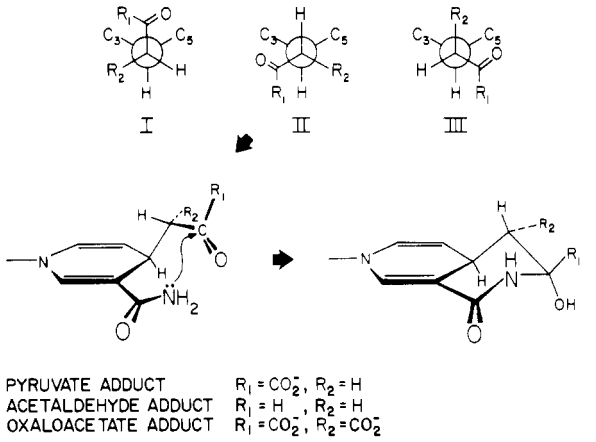

FIGURE 9: The conformation of the intermediate in adduct formation which accounts for the stereochemistry of the cyclized adducts. Rotamer populations I and II about the N-4–N-10 bond are the only conformations which can lead to ring closure (in rotamer III the carbonyl and amido groups are distal to each other). In all cases, rotamer II is preferred to rotamer I, since the later rotamer eclipses the carbonyl and its substituent with the dihydropyridine ring. At the same time, the conformation about the N-9–N-10 bond prefers R_1 gauche to R_2 and the N-10 proton (Karabatsos & Fenoglio, 1969). The attack of the amido group on the carbonyl in this preferred conformation leads to the stereochemistry we observe for the cyclized adducts; note that R_1 is gauche to R_2 and the N-10 proton.

the NAD–pyruvate adduct bear a close resemblance. Alternatively, the adducts may be able to induce a fit with the residues in the active site.

The dihydropyridine nature of these adducts is probably a further factor responsible for their high binding affinity to dehydrogenases since dihydropyridine nucleotides normally bind much more tightly to dehydrogenases than do their oxidized derivatives (Everse & Kaplan, 1973; Danenberg et al., 1978). Clearly, however, the carboxyl group at N-10 of the NAD–pyruvate adduct is also important for the specific binding of the adduct to LDH since other adducts with either additional substituents or lacking the carboxylate show little affinity for LDH (Everse et al., 1971). By inference, then, lactate dehydrogenase must have a residue that can bind a carboxylate. X-ray studies reveal that there is an arginine located near this region of the bound coenzyme (Adams et al., 1973) and it is probably this group that primarily "recognizes" the carboxyl group of the NAD–pyruvate adduct.

The two forms of the cyclized NAD–pyruvate adduct have also been observed to inhibit the L-lactate dehydrogenases from chicken and the D-lactate dehydrogenases from horseshoe crab (*Limulus*) and the sea worm (*Nereis*) with identical inhibition constants (Long & Kaplan, 1973). As discussed above, these results are consistent with the proposal that the "recognition" of the cyclized NAD–pyruvate adducts by LDH is independent of their precise stereospecific configuration.

Finally, the cyclized adduct can be employed as a tool for studying the stereochemistry of formation of the postulated acyclic ternary complexes. Arnold & Kaplan (1974) have shown that chicken muscle LDH can catalyze formation of the cyclic NAD–pyruvate adduct. The proposed mechanism involves formation of the acyclic adduct which dissociates from the enzyme and then cyclizes. This enzymatically produced adduct is unique and corresponds to the *R* form (Arnold & Kaplan, 1974). Note that this adduct is that which results from the addition of pyruvate to the *pro-R* (A side) of the nicotinamide ring. This is the same side to which LDH transfers hydride during the normal reaction with lactate (Loewus et al., 1953) and, thus, is consistent with the concept that the substrate is bound toward the A side of the nicotinamide ring.

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