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Characterization of a Long-Wavelength Feature in the Absorption and Circular Dichroism Spectra of β-Nicotinamide Adenine Dinucleotide. Evidence for a Charge Transfer Transition†

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ABSTRACT: The absorption spectrum of β-NAD^+ has a weak, broad feature extending to wavelengths well above 300 nm. A corresponding positive band is observed in the circular dichroism (CD) spectrum. α-NAD^+ has a somewhat stronger long-wavelength band in absorption and a negative CD band at 312 nm, opposite in sign to the main 260-nm band. These long-wavelength features are markedly diminished by lowering the pH, raising the temperature, or adding organic solvents. Enzymatic cleavage of both β- and α-NAD^+ abolishes the long-wavelength absorption and CD bands. We have also studied pyridine-3-aldehyde adenine dinucleotide, 3-acetylpyridine adenine dinucleotide, deamino-NAD^+, and $NADP^+$. All of these analogues exhibit long-wavelength absorption and CD bands analogous to those of NAD^+. The breadth of these features and their dependence on external conditions and on the integrity of the dinucleotide all support the assignment of this band to an intramolecular charge transfer transition, with the adenine moiety as the donor and the nicotinamide as the acceptor. The existence of such a charge-transfer transition in β-NAD^+ and related molecules provides conclusive evidence for a significant population of conformations in which the adenine and nicotinamide rings are stacked. Comparison of the extinction coefficient of N^1-methylnicotinamide-adenosine complex with that of β-NAD^+ at 320 nm provides an estimate of 40% stacking in β-NAD^+. In addition to characterizing the long-wavelength band of β-NAD^+ and various analogues, higher energy transitions have been characterized and some assignments made. In particular, the $n\pi^*$ transitions characteristic of the 3-substituent of the pyridinium ring are assigned by analogy to the corresponding substituted benzene derivative.

The pyridine nucleotide coenzyme,[1] β-NAD^+, is required as an electron carrier in metabolic processes in all known forms of life. The conformation of the coenzyme when bound to several enzymes has been determined by x-ray diffraction studies (Brändèn et al., 1975; Moras et al., 1975; Rossmann et al., 1975; Holbrook et al., 1975; Liljas & Rossmann, 1974;

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[1] Abbreviations used: β-NAD^+, β-nicotinamide adenine dinucleotide; α-NAD^+, α-nicotinamide adenine dinucleotide (in α-NAD^+ the anomeric carbon of the ribose bound to the nicotinamide is in the α conformation); β-NMN^+, β-nicotinamide mononucleotide; α-NMN^+, α-nicotinamide mononucleotide; AMP, 5′-adenosine monophosphate; ε-NAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; paNAD$^+$, 3-pyridinealdehyde adenine dinucleotide; acNAD$^+$, 3-acetylpyridine adenine dinucleotide; deamino-NAD^+, nicotinamide hypoxanthine dinucleotide; CD, circular dichroism; NMR, nuclear magnetic resonance; ^{1}H NMR, proton magnetic resonance; UV, ultraviolet.

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Eklund et al., 1976) to be of an "open" or extended type. A recent x-ray study of the crystalline Li^+–NAD^+ complex (Saenger et al., 1977) also shows an extended conformation but intermolecular base stacking is observed. The nicotinamide ring from one molecule is in close contact above and parallel to the adenine ring of a neighboring dinucleotide. The free solution conformation of the coenzyme has not been explicitly defined but a number of physical properties have given some insight into the structure (Sarma & Mynott, 1972a).

Early studies of β-NADH with fluorescence (Weber, 1957) led to the proposal of an intramolecular complex in which the adenine and dihydronicotinamide moieties were stacked. Similar work by Shifrin & Kaplan (1960) led to a conformation where the bases were in the same plane and hydrogen bonded. It is now fairly well established (Meyer et al., 1962; Czerlinski & Hommes, 1964; Jardetzky & Wade-Jardetzky, 1966; Miles & Urry, 1968; Cross & Fisher, 1969; Catterall et al., 1969; Sarma & Mynott, 1972a) that NADH and NAD^+ exist in an equilibrium between an "open"[2] conformation and a "folded" conformation.

Thermodynamic studies by means of proton magnetic resonance on β-NAD^+ in concentrated solutions suggested that the amount of the folded form was from 15% to 40% (Jardetzky & Wade-Jardetzky, 1966). The fluorescent derivative ε-NAD^+ gave an upper limit of 45 ± 5% for the oxidized coenzyme (Gruber & Leonard, 1975).

In our studies of dehydrogenases, it became apparent that β-NAD^+ has a tailing absorption on the long-wavelength side of the 259-nm absorption maximum. The CD of NAD^+ also shows a long-wavelength tail extending to wavelengths past 350 nm. Previous studies have shown the presence of this long-wavelength absorption (Siegel et al., 1959; Ungar & Alivisatos, 1961) and CD feature (Bayley, 1973; Eberhardt & Wolfe, 1975) and it has also been shown to be present in the "abbreviated" analogues synthesized by Secrist & Leonard (1972). Little attention has been paid to these spectral features and no explanation has been offered for their existence.

In the work described here we have undertaken the characterization of this long-wavelength feature. By applying circular dichroism, absorption, and difference spectroscopy and environmental perturbations to different analogues of the nicotinamide coenzyme, we have been able to conclude that the long-wavelength band is best explained as an intramolecular charge transfer transition. While this is the main endeavor of this paper, assignments of other spectroscopic transitions in β-NAD^+ and its analogues are also proposed.

Experimental Section

Chemicals. β-NAD^+, α-NAD^+, β-$NADP^+$, β-NMN^+, α-NMN^+, adenosine, AMP, paNAD^+, acNAD^+, deamino-NAD^+, N^1-methylnicotinamide chloride, DEAE-cellulose (medium-mesh), and snake venom phosphodiesterase (EC 3.1.4.1) were products of Sigma Chemical Co. β-NMN^+ was also purchased from P-L Biochemicals. All of the compounds were used without further purification, except for β-NAD^+, α-NAD^+, and β-NMN^+. Over the period of the study at least two different lots of each compound were used with no effect on results outside of experimental error. Sodium phosphate (mono and dibasic) and dioxane were analytical reagent grade products of Mallinckrodt. Sodium hydroxide was Baker Analyzed and phosphoric acid was Fisher reagent grade. Glass-distilled water was used in the preparation of all solutions.

Spectroscopic Determinations. Circular dichroism measurements were obtained on a JASCO J41C spectropolarimeter. Cell pathlengths, time constants, scanning speeds, and sensitivity settings were varied to obtain the highest possible signal-to-noise ratio. In some cases, to increase the signal-to-noise ratio, multiple scanning with baseline subtraction was done using the data processor which accompanies the JASCO J41C. Calibration of the spectropolarimeter was done before each scan with a standard aqueous solution of (+)-10-camphorsulfonic acid. This standard solution was assumed to have a molar ellipticity at 290.5 nm of 7775 deg cm^2 $dmol^{-1}$ (Krueger & Pshigoda, 1971). Cells were fixed in the cell compartment and solutions changed without movement of the cells to eliminate baseline shifts. The average of at least two scans was used in the determination of each spectrum. Deviations between duplicate scans were less than 5% above 225 nm and not more than 15% below that wavelength.

Absorption spectra were obtained on Cary 17 and Cary 118 scanning spectrophotometers. All scans in CD and absorbance measurements were done at 25.0 ± 0.4 °C unless otherwise stated. Temperature was controlled by the use of a Lauda Bath or a Forma-Temp Junior. A thermocouple (Yellow Springs Instrument Co.) was placed in the cell for calibration of the temperature in the solution.

Difference spectra were obtained using 1-cm tandem cylindrical cells (Pyrocell Mfg. Co.) placed in a thermostatable cell holder made for a Cary 17. All solutions were accurately made up with volumetric pipets and volumetric flasks.

Concentration Determination. For a specific concentration, solutions were made up by weighing out the appropriate amount of compound. Accurate concentrations were determined by absorbance using the following molar extinction coefficients: β-NAD^+, $\epsilon = 18.3 \times 10^3$ at 259 nm at pH 7.0; α-NAD^+, $\epsilon = 17.0 \times 10^3$ at 259 nm at pH 7.0; β-$NADP^+$, $\epsilon = 17.6 \times 10^3$ at 259 nm at pH 7.0; α-NMN^+, $\epsilon = 4.7 \times 10^3$ at 266 nm at pH 7.0; β-NMN^+, $\epsilon = 4.9 \times 10^3$ at 266 nm at pH 7.0 (Suzuki et al., 1967); 3-pyridinealdehyde-NAD^+, $\epsilon = 18.3 \times 10^3$ at 260 nm at pH 7.5; 3-acetylpyridine-NAD^+, $\epsilon = 16.4 \times 10^3$ at 260 nm at pH 7.5; deamino-NAD^+, $\epsilon = 14.7 \times 10^3$ at 249 nm at pH 6.0 (Siegel et al., 1959); AMP and adenosine, $\epsilon = 15.4 \times 10^3$ at 259 nm at pH 7.0 (Pabst Laboratories, 1956); and N^1-methylnicotinamide chloride, $\epsilon = 4.44 \times 10^3$ at pH 7.0 (Tillotson & Ziporin, 1960). The pH of all solutions was checked before scanning and the pH adjusted with negligible quantities of concentrated sodium hydroxide or phosphoric acid. All spectra are of solutions at nucleotide concentrations of 3 mM or less in 0.01 M sodium phosphate buffer at pH 7.0 unless otherwise stated.

Purification of β-NAD+, α-NAD+, β-NMN+. β-NAD^+ was purified on DEAE-cellulose by the method of Dalziel (1963) and by paper chromatography (Suzuki et al., 1967) using Whatman No. 1 paper. α-NAD^+ and β-NMN^+ were also purified by paper chromatography by the latter method.

Determination of K and ϵ_c. The values of K, the equilibrium constant, and ϵ_c, the extinction coefficient, for the N^1-methylnicotinamide–adenosine complex were determined according to the method of Towell & Woody (manuscript in preparation).

Cleavage with Snake Venom Phosphodiesterase. The phosphodiesterase (1.3 units) from *Crotalus adamanteus* venom (lyophilized powder) was dissolved in 5 mL of 0.01 M sodium phosphate buffer (pH 7.0) and was dialyzed against the same buffer for over 24 h. An equal volume of the phos-

[2] The terminology "folded" conformation as used in this paper refers to a mixture of conformations where the adenine and nicotinamide rings are stacked. The "open" conformation refers to a mixture of extended conformations in which the adenine and nicotinamide moieties interact only very weakly.

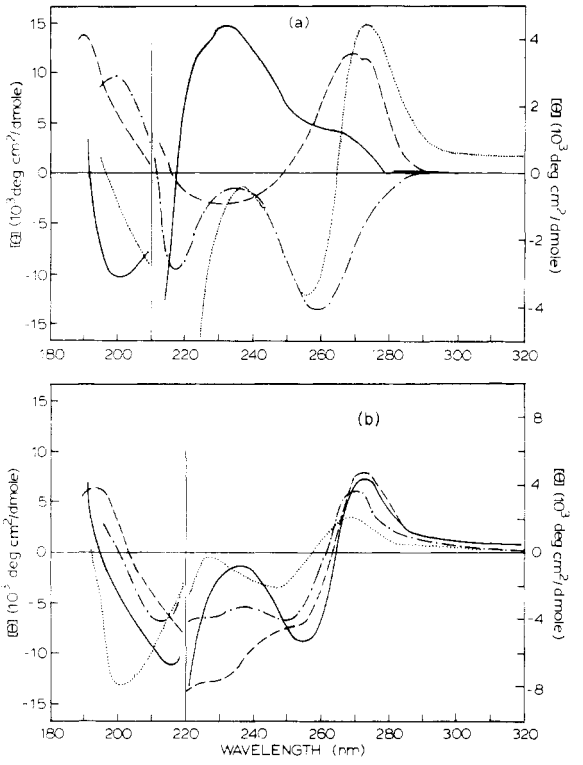

FIGURE 1: (a) The circular dichroism spectra of β-NAD$^+$ (• • •), AMP (- • - •), β-NMN$^+$ (—) and α-NMN$^+$ (- - -). (b) The circular dichroism spectra of NADP$^+$ (—), deamino-NAD$^+$ (• • •), acNAD$^+$ (- - -), and paNAD$^+$ (- • - •). Solvent used was 0.01 M sodium phosphate, pH 7.0, at 25.0 °C.

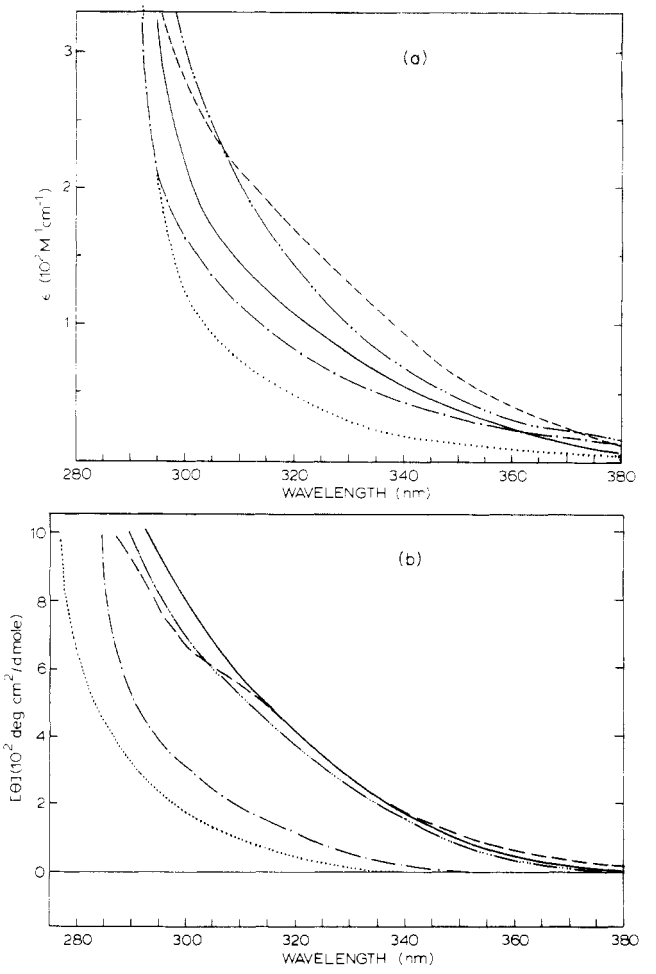

FIGURE 2: The absorption spectra (a) of β-NAD$^+$ (—), acNAD$^+$ (- - -), paNAD$^+$ (- • - •), deamino-NAD$^+$ (• • •), and α-NAD$^+$ (- • • - • •). The absorption spectra of β-NAD$^+$ and β-NADP$^+$ were the same within experimental error. The CD (b) of β-NAD$^+$ (—), acNAD$^+$ (- - -), paNAD$^+$ (- • - •), deamino-NAD$^+$ (• • •), and β-NADP$^+$ (- • • • • • • • • •).

phodiesterase was mixed with the coenzyme or coenzyme analogue (concentration approximately 8.0 mM in sodium phosphate buffer) and allowed to stand at room temperature for 3 h. It was found that the optical properties of the solutions had stabilized in this time period.

Molecular Orbital Calculations. The calculations were done according to previously described procedures (Smith & Woody, 1976) based on the Pariser–Parr–Pople approximation to π-electron systems (Parr, 1963; Murrell & Harget, 1972). Bond lengths and bond angles for nicotinamide and 3-acetylpyridine were slight modifications of the x-ray results of Voet (1973). The modifications ensured symmetry and planarity of the molecules. For the benzene ring of acetophenone and benzamide a bond length of 1.38 Å and bond angle of 120° were used. The bond lengths and angles for the substituent were from the crystallographic study of Voet (1973). The methyl group of acetophenone and 3-acetylpyridine was neglected because of its small effect on transition energies and polarizations. The parameters used are from the variable β approximation of Nishimoto & Forster (1965, 1966). Configuration interaction was considered including the 16 singly excited configurations resulting from transitions from the four highest filled orbitals to the four lowest unfilled orbitals.

The extinction coefficients of the analogues were calculated using the geometric mean of the oscillator strength determined from the dipole moment and dipole velocity (Hansen, 1967). We assume Gaussian bands with a half bandwidth at $1/e$ of the maximum of 11 nm for all the transitions from 180 nm to 300 nm. The 11-nm value was taken from the experimentally determined spectrum of β-NMN$^+$.

Results

The CD of β-NAD$^+$ and of a number of analogues of the coenzyme are presented in Figure 1. The spectra are in good agreement with previous studies below 300 nm (for β-NAD$^+$, Miles & Urry, 1968; Eberhardt & Wolfe, 1975; for AMP, Follmann et al., 1975) with only slight differences in the magnitudes of the CD and absorption bands. The spectra are included here for convenient comparison. β-NAD$^+$ has a positive CD band at 272 nm and a negative band of approximately equal magnitude at 255 nm (Figure 1a). The CD spectrum of the coenzyme is not the sum of the two components, AMP and β-NMN$^+$, as seen in Figure 1a. The two bands of opposite sign, referred to as a couplet (Schellman, 1968), can be attributed to overlapping of the ππ* transitions of adenine and nicotinamide. The analogy to the CD spectra of dinucleoside phosphates such as ApA (Van Holde et al., 1965) led Miles & Urry (1968) to suggest that the adenine and nicotinamide are "stacked" and that the coenzyme exists to some extent in a folded conformation in solution.

paNAD$^+$, deamino-NAD$^+$, acNAD$^+$, and NADP$^+$ also have a couplet in the region around their respective absorption maxima. Deamino-NAD$^+$ (Figure 1b) has a positive CD band maximum at 268 nm with a negative band of approximately equal intensity at 248 nm. β-NADP$^+$ (Figure 1b), with a positive band at 272 nm and a negative band at 255 nm, has a CD very similar to that of β-NAD$^+$. paNAD$^+$ and acNAD$^+$ have maxima at 270 and 273 nm, respectively. paNAD$^+$ shows a minimum at 250 nm, whereas acNAD$^+$ has a distinct shoulder at 254 nm. The appearance of CD couplets in these analogues is evidence for a folded conformation.

TABLE I: Spectrophotometric Data for the Long Wavelength Feature of β-NAD+ and Derivatives.

Derivative[a]	λ (nm)	[θ] (deg cm² dmol⁻¹)	ε (M⁻¹ cm⁻¹)	Δε/ε (10⁻⁴)
β-NAD+	320	416	103	12.3
β-NADP+	320	362	110	10.0
α-NAD+	320	−273	140	−5.9
acNAD+	320	418	175	7.2
paNAD+	320	116	83	4.2
Deamino-NAD+	310	101	75	4.1

[a] All values were obtained at 25.0 °C in pH 7.0, 0.01 M sodium phosphate buffer.

Below 300 nm, our measurements of the CD of α-NAD+ agree closely with those reported by Miles & Urry (1968). The CD spectrum of α-NAD+, as noted by Miles & Urry, differs markedly from the sum of its components, AMP and α-NMN+. It also differs from β-NAD+ in not having a couplet centered at 260 nm, but instead a single positive band is observed with a maximum at 262 nm. Even though α-NAD+ does not have a couplet, NMR analysis of the isomer is consistent with a folded conformation (Sarma et al., 1968; Blumenstein & Raftery, 1973). Miles & Urry (1968) also proposed a folded conformation for α-NAD+.

β-NAD+ and the coenzyme analogues which have a CD couplet near 260 nm also show a positive CD band extending beyond 310 nm (Figure 2b). A similar "tail" is seen in the UV absorption spectrum for each analogue (Figure 2a). α-NAD+ has a long-wavelength absorption feature which is stronger than that of β-NAD+ and the CD of α-NAD+ has a negative band with a maximum at 312 nm (Figure 3c). The observation of a CD band at long wavelengths which differs in sign from that of the 260–270-nm CD band is convincing evidence that in α-NAD+, at least, the long-wavelength feature results from a distinct electronic transition, rather than a broadening of the main band. The long-wavelength band probably has its maximum below 300 nm but it is partially masked by the main 260-nm band of the coenzyme. We have focused our attention on this long-wavelength feature and some relevant spectral data have been accumulated in Table I.

Purification of β-NAD+, α-NAD+, and β-NMN+ resulted in rather small changes in the optical properties of the compounds. The only noticeable change was a slight increase (<3%) in the magnitudes of all the bands in CD. There was no loss of the long-wavelength feature of β- or α-NAD+ in UV absorption or CD.

The CD spectrum for β-NMN+ (Figure 1a) is quite different from that reported by Miles & Urry (1968). We observe a strong positive band at 230 nm with a shoulder at 225 nm, whereas the earlier results showed a negative band at 240 nm. The spectrum given here is of purified β-NMN+ from both Sigma Chemical Co. and P-L Biochemical Inc. The 100-MHz NMR spectrum (data not shown) of β-NMN+ agrees very well with that of Sarma & Mynott (1973a). The CD spectrum for β-NMN+ shown here is also in good agreement with one which has been reported by Bayley (1973). The large discrepancy between the recent CD measurements on β-NMN+ and the earlier ones could be due to impurities in the sample or to instrumental artifacts affecting the measurements of Miles & Urry. Our results agree well with theirs in all other areas of overlap, and the discrepancy in the case of β-NMN+ does not affect their overall conclusions in any way.

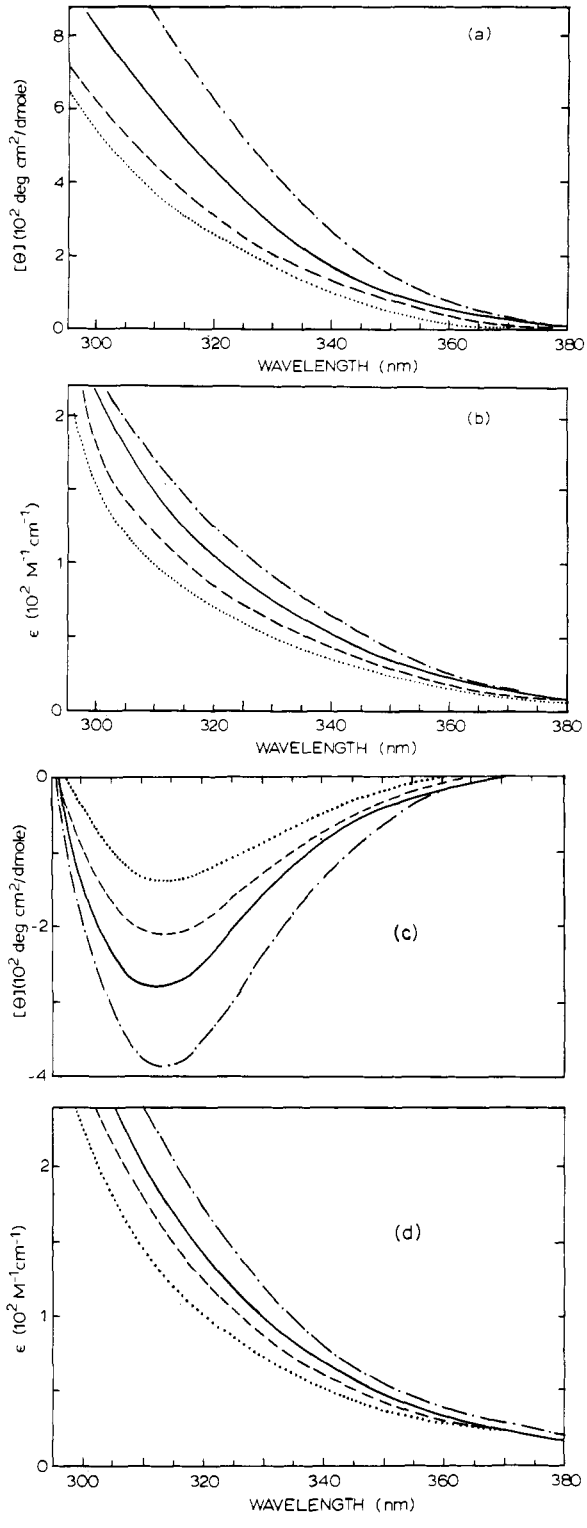

FIGURE 3: Temperature dependence of the long-wavelength CD (a) and absorption spectra (b) of β-NAD+. Measurements were made using 0.01 M sodium phosphate buffer (pH 7.0), at 7.0 °C (- · - ·), 25.0 °C (—), 38.0 °C (- - -), and 54.0 °C (· · ·). Temperature dependence of the long wavelength CD (c) and absorption spectra (d) of α-NAD+. Measurements were made using 0.01 M sodium phosphate buffer (pH 7.0), at 6.0 °C (- · - ·), 25.0 °C (—), 39.0 °C (- - -), and 58.0 °C (· · ·).

AMP has neither absorbance nor CD at wavelengths above 300 nm (Figure 1a). The spectra of β-NMN+ and α-NMN+ also show no CD or absorbance at wavelengths longer than 315 nm, making it difficult to attribute the long-wavelength tail to either the adenine or the nicotinamide moiety of the coenzymes. The absorbance and CD spectrum of β-NAD+ at 320

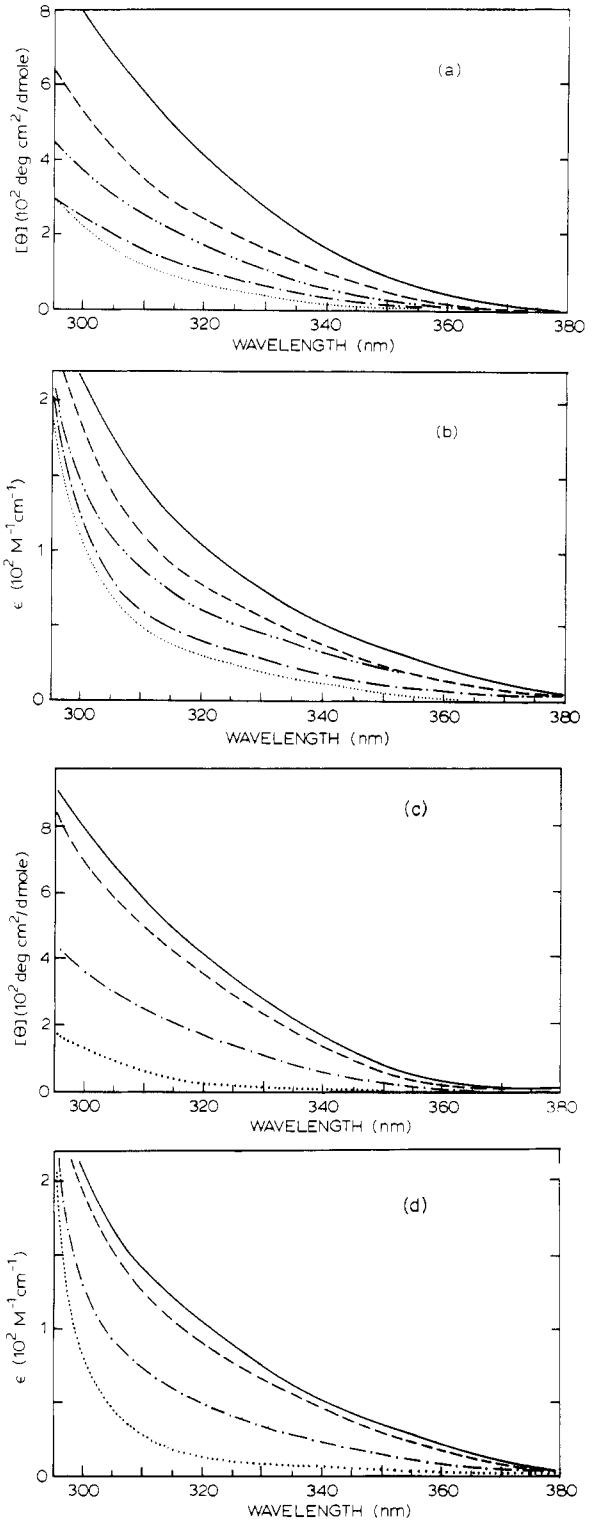

FIGURE 4: Effects on the CD (a) and absorption spectra (b) of β-NAD^+ of adding dioxane. Measurements were made at 25.0 °C with mixtures of 0:10 (v/v) (—), 1:9 (v/v) (- - -), 2:8 (v/v) (- · - ·), 3:7 (v/v) (- · -), 5:5 (v/v) (· · ·) of dioxane and 0.01 M sodium phosphate buffer (pH 7.0). pH dependence of the CD (c) and absorption spectra (d) of the long-wavelength feature of β-NAD^+. Measurements were made at 25.0 °C using 0.01 M sodium phosphate, pH 7.0 (—), pH 4.5 (- - -), pH 3.6 (- · - ·), and pH 2.6 (· · ·).

nm is linear in concentration from 5.0×10^{-5} M to 5.0×10^{-3} M, implying that this band is an intramolecular transition and not due to intermolecular interactions.

The CD and UV absorbance spectrum of β-NAD^+ is greatly influenced by changes in temperature. With increasing temperatures the couplet at 260 nm is diminished (Miles & Urry, 1968) and likewise the "tail" decreases in magnitude (Figure 3). There is a hyperchromic effect on the 259-nm peak observed in the absorption spectrum (data not shown) and a decrease in the absorption tail upon raising the temperature (Figure 3c). Decreasing the temperature to 7.0 °C causes an increase in the long-wavelength feature in the CD and UV absorption spectra. The same types of effects are also seen in the spectra of α-NAD^+ (Figure 3). Temperature effects were found to be reversible up to, but not above, 65.0 °C. Irreversible contributions above 65 °C probably result from hydrolytic reactions.

Variations in the dielectric constant of the solvent also have marked effects on the long-wavelength feature of α- and β-NAD^+. Figure 4 shows a decrease in the long-wavelength feature in both the CD and the absorption spectra of β-NAD^+ on adding dioxane. There is also a loss of the 260-nm couplet accompanying the addition of organic solvent (Miles & Urry, 1968). Similar solvent effects occur in the spectra of α-NAD^+ (data not shown). There are no spectral changes above 315 nm for adenosine, β-NMN^+, nor α-NMN^+ in solutions of up to 50% dioxane.

The N^1 nitrogen of the adenine moiety becomes protonated at a pH of 3.88 (Moore & Underwood, 1969; Blumenstein & Raftery, 1973). There is a complete change in the CD of β-NAD^+ in the region from 280 nm to 260 nm (Miles & Urry, 1968) accompanying protonation. This change has been attributed to the unfolding of the coenzyme due to repulsion of the positively charged nicotinamide moiety with the positively charged adenine moiety (Miles & Urry, 1968). Figure 4 shows a reversible decrease in the long-wavelength feature in the UV and CD spectra for β-NAD^+ when the pH is lowered. There are similar decreases in the spectra of α-NAD^+ (data not shown). With this decrease in the long-wavelength features of β-NAD^+ and α-NAD^+, the titration curves for the coenzymes were determined. β-NAD^+ has a pK_a of 3.76 ± 0.09 in good agreement with the potentiometric titration value of 3.83 (Moore & Underwood, 1969) at comparable ionic strengths. α-NAD^+ has a pK_a of 3.65 ± 0.10, somewhat lower than the pK_a of β-NAD^+.

The long-wavelength tails in CD and absorption spectra are dependent on the juxtaposition of the adenine and nicotinamide moieties for the pyridine coenzyme as demonstrated by the pH, temperature, and solvent effects. When β-NAD^+ is cleaved to AMP and β-NMN^+ with snake venom phosphodiesterase, there is a complete loss of CD above 290 nm (data not shown) and a large decrease in absorbance at wavelengths longer than 300 nm (Figure 5). Analogous behavior is seen in the spectral properties of α-NAD^+.

In Figure 5 it may be noted that the absorption spectrum of acNAD^+ after phosphodiesterase cleavage shows a weak but distinct band at 312 nm. The CD of the cleaved analogue has a negative band with a peak at 315 nm (data not shown). A corresponding shoulder is observed in the absorption and CD spectra of the intact pyridine modified coenzyme (Figure 2). We attribute this to an $n\pi^*$ transition of the 3-acetylpyridinium moiety of the analogue due to its low absorption maximum ($\epsilon_{312} \sim 25$ M^{-1} cm^{-1}), the relatively large negative band observed in the CD of the cleaved analogue, and the fact that acetophenone has an $n\pi^*$ transition in this wavelength region (Suzuki, 1967).

Dinucleoside monophosphates are known to be stacked in neutral solution. Powell et al. (1972) have used a thermodynamic approach for the determination of the equilibrium constant for a two-state system, involving stacked dinucleotides in equilibrium with unstacked dinucleotides. By using a non-

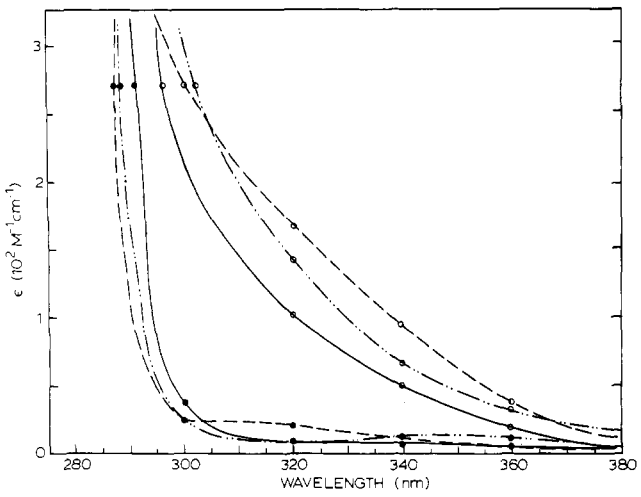

FIGURE 5: Effects on the long-wavelength absorption spectra of β-NAD+ (—), acNAD+ (- - -), and α-NAD+ (- · · ·) upon cleavage with snake venom phosphodiesterase. Measurements were made using 0.01 M sodium phosphate (pH 7.0) at 25.0 °C, with phosphodiesterase (●) and without phosphodiesterase (○).

linear least-squares fit to eq 1

$$\ln \frac{\alpha_u - \alpha}{\alpha - \alpha_s} = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \qquad (1)$$

Powell and co-workers were able to determine the thermodynamic parameters ΔS° and ΔH°. Here α is a measured optical value at temperature T; α_s and α_u are the optical parameters for the stacked and unstacked dinucleotides; and ΔH° and ΔS° are the standard thermodynamic properties for the stacking process. The criteria for a two-state noncooperative system include agreement of the thermodynamic parameters using optical values determined at several wavelengths, linear van't Hoff plots using an equilibrium constant derived from eq 1, and isosbestic points generated by a family of curves at various temperatures.

We have measured the absorbance and CD properties at 320 nm, 270 nm, and 254 nm through a temperature range of 4 to 65.0 °C for β-NAD+. Figure 6 gives an indication of the linearity of the temperature plots for CD and absorption at 320 nm. Attempts to fit these data to eq 1 did not lead to consistent thermodynamic parameters (inset, Figure 6). This failure indicates that a two-state model is inapplicable and that several different conformational states for the coenzyme are involved.

Anderson & Reynolds (1966) have shown that N^1-methylnicotinamide and adenosine in concentrations of 10^{-3} M show a long-wavelength feature in the absorption spectrum similar to what we observe in β-NAD+ and related molecules. The difference titration of adenosine with N^1-methylnicotinamide is given in Figure 7. The long-wavelength band continues to increase with increasing concentrations of N^1-methylnicotinamide. The CD of the complex (Figure 7) has a negative band suggesting that the N^1-methylnicotinamide–adenosine complex has a preferred orientation in solution. The titration of high concentrations of N^1-methylnicotinamide with adenosine gave rise to a family of curves presented in Figure 8. The slopes of these curves are used in a plot of slope/[N^1-methylnicotinamide] vs. slope (Figure 8b) for determination of the ϵ_c and K for the complex (Towell & Woody, manuscript in preparation). In 0.01 M sodium phosphate, pH 7.0, at 25 °C the extinction coefficient for the weak complex is 255 ± 18 M^{-1} cm^{-1} at 320 nm with an equilibrium constant of 1.40 ± 0.09 M^{-1}. The equilibrium

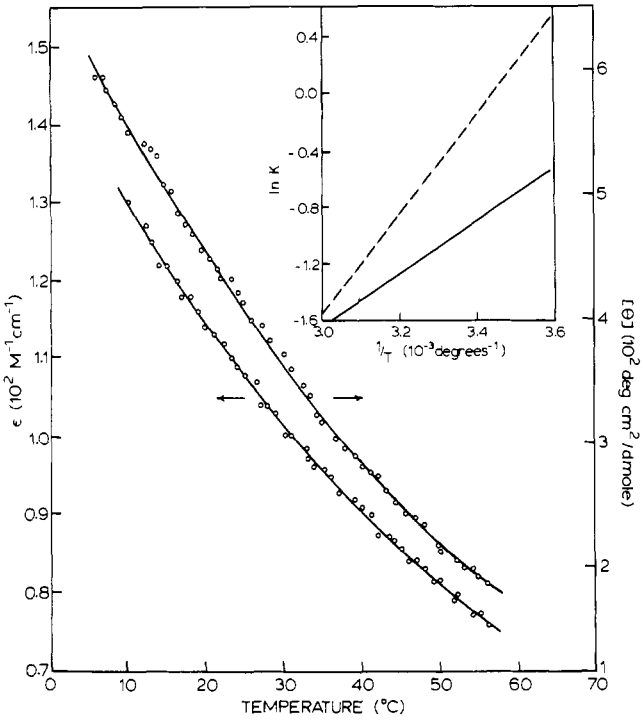

FIGURE 6: Temperature–absorbance and –ellipticity profiles for β-NAD+ at 320 nm. Open circles are experimentally determined points, while the curves are calculated by least-squares analysis for a two-state process (see text). The inset is the van't Hoff plot for ellipticity (- - -) and absorbance (—) determined from the thermodynamic values obtained from the temperature profiles.

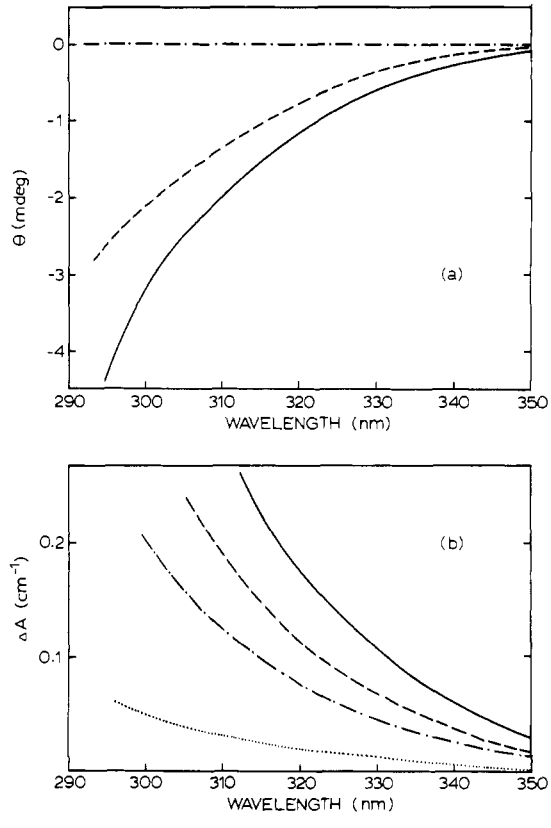

FIGURE 7: UV and CD spectra of N^1-methylnicotinamide–adenosine complex. The CD spectrum (a) of N^1-methylnicotinamide (6.30×10^{-2} M) with adenosine (8.56×10^{-3} M), —; of N^1-methylnicotinamide (5.1×10^{-2} M) with adenosine (6.87×10^{-3} M), - - -; and of adenosine (8.56×10^{-3} M), - · · ·. The absorption spectrum (b) of adenosine (8.56×10^{-2} M) with N^1-methylnicotinamide, 0.613×10^{-2} M, · · ·; 2.45×10^{-2} M, - · · ·; 3.68×10^{-2} M, - - -; and 6.30×10^{-2} M, —.

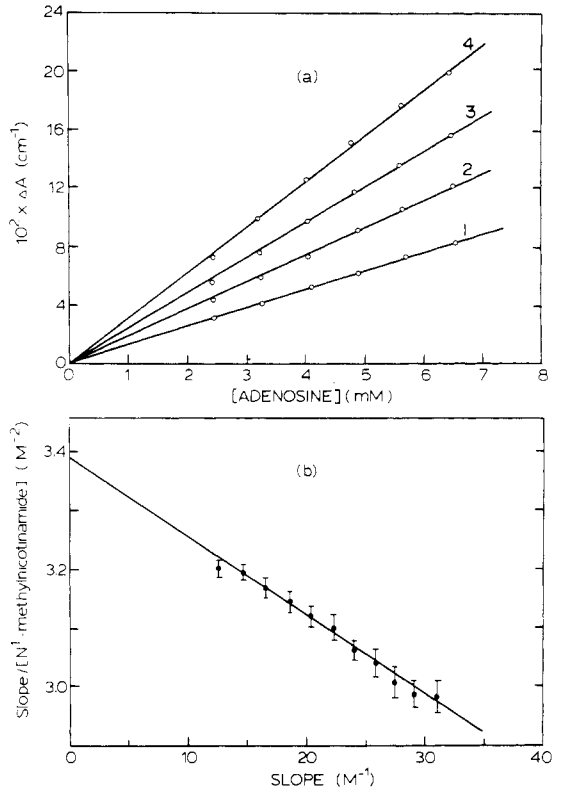

FIGURE 8: (a) Absorbance titration at 320 nm of (1) 3.96×10^{-2} M; (2) 6.56×10^{-2} M; (3) 7.84×10^{-2} M; and (4) 10.4×10^{-2} M N^1-methylnicotinamide with adenosine. (b) The replot of the slopes for determination of ϵ_c and K of the N^1-methylnicotinamide-adenosine complex.

constant is somewhat lower than the value (2.06 M^{-1}) obtained at 325 nm by Anderson & Reynolds (1966). We also determined an extinction coefficient of 180 M^{-1} cm^{-1} at 325 nm, greater than the 143 M^{-1} cm^{-1} of Anderson & Reynolds (1966). Some of the differences between these values may result from the differences in solvents used (0.01 M pyrophosphate buffer (pH 8.2) vs. 0.01 M phosphate buffer (pH 7.0)) and the method of determination of the constants.

We have invoked an analogy between acNAD$^+$ and acetophenone in assigning an $n\pi^*$ transition at 315 nm in the acetylpyridinium derivative. In general, one might expect similarities between the spectra of 3-substituted pyridinium derivatives and the corresponding substituted benzenes. Such relationships would be useful in locating and assigning the $n\pi^*$ transitions in nicotinamide and related molecules. To provide a theoretical basis for such comparisons and analogies, we have performed π-molecular orbital calculations on the following molecules: N^1-methylnicotinamide cation, benzamide, N^1-methyl-3-acetylpyridinium cation, and acetophenone.

The theoretical results are compared with available experimental data in Table II. The calculated wavelengths agree well with observed transitions, although generally the theoretical values are blue-shifted by 2–12 nm relative to the observed ones.

The predicted intensities are not in as good agreement with experimental values, being 1.5–3-fold higher. However, the relative intensities are correctly predicted. The quantitative discrepancies may be attributed to arbitrary assumptions about band shape and width, the use of the geometric mean of the dipole length and dipole velocity oscillator strengths (Hansen, 1967) rather than the lower dipole velocity value, and the general difficulties of parameterizing the Pariser–Parr–Pople theory for complex systems with hetero atoms.

Even though the agreement of the intensities of the transitions is poor, some general trends are observed from the data. There are three major transitions in all the derivatives, centered around 265 nm, 220 nm, and 190 nm. For the pyridinium derivatives, the two lowest energy transitions are of comparable intensity. In contrast, benzamide and acetophenone show the transition at 220 nm with an extinction coefficient 10 times that of the lowest energy transition.

Discussion

We have observed and characterized a feature in the absorption and CD spectra of β-NAD$^+$ and its analogues, and in α-NAD$^+$, which extends to wavelengths well above 300 nm. We assign this band to an intramolecular charge transfer transition between the adenine (donor) and the nicotinamide (acceptor). The observation of a transition of this type provides strong evidence that some fraction of ground state NAD$^+$ molecules must exist in a stacked conformation at any given time. In making the assignment of a charge-transfer *transition*, we do not wish to imply that NAD$^+$ is a charge-transfer *complex* in the sense of Mulliken (1952). The mixing of the charge-transfer configuration with the ground state probably contributes little to the stability of base-stacked conformations, compared with dipole–dipole, dipole–induced dipole, van der Waals, and hydrophobic interactions. Before this assignment can be accepted unequivocally, we must consider various alternative interpretations for our results.

First, are these long-wavelength features real or are they due to impurities? We are not the first to report such features, as they are apparent in published absorption (Siegel et al., 1959; Ungar & Alivisatos, 1961) and CD (Bayley, 1973; Eberhardt & Wolfe, 1975) spectra. "Abbreviated" analogues of NAD$^+$ in which the nicotinamide and adenine are bonded to the 1′ and 5′ positions of a single ribose show distinct long-wavelength tails in absorption and CD (Secrist & Leonard, 1972). No indication of such features appears in the thorough CD and absorption studies of NAD$^+$ by Miles & Urry (1968). However, they apparently did not extend their studies above 300 nm.

Several purification procedures and different lots of β-NAD$^+$ and α-NAD$^+$ give essentially identical results. In addition, if the effects are due to impurities, these impurities must be affected by snake venom phosphodiesterase and must respond to pH, temperature and solvent composition in the same way as β-NAD$^+$ itself. For these reasons, we are confident that the long-wavelength features we observe are intrinsic to NAD$^+$ and its analogues and do not result from impurities.

Second, are these features intramolecular? The long-wavelength features studied here obey the Beer–Lambert law (and its CD analogue) through the concentration range of 5.0×10^{-5} M to 5.0×10^{-3} M, which includes the concentrations of all the solutions used in this study. Since the extinction coefficient and CD are independent of concentration over this 100-fold concentration range which extends to concentrations where only very stable intermolecular complexes would remain undissociated, we conclude that we are observing an *intra*molecular phenomenon, not an *inter*molecular one.

Third, could these long-wavelength features result from a broadening of the main band(s) at 260 nm rather than from a distinct electronic transition? The most convincing argument for a distinct electronic transition is provided by the negative sign of the long-wavelength CD band in α-NAD$^+$, which clearly distinguishes it from the positive CD band at 259 nm. It is true that vibronic effects can cause changes in sign within the CD band of a single electronic transition (Weigang, 1965), but a theorem of Moffitt & Moscowitz (1959) limits such behavior to electrically forbidden transitions, which certainly

TABLE II: Energy, Oscillator Strengths (f), and Extinction Coefficients for Nicotinamide and Derivatives.

Molecule	Calculated λ (nm)	f^b	ϵ^c (M^{-1} cm^{-1})	Observed[a] λ (nm)	f	ϵ (M^{-1} cm^{-1})
N^1-Methylnicotinamide	258	0.214 (0.087)	10 500	263		5070
	221	0.016 (0.004)				
	212	0.191 (0.098)	8 000	209		6550
	202	0.052 (0.015)				
	182	0.766 (0.347)	35 400			
	179	0.658 (0.271)				
Benzamide	257	0.009 (0.003)	410	267		610
	216	0.330 (0.166)	13 900	226		8600
	208	0.060 (0.022)				
	188	0.235 (0.098)				
	184	0.850 (0.404)	41 300			
	182	0.563 (0.222)				
N^1-Methyl-3-acetylpyridine	262	0.252 (0.101)	12 800	264		3910
	224	0.288 (0.138)	11 700	224		5790
	190	0.430 (0.179)	22 000			
	186	0.413 (0.186)				
Acetophenone	264	0.018 (0.006)	832	275	0.02	950
	231	0.471 (0.238)	21 000	231	0.28	13 000
	195	0.368 (0.144)	24 270			
	188	0.589 (0.271)				

[a] Observed values for nicotinamide are from Kračmar et al. (1968) determined in 0.1 N HCl. Observed extinction coefficients for benzamide were determined in H_2O by Baramki et al. (1973). 3-Acetylpyridine extinction coefficients are those from Swain et al. (1949) determined in acidified ethanol. Acetophenone values from Kimura & Nagakura (1965) determined in the vapor phase. [b] The oscillator strength calculated from the transition dipole moment or, in parentheses, the transition dipole velocity. [c] Extinction coefficients calculated from the geometric mean of the dipole length and dipole velocity oscillator strengths (see text; Hansen, 1967).

excludes the 259-nm band of adenine and the 266-nm band of nicotinamide.

Could some broadening effect on the 260-nm band(s) lead to these long-wavelength features? The long-wavelength band and the 260-nm band are affected in opposite ways by changes in temperature, pH, and solvent composition, with the long-wavelength band increasing in intensity while the 260-nm band decreases, and vice versa. An increase in temperature increases the 260-nm absorption intensity without a sizable shift in the maximum, while it decreases the absorption at 320 nm. This is contrary to the expected effects of temperature increases on band broadening. Addition of dioxane shifts the 260-nm band to the red, which should increase the long-wavelength absorption if this feature is simply due to broadening of the main band. On the contrary, the long-wavelength band decreases markedly with increasing dioxane content.

Thus we conclude that the long-wavelength feature represents a distinct intramolecular electronic transition in β-NAD^+, α-NAD^+, and various related molecules. The next question is how to assign this transition. No ππ* transitions are observed in this region in either of the bases which make up NAD^+. The transition is observed in absorption and circular dichroism which by the Frank–Condon principle must reflect ground state geometries. Therefore exciplex phenomena can be excluded. The only plausible alternatives are (a) an nπ* transition which is intensified or shifted markedly in the native NAD^+ conformation relative to the component nucleotides or to "denatured" NAD^+, or (b) an intramolecular charge transfer transition.

The subject of nπ* transitions in adenine derivatives has been much debated. The best experimental evidence for such a transition is the observation of a 270 nm band in various pentofuranosides of adenine (Ingwall, 1972) which has been tentatively assigned as an nπ* transition. Although base stacking in NAD^+ could at least partially shield the adenine from water and accordingly lead to a red shift in the nπ* transition, it is difficult to believe that such an effect could lead to a shift of 30 nm or more as well as a marked broadening. Furthermore, one would expect 50% dioxane to be at least as effective in red shifting the nπ* band as is the rest of the NAD^+ molecule. However, in 50% dioxane neither NAD^+ nor AMP itself exhibit significant absorption or CD above 300 nm.

To our knowledge nπ* transitions in nicotinamide and its analogues have never been identified. One would expect the lowest energy nπ* transition in nicotinamide to be analogous to the amide transition which occurs at 210–230 nm. A useful guide to the effect of conjugating the amide group to a quaternary pyridine ring is provided by the corresponding benzene derivative, benzamide.

The validity of this procedure is indicated by the observation in acNAD+ and in its phosphodiesterase cleavage product of a transition at 315 nm which has the characteristics of an nπ* transition: low intensity in absorption and relatively strong CD. The benzene analogue of 3-acetylpyridinium ion, acetophenone, has a well-characterized nπ* transition at 320 nm (Suzuki, 1967).

Following this analogy, which is further discussed below, we expect the nπ* transition of nicotinamide at about 235–240 nm because the corresponding band has been observed at 238 nm for benzamides (Krueger et al., 1971). This and the fact that neither α-NMN^+ nor β-NMN^+ show increase in absorbance in 50% dioxane solutions at wavelengths above 310 nm allow us to rule out the possibility that the long-wavelength feature of NAD^+ could result from an nπ* transition of the nicotinamide ring.

The broad featureless band observed here in CD and absorption spectra is quite similar to that of known π-system charge transfer complexes. Shifrin (1968) has shown that nicotinamide can act as an acceptor in intramolecular charge transfer complexes with various electron donors. N^1-Methyl-

nicotinamide is an acceptor in intermolecular charge transfer complexes with known electron donors such as ascorbic acid and chlorotheophylline (Brooke & Guttman, 1968a, b). All of the complexes mentioned with the nicotinamide moiety as an acceptor are characterized by a broad absorption band above 300 nm. Adenine, adenosine, and other purine derivatives complex with tryptophan (Slifkin, 1971; Monteray-Garestier & Hélène, 1968), riboflavin (Weber, 1950), and actinomycin D (Behme & Cordes, 1965). Cilento & Schreier (1964) have postulated an intramolecular charge transfer *complex* in NAD^+ with adenine as the donor and nicotinamide as the acceptor. The results presented here support the idea of a low-lying charge transfer transition in NAD^+, but we believe that this transition is a *consequence* of base stacking, rather than a *cause*.

A charge-transfer transition between the nicotinamide and one of the oxygens of the bridging pyrophosphate is improbable. Blumenstein & Raftery (1972) suggested an electrostatic interaction between the negatively charge diphosphate backbone and the positively charged N^1 of the nicotinamide ring of β-NAD^+. They also found this interaction to be pH independent whereas the long-wavelength feature (Figure 8) is pH dependent. The N^1-methylnicotinamide–adenosine complex gives a similar long-wavelength feature to that observed in β-NAD^+, and neither compound has phosphate covalently bound. From these two points we conclude that the transition is dependent on the heterocyclic ring systems of adenine and nicotinamide.

More conclusive evidence of a charge-transfer assignment would be demonstration that the wavelength of the transition increases with decreasing ionization potential of the donor, increasing electron affinity of the acceptor and increasing dielectric constant of the solvent. Unfortunately, such definitive experiments are precluded in this system by the fact that changes in the bases and in the solvent will not only affect the energetics of the charge-transfer transition but also the geometric relationships between the bases. A further complication is that we cannot precisely locate the absorption or CD maximum because the long-wavelength band is overlapped at higher energies by the main $\pi\pi^*$ bands.

Although we are unable to provide these confirming types of evidence for a charge-transfer assignment, the exclusion of reasonable alternatives, the breadth of the observed bands, and the fact that similar bands which are unambiguously of the charge-transfer type are observed in various model systems provide convincing evidence for our assignment.

Folded conformations of β-NAD^+ have been strongly implied by previous work in CD (Miles & Urry, 1968), ^1H NMR (Bose & Sarma, 1975; for a review of all the literature before 1972 see Sarma & Mynott, 1972a), and fluorescence quenching in the ethenoadenine analogue of NAD^+, ϵ-NAD^+ (Gruber & Leonard, 1975). ^{13}C and ^{31}P NMR (Ellis et al., 1973; Blumenstein & Raftery, 1972, 1973; Sarma & Mynott, 1972b, 1973b; Williams et al., 1976) studies have been interpreted as supporting evidence for an intramolecular interaction between the adenine and nicotinamide moieties. However, the experimental requirement for concentrated solutions, above 0.05 M, in NMR experiments make conclusions regarding *intra*molecular vs. intermolecular interactions somewhat ambiguous. Sarma & Mynott (1972a, 1973a) used ^1H Fourier transform NMR to demonstrate concentration-dependent chemical shifts in β-NAD^+ above 5 mM. However, they concluded that base stacking persists at concentrations below 5 mM, where it is almost certainly *intra*molecular in character. Jacobus (1971) has questioned the interpretations of ^1H NMR data on NAD^+ and has pointed out that, although intramolecularly stacked conformations are consistent with the ^1H NMR data, they are not required by it.

We have shown the existence of an *intra*molecular charge-transfer transition between adenine and the nicotinamide moieties of NAD^+ at concentrations well below those required for intermolecular interactions. This intramolecular charge transfer transition provides conclusive evidence that conformations in which the adenine and nicotinamide rings are in close proximity, most likely stacked, are of significance in NAD^+ and the derivatives.

Scale molecular models of β-NAD^+ show that it can assume a folded conformation (Sarma et al., 1968) with very little strain or perturbation of the conformation of the sugar moieties. We have also built space filling models of β-NAD^+ and α-NAD^+. α-NAD^+ can easily assume a number of folded conformations somewhat like those of β-NAD^+, with no effect on the ribose units of the molecule. The slight differences in the folded conformations of β-NAD^+ and α-NAD^+ contribute to the differences in the CD spectra in both the short-wavelength region below 300 nm and the reversal of sign in the long-wavelength charge-transfer transition.

Our evidence for intramolecular base stacking in NAD^+ solutions is not inconsistent with the x-ray crystallographic study of the Li^+ salt of NAD^+ (Saenger et al., 1977), which does not show *intra*molecular stacking but an extended conformation with *inter*molecular stacking. In a flexible molecule of the size of NAD^+, the conformation in the crystal can be strongly influenced by the nature of the counterion and by crystal packing effects. The observation of intermolecular stacking supports the idea that there is a strong π interaction between the adenine and nicotinamide ring systems. Our observations require that this interaction occurs intramolecularly in dilute solution.

We were not able to fit thermodynamic quantities to a simple noncooperative two-state system with a "folded" conformation in equilibrium with an "open" conformation. Presumably more than one conformation of a folded type is in equilibrium with a broad range of unfolded conformations. Similar conclusions were drawn by McDonald et al. (1972) based on ^1H NMR.

We have used the complex of N^1-methylnicotinamide and adenosine as a model of the folded conformation of β-NAD^+. The extinction coefficient for the complex is 255 M^{-1} cm^{-1}, while the extinction coefficient for β-NAD^+ at the same wavelength is 10^3 M^{-1} cm^{-1}, giving an approximate value of 40% of folded conformation. This is only a very rough estimate of the amount of folded β-NAD^+ due to the possibility of large structural differences between an intermolecular complex as compared with the intramolecular complex in NAD^+. The mean distance and the average orientation are almost certainly different, as well as the distribution about these means. Even so, this value is in fairly good agreement with those of Gruber & Leonard (1975) and Jardetzky & Wade-Jardetzky (1966).

We have also made some assignments of other electronic transitions in NAD^+ and its analogues. The location of $n\pi^*$ transitions in $acNAD^+$ at ca. 315 nm and in NAD^+ at ca. 230–240 nm has been discussed previously. One would also expect an $n\pi^*$ transition in the 310–320-nm region of $paNAD^+$, by analogy to benzaldehyde. There are no inflections in either the CD or absorption spectrum in this region. Comparing the long-wavelength CD and absorption of $paNAD^+$ with that of β-NAD^+ (Figure 2), it will be noted that the difference is more marked in CD than in absorption and the shape of the CD curve of $paNAD^+$ might indicate a *negative* $n\pi^*$ transition near 310 nm superimposed on the positive charge-

transfer band. However, this must be regarded as tentative.

Turning to the shorter wavelength region, we note negative CD bands near 230 nm in acNAD$^+$ and paNAD$^+$ (Figure 1b). These do not coincide with absorption maxima, but are near an absorption minimum. By analogy to acetophenone and benzaldehyde, one might assign these bands to the L_a band of the substituted pyridinium ion, since the model compounds have their L_a bands at 230–240 nm. The intensities of the L_a band in the benzene analogues might argue against this, however. Acetophenone has an ϵ_{max} of 13 000 at 231 nm (Table II), which is considerably larger than the combined absorption of the acetylpyridinium and adenine moieties of acNAD$^+$ at 230 nm (9000). The MO calculations summarized in Table II overcome this objection, however. They show that the substitution of a ring carbon by a quaternary nitrogen has little effect on the energy of the major $\pi\pi^*$ transitions but it does affect the relative intensities profoundly. The pyridinium derivatives have L_b (260 nm) and L_a (230 nm) bands of comparable intensity, while the benzene derivatives have L_a bands an order of magnitude stronger than the L_b bands. Thus we assign the 230-nm bands of acNAD$^+$ and paNAD$^+$ to the L_a bands of their 3-substituted pyridinium rings.

A weak shoulder in the CD of both α- and β-NAD$^+$ (Figure 1) near 230 nm could be assigned to either the $n\pi^*$ or the L_a transition of the nicotinamide. The $n\pi^*$ transition of the nicotinamide is almost certainly responsible for the strong CD band at 231 nm in the spectrum of β-NMN$^+$ (Figure 1), while the shoulder at 223 nm corresponds to the L_a band. The absorption spectrum of β-NMN$^+$ shows the L_a band as an inflection near 220 nm, with no noticeable feature corresponding to the major CD peak. The absorption spectrum of α-NMN$^+$ is nearly identical with that of β-NMN$^+$, but the CD is quite different. However, the broad negative band between 220 and 240 nm is probably due to overlapping contributions of the $n\pi^*$ and L_a bands, with the $n\pi^*$ transition lying at longer wavelengths.

The CD spectrum of α- and β-NMN$^+$ shows bands at 200 nm which probably correspond to the weak transition predicted by the MO calculations to lie in this region. The absorption spectrum also shows an inflection near 200 nm, and a sharp rise at shorter wavelengths as the fully allowed $\pi\pi^*$ transitions are approached. In the case of NAD$^+$, the far-ultraviolet CD and absorption are especially complex due to contributions from both the purine and the nicotinamide ring and we shall not attempt assignments in this region.

In summary, we have characterized the weak and broad absorption and CD band of β-NAD$^+$ and its analogues which extends to wavelengths well above 300 nm. We have presented strong evidence that it is a charge-transfer transition from the adenine to the nicotinamide, which implies that base-stacked conformations are of significance in NAD$^+$. Furthermore, we have assigned $n\pi^*$ transitions in several NAD$^+$-related molecules and have discussed other aspects of the electronic structure of these biologically important molecules.

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Studies on the Anomalous Thermotropic Behavior of Aqueous Dispersions of Dipalmitoylphosphatidylcholine–Cholesterol Mixtures†

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ABSTRACT: Examination of the thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine–cholesterol mixtures by high-sensitivity scanning calorimetry has revealed that the phospholipid gel to liquid-crystalline phase transition consists of two components. One, a relatively sharp transition centered at 39.6–40.7 °C, exhibits a transition enthalpy change which decreases linearly with increasing cholesterol content, approaching zero at a cholesterol content of about 25 mol %. The other, a broad, lower intensity transition centered at approximately 41.5 °C for cholesterol concentrations of 20 mol %, displays an enthalpy change which is maximal at about 20–25 mol % cholesterol and which decreases as the cholesterol content decreases to zero or increases above 25 mol %. The origin of these two transitions is discussed in terms of a separation of these lipid mixtures into cholesterol-rich and cholesterol-poor domains.

Since the discovery by Leathes (1925) that cholesterol causes an apparent condensation of phospholipid monolayers, the interaction of this steroid with phospholipids has been the subject of considerable research (for recent reviews, see Demel and de Kruyff 1976; Jain, 1975). In the last decade this research has increasingly taken the form of physical studies aimed at elucidating the thermodynamic properties of cholesterol–phospholipid mixtures. Ladbrooke et al. (1968) initiated this approach with a scanning calorimetric study, demonstrating that the addition of increasing amounts of cholesterol to bilayer arrays of phosphatidylcholines concomitantly diminishes the apparent enthalpy change of the gel to liquid-crystalline phase transition. Subsequent studies have essentially confirmed this result, although a consensus on the

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