The Alkaline Reaction of Nicotinamide Adenine Dinucleotide, a New Transient Intermediate*

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SUMMARY

In basic solutions NAD⁺ reversibly produces a 370 nm absorbing transient intermediate which irreversibly decomposes to a 340 nm absorbing species. The concentration of the transient produced is proportional to the hydroxide concentration up to 5 M NaOH. NAD⁺ also undergoes an ionization reaction of the carboxamide NH₂ group, the Kₐ of which is 7.3 × 10⁻¹³ M. NMN undergoes spectral changes in alkaline solutions entirely analogous to those of NAD⁺, and has a Kₐ of 5.6 × 10⁻¹³ M.

NAD⁺ in alkaline solutions reversibly increases its absorbance in the 290 nm region (1). A reaction also takes place to produce an intermediate which absorbs maximally in the 340 nm region. This intermediate is replaced by a final fluorescent product which absorbs maximally at 360 nm in its basic form and at 340 nm in its acidic, nonfluorescent form (2). The pKₐ of the 360 nm product as measured by acid quenching of fluorescence (2) and spectrally (3) is about 9.6. This fluorescent product is the basis of an analytical assay for NAD⁺ (4). N-Methylnicotinamide cation has been studied in alkaline solutions as a model for the alkaline NAD⁺ reaction and has been found to undergo reversible ionization at the carboxamide group with a dissociation constant of 6.8 × 10⁻¹⁴ M (5). We have recently found that N-methylnicotinamide cation undergoes reversible changes in solutions more alkaline than 2 M NaOH to produce materials absorbing maximally at 320 nm (3). We have interpreted this additional equilibrium reaction to be due to a ring-opening reaction of N-methylnicotinamide cation. A more readily accessible model of ring-opening reactions is available in the alkaline ring-opening reaction of N,N-dimethylcarbamoylnicotinamide cation (3) or of N,N-dimethylcarbamoylpyridinium ion (6). These reactions, which are kinetically second order in hydroxide, are essentially irreversible in that the reversal reaction is so much slower than the rate of formation under alkaline conditions that the ring-opening product can be isolated.

In exploring the possibility of the occurrence of ring-opening reactions of NAD⁺ we have looked very closely at the initially produced spectrum when NAD⁺ is introduced into alkaline solutions. A transient intermediate which absorbs strongly at 360 to 370 nm has been observed. We now report the properties of the transient as well as the equilibrium properties of NAD⁺ and NMN in alkaline solutions.

EXPERIMENTAL PROCEDURE

Materials and Methods—NAD⁺ and NMN are products of P-L Biochemicals. Previously prepared carbonate-free sodium hydroxide solutions from Fisher or their dilutions were used for the rapid kinetic work. For determining the ionization constants of NAD⁺ and NMN the ionic strength of the alkaline solutions was maintained at 0.60 M with reagent grade potassium chloride.

Cary models 14 and 15 recording spectrophotometers were used to scan reacting mixtures of NAD⁺ or NMN. In this procedure a 1-cm quartz cuvette is filled with 3.00 ml of the sodium hydroxide solution, and its spectrum is recorded, with air as a reference. Then 10 μl of 0.028 M NAD⁺ are introduced into the cell with an Eppendorf automatic micropipette, at which time a stopwatch is activated with a foot-operated starting device, and the contents are rapidly mixed. In this manner the recording of the spectrum can be started about 8 to 15 sec after the introduction of reagent. The reaction solution is repeatedly recorded with air as a reference. In the interesting areas of the NAD⁺ spectra the solvent spectrum is manually subtracted to give the true spectra of NAD⁺ and its reaction products.

Kinetic determinations were made at 27 ± 1.5°C from the scanned spectral data from the Cary 15 spectrophotometer, or from constant wave length obtained with a Beckman DU-2 spectrophotometer equipped with a constant temperature bath thermostated to 25.0°C. The integrated form of the rate equation was used to obtain rate constants (7). A plot of log (A₁ - A₄) or of log (A₂ - A₃) against time gives the slope as -k/2.303, where k is the rate constant. Kinetic plots were obtained at 370 to 380 nm for the decay of the 370 nm transient, at 340 nm for the build-up of the 340 nm product, and at 340 and 360 nm for the decay of the 340 nm product. The initial absorbance of the 370 nm intermediate was taken as A₀ from the intercept of

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the kinetic plots of the 370 nm data. A Durrum stopped flow spectrophotometer was used to study the rate of production of the transient 370 nm intermediate. The maximum excursion of the oscilloscope trace is recorded and the resulting transmittance value is converted to $A_{\text{max}}$. The transmittance values resulting from the build-up of the transient are converted to absorbance values and a plot of $\log (A_{\text{max}} - A)$ against time is made and the rate constant is obtained from the slope. All plots obtained in the stopped flow work are linear to 90% reaction.

A Cary 16 spectrophotometer thermostated at 25.0 ± 0.1° was used for the determination of the equilibrium constants of NAD+ and NMN in dilute alkali. A previously measured amount of sodium hydroxide solution is placed in a 1-cm glass-stoppered cuvette and the cuvette is allowed to come to temperature equilibrium in the thermostated cell compartment. Manual measurements are made at 290 nm, giving the absorbance value of the cell and its contents with air as a reference. The cell is always placed in the same position in the cell holder. A measured quantity of NAD+ or NMN is added to the cell and the total contents are mixed. The cell is then carefully replaced and measurements of the absorbance at 290 nm are recorded as a function of time for 3 min. Linear extrapolation to zero time gives $A_0$.

Acid quenching of basic NAD+ solutions was carried out in the following manner: 10 μl of 0.2 M NAD+ were added with stirring to 2.0 ml of 5 M NaOH or to 2 ml of 1 M NaOH. After measured time intervals the reactions were quenched in an ice-salt bath with 1.7 ml of a mixture of 23 ml of concentrated HCl and 10 ml of 1 M Tris, or with 8 ml of 0.6 M Tris·HCl, respectively. The final pH is near 8.2. The spectrum is taken and the NAD+ content is measured by reducing the remaining NAD+ to NADH with ethanal catalysed by alcohol dehydrogenase and measuring the absorbance change at 340 nm (8). The amount of absorbance change at 340 nm obtained in the quenched samples is compared with that obtained when the same amount of NAD+ is added to the acid and basic components previously mixed together. From these data the percentage of NAD+ remaining is calculated.

**RESULTS**

The spectrum of NAD+ or NMN in alkaline solutions, if taken immediately after the NAD+ or NMN is introduced into the alkaline medium, shows an unstable intermediate absorbing maximally at 370 nm. This intermediate is more easily seen in concentrated alkali because more of it is formed. A typical sequence of events is as follows. In 3.0 ml of 5.0 M NaOH solution 10 μl of 0.028 M NAD+ are introduced, the solution is mixed, and scanning is started 11 sec after mixing. Scanning downward from 400 nm, the 370 nm region is reached 23 sec after mixing and a large absorption band is apparent, as well as a second small band at 331 nm. The second scan is started 123 sec after the original mixing and shows very little of the distinct 370 and 331 nm bands. Instead, a 342 nm band is building up, reaching maximum absorbance 6 min after the initial mixing. Thereafter the 342 nm band slowly shifts to longer wave lengths and decreases in intensity. A stable final spectrum, which has a 360 nm maximum, is obtained in 2 hours. This material is highly fluorescent as described by Kaplan, Colowick, and Barnes (2), and is maximally excited at 360 nm and maximally emits at 450 nm (3). Similar results are obtained in solutions more alkaline and less alkaline than above 5 M NaOH solution. Even in 0.3 M NaOH a small amount of the 370 nm transient can be seen in the very first scan if a large amount of NAD+ is used. In solutions less alkaline than 5 M NaOH, after the disappearance of the 370 nm intermediate, a material absorbing at 330 nm appears to be formed and subsequent spectral scans show increasing absorption as well as increasing wave lengths of maximal absorption until the reasonably stable 342 nm intermediate is obtained. The 342 nm band always subsequently slowly decreases in intensity and the wave length of maximal absorption is shifted to higher values until the final 360 nm product is formed.

Rapid repetitive scanning of reacting basic solutions of NAD+ or NMN shows the presence of an isosbestic point between the 370 nm transient and the 340 nm product, as shown in Fig. 1. The isosbestic condition lasts for about 2 min, corresponding to about 75% reaction in the same studied here, and thereafter the isosbestic point shifts to a slightly lower wave length. The instability of the isosbestic point after 75% reaction is probably due to the decomposition of the 340 nm product to the final 360 nm product. The position of the isosbestic point varies with basicity as follows: (given is molarity of NaOH, isosbestic point for NAD+, isosbestic point for NMN) 2.5 M NaOH, 358 nm, ----: 5 M NaOH, 363 ± 5 nm, 366 nm: 10 M NaOH, 355 nm, 356 nm: 15 M NaOH, ----, 335 nm. Semilog plots for the decomposition of the 370 nm material and for the formation of the 340 nm material show similar or identical rates of decomposition or formation. These results are included in Table 1.

The amount of 370 nm material initially formed was determined by extrapolating to zero time in a semilog kinetic plot or
directly with the stopped flow method. These results indicate a linear increase in yield of the 370 nm product with increasing alkalinity up to 5 M NaOH for both NAD and NMN as shown in Fig. 2. The lower limit of the extinction coefficient of the 370 nm product from NAD is 25,000 M$^{-1}$ cm$^{-1}$ and from NMN, 20,000 M$^{-1}$ cm$^{-1}$. Above 5 M NaOH a leveling in the yield of the 370 nm product occurs which may reflect either an approach to complete conversion of NAD$^+$ to the 370 nm material or specific salt effects. The yield data in Fig. 2, if plotted against the alkalinity function of Schwarzenbach and Sulzberger (9), show a definite leveling off in the yield of the 370 nm intermediate as shown in Fig. 3. The maximal yield of the 340 nm product also levels off above 5 M NaOH, as seen in Fig. 2. A similar plot is obtained with the NMN yield data.

The rates of formation of the 370 nm intermediate, decomposition of the 370 nm transient, formation of the 340 nm absorbance, and decrease of the 340 nm absorbance are given in Table I. The rate of decrease of the 340 nm absorbance is identical with the rate of decrease of 360 nm absorbance and probably reflects the decay of the 340 nm product to the 360 nm product. The 370 nm transient is formed much more rapidly than it decomposes to the 340 nm product and the 340 nm product decomposes at an even slower rate to the 360 nm product.

**Rapid Kinetic Studies—**NAD$^+$ when rapidly mixed with weakly alkaline solutions and followed at various wave lengths shows two distinct kinetic phenomena. At high wave lengths a transient increased absorption is observed; at low wave lengths a nontransient increased absorption is observed. For example, in 0.05 M NaOH solutions, NAD$^+$ shows a "slow" ($t_{1/2} \approx 0.5$ sec) increased absorbance in the 340 to 390 nm range with maximal absorbance at 360 to 370 nm. After its formation the transient slowly produces more highly absorbing materials. At wave lengths below 335 nm no transient phenomena are encountered: the increased absorbance of NAD$^+$ at low wave lengths first noticed by Burton and Kaplan (1) is produced with a half-life less than 0.012 sec, which is the limit of the stopped flow cuvette used in these experiments. This nontransient behavior was observed from 335 to 290 nm.

**Nontransient Increase in Absorbance—**Spectral observations of NAD$^+$ in dilute alkali have led to the conclusion that NAD$^+$ increases its absorbance maximally at 290 nm (1). The difference in absorbance of NAD$^+$ and NMN in alkaline solutions versus water at 290 nm is shown in Fig. 4. The maximal change in absorbance comes in the 0 to 0.04 M NaOH range of concentrations for both NAD$^+$ and NMN. Since the behavior of these two compounds is so strikingly similar, the possibility of an adenyl ionization may be discounted in the case of NAD$^+$. The increased absorbance may be interpreted as an ionization of the carboxamide NH$_2$ group in analogy with the carboxamide ionization of N-methylnicotinamide cation (5, 10).

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**Table I**

| NaOH (M) | Rate of formation of 370 nm intermediate$^a$ | Rate of decay of 370 nm intermediate$^a$ | Rate of formation of 340 nm product$^b$ | Rate of decay of 340 nm product$^b$ |
|----------|---------------------------------------------|------------------------------------------|----------------------------------------|----------------------------------|
| 0.04     | 93                                          | 0.031                                    |                                        |                                  |
| 0.05     | 101                                         | 0.054                                    | 0.13                                   |                                  |
| 0.10     | 104                                         | 0.213                                    |                                        |                                  |
| 0.20     | 104                                         | 0.30                                     | 0.13                                   |                                  |
| 0.30     | 80                                          | 0.40                                     | 0.31                                   | 0.45$^c$                         |
| 0.48     | 80                                          | 0.40                                     | 0.31                                   | 0.45$^c$                         |
| 0.60     | 80                                          | 0.40                                     | 0.31                                   | 0.45$^c$                         |
| 1.0      | 75                                          | 0.61                                     | 0.46                                   | 0.69                             |
| 1.5      | 75                                          | 0.68                                     | 0.77                                   |                                  |
| 2.0      | 75                                          | 0.68                                     | 0.77                                   |                                  |
| 2.5      | 96                                          | 0.82                                     | 0.74                                   | 0.63                             |
| 5.0      | 96                                          | 0.82                                     | 0.66                                   | 0.71                             |
| 7.5      | 96                                          | 0.57                                     | 0.33                                   |                                  |
| 10.0     | 96                                          | 0.55                                     | 0.42                                   |                                  |

$^a$ Determined by the stopped flow method.
$^b$ Determined from Cary wave length scans.
$^c$ First column: determined with a Beckman DU-2 spectrophotometer thermostated at 25.0°C. Second and third columns: determined from Cary wave length scans.

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**Fig. 2.** Maximal yield of 370 nm transient and 340 nm product as a function of alkalinity. Initial concentration of NAD$^+$ is 10$^{-4}$ M. O, absorbance of 370 nm transient; •, absorbance of 340 nm product.

**Fig. 3.** Maximal yield of the 370 nm transient plotted from NAD$^+$ against the alkalinity function of Schwarzenbach and Sulzberger (9).
Fig. 4. Difference spectra of NAD+ and NMN at 290 nm as a function of NaOH concentration at 25.0°C, and ionic strength 0.6 M. Lower line, 0.95 x 10^-4 M NAD+ in alkali minus NAD+ in water; upper line, 1.63 x 10^-4 M NMN in alkali minus NMN in water.

Fig. 5. Rate of disappearance of NAD+ from acid-quenched solutions, originally 1 M in NaOH.

The treatment of the data in Fig. 4 is as follows. The ionization constant of NAD+ is given by Equation 2 in which N+ is the positively charged species of NAD+, and N is its neutral species resulting from ionization of NAD+. No is the total of N+ and N.

\[ K_1 = \frac{[N]}{[N^+][OH^-]} = \frac{1}{[N] - [N^+]} \] (2)

The absorbance of a solution of N is given by:

\[ A = e_1(N^+) + e_2(N) = e_1(N) - (N) + e_2(N) \] (3)

where \( e_1 \) and \( e_2 \) are the extinction coefficients of \( N^+ \) and \( N \), respectively. It follows that:

\[ (N) = \frac{A - A_0}{e_2 - e_1} \] (4)

where \( A_0 \) is \( e_2 N_0 \) or the absorbance of an aqueous solution of \( N^+ \).

Substituting this value of \( (N) \) into Equation 2 yields:

\[ K_1 = \frac{1}{(e_2 - e_1)A_0 + 1} \] (5)

A plot of the left-hand side of Equation 5 against \( 1/(OH^-) \) should yield a straight line with \( 1/(e_2 - e_1) \) as the intercept and \( 1/(e_2 - e_1)K_1 \) as the slope. The value of \( K_1 \) is then given as the intercept divided by the slope. The data in Fig. 4 when plotted in this way give a linear regression. A least squares analysis of these data yields \( K_1 \) values of 72.7 ± 8.3 M^-1 for NAD and 56.4 ± 6.2 M^-1 for NMN. This corresponds to acid dissociation constants of \( 7.3 \times 10^{-5} \) M and \( 5.6 \times 10^{-4} \) M for NAD+ and NMN, respectively.

**Quenching Experiments**—With increasing time in basic solutions, increasing amounts of a 375 nm product (seen at pH 8.1) are formed and decreasing amounts of NAD+ remain. Semilog plots of the percentage of NAD+ remaining against time are linear (Fig. 5), as are semilog plots for the appearance of the 375 nm product. The 375 nm material is the acidic form of the 340 nm product (pK ~10), as determined by reversible titration of this material. The rate constants for the disappearance of NAD+ in 1 M NaOH and 5 M NaOH are 0.31 and 0.69 min^-1, respectively. The rate constants for the appearance of the 375 nm product in the quenching experiments are 0.68 and 0.92 min^-1. Although the rate constants for the disappearance of NAD+ and appearance of the 375 nm product are not exactly the same, upon consideration of the room for error in the quenching experiments, it is probable that the rates are equal. In basic solution the disappearance of NAD+ parallels the appearance of the 340 nm product.

**DISCUSSION**

**Kinetics and Stoichiometry**—At the alkalinity levels at which the appearance of the 370 nm material is studied, the NAD+ molecule exists primarily as the amide anion. The formation of this amide anion undoubtedly protects NAD+ from hydrolysis at the amide group as in the case of N-methylnicotinamide because the amide hydrolysis in highly alkaline media is retarded and other alkaline processes which are not inhibited by the amide equilibrium can become predominant (10). Another reaction that must be considered with NAD+ in alkaline solution is cleavage at the nicotinamide-riboside bond, which has been shown to occur by Kaplan et al. (2), and to be first order in hydroxide in the pH 8 to 9 region (11). The riboside cleavage reaction is more sensitive to alkaline than is the pyrophosphate cleavage in 0.1 to 1 M NaOH (1). In 0.17 M KOH, the rate of disappearance of the riboside-nicotinamide linkage determined by the cyanide addition reaction is about equal to the rate of the loss of functional NAD+ as determined by ethanol oxidation catalyzed by alcohol dehydrogenase (1). This means that the cleavage of the nicotinamide riboside bond is the predominant process which determines the destruction of NAD+, as this is the process which destroys the positive charge on the nicotinamide ring nitrogen necessary for the cyanide addition reaction.

The behavior of NAD+ in basic solutions 1 to 15 M in NaOH can be summed up as follows.

1. NAD+, in alkaline solutions in which it exists mainly as the amide anion, undergoes a very rapid reaction (t1/2 ~0.5 sec) to form a highly absorbing species absorbing maximally at 370 nm, \( \epsilon >25,000 \) M^-1 cm^-1.

2. The formation of the 370 nm species is reversible and becomes complete at high alkalinities.

3. The 370 nm species undergoes an irreversible conversion 

   Any species in equilibrium with this species could be undergoing the irreversible reaction. Because of known ring-opening reactions of analogous compounds (3, 6) we believe that the 370 nm species is undergoing the irreversible reaction.
to a 340 nm product and to another substance (or substances) which is transparent in the near ultraviolet. The half-life for this process is about 1 min.

4. The 340 nm material undergoes an irreversible conversion to a highly fluorescent material which absorbs maximally at 360 nm. The half-life for this conversion is about 20 min.

The behavior of NMN in base is very similar to that of NAD$^+$. Equation 6 illustrates the above points.

\[
[\text{Anionic NAD}^+] + k_1[\text{OH}^-] \rightarrow (370) \quad k_2 \rightarrow \text{II(340)} \rightarrow (360)
\]

(6)

Reversibility is supported by the fact that in 5 M NaOH, in which the conversion of NAD$^+$ to the 370 nm substance is nearly complete, quenching with acid 5 sec after the introduction of NAD$^+$ to alkali nearly quantitatively regenerates NAD$^+$.

The variation with basicity of the isosbestic point between the 370 nm and 340 nm species, not accountable by the pK$\alpha$ values of these substances at pH values greater than 14, supports the contention that the 370 nm substance is converted to the 340 nm species as well as optically transparent materials. If the 370 nm species (I) decomposed according to Scheme 6 by consecutive reactions (12) to the absorbing 340 nm species (II) and to transparent materials (T), the ratio of (T):(II) at any time is given by the ratio of the corresponding rate constants for the formation of these materials from I, $k_{a}/k_{s}$. The value of $x$ is the ratio of the corresponding rate constants for the formation of these materials from I, $k_{a}/k_{s}$. The value of x will be constant at constant pH, and an isosbestic will be obtained at the wave length at which $\varepsilon_{II} = \varepsilon_{I} + x$. The optical density at this wave length of a reacting mixture equals $\varepsilon_{II} + x$. The higher x is, that is, the lower the yield of the 340 nm species from the 370 nm species, the lower the wave length at which the isosbestic point occurs. A plot of the ratio of the maximal absorbance of the 370 nm species to absorbance of the 340 nm products gives a linear relationship with hydroxide concentration, indicating that the transition state for the production of the 340 nm species contains more proton than the transition state for the production of the transparent products from the 370 nm species.

The 340 nm product appears to be converted to the final fluorescent 360 nm product, because the rate of decomposition of the 340 nm product is independent of the wave length at which this reaction is studied. The wave lengths used were 340 and 360 nm. There are, however, no material balance data to prove this point with certainty. Further support for the idea that the 340 nm material converts to the 360 nm product is obtained from the data of Kaplan et al. (2) which show that the yield of the 360 nm product is linear in hydroxide up to 5 M, and after 5 M NaOH the yield of the 360 nm product levels off (4). This behavior closely follows the behavior of the yield of the 340 nm material, suggesting that the amount of the final 360 nm material produced depends upon the amount of 340 nm material produced under a given condition.

The fact that a product with spectral, fluorescence, and pK$\alpha$ properties identical with the 380 nm product of NAD$^+$ is obtained from the ring-opening reaction of N,N-dimethylcarbamoyl nicotinamide cation (3) suggests strongly that NAD$^+$ is also undergoing a ring-opening reaction.

The previously studied pyridinium ring-opening reactions (3, 6) involve a pseudo-base intermediate and follow the mechanism given in Equation 7.

\[
\text{NAD} \rightarrow \text{RI} \rightarrow \text{NAD}^+ \rightarrow \text{RIBOSIDE CLEAVAGE PRODUCTS}
\]

(7)

Assuming the steady state for the pseudo-base intermediate III, the rate constant for approach to equilibrium between NAD$^+$, anionic NAD$^-$, and the 370 nm species I is given by Equation 8.

\[
k_{obs} = \frac{k_{a}k_{b}([OH^-])}{[k_{a} + k_{b}]} \quad \text{(8)}
\]

Knowing that $k_{a}$ is 73 M$^{-1}$s$^{-1}$ the $K_{b}(\text{OH}^-)$ term is much larger than 1 in the pH range studied, and Equation 9 results. In the pH range of interest, the value of $k_{a}k_{b}(\text{OH}^-)$ is greater than $k_{-1}k_{-2}$. This is because the ratio of I (370 nm) to (NAD$^+$) is equal to $K_{a}K_{b}([\text{OH}^-]) = \frac{k_{a}k_{b}([\text{OH}^-])}{k_{-1}k_{-2}}$. Equation 9 becomes Equation 10. Because the rate observed (Table I) for the formation of the 370 nm species is pH-independent, the hydroxide term must dominate the denominator, so that the observed rate constant is equal to $k_{a}/73$. The value of $k_{a}$ calculated from the present data is 6600 M$^{-1}$min$^{-1}$.

The important equilibrium and rate relationships involved in the alkaline destruction of NAD$^+$ are illustrated in Equation 11, assuming that the riboside cleavage and ring-opening path-
ways are the most important ones. The rate constant for the disappearance of NAD\(^+\) according to this scheme is given by:

\[
k_{obs} = \frac{k_1(OH^-)}{1 + K_d(OH^-) + K_d(OH^-)^2} + \frac{k_2(OH^-)}{1 + K_d(OH^-) + K_d(OH^-)^2} + \frac{1}{1 + K_d(H^+) + K_d(H^+)^2} + \frac{k_4}{1 + K_d(H^+) + K_d(H^+)^2} (12)
\]

The rate constant as a function of pH would be expected to be quite complex. The first, second, and third terms in Equation 12 describe processes originating from NAD\(^+\), anionic NAD\(^+\), and I (370 nm), respectively. The last term has more higher order hydroxide terms than does the second. The first has none. At higher alkalinities, therefore, destruction reactions originating from the 370 nm intermediate will be more important than reactions originating from NAD\(^+\) or anionic NAD\(^+\).

**Structural Considerations**—The effect of the ribose group in increasing the acidity of the carboxamide moiety over that of N-methylnicotinamide cation IV is at least 10-fold. Martin and Hull (5) report a value of 6.8 \(\pm 1 \times 10^{-14}\) M for the acid dissociation constant of N-methylnicotinamide cation at 25\(^\circ\), whereas the value of Brooke and Guttman (10) extrapolated to 25\(^\circ\) is 3.53 \(\pm 0.2 \times 10^{-14}\) M. The ribose linkage at its aldehyde level of oxidation is more electron-withdrawing than the N-methyl group and is responsible for the acidity of the carboxamide group of NAD\(^+\). It is possible that the ribose linkage plays an important role in NAD\(^+\) reactions and that N-alkylated nicotinamide cations are very poor models for NAD\(^+\). The value of \(k_4\), the rate constant for hydroxide addition to NAD, is 200 times smaller for the same process with \(N\),\(N\)-dimethylcarbamoyl- nicotinamide cation V (3), but 5 times larger than the same process with the unsubstituted \(N\),\(N\)-dimethylcarbamoylpyridinium ion VI (6).

An important difference between NAD and V is that the ring-opened form of NAD is rapidly reversed to NAD, as is also the ring-opened form of IV easily closed to IV, where as the ring-opened form of V is only very slowly acid-closed (3). The ease of reversibility is undoubtedly related to the basicity of the amino nitrogen in the ring-opened form. The more basic the nitrogen, the more rapidly the nitrogen adds to the aldehyde group, and the more rapidly the reversal reaction takes place. V is not a reversible system whereas NAD and IV are. In this respect IV is a good model for NAD.

The ultraviolet absorption characteristics of the 370 nm intermediate are what would be expected for a ring-opened intermediate. Analogies are the ring-opened product of \(N\),\(N\)-dimethylcarbamoyl- nicotinamide cation which absorbs maximally at 392 nm (3) and the quinolinic acid precursor 2-acroyethyl-3-amino fumarate, VII, which absorbs maximally at 360 nm at pH 7 (13).

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