# Pyridine Nucleotide Chemistry. A New Mechanism for the Hydroxide-Catalyzed Hydrolysis of the Nicotinamide–Glycosyl Bond

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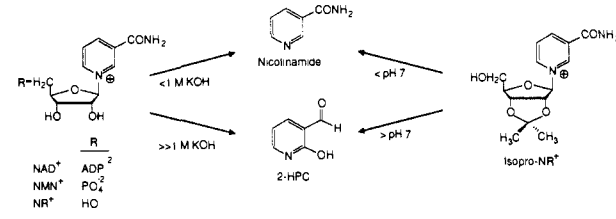
**Abstract:** The mechanism of hydroxide-catalyzed hydrolysis of the glycosyl bond of β-$NAD^+$ has been reinvestigated. The pH dependence of the rate of hydrolysis of β-$NAD^+$ and related compounds has been determined over a range from pH 4.3 to 13.5. Between pH 8.5 and 11 the log of the rate constant is linearly dependent on pH with a slope of unity. Below pH 6.5 and above pH 12.5 the reaction becomes pH independent. The product of the reaction over the entire pH range is nicotinamide. A nonlinear least-squares fit of the data yields a $pK_{eq}$ corresponding to the $pK_a$ of ionization of the nicotinamide ribose diol, which has been determined independently by $^{13}C$ NMR. Methanolysis of β-$NAD^+$ yields a ratio of 3.7:1 for the β and α anomers of 1′-*O*-methyl-ADP ribose. Hydrolysis in a methanol/water mixture shows no selectivity for attack on the basis of the nucleophilicity of the attacking species. The importance of the ribose diol in the hydrolysis reaction was investigated with the isopropylidene derivative of β-nicotinamide riboside. Hydroxide-catalyzed decomposition of β-2′,3′-*O*-isopropylidene nicotinamide riboside is pH independent below pH 7 and linearly dependent on hydroxide concentration above pH 10. In contrast to the results for β-$NAD^+$, no pH-independent region is observed at high pH and the product of the pH-dependent reaction is 2-hydroxy-3-pyridinecarboxaldehyde; i.e., no detectable hydroxide-catalyzed release of nicotinamide is observed. On the basis of these data, as well as solvent isotope effects and data from previous investigations, we propose a new mechanism in which dissociative cleavage of the nicotinamide–glycosyl bond is facilitated by the nicotinamide ribose diol anion through noncovalent stabilization of an oxo carbocation intermediate.

Although $NAD^+$ [1] is commonly associated with its pivotal role in cellular oxidation–reduction processes, its significance as a potential effector of cellular metabolism in nonredox reactions has also recently been recognized. These nonredox reactions are characterized by the cleavage of the nicotinamide–glycosyl bond and are catalyzed by a series of enzymes including polyADP-ribose synthase, various ADP(ribose) transferases (both endogenous proteins and toxins), and NAD-glycohydrolases.[2-4] The mechanism of the chemical and enzymatic cleavage of the nicotinamide–glycosyl bond, however, has not been fully characterized.

The oxidized pyridine coenzymes have long been known to be stable in acid and unstable in base.[5] The rate constants for hydrolysis of $NAD^+$ at 100 °C over the range pH 3–9 have been measured by Colowick et al.[6] They found the rate of hydrolysis to be pH independent below pH 6.0 and hydroxide-catalyzed (i.e., first order in hydroxide) at higher pH. Colowick and co-workers also reported a buffer dependence of the base-catalyzed hydrolysis of the nicotinamide–ribose linkage; however, this was subsequently shown by Anderson and Anderson[7] to be due to the different sensitivities of the buffers to temperature-dependent pH changes.

The chemistry of $NAD^+$ under alkaline conditions is known to involve two different reaction pathways depending upon the hydroxide concentration. Kaplan et al.[8] demonstrated the quantitative recovery of nicotinamide when $NAD^+$ was incubated in 0.1–1.0 N KOH (Scheme I). In 5 N KOH, however, the yield of nicotinamide was only 30% of theoretical, and an unidentified

Scheme I




fluorescent product with a $\lambda_{max}$ at 360 nm was observed. Burton and Kaplan[9] reported that $NAD^+$, $NMN^+$, and $NR^+$ all released this fluorescent product when incubated in solutions of KOH, whereas nicotinamide itself did not react. Subsequent work by Johnson and Morrison[10] and Guilbert and Johnson[11] characterized the main product isolated from the reaction of $NAD^+$ in concentrated base as 2-hydroxy-3-pyridinecarboxaldehyde (2HPC), as shown in Scheme I. This new product arises from hydroxide addition to the nicotinamide ring of the intact dinucleotide followed by ring opening, elimination, and subsequent recyclization.[11b] The change in mechanism from nicotinamide release to 2HPC formation has been attributed to a second-order dependence on hydroxide for the latter reaction;[10] however, experimental evidence for this higher order dependence is lacking. Indeed, both the hydroxide-catalyzed reactions of model compounds[11a] and the formation of the chromophore that leads to 2HPC[11b] have been shown to be first order in hydroxide. These results present a mechanistic dilemma: how the mechanism changes from nicotinamide release to 2HPC formation when both reactions have been shown to be first order in hydroxide.

The mechanism of the enzymatic and nonenzymatic cleavage of the glycosyl bond of $NAD^+$, $NMN^+$, and $NR^+$ has been investigated by measurement of kinetic α secondary deuterium isotope effects.[12,13] At pH 4, where the nonenzymatic hydrolysis of $NAD^+$ is pH independent, the kinetic isotope effect, $k_H/k_D$,

(1) Abbreviations used: $NAD^+$, β-nicotinamide adenine dinucleotide; $NMN^+$, β-nicotinamide mononucleotide; $NR^+$, β-nicotinamide riboside; isopro-$NR^+$, β-2′,3′-*O*-isopropylidene nicotinamide riboside; 2-HPC, 2-hydroxy-3-pyridinecarboxaldehyde; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl) ether-*N*,*N*,*N*′,*N*′-tetraacetic acid; FID, free induction decay; INEPT, insensitive nuclei enhancement by polarization transfer; TMS, tetramethylsilane.

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is 1.11 ± 0.01. At pH 9.6 and 10.4, where hydrolysis is hydroxide catalyzed, the isotope effect is 1.15 ± 0.01. An $\alpha$ secondary deuterium isotope effect greater than unity is characteristic of reactions where rehybridization from $sp^3$ to $sp^2$ occurs in the transition state.[14] The magnitude of the $\alpha$ secondary isotope effects observed for hydrolysis of $NAD^+$ indicates significant $sp^2$ character at the anomeric carbon in the transition state. These results are consistent with both the pH-independent and the hydroxide-catalyzed hydrolysis reactions proceeding by a dissociative mechanism involving an oxo carbocation intermediate. Recent work by Tarnus and Schuber with pyridinium analogues of $NAD^+$ has shown that, at pH 6.4, both the nonenzymatic and enzymatic hydrolysis reactions occur with transition states close to an oxo carbocation intermediate.[15] These results pose another mechanistic dilemma. The kinetic isotope effect data indicate that the hydrolysis of the nicotinamide–glycosyl bond is dissociative, yet above pH 7 the reaction is clearly hydroxide catalyzed (first order in $NAD^+$ and first order in hydroxide, second order overall). The question of how one accounts for the clear hydroxide dependence in a reaction that is dissociative in character arises.

In this paper we report the results of experiments directed at resolving the mechanistic inconsistencies of the alkaline chemistry of pyridine nucleotides through investigations of the base-catalyzed decomposition of $NAD^+$, $NMN^+$, $NR^+$, and $\beta$-2′,3′-isopropylidene nicotinamide riboside (isopro-$NR^+$). On the basis of the results of these experiments, we propose a new mechanism for the hydroxide-catalyzed hydrolysis of the nicotinamide–glycosyl bond in $NAD^+$. This mechanism involves loss of nicotinamide with stabilization of the resulting oxo carbocation intermediate by the diol anion of the nicotinamide ribose moiety. This stabilization leads to a rate acceleration of greater than 4 orders of magnitude relative to the water-catalyzed reaction at neutral pH.

## Experimental Section

**Materials.** $NAD^+$ was obtained from Kyowa Hakko Kogyo Co., Ltd. $NMN^+$, nicotinamide, EGTA, and CAPS and MES buffers were obtained from Sigma Chemical Co. and used without further purification. The 2-hydroxy-3-pyridinecarboxaldehyde was prepared from $NAD^+$ by the method of Guilbert and Johnson.[11a] Nicotinamide riboside ($NR^+$) was prepared from $NMN^+$ by the action of alkaline phosphatase.[16] $\alpha,\beta$-2′,3′-*O*-isopropylidene nicotinamide riboside was prepared according to the procedure of Jeck et al.[17] Pure $\beta$ anomer was isolated by HPLC on a Whatman Partisil 5 ODS-3 (25-cm) reversed-phase column eluting with 10 mM ammonium bicarbonate containing 25% (v/v) acetonitrile.

**Kinetics.** Hydrolytic reactions below pH 5.0 were conducted in 0.2 M acetate buffer. In the range pH 5.2–7.5, 0.2 M MES buffer was used, and for the range pH 7.0–12.5, 0.2 M CAPS buffer was used. Above pH 12.0, 0.2 M KOH, titrated to the appropriate pH with HCl, was used. The ionic strength of all solutions was adjusted to 1.2 M with KCl. The initial concentration of $NAD^+$ and related compounds was 2 mM and was determined spectrophotometrically on an Hitachi 100-80 UV/visible spectrophotometer. Reaction mixtures were incubated in 1-mL septum-sealed vials and maintained at constant temperature (±1 °C) in a block heater. The hydrolysis of the pyridinium–glycosyl bond was monitored by a discontinuous spectrophotometric assay based on the cyanide addition reaction.[18] Aliquots of the reaction mixtures were diluted 1:10 into a solution of 1 M KCN in 10 mM $K_2CO_3$ (pH ≈13) at appropriate time intervals, and the absorption due to formation of the cyanide adduct was determined at the $\lambda_{max}$ for each addition product. The first-order rate constants for $NAD^+$ hydrolysis over the entire pH range were calculated by a least-squares fit of ln (absorption) vs time.

The cyanide assay used for monitoring the disappearance of isopro-$NR^+$ in the pH-dependent region is complicated by the concomitant appearance of a weaker absorption by the reaction product (2HPC) at the $\lambda_{max}$ for the isopro-$NR^+$ cyanide adduct (320 nm). The 2HPC also reacts reversibly with cyanide in the assay mixture with an isosbestic point at 320 nm.[19,20] This reaction of 2HPC with cyanide can, however, be ignored due to the presence of the isosbestic point at the assay wavelength, and the data can be treated as if only 2HPC and the cyanide adduct of isopro-$NR^+$ absorb. The experimental data in the pH-dependent region were therefore fit to a function accounting for this absorption using nonlinear regression analysis. The equation used in the analysis was

$$^{320}A_t = \epsilon_{\text{NR-adduct}}[\text{isopro-NR}^+]_0 e^{-kt} + \epsilon_{\text{2HPC}}[\text{isopro-NR}^+]_0(1 - e^{-kt})$$

where $\epsilon_{\text{NR-adduct}}$ and $\epsilon_{\text{2HPC}}$ are the extinction coefficients of the isopro-$NR^+$ cyanide adduct and 2HPC (and its cyanide adduct) at 320 nm, respectively, $[\text{isopro-NR}^+]_0$ is the initial concentration of the nicotinamide riboside, and $k$ is the first-order rate constant. The value used for the extinction coefficient of the isopro-$NR^+$ cyanide adduct at 320 nm was 5900 $M^{-1}$ $cm^{-1}$.[18] The value used for the extinction coefficient of 2HPC at 320 nm was determined from a UV spectrum of authentic 2HPC at pH 13 using the literature value for the extinction coefficient at the $\lambda_{max}$ (360 nm)[11b] and was found to be 2080 $M^{-1}$ $cm^{-1}$.[20] First-order rate constants for the reaction of isopro-$NR^+$ in the pH-independent region where the reaction exhibits water catalysis with formation of nicotinamide were calculated by a least-squares fit of ln (absorption) vs time.

Methanolysis of $NAD^+$ was performed on a reaction mixture consisting of 50% (v/v) water containing 0.2 M CAPS buffer, pH 10.5, and 50% methanol, corresponding to a molar ratio of water to methanol of 2.3:1. The reaction was conducted at 55 °C and was allowed to proceed for approximately 2 half-lives before being quenched with dilute HCl to pH 8. The solvent was removed by lyophilization, the residue was lyophilized twice from 99.8% $D_2O$ and dissolved in 100.0% $D_2O$, and the $^1H$ NMR spectrum was obtained. Assignments for ADP-ribose and 1-*O*-methyl-ADP-ribose were made by comparison with the spectra of authentic samples.[21,22] The relative amounts of ADP-ribose, 1-*O*-methyl-ADP-ribose, and the anomeric forms of the latter were determined by integration of the appropriate resonances.

**Solvent Isotope Effects.** Hydrolytic reactions were conducted in the same manner as described above utilizing buffers and $NAD^+$ solutions prepared in $D_2O$ (99.8 atom %). Determination of the pD of the $D_2O$ solutions used the standard pH electrode correction for deuterium:[23] pD = meter reading + 0.4.

**NMR Spectroscopy.** Routine $^1H$ spectra for product analyses were acquired at 500 MHz on a General Electric GN-500 instrument operating in the Fourier transform mode using an internal deuterium lock. The samples (initially 2 mM in pyridinium) were prepared to a final volume of 0.35 mL in 5-mm NMR tubes and contained 50 $\mu$M EGTA to suppress paramagnetic line broadening. Except for the product from $NAD^+$ methanolysis, all $^1H$ NMR samples were prepared in $H_2O$ with 5% $D_2O$ added to provide a lock signal. The sample temperature was maintained at 25.0 °C. The pulse width was set to correspond to a 75° tip angle, and no postacquisition delay was used except when the spectra were to be integrated. In those cases a delay of 10 s was used to assure equilibrium intensities. The spectral width was ±3000 Hz, and quadrature phase detection was employed. The free induction decays were acquired with a 16-bit digitizer and stored in 16K data points. The FID was apodized with a single exponential before Fourier transformation, resulting in a line broadening of 0.2 Hz. For samples prepared in $H_2O$ a 1:3:3:1 pulse sequence[24] was used to suppress the water peak. Chemical shifts were referenced to internal 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid (TSP).

Natural-abundance $^{13}C$ spectra were obtained at 125 MHz with an INEPT pulse sequence to enhance the carbon sensitivity.[25] $NAD^+$ samples (40 mM) were prepared in the appropriate buffer solution with 10% $D_2O$ added to provide a lock signal. The samples also contained 20 mM 1,4-dioxane as an internal reference ($\delta$ 66.5) as well as 50 $\mu$M EGTA and were prepared immediately before insertion into the spectrometer. The sample volume was 5 mL in order to avoid the use of vortex plugs, and 10-mm Wilmad flat-bottom NMR tubes were used. The pH was measured after spectral acquisition. The time from sample preparation to completion of data acquisition was typically less than 5 min so that during data acquisition there was less than 25% hydrolysis. The delays in the INEPT pulse sequence were adjusted to maximize the enhancement of methine carbons.[25c] Broad-band proton decoupling employing the WALTZ-16 decoupling scheme was applied during the ac-

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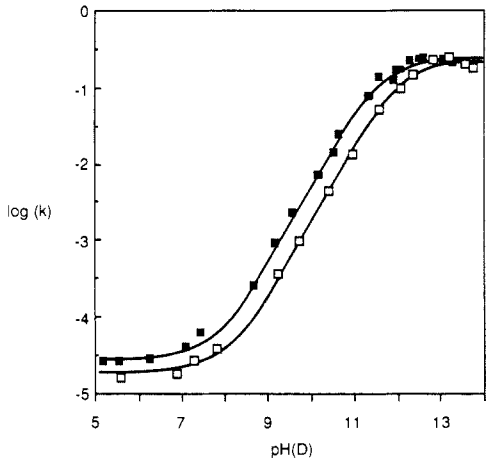


**Figure 1.** pH-rate profiles for $NAD^+$ at 37 °C in aqueous (■) and deuteriated (□) buffer systems. A nonlinear least-squares analysis of the rate constants yields a $pK_{eq}$ of 11.7 ± 0.1 for $H_2O$ and 12.1 ± 0.1 for $D_2O$.

quisition time using 2.1-W decoupler power.[26] The spectral width was set to ±12 500 Hz, and quadrature phase detection was used. Typically, 128 scans were acquired. A postacquisition delay of 2 s was used to allow for proton relaxation. Exponential multiplication of the FID, resulting in a line broadening of 1.0 Hz, was used to enhance the signal to noise ratio. Titration of $NAD^+$ was conducted at 5, 12, and 20 °C, and constant temperature was maintained during acquisition by the spectrometer temperature controller. All $NAD^+$ and buffer solutions were preequilibrated to the appropriate temperature before sample preparation and data acquisition. Assignments of the $^{13}C$ resonances are based on literature values for $NAD^+$ chemical shifts.[27] Chemical shifts are reported relative to TMS and are accurate to 0.02 ppm.

**Nonlinear Regression Analysis.** Nonlinear regression analyses were performed with the algorithm described by Cleland.[28] Standard errors of the calculated parameters were obtained from the standard deviation of the fit. The data for the pH–rate profiles for the hydrolysis of $NAD^+$, $NMN^+$, and $NR^+$ were fit to eq 1, where $k_w$ is the rate constant for the

$$\log k_{obsd} = \log \left( k_w + \frac{k_{ind}}{1 + H^+/K_{eq}} \right) \quad (1)$$

pH-independent water-catalyzed reaction, $k_{ind}$ is the pH-independent rate constant for hydrolysis at high pH, and $K_{eq}$ is the equilibrium constant for the reversible reaction of hydroxide with the nucleotide. This equilibrium constant cannot a priori be assigned to diol ionization.

The data for the pH–rate profile for isopropylidene-$NR^+$ in the range pH 4.3–11.7 were fit to eq 2, where $k_w$ is the rate constant for the pH-independent water-catalyzed reaction and $k_{OH}$ is the second-order

$$\log k_{obsd} = \log (k_w + k_{OH}[OH^-]) \quad (2)$$

rate constant for attack of hydroxide on the nicotinamide ring (the first reaction in the mechanism for 2HPC formation). Data were plotted as log $k_{obsd}$ vs pH to maintain consistency between these and the other pH–rate profiles.

$^{13}C$ NMR titration data were fit to eq 3, where $\delta_{obsd}$ is the observed chemical shift, $\delta_{HA}$ is the chemical shift of the carbon atom in the unionized diol, $\delta_A$ is the chemical shift of the carbon atom in the ionized diol, and $K_{eq}$ is the equilibrium constant for the ionization.

$$\delta_{obsd} = \frac{\delta_{HA}}{1 + H^+/K_{eq}} + \frac{\delta_A}{1 + K_{eq}/H^+} \quad (3)$$

## Results

**pH Dependence of Hydrolysis.** The first-order rate constants for the hydrolysis of $NAD^+$ have been determined over the range pH 5.2–13.5 at 37 °C. The reaction was monitored by measuring the time-dependent decrease in the formation of the cyanide addition complex at 327 nm for aliquots of the incubation mixture added to 1 M KCN solution. The reaction was found to be first order to nearly 4 half-lives over the entire pH range.[20]

A plot of the log of the rate constant vs pH for hydrolysis of $NAD^+$ at 37 °C is shown in Figure 1. As can be seen, hydrolysis is pH independent (water catalyzed) below pH 6.5. In the range from pH 8.5 to 11.0, however, the reaction becomes first order in hydroxide. These results are fully in accord with the previous observations made over a narrower range of pH.[6,7] By extending our study to higher pH, however, we now observe that above pH 12.5 the reaction again becomes pH independent. Analysis of the $^1H$ NMR spectra of the products of the reaction at pH 13.5 (the upper limit of the rate profile) shows that nicotinamide remains the only product generated upon cleavage of the glycosyl bond.[29] When these data are fit to eq 1, a $pK_{eq}$ value of 11.7 is obtained. Similar $pK_{eq}$ values are obtained at 37 °C for the pH-dependent hydrolysis of $NMN^+$ and $NR^+$, and these $pK_{eq}$ values, along with the kinetic data, are summarized in Table I.

**Table I.** Kinetic Parameters for the Hydrolysis of Pyridine Nucleotides at 37 °C[a]

| compd | $pK_{eq}$ | $k_w \times 10^7$, $s^{-1}$ | $k_{ind} \times 10^3$, $s^{-1}$ |
|---|---|---|---|
| β-$NAD^+$ | 11.7 ± 0.1 | 5.0 | 4.0 |
| β-$NMN^+$ | 11.5 ± 0.1 | 5.0 | 4.5 |
| β-$NR^+$ | 11.7 ± 0.1 | 16.8 | 11.8 |

[a] The kinetic parameters were determined by fitting the kinetic data to eq 1.

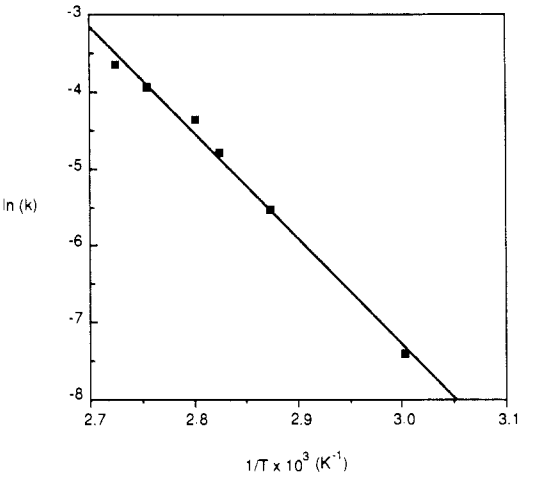


**Figure 2.** Temperature dependence of the rate of hydrolysis of the nicotinamide–glycosyl bond of $NAD^+$ at pH 6.2.

The leveling off of the hydrolytic reaction above pH 12.5 provides an explanation for the change in mechanism observed at higher pH. Because *hydrolysis* of the glycosyl bond becomes pH independent at high pH, the first-order dependence on hydroxide for the slower associative formation of 2HPC allows this latter reaction to eventually dominate the chemistry above pH 14.

**Temperature Dependence of $NAD^+$ Hydrolysis.** The temperature dependence of the rate of hydrolysis of the nicotinamide–glycosyl bond has been measured at pH 6.2, where the reaction undergoes pH-independent water-catalyzed hydrolysis. The temperature dependence has also been measured at pH 13.4, where the reaction is again pH independent. The results of these experiments are shown in Figures 2 and 3. Transition-state theory allows the enthalpy and entropy of activation for the reactions at the two pH values to be calculated[30] from which the $\Delta G^\ddagger$ for each reaction can be determined. At pH 6.2 the values for $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are calculated to be 25.2 ± 1.7 kcal/mol and −3.3 ± 2.5 eu, respectively, giving a $\Delta G^\ddagger$ of 26.2 ± 2.5 kcal/mol. At pH 13.4

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(29) The glycosyl bond is not the only base-labile bond in the molecule. Above pH 13 small amounts, ≤7%, of nicotinic acid adenine dinucleotide can be detected in NMR spectra, indicating that some hydrolysis of the carboxamide group also occurs. Decomposition elsewhere in the molecule was not observed under the experimental conditions.
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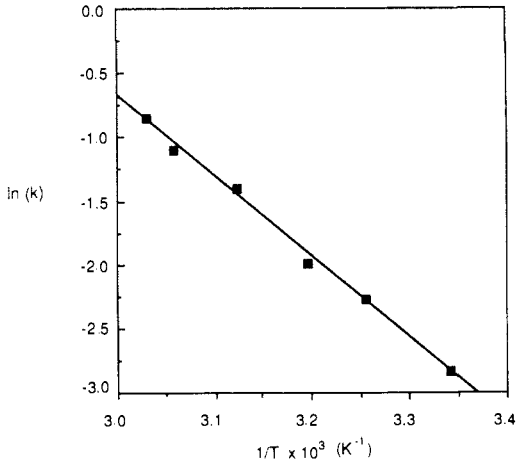



Figure 3. Temperature dependence of the rate of hydrolysis of the nicotinamide-glycosyl bond of $NAD^+$ at pH 13.4.

Table II. $^{13}C$ NMR Titration Data for $\beta$-$NAD^+$ at 20 °C[a]

| resonance | $pK_a$ | $\delta_{HA}$ | $\delta_A$ | $\Delta\delta$ |
|---|---|---|---|---|
| nicotinamide C2′ | 12.17 ± 0.03 | 77.47 | 81.96 | 4.49 |
| nicotinamide C3′ | 12.13 ± 0.03 | 70.28 | 71.77 | 1.49 |
| adenosine C2′ | 12.78 ± 0.05 | 73.89 | 75.30 | 1.41 |
| adenosine C3′ | 12.75 ± 0.05 | 70.29 | 71.36 | 1.07 |

[a] Chemical shifts are reported (ppm) downfield from TMS. The values of $\delta_{HA}$ and $\delta_A$ are the extrapolated chemical shifts of the protonated and deprotonated forms of the ribose diol, and $\Delta\delta$ is the difference in chemical shift caused by diol ionization.

these values are 11.8 ± 0.5 kcal/mol and −30.8 ± 1.5 eu, respectively, giving a $\Delta G^{\ddagger}$ of 21.0 ± 0.9 kcal/mol. The difference in $\Delta G^{\ddagger}$ for the hydrolytic reaction at the two different pH values is therefore calculated to be $\delta\Delta G^{\ddagger}$ = 5.2 ± 1.6 kcal/mol.

**Determination of the $pK_a$ of the Ribose Diols.** The $pK_a$s of the ribose diols of $NAD^+$ have been determined from pH-dependent changes in chemical shifts of the sugar carbons by natural-abundance $^{13}C$ NMR.[31] A plot of the $^{13}C$ chemical shifts for both nicotinamide and adenine ribose 2′- and 3′-carbons over the range from pH 9.3 to 12.9 at 20 °C is shown in Figure 4. A nonlinear least-squares analysis of the data for the nicotinamide ribose 2′-carbon in $NAD^+$ at 20 °C yields a $pK_a$ of 12.17 ± 0.03. This result and a nonlinear least-squares analysis of the data for the other resonances provide the $pK_a$ values listed in Table II. The $pK_a$ values of 12.78 and 12.75 for the adenosine C2′- and C3′-carbons are both considerably higher than the values obtained for the corresponding nicotinamide ribose ionization. This result is in accord with the expected greater electron withdrawal by the positively charged pyridinium moiety. The observed values of the $pK_a$s are also consistent with those observed for other nucleotide and sugar diols.[32]

The extrapolated chemical shifts of the $^{13}C$ ribose diol resonances of $NAD^+$ are also listed in Table II. The largest pH-dependent chemical shift changes are observed for the 2′-carbon of the nicotinamide and adenine ribose groups. The nicotinamide C2′ shows an ionization-dependent downfield shift of 4.5 ppm whereas the nicotinamide C3′ shifts only 1.5 ppm. This suggests that the charge of the ionized diol may not be shared equally among the oxygens on the 2′- and 3′-carbons. Interestingly the adenosine 2′- and 3′-carbons shift only 1.4 and 1.1 ppm downfield,

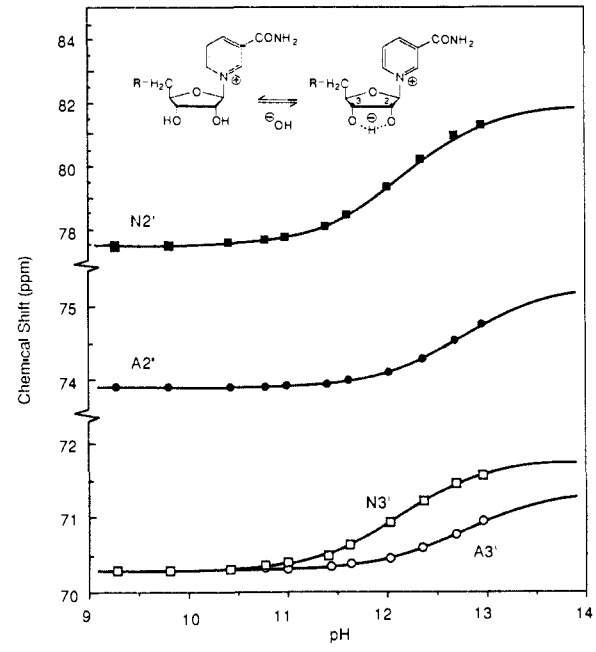



Figure 4. $^{13}C$ chemical shift vs pH for the nicotinamide ribose 2′ (N2′, ■) and 3′ (N3′, □) carbons and the adenine ribose 2′ (A2′, ●) and 3′ (A3′, ○) carbons of $NAD^+$ at 20 °C. Spectra were acquired as described in the text. Nonlinear least-squares analysis of the titration data yields a mean $pK_a$ of 12.15 ± 0.01 for the nicotinamide ribose diol and 12.77 ± 0.03 for the adenine ribose 2′- and 3′-carbons.

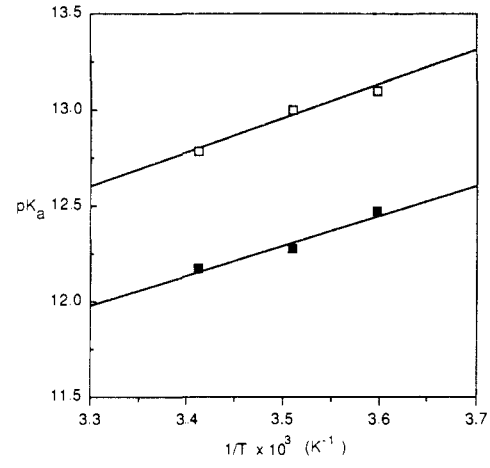



Figure 5. Temperature dependence of the $pK_a$ for the nicotinamide ribose diol (■) and the adenosine ribose diol (□) of $NAD^+$ as determined by $^{13}C$ NMR titration. Extrapolation of the $pK_a$ for the nicotinamide ribose diol up to 37 °C gives a $pK_a$ of 11.9 ± 0.1, consistent with the $pK_{eq}$ obtained at 37 °C from the pH–rate profile.

respectively. The reason for the smaller chemical shift differences for the adenosine ribose remains to be explored.

The $pK_a$ of the ribose diol carbons could not be measured directly at the temperature at which the pH–rate profile data was obtained (37 °C) since the rate of hydrolysis above pH 11 is too fast to permit acquisition of the $^{13}C$ NMR spectra. In order to extrapolate the $pK_a$s of the nicotinamide and adenine ribose diols to 37 °C, their temperature dependence has been determined.[33] Figure 5 shows a plot of the temperature dependence of the diol $pK_a$ obtained from the pH-dependent shifts of the adenosine and nicotinamide ribose 2′-carbons for $NAD^+$ at 5, 12, and 20 °C. From these data the $pK_a$ for the nicotinamide ribose diol of $NAD^+$ is extrapolated to be 11.9 ± 0.1 at 37 °C. This value is in good

(31) $^{13}C$ NMR was used rather than $^1H$ NMR spectroscopy because the 2′- and 3′-protons overlap with the water resonance and thus are not readily observable in aqueous buffers. The nicotinamide anomeric proton is sensitive to both the titration of the nicotinamide ribose diol and to alterations in base stacking that occur when the adenine ribose titrates. This makes an accurate deconvolution of the $pK_a$ for each group difficult. Since the effects from destacking involve alterations in ring current anisotropic shielding, their influence on the $^{13}C$ NMR spectrum is negligible relative to the magnitude of the pH-dependent $^{13}C$ NMR chemical shift changes due to ionization.

(32) Izatt, R. M.; Rytting, J. H.; Hansen, L. D.; Christensen, J. J. *J. Am. Chem. Soc.* **1966**, *88*, 2641–2645.

(33) The temperature dependence of the $pK_a$ is given by $pK_a = (\Delta H_{ion}/2.303R)(1/T) - \Delta S_{ion}/2.303R$, where $\Delta H_{ion}$ and $\Delta S_{ion}$ are the enthalpy and entropy for the ionization, $R$ is the gas constant, and $T$ is the absolute temperature. The linear dependence on $1/T$ allows extrapolation of the $pK_a$ to 37 °C.

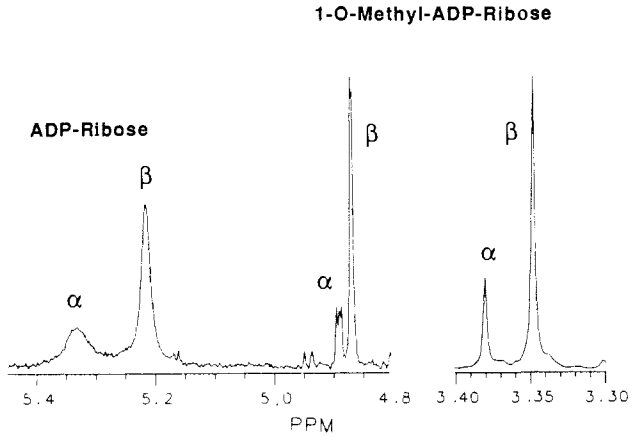



**Figure 6.** Portions of $^1H$ NMR spectra showing the anomeric (4.8–5.4 ppm) and *O*-methyl (3.33–3.39 ppm) resonances for the products isolated from the reaction of $NAD^+$ in 50% methanol–50% water containing 0.2 M CAPS buffer, pH 10.5. The vertical scale for the anomeric proton resonances is 3 times greater than that for the methyl resonances.

agreement with the $pK_{eq}$ value of 11.7 ± 0.1 observed for the hydrolysis reaction at 37 °C, suggesting that diol ionization is responsible for the pH-independent region at high pH.[34]

**Solvent Deuterium Isotope Effects.** Solvent isotope effects have been determined for the hydrolysis of $NAD^+$ in $D_2O$ over the range from pD 5.6 to 13.7. The plot of the log of the rate constants vs pD parallels the results obtained in $H_2O$, as shown in Figure 1. Below pD 7 the reaction is pD independent with a solvent isotope effect of 1.5 ± 0.1. In the range from pD 9.3 to 11.6, the reaction rate has a first-order dependence on deuteroxide concentration and the isotope effect is 2.7 ± 0.1. Above pD 12, where the reaction again becomes independent of pD, the solvent isotope effect is 1.1 ± 0.1. The data for the hydroxide-catalyzed hydrolysis in $D_2O$ were fit by the same method as outlined for the reaction in $H_2O$ and yields a $pK_a$ of 12.1 at 37 °C, consistent with the increase in $pK_a$ expected for deuterium substitution of a weak hydroxy acid.[35] The observed deuteroxide-dependent solvent kinetic isotope effect in the pH-dependent region can therefore be accounted for solely by the increase in the $pK_{eq}$ of the diol brought about by deuterium substitution of the ionizable diol protons.[36]

**Methanolysis versus Hydrolysis.** The solvent dependence of the cleavage of the nicotinamide–glycosyl bond has been investigated for $NAD^+$. Incubation of 4 mM $NAD^+$ in 20 mM CAPS buffer at pH 10.5 in 50% aqueous methanol at 55 °C leads to a first-order decrease in $NAD^+$ concentration with a rate constant comparable to the reaction in the absence of methanol. The $^1H$ NMR spectra of the isolated products reveal a mixture of ADP-ribose and 1-*O*-methyl-ADP-ribose as shown in Figure 6. On the basis of integration of the anomeric proton resonances at 4.8–5.4 ppm, the ratio of hydrolysis to methanolysis (ADP-ribose to 1-*O*-methyl-ADP-ribose) is 2.4:1. This result corresponds to the molar ratio of water to methanol used in the reaction mixture (2.3:1). The ratio of $\beta$ to $\alpha$ 1-*O*-methyl-ADP-ribose can be measured from the same spectrum. Integration of the *O*-methyl resonances at 3.38 and 3.35 ppm yields a $\beta$ to $\alpha$ ratio of 3.7:1.

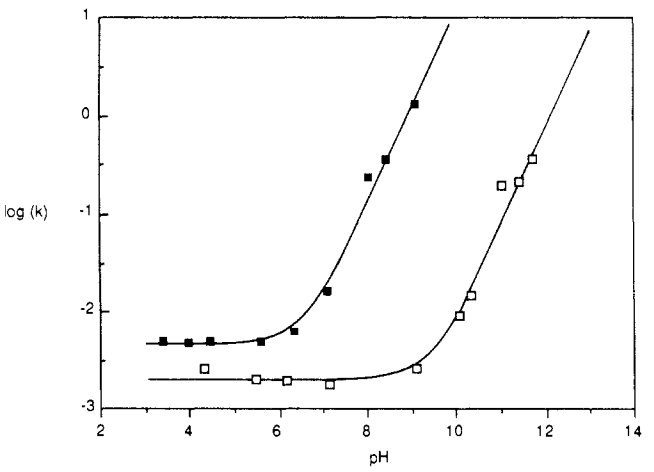



**Figure 7.** pH–rate profiles (low-pH region) for isopro-$NR^+$ (□) and $NAD^+$ (■) at 80 °C. Below pH 7 the product of the reaction of isopro-$NR^+$ is nicotinamide. Above pH 7 the product is 2-HPC. The product of $NAD^+$ hydrolysis is nicotinamide over the entire pH range.

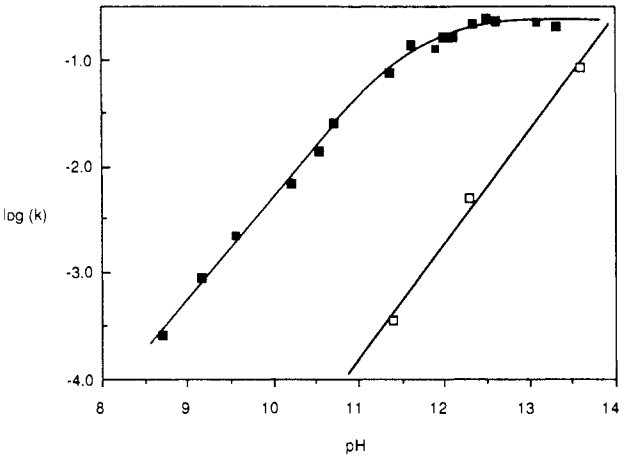



**Figure 8.** pH–rate profiles (high-pH region) for isopro-$NR^+$ (□) and $NAD^+$ (■) at 37 °C. Data for the $NAD^+$ pH–rate profile are taken from Figure 1. The product of the reaction of isopro-$NR^+$ is 2HPC, and the product of the reaction of $NAD^+$ is nicotinamide.

**Hydrolysis of 2′,3′-*O*-Isopropylidene Nicotinamide Riboside.** The role of the ribose diol in the hydrolysis of the nicotinamide–glycosyl bond has been further investigated by examining the base-catalyzed decomposition of 2′,3′-*O*-isopropylidene nicotinamide riboside. The rate of the loss of pyridinium in isopro-$NR^+$ has been determined over the range pH 11.4–13.6 at 37 °C. Figure 7 shows the comparison of isopro-$NR^+$ and $NAD^+$ kinetics over this pH range. The pH–rate profile for $NAD^+$ shows that the reaction becomes pH independent above pH 12 as the nicotinamide ribose diol becomes fully ionized. Furthermore, the reaction of $NAD^+$ over the entire pH range yields only nicotinamide as shown by $^1H$ NMR analysis of the products. In contrast, the rate of reaction of isopro-$NR^+$ has a first-order dependence on hydroxide over the entire pH range, with no indication of any leveling off of the rate. Moreover, the product of the isopro-$NR^+$ reaction is exclusively 2HPC; no nicotinamide is detected.

Experiments to measure the reaction at lower pH values (4.3–11.7) required monitoring of the reaction at higher temperature (80 °C) due to the greater stability of isopro-$NR^+$. The kinetics of hydrolysis of isopro-$NR^+$ relative to $NAD^+$ under these conditions are compared in Figure 8. At pH 4.3, where both reactions are pH independent, the rate of isopro-$NR^+$ hydrolysis is only 4.5 times slower than for $NAD^+$. Analysis of the products for both reactions by $^1H$ NMR shows that nicotinamide is the

(34) The reversible formation of a nicotinamide–hydroxyl adduct can be precluded as an alternative explanation for the pH-dependent changes in chemical shift on a number of grounds: (a) There is no spectrophotometric evidence for significant concentrations of the hydroxyl adduct pseudobase below pH 14, let alone full conversion to the pseudobase by pH 12.5.[9] (b) The pseudobase is either a 1,2- or 1,4-dihydronicotinamide species and thus is a structural analogue to the cyanide adducts. Such species are easily characterized by either $^1H$ [Oppenheimer, N. J.; Arnold, L. J.; Kaplan, N. O. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 3200–3205] or $^{13}C$ [Oppenheimer, N. J., unpublished results] NMR spectroscopy. The range of upfield shifts of the nicotinamide ring resonances upon adduct formation would be 2–4 ppm for protons and 20–100 ppm for $^{13}C$. Such shifts are not observed. (c) The pH–rate profile for isopro-$NAD^+$ does not level off at high pH as does $NAD^+$ over the same pH range; thus, there is no $pK_{eq}$ involving hydroxide below pH 13.5 for this compound although it contains an analogous nicotinamide–glycosyl bond.

(35) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; pp 225–283.

(36) The mechanistic implications of a moderate solvent isotope effect of 1.5 observed for the pH-independent reaction below pH 7 remains to be explored.

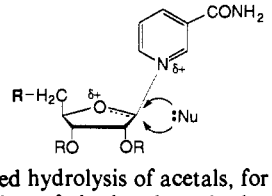
sole product resulting from water-catalyzed hydrolysis of the glycosyl bond. Above pH 7 the reactions of isopro-$NR^+$ and $NAD^+$ both become first order in hydroxide but with significantly different results. The $^1H$ NMR spectra of the products for reactions of isopro-$NR^+$ conducted at pH 8.5 and 12.5 show that only 2-HPC is formed. For $NAD^+$, however, the product of the reaction in the pH-dependent region is nicotinamide. These experiments demonstrate that blocking the hydroxyls of the nicotinamide ribose moiety in isopro-$NR^+$ leads to a complete change in the hydroxide-catalyzed chemistry. Direct attack on the nicotinamide ring with subsequent formation of 2HPC becomes favored above pH 7, and the reaction remains first order in hydroxide up to pH 13.6.

## Discussion

Any proposed mechanism for the alkaline hydrolysis of $NAD^+$ must explain the following results: (i) The pH–rate profile for hydrolysis of $NAD^+$, above pH 8.5, shows a region of first-order dependence on hydroxide concentration. At higher pH the rate of the hydrolysis reaction becomes pH independent. The observed $pK_{eq}$ coincides with the $pK_a$ observed for ionization of the nicotinamide ribose diol. (ii) The ratio of hydrolysis to methanolysis (ADP-ribose to 1′-*O*-methyl-ADP-ribose) corresponds to the molar ratio of water to methanol in the reaction mixture. These results establish the inability of the reaction intermediate to discriminate on the basis of the nucleophilicity of the attacking solvent molecule.[37] (iii) Both $\beta$ and $\alpha$ anomers of 1′-*O*-methyl-ADP-ribose are generated in a ratio of 3.7:1 during solvolysis of $\beta$-$NAD^+$ in 50% methanol. (iv) Isopro-$NR^+$ does not undergo hydroxide-catalyzed *hydrolysis*, and no region of pH independence is observed at high pH. (v) The secondary kinetic deuterium isotope effects measured by Cordes et al. demonstrate the reaction to be dissociative in character with significant rehybridization of the anomeric carbon in the transition state for both the pH-independent and pH-dependent reactions.[12]

The preponderance of evidence presented above is consistent with a mechanism involving the dissociative cleavage of the glycosyl bond. These results would usually be analyzed in terms of either nucleophilic participation in an "exploded" transition state[38] or by anchimeric assistance via a 1,2-anhydro sugar intermediate.[39] However, on the basis of the following analyses, neither of these mechanisms in our view can fully account for the observed data.

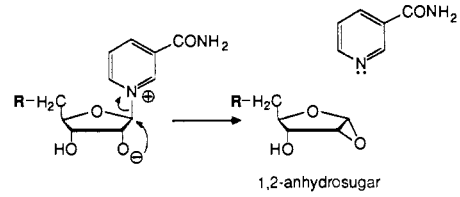
**Exploded Transition State.** The concept of an "exploded" transition state has been presented to explain nucleophilic participation in reactions where secondary kinetic isotope effect data indicate major rehybridization in the transition state.[38]




In the acid-catalyzed hydrolysis of acetals, for example, a nearly complete dissociation of the bond to the leaving group in the transition state is proposed with only a small degree of participation by the ultimate nucleophile. Such transition states explain dependence of a reaction on the exogenous nucleophile whereas product ratios reflect reactions occurring with both retention and inversion of configuration.

The results obtained for the reaction of isopro-$NR^+$ are inconsistent with the nucleophilic participation of hydroxide for the following reasons. Below pH 7, isopro-$NR^+$ is hydrolyzed only 4.5 times slower than for $NAD^+$; thus, derivatization of the ribose diol clearly does not have a major impact on the rate of water-catalyzed hydrolysis. At higher pH, however, no hydroxide-dependent *hydrolysis* of the nicotinamide–ribosyl bond is observed; only the much slower hydroxide addition/elimination reaction due to direct attack of hydroxide on the pyridinium moiety. At pH 10.5 we estimate the rate of isopro-$NR^+$ *hydrolysis* to be $<10^{-3}$ times slower than the rate of $NAD^+$ hydrolysis, i.e. corresponding to the rate of the pH independent water-catalyzed hydrolysis alone.[40] Therefore, invoking nucleophilic participation by water is inconsistent with the lack of catalysis by hydroxide, the stronger nucleophile.

Scheme II




**1,2-Anhydro Sugar Intermediates.** The mechanism generally invoked for hydrolysis of alkaline-labile glycosyl bonds in compounds containing a hydroxyl trans to the aglycon involves formation of a 1,2-anhydro sugar as a transient intermediate (Scheme II).[41] The origins of this mechanism date to the synthesis and characterization of 3,4,6-tri-*O*-acetyl-1,2-anhydro-α-D-glucopyranose (Brigl's anhydride), and such reactions have been used as paradigms of anchimeric assistance.[42]

There are three primary criteria for invoking anchimeric assistance in chemical reactions: (i) observation of a rate acceleration that is correlated to the presence of a neighboring group suitably aligned to participate covalently; (ii) stereochemical outcome of a reaction that is consistent with the expected intervention of the putative intermediate; (iii) chemical properties of an intermediate that are consistent with the overall reaction (e.g., above all else the putative intermediate must be kinetically competent).

Anchimeric assistance via a 1,2-anhydro sugar intermediate has been invoked in the base-catalyzed hydrolysis of pyridine–glycosyl bonds in a number of pyranose and furanose nucleosides, most recently in the hydrolysis of pyridine arabinosides.[43] The questions remain of how appropriate this intermediate is and what its literature precedents are. Although they are extremely labile in acid, 1,2-anhydro sugars are relatively stable in base or toward nucleophilic attack. For example, 1,2-anhydro sugars and related compounds are generally synthesized in a base-catalyzed intramolecular displacement of chloride by an adjacent trans hydroxyl group.[42,44] For small molecules, the resulting 2,3-epoxytetrahydrofurans have even been distilled.[45] Indeed, benzylic epoxy ethers require "gentle heating" and incubation for 24 h in 0.1 M sodium methoxide in anhydrous methanol for methanolysis[46] and Brigl's anhydride[42] as well as other 1,2-anhydropyranoses are crystalline solids.[44]

An extensive search of the literature cited as precedent for anchimeric assistance of alkaline hydrolysis via 1,2-anhydro sugar intermediates has not revealed any examples where either direct evidence for the participation of such intermediates has been reported or, more importantly, where the corresponding anhydro sugar has been tested for kinetic and stereochemical competency. Moreover, in the hydrolysis of the nicotinamide–ribose linkage there are severe mechanistic constraints for direct participation of the diol anion via anchimeric assistance.

(37) Sinnot and Viratelle have demonstrated that methanol is about 100 times more reactive than water toward esters and other carbonyl-type carbons under similar conditions: Sinnott, M. L.; Viratelle, O. M. *Biochem. J.* **1973**, *133*, 81–87.

(38) (a) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161–169. (b) Knier, B. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 6789–6798.

(39) Capon, B. *Chem. Rev.* **1969**, *69*, 407–498.

(40) Based on the detection limit of nicotinamide relative to 2HPC by $^1H$ NMR spectroscopy.

(41) Ballou, C. E. *Adv. Carbohydr. Chem.* **1954**, *9*, 59–94.

(42) Brigl, P. Z. *Z. Physiol. Chem.* **1922**, *122*, 245–262.

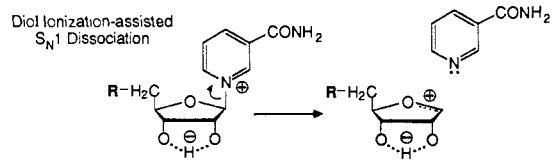
(43) Sinnott, M. J.; Wijesundera, W. S. *Carbohydr. Res.* **1985**, *136*, 357–368.

(44) (a) Sondheimer, S. J.; Yamaguchi, H.; Scheurch, C. *Carbohydr. Res.* **1979**, *74*, 327–332. (b) Wakselman, C.; Wartski, L. *Bull. Chim. Soc. Fr.* **1967**, 2242–2243. (c) Decor, J. P.; Descotes, G. *Bull. Chim. Soc. Fr.* **1970**, 2370–2376.

(45) Birkofer, L.; Dutz, R. *Justus Liebigs Ann. Chem.* **1957**, *608*, 17–21.

(46) (a) Stevens, C. L.; Farkas, S. E. *J. Am. Chem. Soc.* **1952**, *74*, 618–620. (b) Stevens, C. L.; Malik, W.; Pratt, R. *J. Am. Chem. Soc.* **1950**, *72*, 4758–4760.

**Scheme III**




(i) The observed secondary kinetic isotope effect requires an initial, dissociative cleavage of the glycosyl bond, with anchimeric participation of the diol anion only contributing a minor amount of bond formation in the transition state. This raises questions as to how such minor participation (essentially the same degree of participation as observed for water catalyzed hydrolysis) causes the $10^4$-fold acceleration in the rate of the reaction.

(ii) Since 1,2-anhydroribose is not detected by $^1H$ NMR during the reaction, the rate of hydrolysis of the putative intermediate must be at least 3 times faster than cleavage of the glycosyl bond; otherwise, the concentration of the anhydroribose intermediate would build to detectable levels. This requirement is inconsistent with the reported stability of 1,2-anhydro sugars and epoxy ethers under alkaline conditions.

(iii) Finally, the epoxide ring would have to undergo a dissociative opening to re-form an oxo carbocation intermediate. This is required by the intermediate's observed lack of discrimination on the basis of nucleophilicity of the attacking solvent molecule as well as the observed formation of a mixture of anomers as products of methanolysis. From these properties we infer that a 1,2-anhydro sugar intermediate would be stable toward nucleophilic attack but readily undergo dissociative ring opening. This is inconsistent with the $S_N2$ mechanism normally associated with nucleophilic attack on epoxides under alkaline or neutral conditions.[47]

Although none of these factors definitively preclude the intervention of 1,2-anhydro sugars as intermediates in alkaline hydrolysis of glycosyl bonds, they do raise serious doubts regarding their general, axiomatic acceptance. In this study we propose an alternative mechanism that can explain the specific results observed for the alkaline hydrolysis of $NAD^+$. The new mechanism is based on a noncovalent electrostatic stabilization of an oxo carbocationic intermediate by the diol anion.

**Transition-State Stabilization by the Diol Anion.** The key observations that provide the basis for this mechanism are the pH independence of the reaction above pH 12.5 and the correspondence of the $pK_{eq}$ for the hydrolytic reaction to the $pK_a$ of the nicotinamide ribose diol. The results of our experiments identify the ionization of the nicotinamide ribose as the source of hydroxide catalysis. Once the effect of diol ionization on the hydrolytic reaction is taken into account, the relationship between many of the diverse aspects of the alkaline chemistry of $NAD^+$ becomes apparent.

On the basis of results presented herein, we propose that ionization of the nicotinamide ribose diol promotes the dissociative cleavage of the pyridine–ribosyl bond by stabilization of the oxo carbocation intermediate, as shown in Scheme III, without the necessity for covalent participation. This mechanism provides for the dissociative cleavage of the glycosyl bond consistent with the secondary kinetic isotope effect data, the presence of an oxo carbocation intermediate which accounts for the methanolysis results, and diol ionization which accounts for the pH–rate profile and solvent isotope effects.

The magnitude of the ionization-dependent stabilization needed to explain the observed rate increase can be readily estimated. On the basis of the temperature dependence of the hydrolytic reaction at pH 6.2 and 13.4, we have calculated a $\delta\Delta G^{\ddagger}$ value of $5.2 \pm 1.6$ kcal/mol. This value represents the stabilization energy provided to the cationic transition state by ionization of the diol relative to the un-ionized diol. It compares favorably to the $\delta\Delta G$ value of 6.4 kcal/mol calculated from the data of Fox and Jencks[48] for the stabilization of a cationic intermediate by an alkoxide anion two bonds away.

Further insight into the mechanism of hydrolysis is provided by the hydroxide-catalyzed reactions of 2′,3′-*O*-isopropylidene nicotinamide riboside. The isopropylidene group in this compound imposes a rigid geometry on the furanose ring and prevents any ionization involving the sugar moiety below pH 16. Schuber et al.[49] have suggested that the stability of the glycosyl bond in $NAD^+$ should be sensitive to the geometry of the lone pairs on the furanose ring oxygen relative to the nicotinamide–glycosyl bond. Clearly, below pH 7 the rigid geometry in isopro-$NR^+$ decreases the rate of hydrolysis by less than a factor of 5 relative to the conformationally mobile nicotinamide riboside and ribotides. Therefore, the rigid geometry provides only modest interference to formation of the oxocarbocation intermediate.[50] The stability of isopro-$NR^+$ toward base-catalyzed *hydrolysis* must therefore be attributable to factors other than steric constraints, namely its inability to generate an anionic form of the sugar.

The consequences of preventing diol ionization are clear. Although a hydroxide-catalyzed reaction of isopro-$NR^+$ is observed above pH 8, it occurs under more vigorous conditions than for either $NAD^+$ or $NR^+$. Furthermore, the hydroxide-catalyzed reaction of isopro-$NR^+$ involves an associative mechanism wherein hydroxide ion adds to the pyridine ring followed by elimination and cyclization reactions to yield 2HPC as the only product; no hydroxide-catalyzed release of nicotinamide is observed. Therefore, in the absence of diol ionization the nicotinamide–glycosyl bond is highly resistant to hydroxide-catalyzed dissociative hydrolysis.

The results of our experiments with pyridine nucleotides demonstrate that ionization of the ribose diol leads to a $10^4$-fold increase in the rate of hydrolysis. This rate enhancement can be best explained through a noncovalent stabilization of the oxo carbocation intermediate by the diol anion.[51] Such stabilization by the diol anion represents a new mechanism for hydroxide catalysis of the cleavage of glycosyl bonds, and its applicability should not be limited to just the pyridine nucleotides. Given the observation that the cleavage of a glycosyl bond is generally dissociative in character, this new mechanism could have far-ranging implications regarding the mechanisms of both related chemical and enzymatic reactions.

**Acknowledgment.** We thank Drs. E. H. Cordes, W. P. Jencks, and F. Schuber for helpful discussions. This research was in part supported by National Institutes of Health Grant GM-22982. We also acknowledge support from grants to the University of California, San Francisco, Nuclear Magnetic Resonance Laboratory (National Institutes of Health Grant RR01668 and National Science Foundation Grant DMB 8406826).

**Registry No.** β-NAD, 53-84-9; NMN, 1094-61-7; $NR^+$, 1341-23-7; isopro-$NR^+$, 14265-48-6.

(47) Rao, A. S.; Paknikar, S. K.; Kirtane, J. G. *Tetrahedron* **1983**, *39*, 2323–2367.

(48) Fox, J. P.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 1436–1449.

(49) Schuber, F.; Travo, P.; Pascal, M. *Bioorg. Chem.* **1979**, *8*, 83–90.

(50) Note only nicotinamide is released in the pH-independent reaction.

(51) The electrostatic stabilization mechanism can explain other results as well. In preliminary investigations we find the glycosyl bond of α-$NAD^+$ to be more resistant to alkaline hydrolysis than β-$NAD^+$ (unpublished data). This result would be explained by the anchimeric assistance mechanism as the inability of the cis 2′-hydroxyl to participate in displacement. Our mechanism predicts that ionization of the diol in the α anomer, which occurs in close proximity to the cationic pyridinium, would electrostatically stabilize the ground state relative to the more charge-separated oxo carbocation intermediate. This should increase the energy barrier to the transition state and decrease the leaving ability of the nicotinamide. In the trans conformation there would be little electrostatic stabilization of the ground state. We note that α-$NAD^+$ [Kaplan, N. O. *Enzymes* **1960**, *3B*, 105–169] and the β-nicotinamide arabinotide analogue [Kam, B. L.; Malver, O.; Marschner, T. M.; Oppenheimer, N. J. *Biochemistry* **1987**, *26*, 3453–3461], where the pyridinium moieties are cis to the 2′-hydroxyl, have redox potentials 20 mV more negative than for the corresponding coenzymes with a trans configuration, consistent with the ability, in these cases, of the neutral hydroxyl to stabilize the adjacent pyridinium.