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Acid-Catalyzed Hydration of Reduced Nicotinamide Adenine Dinucleotide and Its Analogues†

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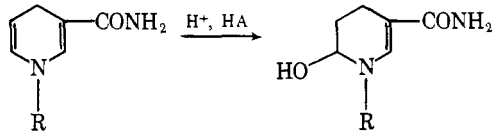
ABSTRACT: The rate of the primary acid modification reaction of 1,4-dihydronicotinamide adenine dinucleotide (NADH) and 1,4-dihydro-3-acetylpyridine adenine dinucleotide (APADH) and their analogues has been studied over a wide pH range (pH 1–7) with a variety of general acid catalysts. The rate depends on $[H^+]$ at moderate pH and becomes independent of $[H^+]$ at low pH. This behavior is attributed to substrate protonation at the carbonyl group (pK of NADH = 0.6). The reaction is general acid catalyzed; large solvent deuterium isotope effects are observed for the general acid and lyonium ion terms. Most buffers cause a linear rate increase with increasing buffer concentration, but certain buffers cause a hyperbolic rate increase. The nonlinear buffer effects are due to complexation of the buffer with the substrate, rather than to a change in rate-limiting step. The rate-limiting step is a proton transfer from the general acid species to the C_5 position of the substrate. Anomerization is not a necessary first step in the case of the primary acid modification reaction of β-NADH, in which β to α anomerization takes place.

NADH[1] and its analogues are unstable in acid solutions or even in buffered neutral solutions because they undergo a general acid catalyzed hydration reaction, according to eq 1,

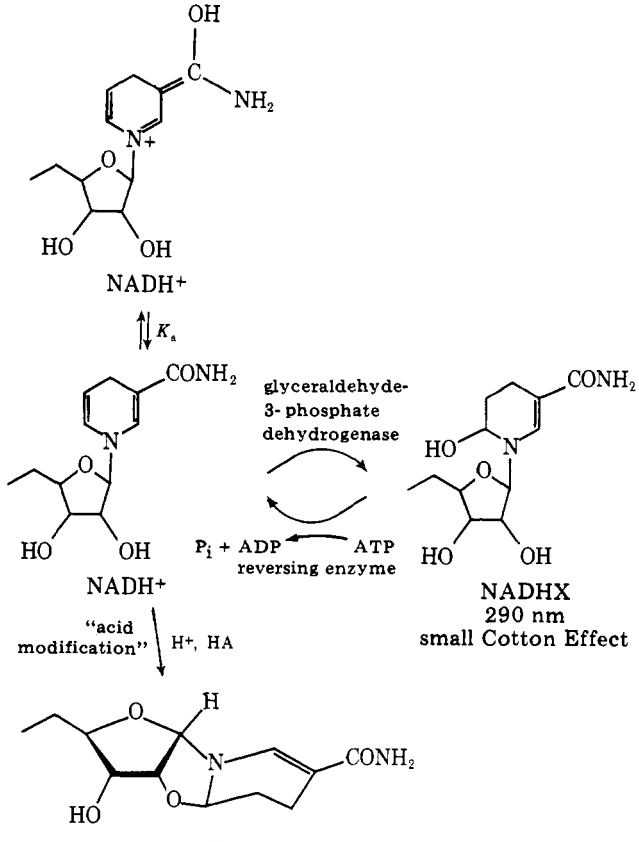


(1)

I

to form the primary acid product, **I**, which is characterized by an absorption band at 290–300 nm. Secondary reactions destroy **I** (Kim and Chaykin, 1968; Stock et al., 1961; Johnston et al., 1963; Alivisatos et al., 1965; Burton and Kaplan, 1963). In the case of the ribose-containing NADH, the primary acid product results from a hydration step, an anomerization at the ribosyl–dihydronicotinamide bond (Miles et al., 1968), and a cyclization reaction to form **II** as shown in Scheme I (Oppenheimer and Kaplan, 1974a). Both α- and β-NADH form **II**, which is characterized by a large positive Cotton effect at 290 nm. NADH also undergoes a hydration reaction catalyzed by glyceraldehyde-phosphate dehydrogenase to form NADHX (Rafter et al., 1954; Chaykin et al., 1956, Hilvers et al., 1966), which is the hydrated form of NADH which has not undergone anomerization at the ribosyl–dihydronicotinamide bond (Oppenheimer and Kaplan, 1974b; Oppenheimer, 1973). NADHX and **II** have very similar spectroscopic properties and

Scheme I



are associated with various enzyme systems as products, inhibitors, or by-products (Huennekens et al., 1955; Pfleiderer and Stock, 1962; Weiland et al., 1960; Gelderman and Pea-

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[1] Abbreviations used are: NADH, dihydronicotinamide adenine dinucleotide; NMNH, dihydronicotinamide mononucleotide; BzNH, 1-benzyl-1,4-dihydronicotinamide; PrNH, 1-propyl-1,4-dihydronicotinamide; APADH, 1,4-dihydro-3-acetylpyridine adenine dinucleotide; BzAPH, 1-benzyl-1,4-dihydro-3-acetylpyridine; MeAPH, 1-methyl-1,4-dihydro-3-acetylpyridine; Pic, 4-picoline; Py, pyridine; Im, imidazole; P_i, inorganic phosphate; PP_i, pyrophosphate; AcOH, acetic acid; ClAcOH, chloroacetic acid; PhP_i, phenyl phosphate.

cock, 1965; Millar et al., 1971). NADHX, but not **II**, is convertible to NADH by the "reversing enzyme" in a reaction which requires ATP (Meinhart et al., 1956), as shown in Scheme I. NADHX can also be formed nonenzymatically in neutral phosphate buffers (Pfleiderer and Stock, 1962), and is converted to **II** by acids (Chaykin et al., 1956).

In order to more fully understand the mechanism of hydration of NADH, we undertook a thorough study of nonlinear buffer effects, general acid catalysis, substituent effects, and solvent isotope effects.

Experimental Section

Materials. β-NADH, α-NADH, APADH, and NMNH were purchased from Sigma Chemical Co. BzNH, PrNH, MeAPH, and BzAPH were prepared by the reduction of the corresponding pyridinium salt with sodium hydrosulfite, according to the procedure of Anderson and Berkelhammer (1958). BzNH was obtained as yellow needles, mp 112–114 °C, after recrystallization from ethanol–water; PrNH was obtained as yellow needles, mp 88–90 °C, after recrystallization from diethyl ether with 1–2% acetone; and BzAPH was obtained as yellow plates, mp 64–67 °C, after recrystallization from pentane. Attempts to recrystallize MeAPH were unsuccessful. The pyridinium salts obtained by reaction of the alkyl halide and nicotinamide or 3-acetylpyridine in anhydrous methanol were all recrystallized prior to reduction to the dihydropyridines. NADHX was prepared according to the method of Krebs (1963).

Reagent grade inorganic salts were used without further purification. Imidazole was recrystallized from benzene, and 4-picoline was redistilled before use. D_2O (99.7%) was obtained from Sigma and DCl was obtained as a 38% solution in D_2O (99%) from International Chemical and Nuclear Corp. Acetaldehyde was obtained from Aldrich, and yeast alcohol dehydrogenase was obtained from Sigma.

Stock buffers were prepared with ionic strength of 1.0 M. Ionic strengths of dilute buffers were maintained at 1.0 M by diluting stock buffers with 1.0 M KCl. Stock solutions ($3–5 \times 10^{-3}$ M) of the dihydropyridines were prepared immediately before use for the kinetic determinations. Solutions in water were made except for BzNH and BzAPH which were dissolved in 5–10% ethanol in water.

Kinetic Studies. Reaction rates were measured by following the decrease in absorbance of the dihydropyridine at 340 or 360 nm, or by following the increase in absorbance due to the acid modification product at 290 or 300 nm. A Beckman DU-2 or a Cary 16 spectrophotometer equipped with a cell compartment thermostated at 25.0 °C was used for these measurements. For rapid reactions ($t_{1/2} < 10$ s), the rate measurements were carried out using a Durrum stopped-flow spectrophotometer. After completion of each kinetic determination, the pH of each of the reaction mixtures was measured with a Radiometer, Type TTT-1d, pH meter coupled with a PHA-630 scale expander. Low-pH studies were carried out in HCl or DCl solutions. For solutions in D_2O, pD was calculated by adding 0.41 to the pH meter reading (Fife and Bruice, 1961). Values of h_0 and d_0 were obtained from Paul and Long (1957) and Högfeldt and Biegeleisen (1960). The water reaction was measured directly in water made slightly alkaline (pH 9–12) with NaOH.

Reaction rates were also measured by following the change in the circular dichroic (CD) spectra of β-NADH with a Cary 60 spectropolarimeter. The disappearance of β-NADH was followed by observing the decrease of the negative Cotton effect at 260 nm; the appearance of the primary acid product was followed by observing the increase in the positive Cotton effect at 285 nm.

Pseudo-first-order rate constants were calculated as $k_{obsd} = 0.693/t_{1/2}$. The half-lives were directly read from semilog plots of $(A_t - A_\infty)$ or $(A_\infty - A_t)$ vs. time, where A_t and A_∞ are the absorbance readings at time t and at infinite time (ten half-lives), respectively. The rate constants for the very slow reactions were calculated from $(dA/dt)/A$.

For a series of buffers at constant pH and constant ionic strength, the observed rate constants were plotted against the concentration of the acid buffer component. The slope and intercept were obtained by the method of least-squares programmed on an Olivetti-P602 microcomputer. The slope is taken as the general acid rate constant, k_{HA}, and the intercept gives a buffer-independent rate constant, k_0, which itself is a pH-dependent term and is equal to the sum of the water and hydronium ion terms, $k_w + k_H[H^+]$.

pK_s and pK_s' Determinations. The pK_s values of the dihydronicotinamides were obtained as slope/intercept from plots of $1/k_{obsd}$ vs. $1/[H^+]$, where $[H^+]$ is derived from pH (pD) or h_0 (d_0).

The pK_s' values of the primary acid modification products were determined from the variation of the absorbance of 290 or 300 nm with pH. A stock solution of the primary acid product was prepared by allowing the dihydropyridine to react in 0.1 M HCl and adjusting the pH to 7–8 with NaOH at the completion of the reaction. Aliquots of the stock solution were added to buffer solutions at several pH values, and the initial absorbance was determined by extrapolating absorbance readings to zero time. The pK_s' values were calculated from eq 2

$$pK_s' = pH + \log\left[\frac{A_i - A}{A - A_u}\right] \quad (2)$$

where A_i, A_u, and A refer to the absorbance of the protonated, unprotonated, and observed form, respectively.

Enzymatic Assay for β-NADH. The concentration of β-NADH in the course of the hydration reaction was determined by assaying with yeast alcohol dehydrogenase, which shows greater than a 1000-fold specificity for β-NADH as compared to α-NADH (Oppenheimer and Kaplan, 1975). The decrease in absorbance, after enzyme and acetaldehyde were added, gives the fraction of total NADH which is β-NADH.

Determination of Complexation Constants, K_c. The values of K_c were obtained from a double-reciprocal plot of k_{obsd}^{-1} vs. $(buffer)_{total}^{-1}$, as the intercept divided by the slope.

Results

General Acid Catalysis and pH Dependence. The rate of the acid-catalyzed disappearance of NADH and its analogues as a function of $[H^+]$ at zero buffer concentration is linearly dependent on $[H^+]$ at moderately low pH, and becomes independent of pH at very low pH. Figure 1 shows an example of this behavior. At high pH (9 and 12), the rate again becomes pH independent. Plots of $\log k_{obsd} - \log(h_0 + K_s)$ vs. a_{H_2O} for all the substrates in acid solution yield slopes (w values) of zero (Bunnett, 1961). The buffer catalysis studied at several pH values demonstrates only general acid catalysis. Equation 3 describes the pH and buffer dependence of the pseudo-first-order rate constant, k_{obsd},

$$k_{obsd} = \frac{k_w + k_H[H^+] + k_{HA}[HA]}{1 + [H^+]/K_s} \quad (3)$$

where K_s is the acid-dissociation constant of the substrate, and k_w, k_H, and k_{HA} are the rate coefficients for the uncatalyzed,

TABLE I: Summary of Kinetic Parameters for the Primary Acid Modication Reaction of NADH and its Analogues.[a]

Substrate	α[b]	pK_s[c]	pK_s'[d]	$10^3 k_{HA}$, M^{-1} s^{-1}														
				H^+ (pK −1.74)	H_2O (pK 15.74)	ClAcOH (pK 2.85)	HCO_2H (pK 3.79)	Lactic Acid (pK 3.86)	AcOH (pK 4.73)	PyH^+ (pK 5.23)	Citric Acid (pK 5.4)	$PiCH^+$ (pK 6.02)	Maleic Acid (pK 6.23)	PP_i (pK 6.5)	HSO_3^-[e] (pK 6.7)	ImH^+ (pK 7.0)	P_i (pK 7.12)	PhP_i (pK 5.65)
β-NADH	0.49	0.62	0.8	2.02×10^3	3.27×10^{-6}		5.38	0.53	1.22	0.23	0.58		0.070	0.15	0.015		0.192	
	(0.46)	(1.2)																
α-NADH	0.39			1.34×10^3			4.13		0.62				0.075	1.6			1.50	
	(0.37)																	
NMNH	0.54	0.72	0.8	1.90×10^3	1.2×10^{-6}		16.6		1.14								0.410	
	(0.43)																	
BzNH	0.54	0.96	2.6	3.35×10^4	1.52×10^{-5}		258		37.6	17.3		4.95				0.283	8.22	79.6
	(0.42)	(1.54)																
PrNH	0.54	1.14	1.3	9.62×10^4	3.87×10^{-5}		988		118	76.0	10.5	13.5		52	48	0.99	40.2	
	(0.39)	(1.50)																
APADH	0.42	0.11		97.6	3.64×10^{-6}	0.997	0.194	0.177	0.029									
BzAPH	0.47	1.13		9.25×10^2	6.0×10^{-6}	25.4	5.82		1.14						3.2		0.313	
	(0.40)																	
MeAPH	0.40	1.75		2.84×10^3		42	12.6		2.22			1.50					1.33	
	(0.40)																	

[a] Conditions: 25 °C, μ = 1.0, water. [b] Values calculated from all the rate data. Values in parentheses calculated from H_3O^+, P_i, AcOH, and HCO_2H data. [c] Values in parenthesis were determined in heavy water. [d] pK_s' values could not be determined for acetylpyridine derivatives because there is no significant variation of absorbance with pH. [e] Values determined by Tuazon and Johnson (1976).

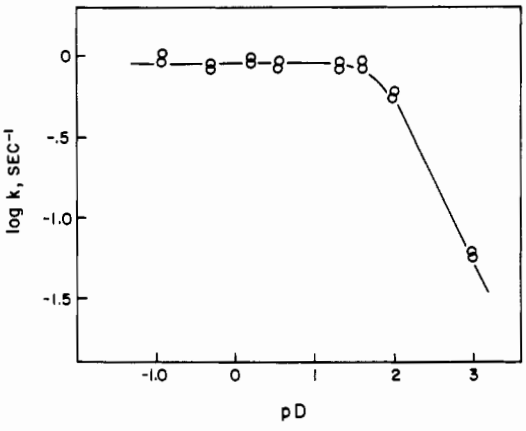


FIGURE 1: pD–rate profile for the primary acid reaction of BzNH at 25 °C and μ = 1.0 M. The solid line was calculated using eq 3.

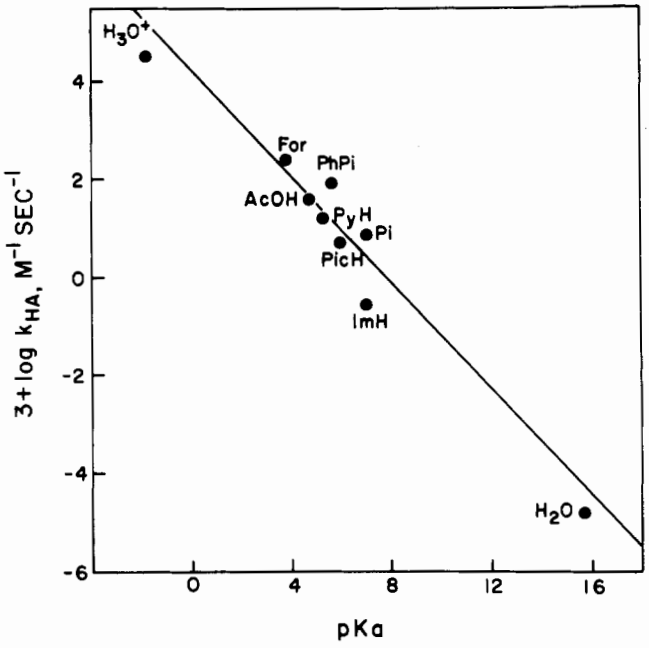


FIGURE 2: Brønsted plot for the primary acid modification reaction of BzNH at 25 °C and μ = 1.0 M.

H^+ catalyzed, and general acid catalyzed reactions, respectively. Table I gives a summary of the parameters in eq 3. Figure 2 is a typical Bronsted plot. Solvent deuterium isotope effects for the various terms are given in Table II.

The values for the rate constants at 25 °C, μ = 1, given in Table I for PrNH reaction with AcOH, P_i, PyH^+, $PicH^+$, H_3O^+, and for the NADH reaction with P_i and H^+, are similar to the rate constant values at 25 °C, μ = 0.48, reported by Johnston et al. (1963). Our values for k_w are 55- and 12-fold lower for NADH and PrNH, respectively, than the values given by Johnston et al. (1963). This difference is because the previous workers extrapolated k_w at H^+ = 0 from a replot of the intercepts at zero buffer concentration vs. $[H^+]$. In contrast, we measured k_w directly at pH 9 and 12, both of which gave identical rate constants. Our values for k_w, therefore, are more accurate. The rate constant for the HSO_3^- reaction with PrNH is threefold lower than the value given by Johnston et al. (1963). This is because we found that the term first-order in $[HSO_3^-]$ is linear in $[H^+]$, and a more detailed treatment of the $[HSO_3^-]$ data was needed than that given previously. The bisulfite reaction of dihydropyridines is the subject of another paper (Tuazon and Johnson, 1977).

TABLE II: Solvent Isotope Effects, k_{HA}/k_{DA}.

	PrNH	BzNH	NADH
L_3O^+	2.52 ± 0.16	3.11 ± 0.20	3.55 ± 0.30
HCO_2L	7.0 ± 1.8	7.4 ± 2.0	4.91 ± 0.72
CH_3CO_2L	5.88 ± 0.30	5.53 ± 0.34	
PyL^+		5.00 ± 0.24	
L_2O	1.63 ± 0.04	1.92	1.64

Salt and solvent effects on k_H were measured. Increasing the ionic strength from 0 to 1.0 does not affect the value of k_H for NADH and increases the k_H value 2.0- and 1.5-fold for BzNH and PrNH, respectively. The k_H value for BzNH is not changed in 10% ethanol.

Nonlinear Buffers. Certain buffers give rise to nonlinear buffer catalysis, a phenomenon first noticed by Johnston et al. (1963) who found negative deviations from linearity with pyridine and imidazole buffers and positive deviations with bisulfite buffers. The linear parts of these bimolecular buffer terms are included in Table I as k_{HA}. We find, in addition, that maleic acid gives negative deviations from linearity. Pyridine buffers were studied in detail: Figure 3 shows a typical result. The plateau levels are hyperbolic with respect to $[H^+]$.

The leveling off of rate at high buffer concentrations could be due to self-association of buffer components, formation of a buffer–substrate complex which is unreactive or less reactive, specific salt effects, or to a change in the rate-determining step. Salt and solvent effects are not a major cause because of the insignificant effect 10% added ethanol has on k_{HA} for the acetic acid reaction with BzNH. A change from $\mu = 0$ to 1.0 has no effect for the acetic acid reaction with PrNH. A change in rate-determining step seems unlikely, in view of the fact that for the same substrate at the same pH some buffers are linear, whereas others are hyperbolic and level off at rates which are well below the rates on the linear portion for linear buffers. Furthermore, if the hydration of NADH is similar to the hydration of enamines, then the linear portion of the curve represents the general acid catalyzed proton-transfer step, and the plateau values represent the hydration step. The plateau values would therefore be expected to be linearly dependent on $[H^+]$ and to have an inverse isotope effect (Guthrie and Jordan, 1972). These phenomena are not observed.[2]

Hydrophobic pyridine buffer self-association has been proposed to account for the lesser reactivity of pyridines at higher concentrations at 38 °C. The negative deviations follow the order: 2,4,6-collidine > 2,6-lutidine > 4-picoline > pyridine (Kirby and Jencks, 1965; DiSabato and Jencks, 1961). The curvature for pyridine at 39 °C is much less marked than that observed here at 25 °C as shown in Figure 3. Because hydrophobic bonding is entropy controlled (Nemathy and Scheraga, 1962), less self-association would be expected at the lower temperature used in this work. In studies reported using conditions very similar or identical to those here (25 °C and 1 M ionic strength), no deviations from linearity are observed in 2,6-lutidine buffers up to 0.25 M or in pyridine buffers up to 2 M, for general base catalyzed reactions (Johnson and Rumon, 1965; Covitz and Westheimer, 1963). Similarly, no deviation from linearity is observed in 1.5 M imidazole buffers for a general base catalyzed reaction (Kirsch and Jencks, 1964). In conclusion, the magnitude and shape of the negative deviations from linearity as in Figure 3 cannot be accounted for by hydrophobic buffer self-association.

[2] At pH 5.7 and pD 6.0, the plateau levels for the pyridinium ion catalysis of PrNH hydration show a solvent isotope effect of 2.0 assuming lyonium ion dependence at this pH (pD).

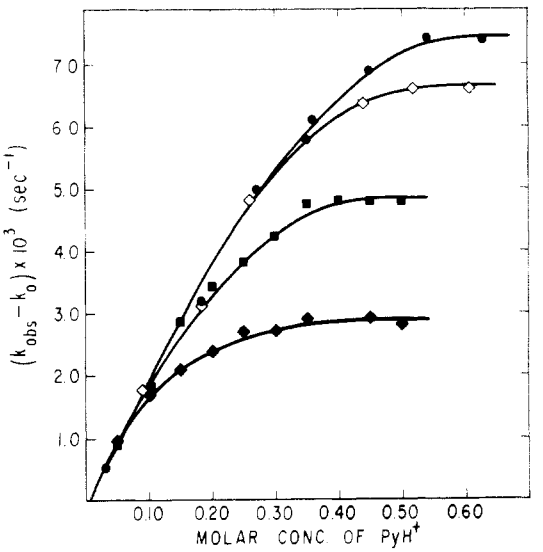


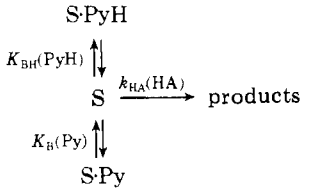
FIGURE 3: Plots of $k_{obsd} - k_0$ against the concentration of PyH^+ for the hydration of BzNH in pyridine buffers at 25 °C and = 1.0 M. (●) pH 4.15, (◇) pH 4.40; (■) pH 5.40, (◆) pH 5.70.

TABLE III: Rate and Complexation Constants in Nitrogeneous Buffers at 25 °C, M = 1.0 M.

	Pyridine		4-Picoline		Imidazole	
Substrate	K_{BH} (M^{-1})	K_B (M^{-1})	K_{BH} (M^{-1})	K_B (M^{-1})	K_{BH} (M^{-1})	K_B (M^{-1})
PrNH	3.2	2.0	2.3	2.0	0.9	1.6
BzNH	2.5	1.3				

The pyridine buffer data can be accounted for by complexation of the substrate (S) with both the basic form (B) and the acidic form (BH) of the buffer, as shown in Scheme II,

Scheme II



where K_B and K_{BH} are the complexation constants for pyridine, Py, and pyridinium ion, PyH, respectively. The observed rate constant for Scheme II is given by eq 4,

$$k_{obsd} = k_0 + \frac{k_{HA}[HA]}{1 + [PyH](K_B K_a/[H^+] + K_{BH})} \quad (4)$$

where k_0 is the rate constant for the solvent terms (hydronium ion plus water) and K_a is the acid dissociation constant for pyridine. In the pyridine buffers (where HA is PyH), the plateau rate constants, $k_{plateau}$, obtained when the second term in the denominator is large compared to 1, is given by eq 5.

$$k_{plateau} = \frac{k_{HA}}{K_B K_a/[H^+] + K_{BH}} \quad (5)$$

Equation 5 predicts hyperbolic behavior with respect to $[H^+]$, as was observed. Table III gives the values of the complexation

TABLE IV: Primary Acid Modification Reaction of PrNH in Mixed Buffers at 25 °C, M = 1.0 M.

Buffer Composition		pH	k_{obsd} (s^{-1})	k_{calcd}[a] (s^{-1})	k_{obsd}/k_{calcd}	K_c[b] (M^{-1})
M_{ImH^+}/M_{Im}	$M_{H_2PO_4^-}/M_{HPO_4^{2-}}$					
0.81/0.09		6.20	0.00108	0.00108	1.00	
	0.05–0.015 M	6.16	0.00235	0.00309	0.76	0.8
	0.033–0.010 M	6.17	0.00190	0.00241	0.79	
	0.017–0.005 M	6.16	0.00147	0.00176	0.84	
0.72/0.08		6.20	0.00102	0.00102	1.00	
	0.05–0.015 M	6.20	0.00226	0.00303	0.75	0.7
	0.033–0.010 M	6.16	0.00187	0.00235	0.80	
	0.017–0.005 M	6.19	0.00144	0.00170	0.85	
0.27/0.03		6.25	0.000608	0.000608	1.00	
	0.05–0.015 M	6.26	0.00218	0.00262	0.83	1.0
	0.033–0.010 M	6.26	0.00165	0.00193	0.86	
	0.017–0.005 M	6.28	0.00117	0.00129	0.91	
0.81/0.09	No phosphate, 7 dilutions of imidazole buffer	6.20				0.9

[a] $k_{calcd} = k_{obsd}$ in imidazole buffer + $k_{H_2PO_4} - x[H_2PO_4^-]$. [b] K_c calculated according to eq 11.

constants obtained by an analysis of several systems according to eq 5.

That the substrate forms an unreactive complex with the buffer is shown by studies of mixed imidazole–phosphate buffers. Phosphate is a less effective buffer catalyst at plateau levels of imidazole than at lower levels of imidazole, or in its absence as shown in Table IV. This indicates that the substrate is less available for the phosphate-catalyzed reaction. In this case, the interaction of buffer components is ruled out because the admixture of imidazole and phosphate buffers of the same pH does not give rise to a pH shift within 0.01 pH units. K_c is given in terms of the total buffer concentration, and is defined by eq 6.

$$K_c = \frac{[S \cdot BH] + [S \cdot B]}{[S]([B] + [BH])} = \frac{K_B[H^+] + K_B K_a}{[H^+] + K_a} \quad (6)$$

The inhibition by the complexing agent imidazole of catalysis by the linear phosphate buffer is treated as follows: the kinetic expression for mixtures of hyperbolic buffers B/BH and linear buffers HZ according to Scheme II is given by eq 7

$$k_{obsd} = k_0 + \frac{k_{HB}[HB] + k_{HZ}[HZ]}{1 + [HB]K_c/(1 + x)} \quad (7)$$

where x is the buffer ratio B/BH of the hyperbolic buffer. If the two buffer components HB and HZ had acted independently, eq 8 would describe the kinetic behavior.

$$k_{calcd} = k_0 + \frac{k_{HB}[HB]}{1 + [HB]K_c/(1 + x)} + k_{HZ}[HZ] \quad (8)$$

The difference between eq 8 and 7 gives the degree of inhibition, Δ, of linear buffer catalysis by complexing buffers.

$$\Delta = k_{calcd} - k_{obsd} = \frac{k_{HZ}K_c[HB][HZ]}{1 + x + K_c[HB]} \quad (9)$$

The degree of inhibition is a linear function of the concentration of the linear buffer and is hyperbolic in HB. Treatment of the mixed imidazole–phosphate buffer data by eq 9 yields values of K_c, as shown in Table IV, which are consistent with the values of K_c obtained with the imidazole buffer alone (Table III).

Products. The product of PrNH with 1 M and 0.1 M HCl is identical to the product from 1 M and 0.1 M acetate buffers, pH 4.5, in its UV spectrum, thin-layer silica gel chromatographic characteristics using a number of solvents (9:1, 3:2, and 1:1 chloroform–methanol, 2% aqueous acetone, and methanol) and in elution from a Bio-Rad AG1-X2 anionic and Dowex AG 50W-X8 cationic-exchange columns. The rates of the secondary reaction of the acetate and HCl products are identical under identical conditions, including 0.1, 0.5, and 1 M HCl, acetate, and formate buffers.

Nonreversibility of Primary Acid Modification Reaction. The primary acid modification reaction cannot be reversed in aqueous or organic solvents or by pH alteration to neutral or alkaline values. For example, no increase in absorbance at 340 nm or decrease in absorbance at 290 nm was observed when the primary acid product of BzNH or NADH was placed in dioxane, acetontrile, ethanol, or water solutions at pH 8 or 12. This is in contrast to the results of Kim and Chaykin (1968).

Anomerization of NADH and NADHX. α- and β-NADH undergo the primary acid modification reaction at the same rates in most buffers, except in phosphate and pyrophosphate buffers, where the rate coefficient of the α isomer is about nine times greater than that of the β isomer (Table I). For β-NADH, in 1.0 M acetic acid buffer, pH 4.0, the observed rate as measured from the increase in the positive Cotton effect at 290 nm is identical to that observed by UV absorption spectrophotometry. Enzymatic analysis with yeast alcohol dehydrogenase for the unreacted NADH in the course of the hydration reaction shows that 95% of the remaining absorbance at 340 nm is β-NADH. It is interesting to note that during the hydration reaction, a strong inhibitor of the enzyme is formed, as evidenced by the slower initial rates. Control experiments with the primary acid product or with α-NADH show that the observed inhibition cannot be fully accounted for by these compounds. Fawcett et al. (1961) observed a material in moist NADH which exhibits inhibitory properties toward some dehydrogenases.

In concentrated phosphate buffer, 3.0 M, pH 7.0, the reaction product exhibits a negative Cotton effect at 260 nm in addition to the positive Cotton effect at 290 nm exhibited by the primary acid product formed in 0.1 M HCl, acetate buffer, pH 4.0, and dilute phosphate buffer, pH 6.4. A mixture of the primary acid product and NADHX forms in concentrated phosphate buffers, since NADHX formed enzymatically, exhibits a negative Cotton effect at 260 nm. NADHX formed

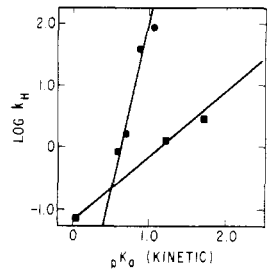
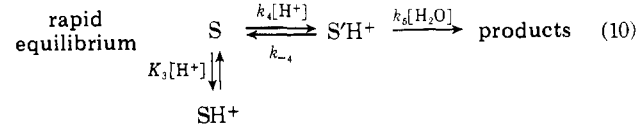


FIGURE 4: Plot of log k_H vs. pK_s for the N-substituted 1,4-dihydronicotinamides (●); and N-substituted 3-acetyl-1,4-dihydropyridines (■).

in the presence of the enzyme or in concentrated phosphate solutions is converted to the primary acid product, as indicated by the increase in the positive Cotton effect at 290 nm and a decrease in the negative Cotton effect at 260 nm with time. In a set of five dilute HCl and phosphate buffers, ranging in pH from 2.7 to 3.8, k_H is 3.0 M^{-1} s^{-1}. In two series of acetate buffers, pH 4.0 and 4.5, 0.01 to 0.50 M in acetic acid, a dependence of k_{obsd} on buffer concentration exists, $k_{HA} = 1 \times 10^{-3}$ M^{-1} s^{-1}. However, acetate buffers are anomalous due to the larger than expected intercepts on the basis of the observed value of k_H.

Discussion

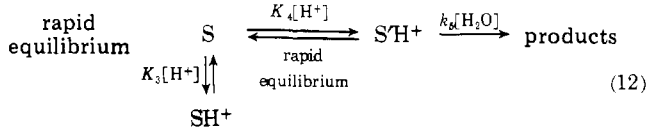
The pH-rate profiles for the primary acid modification reaction, as shown by Figure 1, indicate that equilibrium protonation changes the substrate to an unreactive form. Enamines can be protonated either on nitrogen or on carbon. The carbon site is more basic than the nitrogen site by one or more pK units; nevertheless, the carbon site undergoes protonation much more slowly than the nitrogen site (Sollenberger and Martin, 1970; Elguero et al., 1965). These pH-rate profiles can be caused by two situations: equilibrium protonation of the substrate at only the more rapidly protonated site, to form unreactive SH^+ according to eq 10 and 11



(10)

$$k_{obsd} = \frac{\left(\frac{k_4 k_5}{k_{-4} + k_5}\right) K_3[H^+]}{K_3 + [H^+]} \qquad (11)$$

or equilibrium protonation of both sites to form SH^+ and $S'H^+$ with $S'H^+$ entering into the reaction according to eq 12 and 13.



(12)

$$k_{obsd} = \frac{k_5 \left(\frac{K_3 K_4}{K_3 + K_4}\right)[H^+]}{\left(\frac{K_3 K_4}{K_3 + K_4}\right) + (H^+)} \qquad (13)$$

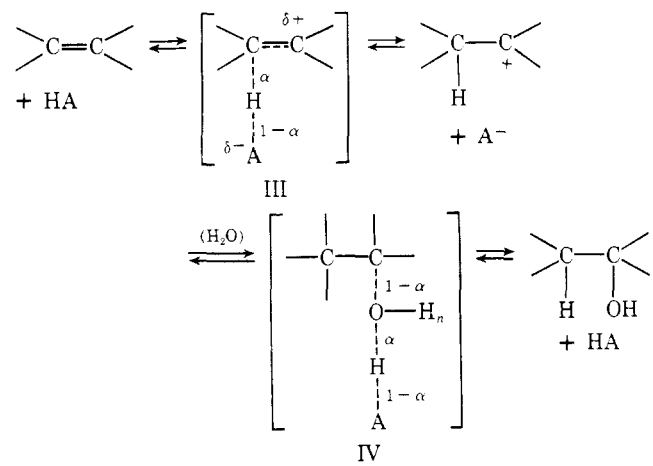
The experimentally determined value of K_s is equal to the acid-dissociation constant, K_3, according to eq 11, whereas, according to eq 13, K_s is ½ the harmonic mean of K_3 and K_4. If pK_3 and pK_4 differ by 1 pK unit or more, K_s represents the more basic of the two sites. Dihydronicotinamides are vinylic amides and protonate more rapidly at the urea center carbonyl oxygen than at the vinyl carbon atom. The pK_3 for dihydronicotinamides (0.6–1.1) resembles that for urea protonation (Jencks, 1969), pK = 0.18, and is similar to the pK of 1.1 for the nicotinamide anion radical (Brühlmann and Hayon, 1974). This indicates that the mechanism in eq 10 prevails for dihydronicotinamides. For mechanism 12, pK_s represents a site more basic than urea. For the 3-acetyl-1,4-dihydropyridines, the pK_s values according to eq 11 should be similar to that for a vinylogous acetamide (Jencks, 1969), −0.5, and should be higher according to eq 13. The values of pK_s obtained, 0.1 to 1.8, vary more than for the dihydronicotinamides and could be accounted for by eq 13 for the substrates with the higher values of pK_s. However, the fact that acetylpyridine radical anion also has an elevated pK of 3.5–4.5 diminishes this possibility (Brühlmann and Hayon, 1974).

Structural Effects on Reactivity. The NADH hydration reaction is, chemically, an enamine hydration type of reaction. Electron donating or basicity-increasing substituents on the enamine nitrogen or carbon increase the hydration reactivity (Sollenberger and Martin, 1970; Stamhuis and Maas, 1965; Maas et al., 1967; Coward and Bruice, 1969; Guthrie and Jordan, 1972; Jordan, 1974). The structural effects on the reactivity of dihydronicotinamides are very marked. Figure 4 gives plots of log k_H vs. pK_s. The slope for the dihydronicotinamides is 5.8; the slope for 3-acetyl-1,4-dihydropyridines is 1.1. The sensitivity of the rate of hydration of dihydronicotinamides to structural parameters is much greater than that observed for other enamines. Five sets of literature data plotted as in Figure 4 give slopes of 0.07 to 1.2 for other enamine families (Sollenberger and Martin, 1970; Stamhuis and Maas, 1965; Coward and Bruice, 1969; Jordan, 1974).

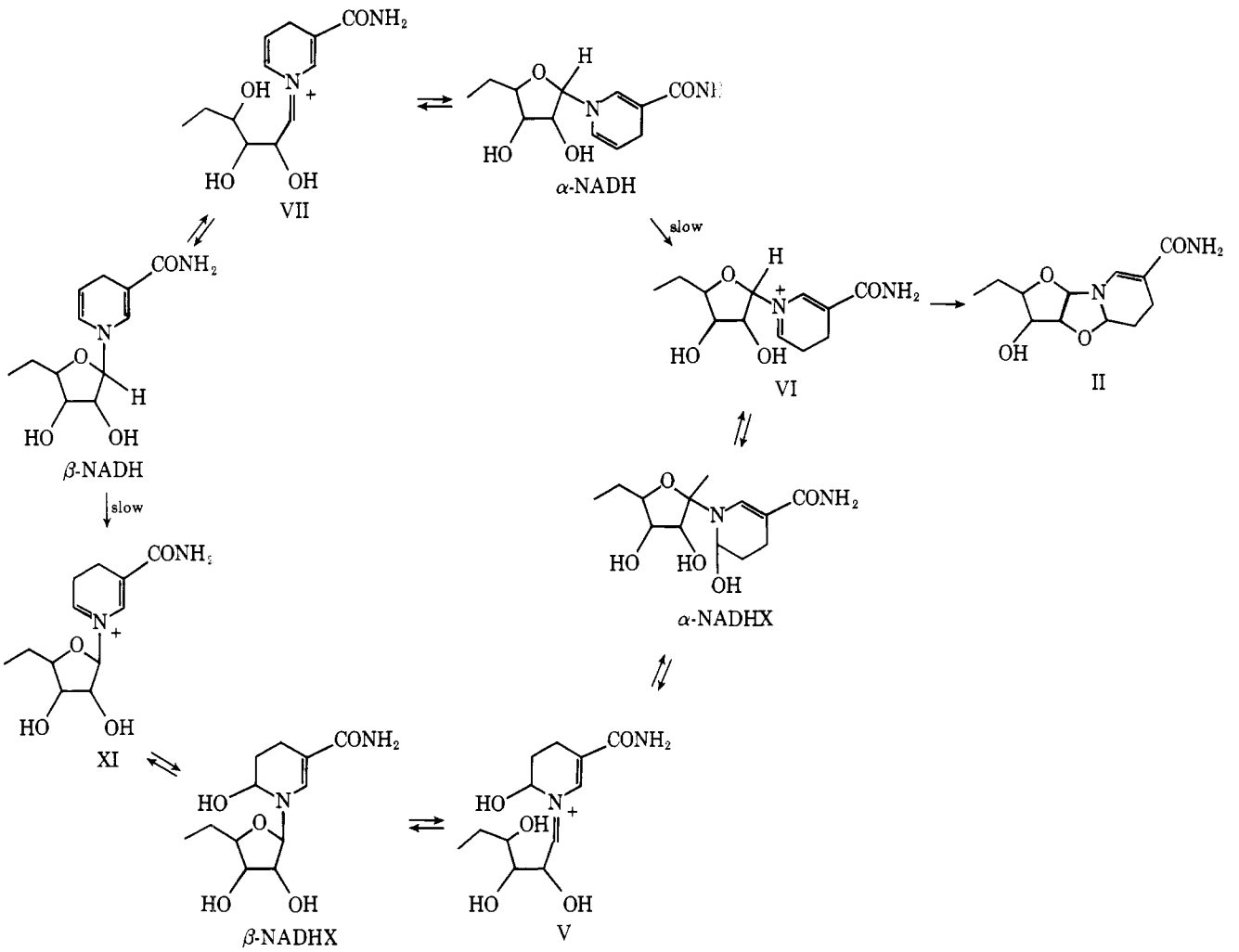
General Catalysis. The Bronsted α value for all the substrates calculated from all the kinetic data is 0.47 ± 0.06. If the points for H_3O^+, acetic acid, formic acid, and phosphate are used in calculating α, a value of 0.41 ± 0.02 is obtained. The typical Bronsted plot shown in Figure 2 shows some scatter according to charge type and nature of catalyst. The characteristic patterns for all the substrates suggest specific transition-state interactions of buffer acids with the dihydropyridine ring.

The substantial isotope effects observed (Table II) are consistent with a rate-limiting proton transfer. Both transition states **III** and **IV** in Scheme III (corresponding to eq 10 and 12, respectively) are possible for general catalysis, assuming a stepwise process. The isotope effects for the water terms are expected to be higher than for the buffer species terms (which,

Scheme *III*



Scheme IV



in turn, should be higher than for the H_3O^+ term, due to the secondary isotope effect contributions of the solvent species H_3O^+). These secondary-effect contributions may be calculated by the approximate method of Schowen (1973). The water-term isotope effects are remarkably low and suggest tighter binding of the solvating-water protons in the transition state than in the ground state. This phenomenon has already been observed in 3,5-dinitroaspirin hydrolysis (Gandour and Schowen, 1974).

The most probable transition state is **III**. Transition state **IV** is unlikely on the basis of the known inability of immonium ions of weak bases to undergo a general base catalyzed reaction with water (Jencks, 1969) and on the basis of the incorporation of only one deuterium in the final product (Oppenheimer and Kaplan, 1974b). The assignment of **III** as the transition state is strengthened by our observation of zero w values, which are characteristic of the rate-limiting proton transfer from H_3O^+ to carbon bases (Bunnett, 1961).

Complexation. The magnitudes of the association constants given in Tables III and IV, 0.7–3.2, are similar to those of dihydronicotinamide–nicotinamide and dihydronicotinamide–flavin systems (Cilento and Schreier, 1964; Blankenhorn, 1975). Dihydronicotinamide complexes with trifluoroacetophenone, acridinium ion, and maleic anhydride have been kinetically or spectrophotometrically detected (Steffans and Chipman, 1971; Creighten et al., 1973; Kosower and Sorenson, 1962). The inhibitory effect of imidazole on PrNH hydration rate is similar in magnitude to that of imidazole on the saponification rate of methyl cinnamate (Conners and Mollica, 1965).

Reaction Path for NADH. The conversion of β-NADH to its primary acid product, **II**, involves both hydration and anomerization reactions. Scheme IV accounts for the reaction pathway. Depending upon the pH and the buffer concentration, anomerization takes place concurrently with, before, or after, the hydration reaction. The anomerizations, which occur via the Schiff-base intermediates **V** and **VII**, are expected to be less sensitive to buffer catalysis than to hydronium-ion catalysis, as compared with the NADH hydration. This is because of the high α values (~1) associated with Schiff-base formation from carbinolamines derived from weak bases (Jencks, 1969), as compared with those for dihydropyridine hydration (0.41–0.47).[3] This is borne out by the trapping of β-NADHX in concentrated phosphate buffers at high pH, where the rate of hydration catalyzed by buffer exceeds the hydronium-ion catalyzed (and weak-buffer catalyzed) anomerizations of β-NADHX. At lower pH, where specific acid catalysis predominates over buffer catalysis, anomerization proceeds faster than hydration. The k_H values for β-NADHX conversion to **II** and β-NADH hydration are 3.0 M^{-1} s^{-1} and 1.1 M^{-1} s^{-1}, respectively. The k_H value for β-NADH anom-

[3] Schiff-base formation from carbinolamines derived from strongly basic amines have α values of ~0.75 (Jencks, 1969). Few studies have been carried out for glycosylamines. One study for arabinosylamide (Isbell and Frush, 1951), a carbinolamine ether derived from a strong base, gives an α value of 0.79.

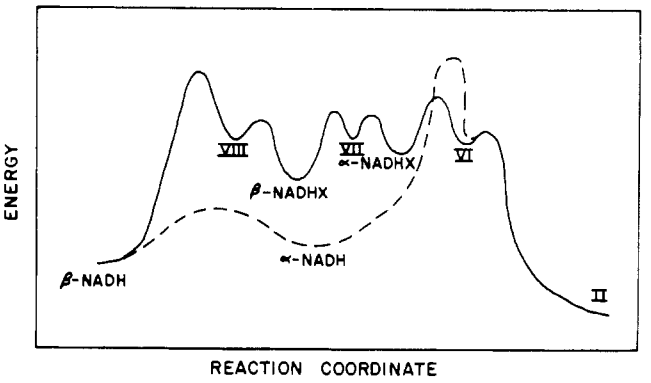


FIGURE 5: Free-energy profile for the primary acid modification of NADH under relatively acidic conditions.

erization is expected to be similar to that for β-NADHX anomerization.[4]

In the lower pH buffers, anomer equilibration between the β and α forms of NADH occurs more rapidly than the hydration reaction, in contrast to the situation in the higher pH buffers, where hydration is faster than anomerization. Evidence for this is given by the equal rate coefficients for β- and α-NADH hydration in low pH buffers (H^+, acetate, formate), and the distinct catalytic coefficients in high pH buffers (phosphate and pyrophosphate); also by the fact that in 1 M acetate buffers, the hydration rate, as measured by UV spectrophotometry, is identical to the rate of formation of the final product II, as measured by CD spectrophotometry. At lower pH values, the rate of β-NADH hydration is being measured, even when starting with α-NADH, because equilibrium anomerization of NADH gives mostly the β form (Oppenheimer and Kaplan, 1975). The energy–reaction coordinate profile of Figure 5 illustrates the situation for low pH buffers, where the energy barrier is less for anomerization than it is for hydration.

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[4] The rate of α to β anomerization of NADH has been measured at 38 °C, pH 7.5 (Oppenheimer and Kaplan, 1975). From these data, assuming only hydronium-ion catalysis at this pH, and an equilibrium ratio of 0.9/0.1 for the β/α forms, a k_H value of 170 M^{-1} s^{-1} is calculated. Using an estimate of 11.5 kcal for ΔE^* (Capon, 1969), a value of 27 M^{-1}·s^{-1} is calculated for the k_H value of β to α anomerization of 25 °C. Anomerization depends upon electron release from the ring nitrogen, because of the formation of the Schiff base intermediate, and, hence, upon the basicity of the nitrogen. The basicity is a function of σ* (Hall, 1957): NADHX and NADH differ by a CH_2OH group which replaces the vinyl group, the σ* values for which are identical. Therefore, the rate of anomerization of NADHX and NADH would be expected to about the same.

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Free Radical and Ionic Reaction of Bisulfite with Reduced Nicotinamide Adenine Dinucleotide and Its Analogues†

Polygena T. Tuazon and S. L. Johnson*

ABSTRACT: 1,4-Dihydronicotinamide adenine dinucleotide (NADH) and its analogues undergo two reactions in sulfite buffers in the pH range 5.5–7.1: (1) an oxygen-mediated free-radical chain reaction which results in the oxidation of the dihydropyridine to the pyridinium salt, and (2) an ionic reaction which results in the hydration of the 5,6 double bond of the dihydropyridine. The free-radical reaction is inhibited by superoxide dismutase (indicating the involvement of superoxide radicals) and by free-radical inhibitors. The ionic reaction is not affected by free-radical inhibitors and follows the rate law: rate = [substrate][HSO_3^-]($k + \Sigma k'$ [HA]), where HA is a general acid or hydronium ion. The occurrence of third-order terms of the type [substrate] × [HSO_3^-][HA] is consistent with the formation of a reactive bisulfite-substrate complex, which undergoes general acid catalyzed hydration.

In our studies on the general acid catalyzed hydration of NADH[1] and its analogues (Johnson and Tuazon, 1977), bisulfite is an anomalous acid catalyst for the reaction for two reasons: it shows (a) an initial fast disappearance of the substrate, followed by a much slower reaction and (b) positive deviations from linear dependence on buffer concentration, in contrast to the linear buffer dependence exhibited by most buffers. Early work by Stock et al. (1961) indicates that oxidation of the dihydropyridine of NADH to the pyridinium salt occurs under certain conditions in bisulfite buffers. Nonlinear buffer catalysis by bisulfite was observed by Johnston et al. (1963), although the complete rate law was not fully investigated at that time.

Recent investigations show that bisulfite reacts by an ionic mechanism as in the addition to the 5,6 double bond of pyrimidine nucleosides (Shapiro et al., 1970; Hayatsu et al., 1970), in its nucleophilic reactions with NAD^+ (Johnson and Smith, 1976), and with flavins (Hevesi and Bruice, 1973; Bruice et al., 1973). Bisulfite also reacts by a free-radical chain mechanism with certain nucleosides and with methionine (Hayatsu and Inoue, 1971; Sono and Hayatsu, 1973; Hayatsu et al., 1972; Inoue and Hayatsu, 1971).

NADH and its analogues undergo nonenzymatic oxidation reactions; the mechanism is of interest for the understanding of the enzyme-catalyzed oxidation of NADH. The majority of the reactions are explained by or are formally analogous to a direct transfer of a hydride ion from the dihydropyridine to riboflavin, certain ketones, and other oxidizing agents (Suelter and Metzler, 1960; Abeles et al., 1957; Norcross et al., 1962; Cilento, 1960; Ludowieg and Levy, 1964; Spiegel and Drysdale, 1960; Cilento, 1960).

On the other hand, oxidations by obligate one-electron acceptors, such as ferricyanide and spirocyclohexylporphyrexide, proceed by a free-radical mechanism (Schellenberg and Hellerman, 1958). Reductions of chloro compounds by dihydropyridines proceed by free-radical chain reactions (Kurz et al., 1961; Dittmer and Fouty, 1964). NADH undergoes a free-radical chain oxidation by superoxide, catalyzed by lactate dehydrogenase (Bielski and Chan, 1975). In addition, lactate dehydrogenase catalyzes a stereospecific hydrogen-atom transfer from NADH to dicarboxylate radicals (Chan and Bielski, 1975).

The autoxidation of sulfite to sulfate is a classical example of a free-radical chain reaction (Abel, 1951; Fuller and Crist, 1941). Traces of metals are responsible for initiating the "spontaneous" reaction. An additional pathway for sulfite oxidation is mediated by superoxide (McCord and Fridovich, 1969a).

In view of the reactivity of bisulfite towards a variety of biologically important molecules which could account for its

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[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; NAD^+, nicotinamide adenine dinucleotide; BzNH, 1-benzyl-1,4-dihydronicotinamide; BzN^+, *N*-benzylnicotinamide cation; PrNH, 1-propyl-1,4-dihydronicotinamide; PrN^+, *N*-propylnicotinamide cation; BzAPH, 1-benzyl-3-acetyl-1,4-dihydropyridine; SOD, superoxide dismutase; HQ, hydroquinone; PyH, dihydropyridine; NAD-SO_3^-, the sulfite complex of NAD^+; EDTA, (ethylenedinitrilo)tetraacetic acid.