chem. Biophys. 166, 526.
Paul, M. A., and Long, F. A. (1957), *Chem. Rev. 57,* 1.
Pfleiderer, G., and Stock, A. (1962), *Biochem. Z. 336,* 56.
Rafter, G. W., Chaykin, S., and Krebs, E. G. (1954), *J. Biol. Chem. 208,* 799.
Schowen, R. L. (1973), *Prog. Phys. Org. Chem. 9,* 275.
Sollenberger, P. Y., and Martin, R. B. (1970), *J. Am. Chem. Soc. 92,* 4261.
Stamhuis, E. J., and Maas, W. (1965), *J. Org. Chem. 30,* 2156.
Steffans, J. F., and Chipman, D. M. (1971), *J. Am. Chem. Soc. 93,* 6694.
Stock, A., Sann, E., and Pfleiderer, G. (1961), *Ann. Chem. 647,* 188.
Tuazon, P. T., and Johnson, S. L. (1977), *Biochemistry 16* (following paper in this issue).
Weiland, T., Duesberg, P., Pfleiderer, G., Stock, A., and Sann, E. (1960), *Arch. Biochem. Biophys., Suppl. 1,* 260.

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

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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.

toxicity (Schroeter, 1961), we investigated the nature of its reaction with NADH and its analogues.

Experimental Procedures

Materials. Reagent grade inorganic salts were used without further purification. Sodium sulfite was from Fisher Scientific Co. β-NADH was purchased from Sigma, and BzNH, PrNH, and BzAHP were prepared as previously described (Johnson and Tuazon, 1977). Superoxide dismutase was purchased from Sigma.

Stock buffers were prepared with ionic strength of 1.0 M. More dilute buffers were prepared from the stock buffers by diluting with 1.0 M KCl to maintain the ionic strength at 1.0 M. Bisulfite buffers were made from sodium sulfite and hydrochloric acid and stock solutions of the dihydronicotinamide were prepared in slightly basic solutions immediately before use for the kinetic determinations.

Kinetic Studies. The decrease in absorbance of the substrate at 340 or 360 nm was followed using a recording Cary 16 spectrophotometer equipped with a cell compartment thermostated at 25.0 °C. The ionic reactions were started by adding 50–100 μL of the stock solution of the dihydropyridine to 3.0 mL of sulfite buffer in a cuvet. The free-radical reactions were initiated by adding measured quantities of sulfite solution to the substrate. The initial absorbance, A_0, of the dihydropyridine was obtained in the appropriate volume of 1 M KCl solution before each kinetic determination. Measurements of pH were immediately made following each kinetic run using a Radiometer, Type TTT-1d, pH meter coupled with a PHA-630 scale expander. Rate measurements of the fast reactions were also carried out using a Durrum stopped-flow spectrophometer.

For the ionic portion of the reaction, pseudo-first-order rate constants (k_{obsd}) were determined from plots of log $(A_t - A_\infty)$ vs. time, where A_∞ and A_t are the absorbances at infinite time and at time t. The rate constants for the very slow reactions were calculated as $(dA/dt)/A_0$, where dA/dt is the initial rate of absorbance decrease. Extrapolation of the semilog plots to zero time gave apparent initial absorbance readings, A_i, which were lower than the actual initial absorbance, A_0, of solutions made in 1 M KCl or very dilute phosphate buffer or water. The difference, $\Delta A = A_0 - A_i$, was taken as a measure of the extent of the initial fast free-radical reaction. ΔA divided by the extinction coefficients of PrNH (A_{max}, 360 nm; ϵ, 7060 M^{-1} cm^{-1}) and BzNH (A_{max}, 355 nm; ϵ, 7250 M^{-1} cm^{-1}) gives the concentration of substrate used up in the free-radical reaction. At the sulfite concentrations used, little sulfite complexation takes place with PrN^+ and BzN^+ (Johnson and Smith, 1976). NAD^+, however, complexes significantly with sulfite to produce NAD-SO_3^- with an absorption band in the 340-nm region, which would mask the disappearance of NADH. ΔA divided by $\epsilon_{NADH} - \epsilon_{NAD\text{-}SO_3} \times K_1[SO_3^{2-}]/(1 + K_1[SO_3^{2-}])$ gives the concentration of NADH used up on the free-radical reaction. At 340 nm, the values of ϵ_{NADH}, $\epsilon_{NAD\text{-}SO_3}$, and K_1 are 6200 M^{-1} cm^{-1}, 3200 M^{-1} cm^{-1}, and 40 M^{-1}, respectively. K_1 is the formation constant of NAD-SO_3^- (Johnson and Smith, 1976). The disappearance of NADH was also examined fluorimetrically, with a Turner fluorimeter, because NADH but not NAD-SO_3^- is fluorescent.

The free-radical reaction rates were studied in detail by adding fresh oxygen-containing solutions of substrate to preprepared (oxygen depleted) sulfite buffers. The reactions were followed spectrophotometrically in stoppered cuvets. The pH was measured before and after the reaction. By varying the ratio of oxygen-depleted sulfite solutions and oxygen-containing substrate solutions, the amount of oxygen available to the substrate is varied. The sulfite buffers were prepared at least 2 h in advance before using.

Product Analysis. Product determinations were carried out for reactions in 0.4 and 0.04 M sulfite buffer, pH 6.5. A reaction solution was made by adding 300 μL of stock PrNH solution (0.005 M) to 3.0 mL of the sulfite buffer. The reaction was carried out for 30 min in a stoppered flask. After the reaction was completed, the solution was made basic with NaOH, introduced into a Bio-Rad AG1-X2 anion-exchange column, and eluted with water.

Superoxide Detection. The presence of superoxide was assayed using cytochrome *c* (McCord and Fridovich, 1969b), and nitroblue tetrazolium (Misra, 1974).

Results

The disappearance of NADH and its analogues in bisulfite buffers in the pH range 5.5–7.1 is the result of an initial fast reaction and a slow reaction. This initial fast reaction is not seen in any of the other buffers used for studying the dihydropyridine hydration reaction (Johnson and Tuazon, 1977).

Time Course and Extent of the Free-Radical Reaction. The fast reaction has the characteristics of a free-radical reaction in that its rate is affected by free-radical reagents, and because erratic and nonreproducible results are obtained from preparation to preparation. The same preparation used on consecutive days gave similar results.[2] The slow (ionic) reaction is reproducible, is unaffected by free radical reagents, and follows a semilogarithmic course. The progress of the fast reaction is variable as follows: (a) an induction period followed by a fast reaction (accelerative), (b) a nearly linear steady rate with an abrupt stop (linear), (c) multiple accelerative steps, and (d) decelerative. Higher concentrations of sulfite or metal ions give accelerative progress curves, and low concentrations of sulfite or metal ions give decelerative curves. The extent of the free-radical reaction depends only on the oxygen concentration, and is independent of sulfite concentration, pH, the presence of free radical reagents, or metal ions. In the case of PrNH and BzNH, the extent of the fast reaction is independent of substrate concentration. For NADH, the extent of the fast reaction, $-\Delta$NADH, is directly proportional to the initial NADH concentration, $[NADH]_0$, according to eq 1, for concentrations of 0.39–1.6 × 10^{-5} M.

$$-\Delta\text{NADH} = 0.26[\text{NADH}]_0 \quad (1)$$

ΔNADH is three-to eightfold smaller than the amount of oxygen available (2.7 × 10^{-4} M) in air-saturated solutions at 25 °C. On the other hand, ΔPrNH and ΔBzNH exceed the amount of dissolved oxygen by a factor of 3–5.

Kinetics of the Free-Radical Reaction. The parameters measured are the initial velocity, V_i, maximal velocity, V_m, induction period, lag, and the time to reach maximal velocity, t_m. The most detailed analysis is for PrNH, and the following results apply to this substrate, unless specified otherwise.

The rate of the free-radical reaction is markedly increased by the metal ions Cu^{2+}, Mn^{2+}, and Fe^{3+}, and slowed by EDTA. The potent inhibitors of sulfite oxidation, ethanol, and mannitol have no effect. Figure 1 shows the effects of EDTA and of sulfite oxidation inhibitors. Over the concentration range 0.66 × 10^{-5} to 3.3 × 10^{-5} M, the initial rate, V_i, varies with the 1.6th power of Cu^{2+}. EDTA has an effect on all the

[2] The inclusion of small quantities of metal ions in the reaction mixture, for example, 6.7 × 10^{-6} M Cu^{+2}, gave more reproducible results.

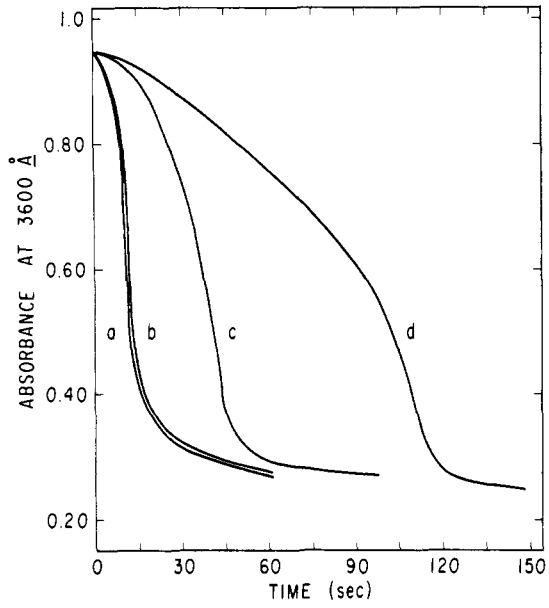


FIGURE 1: Effect of free-radical inhibitors on the rate of PrNH oxidation in 0.02 M sulfite, pH 6.5, at 25 °C. Reaction was initiated by adding 1.5 mL of stock PrNH to 1.5 mL of 0.04 M bisulfite, pH 6.5, containing (a) no inhibitor; (b) 200 μL of ethanol or 100 μL of 0.5 M mannitol; (c) 20 μL of 0.2 M EDTA; (d) 100 μL of 0.2 M EDTA.

kinetic parameters: the lag is directly proportional to EDTA concentration; and V_i^{-1}, V_m^{-1}, and t_m are linear in EDTA concentration with an EDTA-independent intercept.

Superoxide dismutase, SOD, increases the lag and t_m, and decreases V_i and V_m in a manner identical to EDTA. The lag is directly proportional to [SOD], while V_i^{-1}, V_m^{-1}, and t_m are linear in [SOD] with a SOD-independent intercept. A similar concentration of bovine serum albumin did not affect the reaction, indicating that the influence of superoxide dismutase to the system is not due to introduced impurities, e.g., "reactive" groups in the protein that can act as scavengers for superoxide radicals. The presence of superoxide in the sulfite buffers was readily detectable with the cytochrome *c* and nitroblue tetrazolium assays.

Hydroquinone inhibits the sulfite mediated PrNH disappearance, with a direct proportionality of V_i^{-1} and [HQ].

The rates (V_i or V_m) of the free-radical reaction are directly proportional to the total sulfite buffer concentration over the range 0.006–0.4 M. The lag and t_m are inversely proportional to the sulfite buffer concentration. This relationship applies to all the free-radical reactions studies, whether spontaneous or Cu^{2+} catalyzed. The pH optimum for the reaction is about 6.5. At pH 7.26, 6.78, and 6.11, the relative slopes of V_i vs. total sulfite are 4.0, 14.6, and 5.7, respectively.

Acetone is an inhibitor; small quantities of acetone, 0.005 M, decreased V_i by a factor of 7.

Oxygen concentration is without effect on the rate above 50% air saturation. A strong dependence is observed at 0.6–3.5% air-saturation levels. The rate is linear in initial substrate concentration with a concentration-independent term according to eq 2.

$$V_i = A + B[\mathrm{PyH}] \qquad (2)$$

The relative contributions of A and B vary with the substrate. The A term for PrNH and BzNH is large so that at the customary concentrations used (10^{-4}–10^{-5} M) little dependence on the substrate concentration is observed. NADH, on the other hand, has a smaller A term, so that V_i has a greater dependence on the initial NADH concentration. The parameters in eq 2 are given in Table I.

TABLE I: Effect of Initial Substrate Concentration on Sulfite-Mediated Free-Radical Oxidation of Dihydropyridines.[a]

	$V_i = A + B$ [PyH]$_0$, M^{-1} s^{-1}		
Substrate	10^7A	10^3B	$10^4A/B$
PrNH	3.5	2.1	1.6
BzNH	3.3	0.63	5.2
NADH	0.21	2.5	0.084

[a] Small measured quantities of substrate, sulfite buffer, and $CuSO_4$ were added to air-saturated water, with the sulfite addition last to initiate the reaction. Final concentration of Cu^{2+} is 6.67×10^{-6} M. The reaction mixture was 25 °C.

Product Analysis. Analysis of the products of the reaction in 0.04 and 0.4 M sulfite buffers, pH 6.5, at 9% air saturation, shows that oxidation of the dihydropyridine to the pyridinium salt has occurred. The pyridinium salt is separated by an anion-exchange resin, where it elutes with the void volume with water, and by a cation-exchange resin, where it elutes last with saturated ammonium chloride solution. The pyridinium salt is identified by comparison of its characteristic UV spectrum (an A_{max} with two peaks on each side) with that of authentic 1-propyl-3-carbamoylpyridinium chloride (A_{max} = 265 nm, ϵ = 3900 M^{-1} cm^{-1}). Based on the absorbance of the pyridinium salt eluted and the initial concentration of PrNH)2.8 $\times 10^{-4}$ M), the pyridinium salt is obtained in 63% yield in 0.04 M sulfite and 49% yield in 0.4 M sulfite. In acetate (pH 4.5) and phosphate (pH 6.5) buffers, as well as in 0.1 M HCl, the amount of dihydropyridine converted to the pyridinium salt is no more than 10%.

The other product of the sulfite reaction has a spectrum similar to the primary acid product obtained in HCl or in acetate buffers (A_{max} = 297 nm). However, the sulfite product differs from the primary acid product as follows. (a) The sulfite product is more stable than the primary acid product in acidic solutions. For example, 50% of the 290-nm absorbance of the primary acid product of NADH disappears in 5 min in 1 M HCl and in 19 min in 0.1 M HCl, whereas only 5% of the bisulfite product disappears in 20 min in 1 M HCl and 11% in 45 min in 0.1 M HCl. (b) The thin-layer chromatographic properties are distinct. On silica gel plates with 1-propanol–water (60:40) as the developing solvent, the sulfite product of NADH has a slightly lower R_f value than the primary acid product. (c) The sulfite product of PrNH is more negatively charged than the primary acid product; it elutes from an anion-exchange later than the primary acid product, the latter coming off immediately with water as eluant.

The primary acid product transforms into the sulfite product by treatment with sulfite. When placed in HCl solutions containing sulfite, the NADH primary acid product is stabilized in a manner similar to that of the sulfite product. For example, in 1 M HCl containing 0.007 M sulfite, the absorbance at 290 nm decreases by only 9% after 29 min; in 0.1 M HCl containing 0.007 M sulfite, a decrease of 2.5% is observed after 53 min. These are to be compared with the expected decreases in HCl solutions without sulfite of 97 and 87%, respectively.

Slow Reaction. The rate of the slow reaction is independent of the manner in initiating the reaction or of the percent air saturation, and is affected by metal ions, EDTA, HQ, SOD, ethanol, or mannitol. In the pH range 5.5–7.1, the reaction

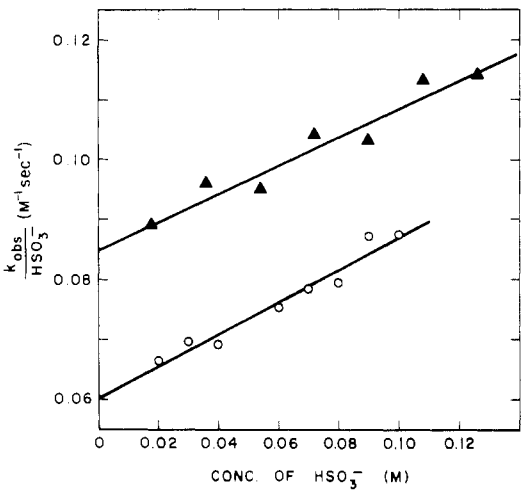


FIGURE 2: Plots of $k_{obsd}/[HSO_3^-]$ against bisulfite concentration for the primary acid modification reaction of PrNH in bisulfite buffers at pH 6.6 (Δ) and pH 7.2 (O) at 25 °C and μ = 1.0 M.

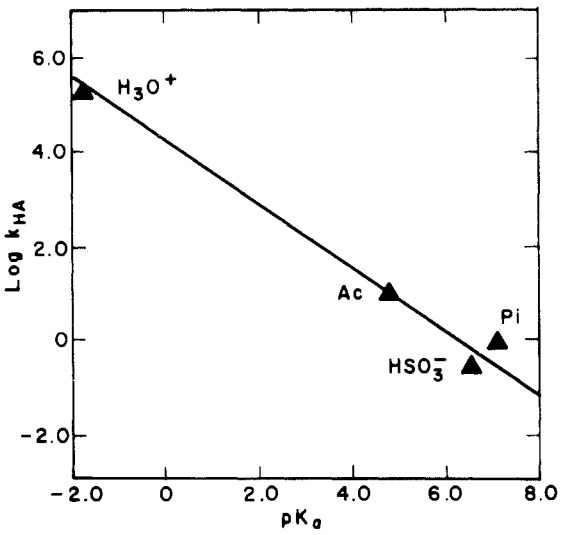


FIGURE 3: Brønsted plot of the $[HSO_3^-][HA]$ terms for PrNH hydration in bisulfite buffers. The α value is 0.67.

TABLE II: Kinetic Constants for the Primary Acid Modification Reaction of Dihydropyridines in Bisulfite Buffers at 25 °C, μ = 1.0 M.

Substrate	k_{HSO_3} ($M^{-1}s^{-1}$)	$k_{HSO_3,H}$ ($M^{-2}s^{-1}$)	$k_{HSO_3,HAc}$ ($M^{-2}s^{-1}$)	k_{HSO_3,P_i} ($M^{-2}s^{-1}$)	k_{HSO_3,HSO_3} ($M^{-2}s^{-1}$)
PrNH	4.8×10^{-2}	1.9×10^5	9.2	1.1	0.29
BzNH		4.8×10^3			0.052
NADH		1.7×10^2			*a*
BzAPH	5.3×10^{-4}	3.2×10^3			*a*

a Second-order bisulfite terms are not observed at the low concentrations of bisulfite used (i.e., up to 0.2 M HSO_3^-), assuming catalytic coefficient ratios similar to those observed for PrNH and BzNH.

follows pseudo-first-order kinetics. Plots of k_{obsd} vs. bisulfite concentration, at constant pH values, exhibit an upward curvature at higher concentrations of bisulfite with PrNH or BzNH as substrate, indicating that the reaction is dependent upon the bisulfite concentration to more than the first power. The data were analyzed according to eq 3,

$$[H^+][HSO_3^-] + k_{HA,HSO_3}[HA][HSO_3^-] \qquad (3)$$

where k_0 is the intercept value. From plots of $(k_{obsd} - k_0)/[HSO_3^-]$ vs. $[HSO_3^-]$ as $k_{obsd} - k_0 = k_{HSO_3}[HSO_3^-] + k_{HSO_3,HSO_3}[HSO_3^-]^2 + k_{H,HSO_3}$ shown in Figure 2, the terms linear in bisulfite are obtained from the intercept, and those proportional to $[HSO_3^-]^2$ are determined from the slope. The slope is pH independent, while the intercepts are pH dependent. Linear plots of k_{obsd} vs. $[HSO_3^-]$ are obtained for BzAPH and NADH with the same range of bisulfite concentrations as were used for PrNH and BzNH.

Reactions of dihydronicotinamides in acetate and dihydrogen phosphate buffers are subject to general acid catalysis and exhibit a linear dependence of k_{obsd} on buffer concentrations at constant pH (Johnson and Tuazon, 1977). When a constant amount of bisulfite is added to variable concentrations of linear buffers at constant pH, the result is linear plots whose slopes are greater than the k_{HA} values obtained in pure acetate or phosphate buffers, as described by eq 4.

$$\text{slope} = k_{HA} + k_{HA,HSO_3}[HSO_3^-] \qquad (4)$$

A summary of the terms in eq 3 and 4 is given in Table II. A Brønsted plot of the $[HSO_3][HA]$ terms for PrNH is shown in Figure 3.

Discussion

NADH and its analogues react in bisulfite buffers in the pH region 5.5–7.1, to form the corresponding pyridinium salt and the hydration product, as evidenced by the UV spectral and ion-exchange characteristics of the isolated products. The oxidation of dihydropyridines occurs in the initial fast part of the reaction and the hydration occurs in the slow reaction. The latter reaction is due to primary acid product formation in acidic solutions where bisulfite is acting as a general acid catalyst.

Oxidation Reaction. The oxidation of the dihydropyridine in bisulfite buffers involves free radicals. The oxidation is inhibited by hydroquinone which acts as a free-radical scavanger, and by EDTA which chelates metal ions, thus preventing free-radical initiation by the metal-sulfite reaction (Abel, 1951). Acetone inhibits by removing bisulfite in a favorable complexation reaction (K = 270 M^{-1} (Gubareva, 1947)). The induction period (Figure 2) is common to free-radical oxidation reactions (Stirling, 1965) and is the time required to generate the necessary concentration of carrier radicals.

The requirements for the free-radical reaction are the simultaneous presence of oxygen and sulfite. The characteristics of the free-radical oxidation of sulfite (Fuller and Crist, 1941; Abel, 1951; McCord and Fridovich, 1969a,b) and of the sulfite-mediated oxidation of dihydropyridines have a number of points in common, suggesting a common chain carrier in both reactions. There are: (a) the pH rate optimum at pH 6.5, (b) the partial involvement of superoxide, and (c) the partial involvement of metal ions. A recent study of sulfur oxide radicals generated by pulse radiolysis led to the most complete mechanism of non-superoxide-mediated sulfite oxidation (Hayon et al., 1972): initiator $\rightarrow SO_3^-\cdot$; $SO_3^-\cdot + SO_3^{2-} \rightarrow SO_4^-\cdot + SO_4^{2-}$; $SO_4^-\cdot + SO_3^{2-} \rightarrow SO_4^{2-} + SO_3^-\cdot$. $SO_3^-\cdot$ (and even more so $SO_5^-\cdot$) is a relatively stable radical compared with the other radical species in the pathway; in particular $SO_3^-\cdot$ does not react with ethanol as does $SO_4^-\cdot$.

$SO_3^-\cdot$ is probably the common carrier in the two oxidation

reactions. A probable sequence of reactions is given below.

Initiation

$$\text{Initiator} + Cu^{2+} \rightarrow R\cdot \quad (5)$$

$$R\cdot + SO_3^{2-} \rightarrow R^- + SO_3^{-}\cdot \quad (6)$$

$$Cu^{2+} + SO_3^{2-} \rightarrow Cu^+ + SO_3^{-}\cdot \quad (7)$$

Propagation

$$SO_3^{-}\cdot + PyH \rightarrow HSO_3^- + Py\cdot \quad (8)$$

$$Py\cdot + O_2 \rightarrow Py^+ + O_2^{-}\cdot \quad (9)$$

$$SO_3^{2-} + O_2^{-}\cdot + 2\,H^+ \rightarrow SO_3^{-}\cdot + H_2O_2 \quad (10)$$

Termination

$$2\,O_2^{-}\cdot + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (11)$$

$$2\,SO_3^{-}\cdot \rightarrow S_2O_6 \quad (12)$$

$$2\,Py\cdot \rightarrow (Py)_2 \quad (13)$$

$$Py\cdot + SO_3^{-}\cdot \rightarrow PySO_3^- \rightleftarrows Py^+ + SO_3^{2-} \quad (14)$$

$$Py\cdot + HO_2\cdot \rightarrow PyO_2H \rightleftarrows Py^+ + HO_2^- \quad (15)$$

$$SO_3^{-}\cdot + HO_2\cdot \rightarrow HSO_5^- \text{ or } HSO_3^- + O_2 \quad (16)$$

In order for $SO_3^{-}\cdot$ to be the carrier radical for PyH oxidation, Py· must effectively compete with it for reaction with O_2. Alcohols inhibit sulfite oxidation, but not PyH oxidation, because radicals other than $SO_3^{-}\cdot$, probably $SO_4^{-}\cdot$, are trapped in sulfite oxidation (Hayon et al., 1972). At high oxygen concentrations, reaction 11 is the most likely termination step. This is because step 9 is very fast (1.0×10^9 M^{-1} s^{-1}, Land and Swallow, 1971), causing the relatively stable superoxide radicals to accumulate for the propagation reaction 8 or for the termination reaction 11 (1.5×10^6 M^{-1} s^{-1}, Czapski, 1971). The rate-limiting step would then be reaction 10, which is independent of substrate concentration. This explains the identity of the substrate-independent rate terms for BzNH and PrNH. A different rate-limiting step involving the substrate, reaction 8, probably occurs for NADH, due to its lesser reactivity.

The requirements for the carrier radical is that it have an oxidation potential, ϵ_{0III}', which is more positive than that for Py·. ϵ_0' for Py· can be calculated from the known ϵ_0' values of PyH and Py^+.

$$Py^+ + 2e + H^+ \rightarrow PH \quad \epsilon_{0I}' \quad (17)$$

$$Py^+ + e \rightarrow Py\cdot \quad \epsilon_{0II}' \quad (18)$$

$$Py\cdot + e + H^+ \rightarrow PyH \quad \epsilon_{0III}' \quad (19)$$

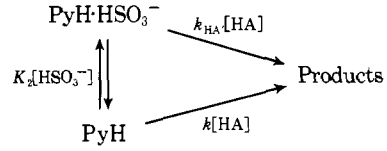
$$\epsilon_{0III}' = 2\epsilon_{0I}' - \epsilon_{0II}' \quad (20)$$

The values of ϵ_{0I}' and ϵ_{0II}' for NAD^+ and *N*-methylnicotinamide cation of −0.34 and −0.75 V (NAD^+) and −0.42 and −0.90 V (*N*-methylnicotinamide cation) allow the calculation according to eq 20 of ϵ_{0III}' (Brühlmann and Hayon, 1974; Sober, 1968). ϵ_{0III}' values for NAD^+ and *N*-methylnicotinamide cation are +0.07 and +0.06 V, respectively. Porphyrexide radicals (Schellenberg and Hellerman, 1958) are able to abstract a hydrogen from NADH because their ϵ_0' values are in the range +0.56 to +0.72 V (Clark, 1960). Because $SO_3^{-}\cdot$ is unable to abstract a hydrogen atom from ethanol, an upper limit can be placed on its oxidation potential of +0.29 V. This value was calculated according to eq 20, where ϵ_{0I}' is for acetaldehyde + 2 e + 2 H^+ → ethanol, −0.197 V (Sober, 1968), and ϵ_{0II}' is for acetaldehyde + e + H^+ → $CH_3CHOH\cdot$, −0.68 V (Rao and Hayon, 1974).

As with sulfite oxidation, sulfite-mediated PyH oxidation involves more than one pathway. Mechanisms involving other oxide radicals of sulfur as the chain carriers are possible. $SO_5^{-}\cdot$ probably does not fulfill this role because it is more stable than $SO_3^{-}\cdot$ (Hayon et al., 1972). Mechanisms involving the reaction: $H^+ + O_2^{-}\cdot + PyH \rightarrow Py\cdot + H_2O_2$, with sulfite involvement in the generation of superoxide, are not possible because (a) this reaction is extremely slow for NADH (k = 27 M^{-1} s^{-1}, Land and Swallow, 1971), (b) NADH would be readily oxidized by ordinary handling because of the ubiquity of superoxide in solutions, other than sulfite solutions, (c) the kinetics require a term 0.5 order in sulfite, 0.5 order in O_2, and first order in PyH. The multiplicity of sulfite and oxygen radical species present in oxygen-containing sulfite solutions suggests that it is reasonable that at least one of these species, probably $SO_3^{-}\cdot$, is responsible for the chain propagation.

Ionic Reaction. The slow part of the reaction of NADH in bisulfite is due to the hydration of the 5,6 double bond of the dihydropyridine where bisulfite acts as a general acid catalyst. The kinetic behavior of bisulfite reactivity to dihydropyridines is described by a $[HSO_3^-]$ term as well as by the higher order terms $[HSO_3^-][H^+]$, $[HSO_3^-][HA]$, $[HSO_3^-]^2$. The simplest explanation of the higher-order terms is given in Scheme I where HSO_3^- forms a more reactive complex with the substrate, which then undergoes general acid catalyzed hydration. The rate expression for the bisulfite terms is given by eq 21.

Scheme I

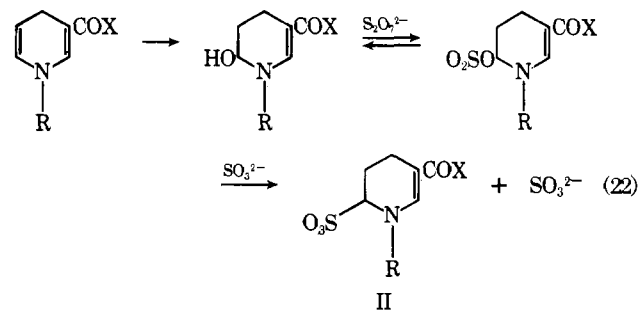


$$k_{obsd} = \frac{[k_{H_2O}' + k_{HSO_3}'[HSO_3^-] + k_{HA}'[HA] + k_{HSO_3}/K_2]K_2[HSO_3^-]}{1 + K_2[HSO_3^-]} \quad (21)$$

If $k_2[HSO_3^-] < 1$ then the observed rate expression would be given by eq 3, by which the data was treated. Each observed $k_{HSO_3,HA}$ term in eq 3 is equal to K_2 multiplied by the appropriate k_{HA} coefficient in Scheme I.

It is proposed here that the complex formed in Scheme I is a bisulfite addition complex at the carbonyl group. Because of the greatly increased reactivity at the 5,6 double bond caused by substituent changes at the 3 position (Stock et al., 1961), the complex is very reactive. In the pH range studied, the equilibrium constant, K_2, depends on the concentration of the HSO_3^- species, rather than on the concentrations of H_2SO_3 or SO_3^{2-}, by analogy with the pH dependence on the bisulfite equilibrium with aldehydes (Steward and Donnally, 1932; Kokesh and Hall, 1975).

The product of the bisulfite reactions is most likely the sulfonate II, which is formed by a series of reactions, as shown in eq 22. Pyrosulfite, $S_2O_7^{2-}\cdot$ is present in all sulfite buffers and



is known to be in rapid equilibrium with bisulfite (Golding, 1960; Betts and Voss, 1970). Pyrosulfite reacts with OH groups to form reactive bisulfite esters (Higuchi et al., 1966), which can then undergo a nucleophilic displacement reaction with sulfite ion (Schroeter, 1962).

References

Abel, E. (1951), *Monatsch. Chem. 82,* 815.

Abeles, R. H., Hutton, R. F., and Westheimer, F. H. (1957), *J. Am. Chem. Soc. 79,* 712.

Betts, R. H., and Voss, R. H. (1970), *Can. J. Chem. 48,* 2035.

Bielski, B. H., and Chan, P. C. (1975), *J. Biol. Chem. 250,* 318.

Brühlmann, U., and Hayon, E. (1974), *J. Am. Chem. Soc. 96,* 6169.

Bruice, T. C., Hevesi, L., and Shinkai, S. (1973), *Biochemistry 12,* 2083.

Chan, P. C., and Bielski, B. H. (1975), *J. Biol. Chem. 250,* 7266.

Cilento, G. (1960), *Arch. Biochem. Biophys. 88,* 352.

Clark, W. M. (1960), in Oxidation Reduction Potentials of Organic Systems, Baltimore, Md., Williams and Wilkins, p 437–438.

Czapski, G. (1971), *Annu. Rev. Phys. Chem. 22,* 196.

Dittmer, D. C., and Fouty, R. A. (1964), *J. Am. Chem. Soc. 86,* 91.

Fuller, E. C., and Crist, R. H. (1941), *J. Am. Chem. Soc. 63,* 1644.

Golding, R. M. (1960), *J. Chem. Soc.,* 3711.

Gubareva, M. A. (1947), *J. Gen. Chem. U.S.S.R.* (Engl. Transl.) *17,* 2529.

Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. (1970), *Biochemistry 9,* 2858.

Hayatsu, H., and Inoue, M. (1971), *J. Am. Chem. Soc. 93,* 2301.

Hayatsu, H., Wataya, Y., Furuichi, Y., and Kawazoe, Y. (1972), *Chemosphere 1,* 75.

Hayon, E., Treinin, A., and Wilf, J. (1972), *J. Am. Chem. Soc. 94,* 47.

Hevesi, L., and Bruice, T. C. (1973), *Biochemistry 12,* 290.

Higuchi, T., McRae, J. D., and Sha, A. C. (1966), *J. Am. Chem. Soc. 88,* 4015.

Inoue, M., and Hayatsu, H. (1971), *Chem. Pharm. Bull. 19,* 1286.

Johnson, S. L., and Smith, K. W. (1976), *Biochemistry 15,* 553.

Johnson, S. L., and Tuazon, P. T. (1977), *Biochemistry 16* (preceding paper in this issue).

Johnston, C. C., Gardner, J. L., Suelter, C. H., and Metzler, D. E. (1963), *Biochemistry 2,* 689.

Kokesh, F. C., and Hall, K. E. (1975), *J. Org. Chem. 40,* 1632.

Kurz, J. L., Hutton, R., and Westheimer, F. H. (1961), *J. Am. Chem. Soc. 83,* 584.

Ludowieg, J., and Levy, A. (1964), *Biochemistry 3,* 373.

Land, E. J., and Swallow, A. J. (1971), *Biochim. Biophys. Acta 231,* 34.

McCord, J. M., and Fridovich, I. (1969a), *J. Biol. Chem. 244,* 6056.

McCord, J. M., and Fridovich, I. (1969b), *J. Biol. Chem. 244,* 6049.

Misra, H. P. (1974), *J. Biol. Chem. 249,* 2151.

Norcross, B. E., Klinedinst, P. E., and Westheimer, F. H. (1962), *J. Am. Chem. Soc. 84,* 797.

Rao, P. S., and Hayon, E. (1974), *J. Am. Chem. Soc. 96,* 1287.

Schellenberg, K. A., and Hellerman, L. (1958), *J. Biol. Chem. 231,* 547.

Schroeter, L. C. (1961), *J. Pharm. Sci. 50,* 891.

Schroeter, L. C. (1962), *J. Pharm. Sci. 51,* 258.

Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Am. Chem. Soc. 92,* 422.

Sober, H. A. (1968), in Handbook of Biochemistry, Cleveland, Ohio, The Chemical Rubber Co.,

Sono, M., and Hayatsu, H. (1973), *Chem. Pharm. Bull. 21,* 995.

Spiegel, M. J. and Drysdale, G. R. (1960), *J. Biol. Chem. 235,* 2498.

Steward, T. D., and Donnally, L. H. (1932), *J. Am. Chem. Soc. 54,* 2333.

Stirling, C. J. M. (1965), in Radicals in Organic Chemistry, London, Oldbourne Press, p 61.

Stock, A., Sann, E., and Pfleiderer, G. (1961), *Ann. Chem. 647,* 188.

Suelter, C. H., and Metzler, D. E. (1960), *Biochim. Biophys. Acta 44,* 23.

Wallenfels, K., and Gellrich, M. (1959), *Ann. Chem. 621,* 149.