Ronzio, R. A., Rowe, W. B., and Meister, A. (1969), *Biochemistry 8,* 1066.
Rowe, W. B., Ronzio, R. A., and Meister, A. (1969), *Biochemistry 8,* 2674.
Rubinstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20,* 67.
Stadtman, E. R., Ginsburg, A., Ciardi, J. E., Yeh, J., Hennig, S. B., and Shapiro, B. M. (1970), *Adv. Enzyme Regul. 8,* 99.
Tate, S. S., and Meister, A. (1973), in The Enzymes of Glutamine Metabolism, Prusiner, S., and Stadtman, E. R., Ed., New York, N.Y., Academic Press, p 77.
Timmons, R. B., Rhee, S. G., Luterman, D. L., and Chock, P. B. (1974), *Biochemistry 13,* 4479.
Villafranca, J. J., Ash, D. E., and Wedler, F. C. (1975), *Biochem. Biophys. Res. Commun., 66,* 1003.
Villafranca, J. J., Ash, D. E., and Wedler, F. C. (1976), *Biochemistry,* preceding paper in this issue.
Villafranca, J. J., and Wedler, F. C. (1974), *Biochemistry 13,* 3286.
Wangsness, R. K., and Bloch, F. (1955), *Phys. Rev. 89,* 728.
Wedler, F. C. (1974), *J. Biol. Chem. 249,* 5080.
Wedler, F. C., and Boyer, P. D. (1972), *J. Biol. Chem. 247,* 984.
Weisbrod, R. E., and Meister, A. (1973), *J. Biol. Chem. 248,* 3997.
Wolfenden, R. (1972), *Acc. Chem. Res. 5,* 10.
Wolga, G. J., and Tseng, R. (1964), *Phys. Rev. 133,* A1563.
Woolfolk, C. A., Shapiro, B., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 166,* 177.

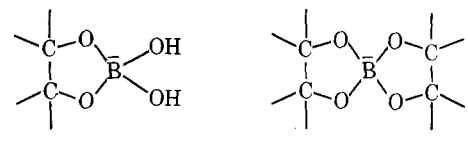
The Interaction of Borate and Sulfite with Pyridine Nucleotides†

S. L. Johnson* and Katherine Woo Smith

ABSTRACT: The kinetics and equilibria of the borate interaction at ribose with NAD^+ and NMN^+ have been measured using as a chromophoric probe the perturbation effect borate has on the addition of sulfite to the 4 position of the nicotinamide ring. NAD^+ and NMN^+ have more favorable borate association constants than do their corresponding sulfite addition complexes. The rate of interaction of the ribose moiety with borate at low borate buffer concentration is dependent on the concentration of both borate and boric acid. At high borate concentration the rate becomes independent of borate concentration, indicating the existence of a two-step process for the interaction of NAD–sulfite with borate with a change of rate-determining step from the interaction of the ribose hydroxyl group with borate at low borate to an elimination of sulfite at high borate concentration. A linear free energy relationship with a slope of 0.94 describes an increased reactivity of the nucleotide for sulfite as the affinity of the nucleotide for sulfite increases.

During an investigation of the rate of interaction of sulfite with NMN^+,[1] and NAD^+ as a function of pH using the technique of stopped-flow spectrophotometry, it was found that in borate buffers, two transients are observed instead of the single transient due to the addition of sulfite to the 4 position of the nicotinamide ring. That this phenomenon is due to the borate–ribose interaction was ascertained by the finding of only one reaction in nicotinamide cation analogues lacking the ribose moiety.

Borate is known to be toxic to living organisms (Thienes and Haley, 1964; Dreisbach, 1971). Few studies have been carried out on the mechanism of interaction of borate with enzymes and coenzymes. Borate is also known to competitively inhibit a number of dehydrogenases (Weser, 1968; Misawa et al., 1966; Roush and Gowdy, 1961; Deitrich, 1967). Because this inhibition is possibly due to the interaction of borate with the coenzyme we have studied the interaction of borate with NAD^+ and NMN^+ in an attempt to better understand the biological effects of borate. The interaction of borate with diols and sugars with adjacent hydroxyl groups is well known. Stable borate complexes of structure type I in dilute sugar and of structure type II, in more concentrated sugar, are formed (Weser, 1967a). The association constants[2] for the formation of I and II from borate, K_1' and K_2', are measured most conveniently by differential potentiometry in which the release of protons from diol solutions upon the addition of borate is measured (Kilpi, 1952). This method is more convenient for measuring K_2' because of the relative concentrations of reactants used.



I II

No measurement of borate–nucleotide association constants K_1' has been made, although K_2' values for two nu-

† From the Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261. *Received June 24, 1975.* This work was supported by Public Health Service Grant GM 16856.

[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NMN^+, nicotinamide mononucleotide; B, borate; BH, boric acid; NADB, borate complex of NAD^+; NAD-SO_3^-, sulfite complex of NAD^+; NADB-SO_3^-, sulfite complex of NADB; NMN-SO_3^-, sulfite complex of NMN^+; NADH, reduced nicotinamide adenine dinucleotide; ADPR, adenosine diphosphoribose.

[2] K_1' is defined as [I]/[diol][borate]; K_2' is defined as [II]/[diol]^2[borate].

cleosides have been determined potentiometrically by Weser (1967b). The perturbation of borate on the NAD-sulfite equilibrium (Pfleiderer et al., 1960) provided us with a very convenient tool for the measurement of K_1' for NAD^+ at enzyme substrate levels of concentration. This method also provided us with a very convenient method for measuring the rate of borate-nucleotide reaction. Save for the studies of the interaction of hydroxide ion and tartaric acid with boric acid, of lactic acid with phenylboronic acid, and of borate polymerization in concentrated borate solutions, few measurements have been made on the rates of borate reactions (Kustin and Pizer, 1969; Friedman et al., 1974; Anderson et al., 1964).

Experimental Section

NAD^+ and NMN^+ are products of Sigma Biochemicals. The concentration of NAD^+ was assayed using yeast alcohol dehydrogenase from Sigma. 1-Benzyl-3-acetylpyridinium chloride was prepared by mixing benzyl chloride with 3-acetylpyridine in benzene at room temperature. Recrystallization from ethanol yields a white product, mp 189–190 °C (uncor). Sodium sulfite and boric acid were used without further purification. To minimize the effect of the slow decomposition of sulfite ions, fresh sulfite solutions were used for all experiments. Sodium borate was prepared by neutralization of boric acid with NaOH. Reagent grade chemicals were used for the preparation of all the buffer solutions which were maintained at an ionic strength of 0.63 M with potassium chloride.

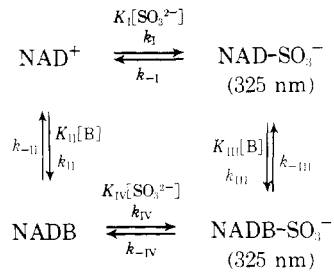
Absorption spectra were recorded with either a Cary Model 14 or a Model 16 recording spectrophotometer, using 3-ml quartz absorption cells. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian Model T-60 spectrometer. The measurements were made with solutions containing 0.14 M NAD^+ and 0–0.42 M sodium sulfite in D_2O. The pH was approximately 6.

The rate of production of $NAD\text{-}SO_3^-$ or $NMN\text{-}SO_3^-$ and their partial decomposition in the presence of borate were measured at 325 nm using a Durrum stopped-flow spectrophotometer. The cuvette for the apparatus is a fused silica tube with 2.0 cm incident light path and 20 mm inside diameter. The instrument dead time is 2.5 msec. Reactions are performed under pseudo-first-order conditions with sulfite concentration in large excess over that of substrate. The transmittance values from the oscilliscope trace are converted to absorbance values and first-order rate constants, k_{obsd}, are calculated from plots of log $(A_\infty - A_t)$ or log $(A_t - A_\infty)$ against time (Frost and Pearson, 1965). All plots are linear to 90% reaction. Second-order rate constants are obtained from the slopes of plots of k_{obsd} against sulfite or borate concentration. The data were treated using a linear least-squares program on the Olivetti P602 microcomputer.

A Cary Model 16 spectrophotometer thermostated at 25.0 ± 0.1 °C was used for equilibrium determinations. 20 μl of 0.044 M NAD^+ (0.046 M NMN^+) is introduced with an Eppendorf micropipet into a 1-cm quartz cuvette containing measured amounts of borate and buffer solution. The total volume is 3.0 ml and the initial ionic strength is 0.63 M. Manual measurements are made at 325 nm for NAD^+ (320 nm for NMN^+), giving the absorbance value, A, of the cell and its contents with air as reference. Aliquots of 0.5 M sodium sulfite are added to the cuvette. The contents of the cuvette is rapidly mixed and measurements of the absorbance are recorded after each sulfite addition. The procedure is repeated for monitoring the pH of the solution before and after addition of sulfite. The absorbance values are corrected for A and for the volume change due to sulfite addition. Double-reciprocal plots of absorbance against sulfite concentrations are made for various concentrations of borate (Benesi and Hildebrand, 1949). Assuming that the molar extinction coefficient, ϵ is the same for the pyridine nucleotide-sulfite complex and the pyridine nucleotide-sulfite-borate complex, ϵ is calculated from the average value[3] of the intercepts and the substrate concentration, c: $\epsilon = 1/(\text{intercept} \times c)$. The equilibrium constant, K_{app}, is calculated from the slope, substrate concentration, and molar extinction coefficient: $K_{app} = 1/(\text{slope} \times \epsilon c)$.

The values of K_{app} and the corresponding values of borate concentration were treated using a nonlinear least-squares curve fitting program on the DEC System-10 computer in order to determine the individual equilibrium constants of Scheme I.

Scheme I



The pH of the solutions was measured using a Radiometer Type TTT1D pH meter with a Type PHA 630T scale expander. The electrode system employed consisted of a G2222C glass electrode and a K4112 calomel electrode. For the kinetics experiments, the pH of the solutions is measured at the completion of the reactions. For equilibrium studies, the procedure for adding aliquots of sulfite to a solution of NAD^+ or NMN^+ in borate and buffer is repeated. The pH is measured before and after each sulfite addition.

Results

The equilibrium between pyridine nucleotide, sulfite, and the sulfite addition complex was measured spectrophotometrically by observing the absorption band of the complex at 320–325 nm characteristic of the complex, as a function of sulfite concentration at constant initial nucleotide concentration. Equilibrium constant determinations repeated in a series of experiments in which a constant amount of borate was present show a decrease in the apparent equilibrium constant for sulfite addition. These results are shown in Table I. Spectral scans of $NAD\text{-}SO_3^-$ show that it has identical spectral characteristics in terms of the wavelength of maximum absorption, in the presence or absence of borate. Borate does not affect the apparent equilibrium of complex formation in the case of non-ribose containing nucleotide analogues such as 1-benzyl-3-acetylpyridinium chloride. The effect of pH on the addition of sulfite to NAD^+ was studied separately at pH 5 and 8. In accordance with Pfleiderer et al. (1960) we observe that the addition of sulfite is dependent only on the dianionic form of sulfite.

Borate can interact at the ribose group with both NAD^+ and $NAD\text{-}SO_3^-$, and conversely sulfite can interact at the nicotinamide ring with both NAD^+ and NADB to form a

[3] The observed absorbance of solutions of $NAD\text{-}SO_3^-$ in the presence of excess sulfite remains constant upon the addition of borate in quantities sufficient to form $NADB\text{-}SO_3^-$.

Table I: Pyridine Nucleotide–Sulfite Equilibria in the Presence of Borate.[a,b]

Substrate	pH (±0.1)	$NaB(OH)_4$ Concn (mM)	K_{app} (M^{-1})	K_I (M^{-1})	K_{II} (M^{-1})	K_{III} (M^{-1})	K_{IV} (M^{-1})
NAD^+	10.4	0	43				
		0.50	28				
		1.2	15				
		2.5	15	40 ± 2	1500 ± 400	250 ± 90	6.7 ± 1.5
		7.5	8.3				
		25	7.4				
		38	8.4				
NAD^+	9.0	0	40				
		1.2	19				
		2.5	15				
		5.0	10	41 ± 1	1700 ± 300	350 ± 70	8.4 ± 0.7
		25	8.8				
		75	9.6				
NMN^+	9.0	0	25				
		1.2	14				
		2.5	12				
		5.0	8.7	25 ± 1	1900 ± 800	720 ± 300	9.3 ± 0.8
		7.5	12				
		25	10				
		75	9.2				

[a] Reactions performed in carbonate/bicarbonate buffer at 25°. Ionic strength varied from 0.63 to 0.70 M upon addition of sulfite. Sulfite concentration range is 3.3×10^{-3} to 5.1×10^{-2} M. Substrate concentration is 3.0×10^{-4} M. [b] Molar extinction coefficient for $NAD-SO_3^-$ and $NMN-SO_3^-$ is 4500 M^{-1} cm^{-1}.

chromophoric complex as shown in Scheme I, where $K_I K_{III} = K_{II} K_{IV}$. The observed absorption at 325 nm is given by:

$$A_{obsd} = \frac{N_0[SO_3^{2-}](K_I\epsilon_1 + K_{IV}\,\epsilon_2\,K_{II}[B])}{1 + K_{II}[B] + K_I[SO_3^{2-}] + K_{II}K_{IV}[B][SO_3^{2-}]} \quad (1)$$

N_0 is the total nucleotide concentration and ϵ_1, ϵ_2 are the extinction coefficients for $NAD-SO_3^-$ and $NADB-SO_3^-$. Considering ϵ_1 and ϵ_2 to be equal to ϵ, eq 1 is simplified to give, in its reciprocal form:

$$\epsilon N_0/A_{obsd} = \frac{1 + K_{II}[B] + [SO_3^{2-}](K_I + K_{II}K_{IV}[B])}{[SO_3^{2-}](K_I + K_{II}K_{IV}[B])} \quad (2)$$

The conventional double-reciprocal plot of $1/A_{obsd}$ vs. $1/[SO_3^{2-}]$ yields an apparent equilibrium constant, K_{app}, as the intercept divided by the slope:

$$K_{app} = \frac{K_I + K_{II}K_{IV}[B]}{1 + K_{II}[B]} \quad (3)$$

The constants derived from eq 3 are presented in Table I. The constants are pH independent from pH 9 to 10.4.

The rate of addition of sulfite to pyridine nucleotides was studied as a function of borate concentration. Figure 1 shows some typical results. In the absence of borate (Figure 1a) only one transient is observed upon mixing NAD^+ with sulfite. In the presence of borate, two different results are obtained, depending upon whether borate is premixed with NAD^+ or with sulfite. Borate premixed with sulfite gives rise to two transients as in Figure 1c. Borate premixed with NAD^+ gives rise to only one transient as in Figure 1d. The presence or absence of borate has no effect on non-ribose containing pyridine nucleotide analogues as shown in Figure 1b in the case of 1-benzyl-3-acetylpyridinium chloride.

The rate of the first transient in Figure 1c increases with sulfite concentration, while that of the second transient increases with borate concentration. These results indicate

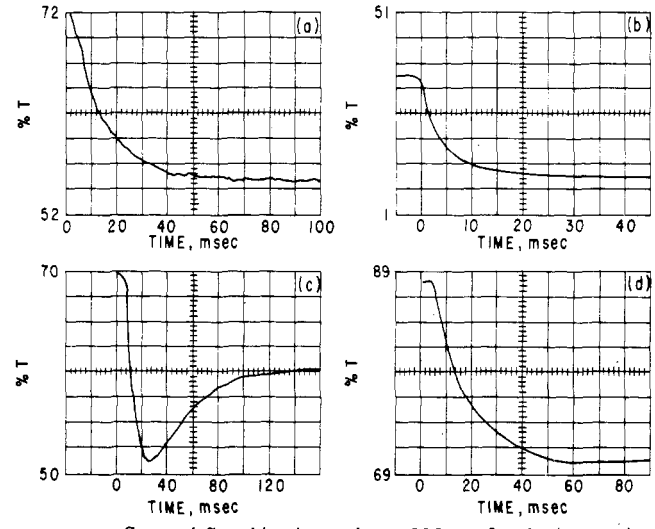


FIGURE 1: Stopped-flow kinetic results at 325 nm for the interaction of sulfite with NAD^+ and analogue. (a) Syringe I, NAD^+, 1.7×10^{-4} M; syringe II, 0.025 M sulfite, pH 8.5; (b) syringe I, 1-benzyl-3-acetylpyridinium chloride, 1.7×10^{-4} M; syringe II, 0.025 M sulfite and 0.15 M borate, pH 8.5; (c) syringe I, NAD^+, 1.7×10^{-4} M; syringe II, 0.025 M sulfite in 0.24 M borate buffer (pH 8.4); (d) syringe I, NAD^+, 1.7×10^{-4} M in 0.25 M borate buffer (pH 8.4); syringe II, 0.025 M sulfite (pH 8.4). Final ionic strength is 0.63 M.

that sulfite reacts rapidly with NAD^+ to produce $NAD-SO_3^-$ in the first phase of Figure 1c. This complex then reacts with borate in the second phase to produce $NADB-SO_3^-$. A new equilibrium between NADB and $NADB-SO_3^-$ is then established in the k_{IV}/k_{-IV} step as shown in eq 4:

$$NAD^+ \underset{k_{-I}}{\overset{k_I[SO_3^{2-}]}{\rightleftharpoons}} NAD\text{-}SO_3^- \underset{k_{-III}[H^+ + H_2O]}{\overset{k_{III}[B+BH]}{\rightleftharpoons}} \times$$

$$NADB\text{-}SO_3^- \underset{k_{IV}[SO_3^{2-}]}{\overset{k_{-IV}}{\rightleftharpoons}} NADB \quad (4)$$

Table II: Rate Constants for the Addition of Sulfite to Pyridine Nucleotides.[a]

Substrate	Buffer	pH (±0.1)	Concn Range, SO_3^{2-} (M)	k_{-I}[b] (s^{-1})	k_I[c] (M^{-1} s^{-1})
NAD^+	SO_3^{2-}, HSO_3^-	8.1	0.020–0.0025		1960 ± 130
		6.6	0.12–0.0050	39 ± 3	1880 ± 10
		5.2[d]	0.012–0.0020		2320 ± 360
NAD^+	0.019 M $NaB(OH)_4$, 0.0038 M H_3BO_3	9.8	0.10–0.010	68 ± 7	1760 ± 140
	0.0060 M $NaB(OH)_4$, 0.0080 M H_3BO_3	8.9	0.10–0.0050		2120 ± 130
NADB[e]	0.060 M $NaB(OH)_4$, 0.030 M H_3BO_3	9.5	0.10–0.0050		520 ± 4
	0.060 M $NaB(OH)_4$, 0.18 M H_3BO_3	8.5	0.10–0.010	56 ± 3	427 ± 26
NMN^+	CO_3^{2-}, HCO_3^-	8.9	0.10–0.0025		1180 ± 30
	SO_3^{2-}, HSO_3^-	7.2	0.18–0.0050	34 ± 5	1230 ± 20
		5.3	0.015–0.0015		1320 ± 100
NMN^+	0.019 M $NaB(OH)_4$, 0.0038 M H_3BO_3	9.7	0.050–0.0050	61 ± 16	2210 ± 360
	0.0060 M $NaB(OH)_4$, 0.0080 M H_3BO_3	8.9	0.10–0.0050		2210 ± 210
NMNB[e]	0.060 M $NaB(OH)_4$, 0.18 M H_3BO_3	8.5	0.10–0.0050	39	455 ± 30

[a] Constant ionic strength of 0.63 M maintained with KCl. Temperature is 25 °C. Substrate concentration is 1.7×10^{-4} M. [b] First-order rate constant obtained from intercept of plot of k_{obsd} *vs.* $[SO_3^{2-}]$. [c] Second-order rate constant for the first relaxation due to the addition of sulfite to the nicotinamide ring. [d] pH 5.2 ± 0.2. [e] NAD^+ or NMN^+ premixed with borate before reaction with sulfite.

Table III: Rate Constants for the Reaction between Borate and NAD^+/NMN^+.[a]

Substrate	pH (±0.1)	Buffer Concn Range (mM) $B(OH)_4^-/H_3BO_3$	$k_{plateau}$[b] (s^{-1})	k_{III}[c] (M^{-1} s^{-1})
NAD^+	9.8	95/19 – 4.7/9.5	22	355
	8.8	75/100 – 0.24/0.32	30	750
NMN^+	9.7[d]	95/19 – 4.7/0.95	35	580
	8.9	37/50 – 1.4/1.9	37	1360

[a] Ionic strength maintained at 0.63 M with KCl. Sulfite and substrate concentrations are 0.025 M and 1.7×10^{-4} M, respectively. The temperature is 25 °C. [b] Plateau value obtained from plot of k_{obsd} vs. borate concentration. The value of k_{-III} obtained from the intercept is 2 ± 1 s^{-1}. [c] Second-order rate constant for the second relaxation attributed to reaction between ribose of pyridine nucleotide and borate. [d] pH 9.7 ± 0.2.

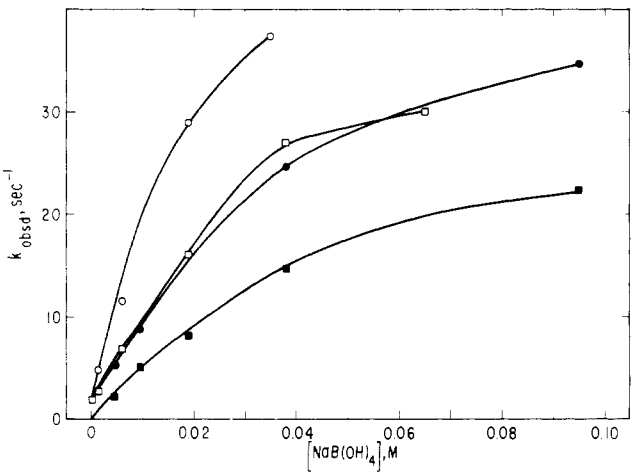


FIGURE 2: The rate of interaction of borate with $NAD\text{-}SO_3^-$ (solid symbols) and $NMN\text{-}SO_3^-$ (open symbols). Squares, 0.025 M sulfite at pH 9.8; circles, 0.025 M sulfite, pH 8.8.

In the absence of borate, a plot of the observed first-order rate constants vs. sulfite concentrations yields a straight line of slope k_I and intercept k_{-I} according to the equation for the rate of approach to equilibrium, eq 4. Values for the rate constants k_I and k_{-I} for sulfite addition to and elimination from NAD^+, NADB, NMN^+, and NMNB are given in Table II. Identical values of k_I are obtained in the presence or absence of borate providing that borate is not premixed with nucleotide, and under the condition $k_I[SO_3^{2-}] > k_{III}[B + BH]$. The absence of a pH dependence on k_I for NAD^+ and NMN^+ indicates that SO_3^{2-} is the only reactive sulfite species. When NAD^+ or NMN^+ is premixed with borate, the borated nucleotide substrate formed reacts 4–5 times less readily with sulfite.

The rate of the second slower reaction of Figure 1c depends more strongly on borate at low borate concentration than at high borate concentration as illustrated in Figure 2. This is indicative of a change in rate-determining step from the k_{III} to the k_{IV} step of eq 4. Starting with $NAD\text{-}SO_3^-$ as the reactant of eq 4 and proceeding to the right, the borate dependence on the overall rate will occur only when the k_{-IV} sulfite expulsion step is faster than the boration step. When the k_{III} step becomes very fast at high borate concentration, $NADB\text{-}SO_3^-$ accumulates and the reaction rate depends upon the rate of equilibration of the k_{IV}/k_{-IV} step, and becomes independent of borate concentration.

The initial slopes of the curves in Figure 2 increase at lower pH values (Table III) indicating a substantial boric acid term according to eq 5 where K_a is the dissociation constant of boric acid. Values for k_B and k_{BH} are as follows: NAD^+, 285 M^{-1} s^{-1}, 350 M^{-1} s^{-1}; NMN^+, 440 M^{-1} s^{-1}, 690 M^{-1} s^{-1}. The observed rate constant for the deboration of $NADB\text{-}SO_3^-$ can be calculated from the rate and equilibrium constants and is given by eq 6. The rate of the hydronium ion term is nearly diffusion limited.

$$\text{rate} = k_B[B] + k_{BH}[BH] = [B](k_B + k_{BH}[H^+]/K_a) \quad (5)$$

$$k_{obsd} = k_B[H^+]/K_{III}K_a + k_{BH}/K_{III} = 1.8 \times 10^9\,[H^+] + 1\ s^{-1} \quad (6)$$

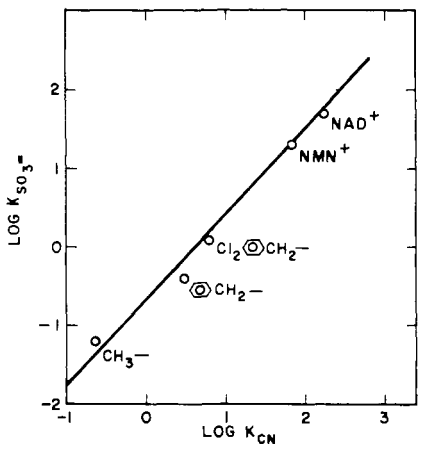


FIGURE 3: Linear free energy relationship between equilibrium constants for addition of cyanide and sulfite to nucleotides and N-substituted nicotinamide analogues. Data taken from Lindquist and Cordes (1968), Pfleiderer et al. (1960), Wallenfels and Diekmann (1959), and Smith and Johnson (unpublished work). Slope is 1.0.

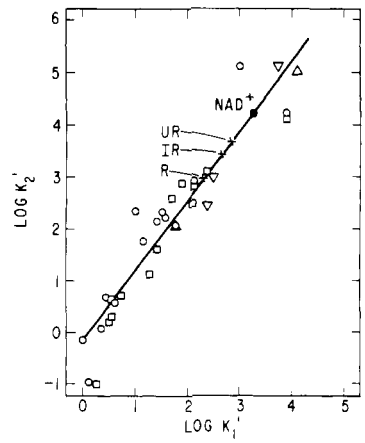
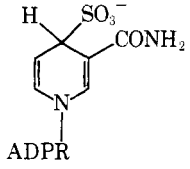


FIGURE 4: The relationship between K_1' and K_2' for a variety of sugars. Data taken from: squares, Roy et al. (1957); circles, Conner and Bulgrin (1967); triangles, Antikainen (1955, 1958) and Antikainen and Tevanen (1959); X, K_2' value only, Weser (1967b); solid circle, K_1' value only, this paper; R is ribose, UR is uridine, and IR is inosine. The line drawn is the least-squares line of slope 1.32 ± 0.59 and intercept −0.130 ± 0.118.

The structure of the sulfite addition complex was investigated by nuclear magnetic resonance. Treatment of NAD^+ with sulfite results in the progressive broadening of the nicotinamide signals at τ 2.31 (H_2); 2.44 (H_6) doublet, J = 7 Hz; 2.78 (H_4) doublet, J = 8 Hz; 3.48 (H_5) multiplet. The two adenine signals at τ 3.20 (H_2') and 3.36 (H_8') remain sharp and unperturbed at all sulfite concentrations. At high sulfite concentration new but broad signals emerge at τ 4.1, 4.9 (v. broad), 6.2, and 6.5. At lower sulfite concentration where the broadened nicotinamide protons were observed, no C–H exchange with the solvent D_2O was observed upon standing for 2 weeks.

Discussion

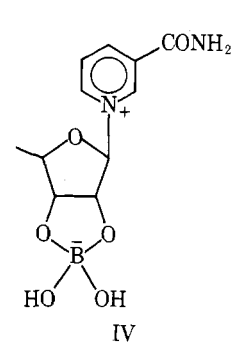
The interaction of sulfite with NAD^+ produces a covalent addition complex, the most probable structure for which is the 1,4-addition complex III, in which a carbon–



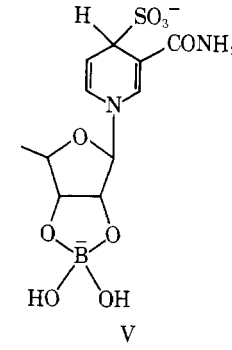
III

sulfur bond is formed to produce a sulfonate, in analogy with the structure of uracil–sulfite complexes (Hayatsu et al., 1970). The existence of only one transient for the formation of $NAD\text{–}SO_3^-$ indicates that no observable isomeric addition products are formed in the process of forming III. The 1,4-addition complex is the most likely complex to be formed in view of the structural work of Lindquist and Cordes (1968) on the cyanide addition compounds of nucleotides and analogues. The existence of a linear free energy relationship between the addition equilibria of cyanide and sulfite (Figure 3) with a slope of 1.0 is consistent with the formation of 1,4-complexes for both sulfite and cyanide reactions. The NMR spectrum of $NAD\text{–}SO_3^-$, though not completely resolved, is similar to the NMR spectra of the cyanide and dithionite complexes of NAD^+ analogues (Lindquist and Cordes, 1968; Diekmann et al., 1964; Caughey and Schellenberg, 1966).

The equilibrium of borate with NAD^+ and $NAD\text{–}SO_3^-$, as with the equilibrium with diols and sugars, is dependent only on borate (not boric acid), indicating that the reaction product has a net increase of one negative charge. The much more favorable equilibria of boration of nucleotide than of nucleotide–sulfite could be explained by electrostatic stabilization of negative charge on the boron by the positive charge of the nicotinamide cation nitrogen, IV, in contrast to the lack of such stabilization in the borated sulfite addition complex, V.



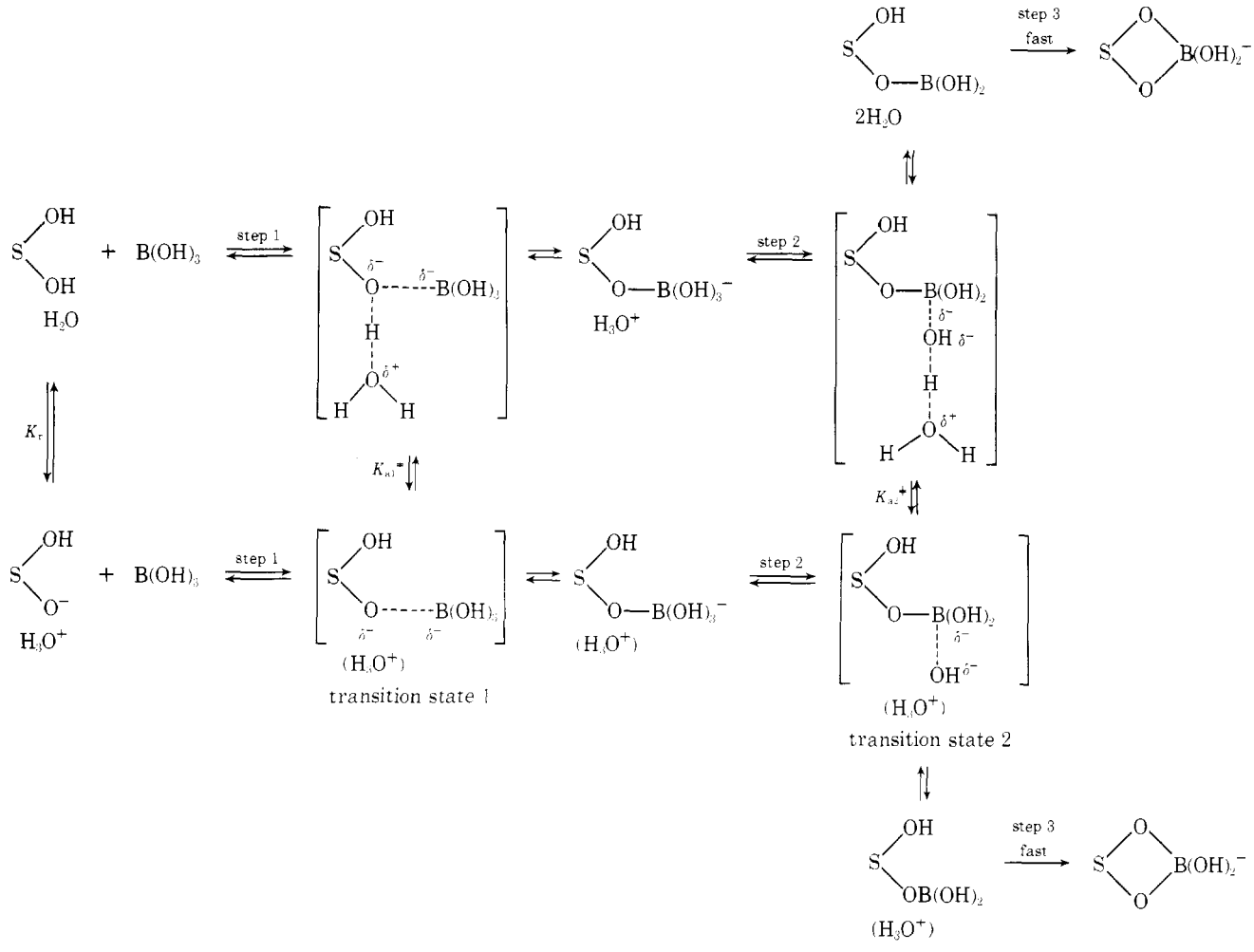
IV



V

The fact that NMN^+ and NAD^+ have nearly identical K_{II} values indicates that under the conditions used, complexation of the ribose adjacent to the nicotinamide moiety rather than to the adenine moiety is being measured in the K_{II} equilibrium. This would indicate that borate prefers to complex with the ribose adjacent to the positively charged nicotinamide than with the ribose adjacent to the neutral adenine. No direct information is available for the K_1' values of adenosine or other neutral nucleosides. The best information is the K_2' values of ribose and of two nucleosides, uridine and inosine, whose values are $10^{3.01}$, $10^{3.68}$, and $10^{3.42}$ M^{-2}, respectively (Weser, 1967b). In order to extrapolate from the more readily available K_2' values to the biologically significant K_1' values, a linear free energy relationship was found between K_2' and K_1' values for the same sugar (Figure 4). The K_1' value for NAD^+ is greater than the extrapolated K_1' values for ribose, inosine, and uridine by factors of 8.0, 3.8, and 2.4, respectively. By this analogy it would also be expected that NAD^+ will complex much more favorably with borate than NADH as is found in the accompanying paper (Smith and Johnson, 1976).

Scheme II



The sixfold greater ability of borate to complex with NAD^+ compared with $NAD\text{-}SO_3^-$ can be stated in an alternate way according to Scheme I: sulfite has a sixfold greater ability to complex with NAD^+ than with NADB. Because the complexation of sulfite with NAD^+ and N-substituted nicotinamide analogues follows a linear free energy relationship with the complexation of cyanide with a slope of 1.0, Figure 3, it would be expected that cyanide (and other addends) will add more readily to NAD^+ than to NADB. The great sensitivity to the charged nature of the ribose moiety in the present case is very similar to that found by Pfleiderer et al. (1960) in the case of addition of sulfite to NAD^+, and to the monoethyl and diethyl phosphate esters of NAD^+. The effect of the esterification of the phosphate groups was to remove one and two, respectively, negative charges from the phosphate-ribose moiety, and to correspondingly increase the addition equilibrium two-fold for each negative charge removed (or net increase in positive charge).

The equilibrium position of addition of sulfite to the nucleotides studied is related to the rate of addition, as shown in Figure 5. Because the reverse reaction shows little dependence on structural variations, the equilibrium constant is determined largely by the sulfite addition step. The slope, 0.94, is in contrast to the slope of 0.54 obtained by plotting the logarithm of the rate constant for addition of cyanide vs. the logarithm of the equilibrium constant, from the data on nucleotide analogues of Lindquist and Cordes (1968). This indicates that in the transition state for formation of III, carbon-sulfur bond making is about 94% complete and for formation of the cyanide adducts, 54% complete.

The observation of both a borate and a boric acid term in the expression for the rate of boration of the ribose moiety of $NMN\text{-}SO_3^-$ and $NAD\text{-}SO_3^-$ indicates that both the ribose anion and ribose interact with boric acid (eq 7 and 8). The alternative mechanism for the borate term, the interaction of ribose with borate anion, has very little likelihood. The microscopic constants for the processes in eq 7 and 8 are 350 M^{-1} s^{-1} and $285K_a/K_r = 1.8 \times 10^6$ M^{-1} s^{-1}, respectively, where K_a is $10^{-9.2}$, the dissociation constant of borate, and K_r is the dissociation constant of ribose, which is approximately 10^{-13} in analogy with the dissociation constants of adenosine monophosphate (Christensen et al., 1970). The latter value of k_B' for the ribose anion is smaller by approximately $10^{3.7}$ than the corresponding value for hydroxide ion (Anderson et al., 1964), and larger by $10^{2.9}$ than the value for tartrate dianion (Kustin and Pizer, 1969).

$$ROH + B(OH)_3 \xrightarrow{k_{BH}} ROB(OH)_3^- + H^+ \qquad (7)$$

$$RO^- + B(OH)_3 \xrightarrow{k_B'} ROB(OH)_3^- \qquad (8)$$

The boration of dihydroxy compounds is a three-step process involving (1) the addition of the first substrate hydroxyl group to boric acid yielding an anionic borate ester

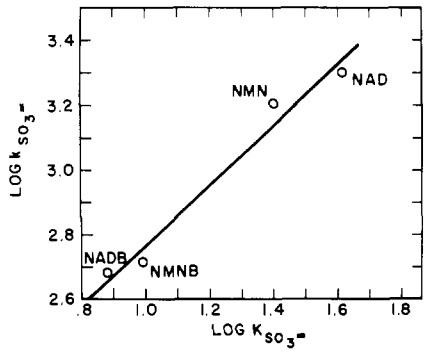
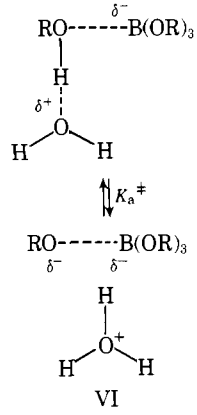


FIGURE 5: Linear free energy relationship between addition rate of sulfite to nucleotides and the equilibrium constant of addition. The slope is 0.94.

$ROB(OH)_3^-$, (2) the elimination of one hydroxyl group from $ROB(OH)_3^-$ to yield $ROB(OH)_2$, (3) addition of the second substrate hydroxyl group to give the cyclic borate ester. These steps and their transition states are shown in Scheme II. It is reasonable to assume that either of the intermolecular first two steps are rate limiting rather than the intramolecular third step. Assuming similar transition states for the borate and boric acid terms, the acid dissociation constant, $K_a^{\ddagger}$ of the transition state VI is equal to $k_B'K_r/k_{BH} = k_BK_a/k_{BH} = 10^{-9.1}$, as calculated according to the method of Kurz (1972). The degree of O-B bond making is about 26% in the first transition state or the degree of O-B bond breaking is about 62% in the second transition state. The result for the boration of pyridine nucleotide for which the ratio k_B'/k_{BH} is 5×10^3, is in marked contrast to the corresponding ratios obtained for the boration of tartrate dianion/tartaric acid, 1/2.2, and for the phenylboration of lactate/lactic acid, 10.1 (Kustin and Pizer, 1969; Friedman et al., 1974). For the α-hydroxy carboxylic acids, the transition state acidities are much greater than for the α-dihydroxy ribose derivative. For the phenylboration of lactic acid, $K_a^{\ddagger}$ is $10^{-2.9}$. These transition state parameters would indicate different transition states for the two classes of compounds forming cyclic borate esters.



VI

In summary, the present kinetic and equilibrium studies on the addition of sulfite and borate to NAD^+ and its analogues show that (1) borate reacts with the ribose adjacent to the nicotinamide cation, (2) both ribose and ribose anion react with boric acid, (3) the transition state for the interaction of sulfite and nucleotide is 94% progressed from reactants to products, and (4) the structural parameters for the equilibrium addition of sulfite to nucleotides have the same characteristics as the addition of cyanide to nucleotides.

Acknowledgment

We are grateful to Mrs. Beatrice Goodman for preparing the manuscript.

References

Anderson, J. L., Eyring, E. M., and Whittaker, M. P. (1964), *J. Phys. Chem. 68,* 1128.
Antikainen, P. J. (1955), *Acta Chem. Scand. 9,* 1008.
Antikainen, P. J. (1958), *Suom. Kemistil. B 31,* 255.
Antikainen, P. J., and Tevanen, K. (1959), *Suom. Kemistil. B 32,* 214.
Benesi, H. A., and Hildebrand, J. H. (1949), *J. Am. Chem. Soc. 71,* 2703.
Caughey, W. S., and Schellenberg, K. A. (1966), *J. Org. Chem. 31,* 1978.
Christensen, J. J., Rytting, J. H., and Izatt, R. M. (1970), *J. Chem. Soc. B,* 1646.
Conner, J. M., and Bulgrin, V. C. (1967), *J. Inorg. Nucl. Chem. 29,* 1953.
Deitrich, R. A. (1967), *Arch. Biochem. Biophys. 119,* 253.
Diekmann, H., Englert, G., and Wallenfels, K. (1964), *Tetrahedron 20,* 281.
Dreisbach, R. H. (1971), Handbook of Poisoning: Diagnosis and Treatment, 7th ed, Los Altos, Calif., Lange Medical Publication, p 306.
Friedman, S., Pace, B., and Pizer, R. (1974), *J. Am. Chem. Soc. 96,* 5381.
Frost, A. A., and Pearson, R. G. (1965), Kinetics and Mechanism, 2nd ed, New York, N.Y., Wiley, p 13.
Hayatsu, H., Wataya, Y., and Kai, K. (1970), *J. Am. Chem. Soc. 92,* 724.
Kilpi, S. (1952), *J. Am. Chem. Soc. 74,* 5296.
Kurz, J. L. (1972), *Acc. Chem. Res. 5,* 1.
Kustin, K., and Pizer, R. J. (1969), *J. Am. Chem. Soc. 91,* 317.
Lindquist, R. N., and Cordes, E. H. (1968), *J. Am. Chem. Soc. 90,* 1269.
Misawa, T., Kaneshima, H., and Akagi, M. (1966), *Chem. Pharm. Bull. 14,* 467.
Pfleiderer, G., Sann, E., and Stock, A. (1960), *Chem. Ber. 93,* 3083.
Roush, A. H., and Gowdy, B. B. (1961), *Biochim. Biophys. Acta 52,* 200.
Roy, G. L., LaFerriere, A. L., and Edwards, J. O. (1957), *J. Inorg. Nucl. Chem. 4,* 106.
Smith, K. W., and Johnson, S. L. (1976), *Biochemistry,* following paper in this issue.
Thienes, C. H., and Haley, T. J. (1964), Clinical Toxicology, Philadelphia, Pa., Lea and Febiger, p 277.
Wallenfels, K., and Diekmann, H. (1959), *Justus Liebigs Ann. Chem. 621,* 166.
Weser, U. (1967a), *Struct. Bonding (Berlin) 2,* 160.
Weser, U. (1967b), *Z. Naturforsch. B 22,* 457.
Weser, U. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 1479.
Zittle, C. A. (1951), *Adv. Enzymol. Relat. Areas Mol. Biol. 12,* 493.