Kinetics and Mechanism of the Cleavage of Thiamin, 2-(1-Hydroxyethyl)thiamin, and a Derivative by Bisulfite Ion in Aqueous Solution. Evidence for an Intermediate

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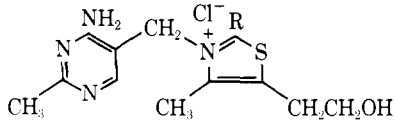
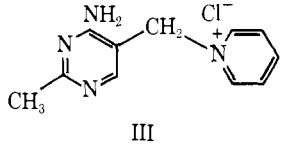
Abstract: Thiamin (I), 2-(1-hydroxyethyl)thiamin (II), and 1-[(2-methyl-4-amino-5-pyrimidinyl)methyl]pyridinium chloride (III) show bell-shaped pD-rate profiles for their second-order cleavage reactions by sulfite ion in D_2O at 25.0 °C and 1 M ionic strength. Products are 2-methyl-4-amino-5-pyrimidinylmethanesulfonic acid and a thiazole from I and II and pyridine from III. The maximum value of the observed second-order rate constant falls near pD 6.5 and the relative reactivity order based on pD-independent second-order rate constants is 1:0.62:2.0 for I, II, and III, respectively. Only III was studied at pD values low enough to determine accurately a rate constant for bisulfite ion; the rate constant ratio for sulfite to bisulfite ion is 1.4×10^4. The presence of azide ion was found to give rise to the formation of 2-methyl-4-amino-5-pyrimidinylmethyl azide, at times as the major product containing the pyrimidine ring. No azide product forms in the absence of sulfite ion and azide ion does not influence the rates of cleavage reactions even when it leads to major amounts of azide product. In order to explain these results, which require separate rate and product determining steps, a novel mechanism is advanced. It consists of the addition of sulfite ion (and bisulfite ion at low pD) to position 6 of a pyrimidine ring of substrate present as its conjugate acid. The intermediate adduct then undergoes reactions which lead to the observed substitution products. The initial rates of competition reactions giving rise to substitution products by the action of sulfite and azide ions reveal that sulfite ion is about 54 times more nucleophilic toward an intermediate than azide ion. It is suggested that in some enzymic reactions involving thiamin, a similar addition mechanism may take place.

Victory in the two-continent race to determine the structure of thiamin (I or vitamin B-1) was assured to Williams when he discovered in 1935 that this molecule was readily cleaved by aqueous solutions of bisulfite ion under mild conditions to give two easily characterizable products.[1,2] In spite of a thorough kinetic study in 1969,[3] a mechanism has never been established for this cleavage reaction or for a related process in which sulfite ion acts as a catalyst to convert thiamin in the presence of nucleophiles to derivatives.[4-6]

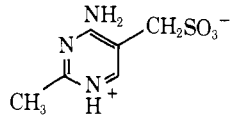
We report the results of a kinetic study involving the cleavage by aqueous sulfite ion of 1, 2-(1-hydroxyethyl)thiamin (11 or HET), and a derivative of these, 1-[(2-methyl-4-amino-5-pyrimidinyl)methyl]pyridinium chloride (III). We observe that azide ion, when added to these reactants, markedly alters the composition of product mixtures without influencing the rates of cleavage. Thus, our study clearly establishes separate rate and product determining steps which demand the formation of an intermediate.



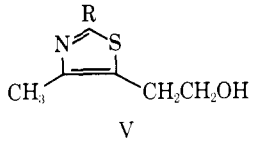
I, R = H
II, R = $CH(OH)CH_3$



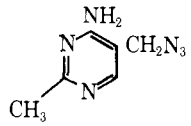
III



IV



V



VI

We are able to establish a mechanism which for the first time reconciles discordant observations appearing in the literature. Our novel mechanism focuses on the pyrimidine ring of substrates. This suggestion stands in contrast to the generally accepted chemistry and biochemistry of vitamin B-1 which deals with its thiazole portion.[7,8] The characteristics of the sulfite ion cleavage reaction are sufficiently similar to those involving the enzyme thiaminase I, thiamin, and nucleophiles[9] that we are lead to suggest a similar mechanism of action for the enzyme.

Results

Rates of Cleavage. An early, detailed study showed that the cleavage of thiamin by sulfite ion to yield the corresponding sulfonic acid IV (shown as the betaine) and thiazole V (R = H) followed second-order kinetics, being first order in substrate and in sulfite ion.[3] The acidity of the medium has a pronounced influence on rates of cleavage; it appears to us that the pH-rate profile is "bell shaped",[10] although rate constants were not obtained at sufficiently high pH to show the symmetry of the "bell" in this early study.

Our first aim was to reconstruct the profile for thiamin. In order to employ NMR as a method of analysis which allows the concentrations of reactants and products to be monitored, D_2O was chosen as the solvent and the ionic strength was 1 M, unlike the early investigation which employed dilute solutions of substrates and salts in H_2O.[3] Working at the high concentrations (0.10-0.12 M) of organic substrates required of this analytical method compelled the use of a pH stat to maintain the pD of the medium at a constant value. As seen in Figure 1, our pD-rate profile clearly shows a bell shape. Comparison of the two studies (25 °C) reveals that the maximum values of the rate constants (taken at the top of the bell shape) are similar, being 1.4×10^{-2} (H_2O) and 6.32×10^{-3} M^{-1} s^{-1} (D_2O), but this maximum falls at pH 5.7 and at pD 6.5. The 2.2-fold larger value for the cleavage at low ionic strength is not unexpected for a reaction involving the combination of two ionic species. The shift in the position of the maximum value for the rate constant also is consistent with the influence of

ionic strength on pK_a values and a change to D_2O solvent which brings about an increase in pK_a.[11]

Rates of cleavage are given by

$$\text{rate} = k_{obsd}[S]_t[SO_3^{2-}]_t = k_2[DS^+][SO_3^{2-}] \quad (1)$$

and the observed second-order rate constant, k_{obsd}, by

$$k_{obsd} = \frac{k_2[D^+]}{([D^+] + K_a)} \frac{K_a^s}{([D^+] + K_a^s)} \quad (2)$$

where $[S]_t$ is the total concentration of substrate, $[SO_3^{2-}]_t$ equals $[SO_3^{2-}] + [DSO_3^-]$, K_a is the dissociation constant of the organic substrate, $[D^+][S]/[DS^+]$, and K_a^s is the dissociation constant of bisulfite ion. Equation 1 indicates that the acidic form of the substrate reacts with sulfite ion.

The pD-rate profile for I was redetermined using azide ion along with sulfite ion. In two cases (pD 4.9 and 5.4) equal amounts of sulfonic acid IV and azido compound VI were formed early in each run while at pD 6.4 13% of VI was produced. No azide product was detected at pD 7.4. The k_2 values, eq 1, calculated from runs with and without azide ion are identical within the uncertainty limits of the experiments.

Similar pD-rate profiles were obtained for II, a key intermediate (as a pyrophosphate) in biochemical transformations involving I and pyruvic acid[8] and for pyridine substrate III, a relative of important antivitamins.[12] With II, azide product was not formed at pD 7.4 but it was produced at pD 5.4 and 6.4. One run involving II at pD 5.4 gave 70% of VI. Yet k_2 calculated according to eq 1 was essentially the same as that obtained when no VI was produced. Similarly, no dependence of k_2 on azide ion concentration was detected in the case of III when the yields of VI varied from 0 to 74%. The profiles for the three substrates shown in Figure 1 contain all the results, those obtained with and without azide ion. Clearly, azide ion does not influence the rate constants for the cleavage of I–III.

Careful analysis of the rate profiles indicates that there is no significant difference between the values of dissociation constants determined titrimetrically and those calculated in fitting a curve to the experimental points in Figure 1. A good fit of the data points is obtained when eq 2 is employed to calculate how k_{obsd} varies with pD.

On comparison with thiamin, II shows a slightly lower reactivity; its maximum observed second-order rate constant is only about 10% less than that of thiamin while pyridine derivative III is about 70% more reactive than thiamin. The position of this maximum is very nearly the same for all the substrates, about pD 6.5.

As the insert in Figure 1 shows, the curve calculated to fit the points at low pD values for III systematically underestimates the values of k_{obsd}. It appears that a second nucleophile, DSO_3^-, must also be reacting with III under these conditions.

$$\text{rate} = k_2[DS^+][SO_3^{2-}] + k_3[DS^+][DSO_3^-] \quad (3)$$

$$k_{obsd} = \frac{[D^+]}{[D^+] + K_a}\left(\frac{k_2K_a^s}{[D^+] + K_a^s} + \frac{k_3[D^+]}{[D^+] + K_a^s}\right) \quad (4)$$

Equation 3 describes the rates of cleavage and eq 4 the dependence of the observed second-order rate constant on the fractional amount of substrate in its acidic form and on the fraction of the total sulfite ion concentration representing the quantities of SO_3^{2-} and DSO_3^-. Inclusion of an additional kinetic term containing rate constant k_3 associated with bisulfite ion now provides a satisfactory fit of the data. All the rate and equilibrium constants used to construct the three profiles are given in Table I. Presumably there are similar k_3 kinetic terms representing reaction of the conjugate acid forms of the two other substrates with bisulfite ion. Our studies did not extend to sufficiently low pD values to establish such terms accurately. The ratios of the pD independent second-order rate constants k_2 are 1:0.62:2.0 for I:II:III. As required,[10] the rate maximum falls at the average of the two pK_a values for substrate and bisulfite ion.

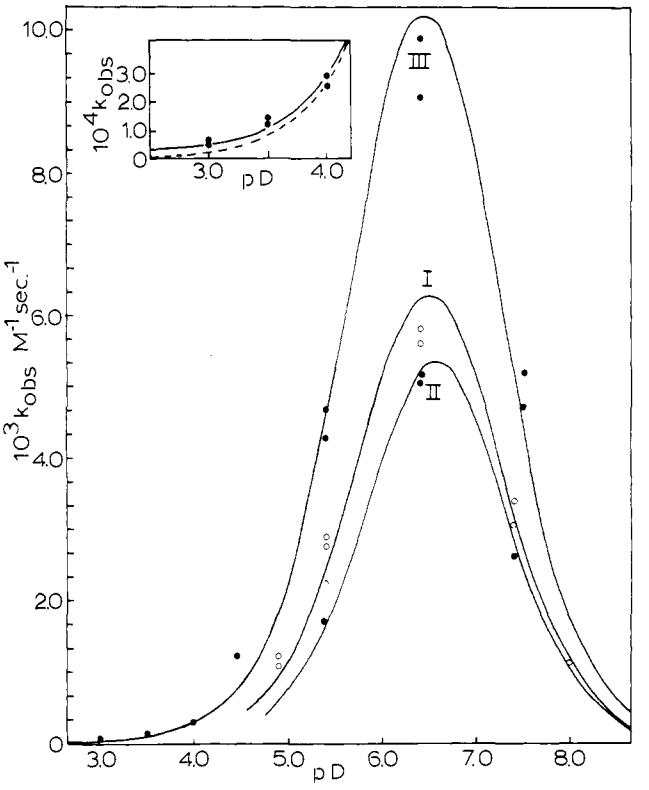
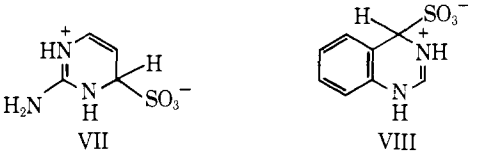


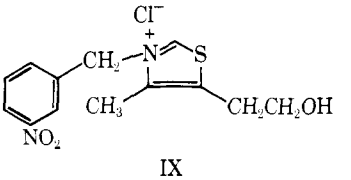
Figure 1. pD-rate constant profiles for the second-order cleavage of I–III by sulfite ion at 25.0 °C in D_2O at 1 M ionic strength. Points are experimental values while the curves are the result of calculations using eq 2 for I and II and eq 4 for III and the rate and equilibrium constants in Table I. The insert shows an expanded portion of the curve for III at low pD. The dotted line is based on eq 2 which neglects the contribution of DSO_3^- while the solid line is calculated using eq 4 which includes a term for DSO_3^-.

The ratio of second-order rate constants for reaction of III with SO_3^{2-} and with DSO_3^-, k_2/k_3, is 1.4×10^4. Comparing this ratio with those reported for other substrates demonstrates similarities. When these two nucleophiles add to protonated 2-aminopyrimidine to give VII the more basic nucleophile is 1.1×10^4 (25 °C) times more reactive;[13] when the nucleophiles compete for protonated quinazoline to give VIII, the value is 1.4×10^5 (25 °C).[14] The similarity between our value and the two others provides additional strong support for our kinetic analysis.



VII

VIII



IX

Compound IX was examined briefly. It contains the same thiazole ring as thiamin, but has a nitrobenzene ring in place of the pyrimidine group. It did not undergo a cleavage reaction either in the presence of sulfite ion or in a mixture of sulfite and azide ions. A similar lack of reactivity toward sulfite ion has been noted for the ortho and para nitrobenzyl derivatives of IX.[4]

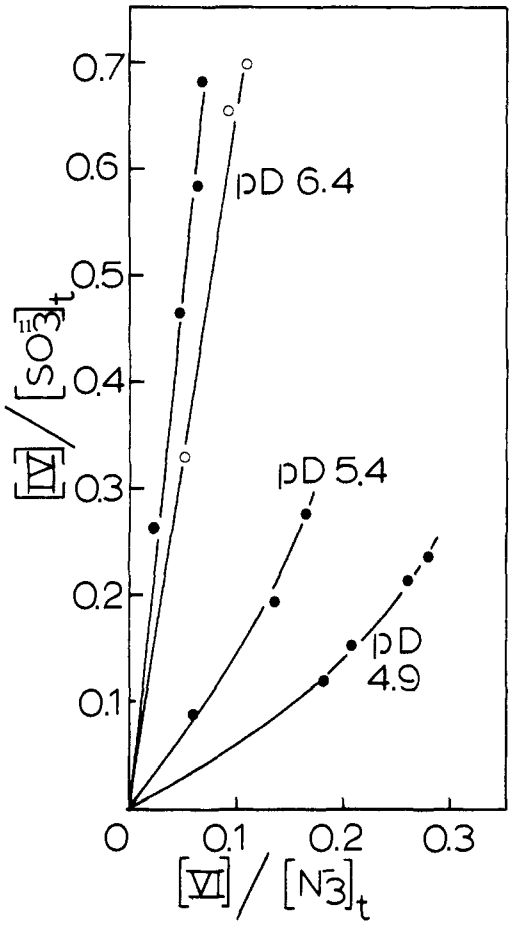


Figure 2. Product ratio plots for thiamin (I) to show the fraction of initial, total sulfite and azide ions converted to their respective products IV and VI as a function of the pD of the medium. The $[SO_3^{2-}]_t/[N_3^-]_t$ ratio at pD 4.9 is 1.29, at 5.4 it is 0.57, and at 6.4, 0.65 (filled circles) and 0.33 (open circles). The initial, total concentrations of sulfite ion are 0.19, 0.13, 0.098, and 0.049 M, respectively.

Trapping an Intermediate. For many years it has been known that thiamin and related compounds such as III react with nucleophiles in the presence but not in the absence of sulfite ion. The group bonded to the methylene carbon atom attached to the pyrimidine ring of these substrates is replaced by a nucleophile which may be, for example, a thiazole, pyridine, or an aniline.[4-6] In the case of substituted anilines, their ability to compete with sulfite ion is related to their basicity.[6] No satisfactory mechanism has ever been advanced to explain the catalytic role of sulfite ion in these transamination reactions.

We carried out preliminary experiments to obtain information to allow us to design competition reactions and to provide data about the possible reversibility of product formation in kinetic studies. It is clear from our preparation of III from I under conditions where the ratio of pyridine to sulfite ions is kept very large that pyridine can compete with sulfite ion. However, a control experiment in which the initial ratio of the concentrations of free pyridine to sulfite ion was 17 failed to show the formation of III from I by NMR analysis. So pyridine is not a very effective competitor of sulfite ion. Therefore, it seems unlikely that in our kinetic studies involving III the liberated pyridine successfully competes with sulfite ion to regenerate III to any significant degree. Even after 2 half-lives when a substantial amount of pyridine has formed and some sulfite ion has been consumed by the formation of IV, the ratio of the concentrations of these two nucleophiles is <20. This includes runs at the lowest pD where pyridine gains an advantage over sulfite ion because, as a weaker base, less of it is converted to its conjugate acid than in the case of sulfite ion.

Table I. Rate and Equilibrium Constants Used to Calculate the pD-Rate Profiles in Figure 1 at 25 ± 0.1 °C and 1.0 M Ionic Strength[a]

Compd	pK_a	k_2,[b] M^{-1} s^{-1}
$NaDSO_3$	7.14 ± 0.05	
DN_3	4.94 ± 0.03	
Pyridine	6.06 ± 0.02	
Thiamin (I)	5.83 ± 0.03	0.192 ± 0.020
HET (II)	6.00 ± 0.04	0.118 ± 0.007
III	5.73 ± 0.03	0.378 ± 0.045
		2.72×10^{-5}[c]

[a] In D_2O. [b] For sulfite ion. [c] For bisulfite ion, k_3.

Reversibility in the case of I and II is also expected to be unimportant under the conditions of our kinetic studies. Thiazoles V are less basic and therefore less nucleophilic than pyridine.[9] Although the lower basicity ensures that a higher fraction of free thiazole than of free pyridine will be present in acidic solution, this concentration advantage is expected to be insufficient to offset decreased nucleophilicity, especially in the case of sterically hindered thiazole V where R = $CH(OH)CH_3$.[15]

The kinetic results themselves suggest that regeneration of starting material in reactions involving liberated thiazole or pyridine is insignificant. Kinetic plots do not show downward curvature indicative of reversibility.

Azide ion was found to be a good competitor of sulfite ion in forming product containing the pyrimidine portion of starting material. But it does not react in the absence of sulfite ion. Thus, a control run showed that I and an excess of azide ion underwent no observable change (NMR) even after 70 h at 60 °C. These conditions are far more severe than those of the kinetic investigations.

We have been unable to displace the sulfonic acid group from IV in the presence of sulfite ion by any of the nitrogen nucleophiles used in this study, even at elevated temperatures. Sulfonic acid IV is the most stable material which contains the pyrimidine ring in our study. Therefore, when sufficient sulfite ion is present, the pyrimidine portion of I-III must eventually be converted entirely to IV in any competition study leading to multiple substitution products.

Seventy percent of the kinetic runs shown in Figure 1 contained azide ion. Thirty-two percent of the total produced azide VI in at least 50% yield. In some cases the ratio of azide VI to sulfonic acid IV was as much as 2 or 3 to 1. Yet in all these cases second-order rate constants had the same value within experimental error as those determined in the absence of azide ion. These results too argue against significant reversible cleavage of substrates I-III. Clearly, azide ion has no influence on the rate of cleavage of substrates I-III but it can have a marked influence on the identity and competition of product mixtures. This is strong evidence for the occurrence of an intermediate.

Figures 2-4 provide quantitative information about the product composition of reaction mixtures involving I-III and azide and sulfite ions as determined by NMR analysis. These plots show the fraction of each nucleophile converted to product containing the pyrimidine ring as a function of pD. They are constructed from information obtained about product composition throughout a kinetic run. Only a small variation in product distribution occurs because analyses were not made late in the run when substantial amounts of VI began to be converted to IV and because a major change in the rate of

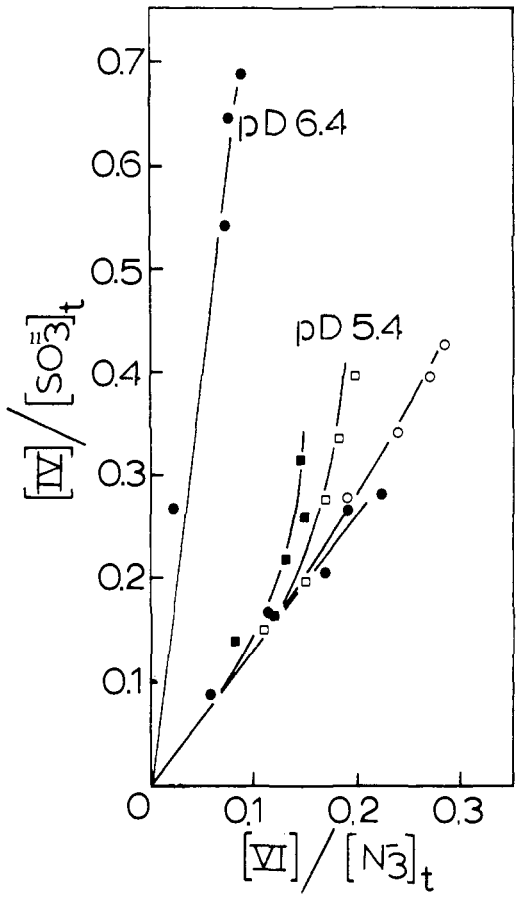


Figure 3. Product ratio plots for HET (II). The $[SO_3^{2-}]_t/[N_3^-]_t$ ratios at pD 5.4 for the four runs are 0.48 (filled squares), 0.28 (open squares), 0.59 (open circles), and 0.82 (filled circles) and at pD 6.4, 0.20. The sulfite concentrations are 0.14, 0.083, 0.085, 0.11, and 0.055 M, respectively.

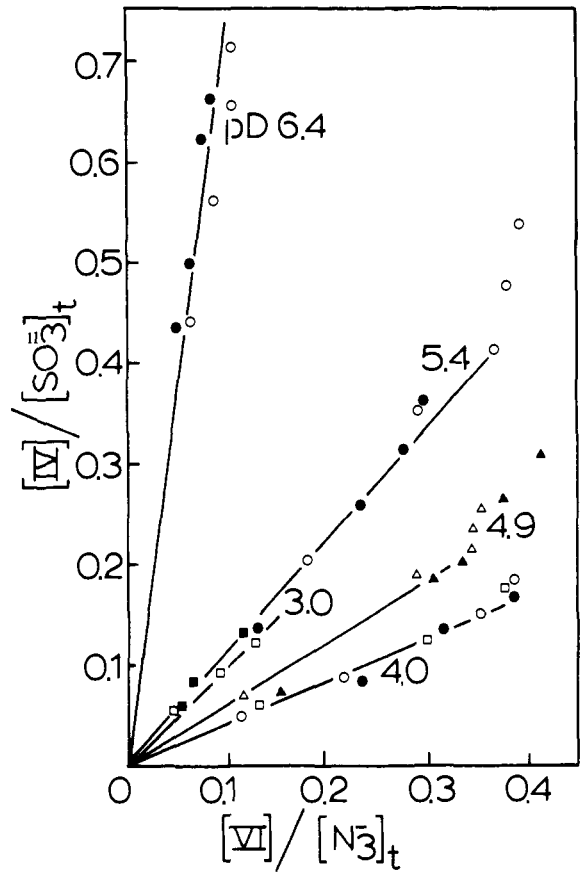


Figure 4. Product ratio plots for III. The $[SO_3^{2-}]_t/[N_3^-]_t$ ratio at pD 3.0 is 1.32 (filled squares) and 1.27 (open squares), at pD 4.0, 0.67 (open circles) 2.11 (open squares), and 1.12 (filled circles), and at pD 4.9, 1.29 (open triangles) and 0.73 (filled triangles), and at pD 5.4, 0.48 (open circles) and 0.73 (filled circles), and at pD 6.4, 0.66 (filled circles) and 0.53 (open circles). The concentrations of sulfite ion are 0.19, 0.18, 0.097, 0.19, 0.17, 0.19, 0.11, 0.073, 0.11, 0.082, and 0.066 M, respectively. For clarity, two runs at pD 3.5 are not shown. Here the initial slope is 0.62 and the concentration ratios are 1.29 and 1.93 with the sulfite ion concentrations being 0.19 and 0.18 M, respectively.

formation of one product must take place before the product ratio established early in a reaction is perturbed markedly.

Product ratio plots show upward curvature at low pD values. This is a result of the conversion of azide VI to sulfonic acid IV. However, at pD 6.4 the plots are linear. This apparent linearity is a consequence of two opposing effects. Because IV initially is forming so much faster than VI, owing to the higher concentration of sulfite ion at the higher pD, the concentration of sulfite ion decreases more rapidly than the concentration of azide ion. This change, if it were acting alone, would give rise to a downward curving plot. However, the conversion of VI and IV with its upward curvature, as seen at low pD, just counterbalances the decreasing rate of formation of IV to give linearity. It should be noted that at high pD the formation of IV from VI occurs earlier in the reaction because the initial sulfite ion concentration is larger.

Product ratio plots for III, Figure 4, the most reactive substrate among I–III, demonstrate linearity over a longer reaction interval at low pD than do I and II. The difference between the rates of cleavage of III and VI is greater than in the case of I or II and VI and so the back reaction of VI to give IV is negligible for a longer reaction interval.

The competition for intermediate must involve two bimolecular processes. Sulfonic acid IV cannot form by an intramolecular, first-order rearrangement in competition with a bimolecular reaction involving azide ion. Because the initial slopes of the plots in Figures 2–4 are independent of the initial concentrations of the two nucleophiles, reactions leading to IV and to VI must have the same kinetic order. This is likely to be first order in intermediate and first order in nucleophile.

The initial slopes of the plots in Figures 2–4 give quantitative information about the relative ability of the nucleophiles bisulfite, sulfite, and azide ions to trap an intermediate to give IV and VI. According to eq 5

$$\text{d(IV)/d(VI) initial slope} \simeq \left(\frac{k^{SO_3^{2-}} K_a^s}{[D^+] + K_a^s} + \frac{k^{DSO_3^-}[D^+]}{[D^+] + K_a^s}\right)[SO_3^{2-}]_t \Big/ \frac{k^{N_3^-} K_a^N}{[D^+] + K_a^N}[N_3^-]_t \quad (5)$$

the initial rate of formation of IV with respect to VI, before significant amounts of VI are converted to IV, provides rate constant ratios. This equation was applied in an equivalent form, eq 6, which is written in terms of product percentages.

$$\frac{\%\ \text{IV}/[SO_3^{2-}]_t}{\%\ \text{VI}/[N_3^-]_t} \simeq \frac{k^{SO_3^{2-}} K_a^s + k^{DSO_3^-}[D^+]}{[D^+] + K_a^s} \Big/ \frac{k^{N_3^-} K_a^N}{[D^+] + K_a^N} \quad (6)$$

These percentages were normalized by dividing them by the initial, total concentrations of appropriate nucleophiles so that plots for several runs at the same pD would show the same initial slope. Rate constant ratios are calculated from the initial slopes after correcting the total concentration of nucleophile for the fractional amount present in a reactive form; K_a^N is the dissociation constant of DN_3, Table I. Before numerical values are considered two comments must be made.

The ability of bisulfite ion to react with an intermediate is clearly revealed in the run at pD 3.0 for III, Figure 4. There is a larger initial slope than would be expected if the competition involved only sulfite and azide ions. The slope is large because there are two pathways leading to IV. The bisulfite term also makes a significant contribution to the run at pD 3.5. Presumably, bisulfite ion would also compete for an intermediate produced from I and II but studies were not carried out at pD values low enough to provide the necessary information.

Although there are two lines involving two different runs at pD 6.4 for I, Figure 2, their slopes give rate constant ratios (42 and 62) within the uncertainty of our measurements. This result shows that at high pD values where one compound is being formed in large perference over the other, rate constant ratios have the largest uncertainty.

The average rate constant ratio $k^{SO_3^{2-}}/k^{N_3^-}$ for I is 54 ± 7, for II it is 55 ± 2, and for III, 53 ± 4. If the values for the pD 6.4 runs are excluded, they become 57 ± 3, 57 (single pD study), and 54 ± 4. Sulfite ion is about 54 times more reactive than azide ion. For III, $k^{DSO_3^-}/k^{N_3^-}$ is 0.0077, indicating that bisulfite is 130 times less nucleophilic than azide ion. Comparison of the two rate constant ratios for III allows $k^{SO_3^{2-}}/k^{DSO_3^-}$ to be evaluated. In competing for the intermediate produced from III, sulfite is 7400 times more reactive than bisulfite ion. This is similar to the value 14 000 obtained from the rate profile in which these two nucleophiles react with the conjugate acid of III.

Discussion

Mechanism. The shapes of the rate profiles are understandable in terms of the degree of protonation of each of the two reactants.[10] On the acid side of the maximum value of k_{obsd}, decreases in the observed second-order rate constant are largely the result of decreases in the concentration of sulfite ion as pD decreases. On the other side of the maximum, decreases in the concentration of substrate in its conjugate acid form with increasing pD largely influence rate constants. In all cases the conjugate acid of substrate reacts with sulfite ion, reaction involving bisulfite ion being important only at low pD values.

Our results clearly establish that the kinetically second-order cleavage of substrates I-III by bisulfite ion in aqueous acid has separate rate and product determining steps. Therefore, one or more intermediates must be present. Thus, while azide ion does not react with I-III in the absence of sulfite ion and while it does not influence the rates of cleavage of these substrates when sulfite ion is present, it does influence the product distribution. Azide VI can be the major product of the reaction along with thiazole V (substrates I and II) or pyridine (substrate III). Even azide VI will eventually react with sulfite ion to give sulfonic acid IV. Competition experiments, Figures 2-4, eliminate as a significant possibility an intramolecular rearrangement of an intermediate to give sulfonic acid IV.

We would emphasize that the results of both the kinetic and competition studies must be considered together in formulating any mechanism. While it is entirely possible to advance a logical mechanism on the basis of the kinetic data alone, this need not be in harmony with the product studies. For example, entirely consistent with the kinetics is a mechanism involving S_N2 attack at the methylene side chain of the substrates in their conjugate acid forms by sulfite ion and at low pD by both sulfite and bisulfite ions. According to this, sulfonic acid IV is a product. But this material is inert to further substitution under conditions which give rise to azide VI. Hence, such a pathway cannot account for the formation of VI and is inconsistent with all the data and must therefore be rejected. Note that sulfite ion will cleave simple benzylammonium salts;[16] this reaction may well take place by a direct displacement mechanism.

The minimum mechanism to explain our rate and product data is given in Scheme I. It shows that D^+, substrate, and

Scheme I

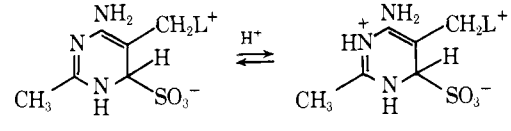
$$D^+ + S + SO_3^{2-} \longrightarrow \text{intermediates} \xrightarrow{SO_3^{2-}} IV \qquad \text{intermediates} \xrightarrow{N_3^-} VI$$

sulfite ion (bisulfite ion should also be included for reactions at low pD) are present in the rate-limiting step to give an intermediate (or even several intermediates). The intermediate then competes for sulfite (and bisulfite ion at low pD) and for azide ions. Note that the formation of sulfonic acid IV actually involves reactions with two sulfite ions, the second taking place after the rate-limiting step and so is not detected kinetically.

What is the identity of the intermediate or intermediates? Although sulfite ion can in principle react either at oxygen or sulfur, we find no suggestion in the current literature dealing with sulfite ion chemistry to suggest that significant reaction takes place at an oxygen atom.[17-21] We therefore suggest intermediates having a carbon-sulfur bond. These result from the addition of sulfite ion to substrate.

Since pyridine substrate III undergoes cleavage as well as thiazole containing compounds I and II and since the second-order rate constants for the three substrates are so similar, differing by only a maximum factor of 3.2, it seems unlikely that sulfite ion adds to the pyridine or thiazole rings. Moreover, a thiamin derivative having a reduced thiazole ring is cleaved by sulfite ion[22] while thiazolium ion IX does not undergo cleavage under conditions at which I and II react. This thiazolium ion is a good model of thiamin. The electron-withdrawing property of the *m*-nitrobenzyl group is similar to that of the protonated pyrimidine ring of thiamin as evidenced by the similarities of the pK_a values for *m*-nitrobenzylammonium (8.6[23]) and for 5-(2-methyl-4-aminopyrimidinyl)methylammonium ions (8.5[24]).

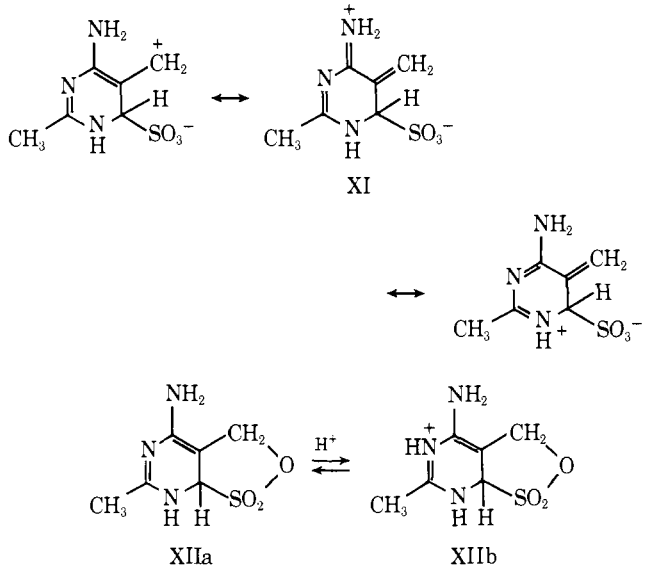
We suggest that sulfite ion adds to the conjugate acid forms of I-III. (The most basic nitrogen atom is N-1′.[25]) Addition is expected to take place at a ring position which is located ortho or para to the positively charged nitrogen atom so as to neutralize this positive charge on CS bond formation. From the results of related compounds[26] we expect addition to unsubstituted position 6 of the pyrimidine ring to be favored over addition to substituted positions. The major sulfite ion-substrate adduct is expected to be X which can be converted to its



X

conjugate acid. The leaving group is designated L^+. (In D_2O NH is converted to ND.)

Three pathways resulting in the cleavage of the bond between the leaving group and the CH_2 side chain following the formation of intermediate X need to be considered. They are (a) an S_N1 type of reaction of X to give charged delocalized intermediate XI which then competes for nucleophiles; (b) an S_N2 type of reaction of X with sulfite and azide ion nucleophiles which takes place after the rate-limiting step; and (c) an intramolecular process involving participation by the sulfonate ion in the elimination of the leaving group to give sultone XII. Direct formation of XI by an S_N2' reaction involving the concerted addition of sulfite ion and expulsion of the leaving group seems unlikely. No adduct of any kind was detected by NMR in our kinetic runs.



XI

XIIa XIIb

The final steps of the mechanism must involve loss of sulfite ion from material which has reacted with a nucleophile. In this way pyrimidine products IV and VI are produced.

We favor the S_N1 mechanism of converting adduct X into its substitution products IV and VI. This pathway allows a logical and straightforward explanation to be advanced for the formation of adduct X. Adduct formation takes place because it transforms the aromatic pyrimidine ring into one which is a much better electron donor. This donor facilitates the expulsion of the nucleofugic leaving group; it stabilizes the transition state which places positive charge on the heterocyclic ring.

For possibility b involving S_N2 reactions of sulfite and azide ions with X, steric and electrostatic factors are expected to be important. Rotation about the bridging methylene bond of X is likely to be severely restricted;[27] the sulfonate ion and leaving group can exist in a syn-anti relationship. In an anti conformation the sulfonate ion hinders attack of an anionic nucleophile while in a syn form, relief of strain facilitates loss of the leaving group.[28] But why should bimolecular substitution take place on adduct X which is more crowded than aromatic starting material?

Again, as with the S_N1 mechanism, the real driving force for adduct formation must be associated with the superior electron-donating properties of the adduct over the aromatic starting material. According to the S_N2 mechanism too, substitution is facilitated by electron donation on the part of the reduced heterocyclic ring. While S_N2 reactions can be influenced by electronic effects,[29] a more reasonable, simpler explanation for adduct formation is found with the S_N1 mechanism. Hence, we favor it.

Possibility c involving a sultone intermediate deserves serious consideration. While a sulfonate ion normally is expected to be a very poor nucleophile, there could be a large kinetic advantage in the case of adduct X which offsets the normal low nucleophilicity. Anchimeric assistance could result because the displacement reaction is intra- rather than intermolecular.[30]

We disfavor as a potential pathway the rate-limiting formation of sultone XII. Our conclusion is based on a consideration of k_2/k_3, eq 3 and 4. The magnitude of this ratio, believed to be associated with the reactions of the conjugate acid of III with sulfite and bisulfite ions, is very similar to those involving the formation of adducts VII[13] and VIII[14] in reactions with the same nucleophiles. However, if a sultone were being formed, k_2 and k_3 are associated with very different reactions, formation of XIIa and XIIb, respectively. The observed rate constants reflect not only the cyclization of X and its conjugate acid in the rate-limiting step but also the preceding steps as well. The close similarity of the k_2/k_3 selectivity ratio for III and those involving VII and VIII is hardly a coincidence. This selectivity suggests that sultone formation does not play a significant role for the substrates and conditions we employed;[31] it instead points to addition of sulfite (k_2) or bisulfite ion (k_3) as the rate-limiting step. In keeping with the well-established principle that the extent of neighboring group participation decreases with increasing stability of a carbonium ion,[32] we suggest that cation XI is sufficiently stabilized so there is no need for participation by the neighboring sulfonate ion in the transition state leading to expulsion of the relatively poor leaving groups found in I-III.[33]

Examination of our rate constant ratios derived from product studies is most interesting. Ritchie has provided a logarithmic scale of N^+ values which is a quantitative measure of the nucleophilicities of ions and molecules toward organic cations.[34] However, while the N^+ value for sulfite ion is clearly established (7.90[35]), that for azide ion is uncertain. The early value of 5.4[36] derived from the rate constant for reaction of azide ion with *p*-nitrobenzenediazonium ion is said to be a lower limit.[34] The larger, more recent value of 7.6[35] derived from results involving tri-*p*-anisylmethyl cation[37] causes azide to show large deviations (ion insufficiently reactive) when correlating other kinetic results.[35] Using the old and new N^+ value for azide ion results in rate constant ratios of 316 and 2, respectively. However, these values pertain to measurements made at low ionic strength. Under our conditions of 1 M ionic strength, the individual rate constants are expected to decrease, a larger decrease being anticipated for sulfite ion. From literature results[37,38] we estimate the calculated ratio to decrease by about a factor of 10 when the ionic strength is increased to 1 M. Our observed value of 54 is in reasonable agreement with the value 316/(~10) or ~32 derived from the old N^+ value and in poor agreement with the new value 2/(~10) or ~0.2. Clarification of the N^+ value for azide ion could provide useful information about the intermediate in the cleavage reactions and the nature of its reactions.

Our rate constant ratios for sulfite and azide ions do not provide a test of an S_N2 mechanism. Although we have varied the nature of the leaving group in the cleavage reactions, the variations, as judged by the pK_a of these groups, are not large. Thus, thiazoles V have $pK_a \sim 3.5$ while pyridine has $pK_a = 5.2$ in H_2O solutions at low ionic strength.[9] Now, rate constants for S_N2 reactions show only a small change as the pK_a of the conjugate acid of the leaving group is varied.[28] Moreover, for a pair of competing nucleophiles, a change in their rate constant ratio will be even smaller as the basicities of leaving groups are varied. The variation in our rate constant ratios obtained from product studies is too great to allow any conclusion about possible leaving group effects to be evaluated. Note that the rate constant ratio for the two nucleophiles in question in an authentic S_N2 reaction is >1.[39]

Little, if any, hydrolysis product has been observed in the cleavage reactions. Commonly, the ability of a carbonium ion to discriminate between water and azide ion nucleophiles is used to measure the selectivity of a carbonium ion.[40]

Consider the following measure of selectivity between azide ion and water nucleophiles involving III at pD 3.0 and a total azide ion concentration of 0.14 M. This run was selected because it minimizes reaction of the intermediate with sulfite ion. If it is assumed for the sake of illustration that as much as 10% of hydrolysis product went undetected when 50% of azide VI was formed, then the rate constant ratio (selectivity) is 2×10^5 in favor of azide ion over water. Interestingly, the logarithm of this value, 5.2, is very similar to Ritchie's lower limit on the N^+ value (5.4[34]). This very high degree of selectivity is consistent with the intermediate being a charge delocalized, resonance stabilized cation, such as XI. However, it may also be

consistent with an S_N2-like reaction of a highly selective sultone intermediate.[39]

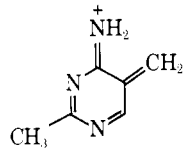
Our work clearly demonstrates that one or more intermediates must form in the cleavage reaction. But more work will have to be done in order to identify conclusively the structures of the intermediates.

Application of the mechanism just considered allows information already in the literature[4-6] to be reevaluated. From a straightforward reinterpretation, a consistent explanation and a clear understanding of rate and product data result.

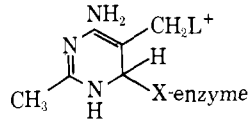
Significance of the Sulfite Ion Adducts. There now is a long list of reactions which involve the addition of sulfite ion to a heterocyclic molecule.[13,14,18-21] In many cases the resultant adduct undergoes further reactions such as deamination,[41] dehalogenation,[20,21,42] or, as in our case, loss of a leaving group from a side chain. In some instances the heterocyclic component of these reactions undergoes similar transformations under biological conditions in the presence of enzymes. In these cases it seems likely that a nucleophilic site, perhaps a thiol group of the enzyme, functions in the same way as sulfite ion in the model reactions.[20]

Thiaminase I, an enzyme destroying thiamin, is capable of inducing a beriberi-like condition. The enzyme is found in some fresh water fish, shellfish, and crustacea. Clams but not oysters, for example, are rich in thiaminase. The nature of the nucleophilic site of the enzyme is not generally known, but in the case of clams the sulfur atom of hypotaurine becomes bonded to the methylene group of the pyrimidine ring[43,44] of the vitamin.

In model studies, thiaminase I cleaves I, III, and related compounds by replacing a heterocyclic ring at the methylene side chain by a nucleophile which may be another heterocyclic ring or even a thiophenoxide ion. Results of kinetic studies suggest the formation of an intermediate involving the enzyme and the pyrimidine portion of substrate minus the leaving group. Two possible types of structures were suggested for the intermediate. One is XIII resulting from loss of the leaving



XIII



XIV

group from aromatic starting material. The other is produced as a result of covalent bond formation between some nucleophilic site in the enzyme and the methylene group bonded to the aromatic pyrimidine ring. The bisulfite ion cleavage reaction was suggested to be a nonenzymatic model for this process in which the enzyme functions as a nucleophilic catalyst.[9] We suggest as a third possibility structure XIV in which the nucleophilic site of the enzyme reacts not at the methylene group but at the pyrimidine ring carbon atom. Further reactions of this adduct then give substitution product in much the same way as we have outlined for the sulfite reactions.

Perhaps such a mechanism is more widespread in biochemical systems than yet recognized.

Experimental Section

Materials. Thiamin chloride hydrochloride (Aldrich), sodium azide (Fisher purified), and sodium metabisulfite (Fisher certified) were used as received. Deuterium oxide (Columbia), 99.8%, was boiled and cooled under nitrogen to remove oxygen. 3-(3-Nitrobenzyl)-4-(2-hydroxyethyl)-5-methylthiazolium chloride was prepared by quaternization, mp 149–152 °C (lit.[45] 152–153.5 °C). 2-(1-Hydroxyethyl)thiamin chloride hydrochloride (HET) prepared from thiamin and acetaldehyde[46] contained unreacted thiamin. Since both materials have similar solubilities purification was effected as follows. Most of the thiamin was removed by heating the solid at reflux with absolute ethanol using about 100 mL of ethanol per 0.5 g of thiamin. The hot suspension was filtered and cooled to give HET (96% by NMR). Recrystallization was accomplished by dissolving 6.26 g of this HET in 225 mL of 95% ethanol, filtering the hot solution, and stirring into the hot filtrate 200 mL of acetone. After standing overnight 4.72 g of HET, mp 233–234 °C dec (lit.[47] mp 234–236 °C), was recovered in 75% yield based on the original amount of thiamin.

An aqueous solution of 2-methyl-4-amino-5-pyrimidinylmethyl azide (VI) was prepared from 0.2 M thiamin, 0.5 M sodium azide, and 0.02 M sodium sulfite at pD 5.6. The mixture then was heated at 60 °C for 250 min, after which time analysis by NMR indicated an 83% conversion to VI. Chemical shifts of H-5 (8.2) and CH_3 (2.6) are very similar to those of thiamin but that for CH_2 (4.6) is considerably higher. No attempt was made to isolate this material. Addition of an equimolar amount of bisulfite converted VI to IV.

1-[(4-Amino-2-methyl-5-pyrimidinyl)methyl]pyridinium Chloride Hydrochloride. Thiamin chloride hydrochloride (16.8 g, 0.0498 mol) was dissolved in 100 mL of water and 15.8 g (0.200 mol) of pyridine was added. The solution was heated on a water bath to ca. 60 °C and 1 mL of a sulfite solution (prepared by dissolving 0.52 g (0.0050 mol) of $NaHSO_3$ in 5 mL of water) was added with stirring. The solution was heated between 65 and 75 °C with 1-mL portions of the sulfite solution being added every 20–30 min until it was all added. After heating a total of 2–2.5 h the solution was cooled, extracted several times with ethyl ether (100 mL total), acidified to ca. pH 2 with hydrochloric acid, and evaporated to dryness under vacuum. The solid residue was digested in ca. 150 mL of absolute ethanol and filtered to give 10.8 g (79%) of white solid, mp 256–258 °C dec, which was ca. 93% pure by NMR analysis. The contaminant is sulfonic acid IV. The salt (1.48 g) was dissolved in 100 mL of 95% ethanol. A small amount of undissolved solid was removed by hot filtration through Celite. The filtrate was reduced to 50 mL in volume and 50 mL of dry acetone was added; after boiling for a few minutes, cooling gave rise to white, scaly crystals, 1.0 g, mp 253–257 °C dec (lit.[48] mp 258 °C dec), in 68% yield. This compound is the dihydrate when allowed to equilibrate with the ambient atmosphere. It can be dried under vacuum over P_2O_5 to the anhydrous form.[9]

Equipment. Kinetic and pK studies made use of a jacketed glass cell of 10 mL total volume. The temperature was maintained at 25 ± 0.10 °C by circulating water from a Lauda constant temperature bath. For kinetic measurements the pD was maintained at a constant value by a Radiometer SBR2 titrigraph combined with a type SBU1 syringe buret and a TTT-1c titrator. A Radiometer combination electrode (GK-2321C) inserted through a rubber serum stopper into the reaction vessel was used for all pD measurements. All pH meter readings were converted to pD by adding 0.40 to the reading. pK_a measurements were made with the same electrode using a Radiometer PHM64 Research pH meter. NMR spectra were obtained on a Varian A-60A spectrometer.

Determination of pK_a Values. The procedure was that of Albert and Serjeant[49] except that 1.0 M ionic strength was maintained with KCl. Titrations of 0.01 M solutions were performed at 25 ± 0.1 °C; the pK_a values so determined from the average K_a values of three titrations are listed in Table I.

Ionic Strength. Contributions to the ionic strength of the medium by the organic substrates in kinetic runs ranged from about 0.4 to 0.6 M. Since substrates may be singly or doubly charged, the concentrations of the two forms were calculated using the pK_a of the substrate, its total concentration, and the pD of the medium. Sufficient KCl was added to make the initial value 1.0 M. During the course of a reaction the ionic strength decreases as divalent reactants are converted to monovalent products. As a result the ionic strength decreases by less than about 20%.

Reagents for Kinetic Studies. All solutions were prepared using boiled D_2O to remove oxygen. Stock solutions of substrates were about 0.2 M and contained 0.1 M benzyl alcohol which served as an NMR standard. Approximately 1.0 M sodium bisulfite was prepared using dried sodium metabisulfite; it contained a few milligrams of Na_2EDTA to retard metal ion catalyzed oxidation. The solution was analyzed periodically for sulfite ion iodometrically.[50] A stock solution of ca. 1.5 M sodium azide was prepared with Na_2EDTA and was stored in the dark. Solutions remained colorless over a period of months.

Control Experiment to Determine the Reactivity of Thiamin toward Azide Ion. When a mixture of 0.2 M thiamin and 0.5 M sodium azide in a D_2O solution which was raised to pD 5.7 by the addition of KOD

was heated at 60 °C for 70 h, no reaction was observed. However, when a sodium sulfite solution (5 mol % relative to I) was added, a 50% conversion to azide VI was apparent after 30 min heating. The NMR signals listed in Table II were used in making the analysis.

Experiment to Determine the Reversibility of a Cleavage Reaction. A solution consisting of 0.099 M total thiamin, 0.080 M total sulfite ion, and 0.097 M total pyridine at pD 4.9 was heated at 25.0 °C using a pH stat to maintain the pD constant. Aliquots were examined as described in the kinetic procedure. Only sulfonic acid IV was detected; no pyridine compound III was detectable by NMR analysis over a period in which 58% of I reacted. The ratio of the concentration of free pyridine to free sulfite ion initially was 17. This result is consistent with a reaction in which the conversion of I to III is insignificant because of the preferred formation of IV. Similar experiments were carried out at pD 5.4 and 6.4 but under these conditions the total pyridine to total sulfite ion concentration ratio was 0.57 and the ratio of free pyridine to free sulfite ion was 3.4 and 1.4, respectively. Again, only IV was detected.

Kinetic Procedure. Three milliliters of stock solution containing the organic substrate and benzyl alcohol as NMR reference was placed in the nitrogen-swept reaction vessel kept at 25 ± 0.1 °C. A weighed amount of KCl was added to give an initial ionic strength of 1.0 M. When azide ion was employed a measured amount of stock solution was added by syringe (Teflon needle) and the combined glass–reference electrode was placed into the solution. The pD was adjusted by adding measured amounts of standard KOD or DCl solutions by syringe; D_2O was then added to give the desired total volume. The reaction vessel was swept briefly with nitrogen and the reaction initiated by adding the appropriate amount of standard $NaDSO_3$ solution by a syringe fitted with a Teflon needle. The final concentration of substrate was 0.10–0.12 M, total sulfite ion 0.05–0.2 M, and azide ion 0.05–0.36 M, when present. A timer and the titrator were started and any minor adjustment to the pD was made, generally in less than 1 min. The reaction vessel was closed with a serum stopper bored to receive the electrode and two Teflon tubes. The tube of 28 gauge bore was attached to the syringe buret and delivered titrant during the reaction to maintain constant pD.

The second tube which terminated in a Luer fitting was used to withdraw aliquots of the reaction mixture for analysis. At appropriate intervals the 0.5-mL aliquot was withdrawn by syringe and quenched by adding it to an NMR tube containing 0.1–0.2 mL of DCl solution of sufficient concentration to lower the pD to ca. 1 or less. After mixing, the sample was analyzed by NMR.

This method of quenching seems adequate. Occasional samples were reanalyzed one or more days after they were obtained and no significant changes in the NMR spectra were found. The chemical shifts of the signals followed during the course of the cleavage reaction are listed in Table II. The phenyl protons of benzyl alcohol were used as the area reference in most cases.

Kinetic Analysis. Observed second-order rate constants for the cleavage reaction by sulfite ion were obtained by the standard plot of $\ln (S_0B/B_0S)$ vs. time where S_0 and S are the substrate concentrations at $t = 0$ and t, respectively, and B_0 and B are the concentrations of total sulfite ion present at the corresponding times. The resultant slope is $(B_0 - S_0)k_{obsd}$.

This treatment requires modification in the reactions where azide product is formed. In these cases the sulfite ion concentration does not decrease at the same rate as that of substrate but at some fraction of this rate. The fraction is given by the mole fraction X of the sulfonic acid formed in the product mixture, i.e., $X = [IV]/([IV] + [VI])$. Inclusion of this factor changes the experimental rate law to that given by eq 7.

$$-dS/dt = k_{obsd}[S][B_0 - X(S_0 - S)] \quad (7)$$

This can be integrated if X is constant. While X, in fact, is not strictly constant, in most runs this fraction was observed to change by only a few percent over the course of the reaction, rarely over 10%. Thus, X is constant to a first approximation compared to the changes occurring in the substrate and sulfite concentrations. Integration assuming X to be constant yields eq 8 and 9.

$$\frac{1}{B_0 - XS_0}\left(\ln \frac{S_0[B_0 - X(S_0 - S)]}{B_0S}\right) = k_{obsd}t \quad (8)$$

$$\frac{1}{B_0 - XS_0}\ln \frac{S_0B}{B_0S} = k_{obsd}t \quad (9)$$

Table II. Chemical Shifts of Protons Used to Analyze Reaction Mixtures during Kinetic Runs[a]

Compd	δ	Type proton
I	5.68	$-CH_2N^+$
II	1.70[b]	$-CH(OH)CH_3$
III	5.80	$-CH_2N^+$
IV	4.16	$-CH_2SO_3^-$
VI	4.59	$-CH_2N_3$
$C_6H_5CH_2OH$	7.34	C_6H_5

[a] At pD ~1. [b] Methyl proton doublet.

The values of B (sulfite ion) used to construct the plots were determined by stoichiometry, i.e., $B = B_0 - X(S_0 - S)$, where X is the mole fraction of IV as determined from the NMR spectrum for each point. For points at which X could not be determined experimentally, e.g., points early in the reaction where the concentration of products was insufficient for accurate NMR integration or late in the reaction in some cases when the sulfonic acid precipitated and therefore precluded integration, values of X closest to the points in question were used. The value of X used in the prelogarithmic term to determine k_{obsd} was taken as the value of X near the midpoint of the reaction.

In general, the plots showed good linearity for close to 2 half-lives of the limiting reagent. The very slow reactions at pD 3.0, however, were followed only to ca. 30% reaction and those at pD 3.5 to ca. 1 half-life.

Two corrections reducing the concentration of free bisulfite ion were not made because they are expected to be <10%. The first considers the equilibrium involving this ion and $S_2O_5^{2-}$ with $K = 0.23\ M^{-1}$ at 25 °C and 1 M ionic strength.[51] The second deals with the conversion of the ion to sulfurous acid. We estimate the pK_a for this process in D_2O at 1 M ionic strength to be about the same as that (1.8[52]) for dilute solutions in H_2O. An increase in the ionic strength with its associated decrease in pK_a is expected to be similar to the increase resulting on changing from light to heavy water.[52]

Unsuccessful Attempts to Cleave 3-(3-Nitrobenzyl)-4-methyl-5-(2-hydroxymethyl)thiazolium Chloride. A sample of the title compound in a 0.06 M sodium bisulfite solution at pD 3 gave no apparent reaction at room temperature over 24 h. Following the addition of solid sodium metabisulfite to the mixture to increase the concentration of sulfite ion to about 0.4 M, and sodium azide (0.25 M) and KOD to raise the pD to 6.6, the sample was heated at 50 °C for 48 h. Again NMR analysis failed to indicate any change.

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Ternary Complexes in Solution. 26.[1] Stacking Interactions in the Mixed-Ligand Complexes Formed by Adenosine or Inosine 5′-Triphosphate, 2,2′-Bipyridyl, and Cobalt(II), Nickel(II), Copper(II), or Zinc(II). Evidence for Phosphate-Protonated Complexes

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Abstract: The formation of a stacked adduct between 2,2′-bipyridyl and the purine moiety of adenosine or ATP, and inosine or ITP, causes a change in absorption that is best observed by UV-difference spectroscopy. Using Benesi–Hildebrand plots the stability of the adducts (log K_{St} = 0.9–1.4) comprising adenosine or inosine and $M(bpy)^{2+}$, where M^{2+} = Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}, can be determined. The stabilities of these adducts are comparable to those formed by 2,2′-bipyridyl and adenosine, inosine, ATP, or ITP. However, the stability of the stacked adducts with ATP or ITP in the presence of M^{2+} is increased by several orders of magnitude. This is the result of the metal ion bridge formed between 2,2′-bipyridyl and the phosphate groups of the nucleotides (=NTP). Studies of the bpy–Zn^{2+}–NTP^{4-} systems by ^{1}H NMR confirm that stacking between the aromatic moieties is enhanced by the coordination of the metal ion. The stability constants for the mixed-ligand complexes $M(bpy)(ATP)^{2-}$ were already known from potentiometric titrations; those for the corresponding ternary systems with ITP have now been determined by the same method. These results agree with the stability constants determined spectrophotometrically. Comparison of the molar absorptivities of all the systems indicates that the stacked isomer dominates in the equilibrium between an opened and stacked form of $M(bpy)(NTP)^{2-}$. This also holds for the protonated ternary complexes $M(bpy)(HITP)^{-}$, for which the intramolecular stacking interactions could also be confirmed by ^{1}H NMR. It is concluded that in $M(bpy)(HITP)^{-}$ the proton is located at the γ-phosphate group. The corresponding complexes with ATP probably also exist but were not observed; this and the sites of proton binding in $M(HATP)^{-}$ are discussed.

Metal ions have many functions in biological systems; a particularly important one is, together with certain nucleotides, as cofactors for various enzymatic reactions;[2] therefore, metal ion–nucleotide complexes have found much attention.[2,4-6] But due to the ambidentate behavior of the nucleotides the structure of many of their complexes, especially in solution, is not yet clear. For binary complexes it is generally agreed that the stability determining factor is the coordination tendency of the phosphate groups,[2,4,7,8] while the nucleic base moieties may or may not be coordinated, depending on the base and metal ion.[9] This leads in solution to equilibria between macrochelates and complexes having only phosphate coordination, as is known, for example, for the Mn^{2+}–adenosine 5′-triphosphate system.[10] A further problem is the site of protonation in these complexes; for example,[11] in $M(HATP)^{-}$ the proton may be located either at N(1) of the adenine moiety or at the terminal