BIOORGANIC CHEMISTRY 22, 1–28 (1994)

REVIEW

Thiamin: A Critical Evaluation of Recent Chemistry of the Pyrimidine Ring

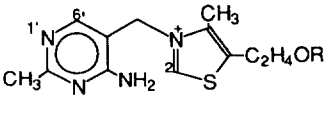
JOHN A. ZOLTEWICZ* AND GEORG URAY†

*Department of Chemistry, University of Florida, Gainesville, Florida 32611-2046; and †Institut für Organische Chemie, Karl-Franzens-Universität Graz, A-8010 Graz, Austria

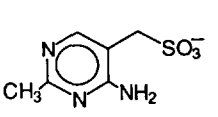
Received April 13, 1993

INTRODUCTION

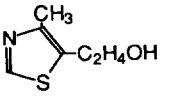
Thiamin (thiamine or vitamin B_1), shown as its simple cation **1**, was isolated from rice bran in 1926. The structure of this vitamin (name derived from "vital amine") was established in 1936 by Williams (*1*) largely due to a lucky accident. After he passed SO_2 into aqueous solutions of the vitamin in order to suppress the growth of bacteria, a white precipitate soon appeared, much to his surprise and good fortune. The solid proved to be the pyrimidine portion having sulfite bonded to the methylene group to give the sulfonic acid salt of **2**. The thiazole leaving group, **3**, remained in the aqueous phase (*2*).



1, R = H
11, R = $P_2O_6^{-3}$



2



3

B_1 is required by all living cells. The classic disease produced by a B_1 deficiency is beriberi but in industrialized societies where it is a common food additive a dietary deficiency is produced by alcoholism which gives rise to neurological and heart disorders (*3*).

SCOPE OF THE REVIEW

A computerized request of the Chemical Abstracts data base seeking information on B_1 indicated over 7,000 entries! This review will be much more selective, dealing mostly with some recent chemistry of the neglected pyrimidine ring. Synthetic

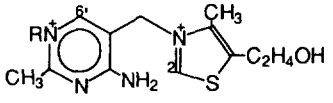
0045-2068/94 $6.00

chemistry will be largely ignored; an excellent review of such literature up to and including 1959 is found in Beilstein (*4*) along with a more recent compilation (*5*). Kluger has reviewed the biochemistry of the thiazolium ring associated with pyruvate decarboxylase (*6*). Good general overviews of the history, chemical properties, synthesis, physiological properties, related antimetabolites, and more are available (*1*, *7–12*).

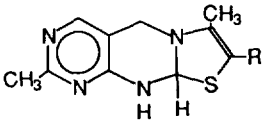
GENERAL OVERVIEW OF THE TRANSFORMATIONS OF B_1

B_1 is a chemical chameleon! Its deceptively simple structure changes with pH. An understanding of the overall chemistry of the vitamin is necessary in order to appreciate the chemistry of the pyrimidine ring and to gain insight as to why some experiments were undertaken. Despite continuing research much still remains controversial.

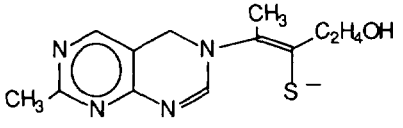
In aqueous acidic solution B_1 exists as its conjugate acid, dication[1] **4a**, but in *water* under alkaline conditions its chemistry is dominated by two main, reversible conversions at room temperature. Intramolecular aminolysis and hydrolysis both involve the thiazolium ring (*14*). The former reaction is faster than the latter.



4a, R = H
4b, R = CH_3



5a, R = C_2H_4OH
5b, R = $COOCH_3$



6

B_1 readily undergoes deprotonation of the 2 position of the thiazolium ring to give an ylide (*15*), a reaction of considerable biological importance, considered subsequently.

(*1*) *Intramolecular Aminolysis*

On quickly raising the pH to 11, for example, the 4′-amino group of B_1 adds to the thiazolium ring to give tricyclic structure **5a** (dihydrothiochrome) that then eliminates a thiolate ion to give the "yellow" form, 5,6-dihydropyrimido[4,5-d]pyrimidine **6**, the kinetic product isolated in 1957 (*16*). This reversible process

[1] The site of protonation of B_1 as N-1′ has been demonstrated in a number of ways, including the use of ^{15}N chemical shifts (*13*).

requires the neutralization of two protons, one at each step. For an aqueous solution the second pKa is smaller than the first and the overall apparent pK_a is 11.6 (19°C) (*16*).

In basic *alcoholic* media the first step, amino group addition, may be observed easily. The white tricyclic, nonionic product **5a** is stable for some time as the major material because now, unlike that for aqueous solution, the first pK_a is smaller than the second (*16*). Moreover, **5a** can be converted to the stable yellow **6** on reaction with an additional equivalent of base (*14*). In the presence of aldehydes and alkoxide base **5a** reacts to produce a 2-hydroxyalkyl thiamin (*17*).

The surprising claim has been made recently in preliminary form that B_1 in water at neutral pH exists largely as **5a**. NMR data provide the basis for the conclusion. Increasing the pH of a phosphate buffer causes the bridging methylene ^{13}C NMR signal to move upfield by 1 ppm. This process is reversible and has an apparent pKa of about 7 (*18*). Further supporting data are awaited because this conclusion is at variance with other studies that indicate B_1 is the major material in neutral solution (*16, 19*).

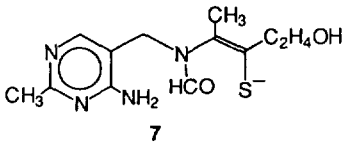
A more robust derivative of the labile **5a** was made in quantitative yield using triethylamine to deprotonate the amino group to give **5b** having an ester substituent in place of the hydroxyethyl side chain. Conjugation between the enamine and the carbonyl group of the ester provides added stabilization over that in **5a**. Tricyclic **5b** does not react with benzaldehyde in the presence of triethylamine in methanol (*20*), a marked contrast to the condensation occurring with **5a** and benzaldehyde (*17*). The bridging methylene protons of **5b** in acetone-d_6 appear as a singlet at 4.45 ppm, but the newly formed tertiary carbon has a proton signal shifted upfield to 6.4 ppm consistent with a change in hybridization (*20*).

The reason for the yellow color of **6** (absorbance at 338 nm (*16*)) has attracted much interest and speculation. When the ene-thiolate group is removed from the nitrogen atom to give a simple dihydropyrimido[4,5-d]pyrimidine this color is bleached out (absorbance now at 298 nm (*21*)).

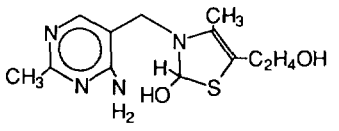
(*2*) *Hydrolysis*

Shortly after the formation of yellow **6** in alkaline solution a new product appears, one in which the thiazolium ring is hydrolyzed to the thermodynamic product, the formamido thiolate, **7** ("thiamin thiolate") with an apparent pK_a of 9.3 (19°C) for a process again requiring two equivalents of hydroxide (*16*), the thiol itself having a lower pK_a of about 6.8 (*22*) (6.8 for a relative having an *N*-benzyl group (*23*) and 7.9 for one with an *N*-methyl group (*22*)). This hydrolysis involves the initial formation of pseudobase **8** that opens to thiolate **7** by the action of a second equivalent of base. For the N-1′-methylated derivative of B_1 (1′-methylthiaminium ion, $NMeB_1$ or **4b**) the pKa for the overall hydrolysis is 8.56 (25°C) (*24*).

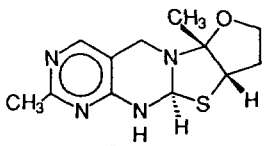
Tetracyclic material **9** arises from the addition of the hydroxyethyl chain to the enamine portion of tricyclic **5a** to generate a tetrahydrofuran ring as part of the *N,O*-acetal (*25*). A crystal structure shows that the material prepared from the salt of thiolate **7** in dioxane by the action of carbon dioxide has the two 5-membered



7



8



9

rings fused *cis* as expected. The material can be converted back to B_1 with acid (*26*).

In unpublished work we find that B_1 when added to an unbuffered alkaline solution with a pH of about 11 undergoes hydrolysis and the pH decreases unexceptionally. But at a higher pH of about 12 the solution shows a gradual *increase* in pH as hydrolysis advances (*27*). At the lower pH only a little of the yellow form **6** is present and it reverts to **7** but at the higher value most of the B_1 is rapidly converted into its yellow form **6** and this then slowly hydrolyzes to **7**, presumably by first reverting back to B_1 (*16*, *19*).

Most workers have taken the formation of the pseudobase by the addition of water or hydroxide ion to be the rate-limiting step in ring hydrolysis and the subsequent ring opening step to be fast (*19*, *23*, *28–30*). But a recent study on 3,4-dimethylthiazolium ion (*31*) and B_1 (*18*) challenges this long held view and claims the rate-limiting step to be ring opening and not pseudobase formation. This conclusion is based on the observation of both general acid and general base catalysis, the former term being crucial to the interpretation of the mechanism. According to this view, the rate-limiting step is the concerted general acid-catalyzed departure of the thiol from the pseudobase or the general base-assisted ring opening of the pseudobase (*18*, *31*). These kinetics were obtained by a special technique involving irreversible trapping of the thiolate by iodine to give a disulfide under conditions where the rate was first order in substrate and zero order in iodine (as triiodide ion) trapping reagent. This approach allowed the forward reaction to be studied at low pH where normally this cannot be done because so little of the hydrolysis product is formed at equilibrium. Control experiments show that similar pyrimidines lacking the thiazolium ring do not undergo a side reaction with the iodine. Moreover, the pK_a value of 6.9 for pseudobase formation on the addition of water to B_1 seems quite low (*31*). The reason for the discrepancy between the conclusions derived using the irreversible method and that obtained by most other investigators studying the reversible reactions is not apparent.

Relaxation kinetics reveal that both the formation of pseudobases of simple thiazolium ions and B_1 as well as their slower decomposition to ring-opened material can be observed separately (*23*, *28*). Moreover, at pH 9.3 about 16% of the total B_1 exists in the pseudobase form **8**. Addition of the first hydroxide to give **8** is some 3400 times slower than the subsequent reaction with the second

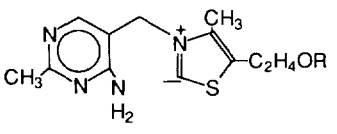
equivalent of hydroxide to open the ring to **6** (*28*). The hydrolysis to **7** is about five times slower than conversion to the yellow structure **6** (*28*).

Thiolate **7** is easily oxidized to its disulfide. The disulfide in turn is readily reduced back to the vitamin and is therefore used as such or preferably in the form of a mixed disulfide as a vitamin substitute (*7*, *11*) because the neutral form passes through membranes more readily than the ionic material (*32*, *33*).

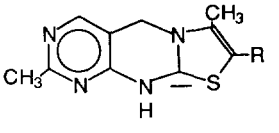
The rate–pH profile produced when thiolate ion **7** or similar thiolate ions from simpler thiazolium ions are added to acidic aqueous solutions has been obtained by two groups using a stopped-flow technique. They extended the study well beyond the usual dilute acid region to low pH. Both obtained the same profiles. The new feature found in these studies is the formation of kinetic and thermodynamic products below pH about 4.5. Both groups agree that the thiazolium ion is the thermodynamic product but they disagree on the identity of the kinetic material. The earlier workers proposed the conversion of the pseudobase, **8** in the case of B_1, to a protonated acyclic material having a formyl group bonded to sulfur and not to nitrogen (*22*). The second group rejected this claim and instead proposed that the pseudobase becomes protonated on its nitrogen atom (pK_a 6.2) and not the enamine carbon to give the kinetic product (*23*). The first group reported that the conversion of the pseudobase to the kinetic product is general acid catalyzed (*22*). Such catalysis is not likely to be consistent with simple protonation on nitrogen and suggests that a bond-breaking process is involved as first claimed.

(*3*) *Ylide Formation*

The seminal discovery concerning the biologically important form of the B_1 was made by Breslow who demonstrated that the reactive site of the enzyme cofactor is the 2 position that readily deprotonates to give ylide **10**. The most recent of



10



12

several estimates of the pK_a for the 2 position is 18.0 (17.7 for the conjugate acid of B_1, both at 30°C) and is based on a kinetic study involving general base-catalyzed deprotonation of B_1 in D_2O (*34*, *35*) and an assumed diffusion-limited reverse protonation (3×10^9 M^{-1} s^{-1} for water) (*36*). It would seem to be the preferred value. The highly controversial claim that the ylide has been isolated as a stable material in the solid state is considered below.

There is widespread disagreement about whether the amino group acts as an intramolecular acid or base in either the chemistry or the enzymology of ylide formation from B_1 (*37–40*). A recent kinetic study dealing with ylide formation in aqueous base failed to detect any catalytic action by the 4′-amino group and therefore concluded that it does not play a role as an intramolecular base to aid ylide generation in the absence of enzymes (*36*). This is not surprising considering

the very low basicity of the amino group; its conjugate acid has been estimated from a linear free energy correlation to have a negative pK_a (*41*).

The small pK_a value found for ylide formation from B_1 provides a dilemma (the "ylide dilemma") when it is realized that at approximately neutral physiological pH the fractional amount of ylide available at equilibrium *in the absence of any special effects* is a mere 10^{-11}. Because of this very low value, considerable speculation has arisen about the way in which nature must overcome this disadvantage in enzymic reactions. One recent study suggests that a discrete ylide may never form; rather, deprotonation and addition of the electrophile may be a concerted reaction in aqueous and in enzymic systems (*42, 43*). This front side substitution has been discredited as examined below (*44*). Still another investigation makes use of a tertiary carbanion, considered subsequently; it too seems to be without foundation.

The main types of enzymic reactions of B_1 present as its pyrophosphate (cocarboxylase or TPP) **11** include the decarboxylation of α-keto acids, both oxidative and nonoxidative, and the chemical equivalent of the acyloin condensation carried out by ketolases. Enzymic reactions require Mg(II) (*37, 45*) as a cofactor (*6, 12*).

WHAT IS THE CORRECT STRUCTURE OF "ISOLATED" THIAMIN YLIDE?

A highly controversial claim that it is possible to isolate thiamin ylide in the solid state after deprotonating B_1 chloride with one equivalent of ethoxide ion in ethanol has been made. The reported proton and carbon spectra appear to be that of a single substance and its ultraviolet spectrum taken using ethanol solvent resembles that of B_1. Under similar conditions a solid material described as tricyclic structure **5a** was isolated much earlier (*16*). The new material has a melting point similar to that of the old **5a** (*46*).

One author states that he is unable to repeat the ylide preparation (*6*) and another that the ylide cannot be detected by NMR in DMSO solution (*47*). Another NMR spectrum is said to show the presence of the yellow form of B_1 (*48*).

The isolated material has curious properties. Attempted alkylation at the ylidic carbon instead gave alkylation at the sulfur atom of **6** (*46*). In aqueous solution where B_1 is estimated to have a pK_a of 18.0 (*36*) for ylide formation the material is said to give an equilibrating mixture of the ylide and ion pair disproportionation products consisting of B_1 monocation and formamido thiolate **7** ("thiaminium thiaminthiolate") (*49*), the latter normally resulting from cleavage of the thiazolium ring. Under nonaqueous conditions in a protic solvent the ylide is said to be in rapid equilibrium with an ion pair consisting of thiaminium ion-free base and the yellow pyrimidopyrimidine thiolate **6** ("thiaminium neothiaminthiolate"). In water the yellow color quickly fades (*46, 50, 51*). Disproportionation is said to be the most important chemical property of the "ylide" (*17, 49*).

The elemental analysis for the ylide is consistent with a number of structures including tricyclic **5a**, tetracyclic **9**, and an ion pair formed from B_1 monocation and the yellow form **6** as the anion but not the ion pair from B_1 and **7**, the latter

possibility having an additional one equivalent of water. The NMR spectrum (DMSO) shows all the protons associated with the thiazole ring are shifted upfield. Most importantly, the bridging methylene group is diastereotopic (4.08 and 4.23 ppm, with J = 15 Hz). This observation alone eliminates a simple ylide structure.

We suggest, in agreement with others (*48*), that the material initially isolated from the alcoholic solvent is the colorless tricyclic structure **5a** isolated by Maier and Metzler (*16*) and not the ylide. It is known to disproportionate partially in methanol and completely in water (*16, 17*). Structure **5a** has the required diastereotopic methylene group. The so-called ylide is not tetracyclic **9** because all the diasterotopic methylene protons should be easily recognized by their multiplicity (*25*) and this complexity is not present (*49*).

LITERATURE SPECULATIONS CONCERNING THE ROLE OF THE 4′-AMINO GROUP IN NONENZYMIC REACTIONS

The chemistry associated with the addition of the amino group to the thiazolium ring has become controversial especially as it pertains to the role of the ylide intermediate and the formation of tricyclic **5a** and yellow **6** and also to the mechanism of isotope exchange at the 2 position in highly alkaline solution.

(*1*) *An Incorrectly Claimed Ylide (Carbene) Insertion into the Amino Group*

A stopped flow kinetic study of the conversion of B_1 to yellow **6** in aqueous solution produced two pKa values for the overall reaction, 12.39 and 10.55 (*52*). The simple average of these two values is 11.5, a value much like that of 11.6 reported earlier (*16*) for the same overall conversion using a simple spectrophotometric method.[2] Moreover, the reported rate constant of 115 $M^{-1}\ s^{-1}$ for the slow step is similar to the value of 99 $M^{-1}\ s^{-1}$ given by others in a later investigation for the formation of **5a** by the action of hydroxide ion (*53*) and thereby establishes the validity of the first study. Unfortunately, the assumed mechanism for the formation of **6** turns out to be incorrect because it employed an ylide intermediate in the reaction pathway. A fast photometric titration of B_1 gave pK_a values of 12.7 (water) and 12.9 (methanol) and these were incorrectly assigned to the formation of ylide **10**, thus prompting the incorrect analysis (*54*).

According to the incorrect mechanism, the formation of **6** is said to take place by the rate-limiting nucleophilic addition of the amino group of B_1 to the ylide (carbene) **10** to give intermediate **5a** in an NH bond insertion reaction. Subsequently, **5a** then ring opens to give **6** (*55*). Later studies using stopped-flow and relaxation techniques at high pH failed to detect the presence of the ylide and thereby discredited this mechanism (*23, 53*).

[2] Such a two proton transfer equilibrium process has traditionally been defined as $K^2 = [H]^2[6]/[B_1]$. This has the advantage that at half conversion when $[6] = [B_1]$, $K = [H]$, just as is the case for a simple, single proton transfer process. Moreover, $2 \times pK$ then equals the sum of the two component pK_a values. The fractional amount of end product base is given by the expression $K^2/([H]^2 + K^2)$ and not the usual $K_a/([H] + K_a)$ as is the case for a monoprotic acid. Thus, the reported pK is the average of the two dissociation constants as suggested here.

The correct mechanism, rejected by the authors, appears to be one involving the rate-limiting addition of the amino group of B_1 to the thiazolium ring to give **5a** and this then is followed by fast elimination to **6**, each step involving a proton transfer. This scheme was advanced in 1957 (*16*).

The pK_a value of 12.7 obtained from the photometric titration of B_1 (*54*) has been attributed to pseudobase formation because this value is similar to those obtained for simple thiazolium ions lacking the pyrimidine ring (*23, 36, 56*). However, the measured value for pseudobase formation from B_1 and water obtained by a relaxation method is 9.7 (*28*). Perhaps the value of 12.7 is for amino group deprotonation without addition to the thiazolium ring.

(2) A Tertiary Carbanion Suggested as a Possible Alternative to the Ylide

A unique mechanism[3] for isotope exchange at the 2 position was proposed with the suggestion that tricyclic **5a** undergoes deprotonation at its tertiary carbon to give carbanion **12** which then eliminates thiolate ion to give anion **6** (*53*). The new mechanism was proposed in part because the second-order rate constant of 5.15×10^6 M^{-1} A^{-1} (25 °C) (*53*) representing the hydroxide-catalyzed conversion of **5a** into **6** is similar to those, 3.4×10^6 M^{-1} s^{-1} (30°C) (*34–36*) and 7.5×10^5 M^{-1} s^{-1} (detritiation, 30°C), (*60*) obtained for the direct formation of ylide by the action of lyate ion deprotonating B_1. Because isotope exchange is so rapid, acidic buffers must be employed, especially when the method of analysis involves proton NMR. Under these conditions B_1 exists essentially as structure **1**.

The overall process for the formation of the tertiary carbanion must also reflect the fractional amount of substrate in the desired reactive form **5a**; at a physiological pH of 7 the fractional amount of **5a** in equilibrium with B_1 is negligible (*16, 53*), excepting a recent and dubious claim to the contrary (*18*). Direct formation of the ylide from B_1, therefore, is a considerably faster process than that by the suggested route via **5a** and the tertiary anion. Moreover, it is not clear how a proton bound to the reacting carbon atom in **5a** becomes reattached to this same carbon atom in product **6** using the tertiary anion mechanism. The similar second-order rate constants are accidental.

An alternate more reasonable mechanism, also consistent with the kinetic data, involving deprotonation at the more acidic nitrogen atom of **5a** and then thiolate ion elimination to give **6** was disfavored (*53*).

Apparent support for the carbanion mechanism is said to be found in a derivative of B_1, claimed to have a sulfamoyl substituent (SO_2NH_2) bonded to the 4′-amino group. Such a substituent is expected to greatly increase the acidity of the NH

[3] This mechanism is reminiscent of a similar one discredited by Haake (*57*) in which others claimed that deprotonation of the 2 position of B_1 took place on the pseudobase of the thiazolium ring to give a tertiary carbanion. In support of the claim, isotope exchange reactions in both aqueous and nonaqueous solutions were observed, alcohols serving as the source of the hydrogen label in the later cases (*58*). Haake suggested that the alcohols acted as their conjugate bases to generate an ylide and did not add to the ring to form a pseudobase. Recent estimates of the relative acidities of alcohols (*59*) support Haake's analysis that a mixture containing *t*-butyl alcohol reacts slower than one having a less sterically hindered alcohol not because of steric hindrance to deprotonation of the pseudobase but because of the reduced acidity of *t*-butyl alcohol and the lower concentration of its conjugate base.

group. Moreover, this compound on forming a cyclic derivative of the **5a** type cannot have an ionizable proton at the annular nitrogen atom. Curiously, the same kinetic pattern of transformations is observed for this material as for B_1, including similar kinetic constants (*61*). Further clarification is required before this claim of support for the tertiary carbanion can be accepted. Perhaps the controversy could be settled by studying **5b**, a stable derivative of **5a**. Similar derivative **5b** does not react with benzaldehyde in methanol containing triethylamine (*20*).

(*3*) *Proposed Hydrolysis via a Pyrimidopyrimidine at High pH*

At high pH, e.g., >11.5, very little B_1 exists as such because it is largely in equilibrium with its various transformation products and so a novel idea was proposed when it was suggested that yellow **6** hydrolyzes directly to the thermodynamically favored formamido thiolate product **7** without the intervention of B_1 as an intermediate. The hydrolysis of the amidine unit in **6** was claimed to be able to provide **7** directly (*62*). An NMR kinetic study using rapid scans of the spectra showed that the rates of disappearance of the yellow form and the appearance of the hydrolysis product at pH about 12.5 are the same. No B_1 was detected (*63*). These observations were said to support the earlier mechanistic conclusion concerning the direct conversion of **6** to **7**. But only a very small amount of free B_1 is expected to exist under such conditions (*16*), making detection by NMR very difficult. The proposal was also discredited by the observation that the same second-order rate constant (19.2 $M^{-1}\ s^{-1}$) representing the slowest step in the overall complex scheme, that associated with the addition of hydroxide ion to B_1 to yield pseudobase, was obtained at high and low pH, thereby showing there is no additional channel for hydrolysis at high pH (*19*). Moreover, at high pH the rate of formation of **7** actually declines as the pH increases, in keeping with the idea that less B_1 is present as the basicity increases (*16*).

The foregoing speculations may be put into perspective by the following considerations based on literature rate and equilibrium values for *aqueous* solutions. If an acidic solution containing B_1 is rapidly raised to pH 11 then the half-life for ylide formation will be about 0.2 ms (*36*), the half-life for conversion to **6** based on initial rates will be about 7 s, while that for the formation of **7** will be approximately 35 s (*53*). The maximum equilibrium concentration of **6** will be about 6% at pH 11 and about 100% at pH 12.5. Eventually all of the B_1 will be converted to its hydrolysis product **7** (*16*). Clearly isotope exchange at position 2 will be at its equilibrium value long before any ring transformations take place. Moreover, at pH 7 essentially all the B_1 will exist as such and the half-life for ylide formation will be 2 s.

THE 4′-AMINO GROUP IN ENZYMIC PROTON TRANSFER

(*1*) *Kinetics*

A series of pyrophosphate derivatives of B_1 outline what structural features are needed for enzymic activity. They include 2- and 4-aminopyridine analogs lacking

the second annular nitrogen atom of the pyrimidine ring as well as a TPP lacking the 4′-amino group. These substances were incubated with the apoenzyme of pyruvate decarboxylase, pyruvate dehydrogenase complex, and transketolase in order to explore their catalytic potential. Only the 4-aminopyridine analog had catalytic activity (65–100%). The 2-aminopyridine and des-amino compounds were inactive. But des-amino substrate does have almost the same binding affinity as B_1. Therefore, N-1′ and the pyrophosphate group seem to be needed for *binding* but not the amino substituent (*64*). The amino group may be involved as a proton donor after the ylide reacts with carbonyl compounds to generate oxide anions. This site would be an even better proton donor to the oxide if N-1′ were protonated to increase its acidity (*65*).

(2) *Enzyme–Substrate Crystal Structures*

An X-ray crystal structure of the complex between the enzyme from baker's yeast and B_1 provides the first real insight into the possible mechanism of enzymic action. Transketolase, which has a dimeric structure, has the pyrophosphate group of B_1 bound in a deep cleft with only the proton at the 2 position of the thiazolium group accessible to solution and the pyrimidine ring in a hydrophobic pocket stacked with phenylalanine. Calcium ion is bound to the pyrophosphate. The conformation of B_1 is not that of the free vitamin. From the positions of the surrounding amino acids the mechanism of action is inferred to be as follows. There is no amino acid close enough to act as a base to form the ylide. The N-1′ position is protonated by a glutamic carboxyl and the 4′-amino group then is deprotonated by imidazole of histidine. The resultant imine then is believed to generate the thiazolium ylide. Close to the positive charge of the thiazolium ring is a stabilizing asparaginate residue. The 4′-amino group is oriented so that it can also protonate the oxide ion formed from a covalently bound carbonyl group following reaction with the ylide. (*66*). Moreover, a crystal structure of the enzyme lacking TPP shows that the cofactor does not induce large changes in the three-dimensional structure of the enzyme (*67*).

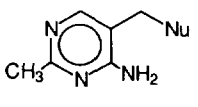
Pyruvate oxidase, a homotetramer, binds the two cofactors TPP and adenine dinucleotide in such a way that the conformation of TPP is similar to that found in transketolase. The 4′-amino group is directed at the C-2 position of the thiazolium ring and there is a neighboring carboxylate group positioned so as to be able to protonate N-1′, again providing evidence to support the speculation that the amino group plays a part in the catalytic process (*68*).

An interesting suggestion that perhaps under biological conditions the 4′-amino group of B_1 might add to the thiazolium ring to give a tricyclic intermediate, presumably **5a**, thereby protecting the ring from hydrolysis has been made (*69*). Such a structure has not yet been verified by these known crystal structures although the conformations in the crystal approach this arrangement. The reaction pathway inferred from the crystal structures has not involved a tertiary carbanion as an intermediate.

NUCELOPHILIC SUBSTITUTION AT THE BRIDGING METHYLENE GROUP

(1) *Sulfite Ion*

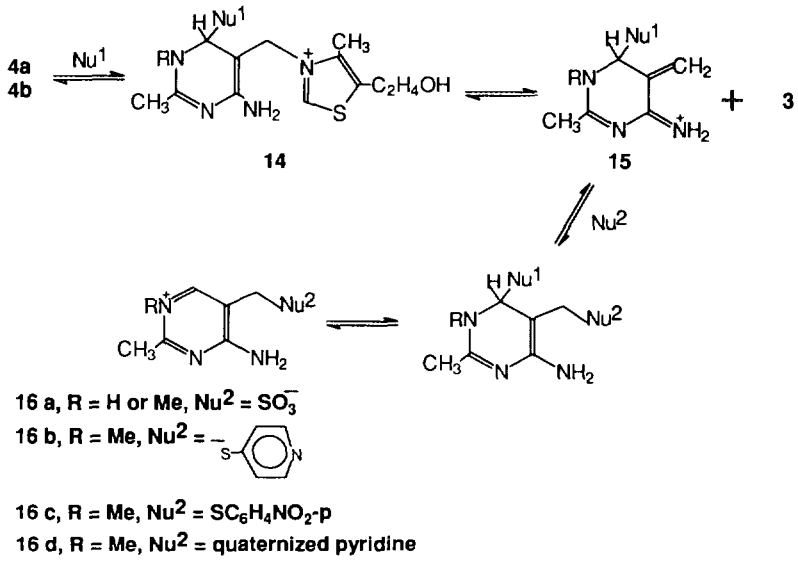
(*a*) *First-order kinetics for* B_1 *and* $NMeB_1$. The mechanism of the reaction behind Williams' accidental discovery in 1934 proved to be highly elusive and controversial. A thorough kinetic study in 1969 (*70*) using sulfite ion in place of SO_2 and another in 1975 (*71*) provided the general outlines of the kinetics but failed to reveal the correct mechanism of cleavage by sulfite ion. The seminal observations that give the important clue to the mechanism had been reported long before these kinetic studies, however. For example, as early as 1951 it had been noted that pyridines (*72, 73*), *provided* they were used in the presence of sulfite ion, bring about nucleophilic substitution of B_1 to give derivatives **13a**. Similarly, a hydrazide gave a substitution product (*74*) and aniline nucleophiles when used together with sulfite ion gave the anilino substitution products **13b** (*71, 75*). These are trapping experiments; they reveal that the mechanism of nucleophilic substitution does not involve the usual S_N2 pathway as observed for benzylic compounds. An intermediate has to be present.



13 a, Nu = quaternized pyridine
13 b, Nu = NHAr
13 c, Nu = N_3
13 d, Nu = NH_2
13 e, Nu = OH
13 f, Nu = H

The correct mechanism was finally demonstrated in 1977 (*76*). The kinetic study made use of competition experiments employing both sulfite and azide ions. In the presence of azide ion, B_1 did not react. When sulfite ion was added, nucleophilic substitution did take place. However, the major substitution product was not sulfonic acid **2** but rather the azide **13c**. With longer reaction times this azide did react with sulfite ion to provide **2** but not initially. Thus, sulfite ion is required for nucleophilic substitution but it does not appear in the initial product. Clearly an intermediate must form prior to the appearance of the product. A generalized mechanism designated $S_N(AE)$ appears in Scheme 1 where AE stands for addition–elimination. Sulfite ion ($Nu^1 = Nu^2$) first adds to the 6′ position of the N-1′-protonated pyrimidine ring of B_1 to give **14**, the leaving group departs to give resonance-stabilized cation **15**, a second sulfite ion traps **15** to give still another intermediate that finally expels the first sulfite ion to give sulfonate **2**, a long and involved process for an apparently simple reaction[4]. Normally, reaction of the second sulfite ion is not kinetically important, thereby failing to provide any clue

[4] Although sulfite ion may react nucleophically at either its oxygen or sulfur atoms, the oxygen atom seldom participates (*77*). When the nucleophilic site is oxygen, the resultant product is expected to eliminate sulfur dioxide and give an alcohol (*78*).



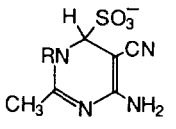
SCHEME 1

of the complexity of the mechanism to early workers. The aqueous solution kinetics show a "bell-shaped" pH (pD)-rate profile indicative of a reaction involving two protonation steps, the conversion of B_1 to its considerably more reactive conjugate acid by protonation at N-1′, and the conversion of bisulfite ion to the more reactive sulfite ion.[5] If B_1 is first converted to its *N*-methyl derivative **4b** (*81*, *82*), the kinetics then are simplified; only a simple pH dependence for the interconversion of bisulfite and sulfite ions now is observed when sulfonate **16a** forms as the final product (*76*).

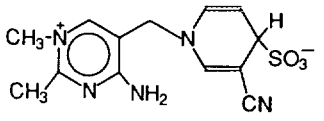
If B_1 is first labeled with deuterium atoms at the 6′ or bridging methylene positions (*83*), then small secondary kinetic isotope effects (KIE) are found. The KIE at 25° for the 6′ position is inverse (0.95) and normal (1.08) for the methylene position. These data provide evidence for the addition to the 6′ position but they do not distinguish between two possibilities concerning the rate-limiting step(s). Either (a) the loss of the first sulfite ion and the loss of the thiazole leaving group from the intermediate are kinetically competitive steps or (b) the addition of sulfite is rapidly reversible and the loss of thiazole is rate limiting. Similar KIE values were found for $NMeB_1$ (*84*).

In order to distinguish between the two mechanistic possibilities an equilibrium isotope effect (EIE) was obtained for adduct **17a**, a pyrimidine analog of B_1 having a deuterium label at position 6 and a cyano substituent at position 5. The equilibrium constants for the addition of sulfite at 25°C are K_H = 26.6 ± 0.26 and K_D = 27.9 ± 0.21 M^{-1}, giving an EIE of 0.95 (*85*), suggesting that for the kinetics of

[5] Bisulfite ion is a mixture of oxygen and sulfur protonated forms with the sulfonic acid tautomer having a protonated sulfur atom as the major contributor (*79*, *80*).



17 a, R = CH_3
17 b, R = $CH_2C_6H_4NO_2$-p



18

cleavage of B_1 the addition of sulfite ion is reversible and the loss of the thiazole-leaving group is rate limiting.

The size of the equilibrium constant for sulfite ion addition to give **17a** provides some insight into the value of the upper limit for such an adduct in the case of B_1, the sulfite adduct of B_1 never having been observed. In comparison with compounds with similar structures such as pyridinium ions that have far larger association constants (*86*), the above addition constants are surprisingly small. Although a cyano group exists at position 5 of **17a** to stabilize the adduct by resonance, this may be largely offset by the electron-donating amino substituent. The bridging methylene group of B_1 must provide far less stabilization than this cyano substituent and so the amount of adduct present at equilibrium in the case of B_1 must be quite small.

The NMR spectrum of an authentic sulfite adduct **17b** in D_2O showed that sulfite ion added to the less sterically hindered, unsubstituted 6 position as expected. In a derivative of **17b** having a hydroxymethyl group at position 5, no such adduct could be detected even in the presence of 3 M sulfite (*87*).

(*b*) *Second- and zero-order kinetics.* Other, strong support exists for complex Scheme 1 in the form of second and zero kinetic orders for sulfite under special conditions. When B_1 itself is treated with very low levels of sulfite ion under spectroscopic conditions trapping of cation **15** by sulfite ion becomes rate limiting (*88*). Now reaction of **15** with the liberated thiazole to return the intermediate back to B_1 is competitive with sulfite ion capture and the kinetics become second order in sulfite ion (*89*).

In the case of substrate **16b** having the anion of 4-thiopyridone as a leaving group, the departing ligand also serves as a nucleophile able to compete for **15** with the highly reactive sulfite. Therefore, second-order kinetics in sulfite ion are observed under a wide variety of conditions. For example, when excess leaving group is present along with sulfite ion (Nu^1) at the start, trapping of **15** by the leaving group (Nu^2) is especially efficient, demonstrating common ion retardation. Under such conditions it is possible to obtain a relative measure of the abilities of the two nucleophiles to trap the intermediate. Thus, the anion of 4-thiopyridone is 250 times more efficient than sulfite ion at trapping **15** (*90*). Under similar conditions *p*-nitrobenzenethiolate ion is 400 times more reactive than sulfite ion (*91*). Clearly these reactions of **15** are not diffusion limited as are those involving carbocations from ketals and sulfite (*78*) and 1-phenylethyl carbocations being trapped by thiolate and azide ions (*92*).

Kinetically zero-order sulfite reactions were observed with 3-cyanopyridine as a special leaving group. Sulfite ion first added reversibly to the pyridine ring to

give sigma adduct **18** and not to the pyrimidine ring largely because the resultant enamine is conjugated with the strongly electron-withdrawing cyano group, thereby providing considerable stabilization. The equilibrium constant for adduct formation has the large value 5×10^6 M^{-1}. The adduct with its poor leaving group does not undergo nucleophilic substitution and so only a small fraction of the total substrate is available in its original form for cleavage. When the amount of sulfite ion at constant pH was increased both the amount of unreactive adduct and the rate of reaction with free substrate increased. This canceling competition resulted in zero-order sulfite kinetics (*87*).

(*c*) *Other kinds of leaving groups*. Large families of *N*-1-methylated B_1 analogs **16** can easily be prepared from $NMeB_1$ (**4b**) simply by heating it in methanol with a variety of nucelophiles (Nu^2) including pyridines, phenoxide ions, and thiolate ions (*81*, *93*), thereby liberating the thiazole. In turn, these analogs may be cleaved to sulfonate **16a** with sulfite. Kinetic studies on these analogs and sulfite ion reveal that substituents on a pyridine-leaving group have a large influence on the rate of cleavage (*94*). By contrast, groups on the phenoxide nucleofuge have a much smaller influence on the cleavage rate (*95*). These contrasting values suggest that the timing of the rate-limiting steps may be different for the two classes of leaving groups, loss of the pyridines participating in the rate-limiting step but not the phenoxide ions.

Figure 1 presents for the first time an extensive linear free energy correlation of much of the kinetic data for sulfite reacting with B_1 and its analogs. The

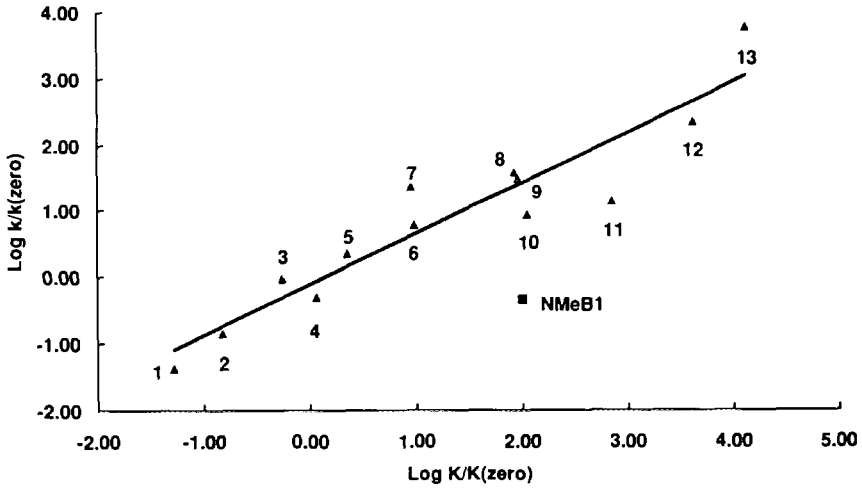


FIG. 1. Log–log plot of the relative reactivities (k/k_0) of 1′-methylated thiamin analogs toward sulfite ion in water at 25°C versus the relative acidities of their conjugate acids (K/K_0). The point for $NMeB_1$ was not considered in calculating the regression line which has slope 0.76 (r = 0.94) and intercept −0.11. The leaving groups are **1**, 3,4-dimethylpyridine; **2**, 4-methylpyridine; **3**, 4-methylphenol; **4**, 4-methylthiophenol; **5**, 3-methoxyphenol; **6**, 3-chlorophenol; **7**, 2,4-dichlorothiophenol; **8**, 4-nitrothiophenol; **9**, 3-cabamoylpyridine; **10**, 4-chlorophenol; **11**, 4-nitrophenol; **12**, 4-cyanopyridine; and **13**, 3-cyanopyridine.