activity by about 50.[7] These changes result in an additional decrease in the reactivity of the sp^3 relative to the sp^2 carbon by an approximate factor of $10^{2.7}$.
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Model Studies of Thiamin Catalysis. Steric Inhibition of Deprotonation of a Hydroxyethyl Side Chain

John A. Zoltewicz* and Larry S. Helmick

Department of Chemistry, University of Florida, Gainesville, Florida 32611

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Rate constants have been obtained for deprotonation of the 2 position of a series of 2-substituted 3-methylbenzothiazolium ions involving formate ion and water as bases in D_2O. Relative rate constants for the two bases are very similar in spite of the reactivity of formate ion being about 3.4×10^4 times greater than that for water. Second-order rate constants for formate ion at 75.0 °C and 1 M ionic strength are: 2-ethyl, 2.28×10^{-2}; 2-hydroxymethyl, 1.10×10^{-2}; 2-isopropyl, 2.28×10^{-4}; and 2-(1-hydroxyethyl), 4.50×10^{-4} M^{-1} s^{-1}. The major influence on reactivity is found in the latter two substances where steric inhibition of resonance is dominant. It is suggested that a similar steric effect will be present in the conjugate base of 2-(1-hydroxyethyl)thiamin and will influence rates and equilibria in nonenzymatic and possibly in enzymatic reactions as well.

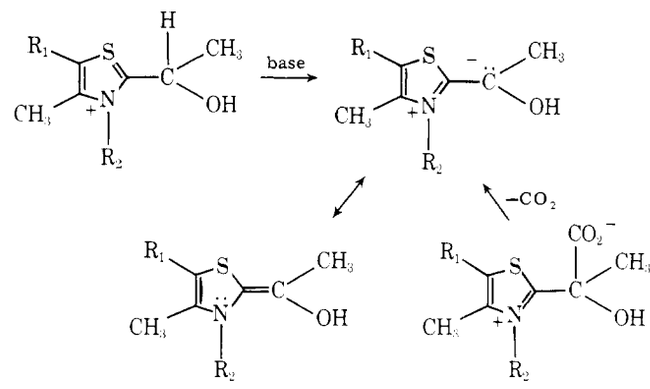
The enzyme cofactor thiamin pyrophosphate participates in a number of significant biological transformations. One very important derivative, an "enamine", is shown in Scheme I where it is formed by deprotonation of the corresponding 2-(1-hydroxyethyl) modification of the cofactor as well as by decarboxylation.[1] Prior to this study information has not been reported above how the methyl and hydroxy groups bonded to the exocyclic carbon atom of the enamine affect its rate of formation.

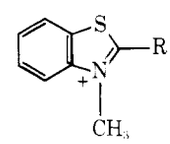
Generally, the effects of methyl and hydroxy or alkoxy substituents on the rates of deprotonation of a carbon atom to which they are bonded are variable and complex. The net effect is likely to be dependent on the hybridization of carbon in the transition state leading to deprotonation. Effects produced when the reactive site is pyrimidal can be different from those when the site is planar. Factors affecting reactivity include inductive and resonance effects, bond strengths as influenced by hybridization,[2] and electron pair repulsion associated both with electrostatic and Pauli exclusion principle effects.[3,4] Examples are known where an alkoxy group hinders[2] as well as facilitates[5] deprotonation of an adjacent sp^3 hybridization carbon.

In addition, the methyl and hydroxy substituents of the thiamin derivative in Scheme I may influence the rate of deprotonation by a steric effect. Interaction of the side chain and R_2 group may prevent the substituents and the ring from adopting a planar configuration, thereby hindering electron delocalization leading to effective charge neutralization. That is, there may be steric inhibition of resonance in the transition state and in the conjugate base.

We have studied a series of 2-substituted 3-methylbenzothiazolium ion model compounds I–V in order to obtain insight into the effects operating in the deprotonation reaction given in Scheme I. Starting with a 2-methyl substituent, the hydrogen atoms have been systematically replaced, first by a methyl and then by a hydroxy group, to yield 2-ethyl (II) and 2-hydroxymethyl (III) substrates. Next, two such substitutions give rise to 2-isopropyl (IV) and 2-(1-hydroxyethyl) (V) model compounds. We believe the effects of these replacements on rates of deprotonation provide considerable insight into the factors affecting reactivity of the enzyme cofactor not only in deprotonation but also in decarboxylation reactions as well.

Scheme I





I, R = CH_3
II, R = CH_2CH_3
III, R = CH_2OH
IV, R = $CH(CH_3)_2$
V, R = $CHOHCH_3$

Results

Hydrogen–deuterium exchange reactions were carried out at 75.0 °C in D_2O solution, generally using a formate buffer.

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Table I. Kinetic Results of Hydrogen–Deuterium Exchange at Position 2 of 2-Substituted 3-Methylbenzthiazolium Ions in Formic Acid Buffers (D_2O) at 75.0 °C and 1.0 M Ionic Strength

Compd	Registry no.	pD[a]	Total buffer, M	Obsd k_ψ, s^{-1}	Calcd[b] k_ψ, s^{-1}	k_B, M^{-1} s^{-1}	k^B_{rel}	$k^{D_2O}_{rel}$
I	40265-71-2	3.22	0.108	2.44×10^{-4}		1.50×10^{-2}		
						(1.62×10^{-2}[c])	1.0	1.0
II	46005-85-0	—	0.1 M DCl	4.18×10^{-5}		(7.73×10^{-6}[d])		
		3.14	0.108	2.56×10^{-4}	3.22×10^{-4}		1.46	1.58
		3.17	0.325	1.02×10^{-3}	9.40×10^{-4}	2.28×10^{-2}		
		3.25	0.0325	1.54×10^{-4}	1.47×10^{-4}			
III	46005-87-2	—	0.1 M DCl	1.34×10^{-5}		(2.48×10^{-7}[d])		
		3.17	0.325	5.09×10^{-4}	4.47×10^{-4}		0.71	0.51
		3.22	0.108	1.49×10^{-4}	1.73×10^{-4}	1.10×10^{-2}		
		3.25	0.0325	6.21×10^{-5}	6.42×10^{-5}			
IV	65102-07-0	—	0.1 M DCl	3.7×10^{-7}		(6.8×10^{-9}[d])		
		3.16	1.20	3.56×10^{-5}	3.30×10^{-5}			
		3.87	0.200	1.99×10^{-5}	1.90×10^{-5}		0.015	0.014
		3.89	0.200	1.56×10^{-5}	1.95×10^{-5}	2.28×10^{-4}		
		3.92	0.600	(3.85×10^{-5})				
V	65102-08-1	—	0.1 M DCl	7.5×10^{-7}		(1.4×10^{-8}[d])		
		3.16	1.20	6.56×10^{-5}	6.51×10^{-5}		0.029	0.028
		3.87	0.200	3.72×10^{-5}	3.76×10^{-5}	4.50×10^{-4}		
		3.92	0.595	(6.92×10^{-5})				

[a] Measured at 25 °C. [b] $[B]_{free} = [buffer]_{tot} \times K_a/([D] + K_a)$ where $pK_a = 4.03$. [c] Reference 6. [d] $k_\psi/[D_2O] = k_{D_2O}$.

Our extensive studies of the 2-methyl substrate have shown that in this buffer both D_2O and formate ion act as catalysts to deprotonate the methyl group; deuterioxide ion does not play an important role kinetically.[6] Therefore, it is assumed that only these two bases are effective with benzothiazolium ions II–V. The pseudo-first-order rate constant for isotope exchange, k_ψ, then is given by eq 1

$$k_\psi = k_{D_2O}[D_2O] + k_B[\text{formate}]_{tot} \times \frac{K_a}{[D] + K_a} \quad (1)$$

where k_{D_2O} and k_B are second-order rate constants for water and formate ions acting as general bases, K_a is the dissociation constant for formic acid ($pK_a = 4.03$), and $[\text{formate}]_{tot}$ is the total buffer concentration.[7]

Because the pK_a of formic acid changes only slightly with temperature[11] the pD of the reaction medium was measured at 25 °C instead of the 75 °C reaction temperature. As a check on this assumption, one kinetic run was carried out on the methyl substrate which we have already investigated extensively.[6] The second-order rate constant was calculated according to eq 1 using the pD measured at 25 °C and the value of k_{D_2O} obtained earlier.[6] The k_B values, Table I, are very similar; the one obtained using pD's determined at 75 °C is only 9.2% smaller than the one derived with pD values measured at 25 °C.

Kinetic data collected on the four other substrates are summarized in Table I. In each case a rate constant was obtained in the absence of any formate buffer; by using 0.1 M DCl the term k_{D_2O} was obtained. The k_B term representing formate ion catalysis then was derived from these results and eq 1. By comparing in Table I the observed and calculated values of k_ψ, the good agreement between these values becomes apparent. The average deviation is about 12% except in the case of V where it is less.

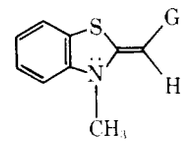
High buffer concentrations were employed with the two less reactive ions IV and V in order to obtain convenient rates. Interestingly, the results obtained for each ion in the presence of the highest free base concentration examined, 0.26 M formate ion, do not agree with those found at lower base concentrations. The first-order rate constants estimated with the aid of data from the other runs at lower formate ion concentrations are about 1.7 times as great as those observed. The deviant results were therefore not used in calculating a k_B value.

Buffer association needs to be considered as a possible reason for the low reactivity at high formate ion concentrations. Formic acid and formate ion from a complex; at 25 °C the association constant for this process is 0.25 M^{-1}.[12] If this value is applied to our data and no consideration is given to temperature differences and the fact that light and heavy water are involved, then it can be calculated that 8% of the formate ion appears as unreactive complex. This value which is likely to overestimate the true amount of complex at the higher temperature employed in our study is too small to provide an explanation of our deviations. Although there are a number of reasons for nonlinear buffer concentration–rate plots,[12] a likely explanation of our deviant data is found in salt effects.

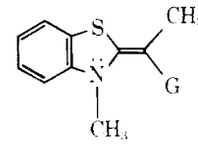
Discussion

Reactivity and Selectivity. The results in Table I in the form of relative rate constants, k_{rel}, using the reactivity of the 2-methyl substrate as a reference show that formate ion and water have very similar selectivities in deprotonating the carbon acids. Therefore in making comparisons an average of the relative reactivities for the pair of bases is considered. On this basis, the spread between the most and least reactive substrate is a factor of 105.

Relative rate constants show that introduction of a single substituent onto the 2-methyl group of the benzothiazolium ion does very little to reactivity. Methyl substitution to give II increases reactivity by about the same extent (50%) that hydroxy substitution to yield III decreases it (70%). The conjugate bases formed from these acids probably have *Z* configuration VI for steric reasons. The exocyclic double bond



VI, G = CH_3, OH



VII, G = CH_3, OH

in these conjugate bases is expected to be well developed. For example, the methylene protons of the base formed on deprotonation of I show nonequivalent NMR signals.[13] This is consistent with the presence of a substantial barrier to rotation.

Precise interpretations of how substituents influence re-

Table II. Elemental Analyses of 2-Substituted 3-Methylbenzothiazolium Perchlorates

Substituent	Mp, °C	% Calcd			% Found		
		C	H	N	C	H	N
C_2H_5	136–138	43.25	4.36	5.04	43.09	4.38	4.98
$(CH_3)_2CH$	141–142	45.28	4.84	4.80	45.14	4.88	4.74
CH_3CHOH	115–116	40.89	4.12	4.77	40.91	4.15	4.83

activity in the case of II and III are difficult to make. If the transition state were highly pyramidal, then the hydroxy group would exert a large rate accelerating effect due to the electron withdrawing character of oxygen. A methyl group, however, would exert a marked rate retarding effect. Neither is observed. Therefore, in the transition state the carbon atom must lose p-orbital character in its σ bonds and begin to approach an sp^2 geometry. Electron–electron interactions associated with the carbon and oxygen centers coupled with a decrease in the strength of the carbon–oxygen bond oppose the inductive activation of the oxygen atom. The bond between the methyl substituent and the reactive carbon gets stronger as p-orbital character is removed from it; this beneficial change opposes the unfavorable inductive effect. Thus, it is possible to rationalize the small net result on reactivity of the two substituents in terms of these opposing effects. Of course, as the reactive bond acquires more p character, more negative charge is delocalized into the positively charged ring resulting in a more developed exocyclic double bond. The double bond when highly developed in the product will be stabilized by both the hydroxy and methyl groups.[14]

In contrast to the very small changes in rate constants resulting from introducing a single substituent onto the 2-methyl group, disubstitution produces much larger changes which are reductions in reactivity. Isopropyl and hydroxyethyl substrates are about 70 and 35 times less reactive, respectively, than reference acid I. The origin of this diminished reactivity can only be steric and reflects inhibition of resonance in the transition state.

A reversal in the relative reactivities of hydroxy substrate and its alkyl counterpart occurs at the levels of mono- and disubstitution. Thus, monosubstitution gives rise to a methyl derivative which is 2.6 times more reactive than the corresponding hydroxy cation, II vs. III. But with disubstituted substrates the methyl derivative is 0.51 times less reactive than its hydroxylated counterpart, IV vs. V. These same results may be considered in another way, in terms of the kinetic effects produced by introducing a methyl substituent. The reactivity of a hydroxy compound is decreased by a factor of 21 (III vs. V) while that of an alkyl ion is diminished by 105 (II vs. IV).

Two explanations seem relevant. First, steric compressions which dominate the reactivites of IV and V may be smaller in the hydroxylated material. The transition state leading to conjugate base may adopt a conformation which positions the smaller hydroxy substituent close to the *N*-methyl group, the site of the energetically unfavorable steric interaction. That is, *E* configuration VII is formed on deprotonation. The extent of the steric inhibition is thereby reduced and the resultant reactivity is greater. Second, and in addition to the first conformational factor, transition states for the two disubstituted compounds could be more pyramidal than those for the monosubstituted substrates. The more a transition state retains sp^3 geometry, the smaller will be steric repulsions which are greatest in the conjugate base where the side chain and ring attempt to become coplanar. Moreover, it is possible that in a pyramidal transition state the hydroxy group exerts an activating inductive effect which opposes and is larger than the destabilizing effect of the methyl group. It should be noted that considerable electron delocalization into a ring can take place from a pyramidal center.[15]

Support for our configurational assignment comes from another source. From an examination of molecular models in conjunction with studies on the decarboxylation of 2-(1-carboxy-1-hydroxyethyl)thiazolium ions, the suggestion was advanced that the enamine derivative will exist in a configuration which minimizes steric interactions,[16] i.e., the *E* configuration.

Significant information becomes apparent when the reactivities of formate ion and water are compared. Formate ion is more reactive than water by an almost constant factor of 3.4×10^4. This corresponds to a difference in free energies of activation of 7.2 kcal/mol. Results can be considered in quantitative terms in the form of a linear free energy relationship which compares the logarithms of the second-order rate constants for formate ion with those for water. This excellent relationship has a slope of 0.995 and a correlation coefficient of 0.998. The free energy relationship shows that steric effects are not dependent on the charge and identities of the two catalysts and must primarily be associated with interactions in the substrate and not between substrate and base.

In giving rise to conjugate base with an *E* configuration substrate must assume a particular conformation in the transition state. A hydroxyethyl reactant, for example, has the hydroxy group positioned close to the nitrogen atom of the thiazolium ion and the methyl substituent close to the sulfur atom so as to minimize steric compressions as product forms. However, this may not be the major conformer in the ground state. X-ray data on 2-(1-hydroxyethyl)thiazolium ions reveal that in the solid state the positions of the hydroxy and methyl groups are reversed to those for the transition state; i.e., the hydroxy group is close to the sulfur atom.[17] If this conformation is also the major one in solution, then proton transfer must proceed thorough a rotomer present as a minor component of the mixture of conformers.

Steric inhibition of resonance should also be kinetically important for deprotonation reactions in which other groups such as hydroxybenzyl[9] are bonded to thiazolium ion rings. Again, the configuration of the intermediate is expected to be *E* because the smaller hydroxy rather than the larger phenyl group is positioned close to the N substituent.

An explanation for an unsuccessful reaction in terms of a steric effect becomes apparent for a heretofore puzzling result. The *N*-benzyl derivative of thiamin serves almost equally as well as thiamin in acting as a catalyst for the nonenzymatic conversion of pyruvate ion to acetoin. However, the α-methylbenzyl derivative is inactive.[18] We suggest that the inactivity is associated with steric hindrance to the formation of the intermediate enamine. The α-methyl group prevents the ring and the side chain from becoming coplanar, thereby substantially reducing conjugation between the two portions.

In view of the constant steric effect toward two bases of very different reactivities in the case of the 2-(1-hydroxyethyl)-benzothizolium ion model compound we make this suggestion: steric hindrance of similar magnitude will be observed in the reactions of 2-(1-hydroxyethyl)thiamin. This steric inhibition of deprotonation will be observed in purely chemical systems involving the substituted enzyme cofactor and in enzyme catalyzed reactions as well, unless the enzyme constrains the intermediate to adopt a pyramidal form. Similar steric in-

teractions will also be present when the intermediate enamine is formed by decarboxylation.

Experimental Section

The reagents and instrumentation employed in this study are listed in the accompanying paper.[6]

Preparation of 2-Substituted 3-Methylbenzothiazolium Salts. 2-Substituted benzothiazoles[19] were made by heating *o*-aminobenzenethiol with an equivalent amount of the appropriate carboxylic acid or acid anhydride in a bomb at temperatures ranging from 120 to 165 °C.[20] They were quaternized with methyl iodide.[21] The 2-hydroxymethyl iodide was used as such, mp 227–228 °C (lit.[20] mp 219 °C), but the others were converted to perchlorates. Perchlorate salts were prepared either by dissolving the iodides in a warm saturated solution of magnesium perchlorate in absolute ethanol or by dissolving in a mixture of ethyl acetate, absolute ethanol, and 70% perchloric acid (36:8:5 by volume). Perchlorate salts crystallized on cooling and were recrystallized from ethanol. Melting points and analyses were recorded in Table II. Analyses were made by Atlantic Microlab, Inc. Chemical shifts (τ, D_2O) of *N*-methyl, *C*-methyl, and CH protons observed during kinetic runs are: C_2H_5, 5.74, 8.36, and 6.45; $(CH_3)_2CH$, 5.69, 8.37, and 6.00; CH_2OH, 5.78 and 4.55; CH_3CHOH, 5.69, 8.23, and 4.18, respectively.

Kinetics of Hydrogen–Deuterium Exchange. Details are similar to those in the accompanying article.[6] Stock solutions of formic acid and sodium formate were employed. Ionic strength was maintained at 1 M using KCl (with iodides) or NaCl (with perchlorate salts). Reactions were followed for 2–3 half-lives except for the slowest runs involving 0.1 M DCl where only 1 half-life was observed. In addition to substrates being examined separately, a run was also made on a pair of substrates with similar reactivities in the same mixture.

Following a kinetic run pD measurements were made at 25.0 °C on both heated and unheated reaction mixtures. Differences were of the order 0.03 except in the case of two runs with IV at the lowest buffer concentrations; one of these also contained hydroxyethyl compound. Curiously, in these cases the difference in pD was 0.12; the pD of the unheated sample was recorded. This makes the free base concentration uncertain by about 15%. A pH reading was converted to pD by adding 0.40.[22]

Control Studies to Determine the Stabilities of Substrates. Each compound was heated in formate buffer in H_2O for a period corresponding to 10–20 half-lives for isotope exchange. No decomposition was detected by NMR; pH differences between heated and unheated samples were no greater than 0.03.

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Kinetics and Mechanism of the Deamination of 1-Methyl-5,6-dihydrocytosine

Sanford Slae and Robert Shapiro*

Department of Chemistry, New York University, New York, New York 10003

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Kinetic studies of the deamination of 1-methyl-5,6-dihydrocytosine (MDC) have been carried out in acidic and basic aqueous solutions at 37 °C, μ = 1.0 (ionic strength). General-base catalysis was observed under acidic but not basic conditions. The Brønsted relationship for this reaction showed β = 0.19. No dependence on hydroxide ion concentration was demonstrable under alkaline conditions. Activation enthalpies and entropies were measured for this reaction in the absence of general catalysts in acidic and basic media for the range 20–47 °C. Direct hydroxide ion attack on the protonated substrate is a plausible mechanism for the reaction in alkaline media. An alternative mechanism involving participation of water as a proton-transfer agent in the transition state with either formation or reaction of the tetrahedral intermediate as the rate-dtermining step is also consistent with all of the kinetic data.

The deamination of cytosine to uracil by bisulfite[1,2] has been applied widely, as a synthetic method in nucleoside chemistry and as a tool for the modification of nucleic acids.[3] Mutations are induced in bacteria and viruses upon treating them with high concentrations of bisulfite and acidic pH.[4] The mutagenic specificity observed indicates that the mutations are caused by cytosine deamination within DNA.[4] The possibility exists that environmental bisulfite and sulfur dioxide may constitute a mutagenic hazard[5] to the general public. To evaluate this hazard, it is necessary to be able to extrapolate deamination rates to low bisulfite concentrations at neutral pH.

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