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(25) It has been argued by a referee that a small change in charge could be consistent with a charge distribution arising from a fast averaging of permutational isomers in a stepwise pathway. If the rate-limiting step were the formation of the intermediate (as might be expected if the permutational isomerization were fast) then the effect "seen" would be that for the addition step and "averaging" would not be "seen". In any case the excess charge would reside on the sulfone oxygens and the sum of this (which would be "seen" as a difference) would always be unity no matter how the charge were disposed.

$N^{1'}$-Methylthiaminium Diiodide. Model Study on the Effect of a Coenzyme Bound Positive Charge on Reaction Mechanisms Requiring Thiamin Pyrophosphate

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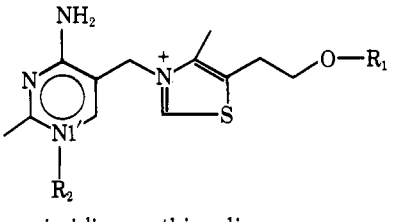
Abstract: Thiamin (Ia), 4-aminopyrimidine (IIa), and 4-amino-5-methoxymethyl-2-methylpyrimidine (IIIa) were converted to the corresponding pyrimidinium methiodides Ib, IIb, and IIIb, respectively. The relative rates by Ib over Ia were found to be 2.5:1 for thiazolium ylide formation; ca. 3:1 for pyruvate decarboxylation (CO_2 loss), ca. 15:1 for acetoin formation from pyruvate, and about 4:1 for thiazolium ring opening. Thus Ib in all respects is a superior model catalyst to Ia. A plausible source of all the demonstrated rate enhancements is an electrostatic field effect of the positive charge in Ib destabilizing the positively charged ground states and/or stabilizing the carbanion and oxyanion-like transition states. The amine deprotonation pK_as of IIb and IIIb were determined to be near 12; hence the positive charge in Ib can be expected to convert the amino group into a weak acid. The possible relevance of these findings to thiamin pyrophosphate requiring enzymatic reactions is discussed.

The thiazolium ring of the coenzyme thiamin pyrophosphate (TPP) forms a covalent intermediate with the substrates of the enzymatic reactions requiring TPP.[1] A chemical role



pyrimidine thiazolium

R_1	R_2	Name
H		Thiamin (Ia)
H	CH_3	$N^{1'}$-Methylthiamin (Ib)
$PO_3PO_3^{3-}$		Thiamin pyrophosphate (TPP)

of the pyrimidine and dimethylenepyrophosphate portions of the coenzyme is still not clear, however. Nevertheless, both moieties have been substantiated to be essential for coenzyme activity. Via a number of elegant synthetic feats Schellenberger's group was able to demonstrate that while the amino group is not needed for the coenzyme to exhibit inhibitory properties, it is essential for catalysis.[2] The rest of the pyrimidine undoubtedly is important in the binding process. In model systems the thiazolium ring was found to form a weak molecular complex with tryptophan.[3] More recently the pyrimidine moiety was also shown to interact with tryptophan according to fluorescence experiments.[4] These results suggest potential binding mechanisms on the enzyme. Schellenberger and coworkers suggested that the amino group functions either as a proton acceptor (normal role for strongly basic amino groups) and/or in the transferral of acetaldehyde from coenzyme to solution.[2] The major difficulty with this proposal is that all related aminopurines, such as adenine,[5] and amino pyrimidines, such as that in TPP[6] and cytosine,[5] possess very weakly basic and weakly nucleophilic amino groups. In fact, all these compounds beyond any doubt protonate and bind metal ions on the ring nitrogens preferentially rather than on the exocyclic amino group. Specifically, according to all x-ray crystallographic,[7] nuclear magnetic resonance,[8] and theoretical results[9] N1′ is the site of proton binding in thiamin and TPP. It is also quite clear now that both soft, such as Ni(II), and hard, such as Mn(II), metal ions bind to the pyrimidine N1′ position in thiamin, albeit the Ni(II) binds stronger than the Mn(II)-[10-13]

Based on these facts, it occurred to us that the enzyme could activate the amino group catalytically, making it in fact a proton donor, as well as enhance the rates of several of the el-

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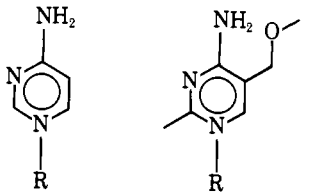
Table I. ^{1}H NMR Chemical Shifts at 100 MHz in Me_2SO-d_6 at 27 ± 1 °C[a]

Nucleus obsd	Chemical shifts										
			Δ^bIb–Ia							Δ^bIIIb–IIIa	
	Ia	Ib	Me_2SO-d_6[c]	H_2O[c]	IIa	IIb	ΔIIb–IIa[b,d]	IIIa	IIIb	Me_2SO-d_6[d]	D_2O[c]
					Pyrimidine						
C2′-H					8.33 (s)	8.78 (s)	0.45				
C2′-CH_3	2.42 (s)	2.68 (s)	0.26	0.23				2.33 (s)	2.59 (s)	0.26	0.23
C5′-H					6.42 (d)	6.84 (d)	0.42				
C6′-H	8.16 (s)	8.41 (s)	0.25	0.05	8.03 (d)	8.26 (d)	0.23	7.95 (s)	8.35 (s)	0.40	0.17
C5′-CH_2	5.43 (s)	5.53 (s)	0.10					4.26 (s)	4.32 (s)	0.06	0.11
C4′-NH_2	7.34 (s)	8.84 (s)	1.50	~1.60	6.77 (s)	8.78 (s)	2.01	6.60 (s)	8.22 (s)	1.62	
		9.29 (s)	1.95						8.98 (s)	2.38	
N1′-CH_3		3.85 (s)				3.83 (s)			3.83 (s)		
O′-CH_3								3.26 (s)	3.35 (s)	0.09	0.00
					Thiazolium						
C2-H	9.57 (s)	9.99 (s)	0.42	0.20							
C4-CH_3	2.53 (s)	2.53 (s)	0.00								
CH_2CH_2O	3.10 (t)	3.10 (t)	0.00								
CH_2CH_2O	3.68 (t)	3.68 (t)	0.00								

[a] s, singlet; d, doublet; t, triplet. [b] Chemical shift difference between methylated and unmethylated analogues. [c] Measured from DSS. [d] Measured from Me_4Si.

ementary steps by attaching a positive charge to the N1′ pyrimidine position.[14] Potentially a proton, or the Mg(II) ion known to be required for catalysis,[1] or a positively charged amino acid side chain from the enzyme could serve this purpose. Since in aqueous model systems any of these positive charges would only be associated with the pyrimidine ring in a mobile equilibrium, we have introduced a methyl group onto the N1′ pyrimidine position to mimic the presence of a single positive charge on this ring. To elucidate the effect of this methyl group on thiamin-catalyzed reactions we also synthesized appropriate pyrimidine models methylated at the corresponding N1 position.



R	
	IIa
CH_3	IIb
	IIIa
CH_3	IIIb

We have found from spectroscopic, thermodynamic, and kinetic measurements that in all respects Ib is a superior model catalyst to Ia.

Experimental Section

Materials. Thiamin mononitrate was obtained from Nutritional Biochemicals Corp., 4-aminopyrimidine (IIa) from Cyclo, methyl iodide from Fisher, α-naphthol and acetoin (3-hydroxy-2-butanone) from Matheson Coleman and Bell, phenylglyoxalic acid and tris(hydroxymethyl)aminomethane from Aldrich, and creatine from Eastman. 4-Amino-5-methoxymethyl-2-methylpyrimidine (IIIa) was a gift from Merck Sharpe and Dohme Research Laboratories, Inc., Rahway, N.J.

Synthesis of N^1-Methyl Salts. IIb and IIIb were synthesized from IIa and IIIa, respectively, according to the typical procedure given below. A given quantity of IIIa was mixed with molar excess methyl iodide in methanol and left to stand at room temperature for 0.5 h. The mixtures were then refluxed at ca. 50 °C for 3 h, after which the solvent was evaporated in vacuo. Upon treatment with ethanol the undissolved product IIIb was filtered, dried, and recrystallized from boiling ethanol. Since IIb was reported elsewhere,[15] it could be identified by comparison of its physical properties with literature values. IIIb gave the following elemental analysis. Anal. Calcd for $C_8H_{15}IN_3O$: C, 32.56; H, 4.78; N, 14.23; I, 43.0. Found: C, 32.64; H, 4.97; N, 14.02; I, 42.87. ^{1}H NMR data on IIb and IIIb are quoted in Table I.

Ib was synthesized by refluxing thiamin mononitrate (Ia) with excess methyl iodide in methanol overnight. The residue which resulted on cooling was filtered and upon partial evaporation of the solvent the filtrate gave a further precipitate. This precipitate was taken up in boiling ethanol and the undissolved material filtered off while the solution was still hot. Upon cooling, the filtrate gave Ib as a precipitate as characterized by ^{1}H NMR (Table I). Anal. Calcd for $C_{13}H_{20}I_2N_4OS$: C, 29.21; H, 3.75; I, 47.56; N, 10.49. Found: C, 29.43; H, 4.23; I, 46.51; N, 10.87. The slight discrepancy is probably due to a 5–10% NO_3^- (from starting material) as the counterion. Ib melted at 214–220 °C with decomposition. The assignment of the structure, including position of methylation at N1′, has been unequivocally confirmed by single-crystal x-ray crystallographic studies performed in this department. Alternatively, Ib could be separated from Ia by first dissolving the product mixture in a minimum amount of water at pH 7.0 and filtering the residue, found to be mainly Ia. The solution pH was then adjusted to 4 and the water was stripped off. Dissolving the mixture at pH 7 and repeating this fractionation about three times afforded pure Ib according to ^{1}H NMR detection limits (>98%).

pK determinations were performed spectrophotometrically on a Beckman Acta III spectrometer at ca. 24 ± 2 °C (room temperature) to avoid any time lapse which may lead to decomposition of the solutions. In a typical experiment 30 μL of 0.01 M stock solution of IIb or IIIb, prepared in absolute ethanol, was added to 3 mL of 0.1 M buffer, the solution was mixed, and absorbance was read. All pH measurements were made on a Radiometer pHm 26 meter. At pH > 11 the G202B and appropriate calomel electrodes were employed. Aliquots of standard 1 M NaOH were used to prepare solutions between 0.01 and 0.1 M in NaOH and the ionic strength was maintained at 0.1 with KCl. All buffers were degassed for at least 30 min with N_2 gas and the pH reading was taken; UV measurements were made within 30 min following termination of degassing. The solutions were found to be stable for several hours below pH 13. The data (Figure 2) were computer fitted to the "best" theoretical titration curve providing the pK_as.

^{1}H NMR Studies. High-resolution data on all compounds were collected in ^2H_2O and Me_2SO-d_6 on a JEOL PS-FT-100 spectrometer operating in the fast Fourier transform made at 27 ± 1 °C. Chemical shifts were recorded against DSS (4,4-dimethyl-4-silapentanesulfonic acid sodium salt) and are reproducible to ±0.003 ppm. On 0.1 M solutions a few transients with 8K data points, 1000 Hz spectral width, and 90° pulse with appropriate repetition time gave noise-free spectra. Chemical shifts in Me_2SO-d_6 were recorded against Me_4Si or DSS.

The rates of chemical exchange of the C2-H in the thiazolium rings

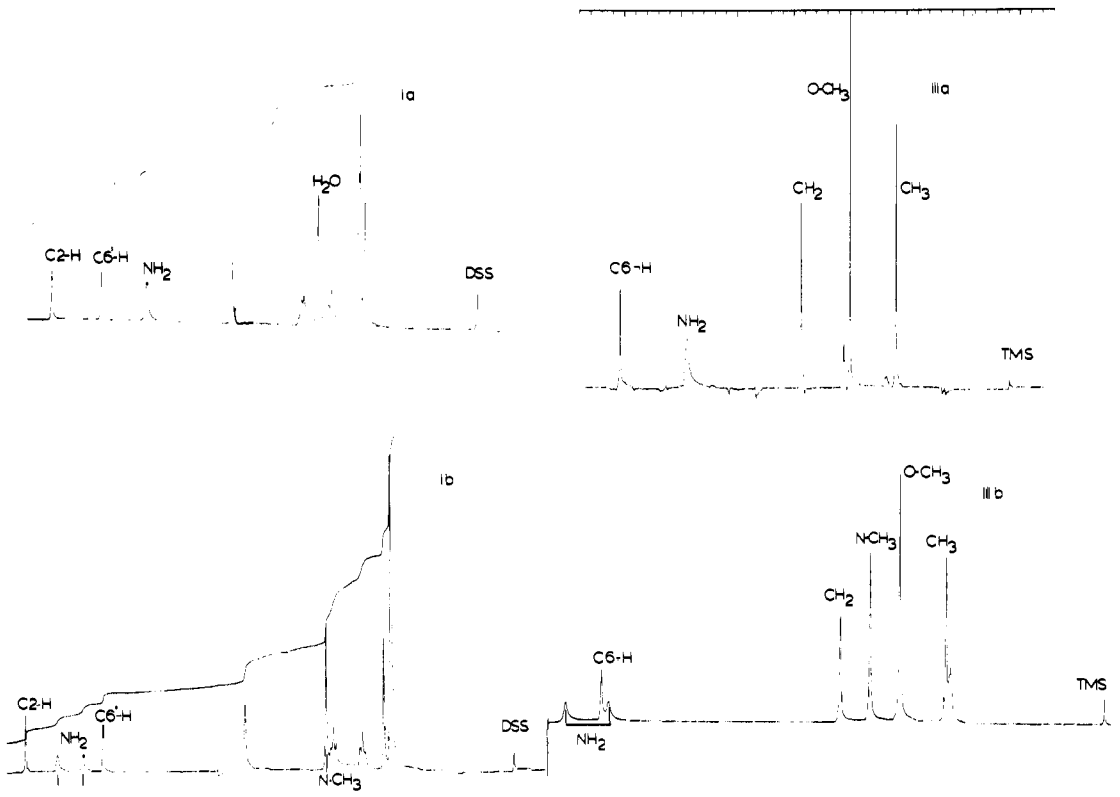
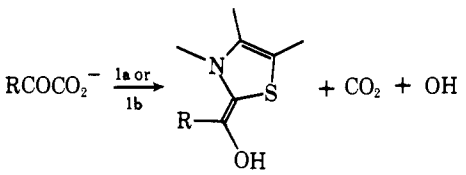


Figure 1. ^1H NMR spectra in Me_2SO-d_6. Ia, Ib, and IIIa recorded at 1250 Hz, IIIb at 1000 Hz spectral width. Chemical shifts are listed in Table I. The peaks near 2.5 ppm were complicated by residual ^1H in the solvent.

of Ia and Ib were determined at room temperature on a Perkin-Elmer R-24A 60-MHz instrument. Solutions (0.25 M) of Ia and Ib were made up in H_2O and adjusted to pH 8.2 ± 0.1 with HCl or NaOH. pHs were recorded before and after the NMR spectra were taken and these pH values agreed to within 0.1 units. The phase of the spectrum was adjusted on the large H_2O signal and the spectrum corresponding to each sample was recorded in both increasing and decreasing field directions. The widths at half-maximal peak height were determined for both C2-H and C4-CH_3 since the latter does not undergo exchange broadening.

Measurement of Decarboxylation Rates. As pointed out before and shown to be experimentally sound,[16] the rate of pyruvate decarboxylation can be followed either respirometrically or employing a pH-stat



unit. We employed a Radiometer pH-stat (pH meter equipped with automatic titrator, autoburet, titrigraph recorder, and circulating temperature bath) to measure the rate of hydroxide ion release, where R = CH_3 or C_6H_5. The initial velocity of these curves of H_3O^+ consumed vs. time reflect the zero-order decarboxylation rates in the thiazolium-catalyzed model reactions, as the proton is consumed at the time of CO_2 loss.

Acetoin formation was followed according to procedures outlined by Westerfeld[17] and Metzler and associates.[18] Mixtures of 0.2 M pyruvate and 0.02 M Ia or Ib were incubated for 2 h at 40 °C under N_2 atmosphere in 0.2 M Tris buffer at pH 7.6 for Ib and pH 7.6, 8.1, and 8.6 for Ia. Aliquots (1 mL) were withdrawn for the acetoin test. The remainder was adjusted to pH 1.5 with HCl (to quench the acyloin reaction) and allowed to incubate for 1 h to decarboxylate acetolactate to acetoin. Both reaction mixtures, before and after acid treatment, were tested for acetoin as follows: 1 mL of the condensation reaction mixture was mixed with 4 mL of H_2O, 1 mL of 0.5% creatine, and 1 mL of 5% α-naphthol. The absorbance at 525 nm was read after the color was allowed to develop for 1.5 h at room temperature. The reference cuvette contained all components except the reaction mixture. Standard samples were made up using 85% acetoin in water. Since thiamin and its ring-opened forms can both interfere with the color reactions, absolute quantities of acetoin cannot be determined this way but relative amounts can by keeping all reaction conditions the same. It was evident that the reaction mixtures near neutral pH produced acetolactate predominantly as the amount of acetoin detected increased ca. eightfold on incubation under acidic conditions.

Determination of the Rate of Thiazolium Ring Opening. A stock solution 0.01 M in Ia or Ib was made up at pH 4. This stock solution (30 μL) was added to a 1-cm UV cuvette containing 3 mL of 0.1 ionic strength carbonate buffer preincubated at 30 °C. The rate of ring opening was determined[19] from pseudo-first-order kinetic plots obtained at 297 nm at pH 9.65 ± 0.1 and at 255 nm (for Ia only) at pH 10.4 ± 0.1; 255 nm could not be used for Ib since only a shoulder is observed at this wavelength for this compound and the absorbance change with time is very small.

Results and Discussion

^1H NMR Chemical Shifts. Table I presents the data in Me_2SO-d_6 for all compounds studied. Figure 1 shows the relevant low-field regions of the spectra. As expected the $N1'$-methiodides exhibit deshielding of all pyrimidine proton nuclei compared to their unalkylated analogues. The unanticipated result concerns the appearance of the amino proton resonances. In Me_2SO-d_6 there are two amino peaks (of equal areas according to integration) in Ib and IIIb only. In accord with previous experiments of this type by McConnell on cytosine derivatives,[20] we attribute this behavior to the slowing down of the rotation of the exocyclic amino group on the NMR time scale so that the two amino hydrogens reflect their different environments. The enhanced double bond character of the C4–N bond on attachment of a positive charge at $N1'$ is evident from a comparison of the crystal structures of thiamins having a neutral[21] and protonated[22] pyrimidine ring. Those studies suggest the following to be the principal resonance contributions.

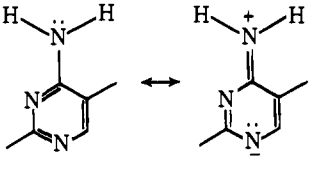
Apparently (see data on IIb) the positive charge above is not

Table II. pK_a's of Thiamin and Related Pyrimidines, ca. 25 °C

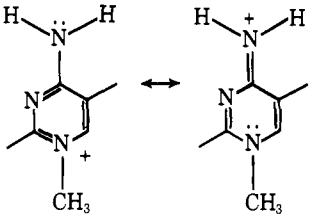
Compd	N1′ (pyrimidine)[a]	NH_2 (pyrimidine)[b]	C2-H (thiazolium)[c]
Ia	4.93[d]		12.6
IIa	5.71[e]		
IIb		12.1[d] + 0.1, 12.2 ± 0.15[e]	
IIIa	5.90[d]		
IIIb		12.7 ± 0.15[d]	

[a] Deprotonation of pK_a of N1 protonated compounds measured potentiometrically. [b] Deprotonation of pK_a of exocyclic amino groups measured spectrophotometrically. [c] Deprotonation of pK_a of thiazolium C2-H to ylide from ref 40 employing temperature jump. [d] This work. [e] Reference 15.

sufficient to give two amino peaks. Two distinct amino hydrogen resonances are only observed by further substitution

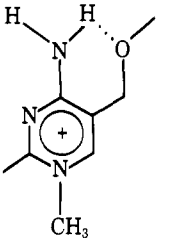


I-IIIa I-IIIa*



I-IIIb I-IIIb*

at C5′. This could be due to the interaction of one amino hydrogen via an intramolecular hydrogen bond. In IIIb this in-



IIIb

teraction is nearly ideal since a six-membered hydrogen bonding network is easily constructed. Such intramolecular hydrogen bonding in neutral aminopyrimidine was first suggested by Mason.[23] Observation of the phenomenon in Ib suggests that an intramolecular hydrogen bonding scheme may exist in this ion as well, or more likely, that the two amino hydrogens participate in intermolecular hydrogen bonds to two different acceptors (i.e., to I^- and β-hydroxyethyl of a second molecule) as suggested by the x-ray studies. Some alternative explanations exist, but are less likely to be valid.[24] In aqueous medium this phenomenon is not observed.

Sable and his co-workers had shown some years ago that the amino hydrogen resonance, a single peak, is visible in aqueous solutions of Ia.[6] They attributed this to relatively slow exchange between these amino and solvent protons. We examined spectra of thiamin at low pH in H_2O and that of Ib in H_2O down to −2 °C. We could observe only a singlet in this entire temperature range substantiating earlier work.[6] Thus, the unusual behavior of Ib and IIIb observed in aprotic but polar Me_2SO-d_6 is not in effect in H_2O. Clearly, the aqueous solvent

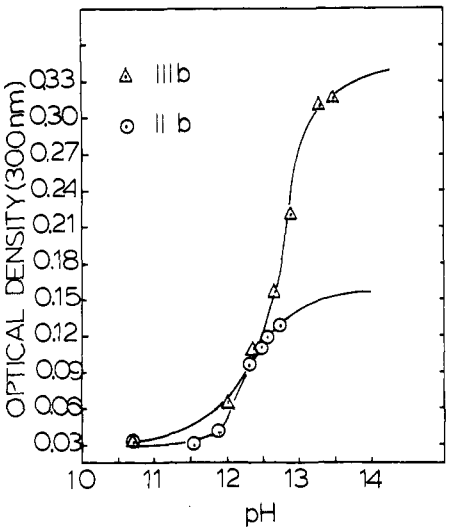


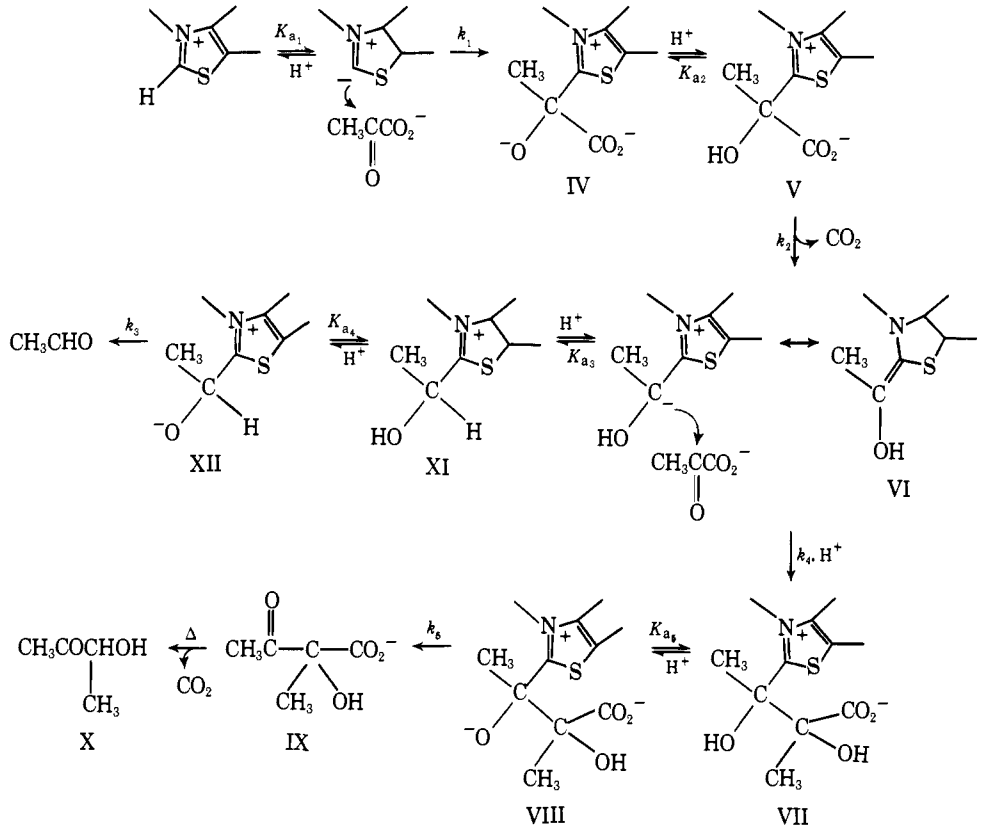
Figure 2. Ultraviolet pK_a titration of IIb and IIIb.

would be an excellent competitor as a hydrogen bond acceptor. The hydrogen bond acceptor property of these amino hydrogens in Ib is clearly demonstrated.

The second finding is the significant deshielding of the C2-H resonance only in the thiazolium ring in Ib compared to Ia. This phenomenon is observed in both Me_2SO-d_6 and in H_2O as solvents and is reminiscent of the behavior of C2-H in thiaminium ion.[8] The C2-H is clearly unique in this respect as is shown by a comparison of the Δ (difference in chemical shift between Ia and Ib) of 0.4 ppm for this nucleus (separated from the pyrimidine ring by four bonds) with Δ of 0.1 ppm for the C5′-CH_2 (separated from the pyrimidine ring by only two bonds) chemical shifts. The other hydrogen nuclei attached to the thiazolium ring are unaffected going from Ia to Ib. The specific deshielding of C2-H in Ib is not due to a through-bond mediated inductive effect since such an effect should be larger for C5-CH_2. Two possible explanations of this deshielding are (a) the conformational disposition of the two aromatic rings with respect to each other changes going from Ia to Ib, such that the pyrimidine ring shields C2-H less in Ib than in Ia; or (b) the conformational disposition of the two aromatic rings remains the same; the deshielding in Ib is simply due to the neighboring positive charge. Whatever the exact origin of this deshielding, the observed phenomenon should be useful in testing the potential binding of a positive moiety to the pyrimidine portion in the enzymatic mechanism.

pK_a of Amino Groups in *N*-Alkylpyrimidinium Substrates. The amino pK_as in IIb and IIIb were determined as models for Ib since the latter also has a thiazolium pK_a and is subject to thiazolium ring opening in strong base. A UV spectrophotometric approach was utilized. These studies also indicated that compounds examined were stable up to a pH of 13, beyond which hydrolysis may have taken place, to the corresponding 4-hydroxyprimidine. The pK_a values obtained are quoted in Table II and the UV titration is shown in Figure 2. Since in IIb and IIIb only there is a pK_a near pH 12 but not in IIa or IIIa, the site of deprotonation in IIb and IIIb can be assigned to the amino group. On account of the similarity of IIb and IIIb to the pyrimidinium ring of Ib, a pK_a near 12 can also be projected for the amino deprotonation process of this compound. Less direct spectroscopic evidence has recently been reported for acidification in the closely related adenine system. McConnell reported ^1H NMR evidence showing that N1 protonation of adenine in 2′,3′-cyclic adenosine monophosphate lowered the amino pK_a from 18–19 to 10.6–11.7.[25] Cross, Brown, and Fisher reported stopped-flow UV evidence for an apparent pK_a of 7.8 for the exocyclic imino tautomer of

Scheme I



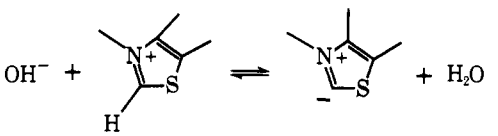
adenosine.[26] Macon and Wolfenden reported a pK of 8.25 for protonation of the exocyclic imino nitrogen in N^1-methyladenosine.[27] A most dramatic effect of a neighboring positive charge on NH acidities was recently demonstrated by Melendez and Vilarrasa[28] who showed that the NH deprotonation pK_a of imidazole shifted from 14.2 in the unsubstituted molecule to 2.6 by introduction of $-N_2^+$ in the C2 position. The weakly acidic nature of the amino group found in Ib (para to the positive charge) is not unreasonable. Thus, while the amino group in Ia–IIIa cannot act as a proton acceptor, in Ib–IIIb it can act as a proton donor, potentially useful as a general acid–base catalyst. From the precedent in adenine derivatives, the amino group in thiamin (or TPP) should have a pK_a of 18–20, whereas its protonation should only occur in strong acid.[5,a29]

Comparative Catalytic Effects of Ia and Ib. A scheme for the catalytic action of thiamin on pyruvate, based on the work mainly of Breslow[30] and Metzler,[18,19] is shown in Scheme I. The rates of various steps in the mechanism could be studied with different techniques. The rate of ylide formation could be ascertained from either the rate of ^2H incorporation into the C2 thiazolium position (only in weak acid) or by exchange broadened line width measurement of the ^1H NMR spectrum. The rate of decarboxylation, all steps up to and including k_2, could be studied either by respirometry or following the rate of hydroxide liberation in the reaction.[16] The rate of acetaldehyde formation could be followed spectrophotometrically at 340 nm employing alcohol dehydrogenase and NADH. Finally, the amount of acetoin formed could be determined employing complexometric techniques.[17,18] We examined all these facets of the model reaction employing Ia and Ib as model catalysts. To allow direct comparison between the catalytic efficiencies of Ia and Ib, the pH of the reactions had to be carefully chosen. The medium had to be basic enough to ensure that the pyrimidine ring of Ia remained neutral (pH >7), and not basic enough to allow significant thiazolium ring opening[19] (pH <9) unless the ring opening rates were to be specifically determined.

Exchange Kinetics. The widths at half-height, $\Delta\nu_{1/2}$, for C2-H in Ia and Ib were measured and compared to the width at half-height of the nonexchanging C4-CH_3 resonance $\Delta\nu_{1/2}^0$. When $\Delta\nu$ is measured in hertz, τ, the mean lifetime (s) of the protons in a particular chemical environment, is given by

$$\tau^{-1} = 2\pi(\Delta\nu_{1/2} - \Delta\nu_{1/2}^0)$$

The value of τ^{-1} is the pseudo-first-order exchange rate constant (in either direction) for the process



The half-lives correspondingly are given by $t_{1/2} = \tau \ln 2$. At pH 8.2 the half-life of C2-H in Ia was 2.5 times longer than in Ib (Figure 3). These exchange kinetic results demonstrate that the positive charge on the pyrimidine ring enhances the kinetic acidity of C2-H. Destabilization of the thiazolium ground state by the neighboring positive charge in Ib relative to the transition state is a reasonable explanation for this observation. At neutral pH the amino hydrogens in Ib were also found to exchange with solvent faster than in Ia, as expected.

Suchy et al.[8] and Gallo and Sable[31] reported a tenfold increase in C2-H $\rightleftharpoons$ C2-D incorporation in thiamin over 4′-deamino-4′-hydroxythiamin (oxythiamin). Under their experimental conditions the thiamin pyrimidine ring was protonated (pK_a = 5.0), whereas that of oxythiamin was not (pK_a = 2.3). Based on our results the faster exchange they observed in thiamin could, at least in part, be attributed to the presence of the protonated pyrimidine ring.

Petzold investigated the kinetics of C2-H exchange in Ia and in thiamin pyrophoshate[32] and suggested a mechanism in-

Table III. Comparative Catalytic Effects of Ia and Ib

Process catalyzed	Temp, °C	pH	k_{obsd} with Ia	k_{obsd} with Ib	Concn	Rel rates rate Ib/ rate Ia
C2-H exchange[a]	24 ± 1	8.1 ± 0.1	30.1 s^{-1}	75.4 s^{-1}	0.25 M	2.5
$PhCOCO_2^-$ decarboxylation[b]	30 ± 1	7.7 ± 0.05	1.69 ± 10^{-6} mol/ min · mol Ia	5.80 × 10^{-6} mol/ min · mol Ib	5 mL 0.05 M $PhCOCO_2^-$ 0.5 mL 0.10 M Ia or Ib	3.4
$CH_3COCO_2^-$ decarboxylation[b]	30 ± 1	7.8 ± 0.05	2.08 × 10^{-6} mol/ min · mol Ia	6.24 × 10^{-6} mol/ min · mol Ib	2.8 mL 0.2 M pyruvate 0.2 mL 0.3 M Ia or Ib	3
Thiazolium ring opening[c]	30 ± 1	9.6 ± 0.1	5.7 × 10^{-4} s^{-1}	2.2 × 10^{-3} s^{-1}	10^{-4} M	3.9
Acetoin formation[d,e]	40 ± 1	7.6 ± 0.1 for Ib 8.6 ± 0.1 for Ia	0.64 (2)	0.92 (3)	2.8 mL 0.2 M pyruvate 0.2 mL 0.3 M Ia or Ib	1.5

[a] ^1H NMR line width measurement. [b] pH-Stat kinetics. [c] UV. [d] Colorimetry; absorbance at 525 nm (see Experimental Section), in parentheses number of determinations. [e] Amount of acetoin detected was assumed to be proportional to the rate of the reaction.

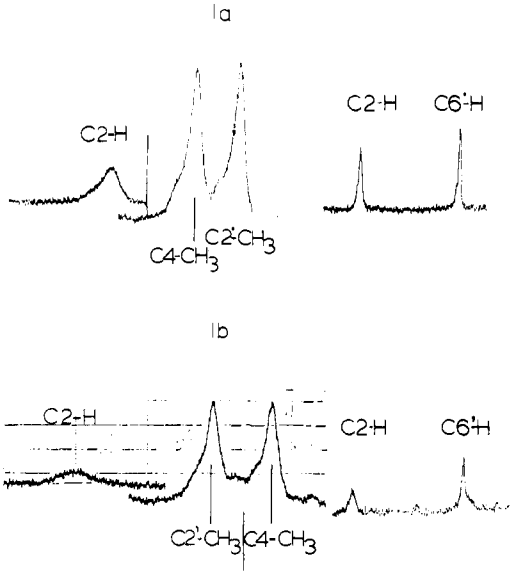
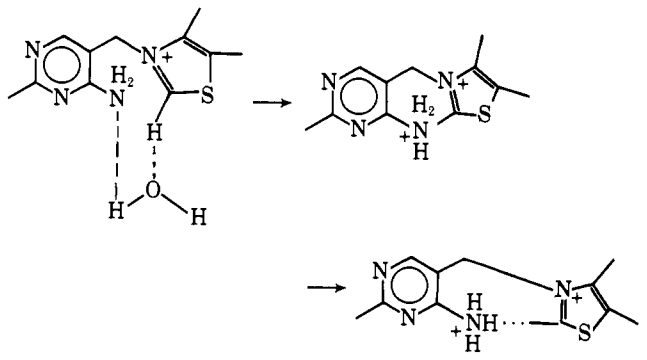


Figure 3. ^1H NMR line broadening studies in H_2O at 60 MHz, pH 8.17 ± 0.02, 0.25 M concentrations of Ia and Ib, identical spectral parameters. Right-hand peaks in normal scan, left-hand peaks show expanded spectrum used for line width measurements. Primed positions pertain to the pyrimidine ring, unprimed to the thiazolium.

volving the amino group. This implies a role for the amino group as a proton acceptor in general acid-base catalysis. If



the amino group had this role the C2-H of Ia should exchange faster than that of Ib (clearly not the case according to our results, Table III) since the amino nitrogen should be less basic in Ib than in Ia. In view of the fractional order dependence of exchange rates on OH^- concentration reported by Petzold,[32]

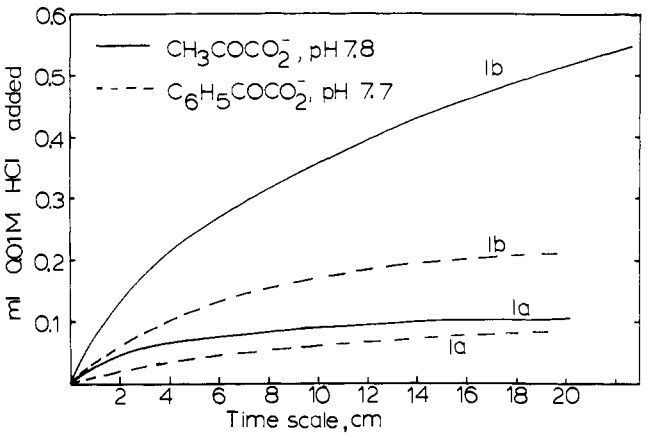


Figure 4. pH-Stat kinetic plots for decarboxylation of pyruvic and phenylglyoxalic acids. Chart speed for pyruvic acid, 2 min/cm; for phenylglyoxalic acid, 1 min/cm, 30 °C.

our comparisons for Ia and Ib at a single pH should still be qualitatively correct.

Decarboxylation and Acetoin Formation. The rates of decarboxylation of pyruvic and phenylglyoxalic ($PhCOCO_2H$) acids were measured by the pH-stat method employing Ia and Ib as catalysts. The experimental data (Figure 4, Table III) show that Ib is a superior catalyst for decarboxylation than is Ia with both substrates. Since the ylide form of the catalyst is needed in the rate expression, part of the decarboxylation catalytic factor is again due to greater stability of ylide in Ib than in Ia. In addition, however, Ib must catalyze better than Ia some other step among the steps culminating in CO_2 loss. This additional catalysis by Ib could occur in the nucleophilic addition or in the decarboxylation step. The major conclusion, beyond experimental error, is that Ib provides a modest additional catalysis for decarboxylation over Ia under our experimental conditions. Ia to date has been the best model catalyst known for this reaction.

That the positively charged pyrimidinium of Ib is more effective in stabilization of all carbanionic and oxyanionic intermediates (also of the transition states preceding them) than is the neutral one of Ia is also evident from the acetoin formation studies. At pH 8.1 thiamin produced only trace quantities of acetoin at 40 °C even under conditions leading to quantitative decarboxylation of acetolactate to acetoin (i.e., subsequent acid incubation, see Experimental Section). Ib on the other hand, was effective in acetoin condensation even at pH 7.6. In fact catalysis of acetoin formation by Ib could not

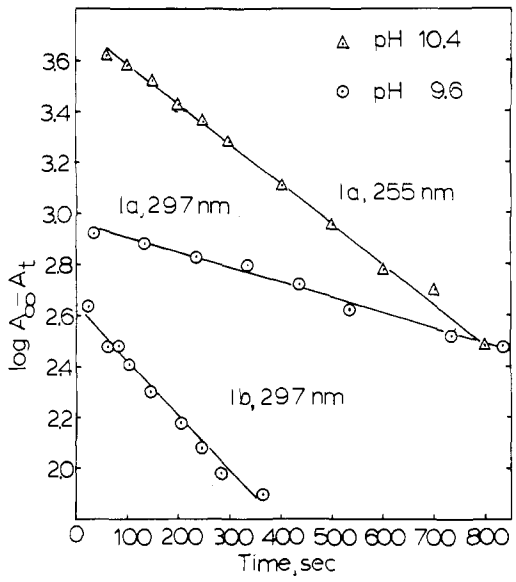


Figure 5. Pseudo-first-order kinetic data for thiazolium ring opening at 30 °C for Ia and Ib.

be studied at pH 8.6, 40 °C (requiring a several hours long incubation period) since Ib gives a pseudobase of the thiazolium ring (vide infra) at a lower pH than does Ia. The ratio of acetoin formed by Ib at pH 7.6 ± 0.1 to that formed by Ia at pH 8.6 ± 0.1 is about 1.5. The pH dependence of the rate of acetoin formation as catalyzed by Ia was demonstrated by others.[18]

It was evident from our experiments that at pH 7.6 acetoin formation is much slower than decarboxylation as catalyzed by Ia since no detectable quantity of acetoin was apparent at this pH. Thus the rate-limiting step for acetolactate (and hence acetoin) formation must be a step subsequent to CO_2 loss, the rate being proportional to either k_4[pyruvate][VI] or k_5[VIII]. Since the concentration of either VI or VIII should vary directly with OH^- concentration (see accompanying K_{a3} and K_{a5}), the ratio of acetoin formed by Ib/Ia at pH 7.6 can be estimated at 15. The model reaction produced no detectable acetaldehyde ($K_3 = 0$) according to the enzymatic reaction

$$CH_3CHO + NADH \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} CH_3CH_2OH + NAD^+$$

and monitoring the NADH absorbance at 340 nm. Accordingly, all pyruvate was probably converted to acetolactate, then to acetoin. While NADH may also reduce the thiazolium ring,[33] this would be a very slow reaction under our conditions, and the NADH absorbance would change in the same direction, i.e., decrease, as with the reaction outlined above. Our failure to detect acetaldehyde is in accord with earlier studies.

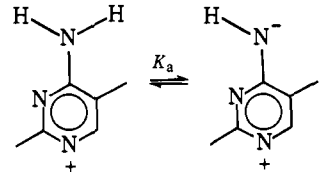
The 15-fold rate enhancement by Ib over Ia in acetoin formation can, at least in part, be attributed to stabilization of carbanionic and oxyanionic transition states related in structure to VI and VIII by the positively charged ring in Ib. In addition, in order to lose acetolactate, the α-hydroxy proton in VII must be removed so as to convert the alcohol to the alkoxide. A pK_a of 11 has been reported for this alcohol group in Ia.[34] The presence of the positive charge in Ib should increase the acidity of this alcohol group, hence facilitate the release of product from the coenzyme.

Thiazolium Ring Opening Reactions. UV spectroscopy was employed to follow the pseudo-first-order rate for thiazolium ring opening.[18] At pH 9.65 ± 0.1 Ib undergoes ring opening at some four times faster rates than Ia (Table III, Figure 5). The presence of the adjacent positive charge in Ib destabilizes the thiazolium ground state compared to Ia. In recent studies Haake and his co-workers suggested that the ring-opened thiazolium ring may function in the transport of thiamin.[35] The faster rate of ring opening found in Ib than in Ia suggests a further potential use of the proposed model.

In fact, as Breslow pointed out,[30] thiamin has optimal activity at a pH high enough to produce significant amount of ylide, and low enough to avoid substantial conversion to the pseudobase and thizolium ring opened forms both of which would be catalytically inactive. Ib accordingly would have a lower optimum pH for decarboxylation than Ia since Ib undergoes thiazolium ring opening at lower pH than does Ia. This we have clearly observed since at pH 8.7 Ib forms hardly any acetoin at 40 °C (optimal pH for acetoin formation catalyzed by Ia is ca. 8.9 at 40 °C).

Possible Relevance of the Model to the Enzyme Mechanism. The role of the pyrimidine ring as an electron-withdrawing group in thiamin action had been recognized before.[6,30] In his early definitive model studies Breslow[30] had shown that the pyrimidyl methyl substituent accelerated both C2-H exchange and acetoin formation compared to a methyl substituent at N3. This study has shown that a positive charge on the pyrimidine can accelerate ylide formation, decarboxylation, and acetoin formation over the natural vitamin. The results are consistent with the idea of either a through-bond electron-withdrawing inductive effect or an electrostatic field effect of the positive charge. It is interesting and important that the model is most effective in acetoin formation. We have shown elsewhere that the rate-limiting step in the pyruvate decarboxylase mechanism is not loss of CO_2.[36] According to previous studies[2,37] and the model systems here reported, it is likely to be loss of acetaldehyde. It is the loss of product in the model and enzyme reactions which requires particular attention. One can speculate on further improvements in our model if one considers the nature of electrostatic interactions. The magnitude of such interactions varies directly with the charges and inversely with distance of charge separation and medium dielectric constant. Were the divalent cation Mg(II) bound to pyrimidine N1′ in the enzymic process, this could double the effect. Lowering of the medium dielectric constant, as shown to be effective for all steps in the pyruvate decarboxylase model reaction[37] and implied by fluorescence studies[38] on the enzyme, could increase the effect by at least an order of magnitude. Conformational inflexibility, especially likely in the enzymic intermediates,[39] could bring the positive center closer to the incipient carbanionic transition states, also increasing the effect here found.

The second potentially important feature of the model here suggested concerns the acid-base properties of the amino group of the coenzyme. It has been demonstrated that the amino group could be converted to a weak acid in Ib with an accompanying strong conjugate base. This acid-base system could



participate effectively in proton transfers involving K_{a2}, K_{a4}, K_{a3}, and K_{a5} in Scheme I. The approximate pK_a of 12 for this amino group is very close to several other important pK_as in the mechanism. These include pK_{a1} = 12.7 (Scheme I) given by Hopmann and Brugnoni,[40] pK_{a4} = 11[34] or 12.0[37a], and pK_{a3} = 17.[37a] A hydrophobic active site would tend to enhance the dissociation shown above in the present model in addition to lowering pK_{a1}, pK_{a3}, and pK_{a4} simultaneously. Hence, the model would supply a viable intramolecular general acid-base

catalyst in the fashion outlined. We have demonstrated how the enzyme could acidify the amino protons. To our best knowledge no mechanism exists currently which would account for making this very weakly basic amino group into a strong base. One should also mention that the lowering of pK_as by enzymes is not unprecedented. Schmidt and Westheimer demonstrated a ca. four pK unit difference between the ϵ-amino group of free lysine and the catalytically important lysine of acetoacetate decarboxylase.[41] While we have not substantiated that the amino group of the model Ib participates as an acid-base catalyst in the model kinetic studies, the possibility of such a mechanism on the enzyme cannot be dismissed. The optimum pH for yeast pyruvate decarboxylase is about 6.0.[16,42] The optimum pH for thiamin-catalyzed reactions is about 8.9. The model system here suggested lowers the optimum pH of all reactions studied to 7–8, clearly in the appropriate direction.

It is noteworthy that all thiamin model catalysts studied to date led to formation of condensation products rather than of acetaldehyde. The pyruvate decarboxylase holoenzyme must in some fashion release acetaldehyde prior to approach of a second pyruvate molecule since only trace amounts of acetoin were reported in the enzymatic reaction.[1] It is the catalysis of acetaldehyde release which to date has no simple chemical analogy and which warrants further investigation.

A major virtue of the hypothesis here presented is that it can be further explored both in enzymatic and model studies.

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