# Thiamin Catalysis. 2. Kinetics and Mechanism of the Generation of the Yellow Form of Thiamin[1]

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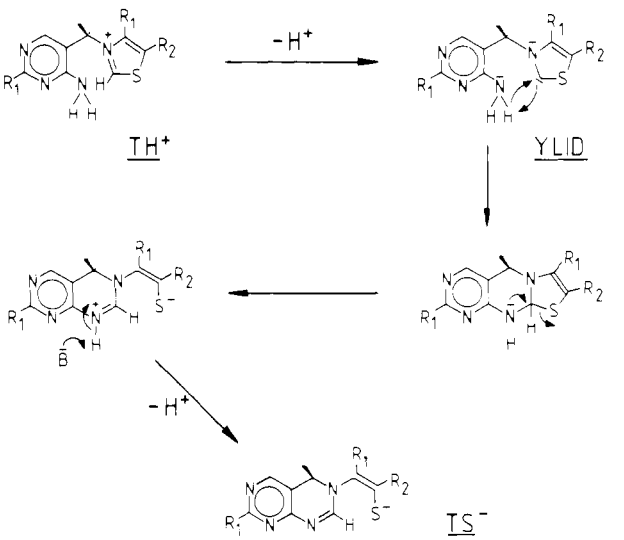
**Abstract:** The kinetics of the hydroxyl ion induced transformation of thiamin to the so-called yellow form were investigated by stopped-flow measurements in dependence of the pH, buffer composition, and ionic strength of aqueous solutions. The pH amplitude and rate profiles are described satisfactorily with the amplitude and rate equations calculated for a model of two consecutive deprotonations, the first being rate limiting. Least-squares curve fitting yielded the following results: $pK_{a1} = 12.39 \pm 0.05$, $pK_{a2} = 10.55 \pm 0.03$ ($pK_a$: acid dissociation constants) and the reverse rate constant $k_{-1} = 2.6 \pm 0.3\ s^{-1}$ with a root mean square standard deviation of 0.15. The pH's employed in this study cause the thiamin ylide (deprotonation of the thiazolium C–H group with a $pK$ of 12.6) to play a role in the transformation. Evidence for this is provided by carrying out the reaction in deuterated ethanol and trapping the yellow form with BrCN. Deuterium for hydrogen exchange is demonstrated at the C(2) site of the dihydropyrimidinopyrimidine ring by IR and $^1H$ and $^{13}C$ NMR spectroscopy. This finding demands extension of the 3-state to a 4-state model, the two deprotonation steps separated by a rate-limiting intramolecular transition. A numerical analysis of the data yielded $k_{-1} = 2.65 \pm 0.25\ s^{-1}$, $pK_{a1} = 14.15 \pm 0.03$, $K_1 = 59 \pm 6$, and $pK_{a2} = 10.55 \pm 0.03$ with a root mean square deviation of 0.15. Both models yield the same $pK_{av} = 11.47$. This $pK_{a1}$ should match the $pK$ of ylide formation, but it differs by the value of $pK_1$. There is no explanation for this situation.

Thiamin (vitamin $B_1$, TH) is a highly labile compound, which undergoes easily transformations induced by alkali. In particular, if TH is dissolved in aqueous solutions with a pH greater than 11, the color turns immediately yellow.[2,3] Zima and Williams[3] suggested for the first time the dihydropyrimidinopyrimidine structure, which was supported by the results of subsequent work[4-6] (see Scheme I, YF, $R_1 = CH_3$, $R_2 = (CH_2)_2OH$). Two equivalents of base is consumed during the reaction (3 equiv if thiamin dichloride is used). By adding 1 equiv of base to an ethanolic solution of TH and isolating the product, Maier and Metzler[4] concluded that this intermediate must possess a tricyclic structure (TC, dihydrothiachromine form of TH). This conclusion rested on a comparative spectroscopic product analysis. Thus, they were able to propose a two-step reaction mechanism for the generation of YF, though stopped-flow measurements have to be performed in order to measure the kinetics because YF formation is rapid.

This paper reports on the kinetics of the generation of YF and on deuterium exchange experiments, which demonstrate deuterium incorporation into YF at the C(2) site of the thiazolium ring of TH. The mechanism by Maier and Metzler[4] precludes this possibility.

Stopped-flow measurements of alkaline solutions of TH have been carried out previously[7] as a fast photometric titration for the determination of the $pK$ of the thiazolium ring C(2)–H group in order to avoid interferences from the undesired hydrolytic and protolytic side reactions, to which TH is subjected at high pH values, inferring that these preexponential amplitudes are due to the formation of the ylide of TH. It is therefore concluded that the TH ylide is the primary intermediate in the alkali-induced generation of YF.

Scheme I




## Experimental Section

Thiaminium dichloride (Merck) was recrystallized from 80% ethanol. All solutions were made up with deionized, doubly distilled water. Stock solutions of TH were of the order of $10^{-4}$ M and contained $10^{-3}$ M potassium dihydrogen phosphate, yielding a pH of 7.3. The ionic strength was adjusted to 0.1 M with NaCl. The stock solutions were mixed with various buffer solutions of 0.1 M ionic strength and adjusted pH in a modified Gibbs–Durrum stopped-flow apparatus. The change of the absorbance was monitored at 335 nm and 25 °C. The absorbance–time profile was transmitted via a 905 Datalab to a PDP 11/40 minicomputer. The exponentials were analyzed by a program, DIALOG, kindly supplied by Dr. A. Labhardt of our department. The effluent mixture of the stop syringe was collected, and the pH was measured with a high-alkali electrode (Ingold KG, Zürich) connected to a Polymetron Precision pH meter type 1501 that was calibrated with NBS standard buffers.[8] The dependence of the rate of transformation on the ionic strength was measured at pH 11.55, where the employed arsenate buffer $AsO_4H^{2-}/AsO_4^{3-}$ has its highest buffer capacity, and at 20 °C. The buffer concentration was $4.4 \times 10^{-4}$ M, and the ionic strength was adjusted by the addition of 0.1 N NaCl and $H_2O$ in various ratios. Similarly, while the ionic strength was kept constant by adding calculated amounts of NaCl, the buffer concentration was varied or amounts of acetate or hydroxyethylamine were added in order to investigate the buffer dependency of the reaction.

The deuterium exchange experiments were performed by closely following Kasahara's preparation of neocyanothiamin,[9] which is the thiocyanate derivative of YF. Under a stream of dry nitrogen and under cooling with a salt/ice mixture, 0.292 g (12.7 mM) of sodium was dissolved in 12 mL of absolute monodeuterioethanol (Fluka AG), and 1.426

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(1) Partly taken from the Ph.D. thesis of G. P. B.; for part 1, see ref 7; for part 3, see ref 10.

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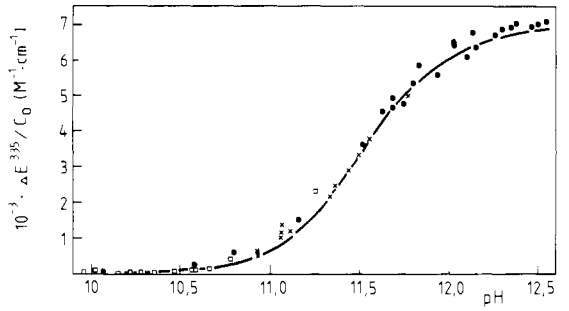


**Figure 1.** Plot of the normalized amplitudes vs. pH adjusted by various buffers (□, hydroxyethylamine; ×, ethylamine; ●, NaCl/NaOH; all with ionic strength 0.1 M). The solid line has been calculated by using the data given in the text for the mechanism of eq 4.

g of TH (4.2 mM) was added in small portions. The bright yellow solution was filtered, under stirring and cooling with a salt/ice mixture, into 2.12 mL of a 2 M ethanolic BrCN solution. The precipitate was filtered, washed twice with acetone (pA, Merck), and recrystallized from dry acetonitrile (0.03% $H_2O$, pA, Merck), needles, mp 150–151 °C (152.5 °C[9]). The reference compound was prepared by repeating the preparation with similar amounts of the substances utilizing nondeuterated ethanol (absolute pA, Merck). The $^1H$ and $^{13}C$ NMR spectra were measured with a Bruker WM 250 (250 MHz) instrument with tetramethylsilane as internal standard in perdeuterated dimethyl sulfoxide at ambient temperatures. The IR spectra were taken with a Perkin-Elmer 237 grating IR spectrophotometer from KBr pellets. The wavenumber calibration was checked with a polystyrene spectrum. The differences between the calibrating peaks, whose wavenumbers were taken from the Perkin-Elmer instruction manual, and the actual readings were never larger than 2 $cm^{-1}$.

## Results

**The Stopped-Flow Kinetics.** Since only TH and hydroxyl ions are participating in the reaction and the hydroxyl ion concentration is buffered, a pseudomonomolecular reaction takes place with a monoexponential time–absorbance profile. Applicable in this case is the general inhomogeneous differential equation:

$$dc/dt = -ac + bc_0 \quad (1)$$

where $c$ stands for the time-dependent molar concentration of TH, $c_0$ for the time-independent (initial) molar concentration of TH, and $t$ denotes time. The coefficients $a$ and $b$ have to be calculated for each proposed reaction mechanism, where $a$ is the reciprocal of the characteristic exponential time constant of a given reaction mixture and is denoted $k_{obsd}$ in this study. For $dc/dt = 0$, i.e., at infinite time, the degree of transformation is

$$\alpha = c_\infty/c_0 = b/a \quad (2)$$

and varies from 0 to 1. The amplitude equation is given by

$$\Delta E/c_0 = d\Delta\epsilon\alpha \quad (3)$$

where $\Delta\epsilon = \epsilon_{products} - \epsilon_{educts}$ ($\epsilon$, molar decadic absorbancy; $d$, pathlength) and $\Delta E$ is the difference of the absorbances read at time $t = 0$ and $t = \infty$. The logarithm of $k_{obsd}$ and the associated amplitudes normalized by $c_0$ are plotted vs. pH in Figures 1 and 2.

The maximal slope at the midpoint of such amplitude plots is, according to theory, $(\partial\alpha/\partial pH)_{\alpha=0.5} = 0.575$ or 1.150 for a monobasic or dibasic acid, respectively. Since that slope in Figure 1 is close to unity, 2 equiv of hydroxyl ions is evidently consumed in this reaction, in agreement with the earlier findings.[4] However, these authors measured the slow spectral changes that occur following the fast primary changes discussed in this study by using a conventional spectrophotometer. Because the generation of YF is much faster than any other alkali-induced transformation of TH, we note that the measured amplitudes belong to the generation of YF and not to the subsequent decay.[10] In the case of a degenerate dibasic acid, it is $pK_{av} = 0.5(pK_{a1} + pK_{a2})$. The $pK_{av}$ = 11.47 found in this study is in good agreement with that reported earlier (11.6, $\mu$ = 0.2, 19 °C).[4]

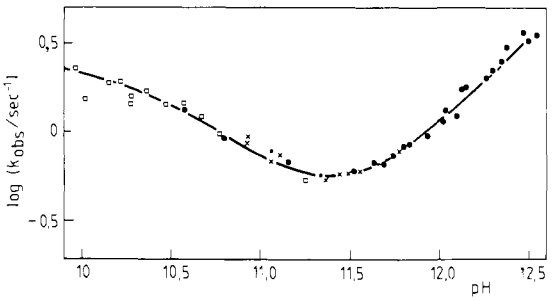


**Figure 2.** Plot of the logarithm of the exponential time constant, log $k_{obsd}$, vs. pH. Conditions as described in the Experimental Section.

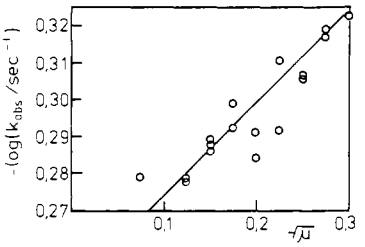


**Figure 3.** Plot of the logarithm of $k_{obsd}$ vs. the square root of the ionic strength $\mu$ according to eq 6. Note the expanded ordinate scale.

Table I. Results of a Simultaneous Least-Squares Curve Fit of the pH Amplitude and Rate Profiles for the Mechanisms of Eq 4 and 7

| quantity | eq 4 | eq 7 |
|---|---|---|
| $\Delta\epsilon$, $M^{-1}$ $cm^{-1}$ | 7000 ± 1000 | 7000 ± 1000 |
| $pK_{a1}$ | 12.39 ± 0.05 | 14.15 ± 0.03 |
| $pK_I$ | | −1.77 ± 0.04 |
| $pK_{a2}$ | 10.55 ± 0.03 | 10.55 ± 0.03 |
| $pK_{av}$ | 11.47 | 11.46 |
| $k_+^a$ | 115 ± 15 $M^{-1}$ $s^{-1}$ | 155 ± 20 $s^{-1}$ |
| $k_-^a$, $s^{-1}$ | 2.64 ± 0.3 | 2.65 ± 0.25 |
| $\sigma^b$ | 0.15 | 0.15 |

[a] $k_+$ and $k_-$ are the forward and reverse rate constants of the rate-limiting step. [b] $\sigma$ is the root mean square standard deviation of the scatter of the data about the fitted lines.

The reaction mechanism as proposed by Maier and Metzler[4] and shown in eq 4 suffices for the description of the kinetics,

$$TH \underset{K_{a1}}{\overset{-H^+}{\rightleftharpoons}} TC \underset{K_{a2}}{\overset{-H^+}{\rightleftharpoons}} YF \quad (4)$$

if it is assumed that step 1 is rate limiting. The derived rate and amplitude equations are:

$$k_{obsd} = k_{-1}\left[\frac{K_{a1}}{a_H} + \frac{a_H}{K_{a2} + a_H}\right] \quad (5a)$$

$$\frac{\Delta E}{c_0} = d\Delta\epsilon\frac{K_{a1}K_{a2}}{K_{a1}K_{a2} + (K_{a1} + a_H)a_H} \quad (5b)$$

The $K_a$'s are the acid dissociation constants, $k_{-1}$ is the reverse rate constant of step 1, and $a_H$ is the potentiometrically measured hydrogen ion activity. The results of a simultaneous digital computer assisted least-squares curve fit of the pH amplitude and rate profiles are collected in Table I under "eq 4".

**Ionic Strength and Buffer Mediation.** The influence of the ionic strength on this kind of reaction can be described by eq 6[11] where

$$\log k_{obsd} = \log k^0 + 2z_Az_B0.4343A\mu^{1/2} \quad (6)$$

$k^0$ is the reaction time constant extrapolated to zero ionic strength and $z_Az_B$ is the product of the charges of the two reacting species.

(10) Hopmann, R.; Brugnoni, G. P. *Angew. Chem.* **1981**, *93*, 1007; *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 961.

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$A$ is a computable constant and its theoretical value from the Debye–Hückel theory is 1.172 at 20 °C.[11] The plot of these data that have been measured with arsenate buffer at 20 °C and pH 11.55 is shown in Figure 3. From the straight line, which was drawn through the data to give the best visual fit, the following results are derived: $k^0 = 0.56\ s^{-1}$, $z_A z_B = -1$, and $A = 0.234$.

The buffer dependency of this reaction was investigated by increasing the buffer concentration at constant ionic strength or by adding various amounts of acetate or hydroxyethylamine. Within experimental error, no systematic trend in dependence of the buffer composition or the concentrations could be detected.

**Spectroscopic Analysis of Deuterium Exchange Experiments.** Asahi[5] has reported the NMR data of YF in 1 N NaOH and 1 N methanolic KOH, and Asahi and Mizuta[6] have published the respective spectrum of a 4 N KOH solution by a low-temperature quenching technique. Compared to our spectrum of unlabeled neocyanothiamin in perdeuterated dimethyl sulfoxide, there are only minor shifts in the resonances. The following assignments are given for unlabeled neocyanothiamin (s, singlet; t, triplet; q, quartet; values in ppm): 2.07 s (C=C*CH*$_3$), 2.47 s (>C*CH*$_3$), 2.67 t (*CH*$_2$CH$_2$OH), 3.65 q (CH$_2$*CH*$_2$OH), 4.67 s (>*CH*$_2$N<), 4.89 t (CH$_2$*OH*), 7.40 s (—N=*CH*N<), 8.20 s (N=*CH*C<). Of foremost interest is the peak at 7.40 ppm, which is easily assigned to the C(2)–H group of the dihydropyrimidine ring because it vanishes largely in the spectrum of the deuterated compound. From the internally normalized peak intensities, the degree of deuteration is estimated to be about 85%.

In the $^{13}C$ spectrum, the band in question of the nondeuterated compound appears at 156.1 ppm. It is missing in the spectrum of the deuterated compound except for a small peak, indicating residual amounts of the nondeuterated compound in the deuterated material. Owing to the quadrupole coupling of the deuterium, the longitudinal relaxation time increases considerably, causing the resonance line to be broadened and to disappear in the background.

Among the many changes observed in the IR spectra of unlabeled and [$^2$H]neocyanothiamin, the most prominent feature is the disappearance of a band at 3030 $cm^{-1}$ and the appearance of a band at 2260 $cm^{-1}$, respectively. The ratio of the two wavenumbers is 1.34, which corresponds well to the expected isotopic shift of a deuterium for hydrogen replacement. These bands are readily assigned to the C(2)–H and C(2)–D stretching modes. Breslow[12a] reported the C–D band of TH to be centered at about 2220 $cm^{-1}$ and an out-of-plane bending mode located at about 910 $cm^{-1}$, the latter disappearing on deuteration. Ullrich and Mannschreck[13] observed three bands in the [$^2$H]TH spectrum, located at 2475, 2280, and 2150 $cm^{-1}$, which were claimed to belong to the OD, ND, and CD stretching modes, respectively. If a solution of YF was acidified with DCl below pH 6 and the product was isolated, we found these three bands, too. If a solution of YF was acidified such that the pH >7, the band at 2150 $cm^{-1}$ was missing. It is therefore concluded that the band at 2150 $cm^{-1}$ is due to the TH pyrimidine ring protonation and the 2280-$cm^{-1}$ band is that of the CD group. A small shift of 1–2% only can be expected for the N–CH to N–CD IR absorption bands of YF. Changes in the band contour of the two broad bands between 1500 and 1600 $cm^{-1}$ are ascribed to deuteration. The many changes in the fingerprint region are not readily assigned, and further discussion is omitted because sufficient evidence is accumulated that deuterium for hydrogen exchange occurs at the C(2) site of the dihydropyrimidinopyrimidine moiety.

## Discussion

The kinetics of the generation of YF can be well reconciled with the minimal reaction scheme of eq 4 provided it is assumed that the first step of it is rate limiting. The lines calculated with the parameter values fit the experimental data well, and the coefficients of correlation indicate that the numerical analysis is well-balanced. From the reverse rate constant and the p$K_{a1}$, a bimolecular reaction rate constant is calculated, $k_1 = 115\ M^{-1}\ s^{-1}$. Since the reaction between TH and the hydroxyl ion must be viewed as a diffusion-controlled rate process, this value appears to be extremely low and calls for an explanation. Furthermore, one would expect that the reaction exhibits some dependency on the kind and concentration of the buffer (general base catalysis!) and on the ionic strength. The reaction was found, however, to be, within experimental error, independent of the buffer but slightly dependent only on the ionic strength, as shown in Figure 3 (note the expanded ordinate scale). On principle, eq 4, which is derived from the Debye–Hückel theory, is not valid in the range of the ionic strength used in this study ($0.01 < \mu < 0.1$) but is valid for very dilute solutions only. Despite this restriction, it is very useful in diagnosing certain features of a reaction. For instance, the product $z_A z_B = -1$ indicates that negatively and positively charged ions are reacting with each other, i.e., a reaction between TH and the hydroxyl ion. On the other hand, the factor $A$ found in this study is well below the theoretical value. This could be due either to a delocalization of the charges or to the possibility that the centers of charge and reaction do not coincide topologically or to the rate-limiting process not being identical with the one that is subject to ionic-strength mediation. The latter is thought to hold true; i.e., the reaction step, in which the two charged species are reacting with each other, is not observed intrinsically. Direct evidence for this conjecture arises from the deuterium exchange experiments.

Transforming TH in deuterated alkali containing ethanol and trapping YF with cyanobromide yield a product that is deuterated at the C(2) site of the dihydropyrimidinopyrimidine moiety of YF according to IR and $^1$H and $^{13}C$ NMR spectral analysis. The two-step mechanism of eq 4 proposed by Maier and Metzler[4] does not leave room for the deuterium for hydrogen exchange at that site, which is the same carbon atom as the thiazolium ring C(2) of TH. The deuterium exchange there is a well-established fact of TH chemistry and was observed at much lower pH's.[12,14] It is a base-catalyzed reaction proceeding via the thiazolium ylide, which is so important biologically.[15]

To determine the p$K$ of the ylide, we carried out a fast photometric titration in the stopped-flow apparatus.[7] The signals, which were found to be dependent on the pH and the TH concentration, were claimed to be due to a protolytic reaction in the submillisecond time range, viz., the generation of the ylide of TH. A p$K$ = 12.6 was deduced from earlier[7] and newer measurements. The signal of that photometric titration is best viewed, in this context, as an amplitude of a preexponential kinetic phase. The deuterium incorporation into neocyanothiamin corroborates the proposition that the first deprotonation step is not the one which limits the rate of TH transformation. This also precludes that the deuterium exchange occurs at a later stage of the transformation, though it is known that diazolium salts can exchange their C(2) proton for deuterium very rapidly.[14,16,17] Thus, the ylide must be rated as the primary intermediate in this reaction. For this, and also because the deprotonation of CH acids is a very fast process with time constants in the submillisecond time range,[18] transformation step 1 will not be rate limiting.

Ylides are known in organic chemistry to be highly reactive intermediates with electrophilic character which can turn to nucleophilicity if substituents of sufficient electron-donor capacity are present.[19] Thus, the next reaction event can be envisaged to be the attack of the ylide onto the amino group of TH, perhaps under concomitant migration of a proton yielding the dihydrothiachromine structure TC (see Scheme I).

This compound should be included in any reaction sequence because of the easy oxidation of TH in highly alkaline solutions

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producing thiochrome. Subsequently, the thiazolium ring could be opened because the thiolate is a good leaving group, and the second proton will be removed finally to yield YF. The mechanism is illustrated in Scheme I. The transition from TC to YF can also be visualized as a base-catalyzed reaction, in which the proton removal from TC induces the ring-opening to yield YF; however, the zwitterion can be of importance in the transformation sequence; its p$K$ was determined to be 6.38.[20] At any rate, whatever the course of transformation from TC to YF will be, while Maier and Metzler[4] provided sufficient evidence for the existence of TC, the present study calls for the ylide as the primary intermediate.

Thus the mechanism by Maier and Metzler[4] is extended by inserting at least one intramolecular transformation step between the two deprotonation steps. Since the second deprotonation step, similar to the first deprotonation step, can be assumed to be very rapid, one is compelled to conclude that the intramolecular transition from the ylide to TC or to the zwitterion must be rate limiting. This is in accord with the general experience that the formation of a covalent bond is usually slower than an elementary rate process like the proton transfer. For the purpose of deriving the rate and amplitude equations, the transformation sequence is summarized in eq 7:

$$\mathrm{TH} \underset{H_{a1}}{\overset{-H^+}{\rightleftharpoons}} \text{ylide} \underset{K_1}{\rightleftharpoons} \mathrm{I} \underset{K_{a2}}{\overset{-H^+}{\rightleftharpoons}} \mathrm{YF} \qquad (7)$$

where I stands for that intermediate following the rate-limiting step. On principle, one can develop formally other reaction sequences and look for their validity; viz., the intramolecular transition still being rate limiting could follow the second deprotonation, or the second deprotonation preceding or following the intramolecular transition is taken to be rate limiting. The rate and amplitude equations were calculated for all these situations and compared to the experimental results, but, in fact, only the reaction sequence formulated by eq 7 is able to describe the data. The rate and amplitude equations are as follows:

$$k_{obsd} = k_{-I}\left[\frac{K_{a1}K_I}{K_{a1} + a_H} + \frac{a_H}{K_{a2} + a_H}\right] \qquad (8a)$$

$$\frac{\Delta E}{c_0} = d\Delta\epsilon\frac{K_{a1}K_IK_{a2}}{K_{a1}K_I(K_{a2} + a_H) + (K_{a1} + a_H)a_H} \qquad (8b)$$

where $k_1$ is the reverse rate and $K_1$ the equilibrium constant of the isomerization step. The results of the simultaneous least-squares curve fitting using eq 8 are shown in Table I in the column headed "eq 7". Note that the sum $pK_{a1} + pK_1$ yields the same value as that for $pK_{a1}$ of the 3-state model. Since the value of $pK_{a2}$ remained unchanged, the $pK_{av}$ is as before, too. This situation leads to the supposition that a correlation must exist between $K_{a1}$ and $K_1$. In fact, looking at eq 8, one can see that, if $a_H$ is not small enough, the two quantities $K_{a1}$ and $K_1$ appear as a product and cannot be determined individually. On the other hand, the coefficients of correlation are balanced and lower than for the 3-state-model calculations. This can be expected if the number of parameters of an underdetermined numerical system is increased.

In conclusion, the value of $pK_{a1} = 14.15$ should be comparable directly with the $pK = 12.6$ determined for the TH ylide.[7] There is no rational explanation for the difference of the two values. Any attempt to resolve the time–absorbance profile at wavelengths of interest into more than the two kinetic phases discussed in this paper, namely the preexponential amplitudes and the monoexponential formation of YF, were unsuccessful. Yet, the participation of another intermediate as discussed above or an additional intermediate cannot be excluded entirely. For instance, one species could arise by proton abstraction from the pyrimidine amino group of TH[21] and be the actual species rearranging to TC. It could also be imputed to be in equilibrium with the ylide. However, there is no direct chemical evidence presently for the existence of such an intermediate. By dissolving 4-amino-2,6-dimethylpyrimidine in 1 N sodium hydroxide and 1 N NaCl of various pH and comparing these solutions spectrophotometrically, we could observe no spectral changes that would lend support to the hypothesis that the amino group of TH loses a proton. Only the pyrimidine ring protonation can be seen. It appears that, on the basis of the present results, the mechanism proposed in eq 7 is the most appreciative to interpret the measured data.

**Acknowledgment.** We are very gratefully indebted to Drs. H. P. Hornisch and G. Rist, Ciba–Geigy AG, Basel, for providing kindly the $^1H$ and $^{13}C$ NMR spectra.

**Registry No.** Thiamin, 59-43-8; YF, 80483-97-2.

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(21) We are grateful to the referee who pointed out this mechanistic possibility.