Hydrolysis of Thiamine

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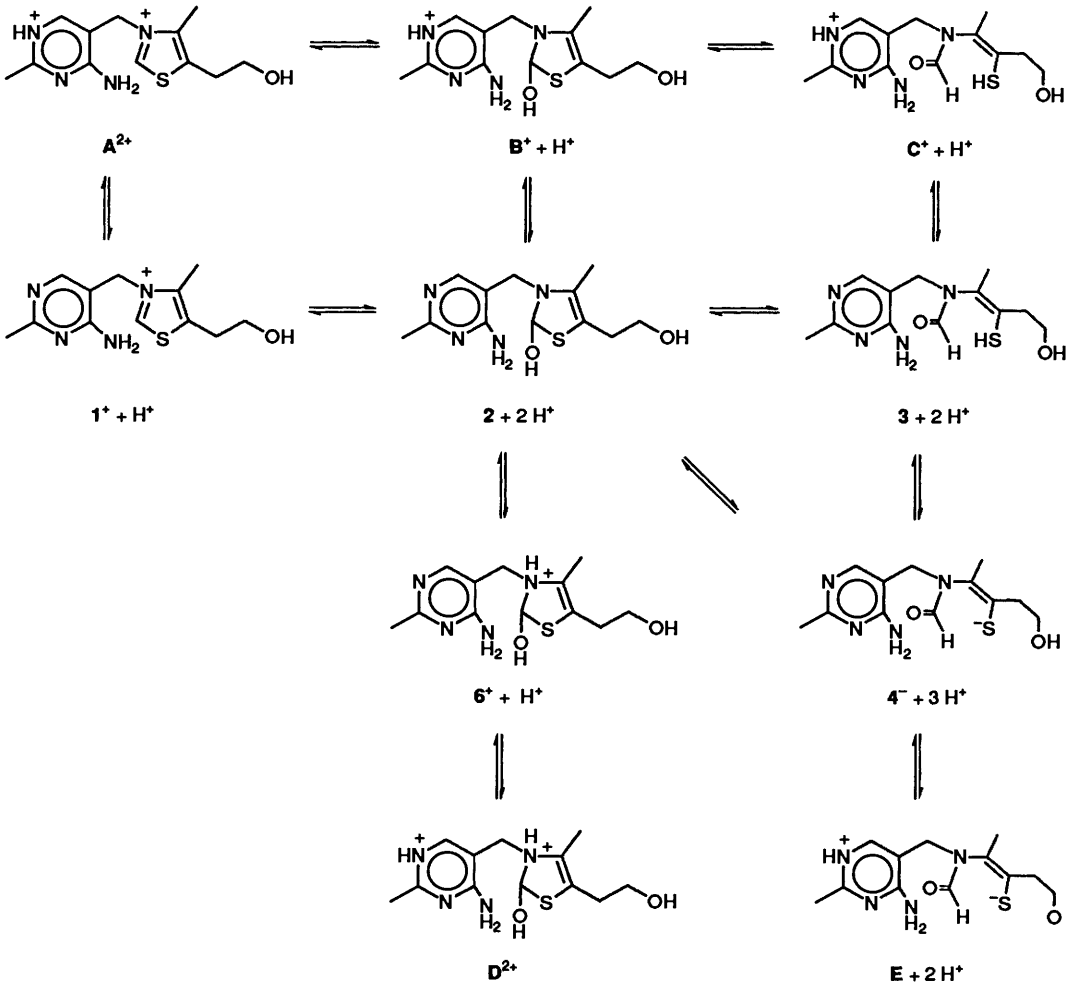
The hydrolysis of thiamine has been studied over the range $0.5 < pH < 10.5$ in aqueous solution at 25 °C using the stopped-flow technique. When the backward reaction from thiazolium anion to thiamine is followed, two relaxation effects are observed and the evaluation of the relaxation amplitudes allows us to determine seven independent equilibrium constants between thiamine, differently protonated pseudo bases and differently protonated thioles. The results are confirmed by the pH-dependence of the relaxation times. The hydrolysis of thiamine proceeds *via* the same mechanism as observed for other thiazolium salts, when the protonation of the pyrimidine ring of thiamine is also taken into account for all species.

Thiamine plays a very important role in the metabolism of carbohydrates[1] and the mechanism of its enzymatic activity is still being intensively investigated.[2,3] For the understanding of its biochemical reactions, the knowledge of the hydrolysis of thiamine, *i.e.* its reactivity with bases and acids, is essential. Although this has been studied by many authors (see *e.g.* refs. 4–10), its mechanism is not yet completely understood. All recent studies on the subject are based on the work of Breslow[11] which has been quoted more than 500 times.[12] In this contribution we report on a thorough stopped-flow investigation, where all possibilities of this technique have been exploited.

In previous investigations[13,14] we studied the hydrolysis of thiazolium salts with substituents which cannot be protonated. For these we confirmed the reaction mechanism proposed by Breslow[11] including species $\mathbf{1}^+$, **2**, **3** and $\mathbf{4}^-$ and the kinetic results showed the existence of the protonated pseudo-base $\mathbf{6}^+$. (Bold numbers and letters refer to Scheme 1.) In the case of thiamine the pyrimidine substituent may also be protonated, leading to $\mathbf{A}^{2+}$, $\mathbf{B}^+$, $\mathbf{C}^+$, $\mathbf{D}^{2+}$ and **E**. These considerations lead to Scheme 1 for the hydrolysis of thiamine. The three different structures (thiazolium cation, pseudo-base and thiole) are horizontally arranged. Protonation at well solvated nitrogen or



Scheme 1

sulfur atoms are vertically arranged. These protonation steps proceed very rapidly at a diffusion-controlled rate and cannot be resolved by the applied technique. Between the columns arrows are drawn only for those steps that could be traced in the course of this investigation.

Furthermore, when base is added to aqueous solutions of thiamine, with pH > 10.5 the so-called yellow form (the only species absorbing in the visible region at $\lambda = 335$ nm) is observed as an unstable intermediate. This reaction proceeds *via* a tricyclic intermediate.[15] However, the present contribution is restricted to the hydrolysis of thiamine to pseudo-bases and thiolates, whereas the formation of the tricyclic and yellow forms will be discussed in a subsequent paper.

Experimental

Thiamine hydrochloride was obtained from Aldrich and used without further purification. All other chemicals were of analytical grade. The solutions were prepared with triply distilled water. Stock solutions of thiamine hydrochloride were prepared weekly. Stock solutions of thiamine hydrochloride with NaOH at pH 12 were prepared daily. The following buffer systems were used (pH range in parentheses): formate (3.1–4.0), acetate (4.1–5.7), citrate (2.7–3.7 and 5.4–6.3), hydrogen phosphate (6.0–8.1), tris(hydroxymethyl)aminomethane (7.7–9.0), ammonium (8.3–10.1), carbonate (9.1–10.8) and phosphate (10.5–11.9). The pH values of the solutions were calculated from the concentrations and the dissociation constants of the buffers[16] and they were measured using a glass electrode. Calculated and experimentally obtained values agreed to within ±0.03 units. All measurements were performed in aqueous solutions at 25 °C using a concentration of 7.25×10^{-5} mol dm^{-3} thiamine, if not indicated otherwise.

Equilibrium constants were obtained by optical titration using a Uvicon 860 spectrophotometer. For the kinetic measurements the stopped-flow technique was applied mixing equal volumes of solutions of thiamine and buffer solutions. The progress of the reaction was followed spectrophotometrically either at a single wavelength or with a diode array set-up for whole spectra. The kinetic measurements were performed in three different ways. (*i*) By adding NaOH the pH value of a solution of thiamine was adjusted to pH 12.0, where the equilibrium is completely shifted to the thiolate anion $\mathbf{4}^-$. In the stopped-flow experiment this solution was mixed with less basic or acidic buffer to reach a final pH between 0.5 and 9.1. In this way the backward reaction from $\mathbf{4}^-$ to the thiamine cation $\mathbf{1}^+$ and the thiaminium dication $\mathbf{A}^{2+}$ was observed at $\lambda = 250$ nm (maximum difference between the absorbances of $\mathbf{1}^+$ and $\mathbf{4}^-$). (*ii*) Some measurements quoted under (*i*) were repeated using the pH indicator Bromophenol Blue ($\mathrm{p}K_\mathrm{s} = 4.23$) in order to determine the number of protons involved in the reaction steps. In these measurements solutions of thiamine at pH 11.0 were mixed with solutions of HCl in order to obtain a final pH of 3.5–4.0, and the reactions were followed at $\lambda = 592$ nm (maximum absorbance of the indicator). (*iii*) An acidic solution of thiamine, where only species $\mathbf{1}^+$ and $\mathbf{A}^{2+}$ exist, is mixed with a solution of NaOH. If the final pH is between 8.4 and 10.5, we observe the forward reaction, *i.e.* the formation of $\mathbf{4}^-$. If the final pH is higher than 10.5 we observe first the formation of the intermediate yellow form and in a subsequent reaction the formation of $\mathbf{4}^-$.

In the sets of experiments (*i*) and (*iii*) the solutions were well buffered. Therefore the reactions proceed under pseudo-first-order conditions and the change in absorbance could be fitted to eqn. (1) for one ($X_\mathrm{s} = 0$) or two superimposed relaxation effects. ($t = 0$ is the time of mixing.) X_i and τ_i are called relaxation amplitudes and relaxation times, respectively. The experiments of set (*ii*) could also be well approximated by eqn. (1), although the pseudo-first-order condition was only poorly fulfilled.

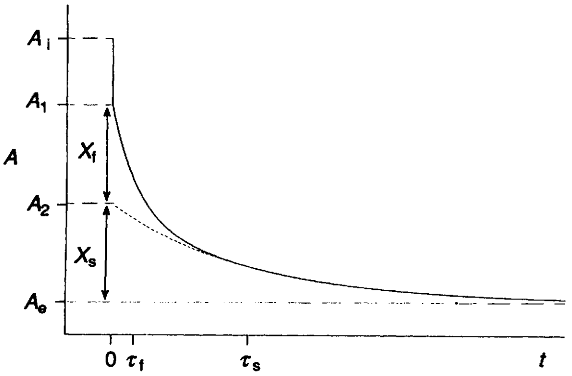


Fig. 1 Change in absorbance of solutions of thiamine after a sudden change in the pH value at $t = 0$ (schematically)

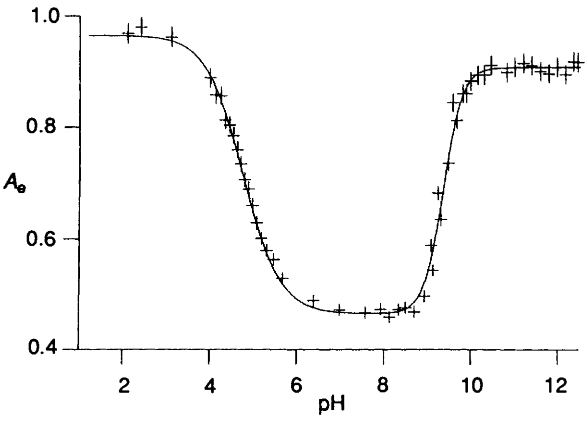


Fig. 2 Absorbance at equilibrium as a function of pH for 7.25×10^{-5} mol dm^{-3} solutions of thiamine at $\lambda = 250$ nm. The curves are calculated with the values of $K_{1\mathrm{A}}$ and K_{41} given in the text.

$$A = X_\mathrm{f}\mathrm{e}^{t/\tau_\mathrm{f}} + X_\mathrm{s}\mathrm{e}^{t/\tau_\mathrm{s}} + A_\mathrm{e} \qquad (1)$$

For a stopped-flow experiment the change of absorbance is shown in Fig. 1 according to eqn. (1), where the symbols have the following meanings. A_e is the absorbance at equilibrium, A_2 is obtained by extrapolating only the slow relaxation effect to the time of mixing at $t = 0$: thus A_2 is the value of the absorbance in the hypothetical state, where the fast relaxation effect has reached equilibrium and the slow relaxation effect has not yet started. A_1 is the absorbance obtained by extrapolating both relaxation effects to $t = 0$; A_1 may differ from the initial absorbance A_i, which is the mean value of the absorbances of the two solutions to be mixed. A_2 is equal to A_1, when a single relaxation effect is observed. The relaxation amplitudes are $X_\mathrm{f} = A_1 - A_2$ and $X_\mathrm{s} = A_2 - A_\mathrm{e}$.

Results

Equilibrium Measurements.—It is well known[15] that at equilibrium only the thiamine cation $\mathbf{1}^+$, the thiaminium dication $\mathbf{A}^{2+}$ and the thiolate anion $\mathbf{4}^-$ exist in detectable concentrations. Thus from the optical absorbance at equilibrium we obtain the dissociation constants defined in eqns. (3) and (4) for reaction (2). The data in Fig. 2 clearly show a protonation step with $\mathrm{p}K_{1\mathrm{A}} = 4.59 \pm 0.05$ and a steep step caused by a double protonation with a point of inflection at pH = 9.34 ± 0.05, yielding $K_{41} = 10^{-18.68}$ mol^2 dm^{-6}. The UV spectra of the different species are shown in Fig. 3. f_I and f_II

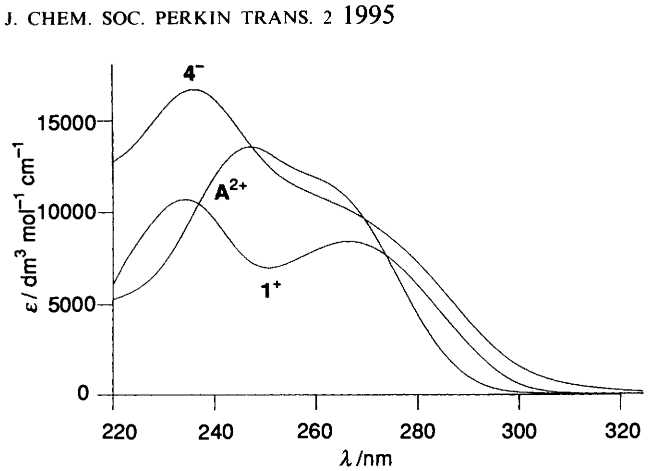


Fig. 3 Spectra of solutions of thiamine at equilibrium: pH 2.0 (A^{2+}), pH 6.0 (1^+), pH 12.6 (4^-)

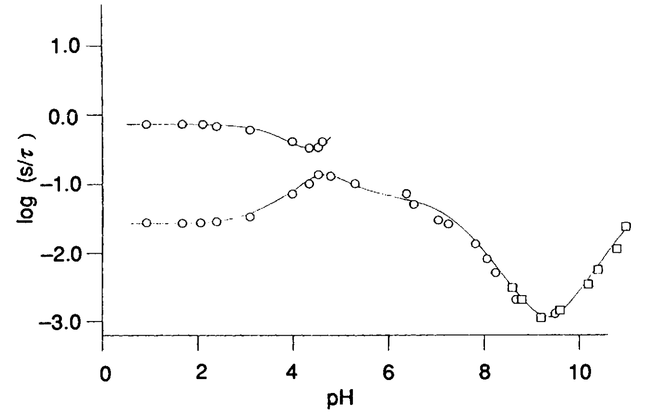


Fig. 4 Log (s/τ) *vs.* pH for the hydrolysis of thiamine. (○) Obtained from experiments (*i*) and (*ii*) (backward reaction); (□) obtained from experiments (*iii*) (forward reaction).

are the activity coefficients for mono- and di-valent ions, respectively, which are calculated according to Davies.[17]

$$A^{2+} \rightleftharpoons 1^+ + H^+ \rightleftharpoons 4^- + 3\,H^+ \qquad (2)$$

$$K_{1A} = \frac{c_1 c_H}{c_A}\,\frac{f_I^2}{f_{II}} \qquad (3)$$

$$K_{41} = \frac{c_4 c_H^2}{c_1} f_I^2 \qquad (4)$$

Backward Reaction.—When studying the backward reaction [experiment (*i*)], we observe a single relaxation effect in the range $5 < \text{pH} < 9.1$ and two relaxation effects in the range $0.5 < \text{pH} \leqslant 5$. The reciprocal relaxation times are plotted in Fig. 4, and the absorbances A_1 and A_2 are shown in Fig. 5.

In these experiments we start with solutions containing the thiolate anion 4^- and A_1 refers to a state in which the equilibrium is reached very quickly (diffusion controlled), where only **3**, 4^-, C^+ and **E** exist. The values of A_1 indicate two protonation equilibria between these species. In the first one, 4^- is protonated to give an uncharged intermediate, which is further protonated to a monovalent intermediate in the second step. By using a fitting procedure the dissociation constants of these two steps are determined to be $pK_{\alpha 0} = 7.27 \pm 0.05$ and $pK_{\alpha 1} = 4.3 \pm 0.5$. (The number in the index refers to the charge of the corresponding acid.)

The constants are related to Scheme 1 by eqns. (5) and (6). A_2 refers to the state where the slow relaxation effect has not yet started and the fast relaxation effect is completed. According to the previous studies,[13,14] in this state the thiolate species (**3**, 4^-, C^+, **E**) are in equilibrium with the pseudo-bases (**2**, 6^+, B^+, D^{2+}), but the thiazolium ions (1^+, A^{2+}) are not yet formed. For pH > 5 we do not observe the fast relaxation effect, *i.e.* in this range the concentration of the pseudo-bases is negligibly small. For pH < 5 there are two relaxation effects and the pH dependence of A_2 indicates a further protonation step, which corresponds to the formation of D^{2+}. Applying A_2 to the three protonation steps yields $pK_{\beta 0} = 7.3 \pm 0.1$, whereas the lower p$K$ values cannot be separated and the fitting yields $pK_{\beta 1} + pK_{\beta 2} = 8.6 \pm 0.1$ with $pK_{\beta 1} \approx pK_{\beta 2}$. The dissociation constants are related to Scheme 1 by eqns. (7)–(9). According to these constants, the spectra of some intermediates were recorded using the diode-array set-up: at pH 2.0, the absorbances of C^+ and D^{2+} are observed as A_1 and A_2, respectively, and at pH 6.0, A_1 is the absorbance of **3** and **E** in equilibrium. The spectra of (**3**, **E**), C^+ and D^{2+} are shown in Fig. 6.

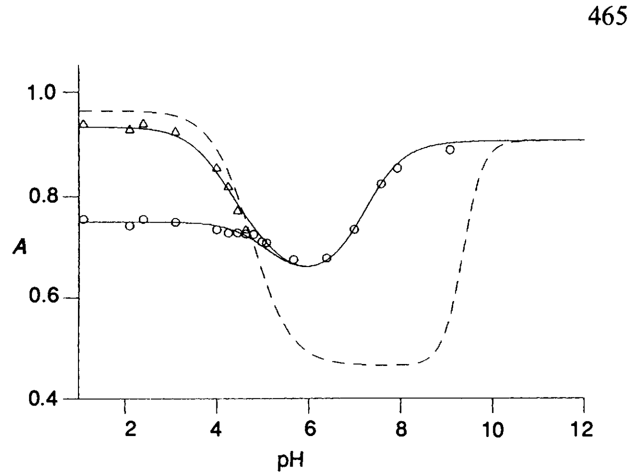


Fig. 5 Absorbances A_1 (○), A_2 (△) and A_e (– – – –) at λ = 250 nm, for experiments (*i*) (backward reaction) for 7.25 × 10^{-5} mol dm^{-3} solutions of thiamine. The curves for A_1 and A_2 are calculated with the constants given in Table 1. The absorbance at equilibrium A_e is taken from Fig. 2.

$$K_{\alpha 0} = \frac{c_4 c_H}{c_3 + c_E} f_I^2 \qquad (5)$$

$$K_{\alpha 1} = \frac{(c_3 + c_E) c_H}{c_C} \qquad (6)$$

$$K_{\beta 0} = \frac{c_4 c_H}{c_2 + c_3 + c_E} f_I^2 \qquad (7)$$

$$K_{\beta 1} = \frac{(c_2 + c_3 + c_E) c_H}{c_6 + c_B + c_C} \qquad (8)$$

$$K_{\beta 2} = \frac{(c_6 + c_B + c_C) c_H}{c_D}\,\frac{f_I^2}{f_{II}} \qquad (9)$$

In the range $4 < \text{pH} < 5$ the two relaxation times differ by less than a factor of five and the errors in the experimentally determined values of A_1 and A_2 are large leading to the high uncertainties in $pK_{\alpha 1}$ and in the discrete values of $pK_{\beta 1}$ and $pK_{\beta 2}$. Therefore we used a pH indicator [set (*ii*) of experiments] in order to confirm the number of protons and the dissociation constants involved in the relaxation effects. In these measurements we added 5 × 10^{-6} mol dm^{-3} Bromophenol Blue (BPB) to 1 × 10^{-4} mol dm^{-3} solutions of thiamine and observed the progress of the reaction at the absorption maximum of BPB

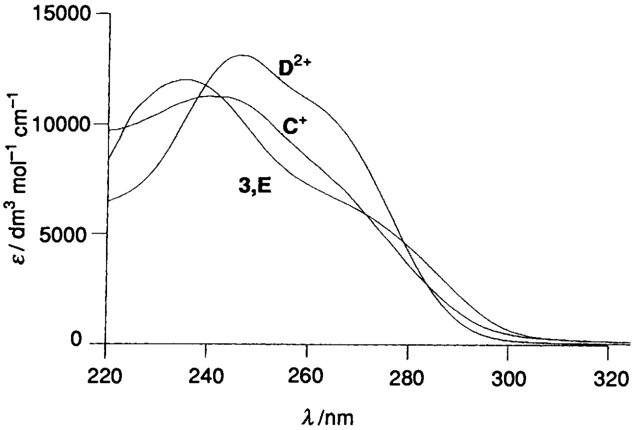


Fig. 6 Spectra of the intermediate species obtained from experiments (*i*): **3** is in equilibrium with **E** (A_1 at pH 6.0), **C**$^+$ (A_1 at pH 2.0) and **D**$^{2+}$ (A_2 at pH 2.0)

(λ = 592 nm). Thus the absorbances A_1, A_2 and A_e allow us to calculate the corresponding proton concentrations $c_{H,1}$, $c_{H,2}$ and $c_{H,e}$. These measurements were performed at 10 different values of $c_{H,1}$ [(1–3) × 10^{-4} mol dm^{-3}]. As an example we report the measurement for $c_{H,1} = 2.04 \times 10^{-4}$ mol dm^{-3}, where we obtained $c_{H,2} = 1.33 \times 10^{-4}$ and $c_{H,e} = 0.98 \times 10^{-4}$ mol dm^{-3} (all values with an error of $\pm 0.07 \times 10^{-4}$ mol dm^{-3}). At these pH values the concentration of the anion **4**$^-$ may be neglected and the conservation laws for thiamine and protons lead to eqns. (10) and (11), respectively, with $T = 1 \times 10^{-4}$ mol dm^{-3}. (The concentration of the indicator is neglected in this discussion.) H can be calculated from $c_{H,e}$, since at equilibrium eqns. (10) and (11) are reduced to eqns. (12) and (13), respectively, and K_{1A} is known from measurements at equilibrium. In state '2' the thiazolium ions **1**$^+$ and **A**$^{2+}$ are not yet present, and here eqns. (10) and (11) are reduced to eqns. (14) and (15), respectively. Inserting into these equations the product $K_{\beta1}K_{\beta2}$, which is known to have a relatively small error, allows us to calculate $c_{D,2}$ and thus separately, the constants $K_{\beta1}$ and $K_{\beta2}$. Finally in state '1' only the thiolate species are present leading to eqns. (16) and (17), from which $K_{\alpha1}$ can be calculated.

$$c_1 + c_2 + c_3 + c_6 + c_A + c_B + c_C + c_D + c_E = T \quad (10)$$

$$c_H + c_1 + c_6 + 2c_A + c_B + c_C + 2c_D = H \quad (11)$$

$$c_{1,e} + c_{A,e} = T \quad (12)$$

$$c_{H,e} + c_{1,e} + 2c_{A,e} = H \quad (13)$$

$$c_{2,2} + c_{3,2} + c_{6,2} + c_{B,2} + c_{C,2} + c_{D,2} + c_{E,2} = T \quad (14)$$

$$c_{H,2} + c_{6,2} + c_{B,2} + c_{C,2} + c_{D,2} = H \quad (15)$$

$$c_{3,1} + c_{C,1} + c_{E,1} = T \quad (16)$$

$$c_{H,1} + c_{C,1} = H \quad (17)$$

These calculations have been made for all 10 measurements and by averaging the results the following values are obtained for the equilibrium constants: p$K_{\alpha1}$ = 4.1 ± 0.2, p$K_{\beta1}$ = 4.6 ± 0.2, p$K_{\beta2}$ = 4.0 ± 0.2.

Forward Reaction.—When mixing a solution of thiamine hydrochloride (pH *ca.* 4.5) with buffer [experiments (*iii*)], we observe a single relaxation effect in the range 8.4 < pH < 10.5; the relaxation times are included in Fig. 4. In these experiments we start with a solution containing **A**$^{2+}$ and **1**$^+$. By jumping to pH > 8.0 the equilibrium is shifted completely towards **1**$^+$ in a very fast reaction, *i.e.* A_1 is the absorbance of **1**$^+$. Up to pH 10.5, the equilibrium between **1**$^+$ and **4**$^-$ is established in a single relaxation process, *i.e.* no further information is obtainable from the relaxation amplitude. The appearance of a second relaxation effect at pH > 10.5 indicates the existence of the intermediate yellow form **Y**$^-$, which will not be discussed here.

Relaxation Times.—The relaxation times are plotted in Fig. 4. Measurements were performed in buffered solutions, where the buffer concentration was not larger than 0.05 mol dm^{-3}. Under this condition the relaxation times do not depend on the buffer concentration, and therefore buffer catalysis does not need to be considered.

When studying the backward reaction, two relaxation effects can be resolved. During the faster one, equilibrium is established between the thioles (last column in Scheme 1) and the pseudobases (middle column). The rate law for this reaction is given in eqn. (18), taking into account that in the timescale of our experiments **C**$^+$, **3**, **4**$^-$ and **E** reach equilibrium by diffusion-controlled reactions. The integration of the rate law yields eqn. (19) for the relaxation time τ_f. K_{ij} is defined as the equilibrium constant, where species 'i' and 'j' are the numerator and denominator, respectively, and where protons are included appropriately. In the slow relaxation process (both for forward and backward reactions) equilibrium is established between the thiazolium cations **1**$^+$ and **A**$^{2+}$ and the other species. For this process, the rate law and relaxation time are given by eqns. (20) and (21), respectively. K_W is the ionic product of water. Eqn. (20) takes into account that in step **1**$^+$ ⟶ **2** species **1**$^+$ may react either with OH$^-$ or with H$_2$O with the release of a proton. Step **A**$^{2+}$ ⟶ **B**$^+$ is treated analogously. The water activity '1' is included in the rate constants $k_{AB}{}^{OH}$ and $k_{12}{}^{H}$.

$$\frac{d(c_3 + c_4 + c_C + c_E)}{dt} = k_{BC}c_B - k_{CB}c_C \quad (18)$$

$$\frac{1}{\tau_f} = k_{CB}\left[\left(1 + \frac{K_{\alpha1}}{c_H} + \frac{K_{\alpha0}K_{\alpha1}}{c_H^2 f_I^2}\right)^{-1} + \left(\frac{c_H f_I^2}{K_{CD} f_{II}} + K_{BC} + K_{6C} + \frac{K_{2C}}{c_H}\right)^{-1}\right] \quad (19)$$

$$\frac{d(c_1 + c_A)}{dt} = -k_{12}^{H}c_1 - k_{12}^{OH}c_1c_{OH}f_I^2 + k_{21}^{H}c_2c_H + k_{21}^{OH}c_2 - k_{AB}^{H}c_A - k_{AB}^{OH}c_Ac_{OH}f_{II} + k_{BA}^{H}c_Bc_H\frac{f_I^2}{f_{II}} + k_{BA}^{OH}c_B \quad (20)$$

$$\frac{1}{\tau_s} = \left\{k_0 + k_Hc_H\frac{f_I^2}{f_{II}} + k_{OH}f_I^2c_{OH}\right\} \times F(c_H) \quad (21)$$

with

$$k_0 = k_{AB}^{OH}\frac{K_W}{K_{1A}} + k_{12}^{H} \quad (21a)$$

$$k_H = \frac{k_{AB}^{H}}{K_{1A}} \quad (21b)$$

$$k_{OH} = k_{12}^{OH} \quad (21c)$$

$$F(c_H) = \left(1 + \frac{c_H f_I^2}{K_{1A} f_{II}}\right)^{-1} + c_H K_{\beta 0}/K_{41} \left(\frac{c_H^2 f_I^2}{K_{\beta 1} K_{\beta 2} f_{II}} + \frac{c_H}{K_{\beta 1}} + 1 + \frac{K_{\beta 0}}{c_H f_I^2}\right) \quad (21d)$$

The slow relaxation time is measured in experiments (*i*) in the range $0.3 \leqslant pH \leqslant 9.1$ and in experiments (*iii*) in the range $8.4 \leqslant pH \leqslant 10.5$. For pH > 6.5, eqn. (21) can be approximated by eqn. (22), *i.e.* in this range k_{12}^{OH} is the only unknown and by

$$\frac{1}{\tau_s} = k_{12}^{OH} f_I^2 \left\{ c_{OH} + \frac{K_W c_H}{K_{41}} \cdot \frac{K_{\beta 0}}{c_H f_I^2 + K_{\beta 0}} \right\} \quad (22)$$

fitting the relaxation times to eqn. (22) we obtain $k_{12}^{OH} = (25 \pm 5)\ dm^3\ mol^{-1}\ s^{-1}$. In the plot log (s/$\tau$) *vs.* pH a minimum is observed at $c_H^2 = K_{41}$ (pH 9.35). On the left-hand and the right-hand side of the minimum the slope is -1 and $+1$, respectively, and for pH < $(pK_{\beta 0} + 1)$ the results deviate from the linear slope, *i.e.* $pK_{\beta 0} \sim 7$.

For pH < 2.5, τ is independent of the proton concentration and eqn. (21) may be reduced to eqn. (23), *i.e.* in this range k_{AB}^{H} =

$$\frac{1}{\tau_s} = k_{AB}^{H} \frac{K_{\beta 0} K_{\beta 1} K_{\beta 2}}{K_{1A} K_{41}} \quad (23)$$

$(2.2 \pm 0.5) \times 10^{-9}\ s^{-1}$ is obtained. Thus in eqn. (21) two of the three rate constants can be evaluated separately. The third one, $k_0 = (2.8 \pm 0.7) \times 10^{-8}\ s^{-1}$, is obtained by fitting the relaxation times for 2.5 < pH < 6.5. The curve in Fig. 4 is calculated with these constants. τ depends in a very characteristic way on the proton concentration and therefore the excellent agreement between measured and calculated values of τ confirms the reaction scheme and the equilibrium constants obtained from the evaluation of the amplitudes.

The faster relaxation effect is observed in experiments (*i*) only for pH < 5. The plot of the reciprocal relaxation time *vs.* pH shows a limiting value at low pH, which corresponds to $k_{CB} = (0.8 \pm 0.2)\ s^{-1}$. According to eqn. (19) the relaxation rate starts to decrease for pH $\approx (pK_{\alpha 1} - 1)$ in agreement with the experimental values. An increase is predicted at pH $\approx (pK_{CD} - 1)$; this allows us to estimate $pK_{CD} \approx 5$.

Discussion

The results presented in the preceding paragraph for the hydrolysis of thiamine may be summarized as follows: at equilibrium only species **1**$^+$, **4**$^-$ and **A**$^{2+}$ exist, and the dissociation constants K_{1A} and K_{41} are evaluated from spectrophotometric measurements. In the kinetic experiments (*i*) and (*ii*) the backward reaction of **4**$^-$ to **1**$^+$ and **A**$^{2+}$ is studied. Here A_1 is measured at a time when only **3**, **4**$^-$, **C**$^+$ and **E** (last column in Scheme 1) exist. These species are in equilibrium with each other and the dissociation constants $K_{\alpha 0}$ and $K_{\alpha 1}$ are determined. Analogously for A_2 equilibrium is established between **2**, **3**, **4**$^-$, **6**$^+$, **B**$^+$, **C**$^+$, **D**$^{2+}$ and **E** (last two columns in Scheme 1) and $K_{\beta 0}$, $K_{\beta 1}$ and $K_{\beta 2}$ are obtained.

Thus only seven independent equilibrium constants are obtained for the description of the nine equilibria between the ten different species involved in Scheme 1. It is not possible to determine separately the equilibria between equally protonated species, where the reactions proceed under diffusion control (equally charged species in the same column in Scheme 1). That means that the two equilibria between **3** and **E** and between **6**$^+$ and **B**$^+$ cannot be evaluated from the experimental equilibrium constants. Further information is obtainable from the spectra in Figs. 3 and 6. Fig. 3 shows that the protonation of **1**$^+$ shifts the absorption maximum of the pyrimidine ring from 234 to 246 nm. The spectrum of (**3** and **E**) in Fig. 6 has the maximum absorption at 236 nm, and therefore we conclude that the pyrimidine ring is not protonated and species **3** predominates. Unfortunately, the spectrum of species (**B**$^+$ and **6**$^+$) cannot be observed separately, since $K_{\alpha 1}$, $K_{\beta 1}$ and $K_{\beta 2}$ have similar values.

By neglecting **E** and considering **B**$^+$ and **6**$^+$, it should be possible to calculate the equilibria between all species involved in the hydrolysis of thiamine. However it turns out, that $pK_{\alpha 0}$ and $pK_{\beta 0}$ have the same value within experimental error, *i.e.* c_2 is small compared with $(c_3 + c_E)$, and for equilibrium constants involving species **2** only a limit can be given. With those considerations the following p*K* values are calculated from the experimental equilibrium constants: $pK_{43} = 7.3 \pm 0.1$ and $pK_{3C} = 4.1 \pm 0.2$ for the thioles; $pK_{2(6B)} > 5.0$ and $pK_{(6B)D} = 4.2 \pm 0.3$ for the pseudo-bases; $pK_{1A} = 4.59 \pm 0.05$ for the thiazolium ions. The stability ratios between the different structures is given by $K_{C1} = 10^{-7.3}$ and $K_{DA} = 10^{-7.3}$. From these values $pK_{CD} = 4.6 \pm 0.3$ is obtained in agreement with the value estimated from the pH dependence of the fast relaxation time.

In the previous contribution, dissociation constants were determined for thiazolium cations, where the pyrimidine ring was replaced by other substituents.[14] There we obtained $6.4 \leqslant pK_{43} \leqslant 7.3$ and $11.0 \leqslant pK_{31} \leqslant 13.4$. The values for thiamine fit well into these ranges. In order to be able to discuss the other data we have to make assumptions concerning c_2/c_3 and c_6/c_B. For this purpose we considered the protonation of the pyrimidine ring: 4-amino-2-methylpyrimidine [where the thiazolium substituent is replaced by a proton in position (5)] has p*K* = 6.53 and this value is reduced by the insertion of electron withdrawing substituents to 5 to 2.[18] For thiamine we could evaluate $pK_{1A} = 4.59$ and $pK_{3C} = 4.1$. Assuming a similar value for the other protonation equilibria ($pK_{2B} \sim pK_{4E} \sim pK_{6D} = 4.4 \pm 0.5$) yields (*i*) $c_3/c_E > 300$, confirming that species **E** cannot be detected, (*ii*) $0.1 < c_6/c_B < 10$, and therefore both pseudo-bases **6**$^+$ and **B**$^+$ have to be considered, (*iii*) $0.1 < c_2/c_3 < 0.3$, taking into account the formerly evaluated result $c_{2/3} < 0.3$. For the other thiazolium salts we obtained $1 \leqslant c_2/c_3 \leqslant 10$.

For the evaluation of the short relaxation time we assume that the thioles react to form the pseudo-bases *via* **C**$^+$ and **B**$^+$ and then we obtain $k_{CB} = 0.8\ s^{-1}$, but other approaches may also lead to the same pH dependence of τ_f being observed. We used the simplest reaction scheme, which is in agreement with the experimental results and which furthermore is favoured by the fact that **C**$^+$ is the predominant thiole species for pH < 5. For other compounds similar values are observed for the reaction of thioles to pseudo-bases[15] ($0.1 \leqslant k_{32} \leqslant 1.5$). For pH > 5, the amplitude X_f is so small that the fast relaxation effect cannot be observed. However, in this range the paths from **2** to **3** or from **2** to **4**$^-$ contribute to the formation of thioles from pseudo-bases. Otherwise this reaction step would become rate determining for high pH values, in contradiction with the experimental results.

For the slow relaxation effect from thiazolium cations to pseudo-bases we have to take into account four parallel reaction paths, eqns. (24)–(27). From the four independent rate constants, the experiments allow us to calculate separately $k_{12}^{OH} = 25\ dm^3\ mol^{-1}\ s^{-1}$ and $k_{AB}^{H} = 2.2 \times 10^{-9}\ s^{-1}$, whereas only a combination of k_{12}^{H} and k_{AB}^{OH} is obtained, see eqn. (21*a*). For other thiazolium cations similar values of k_{12}^{OH} have been reported in the previous publication.[14] The small differences may be attributed to substitution effects as shown in Fig. 7, where the logarithm of the rate constant is plotted *vs.* the Hammett σ factor for different N(3)-substituted thiazolium cations.[19,20] Regarding k_0, it should be mentioned that its value

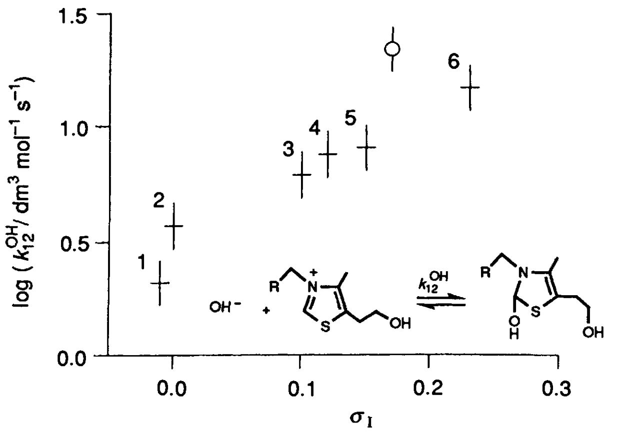


Fig. 7 Log k_{12}^{OH} *vs.* σ_I for thiazolium salts with different substituents R. 1: CH_3; 2: H; 3: 4-H_3C–C_6H_4; 4: C_6H_5; 5: 4-Cl–C_6H_4; 6: 4-O_2N–C_6H_4; ○: thiamine.

Table 1 Dissociation and rate constants obtained for the hydrolysis of thiamine at 25 °C compared with the corresponding values taken from ref. 14 for simple thiazolium salts

	Thiamine	Other salts
pK_{21}	12.1 ± 0.4	11.0–13.0
pK_{31}	11.4 ± 0.2	11.2–13.4
pK_{26}	5.0 ± 1.0	6.0–7.4
pK_{43}	7.3 ± 0.1	6.4–7.3
pK_{1A}	4.59 ± 0.03	
pK_{3C}	4.1 ± 0.2	
$pK_{(6B)D}$	4.2 ± 0.3	
pK_{2B} [a]	4.4 ± 0.5	
pK_{4E} [a]	4.4 ± 0.5	
pK_{6D} [a]	4.4 ± 0.5	
log (k_0/s^{-1})	7.55 ± 0.10	
log (k_{12}^{OH}/dm^3 mol^{-1} s^{-1})	1.40 ± 0.05	0.32–1.48
log (k_{AB}^{H}/s^{-1})	−8.7 ± 0.1	
log (k_{CB}/s^{-1})	−0.10 ± 0.05	

[a] Estimated value.

is similar to k_{12}^{H} as well as $k_0\ K_{1A}/K_W$ is similar to k_{12}^{OH} observed for other thiazolium cations and therefore both paths (25) and (26) may contribute to the overall reaction.

$$\mathbf{A}^{2+} + H_2O \xrightleftharpoons{k_{AB}^{H}} \mathbf{B}^{+} + H^{+} \tag{24}$$

$$\mathbf{A}^{2+} + OH^{-} \xrightleftharpoons{k_{AB}^{OH}} \mathbf{B}^{+} \tag{25}$$

$$\mathbf{1}^{+} + H_2O \xrightleftharpoons{k_{12}^{H}} \mathbf{2} + H^{+} \tag{26}$$

$$\mathbf{1}^{+} + OH^{-} \xrightleftharpoons{k_{12}^{OH}} \mathbf{2} \tag{27}$$

Finally our results should be compared with data given in the literature for the hydrolysis of thiamine. The dissociation constants K_{1A} and K_{41} relate to species that exist at equilibrium and our values agree well with those determined by other authors.[4,9,15] All other constants have to be determined by kinetic methods. Although for this reaction kinetic experiments have been performed frequently,[6,15,21-23] so far no agreement about its detailed mechanism exists. k_{12}^{OH} is the only rate constant that may be compared directly and here all authors obtain similar values between 8 and 25 dm^3 mol^{-1} s^{-1}. Chahine and Dubois[6] report values for k_{12}^{H} and K_{42} and also Hopmann[21] gives a value for K_{42}, but these were evaluated assuming simpler reaction mechanisms and therefore cannot be compared with our results. In more recent publications Washabaugh and co-workers[23,24] discuss whether the reaction of the pseudo-bases to form the thioles may be rate determining. This clearly contradicts our results and those of some other authors.[6,11,15,21]

Summary

The hydrolysis of thiamine is studied extensively between pH 0 and 10.5. All results agree quantitatively with Scheme 1 and the constants given in Table 1. Scheme 1 is based on results obtained for simpler thiazolium salts,[13,14] where only species $\mathbf{1}^+$, **2**, **3**, $\mathbf{4}^-$ and $\mathbf{6}^+$ exist. Protonation of the pyrimidine ring of these species leads directly to Scheme 1. Equilibrium constants are evaluated from the amplitudes of relaxation effects of stopped-flow experiments and they are confirmed by the pH dependence of the relaxation times. Rate and equilibrium constants of thiamine differ by less than one order of magnitude from the corresponding values of other thiazolium salts. No indication is given for the existence of the ylide, which has been postulated to be the active catalytic species by Breslow.[11] This agrees with pK values in the range 17–19 reported for the ylide of different thiazolium salts.[25] For the three intermediate forms **3**, $\mathbf{C}^+$ and $\mathbf{D}^{2+}$ the UV–VIS spectra have been recorded.

Erratum

In the previous publication[14] Table 2 contains two errors: pK_{32} has to be replaced by pK_{31}, and k_1^{OH} has to be replaced by k_{12}^{OH}.

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