Kinetics and Thermodynamics of the Structural Transformation of Thiamine in Neutral and Basic Media. Part 2.[1] Ultraviolet Spectrum of the Intramolecular Adduct

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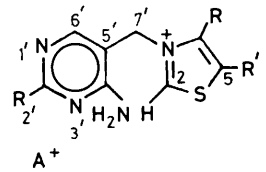
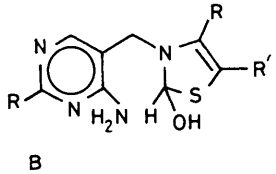
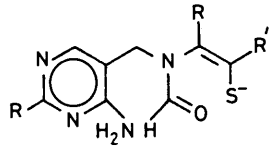
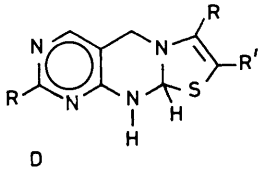
Through chemical relaxation methods, it is shown that, in basic media, the thiamine yellow form J^- is transformed into thiolate C^- *via* intramolecular σ-adduct D, cation A^+, and pseudo-base B. The formation of the pseudo-base B by the hydroxylation of the cation A^+ is the rate-limiting step of the structural transformations of thiamine in aqueous media, with a second-order rate constant k_{14} 19.2 l mol^{-1} s^{-1} which is in very good agreement with the rate constant measured earlier in mildly basic media where J^- is not involved. The reversible transformation of J^- into A^+ was also analysed in neutral media where the yellow form J^- yields cation A^+ by two distinctive steps. (i) The first is very fast and corresponds to the formation and accumulation of σ-adduct D. (ii) The second one is fast and corresponds to the acid-promoted ring-opening of σ-adduct D to yield cation A^+, with a second-order rate constant k_{61} 4.15 × 10^5 l mol^{-1} s^{-1}. σ-Adduct D is isolated as a kinetic product and its u.v. spectrum is reported. Thus, in neutral media (which are close to natural media) thiamine is in a very rapid equilibrium with species B and D. This means that these two tetrahedral adducts are available, for any biological reaction requiring their assistance, by shifting *via* A^+ the global thiamine equilibrium in neutral media.

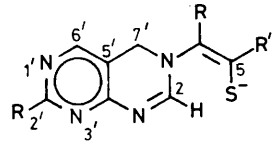
Thiamine, the vital coenzyme vitamin B1 found in all living species, is responsible for many metabolic processes involving the formation and cleavage of carbon–carbon bonds adjacent to a carbonyl group,[2] *e.g.* pyruvate decarboxylation.[2–8] In Part 1 we have shown that, in neutral media, the thermodynamic species A^+ would undergo covalent hydration with proton loss, whereas it was known that, in mildly basic media, A^+ would undergo hydroxylation,[1,3,5,6] thereby leading (in both cases) to the formation of the pseudo-base intermediate B which, by deprotonation and ring-opening, would change into thiolate C^- (the predominant form in basic media) [reactions (1)–(3)].



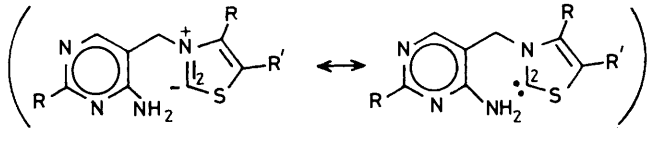








R = CH_3
R′ = C_2H_4OH



$$A^+ + H_2O \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} B + H^+ \quad (1)$$

$$K_1 = [B][H^+]/[A^+] = 1.95 \times 10^{-10} \text{ mol l}^{-1}$$
with k_{23} 1.15 × 10^6 l mol^{-1} s^{-1}

$$A^+ + OH^- \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} B \quad (2)$$

k_{34} 19.6 l mol^{-1} s^{-1}

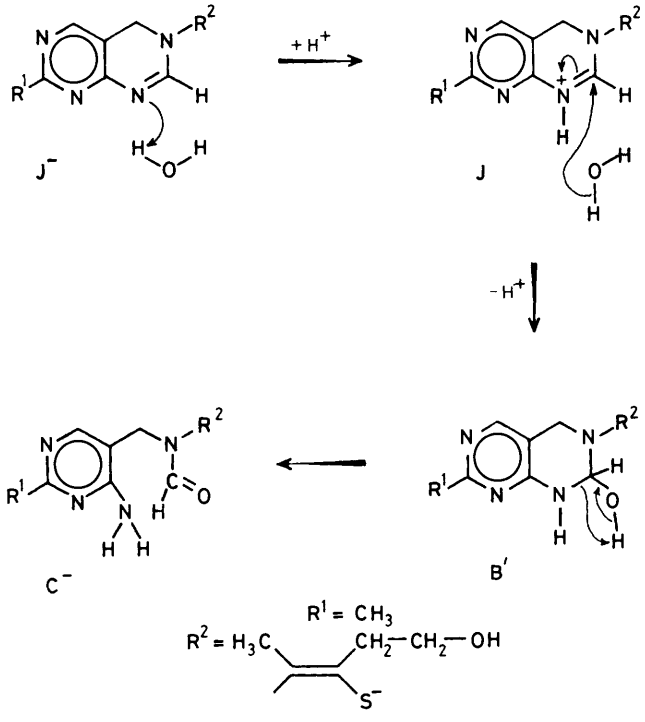
$$B + OH^- \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C^- \quad (3)$$

$$K_{2b} = [B][OH^-]/[C^-] = 1.15 \times 10^{-9} \text{ mol l}^{-1}$$
with k_{12} 6.75 × 10^4 l mol^{-1} s^{-1} at 5 °C

$$A^+ + OH^- \rightleftharpoons D \qquad K_{3b} = [A^+][OH^-]/[D] = K_3[H^+]/[A^+] \quad (4)$$

$$D + OH^- \rightleftharpoons J^- \qquad K_{4b} = [D][OH^-]/[A^+] = K_4[J^-][H^+][D] \quad (5)$$

In media with basicity above pH 11, thiamine changes into its yellow form J^- which in turn slowly yields C^-.[9–12] Maier and Metzler previously postulated that the $A^+ \rightleftharpoons J^-$ transformation occurs *via* a σ-adduct D resulting from intramolecular nucleophilic addition of the aminopyrimidine at position 2 of the thiazolium and that J^- is reversibly transformed into C^- *via* D, A^+, and B.[9] This mechanism was partly confirmed by Hopmann *et al.*, who measured a second-order rate constant of



Scheme.

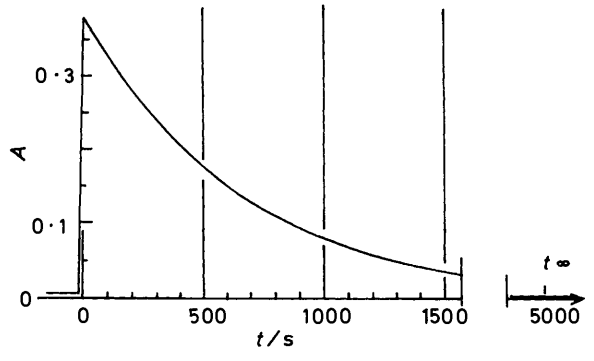


Figure 1. Absorbance change at 339.5 nm, for solution of thiamine when subjected to a fast pH-jump from neutral (pH *ca.* 6) to very basic (pH *ca.* 13.60) at 25 °C and 0.2M ionic strength (thiamine concentration *c* 4.90 × 10^{-5}M)

115 l mol^{-1} s^{-1} for the formation of D.[10,11] However, these authors expanded the mechanism of J^- formation by a step involving the deprotonation of A^+ into A_{-H} (Breslow's catalytic species[8]), and considered that the transformation of J^- into C^- was semi-irreversible, taking place *via* two new thiamine species B′ and J (Scheme).[11,12]

J^- is a kinetic species, whose evolution into C^- is still not very clear, in particular because no spectroscopic or kinetic evidence confirms either of the two transformations of J^- into C^-.[9-12] The purpose of this paper is to elucidate the kinetics and equilibria controlling formation and conversion of J^- and C^-. All these equilibria are related to H^+- and/or OH^--assisted opening and formation of two different rings. The method of chemical relaxation[13-15] allowed us to achieve better understanding of the kinetic processes involved as we could isolate adduct D as a kinetic product.

Results

Since the u.v. spectra of the thiamine species are well known[1,9-12] results were monitored by spectrophotometry.

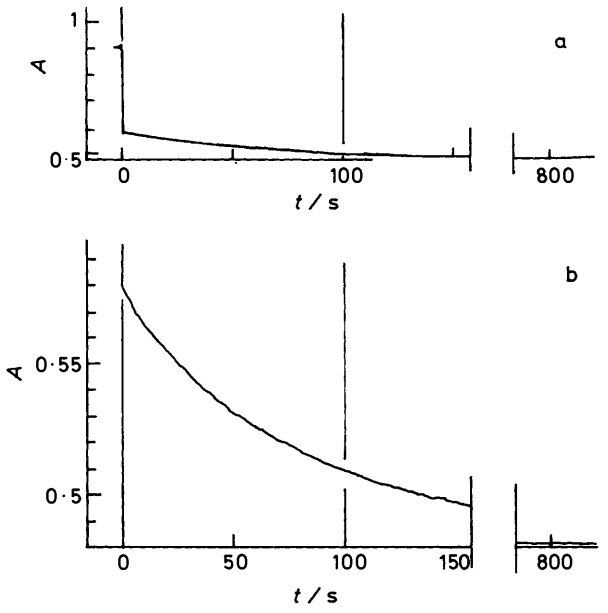


Figure 2. Absorbance change at 249 nm, for a solution of the thiamine yellow form J^- when subjected to a fast pH-jump from basic (pH *ca.* 12.5) to neutral (pH *ca.* 7.70) at 25 °C and 0.2M ionic strength, *c* 8.15 × 10^{-5}M

Kinetic Phenomena.—When the basicity of a neutral solution of thiamine is made to rise quickly (pH >11), at least two kinetic phenomena are detected at 339.5 nm[9-12] (Figure 1). (a) The first, a sharp increase in absorbance, was ascribed to the $A^+ \rightleftharpoons J^-$ transformation.[10,11] (b) The second phenomenon is a slow exponential decrease of absorbance which practically disappears at t_∞. This phenomenon was ascribed by Maier and Metzler to the reversible transformation of J^- into C^- *via* D, A^+, and B.[9] The same phenomenon was ascribed by Hopmann *et al.* to a semi-irreversible process (Scheme).[11,12]

When, after a fast decrease in acidity from neutral to pH *ca.* 12.5 (after the formation of J^- and before its slow evolution into C^-), a solution of thiamine is subjected to rapid neutralization (7.4 < pH < 8.3) by micro-injection of Na_3PO_4–HCl, at least two kinetic processes are detected in the 220—300 nm range (Figure 2). (i) The first phenomenon is a sharp decrease of absorbance. (ii) The second is an exponential decrease of absorbance with time. The amplitudes of both processes are pH-independent in the vicinity of neutrality. Above 300 nm the amplitude of the slow phenomenon becomes negligible.

Relaxation Processes and Mechanistic Considerations.—Since all the observed kinetic phenomena were pure exponentials,[13] they were considered as relaxation processes.[14] Reciprocal relaxation equations were derived by chemical relaxation methods,[13] and the use of these methods was justified by calculating a thermodynamic term *r* which accounted for the amount of perturbation admissible in a chemical relaxation experiment.[15]

Amplitude of the fast phenomenon (basic media). At 339.5 nm J^- is the only thiamine species to have an absorbance spectrum. Thus, the Beer–Lambert law can be expressed as (6) where

$$A = \varepsilon_{J^-}[J^-]l \qquad (6)$$

l = 1 cm and ε_{J^-} = 7 000 l mol^{-1} cm^{-1}.[9-11] When J^- is replaced in equation (6) by its expression as a function of $[OH^-]$, $\bar{K}_b$, K_{4b}, and the analytical concentration of thiamine *c*, *A* is described by equation (7). $\bar{K}_b = [OH^-]^2[A^+]/[J^-] = 1 \times 10^{-5}$ mol^2 l^{-2} (measured at 25 °C, see Experimental section).

Table 1. Relaxation amplitudes related to the formation of J^-. A_E designates experimentally measured amplitudes, A_C amplitudes calculated with equation (7) for pK_4 10.90 at 25 °C and 0.2M ionic strength

Concentration (M)	pH	A_E	A_C
5.75×10^{-5}	12.08	0.39	0.37
5.75×10^{-5}	11.68	0.25	0.27
5.75×10^{-5}	11.30	0.11	0.12
5.75×10^{-5}	11.71	0.28	0.28
5.75×10^{-5}	11.56	0.23	0.22
5.75×10^{-5}	11.45	0.19	0.18
5.75×10^{-5}	11.66	0.27	0.26
5.75×10^{-5}	11.85	0.29	0.32
1.23×10^{-4}	11.75	0.66	0.63
1.23×10^{-4}	11.57	0.47	0.49
1.23×10^{-4}	11.69	0.56	0.58
1.23×10^{-4}	11.85	0.70	0.69

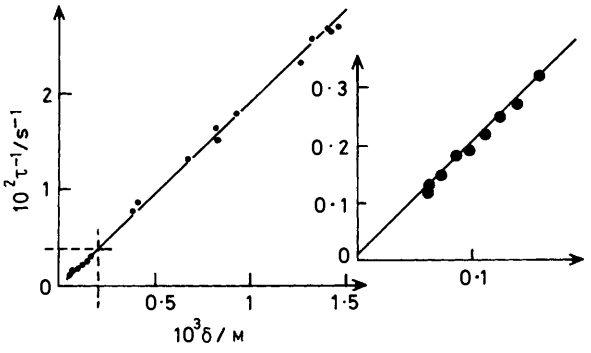


Figure 3. Plot of τ_2^{-1} against $k_{34}\delta$: intercept $(4 \pm 6) \times 10^{-4}$ s^{-1}, slope (19.20 ± 0.80) l mol^{-1} s^{-1}, r 0.9978 for 20 experimental points $\{\delta = K_{3b}\bar{K}_b[OH^-]/(K_{3b}\bar{K}_b + K_{3b}[OH^-]^2 + \bar{K}_b[OH^-])\}$. The scale is expanded six times for the first nine points

$$A = \frac{[OH^-]^2}{[OH^-]^2 + K_{4b}[OH^-] + \bar{K}_b}\varepsilon_{J^-}c \qquad (7)$$

Computer simulation of equation (7), using an iteration step, ΔpK 0.01, for pK_4 in the pH range 10.3—11.3 (for 12 experimental amplitudes for the formation of J^-) yields pK_4 = 10.88 ± 0.02 (Table 1). pK_3 12.1 ± 0.05 can be calculated from pK_4 and from p$\bar{K}$.

Structural Transformation of J^- into C^-. Reciprocal relaxation time equation. The mechanism in the Scheme is hard to investigate since there is no thermodynamic or kinetic evidence for the formation of the two newly proposed species J and B′.

If J^- yields C^- *via* D, A^+, and B, the slow kinetic process in Figure 1 can be ascribed to the slowest reaction involved in the structural transformations of thiamine in basic media, which is the hydroxylation of cation A^+ into pseudobase B [reaction (2)],[1] all the other fast reactions (3)—(5) being in constant equilibrium. Since at pH > 12 the observed species are J^- and C^-, the kinetic equation of the $J^- \rightleftharpoons C^-$ transformation is set as equation (8).* The conservation of matter and charge implies that equations (9) and (10) hold. Since reactions (4), (5), and (3)

$$-d[C^-]/dt = -k_{34}[OH^-][A^+] + K_{43}[B] \qquad (8)$$

$$\Delta[J^-] + \Delta[D] + \Delta[A^+] + \Delta[B] + \Delta[C^-] = 0 \qquad (9)$$

$$\Delta[J^-] + \Delta[C^-] + \Delta[OH^-] = \Delta[A^+] \qquad (10)$$

are in constant equilibrium during the $J^- \rightleftharpoons C^-$ transformation, one can write equations (11)—(13).

$$\Delta[C^-] = [B]\Delta[OH^-]/K_{2b} + [OH^-]\Delta[B]/K_{2b} \qquad (11)$$

$$\Delta[D] = [A^+]\Delta[OH^-]/K_{3b} + [OH^-]\Delta[A^+]/K_{3b} \qquad (12)$$

$$\Delta[J^-] = [D]\Delta[OH^-]/K_{4b} + [OH^-]\Delta[D]/K_{4b} \qquad (13)$$

In our experimental conditions relationship (14) holds. From equations (11)—(13) and from (9) one can derive the reciprocal relaxation equation (15) for reaction (2) in very basic media. Since k_{43} is very low,[1] in our experimental conditions equation (15) becomes (16).† A linear least-squares regression of the data

$$[OH^-] \gg [A^+], [B], [D], [C^-], [J^-] \qquad (14)$$

$$\tau_2^{-1} = k_{34}\frac{K_{3b}\bar{K}_b[OH^-]}{K_{3b}\bar{K}_b + \bar{K}_b[OH^-] + K_{3b}[OH^-]^2} + k_{43}\frac{K_{2b}}{[OH^-]} \qquad (15)$$

$$\tau_2^{-1} = k_{34}\frac{K_{3b}\bar{K}_b[OH^-]}{K_{3b}\bar{K}_b + \bar{K}_b[OH^-] + K_{3b}[OH^-]^2} \qquad (16)$$

employing equation (16) gave at, 25 °C and at 0.2M ionic strength, k_{34} (19.2 ± 1.7) l mol^{-1} s^{-1} (Figure 3), which is in good agreement with the k_{34} value measured in mildly basic media.

What stands out is that the *pathway of Maier and Metzler seems to be preferred to that of Hopmann* et al. (Scheme), as the latter contribution to the observed relaxation time is negligible. If it were not negligible, the fit of the data could not have been achieved as in Figure 3, and the intervention of a direct pathway would at least have affected the intercept of the linear regression line.

Slow relaxation. Reciprocal relaxation equation (neutral media). When a solution of J^- (very basic) is rapidly neutralized by micro-injection of Na_3PO_4–HCl, J^- is totally transformed into A^+ in at least two steps (Figure 2) whose amplitudes are pH-independent. This indicates that the pK values of both chemical processes are basic. Otherwise (p$K \ll 8$) the two observed amplitudes would have been pH-dependent. Thus, J^- generates a kinetic intermediate which accumulates at the end of the sharp decrease of absorbance (Figure 2). This kinetic product produces A^+ with a relaxation time τ_3.

We ascribe the first rapid process depicted in Figure 2 to the $J^- \rightleftharpoons D$ transformation. This being so, the second process

* If the $J \rightleftharpoons C^-$ transformation takes place through D, A^+, and B, the thermodynamic factor r (introduced in ref. 15) which, for a chemical relaxation experiment, should be lower than 0.1, is expressed in our experimental conditions by equation (i). In the pH-jump experiments

$$r = (2K_{3b}\bar{K}_b + \bar{K}_b[OH^-])\Delta[C^-]/(\bar{K}_bK_{3b} + K_b[OH^-] + K_{3b}[OH^-]^2)[OH^-] \qquad (i)$$

the perturbation cannot exceed the concentration of J^- in the medium which, at most, is equal to the analytical concentration of thiamine (c). In the most unfavourable case, r is 2.65×10^{-2}. Thus, in our experimental conditions, the approximation of chemical relaxation can be very safely applied to the $J^- \rightleftharpoons C^-$ transformation in basic media.

† As pointed out by a referee, rate equation (16) can be derived without the use of chemical relaxation methods. However, we have always considered that the use of these methods (when possible) usually simplifies the kinetic analysis and gives direct access to the elementary rate constants which is not always evident with a more 'classical' kinetic analysis.

Table 2. Comparison of our rate constants with those in the literature

Reaction	Our values: Second-order rate constant (l mol^{-1} s^{-1})	Our values: Reverse rate constant (s^{-1})	Literature values: Second-order rate constant (l mol^{-1} s^{-1})	Literature values: Reverse rate constant (s^{-1})
$B + H^+ \rightleftharpoons A^+$	1.15×10^5	2.15×10^{-4}	----------	
$A^+ + OH^- \rightleftharpoons B$	19.2—19.6	1.15×10^{-3}	7.2—13[b]	
$B + OH^- \rightleftharpoons C^-$	6.75×10^4	8.85×10^{-2}	Considered as acid catalysed	
$D + H^+ \rightleftharpoons A^+$	4.60×10^5	*ca.* 3×10^{-2}	----------	
$A^+ + OH^- \rightleftharpoons D$			115[c]	2.65

[a] Measured at 5 °C. [b] Refs. 5 and 9. [c] Ref. 10.

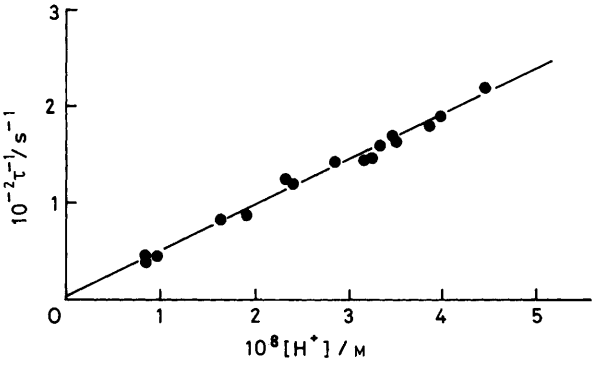


Figure 4. Plot of τ_3^{-1} against $k_{16} + k_{61}[H^+]$: intercept $(3 \pm 7) \times 10^{-4}$ s^{-1}, slope 4.60×10^5 l mol^{-1} s^{-1}, r 0.9953 for 16 experimental points

can be ascribed to the transformation of adduct D into A^+ which, in basic media, is known to be slower than the $D \rightleftharpoons J^-$ transformation.[10]

Near-neutrality, the transformation of D into A^+ can occur by two possible routes, acid-promoted pathway and base-promoted pathway [reactions (18) and (4)].

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \quad (17)$$

$$A^+ \underset{k_{61}}{\overset{k_{16}}{\rightleftharpoons}} D + H^+ \quad (18)$$

Since the observed slow kinetic processes (Figure 2) are pure exponentials and since the buffer concentration is *ca.* 100 times the concentration of thiamine, the observed slow phenomena can be safely considered as relaxation modes.[15] The reciprocal relaxation time equation (τ_3^{-1}) of reactions (18) and (4) is expressed as (19).[13] All observed relaxation times only obey that

$$\tau_3^{-1} = k_{61}[H^+] + k_{16} + k_{14}[OH^-] + k_{14} \quad (19)$$

part of equation (19) which deals with the acid-promoted ring opening of D ($\tau_3^{-1} = k_{61}[H^+] + k_{16}$).*

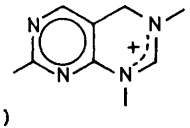
A linear least-squares regression of the data against that part of equation (19) which deals with the acid-promoted pathway gave (at 25 °C and 0.2M ionic strength) k_{61} $(4.60 \pm 0.30) \times 10^5$ l mol^{-1} s^{-1} and k_{16} *ca.* 0, which is normal because $k_{16}/k_{61} = K_3 = 9 \times 10^{-13}$ mol l^{-1}. So, k_{16} is too small to be measured here (Figure 4).

In the vicinity of neutrality, J^- is rapidly transformed into D, which by acid-promoted ring opening engenders cation A^+, the thermodynamic species.

Discussion

In Table 2, we summarize our findings and compare them when possible with those of the literature. Table 2 shows some evidence required for better understanding of the general mechanism of the structural transformations of thiamine over a wide range of pH in basic and in neutral media which are close to natural media.

Transformation of J^- into C^-.—The transformation of J^- into C^- is reversible and occurs *via* σ-adduct D, cation A^+, and the rate-limiting step of pseudo-base B formation from cation A^+ [reaction (2)]. The rate constant k_{34} 19.2 l mol^{-1} s^{-1}, measured here for this reaction, is in very good agreement with the same constant measured earlier in mildly basic media[1] (where [J^-] *ca.* 0). This constant can also be compared to Maier and Metzler's estimated hydroxylation constant at 19 °C (7.9 l mol^{-1} s^{-1}).[9] The transformation of J^- into C^- (Scheme)[11,12] does not seem to occur since its contribution to the observed data is negligible. It should be noted here that the average pK of the $A^+ \rightleftharpoons C^-$ equilibrium is 9.3,[1,3,4,6] while that of the $A^+ \rightleftharpoons J^-$ equilibrium is 11.5 at 25 °C (11.6 at 19 °C[9]). This means that J^- is thermodynamically extremely underprivileged compared with C^-, the thermodynamic product in basic media. However (in the totally equilibrated state), J^- is still present in these media but at no detectable concentration, since [J^-]/[C^-] = 5×10^{-5}. However, this does not mean, as stated by Hopmann *et al.*,[11,12] that this transformation does not exist. It will always take place through the different reactions (1)—(3) even if the direct pathway depicted in the Scheme, and shown here as highly improbable, takes place. In order to justify their proposals (Scheme), Hopmann *et al.*[11,12] synthesized molecule (**1**) which has a similar heterocyclic skeleton to that of J^-, but which bears two methyls at positions 1 and 3.



(**1**)

Methylation at N(1) of (**1**) transforms the proposed structure

* The possibility of buffer catalysis (ii) of the $D \rightleftharpoons A^+$ transformation in the neutral media is considered here. In our experimental conditions,

$$A^+ + HPO_4^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D + H_2PO_4^- \quad (ii)$$

the reciprocal relaxation equation associated with the intermolecular thiamine–phosphoric acid reaction is expressed as (iii) where c' is the

$$\tau^{-1} = k_1 \frac{K}{[H^+] + K} c' + k_{-1} \frac{[H^+]}{[H^+] + K} c' \quad (iii)$$

analytical concentration of the buffer and $K = [HPO_4^{2-}][H^+]/[H_2PO_4^-]$. This equation is not fitted by our experimental data: 10^{-2}M < c' < 10^{-3}M.

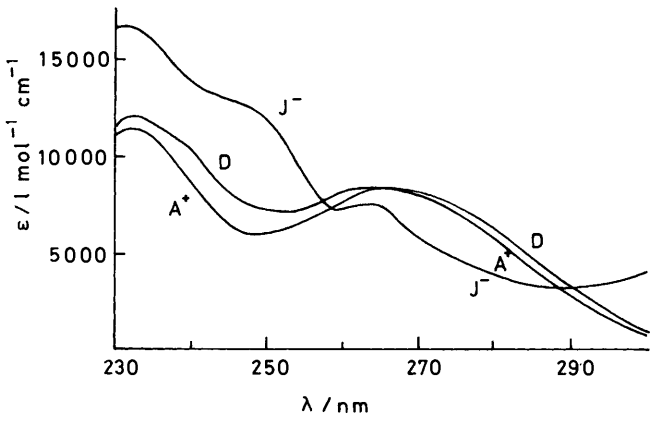


Figure 5. U.v. spectra of σ-adduct D, cation A^+, and yellow form J^-. The molecular extinction coefficient of each species is plotted against the wavelength

into a cation and renders position 2 a nucleophilic addition centre,[16] which can be attacked by water to form a pseudo-base, while J^- is an anion the protonation of which at the imine should have a low pK as in quinazolinium or other pyrimidines and pyridines in polycyclic and aromatic structures.[16,17] Thus, the fact that (**1**) gives a pseudo-base does not mean that J yields a pseudo-base under the same conditions, expecially as, apart from (**1**), there is no evidence for the existence and the involvement of J in the transformation of J^- into C^-.

Transformation of J^- into A^+.—When J^- is placed in a neutral medium, it will, at first, yield very rapidly the σ-adduct D, which will slowly yield the thermodynamic product A^+ by the acid-promoted reaction (18), with a second-order rate constant k_{61} of 4.60×10^5 l mol^{-1} s^{-1}. Since pK_3 and pK_4 are basic and since reaction (5) is faster than (4), when placed in a neutral medium, J^- is instantly and totally transformed into D which slowly yields A^+. Therefore, in Figure 2, after the sharp decrease of absorbance and before the beginning of the slow phenomenon, the absorbance is due entirely to the intramolecular adduct D. This experiment repeated for different wavelengths, for identical pH-jumps from basic to neutral, allowed us to measure the u.v. spectrum of D (Figure 5). This spectrum (save probably the solvent effect) is similar to that calculated previously by Maier and Metzler in methanol,[9] and is very different from the spectrum reported by Sugimoto and Hirai for A_{-H} in ethanol.[18]

Conclusions

Aside from its biological importance, thiamine, from a structural vantage point, is an interesting model compound with which it is possible to observe most reactions that an unsaturated heterocyclic compound can undergo in water. The work described here shows that, in very basic media (pH > 11), thiamine changes into a yellow form J^- which is transformed into thiolate C^- *via* σ-adduct D, cation A^+, and pseudo-base B. In neutral media, J^- gives A^+ by two steps. The first corresponds to the formation and accumulation of σ-adduct D whose u.v. spectrum has been measured and reported; the second corresponds to the acid-promoted ring opening of D to yield A^+.

The pH of biological media is considered to be neutral. Thus, thiamine catalyses pyruvate decarboxylation with a basic species but in neutral media.[4] This means that in thiamine-catalysed acetoin formation from pyruvate,[19] catalyst A_{-H} achieves the needed concentration for the reaction to be accomplished, and fast thiamine deprotonation allows A_{-H} to achieve the needed catalyst concentration in the medium. What seems interesting in these results is the fact that, in neutral media, thiamine is in very rapid equilibrium with species B′ and D. This means that any reaction which needs these two species in neutral media can shift the global thiamine equilibrium; A^+ thus constitutes a reservoir for these two species.

Experimental

Thiamine (Aldrich) was purified as described before.[1] NaOH (Merck Titrisol), KCl, and Na_3PO_4 (Merck) were used without further purification. Water was twice distilled and degassed with argon.

Stock Solutions.—Fresh solutions of thiamine were used in concentrations ranging from 4×10^{-5} to 1.5×10^{-4}M.

pH *Measurements.*—The pH was measured before and after the concentration jump with a Radiometer pH-meter (equipped with a Metrohm combined electrode for pH < 12, but with a B-type combined electrode for pH > 12) under nitrogen or argon directly in the sample cell at (25 ± 0.5) °C.[1] All values were adjusted with respect to the activity coefficient of H^+ and OH^- in the media,[1] and the post-perturbation ionic strength was always adjusted to 0.2M with KCl.

Kinetic Measurements.—For the $J^- \rightleftharpoons C^-$ transformation, the neutral solutions of thiamine were perturbed by injecting microvolumes of NaOH according to previously described methods.[1] Kinetic measurements were performed on a nitrogen-purged Cary C 210 u.v.–visible spectrophotometer. The sample cell was thermostatted at (25 ± 0.5) °C by a previously described method.[1] For the $J^- \rightleftharpoons A^+$ transformation in neutral media, neutral thiamine solutions were at first rendered strongly basic (pH > 12.5) by microinjections of concentrated NaOH directly in the sample cell. Immediately after the formation of J^- the solutions were neutralised by injection, in the same sample cell, of weighed microvolumes of concentrated Na_3PO_4–HCl solution. The concentrations of the final neutral thiamine solutions in Na_3PO_4 ranged from 1×10^{-2} to 1×10^{-3}M.

Measurement of $\bar{K}_b$, the Average Constant of the Overall $A^+ \rightleftharpoons J^-$ Equilibrium.—$\bar{K}_b = [OH^-]^2[A^+]/[J^-]$ was measured by standard procedures[9,10] as $9.5 \pm (0.5) \times 10^{-6}$ mol^2 l^{-2} (25 °C; 0.2M ionic strength)

Signal Analysis.—Reciprocal relaxation times were obtained from the experimental signals by the semi-log methods with least-squares adjustments.[1,14] All the signals with uncertainties > 6% were not taken into consideration.

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