Kinetics and Thermodynamics of the Structural Transformations of Thiamine in Basic Aqueous Media. Part 3.[1,2] Interpretation of the Lability of the 2-Proton *via* an Intramolecular σ-Adduct

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The structural transformations of thiamine into its yellow form J^- in very basic media (pH > 11) have been investigated by pH stopped-flow jump and temperature-jump techniques. This transformation *via* the deprotonation of the thiazolium 2-position to give the resonance-stabilized carbene A_{-H}, hitherto considered as the biocatalyst of vitamin B1 metabolic activity, is questioned, since deprotonation of the thiamine 2-position is unlikely for $pK < 15$. Formation of the J^- species occurs through the σ-adduct D resulting from intramolecular NH_2 base-promoted nucleophilic addition at the thiazolium 2-position. This ring formation occurs with a second-order rate constant k_{14} of 99 l mol^{-1} s^{-1}. σ-Adduct D generates J^- by a base-promoted reaction: k_{45} 5.15 ×10^6 l mol^{-1} s^{-1}. The deuterium isotope effect $k_{45(H_2O)}/k_{45(D_2O)}$ 2.05 and ΔpK_4 0.75 for this $D \rightleftharpoons J^-$ reaction imply the involvement of several proton transfers. It is surmised that this $D \rightleftharpoons J^-$ transformation takes place through the deprotonation of the 2-position of D into a carbanion intermediate D^- which by prototropic ring-opening yields J^-. This hypothesis accounts for the half-life of the 2-proton, known from n.m.r. measurements in neutral aqueous media. This tentative mechanism answers a long standing question on the role played by the amino pyrimidine moiety in the biocatalytic activity of thiamine. The existence of such a carbanion D^- species as a candidate among the catalysts which monitor the metabolic activity of vitamin B1 is, therefore, proposed with caution.

Thiamine or vitamin B1 is a coenzyme involved in carbohydrate metabolism before the beginning of the respiratory cycle.[3] Thiamine is also present in the brain, where it can play an important role.[4] A general mechanism for the structural transformations of thiamine in neutral, mildly basic, and basic media has been proposed (Mechanism).[1,2,5,6]

$$A^+ \rightleftharpoons B + H^+ \quad K_1 = [B][H^+]/[A^+] \text{ (fast)} \quad (1)$$

$$A^+ + OH^- \rightleftharpoons B \quad \text{(rate-limiting)} \quad (2)$$

$$B + OH^- \rightleftharpoons C^- \quad K_2 = [C^-][H^+]/[B] \text{ (fast)} \quad (3)$$

$$A^+ + OH^- \underset{k_{41}}{\overset{k_{14}}{\rightleftharpoons}} D \quad \text{(fast)} \quad (4)$$

$$D + H^+ \rightleftharpoons A^+ \quad K_3 = [D][H^+]/[A^+] \text{ (fast)} \quad (5)$$

$$D + OH^- \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} J^- \quad K_4 = [J^-][H^+]/[D] \text{ (very fast)} \quad (6)$$

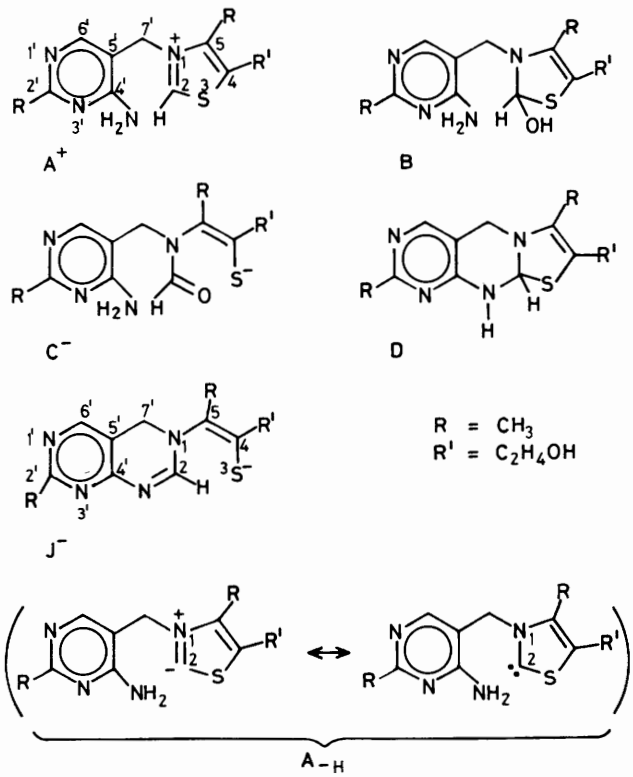
Mechanism.

Hopmann *et al.* showed by n.m.r. that the 2-proton of neocyanothiamine is exchanged with the deuterons of a deuteriated medium during transformation of neocyanothiamine into its yellow form.[4,5] This observation led these authors to expand reaction (4) by a step involving the deprotonation of A^+ into A_{-H}, the structure proposed by Breslow[7,8] and considered responsible for the biocatalytic activity of thiamine in pyruvate decarboxylation.[9–14]

$$A^+ + OH^- \rightleftharpoons A_{-H} \quad (7)$$

$$A_{-H} \rightleftharpoons D \quad (8)$$

It should be noted here that A_{-H} can be considered as a resonance-stabilized carbene species,[15] which seems to have been recently isolated in very basic ethanolic medium.[16] To rationalize the involvement of A_{-H} in the transformation of A^+



into J^-, Hopmann *et al.*[5] ascribed a pK of 14.15 to the deprotonation of A^+ which, they admitted, is much higher than the pK of 12.6 they had previously reported.[13]

Physiological media are neutral, and if the deprotonation of cation A^+ has p$K > 12.6$,[5,14] this would indicate that the key to the biocatalytic activity of thiamine lies elsewhere, in its structural transformations in basic media. These observations led us to this chemical relaxation study of the structural transformations of thiamine in basic media, as well as to a thorough investigation of the lability of the 2-proton.

Experimental

Thiamine (Merck) was kept under vacuum and used without further purification, m.p. 248—250 °C.[1] NaOH and HCl (Merck Titrisol) and KCl and Na_3PO_4 (Merck très pur) were used without further purification. Heavy water (99.9%), NaOD (40%), and DCl (20%) were Aldrich gold label products. Water was twice distilled and degassed with argon.

Stock Solutions.—Fresh solutions of thiamine were used in concentration (c) ranging from 10^{-3} to 10^{-2}M for kinetic measurements and from 0.5 to 1M for n.m.r. experiments.

pH *Measurements.*—For temperature-jump experiments the pH and the pD (pD 0.41 + pH[17]) were measured with Radiometer pH-meter equipped with a Metrohm E.A. 125 combined electrode in the circulation reservoir of the temperature-jump spectrophotometer. The buffers used for pH standardization were pH 6.86 and 10.01 NBS standards (Beckman). pOH and pOD were determined from pH and pD with pK_w 14.00 for water and 14.93 for heavy water. These measurements were performed in solutions of 0.5M ionic strength where the Debye–Hückel relation is not respected.[17] Therefore, $[OH^-]$ and $[OD^-]$ used in the temperature-jump analysis are more related to activities than to concentrations.

Stopped-flow Measurements.—Kinetic measurements were performed under argon on an Sf-3A Canterbury stopped-flow Nortech spectrophotometer equipped with a thermostatted bath held at 25 ± 0.5 °C and with a Hewlett–Packard memory unit. Output voltage was adjusted to 1 V for zero absorbance. Equal volumes of solutions of NaOH (4×10^{-2}—2M) and of neutral thiamine (1—2.3×10^{-3}M) were simultaneously injected into the mixing chamber (mixing time $< 5 \times 10^{-3}$ s). The experimental signals were accumulated three times for each experiment. All kinetic phenomena measured at 339.5 nm with final ionic strength of 0.2M were taken into account; those with a final ionic strength above 0.2M (detected between 280 and 400 nm) were not used. Final $[OH^-]$ were deduced from the diluted NaOH + thiamine mixture by taking into account the need for two equiv. of OH^- for transforming A^+ into J^-. The final (post-perturbation) ionic strength was adjusted, when possible, to 0.2M with KCl.

Temperature-jump Measurements.—A Messanlagen and Studiengesellschaft Joule effect temperature-jump spectrophotometer equipped with an external reservoir (at 2 ± 0.5 °C) connected to the temperature-jump cell *via* a peristaltic pump[18] was used to analyse the transformation of J^- into D. Neutral solutions of thiamine and Na_3PO_4 in H_2O and D_2O were introduced into the reservoir and into the cell; at thermal equilibrium, the thiamine–Na_3PO_4 solution was rendered basic by microinjecting a 10M solution of NaOH into the reservoir (a 30 s mixing time was measured as a function of the outflow from the peristaltic pump). Once J^- was generated (at the end of mixing) a 3.5—4 °C temperature jump was produced by discharging a 0.02 μF condenser charged at 30 kV. The absorbance at 300—400 nm was recorded as a function of time on a PDP 11 computer by the published procedures.[18] The relaxation signals were acquired from pH 10.7 to 11.7 and from pD 11.3 to 12.2. When the pH was near 13 the absorbance change was indistinguishable from heating (<2—3 μs). It should be noted that the transformation of J^- into C is slow near pH 11. Moreover, this transformation was considerably slowed by the temperature drop which allowed us to perform some temperature jumps around pH 11.5. (Here $J^- \rightleftharpoons C^-$ is fastest with a lifespan of a few minutes for J^- at 2 °C.) In these experiments, the ionic strength was adjusted to 0.5M with KCl.

N.m.r. Analysis.—N.m.r. spectra were recorded on a 200 MHz Fourier transform Bruker spectrometer. The concentration of thiamine in D_2O was *ca.* 0.5—1M. After subjecting a thiamine solution to a fast rise in basicity by injecting microvolumes of 40% NaOD solution, the ^1H n.m.r. spectrum of J^- was rapidly recorded at 5 °C *ca.* 30 s after the formation of J^-. In very basic media ($[OH^-]$ *ca.* 0.5M) the transformation of J^- into C^- is slowed down considerably, thereby allowing the spectrum of J^- to be recorded.

Signal Analysis.—The temperature-jump signals were acquired by a PDP 11 computer and analysed by the semi-log and Guggenheim methods with least-squares adjustments; they were also checked by the phase plane and by the Padé–Laplace methods.[19] Uncertainty on relaxation times ranged from 4 to 8%. Stopped-flow signals were analysed by the methods stated above and the uncertainty in these experimental relaxation times ranged from 3 to 8%. (The Padé–Laplace method is used to analyse a signal containing one or more exponentials. It gives the exact number of exponentials contained in an experimental signal. This method showed that all signals reported in this paper were pure exponentials.)

Results

Two types of kinetic experiments were performed: pH-jump by stopped-flow techniques and temperature-jump by the Joule effect. Detection was spectrophotometric.[1,2]

Kinetic Phenomena.—When the basicity of a neutral solution of thiamine (containing A^+) is made to rise quickly (pH > 11), at least two kinetic phenomena are detected at 339.5 nm.[2,5,6]

The first kinetic process was time-resolved by the stopped-flow technique[5] (Figure 1); it is an exponential increase of absorbance and is ascribed to the rate-limiting step of the $A^+ \rightleftharpoons J^-$ transformation in basic media.[4,5] For solutions with $\leqslant$1M-$[OH^-]$, a single kinetic phenomenon is always detected in the 280—400 nm range by the stopped-flow technique. The amplitude of this single phenomenon becomes OH^--independent for $[OH^-] \geqslant 3 \times 10^{-2}$M.

The second kinetic phenomenon was ascribed to the indirect transformation of J^- into C^- through species D, A^+, and B.[2]

When a solution of thiamine and Na_3PO_4, immediately after fast basification, is subjected to a rapid temperature jump, a single relaxation process is observed in the 300—400 nm range. This kinetic process is a fast exponential increase of absorbance (Figure 2) the amplitude of which is wavelength- and pH-dependent in the vicinity of pH 11. This kinetic process is slowed 6—7 times in basic D_2O.

^1H *N.m.r. Spectroscopy of Thiamine in Heavy Water.*—^1H N.m.r. spectra of AH^{2+} (A^+ protonated at the 1′-position) and J^- (previously recorded by Azahi and Mizuta[20] using a continuous flow procedure for the spectrum of J^-) were recorded in heavy water with a Fourier transform spectrometer:

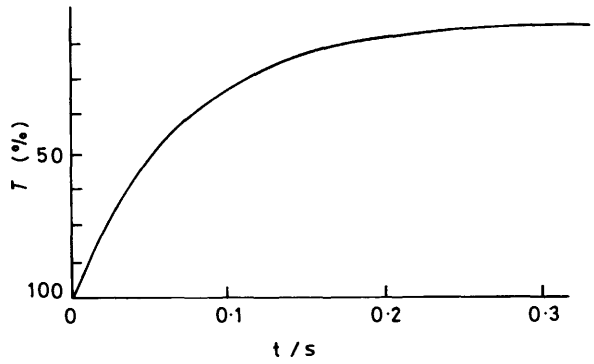


Figure 1. Transmittance change in a thiamine solution at 339.5 nm after a stopped-flow $[OH^-]$-jump from neutrality to $[OH^-]$ 9.83 × 10^{-2}M

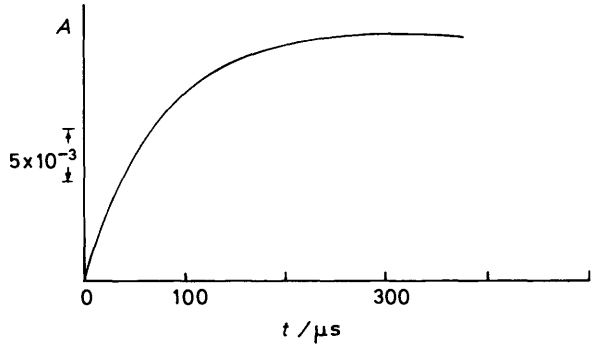


Figure 2. Absorbance change at 370 nm as detected after a temperature-jump of 3.5—4 °C performed on a J^- solution at 2 °C, 0.5M ionic strength, pH 11.24 and c 1.2 × 10^{-3}M

Table 1. Chemical shifts δ of thiamine protons according to structure

Proton	2	6′	6	7	8′	8	7′
$\delta(AH^{2+})$	9.83	8.19	3.30	3.90	2.69	2.79	5.69
$\delta(J^-)$	7.54	8.14	2.90	3.81	2.54	1.90	solvent

the spectrum of J^- was rapidly acquired 30 s after the basicity of a solution of AH^{2+} had been sharply increased (Table 1).

In heavy water AH^{2+} becomes AD^{2+} and produces a spectrum identical to that previously recorded in water.[20] However, the spectrum of J^- recorded in heavy water no longer displays a peak at δ 7.45 (2-H). The n.m.r. spectrum of a solution of AH^{2+} in D_2O (obtained by neutralizing a solution of J^- with DCl) no longer displays a peak for 2-H at δ 9.83. These observations, like those on neocyanothiamine by Hopmann *et al.*,[5] can indicate the occurrence, during the $A^+ \rightleftharpoons J^-$ transformation, of an exchange between the thiamine 2-H and the heavy water deuterons. They can also indicate the occurrence of a very fast proton–deuteron exchange at the 2-position of thiamine before the formation of J^- (Scheme 1).

$$A^+ + OH^- \rightleftharpoons A_{-H} \quad \text{(very fast)}$$
$$\Big\Updownarrow \text{(fast)}$$
$$D + OH^- \rightleftharpoons J^- \quad \text{(fast)}$$

Scheme 1.

The Structural Transformation $A^+ \rightleftharpoons J^-$.—All the observed kinetic phenomena are pure exponentials,[21] and were considered as relaxation processes.[1,2,22] The use of chemical relaxation methods[21] was justified by calculating a thermodynamic factor r which accounted for the perturbation admissible in a chemical relaxation experiment.*

The fast stopped-flow relaxation. The fast relaxation of Figure 1 is ascribed to the structural transformation of cation A^+ into its yellow form J^-. This process displays a single relaxation amplitude in the 280—400 nm range, even for a final $[OH^-]$ of *ca.* 1M. This single observed process is too slow to be ascribed to an acid–base reaction in basic media, even if it is not diffusion-controlled.[24,25] The amplitude of this process always corresponds to a variation in absorbance between that of A^+ (initial species) and that of J^- (intermediate species) (Figure 1). Acid–base reaction (7), the deprotonation of A^+ into the resonance-stabilized carbene species A_{-H}, even if not diffusion-controlled, is too fast to be ascribed to the stopped-flow observed phenomenon.[12,24] The high $[OH^-]$ of some of our experiments implies that, if the formation of a resonance-stabilized carbene [reaction (7)] is to occur during the $A^+ \rightleftharpoons J^-$ transformation, it would have $pK > 15$. Since reaction (7) is faster than the observed kinetic process of Figure 1, when the pH jump, from neutral to around the pK value of reaction (7), is performed, A_{-H} would start accumulating as a kinetic product. This should be revealed by a pre-exponential phenomenon observable by the stopped-flow technique. However, we only observe a single kinetic process for the $A^+ \rightleftharpoons J^-$ transformation. To observe the amplitude of deprotonation of A^+, if the pK of reaction (6) is >15, the final $[OH^-]$ (after the pH jump) should be *ca.* 10M. Nevertheless, we cannot, on this basis only, preclude the involvement of reaction (7) in the structural transformation of A^+ into J^-.

Involvement of A_{-H}? The difference between pK_3 and pK_4,[2] together with the concentrations of OH^- used in our experiments,† show that the rate-limiting step in the $A^+ \rightleftharpoons J^-$ transformation is the formation of σ-adduct D.[5]

By taking into account the deprotonation of A^+ into A_{-H} [reaction (7)] during the formation of σ-adduct D, the rate-limiting-step of the $A^+ \rightleftharpoons J^-$ transformation, according to Hopmann *et al.*, is the transformation of A_{-H} into D [reaction (8)].[5]

$$A^+ + OH^- \rightleftharpoons A_{-H} \qquad (7)$$

$$A_{-H} \underset{k_{-1}}{\overset{k''_1}{\rightleftharpoons}} D \quad \text{(rate-limiting)} \qquad (8)$$

$$K_b = [A^+][OH^-]/[A_{-H}]$$

Since reaction (8) is slower than (7) and (6) and since J^- is the semi-thermodynamic product, reactions (7) and (6) are in a constant equilibrated state during reaction (8). Substitution

* Brouillard *et al.*[23] introduced a thermodynamic factor r which accounted for the amount of a perturbation and which, in a chemical relaxation experiment, should be <0.1. In the case of the transformation of A^+ into J^- A^+ is placed, by the stopped-flow technique, in a basic medium where it will yield J^-. In our experimental conditions ($[OH^-] \gg [A^+]$, $[D]$, and $[J^-]$), $r = (2K_{4b} + [OH^-])[OH^-]\Delta[A^+]/([OH^-]^2 + K_b)(K_{4b} + [OH^-])$ and can never exceed 0.06 for a perturbation amount equal to the analytical concentration of thiamine in the medium. Thus, the methods of chemical relaxation can be very safely applied to the $A^+ \rightleftharpoons J^-$ structural transformation in basic media.

† It should be noted that if the $D^- \rightleftharpoons J^-$ transformation is considered as rate limiting (the formation of D cannot be detected above 320 nm[2]), the equation for reciprocal relaxation time 4 is $\tau_4^{-1} = k_{45}[OH^-]^2/K_{3b} + [OH^-]) + k_{54}$. A linear least-squares regression of the data against this equation gave k_{45} 94 l mol^{-1} s^{-1} and k_{45} 1.5 s^{-1}, which is practically impossible, since in that case $k_{54}/k_{45} = K_{4b} = 1.6 \times 10^{-2}$ mol l^{-1}. This means that D, not J^-, would be the semi-thermodynamic product.

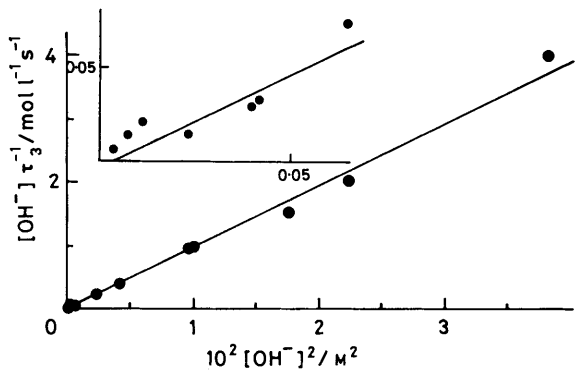


Figure 3. Plot of $[OH]^{-}\tau_3^{-1} = k_{14}[OH^-]^2 + k_{41}K_{4b}$; intercept ($-1.5$—$3.0$) $\times 10^{-2}$ s^{-1}, slope (99 ± 7) l mol^{-1} s^{-1}, r 0.9982 for 15 experimental points

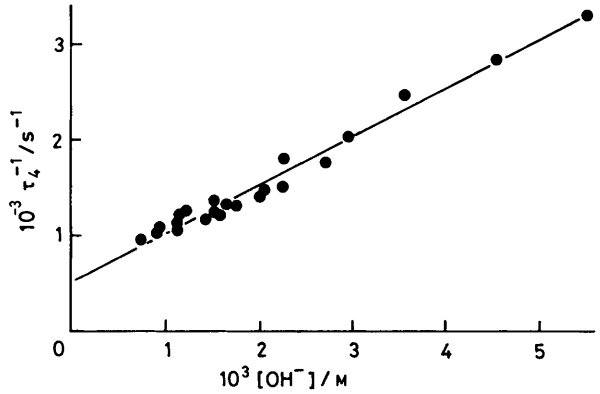


Figure 4. Plot of $\tau_4^{-1} = k_{45}[OH^-] + k_{54}$; intercept ($5.25 \pm 0.40$) $\times 10^3$ s^{-1}, slope (5.15 ± 0.65) $\times 10^6$ l mol^{-1} s^{-1}, r 0.9908 for 22 experimental points

methods can be used to derive the reciprocal relaxation equation of rate-limiting reaction (8), the kinetic equation of which is expressed as (9).

$$-d[J^-]/dt = -k''_1[A_{-H}] + k''_{-1}[D] \quad (9)$$

The conservation of matter and charge after the formation of J^- and before its evolution into C^- implies that equations (10) and (11) hold.

$$\Delta[A^+] + \Delta[A_{-H}] + \Delta[D] + \Delta[J^-] = 0 \quad (10)$$

$$\Delta[A^+] = \Delta[J^-] + \Delta[OH^-] \quad (11)$$

* The possibility of buffer catalysis of the $J^- \rightleftharpoons D$ transformations in basic media is considered here [reaction (i)]

$$J^- + HPO_4^{2-} \rightleftharpoons D + PO_4^{3-} \quad (i)$$

In our experimental conditions (PO_4^{3-}, $HPO_4^{2-} \gg D$, J^-), the reciprocal relaxation equation of such a reaction is expressed as (ii)

$$\tau^{-1} = k[H^+]c'/([H^+] + K) + k_{-1}Kc'/([H^+] + K) \quad (ii)$$

where c' is the analytical concentration of the buffer in the media and $K = [H^+][PO_4^{3-}]/[HPO_4^{2-}]$. This equation is not fitted by our experimental data.

The differentiation of equilibria (7) and (6) implies that relations (12) and (13) hold.

$$\Delta[A_{-H}] = [A^+]\Delta[OH^-]/K_b + [OH^-]\Delta[A^+]/K_b \quad (12)$$

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

From equations (10)—(13) one can derive the relaxation time equation (14) for reaction (8). The experimental data do not fit

$$\tau^{-1} = k''_1[OH^-]/(K_b + [OH^-]) + k''_{-1}K_b/[OH^-] \quad (14)$$

this equation for $K_b < 10$ mol l^{-1} ($pK < 15$). Thus, although we would agree with Hopmann *et al.* that the formation of D is rate-limiting,[5] we rule out the involvement of reaction (7) (the formation of A_{-H}) with a $pK < 15$ in the transformation of cation A^+ into yellow form J^-.

The $A^+ \rightleftharpoons D$ *transformation. Reciprocal relaxation time.* Assuming that reaction (4) is the rate-limiting step in the transformation of A^+ into J^-, as expressed by kinetic equation (15), substitution methods (21) can be used to derive the reciprocal relaxation time equation for reaction (4).

$$-d[A^+]/dt = k_{14}[A^+][OH^-] - k_{41}[D] \quad (15)$$

At the partial A^+ to J^- equilibrium (just after the fast formation of J^- and before its evolution into C^- [2]), the solution contains practically nothing but A^+, D, and J^-. The conservation of matter and charge then implies that equations (16) and (17) hold.

$$\Delta[A^+] + \Delta[D] + \Delta[J^-] = 0 \quad (16)$$

$$\Delta[J^-] + \Delta[OH^-] = \Delta[A^+] \quad (17)$$

Since reaction (6) is in a constant equilibrated state during reaction (4), we have equation (18).

$$\Delta[J^-] = [D]\Delta[OH^-]/K_{4b} + [OH^-]\Delta[D]/K_{4b} \quad (18)$$

The equation for the reciprocal relaxation time is deduced from equations (15)—(18). It can be written as (19).

$$[OH^-]\tau_3^{-1} = k_{14}[OH^-]^2 + k_{41}K_{4b} \quad (19)$$

A linear least-squares regression of the data against equation (19) (Figure 3) gave: $k_{14} = (9.9 \pm 0.7) \times 10$ l mol^{-1} s^{-1}, a value compatible with that given in the literature.[4,5] The intercept should be equal to $k_{14}K_{4b} \sim 1 \times 10^{-3}$ mol l^{-1} s^{-1} [since $K_b = 1 \times 10^{-5}$ mol^2 l^{-2} (ref. 2)]; this value is too small to be measured. Thus, the rate-limiting step of the structural transformation of A^+ into J^- is the formation of σ-adduct D but with no noticeable involvement of the direct deprotonation of A^+ into A_{-H} [reaction (7)].

The fast temperature-jump relaxation. When the pH of the neutral thiamine solution rises to *ca.* 11, A^+ is partially and rapidly transformed into J^-,[2] which slowly produces C^-.[2] At 2 °C and near pH 11, the $C^- \rightleftharpoons J^-$ transformation [2] is slow enough to enable us to perform a temperature-jump on a solution which contains A^+, D, and J^- before C^- starts to reach a significant concentration in the medium. The relaxation of Figure 2 is too fast to be attributed to the $A^+ \rightleftharpoons J^-$ transformation seen earlier (Figure 1). We ascribe it then to reaction (6) in buffered media [reaction (20)].

$$HPO_4^{2-} + OH \rightleftharpoons PO_4^{3-} \quad (20)$$

The reciprocal relaxation time equation associated with reaction (6) is expressed as (2).[21,*]

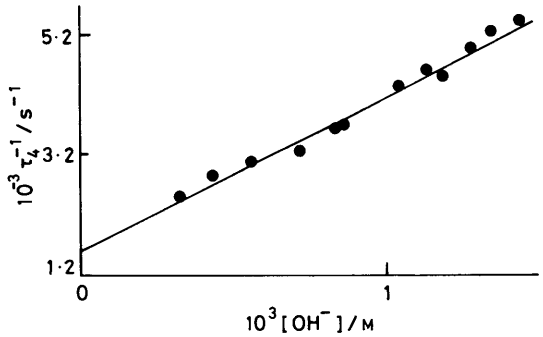


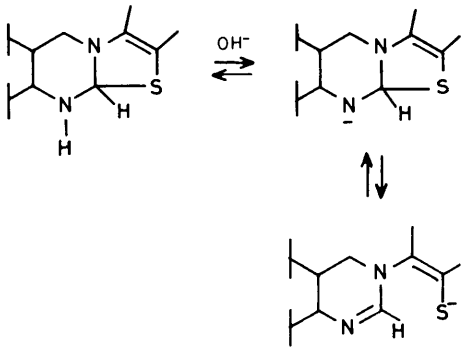
Figure 5. Plot of $\tau_4^{-1} = k_{45}[OD^-] + k_{54}$ in heavy water; intercept (1.7 ± 0.3) × 10^3 s^{-1}, slope (2.5 ± 0.25) × 10^6 l mol^{-1} s^{-1}, r 0.9908 for 12 experimental points

$$\tau_4^{-1} = k_{45}[OH^-] + k_{54} \quad (21)$$

A linear least-squares regression of the data against equation (21) gave (at 6.5 °C and 0.5M ionic strength) (Figure 4): k_{45} (5.15 ± 0.25) × 10^6 l mol^{-1} s^{-1}, k_{54} (5.25 ± 0.3) × 10^3 s^{-1}, and $K_{4b} = k_{54}/k_{45}$ = (1.05 ± 0.15) × 10^{-3} mol l^{-1}. The K_{4b} value measured at 6.5 °C is in good agreement with that reported previously at 25 °C.[2] The transformation of D into J^- is fast probably because it involves a proton transfer.

The fast $D^- \rightleftharpoons J^-$ *transformation. Kinetic isotope effect.* The deuterium isotope effect measured in a J^- solution in D_2O would provide interesting information about the nature of the proton transfer[26] involved in the $D \rightleftharpoons J^-$ step.

If the $D \rightleftharpoons J^-$ transformation occurs according to the expected mechanism involving deprotonation of the secondary amine and intramolecular reaction of ring-opening of the thiazolium of the resulting anion[27] (Scheme 2), the



$$D + OH^- \rightleftharpoons \text{(very fast)} \quad (22)$$

$$D^- \underset{k''_{54}}{\overset{k''_{45}}{\rightleftharpoons}} J^- \quad \text{(fast)} \quad (23)$$

Scheme 2.

system will be revealed by two relaxation modes.[21] In this work the signals are detected above 290 nm (because of the high thiamine concentration used in these experiments in order to achieve the needed concentrations of J^- to perform kinetic runs). At these wavelengths D does not have an absorbance spectrum;[2] the observed signal (Figure 2) will then correspond to the $D \rightleftharpoons J^-$ ring chain tautomerism which is slower than acid–base reaction (22).[18,21,23,24] The reciprocal relaxation time equation associated with the rate-limiting transformation of D^- into J^- is expressed as (24) with $[OH^-] \gg [D]$.[20b]

$$\tau'^{-1} = k''_{45}(k'_{45}[OH^-] + [D])/(k'_{45}[OH^-] + k'_{54}) + k''_{54} \quad (24)$$

To make this equation similar to equation (21), the rate equation associated with the transformation of D into J^- (Figure 4), $k''_{45}[OH^-]$ should be neglected when compared with k'_{54}.* In this case, equation (24) is expressed as (25) where

$$\tau'^{-1} = k''_{45}[OH^-]/K'_{4b} + k''_{54} \quad (25)$$

$K'_{4b} = [D][OH^-]/[\mathbf{D}] = k'_{54}/k'_{45}$ and $k''_{54}/k'_{4b}k''_{45} = K_{4b}$.

The value of k_{45} is then equal to k''_{45}/K'_{4b}. The ratio $k_{45(H_2O)}/k_{45(D_2O)}$ (k_{45} measured in water and in heavy water respectively) would be equal to $(k''_{45}/K'_{4b(H_2O)})/(k''_{45}/K'_{4b(D_2O)})$. If we assume that the solvation of OH^- is much greater than that of D, D^-, and J^- and that reaction (23) is intramolecular, we can consider that the major contribution to the solvent isotope effect would be provided by the reaction involving the proton transfer[26] [reaction (22)]. We can, therefore, write that $K'_{4b(H_2O)}/K'_{4b(D_2O)} \sim K_{4b(H_2O)}/K_{4b(D_2O)}$. In this case, the kinetic isotope effect on k''_{45} is expressed as: $k''_{45(H_2O)}/k''_{45(D_2O)} \sim k_{45}K_{4b(H_2O)}/k_{45}K_{4b(D_2O)}$.

If the transformation of D into J^- occurs according to Scheme 2, the kinetic isotope effect on intramolecular reaction (23) should not differ much from unity. The temperature-jump measurements performed in heavy water gave (Figure 5) $k_{45(D_2O)}$ (2.50 ± 0.25) × 10^6 l mol^{-1} s^{-1}, $k_{54(D_2O)}$ (1.7 ± 0.18) × 10^{-3} s^{-1}. Hence, $k_{45(H_2O)}/k_{45(D_2O)}$ 2.05 ± 0.10 and ΔpK_4 0.75 ± 0.05 (ΔpK_4 is the difference between pK_4 measured in heavy water and pK_4 measured in water). Thus the kinetic isotope effect $k''_{45(H_2O)}/k''_{54(D_2O)}$ is 3.4 ± 0.4 which is much higher than unity. This imples that Scheme 2 does not totally describe the facts and that reaction (24) may also involve a proton transfer. Moreover, the ratio $k_{45(H_2O)}/k_{45(D_2O)}$ 2.05 and ΔpK_4 0.75 are much too high when compared with isotope effects in other base-catalysed reactions.[26] This also indicates that there are probably more than one proton transfers involved in reaction (6).† However, so far we cannot on the basis of these indirect investigations exclude Scheme 2.

Table 2. Rate constants involved in the mechanism of the structural transformations of thiamine in neutral and basic media

Reactions	Second-order rate constant (l mol^{-1} s^{-1})	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}
$B + OH^- \rightleftharpoons C^-$	6.75 × 10^4	8.85 × 10^{-2}
$D + H^+ \rightleftharpoons A^+$	4.60 × 10^5	~3 × 10^{-2}
$A^+ + OH^- \rightleftharpoons D$	99	~1.5
$D + OH^- \rightleftharpoons J^-$	5.15 × 10^6	5.25 × 10^3

Discussion

The kinetic and thermodynamic data measured in this work and in our previous articles[1,2] are reported in Table 2. The value of

* Around pH 11, estimating k'_{45} at 10^7—10^{10} l mol^{-1} s^{-1}, k'_{54} should at least be 10^7—10^9 s^{-1}. This would give a deprotonation pK of D > 12. Hence, the value of $[J^-]/[D^-]$ would be >10. This indicates that, if a proton transfer is involved in the $D \rightleftharpoons J^-$ transformation, it will not globally affect the reciprocal relaxation time [equation (21)].

† The expected isotope effect on pK can be empirically estimated by Bell's relation $\Delta pK = 0.41 + 0.020\ pK$.[26] This gives a ΔpK_4 value of 0.60, while the measured ΔpK_4 is 0.75, because the ring-opening of D is a prototropic tautomerism. In this case, the ΔpK_4 measured here will include the contribution of isotope effects from both reactions (20) and (21) (see ref. 28).

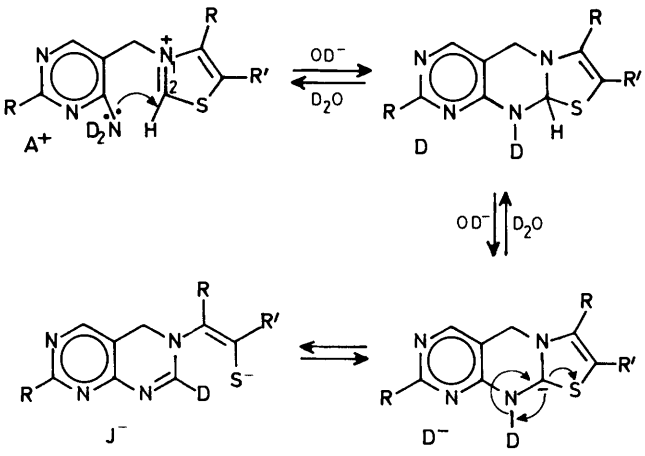
k_{14} = (115 l mol^{-1} s^{-1}) reported by Hopmann *et al.*[4,5] for the transformation of A^+ into adduct D is confirmed and corresponds to the elementary rate constant measured here by chemical relaxation.

The $A^+ \rightleftharpoons J^-$ *Transformation and* 2-H *Lability.*—For the structural transformation of A^+ into J^- on spectroscopic and kinetic bases, we preclude the involvement of the deprotonation of A^+ into A_{-H} in the formation of adduct D unless the deprotonation pK of A^+ is >15. Like certain authors[14] but unlike Hopmann *et al.*,[4,5] we have shown here that reaction (7) can occur only with pK > 15. Should it indeed occur, the nucleophilic addition site, the 2-position, would be deactivated. Although resonance-stabilized carbene A_{-H} can display dual electrophilic–nucleophilic nature,[15] the nucleophilic aspect seems predominant in A_{-H}. Indeed, nucleophile A_{-H} supposedly attacks the carbon atom of the carbonyl group (bearing a partial positive charge) of pyruvic acid.[9] We do not quite understand why, in basic media, as proposed recently,[4,5] nucleophile NH_2 would attack the nucleophilic entity A_{-H} and convert it into the intramolecular σ-adduct D. Therefore, the deuteriation of the J^- 2-position, reported by Hopmann *et al.*, does not occur during the formation of D. It can take place either during the transformation of D into J^- or before the structural transformation of A^+ into D as shown in Scheme 1.

In the vicinity of neutrality, J^- is very rapidly transformed into σ-adduct D, and D in turn yields A^+ by acid-promoted ring-opening with a second-order rate constant k_{61} 4.6 × 10^5 l mol^{-1} s^{-1}) [reaction (5), Table 2].[2] This value is very close to the deuteriation rate constant of thiamine (7.5 × 10^5 l mol^{-1} s^{-1}) estimated by Kemp and O'Brien at 30 °C.[29] Both constants are compatible with a ring-opening reaction[27] which can be diffusion-controlled,[30] especially when it involves a proton transfer.[27]

If 2-H exchange takes place *via* the very fast $J^- \rightleftharpoons D$ transformation [reaction (6)], (which occurs with a second-order rate constant k_{45} 5.15 × 10^6 l mol^{-1} s^{-1}), it would be controlled in neutral and mildly basic media by the slow formation of A^+ from D [reaction (5)] the second-order rate constant of which was measured by slow mixing techniques.[2] In n.m.r. proton exchange experiments, the typical concentration of thiamine would, for technical reasons, be *ca.* 0.1—1M. Under these conditions, the concentration of D in the vicinity of neutrality would be *ca.* 10^{-5}—10^{-6}M (Figure 5), a value much higher than [H^+], and the reciprocal relaxation equation of σ-adduct D formation [reaction (5)] is expressed as[21] $\tau_3^{-1} = k_{61}[D] + k_{16}$. This equation is not suited to acidic media, where thiamine protonates at the 1′-position with pK 5.22.[1] For a typical analytical concentration of thiamine ranging from 0.1—1M, $t_{1/2}$ = 0.69 τ would vary at pH 7 from 0.2 to 2 s and at pH 8 from 0.02 to 0.2 s. This compares well with the (n.m.r.-measured) half-lifetimes of 2-H of thiamine and thiamine pyrophosphate[31] at 28 °C (4 s at pH 7 and 0.2 s at pH 8). It also agrees with the rate value measured by Kemp and O'Brien for 2-H lability.[29]

Does this mean that in the typical case of thiamine pyrophosphate and of thiamine, the rate of the 2-H lability in neutral media is controlled by the $A^+ \rightleftharpoons D$ equilibrium during the fast $D \rightleftharpoons J^-$ step? If so, how can we explain it knowing that the classical ring-opening of D into J^-, which involves the deprotonation of the secondary amine and the ring opening of the thiazole of the resulting anion (Scheme 2), cannot explain this 2-H exchange during the $A^+ \rightleftharpoons J^-$ step. In particular, the acidity of a singly bonded CH is usually lower than that of an amine.[26] (The pK of CH_4 is *ca.* 45, while the pK of NH_3 is *ca.* 35.[26]) However, C-2 in D is linked to three electronegative atoms, two nitrogens and one sulphur. The nitrogens of the secondary amine is linked to an aromatic group, and the other nitrogen and the sulphur are both engaged in a conjugated thiazole heterocycle. We know that the deprotonation pK of C–H linked to one or more electronegative or aromatic groups can be comparatively low.[32] Furthermore, pK decreases dramatically with the number of electronegative groups linked to the C–H.[26a] The pK is greatly decreased for an electronegative substituent R than for H, >13 pK units for $(MeSO_4)_3CH$ and *ca.* 15 units for $(CN)_3CH$. The pK values of these two R_3CH acids are negative, but these are extreme cases.[26a] Thus, C-2 could be more acidic than the secondary amine,* and it is not unwise to speculate on the deprotonation of the 2-position of D to yield an unstable carbanion



Scheme 3.

intermediate D^- † which, by ring-opening, would generate J^- (Scheme 3). Such a carbanion would explain the exchange of the thiamine 2-H with the heavy water deuterons, observed by ^{1}H n.m.r. during the $A^+ \rightleftharpoons J^-$ transformation. It would also explain the similarity between the deuteriation rate constant of C-2[29] and the rate constant of D ring-opening into A^+ in neutral and mildly basic media. Moreover, it concurs with the kinetic isotope effects which seem to indicate that more than one proton transfer is involved in the $D \rightleftharpoons J^-$ step. This C-2 deprotonation in D is based only on indirect arguments which at this stage need to be more firmly confirmed. Therefore, even if the direct deprotonation of C-2 in A^+ is the most likely, our arguments imply that in the typical case of thiamine, the competitive deprotonation of adduct D may occur with a lower pK value than C-2 deprotonation in other thiazolium salts. This assumption permits us to understand the high catalytic efficiency of thiamine in pyruvate decarboxylation, when compared with other thiazolium salts.[10]

According to the recent results of Karimian *et al.* the lability of 2-H in water supposedly occurs *via* pseudobase B (Scheme 4).[35] However, B is not involved in the $A^+ \rightleftharpoons J^-$ transformation and its concentration in the medium when J^- is formed, before the formation of C^-, is nil.[2] Furthermore, under these conditions, in basic media the rate of exchange of 2-H would obviously be controlled by the rate-limiting formation of B which occurs with a second-order rate constant of 19.2 l mol^{-1} s^{-1}.[1] This contradicts our n.m.r. analysis which shows that

* The acidities of amines are also lowered when these are linked to electronegative groups. Thus, the deprotonation pK of 2,4-dinitroaniline is *ca.* 15.[33] However, in dinitroaniline the amine is attached to a very important electron-withdrawing group which is not the case of the secondary amine in D linked to a pyrimidine and to a thiazole ring. These are not considered as particularly electron-attractive groups.

† In dialkylformamide acetals the C–H attached to the three oxygens is known to exchange its proton in deuteriated media.[34]

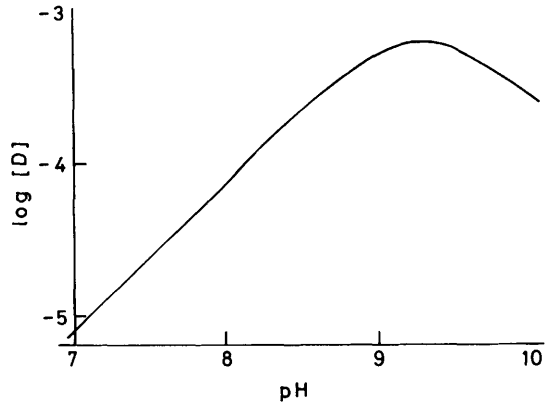
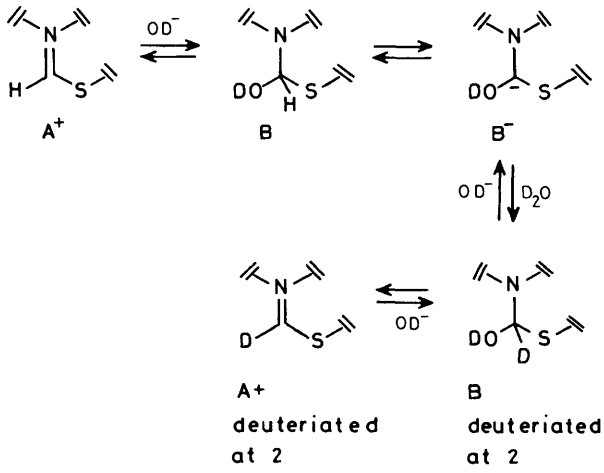


Figure 6. log[D] *versus* pH, for a thiamine concentration of 1M



Scheme 4.

the 2-H of J^- is rapidly exchanged with those of the aqueous basic medium. This proton exchange is, as observed by Haake,[36] much faster than pseudobase formation in basic media.[2] Thus, in the case of the $A^+ \rightleftharpoons J^-$ transformation, we can exclude 2-H lability through the pseudobase.

Role of the Aminopyrimidine Ring in the Catalytic Activity of Vitamin B1.—Although thiazolium salts with no aminopyrimidine group (AP) can catalyse the addition of aldehydes to activated double bonds,* 2-H exchange in these salts remains difficult[29] and probably occurs by direct deprotonation of the cation [reaction (7)]. However, in thiamine the AP is responsible for the formation of σ-adduct D in which C-2 becomes attached to three electronegative groups which, according to our proposals, favour its direct deprotonation. This AP intervention can explain the isotope effects and n.m.r. half-life measurements.[31] Furthermore, the AP, without which the biocatalytic activity of thiamine is drastically inhibited,[10,38] is responsible for the formation of J^- with pK 11.5. The pK of formation of A_{-H} is >15. Therefore, the AP favours the $A^+ \rightleftharpoons J^-$ step at the expense of $A^+ \rightleftharpoons A_{-H}$. D^- As a possible biocatalyst would explain the dependence of the biocatalytic activity of thiamine on the aminopyrimidine moiety.

* Stetter[37] used thiazolium salts to catalyse the addition of some aldehydes to activated double bonds. These experiments were performed in alcohol, dimethylformamide, and dioxane in the presence of triethylamine or sodium acetate. They necessitated 6—15 h at 60—80 °C. These conditions are very different from those of Yount and Metzler[10] and from those of natural biological media.

[D] Reaches a maximum at pH *ca.* 9.2 (Figure 6). The same is true for A_{-H}. However, since its pK of formation is >15 its concentration would never exceed $1/10^4$ [D]. Moreover, [D] closely follows the variation of the catalytic activity of thiamine in acetoin formation from pyruvic acid which reaches a maximum at pH *ca.* 9.2.[39] Thus, D^-, as an intermediate, seems a reasonable candidate in the catalytic activity of thiamine during acetoin formation.

Conclusions.—Since Breslow's important discovery of 2-H lability and his proposal for A_{-H} as biocatalyst,[7] the 2-position of thiamine has been considered the promoter of vitamin B1 activity. This activity might also stem from some nucleophilic thiamine species other than A_{-H}, including those arising from structural transformations involving the 2-position. Unlikely as it seems, there is enough circumstantial evidence consistent with the involvement of D^- in the exchange of thiamine at the 2-position to encourage further work along those lines. Albeit speculative, the proposal of the intermediate D^- as a possible biocatalyst renders many observations coherent: the role of the aminopyrimidine moiety of thiamine in this biocatalytic activity, the observed 2-H lability in the transformation of A^+ into J^-, the isotope effects associated with the D ⟶ J^- step, the similarity between the half-lives of 2-H as measured by n.m.r. in neutral and mildly basic media and those simulated for the acid-promoted ring-opening of D into A^+, and finally the similarity between the rate constant of this ring-opening and that of the deuteriation of C-2 are all thereby explained. Since this ring-opening of D is the rate-limiting step in the structural transformation of J^-, it controls 2-H lability in neutral media, namely in conditions approximating those of natural biological systems. In addition to physicochemical and mechanistic considerations, the existence of the D^- intermediate can also be approached through its potential catalytic action in pyruvate decarboxylation in the presence of the required enzymes. Therefore, it is logical to look for additional, mainly biological, arguments in support of the proposals put forth in this work. Thus far, the use of the methods and techniques of chemical relaxation has made for a real step forward in understanding the detailed mechanism of the structural transformations of thiamine in neutral and basic aqueous media. However, our work on thiamine answers some questions and generates more. Above all it calls for further investigation, which is currently in progress.

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