# 167. Fast-Atom-Bombardment Mass Spectrometry and $^1$H-NMR Spectrometry in Studies of the Transformations of Thiamine in Strongly Basic Solutions

by **Ian Horman**

*Nestlé* Products Technical Assistance Co. Ltd., CH-1800 Vevey

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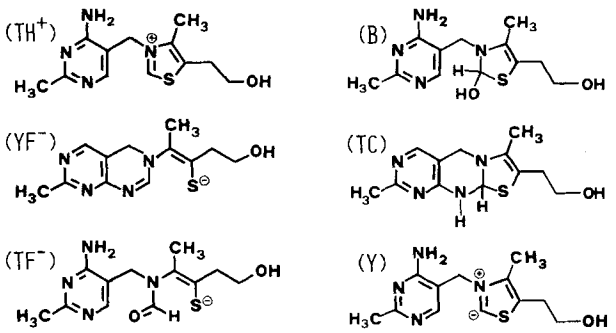
*Summary*

Fast-atom-bombardment (FAB) mass spectra have been used to establish or confirm the nature of the yellow-product and open-chain forms of thiamine produced in the presence of excess (5 mol-equiv.) of strong base. Time-dependent changes in $^1$H-NMR spectra show that the rates of disappearance of the yellow form and of formation of the open-chain form are the same at pH 12.5, having a pseudo-first-order rate constant of $k = 2.2 \times 10^{-3}$ sec$^{-1}$. The implications of these results are discussed in the light of current ideas on the acid-base dependence of thiamine structures.

**Introduction.** – After almost half a century since *Williams & Rüehle* [1] elucidated the structure of thiamine ($TH^+$), the chemistry of this compound is still a source of confusion in the literature. Early work on pH-dependent structural transformations of $TH^+$ in basic solution [2] [3] proposed that it was converted rapidly and reversibly into a yellow form ($YF^-$), and simultaneously in a slow reversible reaction *via* a tetrahedral pseudobase (B) into a stable ring-opened thiol form ($TF^-$). A tricyclic intermediate (TC) was also described [3]. Two recent studies report the kinetics of these base-induced transformations from time-dependent changes in UV/visible spectra. The first [4] concludes that in addition to the reactions outlined above, $YF^-$ is also converted directly and irreversibly into $TF^-$. The second [5] assumes that only a single intermediate exists between $TH^+$ and $TF^-$, namely B. The UV spectra reported in both studies are essentially identical, although in the kinetic analysis they are assigned to different species. Other authors have invoked B as an intermediate in the H-D exchange of the thiazolium C(2) proton in weakly acidic and neutral solutions [6] in opposition to the mainstream of the literature which ties this exchange to an ylid intermediate (Y) [7], the key intermediate in the current theory of the mode of action of thiamine in pyruvate decarboxylation [8]. However, a $pK_a$ of 12.6 is reported for the formation of Y [9], and as such, the concentration of Y in weakly acidic or neutral solution would be expected to be negligible. Further, the role of the pyrimidine $NH_2$-group in the physiological and chemical catalytic action of thiamine is not yet defined. These considerations led to the conclusion at a recent meeting devoted to the chemistry and mechanism of action of thiamine that 'the number of theories on thiamine is proportional to the ignorance' [10], and that 'it is clear from the number of theories that people propose that there are at least another 25 years of experiments to be done' [11].




To clarify this situation, new analytical methodology is called for. Thus, in this publication, restricted to the chemical transformations of $TH^+$ in strong base, fast-atom-bombardment (FAB) mass spectra are used to confirm the identities of reactant, intermediate and product species, and high-resolution $^1$H-NMR spectroscopy is employed to investigate reaction kinetics.

**Results.** – The FAB mass spectrum of thiamine chloride hydrochloride in a neutral matrix is shown in *Fig. 1 (a)*, where the peak at *m/z* 265 corresponds to the $TH^+$ ion [12]. *Fig. 1 (b)* and *(c)* are negative-ion FAB mass spectra of thiamine chloride hydrochloride dissolved in a strongly basic glycerol matrix. *Fig. 1 (b)* was run on the bright yellow solution formed immediately on mixing, and shows abundant peaks at *m/z* 263 and 147, corresponding to the parent ion and to a logical fragment ion of the structure $YF^-$. *Fig. 1 (c)* was run on the same sample 10 min later, without taking it out of the mass spectrometer, and the peak at *m/z* 281 was seen to have increased at the expense of *m/z* 263 and 147, corresponding to the transformation of $YF^-$ into $TF^-$.

300-MHz $^1$H-NMR spectra showing the evolution of a basic aqueous solution of thiamine are presented in *Fig. 2*. Signals assigned to the methyl and oxyethyl protons of $YF^-$ and $TF^-$ are seen, and only these two forms are apparently present, all signals for $TH^+$ having disappeared relative to the spectrum in neutral solution [13]. Because neither acid nor base is consumed in the transformation of $YF^-$ to $TF^-$, the pH of the solution remains constant, and it is appropriate to interpret this in terms of a pseudo-first-order unimolecular transformation. Values of rate constants determined from the changes in intensity with time of peaks in *Fig. 2* are reported in the table accompanying *Fig. 2*. It is evident that within experimental error, the rate of disappearance of $YF^-$ is the same as the rate of formation of $TF^-$.

**Discussion.** – Evidence for the structure $YF^-$ as representing the yellow form of thiamine dates back to 1940, and to elemental composition determinations carried out on the yellow sodium salt formed by treating thiamine with sodium ethoxide in absolute alcohol [2]. However, the salt was found to be hygroscopic and unstable on isolation from its anhydrous medium. It was necessary to postulate the presence of three molar equivalents of water to account for the elemental composition observed, and the result was not claimed to be absolutely certain. FAB mass spectrometry has recently been shown to be of great value in analysing naturally ionic compounds, and provided an elegant confirmation of the structure $YF^-$, as well as giving spectra for $TH^+$ and

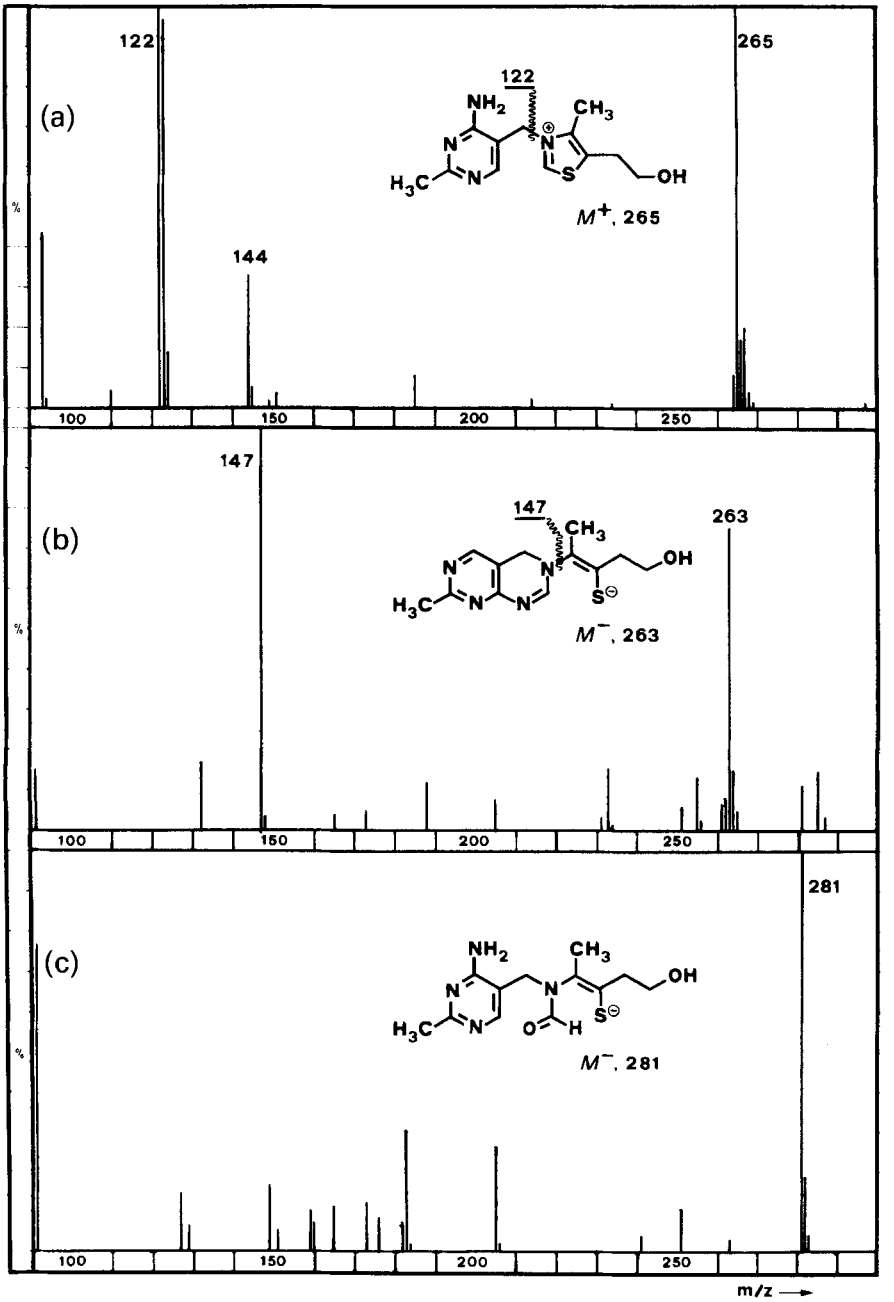
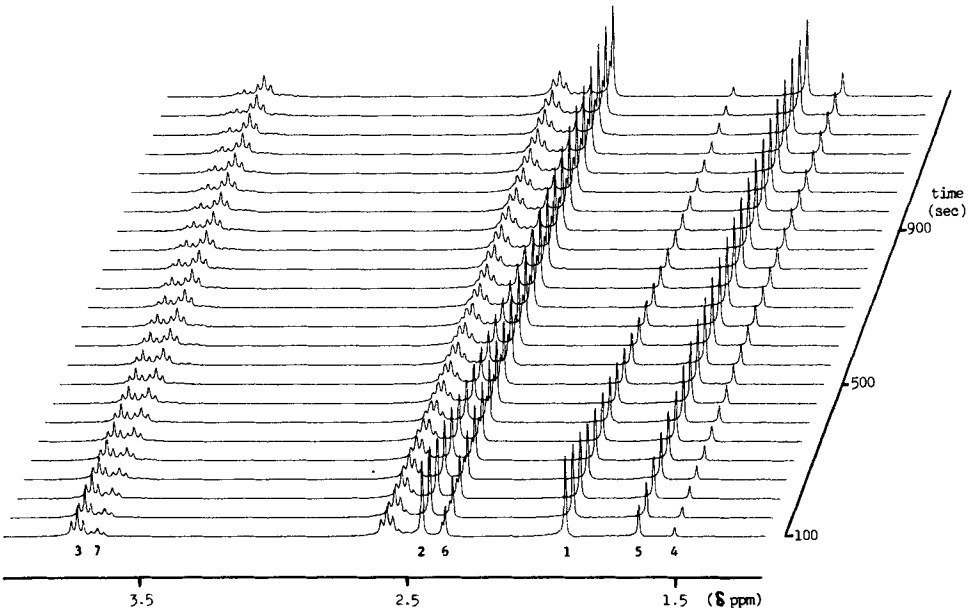



Fig. 1. *Fast atom bombardment mass spectra. a)* The positive-ion spectrum of thiamine in a neutral matrix; *b) the negative-ion* spectrum of the yellow product formed instantaneously on adding excess strong base to a neutral solution of thiamine; *c)* the negative-ion spectrum of the product formed in basic solution by decay of the yellow product. Spectra run from a glycerol matrix with 6–8 kV Xe atoms.




| Peak | Assignment | $k \times 10^3 s^{-1}$ | |
|---|---|---|---|
| 1 | $CH_3$, allyl, $YF^-$ | 2.16 | |
| 2 | $CH_3$, pyrimidine, $YF^-$ | 2.07 | $k_{av} = (2.15 \pm 0.07) \times 10^{-3}\ s^{-1}$ |
| 3 | $CH_2$-OH, $YF^-$ | 2.21 | |
| 4 | $CH_3$, allyl *(anti)*, $TF^-$ | 2.37 | |
| 5 | $CH_3$, allyl *(syn)*, $TF^-$ | 2.39 | $k_{av} = (2.22 \pm 0.14) \times 10^{-3}\ s^{-1}$ |
| 6 | $CH_3$, pyrimidine, $TF^-$ | 2.07 | |
| 7 | $CH_2$-OH, $TF^-$ | 2.07 | |

Fig. 2. *The aliphatic region of the 300-MHz $^1$H-NMR spectra recorded at 50 sec time intervals on a $D_2O$ solution of thiamine chloride hydrochloride plus five equivalents of NaOD.* Final $[OD^-] \simeq 0.04$M. Spectra were recorded at 25°. The table shows assignments of the peaks, and presents pseudo-first-order rate constants ($k$) calculated from peak intensity changes with time. Average values of $k$ along with standard deviations are shown for the decay of $YF^-$ and the formation of $TF^-$. For comparison, a constant $k = 2.33 \times 10^{-3}$ sec$^{-1}$ is obtained from kinetic measurements by UV/VIS spectroscopy at 366 nm for the same solution. (Spectra run by Dr. *R. Schimpf, Bruker*, Wissembourg, France.)

$TF^-$. In FAB-MS, the sample is introduced into the spectrometer dissolved or dispersed in a small droplet of a liquid matrix. In the present study, when thiamine chloride hydrochloride was dissolved in glycerol containing about 10% of 2N NaOH solution, the intense yellow product formed immediately as in aqueous basic solution, and the FAB mass spectrum evolved from *Fig.1 (b)* to *Fig.1 (c)* over a period of 10 minutes as the yellow product in the droplet transformed into the base stable form, namely $TF^-$. This mode of using FAB-MS in order to follow solution kinetics in real time has been applied to enzyme catalysis [14]. Thus, we establish $YF^-$ rather than B as the structure of the major intermediate in strongly basic solution, at the same time confirming $TH^+$ and $TF^-$ as the stable forms of thiamine at neutral and strongly basic

pH. $TF^-$ is also confirmed by evidence from elemental composition determinations [2], from $^1$H-NMR [15], and from ammonia-chemical-ionisation MS of its *S*-methyl derivate [16].

The earlier kinetic studies cited [4] [5] used time-dependent changes in UV/visible spectra to determine rate constants for the multiple equilibria involved in the respective mechanisms proposed. However, at any given wavelength, reactant, intermediate and product forms of thiamine all provide their contribution to the measured absorbance, and spectra of intermediate forms are always obtained by subtracting the contributions coming from estimated concentrations of the other forms. Moreover, the spectra of all forms are very similar. NMR overcomes this problem because clearly defined signals are observed for the different species. In *Fig. 2,* signals are seen for $YF^-$ and for two forms of $TF^-$ having *syn-* and *anti-*conformations of the *N*-formyl group [15]. The equal rates observed for the disappearance of $YF^-$and for the appearance of the two $TF^-$ conformers indicates that if an intermediate exists between $YF^-$ and $TF^-$ at pH 12.5, it is short-lived. This would support the theory of a direct acid-catalyzed decay of $YF^-$ to $TF^-$ [4], rather than the earlier suggested mechanism involving the multistep sequence $YF^- \rightarrow TH^+ \rightarrow B \rightarrow TF^-$ [3]. Further, since $YF^-$ is seen to be formed as a transient intermediate even in mildly basic solutions (pH 9), its contribution in any kinetic treatment cannot be ignored as in [5].

Thiamine, being a vitamin, makes an important contribution to the life process, and as such a correct understanding of its chemistry is to be desired. The results presented contribute to this understanding, and demonstrate the possibilities offered by new instrumental methodology in a field where analyses are predominantly carried out using more classical techniques. We are extending this study to other pH values, particularly in the mildly basic region which is of more direct interest from a physiological viewpoint. Preliminary observations show that several intermediates are present, possibly corresponding to structures such as B, Y and TC. A complete analysis will however require measurements at several pH values in the range 8.5 to 11, since the rates of interconversion of the different species vary markedly with pH in this range.

FAB-MS has one major limitation in this type of investigation. It is difficult to control conditions of temperature, pH and sample concentration within the droplet of matrix on the MS introduction probe. Further, water alone cannot be used as the matrix, and as such, kinetic measurements are at best semiquantitative. This difficulty can be overcome by introducing an aqueous solution of thiamine at a specific pH by aspiration through a thermospray introduction system, such as is more commonly used as a liquid chromatography-mass spectrometry interface, and we are currently exploring this possibility to obtain precise quantitative kinetic data on triansient intermediates by mass spectrometric monitoring methods.

**Experimental.** – *General.* Thiamine chloride hydrochloride (*Merck,* Darmstadt, BRD) was crystallized from distilled water, washed with EtOH, and dried under vacuum, m.p. 248–249° ([17]: 248–250°). $D_2O$ and NaOD (*Stohler Isotopes,* Innerberg, CH) were 99.8% deuterated and were used without further purification.

*Mass Spectrometry.* FAB-MS were recorded on a *Kratos MS 50* instrument equipped with a commercial *Kratos* FAB source. Conditions of recording were as described in [12], except that 6–8 kV Xe atoms were used as the bombarding particles. Glycerol, or glycerol plus 10% 2M NaOH, was used as the sample matrix.

*NMR Spectrometry.* $^1$H-NMR spectra were recorded on a *Bruker AM-300* instrument at 300 MHz and at 25°. $D_2O$ to which NaOD was added to adjust the pH as desired was used as solvent.

*Kinetic Measurements.* Typically, 3.37 mg (0.01 mmol) of thiamine chloride hydrochloride were weighed into a 5-mm (OD) NMR tube, and 0.5 ml of a 0.1M $D_2O$ solution of NaOD (0.05 mmol) were added at r.t. After mixing, the sample was introduced into the NMR spectrometer, the field optimised, and data aquisition begun some 40 to 50 sec after the time of mixing. Spectra were recorded without lock, using a 5 μs (45°) pulse width and an aquisition time of 2.6 sec, as the average of four transients. Time-dependent changes in spectra were followed by recording successive groups of four transients at regular time intervals. Pseudo-first-order rate constants were calculated from changes in signal intensities with time, using a first-order kinetics program which is part of the standard software of the NMR instrument, and is more usually employed to calculate nuclear relaxation times. Measurements of pH were made directly in the $D_2O$ solutions using a standard pH electrode, and are uncorrected.

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