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Kinetics and Mechanism of the Hydrolysis of Thiaminepyrophosphate (Cocarboxylase)

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The kinetics of the non-enzymic spontaneous hydrolysis of thiaminepyrophosphate (TPP) in aqueous solution is reported at 56, 64 and 78° over a broad pH range of 3.0–9.0 and a constant ionic strength of 0.1 M (KNO_3). The pH rate profiles were analysed and the overall rate constant resolved into individual specific rate constants relating to various ionic species in solution. Activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ for the specific rate constants of the various ionic species of TPP are reported. The possible mechanism for the hydrolysis of TPP is discussed.

Introduction

Thiaminepyrophosphate (TPP) which is the coenzyme form of vitamin B_1, is an important biochemical entity involved in oxidation and decarboxylation reactions. Study of the kinetics and mechanism of the hydrolysis of TPP is a subject matter of much fundamental interest in biochemistry, since it gives an insight into the more complicated phosphate transfer reactions in biochemical systems. A homogenate of isolated small intestinal mucosal cells of rat was reported to hydrolyse TPP [1]. Though there are reports in the literature on the hydrolysis of various nucleoside phosphates [2–4] and some simple organic phosphates [5, 6], no work has been done on the non-enzymic hydrolysis of TPP. In the present paper a detailed kinetic study of the spontaneous hydrolysis of TPP at 56, 64 and 78° and a broad pH range of 3.0–9.0, at a constant ionic strength of 0.1 *M* (KNO_3), is reported.

Experimental

TPP (as hydrochloride) used in this investigation was obtained from United States Biochemical Corporation, U.S.A. Fresh solid TPP was weighed out for each kinetic run to obtain a solution of 2.0×10^{-3} *M*. The ionic strength of the solution was maintained constant by using 0.1 *M* KNO_3. The pH of the solution was maintained constant at the required value by the addition of enough acid or alkali during a kinetic run. Buffers were not used to maintain a constant pH since most buffers have specific effects on hydrolysis rates [2]. The pH of the solution was checked from time to time by using a combination glass–calomel electrode. The pH meter was calibrated by standard methods [7]. The experimental set up is essentially the same as that used by Khan and Mohan [2] in maintaining the reaction mixture at a desired constant temperature. The reactants were stirred continuously with a magnetic stirrer and the kinetic runs were conducted under nitrogen atmosphere.

Samples of 2 ml of the reaction mixture were removed at definite time intervals and the reaction quenched by cooling the reaction mixture to ice temperature. In the analysis of samples for inorganic orthophosphate a procedure was adopted where the orthophosphate was precipitated with the help of calcium chloride. The precipitate of calcium phosphate was separated by centrifugation and dissolved in 60% perchloric acid [8]. The solution thus obtained was used for the determination of orthophosphate by the spectrophotometric technique [9]. First order rate constants were evaluated in terms of orthophosphate production assuming the liberation of one phosphate ion per molecule of hydrolysed TPP.

Results and Discussion

A series of runs were carried out to determine the order of the reaction with respect to TPP concentration. Different initial concentrations were used at a constant pH and the reaction carried out as described above. The results are expressed in a plot of rate *vs.* $[TPP]_0$ in Fig. 1. It is observed that the rate of hydrolysis of TPP is first order with respect to TPP concentration at pH 3.00 and 56° ($\mu = 0.1$ *M* KNO_3). This result is assumed to be valid at other pH values and temperatures.

Keeping $[TPP]_0$ constant at 2.00×10^{-3} *M*, the rate of hydrolysis of TPP was determined at 56, 64, and 78° over a broad pH range of 3.0–9.0. The ob-

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TABLE I. First Order Rate Constants of the Hydrolysis of TPP.

pH	56 °C		64 °C		78 °C	
	k_{obs} $s^{-1} \times 10^7$	k_{calc} $s^{-1} \times 10^7$	k_{obs} $s^{-1} \times 10^6$	k_{calc} $s^{-1} \times 10^6$	k_{obs} $s^{-1} \times 10^6$	k_{calc} $s^{-1} \times 10^6$
3.00	1.93	1.93	0.53	0.53	1.22	1.22
4.00	1.56	1.83	0.30	0.50	0.75	1.08
5.00	1.00	1.25	0.27	0.33	0.60	0.66
6.00	0.70	0.70	0.18	0.26	0.52	0.52
7.00	6.53	6.91	1.28	1.93	3.58	5.00
8.00	8.33	8.73	2.50	2.53	5.12	6.33
9.00	9.00	9.00	2.60	2.60	6.53	6.53

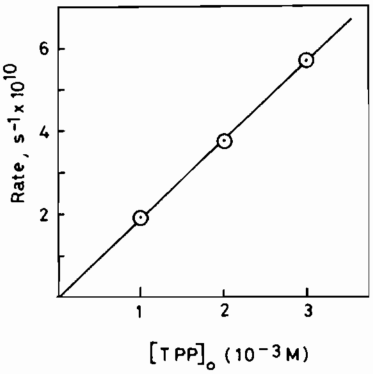


Fig 1. Determination of the order with respect to TPP concentration; pH = 3.00; t = 56 °C.

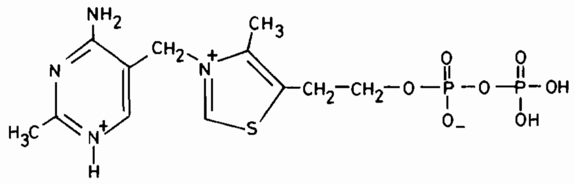


Fig. 2. TPP (fully protonated form).

served and calculated rate constants are presented in Table I. The same trend in pH dependency was observed at all temperatures studied. The rate decreases slowly with an increase in pH up to pH 6 and then increases comparatively rapidly with increasing pH. In the pH range investigated, TPP exists in the form of four ionic species H_3L^+, H_2L, HL^-, L^{2-} respectively. The fully protonated form of TPP may be represented as H_3L^+ (Fig. 2) and the net charge is balanced by a Cl^- ion [10, 11]. In the elucidation of the pH dependency of the overall rate of TPP, specific rate constants were assigned to the various ionic species of TPP according to the equation:

$$k_{obs} = k_0M_0 + k_1M_1 + k_2M_2 + k_3M_3 \qquad (1)$$

where k_0, k_1, k_2, k_3 are the specific rate constants and M_0, M_1, M_2, M_3 are the mol fractions of the species H_3L^+, H_2L, HL^- and L^{2-} respectively. The values of M_0, M_1, M_2, M_3 as a function of pH were calculated by using the dissociation constants obtained from the data reported earlier [12] (pK_a = 1.53; pK_{2a} = 4.81; pK_{3a} = 6.53). In the experimental pH range the concentration of H_3L^+ is only about 3% and hence the term k_0M_0 may be neglected. Equation (1) may then be rewritten as:

$$k_{obs} = k_1M_1 + k_2M_2 + k_3M_3 \qquad (2)$$

TABLE II Mol Fractions of Various Ionic Species; t = 56 °C.

pH	M_0	M_1	M_2	M_3
3.00	0.032	0.953	0.015	–
4.00	0.003	0.863	0.134	–
5.00	–	0.385	0.597	0.018
6.00	–	0.047	0.736	0.217
7.00	–	0.002	0.253	0.745
8.00	–	–	0.033	0.967
9.00	–	–	0.003	0.997

TABLE III Specific First Order Rate Constants for the Hydrolysis of Various Species of TPP.

Temp	k_1 $s^{-1} \times 10^7$	k_2 $s^{-1} \times 10^7$	k_3 $s^{-1} \times 10^6$
56 °C	2.03	0.82	0.90
64 °C	5.57	2.25	2.60
78 °C	12.20	6.32	6.57

The values of k_1, k_2, k_3 were determined by analysing the pH-rate profile and the mol fractions calculated as mentioned above. The mol fractions of the species and their variation with pH at 56° are given in Table II. The specific rate constants for the various ionic species at different temperatures are given in Table III. From the evaluation of specific rate constants for the ionic species of TPP and the mol fractions of different ionic species of TPP a theoretical

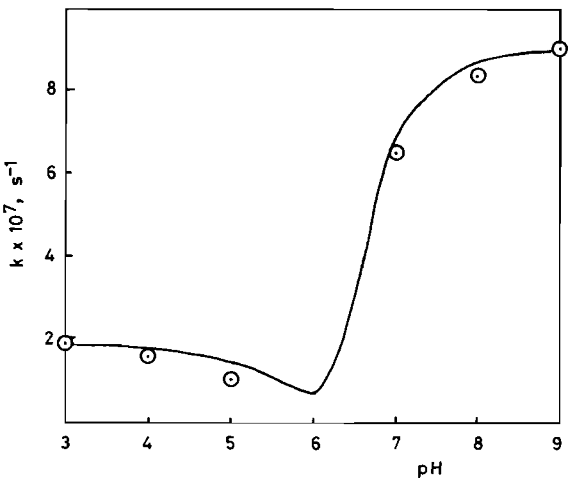


Fig. 3. pH–rate profile; t = 56 °C.

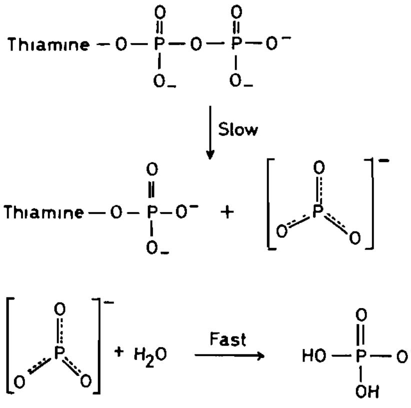
pH-rate profile was drawn. The calculated and the observed values are in good agreement (Table I and Fig. 3).

From the study of the pH dependency of the rate of hydrolysis of TPP, it is observed that the fully deprotonated form (L^{2-}) is the most active species of TPP in its hydrolysis. The activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ corresponding to the specific rate constants of different ionic species of TPP were calculated by using the usual thermodynamic relations and are presented in Table IV.

TABLE IV. Activation Parameters for the Hydrolysis of TPP.

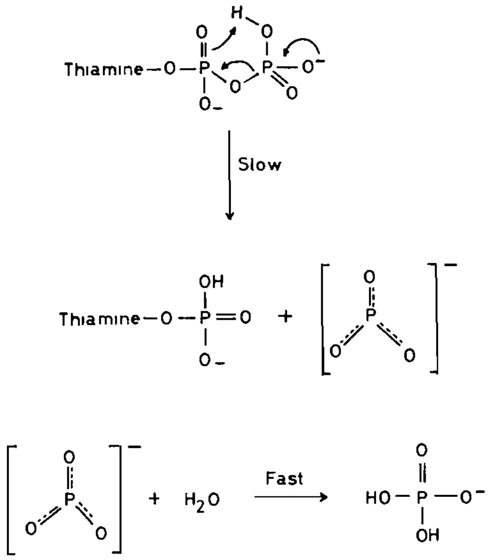
Ionic species of TPP	$\Delta H^{\ddagger}$ (Kcal/mol)	$\Delta S^{\ddagger}$ (56 °C) (e.u.)	$\Delta G^{\ddagger}$ (56 °C) (Kcal/mol)
H_2L	19.3	0.2	19.2
HL^-	21.7	5.7	19.8
L^{2-}	21.1	8.8	18.2

Based on the activation parameters a probable reaction mechanism for the hydrolysis of TPP may be proposed to take place via S_N1 route (Scheme I). The S_N1 route is proposed on the basis of a small positive entropy of activation observed in the reaction. The S_N2 bimolecular reactions are usually accompanied by large negative entropies of activation [13]. Accordingly, the reaction may be shown to proceed by the intermediate formation of a transient metaphosphate from the fully dissociated anionic species L^{2-} in a slow step, the metaphosphate then rapidly reacts with water to give the inorganic phosphate. The same pathway was proposed for the hydrolysis of acetyl phosphate dianion [14]. The recent evidence on the formation of monomeric metaphosphate in the hydrolysis of various phosphate esters was summarized by Westheimer in his review [15].



Scheme I

However, in the case of the other two ionic species of TPP, H_2L and HL^- the hydrolysis may proceed by an internal proton transfer through a cyclic six-membered ring formation, without the solvent participation as in the case of the monoanion of acetyl phosphate [14] (Scheme II). The same type of mechanism was proposed for the hydrolysis of H_2ATP^{2-} and $HATP^{3-}$ [16].



Scheme II

The cleavage of O–P bond in the two processes (Scheme I, II) involves the same energy as exhibited by more or less the same enthalpy of activation of the three ionic species (Table IV). The hydrolysis of the fully dissociated species has however the most favourable entropy when compared to the other two protonated species. The rearrangement of the proton in the rate determining step (Scheme II) costs some entropy, which is exhibited in a lower entropy of activation for the protonated species as compared to the deprotonated one. There is a marked decrease (5.5 e.u.) in the entropy of activation of the diprotonated species as compared to the monoprotonated one. The lowest entropy of activation for H_2L is expected due to the maximum rearrangement of solvation shells resulting from the rearrangement of two protons in the transition state of the species.

References

1 G. D'Andrea, G. Cusaro and V. Basilico, *Boll. Soc. Ital. Biol. Sper., 54,* 297 (1978)
2 M. M. Taqui Khan and M. Srinivas Mohan, *J. Inorg. Nucl. Chem., 36,* 709 (1974).
3 H. Sigel, D. H. Bruisson and B Prijs, *Bioinorg. Chem., 5,* 1 (1975)
4 H. Sigel and P E Amsler, *J. Am. Chem. Soc., 98,* 7390 (1976).
5 J. D. Chanley, E. M. Gindler and H. Sobotka, *J. Am. Chem. Soc., 74,* 4347 (1952).
6 Y. Murakami and A. E. Martell, *J. Am. Chem. Soc., 86,* 2119 (1964).
7 H. S. Harned and B. B. Owen, 'Physical Chemistry of Electrolytic Solutions', 3rd. Edn., Reinhold, New York (1958).
8 G. E Delory, *Biochem. J., 32,* 1161 (1938).
9 E J. King, *Biochem. J., 26,* 292 (1932).
10 J. Pletcher and M. Sax, *Science, 154,* 1331 (1966)
11 H B. Kartz and K. Kustin, *Biochim. Biophys. Acta, 313,* 235 (1973).
12 Mrs Badar Taqui Khan, P. Nageswara Rao and M. M. Taqui Khan, *Indian J. Chem., 20A,* 857 (1981)
13 L L. Schaleger and F. A. Long, 'Advances in Physical Chemistry, Vol I, p. 1, Ed. V. Gold, Academic Press, New York (1963).
14 T. C Bruice and S. J Benkovic, 'Bioorganic Mechanisms', Vol II, p. 22, W. A. Benjamin, New York (1966)
15 F. H. Westheimer, *Chem. Rev., 81,* 313 (1981).
16 M. Srinivas Mohan, *Ph. D. Thesis,* submitted to IIT Madras (1974), India