Studies on the Degradation and the Synthesis of Thiamine Phosphates. (I)

The Degradation of the Thiamine Triphosphate

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Among thiamine monophosphate (TMP), thiamine diphosphate (TDP) (cocarboxylase) and thiamine triphosphate (TTP), it is generally known that TDP has an important biochemical significance as cocarboxylase for the decarboxylation of pyruvate[1] or α-ketoglutarate[2]. It must be remarked here that lipothiamide pyrophosphate[3] has been recently supposed to play a rôle in the oxidative decarboxylation of pyruvate. Nothing definite is known at present about the physiological significance of mono- and triphosphate of thiamine. Synthesis of triphosphate is now readily available by Karrer's method and little is known about the degradation products of this unstable triphosphate[4]. This report concerns the course of spontaneous and enzymatic degradation of TTP to TDP, TMP and thiamine.

EXPERIMENTAL

Paperchromatographic estimation of TTP, TDP, TMP and thiamine. TTP was synthesized from thiamine and phosphoric acid by Karrer's method[4].

TTP, TDP, TMP and thiamine were identified by partition paperchromatography (Toyo Roshi No. 50). They were developed with 80% EtOH for 18 hours in a refrigerator and colored by Dragendorff's reagent[5]. These Rf values were 0.05 (TTP), 0.13 (TDP), 0.35 (TMP) and 0.5 (thiamine), respectively.

The amounts of inorganic phosphorus were estimated by Allen's method[6].

Preparation of takapyrophosphatase and potato-apyrase. For the separation of phosphatases by partition paperchromatography the filterpaper of Toyo Roshi No. 50 (40×40 cm.) was employed.

The purification of pyrophosphatase from Takadiastase was performed as follows. Five g. of Takadiastase (Sankyo Co.) were dissolved in 5 ml. of water and dialyzed in a cellophane sack against distilled water at pH 7.0 for 40 hours. To the dialyzed solution 0.05 ml. of $Al(OH)_3$ $C\gamma$ suspension (3% dry weight) were added, and the mixture was centrifuged. The precipitate was discarded. Then 25 ml. of $Al(OH)_3$ $C\gamma$ suspension were added further to the supernatant, and the mixture was centrifuged again. The precipitate was eluted with 50 ml. of a mixed solution of 10% $(NH_4)_2SO_4$ and 3% $NaHCO_3$ and then the eluate was centrifuged. The separated enzyme solution was adjusted to pH 5.0 with CH_3COOH under stirring. This separated eluate was saturated with solid $(NH_4)_2SO_4$ and filtered through the hardened filterpaper. The sediment was dissolved in 10 ml. of water and dialyzed overnight. This dialyzed solution was adsorbed by 10 ml.

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of $Ca_3(PO_4)_2$-gel, eluted with 30 ml. of the mixture of 10% $(NH_4)_2SO_4$ and 3% $NaHCO_3$ solution and adjusted with diluted acetic acid solution to pH 5.0 as mentioned above. The enzyme solution was saturated again with solid $(NH_4)_2SO_4$. The resultant precipitate was dissloved in 10 ml. of water and dialyzed overnight. This solution showed a stronger activity of pyrophosphatase than the original Takadiastase solution.

Potato apyrase was prepared according to Velluz et al.[8] Potato was homogenized with the same amount of water, and filtered. To the filtrate was added the twice amount of acetone, and fractionated between 0.4 and 0.7 saturation with $(NH_4)_2SO_4$. The dialyzed solution was used as an enzyme source.

RESULTS

1. Spontaneous decomposition products of TTP. TTP was very hygroscopic and unstable. When it was standed in the air at room temperature in summer (about 25°C), it absorbed the moisture of the air and decomposed spontaneously. During about 15 days standing, it remained as a clear and colorless jelly, then became brownish in 15 days thereafter. This was demonstrated to be composed chiefly of TMP. In the H_2SO_4-desiccator, it seemed to absorb the moisture slowly and to decompose and remained as a clear and colorless jelly after 3 months reservation, and after two more months, it turned to a brownish and clear substance, which consisted mostly of TMP.

TTP was also decomposed to TMP in 2-N HCl solition at room temperature in a short time. For the purpose of the comparative observation of TDP formation, a syrupy state of TTP was laid either in the H_2SO_4 desiccator, in the air or in 2-N HCl at room temperature. It was then taken out when they became a clear and colorless jelly, or after it was treated with 2-N HCl at the room temperature for 2 hours, and dissolved in a small quantity of water. An acetone-alcohol mixture (1 : 1) was added to solution and the resulted precipitate was washed with the solvent mixture several times. After inorganic phosphorus compounds were removed completely by washing, the residue was developed by partition paperchromatography in the solvent of 80% EtOH for 18 hours in the refrigerator. Each spot of TMP, TDP and TTP was cut off and the amount of total phosphorus was determined, and then the ratio of TMP, TDP and TTP was caluculated. As shown in Table 1, the decomposition products of TTP in H_2SO_4-desiccator contained TDP in a yield of 65%, and those of TTP by 2-N

Table 1. Decomposed products of TTP by standing.

	In H_2SO_4-desiccator (for 30 days)			In the air (for 15 days)			With 2-N HCl at the room temperature (for 2 hours)		
Products	TMP	TDP	TTP	TMP	TDP	TTP	TMP	TDP	TTP
Total phosphorus	13μg	66 μg	22μg	13μg	27μg	16μg	12μg	38μg	10μg
Ratio of molecules (P)	13	33	7	13	13	5	12	19	3
Yield of TTP		65%			42%			56%	

HCl contained it in a yield of 56%. The lowest yield of 42% of TDP was achieved on the decomposition of TTP in the air. From these results, it might be concluded that TDP would be prepared in a good yield by standing TTP in H_2SO_4-desiccator (Table 1).

2. Enzymatic hydrolysis of TTP by Takadiastase. As shown in Table 2, TTP was hydrolyzed completely to thiamine by Takadiastase at pH 5-6, 37°C for 4 hours (0.15 ml. of 0.001 M TTP, 0.20 ml. of 1% Takadiastase, 0.35 ml. of 0.5 M acetate buffer of pH 5). But even an excess amount of the pyrophosphatase

Table 2. Hydrolysis of TTP by Takadiastase at several pH values. (0.15 ml. of 0.001 M TTP, 0.20 ml. of 1% Takadiastase, 0.35 ml. of 0.5 M acetate buffer of pH 3-8)

pH	3	4	5	6	7	8	control	control
Buffer ml.	0.35	0.35	0.35	0.35	0.35	0.35	0.50	0.55
1% Takadiastase solution ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.0012 M TTP solution (total P are 17μg in 0.15 ml.)	0.15	0.15	0.15	0.15	0.15	0.15	0	0.15
Incubated at 37°C for 4 hours								
Amount of inorganic Pμg found.	79	82	85	84	79	76	64	4
Liberated Pμg	11	14	17	16	11	8		

The sum of Pμg in both controls was reduced.

Table 3. Hydrolysis of TTP by purified pyrophosphatase.

Buffer (pH 5.5)	0.5 ml.
Pyrophosphatase	0.8 ml.
0.0012 M TTP solution (total P are 23μg/0.2 ml.)	0.2 ml.
Incubated at 37°C for 4 hours	
Liberated inorganic P	15μg (70%)

purified from Takadiastase did not hydrolyze TTP to thiamine completely (Table 3). About 70% of total phosphorus of TTP were liberated. So it seems that TTP might be attacked by two or more sorts of phosphatases of Takadiastase.

3. Separation of Takadiastase by partition paperchromatography. The separation of phosphatases of Takadiastase was examined by partition paperchromatography.

The spots were revealed by Dragendorff's reagent.

Two lines, apart 5 cm. each from two adjacent edges of the paper, were drawn and the intersecting point was decided as the origin.

At first a dialyzed 5% Takadiastase solution was spotted at the origin, and developed with 2% NaCl solution in a refrigerator for 6-7 hours.

Then a solution of 75 mg. of TTP in a mixture of 0.75 ml. of acetate buffer

(pH 5.5) and 0.75 ml. of glycerol was applied on the developed line of enzymes. After keeping the paper in the saturated water vapour at 37°C for 1 hour, it was developed in 80% EtOH at right angles with the developed line of enzymes for 17 hours in a all-glass apparatus. The spots were revealed by Dragendorff's reagent. The spots of thiamine appeared in two places, where Rf values were 0 and 0.3-0.4, and that of TMP over the range Rf 0-0.7. The spots of TTP and perhaps TDP appeared to be observed on the whole applying line (Fig. 1).

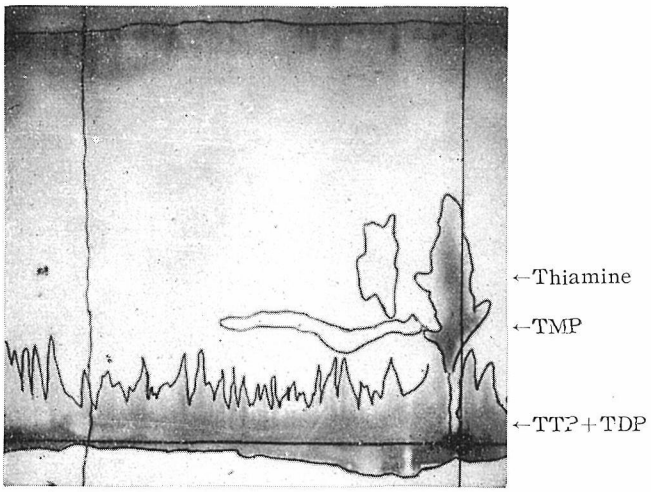

Fig. 1. Paperchromatogram of Takadiastase dialyzed.

Organic pyrophosphatase which was able to hydrolyze TDP to TMP developed in the range of Rf 0-0.7, and phosphomonoesterases which were to hydrolyze TMP to thiamine had the values of Rf 0 and 0.3-0.4.

The purified pyrophosphatase of Takadiastase hydrolyzed TTP to TMP (Rf= 0-0.7), but not to thiamine (Fig. 2). The purified urinary phosphomonoesterase[6]

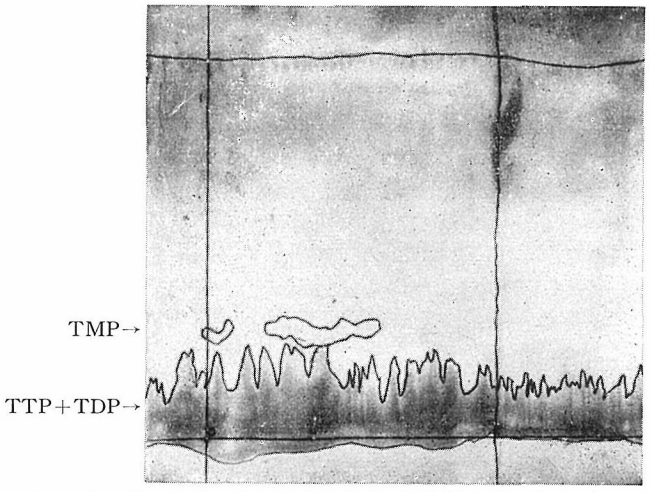

Fig. 2. Paperchromatogram of organic pyrophosphatase purified.

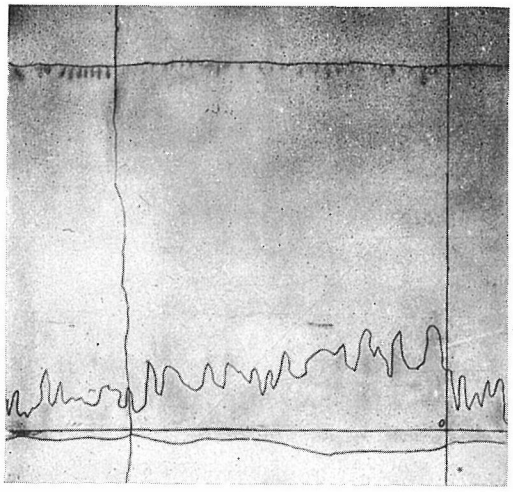

Fig. 3. Paperchromatogram of urinary phosphomonoesterase.

appeared not to contain the enzyme which could hydrolyse TTP to TMP (Fig. 3). This results show that the enzyme which hydrolyzed TTP to TMP and the enzyme which hydrolysed TTP to thiamine had the different Rf values, so it might be concluded that they were not the same. But the question whether the enzyme that hydrolyzed TTP to TDP and the enzyme that hydrolyzed TDP to TMP were the same or not, was not yet answered.

4. The effect of fluorine compounds on the enzymes. As shown in Table 4, the action of Takaphosphatase on TTP was inhibited by NaF, $NaHF_2$ and Na_2F_6Si. Potato apyrase which is able to hydrolyze TTP at pH 6.5 was also inhibited by the fluorine compounds. Even though the enzyme had been deactivated by the addition of the fluorine compounds, it was reactivated by adding excess of Mn^{++} (Table 5).

Table 4. Inhibitory effect of fluorine compounds on the hydrolysis of TTP by Takaphosphatase (pH 5.5, 37°C, for 30 min.) Incubation mixture : 0.15 ml. of 0.003 M TTP, 0.2 ml. of 1% Takaphosphatase and buffer with or without F-compound.

Final concentration in M	Liberated inorganic Pμg
Control (without F-compound)	55μg
NaF (0.05 M)	11μg
$NaHF_2$ (0.01 M)	17μg
Na_2SiF_6 (0.005 M)	48μg

Table 5. Ratio of activity of potato apyrase inhibited by NaF.

Mixture of 0.6 ml. of enzyme and 0.6 ml. of buffer	0.2 ml.	Mixture of 0.6 ml. of enzyme and 0.6 ml. of 0.1 M NaF	0.2 ml.
0.001 M TTP solution	0.1 ml.	0.001 M TTP solution	0.1 ml.
0.5 M acetate buffer of pH 6.5	0.7 ml.	The same buffer	0.7 ml.
Liberated P (37°C, 4 hrs.)	35μg	Liberated P (37°C, 4 hrs.)	20μg
Ratio of activity		100 : 57	

Influence of $MnCl_2$ on NaF inhibition

Mixture of 1.0 ml. of enzyme solution mentioned above and 1.0 ml. of buffer	0.4 ml.	Mixture of 1.0 ml. of enzyme solution contained NaF mentioned above and 1.0 ml. of 0.1 M $MnCl_2$	0.4 ml.
0.001 M TTP	0.1 ml.	0.001 M TTP	0.1 ml.
Buffer (pH 6.5)	0.5 ml.	Buffer (pH 6.5)	0.5 ml.
Liberated P (37°C, 4 hrs.)	36μg	Liberated P (37°C, 4 hrs.)	34μg

Ratio of activity 100 : 95

DISCUSSION

The amount of the degradation products of TTP differs according to the condition of procedure, which was carried out in the same manner as the decomposition of inorganic triphosphate[10], though the difference was not so great. Since the separation of the decomposition products is achieved very easily by using an ion exchange resin (IRC-50[11] or XE-64[12]), the rapid decomposition by 2-N HCl is most useful for the preparation of TDP.

Concerning the hydrolysis TTP by Takaphosphatase, three manners might be considered. Firstly, it must be supposed that three orthophosphate molecules should be liberated one after another. Secondly, one pyrophosphate molecule might be separated from TTP and then TMP left was decomposed further. Lastly, triphosphate and thiamine may be produced directly from TTP. Since TMP was demonstrated to be the intermediate, the first or the second course might be thought to be most probable.

As the inhibition of fluorine compounds on the phosphatase of Takadiastase, it might be also supposed to be due to the formation of Mg-F complex. These data resembled to those of Anagnostopoulos and Courtois[13]. In their data, renal phosphomonoesterase was inhibited by NaF. If NaF was removed by dialysis, by acetone fractionation or by ammonium sulfate fractionation, the activity of of the enzyme was recovered. The inhibiting effect of NaF on hydrolysis TTP by potato apyrase was checked by the addition of $MnCl_2$.

SUMMARY

1. TTP was spontaneously hydrolyzed to TMP. To produce TDP effectively as an intermediate, TTP should be decomposed in a H_2SO_4-desiccator.

2. TTP was hydrolyzed enzymaticaly to TMP and thiamine, which might be resulted by two or more sorts of phosphatases in Takadiastase. It was examined to separate these phosphatases by partition paperchromatography.

3. Potato apyrase which hydrolyzed TTP was inhibited by NaF. By adding an excess amount of Mn^{++}, the inhibitory effect of NaF was excluded.

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