Studies on the Enzymatic Synthesis of Thiamine by *Staphylococcus Aureus*

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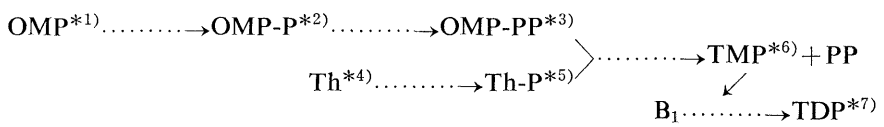
(Received November 28, 1963)

According to the recent enzymatic study of yeasts, the synthetic pathway of thiamine is as follows[1), 2), 3)].



Some species of bacteria are also known to synthesize the vitamin inside the cells and Shimomura[4)] reported that washed cells of *Staphylococcus aureus* coupled OMP and Th to synthesize thiamine.

This report will describe the studies on the mechanism of the synthesis by acetone-dried cells and a cell-free extract obtained from a strain of *Staphylococcus aureus*.

METHODS

1. Acetone-dried cells.

Staphylococcus aureus FDA 209-P grown for 18 hours at 37°C on a nutrient agar composed of 1% Polypepton (Takeda), 1% beef extract and 2% agar was harvested, washed with saline three times, and dried by a treatment of acetone and ether under cooling. The acetone-dried powder thus obtained was stored at 4°C in a vacuum desiccator until was used.

2. Crude enzyme preparation.

The suspension of the acetone-dried powder in 0.04 *M* Tris buffer was submitted to the action of sonic vibrations at a frequency of 10 KC per second for 30

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* 1) 2-Methyl-4-amino-5-hydroxyl-methyl pyrimidine (Oxymethyl pyrimidine)
* 2) Oxymethyl pyrimidine monophosphate
* 3) Oxymethyl pyrimdine diphosphate
* 4) 4-Methyl-5-β-hydroxyethyl-thiazole (Thiazole)
* 5) Thiazole monophosphate
* 6) Thiamine monophosphate
* 7) Thiamine diphosphate

minutes and then was centrifuged at 10,000 rpm for 15 minutes. Resulting faintly yellow transparent supernatant fluid was used as a crude enzyme preparation.

3. Protein determination of enzyme preparations and specific activity of the enzyme.

The protein content of the enzyme was estimated by the biuret method[5]. The spceific activity was defined as mμM of thiamine synthesized per 1 mg of the protein per 3 hours from 0.5μM of OMP-PP and 0.5μM of Th-P (or Th or Th-PP).

4. Enzymatic synthesis of thiamine.

Unless otherwise stated, the reaction system contained 2 ml of Sörensen's phosphate or 0.2 *M* Tris buffer, pH 7.0, each 0.5 ml of pyrimidine and thiazole, and 1 ml of an enzyme preparation. The total volume was 5.0 ml and the mixture was incubated at 37°C for 3 hours.

After the incubation, the reaction was stopped by the addition of 1 ml of 1 *N* HCl, followed by the heating at 80°C for 15 minutes. The solution was then adjusted to pH 4.5 with 1 *N* NaOH and centrifuged at 10,000 rpm for 15 minutes. Thiamine content of the resulting clear supernatant fluid was measured by the thiochrome method.

For the measurement of total thiamine, 1 ml of 6% Takadiastase solution, pH 4.5, which had been treated by "Supper Hyfro Cells" to remove thiamine included, was added to 4 ml of the supernatant fluid and incubated for 2 hours at 45°C. Free thiamine was measure by the method of Matsui and Fujiwara[6], whereby the Yagi fluorometer was used for the thiochrome fluorometry.

5. Preparation of OMP-monophosphate, OMP-diphosphate, Th-monophosphate and Th-diphosphate.

OMP-P, OMP-PP and Th-PP were prepared by the method of Nose and others[3] with slight modification as follows:

5 g of P_2O_5 and 0.7 ml of water was mixed in an ice bath and immerced in a 150°C oil bath for 1.5 hours. After allowing to cool to 100°C, 500 mg of OMP or Th was added to the mixture, which was then incubated for 30 minutes at 120°C with gentle stirring.

The reaction product which had been stood for a week at 25°C was dissolved in a small volume of cold water and added to cold acetone. The resulting precipitate was dissolved in a minimum volume of cold water and was passed through a Dowex 1-X2 formate column. After the column had been washed with water, the OMP-P fraction was obtained by eluting it with 0.5 *M* ammonium formate solution, while the OMP-PP or Th-PP fraction was obtained by the elution with 1.0 *M* ammonium formate. The subsequent purification procedure was just the same as that of Nose, and pure crystals of OMP-PP, OMP-P and Th-PP were obtained. Th-P was pre-

pared by the method of Miyakawa[2].

6. Preparation of DEAE-cellulose column.

10 g of DEAE-cellulose was suspended in 300 ml of 0.1 *M* EDTA. After having been adjusted the suspension to about pH 10 with 0.1 *N* NaOH and agitated for 20 minutes, the cellulose was collected on a glass filter, washed with 100 ml of water, resuspended in 300 ml of water and stirred for 15 minutes. The adsorbents were again collected on a glass filter, washed with water until the washings were about pH 6, and suspended in 300 ml of water, which was then adjusted to pH 7 with a saturated Na_2HPO_4 solution. The suspension was allowed to settle for 10 minutes, and the supernatant fluid was decanted off with non-sedimenting material. The DEAE-cellulose was then resuspended in 300 ml of 0.005 *M* sodium phosphate buffer, pH 7.0, containing 0.005 *M* EDTA and stored at 4°C until was used. A DEAE-cellulose column was prepared by packing about 10 g of DEAE-cellulose into a column of 2.0 × 20 cm.

7. Method of the enzyme purification.

Fractionation of the crude enzyme with ammonium sulfate : Ammonium sulfate crystal was added to the crude enzyme solution with stirring under cooling. The solution was kept neutral by adding 2.0 *N* KOH containing 0.2 *M* EDTA, and the protein precipitated between 25% and 50%, 50% and 75%, and, 75% and 100% of saturation was collected by centrifugation. Each precipitate was dissolved in 0.2 *M* tris buffer at pH 7.0 and dialyzed for 3 hours against 0.005 *M* Tris buffer, pH 7.0, containing 10^{-4} *M* EDTA in the cold before the assay for thiamine synthesizing activity.

Chromatography on DEAE-cellulose column : The dialized enzyme solution obtained from the fraction precipitated between 50% and 75% of ammonium sulfate saturation was poured into the DEAE-cellulose column, which then was washed with 500 ml of 0.005 *M* sodium phosphate buffer, pH 7.0, containing each 0.005 *M* of EDTA and cysteine. The column was then mounted above a fraction collector and the enzyme was eluted with successive appropriate quantities of the phosphate buffer containing EDTA and cysteine and increasing concentrations of NaCl. The concentrations of NaCl were 0.005 *M*, 0.05 *M*, 0.1 *M*, 0.5 *M* and 1.0 *M*. The protein equivalents of the each eluted fraction were checked with Beckmann spectrophotometer at 280 mμ.

8. Paper chromatography and bioautography of reaction products.

1 ml of the partially purified enzyme solution was added to 1 ml of 0.2 *M* tris buffer, pH 7.0, containing 5 μ*M* of OMP-PP, 5 μ*M* of Th-P and 0.5 m*M* of Mg^{++}. After incubating the mixture for 3 hours at 37°C, the reaction was stopped with 1 ml of 1 *N* HCl. The solution was then adjusted to pH 4.5 with 1 *N* NaOH

and centrifuged. The supernatant fluid, some of which was treated with Taka-diastase solution, was concentrated by a lyophilization apparatus. The concentrated mixture was then spotted on a strip of No 50 Tōyō filter paper, and was developed with isopropanol-0.5 *M* acetate buffer, pH 5.0-distilled water mixture (65 : 15 :20) by an ascending method at 20°C for 14 hours. After drying the strips, the spots were determined by bioautography using thiamine-less *E. coli* mutant 70–23.

RESULTS

The acetone-dried powder catalyzed the synthesis of thiamine from a phosphorylated OMP preparation and Th or its phosphorylated derivatives. Whereas the same powder synthesized only a negligible quantity of thiamine from free OMP and Th derivative, whether the latter was free or phosphorylated (Table 1). The phophorylated preparation of OMP and Th used in this case contained both of the mono- and di-phosphate in equal molar concentrations.

Table 1. Thiamine-synthesis by acetone-dried cell of *Staphylococcus aureus* FDA 209-P

Reaction mixtures contained: acetone-dried cells, 200 mg; each substrate, 5μM; 2 ml of $M/15$ phosphate buffer, pH 7, and 2 ml of distilled water.
Incubation was for 2 hours at 37°C.

Substrate	Thiamine synthesized (mμ moles/2 hours)
OMP + Th	2.2
OMP + Phosphorylated Th	1.6
Phosphorylated OMP*1) + Th	85.2
Phosphorylated OMP + Phosphorylated Th*2)	116.2

*1) a mixture containing OMP-P and OMP-PP in equal moles
*2) a mixture containing Th-P and Th-PP in equal moles

Essentially the same result was obtained with the cell free extract obtained by shaking the acetone-dried cells for 1 hours with glass powder in a phosphate buffer in the cold (Table 2).

The result of Table 3 shows that OMP-PP, rather than OMP-P, is the active intermediate of thiamine synthesis. Although the greatest quantity of thiamine was synthesized from OMP-PP and Th-P by the crude cell-free extract, considerable quantity of the vitamin was also synthesized when free Th or Th-PP was used in combination with OMP-PP.

The cell-free extract used subsequent experiments was prepared by treating the acetone-dried powder with sonic vibrations around 10 KC per second. An example of the thiamine synthesizing property of this extract is presented in Fig. 1.

Most of thiamine found after incubating the substrates with the crude enzyme was free form as shown in Table 4. Bound thiamine increased slightly after 10

Table 2. Thiamine synthesis by cell-free extract of *Staphylococcus aureus* FDA 209-P (I)

Reaction mixtures contained: 1 ml of cell-free extract*; each substrate, 5μM; 2 ml of M/15 phosphate buffer, pH 7, and 2 ml of distilled water.
Incubation was for 2 hours at 37°C.

Substrate	Thiamine synthesized (mμ moles/2 hours)
OMP + Th	0.9
OMP + Phosphorylated Th	1.6
Phosphorylated OMP+Th	37.9
Phosphorylated OMP+Phosphorylated Th	34.9

* obtained by centrifuging the suspension containing each 5 g of the acetone-dried cell and glass powder in 25 ml of 0.007 M phosphate buffer, pH 7.0, after vigorous shaking for 1 hour in the cold.

Table 3. Thiamine synthesis by cell-free extract of *Staphylococcus aureus* FDA 209-P (II)

Reaction mixtures contained: 3 ml of cell-free extract*; each substrate, 5μM; 2 ml of M/15 phosphate buffer, pH 7, and 1 ml of distilled water.
Incubation was for 1 hour at 37°C.

Enzyme	Substrate		Thiamine formed (mμ moles/hour)
cell-free extract	OMP-P	Th	0.6
		Th-P	3.1
		Th-PP	0.7
	OMP-PP	Th	75.8
		Th-P	97.8
		Th-PP	91.7
cell-free extract heated at 100° for 15 min.	OMP-P	Th	0.4
		Th-P	2.5
		Th-PP	0.6
	OMP-PP	Th	1.2
		Th-P	3.1
		Th-PP	1.9

* see Table 2

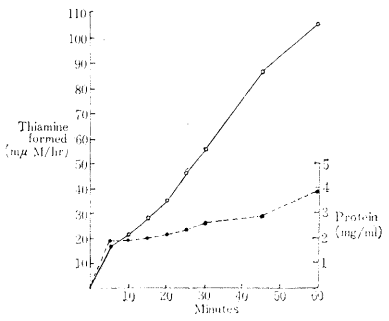

Fig. 1. Effect of the disintegration by sonic vibrations on the thiamine-synthesizing enzyme

5 g of acetone-dried cells, suspended in 50 ml of 0.01 M Tris buffer, were submitted to the action of sonic vibrations at a frequency of 10 KC per second, and the clear supernatant fluid of the crude enzyme was obtained by centrifuging at 10,000 rpm for 15 min.

Reaction mixtures contained: 5μM OMP-PP, 5μM Th-P, 1 ml of the crude enzyme solution and 2 ml of 0.2 M Tris buffer, pH 7, in a total volume of 5 ml. Incubation was for 1 hour at 37°C.

minutes of incubation, but decreased thereafter. The fact that the bound thiamine originally existed in the extract also disappeared might indicate that bound thiamine, if was produced, had been hydrolized by an enzyme existed in the extract.

In the presence of ATP and Mg^{++}, the crude extract also converted the combination of OMP and Th to thiamine, the quantity of which was, however, only one-tenth of that produced by the same extract from OMP-PP and Th-P in the absence of ATP and Mg^{++} (Table 5).

Table 4. Thiamine-synthesis by the crude enzyme* of *Staphylococcus aureus* FDA 209-P

Reaction mixtures contained : 0.5μM OMP-PP, 0.5μM Th (or Th-P), and 4.78 mg of the protein and 0.08 M Tris buffer, pH 7, in a total volume of 5 ml. Incubation was carried out at 37°C.

Substrate	Time (minutes)	Thiamine produced (mμ moles)		
		Free	Total	Combined thiamine calculated
OMP-PP+Th	0	0.47	0.88	0.41
	10	1.04	2.08	1.04
	30	2.49	3.19	0.70
	60	3.60	4.45	0.85
	120	5.78	6.48	0.70
	180	7.69	7.56	—
OMP-PP+Th-P	0	0.50	0.88	0.38
	10	0.76	2.11	1.35
	30	1.92	2.78	0.86
	60	2.58	3.22	0.64
	120	3.51	3.52	0.01
	180	4.05	4.02	—

* extracted from the acetone-dried powder by a treatment with sonic vibrations (10 KC per second) for 30 minutes.

Table 5. Effect of ATP on thiamine-synthesis by the crude enzyme of *Staphylococcus aureus* FDA 209-P

The complete system contained : 0.5μM each substrate, 50μM ATP, 50μM Mg^{++}, 9.8mg of the protein and 0.08M Tris buffer, pH 7, in a total volume of 5ml.
Incubation was for 3 hours at 37°C.

Substrate	Thiamine synthesized (mμ moles/3 hours)
OMP+Th+ATP+Mg^{++}	3.3
OMP+Th	0.3
OMP-PP+Th-P	29.1

The cell-free extract obtained from the acetone powder by disintegration with sonic vibrations was then fractionated with ammonium sulfate and the thiamine synthesizing activity of each fractions was tested.

The results of the experiment presented in Table 6 show that the most active

fraction was that precipitated between 50% and 75% of saturation with ammonium sulfate, and the most active substrate was the combination of OMP-PP and Th-P, though the combination of OMP-PP and Th-PP had also a considerable activity.

The fraction that precipitated between 50% and 75% of ammonium sulfate saturation was further purified by a DEAE-cellulose column and two fractions were obtained as shown in Fig. 2.

Table 6. Thiamine-synthesizing acitivities by ammonium sulfate fractions of crude extract

Reaction mixtures containing 2.5μM OMP-PP, 2.5μM Th-P, 25μM Mg^{++}, 1 ml of each enzyme fraction and 0.08 *M* Tris buffer, pH 7, in a total volume of 5 ml were incubated for 3 hours at 37°C.

Saturation with $(NH_4)_2SO_4$ (%)	Enzyme protein (mg/ml)	Substrate	Thiamine synthesized (mμ moles/3 hours)	
			Free	Total
25%- 50%	9.90	OMP-PP, Th	0.24	1.40
		OMP-PP, Th-P	0.42	4.99
		OMP-PP, Th-PP	0.21	4.04
50%- 75%	5.78	OMP-PP, Th	1.24	2.12
		OMP-PP, Th-P	5.30	16.38
		OMP-PP, Th-PP	1.63	9.75
75%-100%	3.22	OMP-PP, Th	0.33	0.40
		OMP-PP, Th-P	1·44	2.78
		OMP-PP, Th-PP	1.27	1.55

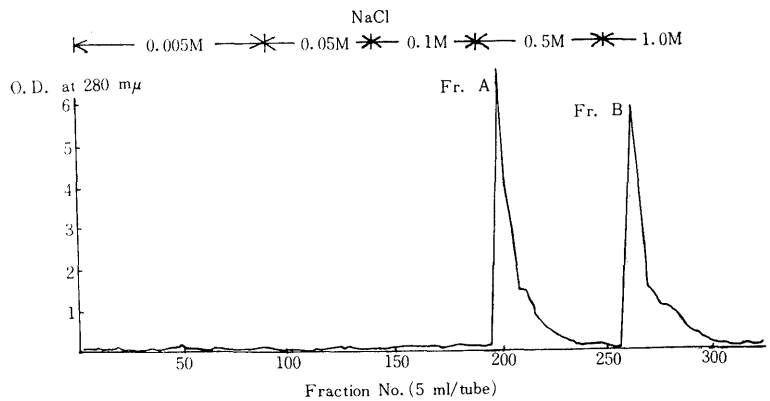

Fig. 2. Chromatographic fractionation on a DEAE-cellulose column of enzyme precipitated by ammonium sulfate between 50% and 75% of saturation.

Column size: 2.0 × 20 cm
Stepwise elution was performed by successive application of 0.005 *M* sodium phosphate buffer, pH 7, containing 0.005 *M* EDTA, 0.005 *M* cysteine and the following concentration of NaCl: 0.005 *M*, 0.05 *M*, 0.1 *M*, 0.5 *M*, 1.0 *M*.
The eluted fractions were assayed by O. D. at 280 mμ.

One fraction, which was named arbitrary as fraction A was eluted from the column with 0.5 *N* NaCl in the phosphate buffer containing EDTA and cysteine, whereas the other fraction, fraction B, was eluted with 1.0 *N* NaCl in the buffer. The fraction A and B were collected, reprecipitated with ammonium sulfate and dialized.

Both of the fractions had thiamine synthesizing activity and the most active combination of substrates was again OMP-PP and Th-P. Although Th-PP was still active for the fractions, the activity of OMP-P and free Th was negligible as shown in Table 7. The specific activity of the enzyme at each steps of purification is shown in Table 8. It would be worth to note that thiamine synthesized by the fractions was essentially combined form.

Table 7. Thiamine-synthesis by enzyme fractions* separated on a DEAE-cellulose column. Reaction mixtures containing 0.5μM each substrates, 50μM ATP, 50μM Mg^{++}, 1 ml of the enzyme solution and 0.08M Tris buffer, pH 7, in a total volume of 5 ml were incubated for 3 hours at 37°C.

Substrate	Thiamine synthesized (mμ moles/3 hours)		
	50%-75% of ammonium sulfate saturation	Fr. A*	Fr. B*
OMP+Th+ATP+Mg^{++}	0.05	0.02	0.00
OMP-P+Th	0.03	0.01	0.01
OMP-P+Th-P	0.04	0.03	0.01
OMP-PP+Th	1.35	0.03	0.01
OMP-PP+Th-P	4.81	15.79	4.78
OMP-PP+Th-PP	1.82	9.42	1.02
Enzyme protein (mg/ml)	1.75	0.89	0.14

* see Fig. 2.

Table 8. Specific activity* of each enzyme solution

Substrate	Specific activity				
	Crude extract	Ammonium sulfate fraction		Fr. A	Fr. B
		20%-80%	50%-75%		
OMP-PP+Th	1.58	0.64	0.64	0	0
OMP-PP+Th-P	1.26	1.90	2.85	19.48	34.20
OMP-PP+Th-PP	0.64	0.95	0.95	10.60	7.28

* defined as mμM of thiamine synthesized per mg of protein per 3 hours from 0.5μM OMP-PP and 0.5μM Th-P (or Th, Th-PP).

The reaction products of the fraction A and B examined by paper chromatography and bioautography were thiamine diphosphate and thiamine monophosphate as shownin Fig. 3.

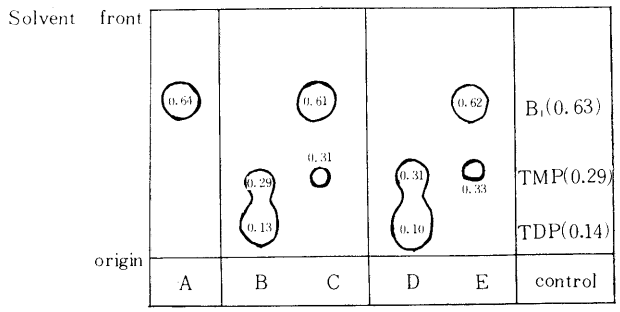

Fig. 3. Composite diagram of bioautographs of chromatographed samples prepared with thiamine less mutant *E. coli* 70-23.

The reaction mixture, containing 1 ml of enzyme solution, 5 μM OMP-PP and 5 μM Th-P, was concentrated in vacuum after an incubation for 3 hours, and spotted on a strip of No 50 Tōyō filter paper. The solvent system was isopropyl alcohol-0.5M acetate buffer (pH 5.0)-water (65 : 15 : 20). Rf value of active zones is given within the zone area.

A: Crude enzyme, precipitated by ammonium sulfate between 20% and 90% of saturation.
B: DEAE-cellulose purified enzyme, fraction A.
C: As B. The reaction products had been hydrolyzed with Takadiastase.
D: DEAE-cellulose purified enzyme, fraction B.
E: As D. The reaction products had been hydrolyzed with Takadiastase.

The activity of the enzyme was promoted by the addition of Mn^{++}, Mg^{++} and Cd^{++}, though Zn^{++}, Cu^{++} and Co^{++} had no effects as shown in Table 9.

The partially purified enzyme was relatively heat stable retaining about half of its activity after heating to 90°C for 10 minutes. The activity was, however, destroyed almost completely after heating to 100°C (Table 10).

Table 9. Effects of divalent cations on thiamine-synthesis by the partially purified enzyme*

Reaction mixtures contained : 0.5μM OMP-PP, 0.5μM Th-P, 4.04 mg of the protein and 0.08 M Tris buffer, pH 7, in a total volume of 5 ml.
The reaction was carried out for 3 hours at 37°C.

Divalent cations	Metallic compounds	Thiamine formed (mμ moles/3 hours)	
		50μ moles cations added	5μ moles cations added
Mg^{++}	$MgSO_4 . 7H_2O$	2.78	2.50
Mn^{++}	$MnCl_2 . 4H_2O$	7.06	5.19
Cd^{++}	$Cd(CH_3COO)_2 . 2H_2O$	2.56	4.12
Cu^{++}	$CuCl_2 . 2H_2O$	0.35	0.53
Co^{++}	$CoSO_4 . 7H_2O$	1.92	0.87
Zn^{++}	$ZnSO_4 . 7H_2O$	0.32	0.55
None		0.65	

* obtained by ammonium sulfate saturation (50~75%)

Table 10. The thermostability of the partially purified enzyme.*

Reaction mixtures contained : 0.5μM OMP-PP, 0.5μM Th-P, 50μM Mg^{++}, 2.44 mg of the protein and 0.08M Tris buffer, pH 7, in a total volume of 5 ml.
Incubation was for 3 hours at 37°C.

Temp. (°C)	Minutes	Thiamine produced (mμ moles/3 hours)
100	30	0.03
100	20	0.05
100	10	0.73
90	10	1.22
80	10	1.62
70	10	1.94
60	10	1.66
50	10	2.14
40	10	2.70
No treatment		2.61

* obtained by ammonium sulfate saturation (50~75%)

DISCUSSION

The experimental data obtained in this experiment indicate that the active form of OMP is OMP-PP, and the result presented in Table 5 suggests that conversion of OMP to OMP-PP requires ATP.

Although free Th was active for the acetone powder and the crude enzyme extracted from the powder, the activity was negligible for the enzyme purified by DEAE-cellulose. On the contrary, both of Th-P and Th-PP were active as a substrate for the enzyme preparation on any purification step. These facts indicate that Th must be phosphorylized before its coupling with pyrimidine moiety of thiamine. From the experimental results obtained here, however, it is difficult to conclude which of the phosphorylated thiazoles is a true active form. The fact that the specific activity of fraction B enzyme for Th-P was about five times as active as the activity for Th-PP might indicate that Th-P and not Th-pp is the active form of Th. However, it is difficult to explain the mechanism of the formation of both of TMP and TDP as coupling products of Th-P with OMP-PP.

In this experiment, it is uncertain whether both of Fraction A and B separated by chromatography on a DEAE-cellulose column are the same thiamine-synthesizing enzyme or not, unless further purification of the enzyme is attempted.

SUMMARY

Studies on the enzymatic synthesis of thiamine by *Staphylococcus aureus* FDA 209-P were carried out and following results were obtained.

1. The active form of pyrimidine was OMP-PP.
2. Free Th as well as its phosphorylated derivatives was active as a substrate for the acetone-dried powder of the organisms and the crude enzyme preparation extracted from the powder.
3. Th-P was the most active form of Th for the enzyme preparation which had been purified by the treatment with ammonium sulfate and chromatography on DEAE-cellulose. The activity of free Th for the partially purified enzyme was negligible.
4. The reaction products from OMP-PP and Th-P of the purified enzyme preparation were TDP and TMP as examined by paper chromatographic and bioautographic method.
5. The activity of the enzyme was increased by the addition of Mn^{++} Mg^{++} or Cd^{++}.
6. The thiamine-synthesizing enzyme was comparatively thermostable.

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