THE ENZYMATIC SYNTHESIS OF THIAMINE MONOPHOSPHATE

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Crude extracts of bakers' yeast readily form thiamine from 2-methyl-4-amino-5-hydroxymethyl pyrimidyl phosphate (PYP) and 4-methyl-5-(β-hydroxyethyl) thiazole[1] (TH) (Harris and Yavit, 1957). With purification of the enzyme, it has been possible to show (Leder, 1959) that the synthesis requires the participation of ATP[2] and that the initial product of the reaction is not free thiamine, but a phosphorylated derivative of thiamine. Free thiamine was determined by the thiochrome reaction (Burch, Bessey, Love and Lowry, 1952) and total thiamine (free plus phosphorylated) by the same reaction after a preliminary incubation with seminal phosphatase or takadiastase. Line 1 of Table I shows that the partially purified enzyme forms no free thiamine and lines 2 and 3 show that thiamine is not phosphorylated when added to the incubation mixture in the presence or absence of the pyrimidine and thiazole substrates.

The enzyme was prepared from Annheuser-Busch bakers' yeast. The purification procedure involved heating a crude extract to 55° for 2 minutes and subsequent fractionation with ammonium sulfate between 50 and 60 percent saturation. The heat step inactivates essentially all of the thiamine phosphate phosphatase present in crude extracts.

That the enzymatically formed thiamine ester was not thiamine pyrophosphate was established by demonstrating its complete inactivity as coenzyme for transketolase[3]. This suggested that the reaction product might be thiamine

[1] This compound and 2-methyl-4-amino-5-hydroxymethyl pyrimidine were kindly supplied by Dr. E. Pierson and Dr. R. D. Babson of Merck and Co.

[2] Abbreviations not indicated in the text are: ATP, adenosine triphosphate; PEP, phosphoenolpyruvate.

[3] We wish to thank Dr. A. G. Datta and Dr. E. Racker for providing crystalline transketolase and the details of their enzymatic procedure for the determination of thiamine pyrophosphate.

TABLE I

THIAMINE ESTER SYNTHESIS

Conditions	Thiamine	
	Free	After Phosphatase
	(millimicromoles)	
1. Complete		
Incubated	0	8.5
Unincubated Control	0	0
2. Complete + Thiamine	9.0	17.0
-PYP, -TH, +Thiamine	8.0	8.3

The complete system contained 25 micromoles of phosphate buffer, pH 7.1; 2.5 µmoles $MgCl_2$; 4 µmoles PEP; 2 µmoles ATP; 20 micrograms pyruvate kinase; 0.55 µmoles PYP; 0.55 µmoles TH; 1 mg. of enzyme in a final volume of 0.5 ml. Approximately 8.5 millimicromoles of thiamine were added where indicated. The reaction mixture was incubated for 30 minutes at 37° and stopped by the addition of 0.5 ml. of 10 percent trichloroacetic acid. Aliquots were assayed for thiamine as described in the text.

monophosphate, which would be formed by the condensation of the pyrimidine moiety with the corresponding thiazole monophosphate, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (THP). The latter was prepared from the analogous pyrophosphate (Weijlard and Tauber, 1938) by acid hydrolysis. The use of THP in place of TH affected neither the yield of thiamine ester nor the requirement of the enzyme system for ATP. Accordingly the pyrimidyl pyrophosphate, 2-methyl 4-amino-5-hydroxymethyl pyrimidyl pyrophosphate (PYPP), was prepared by the pyrophosphorylation of 2-methyl-4-amino-5-hydroxymethyl pyrimidine. Table II demonstrates that the substitution of PYPP for PYP greatly increased the amount of thiamine ester formed and when incubated with THP completely eliminates the requirement for ATP.

Approximately 1.5 micromoles of the phosphorylated thiamine were prepared by incubating a mixture containing 4 micromoles each of PYPP and THP, 2.5 µmoles $MgCl_2$, 25 µmoles glycylglycine buffer, pH 7.1 and 4 mg. of enzyme for 3 hours at 37°. Using procedures described by Siliprandi and Siliprandi (1954), the

TABLE II

THIAMINE ESTER SYNTHESIS FROM PYPP

Conditions	Substrates		Thiamine Ester
			millimicromoles
Complete	PYP	TH	10.7
		THP	9.9
	PYPP	TH	71.0
		THP	90.0
Incomplete - ATP - PEP	PYP	TH	0
		THP	0
	PYPP	TH	0
		THP	89.0

Except for the use of glycylglycine buffer instead of phosphate buffer, the conditions were essentially as described for Table I.

product was isolated and purified by ion exchange and paper chromatography and by paper electrophoresis. The following properties of the compound indicate that it is thiamine monophosphate:

1. It was adsorbed by Amberlite carboxylic acid resin CG-50 which does not bind thiamine diphosphate or thiamine triphosphate.
2. It migrated towards the cathode in paper electrophoresis at pH 5.4.
3. It had the same R_F as thiamine monophosphate in two solvents which readily separate thiamine and its three esters (N-propanol-water-1M acetate buffer, pH 5; 70:20:10) and (N-propanol-0.5M acetate buffer, pH 4.5; 60:40).
4. When mixed with authentic thiamine monophosphate it was not separable by paper chromatography or electrophoresis.
5. It contained 1 mole of total phosphorous per mole of thiamine and no easily hydrolyzable phosphorous.

These results indicate that the condensation of the two heterocyclic moieties of thiamine proceeds as follows:

$$\text{PYPP} + \text{THP} \longrightarrow \text{Thiamine monophosphate} + \text{pyrophosphate}$$

Dephosphorylation of thiamine phosphate by a phosphatase could account for the synthesis of free thiamine in crude extracts (Harris and Yavit, 1957). Phosphorylation of thiamine monophosphate would lead to the formation of cocarboxylase

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