PARTIAL PURIFICATION AND PROPERTIES OF THIAMINOKINASE FROM YEAST*

by

ELIZABETH P. STEYN-PARVÉ

Laboratory for Physiological Chemistry, University, Utrecht (Netherlands)

INTRODUCTION

It has been known for some time that living yeast is able to take up large amounts of thiamine, thereby converting this compound into thiaminepyrophosphate (TPP)[1,2,3,4,5]. Normally, most of the thiamine is found to be present as TPP in yeast[6]; part of the latter compound can be accounted for as carboxylase. Very little additional carboxylase is formed when fresh yeast is incubated with thiamine[7]; most of the accumulated TPP is bound in some other manner[5].

A few years ago we have studied the synthesis of TPP from thiamine by living yeast[5] and have realized that it is a complicated process. We have thought to gain more insight by first studying a more simple system, namely the phosphorylation of thiamine by the isolated enzyme in cell-free extracts or purified preparations.

This field has already been explored by several workers. WEIL-MALHERBE[8] prepared a protein fraction from Lebedew juice of dried brewers' yeast which could transform thiamine into thiaminepyrophosphate and was the first to show that this phosphorylation required adenosinetriphosphate (ATP) as phosphate donator. His work was confirmed and extended by NGUYEN VAN THOAI AND CHEVILLARD[9], who prepared the enzyme phosphorylating thiamine from brewers' yeast and also from dog liver. Recent investigations by NIELSEN AND LEUTHARDT[10] should also be mentioned although they do not concern the yeast enzyme; these authors used preparations from rat liver and showed that the phosphorylating enzyme is a soluble protein.

We have extracted the enzyme from fresh bakers' yeast and have studied the phosphorylating activity of these yeast extracts and of partially purified protein fractions. The enzyme proved to require Mg^{++} ions. Small amounts of inorganic phosphate, though not indispensable, greatly enhanced the enzymic activity. As regards the influence of the pH, a broad optimum was found between pH 6 and 8.

Further purification of the enzyme is in progress, but we have deemed it useful to set down here the most important observations so far obtained.

In keeping with LIPMANN's nomenclature[11], we propose to give this enzyme, which transfers phosphate from ATP to thiamine, the name of thiaminokinase.

* This work forms part of investigations on thiamine metabolism by H. G. K. WESTENBRINK and collaborators.

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EXPERIMENTAL

1. *Materials and methods*

a. *Enzyme preparations.* The preparation of the enzyme will be treated in a following section. As starting material fresh bakers' yeast ("Koningsgist", Delft) was mostly used. In a few cases fresh or freshly dried brewers' yeast (from the "Amstel" Brewery, Amsterdam) was employed.

b. *Adenosinetriphosphate.* The ATP used was prepared in the laboratory according to a slightly modified version of the prescription by DOUNCE *et al.*[11]*. The monobarium salt was dissolved in 0.1 *N* HCl and converted into the potassium salt with K_2SO_4 before use. The final, neutralized solution was always approximately 0.02 *M*. The content of ATP was estimated by determining the amount of inorganic phosphate liberated during 10 minutes' hydrolysis in *N* HCl at 100°.

c. *Determination of TPP.* TPP was determined as described by WESTENBRINK AND STEYN PARVÉ[12], except that 3.75 mg of 2-methyl-4-amino-5-aethoxymethylpyrimidine ("pyrimidyl") were added to each g of alkaline washed yeast instead of 0.2 mg of thiamine. The latter compound was not employed as inhibitor of the yeast phosphatase in the determination of TPP, as preliminary experiments had shown that the alkaline washed yeast can synthesize small amounts of TPP during the manometric measurement from ATP present in the extract and the added thiamine. Moreover, the excessive dose of "pyrimidyl" smoothed out the difference in thiamine content between the extracts and the standard TPP solutions.

d. *Determination of inorganic phosphate.* Carried out according to SUMNER's method[13].

e. *Determination of magnesium.* Performed as indicated by LANG[14].

2. *General procedure for the phosphorylation experiments*

These were always carried out in a reaction mixture of 5 ml, containing: thiamine, 5 mg; 0.02 *M* K–ATP, 0.5 ml; 0.1 *M* $MgSO_4$, 0.25 or 0.5 ml (if required); an appropriate amount of enzyme solution and 0.1 *M* phosphate buffer (usually of pH 6.2) to volume. Other additions were made if necessary for the experiment in question. Incubation took place at 27.5° C for the desired length of time (usually 60 minutes). At the beginning and at the end of each experiment 1 ml of the reaction mixture was pipetted into 5 ml boiling 0.05 *N* HCl and the boiling continued for one minute, to stop the reaction. After cooling, the acid mixtures were neutralized to pH 6.2 with 10% KOH and made up to 10 ml with 0.1 *M* phosphate buffer, pH 6.2. After spinning down the protein precipitate, an aliquot was taken for the determination of TPP. The latter determination was always performed in duplicate.

3. *Extraction of thiaminokinase from yeast*

Proteins can be more easily extracted from brewers' yeast than from bakers' yeast, so the former would seem to be the most likely starting material to prepare the enzyme from. However, fresh bakers' yeast can synthesize much more TPP from thiamine than fresh brewers' yeast, so we preferred to try to extract the enzyme from bakers' yeast. Later our choice appeared to be warranted, because extracts of brewers' yeast proved to contain many other proteins besides thiaminokinase, as will be described below, and so were less pure than the bakers' yeast extracts. Bakers' yeast is extremely difficult to macerate, and drying and subsequent maceration of the yeast entail very considerable losses of enzyme activity in general, so we resolved to try the effect of plasmolyzing the fresh yeast.

Plasmolysis of bakers' yeast with ether or by freezing at −70° C and thawing, followed by maceration of the plasmolyzed yeast at 37°, did not yield active extracts. However, we did succeed in obtaining active extracts by macerating the plasmolyzed yeast in 0.5 *M* KCl at 37°. Plasmolysis with salt, followed by incubation with salt at 37°,

* I wish to express my gratitude to Dr TH. GERRITSEN of our laboratory, who generously supplied me with ATP prepared by him.

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gave negative results with bakers' yeast, although it was successfully employed by THOAI AND CHEVILLARD[9] for brewers' yeast.

After numerous experiments the following procedure was adopted for the extraction of thiaminokinase from bakers' yeast:

Fresh, pressed bakers' yeast, as delivered by the factory, is washed three times with 10 volumes of distilled water. Portions of 10 g of the washed yeast, contained in metal centrifuge tubes, are immersed in a mixture of acetone (or alcohol) and solid CO_2 (temperature approx. $-70°$ C) for 15 minutes and then thawed by dipping the tubes in cold water. This process of alternate freezing and thawing is repeated twice. Solid KCl is then added to the liquefied yeast to a molarity of 0.5, and the mixture is shaken in a water-bath of 37° for 3 hours. After standing overnight in a refrigerator, the yeast macerate is centrifuged and a slightly opalescent, light brown fluid, with a good thiaminokinase activity, is obtained. 40 g of fresh yeast yield about 25 ml of extract.

It is also possible to freeze larger amounts of yeast at a time, *e.g.*, 200 g of yeast, in a metal container, but then the freezing time must be lengthened considerably, and the extracts are not so active as when small portions are frozen. The rapid decrease of temperature in the yeast seems to be of importance if sufficient protein is to be extracted.

Some enzyme could also be extracted by macerating in 0.5 *M* KCl after plasmolysis with ether, but the extracts were less active than those obtained after freezing, and deteriorated rapidly.

Experiments to disengage the proteins from the cell structure by subjecting the plasmolyzed yeast to ultrasonic vibrations have not met with much success so far. Although a solution rich in protein was obtained, it displayed very little thiaminokinase activity.

Lebedew juice of freshly dried brewers' yeast also has a good thiaminokinase activity. It was rejected, however, because its activity per mg of protein nitrogen is much lower than that of the extracts of bakers' yeast: in one experiment 2.8 γ TPP as against 12.8 γ TPP were formed per mg protein nitrogen in 60 minutes. So Lebedew juice harbours many other undesired proteins. Among these is carboxylase, and this constitutes a further disadvantage: Lebedew juice contains about 6 γ of preformed TPP per ml, bakers' yeast extracts only 1–2 γ; so an increase in TPP can best be measured with the latter.

4. *Experiments with the crude yeast extract*

A study of the thiaminokinase activity of the crude extract of bakers' yeast, obtained in the above-mentioned manner, led to a number of observations which are described here.

a. *Time-course of the thiaminokinase reaction.* The progress of the thiaminokinase reaction with time is represented in Fig. 1. The process hardly slows down in the first 2 hours when substrate and ATP are present in excess.

b. *Influence of dialysis.* After dialysis against 0.02 *M* KCl for 24 hours at 2° C, the yeast extract has lost its thiaminokinase activity. It could be reactivated to some extent by adding some boiled yeast extract, but better still by adding Mg ions (Table I). So the thiaminokinase appears to require Mg ions*.

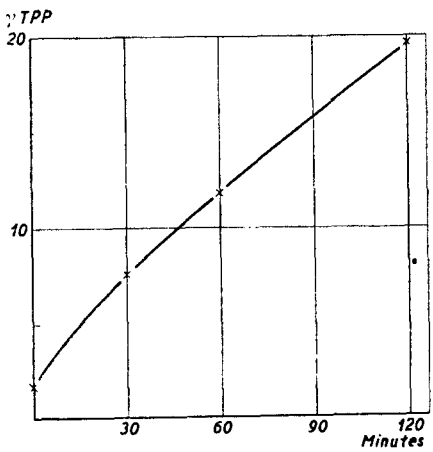

Fig. 1. Time-course of the thiaminokinase reaction. Experiment performed with crude yeast extract, 1 ml per 5 ml reaction mixture

* Some 6 months after we had established this fact for yeast thiaminokinase, a paper by NIELSEN AND LEUTHARDT[10] came to our notice, in which the same observation was made for thiaminokinase from rat liver.

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TABLE I

THIAMINOKINASE ACTIVITY OF DIALYZED YEAST EXTRACT

Experiments performed with Lebedew juice of dried brewers' yeast
Composition of reaction mixtures: thiamine 5 mg; 0.02 *M* K–ATP, 0.5 ml; additions as mentioned in the table and 0.1 *M* phosphate buffer, pH 6.2, to complete the volume to 5 ml

No.	Dialyzed extract	$MgSO_4$ 0.1 *M*	Boiled extract	γ TPP formed in 60 min
1	1 ml	—	—	0.7
2	1 ml	0.25 ml	—	9.2
3	1 ml	—	0.5 ml	3.6
4	—	—	1.0 ml	0

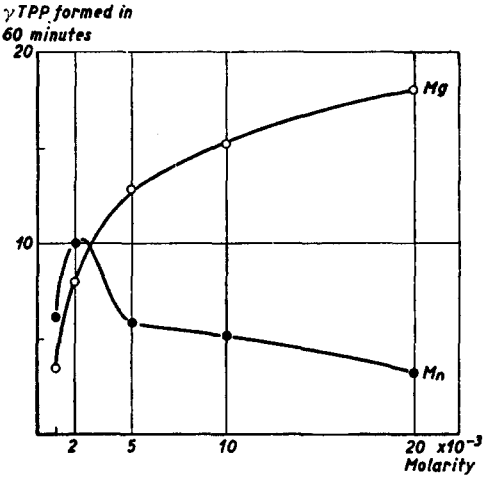

Fig. 2. Activation of thiaminokinase by Mg^{++} and Mn^{++}. Experiment performed with fraction of yeast proteins precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$. 0.58 mg protein nitrogen per 5 ml reaction mixture

c. *Optimal concentration of* Mg. Determination of Mg in some yeast extracts showed that they contain 5–10 μM of Mg per ml. The thiaminokinase activity of the undialyzed extracts is slightly enhanced by adding Mg, as can be seen from Table II, which gives the thiaminokinase activity of both dialyzed and undialyzed extract after addition of varying amounts of Mg. Experiments with a purified enzyme preparation showed that maximal activation is obtained when the concentration of Mg is $2 \cdot 10^{-2}$ molar. Mn ions also activate the thiaminokinase; in low concentrations even more so than Mg ions. At a concentration of $5\text{–}10^{-3}$ *M*, however, inhibition by Mn is already observed, and so the enzymic activity never reaches the same height as is the case with Mg (Fig. 2).

TABLE II

ACTIVATION OF THIAMINOKINASE BY MAGNESIUM

Experiment performed with extract of bakers' yeast, containing 5 μM Mg per ml
Dialysis against 0.02 *M* KCl for 24 hours at 2° C. Increase of volume 20%
Composition of reaction mixtures: thiamine, 5 mg; 0.02 *M* K–ATP, 0.5 ml; yeast extract, 1 ml or dialyzed extract, 1.2 ml; 0.1 *M* $MgSO_4$, 0–0.4 ml; H_2O, 0.4–0 ml; 0.1 *M* phosphate buffer, pH 6.2, to complete volume to 5 ml

$MgSO_4$ added μM	γ TPP formed in 60 min	
	Undialyzed extract	Dialyzed extract
—	5.8	—
10	8.5	18.3
20	8.5	23.9
40	10.5	32.3

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d. *Inhibitors of thiaminokinase.* It will be seen from Table II that after activation with Mg the thiaminokinase activity of the dialyzed extract is several times greater than that of a comparable amount of the undialyzed extract. This observation has repeatedly been made, not only with dialyzed extracts but also with a dialyzed solution of the proteins precipitated from the extract by saturating with $(NH_4)_2SO_4$ (*cf.* Table VII). So the crude yeast extract would seem to contain one or more factors inhibiting the enzyme. In an attempt to elucidate this point experiments were carried out in which the dialyzed extract was supplemented with either the concentrated fluid against which dialysis had been performed, the ash of the same, dissolved in dilute HCl and neutralized to pH 6.2, or a solution of the ash of the undialyzed extract. In all cases the amounts added corresponded to those originally present in the extract. The respective enzymic activities were compared to those of the undialyzed extract and the dialyzed extract without supplements. The results are given in Table III. It is evident that addition of a comparable amount of concentrated dialysis fluid reduces the enzymic activity of the dialyzed extract to that found before dialysis. The ash of the extract and of the dialysis fluid also depress the enzymic activity to some extent, although not nearly as much. So it would seem that the yeast extract contains at least two inhibitors of thiaminokinase, one organic and thermostable, the second inorganic.

TABLE III

INHIBITION OF THIAMINOKINASE BY FACTORS IN THE YEAST EXTRACT

10 ml yeast extract dialyzed against 465 ml distilled water for 24 hours.
Proteins of dialyzed extract dissolved by adding 40 mg solid KCl. Volume: 13 ml.
Outer fluid concentrated to 10 ml by evaporation at 100° C. 5 ml of concentrate dried and ashed. Ash dissolved in 5 ml 0.1 *N* HCl, neutralized with KOH.
5 ml undialyzed yeast extract dried and ashed; dissolved as above.
Volume of reaction mixtures: 5 ml, each containing: thiamine, 5 mg; 0.02 *M* K–ATP, 0.5 ml; 0.1 *M* $MgSO_4$, 0.5 ml; 0.1 *M* phosphate buffer, pH 6.2, 1.7 ml; undialyzed extract, 1 ml or dialyzed extract, 1.3 ml; supplements as in table, 1 ml; H_2O to volume

γ TPP formed in 60 minutes				
Undialyzed extract	Dialyzed extract			
	—	+ conc. outer fluid	+ ashed outer fluid	+ ashed extract
17.0	39.1	18.3	25.1	30.1

As it was thought that the inhibitory effect of the ash might be due to pyrophosphate formed from orthophosphate by ignition, the effect of inorganic pyrophosphate upon the thiaminokinase activity of a dialyzed extract was also investigated. Two concentrations were tested, one corresponding to the amount of orthophosphate in the extract, the second corresponding to the orthophosphate content of a usual reaction mixture containing 3 ml of 0.1 *M* phosphate buffer. However, as Table IV shows, inorganic pyrophosphate does not depress the enzymic activity in the concentrations employed. So the nature of the inorganic inhibitor is as yet unknown.

Regarding the organic inhibitor, it might be surmised that some other phosphate acceptor in the extract would compete with thiamine for the ATP phosphate. If so, this competitor is not a hexose, for we could not detect any such substance in the concentrated outer fluid after dialysis.

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TABLE IV

INFLUENCE OF INORGANIC PYROPHOSPHATE UPON THIAMINOKINASE ACTIVITY

Yeast extract dialyzed against 0.02 *M* KCl for 24 hours. Phosphate content before dialysis: 1.8 mg P/ml (58 μM).

Volume of reaction mixtures 5 ml, each containing: thiamine, K–ATP and $MgSO_4$: as in exp. of Table III; 0.1 *M* phosphate buffer, pH 6.4, 1.75 ml; dialyzed extract, 1.25 ml; solution of $Na_4P_2O_7 \cdot 10\ H_2O$ containing 150 μM per ml: 0, 0.2 and 1.0 ml; H_2O to volume.

Inorganic pyrophosphate (μM)	—	30	150
γ TPP formed in 60 minutes	58.0	57.5	56.3

e. *Optimal concentration of inorganic phosphate.* As the experiment recorded in Table V shows, the thiaminokinase can still function in the absence of inorganic phosphate, but its activity is greatly enhanced by small traces of this compound. Concentrations above $2 \cdot 10^{-1}$ *M* markedly inhibit the enzyme. Addition of a few ml of 0.1 *M* phosphate buffer does not affect the enzymic activity of the undialyzed yeast extract.

TABLE V

INFLUENCE OF INORGANIC PHOSPHATE UPON THIAMINOKINASE ACTIVITY

Phosphate content of undialyzed extract: 58 μM per ml.

Yeast extract dialyzed against 0.02 *M* KCl for 24 hours.

Phosphate added in various concentrations of potassium phosphate buffer, pH 6.5.

Volume of reaction mixtures: 5 ml. All contained thiamine, 5 mg and 0.02 *M* K–ATP, 0.5 ml.

Experiments with undialyzed extract further contained: extract, 1 ml; 0.1 *M* $MgSO_4$, 0.25 ml; K-phosphate as in table; H_2O to volume. Experiments with dialyzed extract: extract, 1.25 ml; 0.1 *M* $MgSO_4$, 0.5 ml; K-phosphate as in table; H_2O to volume.

	Undialyzed extract		Dialyzed extract				
Conc. of phosphate (*M*)	$1.16 \cdot 10^{-2}$	$7.66 \cdot 10^{-2}$	—	$2 \cdot 10^{-3}$	$2 \cdot 10^{-2}$	$2 \cdot 10^{-1}$	1
γ TPP formed in 60 minutes	15.9	17.1	37.4	76.6	56.6	48.6	6.2

5. *Purification of thiaminokinase; localization of the enzyme activity*

In the first attempts to localize the thiaminokinase in the mixture of proteins contained in the yeast extract, the proteins were precipitated from the extract by saturation with solid ammonium sulphate. They were dissolved by adding water and dialyzed against 0.02 *M* KCl at $+2°$ C to remove $(NH_4)_2SO_4$. The protein solution thus obtained was fractionated according to DERRIEN[15,16] by adding varying amounts of saturated $(NH_4)_2SO_4$ and after dialysis the filtrates of the extract were tested for thiaminokinase activity. Their composition was also analyzed by determining the nitrogen contents or the absorption in ultra-violet light of 275 mμ wave-length*.

* Ammonium sulphate was preferred to potassium phosphate for the fractional analysis because it is more practical for preparative purposes: it is very difficult to separate a protein precipitate by centrifugation in a concentrated solution of potassium phosphate, but in saturated ammonium sulphate this can be accomplished in 15 minutes in a centrifugal field of approx. 10,000 × *g*. As high salt concentrations inhibit the thiaminokinase, protein fractions containing ammonium sulphate had to be dialyzed before the enzymic activity could be measured.

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The first crude fractionation showed that the enzyme precipitates between 50 and 70% saturation with $(NH_4)_2SO_4$ (Table VI). Further analysis according to DERRIEN of the protein mixture precipitating in this range pointed to the presence of at least 4–6 components. Most of the thiaminokinase activity disappears from the solution when the $(NH_4)_2SO_4$ concentration is raised above 58% saturation (Fig. 3). The fact that the protein components are not well separated in the diagram indicates that, should one of them be identified with the enzyme protein, it will be very difficult to isolate it by fractional precipitation without incurring enormous losses.

TABLE VI

FRACTIONATION OF THE PROTEINS FROM YEAST EXTRACT WITH $(NH_4)_2SO_4$

For preparation of protein solution see text.

2 ml protein solution diluted to 10 ml by adding varying amounts of saturated $(NH_4)_2SO_4$ and H_2O. Incubated at + 1° for 15 hours, 5 ml of filtrates dialyzed against 0.02 *M* KCl at + 2° and diluted to 10 ml.

Enzyme activity tested in reaction mixtures of 5 ml, containing: thiamine, 5 mg; 0.1 *M* $MgSO_4$, 0.25 ml; 0.02 *M* K–ATP, 0.5 ml; 0.1 *M* phosphate buffer, pH 6.2, 1.25 ml; diluted filtrate, 3 ml.

Percentage saturation with $(NH_4)_2SO_4$	40	50	60	70	80
γ TPP formed in 60 minutes	14.7	13.9	6.6	0	0
Nitrogen in mg per ml dialyzed filtrate	0.320	0.300	0.215	0.058	—*

* Not determined as filtrate still contained a trace of NH_4^+

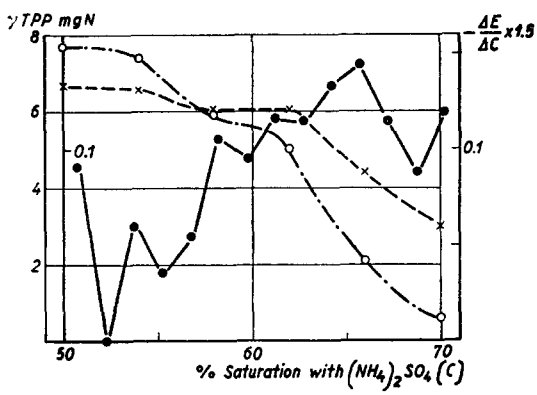

Fig. 3. Analysis of the proteins precipitating between 50 and 70% saturation with $(NH_4)_2SO_4$. Analysis performed on dialyzed solution of total protein precipitated from yeast extract. Fractionation and test of enzymic activity carried out as in Table VI. Ordinates, from left to right: γ TPP formed in 60 minutes per 5 ml reaction mixture, containing 3 ml diluted filtrate; mg nitrogen per ml dialyzed filtrate; $-\left(\frac{\Delta E}{\Delta C}\right)\cdot 1.5$, = difference in extinction between two successive fractions, interval ΔC = 1.5% saturation with $(NH_4)_2SO_4$. (λ = 275 mμ, d = 1 cm).

o–·–·–o γ TPP formed by dialyzed filtrates
×– – –× mg N per ml dialyzed filtrate
●———● "differential" curve for absorption at 275 mμ by filtrates diluted 1:5

The results of a comparative study of thiaminokinase in crude yeast extract, the protein solution obtained from same, and the protein fraction precipitating between 50 and 70% saturation with $(NH_4)_2SO_4$ are assembled in Table VII. The synthetic activity per mg protein nitrogen is considerably increased by the successive treatments applied to the crude extract. The apparent increase of total enzyme activity of the total proteins as compared to the crude extract is not real, but, as mentioned previously, is caused by the removal of inhibitors present in the extract, for upon dialysis of the extract the enzyme activity is greatly augmented, as is seen in experiment No. 2. The enzyme becomes increasingly unstable with progressive purification; this and the time required for its preparation will account for most of the loss of total enzyme activity in fraction

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50–70 as compared to the total proteins. Owing to this instability it is not possible to subject fraction 50–70, obtained in this devious manner from the preparation of total proteins, to further analysis by fractionation with ammonium sulphate. To enable such an analysis the fraction must be isolated more rapidly by direct fractional precipitation from the crude extract.

TABLE VII

THIAMINOKINASE ACTIVITY OF YEAST EXTRACT AND DERIVED PROTEIN SOLUTIONS

Solution of total proteins obtained by saturating yeast extract with $(NH_4)_2SO_4$, dissolving pp. in H_2O and dialyzing against 0.02 *M* KCl for 24 hours.

Protein fraction 50–70 obtained from solution of total proteins by fractional precipitation with saturated $(NH_4)_2SO_4$ between 50 and 70% saturation. Dialyzed as above.

Volumes of solutions listed are those obtained from the volume of solution mentioned previously; *e.g.*, 45 ml total protein solution derived from 95 ml yeast extract, etc.

Exp. No.	Preparation	Volume ml	Protein-N mg/ml	γ TPP in 60′ per mg N	Total activity expressed in γ TPP formed
1	Crude extract	95	1.51	6.4	915
	Total proteins	45	1.66	30.0	2250
	Protein fraction 50–70	14.6	1.46	56.0	1195
2	Crude extract	96	1.76	4.8	810
	Dialyzed extract	115	1.39	22.3	3560
	Total proteins	38	2.13	28.5	2310
	Protein fraction 50–70	8.6	1.71	37.0	540

Matters are complicated because the yeast extract dissolves much less $(NH_4)_2SO_4$ when saturated with this compound than the same volume of a watery dilute protein solution. To ascertain the range in which thiaminokinase precipitates from the crude extract it first had to be fractionated with increasing amounts of the salt, in the manner as described above for the solution of total proteins. The results, given in Table VIII, show that the enzyme precipitates between 40 and 60% saturation with $(NH_4)_2SO_4$; most of it leaving the solution between 50 and 60%. As was to be expected from the diminished solubility of $(NH_4)_2SO_4$ in the extract, the enzyme precipitates at a lower concentration of the salt than in the case of isolation from the protein solution.

TABLE VIII

FRACTIONATION OF YEAST EXTRACT WITH AMMONIUM SULPHATE

3 ml yeast extract diluted to 10 ml with varying amounts of saturated $(NH_4)_2SO_4$ and H_2O.

Incubated at + 1° C for 15 hours. 5 ml of filtrates dialyzed against 0.02 *M* KCl at + 2° and diluted to 10 ml. Enzyme activity tested in reaction mixtures of 5 ml, containing: thiamine 5 mg, $MgSO_4$ (0.1 *M*) 0.25 ml, K–ATP (0.02 *M*) 0.5 ml, phosphate buffer (0.1 *M*, pH 7.0) 1.25 ml, diluted filtrate 3 ml.

Percentage saturation with $(NH_4)_2SO_4$	20	30	40	50	60	70
γ TPP formed in 60 minutes	26.2	22.5	23.4	17.3	1.3	0.7
Protein-nitrogen in mg per ml filtrate	0.20	0.18	0.21	0.13	0.10	0.06

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The protein fraction precipitating between 45 and 60% saturation with $(NH_4)_2SO_4$ was now isolated from crude yeast extract. After dialysis this protein solution was analyzed by fractionation with $(NH_4)_2SO_4$ between 50 and 70% saturation with intervals of 2%. The filtrates were tested for light absorption at 275 mμ, and after dialysis their thiaminokinase activity and nitrogen contents were determined. The results are represented in Fig. 4 (a, b and c). The bulk of the enzyme precipitates between 56 and 66% saturation. Between these concentrations the disappearance of thiaminokinase from the solution keeps pace with the disappearance of protein (*cf.* Figs. 4a and 4c). Application of FALCONER AND TAYLOR's "specific property solubility test"[17] in this range would point to a comparatively pure enzyme, but the "differential curve" in Fig. 4a shows that the protein composition is anything but homogeneous. At least two, perhaps more, proteins precipitate between 56 and 65% saturation. There is no reason

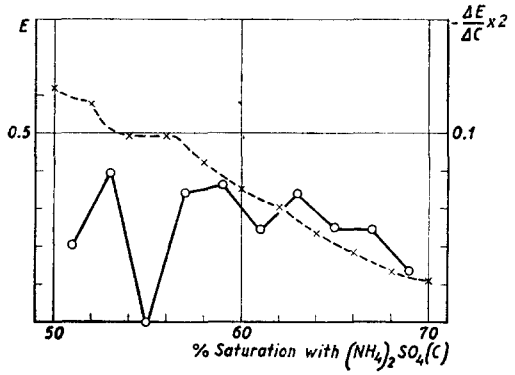

Fig. 4. Fractionation of a protein preparation obtained from yeast extract. For preparation of protein fraction see text. Protein nitrogen: 2.96 mg per ml; TPP formed in 60′ per mg N: 39 γ. 2 ml protein solution diluted to 10 ml by adding amounts of saturated $(NH_4)_2SO_4$, varying from 5 to 7 ml, and H_2O. Incubated at + 1° C for 15 hours. 5 ml of filtrates dialyzed against 0.02 *M* KCl at + 2° and diluted to 10 ml. Composition of reaction mixtures for testing thiaminokinase: see Table VIII. Light absorption at 275 mμ measured with Beckman spectrophotometer on undialyzed filtrate in dilution 1:10

Fig. 4a. Ordinates: left, extinction (E); right, $-\left(\frac{\Delta E}{\Delta C}\right)\cdot 2$. ($\Delta C = 2$)
×---× decrease of extinction of filtrates as a function of $(NH_4)_2SO_4$ concentration
o——o "differential curve" of extinction as a function of salt concentration

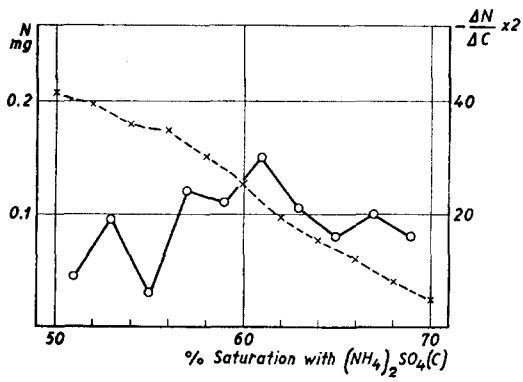

Fig. 4b. Ordinates: left, mg nitrogen per ml filtrate, right, $-\left(\frac{\Delta N}{\Delta C}\right)\cdot 2$. ($\Delta C = 2$)
×---× decrease of nitrogen in filtrates as a function of salt concentration
o——o "differential curve" of nitrogen as a function of salt concentration

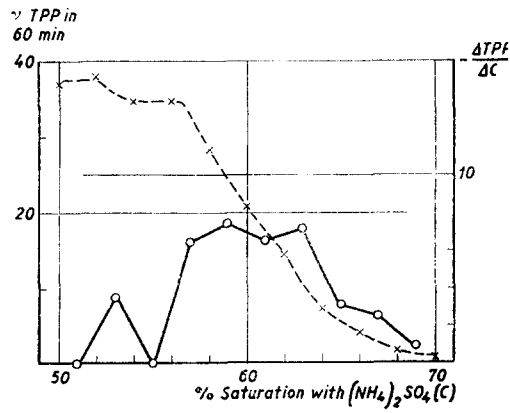

Fig. 4c. Ordinate: left, γ TPP formed in reaction mixture; right, $-\left(\frac{\Delta \text{TPP}}{\Delta C}\right)\cdot 2$. ($\Delta C = 2$)
×---× decrease of TPP formed by filtrates as a function of salt concentration
o——o "differential curve" of TPP as a function of salt concentration

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for designating one of them as the enzyme protein. On the contrary, it seems more probable that they are inert proteins that have absorbed the enzyme to the same extent. For a rough calculation of the "turnover number" of this protein, assuming all the protein to be enzyme, with a hypothetical molecular weight of 60,000, gives a value of only 0.05. So the enzyme probably only constitutes a very small fraction of the proteins precipitating in this range.

Continuing on these lines, a protein fraction precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$ was isolated. This preparation was completely free of phosphatase (see Table IX), while protein fraction 50–70 sometimes still contains some phosphatase. With fraction 55–65 it is possible to study the synthesis of TPP with much smaller amounts of thiamine, which is no longer also required to inhibit the decomposition by the yeast phosphatase of TPP formed (see Discussion).

TABLE IX

TEST OF YEAST PROTEIN FRACTION 55–65 FOR PHOSPHATASE ACTIVITY

Enzyme preparation: protein fraction precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$. Protein nitrogen 1.16 mg per ml.

Reaction mixtures: TPP (10 γ/ml), 0.5 ml; enzyme solution, 1.0 ml; 0.1 *M* $MgSO_4$, 0.5 ml; 0.1 *M* phosphate buffer, pH 6.2, 3.0 ml. Thiamine added as in table.

Incubation temperature: 27.5° C. Time: 50 minutes.

Reaction stopped by transferring 1 ml of the mixture into 5 ml boiling 0.05 *N* HCl and boiling for one minute. If the reaction mixture did not contain thiamine, 1 mg of this substance was added to 5 ml of the 0.05 *N* HCl. After neutralizing, making up the volume to 10 ml with 0.1 *M* phosphate buffer of pH 6.2 and centrifuging, an aliquot of the supernatant was analyzed for TPP.

Time in minutes	γ TPP	
	No thiamine	5000 γ thiamine
0	5.7	5.8
60	5.7	5.7

6. *Experiments with purified enzyme preparations*

a. *Influence of pH on thiaminokinase activity.* Protein fraction 50–70 was employed to establish the optimal pH for the thiaminokinase reaction. A first experiment showed that there is a good activity at pH 6–8, but hardly any below pH 6 (Table X). From further investigation between pH 6 and 8.5 it appeared that there is a broad optimum for the reaction between pH 6 and 8 (Fig. 5). The pH of 6.2, arbitrarily chosen in the first experiments, was therefore not abandoned later, for comparative purposes. Sometimes, however, the pH of the medium was fixed at 7.0.

TABLE X

INFLUENCE OF pH ON THIAMINOKINASE ACTIVITY

Enzyme preparation: protein fraction precipitating between 50 and 70% saturation with $(NH_4)_2SO_4$.

Composition of reaction mixture: thiamine: 5 mg, $MgSO_4$ (0.1 *M*) 0.5 ml, K–ATP (0.02 *M*) 0.5 ml, enzyme solution 0.5 ml, phosphate buffer (0.1 *M*) of varying pH, 3.5 ml.

pH	4	5	6	7	8
γ TPP formed in 60 min	3.3	3.3	14.6	15.8	18.8

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b. *Comparison of synthesis of TPP from thiamine and from thiamine monophosphate.* For this experiment a protein fraction precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$, free of yeast phosphatase (see above), was used. Thiamine monophosphate (TMP) was prepared from TPP by hydrolyzing 5 mg for 15 minutes in 20 ml boiling *M* HCl. After neutralization the solution was made up to 50 ml. Fig. 6 shows that the rate of synthesis of TPP from TMP is approximately half of that from thiamine. This agrees with the observation made by WEIL-MALHERBE for his preparation of yeast protein[8].

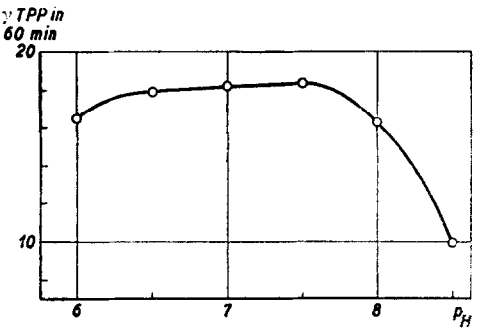

Fig. 5. pH activity curve of thiaminokinase. For details of experiment see Table X

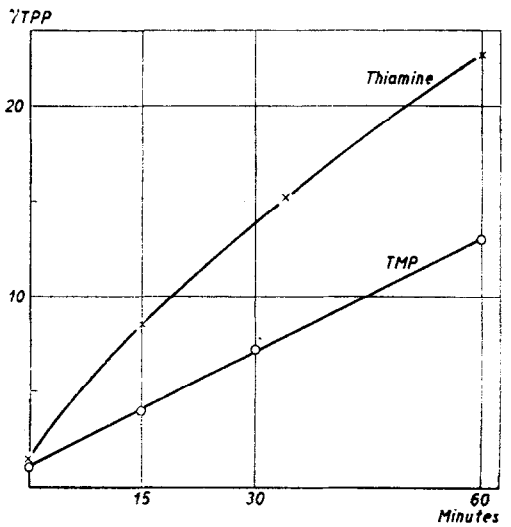

Fig. 6. Comparison of synthesis of TPP from thiamine and from thiamine monophosphate (TMP). Experiment performed with protein fraction precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$. Composition of reaction mixtures: 0.1 *M* $MgSO_4$, 0.5 ml; 0.02 *M* K-ATP, 0.5 ml; 0.1 *M* phosphate buffer pH 7.0, 1.0 ml; enzyme solution, 1 ml (containing 1.16 mg protein-N); thiamine 140 γ or TMP, equivalent of 200 γ TPP, H_2O to complete volume to 5 ml

c. *Influence of* Mg^{++} *and* Mn^{++}. As mentioned previously, experiments with purified enzyme preparations confirmed the observations made with dialyzed yeast extract regarding the concentration of Mg required for optimal activity (*cf.* p. 313, Table II and Fig. 2). The possibility of substituting Mn for Mg was also tested on a purified preparation (Fig. 2).

DISCUSSION

When studying a reaction catalyzed by an enzyme one must always endeavour to provide the best conditions possible for its undisturbed action. As regards the thiaminokinase, two major points must be taken into consideration.

Firstly, the amount of ATP in the reaction mixture must not be a factor limiting the synthesis of TPP during the time of observation. Now the crude yeast extract indeed contains a phosphatase which decomposes ATP under the prevailing experimental circumstances, as appears from the experiment recorded in Table XI. However, the amount of ATP remaining unaffected is still sufficient for the reaction, as shown by the practically undiminished rate of synthesis during two hours (Fig. 1).

Secondly, care must be taken that the TPP synthesized by the thiaminokinase is

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TABLE XI

DECOMPOSITION OF ADENOSINE TRIPHOSPHATE BY DIALYZED YEAST EXTRACT

Reaction mixture: 0.02 *M* K–ATP, 0.5 ml; dialyzed yeast extract, 1 ml; 0.1 *M* acetate buffer, 3.5 ml. pH 6.2. Temp. 27.5° C.

Reaction stopped by transferring 1 ml of the mixture into 5 ml boiling 0.04 *N* HCl and boiling for one minute. After neutralizing, making up the volume to 10 ml with H_2O and centrifuging, an aliquot of the supernatant was analyzed for inorganic P.

100% decomposition of ATP liberates 124 γ P.

μM ATP added	γ P liberated in 60 min	pCt ATP unattacked
2	46.5	61.5

not decomposed again, thus obscuring the phosphorylation reaction. The yeast phosphatase just mentioned also decomposes TPP under the conditions prevailing during the phosphorylation of thiamine, but it is completely inhibited by adding 5 mg thiamine to each 5 ml of reaction mixture containing 1 ml yeast extract, as can be seen from Table XII (see also [18]). This is the reason why all our phosphorylation experiments always contain such a large amount of thiamine. Only with enzyme preparations that have been purified to the exclusion of phosphatase small amounts of thiamine may be used without risk of decomposition of TPP formed (*e.g.*, the experiment of Fig. 6).

TABLE XII

DECOMPOSITION OF THIAMINEPYROPHOSPHATE BY YEAST EXTRACT

Reaction mixtures: TPP (10 γ/ml), 0.5 ml; yeast extract, 1 ml; 0.1 *M* phosphate buffer, pH 6.2, 3.5 ml. Thiamine added as in table. Incubation temperature: 27.5° C. Time: 30 minutes. Further treatment as recorded in Table IX.

γ TPP added	γ thiamine added	Percentage TPP decomposed
5	—	100
5	5000	0

The investigations described in this paper on yeast thiaminokinase call forth some comment in regard to the observations of other workers in this field.

We have for instance, always stopped the phosphorylation by deproteinization before determining the amounts of TPP formed, which others have omitted to do. In our opinion this is necessary, because otherwise phosphorylation will continue during the next operations, and depending upon the time taken up by its assay, the amount of TPP found will vary. In this manner it is not possible to evaluate with certainty the extent of phosphorylation within a certain period of time.

NGUYEN VAN THOAI AND CHEVILLARD[9] isolated a protein preparation with thiaminokinase activity from an autolysate of brewers' yeast by fractionation with ammonium sulphate between 45 and 50% saturation. We, however, find that to isolate the enzyme from bakers' yeast extract we must fractionate between 50 and 60%; while investigation of the proteins in solution shows that the enzyme precipitates between 55

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and 65% saturation. It would seem that the solubility of brewers' yeast thiaminokinase differs markedly from that of the bakers' yeast enzyme.

We have further not experienced the necessity of adding boiled yeast extract to our phosphorylating reaction mixtures in addition to Mg^{++}, as is done by the above-mentioned authors. On the contrary, Mg^{++} was much more effective than boiled extract (Table I).

Our preparation maintains a practically undiminished rate of phosphorylation for two hours at least (Fig. 1), in contrast to that of THOAI AND CHEVILLARD, who report that in their experiments the reaction stops after 10 minutes.

It is remarkable that WEIL-MALHERBE's preparation phosphorylates so little thiamine. This cannot only be explained by assuming that synthesis of TPP stops when the carboxylase protein, present in his preparation, is saturated with this coenzyme, for very much more CO_2 is evolved from pyruvic acid by adding larger amounts of TPP than can be formed from the added thiamine in his experiments.

The short note by NIELSEN AND LEUTHARDT[10] on liver thiaminokinase calls for little comment. They use very little thiamine in their experiments, with the disadvantage that very little TPP can be formed, which makes the determination of the latter much more difficult. Their use of WEIL-MALHERBE's apocarboxylase preparation, which also contains thiaminokinase, for the determination of TPP would be censurable if their reaction mixtures contained more thiamine. We believe it is always safer to use alkaline washed dried yeast as apocarboxylase.

Knowing that thiamine is phosphorylated by ATP, we understand why the phosphorylation of thiamine by living yeast is stimulated so strongly by the addition of glucose[5]: its breakdown will produce more ATP than is provided by the endogenous metabolism of the yeast cell and so increase phosphorylation.

The observation made by WEIL-MALHERBE with his enzyme preparation and also by us for our preparation, that the rate of synthesis of TPP from thiamine monophosphate (TMP) is less than that from thiamine, was interpreted by the first as meaning that TPP is formed by transfer of pyrophosphate from ATP to thiamine, and not via TMP by successive transfer of one orthophosphate group from 2 molecules of ATP. According to WEIL-MALHERBE TMP would first be hydrolyzed to thiamine prior to synthesis of TPP. We doubt if this will be the case. Hydrolysis of TMP must be accomplished by a yeast phosphatase. Now WEIL-MALHERBE's preparation does not contain the acid phosphatase, as he observes no stimulating effect of thiamine on carboxylase action when this is added together with TPP to his protein fraction, which also contains apocarboxylase. And we have reason to believe that the alkaline phosphatase has very little affinity for TPP. Our thiaminokinase preparation displays no phosphatase activity under the circumstances with which the synthesis of TPP from thiamine and from TMP was compared. So it seems to us that the thiaminokinase preparation is capable of catalyzing two processes, at different rates, *viz.*, the phosphorylation of thiamine to TPP and that of TMP to TPP. The exact mechanism of the first process still awaits elucidation.

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SUMMARY

Thiaminokinase, the enzyme catalyzing the synthesis of thiaminepyrophosphate from thiamine and adenosinetriphosphate, has been extracted from fresh bakers' yeast by plasmolysis by freezing at $-70°$ C and thawing, followed by maceration at 37° in 0.5 *M* KCl.

The enzyme has been partially purified by fractionation of the yeast extract with ammonium sulphate. The active protein fraction has been subjected to fractional analysis according to DERRIEN. It is pointed out that the protein leaving the solution in the same range of salt concentration as does the enzymic activity is not homogeneous and probably does not represent the enzyme protein, but inert proteins which have adsorbed the enzyme—which will only constitute a small fraction of the precipitating protein—to the same extent.

Thiaminokinase requires Mg for its action. Optimal activation is obtained with concentrations of $2 \cdot 10^{-2}$ *M*. Low concentrations of Mn also activate the enzyme, even more so than comparable amounts of Mg; but concentrations of Mn above $2 \cdot 10^{-3}$ *M* markedly inhibit the phosphorylation of thiamine.

The action of the enzyme is stimulated strongly by small amounts of inorganic phosphate. Maximal effect is obtained with a concentration of $2 \cdot 10^{-3}$ *M*.

The crude yeast extract contains at least two factors inhibiting thiaminokinase, which can be removed by dialysis, and are thermostable. One of these is of inorganic nature.

The enzyme deploys its maximal activity in the pH range of 6 to 8.

The purified preparation synthesizes thiaminepyrophosphate at a higher rate from thiamine than from thiamine monophosphate.

RÉSUMÉ

La thiaminokinase, l'enzyme qui catalyse la synthèse du pyrophosphate de thiamine à partir de la thiamine et de l'acide adénosinetriphosphorique, a été extraite de la levure des boulangers fraiche par plasmolyse: la levure a été congelée à $-70°$, puis dégelée et finalement macérée à 37° dans le KCl 0.5 *M*.

L'enzyme a été purifié partiellement par fractionnement de l'extrait de levure au sulphate d'ammonium. La fraction protéinique active a été soumise à l'analyse fractionnée d'après DERRIEN. Nous attirons l'attention sur le fait que la protéine qui précipite de la solution dans le même intervalle de concentration saline que l'activité enzymatique n'est pas homogène et ne représente probablement pas la protéine de l'enzyme, mais plutôt des protéines inertes ayant adsorbé l'enzyme. Celui-ci ne constituera qu'une faible fraction de la protéine précipitée.

La thiaminokinase a besoin de Mg pour exercer son activité. L'activation optimale est obtenue par des concentrations de $2 \cdot 10^{-2}$ *M*. De faibles concentrations de Mn activent également l'enzyme et même plus fortement que des quantités comparables de Mg; cependant des concentrations de Mn supérieures à $2 \cdot 10^{-3}$ *M* inhibent considérablement la phosphorylation de la thiamine.

L'action de l'enzyme est fortement stimulée par de petites quantités de phosphate inorganique. On obtient des effets maximum à une concentration de $2 \cdot 10^{-3}$ *M*.

L'extrait de levure brut contient au moins deux facteurs inhibiteurs de la thiaminokinase; ils peuvent être éliminés par dialyse et sont thermostables. L'un d'eux est de nature inorganique.

L'enzyme exerce son activité maximale dans l'intervalle de pH 6 à 8.

La préparation purifiée synthétise le pyrophosphate de thiamine à une vitesse plus élevée à partir de la thiamine qu'à partir du monophosphate de thiamine.

ZUSAMMENFASSUNG

Thiaminokinase, das Enzym, welches die Synthese von Thiaminpyrophosphat aus Thiamin und Adenosintriphosphat katalysiert, wurde aus frischer Bäckereihefe durch Plasmolyse: Gefrieren bei $-70°$ und Auftauen, gefolgt durch Mazeration bei 37° in 0.5 *M* KCl, extrahiert.

Das Enzym wurde durch Fraktionieren des Hefeextraktes mit Ammoniumsulfat teilweise gereinigt. Die aktive Proteinfraktion wurde der fraktionierten Analyse nach DERRIEN unterworfen.

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Es wird darauf hingewiesen, dass das Protein, welches im selben Bereich der Salzkonzentration wie die Enzymaktivität aus der Lösung ausfällt, nicht homogen ist. Es stellt wahrscheinlich nicht das Enzymeiweiss dar, sondern inerte Proteine, welche das Enzym adsorbiert haben; das Enzym wird nur einen kleinen Teil des gefällten Eiweisses ausmachen.

Thiaminokinase benötigt Mg für seine Wirkung. Die höchste Aktivität wird bei Konzentrationen von $2 \cdot 10^{-2}$ *M* erreicht. Geringe Konzentrationen von Mn aktivieren das Enzym auch, selbst mehr als vergleichbare Mengen von Mg; aber Konzentrationen von Mn, welche höher sind als $2 \cdot 10^{-3}$ *M* hemmen die Phosphorylierung von Thiamin deutlich.

Die Enzymwirkung wird durch geringe Mengen von anorganischem Phosphat stark angefacht. Der maximale Effekt wird bei einer Konzentration von $2 \cdot 10^{-3}$ *M* erreicht.

Der rohe Hefeextrakt enthält mindestens zwei Faktoren, welche Thiaminokinase hemmen; sie können durch Dialyse entfernt werden und sind thermostabil. Eine von ihnen ist anorganisch.

Das Enzym entwickelt seine grösste Aktivität in dem pH-Bereiche 6 bis 8.

Das gereinigte Präparat synthetisiert Thiaminpyrophosphat schneller aus Thiamin als aus Thiamin-monophosphat.

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