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Synthesis of Thiamine Phosphates in Baker's Yeast

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The synthesis of thiamine di- and triphosphate has been investigated for Swedish and English baker's yeast. Four different types of buffers were used. The most suitable medium for both compounds was a succinate buffer, pH 3.7.

The biosynthesis of thiamine triphosphate was found to start with the formation of thiamine diphosphate. Then, by addition of another orthophosphate, this compound was transformed into thiamine triphosphate.

As to the biosynthesis of thiamine diphosphate the results agree with the earlier opinion that the compound is formed mainly by a transfer of a pyrophosphate group to thiamine.

The results give no definite information about the biosynthesis of thiamine monophosphate. Different alternatives are discussed.

The synthesis of thiamine diphosphate * by yeast enzymes has been thoroughly investigated in intact and plasmolyzed yeast cells [1–4] as well as in purified enzyme systems [5–9]. The major amount of TDP is synthesized in yeast by a transfer of a pyrophosphate group from ATP to thiamine [1,5,6,8]. Only a smaller amount of TDP can be synthesized by transfer of an orthophosphate group to TMP [4].

About the biosynthesis of TTP not very much has been published. It was shown that baker's yeast in addition to TDP also accumulates TTP, when incubated with an excess of thiamine [10,11]. Further Greiling [9] recently showed that a partly purified enzyme system from brewer's yeast could synthesize TTP from thiamine and ATP.

In the present paper the synthesis of TMP, TDP, and TTP in intact baker's yeast has been investigated.

MATERIALS AND METHODS

Yeast. The yeasts used are the English baker's yeast from the Distillers Company Ltd, London **, and the Swedish baker's yeast from The Yeast Company at Rotebro.

Incubation. 20 g of baker's yeast were suspended in a 0.1 M buffer, 0.2 g thiamine added, and the suspension incubated at 27°C with oxygen as the gas phase.

* Abbreviations: T thiamine, TMP thiamine monophosphate, TDP thiamine diphosphate, TTP thiamine triphosphate, AMP adenosine monophosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, TCA trichloroacetic acid, $^{32}P_i$ radioactive inorganic phosphate.

** Kindly supplied by Dr. E. R. Dawson, The Distillers Company Ltd, London.

When searching for the best medium for the biosynthesis of TTP different buffers were used: sodium succinate buffer 0.1 M, pH 3.7 or 6.2, pyrophosphate buffer 0.1 M, pH 6.2, prepared from sodium pyrophosphate and HCl, and monophosphate buffer 0.1 M, pH 6.2, compounded from Na_2HPO_4 and NaH_2PO_4.

Samples taken from the yeast incubations were immediately centrifuged in the cold, the supernatant discarded, and the yeast cells suspended in cold 40 % TCA to a final concentration of 8 %. After 15 min at −10°C the yeast suspension was stirred strongly, centrifuged, and TCA removed from the yellow extract with ether.

Isolation of thiamine phosphates. From 20 g of yeast a final extract of about 15 ml was obtained. 0.25 ml were spread as a narrow line on a Whatman 52 filter paper washed with versene according to Eggleston and Hems [12]. The chromatograms were developed according to Kiessling [13], and the compounds localized by cutting off a strip from each chromatogram and spraying it with an alkaline potassium ferricyanide solution. The areas, containing TTP, TDP, and TMP were cut out, the compounds removed with water, and then re-chromatographed in the same solvent. The chromatography was repeated a third time in the solvent used by Greiling [14] or according to Siliprandi and Siliprandi [15] (*n*-butanol — 1 M HAc pH 5 — H_2O, 70 : 10 : 20). Finally TTP was purified by paper electrophoresis on Whatman 52 washed as described above. The solvent was an acetate buffer pH 5,4 (*cf.* Ref.[15]), the current 3 mA and the time 9 h.

Isolation of ATP and ADP. ATP and ADP were isolated by ion exchange chromatography according to Herbert and Potter [16].

Phosphate determination. Orthophosphate was determined according to Ernster *et al.*[17] whose method is a modification of that of Martin and Doty [18]. Hydrolyzable phosphate of TDP was determined after 15 min at 100°C in 0.5 M H_2SO_4, non-hydrolyzable phosphate of TTP, TDP, and TMP after wet ashing in 0.3 ml conc. $HClO_4$. γ and βP of TTP were removed by hydrolysis by means of potato apyrase according to Kiessling [11], or by hydrolysis for 3 min at 100°C in 0.1 M HCl for γP and for 15 min in 0.5 M H_2SO_4 for $\gamma + \beta$P.

The radioactivity of the phosphate was measured with an M-6 liquid counter tube on the samples used for ^{32}P determinations after dilution to 10 ml with ethanol.

Thiochrome method. In order to determine the amounts of TTP, TDP, and TMP synthesized in yeast after different time intervals the compounds were isolated by paper chromatography, hydrolyzed to thiamine by a yeast phosphatase according to Westenbrink *et al.*[19], transformed into the thiochrome form according to Hjarde, [20] and measured in a Beckman spectrophotometer with a special fluorescence attachment.

RESULTS AND DISCUSSION

Westenbrink has shown that, in the presence of an excess of thiamine, Dutch baker's yeast could go on synthesizing TDP for several hours [21]. Also for the Swedish baker's yeast I found this to be the case for at least 14 h (Fig. 1).

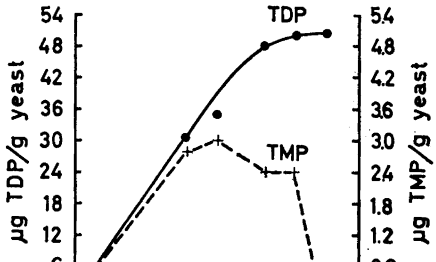

Fig. 1. Synthesis of TDP and TMP in baker's yeast. The yeast is the Swedish baker's yeast, incubated with an excess of thiamine in a 0.1 M succinate buffer, pH 3.7, at 27°C with oxygen as the gas phase. The compounds have been separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

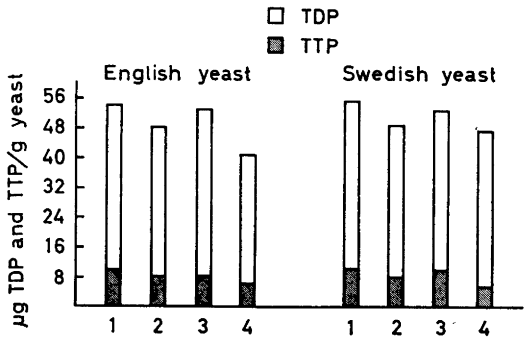

Fig. 2. Synthesis of TTP and TDP in baker's yeast. The yeasts used are an English and a Swedish baker's yeast. The yeasts have been incubated with an excess of thiamine in different buffers (1—4) during 16 h at 27°C with oxygen as the gas phase. The buffers are:

1. 0.1 M succinate buffer, pH 3.7
2. 0.1 M succinate buffer, pH 6.2
3. 0.1 M monophosphate buffer, pH 6.2
4. 0.1 M pyrophosphate buffer, pH 6.2

The compounds have been separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

At the same time the Swedish yeast as well as the English baker's yeast has been shown to synthesize TTP [10,11]. Without addition of special substrates the best medium for synthesis of TTP in the Swedish baker's yeast was found to be either succinate buffer, pH 3.7, or a monophosphate buffer, pH 6.2, and in the English yeast succinate buffer, pH 3.7 (Fig. 2). The same media were also the most favourable for the biosynthesis of TDP (Fig. 2). Buffers tested were succinate buffers, pH 3.7 and 6.2, monophosphate buffer pH 6.2 and pyrophosphate buffer, 6.2, all 0.1 M. In the following experiments with Swedish baker's yeast 0.1 M succinate buffer, pH 3.7, has been used.

Addition of thiamine phosphates to yeast cells immediately results in a hydrolysis of the phosphate by phosphatases, very likely situated at the cell

Table 1. Radioactivity of the different phosphate groups in thiamine phosphates isolated from baker's yeast. Incubation at 27°C with oxygen as the gas phase in a 0.1 M succinate buffer, pH 3.7 and with an excess of thiamine. $^{32}P_i$ was added after 4.5 h incubation, and samples for analysis were taken after another 2 and 4 h, respectively.

Thiamine compound	4.5 + 2 h (counts/5 min/µg P)			4.5 + 4 h (counts/5 min/µg P)		
	αP	βP	γP	αP	βP	γP
TTP	485	819	2 265	1 188	1 690	6 100
TDP	1 315	1 765	—	3 260	3 360	—
TMP	140	—	—	1 440	—	—

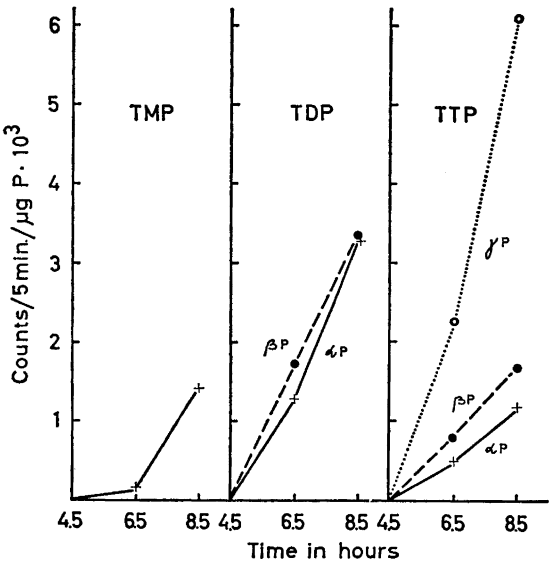

Fig. 3. Radioactivity of α, β and γP from TMP, TDP and TTP. Swedish baker's yeast was incubated with an excess of thiamine in 0.1 M succinate buffer, pH 3.7, with oxygen as the gas phase at 27°C. The first part of the incubation (up to 4.5 h) was performed in the absence, and the second part in the presence of radioactive phosphate. The compounds have been separated by means of paper chromatography.

surface. Besides it is doubtful, whether or not the phosphorylated compounds could penetrate the cell membrane. The yeast cells, however, transfer added thiamine, which easily penetrates the cell membranes, into thiamine phosphates which give origin to starting-points for further synthesis. Thus yeast was allowed to accumulate thiamine phosphates for a certain interval. Then radio-

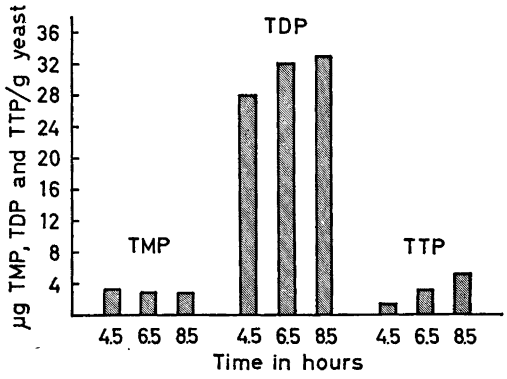

Fig. 4. Amount of TMP, TDP and TTP in baker's yeast after different time intervals. Incubation as in Fig. 3. The compounds were separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

active inorganic phosphate was added, and the incubation continued. Samples were taken after different time intervals, the thiamine phosphates isolated, and the radioactivity of the different phosphate groups determined. This permits to see how TTP and possibly also TDP are synthesized from already formed thiamine phosphates.

Results from an experiment are shown in Table 1 and Fig. 3. After 4.5 h incubation in the absence of $^{32}P_i$, radioactive phosphate was added, and samples taken after another 2 and 4 h incubation. Fig. 4 shows the amounts of TMP, TDP, and TTP at the end of the different intervals.

Two ways are open for the biosynthesis of TTP: either by a transfer of a pyrophosphate group to TMP or a monophosphate group to TDP. The biosynthesis of TDP is thought to be brought about by a transfer of a pyrophosphate group to thiamine. About the way of formation of TMP nothing is known, but it is generally assumed to be generated by hydrolysis of TDP.

From Table 1 and Fig. 3 it is seen that the radioactivity of γP of TTP increases much more rapidly than that of β and αP. As ATP has been shown to be the phosphate donor at the synthesis of TDP from thiamine as well as at the synthesis of TTP in isolated systems, the synthesis of TTP in intact yeast cells, according to Table 1 and Fig. 3 very probably may be written:

$$TDP + ATP \longrightarrow TTP + ADP$$

that is, TDP, already synthesized from thiamine and ATP, receives a third phosphate from ATP.

Another possibility for the synthesis of TTP is:

$$TMP + ATP \longrightarrow TTP + AMP$$

This possibility is, however, excluded by the circumstances that βP in TTP is much less labelled than γP. Very probably the βP of ATP becomes labelled somewhat slower than the γP, but the differences can't suffice for the explanation of the different labelling of β and γP of TTP. This becomes obvious from the synthesis of TDP. From experiments with partly isolated thiamino kinases [5,6,8,9], and with autholyzed yeast cells [4] the synthesis of TDP was found mainly to proceed according to the reaction:

$$T + ATP \longrightarrow TDP + AMP$$

The results in Table 1 and Fig. 3 show that αP of TDP is only slightly less radioactive than βP. The differences can be explained by one of two assumptions: either that βP of ATP does not exchange its phosphate as rapidly as γP, or that TDP to a small extent is also synthesized from TMP. Whatever the explanation may be the fact remains that the differences in radioactivity between βP and γP of ATP are too small to account for the differences between β and γP of TTP. It therefore seems most satisfactory to explain the results by ascribing the synthesis of TTP to a transfer of a phosphate group to previously formed TDP.

It is difficult to give a satisfactory explanation for the figures in Table 1 on the radioactivity of TMP. The general opinion is that TMP is more likely formed by hydrolysis of TDP than by a direct phosphorylation of thiamine. On the contrary Greiling reported that he had found an enzyme in brewer's

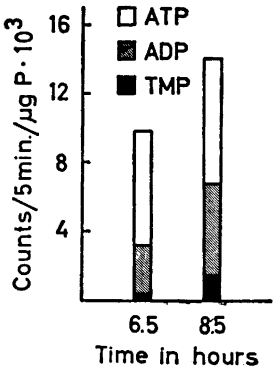

Fig. 5. Radioactivity of TMP and of hydrolyzable phosphate of ATP and ADP after 2 and 4 h incubation respectively. The samples are from the experiment described in Fig. 3. ATP and ADP were isolated on a Dowex 1 column according to Herbert and Potter [16], and the radioactivity measured on the phosphate hydrolyzed during 10 min at 100°C in 1 M H_2SO_4. TMP was isolated by paper chromatography (see Methods), and the radioactivity determined after wet ashing in $HClO_4$.

yeast which, at most to a small extent, is able to synthesize TMP from thiamine and ATP [9].

TMP accumulates only during the first hours, when yeast is incubated with thiamine (Figs. 1 and 4). Further incubation causes a decrease of the amounts of TMP. The decrease may either depend on the fact that TMP is hydrolyzed to thiamine or that it is transformed into another thiamine phosphate (TDP or TTP). However, it does not necessarily mean that TMP is no longer formed. The increasing radioactivity of TMP, isolated from the yeast during periods when the total amount of TMP is decreasing, indicates that a formation of the compound still takes place.

If TMP, as is generally believed, is formed from TDP, the quantity and also the radioactivity, as with αP of TDP, should increase altogether continuously. This is, however, not the case (Figs. 1 and 3). If TMP is synthesized by a transfer of a phosphate from ATP to thiamine, the radioactivity should likewise increase nearly straight-lined as in βP of TDP (the decrease in the total amount of TMP should cause the curve to bend only slightly upwards). The results in Fig. 3 makes, however, ATP a less probable phosphate donor at a possibly occurring synthesis of TMP.

Table 2. The radioactivity of TTP and TDP from yeast after further incubation with an excess of non-radioactive phosphate. First part of the incubation was performed as described in Table 1. Incubation time 16 h. $^{32}P_i$ was added after 8 h incubation and a sample taken after another 8 h. The remaining yeast was then washed twice with a succinate buffer, and the incubation continued in the presence of an excess of non-radioactive phosphate. Samples were taken after 1 and 2 h, respectively.

Thiamine compound	Counts of hydrolyzable P/5 min/µg P		
	16 h	17 h	18 h
TTP	743	762	808
TDP	440	503	495

From Fig. 5 it is seen that ADP becomes labelled at a slower rate than ATP. The increase along a straight line of the radioactivity of ADP eliminates also ADP as a possible phosphate donor.

Thus the results give no information about the way in which TMP is formed in baker's yeast. The comparison of the curve for TMP and αP of TDP (Fig. 3) allows only the conclusion that TMP is not at all, or only to a small extent, an intermediate in the biosynthesis of TDP from thiamine in intact baker's yeast when present in biological concentrations (*cf.* Ref.[4]).

Previously it has been shown that γP in yeast TTP rapidly becomes radioactive, when glucose is added together with radioactive phosphate to a yeast suspension.[22] This is not the case in the absence of glucose. The increase of radioactivity in the γP of TTP in the presence of glucose was therefore looked upon as depending on a turnover of the terminal phosphate in already existing TTP rather than on a further synthesis of TTP.

In order to make sure that in the present experiments (Table 1), performed in the absence of glucose, the high radioactivity of γP in TTP compared with that in β and αP did not depend on a turnover of the type described above, the following control was made: yeast was incubated 16 h as described in Table 1. After 8 h radioactive inorganic phosphate was added. At 16 h the yeast was washed twice with succinate buffer to remove excess of thiamine and $^{32}P_i$. It was then suspended in a new buffer consisting of three parts 0.1 M succinate buffer and one part 0.1 M phosphate buffer, pH 3.7. Samples were taken after 1 and 2 h incubation. The results are seen in Table 2. No decrease of the radioactivity in the hydrolyzable phosphate of TTP and TDP was found. Neither does any remarkable synthesis of TTP take place any longer, and the relatively constant radioactivity in its phosphate indicates that no exchange of the terminal phosphate is going on.

Greiling recently reported that he had been able to synthesize TTP from thiamine with a purified enzyme system from brewer's yeast [9]. He used ATP labelled in the terminal phosphate, and found the ratio between the activities of γ, β and αP in TTP to be 3:3:1. In TDP the ratio between β and αP was 3:1. The conclusions drawn from his results agree with those for the synthesis of TTP in intact yeast cells reported in the present paper.

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