STUDIES ON THIAMINE TRIPHOSPHATE

by

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Thiamine triphosphate* has been synthesized by VELLUZ, AMIARD AND BARTOS[1] and VISCONTINI, BONETTI AND KARRER[2]. Experiments of VELLUZ, AMIARD AND BARTOS[3] indicate that chemically synthesized TTP has about 25% the activity of TDP in a pyruvate decarboxylating system.

In order to ascertain whether synthetic TTP contains any TDP, synthetic material was examined paper-chromatographically by KIESSLING AND LINDAHL[4] and SILIPRANDI AND SILIPRANDI[5]. Appreciable amounts of TDP were always found in the samples examined. Paper chromatography was also used by KIESSLING AND LINDAHL[4] and SILIPRANDI AND SILIPRANDI[5] to isolate TTP. Purified TTP showed the same activity as TDP in a pyruvate decarboxylating system of washed yeast[5] but it was inactive with purified carboxylase (GERTRUDIS DE LA FUENTE AND DIAZ-CADAVIECO)[6].

In 1953 it was shown by the present author that a thiamine phosphate, very likely a thiamine triphosphate, was synthesized by living baker's yeast (KIESSLING[7]).

This paper describes the results of further studies on the isolation of yeast TTP by paper chromatography, the action of potato apyrase on TTP from yeast and on chemically synthesized TTP, and the activity of TTP, synthesized enzymically by yeast and synthesized chemically, in an isolated pyruvate decarboxylating system.

EXPERIMENTAL AND RESULTS

Special chemicals

TTP and TDP were commercial samples (Roche Products, Ltd.) synthesized according to VISCONTINI[5], BONETTI[5] AND KARRER[2]. TTP was always purified by paper chromatography before use as described below.

Pure sodium pyruvate was prepared by a modification of Robertson's method (BARTLEY AND DAVIES[8]).

The baker's yeast was partly from Uppsala Ångkvarn, Uppsala, Sweden, partly from the United Yeast Company, Ltd., Manchester.

Chemical estimations

The thiamine phosphates were converted into free thiamine by enzyme hydrolysis of the phosphate groups with a yeast phosphatase prepared according to WESTENBRINK AND STEYN-PARVÉ[9].

The thiamine formed was oxidized with alkaline ferricyanide to thiochrome and the fluorescence was estimated in a Beckman spectrophotometer with a special fluorescence attachment.

The thiamine phosphate spots on the chromatograms were localized by spraying the papers with an alkaline ferricyanide solution and viewing them under UV of 340 mμ. Phosphate was estimated after wet ashing (HANES AND ISHERWOOD[10]) by the method of BERENBLUM AND CHAIN[11].

* Abbreviations: TTP thiamine triphosphate, TDP thiamine diphosphate, TMP thiamine monophosphate.

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Synthesis and isolation of thiamine phosphates from baker's yeast

100 g of baker's yeast, without added substrate were incubated at 27° with 300 ml 0.1 *M* pyrophosphate buffer, pH 6.5 ($Na_4P_2O_7$ brought to pH 6.5 with HCl) and 0.3 g of thiamine. After 16 hours the reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 8%. After 30 minutes at —10° pH was adjusted to 6 and the sample centrifuged. From the clear yellow extract the thiamine phosphates were adsorbed on Fuller's earth (10 g) and then eluated by pyridine-acetic acid-H_2O (4:0.1:1). 4 ml pyridine mixture was used per gram Fuller's earth. The pyridine was removed by ether and the phosphates were precipitated by 100 ml alcohol and ether (4:1). After the precipitate had been dissolved in 3 ml water the thiamine phosphates were separated by means of paper chromatography. Different methods are available (BARTLEY[12], SILIPRANDI AND SILIPRANDI[5], KREBS AND HEMS[13], KIESSLING AND LINDAHL[4]). In view of the fact that the last mentioned method contains a rather acid solvent it was unsuitable for quantitative analysis as hydrolysis might occur. The method used by KREBS AND HEMS[13] for the separation of nucleotides proved very satisfactory for separating TTP from TDP, TMP, flavines and nucleotides. This method was therefore adopted in the present investigation when TTP was to be isolated. However it fails to separate TDP quantitatively from TMP, for which purpose the method of BARTLEY[12] or SILIPRANDI AND SILIPRANDI[5] was used.

The thiamine phosphates separated with the methods mentioned were extracted from the chromatograms with water or, if the mixture had been put on as a line, the lines were cut out and the compounds run off the strips with water in the same way as described for ATP by HEMS AND BARTLEY[14]. Thus a sample of TTP containing about 200 μg per ml could be obtained. By repeating the chromatography of the TTP sample, it was shown to contain only a very slight impurity of TDP which was removed by the second chromatography (as a third repetition of the procedure showed). The sample then gave only one spot with an R_F value of 0.64. Chemically synthesized TTP gave a value of R_F of 0.66. Chromatographed together with yeast extract both forms gave R_F values of 0.64.

The chromatograms also showed that in addition to TTP and TDP (the synthesis of the latter compound by yeast has been extensively investigated by WESTENBRINK, STEYN-PARVÉ AND VELDMAN[15]) TMP was also formed when yeast was incubated with thiamine. Under the test conditions the ratio between TTP:TDP:TMP was 22:100:13. TMP may have been formed by direct phosphorylation of thiamine, as suggested by STEYN-PARVÉ[16] and demonstrated by VAN THOAI AND CHEVILLARD[17] for brewer's yeast. However TMP may also have been formed through primary pyrophosphorylation of thiamine, followed by a dephosphorylation of TDP.

Action of apyrase on TTP from yeast and on chemically synthesized TTP.

A selective hydrolysis of the γ-P of ATP has been described by LEE AND EILER[18] who showed that incubation of a potato apyrase preparation with ATP solution at 0° resulted in the quantitative hydrolysis of the γ-P. At 40° both γ-P and β-P were hydrolysed.

Potato apyrase prepared according to LEE AND EILER[18] was incubated with chemically synthesized TTP in the same way as described for ATP (LEE AND EILER[18]). After 60 minutes at 0° nearly all TTP had been hydrolysed to TDP as shown by paper chromatography, phosphate estimations from a chromatogram (Table I) and estima-

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TABLE I

HYDROLYSIS OF CHEMICALLY SYNTHESIZED TTP AND TTP FROM YEAST BY POTATO APYRASE AT DIFFERENT TEMPERATURES

To the TTP samples (about 0.25 ml) were added 0.1 ml of enzyme, 0.09 ml of 0.11 *M* $CaCl_2$, 0.1 ml of 0.1 *M* succinate buffer pH 6.5 and water to a final volume of 1.5 ml. The reaction was stopped by addition of TCA to a final concentration of 9%, the proteins were removed by centrifugation and the pH of the supernatant solution was adjusted to 6.5. The compounds were separated by means of paper chromatography, the spots cut out and phosphate estimated after wet ashing. The figures are given as μg P. They refer to 40 μl solution.

	Chemically synthesized TTP			*Yeast TTP*		
	0 min	*60 min* 0°	*50 min* 40°	*0 min*	*120 min* 0°	*120 min* 40°
TTP	1.14	0.14	0.06	1.49	0.42	0.21
TDP	0.09	0.74	—	0.12	0.65	0.24
TMP	—	0.06	0.49	—	0.15	0.38

tions of hydrolysed phosphate in samples taken at different times (Fig. 1). After the same time at 40° most TTP had been converted to TMP (Table I and Fig. I). TTP isolated from yeast was hydrolysed in the same way, except that the reaction was slower (Fig. 2). Even after 120 minutes at 40° some TTP could still be detected on the chromatograms. At 0° the hydrolysis of the enzymically prepared TTP yielded TMP more rapidly than the chemically synthesized TTP (Table I).

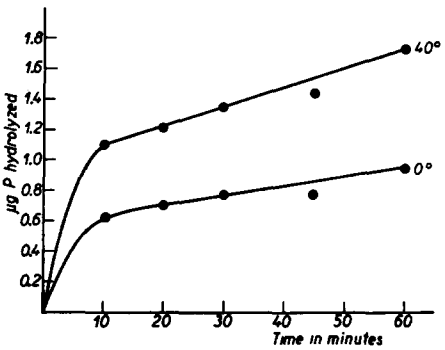

Fig. 1. Incubation of chemically synthesized TTP with potato apyrase at 0° and 40°. The reaction mixture contained 0.24 ml enzyme, 0.18 ml 0.11 *M* $CaCl_2$, 0.20 ml 0.1 *M* Na-succinate pH 6.5, TTP and H_2O to a final volume of 2.92 ml. Samples (0.4 ml) containing 3.07 μg P as TTP-phosphate were removed at the intervals indicated, pipetted into 5 ml *N* H_2SO_4 and anlysed for phosphate by the procedure of BERENBLUM AND CHAIN[11].

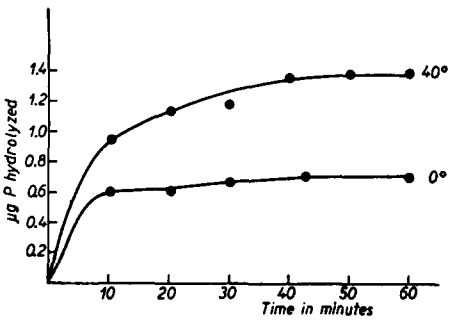

Fig. 2. Incubation of yeast TTP with potato apyrase at 0° and 40°. Experimental conditions as in Fig. 1. The samples removed contained 3.33 μg P as TTP-phosphate.

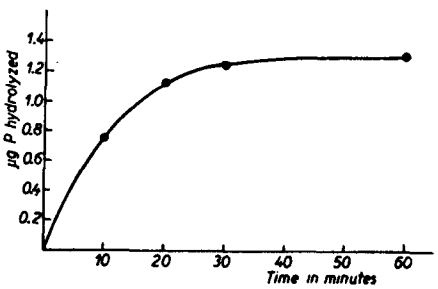

Fig. 3. Incubation of equal parts of yeast TTP and chemically synthesized TTP with potato apyrase at 40°. Experimental conditions as in Fig. 1. The samples removed contained 3.20 μg P as TTP-phosphate.

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The Table shows that small amounts of TDP existed already in the control sample. This sample had been treated in the same way as the samples incubated for 60 minutes except that TCA was added before the enzyme to prevent enzymic action. The TDP must therefore have been formed by acid hydrolysis of TTP as it did not exist in the TTP sample before TCA addition.

A mixture of equal parts of yeast TTP and chemically synthesized TTP incubated with potato apyrase at 40° (Fig. 3) shows a rate of hydrolysis equal to that of yeast TTP alone.

The capacity of TTP from yeast and chemically synthesized TTP to replace TDP in a pyruvate decarboxylating system

VELLUZ, AMIARD AND BARTOS[3] found that chemically synthesized TTP could replace TDP in a washed yeast system, but 4 times more TTP was required. Their TTP was not purified chromatographically. SILIPRANDI AND SILIPRANDI[5] found in a washed yeast system that chromatographically purified chemically synthesized TTP had the same activity as TDP, while GERTRUDIS DE LA FUENTE AND DIAZ-CADAVIECO[6] found no activity with TTP purified in the same way, and tested with purified apocarboxylase.

This result suggests that the positive effect observed by VELLUZ, AMIARD AND BARTOS[3] was due to contamination of their TTP with TDP and those of SILIPRANDI AND SILIPRANDI[5] to the enzymic formation of TDP from TTP in their crude yeast preparation.

This is borne out by the following experiment. Carboxylase was prepared according to GREEN, HERBERT AND SUBRAHMANYAN[19]. Yeast TTP, and chemically synthesized TTP purified by chromatography three separate times as described earlier were added to the carboxylase with pyruvate as a substrate. The CO_2 formed was measured manometrically in a Warburg apparatus at 30°. The carboxylase was also tested for phosphatase activity by incubating it with TTP for 40 minutes without other substrate, and then chromatographing the sample. No visible hydrolysis of TTP had taken part during this time. Results from a manometric experiment are reproduced in Fig. 4 which shows that TTP from yeast, as well as chemically synthesized TTP has no significant ability to replace TDP in a pyruvate decarboxylating system.

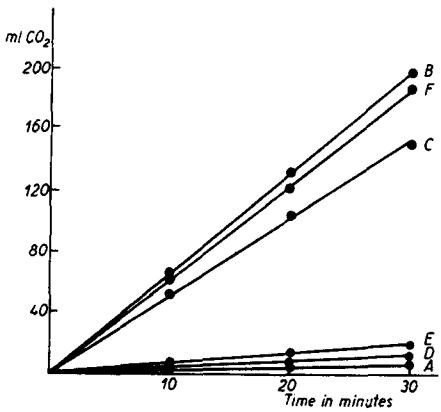

Fig. 4. Activity of TTP and TDP from yeast and chemically synthesized TTP and TDP in a yeast carboxylase system. Each manometer vessel contained 0.3 ml of enzyme, 0.5 ml of *M* pyruvate, 0.3 ml of 0.5 *M* citrate buffer pH 6, 0.3 ml of 0.1 *M* $MnCl_2$ in a total volume of 3.3 ml. The concentrations of the thiamine phosphates are all 3 μg determined as free thiamine with the thiochrome method. A, blank; B, chemically synthesized TDP; C, TDP isolated from yeast; D, chemically synthesized TTP; E, TTP isolated from yeast; F, chemically synthesized TDP + TTP isolated from yeast.

TDP prepared from yeast, tested in the same experiment showed a good activity although slightly lower than chemically synthesized TDP. This indicates that the inability of yeast TTP to replace TDP is not a result of solvent impurities eluted from the chromatograms as the yeast TDP would

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be contaminated to the same degree. Yeast TTP added together with TDP did not increase the activity compared with the same amount of TDP alone. On the contrary it had a slight inhibiting effect Fig. 4).

The yeast TTP became active in the decarboxylation test after treatment with the potato apyrase at 0°. Fig. 5 indicates that the TTP has partly been hydrolysed to TDP by the apyrase; this is in agreement with the results obtained in the paper chromatographic investigation of the apyrase activity and with the estimations of hydrolysed phosphate in the apyrase experiments at 0°.

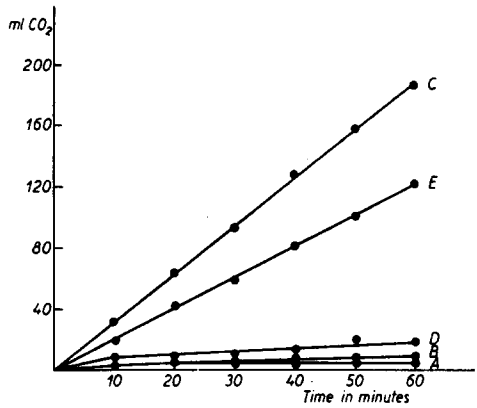

Fig. 5. Activity of TTP from yeast in a yeast carboxylase system after incubation with potato apyrase for 60 minutes at 0°. The manometer vessels contained the same amounts of additions as is described in Fig. 4. A, blank; B, blank + apyrase; C, TDP isolated from yeast; D, TTP isolated from yeast (not preincubated with apyrase); E, TTP isolated from yeast and preincubated with apyrase for 60 minutes at 0°.

DISCUSSION

Baker's yeast has been shown to synthesize considerable quantities of a thiamine phosphate in the presence of thiamine and pyrophosphate, which is very similar to, if not identical with the synthetic TTP made by the synthetic method of VISCONTINI, BONETTI AND KARRER[2]. There are differences between the synthetic and enzymically prepared substances. The R_F values for the purified compounds are not exactly the same (yeast TTP 0.64 and chemically synthesized TTP 0.66) and the hydrolysis of enzymically prepared TTP by potato apyrase does not proceed in quite the same way as with the chemically synthesized form. These differences may be due to an impurity from the yeast, which is not removed by the methods used. In the case of the R_F values this explanation is supported by the fact that the R_F values became the same (0.64) for yeast TTP and chemically synthesized TTP when the two compounds were chromatographed together with yeast extract. In the case of phosphate hydrolysis by potato apyrase the hydrolysis rate in the mixture of yeast TTP and chemically synthesized TTP was about the same as for yeast TTP alone. Thus the hydrolysis of chemically synthesized TTP was inhibited to the same degree by impurities in the yeast TTP sample as the hydrolysis of enzymically synthesized TTP.

When tested for cocarboxylase activity in a pyruvate decarboxylating system neither TTP from yeast nor chemically synthesized TTP had any appreciable activity. With TTP from yeast no investigation has been made up to now, but in the case of chemically synthesized TTP different results are given varying from no ability to replace TDP (GERTRUDIS DE LA FUENTE AND DIAZ-CADAVIECO[6]) to 100 percent ability (SILIPRANDI AND SILIPRANDI[5]). When activity was reported the investigation had been made with a washed yeast system. In the present paper a partly purified isolated carboxylase has been used, and the inability of chemically synthesized TTP to replace TDP is in agreement with the results of GERTRUDIS DE LA FUENTE AND DIAZ-CADAVIECO[6] using the same system.

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SUMMARY

1. The thiamine phosphates formed when baker's yeast was incubated with thiamine and pyrophosphate were analysed by paper chromatography. In addition to thiamine diphosphate (cocarboxylase) and thiamine monophosphate, a compound containing thiamine and phosphate accumulated, which had almost the same R_F value as synthetic thiamine triphosphate. It was taken to be enzymically synthesized TTP.

2. Chemically synthesized TTP incubated with a potato apyrase was hydrolysed in the same way as ATP suggesting that the arrangement of the phosphate groups in the two compounds is the same. Enzymically synthesized TTP incubated with potato apyrase was hydrolysed in the same way but more slowly and the two hydrolysing steps were not so distinct as in the case of the chemically synthesized TTP. These differences were shown to be very probably caused by an inhibitor contaminating the yeast TTP.

3. Neither the enzymically synthesized TTP nor the chemically synthesized TTP can replace cocarboxylase in a purified pyruvate decarboxylating system. Only after preincubation of the TTP with apyrase at o°, which caused the removal of the γ-phosphate of TTP, was cocarboxylase activity obtained.

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