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Incorporation of Radioactive Phosphate into Thiamine Phosphates in Yeast

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The incorporation of radioactive phosphate into thiaminetri-, di-, and monophosphate in baker's yeast has been examined. Yeast containing only its natural content of thiaminephosphates was used as well as yeast made rich in these compounds by preincubation with excess of thiamine.

In both cases radioactive phosphate was incorporated to a higher degree into thiamine triphosphate than into the other thiamine phosphates. The incorporation into thiamine triphosphate was localized especially to the terminal phosphate.

No appreciable synthesis of thiamine phosphates was going on during incorporation. Therefore the high turnover rate of radioactivity in the terminal phosphate of thiamine triphosphate with glucose as a substrate has been interpreted as caused by the compound in question taking part in the phosphate transport in the metabolism.

Yeast has a considerable ability of transforming added thiamine into thiamine diphosphate * (cocarboxylase) [1-4].

The role of TDP has long been known to be that of a decarboxylating coenzyme. Moreover Kiessling and Lindahl [5] found a turnover of the phosphate groups in TDP with a liver cyclophorase system when pyruvate was the substrate. Bartley [6] could not verify these results when using a kidney system and adding TDP, differing thereby from our experiments without added TDP. In a paper by Ogata *et al.*[7], however, it was shown that a rapid transfer of phosphate occurred from ^{32}P labelled TDP into ATP by a rat liver homogenate as well as by a cyclophorase system.

In 1954 it was shown that baker's yeast synthesizes besides TDP also thiamine triphosphate [8]. TTP exists likewise in yeast not preincubated with an excess of thiamine, but to a very small extent as compared with TDP.

* Abbreviations: T thiamine, TMP thiamine monophosphate, TDP thiamine diphosphate, TTP thiamine triphosphate, ATP adenosine triphosphate, ADP adenosine diphosphate, DPN diphosphopyridine nucleotide, Pi inorganic phosphate.

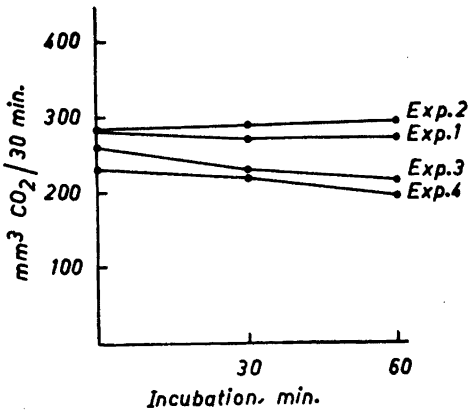

Fig. 1. TDP content of yeast during incubation with glucose and ^{32}P. The yeast had been preincubated with excess of thiamine.

Samples of 2 ml were taken at 0, 60 and 120 minutes, and added to 5 ml 0.1 M HCl at 100°, boiled for 1 minute, cooled, and centrifuged. The clear extracts were neutralized to pH 6, and TDP determined according to Westenbrink *et al.* with the Warburg method [13].

The question soon arose whether TTP could replace TDP in a decarboxylating system. Up to now only a system decarboxylating pyruvate has been examined. It has been shown by Gertrudes de la Fuente [9] * that chemically synthesized TTP, and by Kiessling [10] that both chemically synthesized TTP and TTP isolated from baker's yeast could not replace TDP as a coenzyme in this enzyme system from yeast.

In the present paper the incorporation of radioactive phosphate into yeast TTP is studied and compared with that into TDP, TMP, and ATP. The experiments have been carried out partly with yeast containing only its natural amount of thiamine phosphates, partly with yeast enriched in thiamine phosphates by preincubation with thiamine.

EXPERIMENTAL

Special chemicals. TDP and T were commercial samples (Roche products Ltd.). TMP was prepared by 20 minutes' hydrolysis of TDP at 100°. Paper chromatography proved all TDP to be transformed into TMP.

Chemical estimations. Phosphate was determined according to Berenblum and Chain [11] in a Beckman spectrophotometer. Measurements of radioactivity were made on samples of the diluted, wet ashed material. A M-6 liquid counter (20th Century Electronics) was used.

Enzyme preparation. Potato apyrase was prepared by Dr. W. Bartley according to Lee and Eiler [12].

* Recently a paper appeared by Rossi-Fanelli *et al.* (*Arch. Biochem. Biophys.* **58** (1955) 237) where the results of de la Fuente were confirmed.

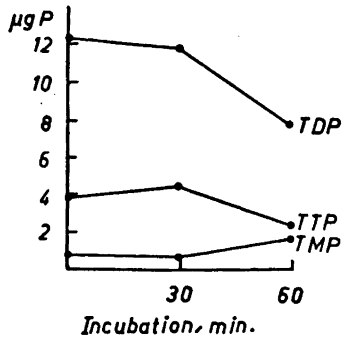

Fig. 2. TTP, TDP, and TMP in yeast rich in thiamine phosphates during incubation with glucose and inorganic phosphate.

Samples were taken at 0, 30 and 60 minutes, the thiamine phosphates isolated, and the phosphate determined after wet ashing.

Preparation and incubation of the particulate suspensions. The content of TTP in biological material in general is very low, but yeast is in my experience a better source than animal tissues. Furthermore, the TTP content of yeast can be very much increased. Preliminary experiments by the present author with animal tissues (liver, heart, brain) never showed any analogous accumulation. Thus yeast has been used in this investigation **. The first part of this paper deals with the naturally occurring TTP in yeast with regard to its capacity of incorporating radioactive phosphate. But even when starting with large amounts of yeast (1 kg) the content of TTP was so small that never sufficient TTP could be isolated to allow an enzymatic analysis of the incorporation of ^{32}P into its different phosphate groups. Thus for the carrying out of such analyses as is treated in the later part of this paper, yeast was used which by preincubation with an excess of T had been made rich in TTP and of course also in TDP and TMP [10]. This preincubation was allowed to go on for 20 hours, after which only a very slow synthesis of thiamine phosphates still occurred. To eliminate this remaining synthesis, the yeast was centrifuged down, mixed with NaCl (20 %), and washed once with succinate buffer pH 5 and twice with distilled water. The NaCl treatment was intended to cause a slight destruction of the cell surface. Excess of T could thus be washed away from the cells, and when yeast pretreated in this way with glucose and phosphate at 27° was incubated in a succinate buffer pH 6, no synthesis of TDP took place (Fig. 1). On the contrary a slight breakdown occurred. The purpose of eliminating the synthesis was to make sure that a possibly occurring incorporation of ^{32}P during the following incubation was not caused by further synthesis of thiamine phosphates from thiamine.

The concentration of TTP, TDP, and TMP throughout an experiment can be seen in Fig. 2. Phosphate was determined after wet ashing. During the first 60 minutes the concentrations are rather constant, but between 60 and 120 minutes TTP and TDP are decomposed (in this experiment TDP was decomposed more rapidly than in the experiments of the same type shown in Fig. 1), and a slight accumulation of TMP takes place.

When examining the incorporation of ^{32}P into the different phosphate groups of the thiamine phosphates 200 g of yeast pretreated as described above was incubated with 0.5 mC inorganic radioactive phosphate and 4 g glucose. Samples containing 50 g yeast were taken at 0, 10, 30, and 60 minutes, and trichloroacetic acid added to a final concentration of 9 %. Then TTP, TDP, and TMP were isolated according to Kiessling [10], and ATP and Pi according to Krebs *et al.*[15]

** The yeast was a baker's yeast from Uppsala Ångkvarn Ltd, Sweden.

When studying the incorporation of ^{32}P into TTP in yeast only containing its natural amount of TTP, fresh baker's yeast was used without adding T and treating it with NaCl, but otherwise with the same additions as to the NaCl treated yeast.

Paper chromatographic separation of thiamine phosphates. For the separation of TTP from other phosphates different paper chromatographic methods have been developed, and the author considers one using *iso*butyric acid-ammonia-versene the most suitable [10]. However, during the first part of this investigation, *viz.* that with yeast only containing its natural amount of TTP, this solvent was not yet known to separate also TTP from other compounds. Instead the method described by Siliprandi and Siliprandi [14] was used. Here ATP and ADP have the same R_F value as TTP, and had to be eliminated prior to chromatography. This was done by adsorbing the thiamine phosphates on Fuller's earth at pH 6, as described before [10]. No ATP, ADP, or other nucleotides then contaminated the thiamine phosphates as was proved by using a paper chromatographic method of Burrows *et al.*[16], separating nucleotides from thiamine phosphates, but not TTP, TDP, and TMP from each other (Fig. 3 A).

Enzymatic hydrolysis of γP and βP from TTP. Lee and Eiler [12] found that a potato apyrase, when incubated with ATP at 0° only splits off the γP, and at 40° both γ and βP. When incubating TTP with this enzyme, Kiessling demonstrated that TTP was attacked in the same way [10]. This enzyme was therefore used in the present investigation to split off the different phosphate groups from TTP in order to compare the incorporation of ^{32}P into them.

RESULTS

Incorporation of ^{32}P into thiamine phosphates in yeast not preincubated with thiamine

In fresh baker's yeast incubated with glucose and radioactive inorganic phosphate the incorporation into TTP, TDP, and TMP is clear from chromatograms reproduced in Fig. 3 B.

It is obvious that the incorporation of ^{32}P into TTP is much more rapid than that into TDP or TMP. No synthesis of TDP occurred during the incubation as evidenced by measurements with the Warburg manometric method.

The incorporation into TTP was dependent on the presence of a substrate. In the experiment shown in Fig. 3 B no substrate was added before the lapse of 20 minutes, and the incorporation of ^{32}P into TTP was very slow. At 20 minutes glucose was added, and the incorporation rate increased immediately.

Hydrolysis of TTP to TDP and TMP. When keeping a TTP sample at room temperature it decomposes only slowly to TDP and TMP. After 15 minutes in N HCl at 100° C it is nearly completely broken down to TMP, but shorter hydrolyzing times give mixtures of TDP and TMP.

A TTP sample isolated from yeast which had been incubated as described above was put as two spots on a chromatogram. A drop of diluted HCl was then added to one of the spots, and dried with hot air. This was repeated until total hydrolysis of the TTP of the spot was supposed to have taken place. Fig. 3 C shows the results. TTP was hydrolyzed, and in its place TDP and TMP spots were formed. The radioactivity of the TDP spot was low, and no appreciable activity was found of the TMP spot. On the other hand the ^{32}P was recovered as inorganic phosphate. From Fig. 3 C it appears that chiefly the γP of TTP becomes radioactive during 20 minutes incubation with substrate.

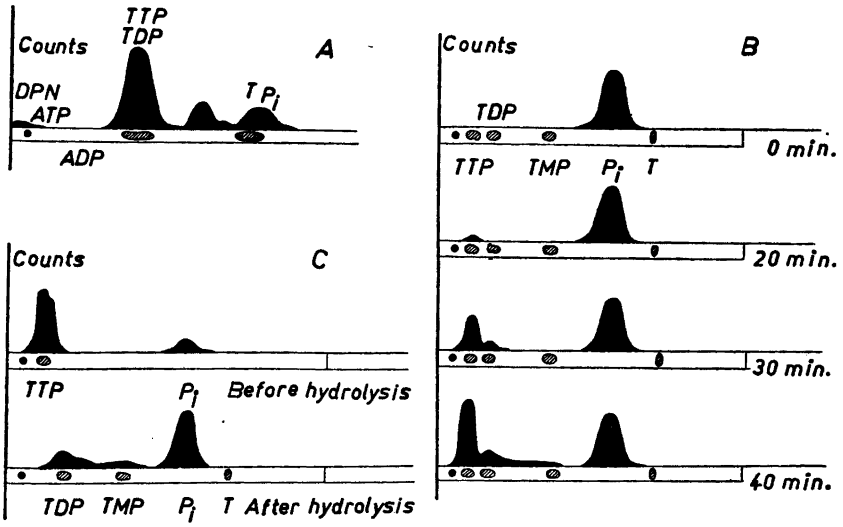

Fig. 3 A. Paper chromatographic separation of thiamine phosphates after removal of ATP, ADP, and DPN.

The chromatographic method is one described by Burrows *et al.*[16] in a solvent containing 60 vol. acetone and 40 vol. 35 % v/v formic acid. The chromatogram shows the absence of radioactivity in the spots corresponding to the R_F-values of the nucleotides. Moreover, when examinating the chromatograms in UV-light 260 mμ no dark spots could be seen. The thiamine phosphates could, however, be detected easily in UV-light after spraying the chromatograms with alcaline $Fe(CN)_6^{3-}$. They are marked out as dark spots in the figure.

B. Paper chromatographic separation of TTP, TDP, TMP, and Pi from yeast.

The yeast was incubated with ^{32}P and glucose in a succinate buffer pH 6. Glucose was not added until after the lapse of 20 minutes. Samples were taken at 0, 20, 30, and 40 minutes, and the thiamine phosphates separated. The paper chromatographic method is one described by Siliprandi and Siliprandi [14]. The thiamine compounds were made visible in UV-light as described in fig. 3 A, and marked out as dark spots.

C. TTP from a yeast sample taken after 40 minutes incubation as described in Fig. 3 B before and after hydrolysis.

As seen from the upper figure this gives only a single spot without hydrolysis. The lower figure shows the same, only here the TTP was hydrolyzed on the paper chromatogram with HCl in hot air before starting the second chromatography. TTP is transformed into TDP and TMP, and the radioactivity is mainly recovered as inorganic phosphate.

The determination of radioactivity on the strips was done according to Lindberg and Hummel [17].

A rough analysis of the radioactivity per μg P was made on TTP and ATP collected from several chromatograms (Table 1). The radioactivity of TTP was about half that of ATP.

Incorporation of ^{32}P into thiamine phosphates in yeast preincubated with an excess of thiamine

In order to obtain in the yeast an amount of TTP sufficient for a real analysis of ^{32}P in the different phosphate groups preincubation with an excess

Table 1. Radioactivity of TTP and ATP from yeast incubated with radioactive phosphate and glucose in a succinate buffer pH 6. The yeast contained only its natural amount of thiamine phosphates, i. e. *no preincubation with thiamine had been carried out before the addition of ^{32}P and substrate. TTP was isolated by paper chromatography (Siliprandi and Siliprandi)* [14], *and ATP according to Krebs and Hems* [15]. *After wet ashing of the isolated compounds radioactivity was determined, and expressed as counts / μg P.*

Exp. 1.	TTP	1 717
	ATP	3 145
Exp. 2.	TTP	1 350
	ATP	3 480

of T was started with, as described above. This preincubation was carried on until no appreciable synthesis occurred, and the thiamine not yet used up was then washed out of the cells. Thereafter the yeast, rich in TTP, TDP, and TMP, was incubated in the same way as described for the fresh yeast containing only its naturally occurring TTP. Glucose was, however, present from the beginning. Fig. 4 shows the ^{32}P per *ug* acid hydrolysable phosphate in TTP and TDP at different intervals. The incorporation into TDP was slow, and ceased after 30 minutes. Into TTP it was much more rapid, and even at 60 minutes ^{32}P was still being incorporated.

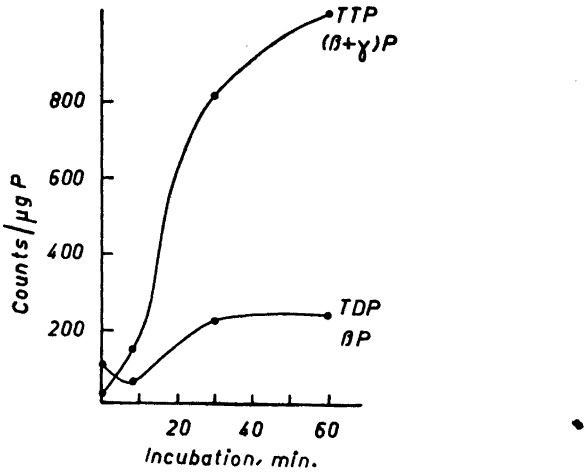

Fig. 4. Radioactivity of hydrolysable phosphate in TTP and TDP from yeast after incubation with ^{32}P and glucose in a succinate buffer pH 6.

The content of thiamine phosphates in the yeast was very high as the yeast had been incubated with an excess of thiamine for 20 hours before ^{32}P and glucose were added. The values are given as counts / μg P, and for TTP this means both βP and γP together, for TDP only βP.

Table 2. Radioactivity of α, β, and γ P of TTP and ATP from yeast after incubation with glucose and radioactive phosphate. Before the addition of ^{32}P and glucose the yeast had been preincubated with excess of thiamine, and thus was very rich in thiamine phosphates during the following incubation. The different phosphate groups were split off by means of a potato apyrase, except for ATP in exp. II, where βP and γP are determined together after acid hydrolysis. The values are given as counts / μg P.

Exp.	Time of incubation (minutes)	TTP			ATP		P_i
		αP	βP	γP	βP	γP	
I	0	0	0	0	0	0	6 310
	10	16	128	280	—	—	—
	30	250	1 102	1 860	3 290	3 820	—
	60	960	1 140	2 400	3 260	2 865	4 070
					(β + γ)P		
II	0	0	0	0	0		39 360
	30	600	1 200	7 320	17 800		—
	60	2 080	2 432	6 992	16 480		20 143

Enzymatic hydrolysis of TTP. When TTP is incubated with potato apyrase at 0° only the γ P is split off, at 40° both the γ and the βP[10]. TTP isolated from samples taken at different intervals of the ^{32}P-incubation was treated with apyrase, and then analyzed for radioactive phosphate (Table 2). In expt. I, Table 2 also ATP is treated in the same way as TTP. In expt. 2, Table 2 β P and γ P from ATP are determined together after acid hydrolysis. Also TDP and TMP (Table 3) were analysed for ^{32}P by acid hydrolysis and wet ashing.

DISCUSSION

This work was started with yeast containing only its natural amount of TTP, *i. e.* no thiamine was added after the yeast left the factory. From yeast TTP could be isolated in amounts detectable on a paper chromatogram. With the chromatographic method used in this part of the investigation only small amounts of yeast extract could be added on the paper to allow a good separation of TTP and TDP. After collecting TTP from several chromatograms it was, however, possible to make preliminary analyses as seen from Table 1. When hydrolyzing the TTP directly on the chromatogram it could be transformed into TDP and TMP. Thus TTP isolated from yeast incubated with ^{32}P and glucose in a succinate buffer gave a spot on the chromatogram which was rather radioactive, whereas the TDP and TMP formed by hydrolysis gave spots exhibiting only very feeble radioactivity (Fig. 3 B and C). It thus seems as if during the comparatively short incubation only the γP of TTP was exchanged. Glucose is necessary for this exchange as is shown in Fig. 3 B. Here no substrate was added until after 20 minutes, and TTP did not incorporate any noteworthy amount of ^{32}P until glucose was present.

Table 3. Radioactivity of α and βP of TDP and αP of TMP from yeast after incubation with glucose and radioactive phosphate. The yeast was the same as that used for investigating TTP and ATP (Table 2). The phosphate groups were split off by acid hydrolysis and wet ashing. The values are given as counts / μg P.

Exp.	Time of incubation (minutes)	TDP		TMP
		αP	βP	αP
II	0	0	0	0
	30	324	490	301
	60	332	408	482

When yeast is incubated with an excess of thiamine in a pyrophosphate buffer a strong synthesis of thiamine phosphates occurs [10]. TTP accumulates to a greater extent in this buffer than for example in a succinate or monophosphate buffer. After about 24 hours the synthesis has almost ceased, and on the removal of most of the remaining thiamine it comes to an end. The use of yeast rich in thiamine phosphates, especially TTP, has certain advantages with regard to the investigation into the rate of incorporation of ^{32}P into the different phosphate groups during the metabolism of glucose. A much greater amount of TTP can be isolated making possible an enzymatic hydrolysis of the different phosphate groups. From Tables 2 and 3 and Fig. 4 it is seen that there is a higher turnover rate of phosphate in TTP than in TDP or TMP. When incubating only for a short time (10 or 30 minutes) mainly the two terminal phosphate groups of TTP are radioactive, and γP in a higher degree than βP. After 60 minutes the radioactivity of αP nearly equals that of βP (Table 3). From this it can be concluded that TTP takes part in the phosphate transport during the metabolism, and this mainly by the turnover of its terminal phosphate. The radioactivity of the most active phosphate group of TTP, the γP, never reaches that of inorganic phosphate or ATP. This may be explained by the hypothesis that during the preincubation with excess of thiamine, the yeast synthesizes more TTP than can take a share in the phosphate transport. This is not the case when using yeast containing only its natural source of TTP. In Table 1 the values of TTP are about half those of ATP, but as is seen from the chromatograms in Fig. 3 C, only γP exhibits a remarkable radioactivity after 20 minutes' incubation. In ATP both βP and γP are nearly equally labelled after this incubation time. Consequently the terminal P of TTP and ATP may be labelled to about the same extent.

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