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Assimilation of Radioactive Orthophosphate by the Yeast *Candida utilis* at a Temperature Higher than Optimal (45°C). Isolation, Identification and Specific Activity of Phosphate Esters Soluble in Cold Trichloroacetic Acid

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Yeast was grown in a nutrient solution containing labelled orthophosphate for 15 min at 45°C. The cells were extracted with cold trichloroacetic acid and the extract fractionated by ion exchange chromatography into groups of a few compounds. These were refractionated by preparative paper chromatography, the separated phosphates identified, and their specific activities determined.

A new nucleotide-sugar, guanosine diphosphofructose, was identified. The highest specific activity was found in trehalose-phosphate, followed by α-glycerophosphate and the hexose phosphates. In these conditions trehalose phosphate evidently could not be synthesized *via* uridine diphosphohexoses or the hexose phosphates.

As the individual reactions in phosphate assimilation are extremely rapid [1] some authors have tried to slow down the rate of the reactions by using a low assimilation temperature.[2,3]

In this work we have studied the specific labelling obtained in different phosphate esters when assimilation of labelled orthophosphate takes place at a temperature higher than optimal (45°C). Paper chromatograms made with the cold TCA extract of such cells showed a distribution of labelling which was quite different from the usual one.[5] Two compounds hardly labelled at all at the optimal temperature (30°C) now dominated the autoradiograms. For the elucidation of their structures it was necessary to isolate them in pure state.

Therefore, the phosphate esters were isolated by ion exchange chromatography and by subsequent paper chromatography. Their structures were

studied in order to identify the major component of each fraction with reasonable reliability and also to be able to determine the specific radioactivity of each compound.

EXPERIMENTAL

Cultivation of the cells and preparation of the TCA extract. Normal cells of the food yeast *Candida utilis* (old name *Torulopsis utilis*) were cultivated as described earlier.[5] Phosphorus-deficient cells were obtained by aerating the normal cells (41 g) for 2 h in a nutrient solution containing 1/10th of the usual amount of phosphate.[5] The yield was 81 g. For the last 20 min the temperature was kept at 45°C. Cells were separated by centrifugation, suspended in a nutrient solution completely devoid of phosphate (500 ml), aerated for 10 min at 45°C, and then 9 mC ^{32}P as orthophosphate with 68 mg KH_2PO_4 carrier was added to make the solution 10^{-3} M with regard to phosphorus. The suspension was aerated for 15 min while the assimilation of labelled orthophosphate took place. After this time the suspension was centrifuged, the cells rinsed once with tap water and extracted with cold 8 % TCA.

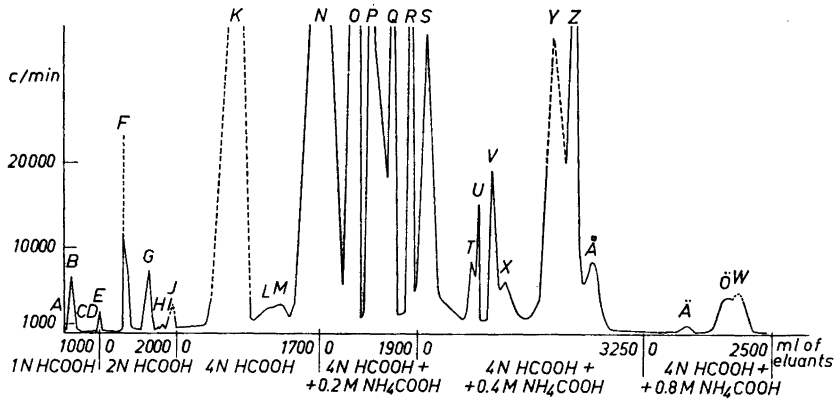

Fig. 1. Dowex-1 ion exchange chromatogram of the soluble phosphates of the yeast *Candida utilis* after 15 min assimilation of radioactive orthophosphate at 45°C. Radioactivity was recorded automatically. Description of peaks in the text.

Fractionation of the TCA extract. After removal of TCA by ether extraction, the extract was lyophilized to about 30 ml and fed into a Dowex-1 × 8 anion exchange column of the size 3.5 cm × 21 cm. The resin (200—400 mesh) was in formate form. The fractionation was performed using the gradient elution described earlier.[5] The volume of the mixing flask was 1 l and it was filled with water at the start of the run.

Elution required 4 days. The radioactivity of the effluent was measured continuously and a graph showing the radioactive peaks was obtained with a recorder (Fig. 1). The fractions were combined into peak groups, lyophilized and made up to 5 ml except for peaks A, C, and D, which were made up to 3 ml, and fraction P, made up to 10 ml.

Identification of the compounds separated. The UV-spectrum of all fractions was determined, and 2-dimensional paper chromatograms were run with a solvent pair suitable for separation of phosphate esters.[5] Co-chromatography was always carried out when possible. Autoradiograms of each paper chromatogram were made. For location of compounds on the paper chromatograms, see Ref. 5. Two typical paper chromatograms are presented in Fig. 2.

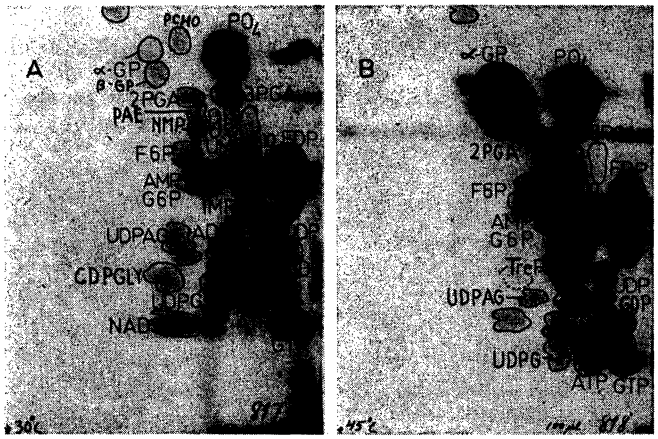

Fig. 2. Autoradiograms of paper chromatograms of the soluble phosphates synthesized in the yeast *Candida utilis* at 30°C (A) and 45°C (B). Of comparable extracts aliquots of 50 µl were used for A, 100 µl for B. For symbols see Ref. 5.
To get the faintest spots visible the autoradiograms are overexposed, which diminishes resolution.

Various additional determinations were carried out with different fractions to verify the identity of the compounds, as has been described earlier.[5]

RESULTS

Of the radioactivity given, 87.2 % was assimilated during the 15-min period, and of the amount assimilated, 65.1 % was TCA-soluble and 34.9 % TCA-insoluble. Thus, in spite of the high temperature, phosphate assimilation and synthesis of TCA-soluble and -insoluble phosphates took place at a seemingly normal pace.

Identification of the main components of the eluate fractions A to W gave the following results (see Fig. 1 and Table 1 for specific activities):

Peak A (Fig. 1). The fraction contained an abundance of free amino acids. A hydroxylamine test carried out for detection of nucleotide peptides was not positive. The phosphorus content of the fraction was high, but the specific activity so low that no active spot could be detected on a 2-dimensional chromatogram. The fraction had the UV-spectrum of adenosine, which was probably caused by the free base eluted in this fraction.

B. *Acetyl-P.* The test with hydroxylamine gave a positive result; according to it 0.4 ml of the concentrate contained 0.3 µmole acetyl phosphate. On the other hand, the total phosphorus was 3.4 µmole, which shows that the bulk of the phosphorus was in some other form. The main compound ran slightly slower than 3-PGA in the first direction and was decomposed in the second (basic) solvent into several spots. This fraction still contained plenty of free amino acids, but the radioactive spot was not ninhydrin-positive.

C to E. CDPEA + CDPCHO + PAE. All these fractions gave a cytidine spectrum and the compounds mentioned were identified by co-chromatography (*cf.* Ref. 5).

NAD—DPN. The compound behaves like DPN on the chromatogram and gives a cyanide complex, as observed spectroscopically.

G. *AMP* was identified by co-chromatography and UV spectrum.

H. *PAE.* The radioactive compound was also ninhydrin-positive, and moved on the paper in a two-dimensional run like PAE.

I. The fraction gave a weak unidentified spot in the glycerophosphate region on a two-dimensional paper chromatogram. The spot coincided with phosphoenol pyruvate but the identity could not be rigorously verified.

J. *R5P* was identified by co-chromatography.

K. *Trehalose phosphate, TreP.* The compound was isolated in a crystalline state (8 mg). It proved difficultly hydrolysable, but in 8 h with 1 N HCl at 100°C in a closed ampoule it was decomposed to glucose-6-phosphate and orthophosphate. Quantitative analysis according to Martin and Doty [6] gave 7.7 % P (theor. 7.31 %) and glucose analysis according to Dische [7] 83 % (theor. 85.2). Thus, the glucose:P ratio was 2:1. Alkaline phosphatase at 37°C liberated orthophosphate and trehalose, identified by paper chromatography using 6 solvent systems and the periodate-benzidine reagent [8] — the only one reacting with trehalose. The identity was finally confirmed by co-chromatography with an authentic sample kindly provided by Mr. MacDonald.

L. *GMP, TPN, trehalose P, F6P, and an unknown.* This fraction gave a cyanide complex spectrum. The compounds were identified by their locations on a 2-dimensional paper chromatogram. GMP was also extracted from the paper strip and its spectrum measured. The unknown compound was only weakly radioactive; it moved rapidly in both directions on a paper chromatogram.

M. *F6P* was identified by co-chromatography.

N. *G6P + F6P* were identified by co-chromatography.

O. *α-Glycerophosphate (α-GP) and ADP.* ADP was identified by its spectrum and location on the paper chromatogram, but α-GP required much investigation. It was first observed that it is not adsorbed by active charcoal and gave a positive reaction in the periodate test with Schiff's reagent [9] for vicinal hydroxyls. The compound was isolated, purified and crystallized as the Ba salt from an ethanolic solution. Quantitative determinations of phosphorus and formaldehyde gave the ratio, 1:1. Phosphorus was determined as above,[6] glycerol by a method originally developed by Zambert and Neish and modified by Korn.[9] In this method free glycerol gives two, but α-glycerides one mole of formaldehyde, which is determined colorimetrically with chromotropic acid. Finally, the identity was fully confirmed by co-chromatography with an authentic preparation obtained from the Nutritional Biochemical Center.

P. *α-GP, GDPM, GMP.* The fraction had a guanine spectrum. Weak spots of GDPM and GMP, and an especially strong one of α-GP, were found on the autoradiogram.

Q. *α-GP, UMP, PCHO and PO_4.* The fraction had a uridine spectrum; the UMP-spot was only weakly visible on the autoradiogram. α-GP and PCHO were very strong.

R. *PO_4 and ADPR.* The fraction had an adenosine spectrum. ADPR is easily decomposed and was found to be barely visible on the first chromatograms; later only PO_4 was detected.

S. *GDP-fructose.* The spectrum of the extract eluted from paper chromatogram strips was a pure guanosine spectrum. The ratio base:ribose:phosphate obtained in the determination was 0.048:0.094:0.090 μmole, which corresponds to 1:2:2. Hexoses also give a positive reaction in the ribose determination, which explains why practically two moles of ribose were obtained. The cysteine-sulphuric acid reaction for hexoses was not successful, probably owing to impurities. For the determination of the sugar component the extract from the spot was hydrolysed by boiling for 5 min in 0.01 N hydrochloric acid. The hydrolysate was chromatographed with butanol-acetic acid-water as solvent. A sugar reacting with aniline hydrogen phthalate and moving in 3 solvents like fructose was obtained. This compound is evidently GDP-fructose, but unfortunately we had no authentic preparation available for co-chromatography.

T. *UDPAG.* Identified on the basis of the spectrum obtained, and the position it took on the paper chromatogram.

U. *UDPGA, UDP, UMP, PEP, and 3-PGA.* The compounds were only identified by co-chromatography.

V. *UDP, UMP, and PEP.* The compounds were identified on the basis of their positions on paper chromatograms. UMP is here, of course, a product of the hydrolysis of UDP.

X. *UDPG and unknown No. 4.* UDP was identified on the basis of its position on the paper chromatogram. The identity of spot No. 4 is not quite established.

Y. *GDP.* Guanosine spectrum, position on paper corresponding to GDP.

Z. *FDP + ATP.* Identified by co-chromatography.

Å. *Adenosine tetraphosphate* gave a pure adenosine spectrum and the radioactive spot was identical with the authentic compound on co-chromatography. Ribose (measured directly from the fraction), and P gave the ratio 0.077:0.304 (μmole) $\simeq$ 1:4.

Ä. *Polyphosphates.* These compounds do not move on the paper at all in the second direction.

Ö. *GTP.* Spectrum and position on the paper identical with the authentic GTP.

W. *UTP.* Spectrum and position on the paper correct.

The specific activities of the purified compounds are listed in Table 1. Orthophosphate gave 6600 cpm/μg P (2.2 % counting effectivity). The second was TreP with 4400 cpm/μg P, the third α-GP with 1800—2300 isolated from different fractions. The latter value may be effected by orthophosphate as an impurity. FDP and G6P followed, both with 2000 cpm/μg P.

In a comparative experiment, after 15 min assimilation at 30°C, DPN had the highest *specific* activity after orthophosphate. Then followed hexose monophosphates, AMP, ADP, UMP, UDP, GMP, GDP, in this order.

Table 1. Specific activity (cpm/μg P) of phosphate derivatives isolated by paper chromatography from fractions of the Dowex chromatography.

Fraction	Compound	cpm/μg P	Fraction	Compound	cpm/μg P
E	CDP-ethanolamine	100	S	GDP-fructose	1060
G	AMP	660	T	UDP-acetylglucosamine	80
J	R5P	850	U	Phosphoenolpyruvate	1390
K	Trehalose phosphate	4400	V	UDP	660
L	TPN	100	X		500
M	F6P	910	Y	GDP	1240
N	G6P	2000	Z	ATP	1600
O–R	PO_4	6600	Å	AtetraP	1400
O,P	α-Glycerophosphate	1800–2300	Ö	GTP	370
P	GMP	230	W	UTP	1000

DISCUSSION

Accumulation of α-glycerophosphate evidently results from inhibition of triose phosphate dehydrogenase at the high temperature. Similar inhibition is effected by such compounds as iodoacetate.[10]

Trehalose phosphate has been known for many years — it was the fourth sugar phosphate to become known, originally isolated by Robison and Morgan [13] in 1928 from the fermentation products of yeast. Its formation in yeast macerates was studied in detail by Veibel [4] in 1931, who showed that it is present in maximum concentration 8 to 32 h after the beginning of fermentation.

Leloir and Cabib [14] in 1953 showed by paper chromatography that trehalose phosphate is synthesized from UDPG and G6P when these are incubated in a yeast extract. Later, the same authors continued their study of the metabolism of trehalose phosphate.[15] Panek [16] and Elander [17] have studied the same question. Panek [16] incubated yeast with radioactive orthophosphate in conditions optimal for trehalose synthesis. She found labelling in UDPG and G6P but none in trehalosephosphate. Our present results on the specific activities (TreP 4400, G6P 2000) are also difficult to interpret in the light of the mechanism for its biosynthesis which was shown to take place in yeast macerates. So far, we have no explanation for this discrepancy.

The accumulation of a new guanosine diphosphate derivative, apparently GDP-fructose, is another interesting result. As far as we know, this compound has not hitherto been found in yeast, although it has been isolated from the mould *Eremothecium ashbyii*.[12] Its significance remains obscure but its accumulation seems not to be connected with the accumulation of α-glycerophosphate or trehalose phosphate.

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