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Trehalose phosphorylase from *Euglena gracilis*

The disaccharide trehalose (1-α-D-glucopyranosyl-α-D-glucopyranoside) is widely distributed in insects, fungi, and yeast[1] and has recently been found in mammalian tissues[2] and in *Euglena gracilis*[3] The only enzyme hitherto described as involved in its utilization was trehalase (trehalose 1-glucohydrolase, EC 3 2 1 28) FREREJACQUE[4] reported in 1941 that a trehalose-splitting enzyme from insects was activated by phosphate and suggested that it was phosphorylase However, this work could not a be confirmed[5]

This paper reports evidence for the presence of an enzyme of *E gracilis*, trehalose phosphorylase, that catalyzes the following reaction

$$\text{Trehalose} + P_i \rightleftharpoons \beta\text{-Glc 1-}P + \text{glucose}$$

Measurement of enzyme activity The activity of this enzyme could be measured in both directions For measurements in the direction of synthesis, the incubation mixture contained imidazole–HCl buffer (pH 7 0), 2 μmoles, glucose, 5 μmoles, α-Glc-1-*P*, 1 μmole, and enzyme in a final volume of 0 04 ml After incubation at 37° for 30 min, the P_i liberated was measured according to FISKE AND SUBBAROW[6] On the other hand, the phosphorolytic cleavage of trehalose was measured as follows phosphate buffer (pH 7 0), 10 μmoles, trehalose, 2 μmoles, and enzyme in a final volume of 0 04 ml The reducing power liberated after 30 min at 37° was determined of 0 04 ml The reducing power liberated after 30 min at 37° was determined by the method of SOMOGYI[7] and of NELSON[8]

Enzyme preparation A photosynthetic strain of *E gracilis* var *bacillaris** was obtained through the courtesy of Dr D E Buetow and Dr W F Danforth The cells were grown in a medium of peptone–yeast extract–ethanol and vitamin B_{12} for 6 days and harvested by centrifugation The cells pellets were suspended in 25% glycerol, 2 mM EDTA, 4 mM phosphate buffer (pH 7 0) and disrupted in a French pressure cell After centrifugation at 10 000 × *g* for 10 min (crude extract) and 105 000 × *g* for 1 h, the trehalose phosphorylase in the supernatant was partially purified by the following steps (a) precipitation between 30 and 60% saturation of $(NH_4)_2SO_4$, (b) absorption on calcium phosphate gel, (c) elution of the gel with 0 15 M phosphate buffer (pH 7 0) The eluates were dialyzed against 25% glycerol–2 mM EDTA–2 mM phosphate buffer (pH 7 0) The specific activity of the enzyme measured in the direction of trehalose synthesis increased about 10-fold as compared to that of the crude extract

Synthesis of trehalose When enzyme extracts were incubated with β-Glc-1-*P* and glucose, a compound was formed which, as is shown in Table I, behaved like trehalose as judged by paper and thin-layer chromatography and by paper electrophoresis None was formed when either β-Glc-1-*P* or glucose was omitted in the reaction

The time curve of hydrolysis in 0 5 M H_2SO_4 of the compound formed enzymatically gave a profile similar to that obtained with authentic trehalose (Fig 1)

* Similar activities were obtained in Strain z and in a non-photosynthetic strain of *E gracilis* The latter was generously supplied by Dr W F Danforth

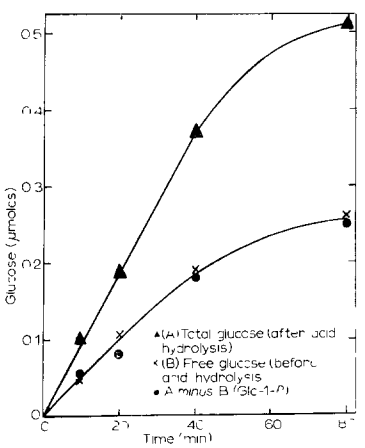
TABLE I

CHROMATOGRAPHY AND ELECTROPHORESIS OF THE PRODUCT OF ENZYME ACTION AND AUTHENTIC TREHALOSE

The analytical runs were carried out on Schleicher and Schull No. 2043a paper. Sugar spots were revealed according to TREVELYAN *et al.*[9]. Solvent 1: isopropanol–acetic acid–water (27 : 4 : 9), Solvent 2: butanol–pyridine–water (6 : 4 : 3). Solvent 3: butanol–acetone–water (4 : 5 : 1). Paper electrophoresis in 0.05 M potassium tetraborate (pH 9.2) for 90 min at 20 V/cm.

Substance	*Paper chromatography*		*Thin-layer chromatography on silica gel*	*Paper electrophoresis*
	Solvent 1 (R_g)	*Solvent 2* (R_g)	*Solvent 3* (R_g)	(M_g)
Enzymatic product*	0.68	0.47	0.75	0.1
Authentic trehalose	0.69	0.45	0.75	0.1

* Conditions for the incubation mixture as described in text.



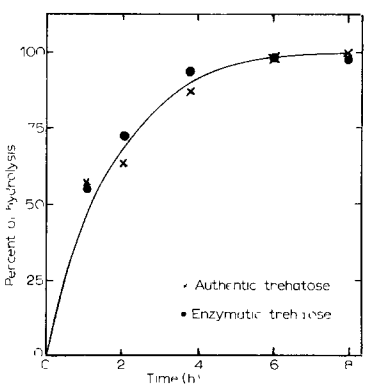


Fig. 1. Time curve of the phosphorolysis of trehalose. The enzyme extract (0.01 ml) was incubated with phosphate buffer (pH 7.0, 10 μmoles) and trehalose (2 μmoles) in a final volume of 0.05 ml at 37°. At the indicated time, the reaction was stopped by heating at 100° for 30 sec, and aliquots were taken to determine free glucose with the glucose oxidase–peroxidase system[10]. In other aliquots glucose *plus* Glc-1-*P* was measured in the same way but after hydrolysis in 0.05 M H_2SO_4. The values given were corrected for small blanks shown by controls without P_i.

Fig. 2. Liberation of reducing power by acid hydrolysis from the product of enzyme action and from authentic trehalose. 5 μmoles (expressed as glucose) of each, enzymatic product and trehalose, were hydrolyzed in 0.5 M H_2SO_4 at 100°. After indicated times, aliquots were neutralized and tested for reducing power by the method of SOMOGYI and of NELSON. The value given by trehalose after 8 h acid hydrolysis was taken as 100%. The enzymatic trehalose was obtained as follows: a 15-fold increased incubation mixture as described in text was incubated for 1 h at 37°. After deproteinization by heating, the precipitate was centrifuged off and the trehalose in the supernatant was adsorbed on a column of charcoal. The latter was washed with water and the disaccharide was eluted with 10% ethanol. The percolate was then dried in vacuum and dissolved in water. Authentic trehalose was submitted to the same treatment.

When enzyme extracts were incubated under the conditions indicated above with α- or β-Glc-1-*P*, a similar amount of P_i was liberated However, after treatment with 1 M NaOH for 20 min at 100°, the compound formed with β-Glc-1-*P* still gave color with the phenol–H_2SO_4 reagent[11], while the products formed with α-Glc-1-*P* gave no color With α-Glc-1-*P*, oligosaccharides of the β-1,3 series were formed due to the traces of laminaribiose phosphorylase and β-1,3-oligoglucan phosphorylase[10] in the enzyme preparation. It is known that β-1,3-glucans are degraded by alkali

Phosphorolysis of trehalose The time curve of phosphorolytic cleavage of trehalose is shown in Fig 2 The amount of free glucose formed during incubation was one-half of the total glucose produced after hydrolysis in 0 05 M H_2SO_4 for 10 min Under these conditions, Glc-1-*P* is completely hydrolyzed, while trehalose is not affected Therefore, the difference in the amounts of glucose measured before and after hydrolysis should be ascribed to Glc-1-*P*

When enzyme extracts were incubated for 30 min with trehalose and phosphate buffer (pH 7 0), the chromatographic analysis of the product gave glucose and a compound which had the same mobility as α- and β-Glc-1-*P* These compounds did not appear in the absence of P_i or trehalose In order to identify the phosphoric ester produced in the reaction, a large-scale mixture with phosphate buffer (pH 7 0, 1 5 mmoles), trehalose (0 8 mmole), and enzyme extract was incubated for 1 h at 37° The phosphoric ester formed (0 15 mmole) was isolated by a method similar to that described for α-Glc-1-*P* (ref 12) Samples of α-Glc-1-*P* and synthetic β-Glc-1-*P* were treated in the same way The three compounds were obtained as potassium salts Although the α derivative crystallized easily from 50% ethanol, attempts to induce crystallization failed with β-Glc-1-*P* and with the enzymatic product[13] The addition of 1 vol of ethyl ether gave a yellow syrup which was converted to a white powder by careful addition of methanol After drying in vacuum, the powders were dissolved in water and aliquots were hydrolyzed in 0 5 M H_2SO_4 producing glucose and P_i in a ratio of about 1 After treatment with muscle phosphoglucomutase[14], the only compound which was converted to an acid stable phosphate ester was α-Glc-1-*P* Other aliquots (12 μmoles) were dried in vacuum and submitted to infrared spectrum analysis (kindly carried out by Dr E A Rúveda) in a KBr disk The spectrum of the enzymatic product was similar to that of synthetic β-Glc-1-*P* The characteristic peaks of the α anomer at 868 and 835 cm^{-1} did not appear either in the authentic β ester or in the labile phosphate enzyme product From the data presented, it can be concluded that the enzyme found in *E gracilis* catalyzes the reversible cleavage of trehalose to β-Glc-1-*P* and glucose

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