Intracellular Trehalase of a Hybrid Yeast

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1. The trehalase found in an extract prepared from a yeast strain that cannot ferment trehalose was studied and characterized. The enzyme is highly specific for trehalose with K_m 1.02×10^{-2} M, and an optimum pH of 6.9. 2. It is inhibited by glucose and by trehalose 6-phosphate, and does not facilitate any significant transglucosylations. 3. pK values 7.7 and 5.8 were detected for the groups associated with binding of the non-ionized substrate to the enzyme. 4. The trehalase was found to be highly labile and was inhibited by thiol-binding reagents. 5. The possible role of this enzyme in the trehalose-dissimilation patterns in the yeast cell was evaluated.

Several strains of yeasts were shown to be able to ferment extracellular trehalose due to its hydrolysis to glucose by a trehalase present in these cells (Fischer, 1895; Bouthilet, Neilson, Mrak & Phaff, 1949; Lukes & Phaff, 1952). Myrbäck and co-workers have observed (Myrbäck, 1949) that old suspensions or dried baker's and brewer's yeasts exhibited a much more intensive trehalose fermentation than that of the fresh cells. Similar phenomena were observed in more recent studies (Frohwein & Leibowitz, 1962; Lukes & Phaff, 1952; Blennemann, Janocha, Keller & Netter, 1963). The substrate, trehalose, is found in large quantities as a reserve carbohydrate in yeasts which usually exhibit trehalase activity (Myrbäck, 1949; Trevelyan, 1958). Myrbäck (1959) concluded that enzyme and substrate in the fresh cells are somehow separated; the trehalase is found in a relatively 'insoluble' state and drying or lysis facilitate the exposure of enzyme to the trehalose molecules.

In a study of α -glucosidase formation by a hybrid yeast (Avigad, 1958a) it was observed that cell extracts exhibited trehalase activity whereas they could not utilize trehalose as an extraneous sugar for fermentation. In addition, when this strain of yeast was grown in absence of an α -glucoside, the only α -glucosidase activity of the cell extracts was that of trehalase. A very active trehalose metabolism in this case was evident from the rapid turnover of this sugar (Avigad, 1960).

In view of a possible role which the intracellular trehalase activity might have in the metabolism of endogenous trehalose in yeast, a study of some properties of this enzyme has been made and is the subject of the present paper.

MATERIALS AND METHODS

Substrates. Trehalose hydrate was purchased from the Mann Research Laboratories Inc., New York, N.Y., U.S.A.,

and the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Trehalose phosphate was prepared from dry brewer's yeast according to the procedure of Robison & Morgan (1928), and purified through the brucine salt (Veibel, 1931). Nucleotides were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. ^{14}C -labelled sugars were purchased from The Radiochemical Centre, Amersham, Bucks.

Enzymes. Alkaline phosphatase was obtained from *Escherichia coli* ML308 after growth on a medium poor in orthophosphate (Garen & Levinthal, 1960); glucose oxidase (Glucostat) was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Other enzyme reagents were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Quantitative determinations. Glucose was measured with glucose oxidase (Huggett & Nixon, 1957) and reducing sugar cuprimetrically (Nelson, 1944; Somogyi, 1945). Total carbohydrate and trehalose were estimated with the phenol-sulphuric acid reagent (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Protein was measured with the phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) and crystalline bovine serum albumin as the standard.

Chromatographic methods. Whatman no. 1 filter paper was used. Solvent systems employed were: I, butan-1-ol-ethanol-water (5:2:2, by vol.) and II, propan-1-ol-ethyl acetate-water (7:1:2, by vol.). The spray reagents used for the detection of reducing sugars were the aniline or *p*-anisidine reagents (Hough, Jones & Wadman, 1950) and aniline-diphenylamine (Bailey & Bourne, 1960). Alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) was used to detect trehalose. Radioautograms for detecting ^{14}C -labelled sugars on paper chromatograms were performed as described previously (Avigad, 1960).

Yeasts. Haploid yeast hybrid 12836 of the genotype MA, MG, su, mz (Hestrin & Lindgren, 1952) has been used. Cells were grown in a nutrient broth as described by Hestrin & Lindgren (1950) with 4% sugar as the carbohydrate source. Growth was carried out in batches of 11. of medium in 21. Erlenmeyer flasks on the Rotatory shaker at 30° for 36 hr. Larger amounts of cells were obtained by growing 101. batches in an air-lift fermenter (Lundgreen & Russel, 1956). Cells were collected by centrifugation and washed

four times with water. Packed cells were spread on filter paper and dried at 37° with a stream of air. Dry cells were kept desiccated at 4°. Yield of cells was usually about 12 g. of fresh packed cells (3 g. dry wt.)/l. of medium.

Preparation of extracts. Fresh cell suspensions (20%, w/v, in 0.02 M-phosphate, pH 7.0, containing 1 mM-EDTA) were disrupted by shaking with glass beads (Ballotini, size 14) in the Nossal (1953) disintegrator at 4° for two 30 sec. periods. The supernatant obtained by centrifugation at 12000g for 20 min. was used for studies of enzyme activities.

Dry cells were extracted with three weights of 0.05 M-sodium bicarbonate, containing 1 mM-2-mercaptoethanol, for 6 hr. at 37°. Cells were precipitated by centrifugation (12000g for 15 min.) and the supernatant was kept in the cold. The precipitate was resuspended in the initial volume of the bicarbonate solution, sonically treated for 10 min. (in the Raytheon 10 kcy./sec. sonic oscillator) and the homogenate stored overnight at 4°. After centrifugation as before, the supernatant solutions were combined and dialysed overnight against 0.5 mM-mercaptoethanol and 1 mM-EDTA at 4°. The solution obtained served for the study of trehalase activity and for further fractionation of the enzyme as described later on. Trehalase activity in such preparations declined about 10% per week of storage under toluene at 4°.

Assay of enzyme activity. The standard assay system contained 0.2 M-trehalose or α -glucoside in 0.05 M-phosphate buffer, pH 6.9, and 0.1 mM-EDTA at 30°. Samples were withdrawn at intervals, immersed for 3 min. in a boiling-water bath and the amount of liberated glucose was determined. When the glucose-oxidase assay was employed, 5 mM-N-ethylmaleimide was added to the assay solution to prevent inhibition of the peroxidase reaction by the mercaptoethanol. In several cases the course of glucose liberation was followed spectrophotometrically at 340 m μ by coupling the trehalase reaction mixture with the hexokinase, ATP, glucose 6-phosphate dehydrogenase and NADP system (Slein, 1963). Each mole of trehalose furnishes 2 moles of glucose upon hydrolysis.

Enzyme activity unit. One unit of enzyme is defined as the amount which hydrolyses 1 μ mole of substrate/hr. under standard conditions of assay.

Growth and fermentation studies. Ability to grow and ferment trehalose or other sugars was studied in test tubes containing a liquid culture medium as described by Wickerham (1951). The synthetic medium was supplemented by 0.1% of yeast extract (Difco). Respiration was studied by conventional procedures in the Warburg apparatus.

RESULTS

Effect of growth conditions on trehalase activity. It was observed that the rate of trehalose hydrolysis was of the same order of magnitude by extracts obtained from cells grown on different carbon sources (Table 1).

Extraction and partial purification of the enzyme. Quantitative extraction of trehalase from dry cells proved to be difficult. Many buffer solutions, various temperatures and toluol were tested in an endeavour to get maximum soluble enzyme activity. The procedure outlined in the Materials and Methods

Table 1. *Effect of substrates for growth on the yield of trehalase activity*

Yeast was grown in carbohydrate-free nutrient medium as described in the Materials and Methods section. Carbon source was added separately to a final concentration of 4%. Washed cells were disrupted mechanically and trehalase activity was determined as described in the text.

Supplement to medium	Yield of cells (g. dry wt./l.)	Trehalase activity (units/g. of dry cells)
None	0.30	57
Glutamate	0.25	60
Acetate	0.75	55
Ethanol	0.50	56
Trehalose	0.15	50
Glucose	3.00	54
Methyl α -D-glucoside	3.00	50
Maltose	3.00	45

section was found to be most efficient and was thus always used. By this method about 0.1–0.2 unit of enzyme/mg. of dry cells could be obtained, which constituted about 80% of the total activity exhibited by the dry cell suspension. Sonic treatment of fresh cell suspensions yielded only about 0.03–0.05 unit of enzyme/mg. of dry cells.

Several procedures were tried to achieve purification of trehalase but with limited success because of rapid inactivation of the enzyme. The procedure carried out at 0° was as follows. Crude extract (about 1 unit/mg. of protein) was treated with protamine sulphate solution of pH 6.0 (50 μ g. of protamine sulphate/mg. of protein) and the precipitate formed was removed by centrifugation. Solid ammonium sulphate was added to the supernatant and the fraction collected between 0.4 and 0.6 saturation was found to contain most of the trehalase activity with two- to three-fold purification. The precipitate obtained was dissolved in 1/20 the volume of the original crude extract volume in 0.04 M-phosphate–0.01 M-histidine buffer, pH 6.9, containing 1 mM-2-mercaptoethanol.

The enzyme could be further purified about three- to five-fold on DEAE-cellulose columns (cf. procedure described in Fig. 1). The enzyme thus obtained was usually very labile in solution and lost more than 60% of its activity overnight at 4°.

Separation between trehalase and α -glucosidase activities. A clear-cut preparative separation between the trehalase activity and the methyl α -D-glucoside-hydrolysing activity in cells grown on methyl α -D-glucoside could be achieved by ammonium sulphate fractionation (Table 2), or by chromatography on a DEAE-cellulose column (Fig. 1).

Fig. 1. Fractionation of α -glucosidase activity on DEAE-cellulose column. All procedures were carried out at 2°. A dialysed enzyme solution (93 ml.; 14000 units of methyl α -D-glucosidase and 87.5 units of trehalase), obtained from 12 g. of dry cells which were grown on methyl α -D-glucoside, was treated with a protamine sulphate solution, pH 6.0, to give 100 μ g./mg. of protein. After the removal of precipitate by centrifugation and addition of mercaptoethanol to 1 mM, neutralized saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was added and the fraction precipitated between 0.4 and 0.8 saturation was collected, dissolved in 2 ml. of 0.01 M-phosphate buffer, pH 6.1, containing 1 mM-mercaptoethanol, and dialysed overnight against a solution of the same composition. A small precipitate was removed by centrifugation and the solution (60 mg. of protein) was applied to a column (21 mm. $\times$ 67 mm.) of DEAE-cellulose-Celite 545 (3:1, w/w), which had been equilibrated with 0.02 M-phosphate buffer, pH 6.9. A gradient was applied with 1 vol. of 0.04 M-phosphate, pH 6.9, containing 1 mM-mercaptoethanol and 0.25 M-KCl in the upper vessel and an equal volume of buffer containing only mercaptoethanol, in the mixing chamber. Fractions (3 ml.) were collected at 2°.

This observation shows that these two α -glucosidase activities should be attributed to two completely separable protein entities.

Effect of pH on enzyme activity. The optimum pH of trehalase activity was found to be between 6.8 and 7.0 (Fig. 2a). Similar rates at pH 6.9 were observed for reactions in phosphate, citrate-phosphate, histidine, maleate, tris-maleate, arsenate or arsenite buffers. 50 mM-EDTA did not inhibit enzyme activity. It was clear that the enzyme was not a phosphorolytic one as the reaction occurred at maximal rate in systems free of orthophosphate.

The stability curve of the trehalase showed a

Table 2. Fractionation of trehalase and α -glucosidase by ammonium sulphate

All procedures were carried out at 2°. Dialysed extracts, prepared from dry cells and containing 1 mM-mercaptoethanol, were treated with protamine sulphate as described in the Materials and Methods section. Neutralized saturated ammonium sulphate solution was added, stirred for 15 min. and the indicated fractions were collected at 10000g for 10 min. The precipitate was dissolved in 5 mM-phosphate buffer, pH 6.9, containing 0.5 mM-mercaptoethanol and glucosidase activities on methyl α -D-glucoside and trehalose were determined.

Fraction (ammonium sulphate, % saturation)	Percentage of initial activity*			
	Expt. 2†		Expt. 3‡	
	Expt. 1† Trehalase	α -Glucosidase	α -Glucosidase	
0-40	1.1	6.8	0.5	5.2
40-50	11.4	11.3	2.3	5.0
50-60	52.3	33.3	6.1	5.6
60-70	0.5	0	52.9	20.2
70-80	0	0	16.9	36.3
80-90	0	0	0	10.3

* 76 units in Expt. 1; 35 units of trehalase and 125 units of α -glucosidase in Expt. 2; 614 units in Expt. 3.

† Extracts from glucose-grown cells without α -glucosidase activity.

‡ Extracts from methyl α -D-glucoside-grown cells.

Fig. 2. Effect of pH on trehalase activity. (a) Standard reaction mixture included appropriate buffer (0.05 M) and 4 units of enzyme/ml. (b) Stability at different pH values: 20 units of enzyme/ml. were incubated in a 0.01 M solution of the appropriate buffer during 80 min. at 0°. Samples were then neutralized and assayed for residual activity at pH 6.9 under the standard conditions. Acetate and citrate-phosphate were used for the range pH 4.0-7.0, phosphate for the range pH 6.8-8.0 and borate for pH 8.0-9.2.

rapid irreversible inactivation by exposure to acid pH (Fig. 2b).

Effect of temperature on the rate of trehalose hydrolysis. A maximal rate of enzyme activity was observed at 30° (Fig. 3a). The enzyme was rapidly

destroyed at temperatures above 40° (Fig. 3b), and this presumably explains the strong decrease in activity observed at high temperatures (Fig. 3a). The energy of activation calculated according to the Arrhenius equation is found to be 7.9 kcal./mole between 1° and 30° (Fig. 3c), which is similar to that found for the silkworm enzyme (Saito, 1960). Under the conditions of assay employed the enzyme was inactivated at temperatures above 32° with an activation energy of -11.5 kcal./mole.

Fig. 3. Effect of temperature on enzyme activity. (a) Rate of hydrolysis at different temperatures of incubation. Enzyme (4 units/ml.) was incubated with trehalose under standard conditions at pH 6.9. (b) Stability of enzyme: 20 units of enzyme/ml. in 0.01M-phosphate buffer, pH 6.9, were incubated for 20 min. at the indicated temperatures. Residual activity was then assayed at 30° under standard conditions. (c) Arrhenius plot for the hydrolysis of trehalose by trehalase. Values are taken from data of curve (a).

Fig. 4. Effect of substrate concentration on the initial rate of trehalose hydrolysis: 2 units of enzyme/ml. of reaction mixture under standard conditions were incubated with varying amounts of trehalose and the amount of glucose liberated was measured as described in the Materials and Methods section. Insert represents a Lineweaver-Burk double-reciprocal plot of the results obtained.

Kinetic studies. A linear dependence of the initial rate of trehalose hydrolysis and enzyme concentration was found in the range 1–15 units of enzyme/ml. under standard assay conditions. The enzyme-substrate affinity constant (K_m) at pH 6.9 was found to be 1.02×10^{-2} M (Fig. 4). The dependence of the K_m on the pH of the reaction mixture is seen from the data in Fig. 5. As the substrate is non-ionizable, the sharp deflexions in the curve most probably represent changes due to ionization of two specific groups on the enzyme molecule with pK values about 7.6 and 5.8.

Glucose, which is the product of the reaction, was found to be inhibitory to enzyme activity. This inhibition was of a non-competitive type (Fig. 6) with $K_i 1 \times 10^{-2}$ M at pH 6.9.

To examine whether apparent inhibition by glucose was due to transglucosylation reaction from trehalose to glucose serving as acceptor, the following experiments were performed: 0.4M-trehalose was incubated with 0.4M-[¹⁴C]glucose (25 mc/m-mole) and 72 units of enzyme/ml. in 0.02M-phosphate buffer, pH 6.9. Samples (25 μl., 0.25 μc) were withdrawn at intervals and separated by chromatography on Whatman no. 3MM filter paper. Incubation was carried out for 24 hr. until about 60% of the trehalose was hydrolysed in the control experiment without added glucose. The chromatograms did not show the appearance of any significant amount of transfer products. Nor did the radioautograms indicate formation of transfer products or any incorporation of [¹⁴C]-glucose into the residual trehalose in the system. The sensitivity of the analytical procedure would

Fig. 5. Dependence of K_m for trehalose on pH of the reaction mixture. Reaction mixture with 4 units of enzyme/ml. was incubated with varying concentrations of trehalose (0.01–0.10M) and 0.05M-buffer (citrate-phosphate for pH 5.2–6.5; phosphate for pH 6.5–7.5; borate for pH 7.5–8.6). Rates of appearance of glucose were determined as described in the Materials and Methods section.

Fig. 6. Inhibition of trehalose hydrolysis by D-glucose. Standard reaction mixtures with 6 units of trehalase/ml. were incubated with varying concentrations of trehalose and glucose. The increase of glucose in the reaction mixture was determined as described in the Materials and Methods section. v , μ moles of trehalose hydrolysed/ml./hr.; $\bullet$, no glucose added; $\circ$, 2.5 mM-glucose; $\blacktriangle$, 5 mM-glucose; $\triangle$, 10 mM-glucose.

Fig. 7. Inhibition of trehalose hydrolysis by trehalose phosphate. Standard reaction mixtures containing 2 units of enzyme/ml. were incubated with varying concentrations of trehalose and trehalose phosphate. v , μ moles of trehalose hydrolysed/ml./hr.; $\bullet$, without trehalose phosphate; $\circ$, 4 mM-trehalose phosphate; $\triangle$, 6 mM-trehalose phosphate.

have allowed the detection of at least 5 mM-[14 C]-trehalose or a 14 C-labelled transfer product by radioautography or by colour reactions.

Inhibition by trehalose 6-phosphate. Trehalose phosphate was not hydrolysed significantly under the standard conditions of assay, but exhibited a competitive inhibitory effect on trehalose hydrolysis (Fig. 7) with K_i 2×10^{-3} M.

Inhibitors of enzyme activity. Fe^{2+} , Fe^{3+} , Mn^{2+} , Mg^{2+} or Ca^{2+} at 1 mM, or sodium fluoride at 10 mM, did not inhibit trehalose hydrolysis by dialysed enzyme solutions. Zn^{2+} at 1 mM inhibited about 20% and Hg^{2+} , Cu^{2+} or Ag^{+} at 1 mM completely inhibited enzyme activity.

The trehalase was completely inactivated by 0.2 mM-*p*-chloromercuribenzoate. About 50% inhibition was exerted by 0.5 mM-*N*-ethylmaleimide and 0.01 mM-*p*-chloromercuribenzoate; however, 10 mM-sodium iodoacetate did not inhibit. 2 mM-Glutathione, -cysteine or -dimercaptopropanol could protect the enzyme from loss of activity by *p*-chloromercuribenzoate or *N*-ethylmaleimide. Tris, which was shown to be a highly efficient competitive inhibitor of yeast α -glucosidase (Avigad, 1958b; Halvorson & Ellias, 1958), did not inhibit trehalase activity at pH 7.0 at concentrations below 0.05 M. However, at 0.1 M- and 0.2 M-tris, 20% and 30% inhibition of trehalose hydrolysis occurred respectively.

Substrate specificity and transglucosylation reactions. Methyl α -D-glucoside, maltose, sucrose, turanose, melibiose, cellobiose or lactose was not hydrolysed under standard conditions (0.1–0.2 M substrate concentrations) by enzyme preparations obtained from glucose-grown cells. In reaction mixtures containing trehalose alone (0.2 M) or with the addition (to 0.1 M) of methyl α -D-glucoside, sucrose, melibiose, lactose, D-galactose or D-xylose or 0.01 M-glucose 1-phosphate and -glucose 6-phosphate, no significant inhibition of trehalose hydrolysis or appearance of transfer products was observed by paper-chromatographic examination of the reaction mixture even after prolonged incubation, which led to more than 60% hydrolysis of the substrate.

Synthesis of trehalose 6-phosphate by the yeast extract. Cell extracts prepared from the hybrid yeast strain exhibited the trehalose-synthetase reaction (Cabib & Leloir, 1958) as is demonstrated in Table 3. Reversal of the reaction, by incubation of trehalose with UDP and other nucleoside diphosphates similar to the conditions employed for the cleavage of sucrose by the 'sucrose-synthetase' system (Avigad, 1964), did not occur.

The product of the reaction was identified as trehalose by the following procedure. A reaction mixture, as described in Table 2, was incubated and then inactivated by immersion for 3 min. in a boiling-water bath. Tris buffer, pH 8.6, was added

Table 3. *Trehalose 6-phosphate synthesis by extracts*

Fresh washed glucose-grown cells (4g.) were suspended in 7 ml. of 0.01M-tris buffer, pH 7.2, disrupted with glass beads and centrifuged as described in the Materials and Methods section. Neutralized saturated ammonium sulphate solution containing 0.01M-EDTA was added to the supernatant to 0.7 saturation. The precipitate was dissolved in 1.0 ml. of 1mM-EDTA and dialysed overnight against the same solution. Reaction mixture (1.0 ml.): tris, pH 7.2, 40 mM; UDP-glucose, 5 mM; glucose 6-phosphate, 10 mM; MgSO₄, 25 mM; EDTA, 1 mM; 0.4 ml. of enzyme solution. After 15 min. at 37° the system was inactivated by 3 min. of immersion in a boiling-water bath and denatured protein was removed by centrifugation. UDP was assayed with the pyruvate-kinase system (Pontis & Leloir, 1962). Glucose 6-phosphate was estimated with the glucose 6-phosphate-dehydrogenase system (Cooper, Srere, Tabachnik & Racker, 1958). Trehalose phosphate assay was performed as follows. The sample was hydrolysed with 0.1N-HCl for 20 min. at 96° to liberate glucose from UDP-glucose and then neutralized with NaOH. Sodium borohydride was added to 0.5% final concentration. After 2 hr., when all reducing hexoses disappeared, the trehalose was estimated colorimetrically with the phenol-H₂SO₄ reagent (Dubois *et al.* 1956). Figures represent μ moles/ml.

	Glucose 6-phosphate	Trehalose phosphate	UDP
Complete system	-3.70	+3.88	+3.44
Without glucose 6-phosphate	-0.09	+0.19	+0.19
Without UDP-glucose	0	0	+0.23

to a final concentration of 0.4M and excess of alkaline phosphatase (100 units) was added. After 15 min. at 37° the reaction mixture was passed through a small column of Dowex-50 (H⁺)-Dowex-1 (CO₃²⁻) mixed-bed resin. The volume of the effluent was reduced by evaporation under reduced pressure at 40°. The appearance of trehalose was confirmed by paper chromatography.

Growth and respiration. When trehalose served as the carbon source the strain of yeast studied showed no gas formation and grew poorly, even after 3 weeks of observation with the Wickerham (1951) procedure. Control cultures on glucose, maltose or methyl α -D-glucoside reached maximum gas formation after 24 hr. Washed cell suspensions were studied for formation of carbon dioxide and uptake of oxygen as described in Table 4. The results clearly indicated that even after the poor growth in the presence of trehalose (Table 1), it could serve only as a very poor substrate, and usually did not facilitate respiration above the endogenous level. The rate of trehalose utilization increased sometimes by about 20–50% on prolonged incubation (4 hr.) in the manometer vessel, presumably due to some lysis and exposure of trehalose to the intracellular hydrolase.

Table 4. *Rate of respiration and fermentation by washed cell suspensions*

Washed cells (equivalent to about 0.5 mg. dry wt./ml.), grown on different sugars, were studied for O₂ uptake and CO₂ formation at 30°, up to 4 hr., in 0.05M-phosphate buffer, pH 6.5, and 0.1M-sugar, by the standard manometric techniques, with air as the gas phase.

Growth sugar	Substrate	Q _{O₂} (uptake)*	Q _{CO₂} (pro- duction)*	R.Q.
Glucose	Endogenous	5	5	1.0
	Glucose	35	90	2.5
	Trehalose	3	2	0.7
Methyl α -D-glucoside	Endogenous	11	8	0.7
	Glucose	35	94	2.7
	Trehalose	5	5	1.0
Maltose	Endogenous	5	4	0.8
	Glucose	21	56	2.7
	Trehalose	3	3	1.0
Trehalose	Endogenous	6	5	0.9
	Glucose	55	125	2.3
	Trehalose	6	10	1.6

* μ l. of gas/mg. of dry cells/hr.

DISCUSSION

Several of the yeast-trehalase properties found in the present studies are significantly different from those described for trehalases obtained from other sources. Thus the optimum pH for trehalase activity from various insects (Kalf & Rieder, 1958; Friedman, 1960; Saito, 1960), *Neurospora* (Hill & Sussman, 1963), intestine (Dahlquist, 1960), yeasts (Myrbäck, 1949; Lukes & Phaff, 1952; Courtois, Petek & Kolahi-Zanouzi, 1962; Panek & Souza, 1964) and other organisms (Myrbäck, 1949; Courtois *et al.* 1962) is between 5.0 and 6.0 and the K_m measured in some of these cases varied between 0.1 and 3.0 mM. In the present study an optimum pH 6.9 and K_m 10 mM was found. For comparison it should be mentioned that the intracellular pH range in yeast cells is considered to be 6.5–7.0 (Kotyk, 1963) and the intracellular concentration of trehalose in the hybrid yeast studied was calculated to be 50–75 mM (Avigad, 1960). In other strains which accumulate larger quantities of trehalose, this value might be even higher. Moreover, it can be argued that if the trehalose is stored in specific compartments within the cell, such as the vacuole, its local concentration would be even higher. Consequently, both the optimum pH and K_m values found *in vitro* for the intracellular trehalase are compatible with maximal rates of activity under conditions when the enzyme and substrate are freely exposed to each other. Nevertheless, it should be noted that the action of an

enzyme *in vivo* might be affected markedly by the micro-environment in which it is embedded (Goldstein, Levin & Katchalski, 1964). At present we know extremely little about the location of trehalose and trehalase in the yeast, or about the cellular mechanisms which induce their maximal mutual interaction.

The possibility that the intracellular yeast trehalase is associated with trehalose breakdown in the cells is further strengthened by the observation that the activity of enzyme is about the same in cells grown on different carbon sources, in contrast with the induced formation of the enzyme described by Lukes & Phaff (1952) and Metzenberg (1962). In addition, the cells could not utilize external trehalose for fermentation unless some lysis or aging had occurred. It should furthermore be noted that the trehalose phosphate synthetase cannot catalyse cleavage of trehalose phosphate or trehalose under standard assay conditions (Cabib & Leloir, 1958; the present work). It is therefore necessary to assume that another reaction is responsible for trehalose breakdown in the cell. The intracellular trehalase may perhaps fill this function.

Trehalose is found as a reserve carbohydrate in many other organisms and its accumulation has recently been studied in fungus spores and insects in particular. As in yeasts (Trevelyan, 1958), analogous patterns of trehalose breakdown or synthesis, as determined by various physiological stimuli, were observed in insects (Candy & Kilby, 1961; Glegg & Evans, 1961; Wyatt, 1961; Saito, 1963) and in *Neurospora* spores (Sussman, 1961; Hill & Sussman, 1964). In all of these, trehalase was also found in the organism and it is likely that it plays there a major role in trehalose catabolism.

Several of the observed properties of the enzyme are of interest. The groups involved in binding the non-ionized substrate, trehalose, to the enzyme were found to have pK values corresponding to those of an imidazole ring (pK 5.6–7.0) and an α -amino group (pK 7.6–8.4) (Dixon & Webb, 1964). As shifts of pK regions of a specific group can occur in a polypeptide chain (Dixon & Webb, 1964) it is also possible that other binding groups are involved. The trehalase is inhibited by reagents reacting with thiol groups and by heavy-metal cations. It did not facilitate any exchange between glucose and trehalose, nor any significant transglucosylation reactions, similar to observations noted with other trehalases (Dahlquist, 1960; Courtois *et al.* 1962). Also of interest is the observation that trehalose 6-phosphate and glucose can inhibit trehalase activity, whereas glucose 6- or 1-phosphate did not exert inhibition. Though the mechanisms of inhibition by trehalose phosphate and by glucose might be different, this effect might be of signifi-

cance as part of regulatory mechanisms of intracellular trehalose metabolism. Trehalose inhibits the trehalose-synthetase reaction (Murphy & Wyatt, 1964) whereas trehalose phosphate is inhibitory to the glycogen synthetase (Goldemberg & Leloir, 1960). However, one has to consider the possibility that trehalose, trehalose phosphate, trehalose synthetase and trehalase are locked in completely separated compartments within the cell such as the vacuole, lysosomes or the cytoplasm. In this case therefore the observed inhibition of trehalase by trehalose phosphate and glucose might not be of physiological importance but only a manifestation of enzyme inhibition *in vitro* by analogous substrates.

Though it can be obtained completely free from other α -glucosidase activities, the relatively low activity of the trehalase of the yeast studied in this work, its lability and its low affinity for trehalose do not favour the use of this enzyme for routine quantitative estimation of trehalose. The highly purified trehalase preparations obtained from insects (Kalf & Reider, 1958; Saito, 1960; Friedman, 1960) or *Neurospora* (Hill & Sussman, 1963) might be more suitable for specific analytical purposes in trehalose determination.

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