JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5518–5522
0021-9193/87/125518-05$02.00/0

Vol. 169, No. 12

Heat-Induced Accumulation and Futile Cycling of Trehalose in *Saccharomyces cerevisiae*

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Received 28 April 1987/Accepted 14 September 1987

Heat shock resulted in rapid accumulation of large amounts of trehalose in *Saccharomyces cerevisiae*. In cultures growing exponentially on glucose, the trehalose content of the cells increased from 0.01 to 1 g/g of protein within 1 h after the incubation temperature was shifted from 27 to 40°C. When the temperature was readjusted to 27°C, the accumulated trehalose was rapidly degraded. In parallel, the activity of the trehalose-phosphate synthase, the key enzyme of trehalose biosynthesis, increased about sixfold during the heat shock and declined to the normal level after readjustment of the temperature. Surprisingly, the activity of neutral trehalase, the key enzyme of trehalose degradation, also increased about threefold during the heat shock and remained almost constant during recovery of the cells at 27°C. In pulse-labeling experiments with [^{14}C]glucose, trehalose was found to be turned over rapidly in heat-shocked cells, indicating that both anabolic and catabolic enzymes of trehalose metabolism were active in vivo. Possible functions of the heat-induced accumulation of trehalose and its rapid turnover in an apparently futile cycle during heat shock are discussed.

The nonreducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is ubiquitously found in fungi, in which it is supposed to function as a reserve carbohydrate (24). In *Saccharomyces cerevisiae*, trehalose accumulates during periods of reduced growth, for example during starvation for nitrogen, phosphorus, or sulfur (13, 19). Trehalose is also abundant in chemostat cultures maintained at low dilution rates (11, 12) and in batch cultures during adaptation to new carbon sources or transition to the stationary phase (13, 18). Under some of these conditions, trehalose can account for up to 23% of the dry weight of the cells (13).

Trehalose biosynthesis in yeast proceeds in two steps. First, trehalose-6-phosphate (P)-synthase (UDP-glucose:D-glucose-6-P-1-glucosyltransferase, EC 2.4.1.15) condenses UDPG and glucose-6-P to yield trehalose-6-P. Second, a specific phosphatase (trehalose-6-P phosphohydrolase, EC 3.1.3.12) cleaves off phosphate from trehalose-6-P (3). Trehalose degradation is mediated by trehalase (trehalose 1-glucohydrolase, EC 3.2.1.28) (24). Two trehalases with different pH optima have been found in *S. cerevisiae* (15). One of them, neutral trehalase, has attracted much attention because its activity is regulated by cyclic AMP (cAMP)-dependent phosphorylation (14, 25–27). Current research has therefore focused on trehalose catabolism, whereas the study of anabolism has been neglected.

It has been reported that yeast cells growing at 37°C continuously contain appreciable amounts of trehalose (7), whereas cells growing at ambient temperature under similar conditions contain only traces of the disaccharide. Moreover, it has been found that one particular heat shock protein is an enzyme of carbohydrate metabolism, namely enolase (8). These findings suggest that heat shock strongly affects carbohydrate metabolism. Physiological studies on this subject are scarce, however. Consequently, we have investigated the key enzymes of trehalose metabolism, trehalose-P-synthase and neutral trehalase, during heat shock and correlated their activity changes with fluctuations and turnover of the trehalose pool.

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MATERIALS AND METHODS

Abbreviations. TCA, Trichloroacetic acid; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; MES, morpholinoethanesulfonic acid; Tricine, tris(hydroxymethyl)-methylglycine; cAMP, cyclic AMP.

Organism and cultivation. *S. cerevisiae* strain C276(a/α [10, 13]) was grown on a synthetic medium containing 2% glucose, 20 mM NH_4Cl, minerals, and vitamins (10). Exponentially growing cultures were used when not stated otherwise. All samples were taken at least four generations before the onset of the diauxic lag phase. At this time, 80% of the initial glucose was still present in the medium. Stationary-phase cultures were obtained by growing cells for 3 days in the standard medium containing only 0.5% glucose.

Induction of trehalose synthesis and determination of trehalose levels. Cultures grown at 27°C were transferred to a water bath at ≥37°C to induce accumulation of trehalose. Cells were collected by filtration (Whatman GF/C), thoroughly washed with ice-cold water to remove external glucose, and resuspended in cold 5% (wt/vol) TCA. Samples were allowed to stand on ice for at least 15 min, and after removal of the filters, they were centrifuged at 2,000 × *g* for 15 min. Trehalose was determined in the supernatants either by the anthrone procedure (13) or by HPLC (in all isotopic tracer experiments). Comparable results were obtained by either technique. When the anthrone assay was used for trehalose determinations, the results were monitored by quantitative TLC. For this purpose, TCA extracts were deionized (Merck, Ion Exchanger V) and loaded on Silica Gel 60 plates (Merck) by using a Linomat III (Camag AG, Muttenz, Switzerland). Plates were developed with acetone-water (86:14, vol/vol) as a solvent. Trehalose spots were visualized and quantified fluorimetrically by the method of Klaus (Kontakte [Merck] 3:24, 1979) with a Shimadzu TLC scanner CS-930 (Shimadzu Inc., Kyoto, Japan). HPLC analyses were performed as described elsewhere (6). Quantitation was carried out with a refractive index detector

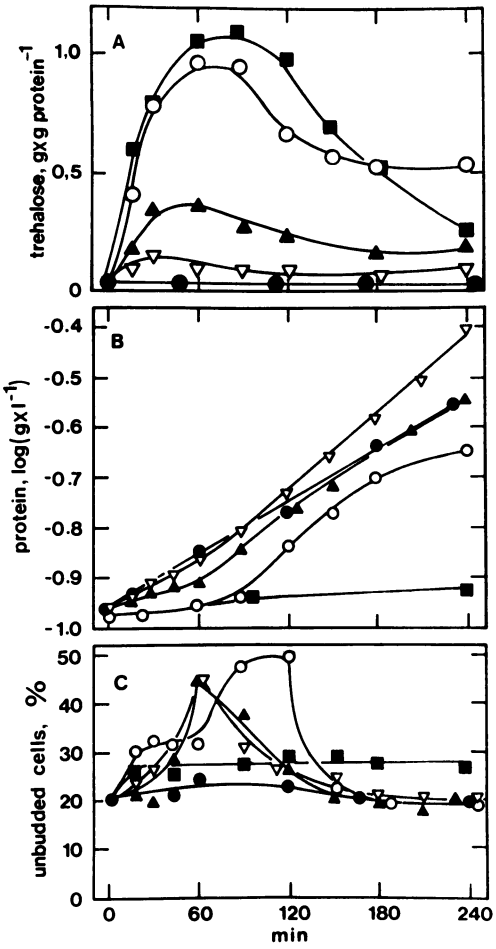

FIG. 1. (A) Heat-induced accumulation of trehalose by log-phase cultures of *S. cerevisiae*. Cultures grown on 2% glucose were heated to 35°C (▽), 37°C (▲), 40°C (○), or 45°C (■) at time zero. Controls were incubated at 27°C continuously (●). (B) Growth (protein) of the same cultures. (C) Accumulation of unbudded cells during heat shock.

(Erma Ltd., Tokyo, Japan) and a Shimadzu CR3A integrator.

Protein. In TCA-fixed samples and in cell homogenates proteins were determined by the methods of Peterson (20) and Bradford (2), respectively. Bovine serum albumin (Fluka, Buchs, Switzerland) was used as a standard.

Enzyme assays. Cells corresponding to ≈15 mg of protein were harvested by filtration (Whatman GF/C filters), washed three times with ice-water, and suspended in 0.6 ml of 50 mM tricine (K^+), pH 7.0, in a 1.5-ml Eppendorf tube. For homogenization, the filter was removed, 900 mg of glass beads (0.5 mm diameter) were added, and the tubes were shaken at 0 to 4°C on a Vibro mixer E1 (Chemap AG, Männedorf, Switzerland) until at least 95% of the cells were broken, as checked by phase-contrast microscopy. The homogenates were desalted on Sephadex G-25 and used for enzyme assays. The activity of neutral trehalase was determined as described (15). Tricine (K^+) (50 mM, pH 7.0) was used instead of MES (K^+). Trehalose-P-synthase activity was measured by the method of Betz (Ph.D. thesis, Ruhr-Universität, Bochum, FRG, 1974). Assay mixtures contained tricine (K^+) (50 mM), pH 7.0, glucose-6-P (10 mM), UDPG, (5 mM), $MgCl_2$ (12.5 mM), enzyme sample, and water in a total volume of 0.4 ml. In controls, glucose-6-P was omitted. Assay mixtures were incubated at 35°C for 5 to 30 min. The reaction was stopped by addition of 0.2 ml of ice-cold 1 N perchloric acid. After neutralization with 0.2 ml of 1 N KOH, the samples were stored on ice for 10 min and centrifuged at 2,000 × *g*. UDP was determined in the supernatants as follows. The assay mixture contained tricine (K^+) (140 mM), pH 7.6, phosphoenolpyruvate (2 mM), NADH (0.31 mM), lactic dehydrogenase (10 μl; ≈20 U), and sample in a total volume of 1.96 ml. The reaction was started by addition of pyruvate kinase (10 μl, 20 U). The decrease in absorbance at 340 nm was recorded and used to calculate the concentration of UDP. The assay was run at 35°C.

Labeling of trehalose. A culture growing exponentially on 1% glucose at 27°C was supplemented with [U-^{14}C]glucose (final specific activity, 200 μCi/mol) and rapidly transferred to a water bath at 40°C. After 45 min, the cells were collected by filtration, thoroughly washed with unlabeled medium at 40°C, and suspended in the same medium warmed to 40°C (note that the cells received fresh glucose [2%], minerals, and vitamins). The washing procedure took less than 3 min. Half of the culture was further incubated at 40°C, and the other half was shifted to 27°C. Samples of cells were withdrawn at intervals. A portion of each sample was used for the determination of trehalose by HPLC. The rest was subjected to TLC as described. The trehalose band was removed from the TLC plate, trehalose was eluted with water, and radioactivity was determined by liquid scintillation spectrophotometry.

Chemicals and enzymes. Chemicals and enzymes were obtained from Sigma. [U-^{14}C]glucose was purchased from Amersham.

RESULTS

Changes in the trehalose level following temperature shifts. At 27°C, cells of *S. cerevisiae* growing exponentially on glucose contained only traces of trehalose. However, they accumulated large amounts of this disaccharide when they were subjected to a heat shock (Fig. 1A). The threshold temperature for trehalose accumulation was 33 to 35°C. While 35°C was still comparatively ineffective, the trehalose pool rapidly expanded at temperatures between 37 and 45°C (Fig. 1A). At 50°C, trehalose accumulation ceased and cells died (not shown). Maximal expansion of the trehalose pool was observed at 45°C (Fig. 1A). However, ≥20% of the cells were killed when incubated at 45°C for 60 min. Therefore, in all further experiments described here, the heat shock was performed at 40°C. At that temperature, up to 1 g of trehalose per g of protein was accumulated and the cells remained viable (1.5% dead cells after 60 min, <5% dead after 100 min).

Remarkably, the accumulation of trehalose started immediately after the cells were shifted to the elevated temperature, and the rate of its net synthesis was almost constant during the first 30 min of heat treatment (Fig. 1A). The trehalose concentration reached its maximum after 60 to 90 min at 40°C. Thereafter, it declined but remained at a much higher level than in control cells kept at 27°C. No trehalose was synthesized when the cells were transferred to medium without glucose prior to the heat shock (not shown).

A large fraction of the glucose taken up at 40°C was spent for the synthesis of trehalose. In a culture labeled with [U-^{14}C]glucose for 45 min, trehalose accounted for ≈32% of the total radioactivity in the cells and ≈46% of the activity in cold TCA extracts. In the neutral fraction of the TCA

extracts, trehalose accounted for approximately 84% of the radioactivity, indicating that no other neutral sugar or polyol was accumulated in substantial quantities during the heat shock. This result was confirmed by HPLC analyses.

Since accumulation of trehalose has been found to coincide generally with periods of reduced growth (24, and references cited therein), we compared the effect of heat on growth rates and on trehalose production. Protein was measured as an indicator of growth. After a shift from 27 to 45°C, growth was completely arrested within 20 min (Fig. 1B). Interestingly, the number of unbudded cells hardly increased, indicating that completion of the cell cycle was impossible at this temperature (Fig. 1C). At 40°C, growth ceased for about 90 min (Fig. 1B). Large amounts of trehalose were accumulated during this period (Fig. 1A). Resumption of growth coincided with a decrease in the trehalose content of the cells, suggesting that trehalose accumulation might indeed be a consequence of reduced growth. The number of unbudded cells increased from 0 to 120 min after the transfer to 40°C and then sharply declined to the level of the control (Fig. 1C). At 37 and 35°C, the protein measurements indicated that growth was not appreciably reduced (Fig. 1B), although considerable amounts of trehalose were accumulated. However, a peak of unbudded cells 40 to 90 min after the shift to elevated temperature indicated a transient G1 arrest of the cells (Fig. 1C), as described by Johnston and Singer (9). Cultures shifted to 35°C grew somewhat faster and contained less trehalose than those at 37°C (Fig. 1A and B). In sum, these data show that in heat-shocked cells of *S. cerevisiae*, accumulation of trehalose is accompanied by growth arrest, reduced growth, or at least transient G1 arrest.

Stationary-phase cells rapidly degraded their large trehalose reserves when fed with glucose (2%) at 27°C (Fig. 2). However, when the same cells were shifted to 40°C, their already high trehalose content doubled within 60 min after glucose had been added. In the absence of external glucose, no further trehalose was accumulated; obviously the cells did not convert their considerable glycogen reserves to trehalose.

The fate of the trehalose pool in heat-shocked cells during recovery was investigated. A log-phase culture grown at 27°C was incubated at 40°C for 45 min. Half of it was then shifted back to 27°C, and the other half was further incubated at 40°C. The cells at 40°C kept their trehalose content high, but those shifted back to 27°C rapidly degraded the trehalose. After 40 min, they contained the same small amount of trehalose as the control cells grown at 27°C throughout (Fig. 3A). Thus, trehalose degradation during recovery from heat shock proceeded about as fast as trehalose accumulation in response to the shock. The possibility of inducing rapid accumulation or degradation of trehalose simply by shifting the temperature above or below 37°C provides an ideal system for studying trehalose metabolism.

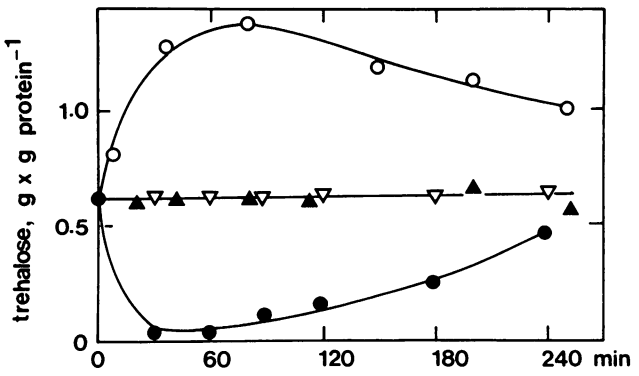

FIG. 2. Changes in the trehalose content of stationary-phase cultures of *S. cerevisiae* as affected by the addition of glucose and by heat shock. Glucose (2%) was added at time zero (○, ●) or not added (▲, ▽). Open symbols, Cultures at 40°C; solid symbols, cultures at 27°C.

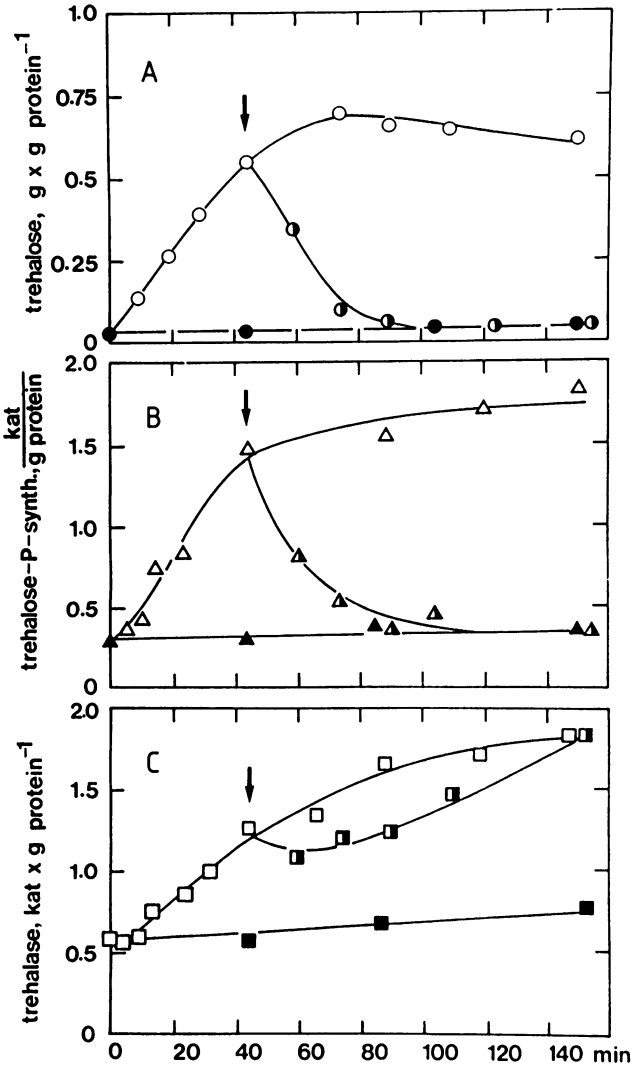

FIG. 3. Trehalose (A), activity of trehalose-P-synthase (B), and activity of neutral trehalase (C) as affected by temperature shifts. Log-phase cultures of *S. cerevisiae* were used. The temperature was shifted from 27 to 40°C at time zero. Open symbols, Cells at 40°C; solid symbols, controls at 27°C; half-open symbols, cells incubated at 40°C for 45 min and then shifted back to 27°C.

Changes in the activities of trehalose-6-P synthase and trehalase following temperature shifts. Heat treatment induced a sixfold increase in the activity of trehalose-P-synthase (Fig. 3B). When the temperature was shifted back to 27°C after the heat shock, trehalose-P-synthase activity rapidly declined and reached the normal low level within about 60 min. On the whole, the changes of trehalose-P-

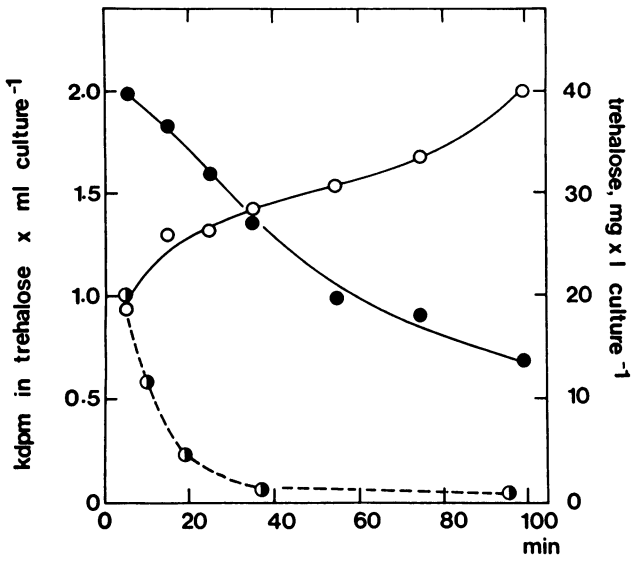

FIG. 4. Turnover of trehalose in log-phase cells of *S. cerevisiae* incubated at 40°C. The cells were labeled with [U-^{14}C]glucose for 45 min at 40°C. At time zero, they were transferred to an unlabeled medium and further cultivated at 40°C. Open symbols, Trehalose content of the cells; solid symbols, total radioactivity in trehalose; half-open symbols, trehalose content of a control culture shifted from 40 to 27°C at time zero.

synthase activity mirrored the fluctuations of the trehalose pool (Fig. 3A and B).

Surprisingly, heat stress also induced an increase in the activity of neutral trehalase (Fig. 3C). When the temperature was reduced to 27°C after the heat treatment, trehalase activity remained approximately constant for about 50 min, although rapid degradation of trehalose was initiated during this period. Thus, the changes in trehalase activity were almost opposite to those one would have expected if the trehalose level were regulated by this enzyme.

Turnover of trehalose during heat shock. Since neutral trehalase and its substrate, trehalose, are both located in the cytosol (10, 27; unpublished results), it was a surprise to find that trehalose was rapidly accumulated by heat-shocked cells even in the presence of high activities of trehalase. It has been reported that it might be difficult to correctly determine the activity of neutral trehalase after a heat shock (23). A brief heat treatment (3 min, 53°C) of *Pichia pastoris* ascospores induced an apparent 10-fold activation of trehalase, but only if the samples were chilled by ice-water. No activation occurred if the samples were injected into liquid nitrogen (23). These results show that trehalase activities measured in vitro must be interpreted with caution. We were unable to obtain reproducible results for trehalase activity with yeast cells frozen in liquid nitrogen. Because of the difficulty in determining the enzyme activity in vitro beyond doubt, we decided to test trehalase activity in vivo by means of an isotopic tracer experiment. In a culture growing exponentially on 1% glucose, the temperature was shifted from 27 to 40°C. Simultaneously, [U-^{14}C]glucose was supplied (1.8 μCi/ml; specific activity, 200 μCi/mol). After 45 min the cells were rapidly collected by filtration, washed with warm standard medium, and suspended in fresh warm medium. Half of the culture was further incubated at 40°C, and the other half was brought to 27°C. In the part kept at 40°C, the radioactivity in trehalose rapidly declined, although the trehalose content of the cells continued to increase (Fig. 4). The rapid loss of labeled trehalose was not due to leakage of the cells; only a small amount of labeled trehalose was found in the culture medium at the end of the experiment. These results show that both trehalose-P-synthase and trehalase were active in the cells. A half-life for trehalose of less than 45 min was calculated from the data in Fig. 4, assuming no synthesis of trehalose from radioactive glucose during the chase. If some of the radioactive glucose liberated by trehalase is reincorporated into trehalose, the true half-life of trehalose is even shorter. The half-life of trehalose in cells recovering from a heat shock was found to be ≤13 min.

DISCUSSION

In recent work, the mobilization of stored trehalose in yeast cells has been shown to generally coincide with activation of neutral trehalase. Trehalase activation is effected by a cAMP-dependent phosphorylation (reviewed in reference 24). In the experiments described in this report, the changes in the activity of trehalase could not explain the dramatic fluctuations of the trehalose pool. It was rather trehalose-P-synthase which played a key role, as its activity changed in parallel to the trehalose content of the cells. Control of the trehalose pool in yeast cells mainly by modulation of the activity of trehalose-P-synthase has been suggested by Panek and Mattoon (19).

The rapid increase in trehalose-P-synthase activity following heat shock raises the question of whether trehalose-P-synthase might be a heat shock protein. At present, this possibility cannot be excluded. However, two recent publications by Panek et al. (17) and Shin et al. (21) imply that the enzyme is activated by heat shock rather than synthesized de novo. According to Panek et al. (17), the activity of trehalose-P-synthase is regulated by cAMP-dependent phosphorylation and dephosphorylation. Dephosphorylation brings about activation of the enzyme. According to Shin et al. (21), heat shock represses cAMP-dependent protein phosphorylation in yeast cells. Together, these findings suggest that the activation of trehalose-P-synthase in heat-shocked cultures of *S. cerevisiae* might be due to dephosphorylation of the enzyme. However, further studies are required to substantiate this hypothesis.

Avigad (1) has reported that trehalose is rapidly turned over in resting cells of *S. cerevisiae*, at least in the presence of a carbon source in the medium. Nevertheless, we were surprised to find such a high rate of trehalose degradation even when the trehalose pool was expanding. Since the biosynthesis of 1 molecule of trehalose from glucose consumes 3 molecules of ATP, whereas no ATP is produced upon hydrolysis of trehalose by trehalase, we conclude that an energy-consuming futile cycle exists between trehalose and glucose.

At 30°C, *S. cerevisiae* contains about 1 μmol of ATP per g (dry weight), whereas at 37°C the content is about five times higher (7), indicating overproduction of ATP. The trehalose cycle might be a system to counteract ATP overproduction at supraoptimal temperatures. If one assumes that the half-life of trehalose is 45 min at 40°C (data from Fig. 4), the total ATP pool of the cultures used in our experiments would be consumed within <1 min by the trehalose cycle. This may seem spectacular. However, it is a long span compared with the normal half-life of ATP, which is about 1 s in microorganisms (22). We therefore favor the speculation that rapid accumulation of trehalose and futile cycling may be essential

for maintaining a constant concentration of glucose in the cytosol. During a heat shock, the glucose concentration in the cells increases up to 50-fold (unpublished results). Glucose can damage cells by reacting nonenzymatically with proteins (4). In addition, glucose taken up in excess would probably interfere with important regulation mechanisms of the cells. It is known that mutant strains of *S. cerevisiae* unable to control their glucose pool during growth on maltose will die when supplied with this carbon source, possibly due to excessive accumulation of glucose and maltose (5). Glucose flowing into the cells in excess of the demand could be efficiently removed by the formation of trehalose, and this reaction could be initiated most rapidly if the cells maintained a relatively high turnover of trehalose.

Finally, the masses of trehalose accumulated in response to a heat shock might prevent the cytosol from being impaired by the heat shock or by dehydration due to prolonged exposure to high temperatures. A protective role for trehalose, in addition to its role as a carbohydrate reserve, has been postulated repeatedly (10, 16), and evidence for such a function has recently been found (7a).

ACKNOWLEDGMENTS

We thank T. Boller and B. Hemmings (Basel) for improving our manuscript. T.H. thanks R. Betz and M. Steden (Bochum) for helpful information concerning the trehalose-P-synthase assay and for the communication that the enzyme activity increases upon a heat shock.

This work was supported by the Swiss National Science Foundation.

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