Microcalorimetric Monitoring of Growth of Saccharomyces cerevisiae: Osmotolerance in Relation to Physiological State

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The importance of the physiological state of a culture of Saccharomyces cerevisiae for tolerance to sudden osmotic dehydration was studied, and it was investigated whether specific osmotolerance factors were demonstrable. The microcalorimeter was used to monitor growth, and different physiological states of the culture were selected and their osmotolerance was tested. In addition to cells in the stationary phase, cells from the transition phase between respirofermentative and respiratory catabolism were osmotolerant. S. cerevisiae exhibited ever-changing metabolism during batch growth on either glucose or ethanol as the carbon source. Instantaneous heat production per biomass formation (dQ/dX) and specific activity of sn-glycerol 3-phosphate dehydrogenase (GPDH) (EC 1.1.1.8) were shown to differ for different physiological states. Neither high respiratory activity nor low total cellular activity, nor factors involved in osmoregulation, i.e., intracellular glycerol or activity of GPDH, correlated with the osmotolerant phenotype.

Different species of yeast vary considerably in response to reduced water activity. While Debaryomyces hansenii and Saccharomyces (Zygosaccharomyces) rouxii are capable of growth in solutions of about 4 M sodium chloride (21, 23), the maximal sodium chloride concentration allowing growth of Saccharomyces cerevisiae is 1.7 M (23).

Osmotolerance is, however, not just species dependent. For S. cerevisiae, osmotolerance was found to vary between cells within an exponentially growing pure culture. About 0.01% of the cells survived plating onto a low-water-activity (a,) agar medium, a phenomenon called water stress plating hypersensitivity (19).

Polyol production and accumulation are proposed to be essential for growth of different microorganisms in high salt concentrations (1, 7, 9, 14, 19). The tolerance of D. hansenii to sudden osmotic dehydration, measured as the length of the lag phase, seemed to increase with enhanced concentrations of intracellular polyols (glycerol plus arabinitol) (2). Glycerol has been found to be the only polyol produced by S. cerevisiae (15).

The cytoplasmic sn-glycerol-3-phosphate dehydrogenase (GPDH) (EC 1.1.1.8) might be a bottleneck in the glycerol-producing pathway (A. Blomberg and L. Adler, submitted for publication). Increased levels of GPDH would provide the cells with a greater capacity for glycerol production. Adaptation of S. cerevisiae to high salinity resulted in the production of elevated amounts of glycerol and increased specific activity of GPDH (11).

The total cellular activity may be a determinant for tolerance, since stationary-phase cells metabolizing at a low rate show an increased resistance to physical and chemical environmental factors (19, 20, 24, 26). Total cellular activity can be studied by microcalorimetry. This technique allows measurements of the heat production rate (dQ/dt) of the culture, which is related to the amount of biomass, the kind of metabolism used, the rate of growth, and the energy spent on reactions not directly coupled to biosynthesis (5, 13).

In order to elucidate the importance of the above-mentioned potential osmotolerance factors, i.e., accumulation of glycerol, activity of GPDH, and total cellular activity, for the response of cells to a sudden osmotic dehydration, reproducible methods in defining the physiological states of the culture during growth are needed. It should be pointed out that during batch growth, the physiological state of the culture will often be composed of a mixture of different metabolic states. A high degree of resolution of the growth process can be achieved by measuring the heat production rate (dQ/dt), which enables observations of fine temporal events during growth (6, 25).

In this study, the continuously registered heat production rate curve has been used as a fingerprint of the growth cycle to collect cells of S. cerevisiae at specific points during growth. The culture has been characterized and its physiological state was shown to be of vital importance to the ability of the cells to tolerate sudden osmotic dehydration.

MATERIALS AND METHODS

Yeast strain, media, and growth conditions. S. cerevisiae Y41 (ATCC 38531) (3) was maintained on nutrient Wickerham agar and cultured during experiments in yeast nitrogen base without amino acids (YNB) (Difco). This medium was supplemented with either glucose or ethanol as the carbon source at a final concentration of 0.5% (wt/vol) except for inoculum cultures, where 1% (wt/vol) glucose was used. The glucose and ethanol were dissolved in 100 ml of distilled water and separately autoclaved or sterile filtered, respectively, and thereafter added aseptically to 900 ml of sterile YNB. Inoculum cultures were grown aerobically in 50 ml of liquid medium with mechanical stirring to the stationary phase (72 h). A 10-ml inoculum of this was added to 250 ml of medium in a conical 1-liter flask to give an initial concentration of 0.5 x 10⁶ to 1 x 10⁸ CFU/ml. All liquid cultures were incubated in a water bath at 30°C and stirred mechanically. During growth, the rate of heat production (μW) and pH were monitored continuously. Samples were withdrawn at selected time intervals or at specific points, designated G/R1, G/R2, G/T, G/R1, G/R2, G/R3, and G/S for glucose-grown cells and E/R1, E/R2, E/R3, and E/S for ethanol-grown cells. Some of the specific points were selected on the basis of the heat production rate (dQ/dt) curve, while others were chosen according to absorbance values at 610 nm. For

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Further information, consult "Identification of physiological states" in the Results section.

**Growth determinations.** Growth was followed turbidometrically at 610 nm, and optical density values were calculated to yield dry weight values by the method of Larsson and Gustafsson (18).

**Microcalorimetry.** The heat production rate (dQ/dt) was measured with a multichannel microcalorimeter (Biactivity Monitor LKB 2277) of the heat conduction type (28), fitted with three channels, each equipped with a flowthrough cell to permit simultaneous recording of three cultures. Each channel contained a measuring and a reference cell. The voltage signal was recorded by a two-channel potentiometric recorder (LKB 2210-022, 1,000 mV range). The flowthrough cell and the connected tubes were sterilized with 70% (wt/vol) ethanol and then rinsed with sterile culture medium until baseline stability was reached. The baseline stability fluctuated less than 1 mW over 24 h in the 300- and 1,000-mW ranges used. For every experiment, the external calibration was performed with an external current supplied to produce 100 or 300 mW. Internal calibration was performed with a chemical reaction (10) and gave an effective volume of the flowthrough cell of 0.331 ml. The microcalorimeter was operated at 30.0°C. The culture was pumped at a rate of 58 ml/h from the growth vessel by a peristaltic pump (Micro Perpex pump LKB 2132) to a T-connection outside the calorimeter, where it was met by a constant flow (32 ml/h) of humidified air (30.0°C) delivered by a second peristaltic pump. After the microcalorimetric measurement it was returned to the growth vessel.

The instantaneous heat production per biomass formation (dQ/dx) was calculated by dividing the specific heat production rate (microwatts per milligram [dry weight]) by the specific growth rate constant (μ).

**Warburg measurements.** CO₂ production and O₂ consumption were monitored by the Warburg technique, and the respiratory quotient (RQ; moles of CO₂ produced per moles of O₂ consumed) was calculated. The equilibration time was 15 min, and measurements over 60-min periods yielded steady-state rates (except for the G/T sample). Measured values are plotted 30 min after sampling (see Fig. 3). For the G/T sample, which showed an ever-changing CO₂ production, values are plotted for every 10 min, with a start value 20 min after sampling.

**Tests for tolerance of osmotic dehydration on solid substrate.** Colony-forming capacity was measured on YEFD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) with appropriate amounts of NaCl added to give final concentrations of 0, 1.0, 1.4, and 1.5 M NaCl. Culture samples were mixed vigorously (Vortex tube mixer) and serially diluted in 0.9% (wt/vol) NaCl solution, and 100-μl portions were spread on plates containing the various NaCl concentrations. Samples were spread immediately to avoid diffusion of NaCl into the sample drop, as this would result in a localized decrease in the agar medium NaCl concentration. The plates were incubated in plastic bags to avoid dessication.

**Tests for tolerance of osmotic dehydration in liquid cultures.** At the specified sampling points, samples were withdrawn from the liquid culture, centrifuged, and inoculated in 20 ml of liquid medium in 100-ml sidearm flasks to give an initial optical density of 0.05 at a wavelength of 610 nm. Different NaCl concentrations (1.0, 1.4, or 1.5 M) were used. The flasks were incubated on a rotary shaker and weighed every day. The liquid volume lost through evaporation was corrected for daily by the addition of sterile water. Growth was followed by measuring the turbidity of the culture.

**Preparation of samples for total glycerol determinations.** Samples (1.5 ml) for analysis of combined intra- and extracellular glycerol were immediately heated in tubes with a pear-drop condenser at 95°C for 10 min and subsequently centrifuged (Eppendorf) for 1 min at 10,000 × g. The supernatants were frozen until analyzed.

**Preparation of samples for extracellular determinations of glucose, ethanol, glycerol, and acetate.** For glucose, ethanol, glycerol, and acetate analyses, samples (1.5 ml) were centrifuged (Eppendorf) for 1 min at 10,000 × g. The supernatants were frozen immediately or, for glycerol analysis, after being heat treated for 10 min as described above.

**Assays.** Concentrations of glucose, ethanol, glycerol, and acetate were analyzed by using enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany). Intracellular concentrations of glycerol were determined by subtraction of the extracellular values from total values.

**Preparation of cell extracts for enzyme activity measurements.** Cells (0.25 to 0.5 g, wet weight) were suspended in 0.5 ml of homogenization buffer (10 mM triethanolamine, 1 mM diithioerythritol [DTE], 1 mM EDTA, pH 7.5). The cell suspension was homogenized at 4°C for 5 min in a bead mill (Vibrogen-Zellmühle, Edmund Büehler, Federal Republic of Germany) with 1 g of glass beads (0.5 mm diameter). The homogenate was collected, and the homogenization vessel was rinsed with 0.5 ml of homogenization buffer. This washing was pooled with the homogenate. The crude extract was centrifuged at 18,000 × g for 15 min. The supernatant was desalted by passage through a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and then assayed for GPDH activity. The final protein concentration of the extract was around 10 mg/ml. Protein was determined by the method of Sedmak and Grossberg (27) with gamma globulin (Sigma) as the standard.

**Assay of enzyme activity.** NAD-dependent GPDH (EC 1.1.1.8) was assayed in a buffer containing 20 mM imidazole hydrochloride (pH 7.0), 1 mM DTE, 1 mM MgCl₂, 0.67 mM dihydroxyacetonephosphate (DHAP), and 0.09 mM NADH. NADH depletion was monitored spectrophotometrically at 340 nm. The assay was performed in a recording Shimadzu UV-240 double-beam spectrophotometer at 25°C in 1-ml cuvettes. The reaction was started by addition of DHAP and was linear for at least 2 min. To calculate the enzyme activity, the reaction rate between 30 and 90 s and an absorption coefficient for NADH of 6.2 × 10³ M⁻¹ cm⁻¹ were used (1 U = 1 μmol of NADH consumed per min). No background NADH oxidase activity was detected. At least five protein concentrations in the range from 0.05 to 0.5 mg/ml in the cuvette were recorded per sample.

Specific activities were dependent on protein concentration in the cuvette (11); the higher the protein concentration, the higher the specific activity. Consequently, from the graph presenting the inverse of specific activity against the inverse of the protein concentration, the line fitted by least-square regression gave the maximum specific activity as the specific activity extrapolated from an infinite protein concentration. This mathematical transformation of the recorded specific activities of a sample into the maximum specific activity of a sample did not change the relative order between different samples, i.e., the fitted lines never crossed each other in the first quadrant. The maximum specific activity will be referred to as the specific activity throughout this article.
RESULTS

Microcalorimetric monitoring of growth. With glucose as the carbon and energy source, S. cerevisiae exhibited diauxic growth (Fig. 1A). During the first phase of growth, glucose was consumed, and ethanol, together with small amounts of glycerol and acetate, was produced (Fig. 1A, C, and D). For most of this glucose-consuming phase, the heat production rate (dQ/dt) increased in parallel with the biomass (Fig. 1A), which resulted in a specific heat production rate of ca. 770 µW/mg (dry weight) (the respirofermentative phase; Table 1). Prior to glucose exhaustion, when the external concentration of glucose reached a level of ca. 2 mM, the heat production rate dropped (Fig. 1A and C). The cause of this drop was almost certainly an inadequate supply of glucose, due to the kinetics of the transport system for glucose: high-affinity Kₘ of 1 to 2 mM (17). A minimum in the specific heat production rate of ca. 380 µW/mg (dry weight) was obtained (the transition phase; Table 1).

During the second phase of growth, the change to ethanol consumption resulted in a reduced rate of biomass production, with a specific growth rate constant of ca. 0.025 h⁻¹ (Fig. 1A; Table 1). The concentration of acetate fluctuated concomitantly with ethanol utilization (Fig. 1C and D). When ethanol was exhausted, a small plateau in the heat production rate curve appeared (Fig. 1A), during which acetate was totally consumed. Simultaneously with the exhaustion of acetate, there was a marked drop in the heat production rate (Fig. 1A), which resulted in a low specific level of 125 µW/mg (dry weight) (Table 1). This point represents the commencement of the stationary phase, at which time growth had ceased.

Great similarities were seen in the shapes of the curves of the heat production rate of ethanol-grown cells (Fig. 2) and the respiratory phase of glucose-grown cells (Fig. 1). A sudden drop in the heat production rate occurred concomitantly with the exhaustion of ethanol (Fig. 2A and C). As previously shown for growth on glucose, a small plateau appeared in the heat production rate curve just before the beginning of the stationary phase and lasted for 1 to 5 h. Acetate (about 1 mM) was utilized during this period (Fig. 2A and D). The maximum specific heat production rate of about 1,100 µW/mg (dry weight) was attained in the middle of the growth period. In accordance with glucose-grown cells, the stationary-phase value of the specific heat production rate was 124 µW/mg (dry weight) (Table 2).

Identification of physiological states. The tested physiological states of glucose-grown cells are stated in Fig. 1 and Table 1 as G/RF1, G/RF2, G/T, G/R1, G/R2, G/R3, and G/S. During growth on glucose, some points were easily defined due to the heat production rate (dQ/dt) curve. Consequently, the continuously recorded heat production rate was used as a marker for sampling in the area of the transition between respirofermentative and respiratory metabolism, i.e., at points G/T (the first minimum), G/R1 (the second minimum), and G/R2 (2 h after the second minimum). The initiation of the stationary phase (G/S) was recognized by the return of

| Phase                      | Sample | µ (h⁻¹) | Specific dQ/dt (µW/mg [dry wt]) | dQ/dX (µW/mg [dry wt] formed) |
|----------------------------|--------|---------|---------------------------------|-----------------------------|
| Respirofermentative        | G/RF1  | 0.29 ± 0.02 | 763 ± 89                        | 9.4 ± 0.8                |
|                            | G/RF2  | 0.29 ± 0.03 | 785 ± 71                        | 9.8 ± 0.5                |
| Transition                 | G/T    | _        | 380 ± 21                        | _                          |
| Respiratory                | G/R1   | 0.03 ± 0.00 | 423 ± 21                        | 51 ± 3                    |
|                            | G/R2   | 0.02 ± 0.005 | 504 ± 31                       | 78 ± 16                  |
|                            | G/R3   | 0.03 ± 0.003 | 560 ± 46                        | 65 ± 4                    |
| Stationary                 | G/S    | 0        | 125 ± 12                        | _                          |

* Data are means ± SD for at least seven independent experiments.
  * * _._. No accurate data could be obtained.
the microcalorimetric signal to a low and steady level. The remainder of the sampling points were more difficult to specify. Sampling at points G/R1, G/R2, and G/R3 was performed at an absorbance of 0.5 (0.4 to 0.6), 1.0 (0.7 to 1.3), and 3.0 (2.9 to 3.1), respectively. Values within parentheses give the accepted sampling range.

The tested physiological states for ethanol-grown cells are referred to as E/R1, E/R2, E/R3, and E/S and are presented in Fig. 2 and Table 2. The only well-defined point in these microcalorimetric measurements appeared at the beginning of the stationary phase (sample E/S). Samplings at points E/R1, E/R2, and E/R3 were performed at an absorbance of 0.5 (0.4 to 0.6), 1.0 (0.7 to 1.3), and 4.0 (no range), respectively. Values within parentheses give the accepted sampling range.

**TABLE 2. Characterization of different physiological states for ethanol-grown cultures of** _S. cerevisiae_

| Phase       | Sample | _µ_ (h⁻¹) | Specific dQ/dt (µW/mg [dry wt]) | dQ/dX (J/mg [dry wt] formed) |
|-------------|--------|-----------|---------------------------------|------------------------------|
| Respiratory | E/R1   | 0.10 ± 0.01 | 928 ± 69                        | 32 ± 3                       |
|             | E/R2   | 0.10 ± 0.01 | 1140 ± 88                       | 40 ± 3                       |
|             | E/R3   | 0.07 ± 0.01 | 1086 ± 129                      | 54 ± 9                       |
| Stationary  | E/S    | 0         | 124 ± 28                        | ∞                             |

*a Data are mean ± SD for eight independent experiments.

**FIG. 3. Changes in respiratory and fermentative parameters of glucose-grown _S. cerevisiae_. (A) Recorded heat production rate (dQ/dt), with an effective volume of the measuring chamber of 0.331 ml. (B) Oxygen uptake rate (O₂ consumed) and carbon dioxide production rate (CO₂ produced). Results from a typical experiment are shown.

**Instantaneous heat production per biomass formation.** By normalizing the heat production rate (dQ/dt) to biomass formed per unit of time, the term instantaneous heat production per biomass formation (dQ/dX) is obtained: dQ/dX = (dQ/dt)/(dX/dt) and dX/dt = µX, where X stands for the biomass and µ represents the specific growth rate constant. Due to the fermentative activity, the respirofermentative phase was characterized by a low dQ/dX of about 9.5 J/mg (dry weight) formed (Table 1). The respiratory phase of glucose-grown cells, however, showed a high value of dQ/dX, 51 to 78 J/mg (dry weight) formed (Table 1). Ethanol-grown cells displayed a continuous rise in the dQ/dX value. At E/R1, 32 J was dissipated per mg (dry weight) formed, which increased to 54 J/mg (dry weight) formed in E/R3 (Table 2).

**Respiratory and fermentative activity.** Throughout the respirofermentative phase, changes in catabolic activities were obtained (Fig. 3). At point G/R2, a mean value of about 20 could be calculated for RQ, while a couple of hours later in G/R2, the RQ value had decreased to 0.6. As can be seen in Fig. 3, this was accomplished by changes in both respiratory and fermentative activities. Glucose was almost depleted in G/T (Fig. 1), which explains the decrease in fermentation. Glucose was exhausted between G/T and G/R1, in contradiction to the results of Brettel et al. (6), who reported glucose to be exhausted at the apex in the heat production rate curve prior to G/T. The respiratory activity is subject to glucose repression (12) and was not fully expressed until point G/R2 (Fig. 3). The respiratory activity of ethanol-grown cells was 4.5, 5.3, and 3.6 µmol of O₂ mg⁻¹ h⁻¹ for E/R1, E/R2, and E/R3, respectively.

**Tolerance to osmotic dehydration.** The colony-forming capacity of the population throughout growth on glucose was enumerated by plating samples on glucose-agar medium containing different concentrations of NaCl (Fig. 4A). The lowest colony-forming capacity on dehydrating medium was obtained for cells transferred from the respirofermentative phase (G/R1 and G/R2), as only 0.01% of the cells formed colonies on 1.5 M NaCl medium. A large fraction of CFU...
were obtained for samples taken from the transition phase (G/T), the beginning of the respiratory phase (G/R1 and G/R2), and from the stationary phase (G/S).

When cells grown on ethanol were plated on agar medium with different salt concentrations, the highest reduction in colony-forming capacity occurred during the first half of the growth period (Fig. 4B). At sampling point E/R1, only 0.01% of the cells formed colonies on 1.5 M NaCl plates. During the latter part of the growth period, there was an increase in the fraction of colony-forming cells, finally showing about 10% colony-forming units in the stationary phase in the presence of 1.5 M NaCl.

Adaptation to liquid salt medium. At the specified physiological states, samples were withdrawn and used as inocula to liquid salt medium with glucose as the carbon source. Growth of the osmotically dehydrated cells was followed turbidimetrically, and the length of the lag phase was taken as a measure of tolerance. At the lowest salt concentration tested (1.0 M NaCl), only minor differences in tolerance were observed regardless of the physiological state of the culture (data not shown). With increased concentrations of salt in the medium, more pronounced differences in tolerance were seen. In 1.4 M NaCl, the lag phases for G/RF1, G/RF2, and G/R3 were 70 to 80 h, while those of G/T, G/R1, and G/R2 were about four times shorter (10 to 20 h). The tolerance of glucose-grown cells to dehydration in 1.5 M NaCl medium is shown in Fig. 5. Inocula from the respirafermentative phase (G/RF1 and G/RF2) and G/R3 in the respiratory phase showed a remarkable reduction in tolerance to the lowered water activity. Cells from the transition phase (G/T), the beginning of the respiratory phase (G/R1 and G/R2), and the stationary phase (G/S) were unsurpassed in tolerance, with G/R2 showing the shortest lag phase.

The cells actively growing on ethanol, E/R1, E/R2, and E/R3, showed lower tolerance than cells from G/RF1 for all the concentrations of sodium chloride tested. Cells in the stationary phase, however, exhibited tolerance comparable to that of stationary-phase cells of glucose-grown cultures: 43-h lag phase in 1.5 M NaCl medium.

Glycerol. Growth on glucose was accompanied by glycerol production. Glycerol was produced during the respirafermentative phase up to point G/T (Fig. 1). During this phase, 1.8 mM glycerol and 0.54 mg (dry weight) of cells per ml were produced. A specific growth rate constant of 0.29 (Table 1) yielded an apparent glycerol production rate of (1.8 \times 0.29)/0.54 = 0.97 \mu mol \cdot g^{-1} \cdot h^{-1}. Only a minor portion of the glycerol that was produced was reutilized by the cells in the respiratory and stationary phases (Fig. 1D), probably due to the fact that the strain used is unable to utilize glycerol as the sole carbon and energy source. No intracellular glycerol was detected for cells grown on either glucose or ethanol (data not shown).

Additions of glycerol (final concentrations of 0.5, 5, 50, and 500 mM) to culture medium containing 1.4 or 1.5 M NaCl had no effect on the adaptation time (length of lag phase) of inoculated cells (data not shown). Contradictory results were obtained with a mutant of D. hansenii with defective glycerol production, for which glycerol additions (0.5 mM) positively affected the adaptation time of the cells in salt medium (4). This difference in response between the two yeasts can be explained by the fact that S. cerevisiae does not have an active uptake system for glycerol, as has been found for D. hansenii (1).

Levels of GPDH activity. Because of the presumptive role of GPDH in osmoregulation, its activity during growth without salt was of interest. For glucose-grown cells, the specific activity of GPDH was ca. 20 mU/mg of protein during the respirafermentative phase (Table 3). When the cells entered the transition phase, the activity rose about threefold. During the respiratory phase of growth, the specific activity stayed at ca. 60 mU/mg of protein. Ethanol-grown cells, however, showed a continuously increasing specific activity of GPDH, with a final value of 115 mU/mg of protein in the stationary phase (Table 3).

**DISCUSSION**

The microcalorimeter records the heat production rate \(dQ/dt\). The value of \(dQ/dt\) is determined by factors such as
TABLE 3. Specific activity of GPDH for different physiological states of *S. cerevisiae*  

| Sample | GPDH sp act (mU/mg of protein) |
|--------|--------------------------------|
| G/RF1  | 18 ± 0.9                      |
| G/RF2  | 21 ± 0.4                      |
| G/T    | 63 ± 15                       |
| G/R1   | 52 ± 3                        |
| G/R2   | 65 ± 7                        |
| G/R3   | 56 ± 9                        |
| G/S    | 64 ± 9                        |
| E/R1   | 17 ± 6                        |
| E/R2   | 32 ± 4                        |
| E/R3   | 85 ± 6                        |
| E/S    | 115 ± 6                       |

* Data are mean ± SD for duplicate independent experiments.

the type of catabolism and anabolism, amount of biomass, rate of growth, and level of ATP-consuming reactions not directly involved in biosynthesis, e.g., ion pumping (5, 13). In order to allocate functional meaning to the dQ/dt data, all these factors have to be considered. Normalization of the heat production rate (dQ/dt) by the rate of biomass formation (dX/dt) will yield the instantaneous heat production per biomass formation (dQ/dX), which is independent of the amount of biomass and rate of growth. The instantaneous heat production per biomass formation (dQ/dX) was shown to differ for different physiological states of glucose-grown *S. cerevisiae*, respiratory-phase cells having a higher value (ca. 60 J/mg [dry weight] formed) than cells from the respirofermentative phase (ca. 9.5 J/mg [dry weight] formed). In a previous study, in which the pH was kept constant at 6.0 throughout growth, the overall heat production per biomass formation for respiratory-phase cells was 30.2 J/mg (dry weight) formed (6). This is in accordance with the value of dQ/dX for E/R1 (Table 2). The pH of our cultures decreased steadily (Fig. 1B and 2B), with a final pH in the stationary phase of ca. 2.5. We therefore propose that increased energy demand for proton pumping is partly the cause of the high dQ/dX values in the respiratory phase of glucose-grown cells as well as the steadily increasing dQ/dX for growth on ethanol.

The heat measurements in combination with other analyses highlighted the ever-changing metabolism of *S. cerevisiae* during this batch mode of cultivation on either glucose or ethanol. Up to the first apex of the heat production rate curve of glucose-grown cells, the yeast cells used respirofermentative catabolism with changing fermentative activity (Fig. 3). The highest fermentative activity was reached between points G/RF1 and G/RF2. At these points, the cells showed a pronounced sensitivity to osmotic dehydration (Fig. 4 and 5). This low tolerance was, however, not due to respirofermentative metabolism per se, since cells from the middle of the respiratory phase (G/R3) also showed pronounced sensitivity to dehydration in liquid salt medium as well as on salt plates. Perpetually lower tolerance to dehydration was shown for ethanol-grown cells. Thus, a high respiratory capacity was shown not to favor a high tolerance to a sudden osmotic dehydration of *S. cerevisiae*. Respiration was shown earlier not to be the osmosensitive cellular function limiting growth under osmotic stress, since respiratory activity was found at salt concentrations that do not permit growth (22).

Glucose-grown cells of *S. cerevisiae* produced all their glycerol in the respirofermentative phase (Fig. 1). It has been suggested that increased concentrations of intracellular polyols decrease the sensitivity of *D. hansenii* to sudden exposure to high salinity (2). Brown et al. (8) and Adler et al. (1) have proposed that glycerol is the main osmoregulator needed by yeast cells, which manage to grow under severe osmotic stress. For cells grown without salt, we show that none of the glycerol produced was retained intracellularly. Thus, glycerol cannot be judged as the determining factor for tolerance to sudden osmotic dehydration of *S. cerevisiae* grown without sodium chloride.

The cytoplasmic GPDH is one of the enzymes in the glycerol-producing pathway. GPDH shows an increased specific activity in yeast cells cultured at high salinity (1, 11). The ability of the cells to rapidly produce required amounts of glycerol may be favored by a high specific activity of GPDH (Blomberg and Adler, submitted). GPDH might thus have a role in the osmoregulation of the cells. During growth in the absence of sodium chloride, an increase in the specific activity of GPDH occurred concomitantly with a pronounced increased tolerance to sudden osmotic dehydration (transition-phase cells [G/T]). However, in spite of the high enzyme activity in cells originating from points G/R3, E/R2, and E/R3, these cells were sensitive to sudden osmotic dehydration. In other words, no correlation could be drawn between the specific activity of the enzyme and the capacity to adapt to or tolerate high concentrations of sodium chloride.

While the increased enzyme activity during the transition phase may be explained by a derepression of the enzyme upon glucose exhaustion, the steadily increasing values during growth on ethanol are not fully understood. Growth on ethanol produces an even higher amount of NADH per biomass formed than in glucose-grown cells (E. Oura. Ph.D. thesis, University of Helsinki, Helsinki, 1972). Besides being involved in glycerol production, GPDH is a constituent of the glycerol 3-phosphate shuttle, coupling cytosolic NADH recycling to mitochondrial respiration (16). The increase in GPDH activity during growth on ethanol might then reflect the enhanced need for balancing the cytoplasmic NADH/NAD pools. The cytoplasmic NADH oxidized in the glycerol 3-phosphate shuttle will yield less ATP than when mitochondrial NADH is oxidized in the respiratory chain. This might partly account for the increase in dQ/dX during growth on ethanol.

Stationary-phase cells with a low specific heat production rate of ca. 125 μW/mg (dry weight) were shown to be osmotolerant. Furthermore, when glucose-grown cells entered the transition phase, the specific heat production rate decreased by a factor of 2 concomitantly with a pronounced increase in osmotolerance (physiological state G/T). It seems that cells with low activity most easily adapted to and tolerated osmotic dehydration. The cells that most easily adapted to high salt concentrations, however, originated from the beginning of the respiratory phase of glucose-grown cultures (point G/R2, Fig. 5). These G/R2 cells showed a specific rate of growth and a specific heat production rate in the same order as for the nonsmotolerant cells from G/R3. Thus, total cellular activity was not a determinant for the osmotolerant phenotype.

This work was initiated to study the influence of the physiological state of *S. cerevisiae* on tolerance to a suddenly increased environmental salt concentration and to investigate whether specific osmotolerance factors were demonstrable. Our results show that large changes in the status of the cells occur within short time periods during growth and that the physiological state of the culture is of
utmost importance for the capacity of the cells to tolerate sudden osmotic dehydration. This was demonstrable by using the microcalorimetry technique, as physiological states could be clearly defined, especially during the rapid course of events in the transition phase and at the entrance to the stationary phase. The existence of a transition phase for glucose-grown \textit{S. cerevisiae} is frequently neglected, which might cause errors in the interpretation and comparison of data. The second purpose was to study the relationship between osmo-tolerance and different conceivable osmotic defense factors. Two of the factors chosen, glycerol and GPDH activity, both have potential roles in the homeostatic cellular functions, trying to neutralize the deleterious effects of osmotic dehydration during growth in or adaptation to osmotic stress. We have not been able to correlate any one of these osmoregulatory factors with the capacity to cope with sudden osmotic dehydration. At present, there seems to be no overlap between the factors involved in osmoregulation and the factors conferring osmotolerance. It might be, however, that another factor(s), in cooperation with these osmoregulatory factors, gives rise to the osmotolerant phenotype.

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**LITERATURE CITED**

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