

## Energy Flux and Osmoregulation of *Saccharomyces cerevisiae* Grown in Chemostats under NaCl Stress

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The energetics and accumulation of solutes in *Saccharomyces cerevisiae* were investigated for cells grown aerobically in a chemostat under NaCl stress and glucose limitation. Changed energy requirements in relation to external salinity were examined by energy balance determinations performed by substrate and product analyses, with the latter including heat measurements by microcalorimetry. In both 0 and 0.9 M NaCl cultures, the catabolism was entirely respiratory at the lowest dilution rates tested but shifted to a mixed respiratory-fermentative metabolism at higher dilution rates. This shift occurred at a considerably lower dilution rate for salt-grown cells. The intracellular solute concentrations, as calculated on the basis of intracellular soluble space determinations, showed that the internal Na<sup>+</sup> concentration increased from about 0.02 molal in basal medium to about 0.18 molal in 0.9 M NaCl medium, while intracellular K<sup>+</sup> was maintained around 0.29 molal despite the variation in external salinity. The intracellular glycerol concentration increased from below 0.05 molal at low salinity to about 1.2 molal at 0.9 M NaCl. The concentrations of the internal solutes, however, changed insignificantly with growth rate and energy metabolism. The additional maintenance energy expenditure for growth at 0.9 M NaCl was, depending on the growth rate, 14 to 31% of the total energy requirement for growth at 0 M NaCl. Including the energy conserved in glycerol, the total additional energy demand for growth at 0.9 M NaCl corresponded to 28 to 51% of the energy required for growth at 0 M NaCl.

Transfer of yeasts to a more saline environment results in an increased energy expenditure which is manifested by changes in the carbon and energy flux (21, 22, 30, 51). These changes are characterized primarily by (i) an increased maintenance energy and (ii) an increased production of glycerol, which both occur at the expense of the cellular anabolism. An increased ATP turnover is expected in saline environments to meet the increased demand to uphold an intracellular ionic composition compatible with cellular function (21, 37, 51). The maintenance requirements at increased concentrations of NaCl have been studied with batch cultures of the yeasts *Saccharomyces cerevisiae* and *Debaryomyces hansenii* (21) and with glucose-limited chemostat cultures of a respiration-deficient mutant of *S. cerevisiae* (51). Since cells in carbon- and energy-rich media may not adjust to minimum energy demands (43), maintenance energy requirements may be greatly overestimated by use of batch cultures. In Watson's chemostat study (51), on the other hand, the maintenance energy requirements were estimated by the use of a Pirt plot (38), in which the substrate fraction used for maintenance is considered constant and therefore independent of the growth rate. In more recent studies, however, maintenance energy requirements were suggested to include both growth rate-independent and growth rate-dependent components (11, 39).

Glycerol is a mandatory end product when *S. cerevisiae* ferments glucose to ethanol. While production of ethanol and CO<sub>2</sub> from glucose is a redox-neutral process, the formation of biomass and oxidized by-products such as acetate generates an excess of reducing equivalents which can be disposed of by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate followed by dephosphorylation to glycerol. Glycerol production also results from exposure of the

cells to reduced water potential. This is considered to be an osmoregulatory response leading to intracellular accumulation of glycerol to maintain turgor, volume, and a biochemically functional internal milieu under conditions of external solute stress (5, 9). Most studies of the osmotic relations of *S. cerevisiae* have been conducted with cells grown in batch culture and have examined either the ionic (27, 36) or glycerol (4, 16, 30) relations of the cells. One of the matters addressed in the present study was the determination of internal levels of both ions and polyols in conditions under which the effects of the external salinity can be clearly separated from effects depending on growth rate and pH, etc. We also analyzed the total energy requirements and the additional maintenance energy requirements for growth at high salinity to gain a better understanding of the cellular demands for growth under NaCl stress.

### MATERIALS AND METHODS

**Organism.** The yeast strain used was *S. cerevisiae* Y41 (ATCC 38531) (2).

**Continuous-culture experiments.** The culture medium used consisted of yeast nitrogen base without amino acids (Difco) and was supplemented with sterile glucose to a final concentration of 0.5% (wt/vol) after being autoclaved. This basal medium, which is designated 0 M NaCl medium in this paper, contained 2 mM NaCl. For NaCl experiments, final concentrations of 0.5 and 0.9 M NaCl were used. Inoculum cultures were grown with shaking at 30°C for 24 h in yeast nitrogen base supplemented with 1% (wt/vol) glucose. A 50-ml inoculum was added to 2,600 ml of culture medium in a chemostat (1601 Ultroferm; LKB Instruments Inc., Rockville, Md.). This culture was grown to stationary phase before continuous substrate feeding. The stirring rate was 300 or 400 rpm, and the temperature was kept at 30°C. The culture was aerated with sterile filtered air (pore size, 0.22

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$\mu\text{m}$ ; Millipore Corp., Bedford, Mass.) at a flux rate of 220 or 280 liters/h. A condenser ( $6^{\circ}\text{C}$ ) was located at the gas outlet, and the pH was kept constant at 4.5 by addition of 1 M NaOH. At dilution rates of up to  $0.09\text{ h}^{-1}$ , the lower stirring and aeration rates were used, while at dilution rates above  $0.09\text{ h}^{-1}$ , the higher rates were adopted to avoid oscillations and to enable true steady states to be reached. The rate of heat production was continuously monitored by microcalorimetry. When the steady state was reached at each dilution rate (see below), samples were withdrawn for determination of dry weight, cell volume, extracellular glucose, ethanol and acetate, total and extracellular polyols and glycerol, intracellular  $\text{Na}^{+}$ ,  $\text{K}^{+}$ , and trehalose.

**Microcalorimetry.** The heat production rate ( $dQ/dt$ ) was measured with a multichannel microcalorimeter (Bioactivity Monitor LKB 2277; Thermometric AB, Järfälla, Sweden) of the heat conduction type (41) equipped with flowthrough cells, as described by Larsson et al. (29). The microcalorimeter was operated at  $30.0^{\circ}\text{C}$ , and the measuring range used was  $1,000\ \mu\text{W}$ . The culture was pumped at a rate of 80 ml/h from the growth vessel by a peristaltic pump (Micro Perpex Pump LKB 2132; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) to a T connection outside the calorimeter, where it was met by a constant flow (40 ml/h) of humidified air ( $30.0^{\circ}\text{C}$ ) delivered by a second peristaltic pump. The flow time from the chemostat to the microcalorimetric flowthrough cell was 1.45 min. During continuous cultivation, the flow after passage through the microcalorimetric cell was discharged. For every experiment, external calibration was conducted with an external current producing 400 or 700  $\mu\text{W}$ . Internal calibration was performed by a chemical reaction (13) which yielded an effective volume of the flowthrough cell of 0.344 ml. A difficulty in using flow microcalorimetry in combination with chemostat cultivation is the time delay caused by the transport of the culture fluid between the fermentor and the measuring flowthrough cell of the calorimeter. During this delay, the limiting substrate (in this case, glucose) may be consumed, resulting in a decreased rate of heat production in the calorimetric cell. To check for this possibility, various tests were performed. (i) To simulate the rate of glucose consumption in the flow line, samples were taken from cultures grown at 0 or 0.9 M NaCl and different dilution rates, for which the glucose levels were 0.3 mM (0 M NaCl) to 0.7 mM (0.9 M NaCl). A linear decrease in the glucose concentration was demonstrated during the first few minutes; however, this decrease was at most a 20% decrease from the initial concentration after a time lapse corresponding to the transport time between the fermentor and the calorimetric cell. (ii) Stopped-flow measurements were made in order to check for glucose availability. The flow through the calorimetric cell was stopped at intervals, and the rate of heat production was monitored. (iii) Two flowthrough cells were connected in series. This resulted in a slightly lowered rate of heat production from the second cell. The decrease in the heat signal with time was extrapolated to time zero, simulating the level of the heat signal in the fermentor. This resulted in a correction of the measured heat signal by a factor of 1.02. Thus, under the prevailing experimental conditions and with the experimental organism, the time delay between the fermentor and the calorimetric flowthrough cell had only a minor influence on the rate of heat production.

**Test for steady-state conditions.** A constant heat production rate, as recorded by microcalorimetry, was used as the criterion for the establishment of steady-state conditions.

The minimum time before sampling always corresponded to  $>3$  volume changes in the fermentor.

**Determination of glycerol.** To determine the total production of glycerol, duplicate samples of 1.5 ml each were heat treated at  $95^{\circ}\text{C}$  for 10 min in tubes equipped with pear-drop condensers to liberate the intracellular polyol. Cell debris was removed by centrifugation ( $10,000 \times g$ ) for 10 min, and the supernatant was kept frozen at  $-20^{\circ}\text{C}$  until it was analyzed. Extracellular glycerol was determined by pelleting the intact cells of duplicate 1.5-ml samples by centrifugation at  $10,000 \times g$  for 10 min. The resulting supernatants were heat treated and frozen as described above. Intracellular concentrations were calculated by subtracting the extracellular concentration from the total concentration. Glycerol was analyzed with enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Mannheim, Germany). To verify that the sole polyol accumulated by *S. cerevisiae* was glycerol, all samples were also analyzed for total polyols as described by Adler and Gustafsson (1).

**Determination of glucose, ethanol, and acetate.** Duplicate samples of 1.5 ml each were centrifuged as described above to remove the cells, and the supernatants were frozen and stored at  $-20^{\circ}\text{C}$  until analysis. Analysis for glucose, ethanol, and acetate was performed with Biochemica Test Combination enzyme combination kits. Ethanol values were corrected for ethanol loss because of aeration. In separate experiments performed under cultivation conditions, the loss of ethanol was determined to be 5% of the actual concentration of the culture.

**Determination of trehalose.** Duplicate samples of 2 ml each were filtered through  $0.45\text{-}\mu\text{m}$ -pore-size filters (Millipore Corp., Bedford, Mass.). The filters were washed with 20 ml of isotonic  $\text{CaCl}_2$  solution, transferred to Kimax tubes containing 4 ml of 80% (vol/vol) ethanol and 24  $\mu\text{g}$  of phenyl  $\beta\text{-D}$ -glucoside as an internal standard, and heat treated at  $75^{\circ}\text{C}$  for 2 h. The filter and cell debris were removed by centrifugation at  $3,500 \times g$  for 5 min. The supernatant was dried, resuspended in 100  $\mu\text{l}$  of 80% (vol/vol) ethanol, and applied to a  $\text{C}_{18}$  cartridge (Bond Elute; Analytichem International, Harbor City, Calif.) which had been preconditioned with 200  $\mu\text{l}$  of methanol and then with 300  $\mu\text{l}$  of ethanol. The sample was eluted with 500  $\mu\text{l}$  of 80% (vol/vol) ethanol, lyophilized, and stored at  $-20^{\circ}\text{C}$  until it was analyzed by gas chromatography (model 5890 chromatograph; Hewlett-Packard Co., Palo Alto, Calif.) with a flame ionization detector. Immediately before analysis, the sample was silylated for 2 h at  $70^{\circ}\text{C}$  by addition of 50  $\mu\text{l}$  of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, 25  $\mu\text{l}$  of pyridine, and 25  $\mu\text{l}$  of trimethylchlorosilane. One microliter of the sample was separated on a 30-m column (DB-1701; J & W Scientific, Rancho Cordova, Calif.) in helium as a carrier gas, with a gas flow of 1.5 ml/min. The column temperature was  $170^{\circ}\text{C}$  when the sample was injected, and the temperature was increased by  $8^{\circ}\text{C}/\text{min}$  to  $275^{\circ}\text{C}$ ; the temperature was then kept at  $275^{\circ}\text{C}$  for 15 min. The injector and detector temperatures were kept at 280 and  $300^{\circ}\text{C}$ , respectively.

**Determination of intracellular  $\text{Na}^{+}$  and  $\text{K}^{+}$ .** Duplicate samples of 2 ml each were filtered through  $0.45\text{-}\mu\text{m}$  Millipore filters, prewashed with isotonic  $\text{CaCl}_2$ , and washed five times with 3 ml of isotonic  $\text{CaCl}_2$ . The filters were then immersed in 3 ml of distilled water, heat treated for 10 min at  $95^{\circ}\text{C}$  in tubes with a pear-drop condenser, and centrifuged for 5 min at  $3,500 \times g$ . The supernatants were frozen and kept at  $-20^{\circ}\text{C}$  until analyzed. Measurements of  $\text{K}^{+}$  and  $\text{Na}^{+}$  content were performed with an atomic absorption spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). For

background values, 2 ml of medium was filtered, and the filters were treated as described above. These background values for  $K^+$  and  $Na^+$  were subtracted from all data.

**Determination of cell volume.** To determine the cellular water volume,  $^3H_2O$  ( $185 \text{ kBq ml}^{-1}$ ) was used to label the total (intra- and extracellular) water, and  $D-[U-^{14}C]$ ribose ( $18.5 \text{ kBq ml}^{-1}$ ) or  $D-[U-^{14}C]$ sorbitol ( $18.5 \text{ kBq ml}^{-1}$ ) was used to label extracellular water. The water-accessible cell volume was calculated as described by Larsson et al. (31).

**Determination of dry weight.** Duplicate samples of 10 ml each were centrifuged for 5 min at  $3,500 \times g$ , washed twice with distilled water, and dried for 48 h at  $110^\circ C$ . This biomass does not contain glycerol; intracellular glycerol was quantitatively lost from the cells during the washing procedure. Ash-free biomass was calculated by subtracting the inorganic ash content (see below).

**Energy calculations.** Energy balance calculations were used to determine the amount of energy consumed for growth at different salinities and dilution rates (for extensive explanations, see references 23 and 50). According to Hess's law of energy conservation, the energy calculations could be performed on the basis of either the total amount of substrate used or the total amount of products formed (including heat), enabling reliability tests of the data. The standard molar enthalpies used (50) for substrates and products refer to their aqueous state, except for the gases, which were considered to be in their gaseous state under all experimental conditions. Since the combustion products of all components were used as the reference state, water (liquid), carbon dioxide (gaseous), oxygen (gaseous), and protons (aqueous) were omitted from the calculations. The enthalpy of combustion, the elemental composition, and the inorganic ash content of the dry biomass of cells grown at different salinities were determined at the Institute of Chemical Engineering, Lausanne, Switzerland, by the method of Gurakan et al. (19). The ammonium consumption was calculated from the biomass production. To normalize energy consumption to biomass, the energy used for growth was expressed per unit of ash-free biomass formed. The additional energy used for growth at high salinity was calculated as the difference in energy consumption between growth at high salinity and growth in basal medium. To express this additional energy consumption in terms of ATP turnover, growth equations were established for the different anabolic and catabolic subreactions by use of the "degree of reduction" (3, 17). By closing the material balance of the growth equations, the stoichiometry of respiration was obtained; this allows determination of the respiratory activity without measurement of the rates of oxygen consumption and carbon dioxide production. In these calculations, the discrepancies in the energy balances were considered. ATP production was calculated from the stoichiometries of product formation, and the contribution from respiration was determined by assuming a P/O ratio of 1.0 (23, 48, 49).

## RESULTS

The effects of external salinity on energy flux and osmotic relations of *S. cerevisiae* in a chemostat under glucose limitation at 0, 0.5, and 0.9 M NaCl were studied.

**Growth yield.** In basal medium (0 M NaCl), growth yield stayed essentially constant as the dilution rate increased from 0.09 to  $0.14 \text{ h}^{-1}$  (Fig. 1). A further increase in the dilution rate caused a drastic decrease in growth yield from the maximal value of  $0.41 \text{ g}$  of biomass per  $\text{g}$  of glucose to a new level at about  $0.23 \text{ g/g}$ . This pattern of behavior is

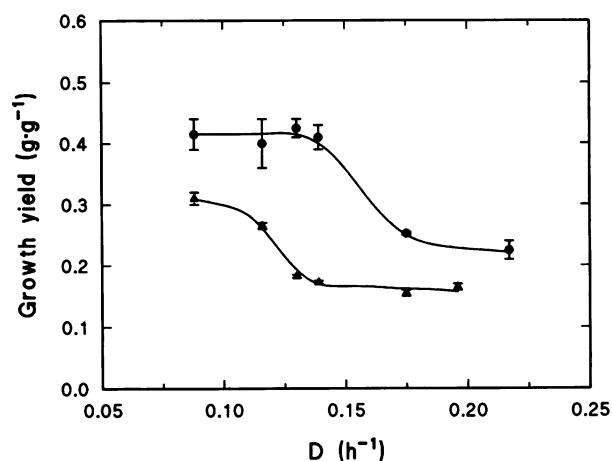


FIG. 1. Growth yield versus dilution rate ( $D$ ) of chemostat-grown *S. cerevisiae* at 0 M (●) and 0.9 M (▲) NaCl. Data are means  $\pm$  standard deviations ( $n = 2$  or 3).

normally observed for *S. cerevisiae* in chemostats, reflecting a shift from respiratory to respirofermentative metabolism as the growth rate increases. During growth at high salinity (0.9 M NaCl), this shift, which in this case was from 0.31 to about  $0.17 \text{ g/g}$ , started at a much lower dilution rate than the shift at 0 M NaCl. Therefore, at intermediate growth rates ( $0.13$  to  $0.14 \text{ h}^{-1}$ ), the growth yield of the high-salinity culture was up to 55% lower than that of the culture in basal medium, whereas at comparable low and high growth rates, the yield was about 25% lower than that at 0 M NaCl (Fig. 1).

**Growth rate and residual glucose concentration.** The residual glucose concentration was less than  $0.4 \text{ mM}$  in basal medium at dilution rates up to  $0.23 \text{ h}^{-1}$  but reached about  $2 \text{ mM}$  at the highest growth rate tested ( $0.31 \text{ h}^{-1}$ ). The residual glucose concentration during growth in 0.9 M NaCl was always higher than during growth in basal medium. It increased with the growth rate from  $0.3 \text{ mM}$  at  $0.09 \text{ h}^{-1}$  to  $2 \text{ mM}$  at  $0.18 \text{ h}^{-1}$  and finally reached  $5 \text{ mM}$  at the highest growth rate ( $0.20 \text{ h}^{-1}$ ) for which a steady state was achieved. At this dilution rate, the growth yield ( $0.17 \text{ g/g}$ ) was about the same as that recorded at the highest dilution rate tested in basal medium ( $0.31 \text{ h}^{-1}$ ). The increase in glucose concentration at both of these dilution rates indicates that the growth rate was nearly maximal.

**Substrate consumption and product formation.** The influence of growth rate on the requirement of energy for biomass production by cells grown in basal medium is illustrated in Fig. 2A. The amount of energy required to produce one unit of ash-free biomass (in kilojoules per gram of ash-free biomass [ $g_{af}$ ]) is determined in two ways, either as the energy content of the total substrate (glucose plus ammonium) consumed or as the sum of the energy contents of all of the products formed (including heat). Because of the first law of thermodynamics, Hess's law of energy conservation, the energy content of the total substrate consumed has to be equal to that of the total products formed. The good agreement between the estimations (the mean energy recovery was  $1.05 \pm 0.06$  [ $n = 6$ ]; a value of 1.00 is optimal) indicates that all reactants and all important products were included in the analysis.

In Fig. 2B, the total energy content of the products formed per unit of ash-free biomass has been split into its individual

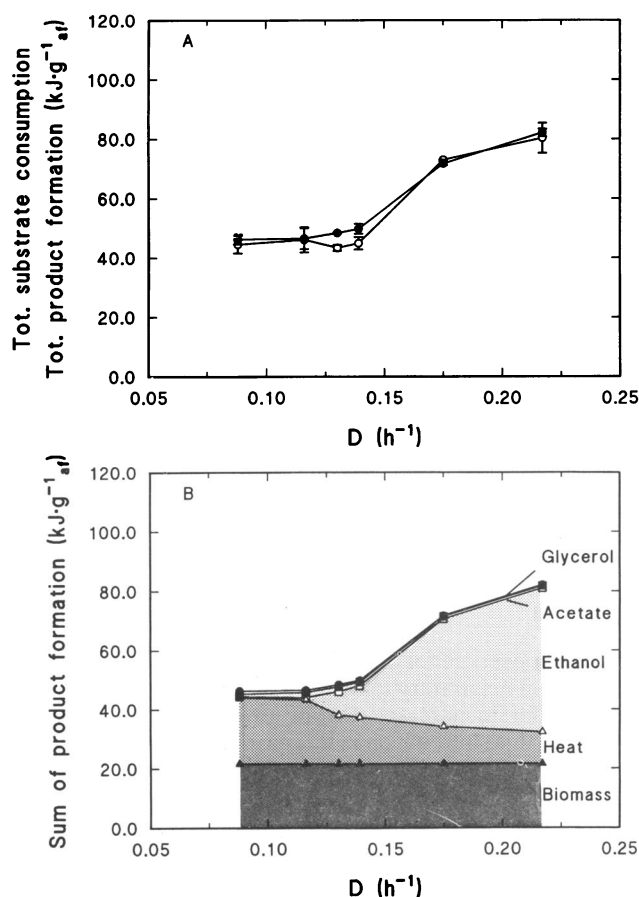


FIG. 2. (A) Total (Tot.) substrate consumption (○) and total product formation (●), expressed in energy units per unit of ash-free biomass formed, versus dilution rate (D) during growth of *S. cerevisiae* in basal medium. The area defined by the nonoverlapping regions of the substrate consumption and product formation curves describes the imbalance in energy recovery (see text for explanation). Data are means  $\pm$  standard deviations ( $n = 2$  or  $3$ ). (B) Sum of product formation during growth of *S. cerevisiae* in basal medium. Symbols: ▲, biomass; △, plus heat; □, plus ethanol; ■, plus acetate; ●, plus total (intracellular and extracellular) glycerol.

components to illustrate the energy contributions from each of the main products formed, i.e., biomass, heat, ethanol, acetate, and glycerol. At low growth rates, biomass and heat were the predominant products, showing that catabolically glucose was almost completely respired to carbon dioxide

and water (see reference 20). Glycerol decreased gradually with increased growth rate from about 1.5 kJ/g<sub>af</sub> (1 mM concentration in the medium) to about 0.3 kJ/g<sub>af</sub> (0.2 mM), while acetate increased transiently to reach about 3 kJ/g<sub>af</sub> (6 mM) at a growth rate of 0.12 h<sup>-1</sup> and decreased to <0.7 kJ/g<sub>af</sub> (1 mM). The critical growth rate for ethanol production to appear was about 0.12 h<sup>-1</sup>, and at higher growth rates there was a gradual increase in ethanol formation and a decrease in the heat production per unit of biomass formed. Complete oxidation of glucose (aqueous) to carbon dioxide (gaseous) and water (liquid) results in heat production of -2,813.6 kJ/mol of glucose, while fermentation of glucose (aqueous) to ethanol (aqueous) and carbon dioxide (gaseous) yields only -100.0 kJ/mol of glucose (50). This partly explains why heat production decreases as fermentation increases. When the growth rate increased from 0.09 to 0.18 h<sup>-1</sup>, the ethanol fermentation increased from being insignificant to accounting for about half of the total carbon consumed. Concomitantly, there was a considerable decrease in anabolism and respiration (Table 1).

During growth in 0.9 M NaCl, the distribution of the energy flux into the different products (Fig. 3) followed a pattern similar to that of cells grown in basal medium (Fig. 2). The main differences were that (i) the shift from an almost purely respiratory catabolism to a mixed respirofermentative catabolism (Fig. 3B) occurred at a lower growth rate at high salinity (about 0.09 h<sup>-1</sup>), (ii) the glycerol production was drastically increased, and (iii) considering all nonanabolic activities to be catabolic, the total catabolic activity was 8 to 13% higher at 0.9 M NaCl (Table 1). At high salinity, glycerol production also showed a dramatic dependence on the dilution rate; it increased strongly with growth rate from about 7 kJ/g<sub>af</sub> at 0.09 h<sup>-1</sup> to 19 kJ/g<sub>af</sub> at 0.20 h<sup>-1</sup> (Fig. 3B). At the highest dilution rate, about 17% of the catabolically consumed carbon source was transformed into glycerol (calculated from data in Table 1). At dilution rates above 0.12 h<sup>-1</sup>, up to 16% of the glucose consumption could not be accounted for by the analyzed products (Fig. 3A); this imbalance indicates the presence of important unidentified products under these growth conditions.

**Additional energy requirements for growth at high salinity.** The total additional energy requirement for growth in 0.9 M NaCl, compared with the energy requirement for growth in basal medium, was calculated to be 12.3 kJ/g<sub>af</sub> in conditions (dilution rate = 0.09 h<sup>-1</sup>) under which the cells conduct an entirely respiratory metabolism (Fig. 4). This additional expenditure is equal to about 28% of the total energy used for growth in basal medium (Fig. 2 and 3), and under these conditions about half of the additional energy (6.2 kJ/g<sub>af</sub>) is conserved in glycerol production. At increased growth rates,

TABLE 1. Fractional distribution of total carbon (glucose) consumption during growth of *S. cerevisiae* at different dilution rates in basal medium containing 0 or 0.9 M NaCl

Dilution rate (h <sup>-1</sup> )	% of total carbon consumption as <sup>a</sup> :									
	Anabolism <sup>b</sup>		Respiration		Ethanol fermentation		Acetate production		Total glycerol production	
	0 M NaCl	0.9 M NaCl	0 M NaCl	0.9 M NaCl	0 M NaCl	0.9 M NaCl	0 M NaCl	0.9 M NaCl	0 M NaCl	0.9 M NaCl
0.09	44	35	51	47	0	0	2	5	3	13
0.12	43	30	50	35	2	20	4	5	1	10
0.18 <sup>c</sup>	26	18	20	18	53	39	1	3	0	14

<sup>a</sup> Data were calculated by constructing growth equations as described by Gustafsson et al. (23).

<sup>b</sup> The anabolic reactions include anabolic CO<sub>2</sub> production to account for the higher degree of reduction of biomass compared with that of glucose.

<sup>c</sup> The energy imbalance for cells grown at this dilution rate was calculated to be 10% at 0.9 M NaCl.

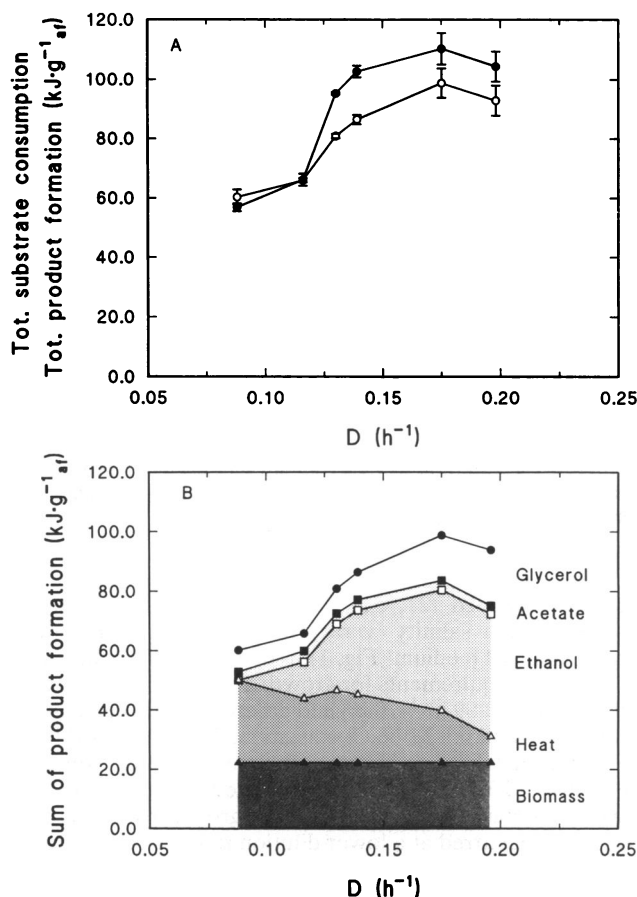


FIG. 3. (A) Total substrate consumption (○) and total product formation (●), expressed in energy units per unit of ash-free biomass formed, versus dilution rate (D) during growth of *S. cerevisiae* at 0.9 M NaCl. The area defined by the nonoverlapping regions of the substrate consumption and product formation curves describes the imbalance in energy recovery (see text for explanation). Data are means  $\pm$  standard deviations ( $n = 2$  to 3). (B) Sum of product formation during growth of *S. cerevisiae* at 0.9 M NaCl. Symbols:  $\blacktriangle$ , biomass;  $\triangle$ , plus heat;  $\square$ , plus ethanol;  $\blacksquare$ , plus acetate;  $\bullet$ , plus total (intracellular and extracellular) glycerol.

during the transition from a purely respiratory catabolism to a respirofermentative metabolism, there was a dramatic severalfold increase in the additional energy requirement for growth in the saline medium (Fig. 4); the requirement peaked at a cost of 57.8 kJ/g<sub>af</sub> at 0.14 h<sup>-1</sup>. At a higher growth rate (0.18 h<sup>-1</sup>), at which fermentative metabolism becomes predominant (Table 1), the additional energy requirement decreased again. At this growth rate, the energy demand imposed by salt corresponded to about 51% of the total energy consumed for growth in basal medium (based on total substrate consumption), and the energy conserved in glycerol production made up about 40% of this salinity-induced energy requirement (Fig. 4).

**Glycerol.** It is clear from the energy relations that there is a considerable investment in increased glycerol production in cells exposed to salt stress. The total glycerol yield increased with salinity and varied with growth rate; it was maintained between 0.1 and 0.5 mmol/g (dry weight) of cells in basal medium and increased to 4 to 10 mmol/g (dry weight) of cells at 0.9 M NaCl. Similarly, and importantly from a

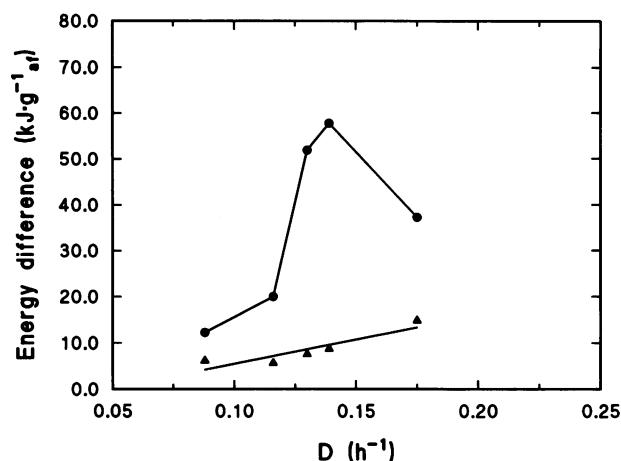


FIG. 4. Additional energy consumption for growth of *S. cerevisiae* at high salinity (0.9 M NaCl) compared with growth in basal medium. Values were calculated as the difference in energy content of the substrates consumed (●) or of the total glycerol produced (▲). D, dilution rate.

physiological point of view, the intracellular glycerol concentrations increased from below 0.05 molal in basal medium to 0.4 molal at 0.5 M NaCl and 1.2 molal at 0.9 M NaCl (Fig. 5A). Although the total glycerol production at high salinity increased strongly with the growth rate, the intracellular glycerol concentration remained fairly constant. Consequently, at high dilution rates the cells suffered considerable losses of glycerol to the surrounding medium; while 66% of the total glycerol produced was retained within the cells at 0.09 h<sup>-1</sup>, the cells retained only 21% at 0.20 h<sup>-1</sup> (Fig. 6).

**Ions.** The intracellular levels of Na<sup>+</sup> and K<sup>+</sup> ions responded insignificantly to changes in the dilution rate but responded differently to changes in salinity (Fig. 5B and C). While the intracellular K<sup>+</sup> level was stably maintained around 0.29 molal regardless of external salinity, the intracellular Na<sup>+</sup> level increased from 0.02 molal in basal medium to 0.10 molal at 0.5 M NaCl and about 0.18 molal at 0.9 M NaCl.

**Trehalose.** Trehalose can be accumulated to high concentrations by *S. cerevisiae* (32, 53). However, in growing cells, the intracellular concentration of trehalose was kept below 25 millimolar, independent of external salinity, at dilution rates of >0.1 h<sup>-1</sup> (Fig. 7). At lower dilution rates, the cells responded by a marked increase in the intracellular trehalose concentration; this increase was more pronounced in high-salinity media.

**Osmotic contribution from glycerol and major cations.** The relative contributions of intracellular glycerol, Na<sup>+</sup>, and K<sup>+</sup> to the intracellular osmotic potential were estimated on the basis of the measured intracellular concentrations of these solutes by using the tables of Harris (26). To calculate the total osmotic potential, the free amino acid concentration was considered to be 125 millimolar (33), and its pool size was considered to be independent of external salinity (cf. reference 10). The remaining pool of metabolic intermediates was assumed to be equal in size to that of the amino acids. These estimations yielded contributions to the total osmotic potential of about 30% for glycerol and about 35% for Na<sup>+</sup> plus K<sup>+</sup> (excluding counterions) in cells grown at 0.5 M NaCl and about 60 and 20%, respectively, in cells cultured at 0.9 M NaCl. 0.5 M NaCl and about 60 and 11%, respectively,

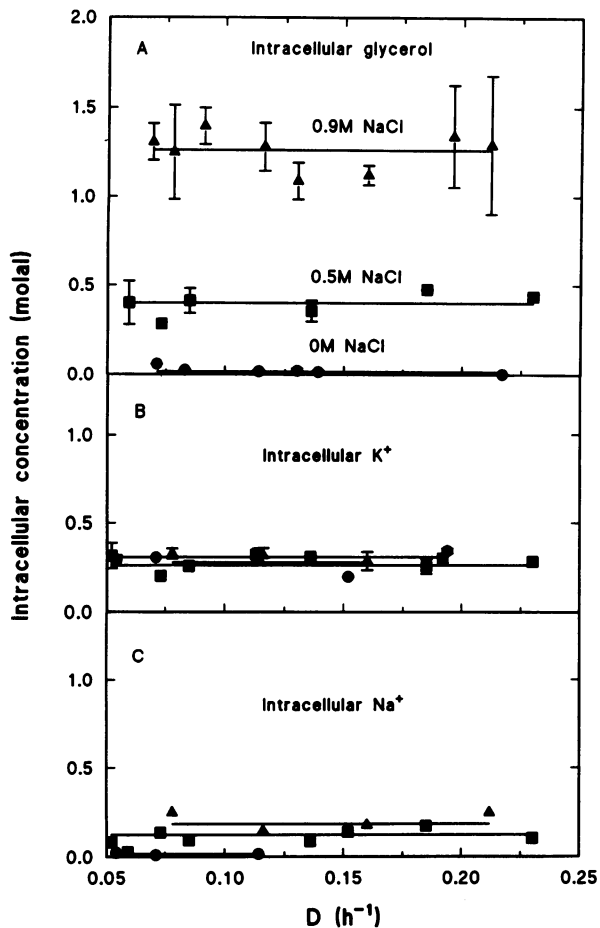


FIG. 5. Intracellular concentrations of glycerol (A), K<sup>+</sup> (B), and Na<sup>+</sup> (C) in *S. cerevisiae* grown in basal medium containing no added NaCl (●), 0.5 M NaCl (■), or 0.9 M NaCl (▲). Data are presented as means  $\pm$  standard deviations ( $n = 2$  to 7). Statistical analysis showed that the dependence on external salinity of the intracellular concentrations of glycerol and Na<sup>+</sup> was significant (calculated  $t$  values are 17 and 7.5, respectively), while their dependence on the dilution rate ( $D$ ) was insignificant (calculated  $t$  values are  $< 2$ ).

in medium containing 0.9 M NaCl. The intracellular osmotic potential determined by these calculations would, at least in principle, allow estimation of the turgor pressure of the cells by subtracting the internal osmotic potential from that of the surrounding medium. Such estimations yielded positive turgor pressure for the cells at all salinities (data not shown). However, recent observations by Cayley et al. (12) indicate that the osmotic coefficients in the cytosol may deviate very strongly from osmotic ideality and that calculations based on osmotic contributions as determined for pure solutions thus may be misleading. Unequal distribution of the major solutes between the vacuole and the cytosol may also influence the osmotic potential of the cell, because of the deviations from osmotic ideality that occur at high solute concentrations (e.g., see reference 5).

#### DISCUSSION

This paper examines the effects of growth medium NaCl concentration on the energetics and osmotic relations of *S. cerevisiae* cultured in a glucose-limited chemostat. As pre-

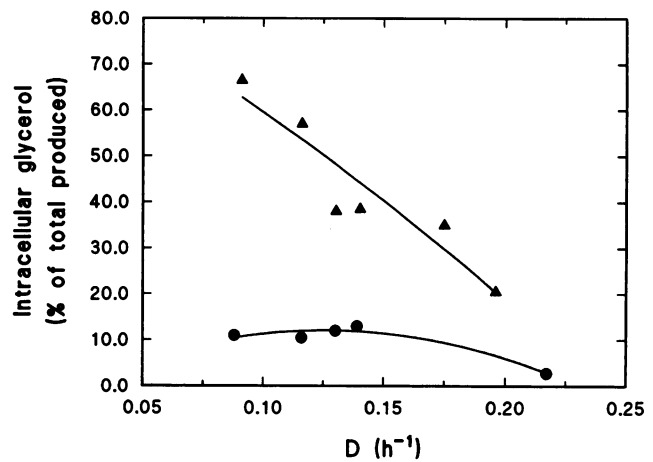


FIG. 6. Distribution of glycerol between medium and *S. cerevisiae* cells during growth in basal medium containing no added NaCl (●) or 0.9 M NaCl (▲).  $D$ , dilution rate.

viously observed (21, 51), the growth yields of cultures grown at high salinity were lower than those of cultures grown in basal medium (Fig. 1), indicating additional carbon and energy requirements for growth under salt stress. At low dilution rate (0.09 h<sup>-1</sup>), the yield difference between cultures grown at 0 and 0.9 M NaCl was about 25%. At this dilution rate, the metabolism of the cells was entirely respiratory (Fig. 2B and 3B; Table 1), but it shifted to a mixed respiratory-fermentative metabolism as the growth rate increased. This shift occurred at a lower dilution rate for cells grown at 0.9 than at 0 M NaCl. Conceivably, the salt-stressed cells have to initiate their fermentative metabolism at a lower dilution rate in order to meet the demand for an increased rate of energy production during growth at high salinity. The price to pay is a drastic yield decrease (Fig. 1) due to the low growth efficiency of the fermentative metabolism. On the basis of total substrate consumption, the additional maintenance energy expenditure for growth at 0.9 M NaCl was calculated to be 6 kJ/g<sub>af</sub> at a dilution rate of 0.09 h<sup>-1</sup> and 22 kJ/g<sub>af</sub> at 0.18 h<sup>-1</sup>. These maintenance values reflect the

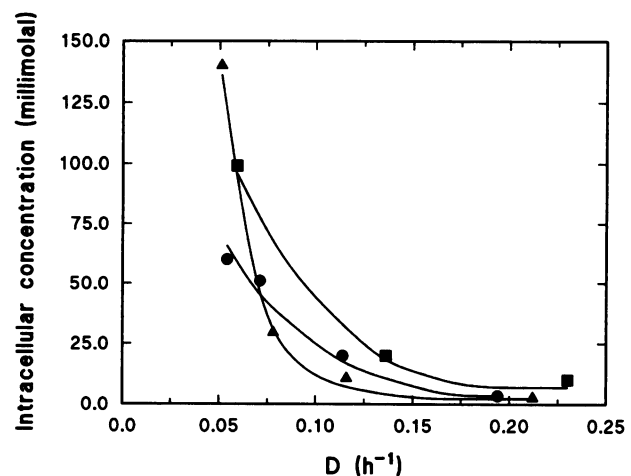


FIG. 7. Intracellular concentration of trehalose in *S. cerevisiae* grown in basal medium containing no added NaCl (●), 0.5 M NaCl (■), or 0.9 M NaCl (▲).  $D$ , dilution rate.

increased catabolic activities but exclude the salt-induced increase in glycerol production. Expressed in another way, the additional maintenance costs for growth at 0.9 M NaCl equal 14 and 31%, respectively, of the total energy used for growth at the corresponding growth rates in basal medium. The higher value is similar to that noted for the additional energy expenditure for growth of *S. cerevisiae* at 0.7 M NaCl in batch culture (21) and in agreement with the hypothesis that energetic efficiency is considerably lower during energy excess than during energy limitation (42, 43, 52). Watson (51) determined the maintenance energy expenditure for growth at 1 M NaCl by use of a respiration-deficient (*petite*) mutant of *S. cerevisiae* to simplify calculations of the amount of ATP generated per glucose molecule utilized. Values were extracted from classical Pirt plots (38), assuming that the maintenance coefficient is invariant with growth rate. This conjecture applied at very low dilution rates (below  $0.07 \text{ h}^{-1}$ ), while deviations occurred at higher dilution rates. The maintenance requirement calculated by this means increased fourfold in the presence of 1 M NaCl, and the estimated increase corresponded to  $1.7 \text{ mmol of ATP g of biomass}^{-1} \text{ h}^{-1}$  at dilution rates of  $<0.07 \text{ h}^{-1}$  (51). In our experiments, which used a respiration-sufficient strain, the increased ATP turnover for maintenance during growth at 0.9 M NaCl was calculated to be  $2.4 \text{ mmol of ATP g}^{-1} \text{ h}^{-1}$  at a low dilution rate ( $0.09 \text{ h}^{-1}$ ), at which respiration is predominant, and  $2.9 \text{ mmol of ATP g}^{-1} \text{ h}^{-1}$  at a high dilution rate ( $0.18 \text{ h}^{-1}$ ), at which respirofermentative metabolism occurs. In these calculations, a P/O ratio of 1.0 (47, 48) was assumed for the respiratory metabolism, and corrections were made for energy-requiring glycerol production. Since our studies refer to dilution rates higher than those of Watson (51), our higher values may be due to a growth rate-dependent component of the maintenance requirements (11, 39). It is also possible that we have overestimated the ATP consumption used for maintenance by not considering the possibility of down regulation of the P/O ratio in response to NaCl stress. Organisms may modify their respiratory chain in response to environmental changes so that the stoichiometry of phosphorylation versus oxidation of reducing equivalents is changed (14).

The increased energy used for maintenance in response to salt stress may be largely explained by a requirement for enhanced ion pumping to lessen the impact of the external salinity on the intracellular physiology. External  $\text{Na}^+$  is relatively effectively excluded from the cells. The intracellular  $\text{Na}^+$  levels increased with external salinity to reach about 0.18 molal at 0.9 M external NaCl; this level is similar to that reported for cells grown at 1 M NaCl (51).  $\text{Na}^+$  is believed to enter the cell through  $\text{K}^+$  carriers and  $\text{Na}^+$ /substrate symporters (6), and the accumulation of inhibitory concentrations of  $\text{Na}^+$  is avoided by active  $\text{Na}^+$  export, which appears to be directly linked to ATP turnover. There is evidence for an  $\text{Na}^+$ -ATPase in *S. cerevisiae* (25) as well as a proton/sodium antiport driven by the plasma membrane ATPase (40). Norrans and Kylin (37) observed that the initial uptake of labelled  $\text{Na}^+$  by *S. cerevisiae* exposed to 1.35 M NaCl attains a value of approximately  $0.5 \text{ mmol of Na}^+ \text{ g}^{-1} \text{ h}^{-1}$ . Thus, depending on the stoichiometry between  $\text{Na}^+$  exported and ATP consumed, a considerable portion of the salt-induced increase in ATP turnover may be used for the enhanced  $\text{Na}^+$  extrusion, as discussed by Watson (51). In contrast, internal  $\text{K}^+$  levels varied little with external salinity (Fig. 5B), and internal  $\text{K}^+$  was not, or was only slightly, exchanged for  $\text{Na}^+$ , as these ions accumulated during growth at high external NaCl concentrations. Two

$\text{K}^+$  transporters have been identified in *S. cerevisiae* (28), and uptake of  $\text{K}^+$  is considered dependent on the proton gradient generated by the plasma membrane ATPase, as indicated by evidence for a  $\text{K}^+/\text{H}^+$  exchange in yeasts (15). Hence, maintenance of  $\text{K}^+$  homeostasis is also expected to require appreciable ATP turnover.

It is thus clear from the data presented that steady-state growth of *S. cerevisiae* in saline media is associated with mechanisms for maintenance of internal ion concentrations lower than the external ion concentrations. The presented results also clearly confirm that the resulting shortfall in intracellular osmotic potential is compensated for by intracellular accumulation of glycerol, consistent with the role of glycerol as a compatible solute in yeasts (5, 8, 9). The intracellular glycerol levels increased dramatically with external salinity (Fig. 5A) and were stably maintained independent of growth rate and type of catabolism (respiratory or respirofermentative), despite the fact that glycerol production varied greatly with the dilution rate (Fig. 3B). It is generally believed that the cells, as a result of glycerol accumulation, adjust cell volume and maintain turgor within a range that can support growth (5, 9). Trehalose, which is accumulated by yeast cells in response to nutrient limitation, desiccation, heat shock, and other forms of stresses (53), does not appear to have a role in osmoregulation of growing *S. cerevisiae* (Fig. 7). The intracellular trehalose concentrations did not respond to salinity, and the attained levels contributed negligibly to the internal osmotic potential. The increase in trehalose that occurs at low dilution rates is in agreement with the reported accumulation of this disaccharide in cells subjected to shortages of energy-yielding substrate (32). Under such circumstances, salinity may stimulate accumulation of trehalose (Fig. 7).

As discussed above, *S. cerevisiae* devotes an increased proportion of its energy source into reactions other than biosynthesis when cultured under salt stress. In addition to the energy spent on increased ATP turnover, which has been called additional maintenance energy in this article, there is also a large expenditure on enhanced glycerol production (Fig. 4), resulting in the total energy demand for growth at 0.9 M NaCl being 28 to 51% higher than that in basal medium. For *S. cerevisiae*, the two main reasons for glycerol production are to provide the cell with a compatible solute for osmoregulation (5, 8) and to serve as a sink for reducing equivalents (34, 35, 44) when the respiratory activity is inadequate or blocked. In the aerated, glucose-limited chemostat culture used for our experiments, NADH dehydrogenases and shuttle systems are expected to oxidize cytosolic NADH and transfer the reducing equivalents to the electron transport chain (54), resulting in a minimal need for glycerol formation for redox adjustments. This explains the low glycerol production observed at all dilution rates in basal medium (Fig. 2B). At higher salinity, an enhanced production is expected because of the induction of glycerol synthesis by the increased external osmolality (4, 8). However, these salt-induced levels depended surprisingly much on the dilution rate. Although the internal glycerol levels were maintained essentially constant (Fig. 5A), the total glycerol production increased from 4 to  $10 \text{ mmol/g}$  (dry weight) of yeast cells when the dilution rate increased from 0.09 to  $0.2 \text{ h}^{-1}$  at 0.9 M NaCl. This might mean that the increased catabolism of glucose which accompanies an increased growth rate (Table 1) generates an excess of reducing equivalents at high salinity through the production of by-products formed by an oxidative process. The existence of unidentified by-products at high growth rates in 0.9 M NaCl

medium is indicated by the shortfall in the energy balance (Fig. 3A). Such by-product formation might lead to increased glycerol production because of a limited capacity of the respiratory chain or its shuttle systems to take care of the surplus NADH produced. Alternatively, leakage of glycerol might increase with growth rate, promoting enhanced glycerol production to compensate for insufficient osmoregulation and ensuing loss in cell volume and turgor as the dilution rate increases.

Batch culture studies have been interpreted to mean that much of the osmotically induced glycerol produced in *S. cerevisiae* is given off to the surrounding medium (8, 30). This mechanism of osmoregulation, based on heavy production to compensate for leakage, has been considered wasteful and contrasted with the conservative mode of osmoregulation exhibited by the osmotolerant yeast *Zygosaccharomyces rouxii* (8, 9). This yeast maintains a fairly constant production over a wide range of water potentials and osmoregulates by changing the proportion of glycerol retained within the cells (9, 46), presumably aided by a transport system for glycerol (45). There are no indications of active transport of glycerol in *S. cerevisiae* (7, 18). However, at 0.9 M NaCl and at low dilution rates ( $<0.12 \text{ h}^{-1}$ ), the cells maintain an up to 600-fold glycerol gradient across the membrane, and about 60% of the total glycerol produced (Fig. 6) is retained within the cells. Under these conditions, *S. cerevisiae* shows a glycerol economy that is comparable to that of *Z. rouxii* (cf. reference 46). *S. cerevisiae* therefore seems capable of limiting glycerol release to the external medium, although the mechanism behind this control remains obscure. One might speculate that the leakage pathway is controlled to a certain extent by turgor-responding membrane channels, as shown for ions in *S. cerevisiae* (24). Such a mechanism would serve to release glycerol produced in excess of the requirement for turgor and volume control and eliminate conflicts due to the dual roles of glycerol in osmotic and redox control.

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