Yeast Water Relations: Physiological Changes Induced by Solute Stress in *Saccharomyces cerevisiae* and *Saccharomyces rouxii*

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A comparison was made of the xerotolerant yeast *Saccharomyces rouxii* and its non-tolerant counterpart *S. cerevisiae* when fully adapted to growth media of high and low water activity (a_w), and during the period of adaptation from a high a_w to a medium containing NaCl (10%, w/v). Comparisons of the fully adapted yeasts were confined to levels of activity of phosphofructokinase and *sn*-glycerol-3-phosphate dehydrogenase. The results confirmed our previous findings obtained with different assay procedures that growth at diminished a_w dramatically increased the level of glycerol phosphate dehydrogenase in *S. cerevisiae* but had little or no effect in that respect on *S. rouxii*. Simultaneously phosphofructokinase activity was about doubled in *S. cerevisiae* and about halved in *S. rouxii*. Differences between the two yeasts were even more conspicuous during the period of response to salt stress. *Saccharomyces rouxii* apparently adapted fully to the salt broth within 6 h (at 30 °C) inasmuch as viability was unaffected by the transfer and glycerol content reached its maximum by then. On the other hand, *S. cerevisiae* took about 140 h to adapt by a process that could be resolved into two stages. Stage 1 was marked by a catastrophic drop in apparent viability and widely different counts on three plating media. Stage 2 was identified by similar counts on all three plating media and a progressive increase in viability up to the level of the original inoculum. Glycerol phosphate dehydrogenase activity increased during this stage but glycerol accumulation began in both species immediately after transfer. Throughout the entire adaptation period, *S. cerevisiae* consumed glucose much more slowly than did *S. rouxii*. The complexity of the response of *S. cerevisiae* to salt stress has focused attention on this species as a reference for studying yeast water relations in general.

INTRODUCTION

The two yeasts *Saccharomyces rouxii* and *S. cerevisiae* differ considerably in their water relations: *S. rouxii* is highly xerotolerant whereas *S. cerevisiae* is relatively intolerant of diminished water activity (a_w). In spite of this, both species osmoregulate with glycerol, which also functions as a compatible solute under relatively extreme conditions of low a_w . There are important physiological differences in the osmoregulatory mechanisms, however: *S. rouxii* responds to a solute stress by increasing the intracellular proportion of a constant amount of glycerol, whereas *S. cerevisiae* responds by retaining a constant intracellular proportion of an increased amount of glycerol which it synthesizes. It is reasonably certain that the major redirection of its carbohydrate metabolism to glycerol production in response to a solute stress is an important factor limiting the tolerance of *S. cerevisiae* to low a_w (Brown, 1978; Edgley & Brown, 1978).

There are some fundamental biochemical differences that are associated with the different osmoregulatory mechanisms used by the two species. For example, Brown & Edgley (1980) reported evidence of differences in the major metabolic pathways to glycerol, differences

Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; MA, malt-extract agar; SHA, synthetic honey agar.

between the species in the response to water stress of those pathways and differences in production of the enzyme *sn*-glycerol-3-phosphate dehydrogenase.

The present paper describes responses of this enzyme and phosphofructokinase to growth of each yeast at several levels of a_w and some of the changes that occur in each species during adaptation from a 'normal' growth medium (a_w 0.997) to one containing NaCl (10%, w/v; a_w 0.936). The responses of the two yeasts during the transition period are very different in several respects and their water relations differ for complex reasons over and above mechanisms of controlling glycerol content.

METHODS

Organisms. *Saccharomyces rouxii* (strain YA, ATCC 38528) and *Saccharomyces cerevisiae* (strain Y41, ATCC 38531), described by Anand & Brown (1968), were maintained at 4 °C on slopes of synthetic honey agar (SHA; containing 48%, w/v, glucose; a_w 0.924; Anand & Brown, 1968) and malt-extract agar (MA; Oxoid) respectively. Experimental cultures were grown in basal liquid medium (a_w 0.997; Anand & Brown, 1968), adjusted where necessary to the stated a_w by addition of NaCl or PEG (av. mol. wt 200), in conical flasks of nominal capacity 2.5 times the volume of the growth medium and incubated at 30 °C with rotary agitation (200 r.p.m.). PEG was added as an aqueous solution (50%) that had previously been deionized on a mixed-bed ion-exchange resin.

For experiments with organisms fully adapted to a specified a_w (steady-state experiments) an inoculation schedule as described by Edgley & Brown (1978) was used. The inoculation and transfer procedures for experiments in which adaptation of a yeast from basal medium to the same medium containing NaCl (10%, w/v) ('salt broth', a_w 0.936) was monitored (transition experiments) were as follows. Basal medium (100 ml) was inoculated by loop from a 1–2 d slope and grown overnight to stationary phase. A new batch of basal medium (400 ml) was inoculated to a level of 10% (v/v) with the first culture and grown well into the exponential growth phase (about 4.5 h and 2.5 h respectively for *S. rouxii* and *S. cerevisiae*). The viable count at this point was normally $6-8 \times 10^6$ ml⁻¹. The culture was chilled in ice and centrifuged briefly at 13200 g at 0 °C. The pellet was resuspended in salt broth (400 ml) at 30 °C, and incubated normally. Aseptic precautions were used throughout the transfer.

Viable counts were made conventionally by the drop plate technique. Some comparisons were made with pour plates. Serial dilutions were made in quarter-strength Ringer's solution for counts of basal-medium cultures and in NaCl (10%, w/v) for salt broth cultures. Suspensions were plated on to MA, SHA and salt-agar [basal medium with NaCl (10%, w/v) and agar (1.5%, w/v)]. Colonies on the three media were counted after, respectively, 3, 5 and 18 d incubation at 30 °C. Salt-agar plates were incubated in plastic bags to restrict evaporation.

Enzyme assays. Cultures (approximately 400 ml) in the mid-exponential growth phase were chilled thoroughly in ice, centrifuged briefly at 13200 g at 0 °C then washed twice by brief centrifugation at 0 °C with either of the following buffer solutions: (i) sodium phosphate buffer (0.067 M, pH 7.0) for subsequent assay of phosphofructokinase; (ii) triethanolamine/HCl (10 mM, pH 7.0) containing EDTA (1.0 mM) and dithiothreitol (1.0 mM) for subsequent assay of *sn*-glycerol-3-phosphate dehydrogenase. The washed yeast was disrupted with a modified Hughes press (Anand, 1969). The broken cells were dispersed in cold wash buffer (0.8–1.0 ml) and centrifuged at 39100 g for 30 min at 0 °C. The extracts were kept at 0 °C and assayed on the day of preparation. If necessary, extracts were diluted with wash buffer.

sn-Glycerol-3-phosphate dehydrogenase was assayed according to Gancedo *et al.* (1968). The method involved assessing the rate of reduction of dihydroxyacetone phosphate (DHAP) by NADH. Specific activity of glycerol-3-phosphate dehydrogenase from *S. cerevisiae* increased with protein concentration as does the corresponding enzyme purified from rabbit muscle (Batke *et al.*, 1980). The relation between protein concentration in crude extracts and specific activity of the *S. cerevisiae* enzyme(s) fitted the Michaelis–Menten equation and we were therefore able to use a double reciprocal plot to determine specific activity at infinite protein concentration. Specific activity of glycerol-3-phosphate dehydrogenase from *S. cerevisiae* only is thus expressed throughout as SA_{max} , that is the specific activity determined by extrapolation to infinite protein concentration. This was not done for the results reported by Brown & Edgley (1980), nor, in the present work, with assays of DHAP reduction by NADPH.

Phosphofructokinase was assayed according to Sols & Salas (1970) using a coupled enzyme system. The supplementary enzymes used in this assay (aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase) were obtained commercially as suspensions in (NH₄)₂SO₄ (3.2 M) and, where indicated, were dialysed overnight at 0 °C against the assay buffer (potassium phosphate, 25 mM, pH 6.5, 200 vol.).

Analytical methods. Protein was estimated by the biuret method (Layne, 1957) using bovine serum albumin standards.

Polyols (total) were estimated by periodate oxidation (Hanahan & Olley, 1958). Glycerol was estimated enzymically (Eggstein & Kuhlmann, 1974) but with NADH at a concentration of 0.1 mM in the assay. The amount

of arabitol in *S. rouxii* extracts was calculated from the difference between total polyol and glycerol content, since these were the only polyhydric alcohols detectable in the extracts by chromatography. Arabitol was not detected in *S. cerevisiae* extracts.

Glucose was estimated enzymically with a commercial glucose oxidase kit (GOD-Perid, Boehringer) used as follows. The glucose oxidase reagent was dissolved in water to a concentration of 5% (w/v). This solution (0.2 ml) was mixed with the sugar solution (0.2 ml) and incubated with shaking for 30 min at 30 °C. Acetic acid (3.6 ml) was added, and the A_{650} was measured. (The wavelength can be changed if the readings are too high.) An A_{650} of about 0.5 is given by 0.5 mM glucose. This modified use of the commercial reagent is based on the method of Davies & Wayman (1973). Trehalose was provisionally identified by descending paper chromatography in butan-1-ol/acetic acid/water (6:1:2, by vol.) and detection with silver nitrate.

Preparation of extracts for analysis. Samples (25 ml) of culture were chilled in ice and centrifuged at 12100 g for 10 min. The supernatant solution was collected and immediately frozen. The pellets were washed twice at 0 °C with water (4.0 ml; samples from basal medium) or 10% NaCl (4.0 ml; samples from salt broth) by centrifuging for 5 min at 12100 g. The washings were pooled and frozen. The pellet was freeze-dried and extracted first by mixing with absolute ethanol (10 ml) and allowing to stand overnight at 0 °C. Water (15 ml, 0 °C) was then added to this slurry, and the aqueous-ethanolic slurry was maintained at 0 °C for 45 min after which it was centrifuged and washed twice in the centrifuge with water (10 ml). The extract and washings were combined and concentrated by rotary evaporation under vacuum.

Intracellular polyol content was calculated from the polyol content of cell extracts plus that lost in washing. Polyol leakage during washing was estimated by subtracting from the amount in the washings the amount contributed by the growth medium retained as interstitial fluid after the first centrifugation. The volume of interstitial fluid was calculated from the amount of glucose in the washings and the concentration of glucose in the medium at the time of harvesting, on the assumption that all glucose in the washings had an extracellular origin.

Dry mass was determined on separate 25 ml samples of culture by centrifuging and washing with water as described above and drying at 110 °C for 24 h.

RESULTS

Enzyme activity in the fully adapted yeast

Table 1 shows glycerol-3-phosphate dehydrogenase activity or, more precisely, specific rates of reduction of DHAP by unfractionated extracts of each yeast, with NADH and NADPH separately as reductants. The salient features of this table are: (i) in *S. cerevisiae* the enzyme activity was about 100 times greater with NADH than with NADPH; (ii) activity with NADH was increased by up to 30-fold, and with NADPH by up to 20-fold, when *S. cerevisiae* was grown in media containing NaCl, the greatest increase occurring with 10% (w/v) NaCl; (iii) the

Table 1. *Effects of solute concentration or a_w of the growth medium on DHAP-reducing activity of crude extracts of Saccharomyces cerevisiae and S. rouxii*

Activities are expressed as SA_{max} (see Methods) for reduction of DHAP with NADH by *S. cerevisiae* extracts and conventionally as specific activity in all other cases. The units are $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. The numbers in parentheses denote the number of preparations assayed. Values are given as mean $\pm$ S.E.M. for four or more preparations and otherwise as the mean and range.

Growth medium	Enzyme activity			
	<i>S. cerevisiae</i>		<i>S. rouxii</i>	
	NADH	NADPH	NADH	NADPH
Basal	154 $\pm$ 28	2.1 $\pm$ 0.2	7.0 $\pm$ 1.0	4.5 $\pm$ 0.8
(a_w 0.997)	(6)	(6)	(4)	(4)
Salt broth	1182 $\pm$ 62	8.1	6.5	2.4
(5% NaCl, a_w 0.968)	(2)	(1)	(1)	(1)
Salt broth	4598 $\pm$ 214	46.5	11.2	2.0
(10% NaCl, a_w 0.936)	(2)	(1)	(1)	(1)
Basal + PEG	990 $\pm$ 74	—	10.3	4.5
(a_w 0.970)	(2)	—	(1)	(1)
Basal + PEG	1340 $\pm$ 24	—	8.8	5.2
(a_w 0.940)	(2)	—	(1)	(1)

Table 2. *Effects of growth in the presence of salt on phosphofructokinase activity in crude extracts of Saccharomyces cerevisiae and S. rouxii*

Units of specific activity are $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Values for basal medium preparations are given as the mean and s.e.m. for three preparations. Values for salt broth are the mean and range for two preparations. Inhibition by 1 mM-ATP was calculated with reference to rates obtained with 0.1 mM-ATP.

Growth medium	Assay conditions		Specific activity		Inhibition by 1 mM-ATP (%)	
	NH_4^+ (16 mM)	[ATP] (mM)	<i>S. cerevisiae</i>	<i>S. rouxii</i>	<i>S. cerevisiae</i>	<i>S. rouxii</i>
Basal (a_w 0.997)	+	0.1	168 $\pm$ 20	145 $\pm$ 11	—	—
	+	1.0	157 $\pm$ 37	51 $\pm$ 8	7	65
	—	0.1	138 $\pm$ 6	10 $\pm$ 1	—	—
	—	1.0	14 $\pm$ 5	2 $\pm$ 1	90	80
Salt broth (10% NaCl, a_w 0.936)	+	0.1	300 $\pm$ 40	92 $\pm$ 5	—	—
	+	1.0	174 $\pm$ 23	36 $\pm$ 4	42	61

activity of *S. cerevisiae* extract was 20–400 times that of *S. rouxii*, depending on growth conditions. In *S. rouxii*, enzyme activity with NADH was 1.5–5.5 times as great as with NADPH and neither was appreciably affected by a_w or salinity.

Table 2 lists phosphofructokinase activity in extracts of the two yeasts after growth in basal medium and salt broth. Features of this table include the degree of inhibition effected by the substrate, ATP, and the influence on that inhibition of lowering the NH_4^+ concentration by dialysing the supplementary enzymes. Phosphofructokinase from many sources is subject to substrate inhibition by ATP (Hofman, 1976) and, for that reason, the enzyme is sometimes assayed with GTP. Inhibition by ATP is reversed, at least for baker's yeast phosphofructokinase, by NH_4^+ (Atzpodian & Bode, 1970).

There was apparently a different type of phosphofructokinase in each species, that in *S. rouxii* being much more susceptible to ATP inhibition than the enzyme(s) from *S. cerevisiae*. This difference was exaggerated when dialysed auxiliary enzymes were used and, without the protection of NH_4^+ , the *S. cerevisiae* preparation was also substantially inhibited by 1.0 mM-ATP. The effect was reversed by restoring NH_4^+ (added as NH_4Cl) to the assay mixture. When the yeasts were grown in salt broth, phosphofructokinase activity was about halved in *S. rouxii* and about doubled in *S. cerevisiae*. The additional activity so produced, however, was sensitive to 1.0 mM-ATP (assayed with undialysed supplementary enzymes), implying two types of phosphofructokinase and a greater potential for regulation of glycolysis when *S. cerevisiae* is grown in 10% NaCl.

Changes during adaptation to salt broth

Viability changes. When *S. rouxii* was transferred to salt broth (containing 10% NaCl), viability, as assessed by plate counts on three agar media, changed very little and there was reasonably close agreement among the three media (Fig. 1*a*). The approximately twofold increase in the population during the period of observation was as expected since the inoculating culture was harvested somewhat more than half-way through its exponential growth phase.

The response of the population of *S. cerevisiae* to transfer to salt broth was totally different (Fig. 1*b*). We have designated the first 45 h, with widely different plate counts, as Stage 1, and the period after 45 h as Stage 2 of the adaptation process. Similar results were obtained when the inoculum was grown from a single colony instead of a slope culture (see Methods).

Polyols and trehalose. Accumulation of intracellular glycerol by each species began immediately after transfer to salt broth, or at least within the sampling time of about 3 min (Table 3; Fig. 2). In *S. rouxii* (Table 3*a*) there was some synthesis of both glycerol and arabitol during the first 5 h or so after transfer. The change in the quotient intracellular/extracellular glycerol reflected initially the delay in accumulation of glycerol outside the cell (over the first

Fig. 1. Viable counts, on three agar media, of *Saccharomyces rouxii* (a) and *S. cerevisiae* (b) after transfer from basal yeast medium to salt broth (containing 10%, w/v, NaCl). The 'before transfer' counts were those of the inoculating culture before harvesting (see text). $\circ$, Count on malt extract agar; $\bullet$, count on synthetic honey agar; $\square$, count on salt agar.

Fig. 2. The partitioning of glycerol in *Saccharomyces rouxii* (a) and *S. cerevisiae* (b) after transfer from basal yeast medium to salt broth (containing 10%, w/v, NaCl). Points above the zero line (dashed) denote intracellular glycerol content and, below the line, yield of extracellular glycerol expressed, in the same units, as a function of the mass of dry yeast. The 'before transfer' values were those of the inoculating culture before harvesting. Note the different time scales in parts (a) and (b). This figure shows graphically for several experiments (denoted by the various symbols) results similar to those listed for single experiments in Table 3(a and b). The stars denote the effective coincidence of four points.

3.5 h) and later the consumption or uptake of extracellular glycerol. The corresponding ratio for arabitol decreased over the period 1–11 h, reflecting a relatively stable internal arabitol content and a progressive increase in the amount of extracellular arabitol. Ultimately external arabitol was also consumed.

Synthesis of glycerol by *S. cerevisiae* in response to the stress was much slower than in *S. rouxii* even though it, too, began immediately after the transfer (Table 3b). Total synthesis continued

Table 3. *Adaptation of Saccharomyces rouxii and S. cerevisiae to salt stress*

The 'before transfer' sample was taken directly from the inoculating culture before the harvesting and washing procedure described in the text. Note that the quotient (I/E) is not a concentration ratio. The data are from a single experiment.

Sampling time (h)	Suspension density (mg dry mass ml ⁻¹)	Glycerol yield [mmol (g dry yeast) ⁻¹]			Arabitol yield [mmol (g dry yeast) ⁻¹]				
		Intracellular (I)	Extracellular (E)	Total (I + E)	Quotient (I/E)	Intracellular (I)	Extracellular (E)	Total (I + E)	Quotient (I/E)
(a) <i>Production and partitioning of glycerol and arabitol by S. rouxii</i>									
Before transfer	—	0.37	1.53	1.90	0.24	0.53	0.38	0.91	1.39
0.05 ('zero time')	0.442	0.53	0.059	0.59	8.98	0.52	0.018	0.53	28.89
1.0	0.464	1.49	0.41	1.90	3.63	1.06	0.032	1.09	33.12
2.0	0.507	1.43	0.64	2.07	2.23	1.22	0.095	1.32	12.84
3.5	0.546	2.44	0.79	3.23	3.09	1.40	0.33	1.73	4.24
5.5	0.548	2.87	0.38	3.25	7.55	1.29	0.39	1.68	3.31
11.0	0.612	2.98	0.15	3.13	19.87	1.01	0.40	1.41	2.52
36.0	2.158	2.22	0	2.22	∞	1.21	0	1.21	∞
(b) <i>Production and partitioning of glycerol by S. cerevisiae</i>									
Before transfer	—	0.010	0.52	0.53	0.019				
0.05 ('zero time')	0.616	0.041	0.014	0.055	2.93				
2.0	0.544	0.068	0.20	0.27	0.34				
20.5	0.539	0.070	2.34	2.41	0.030				
44.5	0.487	0.057	4.14	4.20	0.014				
70.5	0.478	0.16	6.72	6.89	0.024				
98.0	0.536	0.55	8.30	8.85	0.066				
140.0	1.423	1.48	1.44	2.93	1.03

Fig. 3. Consumption of glucose by *Saccharomyces rouxii* (a) and *S. cerevisiae* (b) after transfer from basal yeast medium to salt broth. The results in (a) are from the same experiment as shown in Table 3(a) and Fig. 1(a) and, in (b) Table 3(b) and Fig. 1(b). Note the different time scales in parts (a) and (b).

Fig. 4. Changes in the specific activity (SA_{max} , see Methods) of glycerol-3-phosphate dehydrogenase after transfer of *Saccharomyces cerevisiae* from basal yeast medium to salt broth (10%, w/v, NaCl). These results are from the same experiment as shown in Table 3(b) and Fig. 1(b).

for about 98 h, however, and led to an amount nearly three times as much as produced by the xerotolerant species. Internal glycerol remained lower than in *S. rouxii*; external glycerol was much higher after the first 2 h and correspondingly the intracellular/extracellular quotient was low, being less than unity for all except the initial ('zero time') sample, when there was scarcely any external glycerol, and the 140 h sample, when external glycerol was being consumed. The quotient for *S. rouxii* was always substantially greater than unity. For *S. cerevisiae*, the quotient decreased during Stage 1 and increased during Stage 2 of the adaptation. A substance provisionally identified as trehalose was detectable by paper chromatography of extracts from 20 h onwards. This has previously been reported for fully adapted cultures of *S. cerevisiae* at low a_w (Brown, 1978). None has been detected in *S. rouxii*.

Glucose consumption. During the period of adaptation to salt broth *S. rouxii* consumed 30% of its extracellular glucose by 11 h and all of it by 36 h (Fig. 3a). For *S. cerevisiae* extracellular glucose concentration was about $9 \mu\text{mol ml}^{-1}$ (more than 30% of the initial concentration) at the end of Stage 1 (45 h), less than $1 \mu\text{mol ml}^{-1}$ at 100 h and totally consumed by 140 h (Fig. 3b).

Glycerol-3-phosphate dehydrogenase. There was no change in glycerol-3-phosphate dehydrogenase activity of *S. cerevisiae* during Stage 1; activity of the enzyme increased with Stage 2 and reached an apparent maximum at about 100 h (Fig. 4). These results are from the experiment

represented in Table 3(b). Similar results were obtained from other experiments summarized in Fig. 2(b). There was no change in activity of *S. rouxii* extracts during the adaptation period.

DISCUSSION

The steady-state situation. The specific activities of glycerol-3-phosphate dehydrogenase shown in Table 1 are much higher for *S. cerevisiae* and slightly higher for *S. rouxii* than reported by Brown & Edgley (1980). The main reasons for this are the use of a different extraction buffer [Brown & Edgley (1980) used phosphate buffer (Sørensen, $\text{Na}^+ + \text{K}^+$, 0.067 M, pH 7.0) in preparation of yeast extracts for enzyme assay], and the use of SA_{max} in reporting the present results (see Methods). Glycerol-3-phosphate dehydrogenase from various sources has regulatory characteristics (e.g. Black, 1966; Tajima & Yoshizumi, 1973; Bewley & Miller, 1979; Nader *et al.*, 1979; Storey & Storey, 1982). The regulatory mechanism seems to include, at least for the enzyme from rabbit skeletal muscle, a capacity for aggregation-disaggregation between a monomeric and dimeric form of the enzyme as a function of both enzyme and substrate concentration. Both forms are active but the dimer is more so. The dimer can also apparently aggregate with aldolase to give a yet slightly more active form (Batke *et al.*, 1980). The enzyme from *S. cerevisiae* is capable of somewhat similar behaviour, at least to the extent of apparently changing its aggregation state as a function of protein concentration. We have no evidence for or against its complexing specifically with another enzyme. *Saccharomyces rouxii* extracts, however, did not respond in this way to protein concentration with either NADH or NADPH as reductant and, at least by this criterion, the glycerol phosphate dehydrogenase from that species is a distinctly different enzyme(s) from that in *S. cerevisiae*.

Nevertheless, the general effects of growth at diminished a_w were essentially as described by Brown & Edgley (1980) inasmuch as the specific activity of the glycerol phosphate dehydrogenase was greatly increased in *S. cerevisiae* (about 30-fold in 10% NaCl) but was scarcely affected in *S. rouxii*. The stimulatory effect of PEG at the same a_w was somewhat less but still substantial. A difference between the two solutes is consistent with various other responses of yeast (Brown, 1978). The major effect of the different assay procedure on the results for *S. rouxii* was to reverse the apparent relative importance of NADPH and NADH in the reduction of DHAP. In the present series of assays activity was higher with NADH than with NADPH, whereas the reverse was true in Brown & Edgley's (1980) results. In view of the difficulties inherent in comparing physiological activities of different reactions, it is reasonable to conclude that DHAP reduction in *S. rouxii* occurs with about equal facility with either NADH or NADPH as reductant and, under all experimental conditions, at a much lower rate than the NADH-specific reduction of DHAP in *S. cerevisiae*. This, together with the substantial rate of reduction of DHA and glyceraldehyde by NADPH and the relatively high levels of activity of the pentose phosphate cycle enzymes in *S. rouxii* (Brown & Edgley, 1980), is consistent with a greater contribution by that cycle to carbohydrate metabolism in *S. rouxii* than in *S. cerevisiae*.

The specific activities of phosphofructokinase in Table 2 also imply that glycolysis is considerably less important for *S. rouxii* than for *S. cerevisiae* and, moreover, is more susceptible to control by ATP inhibition in the xerotolerant species. Furthermore, production of this enzyme, like that of glycerol phosphate dehydrogenase, was enhanced by growth in 10% NaCl only in *S. cerevisiae*. The additional kinase so produced, however, had different substrate (ATP)-inhibition characteristics from the dominant enzyme in the yeast grown on basal medium.

Thus important differences between the two species include not only the relative contributions of the possible metabolic pathways to glycerol and the major response of glycerol synthesis to a_w in only one of these species, but also effects of water activity or salinity on the production by *S. cerevisiae* of at least two enzymes involved in glycerol synthesis.

The transition. The transitional behaviour of *S. cerevisiae* compared with that of *S. rouxii* is a splendid example of what not to do if you want to be xerotolerant. *Saccharomyces cerevisiae* clearly 'recognized' a need for intracellular glycerol but its response to that need was much more complex and less effective than the simpler tactic used by *S. rouxii*.

Nowhere is the difference between the two species more conspicuous than in the viability changes that occurred during the period of adaptation to salt broth. Whereas the population of *S. rouxii* was unruffled by the transfer, that of *S. cerevisiae* experienced a major trauma.

The information contained in Fig. 1 (*a* and *b*) is quite complex. For a start, there are several time factors implicit in the two figures. These are: (i) the time shown on the abscissa, which is the period spent by the yeast in the salt broth up to the moment of sampling; (ii) a period of minutes during the serial dilutions in 10% NaCl; (iii) the period of incubation of the plates which ranged from 3 d for MA to 18 d for salt agar. Thus the results reflect not only the state of adaptation of the population to the salt broth at the time of sampling but also, where appropriate, a continuation of the adaptation process in the discrete colony-forming units once plated on to the agar.

Plating from salt broth on to MA subjected the yeasts to a dilution stress (an osmotic shock) whereas plating on to salt agar or SHA did not. It is noteworthy in this context that, for the first 2 h after transfer, the count of *S. cerevisiae* on MA did not drop whereas on salt agar it did so catastrophically (by $>10^4$). Conversely, as the adaptation of *S. cerevisiae* progressed during Stage 1 the MA count dropped while that on salt agar (and, with some minor variations, on SHA) increased. This raises two questions, namely: (a) was the drop in the MA count during Stage 1 merely the result of a dilution stress as the adaptation progressed or was it a genuine expression of diminished total viability? (b) did the small residue of cells viable on salt agar and SHA represent a 'rump' of the population that was viable in salt broth and whose subsequent multiplication was actually the adaptation process or did it reflect merely the heterogeneity of a population that was severely stressed but which, *in toto*, adapted physiologically?

We do not yet know the answer to question (a), but the decrease in cell mass during the first 70 h or so of the adaptation of *S. cerevisiae* (Table 3*b*) indicates some loss of viability during this period. The declining count on MA, however, was accompanied by increasing counts on the other two media. The correlation between these two opposing trends must reflect in some way the physiological states of the population during Stage 1. Thus Stage 1 seems to be a series of 'points of no return' beyond which part of the population sacrifices its recovery potential (count on MA) in the interests of increasing its apparent viability in the salt medium. Once into Stage 2, this trade-off is no longer necessary.

There is some evidence relevant to question (b). First, the apparent exponential growth rate of the population during late Stage 1 and early Stage 2 was about 0.14 generations h^{-1} , which is low enough to be consistent with either multiplication or physiological adaptation. Second, K. F. McKenzie & A. D. Brown (unpublished) have shown that, in the course of a normal growth cycle in basal medium *S. cerevisiae*, but not *S. rouxii*, passes through a period in which counts on SHA and salt agar are at least 10^3 below counts on MA. The period coincides with bud formation and recovery is rapid when the buds separate. This observation seems to embody part of an explanation of the events depicted in Fig. 1 (*b*) and, for the present at least, we regard the apparent population changes of Stage 1 as a reflection of a general physiological adaptation of the whole population rather than simply the multiplication of a small viable residue.

Another factor that is relevant to this question is the glycerol content of the yeast if glycerol is produced only by those cells that are viable by any of the criteria that might be invoked from Fig. 1 (*b*). When this is done, taking the average mass of a wet yeast cell to be about 2×10^{-10} g and the water content to be about 60%, then apparent intracellular glycerol concentrations (from Table 3*b*) for viable cells are as follows: 2 h, MA count (as index of viability), 0.03 molal; salt agar count, 3×10^3 molal; 44.5 h, MA count, 23 molal. Except when the 2 h count on MA was used as the number of viable cells, these values are patently impossible. We must therefore conclude that glycerol is produced essentially by the whole population of *S. cerevisiae* and not solely by that portion that is viable by any of the criteria employed.

Although the anomalies in viability that are characteristic of the early period of the transition were overcome by the end of Stage 1, Stage 2 was not simply a period of multiplication by the fully adapted organisms since the yeast produced both additional glycerol and glycerol phosphate dehydrogenase during this period (Table 3*b*; Fig. 4). Although we have not followed changes in phosphofructokinase activity during the transition, it is reasonable to assume that the

additional enzyme indicated in Table 2 is also produced during Stage 2. Table 2 implies that the extra phosphofructokinase produced by the fully adapted yeast is an isoenzyme of that produced in basal medium, since it was affected differently by ATP. It is therefore pertinent to ask whether the additional glycerol phosphate dehydrogenase produced in response to salt stress was more of the same or also an isoenzyme of that produced in basal medium. We cannot yet answer this question but isoenzymes of glycerol phosphate dehydrogenase are common in other organisms, especially when activity is increased in some tissues at certain periods of development (e.g. Bewley & Miller, 1979).

Although we do not yet have direct evidence in a number of relevant areas, it is obvious that, during Stage 1, *S. cerevisiae* is capable of glycerol production, and hence energy metabolism, but may well be incapable of protein synthesis. We have not yet determined whether the additional glycerol phosphate dehydrogenase activity that appeared in Stage 2 was the result of protein synthesis or of a process such as the activation of a zymogen. For the present we assume, as an hypothesis, that it was *de novo* synthesis. Glycerol production continued during Stage 2 but its content for much of the time was less than that appropriate for the prevailing a_w . We must assume, therefore, that intracellular physical chemistry was more inhibitory than in the fully adapted yeast but, of course, less inhibitory than in the early part of Stage 1. In spite of this, not only is energy metabolism possible but so is enhancement of enzyme activity.

Thus the overall response of *S. cerevisiae* is determined by at least two environmental signals, an immediate one redirecting carbohydrate metabolism to glycerol production and a later one leading to an increase in enzyme activity. We have already suggested that the former might be K^+ depletion and the latter increased intracellular glycerol concentration (Brown & Edgley, 1980).

In comparison with the complexity of the attempt by *S. cerevisiae* to adapt to 10% NaCl, there is little to be said about the effortless transition of *S. rouxii*. Viability was not affected by the transfer. We have previously reported (Brown, 1978; Edgley, 1980) that fully adapted *S. rouxii* does not significantly vary the amount of polyol it synthesizes in relation to the a_w of its growth medium and that it varies its glycerol content merely by controlling the proportion it retains of an approximately constant amount synthesized. The present results (Table 3a) show that it did, in fact, synthesize both arabitol and glycerol during the transition although their combined amount was less than half the glycerol production of *S. cerevisiae*. In spite of this, *S. rouxii* produced its glycerol much faster than *S. cerevisiae* during the transition. Since the culture conditions used in the present work were quite different from those used previously for the fully adapted yeast in steady state, and because the extracellular glycerol load of the inoculating culture had been depleted, differences between the two sets of conditions in the several variables studied are to be expected. The constant factor is that, although *S. rouxii* did synthesize some glycerol during adaptation to salt stress, it made use of enhanced retention as a major contribution to raising its glycerol content as it did in steady state. This was not done by *S. cerevisiae* under either circumstance.

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