

Microbial Water Relations. Effects of Solute Concentration on the Respiratory Activity of Sugar-tolerant and Non-tolerant Yeasts

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SUMMARY

The respiratory activity of the sugar-tolerant (osmophilic) yeast, *Saccharomyces rouxii*, and the non-tolerant species, *Sacchchromyces cerevisiae*, were compared after growth in a complex basal medium, the medium supplemented with polyethylene glycol (mol. wt 200) to give a water activity of 0.95, and the medium supplemented with glucose (24 and 36 %, w/v). The properties compared were Q_{O_2} (glucose), NADH oxidase activity of isolated mitochondrial fractions, and cytochrome content. When grown in the basal medium *S. cerevisiae* was somewhat more active than *S. rouxii* by all criteria. Growth in the media supplemented were high glucose concentrations produced catabolite repression of respiration in *S. cerevisiae* but not in *S. rouxii*. The implications of this difference for polyol biosynthesis and the water relations of the sugar-tolerant species are discussed.

INTRODUCTION

Apart from their water relations, the sugar-tolerant ('osmophilic') yeasts differ from related non-tolerant species in several ways. Their growth rates under optimal conditions are substantially less than the corresponding growth rates of non-tolerant species (Anand & Brown, 1968). Further, the tolerant yeasts produce polyhydric alcohols, sometimes in high yields (Onishi, 1963; Spencer, 1968). The polyols are substantially retained by the yeasts and can reach a high intracellular concentration. In their role as compatible solutes, they are a major determinant of the water relations of the tolerant species (Brown & Simpson, 1972; Brown, 1974). There is, however, an apparent biochemical anomaly in polyol production by these organisms. In spite of the fact that the polyhydric alcohols are essentially fermentation end-products, their synthesis requires an adequate supply of oxygen; at low oxygen tension and sugar-tolerant yeasts revert to a normal ethanolic fermentation (Onishi, 1963; Spencer, 1968).

These observations, together with the conspicuous ability to grow well in high concentrations of many types of sugar, including glucose, where catabolite repression might be expected, suggested that the composition and/or biosynthesis of the respiratory apparatus of the tolerant yeasts might differ from that of their non-tolerant counterparts.

This paper compares the formation and function of the respiratory apparatus of the sugar-tolerant yeast, *Saccharomyces rouxii*, with that of the closely similar non-tolerant *S. cerevisiae*.

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METHODS

Organisms. *Saccharomyces rouxii* (strain YA: Anand & Brown, 1968) and *S. cerevisiae* (strain Y41; Anand & Brown, 1968) were used throughout. Stock cultures were maintained at 2 to 4 °C on 'synthetic honey agar' and malt agar respectively (Anand & Brown, 1968). The yeasts were cultured on a rotary shaker (200 rev./min) at 30 °C to mid-exponential phase in basal medium (Anand & Brown, 1968) and in the same medium supplemented with glucose as indicated or with polyethylene glycol [mol. wt 200, 33.5 % (w/w), water activity (a_w) 0.95]. Polyethylene glycol was deionized (Anand & Brown, 1968) before use. Conical flasks (2 l nominal capacity) were used throughout and contained 1 l growth medium. Before harvesting, cultures were cooled in ice and then centrifuged briefly at 13 200 g and 0 °C. Harvested pellets were washed for specific purposes as described below.

Whole yeast respiration. Each culture was halved. After growth in basal medium one half was washed three times at 0 °C in phosphate buffer ($\text{Na}^+ + \text{K}^+$, 0.067 M, pH 5.9, a_w 0.997) and resuspended in the buffer at 0 °C (suspension A). The other half was washed in phosphate buffer, in buffer containing 16.8 % (w/w) polyethylene glycol (PEG, mol. wt 200) and in buffer plus 33.5 % (w/w) PEG (a_w approximately 0.95); it was finally suspended in buffer plus 33.5 % PEG (suspension B). After growth in media containing glucose (36 or 48 %, w/v) or PEG, suspension A was prepared by washing once in buffer plus 33.5 % (w/w) PEG, once in buffer plus 16.8 % PEG, and once in buffer; it was finally resuspended in buffer. Suspension B was prepared by washing three times, as after growth in basal medium, and suspending in buffer plus 33.5 % PEG. After growth in 24 % (w/v) glucose, suspension A was washed once in buffer plus 16.8 % PEG, once in buffer plus 8.4 % PEG and once in buffer, and finally resuspended in buffer. The other half was washed three times, as for suspension B after growth in basal medium, and resuspended in buffer plus 16.8 % PEG (suspension C).

For each wash the suspension was centrifuged briefly at 12 000 g. The yeast concentration in the final suspension was usually within the range 6 to 18 mg dry wt/ml. The respiration rate (Q_{O_2}) was determined at 30 °C in the presence of glucose by conventional manometric techniques. The suspending buffer was always the same as that used for the final suspension of the yeast (suspension A, buffer; suspension B, buffer plus 33.5 %, w/w, PEG; suspension C, buffer plus 16.8 %, w/w, PEG).

NADH oxidase assay. NADH oxidase was assayed in mitochondrial fractions prepared as follows. Yeast grown in basal medium was harvested, washed once in phosphate buffer ($\text{Na}^+ + \text{K}^+$, 0.015 M, pH 7.4) and once in tris-HCl (0.02 M, pH 7.2) containing the following substances added to the buffer in the order stated: sucrose (0.5 M), EDTA (sodium salt, 0.002 M) and magnesium chloride (0.002 M). Yeast grown in media containing polyethylene glycol or glucose at a high concentration was washed in graded solutions of buffered polyethylene glycol, as for the respiration experiments, once in phosphate buffer alone, and finally in the tris-HCl+sucrose buffer as above. The yeast pellet was transferred to a press (see Anand, 1969) at the temperature of solid CO_2 and disrupted. The ruptured yeast was thawed by resuspending in a minimal volume of the tris+sucrose buffer at 0 °C and thoroughly dispersed with a glass-Teflon homogenizer. The suspension was centrifuged at 0 °C briefly at 7700 g and the supernatant retained. The pellet was resuspended and washed once more in the centrifuge under the same conditions. The two supernatant fractions were combined and centrifuged for 15 min at 70 000 to 80 000 g. The pellet was resuspended with a glass-Teflon homogenizer to a protein concentration of 7 to 11 mg/ml in tris+sucrose buffer. This suspension was the mitochondrial fraction used for the assay of NADH oxidase.

It was stored frozen if necessary; there was no loss of activity in the frozen state for at least two weeks.

NADH oxidase was assayed conventionally at 30 °C by measuring the rate of diminution of extinction at 340 nm. The assay mixture contained (ml): tris-HCl buffer (0.1 M, pH 7.70), 0.50; EDTA (sodium salt, 2.0 mM), 0.20; MgCl₂ (0.01 M), 0.05; water, 0.20; enzyme (8.5 to 11.1 mg protein/ml), 0.03; NADH (2.5 mM), 0.02. The constituents were added in the order stated. The reaction mixture, except for NADH, was pre-incubated for 3 min in a water bath at 30 °C then transferred to the water-jacketed compartment (at 30 °C) of a Zeiss PMQ II spectrophotometer. The reaction was started by adding NADH.

Cytochrome composition. The cultures were chilled, harvested and washed twice in water at 0 °C, resuspended in water to a nominal cell density of 40 to 50 mg dry wt/ml and frozen. Difference spectra (dithionite reduced versus ferricyanide oxidized) were determined on yeast prepared as follows. The frozen suspension was thawed and mixed thoroughly. Separate portions (0.4 ml) were incubated for 2 min at room temperature with sodium dithionite (50 mg/ml in 1.0 M-tris buffer pH 7.6; 10 μ l) and with potassium ferricyanide (0.5 M; 5 μ l). Difference spectra were measured at the temperature of liquid nitrogen in a split-beam spectrophotometer designed and built at the Johnson Foundation, University of Pennsylvania (Chance, 1957). The low temperature cuvette (Bonner, 1961) had a path length of 2 mm.

For all purposes the mass of yeast was determined on suspensions which had been washed twice with water and heated at 85 °C for 3 days or 105 °C for 2 days.

Electronmicroscopy. The cultures were harvested after chilling, washed and fixed in glutaraldehyde, post-fixed with osmium tetroxide, and stained with uranyl acetate. The preparations were dehydrated in ethanol, embedded in Araldite and sectioned. Yeasts which had been grown in the presence of polyethylene glycol or glucose (36 %, w/v) were initially washed in buffer containing 'half-strength' polyethylene glycol (16.6 %, w/w), a procedure found necessary to prevent extensive vacuolation.

RESULTS

Respiration

Table 1 summarizes respiration measurements with whole yeast in phosphate buffer and in buffer supplemented with polyethylene glycol. After growth in basal medium the Q_{O_2} (glucose) of *S. cerevisiae* was 22 to 23 % higher than that of *S. rouxii*, whether measured in the presence or absence of added polyethylene glycol. Incubating either yeast in the presence of polyethylene glycol approximately halved the Q_{O_2} . Growth at 0.95 a_w in polyethylene glycol had little effect on the Q_{O_2} of *S. rouxii* in phosphate buffer but reduced the Q_{O_2} of *S. cerevisiae* by about 25 %. Both yeasts respired faster in buffered polyethylene glycol, however, giving Q_{O_2} values of about 80 % of that obtained in buffer alone. Expressed another way, the Q_{O_2} in buffered polyethylene glycol was, for both yeasts, 30 to 60 % higher after growth in polyethylene glycol than after growth in basal medium.

Growth in 24, 36 and 48 % glucose reduced the Q_{O_2} of *S. rouxii* by about 20 % when measured in phosphate buffer. Within that range there was no effect which could be attributed to the concentration of glucose in the growth medium. The corresponding reduction in the Q_{O_2} of *S. cerevisiae* was about 75 % after growth in 24 and 36 % glucose (it does not grow in 48 % glucose). The ratio of Q_{O_2} in polyethylene glycol to Q_{O_2} in buffer alone was again higher for both yeasts than when they were grown in basal medium. For *S. rouxii* the Q_{O_2} in polyethylene glycol was absolutely higher than when the yeast was grown in basal medium.

Table 1. *Respiration rates of whole yeast*

Growth medium	Yeast	Q_{O_2} (glucose)* measured in:	
		Buffer (0.997 a_w)	Buffer and PEG (0.95 a_w)
Basal	<i>S. cerevisiae</i>	95	44
	<i>S. rouxii</i>	77	36
Basal+PEG (33.5 %, w/w; 0.95 a_w)	<i>S. cerevisiae</i>	73	57
	<i>S. rouxii</i>	76, 106	60, 95
Basal+glucose (24 and 36 %, w/v)	<i>S. cerevisiae</i>	22	18
(24, 36 and 48 %, w/v)	<i>S. rouxii</i>	63	42 (51)†

The reaction vessels contained: phosphate buffer or phosphate buffer+PEG (33.5 %, w/v) (see text), 2.3 ml; yeast suspension (see text), 0.4 ml; glucose (10 %, w/v) 0.3 ml, side arm; KOH (20 %, w/v), 0.2 ml, centre well.

* The overall standard deviation for Q_{O_2} was ± 3 % of the stated value. An exception was *S. rouxii* grown in basal+PEG, under which conditions duplicate preparations gave the values shown.

† Grown in 24 % (w/v) glucose and measured in 16.8 % (w/w) PEG. (With *S. cerevisiae* there was no difference in Q_{O_2} between yeast grown in 36 % glucose and measured in 33.5 % PEG, and yeast grown in 24 % glucose and measured in 16.8 % PEG.

Table 2. *NADH oxidase activity of mitochondrial preparations*

Growth medium	Yeast	NADH oxidase activity* (μ moles/min/mg protein)
Basal	<i>S. cerevisiae</i>	35.8
	<i>S. rouxii</i>	31.9
Basal+PEG (33.5 %, w/w)	<i>S. cerevisiae</i>	15.8–19.4
	<i>S. rouxii</i>	20.1–34.2
Basal+glucose (36 %, w/v)	<i>S. cerevisiae</i>	3.5
(36 %, w/v)	<i>S. rouxii</i>	21.9
(48 %, w/v)	<i>S. rouxii</i>	14.4

* Replicate preparations normally gave values within 5 % of the mean. Organisms grown in basal+PEG were exceptional and gave preparations with activities in the ranges shown (cf. Table 1).

NADH oxidase

A similar general trend is shown in Table 2 for the NADH oxidase activity of mitochondria preparations. The specific activity of preparations from *S. cerevisiae* was about 12 % higher than corresponding preparations from *S. rouxii* when both yeasts were grown in basal medium. As with Q_{O_2} , there was more variation among preparations of yeast grown in the presence of polyethylene glycol. *Saccharomyces rouxii* preparations were probably not affected by growth in polyethylene glycol; they were certainly not significantly affected. The NADH oxidase activity of *S. cerevisiae* mitochondria was lowered by 46 to 56 % under these conditions.

Unlike Q_{O_2} , NADH oxidase activity of mitochondrial preparations from *S. rouxii* responded to the glucose concentration of the growth medium within the range of 36 to 48 % (w/v). Growth in 48 % glucose halved the activity; growth in 36 % glucose reduced it

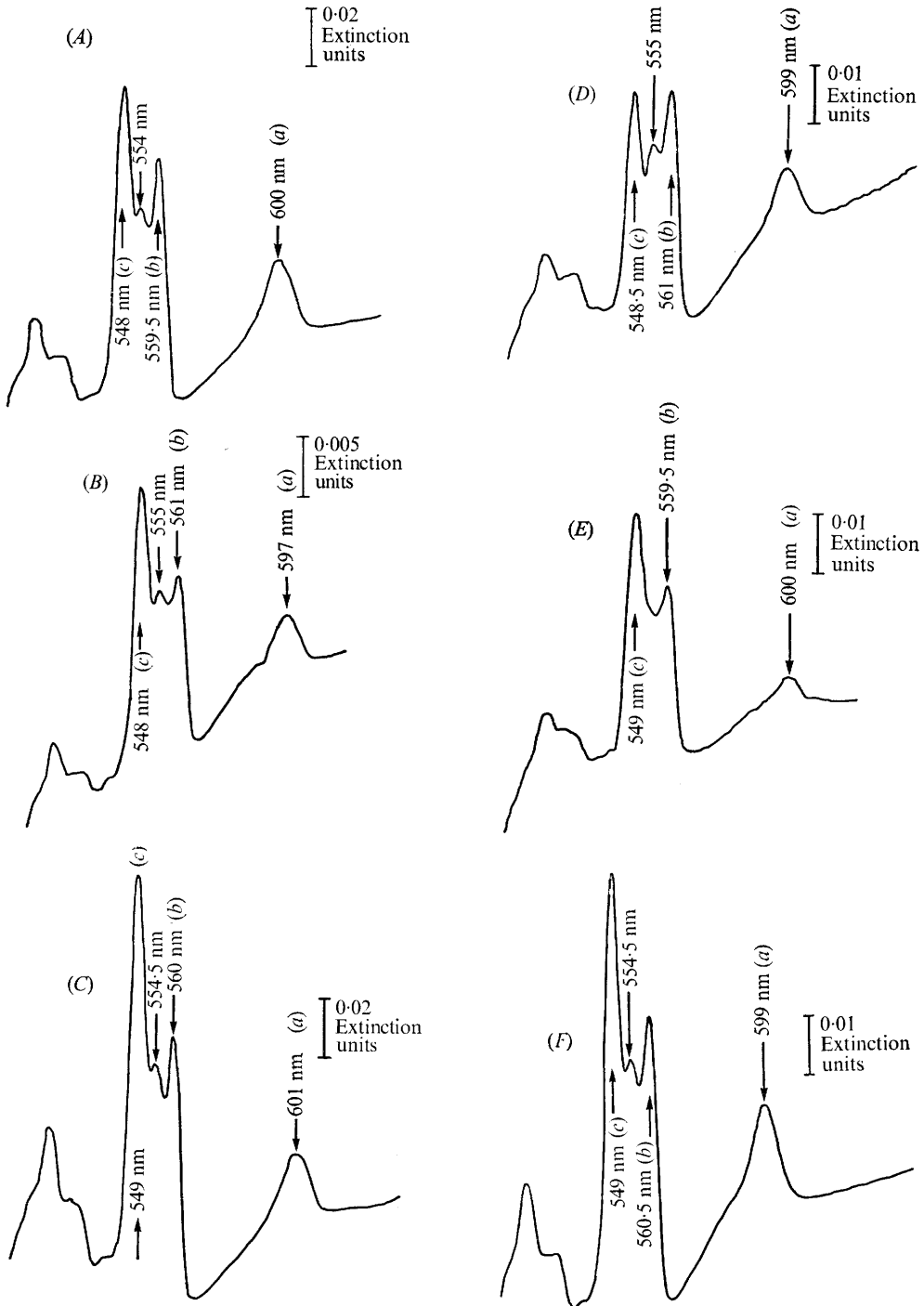


Fig. 1. Absorption spectra (dithionite reduced versus ferricyanide oxidized) of yeast suspensions at 77 °K. (A) *Saccharomyces cerevisiae* grown in basal medium. Suspension density 45.3 mg/ml. (B) *Saccharomyces rouxii* grown in basal medium. Suspension density 38.5 mg/ml. (C) *Saccharomyces cerevisiae* grown at 0.95 a_w in polyethylene glycol. Suspension density 43.0 mg/ml. (D) *Saccharomyces rouxii* grown at 0.95 a_w in polyethylene glycol. Suspension density 51.6 mg/ml. (E) *Saccharomyces cerevisiae* grown in 36% (w/v) glucose. Suspension density 40.0 mg/ml. (F) *Saccharomyces rouxii* grown in 36% (w/v) glucose. Suspension density 40.0 mg/ml.

Table 3. *Relative cytochrome contents of the two yeasts*

Growth medium	Yeast	'Cytochrome c' (units*/100 mg)	'Cytochrome b' (units*/100 mg)
Basal	<i>S. cerevisiae</i>	0.238	0.185
	<i>S. rouxii</i>	0.057	0.037
Basal+PEG (33.5 %, w/w)	<i>S. cerevisiae</i>	0.344	0.212
	<i>S. rouxii</i>	0.076	0.076
Basal+glucose (36 %, w/v)	<i>S. cerevisiae</i>	0.103	0.072
	<i>S. rouxii</i>	0.186	0.123

* Units for 'cytochrome c': extinction units, 548 or 549 to 570 nm; units for 'cytochrome b': extinction units, 560 or 561 to 570 nm.

by about 30 %. In marked contrast, however, growth in 36 % glucose reduced the NADH oxidase of *S. cerevisiae* mitochondrial preparations to less than 10 % of the original value.

Cytochrome absorption spectra

The cytochrome composition of the yeast is illustrated in Fig. 1. The two species were qualitatively similar although there were some minor differences in the wavelength of the α -bands of cytochromes *a* and *b*. There were, however, major quantitative differences, approximately summarized in Table 3, for cytochromes *b* and *c*. When grown in basal medium *S. cerevisiae* had 4 to 5 times as much cytochrome *b* and *c* as did *S. rouxii*. When they were grown at 0.95 a_w in polyethylene glycol, the cytochrome content of each yeast was increased. The proportional increase in cytochrome *c* was similar in both yeasts, but the cytochrome *b* content of *S. rouxii* was about doubled and its peak height equalled that of cytochrome *c*.

The two yeasts had opposite responses to growth in 36 % glucose. The cytochrome *b* and *c* content of *S. cerevisiae* was halved and the small peak of about 555 nm disappeared, whereas the apparent overall cytochrome content of *S. rouxii* increased about threefold and the content of cytochromes *b* and *c* was about twice as great as that of *S. cerevisiae*.

Electronmicroscopy

The major structural changes which were observed occurred in *S. cerevisiae*; similar changes have been amply described and illustrated by other authors (e.g. Linnane, Vitols & Nowland, 1962; Yotsuyanagi, 1962; Wallace, Huang & Linnane, 1968). *Saccharomyces rouxii* responded only to a minor extent to variations in the composition of the growth medium. When they were grown in basal medium the two yeasts had a similar fine structure and contained a similar number of mitochondrial profiles [*S. cerevisiae* 8.9 ± 1.7 (S.E.M.) and *S. rouxii* 7.4 ± 0.8 /equatorial section]. When grown in the presence of polyethylene glycol at 0.95 a_w some differences were evident. *Saccharomyces cerevisiae* had more mitochondria (15.8 ± 1.2) than *S. rouxii* (6.1 ± 0.7) but the mitochondria were somewhat larger in *S. rouxii*. When grown in 36 % glucose, mitochondrial numbers were again similar and not very different from those of the yeasts grown in basal medium (*S. cerevisiae*, 7.2 ± 1.0 ; *S. rouxii*, 6.6 ± 0.7). The 'mitochondrial' profiles in *S. cerevisiae* had little or no internal structure, however, whereas those of *S. rouxii* appeared normal and contained cristae.

DISCUSSION

There is nothing novel in the observation that growth in a relatively high glucose concentration represses respiratory activity and mitochondrial formation in *Saccharomyces cerevisiae* (Slonimski, 1953; Ephrussi, Slonimski, Yotsuyanagi & Tavlitzki, 1956; Yotsuyanagi, 1962; Tustanoff & Bartley, 1964; Utter, Duell & Bernofsky, 1968). The comparison with the related species, *S. rouxii*, is of interest for several reasons, one being the common assumption that this type of catabolite repression is characteristic of the genus *Saccharomyces*.

The high glucose concentrations used in the present series of experiments presumably affected the results by at least two processes of major significance, one involving the relatively non-specific physico-chemical effects of a concentrated non-electrolyte in the growth medium, the other the much more specific effects of catabolite repression. Although it is now clear that the role of non-electrolytes in the apparent water relations of yeast is more than simply that of a non-specific solute which produces a certain water activity (a_w) in the extracellular fluid (Anand & Brown, 1968; Brown & Simpson, 1972), the response of any one organism to changes in extracellular solute concentration is usually qualitatively similar for many different non-electrolytes.

Thus it is reasonable to assume that incorporation into the growth medium of polyethylene glycol, which is not metabolized, produced an effect which, at least as a first approximation, resembled the purely physico-chemical influences of high glucose concentration. At this level there were differences between the two species but they were of relatively minor proportions. Growth in polyethylene glycol slightly lowered Q_{O_2} in *S. cerevisiae* but not in *S. rouxii*, halved NADH oxidase activity in *S. cerevisiae* but not in *S. rouxii*, and increased the cytochrome *b+c* content of both species. It also caused in both species a degree of adaptation to polyethylene glycol as reflected in the enhanced respiration rates of yeast suspended on polyethylene glycol.

In some respects growth in high concentrations of glucose resembled growth in polyethylene glycol but, on balance, it caused very large differences between the two species. Following on from the comments above, a major factor in these differences can be assumed to be different responses of the two species to the specific repressive action of glucose metabolism. The similarities between the two solutes were evident in the adaptation by both yeasts to polyethylene glycol as a suspending solution of O_2 -uptake measurements. This adaptation is reflected in Table 1 in the ratio of Q_{O_2} in polyethylene glycol to Q_{O_2} in buffer alone after growth in the three media. The nature of the adaptation mechanism is not known but increased permeability to glucose of the yeasts and/or their mitochondria is a possibility. The other important similarity is that growth in a high glucose concentration increased the cytochrome content of *S. rouxii*; indeed, the increase was much greater than produced by polyethylene glycol.

In this and all other measured properties, however, the two yeasts differed profoundly from each other after growth in high concentrations of glucose. By all the criteria used, the capacity of *S. cerevisiae* to respire was greatly reduced by 24 and 36 % (w/v) glucose in the growth medium, as was to be expected (see above). The corresponding effect on the Q_{O_2} of *S. rouxii* was minor, and was associated with mutually opposing trends in the response of NADH oxidase activity on the one hand and cytochrome content on the other.

Part of the effect of glucose on *S. rouxii* can be assumed to have been caused by direct physico-chemical effects of a non-electrolyte and part by the differences which must inevitably occur in any system when the quantitative details of metabolism are changed. Whether or not catabolite repression specifically contributed to the slight reduction in Q_{O_2}

and somewhat greater reduction in NADH oxidase activity caused by glucose in *S. rouxii* is doubtful. If it did, the contribution was obviously of minor significance.

The failure of glucose metabolism to repress the respiratory capacity of *S. rouxii* is potentially explicable in two ways: the mechanism of gene expression, at least for the biogenesis of the respiratory chain, is different in the two species and, in *S. rouxii*, is not inherently susceptible to catabolite repression; or the repression mechanism is overcome by arabitol. Arabitol accumulates to a high concentration in *S. rouxii* (Brown & Simpson, 1972; Brown, 1974) and protects enzymes against inactivation at low levels of water activity (Brown & Simpson, 1972) as does glycerol in halophilic algae (Borowitzka & Brown, 1974) and K⁺ (and KCl) in halophilic bacteria (Aitken & Brown, 1972). It is conceivable that arabitol at such high concentrations as exist in *S. rouxii* could change the overall kinetics of gene expression in such a way that catabolite repression, of the kind under discussion, does not occur. Indeed, according to one report (Wallis, Ottolenghi & Whittaker, 1972), incubation of *S. cerevisiae* in 2 % glycerol can induce mutation of the yeast to a petite form. The possibility that arabitol does modify gene expression is amenable to investigation and clearly warrants it.

The sugar-tolerant yeasts must be able to resist catabolite repression of respiration if they are to thrive at the very high sugar concentrations which can occur in their normal habitats. To grow under such conditions they must produce their compatible solute, a polyol. Although the detailed mechanisms of polyol production are obscure it is an empirical observation that polyol formation is favoured by aerobic conditions and hindered by an oxygen deficiency (Onishi, 1963; Spencer, 1968). Since repression or inhibition of respiration would be tantamount to anaerobiosis, the yeast must be able to retain its respiratory capacity if it is to synthesize its compatible solute. It thus provides another example of the ramifications of adaptation to an extreme environmental situation.

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