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Microbial Water Relations: Features of the Intracellular Composition of Sugar-Tolerant Yeasts

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Several factors contributed to differences in intracellular composition between sugar-tolerant (osmophilic) and nontolerant species of yeast. One such factor was the difference in accumulation of those nonelectrolytes whose uptake was not dominated by vigorous metabolism. In such cases (lactose and glycerol), the sugar-tolerant species had a much lower capacity for the solute than did the nontolerant species. Sucrose uptake was consistently different between all sugar-tolerant strains on the one hand and all nontolerant strains on the other. The difference was attributable in part to metabolism of sucrose by the nontolerant yeasts. The major difference between the two types of yeast, however, was the presence of one or more polyhydric alcohols at high concentrations within each of the sugar-tolerant strains but none of the nontolerant strains. In most cases the major polyol was arabitol. The solute concentration (and, hence, water availability) of the growth medium affected both the amount of arabitol produced by *Saccharomyces rouxii* and the proportion retained by the yeast after brief washing with water at 0 C. When the yeast was suspended in a buffer at 30 C, the polyol leaked out at a slow, constant, reproducible rate. The polyene antibiotic amphotericin B caused rapid release of polyol by the yeast, the rate being proportional to amphotericin concentration. Contact of the yeast with glucose (1 mM) caused an extremely rapid ejection of polyol which lasted less than 40 s. Some implications of these results are discussed, as is the role of the polyol as a compatible solute in determining the water relations of the yeast.

The so-called osmophilic yeasts and the xerophilic molds form a group which can thrive at lower levels of thermodynamically available water than any other microorganisms. In most cases, the peculiar water relations of these organisms are the result of a tolerance of rather than a requirement for high solute concentrations. In this respect, they differ fundamentally from the halophilic bacteria, which also thrive at low levels of water activity but do so partly by virtue of an absolute requirement for high concentrations of sodium chloride.

The limits of water activity (a_w) tolerated by the yeasts are affected by the nature of the extracellular solutes present (2). These solutes are commonly sugars or related nonelectrolytes, but salt tolerance is also occasionally encountered (10).

In somewhat oversimplified terms, two explanations for the physiology of sugar tolerance are possible. The first is that the proteins of tolerant organisms are intrinsically different from the corresponding proteins of the nontolerant organisms and, because of this difference, they can function effectively at low levels of water activity (or high solute concentrations). This is the situation with halophilic bacteria (1, 4). A physiologically functional halophil enzyme or enzyme system could be selected at random and be recognized as distinctive because of its salt requirements. The second major hypothesis is that, in general, enzymes are fundamentally similar in both tolerant and nontolerant organisms, but intracellular compositions are different in such a way that the impact of the environment on the intracellular physiology of tolerant species is lessened. This is apparently the type of mechanism primarily responsible for the peculiar survival characteristics of bacterial endospores. The second hypothesis, however, does not and cannot include the possibility that the interior of a sugar-tolerant yeast remains dilute relative to the extracellular environment. Unicellular microorganisms must adjust thermodynamically to their environment, and they have no way of preventing loss of water against a water activity gradient (4).

The nicotinamide adenine dinucleotide phosphate (NADP)-specific isocitrate dehydrogenase from a strain of the sugar-tolerant yeast

Saccharomyces rouxii has been shown (unpublished data) to be indistinguishable from the corresponding enzyme of the nontolerant yeast *S. cerevisiae* in its kinetics, water relations, and electrophoretic properties. Even though only one enzyme was used in this comparison, it would seem reasonably certain that the first hypothesis is not valid, i.e., the water relations of the sugar-tolerant yeast are not the result of a general peculiarity of its protein chemistry or enzymology. Furthermore, the salt relations of a halophil isocitrate dehydrogenase (1) and the effects of various nonelectrolytes on yeast isocitrate dehydrogenases (5; J. R. Simpson and A. D. Brown, manuscript in preparation) leave no doubt that the primary determinant of the apparent water requirements of at least these enzymes is not the amount of thermodynamically available water (a_w), but is instead the type and concentration of solute(s) which are present.

It was to be expected, therefore, that a fundamental difference exists in the intracellular composition of the two types of yeast. This paper, which describes some aspects of such a difference, gives the results of three sets of experiments. The first deals with the uptake of various nonelectrolytes by the two yeasts. The second describes the accumulation within the sugar-tolerant strains of one or more polyhydric alcohols. The third describes aspects of polyol release from a sugar-tolerant species. A preliminary account of some of the essential findings has been published (5).

MATERIALS AND METHODS

Organisms. Most of the experimental work used the sugar-tolerant yeast *S. rouxii* strain YA (2) and the nontolerant species *S. cerevisiae* strain Y41 (2). All the other strains designated by Brown and Simpson (5) were used in comparative studies.

Growth media. For experimental purposes, the yeasts were grown with rotary agitation (200 rpm) at 30 C in basal medium, basal medium supplemented with polyethylene glycol (molecular weight 200) to the required water activity, or synthetic honey broth (2) as specified. Unless stated otherwise, they were harvested in mid-exponential growth phase by centrifugation at 0 C and washed twice with water or a buffered solution.

Measurement of solute uptake. Solute uptake was estimated with ^{14}C-labeled compounds; unless indicated otherwise, the label was uniformly distributed. Radioactivity was estimated in a Nuclear-Chicago gas-flow counter. Suspensions of yeasts at a concentration of 11 to 17 mg(dry mass)/ml were incubated with several concentrations of nonelectrolytes of constant specific radioactivity. After incubation for 5 min, a sample (usually 1.0 ml) was withdrawn and centrifuged briefly at 7,700 $\times$ *g*; preliminary experiments on the rate of uptake had shown that the labeled solute reached its maximal intracellular concentration under these conditions. The supernatant fluid from the centrifugation was retained and, after suitable dilution, its radioactivity was estimated. The yeast pellet was washed twice in buffer containing unlabeled solute at the same concentration as in the reaction mixture and finally suspended in water to the same volume as the original sample. Portions (0.1 ml) of the final, washed suspension were spread on planchettes and the radioactivity was estimated. Within the range of yeast concentrations used, errors caused by self-absorption were negligible. Solute uptake was expressed relative to the mass of organisms washed twice with water in the centrifuge and dried at 85 C for 36 to 48 h. All statements of the mass of yeast refer to dry mass determined in this way. Radioactivity counts were normalized by adjusting them for the count of the buffer in which the yeasts were suspended.

Preparation of cell walls. Yeasts were harvested and washed with water at 0 C. The pellets were transferred to a stainless-steel press at the temperature of solid CO_2. The press, which disrupts frozen yeast in the manner of a Hughes press (6), is constructed differently and is stronger than a Hughes press. It was designed and made by D. W. Wynne and has been described in detail (J. C. Anand, Ph.D. thesis, Univ. of New South Wales, Kensington, New South Wales, Australia, 1969). The paste of disrupted yeast was diluted with water and subjected to alternate high- and low-speed centrifugations in the manner conventional for the isolation of microbial walls. The conditions actually used were (i) momentarily at 270 $\times$ *g* and (ii) 10 min at 27,000 $\times$ *g*.

Preparation of yeast extracts. Yeasts were harvested, washed twice in the centrifuge with water (30 ml; mass of yeast, 0.2 to 0.7 g) at 0 C, and freeze-dried. The dried yeast was extracted by either of the following two methods. (i) The yeast was suspended in ethanol (10 ml) at 0 C overnight. To the suspension was added cold water (about 15 ml); the slurry was maintained at 0 C for another 45 min, centrifuged, and washed twice in the centrifuge with water (10 ml) at 0 C. The extract and both washings were combined, concentrated, and freeze-dried. (ii) The freeze-dried yeast was suspended in aqueous trichloroacetic acid (5%, wt/vol; 20 ml) overnight at 0 C. The suspension was centrifuged and washed twice with water as above. The extract and washings were combined. Trichloroacetic acid was removed by extraction with diethyl ether; the residual aqueous solution was concentrated and freeze-dried.

Polyol release from S. rouxii. The rates of release of polyol from *S. rouxii* were measured in two types of experiment. In the first type, the suspension (5.0 ml) was shaken at 30 C in glass centrifuge tubes of about 1.3-cm internal diameter. At the stated times, tubes were removed from the water bath and transferred to a bench centrifuge with a capacity for 16 tubes. The samples were centrifuged for about 3 min at 1,000 to 1,500 $\times$ *g*. The supernatant fluid was immediately decanted and frozen until analyzed. (Polyol concentration decreased even in frozen samples over the course of several days. Polyol was always estimated within 18 h of collection; over this period the change in concentration was negligible.) In this type of

experiment, there was a time interval of 5 to 6 min between removing the sample from the water bath and decanting the supernatant fluid.

In the second type of experiment, the suspension (20 or 30 ml) was shaken in a 250-ml conical flask at 30 C. At the stated times, samples (2.0 ml) were collected with a pipette and rapidly filtered under pressure through membrane filters capable of retaining the yeast. The filters, which were mounted in groups of eight, discharged directly into test tubes. The samples were frozen immediately and stored until analyzed. In this type of experiment, the time interval between withdrawing the sample and completing the filtration was 30 to 40 s. For both types of experiment the reaction was started by adding a small volume of yeast suspension to the reaction mixture at 30 C.

Chemical analyses. Polyols were identified and estimated as described previously (5). The identity of arabitol was confirmed by gas chromatography of the acetylated polyol in a 10-ft (304.8-cm) column packed with ECNSS-M (1.5%, wt/wt) on 80- to 100-mesh Gas Chrom Z. When glucose was present in concentrations comparable to those of the polyol, the estimation of the latter was corrected for the color given by glucose in the reaction. This was roughly equal to the color equivalent of polyol at one-tenth the glucose concentration. There was neither synergistic reaction of glucose and polyol in the estimation nor inhibition by glucose of the reaction of the polyol. When glucose was present at very high concentrations, as in synthetic honey broth, the glucose was oxidized to gluconic acid with glucose oxidase and catalase. The gluconic acid was removed with an ion-exchange resin; a mixed-bed resin was used since this simultaneously removed buffer ions, etc. Glucose was estimated with glucose oxidase preparations (Boehringer and Worthington). All reagents were commercial reagent-grade products. All ^{14}C-labeled compounds were chromatographically homogeneous with respect to the label. The polyene antibiotic amphotericin B (methyl ester) was obtained through E. F. Gale from C. P. Schaffner of Rutgers University.

RESULTS

Solute uptake by the two types of yeast. Sucrose is frequently encountered at high concentrations in the habitats of sugar-tolerant yeasts and is, therefore, potentially important as a factor affecting their intracellular composition. Preliminary experiments on the rate of label uptake from a buffered, ^{14}C-labeled sucrose solution (0.1%, wt/vol; 0.02 μCi/ml) revealed active uptake by *S. cerevisiae* and apparent exclusion by *S. rouxii*. All the sugar-tolerant yeasts in our collection behaved indentically under these conditions. Similar results were obtained with *S. rouxii* suspended in buffered sucrose solution adjusted to 0.95 a_w with polyethylene glycol (molecular weight 200), when harvested in the stationary phase of growth and after growth in a medium adjusted with sucrose to 0.98 a_w.

Figure 1 shows, however, that the tolerant species, *S. rouxii*, does indeed equilibrate with extracellular sucrose, whereas the nontolerant species, *S. cerevisiae*, produced a regression which did not extrapolate through the origin and is characteristic of a metabolite. The difference between the two species is in fact largely a matter of sucrose metabolism by one of them. *S. cerevisiae* produced an invertase (488 to 706 nmol of sucrose hydrolyzed per min per mg of protein in crude cell-free preparations); *S. rouxii* did not. All the label was recovered as sucrose from *S. rouxii*, but it was recovered only as breakdown products from *S. cerevisiae*. With an assumed cell water content of 60% (i.e., 1.5 g of water per g of dry cell substance), the equilibrium sucrose concentration in *S. rouxii* (Fig. 1) is about 40% of the extracellular concentration. Under similar conditions, the isolated walls of the two yeasts behaved identically to each other and accumulated the isotope to about one-half of the concentration (wt/wt) reached in whole *S. rouxii*.

Lactose is not metabolized by either yeast and gave intracellular concentrations shown in Fig. 2. Under these conditions, the concentration within *S. cerevisiae* was about 70% of the extracellular concentration; in *S. rouxii* it was about 10%. Uptake of [1-^{14}C]lactose by isolated

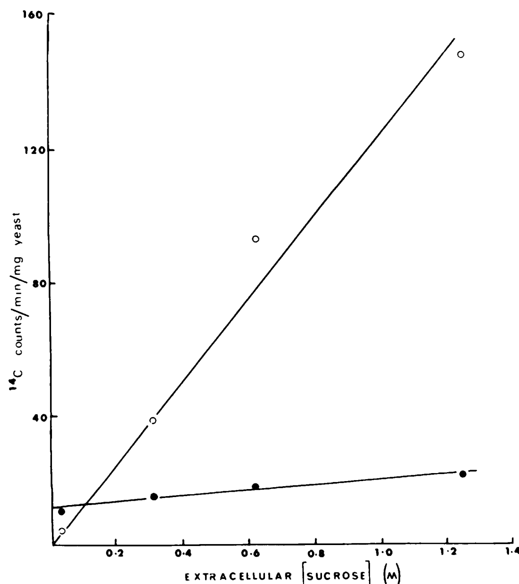

FIG. 1. *Extent of uptake of ^{14}C from solutions of [^{14}C]sucrose by Saccharomyces cerevisiae (●) and S. rouxii (○). The extracellular sucrose solutions had a specific activity of 0.534 mCi/mol. The intracellular sucrose concentrations in S. rouxii represented by this figure are about 40% of the corresponding extracellular concentration.*

walls was the same for both species. An assumed water content of 60% for the walls gave intra-wall concentrations equivalent to about 16% of that in the suspending solution.

The uptake of glycerol as a function of extracellular glycerol concentration is shown for both yeasts in Fig. 3. Again there was a substantial difference between the two species, with greater solute accumulation in *S. cerevisiae*. At the lower concentrations, where the regression was approximately linear, intracellular concentration in *S. cerevisiae* approximately equalled

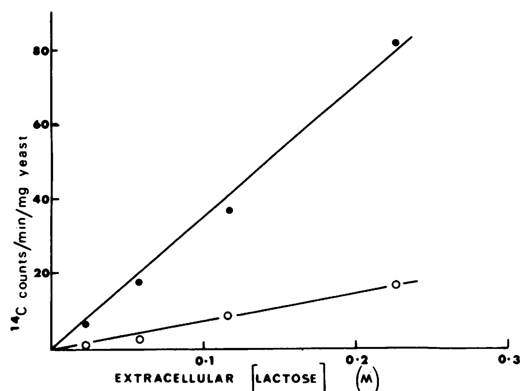

FIG. 2. *Extent of uptake of [^{14}C]lactose by Saccharomyces cerevisiae* (●) *and S. rouxii* (O). *The extracellular lactose had a specific activity of 0.573 mCi/mol. The intracellular lactose concentration of S. cerevisiae was about 70% and of S. rouxii about 10% of the extracellular concentration.*

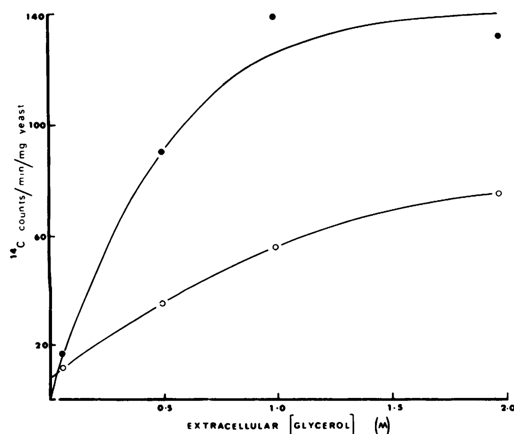

FIG. 3. *Extent of uptake of ^{14}C from solutions of [1-^{14}C]glycerol by Saccharomyces cerevisiae* (●) *and S. rouxii* (O). *The extracellular glycerol had a specific activity of 0.337 mCi/mol. At low glycerol concentrations where the regression is approximately linear, the intracellular glycerol concentration in S. cerevisiae was approximately equal to the extracellular concentration.*

extracellular concentration. The curve for *S. cerevisiae* extrapolated through the origin, but that for *S. rouxii* did not. This implies some degree of active glycerol uptake, presumably by metabolism, in *S. rouxii*.

Accumulation of ^{14}C as a function of extracellular glucose concentration was the same in each yeast and was typical of a metabolite. Maximal uptake was equivalent to 60 counts per min per mg for glucose with a specific activity of 0.48 mCi/mol.

Intracellular polyol accumulation. Chromatographic examination of yeast extracts revealed in *S. rouxii* but not in *S. cerevisiae* a large quantity of a substance eventually identified as a pentitol. As already reported (5), all the sugar-tolerant strains, but none of the nontolerant strains, contained a polyhydric alcohol as the major trichloroacetic acid-soluble constituent. In all but one strain the polyol was indistinguishable from arabitol; its identity as arabitol was confirmed by gas-liquid chromatography for strain YA. Gas chromatography of YA extracts also revealed traces of glycerol which were not detected on paper chromatograms. In strain YE (2) arabitol was the major constituent, but there were also smaller but significant quantities of glycerol and a hexitol (probably mannitol). In strain YO, which is slightly less sugar-tolerant than the others, the solute was a hexitol (probably mannitol).

The polyols extracted from freeze-dried yeasts were those remaining after the organisms had been washed twice in the centrifuge with water at 0 to 4 C. In two experiments in which extracts were dried and weighed, arabitol accounted for 38% of the material extracted from yeast grown in basal medium and for 45% of material extracted from yeast grown in synthetic honey broth. Table 1 lists the arabitol content of *S. rouxii* strain YA, the growth media, the washings, and the extracts. Less total polyol was produced in basal medium than in media adjusted to lower levels of water activity, but yeasts grown in basal medium retained a higher proportion of their intracellular polyol during the water washing. A wide range of apparent intracellular concentrations was deduced for organisms grown in synthetic honey broth. In the second of the two examples, the results were affected by significant carryover of arabitol from the culture medium into the first wash. The two preparations were also quite different in the amount of arabitol removed in the second wash and finally by trichloroacetic acid extraction. The table also shows that yeast grown in media adjusted with polyethylene glycol lost a high proportion of its

TABLE 1. *Quantities of arabitol in media and extracts of Saccharomyces rouxii strain YA*

Growth medium	Arabitol content (% [wt/wt] of yeast)[a]				Arabitol concn in medium (M)	Arabitol concn, intracellular (molal)[b]		
						After 2nd washing	Before washing[c]	
	Medium	1st wash	2nd wash	Trichloro-acetic acid extract			Whole yeast	Proto-plast[d]
Basal (a_w = 0.997)	74	0.95	0.07	9.8–14.1	0.002	0.8	0.4–0.7	0.6–0.9
Synthetic honey (48% glucose; a_w = 0.95)	(i)	8.8	2.7	15.6		0.7	1.3	1.9
	(ii) 5,100	145	0.7	9.3	0.1	0.4	6.8	9.7
Basal + PEG[e] (a_w = 0.95)	854	17.9	5.0	11.2	0.04	0.5	1.6	2.3
Basal + PEG (a_w = 0.91)		72	0.4	3.5		0.2	3.7	5.3

[a] Calculated on the mass of freeze-dried yeast after two washes with water and before extraction with trichloroacetic acid.
[b] Assuming a water content of 60% in basal medium and proportionately less in media of lower a_w.
[c] Calculated from the sum of the quantities extracted by washing and by trichloroacetic acid, assuming no contamination of the washings by residual medium.
[d] Corrected for an average wall volume of 30% of the yeast, assuming all polyol was within the protoplast.
[e] PEG, Polyethylene glycol.

polyol during washing; this was especially true of yeast harvested from a medium of 0.91 a_w.

The last column of Table 1, which gives an apparent concentration of arabitol within the yeast protoplast, was based on the assumption that intracellular polyol was in fact retained within the protoplast and not within the occluded wall water. For the purpose of this calculation, the contribution of the cell wall to the total volume was deduced from electron micrographs of sectioned yeast.

Polyol release from S. rouxii. Some factors affecting the retention of arabitol by *S. rouxii* were investigated. The yeasts were harvested as usual in the mid-exponential growth phase. In preliminary experiments, they were washed in the centrifuge at 0 C once in phosphate buffer (Na^+ plus K^+, 0.05 M, pH 6.98) and once in water and resuspended in water to a density which was usually within the range of 45 to 50 mg (dry weight) of yeast per ml. The yeast was incubated at a density of about 2 mg/ml for 30 min at 30 C in the following solutions: water, phosphate buffer (Na^+ plus K^+; 0.05 M; pH 5.75, 6.98, and 7.90), barbiturate buffer (0.05 M, pH 7.0), calcium chloride (10^{-1} and 10^{-3} M), and urea (0.5 and 0.05 M). All solutes slightly diminished polyol leakage from the yeast, and there was evidence of a small effect of pH in the phosphate buffer, polyol release becoming slightly less with the change from pH 5.75 to 7.90. Exploratory measurements with amphotericin in the same phosphate buffers showed, after 30 min, a minimal extracellular polyol concentration at pH 6.98; in those experiments, the yeast was washed twice in the centrifuge at 0 C with the buffer and finally resuspended at 0 C in the same buffer to a cell concentration which was usually within the range of 45 to 50 mg/ml. The rate of polyol leakage from *S. rouxii* at 30 C in this buffer with no other additions was equivalent to 2.83 ± 0.83 (standard error) μmol per min per g (dry per weight) of yeast for a suspension density in the vicinity of 2 mg/ml and an arabitol content in the yeast of 11 to 13% (wt/wt). (These experiments were done in the Department of Biochemistry, Cambridge, England. Arabitol contents were consistently within this range, which is narrower than that represented by the earlier results shown in Table 1 and significantly less than those reported previously (5). The reason for this last discrepancy is not known.)

Effects of amphotericin and glucose. The polyene antibiotic amphotericin B (methyl ester) accelerated polyol release in direct proportion to amphotericin concentration in the range of 2 to 8 μg/ml (Fig. 4). Under these circumstances, polyol leakage induced by amphotericin continued to equilibrium with the extracellular fluid.

Since energy metabolism is commonly associated with solute fluxes across cell membranes, effects of glucose on arabitol release were stud-

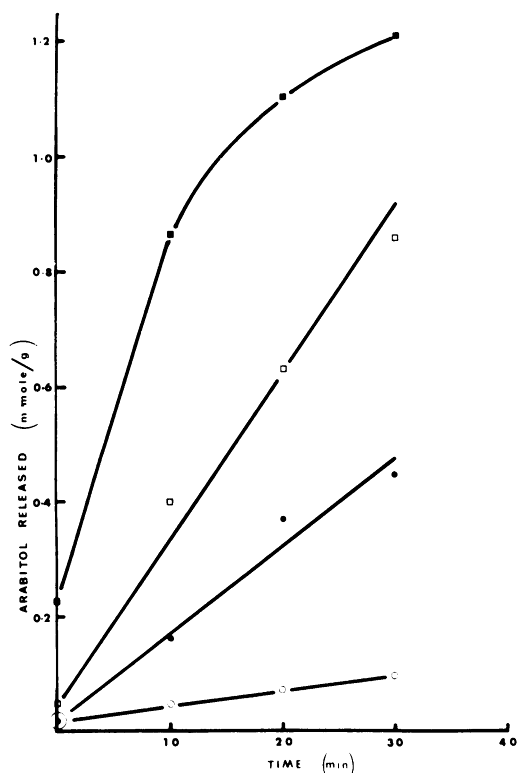

FIG. 4. *Effect of amphotericin B (methyl ester) on release of arabitol from Saccharomyces rouxii. The yeast was grown in basal yeast medium and, in the reaction mixture, was suspended in phosphate buffer (0.05 M, pH 6.98) at 30 C to a cell concentration of 1.44 mg/ml. The regressions from bottom to top were obtained, respectively, with amphotericin at concentrations of 0, 2, 4, and 8 μg/ml. They represent release of polyol at rates equivalent to 3, 15, 29, and 63 μmol per g of yeast per min.*

ied. The results of an experiment in which samples were collected by centrifugation are shown in Fig. 5. This figure shows the following. (i) Amphotericin had, at most, a minor effect on glucose consumption. (ii) Glucose induced a rapid early appearance of polyol in the extracellular fluid in both the presence and absence of amphotericin. The initial magnitude of the extracellular polyol concentration was much greater than could be explained by metabolic conversion of glucose to arabitol and must have been caused by release from the yeast. (iii) The high initial extracellular polyol concentration induced by glucose subsequently diminished. (iv) Glucose caused a slight increase in the rate of polyol release which occurred over 30 min in the presence of amphotericin. (The effect illustrated in Fig. 5 is small, but it was consistent throughout replicates of this experiment.) The excess of polyol in the amphotericin-plus-glucose supernatant over the amphotericin-without-glucose supernatant increased from 0.29 mM at zero time to 0.62 mM at 30 min. The increment in the excess, namely 0.33 mM polyol, was only slightly greater than the glucose consumption over the same period (equivalent to 0.31 mM) and could thus have been caused largely by fermentation of the glucose to polyol.

The results represented by Fig. 5, however, contained a time lag of 5 to 6 min before the supernatant fluid was separated from the yeast. An explanation of the apparently instantaneous glucose-induced release of polyol, therefore, required resolution of the first 5 or so min of the incubation period. This was done with filtered samples as described above. Results are shown in Fig. 6 and 7 for experiments in which the initial glucose concentrations were 1.0 and 0.1 mM, respectively.

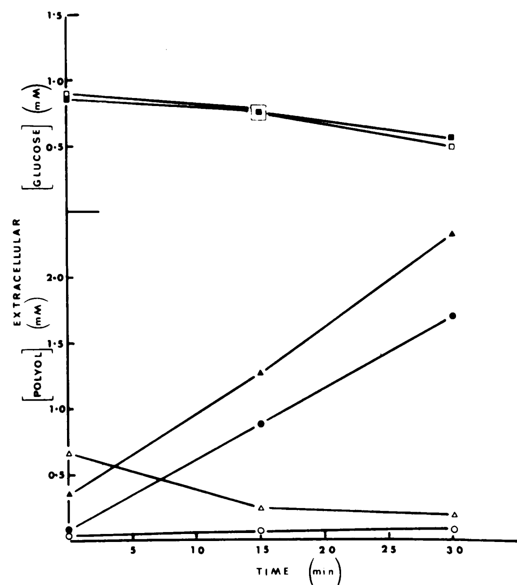

FIG. 5. *Effects of glucose on release of polyol from Saccharomyces rouxii in the presence and absence of amphotericin B (methyl ester) (4 μg/ml). The yeast was grown in basal medium and, in the reaction mixture, was suspended in phosphate buffer (0.05 M, pH 6.98) at 30 C to a concentration of 2.0 mg/ml. The upper panel shows the glucose concentration in the extracellular fluid (□, suspension without amphotericin; ■, suspension with amphotericin). The lower panel shows the polyol concentration in the extracellular fluid (○, suspension in buffer; △, suspension in buffer plus 1 mM glucose; ●, suspension plus amphotericin; ▲, suspension plus amphotericin plus glucose). Sampling times depicted in this figure denote time of removal of the suspension from the water bath. The extracellular fluid was separated by centrifugation, and final decantation occurred 5 to 6 min after the stated time (see text).*

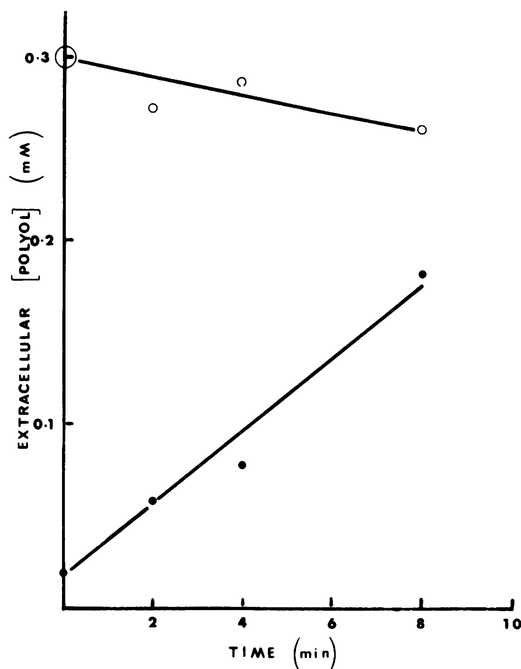

FIG. 6. *Effect of glucose on release of polyol from Saccharomyces rouxii. The yeast was grown in basal medium and, in the reaction mixture, was suspended in phosphate buffer (0.05 M, pH 6.98) with or without glucose (1.0 mM) at 30 C to a concentration of 3.4 mg/ml. The upper regression (O) represents the change in extracellular polyol concentration in the presence of glucose. The glucose concentration dropped from 1.0 to 0.9 mM during the 8 min. The lower regression (●) shows the change in extracellular polyol concentration in the absence of added glucose. The extracellular fluid was collected by filtration which was completed 30 to 40 s after the times depicted in this figure (compare Fig. 5 and see text).*

Under both conditions, glucose stimulated a very rapid release of polyol from the yeast, the magnitude and hence the apparent rate being much greater with the higher concentration of the sugar. At zero time the excess polyol concentration over that in the control suspension was 83 μmol of polyol per g of yeast for 1.0 mM glucose (Fig. 6) and about 10 μmol of polyol per g of yeast for 0.1 mM glucose (Fig. 7). Furthermore, at the higher glucose concentration, polyol was released so rapidly that the process was effectively complete within the sampling time (30 to 40 s); the experiment measured only the subsequent disappearance of polyol. At the lower glucose concentration, most of the extracellular polyol appeared within the zero sampling time, but its concentration continued to increase at a rate which initially paralleled that of the control suspension. After about 10 min, when the apparent rate of glucose metabolism was increasing, the rate of polyol release leveled off.

DISCUSSION

The intracellular compositions of the two types of yeast were substantially different under all experimental conditions and can reasonably be assumed to be so under most conditions. The differences are consistent with the water relations of the yeasts and are attributable partly to peculiarities of nonelectrolyte uptake and largely to the high intracellular polyol concentrations in the sugar-tolerant species.

Different factors contributed in each case to the response of the two yeasts to the four extracellular nonelectrolytes. Inability to metabolize sucrose was the major determinant distinguishing the response of the sugar-tolerant species from that of *S. cerevisiae*. Lactose, which is not metabolized by either species, accumulated to a much greater extent in *S. cerevisiae* than in *S. rouxii*. The small uptake by the latter species might be wholly attributable to diffusion of lactose into cell wall water, implying total exclusion from the protoplast.

Glycerol also produced different effects, because it accumulated to a greater extent in the non-sugar-tolerant species. Glycerol, of course, is a metabolite for most yeasts. The shape of the uptake curve for *S. cerevisiae* (Fig. 3) shows, however, that metabolism was of negligible importance in glycerol accumulation by that yeast. For all practical purposes this also represents an expression of the capacity of the yeast for the solute. In *S. rouxii* an active process (presumably metabolism) was significant, but

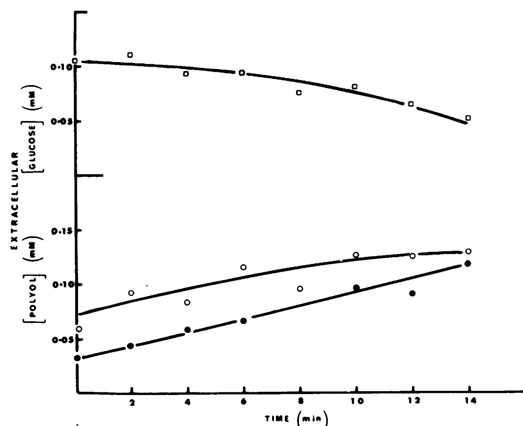

FIG. 7. *As for Fig. 6 except for the initial glucose concentration, which was 0.1 mM. The yeast concentration was 2.3 mg/ml. Symbols: O, extracellular polyol concentration in the presence of glucose; ●, extracellular polyol concentration in the absence of added glucose; □, extracellular glucose concentration.*

of minor proportions, in relation to the total glycerol uptake, which was also much less than in *S. cerevisiae*. (The evidence of significant glycerol metabolism in *S. rouxii* is consistent with the presence in that species of an NADP-specific polyol dehydrogenase; J. R. Simpson, unpublished results).

The essentially identical steady-state levels of isotope accumulation by the two yeasts exposed to glucose solutions in themselves give no indication of the differences in composition which can result from metabolism of this sugar. The metabolic end products are different; arabitol accumulates in one species but not the other and, moreover, there is a rapid release of preformed arabitol which occurs on first contact of *S. rouxii* with glucose.

It can also be assumed, for thermodynamic reasons which will be discussed elsewhere, that the arabitol content of *S. rouxii* lowered its capacity for nonelectrolytes in general, and this was reflected in its response to glycerol and possibly also to lactose.

The production of polyhydric alcohols by yeasts and molds is well known and well documented (8, 11). Spencer (11) has reported at length the effects of growth conditions on yields (extracellular) of various polyols given by many species of osmophilic yeasts. It is not the purpose of this paper to discuss the results in that context. What does not seem to have been recognized, however, is the high intracellular concentration achieved and the significance of accumulated polyols in the water relations of the microorganisms which synthesize them.

The results in Table 1 are of a preliminary nature and intended primarily to show trends. Factors affecting polyol accumulation are currently under systematic investigation. Some of the variability in Table 1 was a result of washing the yeast before analysis. Washing the yeast could not be avoided because of the accumulation of arabitol in some growth media, especially synthetic honey broth. This medium, which was highly viscous, gave significant carryover of growth medium into the first wash; this was especially true of the second of the two examples quoted. Nevertheless, some tentative conclusions are possible. The first is that the amount of polyol extracted from washed yeast did not vary greatly with growth conditions, except for incubation at 0.91 a_w in the presence of polyethylene glycol. Under those conditions, there was polyol production but little or no cell division during the incubation. It is likely that the yeast had autolyzed to some extent.

On the other hand, the total arabitol production did vary greatly with growth conditions; the evidence suggests that a high extracellular solute concentration (low a_w) was associated with a high intracellular polyol concentration. This is to be expected. Marine and halophilic species of the unicellular alga *Dunaliella* accumulate glycerol in direct response to extracellular salt concentration (3a). The relative uniformity in polyol content of the washed yeast was thus a consequence of widely varying degrees of polyol loss during the wash with water. Much more polyol was lost in washing yeast which had been grown at a low a_w than from yeast grown in basal medium. This difference persisted to the second wash, where contamination by the growth medium had been reduced to negligible proportions. Whereas the two examples cited for synthetic honey broth experiment illustrate the range of variability encountered in yeast grown in this medium, incubation in polyethylene glycol at 0.91 a_w gave an organism notably different in its physiology from those grown under the other conditions listed in Table 1. Autolysis was likely under these conditions, and permeability changes can reasonably be assumed to explain the massive loss of polyol during the first wash. The results for other growth conditions suggest that an arabitol content in the range of 9 to 15% is to be expected when the yeast is in a relatively stable condition in water or dilute buffer. This is the level of concentration associated with a leakage rate of 2.8 μmol per min per g in phosphate buffer at 30 C (see above).

High concentrations of polyols have been reported in other types of microorganisms. Thus, Lewis and Smith (8) have cited reports of threitol occurring in the mycelium of the fungus *Armillaria mellea* in amounts of up to 13% of the dry mycelium. Other polyols were reported to reach up to 20% of some fungal mycelia; polyol contents of up to 10% have been encountered in lichens. In all these cases, the polyol can be assumed to have been predominantly intracellular, a situation which is perhaps less noteworthy in a filamentous organism with a large aerial component than in a submerged unicellular microorganism.

The factors that affect polyol retention at such a concentration are of interest since arabitol must be retained in order to function as a compatible solute (5). Arabitol is capable of rapid diffusion across the yeast membrane, however, as shown by the extent of its release during washing (Table 1). The experiments with amphotericin (Fig. 4) leave little doubt that membrane integrity is a major requirement for retaining arabitol. The polyene antibiotics are known to impair membrane permeability of susceptible organisms, apparently by complexing with membrane sterols (7, 9). One of the

earliest detectable consequences of the action of amphotericin B (methyl ester) on the yeast *Candida albicans* is the release of K^+. This occurs at rates that are proportional to amphotericin concentration and are of similar magnitude to those reported above for polyol release (E. F. Gale, personal communication). It appears likely, therefore, that the early leakage caused by amphotericin is nonspecific, at least for solutes of low to moderate molecular weight.

There are reasons for suspecting, however, that factors in addition to membrane permeability are involved in arabitol retention by the yeast. The remarkably rapid ejection of polyol that follows contact of the yeast with an energy source (glucose) is not easy to reconcile with a process which is located exclusively in the membrane, notwithstanding any mechanisms such as "pumps" which might be invoked. The rate of polyol loss under these circumstances was clearly many times greater than that resulting from membrane damage caused by amphotericin (Fig. 4 and 6). Contact of some microorganisms with an energy source such as glucose causes an immediate and very rapid production of heat, the rate of heat evolution having a time scale similar to that implied above for the rapid efflux of polyol (3; W. W. Forrest and R. L. Berger, personal communication). The initial rapid heat evolution can be accompanied by an uptake of K^+ (12) but is not necessarily tied to it (W. W. Forrest and R. L. Berger, personal communication). The present results suggest that, in addition to whatever effect they might have on membrane properties, the processes causing rapid heat evolution might effect polyol retention through a change in an essentially cytoplasmic property such as the effective activity coefficient of the solute (arabitol) or solvent water. In the light of these observations, thermodynamic studies of solute retention and fluxes in microorganisms are warranted.

Although the polyol must contribute to the osmotic status of the yeast, its major physiological role in the yeasts' water relations is evidently that of a compatible solute (5). Compatible solutes function by being very poor enzyme inhibitors and, for this reason, protect enzymes against inhibition or inactivation which would otherwise occur at biologically low levels of water activity. This function of polyols in relation to yeast isocitrate dehydrogenase has been described briefly by Brown and Simpson (5) and will be discussed in detail for a series of nonelectrolytes by Simpson and Brown (manuscript in preparation). Potassium ions or salts have a similar role in halophilic bacteria (1), as has glycerol in halophilic algae (3a).

One may conclude at this stage that there is no evidence that the different water relations of sugar-tolerant and nontolerant yeasts are a result of generalized differences in water relations of their enzymes. On the other hand, there are considerable systematic differences in the intracellular composition of the two types of yeast. These differences are reflected partly in patterns of uptake of nonelectrolytes but largely in the occurrence at a high concentration of a polyol, usually arabitol, in the sugar-tolerant species.

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