LOCALIZATION OF SUCROSE AND MALTOSE FERMENTING SYSTEMS IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Isolated cell walls of bakers' yeast (*Saccharomyces cerevisiae*, strain LK2G12) contain large amounts of invertase (β-fructosidase). Maltase (α-glucosidase) could not be detected in these preparations. Glucose and sucrose were fermented by cells grown on glucose, sucrose or maltose. Enzymic removal of the cell wall was paralleled by the release of soluble invertase and produced protoplasts which had lost the ability to ferment sucrose but not glucose. These protoplasts excreted substantial quantities of a soluble invertase during incubation with sucrose or glucose and the snail enzyme preparation. Maltose was fermented only by cells grown on this sugar. Protoplasts from these cells retained the ability to ferment maltose and released α-glucosidase upon lysis. Only small amounts of α-glucosidase were detectable in cells raised in a synthetic medium containing glucose as the only sugar. If the medium contained glucose or sucrose and low levels of maltose, the resulting cells did not ferment maltose nor did the derived protoplasts. Lysis of these protoplasts released substantial amounts of α-glucosidase. These results provide additional evidence that the major portion of the invertase is in the cell wall; in strain LK2G12 the rest is intracellular and not readily accessible to sucrose. Maltase is apparently only intracellular and becomes accessible to maltose through the action of an inducible system in the membrane.

INTRODUCTION

It is generally accepted that the first step in fermentation of sucrose by bakers' yeast is hydrolysis to form fructose and glucose[1]. The rate generally exceeds that for the subsequent fermentation and leads to extracellular accumulation of the hexoses.

Indirect evidence has been presented for a peripheral localization of invertase in *Saccharomyces cerevisiae*[2,3]. More direct evidence has recently been obtained through the use of digestive enzymes of the snail *Helix pomatia* to remove the yeast cell wall. EDDY AND WILLIAMSON[4] prepared protoplasts of *Saccharomyces carlsbergensis* and showed that growing protoplasts formed fragile aberrant walls which were devoid of invertase[5].

FRIIS AND OTTOLENGHI[6] working with a hybrid of *Saccharomyces* species observed

Abbreviation: TPP, thiamine pyrophosphate.

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that the invertase of cells grown on glucose was exclusively intracellular and was released upon lysis of protoplasts. With sucrose as the carbohydrate source, organisms were obtained from which about 75% of the external invertase could be removed with snail enzyme. The resulting protoplasts retained some activity in fermenting sucrose and substantial amounts of invertase were released upon lysis. With other strains of bakers' and brewers' yeast, BURGER, BACON AND BACON[7] concluded that invertase was present in a soluble form external to the cell membrane but in some manner retained by the cell wall. Treatment of the cells with snail enzyme released essentially all of the invertase even when 0.2 *M* lactose was present to prevent lysis of the protoplasts. These investigators did not observe synthesis of invertase during the removal of the cell wall by the snail enzyme.

SUTTON, MARINI AND LAMPEN[8] have reported briefly that formation of protoplasts from a strain of *S. cerevisiae* was accompanied by the loss of ability to ferment sucrose, although the intracellular sucrose-cleaving activity was retained. The present communication demonstrates that lysates of protoplasts contain two enzymes capable of hydrolyzing sucrose: invertase (β-fructosidase) and, under certain conditions, α-glucosidase. The cellular localization pattern and accessibility of the two enzymes have been compared.

MATERIALS AND METHODS

Preparation of cells and protoplasts

Log phase cells of *Saccharomyces cerevisiae*, strain LK2G12 were grown on Wickerham's peptone–yeast extract–malt extract medium as described by MARINI, ARNOW AND LAMPEN[9]. The carbohydrate was glucose, unless otherwise stated.

Walls were prepared by rupturing log phase cells with glass beads[10]. Eight millilitres of packed cells and 15 ml of beads, suspended in 15 ml of 0.05 *M* potassium phosphate buffer pH 6.8 with 0.2 mg of 2,3-dimercaptopropanol were blended intermittently at maximum speed in a Lourdes Multimixer for a cumulative treatment of 12 min. By immersing the cup in a beaker of salt and ice the temperature inside the cup was maintained below 10° during treatment. Beads and some cells were removed by centrifugation for 5 min at 800 $\times$ *g*; walls were packed in 10 min at 1500 $\times$ *g*. The crude homogenates contained less than 10% whole cells, most of which appeared to be damaged. Three centrifugation cycles between 800 and 1500 $\times$ *g* (the bottom fourth of each sediment at 1500 $\times$ *g* was discarded) yielded a preparation containing a preponderance of empty walls. The fraction did not catalyze CO_2 production or the disappearance of glucose, nor did it appear to contain undamaged cells, although 2–5% had not suffered complete rupture. Further purification of small quantities of cell walls was achieved by centrifugation in a density gradient between 10 and 25% sorbitol.

For preparation of protoplasts, log phase cells from 100 ml of culture were suspended in 10 ml of McIlvaine's sodium citrate–phosphate buffer[11] at pH 5.8 containing 0.8 *M* mannitol. After addition of 0.1 volume of snail enzyme the cells were incubated at 28°. Protoplast formation was usually complete in 7 h although 12 to 14 h incubation was used in these studies. The yield of protoplasts was 60%. This probably is a minimum value since buds were counted as individual cells and some of these may have coalesced following removal of the cell wall. The suspension was centrifuged for 5 min at 800 $\times$ *g*. For some experiments the supernatant fluid, containing materials solubilized during protoplast formation, was clarified by centrifugation at 20000 $\times$ *g*

for 15 min. The protoplasts were washed in three changes of buffered mannitol before use. The final yield was 40%. Lysates were made by suspending protoplasts in water or buffer without mannitol.

Other materials

The snail enzyme (Suc digestif d'*Helix pomatia* stabilisé, from L'industrie Biologique Française, 35–49 Quai du Moulin de Cage, Gennevilliers, Seine, France) was clarified by 15 min centrifugation at 20000 × *g*. One-tenth volume of 1% cysteine hydrochloride solution was added to inactivate the merthiolate present as a preservative.

ATP, DPN, TPP, and glucose oxidase were purchased from Sigma Chemical Co., St. Louis, Mo. A highly purified preparation of nystatin (Lot HV942) was generously furnished by the Squibb Institute for Medical Research, New Brunswick, N.J.

Analytical methods

Conventional manometric techniques were employed to measure anaerobic fermentation of sugars. Glucose was determined manometrically by oxidation with glucose oxidase in the presence of catalase and ethanol[12]. Reducing sugar was estimated by the colorimetric method of NELSON[13]. Invertase activity was measured in the presence of α-glucosidase by the formation of reducing sugar from sucrose at pH 4.7, where α-glucosidase is inactive (see text), or by hydrolysis of the specific substrate raffinose. Activity of α-glucosidase was determined by cleavage of maltose or melezitose at pH 6.6, the optimum for α-glucosidase (see ref. 14 and Fig. 3).

RESULTS

Metabolic activities of cell walls and non-glycolyzing cells

The wall fraction contained a substantial amount of invertase activity of which only a part was retained during purification. For example one fraction split 34 μmoles of sucrose/h/mg dry wt. at the optimum pH of 4.7. K_m of the reaction was 0.02 *M*. Centrifugation in a density gradient achieved marked purification of the cell walls (as observed visually), but there was a decrease in the specific activity to 14.5 μmoles/h/mg. The invertase activity of several batches of intact cells ranged from 14 to 35 μmoles/h/mg (Tables II and IV), thus the wall fractions did not represent any definite

TABLE I

GLYCOLYSIS AND INVERTASE ACTIVITY IN PRESENCE OF NYSTATIN

pH	*Nystatin (μg/ml)*	*μmoles product from 30 μmoles sucrose**	
		Carbon dioxide	*Glucose*
5.8	30	< 1	26
5.8	3	3	17
4.0	3	0	25

* Each flask contained $1 \cdot 10^8$ cells, 1.5 ml sodium citrate–phosphate buffer, sucrose and nystatin as indicated, and water to 2.0 ml. After 60 min incubation under nitrogen, flasks were flushed with air and glucose oxidase added from a sidearm for measurement of the glucose formed.

purification of the enzyme. Because of this low activity and the inherent difficulties of achieving quantitative separation of fractions from broken yeast, other approaches were used to study distribution of disaccharidases between parts of the yeast cell.

The antifungal antibiotic nystatin inhibits fermentation of glucose, sucrose and fructose by log-phase cells[9]. In contrast, the invertase of the wall fractions and the soluble invertase released upon lysis of protoplasts were insensitive to nystatin at levels as high as 50 μg/ml. Because of this differential sensitivity, invertase in intact cells could conveniently be measured by adding nystatin to prevent removal of the hexose formed through sucrose cleavage (Table I). At pH 5.8, glycolysis was blocked completely by 30 μg/ml of nystatin, whereas invertase activity was unimpaired. With 3 μg of nystatin/ml glycolysis was inhibited only after a short lag. The decreased accumulation of free glucose in this experiment probably reflects the formation of fermentation intermediates which is known to occur under these conditions[15]. At pH 4, a level of 3 μg/ml nystatin was sufficient to block glycolysis completely. Again invertase was not inhibited. It should be noted that the rate of hexose formation from sucrose by strain LK2G12 under our conditions exceeds the rate of hexose fermentation by uninhibited cells. Thus, invertase activity does not appear to be the factor limiting the rate of utilization of sucrose.

Since sucrose cleavage could be achieved by α-glucosidase as well as by invertase, the substrate specificities of nystatin-inhibited cells and of wall fractions (from untreated cells) were examined (Table II). The presence of β-fructosidase was demonstrated by the hydrolysis of raffinose and of sucrose at pH 4.7. Neither maltose nor melezitose was cleaved whether walls of cells grown on glucose were used (Table II) or those from cells grown on maltose. It is of special interest that cells grown on maltose and capable of fermenting this sugar without lag failed to form significant quantities of glucose from maltose in the presence of 30 μg/ml of nystatin.

Effect of removal of the cell wall on metabolic activities

Log phase cells of *S. cerevisiae* LK2G12 grown on glucose, sucrose, or maltose, fermented glucose, fructose, and sucrose at about equal rates. Only cells grown on or adapted to maltose fermented this disaccharide.

TABLE II

SUBSTRATE SPECIFICITY OF WALL FRACTION AND NYSTATIN-TREATED CELLS

Two milligrams of wall fraction were incubated with 30 μmoles of substrate in 1.3 ml citrate-phosphate buffer for 120 min. Two milligrams of cells were treated with 15 μg nystatin and incubated 15–60 min in 3 ml buffer with 90 μmoles substrate. Since maltose is a reducing sugar, the criterion of its cleavage was the formation of glucose as measured by glucose oxidase.

Substrate	*pH*	*Reducing sugar formed (μmoles/h/mg)*	
		Walls	*Nystatin-treated cells*
Sucrose	6.6	10.0*	14.2*
Sucrose	4.7	12.4*	24.3*
Raffinose	6.6	2.2	2.1
Raffinose	4.7	2.7	3.1
Maltose	6.6	$<$ 0.3	$<$ 1.0
Melezitose	6.6	$<$ 0.01	$<$ 0.1

* Sucrose cleavage had proceeded too far to represent the maximum rate.

Removal of the cell wall had a relatively minor effect on the rate of glycolysis of hexoses (Table III). Further, in most experiments, the loss of the wall did not significantly influence fermentation of maltose by cells grown on maltose. Maltase occasionally became cryptic during this procedure. In such cases the addition of a small amount of glucose initiated maltose fermentation[14]. Removal of the yeast cell wall was accompanied, however, by an almost total loss of the ability to ferment sucrose. Table III shows the lack of activity on sucrose after maltose-grown cells were treated with snail enzyme; similar results were obtained with cells grown on sucrose or glucose.

Sucrose fermentation could be restored by combining a suspension of protoplasts with a cell wall fraction prepared from broken yeast (Fig. 1A). The wall fraction alone did not ferment glucose or sucrose. Removal of the cell wall by snail enzyme during protoplast formation released invertase in a form which was not sedimented in 20 min at 20000 $\times$ g. Addition of the solubilized invertase to washed protoplasts also reconstituted a system capable of fermenting sucrose (Fig. 1B). The volume of supernatant was that in which the protoplasts had been made, in effect restoring their original wall material in soluble form. Sufficient invertase was present in the supernatant that the rate of CO_2 production from sucrose equalled the rate obtained with the constituent hexoses.

It is evident from Fig. 1B that the snail enzyme preparation contained some sucrose-splitting activity, but the amount was less than 15% of that released from the yeast cells during protoplast formation. Since the preparation was almost inactive at pH 4.7 and split melezitose but not raffinose, it appears to contain α-glucosidase rather than invertase.

Glycolytic and saccharidase activities of protoplast lysates

The protoplasts lysed readily on suspension in hypotonic media. Glycolysis by lysates required ATP, DPN, TPP, Mg^{2+}, K^+ or NH_4^+, and an oxidant (pyruvate was generally used) for maximum activity.

Protoplasts from cells grown on the Wickerham medium with glucose or sucrose fermented sucrose and maltose very slowly (some maltose is present in the malt extract). Lysed protoplasts supplemented with the necessary cofactors, fermented sucrose and maltose as rapidly as glucose and fructose (Fig. 2). Addition of pyruvate and the cofactors did not initiate fermentation of disaccharides by intact protoplasts, nor did protoplasts decarboxylate pyruvate under these conditions. The initial incubation of intact protoplasts with disaccharides (as in the experiment of Fig. 2) was not essential for the subsequent fermentation of sucrose or maltose by their lysates.

TABLE III

FERMENTATIONS BY LOG-PHASE CELLS AND BY PROTOPLASTS

The sugars (60 μM) were incubated under nitrogen with $1 \cdot 10^8$ protoplasts or cells in 3.0 ml buffered mannitol, pH 5.8.

Substrate	*CO_2 production ($\mu l/h/10^8$)*	
	Log-phase cells	*Protoplasts*
Glucose	675	502
Sucrose	675	0
Maltose	671	575

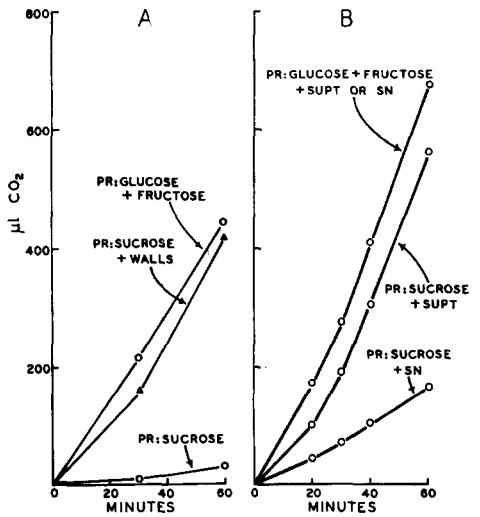

Fig. 1. Reconstitution of sucrose fermentation. A. Each vessel contained $8 \cdot 10^7$ protoplasts (PR) in a final volume of 2 ml 0.03 *M* sodium citrate–phosphate buffer, pH 7.0, 0.8 *M* in mannitol. The flask indicated also received 2 mg of cell wall fraction (WALLS). After equilibration under nitrogen, 30 µmoles of the substrates were tipped in. B. Protoplasts ($1 \cdot 10^8$) were tested for fermentation of sucrose and hexoses as in A. The flasks indicated also contained 0.3 ml supernatant from protoplast formation (SUPT) representing the solubilized material of 10^8 protoplasts, or the equivalent amount of snail enzyme (SN).

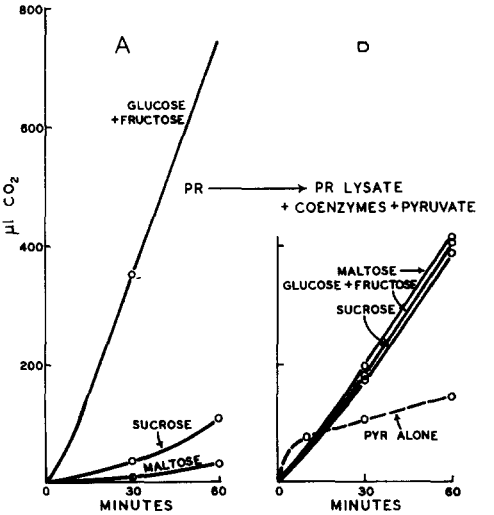

Fig. 2. Glycolytic activity of protoplasts and lysates. A. Intact protoplasts (PR) ($1 \cdot 10^8$) were incubated under nitrogen in 1.2 ml of 0.03 *M* citrate–phosphate buffer pH 7.0 containing 0.8 *M* mannitol and 0.02 *M* $MgCl_2$. The substrates (30 µmoles in 0.1 ml) were tipped in at zero time. B. After 60 min the protoplasts were packed by centrifugation (1500 × *g*) and lysed by suspension in 1.2 ml 0.03 *M* citrate–phosphate, 0.02 *M* $MgCl_2$. After equilibration under nitrogen, the cofactors, pyruvate, and substrates (as in A) were added. The amounts were, in µmoles: 30 sugar, 5 pyruvate (PYR), 5 ATP, 2.5 DPN, 2.5 TPP and 7.5 NH_4Cl; the final volume was 1.5 ml. One lysate from protoplasts preincubated in glucose and fructose contained all additions but sugar (PYR alone).

The protoplast lysates contain both α-glucosidase and β-fructosidase as was shown by the cleavage of melezitose and of raffinose (Fig. 3). Cells grown on a defined medium with glucose as the only carbohydrate contain relatively low levels of α-glucosidase. There was a high level of cryptic α-glucosidase in cells raised on the Wickerham medium, probably as a result of the presence of small amounts of α-glucosides in this crude medium. It is assumed that the pH activity curve of intracellular invertase on sucrose is similar to that on raffinose (Fig. 3) or to that determined for the enzyme in the walls. One can then estimate that invertase contributed approx. 60% and α-glucosidase approx. 40% of the sucrose-splitting activity at pH 6.6 with the protoplasts used in most of this work.

Distribution of invertase

Invertase activity was determined on four preparations of glucose-grown cells, and on the solubilized material and protoplasts derived from them (Table IV). Although the intact protoplasts manifested less than 5% of the sucrose-fermenting ability of the cells (Table III), 10 to 30% of the total invertase was found to be contained within the protoplasts. Most of the intracellular invertase released by lysis

remained in the supernatant fluid after 15 min centrifugation at 20000 × *g*. Particles the size of mitochondria and remnants of the membrane were visible after lysis; these sedimentable structures were low in invertase activity.

Synthesis of invertase

The invertase activity solubilized during the conversion of cells to protoplasts was consistently greater than the invertase activity of the original cells (Table IV). Addition of snail enzyme did not increase the apparent invertase activity of nystatin-treated cells, hence the increased activity appears to represent a true synthesis.

Evidence that washed protoplasts synthesize invertase during incubation with the snail enzyme is given in Table V. The quantity excreted during a 2-h incubation

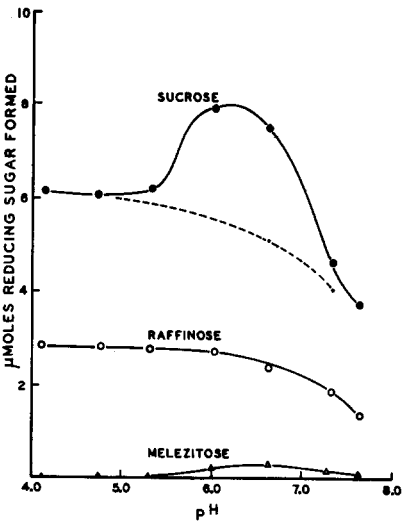

Fig. 3. Effect of pH on invertase and α-glucosidase activity of protoplast lysates. Lysates were incubated in 1.5 ml 0.03 *M* citrate–phosphate buffer for 60 min with 30 μmoles sucrose, or for 150 min with 30 μmoles raffinose or melezitose. The broken line indicates the expected cleavage of sucrose by invertase alone (see text).

TABLE IV

DISTRIBUTION OF INVERTASE IN CELLS AND PROTOPLASTS

$7 \cdot 10^7$ cells (glycolysis blocked with 30 μg/ml nystatin), $7 \cdot 10^7$ protoplasts (lysed upon suspension in the buffer), the supernatant in which $7 \cdot 10^7$ cells had been converted to protoplasts, or an equal volume of 10% snail enzyme, were incubated with 30 μmoles sucrose in 3 ml 0.05 *M* citrate–phosphate buffer pH 4.7. Similar preparations were incubated with 30 μmoles raffinose in pH 6.6 buffer. Reducing sugar was determined after 20 to 100 min. Preparations incubated without sucrose had negligible reducing values. Each column presents data for an individual batch of cells.

Invertase preparation	*Reducing sugars formed (μmoles/h)*			
	From sucrose		*From raffinose*	
Cells (nystatin-treated)	47.6	26.1	6.4	7.2
Protoplasts (lysed)	6.8	8.7	1.8	0.8
Supernatant from protoplast formation	66.8	38.4	12.1	—
Snail enzyme	4.7	4.9	0.7	—

TABLE V

BIOSYNTHESIS OF INVERTASE BY PROTOPLASTS

The incubation mixtures contained $3 \cdot 10^8$ protoplasts and 30 μmoles of sugar where indicated in 1.1 ml of pH 5.8 sodium citrate–phosphate buffer with 0.82 *M* mannitol and 10% (by volume) of the snail enzyme. After 2 h incubation the protoplasts were separated from their supernatants by centrifugation and lysed by suspension in water. For assay of invertase, samples of supernatant and lysate were incubated 45 min in the absence or presence of 90 μmoles sucrose in 3.0 ml 0.03 *M* sodium citrate–phosphate buffer pH 4.8. Activity is expressed as μmoles reducing sugar formed/h. Values are corrected for the sucrose-cleaving activity present in the snail enzyme and for the reducing sugar formed in the absence of sucrose.

Sugar	*Temperature*	*Invertase per 10^8 protoplasts*	
		Protoplast lysates	*Supernatants*
None	4°	9.4	1.0
None	28°	8.8	1.8
Sucrose	28°	13.2	59.4
Maltose	28°	12.0	32.4

TABLE VI

REQUIREMENT OF SNAIL ENZYME FOR INVERTASE FORMATION

Experimental procedure as in Table V. Snail enzyme was omitted from certain mixtures and only the excreted invertase was determined.

Sugar	*Snail enzyme*	*Invertase activity excreted per 10^8 protoplasts (μmoles reducing sugar/h)*
None	—	1.5
Sucrose	—	3.4
None	+	1.8
Sucrose	+	36.0
Glucose	+	20.3

with snail enzyme and sucrose was approximately 7 × that originally present in the protoplasts. When sucrose was replaced by maltose or by glucose the quantity excreted was somewhat less. Invertase-forming activity was eliminated by lysis of the protoplast preparation. There was relatively little increase in intracellular invertase as measured in lysates of the sedimented protoplasts.

The snail enzyme is essential for invertase biosynthesis under these test conditions (Table VI). There was no significant increase in invertase if the snail enzyme was boiled or was replaced by a casein hydrolysate (40 mg/ml) or if 10 μg of nystatin/ml was present together with the snail enzyme.

DISCUSSION

The results obtained with *S. cerevisiae* LK2G12 are in agreement with those obtained by FRIIS AND OTTOLENGHI[6] and by BURGER *et al.*[7] with related yeasts in that the major portion of the invertase was found to be located in the cell wall structure. In contrast to the organism used by FRIIS AND OTTOLENGHI, which did not contain external invertase after growth on glucose, most of the invertase of our yeast was in the wall

under these conditions. Like their organism and in contrast with that of BURGER *et al.*, 10 to 30% of the total invertase was intracellular and not accessible to external substrate. Also, significant quantities of invertase were synthesized and released into the medium under our conditions of protoplast preparation. The lack of internal invertase in the protoplasts prepared by BURGER *et al.* is surprising and may result from the use of 0.2 *M* lactose as the osmotic stabilizer, a concentration which we have found suboptimal for maintenance of our protoplasts. It should be noted that it has been possible to remove external invertase completely from our protoplasts, that is, they do not ferment sucrose during an interval of at least three hours in the presence of purified sucrose[16] (free of glucose).

While the function of cell wall invertase in sucrose fermentation is well defined, that of intracellular invertase is not clear. A role in synthesis of cellular β-fructosides is not excluded, though such substances have not been found in bakers' yeast. A more likely hypothesis is that the internal invertase represents the enzyme at its site of formation. The invertase released from protoplasts by lysis and that released during removal of cell walls by snail enzyme are both soluble at 20000 × *g* and possess identical substrate specificities and pH optima. From the requirement of the snail enzyme preparation for the excretion of invertase one may suggest that the newly formed invertase is ordinarily attached to the cell wall and that snail enzyme removes the enzyme continuously from its site of attachment.

In contrast with the localization of invertase, α-glucosidase appears to be present only within the semipermeable membrane of the cell. It was not detected in cell wall fractions but was released by osmotic lysis of protoplasts. ROBERTSON AND HALVORSON[17] demonstrated that both α-glucosidase and α-glucoside permease must be functional for fermentation of maltose. Maltose-grown cells of *S. cerevisiae* LK2G12 incubated with glucose lost the ability to ferment maltose while the internal content of maltose-splitting enzymes remained high. They concluded that this crypticity was due to loss or inactivation of a specific α-glucoside permease. In the present experiments, the protoplasts prepared from cryptic cells were also cryptic, and released active α-glucosidase upon osmotic lysis. As one would anticipate, control of maltose accessibility appears then to be a characteristic of the cell membrane.

A surprising indication of the crypticity of α-glucosidase is the lack of hydrolysis of maltose by maltose-adapted cells treated with nystatin. These cells lose essentially all their K^+ and NH_4^+, leak ultraviolet absorbing material and some protein, and decarboxylate pyruvate anaerobically at neutral pH (see refs. 9, 18), yet maltase is not accessible to external substrate. Since the original cells fermented maltose without a lag, this phenomenon is additional evidence for the intracellular nature of α-glucosidase, and an active transport system for maltose uptake.

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