An Inducible System for the Hydrolysis and Transport of β-Glucosides in Yeast

I. *Characteristics of the β-glucosidase activity of intact and of lysed cells*

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ABSTRACT A strain of bakers' yeast was isolated which could utilize cellobiose and other β-D-glucosides quantitatively as carbon and energy sources for growth. Cellobiose-grown cells contained a largely cryptic enzyme active against the chromogenic substrate *p*-nitrophenyl-β-D-glucoside. The patent (intact cell) activity of such cells was inhibited by azide and, competitively, by cellobiose; neither agent inhibited the β-glucosidase activity of lysed cells or of extracts. The enzyme induced by growth in cellobiose medium had no affinity for cellobiose as either substrate or inhibitor; its substrate specificity classifies it as an aryl-β-glucosidase. It was concluded that growth in cellobiose also induced the formation of a stereospecific and energy-dependent system whose function determined the rate at which intact cells could hydrolyze substrates of the intracellular β-glucosidase.

INTRODUCTION

A number of studies from this laboratory have attempted to define, as precisely as possible, the state of the intracellular enzyme catalase and the conditions which govern its activity *in vivo* (1). This enzyme exists in two distinct forms within the living yeast cell. One of these forms, called the *patent* fraction since it is active *in vivo* against external substrate, is highly resistant to inactivation by heat (2) and is, at least in large part, localized at the cell membrane (3). The other fraction, called *cryptic* since its activity can be detected only

after lysis of the cells with an appropriate physical or chemical agent, is relatively sensitive to heat inactivation (2) and is localized in the cell interior (3).

Isolation of a strain of bakers' yeast with high inducible β-glucosidase activity has made possible a study of the control of the activity of this enzyme within the living cell and thus permits comparison with the catalase of the parental strain. Preliminary reports of part of this work have been published (4, 5).

EXPERIMENTAL

A colony of bakers' yeast, *Saccharomyces cerevisiae*, was isolated by virtue of its ability to utilize the β-glucoside cellobiose as carbon and energy source; we designated the descendants of the original colony as strain C. Cells were grown in a medium described previously (1); if glucose was the carbon source its concentration was 10 gm/liter, and if cellobiose, 5 gm/liter, unless otherwise specified. Cultures were shaken vigorously during growth at 30°C. The buffer used throughout was M/15 phosphate, pH 6.8.

Assay of β-glucosidase utilized the chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and was discontinuous; for each assay, two samples were withdrawn at different times of incubation at 30°C, and the *p*-nitrophenol released was determined colorimetrically. Reduced glutathione was included in the assay mixture, following Duerksen and Halvorson (6). Patent (intact cell) β-glucosidase activity was determined at a density of approximately 1 mg/ml (dry weight). Total β-glucosidase activity (patent + cryptic) was determined at a cell density of approximately 0.03 mg/ml, after lysis with *n*-butanol which exposed the cryptic activity (1). Specific activities are reported in units of micromoles *p*NPG hydrolyzed per minute per milligram yeast. In the studies of the effect of inhibitors, these agents were dissolved in the substrate solution. Crude enzyme extracts were prepared by grinding a viscous slurry of cells with powdered glass and extracting with buffer.

Molar growth yields, defined as net growth of yeast per millimole carbon source originally present in the medium, were determined by inoculating cells in both a complete medium (*i.e.*, containing the carbohydrate to be tested) and in a medium lacking this agent but identical in other respects. The difference in cell density between the two flasks gave the net growth for calculation of the molar growth yield.

Enzymic reversion, defined by Pollock (7) as the process by which preinduced cells with high levels of an inducible enzyme return to the non-induced, low level (basal enzyme), was studied by methods previously described (1). In these experiments, cells were preinduced by growth in cellobiose medium, and enzymic reversion occurred as the cells were allowed to grow in glucose medium.

RESULTS

A. *Growth and Induction*

Fig. 1 shows the net growth of cells of strain C as a function of the concentration of cellobiose in the medium. The curve is linear up to about 0.2 gm per cent. From the linear portion of the curve, one calculates a growth yield of

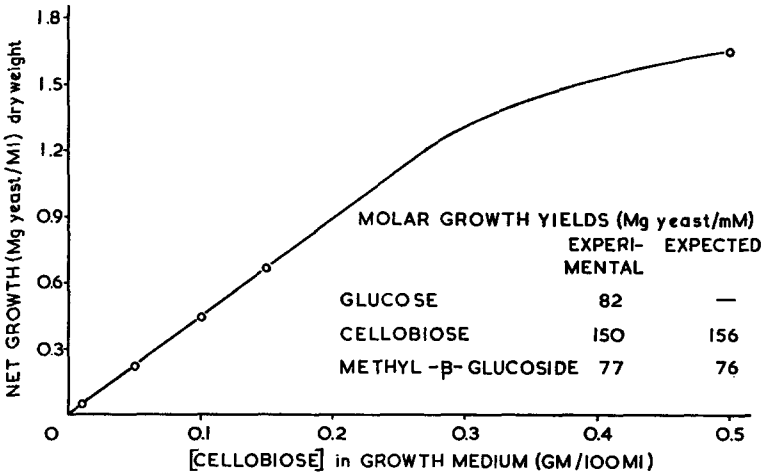

FIGURE 1. Net growth of strain C yeast *versus* initial cellobiose concentration in the growth medium. Experimentally observed molar growth yields for glucose, cellobiose, and BMG, and yields calculated for cellobiose and BMG on the basis of the observed value for glucose, are shown on the figure. It was assumed that only the glucose moiety of BMG was utilized for growth.

150 mg cells per mM cellobiose. On the basis of the experimentally determined growth yield with glucose (82 mg per mM), one can calculate the growth yield for cellobiose to be expected if this compound were quantitatively utilized. The close agreement of experimental and theoretical values (150 and 156 respectively) demonstrates the quantitative utilization of cellobiose as energy and carbon source for growth.

TABLE I

SPECIFIC PATENT AND TOTAL β-GLUCOSIDASE ACTIVITIES OF STRAIN C CELLS GROWN ON VARIOUS CARBON SOURCES AFTER PRIOR GROWTH ON GLUCOSE MEDIUM

Cells were harvested in stationary phase. Units are μM *p*NPG split/min./mg yeast. Total activity was determined after treatment with *n*-butanol.

A.	0.03 M cellobiose	0.06 M Methyl-β-glucoside	0.06 M Phenyl-β-glucoside	0.015 M glucose
1. Patent activity	0.037	0.019	0.002	0.002
2. Total activity	0.498	0.082	0.052	0.010
B.	**0.015 M cellobiose**	**0.015 M amygdalin**	**0.015 M esculin**	**0.015 M salicin**
3. Patent activity	0.054	0.009	0.072	0.045
4. Total activity	0.455	0.031	0.218	0.075
5. Net growth, *mg yeast/ml*	1.17	0.18	1.20	0.70

The ability of various carbohydrate carbon sources to induce patent and total β-glucosidase activity is shown in Table IA. Cellobiose was by far the best inducer of this enzyme, inducing levels 5 to 10 times greater than did methyl-β-D-glucoside (BMG). Phenyl-β-D-glucoside (BPG) was, like BMG, utilized quantitatively for growth, but was a poor inducer of β-glucosidase. It will be noted that the activity induced by all these substances was largely cryptic.

The natural β-glucosides, amygdalin, esculin, and salicin, were also tested as inducers and as growth substrates (Table IB). Amygdalin did not support

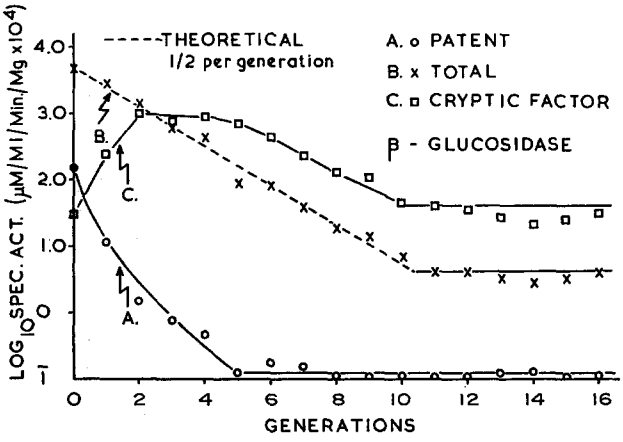

FIGURE 2. Reversion of A, patent specific β-glucosidase activity, B, total specific β-glucosidase activity, determined after lysis, and C, cryptic factor (ratio of B/A), during growth of preinduced cells in glucose medium. The broken line is that which one would observe if the total specific activity per cell were to dilute out by ½ per generation.

growth nor did it induce significant quantities of the enzyme. Final levels of growth in esculin medium were comparable to those in cellobiose, but the growth rate was considerably slower and the level of total enzyme induced somewhat lower. Salicin induced a patent β-glucosidase activity about equal to that induced by cellobiose, but the total induced enzyme activity was only about 10 to 15 per cent of that induced by cellobiose.

Enzymic reversion of preinduced cells is shown in Fig. 2. It will be noted from the close fit of the experimental points to the theoretical line of curve B that the mechanism of enzymic reversion was a dilution of the intracellular β-glucosidase. Curve A shows that the patent activity declined with each division much more rapidly than did the total activity; the former reached its basal level at about the fifth generation. The effect of this rapid decline of patent activity was to increase the cryptic factor (defined as the ratio of total to patent activities, and shown in curve C), from about 30 in the fully induced cells to about 1,000 at the second, third, and fourth generations of enzymic

reversion. The basal level of cryptic enzyme was reached at about the tenth generation.

Fig. 3 shows an experiment in which the preinduced cells were grown in glucose for seven generations and then were washed and resuspended in cellobiose medium. In the case of the patent activity, an immediate increase was noted with the first generation in cellobiose medium (curve A); in three generations, the patent activity reached or in some experiments exceeded, its original level. The cryptic activity (curve B), after a delay of one generation, increased and ultimately reached its original level.

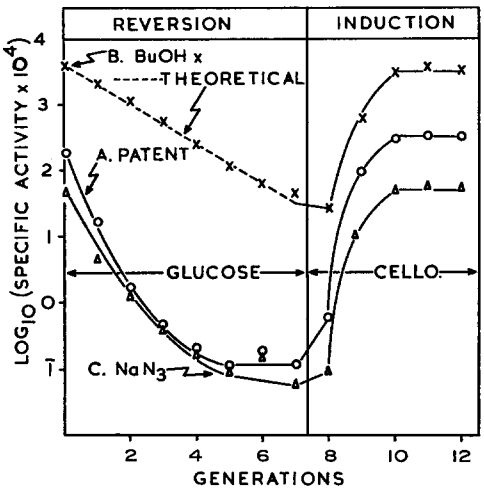

FIGURE 3. A and B are as in Fig. 2. C shows the effect of 10^{-3} M NaN_3 during assay of patent activity. After the seventh generation of enzymic reversion in glucose medium, the cells were washed twice and resuspended in cellobiose medium.

B. *The Inhibition of β-Glucosidase Activity by Metabolic Inhibitors and β-Glucosides*

Sodium azide, at its optimal concentration of 10^{-2} M, reduced the patent activity of an induced yeast suspension to less than 5 per cent of the uninhibited level. 10^{-3} M NaN_3, the concentration habitually used in these experiments, caused an inhibition of the patent activity of about 80 per cent. Cyanide had the same effect, at a somewhat higher concentration. Neither azide nor cyanide had any effect on the β-glucosidase activity of lysed cells or of crude extracts. Patent activity thus depends, in some manner, on the availability of metabolic energy. This dependence diminished sharply during enzymic reversion, as shown in Fig. 3C, but the high sensitivity to azide inhibition reappeared as the patent activity of the cells increased during induction by cellobiose. The inhibition by azide was not competitive as illustrated in the double reciprocal plot of Fig. 4.

Cellobiose and BMG were found to be strong inhibitors of the patent activity of induced cells and, like azide, were also without effect on the hydro-

lytic activity of lysed cells or of extracts (Fig. 5), even at a concentration, in the case of cellobiose, of 10^{-1} M. The inhibitory effect of both substances was competitive as illustrated, for cellobiose, in Fig. 6. When optimal concentra-

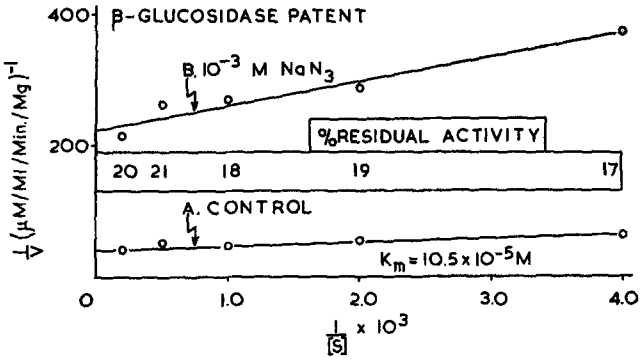

FIGURE 4. Double reciprocal plot showing inhibition of patent β-glucosidase activity by N_3^-; cellobiose-grown cells.

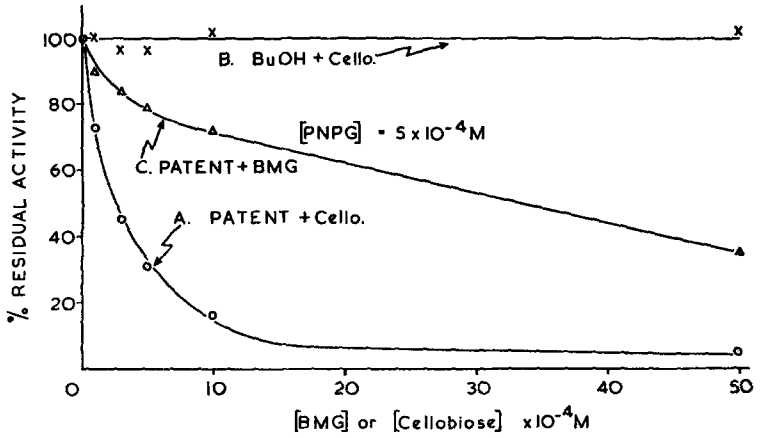

FIGURE 5. The effect of concentration of cellobiose on A, patent activity, B, activity of butanol-lysed cells, and C, the effect of concentration of BMG on patent activity. The effect of BMG on activity of lysed cells is the same as that of cellobiose, shown in B. Cells were grown in cellobiose, and β-glucosidase was assayed in the presence of varying concentrations of cellobiose and BMG.

tions of both azide and cellobiose were both present during assay, patent activity was inhibited by 97 to 98 per cent. The inhibitory effects of azide and cellobiose were quite independent, as shown in Table II. At least two different components, neither of which is the β-glucosidase itself, would thus seem also to be involved in the patent enzyme activity of induced cells. During the first divisions of enzymic reversion the sensitivity of the patent activity to cellobiose diminished, and had vanished completely by the second or third

generation. On the other hand, sensitivity to azide was more variable during the later divisions of enzymic reversion and often reappeared, even if relatively weak.

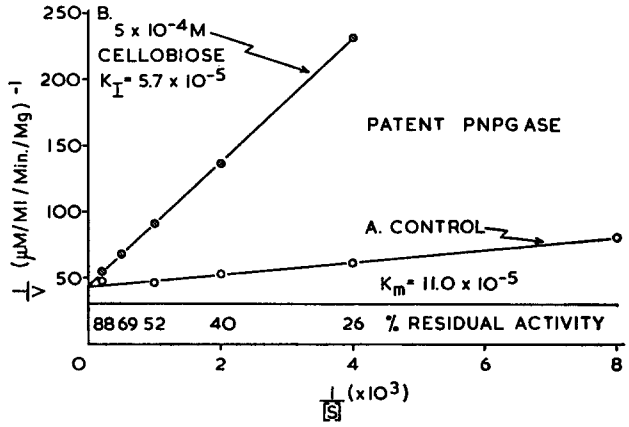

FIGURE 6. Double reciprocal plot showing competitive inhibition of patent β-glucosidase activity by cellobiose; cellobiose-grown cells.

TABLE II

ADDITIVE EFFECTS OF CELLOBIOSE AND AZIDE ON PATENT β-GLUCOSIDASE ACTIVITY OF CELLOBIOSE-GROWN CELLS

In the assay mixture, final concentrations of cellobiose and azide were 5×10^{-4} M and 10^{-3} M respectively.

Category	Specific activity
1. Controls	0.0377
2. With cellobiose alone	0.0131
3. With azide alone	0.0064
4. With both cellobiose and azide	0.0025
A. Inhibition of control activity by cellobiose (comparison of lines 1 and 2), *per cent*	65
B. Additional inhibition caused by cellobiose in the presence of azide (lines 3 and 4), *per cent*	61
C. Inhibition of control activity by azide (lines 1 and 3), *per cent*	83
D. Additional inhibition caused by azide in the presence of cellobiose (lines 2 and 4), *per cent*	81
E. Inhibition of control activity caused by both cellobiose and azide (lines 1 and 4), *per cent*	93

A Lineweaver-Burk plot for the β-glucosidase activity of lysed cells is illustrated in Fig. 7; curves for the activity of extracts were virtually identical. The data from many such experiments are summarized in Table III. It will be noted that the K_M of patent activity was the same as that for lysed cell and extract activity, about 10^{-4} M.

BPG was found to inhibit competitively both the patent and lysed cell ac-

tivities, as did glucose at very high concentration. The K_I values for these compounds, as well as for cellobiose and BMG, are summarized in Table IV. It is striking that the K_I values for the inhibition of patent and of lysed cell

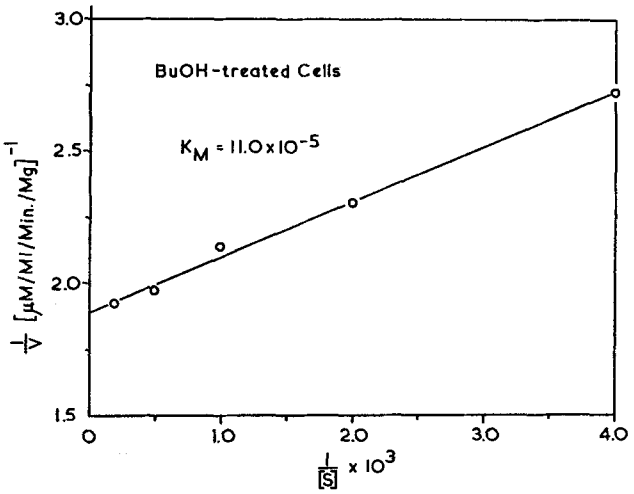

FIGURE 7. Double reciprocal plot of β-glucosidase activity of butanol-treated, cellobiose-grown cells.

TABLE III

DATA FROM LINEWEAVER-BURK PLOTS

Standard deviations are shown. n represents the number of separate determinations whose mean is shown.

Category	$\bar{K}_m$ ($\times 10^{-5}$ M)
A. Patent ($n = 9$)	10.0±3.0
B. Lysed cell ($n = 7$)	10.5±1.6
C. Extract ($n = 2$)	10.8±0.1

Differences are not significant.

activities by BPG are identical; the same is the case with glucose inhibition of patent and lysed cell activities.

C. *Substrate-Specificity of β-Glucosidase*

The substrate specificity of the enzyme was investigated by incubating cellobiose-grown, butanol-lysed cells with various β-glucosides; free glucose was determined by the glucose oxidase method. Results are summarized qualitatively in Table V, A and B. There was no detectable hydrolysis of

TABLE IV

DATA FROM LINEWEAVER-BURK PLOTS

Standard deviations are shown. *n* represents the number of separate determinations whose mean is shown.

A. Effect of β-phenyl glucoside Category	K_I ($\times 10^{-4}$ M)
Patent ($n = 4$)	5.1±1.0
Lysed cell ($n = 4$)	4.7±1.5

Difference is not significant.

B. Effect of glucose Category	K_I ($\times 10^{-1}$ M)
Patent ($n = 2$)	4.0±0.7
Lysed cell ($n = 2$)	4.0±0.1

C. Effect of cellobiose on patent activity ($n = 3$)	$K_I = 5.9 \pm 0.6 \times 10^{-5}$ M
D. Effect of β-methyl glucoside on patent activity ($n = 2$)	$K_I = 3.4 \times 10^{-4}$ M

TABLE V

EFFECT OF VARIOUS CARBOHYDRATES ON THE ACTIVITY OF β-GLUCOSIDASE OF INTACT CELLS AND OF LYSED CELLS

Only 6 of the substances listed were tested as substrates, as indicated in sections A and B. In the case of negative results, the maximum concentration tested is indicated in parentheses.

A. *Substrates*
*p*NPG, BPG, esculin

B. *Not substrates*
Cellobiose* (10^{-2} M); BMG (10^{-2} M); *p*-nitrophenyl-β-D-glucuronide (5×10^{-3}M).

C. *Competitive inhibitors of patent activity, but not of lysed cell or extract activity*
Cellobiose (10^{-1} M); BMG (5×10^{-3} M).

D. *Competitive inhibitors of both patent and lysed cell (and extract) activities*
BPG; amygdalin; salicin; esculin‡; glucose.

E. *Not inhibitors*
Lactose (10^{-3} M); maltose (10^{-3} M); methyl-α-D-glucopyranoside (10^{-2} M); tetraacetyl-BMG (10^{-3} M).

* Traces of cellobiase activity were noted.

‡ The inhibition caused by esculin was probably competitive but this has not been established, owing to technical difficulties in the assay procedure.

BMG. The rate of hydrolysis of cellobiose was so low as to be at the limit of detection of the method; the maximal rate observed was 4.6×10^{-4} μM/min./mg. On the other hand, hydrolysis of BPG was found to be about the same as

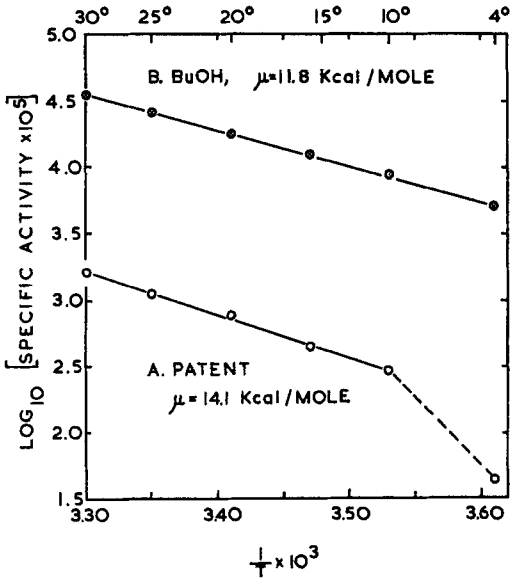

FIGURE 8. Arrhenius plot of the effect of temperature on the hydrolysis of *p*NPG by A, intact cells and B, cells treated with butanol. Cells were cellobiose-grown.

that of *p*NPG, 5.0×10^{-1} μM/min./mg. BMG and cellobiose thus appear to induce β-glucosidase activity gratuitously, since they are not substrates of the enzyme which they induce; the enzyme would seem to be an aryl-β-glucosidase.

The effect of a variety of carbohydrates on patent and lysed cell or extract activities is shown in Table V. It will be noted that α-glucosides and β-galactosides had no inhibitory effect. Acetylation of the glucose moiety of BMG eliminated its inhibitory effect on patent activity. When the aglycone was of the aryl type, the agent inhibited both patent and lysed cell activities, and was a substrate of the hydrolase.

D. *Effect of Temperature on Enzyme-Substrate Systems*

The effect of temperature on patent and lysed cell activities of induced cells is illustrated in the Arrhenius plot of Fig. 8. The former is somewhat more temperature-dependent than the latter and the curve showed an inflection at low temperature not seen in the case of butanol-treated cells. The Arrhenius curves for extracted enzyme were identical to those for the lysed cell suspensions. Data from such experiments are summarized in Table VI. It will be

noted that following lysis of the cells, or extraction of the enzyme, there was a drop in the activation energy of the enzyme-substrate system of about 3,000 cal/mole.

TABLE VI

MEAN EXPERIMENTAL ACTIVATION ENERGIES (μ) FOR THE β-GLUCOSIDASE ACTIVITIES OF INTACT CELLS (PATENT ACTIVITY), LYSED CELLS, AND CRUDE EXTRACTS

Data are in kcal/mole. Standard errors are shown.

Category	u
A. Patent (n = 4)	15.0±0.91
B. Butanol-lysed (n = 6)	11.8±0.54
C. Extract (n = 3)	12.1±0.54
Differences between means	
D. A − B	3.2±1.1*
E. Average difference (A − B) of four paired experiments	3.1±0.55‡
F. C − A	2.9±0.95*
B and C are not significantly different.	

* Significant at p = 0.05.
‡ Significant at p = 0.01.

DISCUSSION

β-glucosidase has been classified as a transosylase, *i.e.* an enzyme which transfers a glycosyl residue from the donor glucoside to an acceptor (8, 9); the action of the enzyme is generally hydrolytic, since the usual acceptor is water (9). Yeast β-glucosidase has been intensively investigated (for a review, see Barnett *et al.* (10)); indeed the wide interspecific variation in the ability of yeasts to grow on natural β-glucosides has been used as a tool for their classification (10). In view of the differing substrate specificities of the β-glucosidases of various yeasts, it has been suggested that there might exist numerous distinct β-glucosidases (10). More recent studies, in yeast by Halvorson and his students (11) and in fungi by Jermyn (12), Youatt (13), and Eberhart *et al.* (14), have shown that two or more β-glucosidases may coexist in cells of the same species.

Despite this variety of β-glucosidases, enzymes with high cellobiase activity have yet to be reported in yeast. None of the six strains of *S. cerevisiae* examined by Barnett *et al.* (10) was able to utilize cellobiose for growth, although two could utilize arbutin and two esculin. Studies from Halvorson's laboratory have dealt with the properties (6) and induction (15) of β-glucosidase in *Rhodotorula minute*, the properties of a constitutive β-glucosidase in the hybrid *S. dobzhanskii* × *S. fragilis* (16), and the genetic control of β-glucosidase formation in *S. lactis* (11, 17). All these enzymes shared one property of the β-glu-

cosidase of *S. cerevisiae* strain C, namely a very low activity against cellobiose. In addition, the enzymes of *R. minute* and of the hybrid had a Michaelis constant very similar to that of *S. cerevisiae* enzyme (8, 9, and 10×10^{-5} M, respectively).

On the other hand, the β-glucosidase of *R. minute* differed from that of *S. cerevisiae* C in a number of important respects: (*a*) it was able to hydrolyze BMG; (*b*) its activation energy was somewhat higher (16,000 cal/mole *versus* 12,000 cal/mole); (*c*) the intact cells of *R. minute* had no detectable patent hydrolytic activity against *p*NPG (15); (*d*) BMG induced levels of β-glucosidase 30 times higher than those induced by cellobiose (15); (*e*) salicin was a better inducer than either cellobiose or esculin (15), whereas it was not an inducer of this enzyme in *S. cerevisiae*.

BMG and cellobiose were both utilized by *S. cerevisiae* as carbon and energy sources for growth with the same efficiency as was glucose. Both BMG and cellobiose were excellent inducers of a β-glucosidase active against aryl-β-glucosides such as *p*NPG, BPG, and esculin. Yet lysed cells and extracts of cellobiose-grown cultures had only traces of cellobiase activity and no measurable activity against BMG.

At least four possible explanations of this paradox suggest themselves: (*a*) a cellobiase (which may also be an alkyl-β-glucosidase) was also induced by growth in β-glucosides, but was destroyed by lysis with butanol, or by the extraction procedure used for assay of β-glucosidase, which seemed to be an aryl-β-glucosidase. A cellobiase, distinct from a general β-glucosidase, has been supposed to exist in yeast (17) and has been demonstrated in the mould *Stachybotrys atra* (13); (*b*) a cellobiose phosphorylase, such as that reported in other microorganisms (18, 19) may have been present, although free glucose is one of the products of the action of this enzyme (20) and thus should have been detected in our assay and was not; (*c*) our aryl-β-glucosidase may have been active *in vivo* against cellobiose and BMG, but may have been altered by lysis so that it became inactive against these two substrates; butanol treatment of *S. cerevisiae* altered certain of the properties of catalase (21), and it also made the β-glucosidase activity of strain C enormously more sensitive to inactivation by heat (unpublished data). Thus, it might well have also altered the substrate specificity of the enzyme; (*d*) the cellobiose- and BMG-splitting enzyme may require an acceptor which is present in proper concentration and location *in vivo*, but not after lysis or disruption of the cell. It is hoped that work currently under way in this laboratory will clarify this question.

It is apparent that the patent β-glucosidase activity of strain C is not limited by the activity of a distinct, membrane-bound fraction of the intracellular enzyme, such as is the case with catalase. This conclusion follows from the potent inhibition of patent activity by azide and cyanide, agents without

effect on the lysed cell or extracted enzyme. On the other hand, it is clear that patent β-glucosidase activity is not limited by the action of the intracellular hydrolase since the latter is insensitive to cellobiose, as well as to azide, agents which strongly inhibit the former. The independence of the action of these inhibitors indicates that the system which limits patent activity consists of at least two distinct components: (*a*) a stereospecific component, which obeys Michaelis-Menten kinetics and is inhibited by β-glucosides and by high concentrations of glucose; (*b*) a component which can be inactivated by metabolic inhibitors.

On the basis of the data presented above, one can conclude that the system which limits patent activity is characterized by a high activation energy, and has a substrate affinity very similar to that of the β-glucosidase, save only for its high affinity to cellobiose and BMG, which is not shared by the latter. The rate-limiting mechanism is also unstable during enzymic reversion, as shown by the fact that after two generations of growth in glucose medium, the patent activity was no longer sensitive to the presence of cellobiose in the assay.

A future paper will present evidence for the existence of a membrane-bound, stereospecific transport system whose function determines the rate of hydrolysis of β-glucosides by intact yeast cells.

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