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# Regulation of sugar transport systems of *Kluyveromyces marxianus*: the role of carbohydrates and their catabolism

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**In *Kluyveromyces marxianus* grown on a glucose-containing synthetic medium four different sugar transporters have been identified. In cells, harvested during the exponential phase, only the constitutive glucose/fructose carrier, probed with 6-deoxy-D-glucose or sorbose, appeared to be active. In cells from the stationary phase three proton symporters can be active, recognizing 6-deoxyglucose (a glucose/galactose carrier), sorbose (a fructose carrier) and galactosides (lactose carrier), respectively. These symporters appeared to be sensitive to catabolite inactivation. This process is induced by incubating cells in the presence of glucose, fructose or mannose. Catabolite inactivation was not influenced by the inhibitor of protein synthesis, anisomycin. Derepression of the proton/sorbose and the proton/galactoside symporters proceeded readily when cells were incubated in a medium without glucose. Activation of the proton/galactose symporter needed, in addition, the presence of specific molecules (inducers) in the medium. The activation of each of these active transport systems was inhibited by anisomycin, showing the involvement of protein synthesis.**

## Introduction

Regulation of metabolic functions in yeast can proceed by a number of mechanisms, among which are catabolite repression and catabolite inactivation. These processes represent, respectively, the inhibition of protein synthesis and the inactivation of already active proteins (see Ref. 1). A number of enzymes of the gluconeogenetic pathway, the tricarboxylic acid cycle and the respiratory chain are subject to glucose catabolite repression and (or) inactivation [1–5]. Some of the sugar transport proteins are also sensitive to catabolite repression and inactivation. This was first observed in *Saccharomyces cerevisiae* for the maltose uptake system [6] and the galactose transporter [7]. More recently similar phenomena have been described for *S. cerevisiae* [8–10] as well as for other yeasts [11–13]. It could be shown, moreover, that proton/sugar symport systems can be influenced by glucose [11,12].

In a previous study similar data were obtained for transport of glucose (and its analogues) in *Kluyveromyces marxianus* [14]. It was found that cells had only one glucose transporter when collected from a medium in which glucose was present at high concentrations. On the other hand, cells separated from a medium with a low glucose

Abbreviations: NPG, *p*-nitrophenyl-β-D-galactoside; TMG, methyl-β-D-thiogalactoside; 6-dGlc, 6-deoxy-D-glucose; $K_i$, inhibition constant.

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level contained two glucose transport systems, one of which appeared to be constitutive. The second system turned out to be a proton/sugar symporter, translocating both glucose and galactose. Though it appeared likely that glucose was involved in the regulation of the activity of this co-transporter, the exact control mechanism was not established. Therefore, in the present study it was attempted to characterize the control mechanisms of sugar transport proteins in *K. marxianus*.

It will be shown that in this yeast at least three proton/sugar symporters can be found. Regulation can proceed by catabolite repression, catabolite inactivation or by induction.

## Materials and Methods

*Kluyveromyces marxianus* CBS 397 was grown on a glucose containing medium, harvested and washed as described before [14].

Sugar transport was measured according to the methods described by Van den Broek et al. [15] (with radio-assays for 6-dGlc, sorbose and TMG), at 25 °C in 2 or 10% aerobic yeast suspensions buffered with 100 mM Tris-maleate. Proton uptake experiments were carried out by methods described previously [16].

ATP was determined by the firefly luciferin/luciferase assay as described by Addanki et al. [17], using Sigma FLE-50 firefly lantern extract.

Anisomycin was utilized to inhibit protein synthesis. The effectiveness of anisomycin was tested by measuring its influence on incorporation of phenylalanine in trichloroacetic acid-precipitable material, and on the induction of β-galactosidase and galactoside transport. The conditions for measuring incorporation of [$^3$H]phenylalanine in trichloroacetic acid-precipitable material were: 2% (w/v) yeast was incubated in 100 mM Tris-maleate (pH 4.5), 2% (v/v) ethanol and [$^3$H]phenylalanine. 0.2 ml samples were drawn after 10 and 30 min of incubation and diluted in 1 ml ice-cold 10% (w/v) trichloroacetic acid. After centrifugation for 1 min in an Eppendorf mini-centrifuge the supernatant was discarded and the pellet resuspended in 0.5 ml 10% trichloroacetic acid. The suspension was heated for three min at 100 °C, cooled to 0 °C and filtered on Schleicher and Schuell GF 92 glass fiber filters. The filters were washed twice with cold trichloroacetic acid and subsequently the radioactivity was determined by liquid scintillation counting. Induction of β-galactosidase was measured by pre-incubating an aerobic 2% (w/v) yeast suspension in 100 mM Tris-maleate (pH 4.5) with 2% lactose for 4 h. After washing the cells three times with ice-cold water, the yeast was permeabilized by the toluene method of Serrano et al. [18]. β-Galactosidase activity was measured by incubating the permeabilized cells in 100 mM Tris-maleate (pH 7.0) with 1 mM NPG. Sampling and measurement of the *p*-nitrophenol formed was done as described before [15]. It appeared that 0.1 mM anisomycin inhibited phenylalanine incorporation and induction of β-galactosidase and galactoside transport in this yeast strain by 85–90%, in accordance with results obtained in a strain of *Saccharomyces fragilis* [19]. This concentration of anisomycin does not change the amount of ATP in the cell. The ATP level of 9 h and 15 h grown yeast remained constant at, respectively, 0.6 and 1.7 μmol ATP/g yeast, for at least $2\frac{1}{2}$ h in aerobic suspensions (+1% ethanol).

L-[U-$^{14}$C]Sorbose and L-[2,3,4,5,6-$^3$H]phenylalanine were obtained from Amersham International, and [$^{14}$C]methyl-β-D-thiogalactoside and $^3$H-labelled 6-deoxy-D-glucose from New England Nuclear. Anisomycin was purchased from Sigma.

## Results and Discussion

### *Characterization of transport systems*

Growth of *K. marxianus* on a glucose containing synthetic medium proceeds, as described before [14], exponentially for about 10 h. In this period the medium glucose is consumed. In the subsequent stationary phase, the yeast first consumes previously excreted metabolites, such as ethanol (10–20 h of culturing), and finally it metabolizes part of the medium citrate, resulting in a drastic increase of the extracellular pH. It was found that the uptake-velocity of 6-deoxy-D-glucose was strongly dependent on the growth phase of the cells [14]. The data of Fig. 1 show, that this dependency does not only hold for 6-dGlc but also for L-sorbose and *p*-nitrophenyl-β-D-galactoside (NPG). Influx of these three sugars is under these experimental conditions slow, or zero (NPG), in the exponential phase (high glucose concentra-

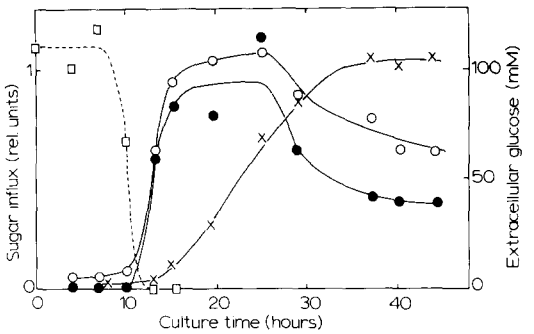


Fig. 1. The influence of the culture time on sugar transport and the glucose level of the growth medium. Extracellular glucose was measured as described before [14]. Uptake of sugars was measured at tracer concentration (sorbose and 6-dGlc) or 1 mM (NPG) in a suspension buffered at pH 4.5. Influx is expressed in relative units representing the amount of radioactivity or NPG absorbed per gram yeast per unit of time. The uptake velocities are expressed as a fraction of the uptake of 6-dGlc in 38-h-grown yeast and of sorbose and NPG in 20-h-grown yeast. Therefore the transport rates are not mutually comparable. The uptake data represent initial influx. □-----□, glucose level; ○——○, sorbose influx; ●——●, NPG influx; ×——×, 6-dGlc influx.

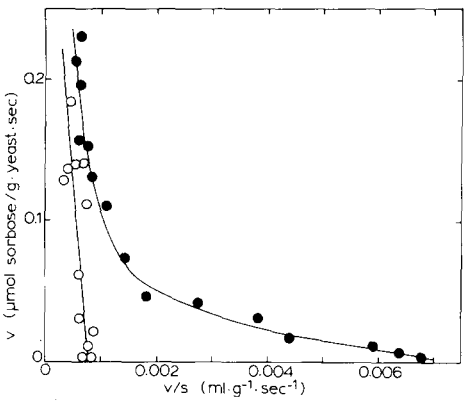


Fig. 2. Eadie Hofstee plot of initial sorbose influx in 9-h-grown (○——○) and 15-h-grown (●——●) yeast. Influx was determined at pH 4.5 from the initial linear part of uptake. The lines represent curve fits of the data with a 1 or 2 component system for, respectively, 9-h- and 15-h-grown yeast.

tion in the medium), whereas in the stationary phase (low glucose level) the uptake velocity is increased. There is an immediate increase in transport velocity for NPG and sorbose as the yeast enters the stationary phase, whereas the velocity increase of 6-dGlc influx has a considerable time lag.

To investigate the cause of the increased uptake rates, the kinetics of transport were studied in cells with (according to Fig. 1), low and high uptake velocities (it should be noted that yeasts with low- or high uptake velocities were harvested at, respectively, 13 h and 38 h for 6-dGlc transport studies, and at, respectively, 9 h and 15 h for sorbose and galactoside uptake). Fig. 2 shows an Eadie-Hofstee plot of the results obtained with sorbose transport in cells grown for 9 h and 15 h. The 9-h-grown yeast exhibited monophasic kinetics, suggesting the existence of a single transporter, whereas the biphasic kinetics observed with 15-h-grown cells indicates that at least two translocators are operative [20]. In previous studies, similar data were obtained for 6-dGlc uptake [14]: monophasic transport in 13-h-grown cells and biphasic kinetics in 38-h-grown cells. In the case of NPG no transport was measurable in exponentially growing yeast (see also Fig. 1), and monophasic uptake was observed in stationary phase cells. Table I gives the kinetic constants for the transport of these three substrates in the different cell-types. It can be concluded that yeast grown for a short period only exhibits low-affinity transport of sorbose and 6-dGlc (respectively, 9-h- or 13-h-grown yeast), whereas in 15–38-h-grown cells high-affinity uptake systems become available for all three substrates.

To establish the specificity of the various transporters, inhibition studies with metabolizable substrates were performed. As shown earlier (Ref. 14), uptake of 6-deoxyglucose in 13-h-grown cells is mainly sensitive to glucose and fructose. This also holds for sorbose uptake in 9-h-grown cells (Table II), indicating that low affinity sorbose and 6-dGlc transport proceeds via the glucose carrier (which is also specific for fructose). Moreover, these data strongly suggest that both 6-dGlc and sorbose are transported by the same translocator, which, according to the data of Table I, probably represents a constitutive transport system.

In 38-h-grown yeast transport of 6-dGlc is mainly inhibitable with glucose and galactose [14], whereas (in 15-h-grown cells), sorbose uptake is highly sensitive to fructose, and NPG influx is most sensitive to lactose (Table II). It can be concluded from these results that *K. marxianus*

TABLE I

KINETIC CONSTANTS OF INITIAL SUGAR INFLUX

Transport was measured in yeast suspensions buffered at pH 4.5. The uptake kinetics were deduced from the initial linear part of uptake. Kinetic constants were obtained from computer simulations of the data as described before [20]. Curve fitting was performed with one or two components in case of, respectively, linear and biphasic kinetics. $K$ in mM; $V$ in $\mu$mol/g per min.

| Growth time | 6-dGlc | | | | Sorbose | | | | NPG | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $K_1$ | $V_1$ | $K_2$ | $V_2$ | $K_1$ | $V_1$ | $K_2$ | $V_2$ | $K$ | $V$ |
| 9 h | | | | | 445 | 26.2 | | | * | * |
| 13 h | 160 | 25.8 | | | | | | | | |
| 15 h | | | | | 560 | 21.3 | 6.0 | 2.40 | 0.75 | 0.70 |
| 38 h | 165 | 13.5 | 0.72 | 1.60 | | | | | | |

* No uptake measurable.

can contain at least four different sugar transport proteins: a constitutive carrier recognizing glucose, fructose, 6-dGlc and sorbose and three transporters specific for, respectively, glucose/galactose (probed with 6-dGlc), fructose, (probed with sorbose), and lactose, (measured with galactosides, such as NPG or TMG). Inhibition studies for this constitutive glucose carrier indicate a high-affinity for the physiological substrates glucose and fructose ($K_i$ approx. 5 mM). Previous experiments suggested that this carrier catalyzed transport-associated phosphorylation of phosphorylatable sugars [14,21]. It is worth noticing that, considering the $V_m$ values of 6-dGlc and sorbose transport, this constitutive transporter has an almost constant capacity throughout growth, and only late in the stationary phase a slight decrease in activity can be observed (Table I).

TABLE II

THE INFLUENCE OF METABOLIZABLE SUGARS ON SORBOSE AND GALACTOSIDE TRANSPORT

Yeast was incubated aerobically in Tris-maleate buffer (pH 4.5). Sugar transport was measured utilizing sorbose at a tracer concentration and NPG at a 1 mM concentration. Metabolizable sugar was added simultaneously with sorbose and NPG (concentration, 5 mM). The uptake velocity was determined from the initial linear part of the uptake curve. Uptake is expressed as a percentage of the control.

| Condition | 9-h-grown cells | 15-h-grown cells | |
|---|---|---|---|
| | sorbose uptake | sorbose uptake | NPG uptake |
| Control | 100 | 100 | 100 |
| + glucose | 65 | 100 | 116 |
| + galactose | 95 | 92 | 42 |
| + fructose | 53 | 17 | 115 |
| + lactose | 85 | 100 | 11 |

It has been described before [14] that low-affinity 6-dGlc transport in 13-h-grown as well as in 38-h-grown cells proceeds through carrier mediated passive transport, whereas the high-affinity transporter of 38-h-grown cells was shown to be a proton/sugar symporter. Transport of sorbose in 9-h-grown yeast was found to be non-accumulative (Fig. 3A), indicating facilitated diffusion. In 15-h-grown cells sorbose and methyl-$\beta$-D-thiogalactoside are accumulated in the cell (Fig. 3), indicating active transport mechanisms. The strong inhibition of transport by uncoupler, as shown in Fig. 3, suggests that the electrochemical proton gradient is involved in energizing transport. This was substantiated by data showing that the sorbose and galactoside influxes were strongly dependent on the proton concentration in the extracellular medium. Moreover, the pH dependence of these sugar transport systems is similar to those observed with some well established proton symport systems in yeast, having a characteristic p$K$ of about 6.5 [20,22,23]. Finally it could be shown that uptake of sorbose and galactosides induced proton uptake (alkalification of the medium), thus establishing that, besides 6-dGlc, also sorbose and galactosides can enter stationary-phase cells by means of proton/sugar symport.

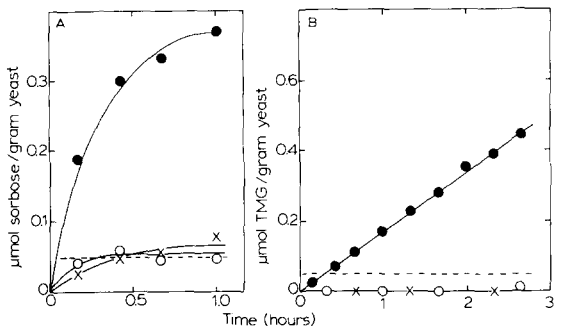


Fig. 3. Uptake of sorbose and TMG in 9-h- and 15-h-grown yeast. 9-h-grown (× ——— ×) and 15-h-grown yeast (●, ○) was incubated at pH 4.5 with 0.1 mM sugar, with (○ ——— ○) or without (● ——— ●, × ——— ×) 0.1 mM carbonylcyanide *m*-chlorophenylhydrazone. The dotted line represents the diffusion equilibrium level.

*Regulation of transport systems*

The results described above reveal that the number of different carriers, operative in translocating sugars, varies during batch-culturing of *K. marxianus*. In the presence of glucose (exponential phase) none of the proton symporters is active (see Fig. 1). This indicates that glucose, or its metabolites, control the activity of these translocators. This was tested by measuring transport parameters in either 15-h- or 38-h-grown cells

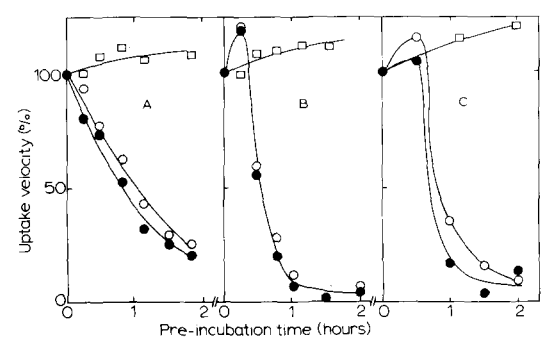


Fig. 4. Inactivation of sugar transport in stationary phase yeast. 2% (w/v) yeast was preincubated aerobically in 0.1 M Tris-maleate (pH 4.5) with 2% ethanol (□ ——— □), 2% glucose (● ——— ●) or 2% glucose plus 0.1 mM anisomycin (○ ——— ○). Samples were withdrawn, washed and subsequently the yeast was tested for sorbose (A), NPG (B) and 6-dGlc (C) uptake. Uptake was measured at pH 4.5 with sorbose and 6-dGlc at tracer and NPG at a 1 mM concentration. The uptake of sorbose and NPG was measured in 15-h-grown yeast and 6-dGlc transport in 38-h-grown cells.

treated with glucose, or in cells lacking active symporters (grown for 9–13 h), incubated in the absence of glucose. The results of experiments in which yeast, containing the symporters, was incubated in buffer with glucose or glucose plus anisomycin showed that transport of the three substrates became rapidly inactivated in the presence of glucose (Fig. 4). Since uptake was measured under conditions where only the activity of the three cotransporters was determined (low sugar concentration in the transport assay), it followed that the glucose pre-incubation resulted in the inactivation of proton symport systems. The fact that the glucose-induced inactivation of the transport system was not sensitive to anisomycin means that protein synthesis was not involved. These results agree with those found for the galactose transporter [7] and the maltose translocator [6] of *S. cerevisiae*. Pre-incubation of the yeast with 2% fructose or mannose instead of glucose gave identical results, whereas galactose, lactose and ethanol did not cause inactivation. Therefore it is likely that catabolite inactivation is caused by a process located ahead of the glycolytic phosphoglucose-isomerase step, indicating either the transport or the hexo- or gluco-kinase step as main factors governing catabolite inactivation. In *Candida utilis* this regulation has been described to be on the level of sugar phosphorylation [13]. However, further experimentation is needed to establish whether this holds for *K. marxianus*.

The reverse experiments, in which cells, which did not contain active symporters, were incubated in a buffer supplemented with ethanol (to energize the yeast) are depicted in Fig. 5. The sorbose and NPG transports are readily stimulated by incubating for some hours in the absence of glucose. The transport stimulation is caused by an increase in the activity of the symport systems, since in both cases uphill transport was restored. Activation of transport as depicted in Fig. 5 is prevented by anisomycin, showing that this process depends on protein synthesis. Also, when these symport systems were first inactivated in 15-h-grown yeast by preincubation with glucose, reactivation of these carriers was observed after incubating the cells under conditions described for Fig. 5. Again this reactivation was prevented by the presence of anisomycin. Inactivation of transport systems has

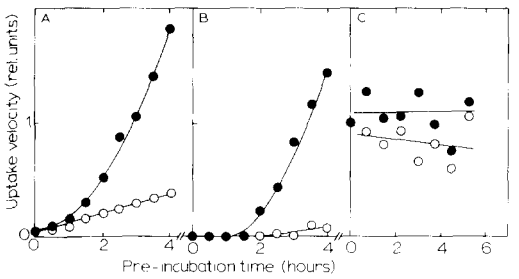


Fig. 5. Activation of sugar transport in 9-h- or 13-h-grown yeast. 2% (w/v) yeast was preincubated aerobically in 0.1 M Tris-maleate (pH 4.5) with 2% ethanol (●———●) or 2% ethanol plus 0.1 M anisomycin (○———○). Samples were withdrawn, and the yeast was washed and tested for transport activity as described for Fig. 1. (A) Sorbose transport (9-h-grown yeast); (B) NPG transport (9-h-grown cells) and (C) 6-dGlc transport (13-h-grown yeast).

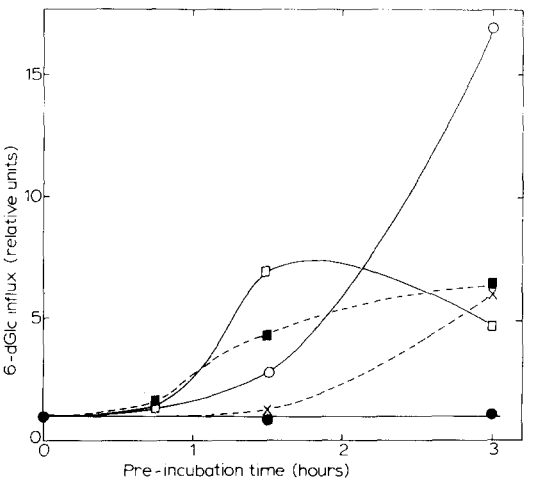


Fig. 6. Activation of 6-dGlc transport in 13-h-grown yeast. 2% (w/v) yeast was preincubated in 0.1 M Tris-maleate (pH 4.5) with 2% ethanol (●———●), 2% galactose (□———□), 2% lactose (■-----■), 2% glycerol (○———○) or 2% pyruvate (×------×). After collecting and washing the yeast, transport was measured as described for Fig. 1. It should be noted that anisomycin cna prevent the observed activations.

been described as reversible [10] and as irreversible [7–9] damage of the respective proteins. The latter experiment, however, indicates that in *K. marxianus* the carriers are irreversibly inactivated. It is likely that proteolytic degradation of (part of) the translocator takes place, maybe after phosphorylation of the protein as described for, e.g., the inactivation of fructose-1,6-bisphosphatase in *Kluyveromyces fragilis* [24].

In contrast to uptake of sorbose and NPG, 6-dGlc transport did not increase within a 6 h incubation period in the absence of glucose (Fig. 5). Apparently the $H^+$/6-dGlc symporter is not regulated by glucose alone, but more factors must be involved. In a previous publication it was suggested that the increase in 6-dGlc transport in the course of growth might be related to processes accompanying the strong increase of the pH of the growth medium [14]. This, however, is not the case: yeast grown in a medium with phthalate instead of citrate showed a similar activation of 6-dGlc uptake as shown in Fig. 1, whereas the medium remained at low pH throughout the stationary phase (not shown). An alternative way to explain that the expression of the proton/6-dGlc symporter needs more than just the absence of glucose, might be the activation of this high-affinity transport system by a specific molecule ('inducer'). This hypothesis was tested by measuring transport in 13-h-grown cells, treated for some time in a buffer with different kinds of substrates.

The results of Fig. 6 show again that 6-dGlc transport is not activated in a medium containing only buffer and ethanol. Ethanol effectively increases the cellular amount of ATP in this yeast, so activation of 6-dGlc transport is not just induced by energization. Activation was caused, however, by galactose, lactose, glycerol and pyruvate. This suggest that this transport increase is due to the presence of specific activators or inducers. This broad 'inducer' specificity was also observed with the galactose transporter of *Kluyveromyces lactis* [25]. So, either the galactose carrier can be induced by more than one kind of molecule, or there is just one inducer which can be formed intracellularly from the beforementioned activators. The fact that this glucose/galactose specific carrier becomes active in the late stationary phase (Fig. 1) indicates that at that time the growth medium also contains an activator of transport.

The data shown in this paper suggest that the fructose and lactose symporters in *K. marxianus* are activated without the obvious interference of a specific inducer. It has, however, been described before [22,26,27] that lactose transport in yeast is inducible with specific inducers, such as lactose

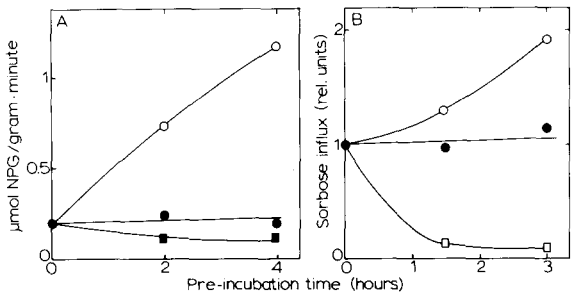


Fig. 7. Induction of NPG (A) and sorbose (B) transport in 15-h-grown yeast. 2% (w/v) yeast was incubated in Tris-maleate (pH 4.5). After sampling and washing the yeast, transport was determined as described for Fig. 1. (A) The yeast was preincubated with 2% lactose (○———○), 2% ethanol (●———●). ■———■, represents the control without extra additions. (B) Yeast was preincubated with 2% ethanol (●———●), 2% ethanol plus 0.05% fructose (○———○) or 2% ethanol plus 1% fructose (□———□).

and galactose. Therefore, it was tested whether activation, as shown in Fig. 5, and induction were identical or separate processes. 15-h-grown yeast was further incubated with either lactose or fructose. After washing the cells transport of respectively NPG or sorbose was measured. Fig. 7A demonstrates that lactose causes a strong, further increase of NPG transport in this maximally 'activated' yeast, indicating induction, superimposed on the activation process demonstrated in Fig. 5. This induction did not occur in the presence of anisomycin, indicating that induction depends on protein synthesis. Induction caused an increase of the $V_m$ from 0.7 μmol NPG/g per min in yeast fully activated in the absence of lactose (Table I) to 8.6 μmol NPG/g per min in induced cells [15]. Induction of the proton/fructose symporter is less straight-forward: pre-incubation of 15-h-grown cells with low fructose concentrations induced an increased sorbose transport, suggesting the induction of the high-affinity fructose carrier. At high fructose concentration, however, the reverse is observed, viz. inactivation of preexisting sorbose transport. Thus, Fig. 7 shows that lactose and fructose can induce transport, and, moreover, that this induced transport exceeds the transport activity formed in the absence of glucose (derepressed conditions). The results, however, do not establish whether these two transport systems i.e. induced and derepressed, are separate or identical entities.

Regulation by glucose of the activity of the sugar transport systems in *Saccharomyces cerevisiae* has been described before [6–10]. More recently this has also been reported for proton/sugar symport in a number of yeasts [11,12]. Most of these glucose effects could be ascribed to catabolite repression or catabolite inactivation. The results shown in this paper can also be described by these processes. In the exponential phase of growth none of the symporters is active, indicating catabolite repression. The activated symporters, present in the stationary phase become readily inactivated when glucose is added to the yeast, indicating catabolite inactivation.

Proton/sorbose symport has been described before in this yeast [20]. In that paper it was concluded from, among others, the observed biphasic uptake kinetics that sorbose transport proceeds by simultaneously occurring facilitated diffusion and proton symport. It was concluded from the kinetic data that separate translocators were involved for both transport mechanisms. These results were obtained with yeast grown for 20 h on glucose. Therefore (see Fig. 1) it can be concluded that the biphasic kinetics were indeed caused by the simultaneous action of two separate carriers, viz. the glucose/fructose carrier and the proton/fructose symporter. It is interesting to note that in *Saccharomyces cerevisiae* it has also been observed that sorbose transport can proceed by way of facilitated diffusion and proton symport [28], and that, as observed here, both transporters are effectively transporting fructose.

The results shown in this paper indicate that the regulation of sugar transport systems in *K. marxianus* apparently is a complex process and further experiments are required to elucidate the biochemical background of the various pathways involved in this regulation.

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