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TRANSPORT AND TRANSPORT-ASSOCIATED PHOSPHORYLATION OF GALACTOSE IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

The galactose transport mechanism in three strains of *Saccharomyces cerevisiae* was investigated in some detail, both in glucose-grown cells and in galactose-induced cells.

In glucose-grown yeast the uptake of galactose and 2-deoxy-D-galactose could be characterized as carrier-mediated facilitated diffusion. Competition and countertransport studies showed that these sugars are taken up *via* the same carrier as glucose and sorbose.

After induction the transport system has changed from a low affinity to a high affinity system, as reflected by a decrease of the K_m by two orders of magnitude. The uphill transport of 2-deoxy-D-galactose after induction indicated that this high affinity system is an active transport mechanism. Competition and countertransport studies showed that this active transport takes place *via* the same carrier as the facilitated diffusion in glucose-grown cells.

In the yeast strain NCYC 240 the induction of the active transport system occurred before the induction of galactose metabolism. During this phase of the induction process galactose was partly phosphorylated, whereas the free sugar concentration in the cells increased to about 50 times the medium concentration. Radioactive pulsing experiments during this interval showed transport-associated phosphorylation of galactose, as demonstrated previously for some other carbohydrates in yeast.

INTRODUCTION

In a previous paper arguments were presented indicating the existence of two transport mechanisms for galactose in *Saccharomyces cerevisiae*, strain Hansen CBS 1172 (ref. 1). It was shown that in glucose-grown yeast cells galactose enters *via* carrier-mediated facilitated diffusion and after induction with galactose *via* an active transport system. One of the arguments for this conclusion was a major change of the transport parameters after induction. The K_m value dropped from about 650 mM in glucose-grown cells to about 5 mM after induction. Further it was suggested, but not proven, that the active transport mechanism of induced cells would be similar to the transport-associated phosphorylation system, operative

in the active transport of glucose[2], 2-deoxy-D-glucose[3] and α-methylglucoside[4] in yeast.

In recent papers Cirillo and co-workers[5-7] reached a quite different conclusion concerning galactose transport in *S. cerevisiae*. According to these authors galactose transport in *S. cerevisiae* always occurs *via* carrier-mediated facilitated diffusion, both before and after induction with galactose. The change of the K_m value by induction as found in our experiments was attributed by these authors to an experimental error[6,7]. In our experiments the initial rate of galactose uptake in uninduced yeast cells was calculated over an initial interval of 10 min. According to Cirillo *et al.* the net rate of uptake falls to zero within 1 min, thus introducing an underestimation of the initial transport rate of two orders of magnitude.

An experimental error of this kind in our previous studies was out of the question, as appropriate control experiments concerning this point had been conducted. Nevertheless it appeared that the fundamental aspects of the contradicting conclusions justified reinvestigation of the problem. In these studies other experimental techniques and other yeast strains were included. The results of these studies are described in the present paper.

METHODS

S. cerevisiae was grown on a liquid medium of the following composition: KH_2PO_4, 1.2 g; citric acid, 2.4 g; *tert*-potassium citrate, 12 g; $(NH_4)_2SO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; KCl, 1 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $FeCl_3 \cdot 6H_2O$, 17.5 mg; $MnSO_4$, 6.25 mg; NaCl, 50 mg; $ZnSO_4$, 50 mg; inositol, 6.25 mg; biotin, 3.5 mg; calcium pantothenate, 6.25 mg; thiamine, 6.25 mg; pyridoxine, 6.25 mg; nicotinic acid, 6.25 mg; riboflavin, 6.25 mg and either glucose, 18.75 g (uninduced cells) or galactose, 18.75 g (induced cells) per l. The yeast was inoculated in about 100 ml of this medium in a culture flask and incubated at 28 °C during 24 h. The content of the flask was subsequently added to 8 l of the same medium in a fermentor (Microferm, New Brunswick) and cultured for another 16–30 h. The yeast was harvested in a continuous flow centrifuge and washed several times with distilled water.

Yeast suspensions were buffered at pH 6.0 with triethylamine–succinate–tartrate[8]. In transport studies cells and medium were separated by Millipore filtration, followed by washing of the cells on the filter with ice-cold water. In control experiments it was found that at the most 2% of the intracellular sugar was lost from the cells by this washing procedure. Yeast extracts for sugar analysis were prepared by treating the cells with absolute ethanol (3 ml per g yeast) for 1 h at room temperature. Subsequently an equal volume of water was added and the extraction was continued for 1 h at 4 °C. An alternative extraction procedure in which the cells were initially extracted with boiling ethanol[7] gave identical results.

Separation and identification of free and phosphorylated intracellular sugars was accomplished as described previously[3]. In most uptake experiments ^{14}C or ^{3}H labeled sugars were used. Radioactivity of the extracts was measured in a liquid scintillation counter, with the liquid scintillator described by Bray[9]. 2-Deoxy-D-galactose and 2-deoxy-D-galactose phosphate were measured chemically, by the method of Waravdekar and Saslaw[10]. To calculate intracellular sugar concentrations, the intracellular water was measured as described before[11].

Fermentation and respiration were measured manometrically, with a differential respirometer.

RESULTS

Galactose transport system in strain Hansen CBS 1172 and in commercial baker's yeast

Galactose uptake in glucose-grown yeast, strain Hansen CBS 1172 is shown in Fig. 1. Over the studied concentration range (1–700 mM) galactose uptake proceeds linearly with time for about 20 min; after longer incubation periods the uptake curve flattens out. This implies that initial rates of uptake can be calculated from the galactose uptake during an initial period up to about 15 min. In accordance, converting the data of galactose uptake over an initial period of 20 s, 1 min, 5 min and 10 min according to the Michaelis–Menten equation gave substantially identical results. Mean values (±S.E.) in 5 sets of experiments were: $V = 0.352$ (±0.025) mmole per g yeast per h; $K_m = 615$ (±60) mM.

Net uptake vanishes after about 200 min, when the intracellular galactose concentration becomes equal to the medium concentration.

The uptake of 2-deoxy-D-galactose in glucose-grown cells proceeds similarly. At equilibrium the intracellular concentration is again equal to the medium concentration. Phosphorylated derivatives of galactose and 2-deoxy-D-galactose were never found in cellular extracts of uninduced cells.

During prolonged incubation with galactose (5 mM) induction takes place. After about 10–12 h the intracellular galactose concentration begins to decrease gradually, falling to zero in the course of about 2 h. During this 2-h interval the capacity for galactose fermentation and respiration is induced, as shown by manometric measurements.

The transport parameters of galactose transport into induced, galactose-grown

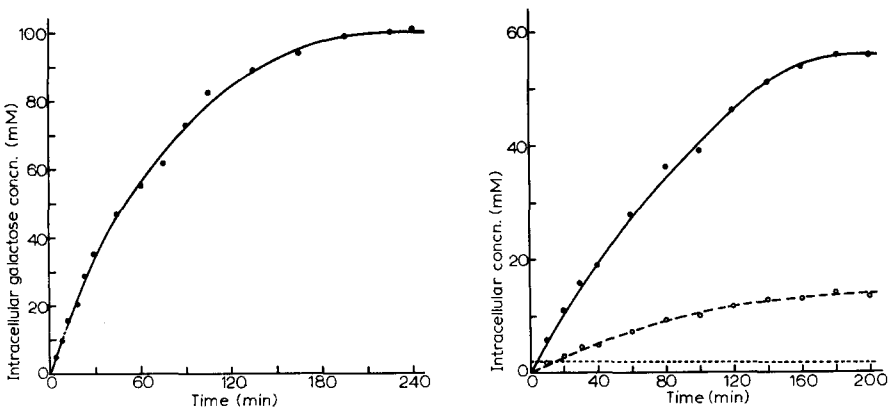


Fig. 1. Galactose uptake in glucose-grown Hansen CBS 1172 at 25 °C. Substrate concn in the medium: 100 mM. Yeast concn: 4 %.

Fig. 2. 2-Deoxy-D-galactose uptake in galactose-grown Hansen CBS 1172 at 25 °C. Initial substrate concn in the medium: 2 mM. Yeast concn: 2.5 %. ●—●, intracellular 2-deoxy-D-galactose phosphate concn; ○---○, intracellular free 2-deoxy-D-galactose concn. The dotted line represents the 2-deoxy-D-galactose concn in the medium.

cells were calculated both from the initial rates of disappearance of galactose from the medium and indirectly, by measuring fermentation rates with the manometric technique. The results with both methods were identical. In 5 sets of experiments a V value of 1.48 ($\pm$0.12) mmoles/g yeast per h and a K_m of 4.6 ($\pm$0.4) mM was found. Transport of 2-deoxy-D-galactose into induced cells is shown in Fig. 2. Inside the cell both 2-deoxy-D-galactose phosphate and the free sugar could be detected. Fig. 2 shows that free 2-deoxy-D-galactose is taken up against a concentration gradient. After 200 min the intracellular concentration of the free sugar is about 10 times the medium concentration.

With commercial baker's yeast ("Koningsgist" from the Gist en Spiritusfabriek, Delft) similar results were obtained. The galactose transport parameters were V = 0.22 ($\pm$0.03) mmole/g yeast per h, K_m = 320 ($\pm$35) mM in uninduced cells and V = 2.12 ($\pm$0.30) mmoles/g yeast per h, K_m = 4.2 ($\pm$0.08) mM in induced cells. 2-Deoxy-D-galactose equilibrated in uninduced cells and was phosphorylated and taken up against a concentration gradient in induced cells.

Galactose transport system in strain NCYC 240

Uptake of galactose and 2-deoxy-D-galactose in uninduced cells of the strain NCYC 240 proceeded somewhat faster than in the strain Hansen CBS 1172. Both substrates are present inside the cell as the free sugar, without phosphorylation. Uptake of galactose is linear with time for about 12 min. From the galactose uptake data over initial periods of 20 s, 1 min and 5 min a K_m value of 510 mM and a V of 0.65 mmole/g yeast per h could be calculated. In induced, galactose-grown cells these parameters were K_m = 4.0 mM and V = 2.11 mmoles/g yeast per h, as calculated from the galactose disappearance from the medium and the fermentation rate.

In induced cells 2-deoxy-D-galactose was recovered in the cellular extracts partly as the free sugar, partly as 2-deoxy-D-galactose phosphate. The free sugar was again taken up against a concentration gradient; the uptake curves were essentially similar to those in the strain Hansen CBS 1172. For this galactose analogue the transport parameters changed from K_m = 410 mM, V = 0.72 mmole/g yeast per h in uninduced cells, to K_m = 3.8 mM, V = 2.23 mmoles/g yeast per h after induction.

To decide whether galactose transport in uninduced and in induced cells proceeds *via* the same carrier or *via* different carriers, the following experiments were conducted. The parameters of glucose and sorbose transport in uninduced and in induced cells were measured with the usual methods. The results are summarized in Table I. In uninduced cells transport of galactose, 2-deoxy-D-galactose and sorbose

TABLE I

TRANSPORT PARAMETERS OF SUGAR TRANSPORT IN UNINDUCED AND IN GALACTOSE-INDUCED CELLS OF THE STRAIN NCYC 240, AT 25 °C.

Substrate	K_m (*mM*)		V (*mmoles/g per h*)	
	Uninduced	*Induced*	*Uninduced*	*Induced*
Galactose	510	4.0	0.65	2.11
2-Deoxy-D-galactose	410	3.8	0.72	2.23
Glucose	5.1	5.1	2.13	2.14
Sorbose	620	610	1.05	1.01

was strongly inhibited by glucose, whereas galactose, 2-deoxy-D-galactose and sorbose showed only a small mutual inhibition. As an example the inhibition of sorbose transport by glucose and galactose is shown in Fig. 3. In uninduced, preloaded cells glucose evokes an extensive countertransport of sorbose, galactose and 2-deoxy-D-galactose against a large concentration gradient. Galactose, in high concentrations, causes a slow, very small countertransport of sorbose and 2-deoxy-D-galactose and, reversely, sorbose causes a slow countertransport of galactose. The sorbose countertransport experiments are depicted in Fig. 4.

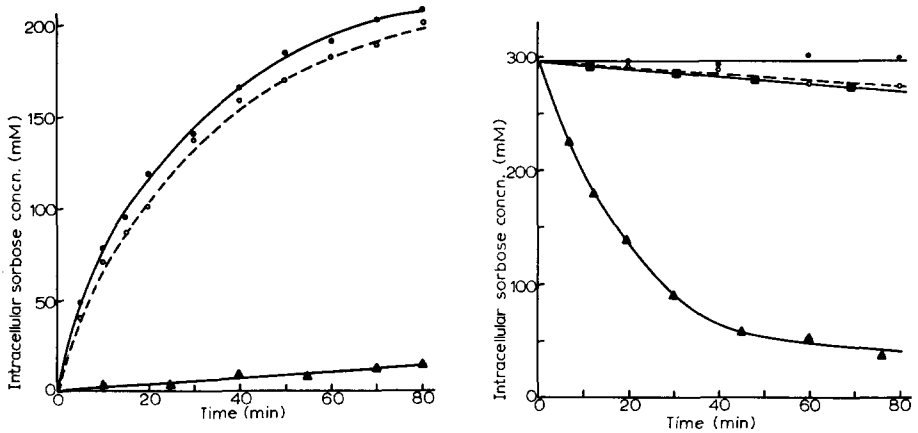


Fig. 3. Inhibition of sorbose transport in uninduced NCYC 240. Sorbose concn: 300 mM. Yeast concn: 4%. ●—●, control; ○---○, 300 mM galactose added; ▲—▲, 50 mM glucose added.

Fig. 4. Sorbose countertransport in uninduced NCYC 240. The cells were equilibrated with 300 mM sorbose by incubating 2.5 h at 25 °C. At zero time 300 mM galactose, 300 mM 2-deoxy-D-galactose or 50 mM glucose was added. ●—●, control; ■—■, 2-deoxy-D-galactose added; ○---○, galactose added; ▲—▲, glucose added.

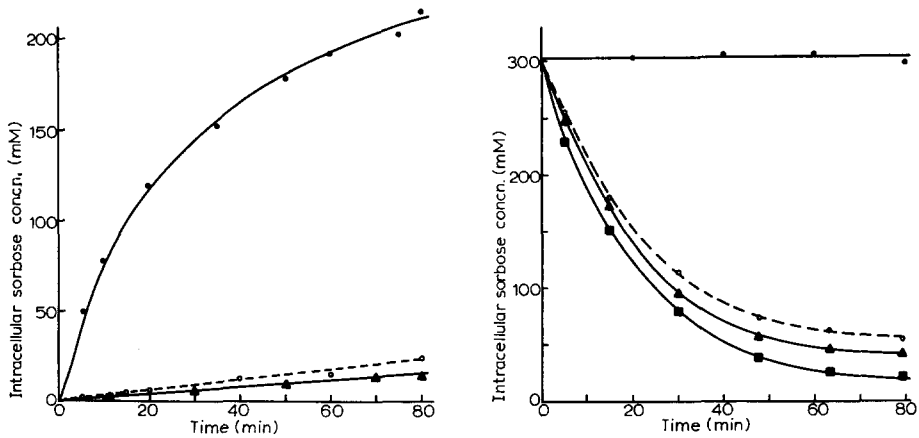


Fig. 5. Inhibition of sorbose transport in galactose-induced NCYC 240. Sorbose concn: 300 mM. ●—●, control; ○---○, 50 mM galactose added; ▲—▲, 50 mM glucose added.

Fig. 6. Sorbose countertransport in galactose-induced NCYC 240. The cells were equilibrated with 300 mM sorbose by incubating 2.5 h at 25 °C. At zero time the second substrate was added. ●—●, control; ○---○, 50 mM galactose added; ■—■, 50 mM 2-deoxy-D-galactose added; ▲—▲, 50 mM glucose added.

After induction sorbose transport is inhibited strongly by glucose, galactose and 2-deoxy-D-galactose, as depicted in Fig. 5. These sugars also cause countertransport of sorbose (Fig. 6), whereas sorbose does no longer cause any countertransport of galactose.

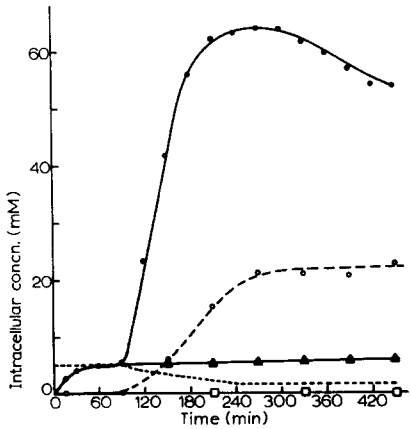
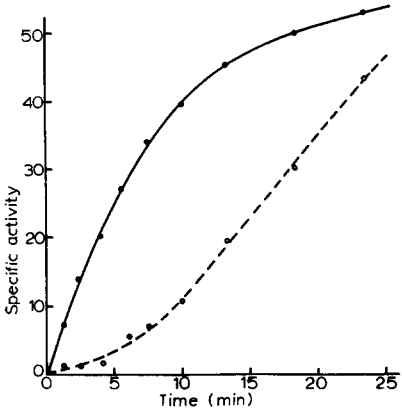


Fig. 7. Galactose uptake in glucose-grown NCYC 240 at 25 °C. Initial galactose concn in the medium: 5 mM. ●—●, free galactose; ○---○, galactose phosphate; ▲—▲, free galactose, in the presence of cycloheximide (5 μg/ml); □—□, galactose phosphate in the presence of cycloheximide. The dotted line represents the galactose concn in the medium in the absence of cycloheximide.

If glucose-grown cells of this yeast strain are incubated with galactose for longer periods, galactose uptake proceeds as shown in Fig. 7. After about 70 min the intracellular free galactose concentration begins to increase, reaching an ultimate value of about 50 times the galactose concentration in the medium. At the same time galactose phosphate appears inside the cells. Manometric experiments showed that no fermentation or respiration of galactose took place during this interval. The first indication of galactose metabolism was found after an incubation period of about 6 h. Both the uphill transport and the phosphorylation of galactose were inhibited completely by cycloheximide (Fig. 7).

In further experiments a pulsing dose of ^{14}C-labeled galactose was added to the medium, after establishment of the uphill galactose concentration gradient. The cell contents were subsequently analyzed at intervals for ^{14}C radioactivity of the free galactose and of galactose phosphate. The total amount of intracellular free and phosphorylated galactose was measured either by double labeling in the same experiment, preincubating with [^{3}H]galactose and pulsing with [^{14}C]galactose, or in a simultaneously conducted parallel experiment under identical experimental conditions, except for the use of ^{14}C-labeled galactose throughout the whole experiment. The results were identical with both methods. The specific activities in both fractions (the ratio of radioactivity/total amount of galactose in that fraction, expressed in arbitrary units) was calculated. The results of a typical experiment are shown in Figs 8 and 9. Similar results were obtained when the galactose concentration during preincubation was varied from 1 to 50 mM; in all experiments the specific activity of the phosphorylated fraction increased faster than the specific activity of the free fraction.



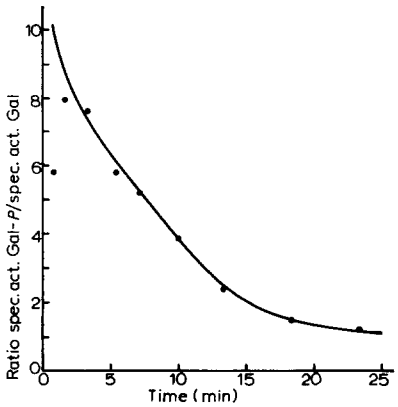


Fig. 8. Specific activity of intracellular galactose phosphate (●—●) and free galactose (○---○) after pulsing with ^{14}C-labeled galactose. Preincubation: 50 ml of a 5 % suspension of glucose-grown NCYC 240 was incubated during 210 min at 25 °C in 5 mM galactose, to induce the active galactose transport system. Subsequently a pulsing dose of [^{14}C]galactose (5 μCi in 0.5 ml) was added (zero time in the figure).

Fig. 9. Ratio of the specific activity of the intracellular galactose phosphate to the specific activity of intracellular free galactose, in the course of time. Experimental conditions: see legend to Fig. 8.

DISCUSSION

In the three yeast strains used in the present investigations the galactose transport mechanism in uninduced cells can be characterized as a carrier-mediated facilitated diffusion. The fact that galactose uptake in the strain Hansen CBS 1172 is linear with time for about 20 min disproves the criticism of Cirillo and co-workers[6,7], that the initial uptake velocities were largely underestimated in our previous experiments[1]. In relatively short-term experiments the intracellular sugar concentration equilibrates with the medium concentration. Similar results were obtained with 2-deoxy-D-galactose. Competition and countertransport studies showed that glucose, sorbose, galactose and 2-deoxy-D-galactose are taken up in the strain NCYC 240 *via* the same carrier, according to the generally accepted criteria of carrier-mediated transport[12-14]. This was shown before in the yeast strain Hansen CBS 1172 (ref. 1). Assuming active transport of glucose[2] and facilitated diffusion of the other sugars *via* this carrier and considering the K_m values summarized in Table I, the kinetics of inhibition were in accordance with the equations for transport inhibition, discussed in a previous paper[15].

After induction competition and countertransport studies demonstrated that glucose, sorbose, galactose and 2-deoxy-D-galactose were still taken up *via* the same carrier in the yeast strain NCYC 240, sorbose entering the cells *via* facilitated diffusion, the other three sugars by active transport. This was also shown for the yeast strain Hansen CBS 1172 in a previous paper[1]. This indicates that the synthesis of an enzyme ("permease") takes place during induction, catalyzing the association of galactose and 2-deoxy-D-galactose with the carrier, in accordance with the permease–carrier model of transmembrane transport[16,17]. The existence of a rather unspecific carrier for many sugars combined with several substrate-specific coupling enzymes has

also been described in bacteria[18], where the aspecific component could be identified as Enzyme I and the specific components as Enzyme II of the phosphoenolpyruvate–phosphotransferase system[19,20].

Comparison of the competition and countertransport data before and after induction demonstrates the large increase of affinity of galactose and 2-deoxy-D-galactose for the carrier. This is also clearly shown by the decrease of the K_m value by two orders of magnitude. The induction of an active transport system is further unequivocally proved by the presented studies on 2-deoxy-D-galactose uptake. This sugar equilibrates in glucose-grown yeast, but is taken up against a concentration gradient of the free sugar after induction.

In fully induced cells, incubated in a galactose containing medium, no free galactose is found, indicating that galactose transport is the rate limiting step of galactose metabolism. In the yeast strain NCYC 240 the synthesis of the active transport system apparently occurs before synthesis of the metabolic enzymes. This results in a period of uphill transport of free galactose (up to about 50 times the concentration in the medium) and galactose phosphorylation, without further metabolic conversions of the sugar. The induction is inhibited completely by cycloheximide, indicating protein synthesis during the induction of the active transport system. In additional experiments, not presented in the results section, it appeared that 2-deoxy-D-galactose also induced the active transport system, with equal potency as galactose.

The dissociation in time of the induction of the active transport system and the metabolic system for galactose allowed further studies of the transport system with ^{14}C pulsing experiments, after establishment of the galactose concentration gradient. If transport of the free sugar would precede intracellular phosphorylation, the specific activity of the free intracellular sugar should increase faster than the specific activity of the phosphorylated fraction after addition of a pulsing dose of ^{14}C-labeled galactose. In the case of transport-associated phosphorylation followed by some intracellular dephosphorylation, however, the radioactivity in the phosphorylated fraction should increase faster, as discussed in detail in previous papers[3,4]. As shown in Figs 8 and 9 galactose phosphate should be considered as the precursor of intracellular free galactose, in accordance with the model of transport-associated phosphorylation.

The length of the lag time between addition of the inducer and the beginning of uphill galactose transport depends on the condition of the yeast. If glucose-grown cells were harvested in the stationary phase, the lag time was about 2.5 h. If harvested in the exponential phase the lag time was shorter, decreasing to 1.2 h for cells in the early exponential phase. In all experiments the beginning of uphill transport and of phosphorylation of galactose coincided in time, as should be expected if the phosphorylation is an integral part of the active transport process.

Apparently this active transport mechanism for galactose is similar to the active, transport-associated phosphorylation system described previously for glucose[2], 2-deoxy-D-glucose[3] and α-methylglucoside[4]. This transport system resembles the phosphotransferase system in bacteria[21,22]. Attempts to establish a possible role of phosphoenolpyruvate in this yeast system failed thus far. Presumably the transport-associated phosphorylation in yeast takes place in a different way, as discussed before[3,15].

These experimental results, demonstrating unequivocally the existence of an active, uphill transport system for galactose and 2-deoxy-D-galactose in these yeast strains, contradict the conclusion of Cirillo and co-workers[5-7], that galactose transport in *S. cerevisiae* always occurs *via* facilitated diffusion. As there are no obvious reasons to question the experimental results of Cirillo and co-workers[5-7], it should presumably be concluded that the characteristics of the transport mechanism for galactose (and presumably other sugars) depend on the experimental conditions and may be different in different yeast strains.

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