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THE TRANSPORT MECHANISM OF α-METHYLGLUCOSIDE IN YEAST EVIDENCE FOR TRANSPORT-ASSOCIATED PHOSPHORYLATION

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SUMMARY

1. α-Methylglucoside transport in *Saccharomyces cerevisiae* Strain NCYC 240 was studied in uninduced and in maltose-induced cells. It appeared that active transport occurred in induced cells, apparently *via* the maltose permease.

2. α-Methylglucoside taken up by induced cells was recovered in cellular extracts partly as the free sugar, partly as a phosphate ester. The ratio free α-methylglucoside/α-methylglucoside phosphate in the cells increased during incubation in the course of time.

3. During de-adaptation the α-methylglucoside-phosphorylating capacity of the cells decreased linearly with the maltose and α-methylglucoside transport capacity.

4. With ^{14}C-labeled α-methylglucoside pulsing experiments, it appeared that shortly after addition of the radioactive pulsing dose the specific activity of the intracellular α-methylglucoside phosphate fraction exceeded the specific activity of the free fraction. This demonstrates that the phosphate ester is the precursor of the intracellular free α-methylglucoside.

5. The experimental results indicated transport-associated phosphorylation of α-methylglucoside, with subsequent release of α-methylglucoside phosphate at the inner face of the membrane. The enzyme catalyzing α-methylglucoside phosphorylation is apparently an integral part of the active transport system.

INTRODUCTION

In previous papers experimental evidence has been presented indicating transport-associated phosphorylation of glucose and 2-deoxy-D-glucose in yeast[1,2]. This active transport mechanism is essentially different from facilitated diffusion, as observed with some other sugars like sorbose[3], although the same carrier is sometimes involved both in active and in facilitated diffusion transport[4–6].

To confirm the hypothesis of transport-associated phosphorylation it appeared worthwhile to study active transport of a nonmetabolized substrate *via* an inducible transport system. The interpretation of experimental results would be further facilitated if the substrate could not be phosphorylated by constitutive enzymes, like hexokinase.

α-Methylglucoside appeared to be a suitable substrate in such studies. *Saccharomyces cerevisiae* Strain NCYC 240 has no constitutive transport system for this sugar.

An active transport system is induced, however, by growth on a maltose-containing medium. In the present paper the active transport of α-methylglucoside in induced yeast will be described.

METHODS

Saccharomyces cerevisiae Strain NCYC 240 was grown at 27° during 48 h on a medium containing 0.5 % peptone, 0.3 % yeast extract, 0.1 % tryptone and either 1 % glucose (uninduced cells) or 1 % maltose (induced cells). The yeast was harvested by centrifugation and washed several times with distilled water.

Yeast suspensions were buffered at pH 5.0 with triethylamine–succinate–tartrate[7]. In uptake studies cells and medium were separated by Millipore filtration, followed by washing of the cells on the filter with ice-cold water. Preparation of yeast extracts and paper-chromatography of these extracts were performed as described previously[2]. Thin-layer chromatograms of cell contents were prepared as described by KABACK[8]. Yeast extracts for ATP determinations were prepared as described by FELDHEIM *et al.*[9]. ATP was measured by the firefly tail luciferin–luciferase system, according to the method of ADDANKI *et al.*[10]. Hexokinase was isolated, purified and assayed according to DARROW AND COLOWICK[11]. Fermentation was measured by the standard Warburg technique. Disintegrated cells for the assay of enzyme activities were prepared by freezing in liquid air and thawing. Maltase activity of disintegrated cells was measured with *p*-nitrophenyl-α-D-glucopyranoside as substrate, as described by KHAN AND EATON[12]. ^{14}C-labeled α-methylglucoside was measured in a liquid-scintillation counter, with the liquid scintillator described by BRAY[13].

De-adaptation was brought about by incubation at 25° in the described glucose-containing nutrient medium.

RESULTS

Uninduced intact and disintegrated yeast cells did not metabolize maltose, as could be shown by manometric measurements. Moreover, no maltase activity could be demonstrated in cell-free extracts. After induction both intact cells and disintegrated cells metabolized maltose; cell-free extracts of this induced yeast displayed a high maltase activity.

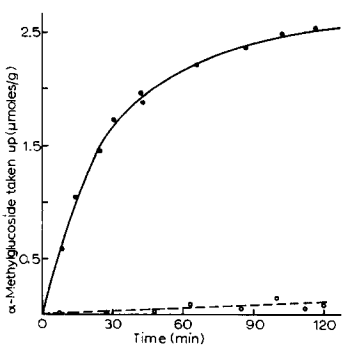
When induced or uninduced yeast cells are incubated with ^{14}C-labeled α-methylglucoside, the total amount of radioactivity in the suspension remains constant for at least 6 h. Apparently this sugar is not metabolized. This could be confirmed by manometric measurements with high yeast concentrations: no trace of fermentation or respiration was found. Cell-free extracts of induced cells did not hydrolyze α-methylglucoside; apparently the maltase activity in induced cells is not the expression of a general α-glucosidase activity.

The mechanism of α-methylglucoside transport is evidently different in different yeast strains[4,5,14]. In Strain NCYC 240 α-methylglucoside appears to be taken up *via* the inducible maltose transport system. This could be shown by measurements of α-methylglucoside uptake in induced and uninduced cells (Fig. 1). Further control experiments, not presented here in detail, confirmed α-methylglucoside uptake *via* the maltose transport system: (1) α-Methylglucoside inhibited maltose transport but

not glucose and sorbose transport (sorbose is taken up *via* the same carrier as glucose in this yeast strain). (2) α-Methylglucoside did not cause sorbose countertransport. (3) as will be discussed in a forthcoming paper, the maltose transport system is much more sensitive to sulfhydryl reagents than the glucose transport system. α-Methylglucoside transport showed the typical sensitivity of the maltose transport system with reference to sulfhydryl reagents. (4) During de-adaptation the α-methylglucoside uptake decreased exactly parallel to the decreasing capacity to take up maltose. During these de-adaptation experiments no substantial change of intracellular maltase and glycolytic enzyme activities took place.

Analysis of extracts of induced yeast cells after preincubation with ^{14}C-labeled α-methylglucoside had the following results. On paper and thin-layer chromatograms of the extracts two distinct spots could be recognized: one corresponding to the free sugar and the second remaining at the origin, like α-methylglucoside phosphate[8]. If the extract was treated with the barium–zinc reagent of SOMOGYI[15] and AUGUSTIN AND HOFMANN[16], part of the radioactivity was precipitated. Chromatography of the supernatant of barium–zinc-treated extracts revealed that the spot corresponding to the free sugar was not influenced by the reagent, whereas the second spot had disappeared completely. After pretreatment of extracts with a highly purified alkaline phosphatase (10 μg per 2 ml extract, 1 h at 37°) a subsequent addition of the barium–zinc reagent caused no precipitation of radioactivity. Moreover, all radioactivity was recovered on chromatograms at the position of the free sugar. In control experiments the phosphatase activity was destroyed by heating the enzyme solution for 20 min in a boiling water bath. This denaturated enzyme had no effect on the extracts (Fig. 2). These experiments indicate that α-methylglucoside occurs inside the cells partly as the free sugar, partly as α-methylglucoside phosphate. The two fractions can be separated by means of the barium–zinc reagent.

At low α-methylglucoside concentrations uphill transport of free sugar occurs in induced cells, as shown in Fig. 3. The ratio of the intracellular concentrations free α-methylglucoside/α-methylglucoside phosphate increased gradually during the



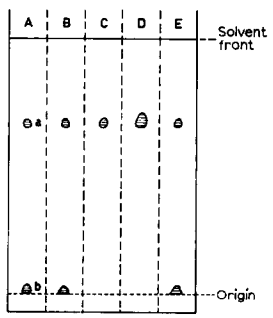


Fig. 1. α-Methylglucoside uptake in uninduced (○---○) and in maltose-induced yeast (●—●) at 25°. Yeast concentration: 5%; α-methylglucoside concentration: 0.8 mM.

Fig. 2. Outline of thin-layer chromatogram of yeast cell extracts. Solvent system: chloroform–methanol–water (60:70:26, by vol.). A, referents (a = free α-methylglucoside; b = α-methylglucoside phosphate, ref. 8); B, normal yeast extract; C, yeast extract treated with barium–zinc reagent; D, yeast extract treated with alkaline phosphatase; E, yeast extract treated with denaturated alkaline phosphatase. With paper chromatography similar results were obtained.

incubation period, without a concomitant change of the ATP concentration (Fig. 4).

Measurement of the initial rate of α-methylglucoside transport at varying substrate concentrations revealed Michaelis–Menten kinetics, as shown in Fig. 5. In fully induced yeast a K_m value of 8 mM and a V_{max} of 0.2 mmole/g per h was found. During de-adaptation a gradual decrease of V_{max} without a concomitant change of K_m took place. Further, it appeared that the ratio of the intracellular concentrations: total α-methylglucoside/α-methylglucoside phosphate, at a fixed α-methylglucoside concentration in the medium and a fixed incubation time, did not change during de-adaptation (Table I). This indicates that the phosphorylating capacity with respect to α-methylglucoside decreased linearly with the transport capacity during de-adaptation.

A few preliminary experiments were performed to localize the α-methylglucoside phosphorylating system. Induced yeast cells, disintegrated by freezing and thawing, caused a significant phosphorylation of α-methylglucoside. After centrifugation the supernatant appeared to contain maltase and the glycolytic enzymes, as shown by

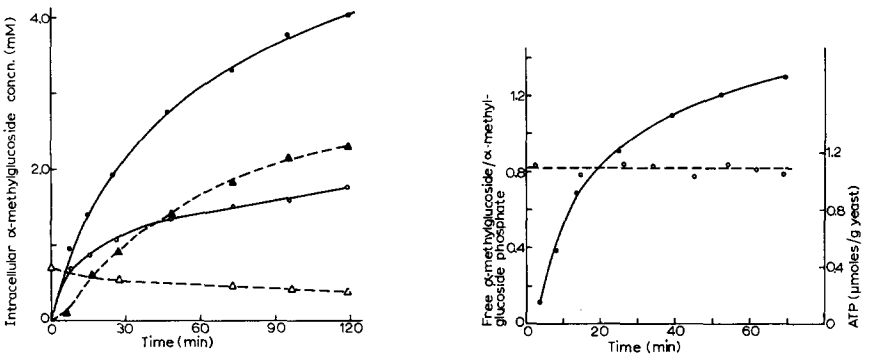


Fig. 3. α-Methylglucoside uptake in the course of time. Yeast concentration, 5%; initial α-methylglucoside concentration in the medium, 0.7 mM; temperature, 25°. ●—●, total intracellular α-methylglucoside concentration; ○—○, intracellular α-methylglucoside phosphate concentration; ▲---▲, intracellular free α-methylglucoside concentration; △---△, α-methylglucoside concentration in the medium.

Fig. 4. The ratio of the intracellular concentrations of free α-methylglucoside/α-methylglucoside phosphate (●—●) and the ATP concentration (○---○) in the course of time. Experimental conditions: see legend to Fig. 3.

TABLE I

TOTAL UPTAKE OF α-METHYLGLUCOSIDE AND THE RATIO TOTAL INTRACELLULAR α-METHYLGLUCOSIDE /α-METHYLGLUCOSIDE PHOSPHATE AFTER VARYING PERIODS OF DE-ADAPTATION

Measurements were made after 10 min incubation with 8 mM α-methylglucoside at 25°.

De-adaptation period (min)	*Total uptake α-methylglucoside (μmoles/g)*	*α-Methylglucoside phosphate (μmoles/g)*	*Ratio total α-methylglucoside/α-methylglucoside phosphate*
0	6.00	2.94	2.04
30	4.32	2.24	1.93
60	3.67	1.86	1.97
120	2.80	1.33	2.10
180	1.94	0.96	2.02

the fermentation of maltose by the supernatant. The supernatant failed, however, to phosphorylate α-methylglucoside. Crystalline hexokinase, prepared from this induced yeast strain, likewise did not catalyze the phosphorylation of α-methylglucoside. Finally, disintegrated uninduced yeast cells did not catalyze the phosphorylation of α-methylglucoside.

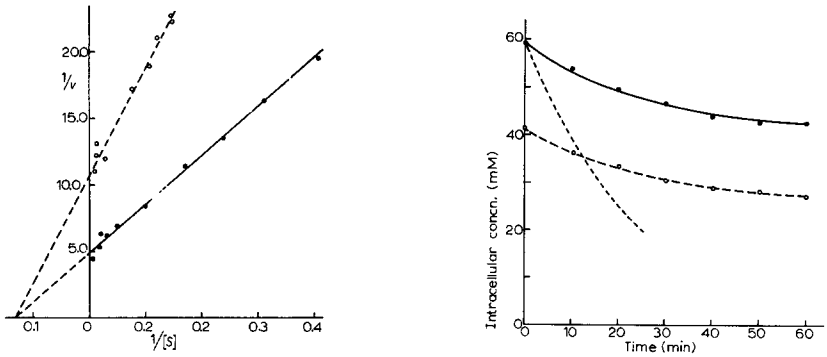


Fig. 5. Kinetics of α-methylglucoside transport at 25°, pH 5.0 The substrate concentration is expressed in mM, the rate of transport in mmoles/g yeast per h, as calculated from initial transport rates over a 30-sec interval. ●—●, induced yeast cells; ○---○, after 2 h de-adaptation.

Fig. 6. α-Methylglucoside efflux at 25°. Yeast cells were preloaded by incubation in 50 mM α-methylglucoside during 60 min and subsequently resuspended (5% cells) in distilled water. ●—●, total intracellular α-methylglucoside; ○---○, free intracellular α-methylglucoside. The dotted line represents the predicted efflux, calculated from Eqn. 1, equating S_1 with the intracellular concentration of free α-methylglucoside.

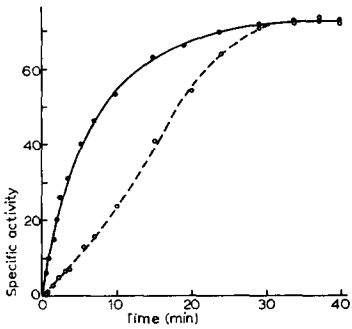
If yeast cells preloaded with α-methylglucoside are resuspended in distilled water, a slow efflux of the sugar occurs (Fig. 6). Adapting the K_m value of 8 mM, found for α-methylglucoside influx, the efflux velocity appeared to be much slower than predicted from the equation of carrier-mediated facilitated diffusion[17]:

$$v = V_{max}\left(\frac{[S_0]}{[S_0] + K_m} - \frac{[S_1]}{[S_1] + K_m}\right) \quad (1)$$

where v = net transport velocity; V_{max} = maximal transport velocity; $[S_0]$ = sugar concentration in the medium; $[S_1]$ = sugar concentration inside the cells; K_m = Michaelis–Menten constant. This is also true if only the intracellular concentration of free α-methylglucoside is considered (Fig. 6).

In further experiments yeast cells were preincubated with varying concentrations of unlabeled α-methylglucoside for periods of 10–60 min, followed by addition of a pulsing dose of ^{14}C-labeled α-methylglucoside to the medium. The cell contents were subsequently analyzed at intervals for radioactivity in the free and phosphorylated α-methylglucoside fractions. The total amount of intracellular free and phosphorylated α-methylglucoside was measured simultaneously in a parallel experiment, in which the experimental conditions were identical except that ^{14}C-labeled α-methylglucoside was used throughout the whole experiment. From the results the specific activities in both fractions in the first experiment could be calculated (defined as the ratio radioactivity/total amount of α-methylglucoside in that fraction, using arbitrary units). A typical experiment is depicted in Figs. 7 and 8. Qualitatively the results

appeared to be independent of the preincubation period, the α-methylglucoside concentration and the amount of radioactive α-methylglucoside used as pulsing dose. In all experiments the specific activity of the α-methylglucoside phosphate fraction was, shortly after addition of the pulsing dose, considerably higher than the specific activity of the free fraction (Table II).



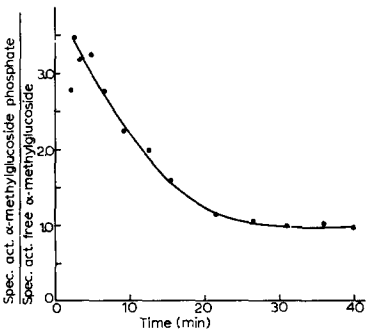


Fig. 7. Specific activity of intracellular α-methylglucoside phosphate (●—●) and free α-methylglucoside (○---○) after pulsing with ^{14}C-labeled α-methylglucoside. Preincubation: 30 min at 25° in 1 mM α-methylglucoside. A pulsing dose of ^{14}C-labeled α-methylglucoside was added at zero time.

Fig. 8. Ratio of the specific activities of intracellular α-methylglucoside phosphate/free α-methylglucoside, after pulsing with ^{14}C-labeled α-methylglucoside. Experimental conditions: see legend to Fig. 7.

TABLE II

RATIO OF THE SPECIFIC ACTIVITIES OF INTRACELLULAR α-METHYLGLUCOSIDE PHOSPHATE/FREE α-METHYLGLUCOSIDE AFTER PULSING WITH ^{14}C-LABELED α-METHYLGLUCOSIDE AT DIFFERENT SUBSTRATE CONCENTRATIONS IN THE MEDIUM

Preincubation: 30 min at 25°. A pulsing dose of ^{14}C-labeled α-methylglucoside was added at zero time.

Concentration α-methylglucoside in medium (mM)	*α-Methylglucoside phosphate/ α-methylglucoside at zero time*	*Ratio specific activities α-methylglucoside phosphate/ free α-methylglucoside after (min):*				
		2	*4*	*10*	*20*	*60*
0.5	8.05	4.1	3.2	1.8	1.0	1.0
1.0	2.52	3.4	2.9	2.0	1.1	1.0
10.0	1.21	3.1	3.0	2.7	1.7	1.0
60.0	0.48	2.9	2.9	2.6	1.8	1.0

DISCUSSION

The existence of an inducible maltose permease system in yeast, Strain NCYC 240 has already been demonstrated by HARRIS AND THOMPSON[14]. α-Methylglucoside uptake studies in induced and uninduced yeast indicated transport of this sugar *via* this maltose permease system. Whereas α-methylglucoside uptake in uninduced yeast is virtually negligible, the uptake in induced cells occurs against a concentration gradient, indicating active transport according to the generally accepted criteria[18,19].

Cell growth on a maltose medium apparently induces both the synthesis of the maltose permease system and the synthesis of maltase. This enzyme appeared to be maltose specific, in the sense that α-methylglucoside is not hydrolyzed by this enzyme. The independent induction of maltase and α-glucosidase in some yeast strains has been described already by LINDEGREN AND LINDEGREN[20]. The only measurable conversion of α-methylglucoside in this induced yeast concerns phosphorylation, as shown in RESULTS. Therefore, α-methylglucoside appeared to be a good substrate to confirm the previously proposed mechanism of transport-associated phosphorylation. The presence of free and phosphorylated α-methylglucoside in cellular extracts can be explained by two possible mechanisms: (1) transmembrane transport of free α-methylglucoside, followed by a partial intracellular phosphorylation; or, (2) phosphorylation of α-methylglucoside as an imperative reaction associated with transmembrane transport, with subsequent release of α-methylglucoside phosphate at the inner face of the membrane, followed by some intracellular dephosphorylation. The close parallel between the transport velocity and the α-methylglucoside-phosphorylating capacity during de-adaptation, as shown in Table I, already suggests an association of these two processes. The shift of the ratio of the intracellular concentrations of free α-methylglucoside/α-methylglucoside phosphate in the course of time (Fig. 4) points to the same conclusion. This shift can hardly be explained by exhaustion of intracellular high-energy phosphate; the ratio always increased, irrespective of the initial α-methylglucoside concentration (0.5–100 mM). Especially at very low α-methylglucoside concentrations the total amount of α-methylglucoside phosphate could hardly have exhausted high-energy phosphate sources, as shown *e.g.* by the constant ATP level (Fig. 4). It seems more likely that the increase of the ratio free α-methylglucoside/α-methylglucoside phosphate is a consequence of the fact that the substrate enters the cell initially as α-methylglucoside phosphate and is subsequently dephosphorylated slowly in the course of time.

More conclusive evidence was obtained in the ^{14}C-labeled α-methylglucoside pulsing experiments. As shown, specific activity measurements of the free and phosphorylated fractions indicated that α-methylglucoside phosphate is the intracellular precursor of intracellular free α-methylglucoside. This is in accordance with transport-associated phosphorylation, followed by intracellular dephosphorylation, and not with transport of free sugar followed by intracellular phosphorylation. The only alternative explanation would be the existence of intracellular compartments with an asymmetric distribution of free and phosphorylated α-methylglucoside. In that case it should be assumed that free α-methylglucoside is initially transported into a distinct intracellular compartment, in which phosphorylation takes place. If free α-methylglucoside can penetrate into other compartments, inaccessible to α-methylglucoside phosphate, the same course of the specific activities after pulsing would be possible. There is no experimental evidence, however, indicating asymmetric intracellular distribution of free and phosphorylated sugars. Moreover, in this case the course of the specific activities would be the kinetic result of several processes: (1) the uptake of sugar in the first compartment; (2) the phosphorylation of the sugar in this compartment; and (3) the penetration of free sugar into other compartments. Then it should be expected that the length of the preincubation interval, the initial α-methylglucoside concentration and the concentration of the ^{14}C-labeled α-methylglucoside pulsing dose would have a pronounced influence on the observed phenomena. This did

not appear to be the case in a series of experiments in which the initial α-methylglucoside concentration was varied from 0.5 to 100 mM, the preincubation period from 10 to 120 min and the pulsing dose from 0.0005 to 20 mM. Finally, in a previous paper experimental results were presented indicating that the existence of intracellular compartments, causing asymmetric solute distribution, is very improbable[21].

Basically two explanations of the observed α-methylglucoside phosphorylation in induced cells would be possible. Either uninduced cells are cryptic with regard to α-methylglucoside phosphorylation and this cryptic quality disappears in the synthesis of the maltose permease, or the phosphorylation is catalyzed by an induced enzyme. As shown, hexokinase isolated from the induced yeast fails to phosphorylate α-methyl glucoside. This is in agreement with the observations of SOLS *et al.*[22]. Moreover, disintegrated uninduced cells did not catalyze the phosphorylation of α-methylglucoside. This rules out the first possibility. Consequently the phosphorylation must be caused by an induced enzyme. According to present knowledge, adaptation of yeast to growth on a maltose medium induces the synthesis of a maltose permease system and of maltase or α-glucosidase. As the observed α-methylglucoside phosphorylation cannot be ascribed to maltase, the most obvious interpretation appears to be that the induced phosphorylating enzyme is an integral part of the permease system. This would be in agreement with the proposed mechanism of transport-associated phosphorylation of actively transported sugars in yeast.

The occurrence of a phosphorylation associated with the transport mechanism means that α-methylglucoside transport cannot be described as a simple facilitated diffusion. The α-methylglucoside efflux measurements as depicted in Fig. 6 indicate concordantly a more complex situation: efflux is much slower as expected from the calculated kinetics of facilitated diffusion.

Apparently the transport mechanism for α-methylglucoside in this induced yeast is quite similar to the transport mechanism for 2-deoxy-D-glucose, as described previously[2]. The situation that α-methylglucoside is not a substrate of hexokinase or an other constitutive phosphorylating enzyme and that the transport system for this sugar is not constitutive but inducible facilitates characterization of the phosphorylating enzyme as part of the permease system.

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