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TRANSPORT-ASSOCIATED PHOSPHORYLATION OF 2-DEOXY-D-GLUCOSE IN *SACCHAROMYCES FRAGILIS*

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SUMMARY

2-Deoxy-D-glucose transport and metabolism was studied in *Saccharomyces fragilis*. Inside the cells four phosphorylated and three non-phosphorylated derivatives were found and identified. Accumulation of phosphorylated 2-deoxyglucose derivatives was balanced by a concomitant decrease of cellular ATP, orthophosphate and polyphosphates.

The free sugar was concentrated against a concentration gradient, contradicting facilitated diffusion. Pulse labeling experiments revealed transport-associated phosphorylation.

Theoretical considerations and analysis of the effects of iodoacetate showed that an intracellular hexokinase activity was not involved in 2-deoxyglucose phosphorylation, although this sugar is a good substrate for the enzyme in in vitro experiments.

INTRODUCTION

In previous papers it was shown that some sugars enter the yeast cell via facilitated diffusion, whereas other sugars are taken up via an active transport system [1–3]. Experimental evidence indicated transport-associated phosphorylation of actively transported sugars in yeast [4, 5]. The hypothesis of transport-associated phosphorylation was based, among others, on pulse labeling experiments with actively transported sugars.

In a recent paper the interpretation of these experiments was questioned by Kotyk and Michaljanicova [6]. Under similar experimental conditions these authors reached different results. These results were interpreted as indicating facilitated diffusion of the free sugar, with subsequent intracellular phosphorylation. To reach this conclusion some unproven assumptions had to be made. Further it was argued that our previous experiments could also be interpreted as indicating facilitated diffusion, assuming that some intracellular metabolic reactions had escaped our attention.

Abbreviations: Glc, glucose; dGlc, 2-deoxyglucose.

To elucidate this problem, further studies on sugar transport were performed. As the transport system of 2-deoxy-D-glucose (dGlc) in *Saccharomyces fragilis* showed some of the characteristics described by Kotyk and Michaljanicova [6], it was studied in detail. The experimental results are discussed in the present communication.

METHODS

S. fragilis was grown and harvested as described before [7]. Incubations with ^{14}C-labeled dGlc were carried out at 25 °C either in 10 mM Tris/citric acid buffer, pH 4.5, or in distilled water. Identical results were obtained in these two media. Cells and medium were separated at appropriate times as described before [7], utilizing S and S BA 85, Selectron, cellulose nitrate filters. Cell extracts for sugar analysis were prepared by treating the cells with 3 vols of absolute ethanol for 1 h at 4 °C, followed by the addition of an equal volume of distilled water. Alternative extraction procedures with boiling ethanol or boiling water gave identical results.

Precipitation of phosphorylated derivatives in the extracts was accomplished with the barium/zinc reagent of Somogyi [8], as described by Augustin and Hofmann [9]. The concentration of the $ZnSO_4$ solution was adjusted to yield a final pH value of 7.0–7.5 in the supernatant of the extract.

Radioactivity of the extracts was measured in a liquid scintillation counter. For paper chromatographic analysis the extracts were freeze-dried. The residue was dissolved in a small volume of water. Aliquots were applied to S and S 2043b paper. Descending chromatography was done with the solvent system *n*-propanol/ammonia/water (6 : 1 : 3, v/v). In a few experiments, to confirm the identity of some of the radioactive derivatives, other solvent systems were used, e.g. ethyl acetate/acetic acid/water (3 : 3 : 1, v/v). Autoradiograms were made by placing the dried chromatogram in close contact with X-ray film for 7–14 days. The chromatograms were subsequently cut into small strips, guided by the blackening of the radiogram. The radioactivity of the strips was measured quantitatively in a liquid scintillation counter.

To calculate intracellular sugar concentrations, the intracellular water volume was measured as described before [10]. A value of 0.53 ml/g yeast (wet weight) was found.

Model experiments on hexokinase were performed with the enzyme isolated from yeast according to Darrow and Colowick [11] and on hexokinase in situ, after treatment of the cells with protamine sulphate as described by Schlenk and Zydek-Cwick [12] and Reitzer and Neet [13]. Enzyme activity was calculated from the production of sugar phosphate, utilizing ^{14}C-labeled sugars as substrate. The enzyme reactions were conducted in 0.1 M triethanolamine buffer, pH 6.6, equal to the intracellular pH. The intracellular pH value was measured according to Borst-Pauwels [14]. Equilibrium isotope exchange experiments with hexokinase were performed as described by Kosow and Rose [15].

Intracellular ATP concentrations were measured as described before [5]. Polyphosphates were assayed according to Lohmann and Langen [16]. Orthophosphate was measured by the method of Fiske and SubbaRow, as modified by Meyerhof and Oesper [17].

dGlc was measured enzymatically with the glucose oxidase method, as modified by Washko and Rice [18], utilizing Glucostat special (Worthington) and chemically

by the method of Waravdekar and Saslaw [19]. 2-Deoxy-D-galactose measurements were performed with galactose oxidase (Boehringer test combination).

Respiration was measured with standard Warburg technique.

2-Deoxyglucose 6-phosphate was prepared from dGlc, with ATP and hexokinase; partial conversion to dGlc-1-phosphate was accomplished by the addition of phosphoglucomutase. dGlc-6-phosphate was converted to 2-deoxygluconic acid 6-phosphate with glucose-6-phosphate dehydrogenase and NADP. 2-Deoxy-D-gluconic acid was prepared either from dGlc, with glucose oxidase, or from 2-deoxy-D-gluconic acid 6-phosphate with alkaline phosphatase.

THEORETICAL

In pulse labeling experiments the yeast cells were preincubated with 1 mM unlabeled dGlc for about 15 min. In an identical parallel experiment, labeled dGlc was present from the beginning, to determine the intracellular concentrations of dGlc derivatives in the actual pulse experiment. After 15 min 90–95 % of the sugar has been transported into the yeast cells. No net uptake occurred during the next 15 min. Inside the cells free dGlc, dGlc-6-phosphate and five other dGlc derivatives were recovered (see Results). As the concentration of these metabolites did not change significantly between the 15th and 20th minute of the incubation period, the system may be considered as a stationary-state system. When a pulse of a chemically negligible amount of ^{14}C-labeled dGlc is applied after 15 min, the following observations are relevant: (1) The radioactive label enters the yeast cells with a virtually constant velocity during about 80 s, without net uptake of sugar from the medium, as judged from the parallel experiment. After 80 s the uptake of radioactivity starts to flatten out gradually. (2) During the first minutes after addition of the pulse the label is recovered in the cells in the free sugar fraction and in the dGlc-6-phosphate fraction mainly. The other fractions are either labeled very slowly or are negligible quantitatively. Assuming uptake of the free sugar, with subsequent intracellular phosphorylation to dGlc-6-phosphate, the system can thus be simplified during short-term pulse labeling experiments to the following model:

$$^{14}S_{out} \xrightarrow{v_0} S_{in} \underset{v_2}{\overset{v_1}{\rightleftharpoons}} S\text{-}P \qquad (1)$$

where S_{in} and S-P are the intracellular pools of free dGlc and dGlc-6-phosphate, respectively. Under stationary state conditions $v_1 = v_2$, thus, irrespective of the reaction mechanism,

$$k_1[S_{in}] = k_2[S\text{-}P] \qquad (2)$$

where k_1 and k_2 are the turnover constants of the pools of free dGlc and dGlc-6-phosphate, respectively. The ratio of the pools can be measured in the parallel experiment. If we put $[S\text{-}P]/[S_{in}] = a$, then:

$$k_1 = ak_2 \qquad (3)$$

If, during the stationary state, a pulse of ^{14}C-labeled dGlc is added to the medium, the isotope enters the cell with a virtually constant velocity during the first 80 s. The influx velocity, v_0, and the intracellular distribution of the label over the

S_{in} and S-P pools ([S'] and [S'P], respectively) can be measured experimentally. According to the described model:

$$v_0 t = [S'] + [S'P], \text{ and:} \tag{4}$$

$$d[S']/dt = v_0 - k_1[S'] + k_2[S'P] \tag{5}$$

From Eqns 3 and 4:

$$d[S']/dt = v_0 + k_2 v_0 t - k_2 (a+1)[S'] \tag{6}$$

Integration of this equation yields:

$$[S'] = \frac{v_0}{k_2(a+1)} \left\{ k_2 t + \frac{a}{a+1} \left(1 - e^{-k_2(a+1)t}\right) \right\} \tag{7}$$

From Eqns 3 and 7 the turnover constants k_1 and k_2 can be calculated.

RESULTS

The uptake of dGlc in *S. fragilis* proceeded linearly with time for about 30 s. After longer incubation periods the uptake curve flattened out. The total amount of radioactivity in the incubation mixture remained constant, even after incubation periods up to 6 h. Apparently no metabolic conversion to volatile products occurred.

Measurements of initial transport velocities (calculated from the uptake in 30 s) at varying dGlc concentrations in the medium revealed Michaelis-Menten kinetics (Fig. 1). From these experiments a V of about 0.5 mM/g per h and a K_m value of 0.9 mM could be calculated.

Paper chromatography of cell extracts, after an incubation period of 1 h, with subsequent autoradiography revealed seven spots with the solvent system *n*-propanol/ammonia/water (6 : 1 : 3, v/v) (Fig. 2). To identify these spots, paper chromatography with different solvent systems, co-chromatography with dGlc derivatives and chemical and enzymatic techniques were used.

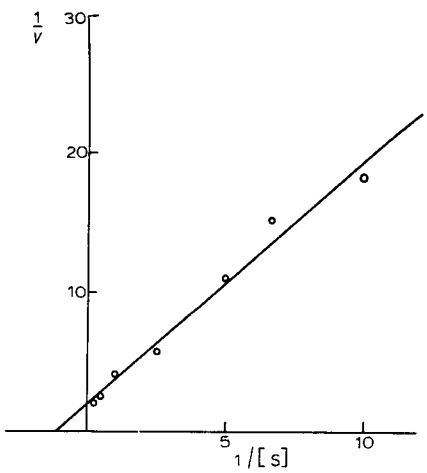

Fig. 1. Kinetics of dGlc transport in *S. fragilis*. Yeast concentration: 10 % (wet weight/volume).

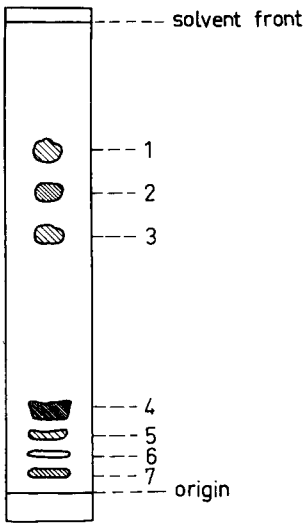

Fig. 2. Outline of paper chromatogram of yeast cell extract, obtained by autoradiography. Spot 1, 2,2′-dideoxy-α,α′-trehalose; spot 2, dGlc; spot 3, deoxy-gluconic acid; spot 4, dGlc-6-*P*; spot 5, dGlc-1-*P*; spot 6, UDP-dGlc; spot 7, dGlc-1,6-P_2.

Spot 1 could be identified as 2,2′-dideoxy-α,α′-trehalose, according to the criteria given by Farkas et al. [20]. Spot 2 is free dGlc, as shown by co-chromatography with pure dGlc, the positive glucose oxidase and thiobarbituric acid reaction of the eluted material and the positive perchloric acid/acetone reaction in situ, according to Weidemann and Fischer [21]. Spot 3 does not react with the perchloric acid-acetone reagent. The material is absorbed to anion exchange resins (DEAE-Sephadex) but is not precipitated by the barium/zinc reagent and not attacked by alkaline phosphatase. It seemed likely, therefore, that this derivative would be 2-deoxy-D-gluconic acid. This was confirmed by control experiments, including co-chromatography in two solvent systems with 2-deoxy-D-gluconic acid, prepared enzymatically from dGlc.

Spots 4–7 disappeared completely after treatment of the extract with an anion exchange resin, with the barium/zinc reagent, or with alkaline phosphatase. In the latter case all radioactivity of these spots shifted to the position of the free sugar. Thus these metabolites are phosphorylated compounds.

Spot 4 appeared to be resistant to hydrolysis in 0.05 M H_2SO_4 at 100 °C during 30 min. In paper chromatography it had the same mobility as enzymatically prepared 2-deoxy-D-glucose 6-phosphate. Therefore spot 4 was identified as dGlc-6-phosphate. Spot 5 was hydrolysed completely within 15 min in 0.05 M H_2SO_4 at 100 °C. Hydrolysis at pH 4 was only partial. This suggested that this fraction might be dGlc-1-phosphate. This was confirmed by co-chromatography with enzymatically prepared dGlc-1-phosphate.

Spot 6 was identified as UDP-dGlc according to the criteria described by Biely and Bauer [22]. Spot 7 is hydrolysed in 0.05 M H_2SO_4 at 100 °C to dGlc-6-phosphate and phosphoric acid. Together with the low chromatographic mobility this indicates that this metabolite is dGlc-1,6-diphosphate.

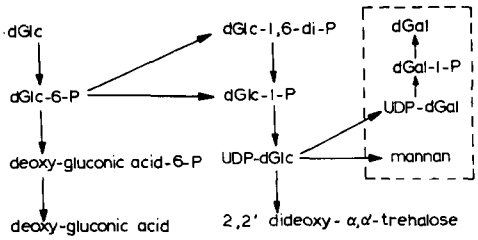

Fig. 3. Scheme of metabolic pathways of dGlc in yeast. The compounds within the dotted lines were not detectable under the present experimental conditions.

With two-dimensional chromatography of the extract with *n*-propanol/ammonia/water (6 : 1 : 3, v/v) in the first and ethyl acetate/acetic acid/water (3 : 3 : 1, v/v) in the second direction it appeared that spot 4 was split up into a main spot, corresponding to dGlc-6-phosphate and a small spot with a lower mobility in the second direction, containing about 4 % of the radioactivity of the original spot. This second spot may be 2-deoxy-D-gluconic acid 6-phosphate, considering the fact that it had the same mobility in both solvent systems as enzymatically prepared 2-deoxy-D-gluconic acid 6-phosphate.

These analytical results can be compared with the possible metabolic conversions of dGlc in yeast cells (see e.g. refs 20, 22 and 23) as depicted in Fig. 3. A crucial problem for the interpretation of our further experimental results was a possible conversion of dGlc metabolites to the corresponding 2-deoxy-D-galactose metabolites, via the Leloir pathway, as it could be shown that the corresponding dGlc and 2-deoxy-D-galactose derivatives would not be separated with the present chromatographic techniques. In model experiments it appeared that 2-deoxy-D-galactose reacted with galactose oxidase. No traces of galactose oxidase-positive material were found in the yeast extracts nor in the eluted material from spot 2. Small amounts of 2-deoxy-D-galactose, added to these samples caused a normal positive reaction, excluding a possible disturbance by the yeast extract. The same results were obtained after treatment of the extracts with alkaline phosphatase. All phosphorylated

TABLE I

INTRACELLULAR CONCENTRATION (μmol/ml) OF dGlc DERIVATIVES AND dGlc CONCENTRATION IN THE MEDIUM, AFTER VARYING INCUBATION PERIODS AT 25 °C

Initial dGlc concentration in the medium: 1.08 μmol/ml. Yeast concentration: 10 % (wet weight/volume).

	Incubation time			
	10 min	60 min	120 min	180 min
dGlc, medium	0.11	0.10	0.10	0.10
dGlc, intracellular	0.30	0.39	0.49	0.54
2-Deoxy-D-gluconic acid	0.07	0.22	0.46	0.95
2,2'-Dideoxy-α,α'-trehalose	0.07	0.77	1.84	3.20
dGlc-6-phosphate	11.34	9.78	8.70	7.24
dGlc-1-phosphate	4.90	5.25	4.69	4.25
UDP-dGlc	0.12	0.36	0.53	0.51
dGlc-1,6-diphosphate	0.22	0.24	0.29	0.31

compounds yielded dGlc as hydrolysis product, without detectable amounts of 2-deoxy-D-galactose. Considering the sensitivity of the galactose oxidase reaction it could be calculated that a conversion of about 2 % of the dGlc or dGlc-6-phosphate to the corresponding 2-deoxy-D-galactose derivative would have been measurable experimentally.

With an initial dGlc concentration in the medium of 1 mM and a yeast concentration of 10 % (wet weight/volume) 90–95 % of the sugar was taken up after 10 min. Beyond this point no further net uptake occurred. The concentration of dGlc derivatives inside the cell after varying incubation periods and the concentration of dGlc in the medium is shown in Table I. It is clear that the intracellular concentration of free sugar exceeds the medium concentration.

Simultaneous measurements of phosphorylated dGlc derivatives, ATP, orthophosphate and polyphosphates in the cell during incubation demonstrated that the accumulation of sugar phosphates is balanced by a decrease of ATP, orthophosphate and polyphosphates (Fig. 4).

Starvation of the cells during 44 h under aerobic conditions prior to the uptake experiments had little effect on dGlc uptake. Both the initial uptake velocity and the total uptake decreased about 8 %. Uptake in normal yeast under anaerobic conditions is inhibited to the same extent. Transport of dGlc into starved cells under anaerobic conditions is inhibited considerably, however (Fig. 5). In Fig. 6 the intracellular concentration of the free sugar is shown in the course of time in normal cells and in starved cells under aerobic and anaerobic conditions.

Uptake and phosphorylation of dGlc had only a limited influence on the intracellular ATP concentration under various experimental conditions and, conversely, the initial ATP concentration and rate of endogenous respiration had only a limited effect on the velocity of dGlc phosphorylation, as shown in Table II. In this table an

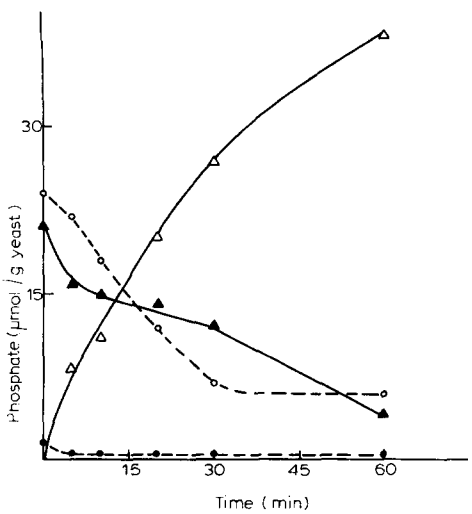

Fig. 4. Intracellular concentrations of dGlc-phosphates (△), orthophoshate (○), polyphosphates (▲) and ATP (●), in the course of time. Yeast concentration 10 %; ethanol concentration 1 %; initial dGlc concentration in the medium, 10 mM.

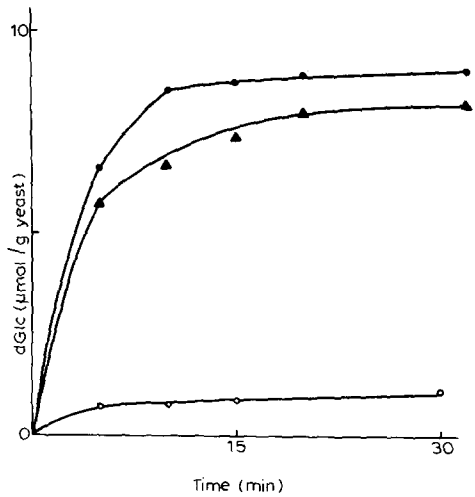

Fig. 5. Total dGlc uptake in normal cells under aerobic conditions (●), starved cells under aerobic conditions (▲) and starved cells under anaerobic conditions (○).

ATP/O quotient of 3 was adopted. If an ATP/O quotient of 2 is assumed (as found in most yeast strains) the discrepancy between ATP production and dGlc phosphorylation in starved cells is even more pronounced. Studies on the activity of hexokinase in situ on the other hand revealed that the enzyme activity is influenced rather strongly by the ATP concentration in this range (Table III).

In countertransport studies, cells preloaded with dGlc were transferred to a medium with 300 mM glucose. The resultant efflux of dGlc is shown in Fig. 7. Paper

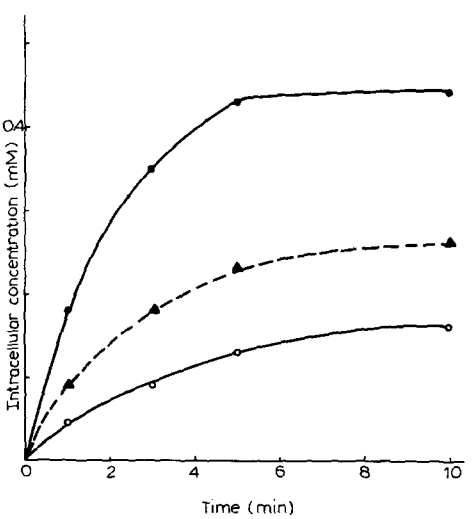

Fig. 6. Accumulation of free dGlc in normal cells under aerobic conditions (●), starved cells under aerobic conditions (▲) and starved cells under anaerobic conditions (○).

TABLE II

ATP CONCENTRATION, ENDOGENOUS RESPIRATION AND dGlc PHOSPHORYLATION UNDER VARIOUS EXPERIMENTAL CONDITIONS

Yeast concentration: 5 %. Endogenous respiration is expressed in ATP production (ATP/O = 3).

Experimental conditions				ATP concn (μmol/g)			ATP production, (μmol/g per min)	dGlc phosphorylation, (μmol/g per min)
Yeast: normal/ starved	Aerobic/ anaerobic	dGlc	Iodoacetate	0 min	3 min	30 min		
Normal	aerobic	–	–	1.60	1.22	1.18	12.5	
Normal	aerobic	10 mM	–	1.62	1.17	0.97	13.5	6.8
Normal	anaerobic	–	–	0.72	0.70	0.65		
Normal	anaerobic	10 mM	–	0.70	0.65	0.40		6.3
Normal	aerobic	–	4 mM	1.61	1.00	0.02		
Normal	aerobic	10 mM	4 mM	1.60	0.63	0.01		
Starved	aerobic	–	–	0.35	0.45	0.35	2.0	
Starved	aerobic	10 mM	–	0.30	0.25	0.10	2.0	6.2

chromatography of cellular extracts revealed a continuous decrease of the dGlc-6-phosphate fraction, with no concomitant loss of free sugar from the cells.

Pulse labeling experiments during the stationary state of cells, preincubated with unlabeled dGlc gave varying results. In some experiments the specific activity of the dGlc-6-phosphate fraction increased faster than the specific activity of the free fraction (Fig. 8). In other experiments, however, the specific activity of the free fraction increased faster initially, to be subsequently surpassed by the specific activity of the dGlc-6-phosphate fraction (Fig. 9). As this is impossible theoretically, an experimental artefact must be present. Assuming the first part of the curve in Fig. 9 to be correct, this would indicate uptake of free sugar, with subsequent intracellular phosphorylation via the hexokinase reaction. In that case the turnover constant k_2 of the phosphorylated fraction in the cells can be calculated from the initial values of the specific activities, according to Eqn 7 (Theoretical). Taking the values 10–15 s

TABLE III

INITIAL HEXOKINASE ACTIVITY OF A YEAST GHOST PREPARATION, AT VARYING ATP AND dGlc CONCENTRATIONS

With 4.0 mM ATP and 4.0 mM glucose an enzyme activity of 10.2 μmol/g per min was measured.

ATP (mM)	dGlc (mM)	Enzyme activity (μmol/g per min)
4.0	4.0	9.8
2.0	4.0	8.7
0.2	4.0	2.9
4.0	0.4	5.9
2.0	0.4	4.8
0.2	0.4	1.6

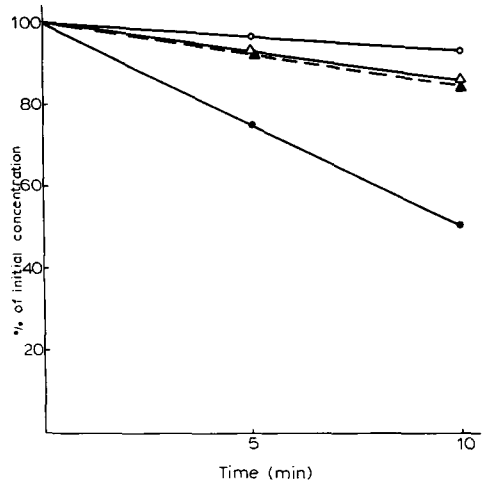

Fig. 7. dGlc counter transport induced by glucose. Intracellular concentrations of dGlc and dGlc-6-*P* were calculated after paper chromatographic separation and subsequent measurement of radioactivity of the individual spots. ○, dGlc-6-*P*, control; △, dGlc, control; ●, dGlc-6-*P*, after addition of 300 mM glucose to the medium; ▲, dGlc, after addition of 300 mM glucose to the medium.

after addition of the pulse, k_2 values of 1.5–1.8 min^{-1} were calculated in seven different experiments. Further, considering the fact that the uptake of radioactivity is virtually linear with time during 80 s, the expected specific activities after varying periods can be calculated with Eqn 7, from the isotope distribution as measured, e.g. 10 s after addition of the pulse. Such a theoretical curve is shown in Fig. 10. Putting the specific activities at unity in the case of complete equilibration, a considerable overshoot must be expected in the free fraction, followed by a gradual decrease to the unity level. A curve with these characteristics was never found experimentally, however.

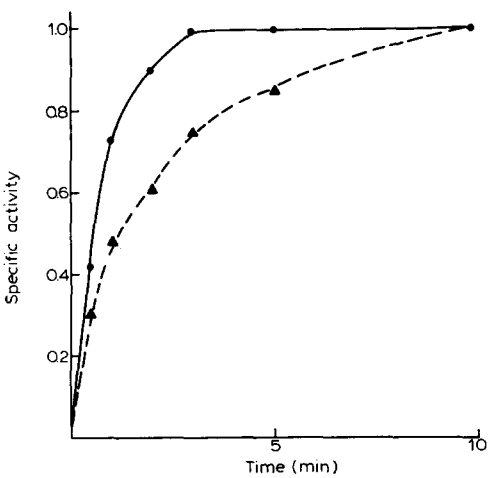

Fig. 8. Specific activity of intracellular dGlc-6-*P* (●) and free dGlc (▲) after pulsing with ^{14}C-labeled dGlc.

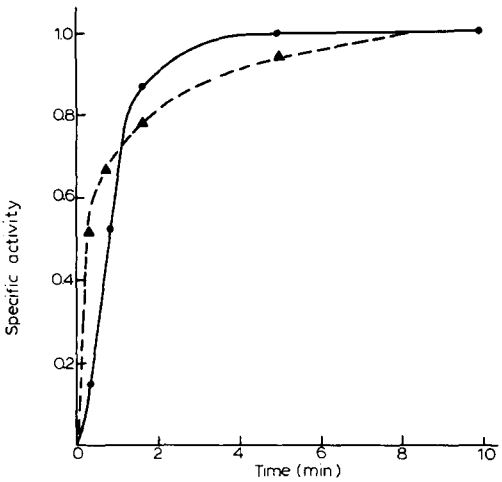

Fig. 9. Specific activity of intracellular dGlc-6-*P* (●) and free dGlc (▲) after pulsing with ^{14}C-labeled dGlc.

In all these experiments the yeast cells and the filter were immersed together in the extracting fluid, as described under Methods. In further experiments two modifications were tried. In some experiments the yeast was removed from the filter with a sharp device, prior to immersion in the extracting fluid. This action took only a few seconds. In other experiments the filter was immersed in the fluid together with the yeast, but removed again after a few seconds of vigorous agitation on a Vortex mixer, to rinse off the yeast cells. In both cases only pulse curves of the type shown in Fig. 8 were found. This indicates that the curves as depicted in Fig. 9 are created by an experimental error, caused by adsorption of radioactivity to the filter. The experimental consequences of this artefact will be discussed below.

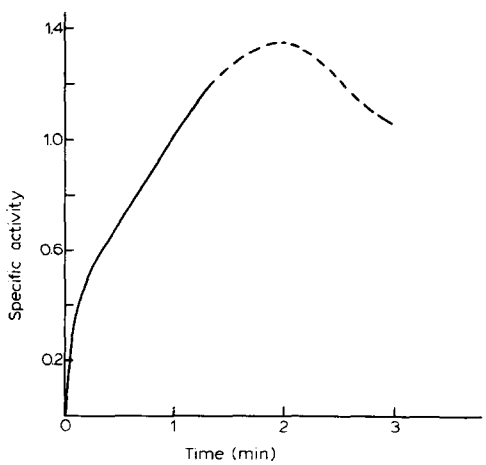

Fig. 10. Theoretical curve representing the specific activity of intracellular free dGlc after pulsing with ^{14}C-labeled dGlc, as calculated from Eqn 7. The dotted part of the curve is an approximation based on experimental data and Eqn 7, considering the fact that the uptake begins to flatten out after 80 s.

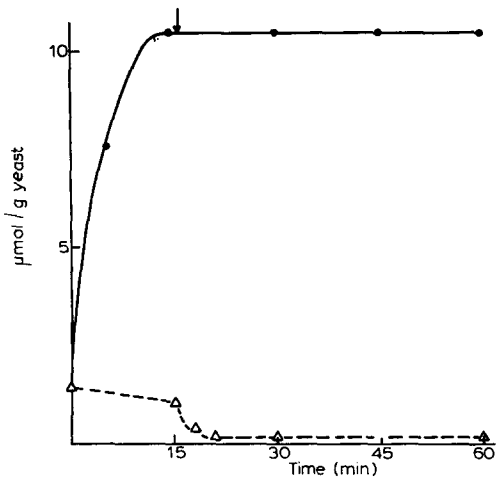

Fig. 11. The influence of iodoacetate on the intracellular concentration of dGlc-6-*P* (●) and ATP (△). 4 mM iodoacetate was added after 16 min (arrow) to a 5% yeast suspension, preincubated with 1 mM dGlc.

In further experiments the influence of iodoacetate was studied. Both with purified yeast hexokinase and with the hexokinase in situ preparations it appeared that iodoacetate did not inhibit the enzyme activity. The equilibrium isotope exchange reaction was also unaffected. If iodoacetate is added to cells, preincubated with dGlc during 10–15 min, no effect on the concentration of the intracellular dGlc-6-phosphate was observed (Fig. 11). The intracellular ATP level decreased to very low levels, however (Fig. 11). A similar decrease was observed in cells not preincubated with dGlc.

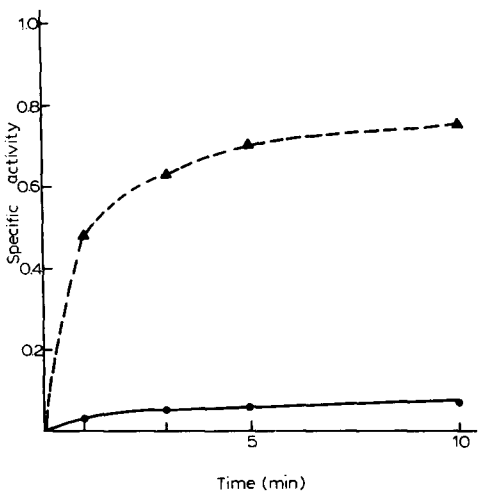

Fig. 12. Specific activity of intracellular dGlc-6-*P* (●) and free dGlc (▲) after pulsing with ^{14}C-labeled dGlc in the presence of 4 mM iodoacetate.

In other experiments cells were preincubated with unlabeled dGlc for 10 min before addition of iodoacetate. 5 min after addition of the poison a pulse dose of ^{14}C-labeled dGlc was added. As shown in Fig. 12, a slow uptake of radioactivity was observed, with much higher specific activities of the intracellular free sugar, as compared to the dGlc-6-phosphate fraction. Apparently a very slow transport of the free sugar takes place in the presence of iodoacetate. The velocity of this transport is two orders of magnitude smaller than the transport velocity in unpoisoned yeast. Moreover, in the absence of metabolic inhibitors this type of transport is presumably almost completely inhibited by the active transport process, as discussed previously [2].

DISCUSSION

Transport and metabolism of dGlc have been described in several yeast strains [4, 9, 20, 22–27]. Apparently there are notable differences between various yeast strains in this respect. Augustin and Hofmann [9] and van Steveninck [4] described transport and phosphorylation to dGlc-6-phosphate in two yeast strains. In a yeast strain described by Bauer and Biely [23] intracellular accumulation of dGlc-6-phosphate, dGlc-1-phosphate, UDP-dGlc, dGlc-1,6-diphosphate and 2-deoxy-D-gluconic acid was found. Only trace amounts of free dGlc if any were found inside the cells. Under aerobic conditions synthesis of 2,2′-dideoxy-α,α′-trehalose took place in this yeast strain [20], whereas incorporation of dGlc into cell wall mannan occurred during growth [27]. Kotyk and Michaljanicova [6] described facilitated diffusion of dGlc in other yeast strains. The strain of *S. fragilis* studied in the present investigations differs apparently from some other yeast strains. At least seven metabolites were recovered intracellularly. The accumulation of phosphorylated dGlc derivatives occurs at the expense of ATP, orthophosphate and polyphosphates, as was described before for the yeast strain Hansen CBS 1172 [4]. In contrast to the yeast strain described by Bauer and Biely [23] free dGlc is present inside the cells and contrary to the results of Kotyk and Michaljanicova [6] with other yeast strains, the C_i/C_0 ratio clearly exceeds unity, contradicting facilitated diffusion. The operation of an active-transport mechanism is indicated by the influence of anaerobic conditions and starvation. Each of these factors has little effect on transport, but combined they cause a pronounced inhibition (Figs 5 and 6). The most obvious interpretation is that, under anaerobic conditions in starved yeast, energy production is too low to support active transport. The inhibition of accumulation of free dGlc under these circumstances (Fig. 6) is especially interesting, taking into account that after 10 min the sugar concentration in the medium is about 0.10 mM with normal yeast under aerobic conditions and, due to the very low accumulation of phosphorylated derivatives, 0.95 mM with starved yeast under anaerobic conditions.

Experimental evidence for an active transport system with transport-associated phosphorylation in other yeast strains has been discussed before [4, 5, 7]. Pulse labeling experiments, designed to investigate whether the same mechanism of energy coupling was operative in this yeast strain were, at first sight, confusing. In some experiments the specific activity of the dGlc-6-phosphate fraction increased faster than the specific activity of the free sugar, indicating transport-associated phosphorylation (Fig. 8). In other experiments a phenomenon as described before by

Kotyk and Michaljanicova [6] was observed: at first the specific activity of the free fraction increased faster, but was exceeded by the specific activity of the dGlc-6-phosphate fraction after some time (Fig. 9). Kotyk and Michaljanicova [6] assumed that the second part of the curve was an artefact, introduced by the presence of another labeled sugar in the cells, that is not labeled rapidly in the pulse experiments. Considering present knowledge of dGlc metabolism in various yeast strains (see Fig. 3) the only obvious possibility would be the formation of free 2-deoxy-D-galactose via the Leloir pathway. In control experiments it appeared that chromatographic separation of dGlc and 2-deoxy-D-galactose would indeed be poor with the solvent systems used. With the galactose oxidase reaction it could be shown, however, that no measurable conversion to 2-deoxy-D-galactose took place during the experiments. Therefore, the experimental artefact could not be explained according to the suggestions of Kotyk and Michaljanicova [6]. The experiments in which the yeast was removed from the filter prior to extraction and the experiments in which the filters were removed quickly from the extraction fluid clearly indicate that the apparent sharp rise of the specific activity in the free fraction during the first seconds was caused by adsorption of radioactivity onto the filters. The yeast cells accumulated about 95 % of the non-labeled sugar during the preincubation period. Thus, the radioactive pulse was added to an extremely low sugar concentration in the medium. Under these circumstances a very small adsorption of sugar onto the filters results in a relatively high adsorption of radioactivity. As verified in model experiments, the appropriate correction for adsorption of radioactivity by the filters depended strongly on the total sugar concentration. Further, it should be considered that the pool ratios of free dGlc/dGlc-6-phosphate were extremely low in these experiments (between 1/20 and 1/40), much lower than the corresponding ratios in experiments discussed in previous papers. Therefore, relatively small experimental errors introduced by adsorption of free dGlc to the filters will have a much more pronounced influence.

After appropriate correction the specific activities increased faster in the dGlc-6-phosphate fractions during the entire pulse experiment. These results support the hypothesis of transport-associated phosphorylation of dGlc in this yeast strain. The other possible explanation for these results (the existence of more than one compartment with an asymmetric distribution of the free and phosphorylated sugar) seems rather unlikely, as discussed in a previous paper [4]. Moreover, other observations contradict the second possibility; transport of free sugar, with subsequent intracellular phosphorylation via the hexokinase reaction. In the first place the rapid uptake of radioactivity in the intracellular free sugar fraction during pulse labeling should result in an overshoot of the specific activity of this fraction in the case of transport of free sugar (Eqn 7, Fig. 10). This overshoot should be considerable, considering the small dGlc/dGlc-6-phosphate ratio, but was never found experimentally. Furthermore, based on the assumption of transport of free sugar, the turnover constant k_2 would be about 1.6 min^{-1}, as shown in the results section. As the intracellular dGlc-6-phosphate content is about 6.12 μM/g yeast after an incubation period of 10 min, the hexokinase activity should be about 9.8 μM/g yeast per min, both in normal and in starved yeast. In Table III the hexokinase activities as measured in a yeast ghost preparation are summarized. The values are in good agreement with values of the optimal enzyme activity given for other yeast strains [13, 15], a K_m with respect to dGlc of 0.3 mM and a K_m with respect to ATP of about 0.5 mM [13].

In dGlc-incubated, starved yeast cells the ATP concentration is about 0.2 mM and the dGlc concentration is about 0.4 mM during the pulse experiments. According to Table III a hexokinase activity of about 1.6 μM/g yeast per min may be expected under these circumstances, significantly lower than the calculated value of 9.8 μM/g yeast per min. Although a possible overestimation of k_2 and an underestimation of the hexokinase in situ activity should be considered, it seems impossible to explain the discrepancy along these lines. Moreover, even a rather small overestimation of k_2 would increase the expected overshoot in the pulse experiments (Fig. 10) considerably. Thus it is very unlikely that the equilibrium exchange phosphorylation would occur via the hexokinase reaction.

The effect of iodoacetate also argues against the participation of hexokinase in dGlc phosphorylation. The enzyme is not inhibited by iodoacetate at 25 °C, in agreement with previous results of Barnard and Ramel [29], who showed that inactivation of yeast hexokinase by bromoacetate proceeded with an appreciable velocity only at temperatures above 30 °C. Iodoacetate addition to yeast cells leads to a fall of the ATP level to vanishing small concentrations. Assuming the equilibrium

$$\text{dGlc} + \text{ATP} \rightleftharpoons \text{dGlc-6-phosphate} + \text{ADP}$$

a continuous dephosphorylation of dGlc-6-phosphate to dGlc should be expected. As shown in Fig. 11, however, no dephosphorylation is observed after iodoacetate poisoning. The remote possibility that the local ATP concentration near the active centre of the hexokinase molecule remains constant, despite the decrease of the total ATP pool, should be considered. The influence of iodoacetate on pulse labeling is not in accordance with this possibility: phosphorylation is inhibited almost completely, whereas labeling of the free intracellular fraction is inhibited to a smaller extent (Fig. 12). Apparently the transport system itself is influenced by iodoacetate, possibly in a way similar to that described in other yeast strains [4].

Finally, the countertransport experiments are in accordance with transport-associated phosphorylation. Efflux of dGlc in these experiments occurred to the expense of the dGlc-6-phosphate fraction, with only a small decrease of the intracellular free sugar pool, although the total efflux concerned an amount of dGlc, several times the total free sugar pool. This can be explained, assuming that dGlc-6-phosphate is the substrate for the carrier at the inside of the membrane, in accordance with the hypothesis of transport-associated phosphorylation.

Considering literature on sugar transport in yeast it seems likely that different transport mechanisms exist. The characteristics of the transport mechanism will depend both on the yeast strain and the sugar. In a forthcoming paper it will be shown, for example, that transport of 2-deoxy-D-galactose in *S. fragilis* proceeds via uptake of the free sugar, with subsequent intracellular phosphorylation.

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