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TRANSPORT OF 2-DEOXY-D-GALACTOSE IN *SACCHAROMYCES FRAGILIS*

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SUMMARY

2-Deoxy-D-galactose (dGal) transport in *Saccharomyces fragilis* is characterized by energy requirement and accumulation of the free sugar against a concentration gradient, indicating active transport.

Besides free sugar dGal-1-phosphate, UDP-dGal and a trehalose-like derivative were found inside the cells. The accumulation of the phosphorylated derivatives was balanced by a concomitant decrease of ATP, orthophosphate and polyphosphates.

With pulse labeling experiments it could be shown that the free sugar is transported into the cells. This conclusion was supported by several other experimental results, e.g. the lack of correlation between the sugar transport parameters and the dGal phosphorylation capacity, and the countertransport of free dGal evoked by galactose in the medium.

The typical differences between this active transport mechanism and the transport-associated phosphorylation system, described previously, are discussed.

INTRODUCTION

2-Deoxy-d-glucose (dGlc) was shown to enter *Saccharomyces fragilis* cells by an active transport mechanism [1]. In this and other yeast strains the energy coupling in dGlc uptake occurs via transport-associated phosphorylation of the sugar, as indicated among others by pulse labeling experiments.

On the other hand the existence of facilitated diffusion systems for many sugars in several yeast strains is well established [2–4]. For some time these two systems: facilitated diffusion and active transport with transport-associated phosphorylation, were the only two sugar transport systems in yeast, described in some detail. Other examples of active transport in yeast have been described [5–8], but the mechanism of energy coupling was not studied.

In recent experiments, 2-deoxy-D-galactose (dGal) appeared to enter *S. fragilis* by active transport (as judged from the energy dependence and the accumulation of

Abbreviations: dGal, 2-deoxy-D-galactose; dGlc, 2-deoxy-D-glucose.

free sugar in the cells) but without transport-associated phosphorylation. A more profound study of this transport system appeared interesting, for several reasons. After incubation with dGal the free sugar, dGal-1-phosphate and some other secondary products could be detected in the cells, similar to the results obtained with dGlc. Kinetic analysis of experiments with these two sugars (one subjected to transport-associated phosphorylation and the other to intracellular phosphorylation, following transport of the free sugar) should reveal essential differences. These differences might facilitate discrimination between the two mechanisms. Further, the analogy with bacterial active sugar transport systems is interesting. In bacteria there are also two groups of active sugar transport systems. The first group, based on the phosphotransferase system, is characterized by transport-associated phosphorylation [9–11]. In the second group the free sugar is taken up against a concentration gradient (see e.g. ref. 12).

Some of the characteristics of dGal transport in *S. fragilis* are discussed in the present paper.

METHODS

S. fragilis was grown on a liquid medium of the following composition: 1.2 g KH_2PO_4; 2.4 g citric acid; 12 g *tert*-potassium citrate; 3 g $(NH_4)_2SO_4$; 0.6 g $MgSO_4 \cdot 7H_2O$; 1 g KCl; 0.3 g $CaCl_2 \cdot 2H_2O$; 17.5 mg $FeCl_3 \cdot 6H_2O$; 6.25 mg $MnSO_4$; 50 mg NaCl; 50 mg $ZnSO_4$; 6.25 mg inositol; 3.5 mg biotin; 6.25 mg calcium pantothenate; 6.25 mg thiamine; 6.25 mg pyridoxine; 6.25 mg nicotinic acid; 6.25 mg riboflavin, and either 20 g glucose, or 20 g galactose, or 20 g per l lactose. The yeast was inoculated in 100 ml of this medium in a culture flask and incubated for 30 h at 29 °C. Then the content of this flask was added to 5 l of the same medium in a fermentor (Microferm, New Brunswick) and cultured for another 20 h at 29 °C with agitation at 240 rev./min and an air flow of 16 l/min. The pH-shift during growth (from 5.2 to about 4.5) was not compensated. The yeast was harvested, utilizing a continuous flow centrifuge and washed three times with distilled water.

When necessary the yeast was starved by incubation of a cell suspension at 20 °C for 44 h under aerobic conditions.

2-Deoxy-[1-^{3}H]galactose was prepared by exchange of the pure sugar with tritium gas and purified by paper chromatography (Amersham). The final product was 98 % pure by paper chromatographic control.

The uptake experiments were conducted at pH 5.2, without buffer. Separation of cells and medium was accomplished by filtration on S and S BA 85 Selectron cellulose nitrate filters. The cells were washed three times with a small volume of ice-cold water. The filter was placed in 3 ml absolute ethanol at 4 °C. After 1 h an equal volume of water was added.

Analysis of the extracts was performed with descending paper chromatography, autoradiography and quantitative measurement of radioactivity in a liquid scintillation counter, as described before [1].

Galactokinase was measured in semi-permeable yeast cells, prepared as described previously [13].

Analytical methods were: dGlc, glucose oxidase method as modified by Washko and Rice [14]; dGal, galactose oxidase (Boehringer); ATP, luciferin-lucif-

erase system, as described before [15]; polyphosphates, according to Lohmann and Langen [16]; orthophosphate, by the method of Meyerhof and Oesper [17], with one modification. Cellular extracts were prepared in alcohol instead of trichloroacetic acid solutions, because dGal-1-phosphate was hydrolysed in the latter solutions at room temperature. This derivative appeared to be stable, however, at pH values above 4 and in alcohol. Respiration was measured with the standard Warburg technique. Intracellular concentrations were calculated as described before [1].

RESULTS

When *S. fragilis* was incubated with [^{3}H]dGal for periods up to 4 h, the total amount of radioactivity in the incubation mixture was constant, indicating that no metabolic conversion to volatile products occurred. The rate of uptake was constant for about 20 s. From the initial rates of transport typical Michaelis Menten kinetics with a V of 0.13±0.04 mM/g per h and a K_m value of 0.35±0.07 mM ($n = 15$) could be deduced. These parameters were not significantly influenced by varying the carbon source in the growing medium (glucose, lactose or galactose).

Respiration rates of glucose, galactose and lactose are summarized in Table I. Apparently the respiration rates are independent of the growing conditions and of the metabolizing sugar, with two exceptions: the respiration rate of galactose and lactose by glucose-grown yeast is about 50 % lower than the other respiration rates.

On paper chromatograms of cell extracts, after incubation with [^{3}H]dGal for 10 min, four distinct spots were found. Spot 1 has an R_f value of 0.62, with *n*-propanol/ammonia/water = 6 : 1 : 3 (v/v) as solvent system, and represents presumably, 2,2'-dideoxy-α,α'-trehalose, according to the criteria of Farkas et al. [18]. Unambiguous identification was impossible, because of the low intracellular concentration of this derivative, hampering purification. This uncertainty appeared to be of no importance for the transport studies. Spot 2, with an R_f value of 0.53, was identified as free dGal. The identification was based on co-chromatography with pure dGal, the positive reaction with galactose oxidase and the reaction with perchloric acid/acetone according to Weidemann and Fischer [19].

Spots 3 and 4 were phosphorylated compounds, as judged from their disappearance after treatment of the extract with alkaline phosphatase, the barium-zinc reagent of Somogyi [20] or with anion exchange resin. After treatment with alkaline phosphatase all the radioactivity of these spots was recovered as free dGal. Spot 3 (R_f value of 0.14) could be identified as dGal-1-phosphate, according to the criteria

TABLE I

RESPIRATION RATE IN μmol O_2/g YEAST PER MIN AT 25 °C, UNDER VARYING CONDITIONS

Carbon source in growing medium	Substrate		
	Glucose	Galactose	Lactose
Glucose	17.8	11.2	8.2
Galactose	18.0	20.0	18.1
Lactose	20.5	21.5	23.0

TABLE II

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

Initial dGal concentration in the medium: 1.08 μmol/ml. Yeast concentration: 10 % wet wt. per volume.

	Incubation time (min)			
	2	10	30	60
Lactose- or galactose- grown yeast				
dGal, medium	0.91	0.39	0.16	0.11
dGal, intracellular	1.01	1.68	0.40	0.43
dGal-1-phosphate	1.94	9.95	14.90	15.61
2,2'-dideoxy-α, α'-trehalose	0.02	0.13	0.21	0.29
UDP-dGal	0.03	0.24	0.48	0.68
Glucose-grown yeast				
dGal, medium	0.91	0.39	0.16	0.11
dGal, intracellular	2.28	6.12	2.24	1.26
dGal-1-phosphate	0.69	5.52	12.96	14.79
2,2'-dideoxy-α, α'-trehalose	0.03	0.24	0.48	0.68
UDP-dGal	0.00	0.00	0.16	0.17

described by Fischer and Weidemann [21]. Spot 4 had an R_f value of 0.12 and showed, after elution, the characteristic absorption spectrum of uridine nucleotide, with maximal absorption at 260 nm. Hydrolysis at pH 7, 100 °C for 15 min yielded only dGal as a radioactive product. Therefore spot 4 was identified as UDP-dGal.

Neither in the free sugar fraction nor in the sugar-phosphate fraction could dGlc be detected under these experimental conditions. After incubation periods of

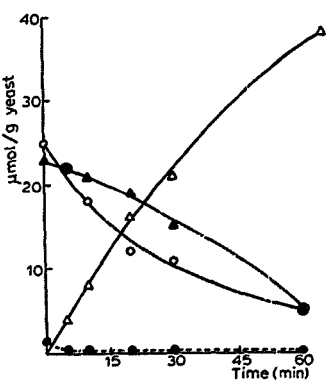

Fig. 1. Intracellular concentrations of dGal-phosphates, (△); orthophosphate, (○); polyphosphates, (▲); ATP, (●), in the course of time. Yeast concentration, 10%; ethanol concentration, 1%; initial dGal concentration in the medium, 10 mM.

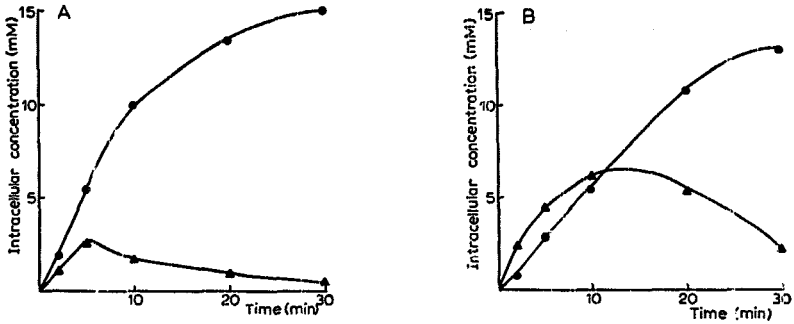

Fig. 2 (A) Uptake of free dGal, (▲); dGal-1-phosphate, (●) in normal cells under aerobic conditions, with yeast grown on galactose, Initial dGal concentration in the medium, 1.08 mM. With yeast grown on lactose similar results were obtained. (B) Uptake in glucose-grown yeast, under the same experimental conditions.

2–4 h, however, trace amounts of dGlc and dGlc-6-phosphate could be detected, apparently formed via the Leloir pathway. The results of two representative experiments are summarized in Table II. In uptake experiments the incubation period never exceeded 60 min. Therefore the possible intracellular conversion of dGal to dGlc derivatives could be neglected.

Phosphate metabolism during sugar accumulation is depicted in Fig. 1. The decrease of the ATP, polyphosphate and orthophosphate concentrations fully accounted for the accumulation of dGal-phosphate.

The intracellular concentrations of free dGal and dGal-1-phosphate during incubation with this sugar are shown in Fig. 2 for yeast, grown on galactose or lactose (Fig. 2A) and for yeast, grown on glucose (Fig. 2B). The higher concentration of free

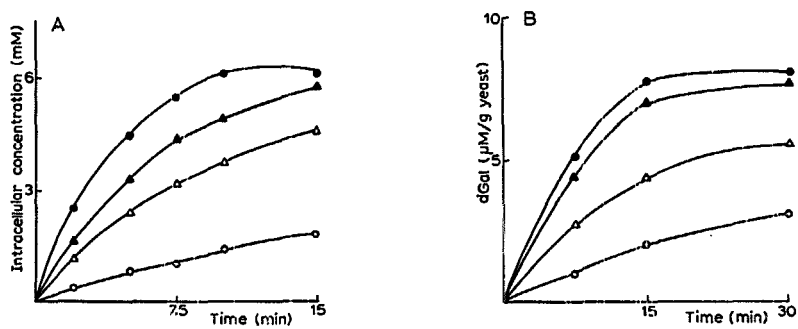

Fig. 3(A) Accumulation of free dGal in glucose-grown yeast, in normal cells under aerobic conditions, (●); in normal cells under anaerobic conditions, (△); in starved cells under aerobic conditions, (▲); in starved cells under anaerobic conditions, (○). (B) Total uptake of dGal in glucose-grown yeast, in normal cells under aerobic conditions, (●); in normal cells under anaerobic conditions, (△); in starved cells under aerobic conditions, (▲); in starved cells under anaerobic conditions, (○).

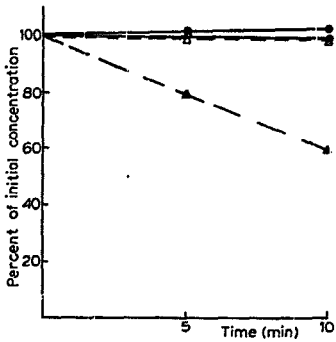

Fig. 4. dGal countertransport induced by galactose. The cells were preloaded with [^{14}C]dGal by incubation of a 10 % yeast suspension with 1 mM dGal for 10 min. Intracellular concentrations were calculated after paper-chromatographic separation and measurement of radioactivity of the individual spots. ○, dGal-1-phosphate, control; △, dGal control; ●, dGal-1-phosphate after addition of 300 mM galactose to the medium; ▲, dGal after addition of 300 mM galactose to the medium.

dGal in glucose-grown cells (as compared to lactose- or galactose-grown cells) after short incubation periods was a highly reproducible phenomenon.

Total uptake of dGal in the course of time in normal and starved cells under aerobic and anaerobic conditions is shown in Fig. 3. The observed inhibitions indicate the energy requirement of the transport process.

In cells, preloaded with dGal, the addition of galactose to the medium evoked countertransport. Analysis of cellular extracts in the course of time revealed that the initial efflux of radioactivity from the cells occurred at the expense of intracellular free sugar (Fig. 4).

Pulse labeling experiments were conducted with yeast cells, preincubated with unlabeled dGal. After 45 min the intracellular concentrations of dGal and its derivatives were virtually constant. A chemically negligible amount of [^{3}H]dGal was added

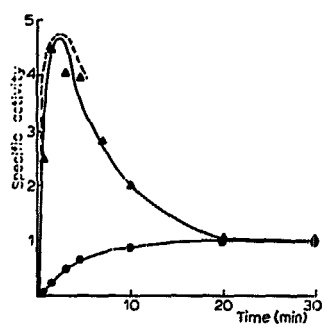

Fig. 5. Specific activity of intracellular dGal-1-phosphate (●) and free dGal (▲) after pulsing with [^{3}H]dGal. The dashed curve represents the theoretical specific activity of free dGal, calculated as explained in the text.

to the medium during this stationary state. The influx of the radioactive label was proportional to time for about 50 s, considerably longer than during the initial uptake. The distribution of the radioactive label over the intracellular free sugar and sugar-phosphate fraction is shown in Fig. 5. The specific activity of the free fraction increased much faster than the specific activity of the dGal-1-phosphate fraction, indicating transport of free sugar with subsequent intracellular phosphorylation.

In the case of uptake of the free sugar with subsequent intracellular phosphorylation, the ^{3}H-concentration in the free fraction can be calculated from the equation:

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

(ref. 1) in which [S'] is the ^{3}H-concentration in the intracellular free sugar fraction; v_0, the influx velocity of the label; k_2, the turnover constant of the sugar-phosphate pool; a, the ratio of the sugar-phosphate/free sugar pools; and t, time in seconds. Calculation of the k_2 value from the observed isotope distribution, 15 s after addition of the pulse, yielded a value of 0.12–0.15 in four different experiments. From v_0 and this k_2 value the expected specific activities after varying periods can be calculated. The expected curve of the specific activity of the free sugar fraction is also shown in Fig. 5. Apparently there is an excellent agreement between the expected and the experimentally observed specific activities.

In model experiments with galactokinase in situ [13] an enzyme activity of about 7 μmol/g yeast per min was found at a galactose concentration of 4 mM and an ATP concentration of 5 mM in lactose-grown yeast. Considering the K_m value of 0.3 mM with respect to galactose [22], this value is very close to the maximal enzyme activity. With dGal as substrate the enzyme activity was much lower. Kinetic analysis revealed a V of 2.8 μmol/g per min and a K_m with respect to dGal of about 20 mM, in accordance with other studies on galactokinase from *S. fragilis* [22]. It appeared that the enzyme was not inhibited by iodoacetate at a concentration of 4 mM at 20 °C.

Addition of iodoacetate to yeast cells, preloaded with dGal, resulted in a fast decrease of the ATP concentration to very low values and a continuous downward

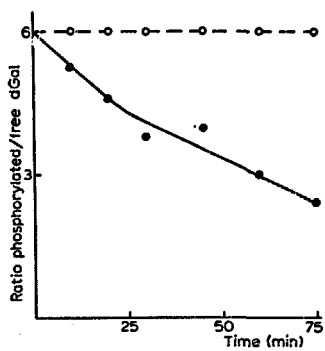

Fig. 6. The influence of iodoacetate (4 mM) on the ratio dGal-1-phosphate/dGal. ●, iodoacetate added; ○, control.

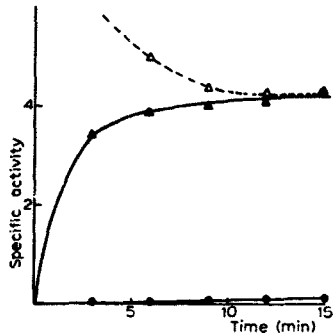

Fig. 7. Specific activity of intracellular dGal-1-phosphate, (●); free dGal, (▲); dGal in the medium, (△) after pulsing with [^{3}H]dGal in the presence of 4 mM iodoacetate.

shift of the dGal-1-phosphate/dGal ratio in the incubation mixture (Fig. 6). More than 50 % of the free dGal that was formed was transported into the medium.

Pulse-labeling experiments on cells, preloaded with unlabeled dGal and subsequently treated with iodoacetate, yielded the results, shown in Fig. 7. The intracellular free sugar pool was labeled rather slowly, reaching the same specific activity as the sugar in the medium after about 15 min. The specific activity of the dGal-1-phosphate fraction, however, increased only very slowly.

DISCUSSION

dGal uptake in *S. fragilis* is apparently an active carrier transport process, considering the Michaelis Menten kinetics and the concentration ratio of the free sugar inside the cells and in the medium. Accumulation rations varied from 10 to 80, depending on experimental conditions. The energy requirement of the transport process is also indicated by the inhibition of transport by anaerobiosis and starvation (Fig. 3). The inhibition cannot be attributed to inhibition of intracellular phosphorylation, considering the cellular and medium concentrations of free dGal. The lower concentrations of free dGal inside the cells caused by anaerobiosis and/or starvation are correlated with lower total uptake of dGal and thus with higher sugar concentrations in the medium. In the experiments depicted in Fig. 3 the C_i/C_0 ratios of the free sugar after 15 min were 26 in normal cells under aerobic conditions, 7 in normal cells under anaerobic conditions, 18 in starved cells under aerobic conditions and 2 in starved cells under anaerobic conditions. This clearly indicates that the transport system itself is energy-dependent.

Inside the cells dGal is partly converted to dGal-1-phosphate, UDP-dGal and, presumably, 2,2'-dideoxy-α,α'-trehalose. Conversion to dGlc and dGlc-phosphates proceeds very slowly. During the first 60 min no dGlc or derivatives of this sugar could be detected. After longer incubation periods trace amounts of dGlc metabolites were found. An extremely slow conversion of dGal via the Leloir pathway was also noted by Fischer and Weidemann in another yeast strain [21]. These authors ascribed this phenomenon to inhibition of the epimerase reaction by UDP-glucose. In the

transport studies described in this paper, the incubation period never exceeded 60 min. Therefore a possible conversion to dGlc derivatives could be ignored

In pulse-labeling experiments the specific activity of the intracellular free dGal increased faster than the specific activity of the other fractions, indicating transmembrane transport of the free sugar with subsequent intracellular phosphorylation. As shown in a previous paper mathematical treatment of this process yields the relationship between the essential parameters, given in Eqn. 1 (ref. 1). With this equation the turnover constant, k_2, of the phosphorylated fraction can be calculated from the isotope distribution e.g. 10 s after addition of the pulse. Then the expected specific activities after varying periods can be calculated with this equation. With small intracellular concentration ratios of the free and the phosphorylated sugar a considerable overshoot of the specific activity of the free fraction should be expected. As shown in Fig. 5 this overshoot was actually found, confirming the transport of free dGal.

From the k_2 value of about 0.14 as calculated from Eqn. 1 and the intracellular dGal-1-phosphate content of about 8 μmol per gram yeast, a galactokinase activity of about 1.1 μmol/g per min can be deduced. This value corresponds very closely to the initial rates of phosphorylation, observed during incubation with relatively high concentrations (10 mM) of dGal. The enzyme activity observed in semipermeable cells is much lower, however. Considering the V of 2.8 μmol/g per min, the K_m of 20 mM with respect to dGal and the intracellular dGal concentration of about 0.4 mM during pulse labeling experiments, the enzyme activity would be about 0.05 μmol/g per min. It seems likely that this discrepancy should be attributed to spatial factors. If e.g. the intracellular localization of the enzyme is close to the inner side of the membrane, the apparent concentration of the transported sugar at the catalytic site will be much higher than the experimentally measured concentration. This could explain the discrepancy between the galactokinase activity in intact cells and in semipermeable cells.

The conclusion that dGal is transported into the cells as the free sugar, without transport-associated phosphorylation, is supported by several other observations. The transport parameters, V and K_m, are equal in glucose-grown and in galactose- or lactose-grown yeast. However, as shown in Fig. 2, the initial rate of phosphorylation of dGal is considerably lower in glucose-grown yeast, as compared to galactose- or lactose-grown yeast. This indicates that there is no direct correlation between the rate of phosphorylation of dGal and the transport system for this sugar. This is quite different from the experimental findings with sugars, entering the cell via transport-associated phosphorylation. With α-methylglucoside transport in *Saccharomyces cerevisiae* NCYC 240, for instance, there was a very close correlation between transport velocity and α-methylglucoside-phosphorylating capacity [15]. The lower rate of dGal phosphorylation in glucose-grown yeast can be attributed to a lower concentration of galactokinase inside the cells. The respiration measurements as summarized in Table I also indicate a lower concentration of enzymes involved in galactose metabolism in glucose-grown yeast.

In countertransport experiments the efflux of dGal from the cells occurred primarily at the expense of the intracellular free sugar (Fig. 4), as should be expected. This is again contrary to the results with sugars, taken up with transport-associated phosphorylation. Countertransport of dGlc from *Saccharomyces fragilis*,

for instance, occurred at the expense of the intracellular dGlc-6-phosphate pool [1].

A further distinction between the two transport systems concerns the effect of iodoacetate on cells, preloaded with sugar. With dGal a gradual conversion of dGal-1-phosphate to dGal is observed, presumably attributable to the galactokinase catalyzed reaction:

$$\text{dGal-1-phosphate} + \text{ADP} \rightleftharpoons \text{dGal} + \text{ATP}$$

The shift to the right should be expected from the vanishing small intracellular ATP concentration after addition of iodoacetate. This effect was not observed with dGlc, contradicting in this case a similar participation of hexokinase in dGlc phosphorylation [1].

Finally, the influence of iodoacetate on pulse labeling is different with the two transport systems. Total transport and phosphorylation are strongly inhibited, in both systems. With dGal, however, the intracellular free dGal pool equilibrated with the sugar in the medium in about 15 min [Fig. 7), whereas free intracellular dGlc equilibrated more slowly [1].

Apparently there are at least three different transport systems for sugars in different yeast strains. The existence of carrier-mediated facilitated diffusion of free sugars in many yeast strains has been well-established [2–4]. The existence of an active transport system, characterized by transport-associated phosphorylation with anorganic polyphosphate as phosphate donor, has been discussed in several preceding papers [1, 15, 23]. This system implies among others the localization of a polyphosphate fraction outside the cytoplasmic membrane [23]. This peripheral localization of polyphosphates in yeast was established by the investigations of Souzu [24, 25] and Kulaev et al. [26]. The participation of polyphosphates in energy-requiring reactions was shown elsewhere, e.g. by Szymona and Ostrowsky [27, 28]. Finally, the involvement of polyphosphates in active sugar transport in *Neurospora crassa*, in a similar way as proposed for yeast, was recently shown by Umnov et al. [29].

The experiments presented in this paper indicate that besides this transport-associated phosphorylation system, another active transport system must be operative in this yeast strain. The characteristics of sugar transport in yeast clearly depend both on the transported sugar and on the investigated yeast strain.

Further experiments, designed to elucidate the energy coupling in dGal transport in *S. fragilis*, are in progress.

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