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TRANSPORT AND TRANSPORT-ASSOCIATED PHOSPHORYLATION OF 2-DEOXY-D-GLUCOSE IN YEAST

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SUMMARY

1. 2-Deoxy-D-glucose taken up by yeast cells was recovered inside the cells partly as free sugar, partly as 2-deoxy-D-glucose 6-phosphate.

2. At low deoxyglucose concentrations in the medium there was an uphill transport of the free sugar. Concentration ratios of 20–30 could be reached, indicating an active transport mechanism.

3. The accumulation of deoxyglucose phosphate was counterbalanced quantitatively by a simultaneous decrease of cellular ATP, orthophosphate and polyphosphate. Intracellular hydrolysis of accumulated deoxyglucose phosphate could be demonstrated.

4. With a [^{14}C]deoxyglucose pulsing technique it could be shown that deoxyglucose phosphate behaved as the precursor of intracellular free deoxyglucose. This indicates that there is not a transport of free deoxyglucose into the cells followed by intracellular phosphorylation by the hexokinase–ATP system, but that phosphorylation of deoxyglucose is associated with transport (followed by partial intracellular hydrolysis).

5. The apparent analogy between the active sugar transport system in yeast and the phosphotransferase-dependent sugar transport in bacteria, described in recent literature, is discussed.

INTRODUCTION

The fermentation of glucose and the respiration of ethanol by yeast is inhibited by 2-deoxy-D-glucose (dGlc)[1–5]. Transported dGlc is recovered from yeast cells partly as free dGlc, partly as dGlc-6-*P*[3,6,7]. Under experimental conditions allowing cell growth, some incorporation of dGlc in uridine nucleotides can be observed[8]. This incorporation seems related to the inhibition of cell growth and the lethal effect of dGlc on growing yeast, *via* an inhibition of the synthesis of structural polysaccharides[5]. Under experimental conditions not allowing growth, however, the only notable conversion of dGlc in yeast cells concerns phosphorylation to dGlc-6-*P* and no lethal effect is observed in short-term experiments. Therefore, dGlc appeared to be a suitable derivative to elucidate the transport mechanism of sugars in yeast.

In previous papers arguments have been discussed that indicate the existence

of two hexose transport mechanisms in yeast: a passive, carrier-mediated facilitated diffusion and an active, metabolically linked transport[9–11]. This active transport was found, *e.g.* for glucose. Experimental evidence indicates a phosphorylating reaction associated with the transport mechanism, that involves polyphosphate as the phosphate donor. This hypothesis contradicts the often encountered assumption of passive, carrier-mediated transport of free glucose, followed by intracellular phosphorylation by the hexokinase–ATP system.

The experiments described in the present communication were undertaken to study the intracellular fate of dGlc in yeast and especially in order to find direct evidence for the postulated active transport mechanism associated with phosphorylation.

METHODS

Saccharomyces cerevisiae, strain Hansen C.B.S. 1172, was grown, harvested and starved as described previously[12]. Phosphorus-deficient yeast was obtained by culturing the yeast on a described medium[12], in which the phosphate concentration is reduced to 20% of the normal level.

Separation of cells and medium was accomplished by millipore filtration followed by a washing of the cells on the filter with ice-cold water. Yeast extracts for chromatography and dGlc analysis were obtained by treating the cells with 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 another hour. dGlc and dGlc-*P* were separated in the extracts by addition of the barium–zinc reagent of SOMOGYI[13], as described by AUGUSTIN AND HOFMANN[6]. Paper chromatography of extracts was performed on S and S-2043b paper, with two solvent systems; ethyl acetate–*n*-propanol–water (20:60:20, by vol.), and ethyl acetate–acetic acid–water (30:30:10, by vol.). dGlc and dGlc-*P* were detected on paper chromatograms by the method described by WEIDEMANN AND FISCHER[14] or, if [^{14}C]dGlc was utilized, by a radioautogram method. Radioautograms were made by placing the dried paper chromatograms in close contact with strips of X-ray film in the dark, for 4–10 days. If quantitative analysis was needed, the chromatograms were subsequently cut into small strips, guided by the blackening of the radioautograms. The radioactivity of these strips could be measured quantitatively in a liquid-scintillation counter. Intracellular concentrations were calculated according to the method described by CIRILLO[15].

Yeast extracts for ATP determinations were prepared according to FELDHEIM, AUGUSTIN AND HOFMANN[3]. ATP was measured with the luciferin–luciferase system, according to the method of ADDANKI, SOTOS AND REARICK[16]. A liquid-scintillation counter was used, recording counts between 15 and 27 sec after mixing. Polyphosphates were extracted and assayed according to LOHMANN AND LANGEN[17]. Orthophosphate was measured by the method of FISKE AND SUBBAROW, as modified by MEYERHOF AND OESPER[18]. dGlc and dGlc-*P* were measured chemically by the method of WARAVDEKAR AND SASLAW[19]. ^{14}C-labelled dGlc was measured in a liquid-scintillation counter, utilizing the liquid scintillator described by BRAY[20]. Hexokinase was isolated from the yeast and assayed as described by DARROW AND COLOWICK[21].

RESULTS

The uptake of dGlc at varying substrate concentrations in the medium is shown in Fig. 1. If yeast extracts are treated with the barium–zinc reagent of SOMOGYI, part of the dGlc-positive material is precipitated. According to AUGUSTIN AND HOFMANN[6], the precipitated fraction is dGlc-6-*P*. As this was an essential feature in all further experiments, this fact was checked carefully. Paper chromatography of yeast extracts after preincubation of the intact cells with ^{14}C-labelled dGlc always showed two distinct spots: one corresponding to free dGlc; the other, to dGlc-6-*P* (Fig. 2). After pretreatment of the extracts with barium–zinc reagent, the spot corresponding to dGlc-6-*P* disappeared completely, whereas the spot corresponding to free dGlc remained unchanged. After pretreatment of the extracts with commercial alkaline phosphatase (100 μg per 2 ml extract, 1 h at 37°) no precipitation of radioactive material took place after subsequent addition of the barium–zinc reagent. On paper chromatograms only one spot appeared, corresponding to free dGlc and with a radioactivity equal to the sum of both spots in untreated yeast extracts (Fig. 2). This proves that the barium–zinc insoluble fraction really is dGlc-*P*.

At low dGlc concentrations in the medium, yeast cells accumulate free dGlc with respect to the medium concentration (Fig. 3). The ratio intracellular concentration/extracellular concentration reached maximal values of 20–30 in these experiments. At higher medium concentrations (over 10 mM), the concentration of free dGlc is equal to or even lower than the concentration in the medium, even after incubation periods up to 2 h. At relatively high dGlc concentrations in the medium, the total uptake of dGlc after longer incubation periods depends on experimental conditions. Uptake is higher under aerobic conditions than anaerobic conditions. Uptake increases if an energy source is added and decreases sharply after iodoacetate poisoning or after previous phosphorus depletion of the cells. After phosphorus de-

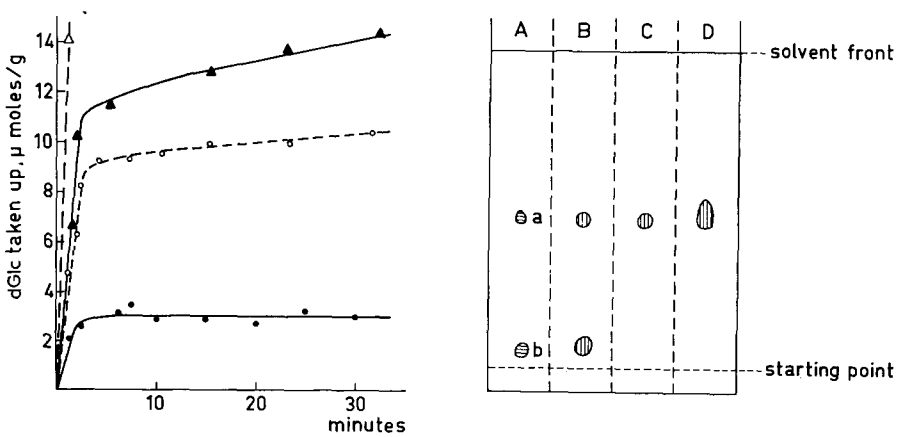
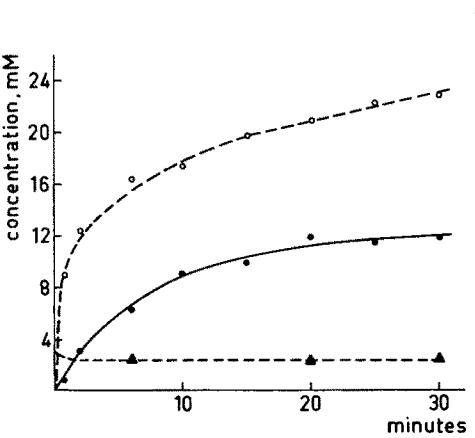


Fig. 1. Total uptake of dGlc in yeast cells, at 25°. Yeast concn., 2%. Initial dGlc concn. in the medium: 0.15 mM (●—●), 0.60 mM (○---○), 1.00 mM (▲—▲), 10 mM (△—△).

Fig. 2. Outline of paper chromatograms of yeast cell extracts. Solvent system: ethyl acetate–*n*-propanol–water (20:60:20, by vol.). A, referents (a, free dGlc; b, dGlc-6-*P*); B, normal yeast extract; C, extract treated with barium–zinc reagent; D, extract treated with alkaline phosphatase. With the solvent system ethyl acetate–acetic acid–water similar results were obtained.

pletion, the uptake velocity can be restored if orthophosphate is added to the medium together with an energy source (Table I).

The effect of iodoacetate poisoning was studied in some detail. If iodoacetate is added to yeast preincubated with dGlc, a prompt decrease of both the phosphorylated and the free dGlc fraction is observed, provided that the intracellular free dGlc concentration exceeded the medium concentration (Fig. 4). If the intracellular



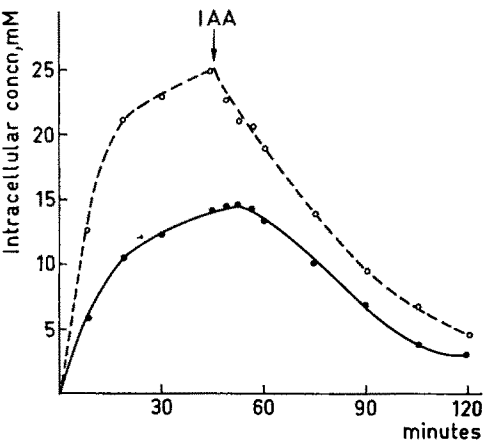


Fig. 3. dGlc uptake in the course of time. Yeast concn., 3%; ethanol concn., 1%; initial dGlc concn. in the medium, 3 mM; temp., 25°. ●—●, intracellular free dGlc concn.; ○---○, intracellular dGlc-*P* concn.; ▲---▲, dGlc concn. in the medium.

Fig. 4. Influence of iodoacetate (IAA) on intracellular dGlc-*P* (○---○) and free dGlc (●—●) concn. Yeast, ethanol and dGlc concns., see Fig. 3. Iodoacetate (1 mM) was added after 46 min.

free dGlc concentration was lower than the medium concentration at the moment of iodoacetate addition, only the phosphorylated fraction decreased, with a concomitant increase of the free dGlc concentration. If, on the other hand, iodoacetate is added to the yeast suspension prior to the dGlc, an initial, rapid uptake still takes place, followed by a gradual decrease of the concentration of the phosphorylated fraction and a concomitant change of the free fraction in the direction of equilibrium with the medium concentration (Fig. 5). The highest concentration of intracellular

TABLE I

TOTAL UPTAKE OF dGlc UNDER VARIOUS EXPERIMENTAL CONDITIONS

Yeast concentration: 5% (wet wt.); temp., 25°; dGlc concn. in the medium: 20 mM. Uptake is expressed in μmoles per g yeast. A: anaerobic conditions; B: aerobic conditions; C: aerobic conditions + 5% glucose; D: aerobic conditions + 2% ethanol; E: aerobic conditions + 2% ethanol, phosphorus-depleted yeast; F: aerobic conditions + 2% ethanol + 20 mM KH_2PO_4, phosphorus-depleted yeast; G: anaerobic conditions, iodoacetate (1 mM)-poisoned yeast.

Total uptake after:	*Conditions*						
	A	*B*	*C*	*D*	*E*	*F*	*G*
10 min	9.8	10.0	10.9	13.1	9.9	9.9	9.8
1 h	15.5	20.3	25.7	41.0	15.6	20.4	9.7
2 h	16.2	25.7	33.1	51.3	18.3	33.5	9.4
3 h	16.1	29.2	39.2	56.6	20.2	48.4	9.4

dGlc-*P* that could be reached under these circumstances was about 5 μmoles per g yeast. This value was never exceeded, not even with very high dGlc concentrations in the medium (up to 500 mM). It should be emphasized that in all experiments with iodoacetate-poisoned yeast, no traces of phosphorylated dGlc could be detected in the medium, either by addition of barium–zinc reagent, or by paper chromatography with subsequent quantitative ^{14}C analysis of the chromatogram. A possible leakage of dGlc-*P* to the medium could be masked by hydrolysis of this compound by the superficial acid phosphatase of the cells. No orthophosphate could be measured in the medium, however.

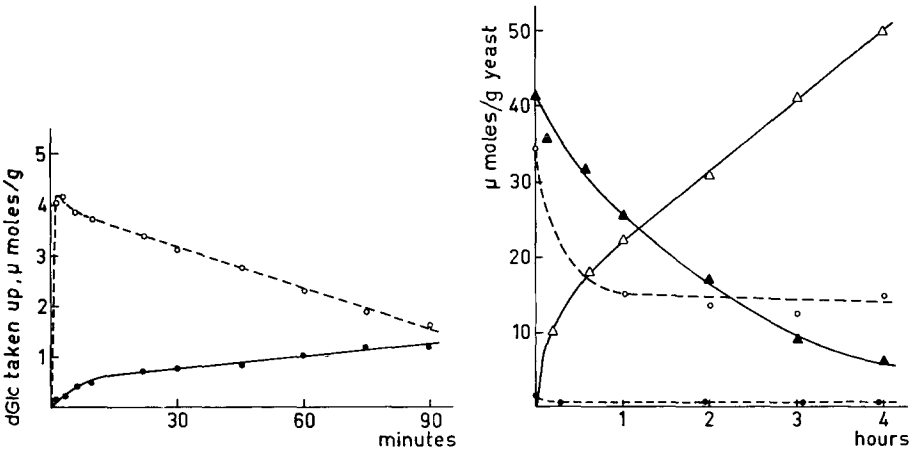


Fig. 5. dGlc uptake in iodoacetate-poisoned yeast. Yeast concn., 2%; dGlc concn. in the medium: 10 mM; temp., 25°. ●—●, free dGlc; ○---○, dGlc-*P*.

Fig. 6. Intracellular concentrations of dGlc-*P* (△—△), ATP (●---●), orthophosphate (○---○) and polyphosphate (▲—▲) in the course of time. Yeast concn., 3%; ethanol concn., 2%; dGlc concn. in the medium, 20 mM; temp., 25°.

If yeast cells are incubated with high dGlc concentrations for several hours in the presence of an energy source, a large percentage of cellular phosphorus becomes fixed as dGlc-*P*. In experiments on this phosphate turnover, simultaneous measurements of intracellular dGlc-*P*, ATP, orthophosphate and polyphosphate were performed. Typical results are given in Fig. 6. In all experiments the accumulation of dGlc-*P* was balanced within the experimental error by the decrease of ATP, orthophosphate and polyphosphate. Varying the incubation time and the dGlc concentration in the medium, it appeared that all three donor fractions always decreased. The ATP concentration decreased from an initial value of about 0.9 μmole to about 0.3 μmole per g yeast within 3 min, irrespective the dGlc concentration. No definite pattern could be detected, however, in the orthophosphate and polyphosphate decrease. Both fractions decreased gradually during the experiments. Sometimes the orthophosphate decrease exceeded the polyphosphate reduction; sometimes the reverse tendency was observed.

Experiments carried out with hexokinase, isolated from the yeast utilized in the present experiments, proved that dGlc is phosphorylated promptly under the conditions described by DARROW AND COLOWICK[21]. On the other hand, according to the hypothesis on sugar transport discussed before, phosphorylation of actively

transported sugars should be associated with the transmembrane transport mechanism. This possibility was studied with dGlc as substrate, utilizing a radioactive pulsing technique.

Yeast cells were incubated with varying amounts of unlabelled dGlc for a period of 10–60 min. After that interval a certain amount of ^{14}C-labelled dGlc was added to the medium. After this addition the cell contents were analysed at intervals for free dGlc and dGlc-*P* and for radioactivity in both fractions. From these experimental results the specific activity in both fractions was calculated (defined as the ratio radioactivity/chemically measured quantity, using arbitrary units). Typical

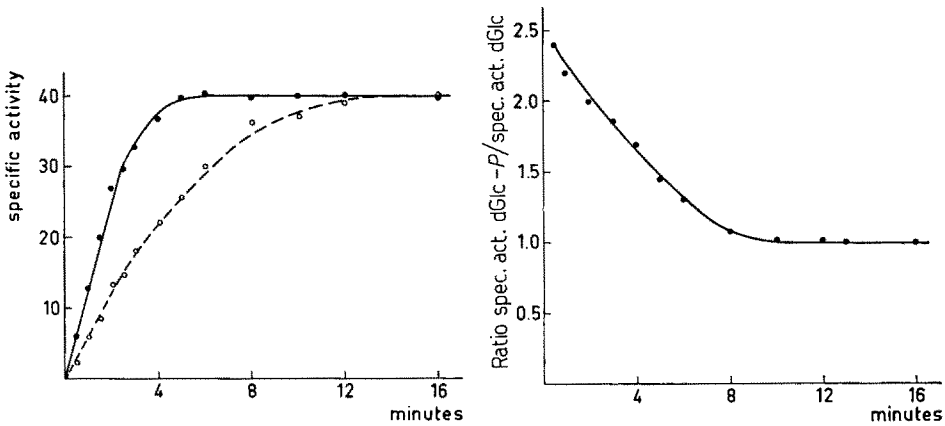


Fig. 7. Specific activity of intracellular free dGlc (○---○) and dGlc-*P* (●—●), after [^{14}C]dGlc pulsing. Preincubation: 3% yeast, 1% ethanol and 3 mM dGlc (unlabelled) at 25° during 20 min. At zero time 0.06 mM ^{14}C-labelled dGlc was added to the suspension.

Fig. 8. Ratio of the specific activity of the intracellular dGlc-*P* to the specific activity of intracellular free dGlc in the course of time. Experimental conditions: see legend to Fig. 7.

results are depicted in Figs. 7 and 8. Similar results were obtained in all experiments, irrespective of the dGlc concentration (over the range 1–100 mM), the incubation period with unlabelled dGlc and the amount of radioactive dGlc added. To exclude experimental errors in the chemical analysis, control experiments were conducted with ^{14}C-labelled dGlc added at the same moment as the unlabelled sugar. Specific activities of free dGlc and dGlc-*P* were constant and equal to each other in these experiments, as should be expected.

DISCUSSION

Uptake and phosphorylation of dGlc by various yeast strains has been described by several authors[1–8] and was confirmed by the present study for the strain Hansen C.B.S. 1172. Because intracellular accumulation of free sugar against a concentration gradient is generally accepted as conclusive evidence for active transport[22,23], the reported results prove the existence of an active transport mechanism for dGlc in this yeast strain (see Fig. 3). This conclusion is supported moreover by the apparent dependence of dGlc uptake on the availability of an energy source (see Table I).

The decrease of the dGlc-*P* concentration in iodoacetate-poisoned yeast, following an initial, rapid accumulation of the phosphorylated sugar, clearly reflects hydrolysis of dGlc-*P* by intracellular phosphatase. It was also shown that free dGlc can be phosphorylated by the intracellular hexokinase–ATP system. This indicates that the intracellular dGlc and dGlc-*P* concentrations must be considered as the reflection of a dynamic equilibrium.

FELDHEIM, AUGUSTIN AND HOFMANN noted that during incubation of yeast cells with dGlc and an energy source, the decrease of ATP and orthophosphate could account for only part of the dGlc-*P* accumulation[3]. As shown in this communication the difference is balanced by a simultaneous decrease of the cellular polyphosphate concentration (Fig. 6). In various microorganisms, enzymes catalysing the following reaction have been described: ADP + $(KPO_3)_n \leftrightarrows$ ATP + $(KPO_3)_{n-1}$[24–26]. This is in accordance with the high-energy character of the polyphosphate bonds. From the experimental results described in this paper, it can not be concluded whether the dGlc phosphorylation out of the cellular polyphosphate pool takes place directly, *via* the above equation through ATP synthesis, or even *via* an initial breakdown to orthophosphate, followed by metabolic ATP synthesis. The first two possibilities seem to be unlikely, however, as no dGlc phosphorylation out of the cellular polyphosphate pool takes place under anaerobic conditions or in iodoacetate-poisoned yeast. This indicates that the chemical energy accumulated in the cellular polyphosphate pool is not directly available to the cells. A similar conclusion was reached by other authors who used quite different experimental approaches[26–29].

In previous papers the existence of an active glucose transport system in yeast has been discussed[9–12]. Experimental evidence indicates that this active transport system is associated with glucose phosphorylation, with a small polyphosphate fraction (physiologically distinct from the bulk intracellular polyphosphate pool) as phosphate donor. With glucose as substrate iodoacetate-poisoned yeast has a residual active transport and phosphorylating capacity of about 5 μmoles per g yeast, significantly exceeding the cellular ATP contents. This proves at least that besides ATP another high-energy phosphate source is available for glucose phosphorylation in iodoacetate-poisoned yeast. As shown in this paper, the maximal amount of dGlc phosphorylated by iodoacetate-poisoned yeast also amounts to 5 μmoles per g yeast, suggesting that glucose and dGlc are transported and phosphorylated by the same system. In the experiments described in this paper, the only notable conversion of dGlc concerns phosphorylation. Therefore, dGlc appeared to be an appropriate substrate for experiments to decide between the two possibilities of transport and phosphorylation, *viz.*: (a) Transport of free sugar, followed by intracellular phosphorylation *via* the hexokinase–ATP system (a–c, in Fig. 9), or: (b) Phosphorylation,

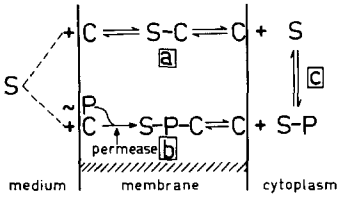


Fig. 9. Model of the two transport possibilities, *viz.*: transport of free sugar (a) with subsequent partial intracellular phosphorylation (c), as opposed to transport-associated phosphorylation (b) with subsequent partial intracellular de-phosphorylation (c).

directly associated with transport, followed by some intracellular hydrolysis (b, c, in Fig. 9).

If there would be no transport-associated phosphorylation, it should be expected that in [^{14}C]dGlc pulsing experiments (after preincubation with unlabelled dGlc) the specific activity of the intracellular free dGlc fraction would rise much faster than the specific activity of the phosphorylated fraction. This effect would even be augmented by the fact that the free fraction is quantitatively much smaller (3–5 times) than the phosphorylated fraction. As shown in Figs. 7 and 8, however, the results are the opposite. The specific activity of the phosphorylated fraction is, shortly after addition of the [^{14}C]dGlc pulsing dose, much higher than the specific activity of the intracellular free fraction. In the course of time this difference diminishes and the ratio of the specific activities in both fractions approaches unity (Fig. 8). In other words, intracellular dGlc-*P* behaves typically as the precursor of intracellular free dGlc. The only obvious interpretation is phosphorylation of dGlc directly associated with transport, as predicted by the discussed hypothesis on active transport. Intracellular free dGlc then, must be the product of the described phosphatase activity*. It should be emphasized that these results do not completely rule out the possibility of the transport of free dGlc, followed by intracellular phosphorylation. If such transport does occur, however, it is apparently quantitatively of minor importance, as compared to the active transport mechanism associated with phosphorylation.

Similar experiments with other non-metabolizable sugars are being performed at the present time and will be discussed in a forthcoming paper.

In recent literature a phosphotransferase system has been described in bacteria, comprised of two enzymes and a heat-stable protein[30,31]. This system catalyses sugar phosphorylation, with phosphoenolpyruvate as phosphate donor. The authors concluded that this phosphotransferase system is involved in transmembrane sugar transport. There appears to be a close resemblance between the properties of this bacterial phosphotransferase system and the active transport system in yeast, described in this and previous papers[9–11]. Therefore it seems highly probable that a similar system will be present in yeast as the molecular basis of the sugar transport system. Further experiments will be designed to study the relationship between the bacterial phosphotransferase system and the active sugar transport system in yeast.

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* The only other possible explanation of the results would be the existence of more than one compartment in the yeast cell with an asymmetry of the distribution of dGlc and dGlc-*P*. Such asymmetry of distribution of free and phosphorylated sugars has never been described, however. Further, it should be expected that the experimental conditions (*e.g.* the amount of labelled and unlabelled dGlc added, the duration of the preincubation with unlabelled dGlc and the presence or absence of metabolizable substrates) would have a pronounced quantitative influence on the results; but this is not the case. Moreover, preliminary experiments to be discussed later, indicate an equal distribution of free and phosphorylated dGlc in the yeast cells.

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