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Facilitated Diffusion of 6-Deoxy-D-Glucose in Bakers' Yeast: Evidence Against Phosphorylation-Associated Transport of Glucose

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6-Deoxy-D-glucose, a structural homomorph of D-glucose which lacks a hydroxyl group at carbon 6 and thus cannot be phosphorylated, is transported by *Saccharomyces cerevisiae* via a facilitated diffusion system with affinity equivalent to that shown with D-glucose. This finding supports the facilitated diffusion mechanism for glucose transport and contradicts theories of transport-associated phosphorylation which hold that sugar phosphorylation is necessary for high-affinity operation of the glucose carrier.

The nature of the glucose transport system of bakers' yeast has remained controversial despite more than two decades of study. At the crux of the controversy is the question whether glucose is transported via a group translocation system in which it is phosphorylated during transport or via a carrier-mediated facilitated diffusion system in which it is phosphorylated by the action of hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2) after it enters the cell. The transport-associated phosphorylation hypothesis, advanced in 1954 by Rothstein (12), was based on observations that (i) intracellular free glucose was not detectable in actively metabolizing cells; (ii) yeast cells were reportedly impermeable to non-metabolizable sugars; and (iii) there was an apparent correlation between substrate specificity for sugar transport and sugar phosphorylation. The facilitated diffusion model gained wider acceptance when it was shown by Cirillo (2–4) and Wilkins and Cirillo (17) that (i) a large number of non-metabolizable sugars that are structural analogs of glucose are in fact transported (but not concentrated) by yeast, although their affinities for transport are much lower than metabolizable sugars, and their transport is competitively inhibited by D-glucose; (ii) glucose itself is taken up as the free sugar by iodoacetate-poisoned yeast; (iii) counterflow can be demonstrated by the addition of D-glucose to cells preloaded with non-metabolizable D-glucose analogs; and (iv) free glucose can be detected in metabolizing cells by counterflow techniques, and the estimated concentration is consistent with predictions based on a carrier-mediated facilitated diffusion model. However, the phosphorylation theory of sugar uptake was revived by Van Steveninck and Rothstein (16) in the form of a hybrid carrier-phosphorylation model, whereby the carrier was presumed to function in a low-affinity facilitated diffusion system in the transport of non-metabolizable glucose analogs (or of glucose itself in iodoacetate-poisoned cells) and in a high-affinity group translocation system for glucose in metabolizing, energy-sufficient cells. Van Steveninck (14, 15) and Jaspers and Van Steveninck (6) supported this model principally with experiments carried out with 2-deoxy-D-glucose (2-DOG), a partially metabolized glucose analog that has a high affinity for the D-glucose uptake system and can be phosphorylated by hexokinase. In pulse-labeling experiments with radioactive 2-DOG designed to determine the time sequence of the appearance of 2-DOG in intracellular pools, they have reported that intracellular pools of 2-DOG phosphate become labeled before free 2-DOG pools do. Moreover, they reported that intracellular concentrations of both 2-DOG and 2-DOG phosphate reach higher levels than external 2-DOG does. Meredith and Romano (11) also found that 2-DOG phosphate is the first species to be detected intracellularly in pulse-label experiments but found no evidence of intracellular concentration of free 2-DOG. More recently, Franzusoff and Cirillo (5) confirmed that 2-DOG first appears in the intracellular sugar phosphate pool, not only in wild-type bakers' yeast, but also in single-kinase mutant strains containing only one of two hexokinase isozymes or glucokinase. This showed that any putative phosphorylation-associated transport mechanism is not a function of a specific kinase. However, they urged caution in interpreting the results of pool labeling experiments with whole cells, in which the problems of compartmentation and multiple pools are difficult to assess.

I now report uptake experiments with 6-

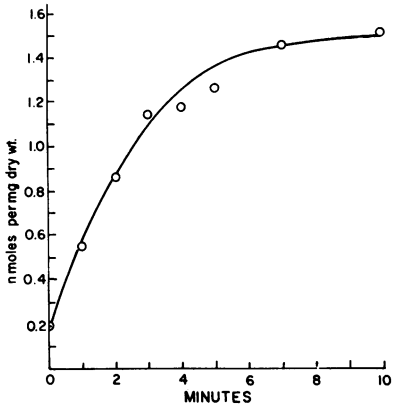

FIG. 1. Uptake of 6-deoxy-D-glucose by *S. cerevisiae*. Cells were incubated with 1 mM 6-deoxy-D-[^{3}H]glucose (2.7 mCi/mmol).

deoxy-D-[^{3}H]glucose, a non-metabolizable analog which is a close structural homomorph of D-glucose, differing from it only in the absence of a hydroxyl group at carbon 6 and therefore incapable of being phosphorylated by hexokinase or glucokinase. This allows a direct test of the notion that a high-affinity D-glucose transport system can operate only with sugars that can be phosphorylated.

Saccharomyces cerevisiae ATCC 9896 (Fleishman strain 139) was grown on GPY broth (2% glucose, 2% peptone, 1% yeast extract) for 24 h on a reciprocal shaker at 30°C. Cells were harvested by centrifugation and then were washed and suspended in 0.05 M phosphate buffer (pH 7.2) containing 0.2 g/liter of $MgSO_4 \cdot 7H_2O$ at a final cell density of 4.2 mg (dry weight) per ml (absorbance, 2.0 at 540 nm).

6-Deoxy-D-[G-^{3}H]glucose was made by New England Nuclear Corp., Boston, Mass., by custom catalytic exchange labeling. The non-radioactive substance used to adjust specific radioactivity to appropriate experimental levels was prepared by hydrolysis of methyl-6-deoxy-β-D-glucopyranoside (Ash Stevens, Inc., Detroit, Mich.) with 2 N H_2SO_4, followed by crystallization from ethyl acetate, by the method of Schmidt (13). For uptake experiments, appropriate volumes of a 6-deoxy-D-[^{3}H]glucose solution (5 mM, 2.7 mCi/mmol) and distilled water were added to 4 ml of cell suspension prepared as described above to give the concentrations specified at a total volume of 5 ml; the cell suspension was incubated at 25°C in a water bath with constant agitation. Samples of 0.5 ml were removed at appropriate time intervals, filtered rapidly through membrane filters (porosity, 0.8 μm; Millipore Corp., Bedford, Mass.), and washed immediately with 5 ml of suspension buffer at room temperature. To determine the total radioactivity, filters with cells on them were transferred to vials containing 10 ml of Bray fluid (1) for counting in a Packard Liquid Scintillation Spectrometer. To determine intracellular free and phosphorylated sugars, cells on the filters were extracted with 2 ml of water at 90°C for 10 min. Extracts were chromatographed on Bio-Rad AG1-X2 anionic exchange resin to separate free and phosphorylated sugars as described previously (11). D-[U-^{14}C]glucose-6-phosphate (ICN Chemical and Radioisotope Division, Irvine, Calif.) was used as a marker for sugar phosphates.

The time course of 6-deoxy-D-glucose uptake at an external concentration of 1 mM is shown in Fig. 1. Uptake was rapid and approached a steady-state level after 10 min, which was equivalent to 78% of the external concentration, when internal concentration was calculated with 1 mg of cells (dry weight) equivalent to 2 μl of intracellular water (11). Thus, there was no concentrative uptake of 6-deoxy-D-glucose.

The results of anion exchange chromatography of extract of cells that had taken up 6-deoxy-D-[^{3}H]-glucose is shown in Fig. 2. It is clear that all radioactivity was recovered in the nonanionic free sugar fraction and none appeared in the anionic fraction, which was marked by glucose-6-phosphate. Thus, there was no phosphorylation or conversion to other anionic products, such as 6-deoxy-D-gluconic acid.

A Lineweaver-Burk plot of the uptake of 6-

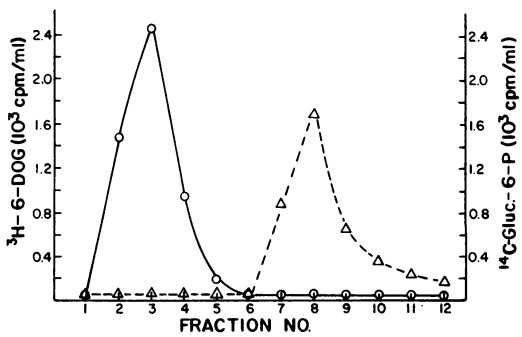

FIG. 2. Ion exchange chromatography of aqueous extracts of cells after uptake of 6-deoxy-D-[^{3}H]glucose. Cells were incubated with 1 mM 6-deoxy-D-[^{3}H]glucose (2.7 mCi/mmol) for 10 min, filtered, washed with uptake buffer, and then extracted with water at 90°C; 0.5 ml of the extract was applied to Bio-Rad AG1-X2 (formate form). Free sugar was eluted with water (fractions 1–6); sugar phosphates were eluted subsequently with 0.5 M ammonium formate in 0.2 M formic acid (fractions 6–12). Symbols: (○) Extract after 6-deoxy-D-[^{3}H]glucose uptake; (△) marker D-[^{14}C]glucose 6-phosphate solution (0.5 ml, 2.5 nCi/ml).

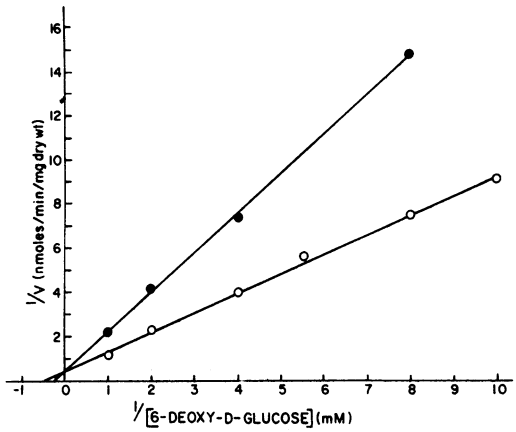

FIG. 3. Lineweaver-Burk plot of the uptake of 6-deoxy-D-glucose by *S. cerevisiae* and its inhibition by D-glucose. Cells were incubated with 6-deoxy-D-[^{3}H]glucose (2.7 mCi/mmol) with no further addition (○) or with 5 mM D-glucose (●).

that these two sugars compete for the same stereospecific carrier. The apparent K_m for 6-deoxy-D-glucose transport calculated from these data is 1.9 mM, and the apparent K_i for the inhibition of 6-deoxy-D-glucose uptake by D-glucose is 2.1 mM. Thus, the affinity of these two sugars for the D-glucose transport system are essentially the same and are also equivalent to that reported for 2-deoxy-D-glucose (4, 11).

These data show that 6-deoxy-D-glucose is transported by yeast via a facilitated diffusion mechanism involving the D-glucose carrier, and they support by analogy the notion that D-glucose enters the cell via the same mechanism. The data negate one of the principal tenets of the phosphorylation theory of transport, namely, that phosphorylation is necessary for the carrier to operate in the high-affinity mode. The previously observed lower affinity of the transport system for analogs such as L-sorbose or 3-*O*-methyl-D-glucose can probably be explained by their reduced structural compatibility with the stereospecific characteristics of the carrier, the reason that also makes them unsuitable substrates for hexokinase and glucokinase. Other support for the phosphorylation mechanism of glucose transport deriving from the studies of pool labeling kinetics described above may be due to the immediate phosphorylation of glucose or 2-DOG by hexokinase or glucokinase upon entry into the cell, and initial free sugar pools may be too small to be detectable by the methods used. It is to be noted that the K_ms for glucose of hexokinase and glucokinase of bakers' yeast are one and two orders of magnitude, respectively, lower than the K_m of glucose or 2-DOG uptake (10). Also, genetic evidence presented by Lobo and Maitra (8, 9) appears to rule out the presence of sugar phosphotransferases other than the soluble two hexokinases and the glucokinase that they have characterized. Of course, these enzymes might be closely, associated with the cell membrane, and the data reported here do not rule out the possibility that glucose is phosphorylated during a transport step after binding to the carrier.

These data also extend previous studies on the specificity of glucose transport in yeast (4, 7) and confirm the importance of a -CH_2OH or -CH_3 substituent at carbon 5 of the glucopyranose chair. The affinity of 6-deoxy-D-glucose is an order of magnitude higher than that reported for D-xylose, which lacks the 5-CH_2OH substituent.

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