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A KINETIC ANALYSIS OF D-XYLOSE TRANSPORT IN *RHODOTORULA GLUTINIS*

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Summary

The kinetics of D-xylose transport were studied in *Rhodotorula glutinis*. Analysis of the saturation isotherm revealed the presence of at least two carriers for D-xylose in the *Rhodotorula* plasma membrane. These two carriers exhibited K_m values differing by more than an order of magnitude. The low-K_m carrier was repressed in rapidly growing cells and derepressed by starvation of the cells.

Several hexoses were observed to inhibit D-xylose transport. In the studies reported here, the inhibitions produced by D-galactose and 2-deoxy-D-glucose were examined in some detail in order to define the interactions of these sugars with the D-xylose carriers. 2-Deoxy-D-glucose competitively inhibited both of the D-xylose carriers. In contrast, only the low-K_m carrier was competitively inhibited by D-galactose.

Introduction

The red yeast *Rh. glutinis* has been reported to accumulate a wide variety of metabolizable and nonmetabolizable monosaccharides against considerable concentration gradients [1]. One of the interesting features of this system is that it is completely inhibited by metabolic inhibitors and uncouplers of oxidative phosphorylation; attempts to demonstrate a passive, facilitated diffusion in the absence of metabolism have been unsuccessful [2,3]. Prior to an examination of the mechanism of energy transduction in the concentrative transport of sugars by *Rhodotorula*, it was decided to characterize the carrier(s) involved.

The number of carbohydrate carriers in the *Rhodotorula* plasma membrane has not been established. Mutual inhibitions between sugar pairs, such as D-arabinose and D-glucose [1], D-xylose and D-glucose [4], and D-xylose and

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L-xylose [1], have been presented as evidence that these sugars utilize the same carrier. On the basis of countertransport studies, Höfer [5] suggested that pentoses and hexoses shared a common carrier in this organism. In 1976, however, Janda and coworkers [6] reported that, although D-xylose and D-galactose were translocated by the same carrier, D-fructose employed a different carrier, and D-glucose appeared to interact with all of the carriers in the membrane. However, none of the studies above presented saturation isotherms for the carbohydrate being transported nor did they provide any evidence that the observed intersugar inhibitions were indeed competitive.

The purpose of the present communication is to present a detailed kinetic analysis of D-xylose transport by *Rh. glutinis* and to describe the modes of inhibition of this process by two hexoses, 2-deoxy-D-glucose and D-galactose.

Methods

Growth and preparation of cells. Rh. glutinis (ATCC 26194) was grown at 30°C in a medium containing per l: 1.0 g K_2HPO_4, 0.66 g of NH_4NO_3, 0.5 g of NaCl, 1.0 g of $MgSO_4 \cdot 7\ H_2O$, 0.33 g of yeast extract (Difco), 0.05 g of $FeCl_3 \cdot 6\ H_2O$, 0.31 g of $CaCl_2 \cdot 2\ H_2O$ and 25 g of glucose [1]. The pH of the medium (without glucose) was adjusted to 5.5 before autoclaving. Five-hundred ml flasks containing 150 ml medium were inoculated with 5 ml of a 24-h culture and incubated in a water-bath shaker for 10 h (mid-log phase). Cells were harvested by centrifugation and washed 3 times with 0.1 M KH_2PO_4 at room temperature. The washed cell suspensions (5% wet weight/volume in 0.1 M KH_2PO_4) were vigorously aerated on a magnetic stirrer for 1.5 h at room temperature, then packed in ice and maintained with gentle stirring at 0°C.

Transport assays. D-Xylose transport was measured by incubating cell suspensions with D-[^{14}C]xylose in a water-bath shaker at 30°C. Uptake was initiated by the rapid addition of 1.0 ml of cell suspension (preincubated at 30°C for 5 min) to an equal volume of 0.1 M KH_2PO_4 containing labeled D-xylose and other ingredients where indicated. At various time intervals, 0.5-ml samples were withdrawn and filtered through a cold prefilter (AP2502500, Millipore)/glass-fiber filter disc (934AH, Reeve Angel) combination. The collected cells and the filters were washed with 4.0 ml of ice-cold 0.1 M KH_2PO_4 and transferred to scintillation vials along with 1.5 ml of 60% (v/v) ethanol and 10 ml of scintillation fluid (Aquasol II, New England Nuclear). Samples were counted in a Beckman Liquid Scintillation Spectrometer (LS-100C).

Data analysis. Initial velocities of D-xylose transport were estimated from the differences in intracellular D-xylose concentration between samples taken after 1 and 4 min of incubation. The progress curves were reasonably linear over this time interval and the calculated initial velocities were directly proportional to cell concentration. The absolute rates of transport, calculated on a dry weight basis, varied somewhat with different cell suspensions. When saturation data from different experiments were combined, the velocities were normalized to a single, reference experiment. For each experiment the ratio of the observed velocity to the reference velocity was calculated at each concentration of D-xylose and the average of these values was employed as the normalization

factor. Equations for the saturation isotherm were fitted to the data set by unweighted, non-linear regression techniques. Computer programs were modelled after those described by Cleland [7].

Materials. D-[U-^{14}C]xylose (2.92 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Illinois. Cycloheximide and non-radioactive sugars were products of Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the highest purity commercially available.

Results

Initial studies of D-xylose transport in *Rh. glutinis* exhibited a variability that precluded any attempts at a detailed kinetic analysis of the transport process. Most of this variability was traced to two factors: (a) difficulty in achieving reproducible filtration rates in the transport assay and (b) time-dependent changes in the transport activity of the cell suspensions. The first problem arose from the use of a membrane filter (Millipore, 0.45 μm porosity) in the transport assays. *Rhodotorula* suspensions frequently clogged this type of filter. Replacement of the membrane filter with a prefilter/glass-fiber filter combination provided for retention of all of the cells while allowing rapid, reproducible rates of filtration. The second factor contributing to the variability of results was found to be the way in which cells were maintained after they were harvested. Fig. 1 demonstrates that the rate of D-xylose transport by washed cell suspensions increases during aeration at room temperature for

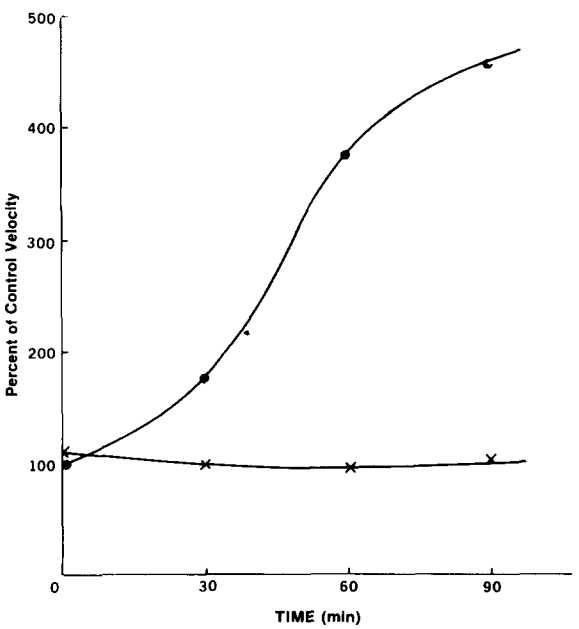

Fig. 1. Time-course of activation of D-xylose transport during starvation of *Rh. glutinis*. Cells were harvested and washed at 0°C. At zero time, cell suspensions were subjected to vigorous aeration at room temperature in the presence (X) and absence (•) of 100 μg/ml of cycloheximide. Transport was asssayed at 30°C in the presence of 1.0 mM D-xylose. Initial velocities were estimated from the differences in intracellular D-xylose concentration between samples taken after 1 and 4 min of incubation.

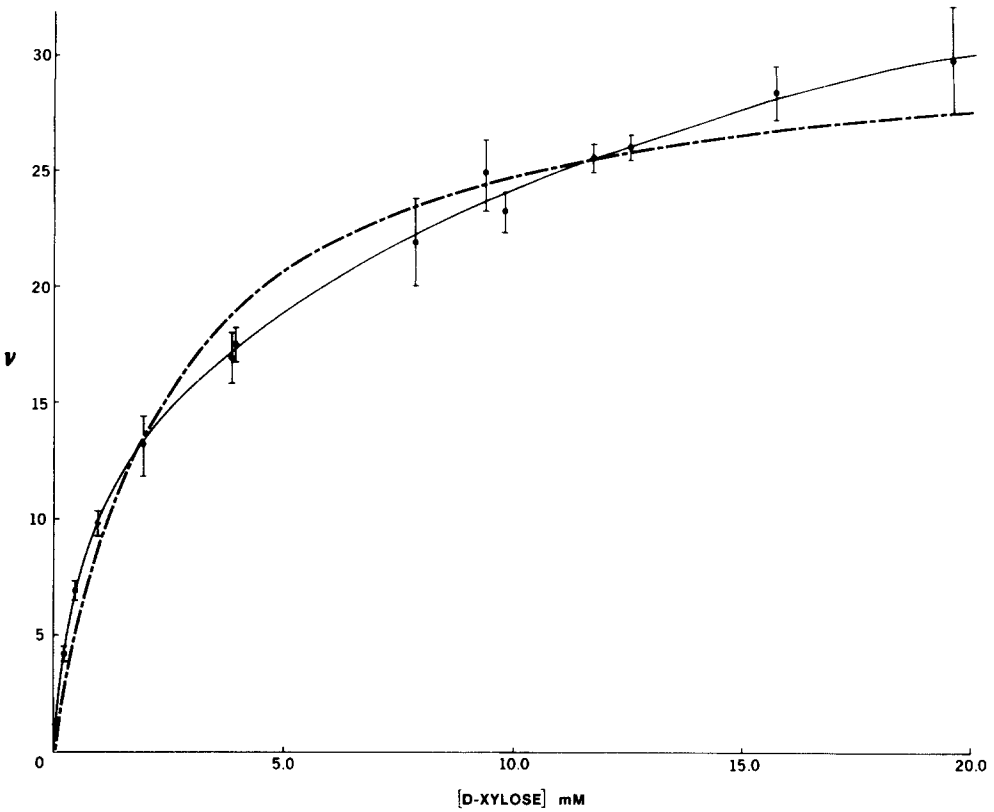

Fig. 2. Saturation isotherm for D-xylose transport by *Rhodotorula*. The curves are from non-linear regression fits to one-carrier (- - - - -) and two-carrier (———) models for the transport process. Velocities are expressed in μmol · min^{-1} · g^{-1} (dry weight). Initial velocities were estimated from the differences in intracellular D-xylose concentration between samples taken after 1 and 4 min of incubation.

1.5 h. Increases in uptake rate were also observed with cells stirring in a refrigerator at 4—6°C, but not when cell suspensions were packed in ice and gently stirred at 0°C. Aerated cells, subsequently packed in ice, maintained their higher rates of transport, unchanged, for several hours. Cycloheximide prevented the increase in transport activity during aeration without significantly affecting the basal rate of D-xylose uptake (Fig. 1). Unless otherwise noted, the studies reported below employed cell suspensions which had been activated by aeration and stored in ice.

The saturation isotherm for D-xylose uptake by *Rh. glutinis* is shown in Fig. 2. This figure represents a composite of results from a dozen separate experiments with different cell suspensions. Fig. 2 contains 105 data points covering an 81-fold range of D-xylose concentrations. The data were tested for consistency with the following models by unweighted, non-linear regressions:

(i) Model I, a Michaelis-Menten function: representing a single, saturable carrier

$$v = \frac{V_1 \cdot [S]}{K_m + [S]} \qquad (1)$$

(ii) Model II, a Michaelis-Menten function plus a linear term: representing a single carrier and a non-saturable diffusion process ('leak')

$$v = \frac{V_1 \cdot [S]}{K_{m_1} + [S]} + k \cdot [S] \tag{2}$$

(iii) Model III, a sum of two Michaelis-Menten functions: representing two saturable carriers in the membrane

$$v = \frac{V_1 \cdot [S]}{K_{m_1} + [S]} + \frac{V_2 \cdot [S]}{K_{m_2} + [S]} \tag{3}$$

where V_1 and V_2 are apparent maximum rates of transport K_{m1} and K_{m2} are kinetic (Michaelis) constants and k is a first-order rate constant. The above models were also considered by Atkins and Gardner [8] in their analysis of intestinal transport kinetics. With all three equations, convergence was attained from several different initial parameter estimates. The converged values of the constants and the variances for the overall fits are presented in Table I. The data were judged to be inconsistent with Model I on the basis of high variance and visual inspection of its inability to fit the data in Fig. 2. Even though a wide range of concentrations was employed in this study the saturation data alone did not allow a clear distinction between Models II and III. The kinetic constants, K_{m1} and K_{m2}, from Model III were less-defined than the K_{m1} and k from Model II, but the variance was somewhat smaller for the former model (Table I). Model III was selected as the better representation of the D-xylose transport system in this organism largely on the basis of its ability to account for the specificity exhibited by the transport system in the inhibition studies described below.

Several hexoses were examined for their ability to inhibit D-xylose (2 mM) transport. D-Glucose, D-fructose, D-galactose and 2-deoxy-D-glucose were found to be inhibitory while L-rhamnose was only slightly inhibitory even at a concentration of 100 mM. D-Galactose and 2-deoxy-D-glucose produced significant inhibition at relatively low concentrations and were selected for further study.

The Dixon [9] plot for 2-deoxy-D-glucose inhibition of D-xylose transport is shown in Fig. 3. The curvature of the graph suggested a parabolic inhibition,

TABLE I

COMPUTED PARAMETERS FROM THE SATURATION ISOTHERM FOR D-XYLOSE

The parameters were obtained from unweighted, non-linear regression analyses of 105 data points. The three models considered were: I, Michaelis-Menten function; II, Michaelis-Menten equation plus a linear term; III, sum of two Michaelis-Menten equations. V is expressed in μmol $\cdot$ min^{-1} $\cdot$ g^{-1} (dry weight), K_m in mM, and k in μl $\cdot$ min^{-1} $\cdot$ g^{-1} (dry weight). Parameters are given ± S.E. The variance is $\Sigma\ (v_{obs}-v_{calc})^2$/(105 minus the number of parameters evaluated).

Parameter	Model I	Model II	Model III
V_1	31 ± 1	19 ± 1	13 ± 2
V_2	—	—	33 ± 4
K_{m1}	2.5 ± 0.2	0.97 ± 0.09	0.56 ± 0.13
K_{m2}	—	—	18 ± 6
k	—	660 ± 40	—
Variance	3.2	1.5	1.3

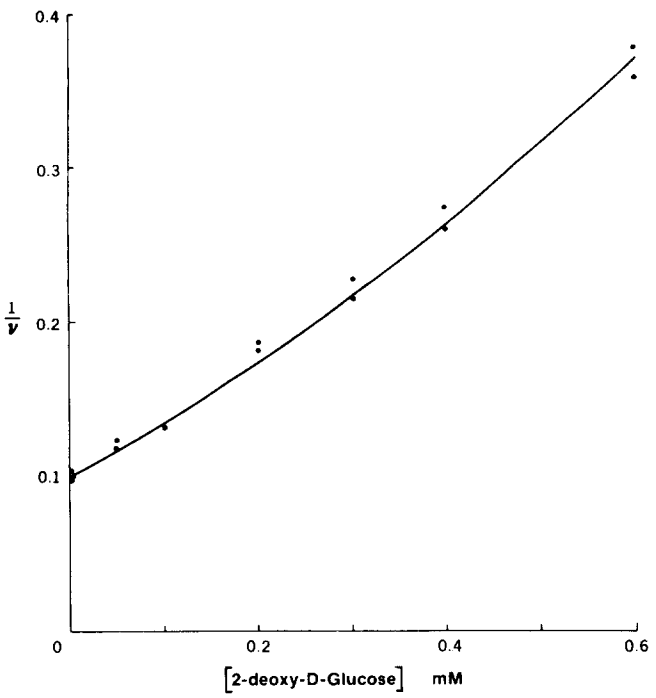

Fig. 3. Dixon plot of the inhibition of D-xylose transport by 2-deoxy-D-glucose. The concentration of D-xylose was 1.0 mM. The solid curve was generated from Eqn. 4 for competitive inhibition of a two-carrier system (Model III). Velocities are expressed in μmol · min^{-1} · g^{-1} (dry weight). Initial velocities were estimated from the differences in intracellular D-xylose concentrations between samples taken after 1 and 4 min of incubation.

probably involving two molecules of inhibitor, and the data were consistent with inhibition of both carriers (Model III) of the transport system. The inhibition was further analyzed by examining the initial rates of transport as a function of the concentration of D-xylose in the presence and absence of 0.2 mM and 0.4 mM 2-deoxy-D-glucose. These data are presented in double-reciprocal form [10] in Fig. 4. The presence of at least two carriers for D-xylose is seen in the distinct curvature of the plots. A strict analysis of the data by non-linear regression comparisons of the fits to equations for competitive, uncompetitive and noncompetitive inhibitions was not feasible because of the limited amount of data (68 points) and the large number of parameters in the appropriate rate equations (e.g. Eqn. 4). Convergence of the data near the reciprocal velocity axis in Fig. 4 strongly suggested that the inhibition was competitive and the lines in both Figs. 3 and 4 were generated from Eqn. 4 with values for V_1, V_2, K_{m1}, and K_{m2} from Table I, and K_{i1} = 0.11 mM, K_{i2} = 0.32 mM, K_{ii1} = 0.17 mM^2 and K_{ii2} = 0.48 mM^2. Eqn. 4 describes a (non-linear) competitive inhibition of both the high- and low-K_m carriers for D-xylose (Model III).

$$v = \frac{V_1 \cdot [S]}{K_{m1} \cdot \left(1 + \frac{[I]}{K_{i1}} + \frac{[I]^2}{K_{ii1}}\right) + [S]} + \frac{V_2 \cdot [S]}{K_{m2} \cdot \left(1 + \frac{[I]}{K_{i2}} + \frac{[I]^2}{K_{ii2}}\right) + [S]} \quad (4)$$

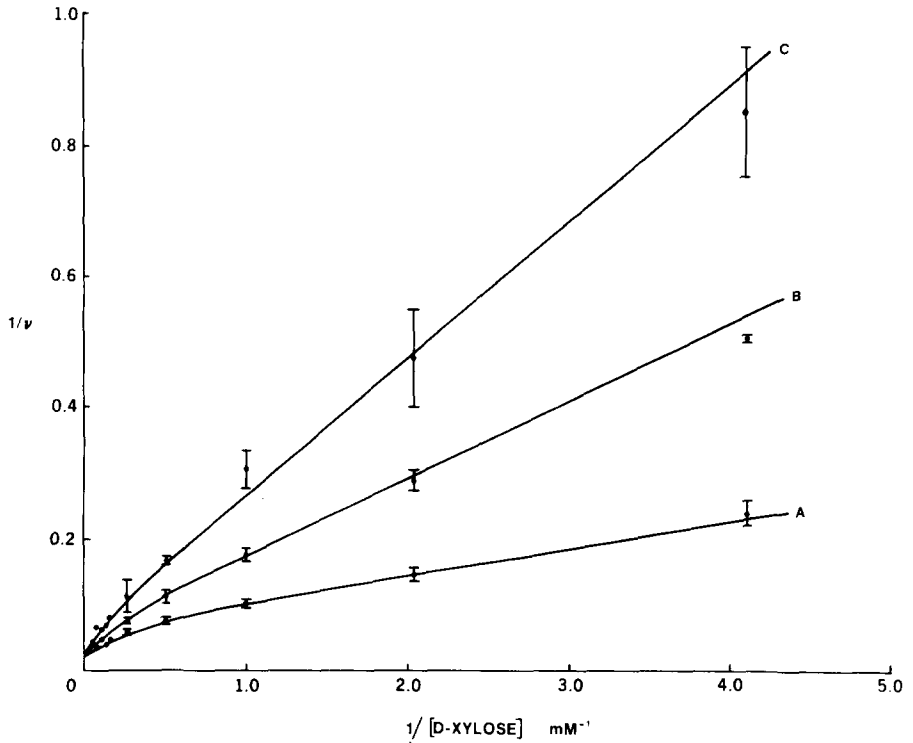

Fig. 4. Lineweaver-Burk plot of the uptake of D-xylose (A) and its inhibition by 0.2 mM (B) and 0.4 mM (C) 2-deoxy-D-glucose. The solid curves were generated from Eqn. 4 for non-linear competitive inhibition of a two-carrier system (Model III). Velocities are expressed in μmol · min^{-1} · g^{-1} (dry weight). Initial velocities were estimated from the differences in intracellular D-xylose concentration between samples taken after 1 and 4 min of incubation.

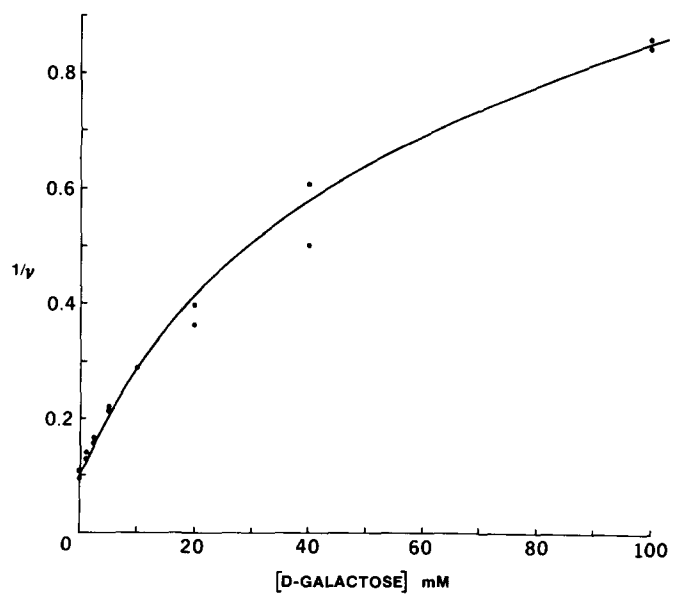

Fig. 5. Dixon plot of the inhibition of D-xylose transport by D-galactose. The concentration of D-xylose was 1.0 mM. Velocities are expressed in μmol · min^{-1} · g^{-1} (dry weight). Initial velocities were estimated from the differences in intracellular D-xylose concentration between samples taken after 1 and 4 min of incubation.

The mode of inhibition of D-xylose transport by D-galactose was significantly different from that observed with 2-deoxy-D-glucose. The non-linear Dixon plot in Fig. 5 indicates that the two carriers differed considerably in their sensitivities to inhibition by D-galactose and very little inhibition was observed at high concentrations of D-xylose. Data from a representative experiment in the presence and absence of 2.5 mM and 5.0 mM D-galactose are presented in double-reciprocal form in Fig. 6. The lines in Fig. 6 were generated from Eqn. 5 which assumes that only the low-K_m carrier was competitively inhibited by D-galactose. Values for the parameters were obtained from Table I and K_{i1} 0.6 mM was used.

$$v = \frac{V_1 \cdot [S]}{K_{m1} \cdot \left(1 + \frac{[I]}{K_{i1}}\right) + [S]} + \frac{V_2 \cdot [S]}{K_{m2} + [S]} \quad (5)$$

In order to further characterize the activation of transport produced by aeration (starvation) of the cell suspensions (Fig. 1), a comparison of the ability of hexoses to inhibit D-xylose transport was made between cells which had been aerated in the usual way and those which had been harvested, washed and maintained at 0°C. Table II demonstrates that the percent inhibition produced

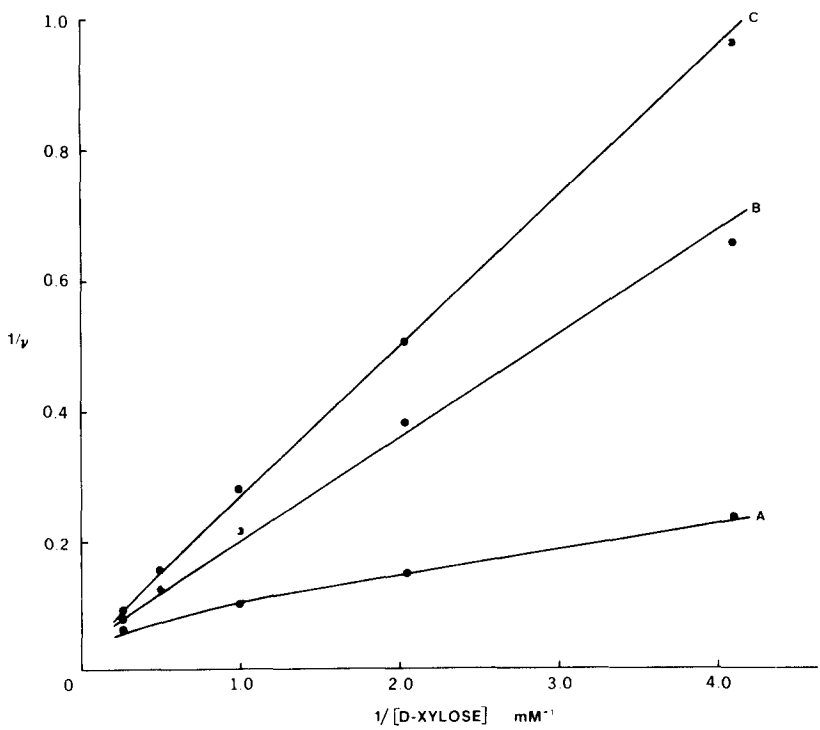

Fig. 6. Lineweaver-Burk plot of the uptake of D-xylose (A) and its inhibition by 2.5 mM (B) and 5.0 mM (C) D-galactose. The solid curves were generated from Eqn. 5 for linear competitive inhibition of the low-K_m carrier in a two-carrier system (Model III). Velocities are expressed in μmol · min^{-1} · g^{-1} (dry weight). Initial velocities were estimated from the differences in intracellular D-xylose concentrations between samples taken after 1 and 4 min of incubation.

TABLE II

COMPARISON OF THE EFFECTS ON INHIBITORS ON STARVED AND NON-STARVED CELLS

Non-starved cells were harvested, washed and maintained at 0°C. Starved cells were harvested, washed and vigorously aerated for 1.5 h at room temperature and then maintained at 0°C.

Inhibitor	[Xylose] (mM)	Percent inhibition of D-xylose transport	
		Non-starved	Starved
0.4 mM	1.94	45	43
2-Deoxy-D-glucose	0.97	63	68
	0.24	66	69
5.0 mM	1.94	6	43
D-Galactose	0.97	30	65
	0.24	43	77

by 0.4 mM 2-deoxy-D-glucose was essentially independent of the way in which the cell suspensions had been prepared. In contrast, the aerated (starved) cells were much more sensitive to inhibition by D-galactose. These results suggest that the activation process increased the proportion of low-K_m carrier in the cells.

Discussion

Although the carbohydrate transport systems of both *Saccharomyces cerevisiae* and *Rhodotorula glutinis* have been extensively studied, the number and nature of the monosaccharide carriers in these organisms is still controversial (see review by Barnett [11]). Careful examination of the literature reveals that much of the information on the carbohydrate carriers in these organisms comes from saturation studies over a narrow (e.g. refs. 5 and 12) or unspecified (e.g. refs. 1 and 12) range of concentrations or from inhibition studies in which a simple competitive relationship is assumed (e.g. refs. 12 and 14) but usually not demonstrated. The results presented in this paper demonstrate the complex behavior that is observed when wide ranges of solute concentrations are employed in transport kinetic studies.

The saturation isotherm for D-xylose uptake by *Rh. glutinis* (Fig. 2) exhibited marked curvature in double-reciprocal form. If only a narrow range of D-xylose concentrations had been examined, the curvature could have been masked by experimental error. A 2-component system had to be invoked to explain the data over the range of concentrations employed. Whether the second component was a saturable (Model III) or a non-saturable (Model II) process could not be determined from the saturation data alone. The specificity exhibited by the second component in the inhibition studies, i.e., inhibition by low concentrations of 2-deoxy-D-glucose compared to little or no inhibition by 10-fold higher concentrations of D-galactose, suggested that this component of the transport was indeed carrier-mediated (Model III). The 2 D-xylose carriers exhibited Michaelis constants differing by a factor of about 30, and the maximum velocity of the high-K_m carrier was about 2.5 times larger than that of the low-K_m system.

Both D-xylose transport systems of *Rhodotorula* appeared to be competitively inhibited by the hexose, 2-deoxy-D-glucose. This type of inhibition is consistent with a model in which either D-xylose or 2-deoxy-D-glucose can occupy the same binding sites on the carriers. The inhibition, however, was not simple. Both the Dixon plot (Fig. 3) and the Lineweaver-Burk plots (Fig. 4) for the inhibition required the $[I]^2$ terms in Eqn. 4 for good correlation between the data set and the rate equation. Any mechanism represented by Eqn. 4 must involve at least two molecules of 2-deoxy-D-glucose in the inhibition of each of the carrier systems. It would be premature to begin to develop such a model until the transport of 2-deoxy-D-glucose itself has been characterized. The inhibition data do suggest, however, that *Rhodotorula* may utilize at least two carriers, with similar K_m values, for the transport of 2-deoxy-D-glucose, and that the kinetics of transport may exhibit some form of cooperativity. Positive cooperativity in the uptake of several sugars by *Rhodotorula* has been suggested by Janda et al. [6].

When D-galactose was tested as an inhibitor of D-xylose transport, the character of the inhibition was distinct from that observed with 2-deoxy-D-glucose. The Dixon plot (Fig. 5) indicated a "partial" inhibition, and very little inhibition was observed at high concentrations of D-xylose. These observations suggested that only the low-K_m system was significantly inhibited by D-galactose. Another distinguishing feature of the inhibition studies with this hexose was a day-to-day variation in the percent inhibition produced by a given concentration of the sugar. On the basis of a model in which only one of the two carriers was inhibited by D-galactose, the observed variations could arise from differences in the relative amounts of the two carriers present in different suspensions of *Rhodotorula*. As noted below, the relative amounts of the carriers may depend on the physiological state of the cells. The effect of that type of variation would be averaged out in the analysis of the saturation isotherm, and the symmetry of Eqn. 4 would make it difficult to detect in the inhibition studies with 2-deoxy-D-glucose. The data in Fig. 6 are from a representative experiment with D-galactose as the inhibitor. These data correlated reasonably well with curves generated from Eqn. 5 assuming competitive inhibition of only the low-K_m carrier. These results suggest that D-xylose and D-galactose may share a common carrier in the *Rhodotorula* plasma membrane. A similar conclusion was reached by Janda et al. [6], although these workers apparently did not observe the second D-xylose carrier which was not inhibited by D-galactose.

The relative activities of the high- and low-K_m systems and the overall rate of D-xylose transport appeared to depend on the nutritional state of the *Rhodotorula* cells. When cells were harvested during vigorous growth on D-glucose and maintained close to 0°C, their transport activity was low. Starvation (aeration) of the washed cells at room temperature for 1.5 h dramatically increased their rate of *D-xylose* transport. A similar stimulation of D-glucose transport during starvation of *Rhodosporidium toruloides* (= *Rh. glutinis*) has been described by Barnett and Sims [15]. Comparison of the inhibitory properties of 2-deoxy-D-glucose and D-galactose on D-xylose transport by starved and non-starved cells suggests that the increased rate of D-xylose transport produced by starvation was due to an increased activity of the low-K_m system.

The effect of cycloheximide on this process is consistent with a derepression of protein synthesis during starvation, but the identity of the protein(s) formed during this period is unknown. The derepression was not limited to cells grown on D-glucose; we have observed a similar process in D-xylose-grown cells. It thus appears that transport of D-xylose (and perhaps other sugars as well) by *Rh. glutinis* is effected by at least two carrier systems, one of which is repressed when cells are growing in the presence of an adequate supply of carbohydrate.

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