J. Membrane Biol. 3, 73 – 82 (1970)

Mobile Membrane Carrier for Monosaccharide Transport in *Rhodotorula gracilis*

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Received 24 February 1970

Summary. Evidence for a mobile membrane carrier mediating the uphill monosaccharide transport in the yeast *Rhodotorula gracilis* is based on two types of observations: (1) Countertransport was found with ^{14}C-labelled D-xylose, L-xylose, L-rhamnose and with L- rhamnose in a cell suspension preincubated with unlabelled D-xylose. This finding indicates, moreover, that both the hexoses and the pentose share the same membrane carrier. (2) The mobility of occupied carrier molecules is higher than that of free carrier molecules. This conclusion has been drawn from: (a) comparison of the initial rates of uptake of a labelled sugar into cells preincubated in the absence and in the presence of unlabelled sugar; (b) comparison on the half-saturation constant of transport with the dissociation constant of the sugar-carrier complex; and (c) comparison of the initial rates of efflux of a labelled sugar into sugar-free and sugar-containing medium.

In an earlier paper (Kotyk & Höfer, 1965), we have shown that the strictly aerobic yeast *Rhodotorula gracilis* transports monosaccharides against their concentration gradients. The metabolically dependent sugar accumulation displayed all the characteristics of a carrier-mediated transport, e.g., saturation kinetics, mutual competition of different monosaccharides, etc. However, one of the very important features of a carrier-mediated transport, the phenomenon of countertransport, was not detectable under the conditions used.

Rosenberg and Wilbrandt (1963) and Silverman and Goresky (1965) have predicted the conditions under which countertransport will occur if the diffusion coefficient of the free carrier and substrate-carrier complex are equal and if the intracellular sugar concentration is a linear function of the extracellular concentration. However, in *R. gracilis*, the intracellular

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sugar concentration does not increase linearly with increasing sugar concentration in the suspending medium (Kotyk & Höfer, 1965; Höfer, *in preparation*), and the mobilities of a free and of a loaded carrier differ. This makes it more difficult to define the conditions for the occurrence of countertransport. Based on the absence of any indication of a countertransport minimum and on the fact that the S_i/S_0 ratio decreases with increasing S_0, eventually reaching values less than one, the postulate was made that there are in the cell membrane of *R. gracilis* two discrete carriers, each of which transports sugar molecules virtually unidirectionally (Kotyk & Höfer, 1965).

Nevertheless, further experiments (the results of which are presented in this paper) provide evidence for the mediation of the monosaccharide translocation in both the inward and the outward directions by a single carrier in the cell membrane. Preliminary reports of the experiments have appeared (Höfer, 1968, 1969).

Materials and Methods

The collection strain used, *Rhodotorula glutinis* 5/Fres/Harrison, was cultivated as described earlier (Kotyk & Höfer, 1965). Cells were harvested after 24 hr, washed three times with distilled water and aerated in a 5% water suspension overnight. The aerated suspension was used for the described experiments carried out aerobically at 30 °C.

Samples of incubated yeast suspension (1.0 ml containing 3 to 5 mg dry weight) were withdrawn at intervals after addition of sugar or after transfer to a new medium, filtered through a membrane filter (pore diameter 0.5 to 0.8 μ; HUFS Synthesia, Uhrineves, Czechoslovakia), placed on a special funnel attached to a water pump, and washed twice with ice-cold water (0.5 ml). The filter with the cell pellet was transferred into a glass tube with 2 ml distilled water and placed for 20 min in a boiling-water bath. The suspension was then deproteinized by successive addition of $ZnSO_4$ and $Ba(OH)_2$, the precipitate spun down and the supernatant used for either sugar analysis or radioactivity counting.

The sugars were determined by standard methods: pentose according to Meijbaum (Umbreit, Burris & Stauffer, 1957), L-rhamnose by the reducing-sugar method of Somogyi (1952) and Nelson (1944), and D-glucose by the blood sugar colorimetric test (GOD Method) of Boehringer GmbH (Mannheim, Germany). Radioactivity was counted either in a methane-flow Frieseke-Hoepfner counter after the samples were dried on aluminium planchets or in a Packard Tri-carb scintillation spectrometer.

The initial rates of sugar fluxes were estimated either from the linear part of the uptake curves or by applying the differentiated form of Newton's formula for equispaced arguments with associated decreasing difference (*cf.* Handbook of Chemistry and Physics, 44th Ed., Chemical Rubber Publ. Co., Cleveland, Ohio, p. 335).

Unlabelled D-xylose and D-glucose were products of E. Merck AG (Darmstadt, Germany); L-xylose of Hoffmann-LaRoche (Basle, Switzerland); L-rhamnose and ^{14}C-L-rhamnose of Calbiochem (Los Angeles, Calif.); ^{14}C-D-xylose was obtained from the Radiochemical Centre (Amersham, Great Britain), and ^{14}C-L-xylose was prepared by Mr. Ivan Benes of this laboratory. All other reagents were commercial products of analytical purity.

Results

The evidence for a mobile membrane carrier for the uphill monosaccharide transport in *R. gracilis* is drawn from two types of experiments.

(1) The flow of one sugar induces a counterflow of labelled molecules of the same kind or of a different sugar, which is transported by the same carrier, due to competition of the two sugars for the exit carrier; consequently, a transient overshoot of the cell label occurs.

(2) In cells preloaded with a given sugar, another sugar (e.g., a radioactive isotope of the former) is transported with a higher initial velocity due to facilitated return of the carrier, if the sugar-carrier complex moves across the cell membrane more rapidly than the free carrier. The former conditions concern the phenomenon of countertransport, the latter the mobility of loaded and free carrier molecules.

Countertransport

This phenomenon of a transient increase or decrease of the cell label is due to competition for the exit or the entrance, respectively, between labelled and "cold" sugars. Under carefully chosen conditions, we were able to obtain distinct countertransport maxima for D-xylose, L-xylose, and L-rhamnose and for L-rhamnose in exchange for D-xylose. The experimental conditions were as follows. The yeast suspension was preincubated with "cold" sugar until a steady state was reached. Then the cells were spun down and at zero time resuspended in a 25-times-lower concentration of ^{14}C-labelled sugar. Samples taken at intervals were then, after extraction and deproteinization (*see* Materials and Methods), counted. A parallel experiment with a yeast suspension preincubated in the absence of added sugar was run as the control. In some experiments, the analytical intracellular concentration of the sugar in question was determined to show that the transient maximum occurred only in the uptake of the label, whereas the analytical content of the sugar had already reached the new steady state level.

Fig. 1 depicts the countertransport maximum obtained with 0.02% ^{14}C-D-xylose after the cells were preloaded with 0.5% "cold" D-xylose. The further increase in cell label after the maximum has been accounted for by incorporation of the labelled D-xylose into cell components. We have therefore attempted to avoid the incorporation by using strictly non-metabolizable sugars such as L-xylose or L-rhamnose. The results are summarized in Fig. 2. The left part of the figure shows the countertransport

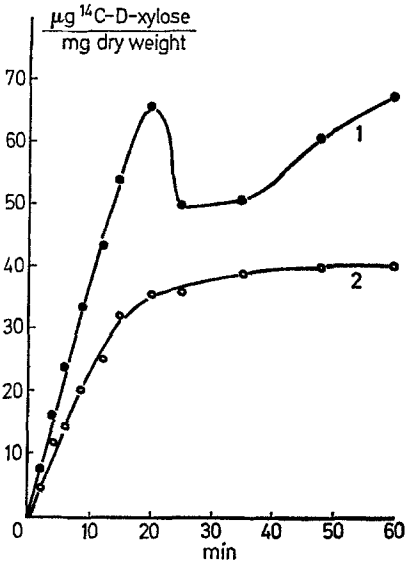

Fig. 1. Countertransport maximum of ^{14}C-D-xylose in *R. gracilis* cells preincubated with unlabelled D-xylose. *Curve 1:* intracellular radioactivity of yeast cells resuspended in 0.02 % ^{14}C-D-xylose after equilibration with 0.5 % "cold" D-xylose. *Curve 2:* intracellular radioactivity of yeast cells incubated in 0.02 % ^{14}C-D-xylose after preincubation in sugar-free medium. For experimental conditions, *see* text

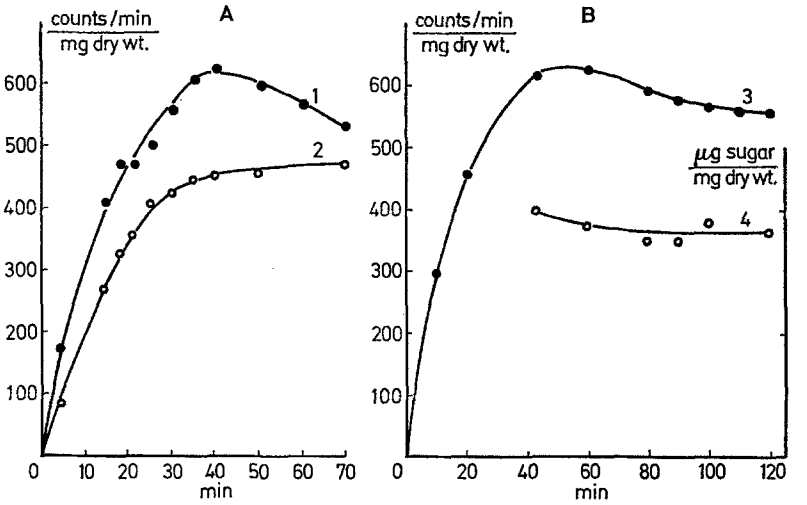

Fig. 2 A and B. Countertransport maximum of ^{14}C-L-xylose (A) and of ^{14}C-L-rhamnose (B) in *R. gracilis* cells preincubated with unlabelled L-xylose and L-rhamnose, respectively. *Curve 1:* intracellular radioactivity of yeast cells resuspended in 0.02 % ^{14}C-L-xylose after equilibration with 0.2 % "cold" L-xylose. *Curve 2:* intracellular radioactivity of yeast cells incubated in 0.02 % ^{14}C-L-xylose after preincubation in sugar-free medium. *Curve 3:* intracellular radioactivity of yeast cells resuspended in 0.01 % ^{14}C-L-rhamnose after equilibration with 0.2 % "cold" L-rhamnose. *Curve 4:* intracellular content of L-rhamnose, measured as reducing-sugar equivalents. For experimental conditions, *see* text

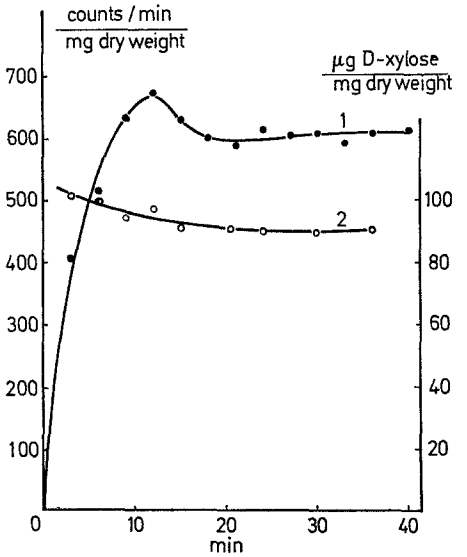

Fig. 3

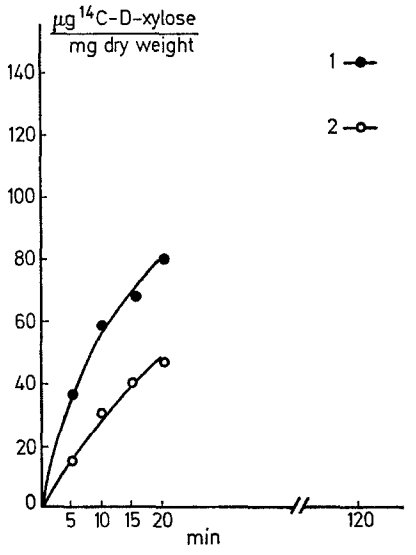

Fig. 4

Fig. 3. Countertransport maximum of ^{14}C-L-rhamnose in *R. gracilis* cells preincubated with unlabelled D-xylose. *Curve 1:* intracellular radioactivity of yeast cells resuspended in 0.01 % ^{14}C-L-rhamnose after equilibration with 0.5 % "cold" D-xylose. *Curve 2:* intracellular content of D-xylose, measured photometrically. For experimental conditions, *see* text

Fig. 4. Acceleration of ^{14}C-D-xylose uptake in *R. gracilis* by preloading the yeast cells with unlabelled D-xylose. *Curve 1:* ^{14}C-D-xylose influx into cells preloaded with "cold" D-xylose. *Curve 2:* ^{14}C-D-xylose influx into non-preloaded cells. The initial rates of uptake were calculated as in Fig. 6. For experimental conditions, *see* text

maximum obtained with ^{14}C-L-xylose (curve *1*). The right part shows the maximum obtained with ^{14}C-L-rhamnose (curve *3*). Curves *2* and *4* both serve as controls. Curve *2* represents the uptake of the label by cells preincubated in the absence of added sugar (under these conditions, no countertransport can occur); curve *4* depicts the analytical concentration of L-rhamnose inside the cells, indicating that during the descending part of the uptake curve, the intracellular concentration has already reached the new steady state level.

Fig. 3 shows the countertransport maximum of ^{14}C-L-rhamnose when the cell suspension was preincubated with unlabelled D-xylose (curve *1*). Curve *2* corresponds to the analytical concentration of the pentose inside the cells, taken as the control of the new steady state reached. The countertransport maximum obtained under these conditions indicates not only the existence of a single membrane carrier but also the capability of this carrier to translocate both pentose and hexoses.

Mobility of Loaded and Free Membrane Carrier Molecules

Koch (1964) has suggested that in *Escherichia coli* the flux of a sugar in one direction could accelerate the flux of another sugar in the opposite direction by facilitating the return of the carrier. This effect, termed "forced diffusion", offers a most valuable test for comparison of the actual mobilities of free and loaded carrier molecules, since in this case the initial rates of uptake of a labelled sugar are measured under identical conditions of external sugar concentration. The experimental procedure was as follows. A sample of the yeast suspension was preincubated with a relatively high sugar concentration until a steady state was reached. Then an analytically negligible amount of labelled sugar was added and the uptake of the label measured. Another sample of the yeast suspension was preincubated in the absence of added sugar. Then the same analytical concentration of sugar together with the same amount of label was added, and again the uptake of the label was measured. The results obtained with ^{14}C-D-xylose are depicted in Fig. 4. The initial rates of uptake were determined for cells preloaded with "cold" sugar and for unpreloaded cells. The values for preloaded and unpreloaded cells were 4.5 and 1.6 µg D-xylose/min/mg dry weight, respectively. Thus, the rate of influx was accelerated by efflux in preloaded cells, or, in other words, the binding of a sugar molecule to the carrier facilitates its movement through the cell membrane.

Kotyk (1967) has derived two kinetic tests relating the mobilities of free and loaded carriers, and has applied them to the equilibrating monosaccharide transport in baker's yeast. One test consists of a comparison of the half-saturation constant for transport, K_T, with the dissociation constant of the sugar-carrier complex, K_{CS}. In the other test, one compares the initial rate of exit of a labelled sugar into sugar-free medium with that into an equilibrium concentration of unlabelled sugar.

Both approaches were also used to estimate the mobilities of the free and the loaded carrier in *R. gracilis*. Fig. 5 shows the first approach. K_T was determined from initial velocities of uptake of ^{14}C-L-rhamnose by means of the Lineweaver-Burk plot (Fig. 5A). K_{CS} was estimated by measuring the half times of uptake of ^{14}C-L-rhamnose, added in an equal amount to all concentrations of unlabelled sugar in the suspending medium, and by plotting the logarithm of the velocity of uptake, $\log v_R$, against the logarithm of external sugar concentration, $\log S_0$; the intercept of extrapolated straight lines corresponds to $-\log K_{CS}$ (Fig. 5B).

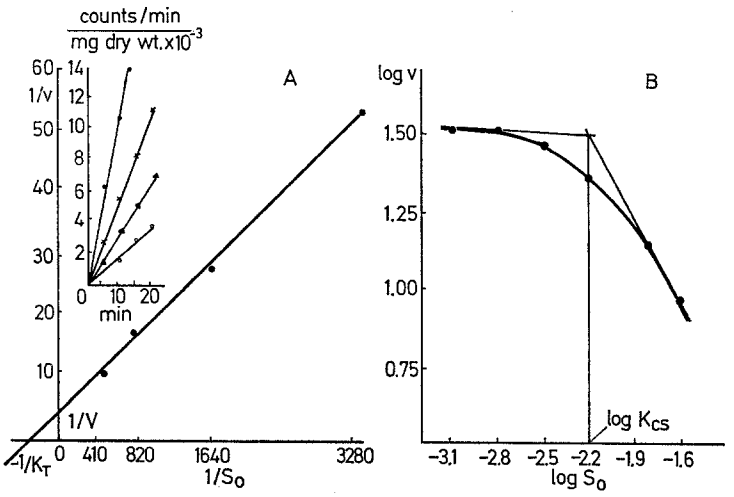

Fig. 5A and B. Estimation of the half-saturation constant of transport, K_T (A), and of the dissociation constant of the sugar-carrier complex (B). (A) Lineweaver-Burke plot of the initial velocities of ^{14}C-L-rhamnose entry into *R. gracilis* cells vs. ^{14}C-L-rhamnose concentration in suspending medium. The intercept on the abscissa corresponds to $-1/K_T$. Inserted figure presents the actual initial velocities of ^{14}C-L-rhamnose entry into yeast cells from suspension of 0.3, 0.6, 1.2 and 2.4×10^{-3} M ^{14}C-L-rhamnose. (B) Logarithmic dependence of the velocity of uptake on the external ^{14}C-L-rhamnose concentration. The intercept of the extrapolated straight lines corresponds to log K_{CS}. *See* text for further details

From Fig. 1, $K_T = 3.4 \times 10^{-3}$ M and $K_{CS} = 6.1 \times 10^{-3}$ M. According to Eq. (8) of Kotyk (1967), the mobility ratio $D_{CS}/D_C = 2\,K_{CS}/K_T - 1$, where D_{CS} is the mobility of a loaded carrier and D_C that of a free one. Making the substitution of *R. gracilis*, one obtains:

$$D_{CS}/D_C = 0.0122 \text{ M}/0.0034 \text{ M} - 1 = 2.6,$$

or, in other words, the loaded carrier moves more rapidly than the free one.

The second test is dealt with in Fig. 6. The cell suspension was preincubated with a high outside concentration of labelled sugar, filtered, washed on the filter twice with ice-cold water and resuspended either in a sugar-free medium or in a medium with an equal concentration of unlabelled sugar. As seen from Fig. 6, the two rates of efflux differ considerably, the one into the sugar-containing medium being greater than that into the sugar-free medium.

According to the simplified Eq. (12) (for very high S_o) of Kotyk (1967), in this case D_{CS}/D_C equals $2/a - 1$, where $a = v_{sf}/v_s$ (v_{sf} is the initial rate of

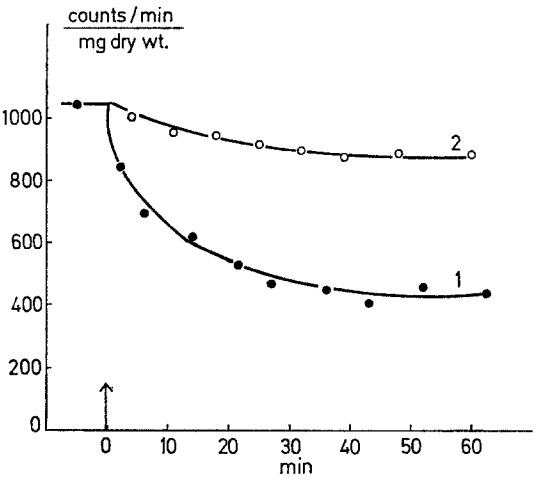

Fig. 6. Comparison of the initial rates of exit from the cells of a labelled sugar into sugar-free and into unlabelled sugar-containing medium. *Curve 1:* efflux of ^{14}C-D-xylose from *R. gracilis* cells equilibrated with 0.5 M ^{14}C-D-xylose into 0.5 M unlabelled D-xylose. *Curve 2:* efflux of ^{14}C-D-xylose from *R. gracilis* cells equilibrated with 0.5 M ^{14}C-D-xylose into sugar-free medium. The initial rates of efflux were calculated by means of the differentiated form of Newton's formula for equispaced arguments with associated decreasing differences (*cf.* Handbook of Chemistry and Physics, Chemical Rubber Publ. Co., 49th Edition, p. A-252). For experimental conditions, *see* text

efflux into sugar-free medium, v_s that into medium containing unlabelled sugar). Substituting the results obtained with *R. gracilis*:

$$D_{CS}/D_C = 2/0.15 - 1 = 12.3.$$

The latter two procedures have, however, been derived for an equilibrating transport, such as that in baker's yeast (Kotyk, 1967) or in mammalian erythrocytes (Miller, 1965). An uphill sugar transport with characteristics as observed in *R. gracilis* (Kotyk & Höfer, 1965; Höfer, 1968, *in preparation*) or in *Candida beverwijkii* (Deák & Kotyk, 1968) makes the conditions more complex. Therefore, the true ratio of carrier mobilities in *R. gracilis* cannot be quantitatively interpreted.

Discussion

The present evidence for a mobile membrane carrier for monosaccharide transport in *R. gracilis* cell membrane is based on the observation of countertransport maxima with various monosaccharides and on the acceleration of radioactive sugar uptake into cells preloaded with unlabelled sugar.

The uphill transport of monosaccharides in various yeast species such as *R. gracilis* (Kotyk & Höfer, 1965) and *C. beverwijkii* (Deák & Kotyk, 1968) possesses the typical characteristic of most uphill transports (Höfer, 1969), namely that the steady state intracellular sugar concentration does not increase linearly with increasing sugar concentration in the suspending medium. This complicates definition of the conditions for the occurrence of countertransport, as discussed by Kotyk and Höfer (1965). Nevertheless, the theoretical treatment has shown that the graph of intracellular labelling, under conditions when countertransport should occur, proceeds through a point with zero tangent also at other than infinite time. However, even in the best case, this point differs only by about 20% from the final new level.

Generally, the phenomenon of countertransport can be observed using two different procedures.

(1) The cell suspension is preincubated with a low concentration of a labelled substrate, then a high concentration of "cold" substrate is added and changes in cell label concentration are measured; a countertransport minimum should occur.

(2) The cell suspension is preincubated with a high concentration of "cold" substrate, the cells are spun down and resuspended in a low concentration of labelled substrate; a countertransport maximum should occur.

Using the former procedure, we failed to obtain a distinct countertransport minimum (Kotyk & Höfer, 1965). Nevertheless the experimental data presented in Figs. 1–3, obtained according to the latter procedure, represent distinct, although rather small, countertransport maxima. The results of Fig. 3 are of special importance, since they show that the influx of a hexose (L-rhamnose) can be transiently stimulated even by competition of a pentose (D-xylose) for the efflux. In other words, both the pentose and the hexoses use the same carrier for their translocation across the cell membrane.

Koch (1964) and Winkler and Wilson (1966) called attention to the possibility of stimulating the influx of a labelled sugar in *E. coli* by preincubating the cells with "cold" sugar, thus facilitating the return of the carrier. The "forced diffusion" (Fig. 4) represents an important criterion of the existence of a mobile carrier system in a biological membrane provided that the free and the occupied carriers display different mobilities.

The two kinetic tests of Kotyk (1967) (Figs. 5 & 6) were derived for equilibrating transport systems. Their application to an uphill-transport system is of limited value since the dissociation of the sugar-carrier complex

can be considerably affected by the coupling with metabolic energy. This is especially valid for *R. gracilis* since its transport system is very tightly coupled with the availability of metabolic energy (Kotyk & Höfer, 1965; Höfer & Kotyk, 1968). Nevertheless, the observed differences between the apparent mobilities of the free and loaded carrier may justify one to draw the conclusion that the sugar translocation in both directions is mediated by a single mobile membrane carrier.

I am indebted to Dr. Arnost Kotyk for stimulating comments on the work described here, to Prof. Dr. Augustin Betz for reading the manuscript and to Mrs. Lida Rihova for skilled technical cooperation.

Part of the work was supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

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