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Evidence for a Proton/Sugar Symport in the Yeast *Rhodotorula gracilis* (*glutinis*)

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1. The uptake of monosaccharides and polyols in the obligatory aerobic yeast *Rhodotorula gracilis* (*glutinis*) was accompanied by proton uptake. 2. The half-saturation constant of transport, K_T, depended on pH, changing from about 2mM at pH4.5 to 80mM at pH8.5 for D-xylose; this change of the effective carrier affinity was reversible. 3. The apparent dissociation constant of the monosaccharide carrier was estimated at pK_a 6.75. 4. At pH8.5, when the pH gradient across the cell membrane vanished, no sugar accumulation was demonstrable. 5. The half-saturation constants of sugar uptake and H^+ co-transport were very similar to each other, the latter obviously being controlled by the former. 6. The H^+/sugar stoicheiometry remained constant under various physiological conditions; it amounted to one H^+ ion per sugar molecule taken up. 7. The data are interpreted as a strong piece of evidence in favour of the active monosaccharide transport in *R. gracilis* (*glutinis*) being an H^+-symport energized by the electrochemical gradient of H^+ across the plasma membrane of the yeast.

Observations on the specific translocations of some amino acids and sugars across the plasma membrane of certain micro-organisms have indicated that the corresponding metabolic-energy-dependent carrier system catalyses a coupled translocation of H^+ and substrate (West & Mitchell, 1972; Seaston *et al.*, 1973; Komor & Tanner, 1974*a*,*b*). The coupling of H^+ translocation with that of substrate has been suggested to be a general mechanism of energization of the active carrier-mediated transport in bacteria (Mitchell, 1967; Harold, 1974). Eddy *et al.* (1970) reported a stimulation of active glycine transport by H^+ in the yeast *Saccharomyces carlsbergensis*, and Eddy & Nowacki (1971) explained it to be the fundamental part of the process. Slayman & Slayman (1974) showed that the system actively transporting sugars in *Neurospora crassa* is a co-transport system driven by the membrane potential and the H^+ gradient.

Considerable support to the concept of H^+/substrate symport has been provided by stoicheiometric studies of H^+/sugar or H^+/amino acid co-transport. The stoicheiometry of H^+/lactose symport in *Escherichia coli* was found to be 1 by West & Mitchell (1973), whereas the uptake of succinate was correlated with the movement of two H^+ ions (Gutowski & Rosenberg, 1975). Slayman & Slayman (1974) reported the stoicheiometry of H^+/glucose symport in *N. crassa* to be 0.8–1.4. Komor & Tanner (1974*a*) measured the stoicheiometry of protons co-transported per molecule of 6-deoxyglucose or glucose in *Chlorella vulgaris* as 1. In *Saccharomyces* species the ratio of protons taken up per molecule of phosphate or L-glutamate transported was 3 (Cockburn *et al.*, 1975).

In attempts to explain the H^+/substrate symport, the protonation of the carrier has been suggested to be the prerequisite for binding the substrate. The pH-dependent alternations of the carrier affinity are due to conformational changes of the carrier molecule after its reversible protonation or deprotonation, as was shown by Komor & Tanner (1975).

The yeast *Rhodotorula gracilis* (*glutinis*) has been reported (Misra & Höfer, 1975) to possess an active H^+-extruding assembly in the plasma membrane capable of maintaining a pH gradient of about 2 pH units across the membrane (outside acidic). The process of H^+ extrusion is energy-dependent and sensitive to dicyclohexylcarbodi-imide. This yeast takes up monosaccharides by an active-transport mechanism (Höfer & Kotyk, 1968). The onset of sugar transport is associated with a transient diminution of the pH gradient, an indication of the function of a H^+-symport mechanism in the active sugar uptake in this yeast (Misra & Höfer, 1975). It was the purpose of the present study to provide further evidence for the existence of the H^+/substrate-symport mechanism in *R. gracilis* (*glutinis*). A preliminary report of this work has appeared (Misra *et al.*, 1975).

Materials and Methods

The growth of the obligatory aerobic yeast *Rhodotorula gracilis* (*glutinis*), listed as *Rhodosporidium toruloides* Banno, mating type *a* at the American

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Type Culture Collection, Rockville, MD, U.S.A. (no. 26194) and at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands (no. 6681), and pH measurements of cell supensions were carried out as described previously (Misra & Höfer, 1975). In all experiments, unless otherwise specified, an aerobic yeast suspension (2.5g wet wt./100ml) in water or 90mM-potassium phosphate buffer was used and D-xylose served as the substrate for measurements of monosaccharide transport. This pentose is taken up rapidly and is not metabolized during the first 30min of incubation until an enzyme system for its catabolism is induced (Höfer *et al.*, 1971). Where experiments were carried out in unbuffered aqueous suspensions, 1mM-$CaCl_2$ was added to enhance the ionic strength of the solution. This electrolyte did not influence the H^+ balance across the plasma membrane. The sugar uptake was measured by using the membrane-filter technique as described by Heller & Höfer (1975). For measurements of the initial transport velocity, samples were withdrawn in 2-min intervals during the first 10min of uptake.

Induction of alditol-transporting system

The procedure was a slight modification of the method described by Klöppel & Höfer (1976). The unbuffered yeast suspension, which contained 5mM-KCl throughout the experiments, was incubated with 10mM-ribitol for 2h at 28°C. The induced cells were then centrifuged (5min, 5000*g*) and aerated in aqueous suspension for about 30min so that the ribitol taken up during the induction period was depleted. The aerated suspension was again centrifuged (5min, 5000*g*) resuspended and used for H^+-co-transport experiments.

Measurements of the effective carrier affinity

The initial velocity of sugar uptake, v_0, was measured at 28°C with widely different D-xylose concentrations, s, at various pH values. Potassium phosphate buffer (90mM) was used throughout these experiments. The half-saturation constants K_T and the maximal transport velocities V_T at each selected pH were estimated by the least-squares method in corresponding double-reciprocal plots ($1/v_0$ versus $1/s$).

Measurements of H^+ co-transport and of H^+/sugar stoicheiometry

The initial velocity of H^+ uptake was measured by recording the pH change of unbuffered yeast suspensions with a PHM-62 instrument (Radiometer, Copenhagen, Denmark) on addition of different sugar concentrations. The initial rate of the pH change was extrapolated graphically to the time of sugar addition. The corresponding amount of protons taken up was then calculated after correcting for the buffer capacity of the yeast suspension as determined by the addition of a known amount of 10mM-HCl. No correction for H^+ recycling was made. This means that our measurements represent the minimum amount of H^+ co-transported with sugar. However, the fixed stoicheiometry of H^+/sugar co-transport measured under various physiological conditions and with different monosaccharides (cf. Table 1) justifies the conclusion that under the experimental conditions used, true H^+ uptake was measured.

When the proton/sugar stoicheiometry was measured, samples of the cell suspension were taken at intervals during the pH measurements to measure concurrently also the initial velocity of sugar uptake. Again, the double-reciprocal plots obtained from the initial-velocity data yielded the half-saturation constant and the maximal velocity for both H^+ and sugar uptake. The V_T values were used for calculation of the stoicheiometry of the H^+/sugar symport.

Determination of the intracellular pH by the distribution of 5,5-dimethyloxazolidine-2,4-dione

The basis of the method is the fact that the distribution of 5,5-dimethyloxazolidine-2,4-dione across the plasma membrane is a function of pH. To a cell suspension (about 15mg dry wt./ml) maintained at known external pH and constant agitation at room temperature (20°C) 0.33mM-5,5-dimethyl-[2-^{14}C]oxazolidine-2,4-dione was added. Samples (1ml) were taken after 7–10 min (this distribution of 5,5-dimethyloxazolidine-2,4-dione was completed in about 3min). The samples were centrifuged on an ECCO-Quick centrifuge (Collatz, Berlin, Germany) at 15000rev./min for 15s and the radioactivity of the supernatant was measured in a Packard model 3380 liquid-scintillation counter in toluene/Triton X-100/ethanol scintillation fluid (5:3:2, by vol.) that contained 0.6% 2,5-dimethyloxazole and 0.06% 1,4-bis-(5-phenyloxazol-2-yl)benzene. The decrease of the radioactivity was taken as a measure of 5,5-dimethyloxazolidine-2,4-dione absorbed inside the cells. In some parallel runs intracellular pH was also determined photometrically by the use of the dye Bromophenol Blue, as described by Kotyk (1963). The following expression was used to calculate the internal pH (Kotyk & Janáček, 1975):

$$pH_i = pH_e + \log\left[\frac{c_i}{c_e}(1 + 10^{pK-pH_e}) - 10^{pK-pH_e}\right]$$

In this equation, pK values of 5,5-dimethyloxazolidine-2,4-dione and of the dye were taken as 6.3 and 4.0, respectively; pH_e (the external pH) was measured in the cell suspension, c_e (external con-

centration of 5,5-dimethyloxazolidine-2,4-dione or of the dye) was determined in the filtrate, and c_i (intracellular concentration of 5,5-dimethyloxazolidine-2,4-dione or of the dye) was calculated from the difference between the initially added concentration and c_e. To calculate c_i, the intracellular water volume was measured by means of the difference between ^3H_2O and hydroxy[^{14}C]methylinulin contents of the cell pellet after incubation of the cell suspension with the two labelled compounds and separation of the cells by filtration. The latter compound served as an impermeable substance for estimation of the extracellular space (a modified method of Harold *et al.*, 1970). The values calculated were in good agreement with those obtained by the haematocrit method (Klöppel & Höfer, 1976) and amounted on average to 2.0 μl of intracellular water per mg dry wt. of yeast.

It should be mentioned here that the values of pH_i given in the present paper represent only an average intracellular pH, neglecting any compartmentation within the yeast cell. Also the relatively low accumulation of the two indicator compounds at high pH_e, where ΔpH appears to be small, makes the pH_i determination less reliable. Both indicators led to comparable results, which were considered only as approximate values.

Chemicals

Hydroxy[^{14}C]methylinulin, [^{14}C]galactose, 2-deoxy-D-[^{14}C]glucose and ^3H_2O were products of The Radiochemical Centre, Amersham, Bucks., U.K. 5,5-Dimethyl[2-^{14}C]oxazolidine-2,4-dione was from New England Nuclear, Boston, MA, U.S.A. Triton X-100 was purchased from Serva Feinbio-

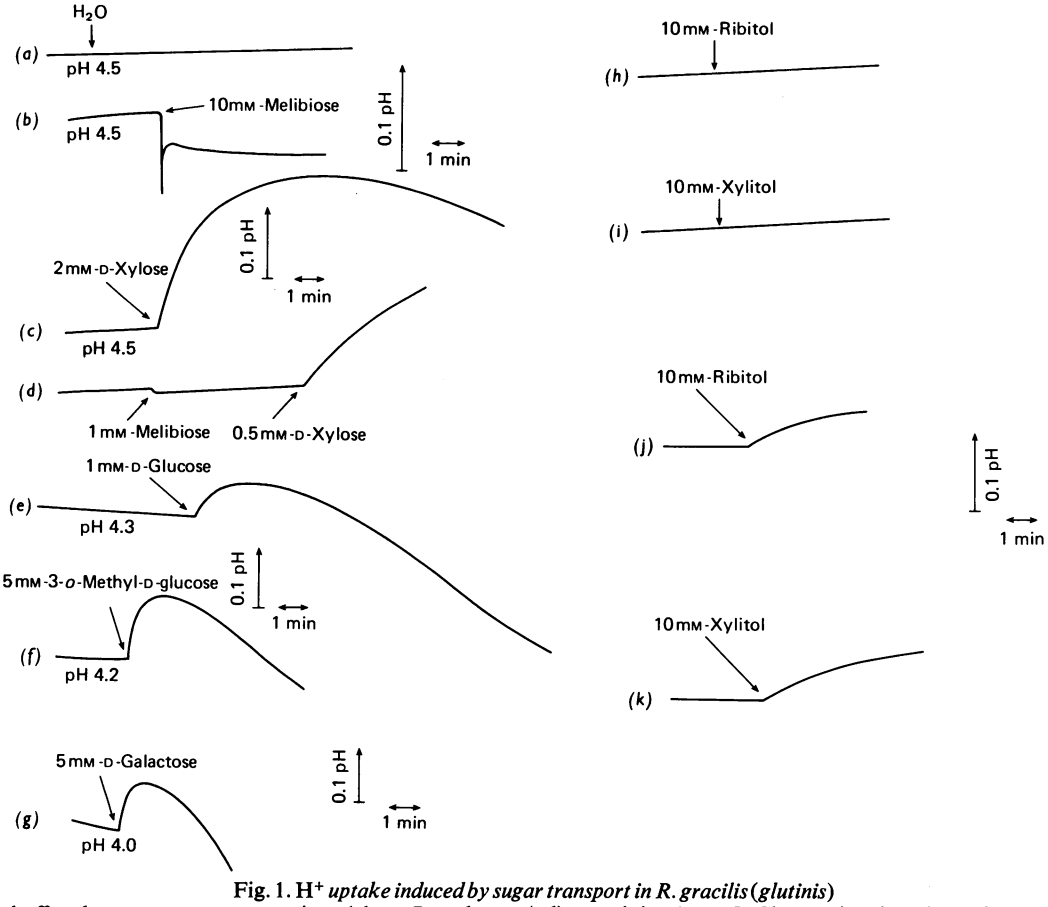

Fig. 1. H^+ *uptake induced by sugar transport in R. gracilis (glutinis)*
Unbuffered aqueous yeast suspensions (about 7 mg dry wt./ml) containing 1 mM-$CaCl_2$ were incubated at 28°C. Curves (*a*), (*b*) and (*d*) serve as control runs; in run (*d*) the addition of a transportable sugar (D-xylose) followed that of a non-transportable one (melibiose). Curves (*c*), (*e*), (*f*) and (*g*) demonstrate H^+ co-transport triggered by the addition of transportable monosaccharides. Curves (*j*) and (*k*) show the H^+ co-transport with two alditols in induced cells; in uninduced cells no proton uptake could be demonstrated (curves *h* and *i*).

chemica, Heidelberg, Germany, yeast extract from Difco Laboratories, Detroit, IL, U.S.A., and all other chemicals were from E. Merck A.G., Darmstadt, Germany. The growth medium contained: NH_4NO_3, 0.066%; K_2HPO_4, 0.1%; $MgSO_4,7H_2O$, 0.1%; NaCl, 0.05%; $CaCl_2,2H_2O$, 0.033%; $FeCl_3,6H_2O$, 0.005%; yeast extract, 0.03%; D-glucose, 4%; pH 5.4.

Results and Discussion

It was suggested previously (Misra & Höfer, 1975) that the transient alkalinization of *R. gracilis* (*glutinis*) suspensions on addition of sugar is due to the functioning of an H^+/sugar symport. However, since all monosaccharides so far tested are taken up actively by this yeast, it was difficult to establish a control experiment to check a possible non-specific pH change caused by the addition of carbohydrates. In the present paper, melibiose was used as a non-transportable sugar (cf. Janda & von Hedenström, 1974) to prove the specificity of H^+ co-transport during sugar uptake (Fig. 1, curves *a–g*). As another control of the coupled H^+ and carbohydrate transport, the inducibility of an active transporting system for alditols (Klöppel & Höfer, 1976) was used. If xylitol or ribitol was added to unbuffered suspensions of induced cells, a distinct increase in the pH of the suspension was observed (Fig. 1, curves *j* and *k*), whereas suspensions of non-induced cells did not respond to added alditols (Fig. 1, curves *h* and *i*). These results confirm the specific coupling of the observed H^+ uptake in co-transport with monosaccharide or alditols in *R. gracilis* (*glutinis*).

Some other predictions of the energization of active transport on the basis of an H^+ symport are as follows.

1. A reversible change of the carrier affinity with altered pH of the external milieu. To test this, the yeast cells were incubated at the same sugar concentration in two parallel runs at two different pH values (potassium phosphate buffer). After a steady-state sugar-uptake value was reached, the cells were removed by centrifugation (5min, 5000*g*) and transferred to new media where the buffers, i.e. the pH values, were just exchanged but the sugar concentration remained the same. The sugar uptake/release was followed until new steady states in both runs were recorded. Fig. 2 demonstrates that the steady-state intracellular sugar concentrations attained at the two different pH values could be reversibly exchanged just by an exchange transfer of the cells from their respective buffered media. This reversibility of steady states is best explained by assuming that the carrier affinity was reversibly changed with altered external pH.

Changes of the carrier affinity for sugar by altered pH was further proved by measurements of the half-saturation constants K_T and the maximal velocities

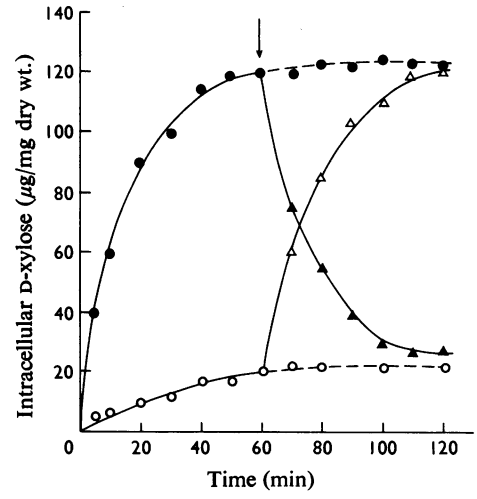

Fig. 2. *Reversible changes of the intracellular steady-state sugar concentrations by varying the pH of the yeast suspension from* 4.5 *to* 8.5 *and vice versa*

Yeast suspensions (about 4mg dry wt./ml) were incubated in 90mM-potassium phosphate buffer (pH either 4.5 or 8.5) at 28°C with 50mM-D-xylose. After 60min (arrow) the cells were separated rapidly by centrifugation (1min, 10000*g* at 2°C) and resuspended in just the buffer of opposite pH containing again 50mM-D-xylose: ●, pH4.5; △, pH8.5→4.5; ▲, pH4.5→8.5; ○, pH8.5.

V_T for D-xylose uptake at different pH values. The data of Fig. 3 indicate that the carrier system is made up of two components. At pH4.5, apparently only a high-affinity component was present. At more alkaline pH, namely 6.5, there appeared two distinct components, one of high and the other of low affinity. The high-affinity component disappeared at pH8.5 and only the low-affinity one was detectable. Under these conditions no D-xylose accumulation could be demonstrated, even if the rate of respiration, as measured by oxygen electrode, was decreased only insignificantly. The sugar uptake was also insensitive to the addition of uncouplers (not shown in Fig. 3). A different pH-dependence of sugar accumulation from that of oxygen consumption had already been reported by Kotyk & Höfer (1965).

If one assumes that the two components of the carrier system correspond to the protonized (high-affinity) and deprotonized (low-affinity) forms of the same carrier molecules, it is possible to determine the apparent dissociation constant of the carrier by measuring the pH-dependence of transport velocity at a given substrate concentration. If it is low enough to exclude any participation on transport of the low-affinity form of the carrier, the decrease in

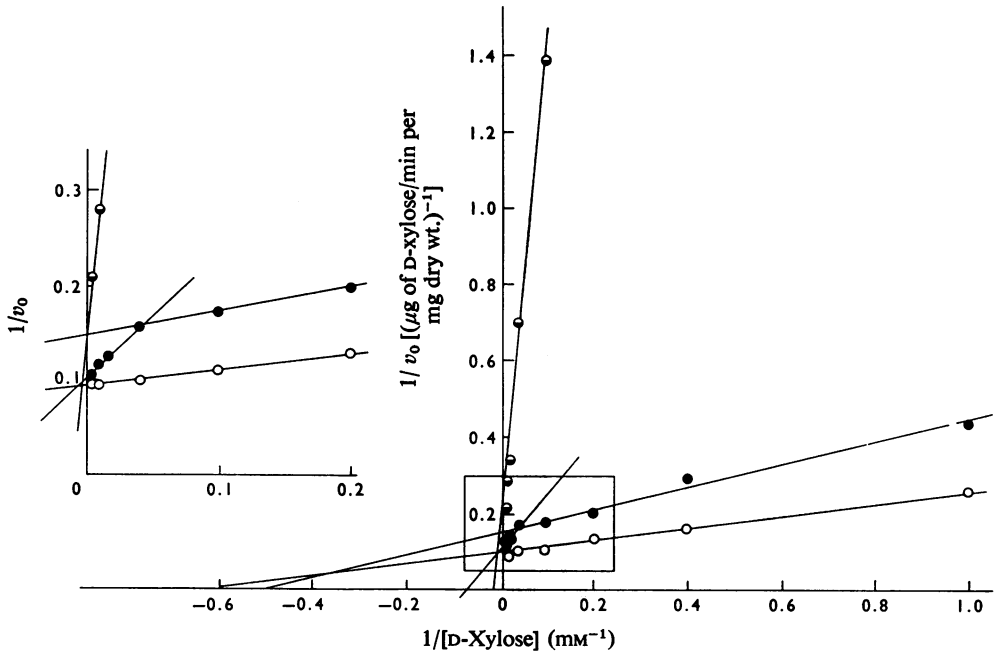

Fig. 3. *Double-reciprocal plots of the uptake kinetics for* D-*xylose at three different pH values:* 4.5 (○), 6.5 (●) *and* 8.5 (◒) Measurements were in potassium phosphate buffer at 28°C. The region of higher substrate concentrations (boxed) is replotted on an enlarged scale in the insert (left). The estimated K_T and V_T values are summarized below.

		High-affinity component		Low-affinity component			
Curve	pH_e	K_{T_h}	V_{T_h}	K_{T_l}	V_{T_l}	v_0	c_i/c_e
○	4.5	1.7	10.5	—	—	10.0	32
●	6.5	2.0	6.7	15	9.7	6.5	14
◒	8.5	—	—	83	6.6	0.7	1

K_T values are expressed in mM, V_T in μg of D-xylose/mg dry wt./min; v_0 corresponds to the initial velocity of uptake at $c_e = 10$mM; c_i/c_e represents the accumulation ratio steady-state intracellular versus extracellular concentration; pH_e = extracellular pH.

the rate of sugar transport can then be taken as a measure of that portion of carrier molecules which is deprotonized. At the half-maximal transport velocity, 50% of the carrier molecules occur in the deprotonized form, and consequently this pH corresponds to the apparent pK_a of the carrier. Fig. 4 shows the results of one such experiment with 2mM-D-xylose as transported substrate. The estimated pK_a of the monosaccharide carrier in *R. gracilis* (*glutinis*) amounted to 6.75±0.04 (average value of four experiments±S.E.M.). This value is very similar to that obtained for 6-deoxyglucose uptake in the green alga *Chlorella vulgaris* by Komor & Tanner (1974*b*).

To obtain more information about the changes occurring in the proton gradient across the plasma membrane when the yeast cells are suspended in potassium phosphate buffer of different pH values, the intracellular pH (pH_i) was measured as described in the Materials and Methods section. Figs. 5(*a*) and 5(*b*) show that pH_i remained more or less constant at the value of 6–6.5 when the pH of the cell suspensions (pH_e) was increased to 5. Further increase of pH_e was followed by a parallel rise of the pH_i. However, on increasing pH_e above 8, no corresponding enhancement of pH_i was observed: rather, pH_i appeared to attain a constant value of about 8.5. This decrease in the pH gradient across the plasma membrane at pH_e around 8.5 is most likely to be the factor involved in the drastically lowered rate of sugar transport (both the velocity and the accumulation) at this pH_e (see insert in Fig. 3). This is in accordance with the general theory of the H^+-symport mechanism, in which the electrochemical gradient of protons is the

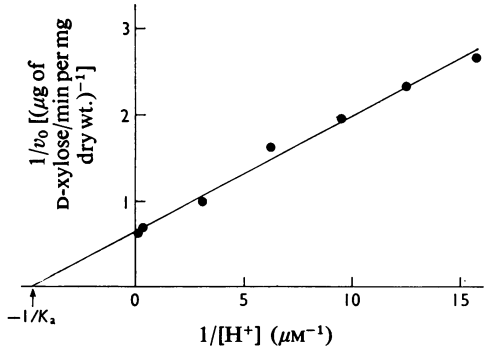

Fig. 4. *Apparent dissociation constant K_a of the monosaccharide carrier in R. gracilis (glutinis).*
K_a was estimated from the double-reciprocal plot of the rates of D-xylose (2mM) uptake against the H^+ concentration of cell suspensions (about 4mg dry wt. of yeast/ml in 90mM-potassium phosphate buffer of various pH values). The experimental temperature was 28°C. In this experiment the value of K_a was 0.2μM, corresponding to a pK_a of 6.7.

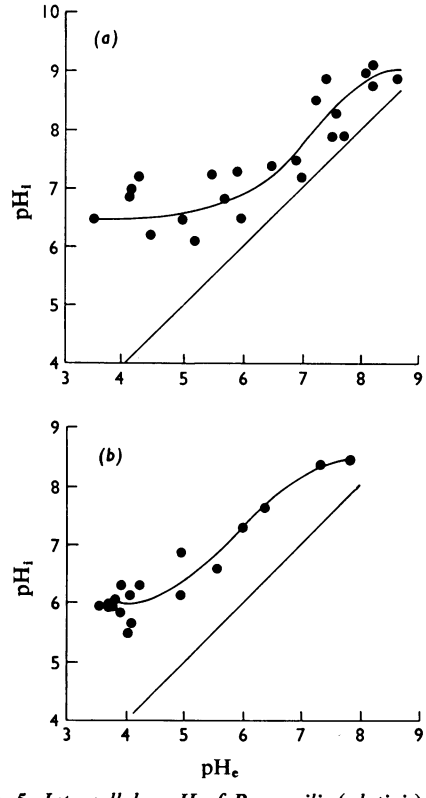

Fig. 5. *Intracellular pH of R. gracilis (glutinis) cells*
The intracellular pH (pH_i) was determined with 5,5-dimethyloxazolidine-2,4-dione (*a*) and with Bromophenol Blue (*b*). For experimental conditions, see the Materials and Methods section.

primary driving force for the active transport (e.g. Mitchell, 1967; Harold, 1974).

2. The identity of the half-saturation constants K_T of both H^+ movement (co-transport) and substrate uptake. In these experiments, after the addition of sugar, H^+ uptake was measured with a pH-meter and concurrently samples of the cell suspension were taken at intervals for determination of sugar uptake. The experiments were carried out at different temperatures in unbuffered aqueous suspensions containing 1mM-$CaCl_2$ (see the Materials and Methods section). Fig. 6 shows one of several such experiments; in each experiment the outside sugar concentration was varied from 1 to 10mM. Table 1 summarizes the results obtained with different yeast batches under various physiological conditions. Even if the K_T values fluctuated from one yeast batch to another, the average K_T for sugar uptake was equal to that for H^+ co-transport.

3. A defined stoicheiometry of H^+ substrate symport. In the present paper the stoicheiometry was calculated from the maximal velocities of both H^+ and sugar transport and is also summarized in Table 1. It amounted to 1 proton disappearing per sugar molecule taken up.

It has been reported previously (Misra & Höfer, 1975) that in unbuffered suspensions *R. gracilis (glutinis)* cells extrude protons until the pH_e reaches a value around 4. The actual pH_e depends on the particular yeast batch used. In the experiments

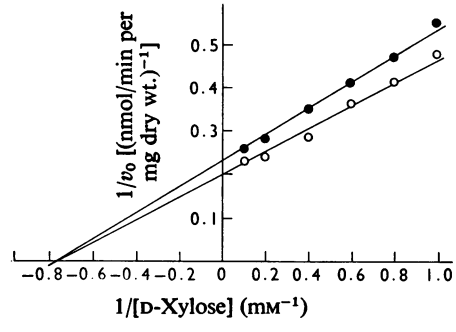

Fig. 6. *Stoicheiometry of the H^+/sugar symport in R. gracilis (glutinis)*
From double-reciprocal plot of D-xylose uptake velocities against D-xylose concentrations both the half-saturation constants (K_T) and the maximal velocities (V_T) of the sugar uptake and of the H^+ co-transport were estimated. ○, D-Xylose uptake (K_T = 1.2mM, V_T = 49.3nmol/min per mg dry wt.); ●, H^+ co-transport (K_T = 1.2mM, V_T = 42.7nmol/min per mg dry wt.). The values of maximal transport velocity were then used to calculate the H^+/sugar stoicheiometry. In this experiment it amounted to 42.7/49.3 = 0.9 H^+ ion per D-xylose molecule. For further data, see Table 1 and the Materials and Methods section.

Table 1. *Transport parameters and stoicheiometry of H+/sugar symport in R. gracilis (glutinis)*
Experimental conditions were as described in the Materials and Methods section. For an interpretation see the text. pH_e is the extracellular pH of unbuffered suspensions containing 1 mM-$CaCl_2$. Values in parentheses are averages ± S.E.M. for the group of results above them or for the numbers of experiments stated. Abbreviation: n.m., not measured.

Sugar	Temperature (°C)	pH_e	K_T (mM)		V_T (nmol/min per mg dry wt.)		$\frac{H^+}{Sugar}$
			Sugar	H^+	Sugar	H^+	
D-Xylose	22	3.9	0.6	0.9	18.0	16.7	0.9
		4.0	0.5	0.6	26.3	32.2	1.2
		5.3	1.0	0.4	17.2	15.9	0.9
			(0.7 ± 0.2)	(0.6 ± 0.2)	(20.5 ± 2.9)	(21.6 ± 5.3)	(1.0 ± 0.1)
	26	3.2	0.7	0.9	31.7	43.1	1.4
		3.4	0.8	1.5	26.7	25.1	0.9
		3.5	1.4	2.4	27.5	22.5	0.8
		3.7	2.6	1.2	30.7	29.5	1.0
		4.2	0.7	0.6	38.0	41.2	1.1
		4.5	1.1	0.4	36.0	29.8	0.8
		5.2	0.4	0.5	28.7	21.7	0.8
			(1.1 ± 0.3)	(1.1 ± 0.3)	(31.3 ± 1.6)	(30.4 ± 3.2)	(1.0 ± 0.1)
	28	3.4	1.4	1.0	40.0	47.2	1.2
		3.9	1.0	0.9	42.5	47.5	1.1
		4.0	1.2	1.2	49.3	42.7	0.9
			(1.2 ± 0.1)	(1.0 ± 0.1)	(43.9 ± 2.8)	(45.8 ± 1.5)	(1.1 ± 0.1)
	40	4.3–5.3	n.m.	n.m.	n.m.	n.m.	(1.1 ± 0.1) (nine expts.)
D-Galactose	28	3.6	1.2	2.2	35.7	26.2	0.7
		4.6	n.m.	n.m.	n.m.	n.m.	(1.0 ± 0.1) (nine expts.)
2-Deoxy-D-glucose	28	3.0	0.3	0.3	12.5	18.9	1.5
		4.5	0.4	0.4	6.0	6.2	1.0

of Table 1 it fluctuated between 3.2 and 5.3; in this pH range the H^+/sugar stoicheiometry did not change significantly. The maximal velocity of transport passed through a maximum at pH_e 4–4.5, in accordance with published data (Kotyk & Höfer, 1965); the H^+ co-transport exhibited the same pattern.

As expected from previous experiments (cf. Heller & Höfer, 1975) the V_T values of sugar uptake were also strongly influenced by enhanced temperature. Again, the maximal velocity of H^+ co-transport was affected correspondingly. The H^+/sugar stoicheiometry was maintained constant.

The fact that the parameters (K_T and V_T) of the H^+ co-transport are influenced in the same way by changing the experimental conditions that are known to affect the sugar transport, without interfering with the given stoicheiometry, is of great importance for the conclusion that the two processes (H^+ co-transport and sugar uptake) are obligatorily coupled to each other. This conclusion was further strengthened by experiments with other monosaccharides, D-galactose and 2-deoxy-D-glucose, the latter having a much lower half-saturation constant than both D-galactose and D-xylose. The two hexoses showed the same stoicheiometry of the H^+/sugar symport (cf. Table 1). Thus the stoicheiometry proved to be independent of the experimental conditions (pH, temperature) and of the monosaccharide transported (K_T). However, the half-saturation constant of the H^+ co-transport was controlled by the K_T value of the particular sugar taken up.

The experimental data in the present study represent a strong piece of evidence in favour of the existence of a H^+/sugar symport as the mechanism of energization of the active sugar uptake in the yeast *R. gracilis* (*glutinis*). A similar line of evidence has been brought by West & Mitchell (1973) for the lactose transport in *E. coli* and by Komor & Tanner (1974*a*,*b*) for the hexose transport in *Chlorella vulgaris*. In *R. gracilis* (*glutinis*) the parallel stimulation of both the sugar uptake and the H^+ co-transport by increasing temperature or by changing pH_e further strengthens this conclusion.

The driving force of the H^+-symport mechanism, the electrochemical H^+ gradient, involves as an indispensable part of it the existence of a membrane potential. Experiments demonstrating the expected electrical potential differences across the cell membrane of *R. gracilis* (*glutinis*) have been carried out in this laboratory (R. Hauer & M. Höfer, unpublished work).

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