Variable H^+ /substrate stoichiometries in *Rhodotorula gracilis* are caused by a pH-dependent protonation of the carrier(s)

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Two carrier-mediated systems transport sugars in the yeast *Rhodotorula gracilis* depending on the pH. One system, with higher affinity for sugars, catalyses a symport of protons with sugar, whereas the other system, having lower affinity, is independent of protons. This was shown in three different ways. (1) At low pH, where only the high-affinity system works, a H^+ /sugar stoichiometry of 1 was found. An increase of the pH and of the sugar concentration, which allowed the low-affinity system to operate, brought about a drop of the stoichiometry to values below 1. (2) During H^+ symport the influx of positive charge was electrically compensated by an equivalent efflux of K^+ from the cells. At high pH and high sugar concentrations this stoichiometry of K^+ and sugar decreased concomitant with the H^+ /sugar stoichiometry. (3) At pH 7.5 both transport systems were operating, as shown by biphasic saturation kinetics. Under these conditions only the high-affinity transport was found to be electrogenic. These results agree with the theory of an electrogenic H^+ /sugar symport where changes in the affinity for substrate are brought about by reversible protonation and deprotonation of the carrier.

Electrogenic symport with H^+ or Na^+ appears to be the most general way used by cells for transporting molecules 'uphill' (Harold, 1976; Eddy, 1978). In this manner the energy stored in the difference of the H^+ or Na^+ electrochemical potential difference across the plasma membrane of bacteria (Harold, 1976), fungi (Eddy, 1978) and plants (Baker, 1978) serves as the driving force for secondary active transport. Although the energetics of this coupling is understood the molecular mechanism of this process remains obscure. Moreover, a large body of kinetic evidence has made it obvious that, on the mechanistic level, the unity of the energetic description, as described above, ceases to exist (Aronson, 1978; Lanyi, 1978; Robertson *et al.*, 1980).

One of the possible mechanisms is a reversible protonation of the carrier, the protonated and deprotonated forms of which display different affinities for a substrate. The protonation of the carrier outside increases the affinity for the substrate and thus increases the influx. On the interior side of the membrane the carrier is deprotonated and returns to its low-affinity form. Thus, efflux is initially slow and becomes appreciable only after accumulation has advanced to some degree.

This model has found a most elegant support in

experiments with an inducible H^+ /hexose transport system in *Chlorella vulgaris* (Komor, 1973; Tanner *et al.*, 1977), where both forms of the carrier could be separated experimentally by means of biphasic kinetics at appropriate pH (Komor & Tanner, 1974). Analogous observations have been made with fungi (Höfer & Misra, 1978) as well as with higher plants (Giaquinta, 1977) and interpreted in a similar way as described above. Hence, the mechanism postulated for *C. vulgaris* may be quite general.

However, a biphasic Lineweaver–Burk plot alone is not sufficient to prove that this type of mechanism is working in a given transport system. The following criteria should be fulfilled as well.

1. Both the high- and the low-affinity transport are catalysed by the same carrier. This is very difficult to prove and could be so far shown only indirectly for the inducible transport system in *C. vulgaris*, where both systems are simultaneously induced by D-glucose (Komor & Tanner, 1974).

2. The high-affinity component, the protonated form of the carrier, is more abundant at low pH, whereas the opposite is true for the low-affinity, deprotonated carrier. This has been shown to be true for *C. vulgaris* (Komor & Tanner, 1975) and for *R. gracilis* (Höfer & Misra, 1978).

3. The high-affinity system catalyses an electro-

genic symport of substrate with protons, whereas the low-affinity transport is electroneutral and without symport of H^+ .

The last criterion has not yet been tested comprehensively for any of the systems mentioned above. It is the purpose of this paper to report a systematic study conducted with the yeast *R. gracilis* concerning this problem.

Materials and methods

For all experiments *Rhodotorula gracilis*, systematically *Rhodospiridium toruloides* mating type *a* (C.B.S. 6681, A.T.C.C. 26194) was grown as described by von Hedenström & Höfer (1974).

Measurement of the H^+ /xylose stoichiometry

A 5% (wet wt./vol.) yeast suspension was aerated for 16–22 h in distilled water before use. Subsequently, the suspension was washed twice and resuspended in doubly quartz-distilled water. This yeast was aerated for at least 2 h before the start of the experiment. For each experimental run, 30 ml of this suspension was taken and stirred at 28°C with 0.1 mM- $CaCl_2$ in order to stabilize the pH electrode. The pH was recorded continuously by a Radiometer GK 201 electrode connected to a PHM 61

Radiometer pH meter and a strip-chart recorder (all three from Radiometer Deutschland, Krefeld, Germany). The experiment was started by adding D-xylose. Samples (1.0 ml) were withdrawn at intervals of 1 min, filtered and assayed for pentose by the orcinol method as described by Heller & Höfer (1975). At the end of each run, 20 μ l of 10 mM-HCl (Titrisol; Merck, Darmstadt, Germany) was added repeatedly in order to determine the buffer capacity of the suspension. For experiments at pH values higher than 5 the suspension was titrated with 10 mM- $Ca(OH)_2$ to the desired value ± 0.2 pH unit.

Measurement of the K^+/H^+ /xylose stoichiometry

The experiments were conducted as described above, except that samples (1.25 ml) were withdrawn 1 and 2 min before and 15, 30, 45 and 60 s after the addition of sugar and sedimented for 10 s at 15 000 g in an Ecco-Quick centrifuge (Collatz, Berlin, Germany). The supernatants were assayed for K^+ by atomic absorption spectrophotometry (model 360, Perkin-Elmer Bodenseewerk, Überlingen, Germany).

Measurement of the membrane potential

The membrane potential and its depolarization were estimated by the distribution of the lipophilic

Table 1. H^+ /sugar stoichiometry as a function of the external pH

A 2.5% (wet wt./vol.) yeast suspension (15 ml) was titrated with 10 mM- $Ca(OH)_2$ to the chosen pH. The initial uptake velocity of D-xylose was determined by withdrawing samples (1 ml) at intervals of 1 min. Sugar was measured as described by Heller & Höfer (1975). The pH was recorded during the experimental and the buffering capacity of the cell suspension was measured as described by Höfer & Misra (1978).

pH	D-Xylose concn. (mM) ...	Stoichiometry							
		0.5	0.7	1.0	2.0	75	150	200	300
4.5		0.77	0.66	0.81	1.75	0.78	0.63	1.10	0.93
		0.88	1.03	1.75	0.89	1.47	1.40	0.75	
		1.00	0.89	0.88	1.50	0.60	0.78		
		1.18	1.28	1.08	0.91	1.45	1.23		
			1.43		0.92				
	Mean	0.96	1.06	1.13	1.19	1.07	1.01	0.93	
5.5		1.24	1.34	1.23	1.32	0.67	0.42	0.37	0.79
		0.76	0.74	0.82	0.88	0.57	0.81	0.65	0.82
		1.30			0.69	0.47	0.50	0.65	
						0.61	1.31		
						0.58	0.76		
	Mean	1.10	1.04	1.03	0.96	0.58	0.76	0.52	0.81
6.5		1.15	1.17	1.92	0.97	0.74	0.40	0.33	0.43
		1.37	0.85	0.91	0.88	0.82	0.51	0.64	0.52
		1.00	0.92	0.88	1.12	0.44	0.80		0.32
		0.65	0.77	0.71	1.00	0.53	0.68		0.24
					1.78	0.36	0.33		
	Mean	1.04	0.93	1.10	1.15	0.80	0.63		
						0.61	0.49	0.48	0.38

cation tetraphenylphosphonium across the plasma membrane as described before (Hauer & Höfer, 1978).

Results and discussion

In our strain of yeast three ways can be thought of for differentiating transport with protons from transport without protons.

The H⁺/sugar stoicheiometry

For the high-affinity transport system an H⁺/sugar stoicheiometry of 1 has been described under conditions where the low-affinity system is inactive (Höfer & Misra, 1978). Herewith, H⁺ is compensated electrically by an equivalent extrusion of K⁺ (Fig. 2). If the low-affinity system transports sugar without H⁺, the stoicheiometry should decrease whenever the system works. This is the case at high concentrations of substrate, where the high-affinity system is already saturated. Moreover, the latter system is working especially at high pH.

Fig. 1. Transport systems of the plasmalemma of *R. gracilis*

A varying participation of carriers can give different H⁺/K⁺/sugar stoicheiometry: (1) H⁺ = K⁺ = sugar if only systems 1 and 5 operate; (2) H⁺ = K⁺ < sugar if either system 2 (at high pH) or systems 3 and/or 4 become operative.

The pK of the carrier has been estimated by Höfer & Misra (1978) to be 6.75.

As shown in Table 1 both predictions proved to be true. The data demonstrate clearly the predicted tendency of decreasing stoicheiometry with increasing sugar concentration and increasing pH. At pH 4.5 the carrier is expected to be about 99% in its protonated form; correspondingly no change of the stoicheiometry was found even at D-xylose concentrations in the 100 mM region.

It should be mentioned quite generally that H⁺/substrate stoicheiometries lower than 1 might be simulated also by a rapid electrogenic extrusion of protons through the H⁺ pump. Moreover, in our

Fig. 2. H⁺/K⁺/sugar stoicheiometry
An unbuffered 5% (wet wt./vol.) yeast suspension was incubated at 28°C. The H⁺ uptake was calculated from continuous pH recordings whereas the fluxes of K⁺ and sugar were determined by taking samples (1 ml) at intervals and measuring the extracellular K⁺ and intracellular sugar concentration as described by Hauer *et al.* (1981). In this experiment the sugar was 30 mM-D-xylose; the initial pH was 3.9. The H⁺/K⁺ stoicheiometry was 0.99 (±0.15 S.E.M., n = 18) and the H⁺/sugar stoicheiometry was 1.0 (±0.18 S.E.M., n = 13). These values were found to be independent of the sugar concentration used (0.5–30 mM). The initial pH was usually between 3.9 and 4.5. O, D-xylose; Δ, H⁺; □, K⁺.

Table 2. K^+ /sugar (and K^+/H^+) stoichiometry as a function of the external pH

The experiments were run as described in the legend to Table 1, except that additional samples were withdrawn 2 and 1 min before and 15, 30, 45 and 60 s after addition of D-xylose and analysed for K^+ by atomic absorption spectrophotometry. The upper values give the K^+ /sugar stoichiometry, the numbers in parentheses below the K^+/H^+ stoichiometry. The average K^+/H^+ stoichiometry of all experiments irrespective of the H^+ /sugar stoichiometry was 0.98 ± 0.04 s.e.m.

pH	D-xylose concn. (mm) ...	Stoichiometry			
		5	10	75	200
4.5		0.81	1.17	0.65	1.27
		(1.06)	(1.25)	(0.89)	(0.97)
		0.84	0.86	1.03	1.29
		(0.88)	(0.93)	(0.98)	(1.03)
		1.05	0.92	0.93	0.82
		(0.89)	(0.86)	(1.03)	(0.98)
Mean		0.90	1.00	0.87	1.13
		(0.94)	(1.01)	(0.97)	(0.99)
6.5		1.02	1.36	0.61	0.18
		(1.06)	(1.30)	(1.04)	(0.72)
		0.83	0.73	0.49	0.29
		(0.87)	(0.76)	(0.86)	(0.91)
			0.86	0.73	0.36
			(0.87)	(1.02)	(0.61)
Mean			1.16	0.66	0.57
			(1.36)	(0.84)	(1.00)
				0.41	0.56
				(0.87)	(0.98)

yeast still another mechanism of compensation of the H^+ co-transport might be effective, e.g. the electro-neutral K^+/H^+ exchange (cf. Fig. 1). However, this kind of interference can be made evident through a stoichiometry much lower than 1 even at low sugar concentrations (0.5–2 mm). For this reason, only experiments giving stoichiometries around 1 at low D-xylose concentrations were included in Table 1. Approximately one out of two experiments had to be rejected. In these cases values such as 0.19, 0.26, 0.37, 0.43 and 0.54 were observed. As predicted, even in these experiments the H^+ influx was electrically balanced by K^+ efflux (results not shown).

The K^+ /sugar stoichiometry

Protons taken up during the initial period of sugar transport are electrically compensated for by an equivalent efflux of potassium ions, as shown in Fig. 2. Consequently, the same predictions and criticisms

Fig. 3. Uptake of tetraphenylphosphonium as a function of the D-xylose concentration

A 4% (about 10 mg dry wt./ml) cell suspension was incubated in 60 mM-Tris/citric acid buffer, pH 7.5. The experiment was started by adding $20 \mu\text{M}$ - $[^{14}\text{C}]$ -tetraphenylphosphonium (8.66 TBq/mol). After 4 min, portions (4 ml) were transferred to vessels containing different D-xylose concentrations: $\circ$, control; $\odot$, 0.5 mm; $\square$, 1 mm; $\triangle$, 2 mm; $\blacktriangle$, 10 mm; $\bullet$, 20 mm; $\blacksquare$, 50 mm. The radioactivity was measured in the supernatant as described by Hauer & Höfer (1978) ($n = 4$).

made for the uptake of protons should also be true for the efflux of K^+ . If the proper controls were made, i.e. the H^+ /sugar stoichiometry was 1:1 at low sugar concentrations (up to 10 mm), then the pattern of the K^+ /sugar stoichiometries conformed with that of the H^+ /sugar stoichiometries (compare Table 1 with Table 2).

Table 2 summarizes the data of both K^+ /sugar and K^+/H^+ stoichiometry. The K^+ /sugar stoichiometry was 1:1 at pH 4.5 and also at pH 6.5 with low sugar concentrations (the low-affinity deprotonated carrier is not operating); it fell to values as low as 0.4 at pH 6.5 with high sugar concentrations. Under all conditions, irrespective of the actual H^+ /sugar or K^+ /sugar stoichiometry the proton influx was mirrored by the K^+ efflux ($K^+/H^+ = 0.98 \pm 0.03$ s.e.m., $n = 28$). The K^+/H^+ stoichiometry of 1 was

maintained even in experiments that were excluded from the Tables because of their low H^+ /sugar stoichiometry at low sugar concentrations (results not shown).

The electrogenicity of sugar transport

Whereas the high-affinity component is electrogenic the low-affinity system should be electroneutral. This can be verified experimentally in the following way. As shown before (Hauer & Höfer, 1978), the electrogenicity of H^+ /sugar symport was shown by a depolarization of the membrane potential which correlated quantitatively with the flow of sugar via the high-affinity system. If the low-affinity system were electrogenic, as well, one should expect a higher degree of depolarization at high sugar concentrations and pH 7.5 (both systems operative). At low concentrations of substrate only the high-affinity component can become saturated.

The extent of depolarization of the membrane potential was measured by observing the effect of different sugar concentrations upon the rate of uptake of the lipophilic cation tetraphenylphosphonium. The higher the electrogenic H^+ /sugar flux the more the uptake of tetraphenylphosphonium is slowed down as the result of an increased depolarization (Hauer & Höfer, 1978). As shown in Fig. 3, the effect of D-xylose upon the membrane potential approaches a maximum value at about

10 mM. This becomes clearer if the values are analysed in the double-reciprocal plot according to Lineweaver and Burk (reciprocal depolarization in arbitrary units as a function of the reciprocal sugar concentration). In this way, a monophasic plot is observed (Fig. 4b) which contrasts with the biphasic kinetics obtained by measurements of sugar transport at pH 7.5 (Fig. 4a). The K_m values for both the depolarization of the membrane potential and the transport of D-xylose by the high-affinity system are identical within the margin of error. Similar results were obtained with D-galactose as a substrate (Hauer & Höfer, 1978).

In this strain of yeast the membrane potential depends strongly on the external pH. At pH 4.5 its value is close to zero (Hauer *et al.*, 1981). For this reason it is not possible to do this type of experiment at pH values lower than 6.5.

All three tests carried out in this work demonstrate that in *R. gracilis* sugars are taken up by two different systems: a high-affinity system which accumulates sugars by means of an electrogenic symport with protons and an electroneutral low-affinity system which catalyses sugar transport independent of ions. The results shown are in accordance with the earlier observations of Komor & Tanner (1974) and of Höfer & Misra (1978) that the high-affinity component is most active at low pH and vice versa.

Fig. 4. Double reciprocal plot of the transport and depolarization kinetics

(a) The transport of D-xylose as a function of sugar concentration at pH 7.5. The experiment was done as described by Höfer & Misra (1978). The K_m of the high-affinity system was in the range 0.8–2 mM ($n = 4$). (b) Replotted results of Fig. 3 (sugar-flux-dependent depolarization of the membrane potential). The differences in the rate of uptake of tetraphenylphosphonium after the addition of sugar were assumed to be linearly related to the degree of depolarization. The ordinate scale is in arbitrary units. The K_m of depolarization was in the range 0.6–2.0 mM ($n = 4$). The shaded areas in (a) and (b) indicate the region where the low-affinity component was found to be operating.

These data conform with the concept originally proposed for sugar transport in *R. gracilis* by a reversible protonation/deprotonation of one carrier protein (Höfer & Misra, 1978). However, this interpretation still remains open to criticism for the following reasons. (1) The concept predicts a passive translocation of sugars catalysed by the deprotonated carrier at high pH. Unfortunately, only a passive efflux from preloaded cells, but never an influx into empty cells, could be found under anaerobic or uncoupled conditions. (2) van den Broek & van Steveninck (1980) criticized the model described above on a kinetic basis. They investigated theoretically all possible combinations of H⁺/sugar symport (by ordered and disordered mechanisms) with passive transport via the same or a different unprotonated carrier. They found that only the combination of H⁺ symport (irrespective of the kinetic type of mechanism) with a molecularly independent passive carrier can give biphasic Lineweaver-Burk plots. However, this criticism may not be valid for *Chlorella* and *Rhodotorula* because in their arguments the possibility of an affinity change as a consequence of protonation or deprotonation of the carrier was excluded.

Our data do not necessarily imply that both transport systems described above interconvert by protonation or deprotonation. They are also compatible with two quite independent processes catalysed by different proteins. A distinction between both possibilities should become possible by selecting suitable mutants.

Recently, a controversy about variable H⁺/substrate stoichiometries has been started in the literature (see Konings & Booth, 1981). Experiments similar to those described in this paper may help to clarify some of the points of issue.

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References

- Aronson, P. S. (1978) *J. Membr. Biol.* **42**, 81–98
- Baker, D. A. (1978) *New Phytol.* **81**, 485–497
- Eddy, A. A. (1978) *Curr. Top. Membr. Transp.* **10**, 279–360
- Giaquinta, R. (1977) *Plant Physiol.* **59**, 750–755
- Harold, F. M. (1976) *Curr. Top. Bioenerg.* **6**, 83–149
- Hauer, R. & Höfer, M. (1978) *J. Membr. Biol.* **43**, 335–349
- Hauer, R., Uhlemann, G., Neumann, J. & Höfer, M. (1981) *Biochim. Biophys. Acta* **649**, 680–690
- Heller, K. & Höfer, M. (1975) *J. Membr. Biol.* **21**, 261–271
- Höfer, M. & Misra, P. C. (1978) *Biochem. J.* **172**, 15–22
- Komor, E. (1973) *FEBS Lett.* **38**, 16–18
- Komor, E. & Tanner, W. (1974) *J. Gen. Physiol.* **64**, 568–581
- Komor, E. & Tanner, W. (1975) *Planta* **123**, 195–198
- Konings, W. N. & Booth, I. R. (1981) *Trends Biochem. Sci.* **6**, 257–262
- Lanyi, J. K. (1978) *Biochemistry* **17**, 3011–3018
- Robertson, D. E., Kaczorowsky, G. J., Garcia, M.-L. & Kaback, H. R. (1980) *Biochemistry* **19**, 5692–5702
- Tanner, W., Komor, E., Fenzl, F. & Decker, M. (1977) in *Regulation of Cell Membrane Activities in Plants* (Marrè, E. & Ciferri, O., eds.), pp. 79–90, Elsevier/North-Holland Biomedical Press, Amsterdam
- van den Broek, P. J. A. & van Steveninck, J. (1980) *Biochim. Biophys. Acta* **602**, 419–432
- von Hedenström, M. & Höfer, M. (1974) *Arch. Microbiol.* **98**, 51–57