Active transport of charged substrates by a proton/sugar co-transport system

Amino-sugar uptake in the yeast *Rhodotorula gracilis*

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1. In the yeast *Rhodotorula gracilis* several amino sugars were actively transported. Glucosamine, which is largely protonated at physiological pH ($pK_{7.75}$) was used as a model substrate. At pH 6.75 its half-saturation constant was 1 mM and the maximal velocity was 50 nmol/min per mg dry wt. 2. Amino sugars were taken up via the monosaccharide carrier. The transport of glucosamine was strongly restricted by monosaccharides. D-Xylose inhibited competitively the uptake of glucosamine. The inhibition constant was 1 mM. Cells preloaded with D-xylose showed exchange transport on subsequent addition of glucosamine. 3. Transport of glucosamine was energized by the membrane potential. Uncoupling agents such as carbonyl cyanide *m*-chlorophenylhydrazide and the lipophilic cation TPP⁺ (tetraphenylphosphonium ion) at concentrations that depolarized the membrane potential inhibited the uptake of glucosamine. Conversely the transport of glucosamine partly dissipated the membrane potential, which was monitored by radioactively labelled lipophilic cations. 4. The translocated charges were electrically compensated by the extrusion of protons and K⁺ (1 glucosamine molecule/0.85 H⁺ + 0.15 K⁺). 5. An increase of the pH in the range 4.75–8.75 lead to a decrease of the half-saturation constant from 5 mM to 1 mM and to an optimum of the maximal velocity at pH 6.75. We suggest that this fair constancy is due to the carrier not distinguishing between the protonated form of glucosamine (pH < 7.75) and the deprotonated form (pH > 7.75). The increase of V_T (maximal transport velocity) between pH 4.75 and 6.75 is due to the increase of the membrane potential; the decrease between pH 6.75 and 8.75 is due to the deprotonization of the carrier.

In 1967 Mitchell proposed that the accumulation of nutrients such as sugars or amino acids is energized by a concomitant flow of protons. Ever since his concept of a substrate/proton co-transport has been verified for many solutes in quite different types of cells (Harold, 1976; Eddy, 1978). The majority of investigations dealt with the transport of uncharged substrates. Only in the case of amino-acid transport in *Staphylococcus aureus* the impact of charge has been systematically tested (Niven & Hamilton, 1974). It was not clear, however, whether in this

organism differently charged substrates were transported by the same system or not. Therefore we decided to look for a charged substrate that is accepted by a transport system for neutral substrates.

We used for our investigation the yeast *Rhodotorula gracilis*, which transports monosaccharides 'uphill' by means of an electrogenic symport with protons (Höfer & Misra, 1978; Hauer & Höfer, 1978). This carrier alternates between a protonated high-affinity form and a deprotonated form with low affinity for sugars. Assuming that both forms are catalytically active, we decided to investigate the transport of a substrate that is itself protonated. As for many other micro-organisms, transport is energized by the pH gradient (ΔpH) and by the membrane potential ($\Delta \Psi$). For the charged deriva-

Abbreviations used: TPP⁺, tetraphenylphosphonium ion; TPMP⁺, trimethylphenylphosphonium ion; K_T , apparent half-saturation constant; V_T , maximal transport velocity; ΔpH , pH difference across the plasmalemma; $\Delta \Psi$, membrane potential.

tives of monosaccharides, however, one can expect a different behaviour (Eddy, 1978). For a cationic substrate one can predict an energization by $\Delta\Psi$ alone. The proton is replaced in this case by the positive charge of the substrate. On the other hand, the transport of an anion should be predominantly energized by ΔpH . In this case the carrier-substrate-proton complex bears no charge and is not subjected to $\Delta\Psi$ (Robin & Kepes, 1973). More complex patterns of charge absorption may occur as well (Eddy, 1978; West, 1980).

In the present investigation we tried to verify these predictions. We started our work with positively charged sugar derivatives and tested the transport of amino sugars.

Materials and methods

The experiments were carried out with the obligatory-aerobic yeast *Rhodotorula gracilis* (*Rhodospiridium toruloides*, mating type a, A.T.C.C. 26194 and C.B.S. 6681). The yeast was grown in a complex medium as described by Misra & Höfer (1975). Cells were harvested after 24 h, washed thrice with water and aerated for 5 h in water (5% wet wt./v). The starved cells were then used for the experiments. In some cases the aerated yeast was centrifuged again and the pellet was stored in the refrigerator overnight. This yeast was used the next day after a further 2 h aeration. Control experiments have shown that this storage does not alter the transport properties.

Transport experiments

All experiments were carried out in Tris/citrate buffer (0.3 M-Tris was adjusted to the appropriate pH with 0.3 M-citric acid). All substrates or inhibitors that were present from the beginning were dissolved at double concentration in this buffer (hereafter referred to as 'incubation medium'). The experiments were started by the addition of an equal volume of aerated yeast suspended in water.

In all runs where the concentration of ^{14}C -labelled substrate was measured in the incubation medium, samples of 0.35 ml were withdrawn and centrifuged for 12 s at 15000 g in an ECCO quick centrifuge (Collatz, Berlin, Germany). A portion (0.2 ml) of the supernatant was mixed with 10 ml of scintillation 'cocktail' (299 M, Packard) and counted for radioactivity in a Packard 3380 liquid-scintillation counter.

Uptake of [^{14}C]glucosamine into the yeast was measured by the filter technique as described by Heller & Höfer (1975). The membrane filters with the yeast cells were either extracted in 5 ml of boiling water and the radioactivity of 1 ml extract was counted in 10 ml of scintillation 'cocktail', or the filters were dissolved in the scintillation 'cocktail'. Both methods led to comparable results.

Measurement of the membrane potential

$\Delta\Psi$ was measured as described by Hauer & Höfer (1978). The distribution of the lipophilic cation [^3H]TPP⁺ was taken as an indicator of the membrane potential. At intervals, samples of 0.35 ml were withdrawn and centrifuged as described above. The radioactivity in 0.2 ml of the supernatant was determined.

Evaluation of measurement of radioactivity

In all cases, when ^3H -labelled substrates were used, the c.p.m. values were corrected for unspecific quenching by the use of an external standard and the channels-ratio method (Brewer *et al.*, 1974).

Continuous measurement of K^+ and H^+

The initial velocity of the H^+ extrusion was measured in the unbuffered yeast suspension by a glass electrode (GK 2401 C; Radiometer, Copenhagen, Denmark), connected to a pH-meter (PHM 62; Radiometer). The buffer capacity of each suspension was measured by adding known amounts of HCl after each run. The K^+ content was similarly recorded by using a K^+ -specific electrode (Orion 9219; Cambridge, MA, U.S.A.) and an Hg/Hg₂Cl₂ reference electrode (K 701; Radiometer). Both electrodes were connected to a dual-pen recorder (PM 8245; Philips, Eindhoven, The Netherlands) in order to monitor K^+ and H^+ fluxes simultaneously. In order to stabilize the electrode response, 0.1 mM-CaCl₂ was added. Control experiments have shown that the addition of Ca²⁺ does not influence the recorded fluxes.

Statistics

For each Figure the number of experiments as described in the legend is given in parentheses (*n*).

Chemicals

[^{14}C]Glucosamine, [^{14}C]galactosamine, [^{14}C]mannosamine and D-[^{14}C]xylose were products of Amersham Buchler, Braunschweig, Germany. TPP⁺ was a customer synthesis by Hoechst, Frankfurt, Germany. Unlabelled amino sugars and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Sigma, Munich, Germany. The emulsifier scintillator (type 299) was from Packard, Frankfurt, Germany. All other chemicals were of Reagent grade and obtained from Merck, Darmstadt, Germany. All solutions were prepared in glass-distilled water.

Results and discussion

In the yeast *Rh. gracilis*, several amino sugars are actively transported. As shown in Fig. 1, glucosamine and mannosamine were taken up and accumulated, whereas no transport of galacto-

Fig. 1. Uptake of glucosamine (●, 10 mM), mannosamine (□, 10 mM) and galactosamine (○, 10 mM) in 0.15 M-Tris/citrate buffer, pH 6.5

Samples of 1 ml were withdrawn, filtered and counted for radioactivity as described in the Materials and methods section. Assuming a content of 2 μ l of cell water/mg dry wt. (Hauer & Höfer, 1978), the intracellular concentration of the substrate ($[S_i]$) was determined. The broken line symbolizes the diffusion equilibrium ($n = 6$).

samine could be detected. Nevertheless, we found a small inhibition of glucosamine transport by galactosamine (Fig. 2). This might indicate that galactosamine, even if it is not transported, can bind to the carrier and thus interferes with glucosamine uptake. The transport of glucosamine is distinctly inhibited by mannosamine (Fig. 2), showing that both substrates share a common system.

For the following experiments we selected glucosamine (pK 7.75) as a model substrate because it is rapidly taken up and is entirely protonated in the physiological pH range (4–7).

Basic properties of the system

(a) *Kinetic parameters.* To determine the transport parameters of the system for glucosamine, the transport rates were measured at concentrations ranging from 0.5 mM to 20 mM. The reciprocal values of transport velocity ($1/v$) were plotted as a function of the inverse substrate concentration ($1/[S]$), (Fig. 3). At pH 6.75 the system showed saturation kinetics

Fig. 2. Effect of *N*-acetylglucosamine (Δ , 50 mM), galactosamine ($\square$, 50 mM), and mannosamine ($\circ$, 50 mM) on the uptake of glucosamine (2 mM initial concentration) at pH 6.5 in 0.15 M-Tris/citrate buffer (●, control without additions)

The experiment was carried out as described in the legend to Fig. 1. The amino sugars were added from a 100-fold concentrated stock and adjusted to the appropriate pH with Tris base. The curves are corrected for dilution effects ($n = 8$).

Fig. 3. Transport parameters of glucosamine uptake. The reciprocal of the transport velocity was plotted as described by Lineweaver and Burk against the inverse substrate (glucosamine) concentration (0.5, 2, 5 and 20 mM). The pH was 6.75 ($n = 6$).

Fig. 4. Inhibition of glucosamine uptake (1 mM initial concentration) by glucose (■) and D-xylose (●) in Tris/citrate buffer (0.15 M, pH 6.5) (○, control without additions)

Samples (0.35 ml) were withdrawn at intervals and centrifuged as described in the Materials and methods section; the radioactivity of 0.2 ml of the supernatant was determined and used for the calculation of the glucosamine concentration in the incubation medium ($n = 5$).

with a half-saturation constant (K_T) of 1 mM (range 0.8–1.2 mM). The maximal velocity (V_T) amounted to 50 nmol/min per mg dry wt. (range 20–50 nmol/min per mg dry wt.). It was fundamental to our investigation that glucosamine shared the transport system for monosaccharides. In order to check this, we carried out competition experiments.

As shown in Fig. 4 both D-glucose and D-xylose inhibited the uptake of glucosamine. In the presence of D-glucose, which possesses a high affinity for the monosaccharide carrier, there was no uptake of glucosamine. D-Xylose, a substrate with transport parameters similar to glucosamine (Höfer & Misra, 1978) was less effective. Vice versa, glucosamine slowed down the uptake of D-xylose (results not shown).

To prove that the inhibition is due to a competition for a common carrier and not for a common energy source, the type of inhibition between D-xylose and glucosamine was determined. In the case of two substrates competing for the same

Fig. 5. Inhibition of glucosamine uptake by D-xylose. The initial velocities of glucosamine uptake in the presence of different D-xylose concentrations were measured as described in the legend to Fig. 3. The experimental data are shown in a double-reciprocal plot. The common intercept on the ordinate indicates a competitive type of inhibition. Experimental details: Tris/citrate buffer (0.15 M), pH 6.75; D-xylose concentrations: ●, 0 mM; □, 1 mM; ■, 2 mM; ○, 5 mM; ▲, 20 mM ($n = 6$).

energy source one would expect a mutual inhibition that could not be overcome by a surplus of one substrate. If two substrates are transported by the same carrier system, the inhibitory effect of the first substrate can be compensated for by increasing the concentration of the second solute.

Fig. 5 shows the effect of different concentrations of D-xylose on the uptake of glucosamine. With increasing concentrations of D-xylose, the apparent

Fig. 6. Exchange transport of glucosamine for accumulation of D-xylose

Cells were incubated with D-xylose (1.5 mM initial concentration) in 0.15 M-Tris/citrate buffer, pH 6.5. At $t = 10$ min (arrow) one half of the suspension was withdrawn and mixed with glucosamine (6 mM initial concentration). The experimental conditions were as described in the legend to Fig. 4. ●, D-Xylose consumption (no addition); ○, D-xylose efflux after addition of glucosamine; △, glucosamine consumption in the presence of D-xylose ($n = 6$).

affinity (K_T) for glucosamine increased, whereas the maximal transport velocity (V_T) remained constant. This competitive type of inhibition suggests unequivocally a common transport system for both substrates. The inhibition constant for D-xylose was estimated by means of the Dixon (1953) plot. It amounted to 1.1 mM, a value that agrees well with the half-saturation constant of D-xylose uptake (Heller & Höfer, 1975).

Moreover, cells preloaded with D-xylose showed exchange transport on subsequent addition of glucosamine. As demonstrated in Fig. 6, a nearly equimolar efflux of D-xylose occurred when glucosamine was taken up by cells preincubated for 10 min with the sugar. Both phenomena, the competitive type of inhibition of glucosamine transport with D-xylose and the exchange transport of D-xylose with glucosamine, are strong evidence for both substrates sharing a common transport system.

(b) *Energetics.* In *Rhodotorula gracilis*, mono-saccharides are taken up by a mechanism strictly dependent on metabolic energy. The uptake of glucosamine is also an energy-requiring process, as demonstrated in Fig. 7. Glucosamine was accumulated inside the cells to a concentration of 80 mM (starting with 2 mM in the medium), and this process was completely inhibited by uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone. Hence the protonmotive force is plausible as energy source for driving the transport of glucosamine

Fig. 7. Inhibition of glucosamine uptake (2 mM initial concentration) by 20 μ M-carbonyl cyanide *m*-chlorophenylhydrazone (△), 5 mM-TPP⁺ (○) and 5 mM-TPMP⁺ (■) [●, control (without additions)]

Experimental conditions were as described in the legend to Fig. 1 ($n = 4$). The broken line represents the diffusion equilibrium.

uphill. If this is true, at least one of its components, the chemical gradient of protons (Δ pH) or the membrane potential (Δ Ψ) must contribute to accumulation.

Conversely, each component contributing should be dissipated by glucosamine transport. The first logical step in such a study is to determine the number of charges translocated for each molecule of substrate taken up. Previous studies (Höfer *et al.*, 1980) have shown that K⁺ serves to maintain the electroneutrality of the electrogenic H⁺/sugar co-transport. However, in the case of glucosamine we found (Fig. 8) that the influx of glucosamine induced an efflux of H⁺ and to a minor degree also of K⁺. The stoichiometry of 1 molecule of substrate/1 (H⁺ + K⁺) ion accounted for the overall electroneutrality of the process. The contribution of each cation varied between experiments, the extreme values being 50 and 85% for H⁺ and 50 and 15% for K⁺. This stoichiometry seems to point to a transport mechanism where the translocation of one molecule of glucosamine is coupled to a transfer of one net charge. However, a symport of one positively charged substrate molecule with one proton cannot be ruled out. The two charges taken up would be compensated predominantly by protons and only to a much smaller degree by K⁺. In this case the extra proton taken up would be

Fig. 8. H^+ and K^+ fluxes accompanying the transport of glucosamine

Glucosamine, which was adjusted to the appropriate pH with Tris base, was added to an unbuffered yeast suspension to give 1 mM-final concentration. As described in the Material and methods section, H^+ fluxes were recorded simultaneously. D-Xylose (10 mM) was added to check on the typical H^+ uptake (alkalinization of the suspension) accompanying the transport of neutral sugars ($n = 10$).

compensated by one proton extruded, and the expected stoichiometry would be decreased to the one observed. This possibility is discussed in more detail below.

The second point worth investigating is whether the transport mechanism itself is electroneutral or electrogenic. An electroneutral process, however, cannot be reconciled with two observations already mentioned: (1) The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone severely inhibited the accumulation at pH 6.75, at which value ΔpH is practically zero (Höfer & Misra, 1978). Under these conditions, carbonyl cyanide *m*-chlorophenylhydrazone can act only by depolarizing $\Delta\Psi$, i.e. the transport must be electrogenic. (2) An electroneutral carrier mechanism would imply that such diverse cations as H^+ and K^+ are accepted by the same carrier to serve as counterions for glucosamine.

Two further experiments support this argument. The transport of glucosamine depolarized the membrane potential. In this set of experiments the membrane potential was monitored by the lipophilic cations introduced by Libermann & Topali (1969). The ions TPP^+ and $TPMP^+$ are accumulated within the cell according to the Nernst equation for the diffusion potential. The final distribution of the ions corresponds to the actual membrane potential. In the case of *Rh. gracilis*, TPP^+ and $TPMP^+$ are accumulated inside the cell, indicating a negative membrane potential of about -120 mV at pH 7.5 (Hauer & Höfer, 1978). As shown in Fig. 9, the addition of glucosamine to a cell suspension incubated with TPP^+ caused an immediate depolarization of the membrane potential.

Fig. 9. Depolarization of the membrane potential induced by glucosamine (20 mM) in Tris/citrate buffer (0.15 M, pH 7.5)

The membrane potential was measured by the uptake of the lipophilic cation $[^3H]TPP^+$ ($20\text{ }\mu\text{M}$) as described by Hauer & Höfer (1978) ($n = 4$).

On the other hand, higher concentrations of TPP^+ and $TPMP^+$ (5 mM), which depolarized the membrane potential in *Rhodotorula gracilis* (Hauer & Höfer, 1978), strongly inhibited the transport of glucosamine as well (Fig. 7). Hence we conclude that glucosamine uptake depends on the membrane potential.

(c) *Mode of transport.* On the basis of what we know about the monosaccharide carrier and what

we have shown above for the accumulation of amino sugars we can envisage three simple models depicted in Fig. 10. A change in the effective proton activity of the medium shifts the ratio of the protonated and unprotonated form of the substrate as well as of the carrier. Consequently, the half-saturation constant, K_T and the maximal velocity, V_T , should change for each model in a characteristic manner. If this is true the following predictions can be made.

(1) According to models 1 or 3 (Fig. 10) an increase in pH, especially when working close to the pK of the substrate, ought to lead to a considerable increase of the apparent K_T , since the concentration of the transported form of the substrate decreases sharply. Concomitantly, in model 3, V_T should increase steadily, because the active form of the carrier is produced by deprotonation of the inactive form.

For model 1 the apparent K_T should behave as expected for model 3, becoming higher with increasing pH of the cell suspension, whereas V_T should decrease at higher pH values as predicted for model 2.

Fig. 10. Three models for glucosamine uptake. Models 1 and 2 show transport via the protonated carrier, whereas in model 3 the substrate is taken up by the unprotonated carrier. Transport of neutral sugars operates as described by model 2.

(2) Conversely, for model 2, a decrease of K_T as well as of V_T can be predicted.

Table 1 gives the theoretical pH-dependency of K_T and V_T . However, these predictions hold only if K_T is not affected by the force driving transport. This is the case if the transport step is rate-limiting. If this is not true, a variation of the driving force would not affect the maximal transport velocity but also the apparent affinity. Fortunately this is not the

Fig. 11. pH-dependency of the transport parameters of glucosamine uptake

The values were obtained from double-reciprocal plots as described in the legend to Fig. 3. Each point is the mean value for six determinations; the S.E.M. is indicated by vertical bars. (a) V_T ; (b) K_T .

Table 1. pH-dependency of K_T and V_T for models 1, 2, and 3 (Fig. 10)

For the calculation of V_T it was assumed that the maximal velocity of transport depends linearly on the concentration of the active form of the carrier (CH^+ in models 1 and 2, C in model 3). The contribution of the membrane potential was neglected here but is discussed in the text. The K_T was normalized to $pH = pK$ of glucosamine, i.e. expressed in multiples of its value at $pH 7.75$. V_T was normalized to $pH = pK$ of the carrier (Höfer & Misra, 1978), i.e. expressed in multiples of its value at $pH 6.75$. $GlcNH_2$ and C, unprotonated form of the substrate and carrier; $GlcNH_3^+$ and CH^+ , protonated forms.

pH	$GlcNH_3^+/GlcNH_2$	K_T		V_T		CH^+/C
		Model 2	Models 1 + 3	Models 1 + 2	Model 3	
4.75	1000:1	500	0.5	2	0.02	100:1
5.75	100:1	50	0.5	1.8	0.2	10:1
6.75	10:1	5	0.55	1	1	1:1
7.75	1:1	1	1.0	0.2	1.8	1:10
8.75	1:10	0.55	5.0	0.02	2	1:100

Fig. 12. Summary of the proposed reactions participating in the transport of amino sugars

Alternative (b) shows that a stoichiometry of 1 H⁺/substrate becomes possible even if both substrate and carrier are protonated.

case in our yeast. This contention is borne out by two lines of evidence: (1) the K_T of D-xylose transport was independent of pH, although the membrane potential changes drastically with pH (Höfer & Misra, 1978). At pH 7 only the membrane potential can drive D-xylose transport, ΔpH being 0. At this pH a close correlation is found for the rate of D-xylose transport and the membrane potential with increasing uncoupler concentration (R. Hauer & M. Höfer, unpublished work).

As can be seen from Fig. 11, neither of the models listed in Fig. 10 is supported by the results of this experiment. Both K_T and V_T were indeed very little affected by the change of the proton concentration by a factor greater than 10^4 . The two parameters remained constant within one order of magnitude. Hence, none of the simple models as discussed above is adequate. The most remarkable discrepancy observed is the near constancy of the apparent affinity in the pH range from 6.75 to 8.75, where the relative concentration of the protonated and unprotonated form of the substrate changed by a factor of 100.

The results of Fig. 11 can be most easily understood if it is assumed that the protonated carrier cannot differentiate between the protonated and the unprotonated form of the substrate. In other words, the transport system has equal affinity for both forms of the substrate. This means that the carrier would operate both as depicted in model 1 and in model 2. Consequently at a pH lower than the $\text{p}K$ of the substrate, a true stoichiometry of 2 H⁺/glucosamine molecule is to be expected.

We have two alternative explanations for the observed stoichiometry of 1. (a) The two charges taken up are compensated predominantly by protons such that the proton translocated by the carrier is compensated by the extrusion of H⁺. In this case, both processes cancel each other out and cannot appear in the charge balance. (b) The true stoichiometry is one. To account for this stoichiometry of 1 we suggest that the substrate becomes rapidly deprotonated on binding and is translocated only after the charge of the carrier-substrate-complex is decreased to one (Fig. 12). At present we have no possibility of deciding which stoichiometry is

correct. The evaluation might be made possible if one could inhibit significantly the extrusion of protons without affecting the rate of active transport. Attempts to do this have failed so far. Both versions agree well with the type of inhibition by D-xylose observed at pH 6.75 (Fig. 5). At this pH, D-xylose is only transported via the protonated system in the range of concentrations used (Höfer & Misra, 1978). Consequently the sugar can inhibit glucosamine transport only if this is mediated by the protonated form of the carrier. The observation of a competitive type of inhibition by D-xylose, as well as the K_T found (1 mM), agree with this concept.

The pH-dependency of V_T can also be explained by the combination of models 1 and 2. An increase of pH causes an increase of the driving force (the membrane potential). This accounts for the increase of V_T in the range pH 4.75–6.75. A further increase of the membrane potential cannot fully compensate the steep decrease of the concentration of the catalyst, the protonated carrier, the $\text{p}K$ of which was estimated to be 6.75 (Höfer & Misra, 1978).

We conclude that, in the yeast *Rhodotorula gracilis*, the basic mechanism for active transport of both neutral sugars and positively charged derivatives (amino sugars) consists in binding the substrate molecules to a common carrier in its protonated form. Amino sugars are bound either in their protonated and unprotonated form without discrimination. Whether these are also translocated with the same impartiality is a subject requiring further study.

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