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THE INFLUENCE OF UNCOUPLERS ON FACILITATED DIFFUSION OF SORBOSE IN *SACCHAROMYCES CEREVISIAE*

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Sorbose uptake in *Saccharomyces cerevisiae*, strain Delft 1, proceeds via mediated passive transport. In the cell sorbose is distributed in at least two compartments. Efflux studies showed that sorbose uptake in one of these compartments is not readily reversible. Uncouplers of oxidative phosphorylation inhibit both transport velocity and steady-state uptake level. It could be shown that these two effects are caused by different modes of action of the uncouplers. None of these two effects could be ascribed to changes of the electrochemical H^+ gradient or of the intracellular pH. It is suggested that the inhibition of uptake velocity is caused by binding of the uncoupler to the sorbose translocator, thus lowering the transport activity. The uncoupler binding site is probably located at the intracellular fragment of the carrier. The second effect, reduction of the steady-state uptake level, is probably due to blocking of sorbose influx into the compartment that exhibits poor reversibility.

Introduction

Uncouplers of oxidative phosphorylation can be regarded as weak acids, able to cross lipid bilayers (for review, see Ref. 1). In the classical view they are supposed to exert their effect by transporting protons across the membrane, thus dissipating the transmembrane electrochemical proton gradient [2–4]. In this way they should uncouple oxidative phosphorylation and inhibit those active transport processes, that depend on the transmembrane electrochemical proton gradient.

However, besides being protonophores, uncouplers can give other reactions. A direct binding to soluble proteins was shown [5] and also more or less specific binding seems to occur to some membrane proteins, such as mitochondrial ATPase [6,7], respiratory chain components [8] and the so-called uncoupler binding proteins in some microorganisms [9]. In yeast it was shown that 2,4-dinitrophenol can bind to cell constituents, probably also proteins [10]. Hoeberichts et al. [11] demonstrated that up to 90% of intracellular 2,4-dinitrophenol is absorbed to cellular components.

Some investigators [6,7,9] even claim an essential role of uncoupler binding to high affinity sites, in the mechanism of uncoupling. Although there is still much debate on the role of uncoupler binding sites, it seems probable, that the effects of uncouplers can not be solely ascribed to the proton translocating properties.

The complex behaviour of uncouplers can also be deduced from the fact that certain transport systems are inhibited by uncouplers in a way not

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCSA, 3,3′,4′,5-tetrachlorosalicylanilide; TPP, tetraphenylphosphonium.

consistent with their mode of action as protonophores. In erythrocytes for example, the passive anion transport, via the band 3 protein, can be inhibited by uncouplers [12,13]. Further in *Bacillus subtilis* inhibition of amino acid transport was explained by a direct interaction of uncoupler with the transporter [14,15]. Finally Komor et al. [16] found that inhibition of hexose transport in *Chlorella vulgaris* was due to an acidification of the cytoplasm, resulting in loss of activity of the transport system.

Because of this complex behaviour of uncouplers it appeared appropriate to evaluate their effects on facilitated diffusion systems of neutral solutes. Therefore an analysis was performed of the influence of uncouplers on passive sugar transport in yeast.

It will be shown that uncouplers can inhibit facilitated diffusion by direct interaction with the translocator. It will also be demonstrated that uncouplers have an influence on the intracellular distribution of the sugar.

Materials and Methods

Saccharomyces cerevisiae (strain Delft 1) was grown, with glucose as carbon source, harvested and washed as described for *Saccharomyces fragilis* [17]. Transport studies were done, with ^{14}C-labeled sorbose under aerobic conditions in 10% (w/v) cell suspensions. In some experiments the cell suspension was buffered with 0.2 M Tris-maleate. Uptake was measured as described earlier [18] with alcohol treatment to extract radioactivity. An alternative, more rapid procedure, was to transfer yeast (30 mg wet weight) after filtration directly into a counting vial with 1 ml 0.5% Triton X-100, followed by addition of scintillation liquid (Picofluor 30, Packard).

To calculate intracellular sugar concentrations, the intracellular water space was determined according to the method of De Bruijne and Van Steveninck [19]. A values of 0.50 ml/g wet weight was measured. The intracellular pH was determined as described by Borst-Pauwels and Dobbelman [20]. After incubation of the cells in buffer the yeast was filtered and washed three times with cold distilled water, prior to measurement of the intracellular pH.

2,4-Dinitrophenol and picrate uptake were followed by measuring the absorbance of the supernatant, after centrifugation in an Eppendorf centrifuge 3200, in a 50 mM Tris-maleate buffer (pH 8) at 362 nm (2,4-dinitrophenol) and 365 nm (picrate).

Pentachlorophenol was obtained from Serva, 3,3',4',5-tetrachlorosalicylanilide from Eastman-Kodak, carbonyl cyanide *m*-chlorophenylhydrazone from Sigma, 2,4-dinitrophenol and picric acid from BDH. All chemicals were analytically pure. L-[^{14}C]Sorbose and inulin-[^{14}C]carboxylic acid were supplied by the Amersham International, Amersham.

Results

To establish facilitated diffusion three criteria were used: equilibrating transport, possibility of counterflow and saturation kinetics.

Fig. 1 shows that sorbose transport in Delft 1 is over a wide range of pH values equilibrating. No accumulation could be observed. Also the initial uptake velocity showed only a slight pH dependence: initial influx at pH 7.5 was about 60–80% of the value at pH 4.6.

Fig. 2 shows the phenomenon of counterflow, a criterion for the involvement of a transporter [21]. Both fructose and glucose induce efflux of intracellular sorbose.

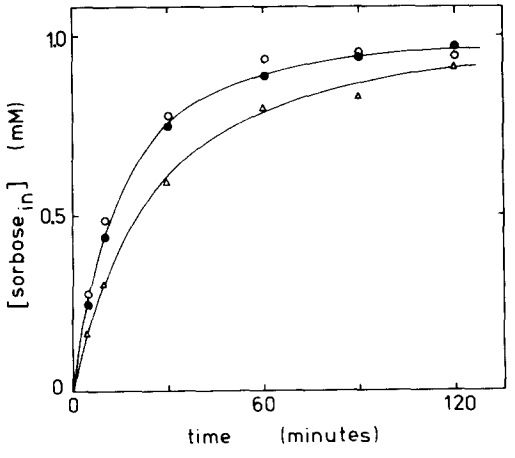

Fig. 1. Influence of extracellular pH on sorbose uptake in Delft 1. Transport was measured in 0.2 M Tris-maleate: pH 3.5 (●——●), pH 5.5 (○——○) and pH 7.5 (△——△). Initial extracellular sorbose concentration 1mM.

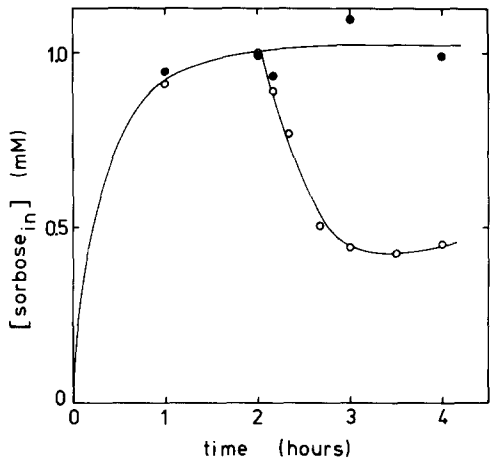

Fig. 2. Counterflow of sorbose induced by D-fructose. Transport was measured in Tris-maleate at pH 4.6. After 2 h of incubation 250 mM fructose was added as powder (○——○). To the control no addition was made (●——●). Addition of 250 mM D-glucose gave an identical effect as fructose. Initial extracellular sorbose concentration 1 mM.

Finally saturation of uptake was shown by analysis of initial influx at pH 4.5. A K_m value of 1250–1450 mM and a V_m of 41–47 μmol sorbose per gram yeast per min were found.

Based on these observations it is concluded that sorbose transport in Delft 1 is a facilitated diffusion system.

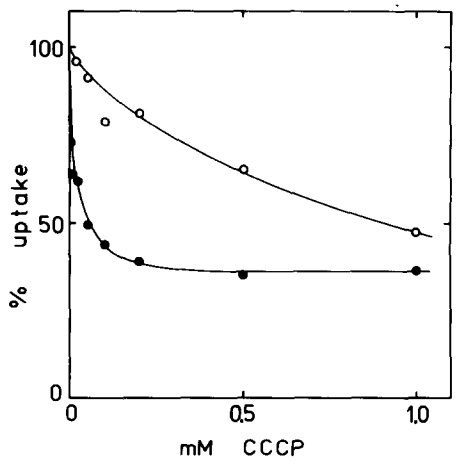

Fig. 3. Dependence of sorbose uptake on the concentration of CCCP. Yeast was incubated at pH 4.0. 1 min after addition of CCCP sugar is added (1 mM). Initial influx is measured after 1 min uptake (●——●), steady-state level after 2 h incubation (○——○).

The influence of uncouplers on sorbose transport is obvious from Fig. 3. The titration curves show that already at low CCCP concentration (± 100 μM) the initial uptake velocity is maximally reduced, to about 30–40% of normal uptake. It is also shown that the steady-state level, determined after 2 h of uptake, is CCCP sensitive, albeit more CCCP is necessary than for inhibition of initial influx velocity. This difference in sensitivity to CCCP suggests that two different mechanisms are involved. Therefore both processes were characterized separately.

Influx velocity could be lowered not only by CCCP but also by 2,4-dinitrophenol, TCSA and pentachlorophenol. This indicates that this effect is probably not due to reaction with S-S bridges in the carrier, as could be reasoned for CCCP, which can act as an SH-reagent [22]. At pH 4.5 uncouplers caused an about 2-fold reduction in uptake velocity, with maximal effect at approx. 100 μM TCSA, 200 μM pentachlorophenol, 400 μM 2,4-dinitrophenol and 100 μM CCCP.

Table I shows that the inhibition of initial influx by CCCP is almost independent of the medium pH and of the presence of buffer. With 2,4-dinitrophenol, however, there is a strong pH dependence: at high pH almost no inhibition is ob-

TABLE I

INFLUENCE OF UNCOUPLERS ON THE INITIAL UPTAKE VELOCITY

Initial transport velocity was determined from uptake experiments 1, 2, 3 and 4 min after addition of 1 mM sorbose. Uncoupler was added 1 min before starting uptake. n.d., not determined. DNP, 2,4-dinitrophenol.

Extracellular medium	Percentage inhibition			
	+CCCP		+DNP	
	100 μM	200 μM	0.3 mM	0.6 mM
Water (pH 5)	56	51	n.d.	n.d.
0.2 M Tris-maleate pH 4.5	64	69	39	39
0.2 M Tris-maleate pH 7.5	44	53	6	19

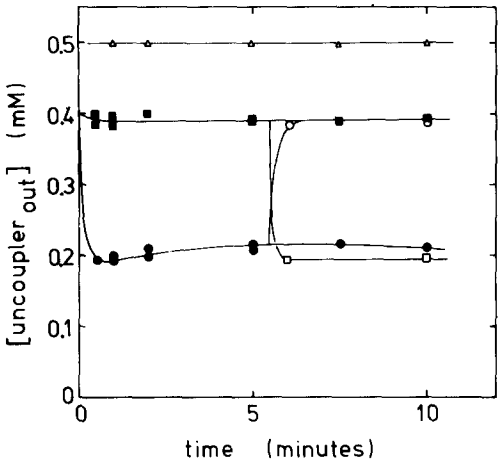

Fig. 4. Uptake of 2.4-dinitrophenol and picrate by Delft 1. Picrate uptake was measured at pH 4.5 (△——△). Initial extracellular concentration was 0.5 mM. 2,4-Dinitrophenol uptake was measured at pH 4.5 (●——●) and pH 7.5 (■——■). Initial extracellular concentration 2,4-dinitrophenol 0.4 mM. Reversibility of uptake was shown by changing pH with NaOH from pH 4.5 to 7.5 (○——○) or from pH 7.5 to 4.5 (□——□).

served, probably because at high pH 2,4-dinitrophenol is not taken up into the cells, as shown in Fig. 4. The fact that also picrate, an uncoupler, which is not taken up under our experimental conditions (Fig. 4), i.e. at membrane potentials negative inside [1,23], does not induce inhibition, suggests that inhibition of facilitated diffusion only occurs after transmembrane transport of the uncoupler.

To evaluate whether uncoupler-induced changes of the transmembrane proton gradient or of the intracellular pH might be involved in the transport inhibition, the intracellular pH was measured after 1 h incubation with and without 1 mM CCCP in media buffered at pH 3.5 and at 7.5. At a medium pH of 3.5 the intracellular pH was 6.1 in the absence and 6.0 in the presence of CCCP. At a medium pH of 7.5 these values were 6.6 and 6.4, respectively. Fresh yeast has a pH_{in} value of 6.0.

The possible role of transmembrane potential was tested by incubating the cells with 5 mM TPP. As shown earlier [24,25] TPP^+ is able to traverse the membrane of *Saccharomyces cerevisiae*, thus depolarizing the membrane at this concentration [25,26]. However, incubation of cells with TPP did

TABLE II

INFLUENCE ON TPP ON INITIAL UPTAKE VELOCITY

Initial transport velocity was determined from uptake experiments 1, 2, 3 and 4 min after addition of 1 mM sorbose. 5 mM TPP was added 1 min before starting uptake. Uptake velocity expressed as μmol sorbose per gram per min.

pH_{out}	Initial uptake velocity	
	Control	+5 mM TPP
4.5	0.090±0.004	0.086±0.003
7.5	0.080±0.013	0.074±0.010

not cause inhibition of sorbose transport: both at pH 4.5 and pH 7.5 TPP reduced the influx velocity only about 5% (see Table II).

The second effect of uncouplers is shown in Fig. 5. At pH 4.5 TCSA, CCCP, 2,4-dinitrophenol and pentachlorophenol (not shown) lower the steady-state level about 50–70% both with 1 mM and with 100 mM sorbose. For TCSA, pentachlorophenol and CCCP this effect is independent of pH, but 2,4-dinitrophenol is ineffective at high pH, as is picrate at pH 5, indicating that this effect is also exerted from the inside of the cell.

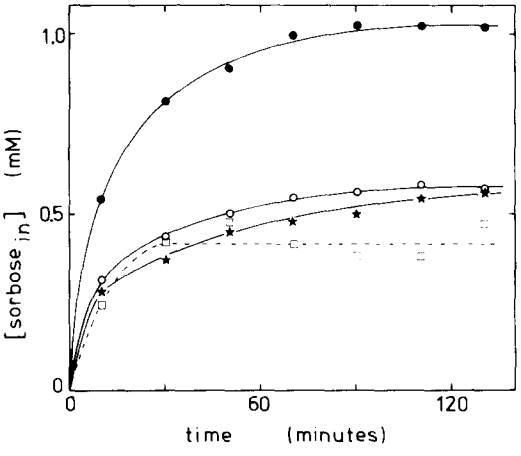

Fig. 5. Influence of uncouplers on sorbose uptake in Delft 1. Yeast was incubated at pH 4.5. 1 min after addition of uncoupler, uptake was started by adding 1 mM sorbose. ●——●, control without uncoupler; ○——○, 1 mM 2,4-dinitrophenol; ★——★, 1 mM CCCP; □——□, 1 mM TCSA.

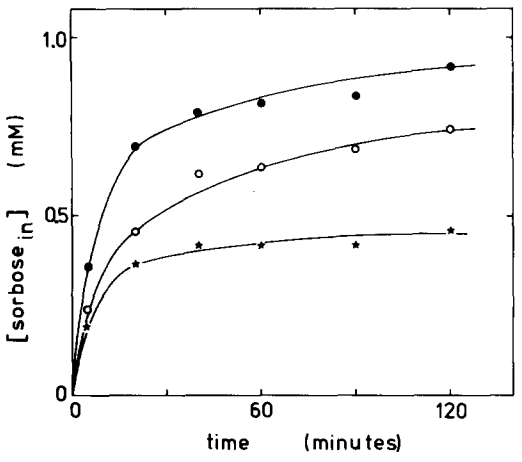

Fig. 6. Role of medium composition in the inhibition of sorbose uptake by CCCP. 1 min after addition of 1 mM CCCP, uptake was started by adding 1 mM sorbose. ●——●, control, without CCCP, in 0.2 mM Tris-maleate pH 5 (an identical result is obtained in unbuffered suspensions, pH 5); ○——○, unbuffered with 1 mM CCCP, pH 5; ★——★, 0.2 M Tris-maleate pH 5 with 1 mM CCCP.

Although not dependent on extracellular pH, this effect is strongly dependent on the composition of the extracellular medium. Fig. 6 shows that the effect in an unbuffered suspension is much less than in 0.2 M Tris-maleate buffered suspensions. Apparently this 'buffer' effect does not correlate with ionic strength, or the presence of Tris ions, since the inhibiting effect is much less in 0.2 M Tris phosphate and in 0.2 M sodium phosphate.

The fact that uncouplers induce non-equilibration suggests that they may render an intracellular compartment impermeable to sorbose. This is also indicated by exchange experiments as shown in Fig. 7. Both 1 mM CCCP and pentachlorophenol give reduced exchange which flattens off at about 50% of the control. This is observed at pH 4.5 and 7.5. This effect cannot be due to uncoupler-induced sorbose efflux, since control experiments showed that after 2 h incubation with sorbose, addition of CCCP or pentachlorophenol gave only a small decrease in cellular sorbose content. About 10% of the cellular sorbose was liberated immediately after addition of uncoupler, suggesting sugar dissociation from cell wall or outer face plasma-membrane adsorption sites.

To evaluate this compartmentilization further, efflux in sorbose free medium was measured. After loading the cells with sorbose, they were spun down and resuspended in sorbose free medium. As depicted in Fig. 8 cells, incubated without CCCP exhibited rapid efflux up to about 40% of the initial value. If cells had been loaded in the presence of 1 mM CCCP, however, a rapid and com-

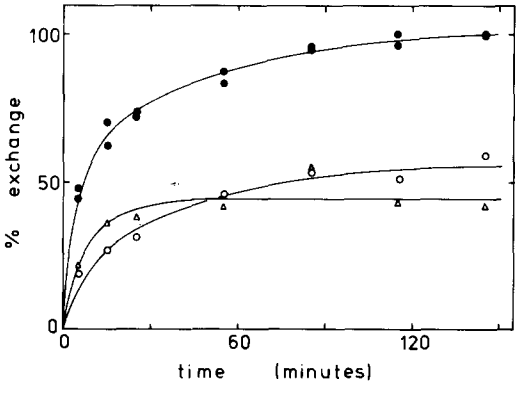

Fig. 7. Influence of pentachlorophenol and CCCP on sorbose exchange. Cells were preincubated, at pH 4.6, with 1 mM sorbose for 2 h. Control experiments showed that at that point a steady-state level was reached. Exchange was started by adding a small amount of [^{14}C]sorbose. ●——●, control without uncoupler; ○——○, 1 min before [^{14}C]sorbose addition, 1 mM CCCP is added; △——△, 1 min before [^{14}C]sorbose addition, 1 mM pentachlorophenol is added.

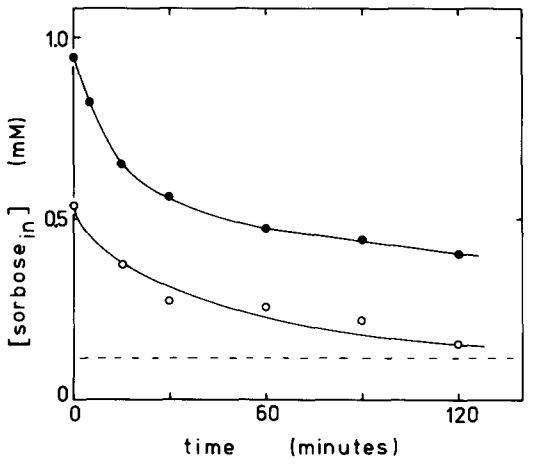

Fig. 8. Reversibility of sorbose transport. Yeast was incubated at pH 4.6, with 1 mM [^{14}C]sorbose in the absence (●——●) or presence (○——○) of 1 mM CCCP. After 2 h, when the steady state was obtained, cells were spun down, supernatant removed and yeast was resuspended in 0.2 M Tris-maleate pH 4.6 without sorbose and CCCP. Subsequently uptake was measured as described in Materials and Methods. The dotted line represents the equilibration level.

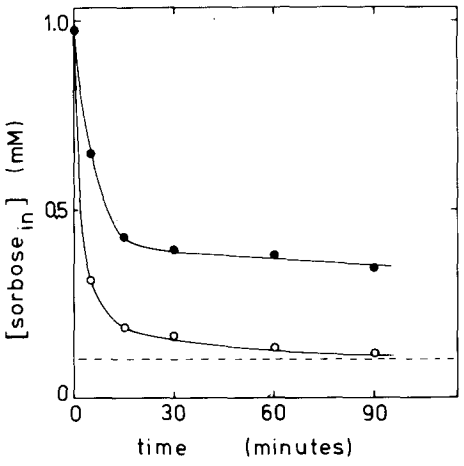

Fig. 9. Influence of chitosan on sorbose efflux. Yeast was incubated with 1 mM [^{14}C]sorbose in an unbuffered suspension. After 2 h cells were spun down, supernatant was removed and yeast was resuspended in water (●——●) or in water with 50 μg chitosan/ml (○——○). The dotted line represents the equilibration level.

plete equilibration took place. This suggests, that sorbose uptake takes place in, at least, two compartments. Uptake in one of these compartments is apparently poorly reversible and, moreover, blocked by uncouplers. This compartment is probably a membrane-closed system. Fig. 9 shows that when membranes are made permeable with chitosan [27] complete efflux of sorbose takes place, also from cells, not incubated with uncoupler. This effect was observed to the same extent with 1 mM and with 100 mM sorbose, suggesting that adsorption of the sugar to intracellular constituents plays no role in these phenomena.

Discussion

In the present paper it is shown that sorbose transport in strain Delft 1 occurs via facilitated diffusion, in accordance with earlier results on several other *Saccharomyces cerevisiae* strains (see, for example, Ref. 28). As no energy coupling takes place in facilitated diffusion, it was a priori expected that sorbose transport in this yeast strain would be unaffected by inhibitors of energy-coupled reactions, such as uncouplers of oxidative phosphorylation. As shown, however, uncouplers interfere with sorbose transport in two different ways: both the initial rate of uptake and the intracellular steady-state concentration are reduced.

Experimental evidence indicates that these two effects are caused by different mechanisms. Maximal reduction of initial rate of uptake is observed at low concentrations of uncouplers, whereas maximal reduction of the steady-state level requires much higher concentrations. Further, the effect of uncouplers on the steady-state level strongly depends on the medium composition, in contrast to the effect on transport velocity.

Due to their effect as protonophores, uncouplers can modify the transmembrane proton gradient, the transmembrane potential and the intracellular pH, depending on experimental conditions. If facilitated diffusion would depend on any of these parameters in a hitherto unknown way, this might possibly explain the influence of uncouplers on facilitated diffusion. However, experimental evidence contradicts this assumption. At a medium pH of 4.5 there exists a transmembrane pH difference of 1.5–2 units. At a medium pH of 7.5 the ΔpH is reversed to -1 unit, whereas the steady-state level is pH-independent and the initial sorbose transport velocity at pH 7.5 is only 20–40% lower as compared to the velocity at pH 4.5. This contradicts an essential role of the transmembrane pH difference in facilitated diffusion. It seems likely that the relatively small difference in transport rates should be ascribed to a pH-effect on the conformation of the sorbose transporter.

Manipulation of the membrane potential by high concentrations of the lipophilic cation TPP, resulting in depolarization [25,26], does not influence the sorbose transport parameters. Thus it may be concluded that facilitated diffusion is not dependent on the membrane potential.

Finally, a decrease of the intracellular pH might inhibit transport activity, as shown e.g. for *Chlorella* [16]. Shifting the medium pH from 3.5 to 7.5 caused an increase of the intracellular pH from 6.1 to 6.6 in the absence, and from 6.0 to 6.4 in the presence of CCCP. Especially the results obtained at a medium pH of 7.5 clearly show that the effect of uncouplers on sorbose transport can not be ascribed to acidification of the cytoplasm.

As the effects of uncouplers on facilitated diffusion of sorbose can not be attributed to their

protonophoric properties, quite different mechanisms should be considered. The inhibition of the transport velocity might be due to binding of the uncoupler to a more or less specific site on the carrier, resulting in a lower transport activity. Over the whole pH range 4.5–7.5 picrate is virtually unable to traverse the plasma membrane. The same is true for 2,4-dinitrophenol at pH 7.5. Coincidingly picrate (over the whole pH range) and 2,4-dinitrophenol (at pH 7.5) do not reduce the sorbose uptake. This suggests that the postulated binding site is localized at the inward-facing fragment of the carrier. An alternative explanation: impossibility of dissociated uncoupler (DNP^-, $pK = 4.0$; $picrate^-$, $pK = 0.3$) to bind to the carrier at the outward facing carrier part seems very unlikely, as inhibition of transport with other uncouplers takes place at $pH > pK$. In this context it is noteworthy that Hoeberichts et al. showed that also for 2,4-dinitrophenol-induced K^+-efflux from yeast the site of action is inside the cells [11]. Assuming a carrier of which the sugar binding site is alternately exposed to the intra- and extracellular medium, it would follow that the uncoupler binding site is not identical to the sugar binding site. Thus inhibition of sorbose transport should not be competitive. Due to the high K_m of sorbose transport it was not possible to test this experimentally.

Considering steady-state sorbose uptake three components can be distinguished: a small adsorption to, possibly, cell walls (constituting about 10% of total uptake at 1 mM sorbose) and transport into two intracellular compartment. Adsorption is readily reversed by uncouplers. Efflux from one of the intracellular compartments is very slow. The fact that this poorly exchangeable sorbose is liberated in the presence of chitosan, both at high and at low sugar concentrations, indicates that this compartment does not represent sorbose bound to cellular constituents, as has been suggested for other sugars [29]. It rather indicates that this compartment is a membrane-surrounded pool of free sorbose. The results shown in Fig. 8 suggest that sugar influx into this compartment is blocked by uncouplers. Although it is tempting to attribute this compartmentalization to the vacuoles, further experiments will be needed to elucidate the nature of these compartments.

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References

1 McLaughlin, S.G.A. and Dilger, J.P. (1980) Physiol. Rev. 60, 825–863
2 Hopfer, U., Lehninger, A.L. and Thompson, T.E. (1968) Proc. Natl. Acad. Sci. U.S.A. 59, 484–490
3 Liberman, E.A., Topaly, V.P., Tsofina, L.M., Jasaitis, A.A. and Skulachev, V.P. (1969) Nature 222, 1076–1078
4 Hopfer, U., Lehninger, A.L. and Lennarz, W.J. (1970) J. Membrane Biol. 3, 142–155
5 Weinbach, E.C. and Garbus, J. (1969) Nature 221, 1016–1018
6 Hanstein, W.G. and Hatefi, Y. (1974) J. Biol. Chem. 249, 1356–1362
7 Katre, N.V. and Wilson, D.F. (1978) Arch. Biochem. Biophys. 191, 647–658
8 Stockdale, M. and Selwin, M.J. (1971) Eur. J. Biochem. 21, 565–574
9 Katre, N.V. and Wilson, D.F. (1980) Biochim. Biophys. Acta 593, 224–229
10 Borst-Pauwels, G.W.F.H. (1968) FEBS Lett. 1, 252–254
11 Hoeberichts, J.A., Hulsebos, T.J.M., Van Wezenbeek, P.M.G.F. and Borst-Pauwels, G.W.F.H. (1980) Biochim. Biophys. Acta 595, 126–132
12 Gunn, R.B. and Tosteson, D.C. (1971) J. Gen. Physiol. 57, 593–609
13 Motais, R., Sola, F. and Cousin, J.L. (1978) Biochim. Biophys. Acta 510, 201–207
14 Brummett, T.B. and Ordal, G.W. (1977) Arch. Biochem. Biophys. 178, 368–372
15 Nicholas, R.A. and Ordal, G.W. (1978) Biochem. J. 176, 639–647
16 Komor, E., Schwab, W.G.W. and Tanner, W. (1979) Biochim. Biophys. Acta 555, 524–530
17 Jaspers, H.T.A. and Van Steveninck, J. (1976) Biochim. Biophys. Acta 443, 243–253
18 Jaspers, H.T.A. and Van Steveninck, J. (1975) Biochim. Biophys. Acta 406, 370–385
19 De Bruijne, A.W. and Van Steveninck, J. (1970) Biochim. Biophys. Acta 196, 45–52
20 Borst-Pauwels, G.W.F.H. and Dobbelman, J. (1972) Acta Bot. Neerl. 21, 149–154
21 Kotyk, A. and Janáček, K. (1975) Cell Membrane Transport. Principles and Techniques, Plenum Press, New York-London
22 Kaback, H.R., Reeves, J.P., Short, S.A. and Lombardi, F.J. (1974) Arch. Biochem. Biophys. 160, 215–222
23 Michels, M. and Bakker, E.P. (1981) Eur. J. Biochim. 116, 513–519

24 Kotyk, A. (1979) in Biophysics of Membrane Transport (Kuczera, J., Gabrielska, J. and Przestalski, S., eds.), Vol. 2, pp. 49–67, Publ. Dept. Agricult. Univ., Wroclaw
25 Barts, P.W.J.A. (1981) Some Electrophysiological Aspects of Solute Transport in Yeast, Ph.D. Thesis, Krips Repro, Meppel
26 Hauer, R. and Höfer, M. (1978) J. Membrane Biol. 43, 335–349
27 Jaspers, H.T.A., Christianse, K. and Van Steveninck, J. (1975) Biochem. Biophys. Res. Commun. 65, 1434–1439
28 Kotyk, A. (1967) in Symposium on Yeast Protoplasts. (Müller, R., ed.), pp. 161–162, Akademie Verlag, Berlin
29 Kotyk, A. and Michaljaničová, D. (1968) Folia Microbiol. 13, 212–220