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On the Existence of H^+-Symport in Yeasts

A Comparative Study

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Abstract. 34 yeast strains representing 22 species and two varieties were investigated for the existence of a proton-sugar symport. The changes in pH of unbuffered cell suspensions on the addition of alkali, acid, transportable sugars and uncouplers were recorded. Responses indicating the existence of an energy dependent proton extrusion and H^+-sugar symport were found in most cases, particularly in *Rhodotorula* but rarely in *Saccharomyces* species. Remarkable differences were found among strains belonging to the same species.

Key words: Membrane transport – Proton-sugar symport – Active transport energization – Yeasts – *Rhodotorula* – *Saccharomyces.*

There is an increasing body of evidence for the role of protons in the transfer of solutes across the cell membrane not only in bacteria (cf. Harold, 1974) but also in various eukaryotic cells (Komor and Tanner, 1974; Slayman and Slayman, 1974) among them yeasts. Seaston et al. (1973) showed, that protons were co-substrates in the transport of amino acids by *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, the latter species as well as *Saccharomyces (Kluyveromyces) fragilis* also took up protons in the presence of certain carbohydrates. Misra and Höfer (1975) and Klöppel and Höfer (1976) demonstrated the existence of an energy-linked proton extrusion across the cell membrane of *Rhodotorula gracilis (Rhodosporidium toruloides)*. The function of active transport processes has also been shown in the case of yeasts mentioned above (Harris and Thompson, 1961; Okada and Halvorson, 1964; Kotyk and Höfer, 1965; Grenson et al., 1970; Kotyk and Rihová, 1972). Since the active transport of monosaccharides occurs widely among various other yeasts (Deák and Kotyk, 1968; Deák and Novák, 1969; Novák and Deák, 1972) it was of interest to look for the possible role of protons in these cases, too.

For comparative purposes the application of some selected tests revealing the basic characteristics of a proton-sugar symport mechanism seemed practicable. If this mechanism operates the following phenomena, among others, have to be measurable experimentally (Seaston et al., 1973; Komor and Tanner, 1974; Slayman and Slayman, 1974; Misra and Höfer, 1975): 1) more acidic external pH than the intracellular one, reflecting the existence of a pH gradient across the cell membrane; 2) regaining the original pH of an unbuffered cell suspension after the addition of alkali but not acid, indicating the unidirectional extrusion of protons to maintain a pH gradient; 3) rapid alkalinization of the cell suspension after the introduction of an uncoupler, indicating the collapse of the pH gradient due to the effect of uncoupler on the membrane permeability and/or on the proton extruding membrane assembly; 4) increase, at least transiently, of the external pH following the addition of a transportable substrate, showing directly the use of the existing pH (electrochemical) gradient to effect substrate transport (i.e. proton-substrate co-transport or symport).

By testing the above phenomena for the existence of a proton gradient across the cell membrane and of its involvement in the symport of substrates a comparative study of various yeast species was undertaken. Data showing a rather general occurrence among yeasts of proton-sugar symport are the subject of this paper.

List of Abbreviations. DNP = 2,4-dinitrophenole; CCCP = carbonyl cyanide m-chlorophenyl hydrazone

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Table 1. Species and strains investigated

Species	Strain CBS no.
Candida albicans	562
Candida claussenii	4908
Candida curvata	570
Candida guilliermondii	566
Candida krusei	573
Candida pseudotropicalis	607
Candida rugosa	613
Candida stellatoidea	1905
Candida tropicalis	94
Candida utilis	621
Hansenula anomala	605
Hansenula anomala	5702[a]
Hansenula anomala	5795
Metschnikowia pulcherrima	5833
Metschnikowia reukaufii	5834
Rhodosporidium toruloides	6681[b]
Rhodosporidium toruloides	14[c]
Rhodotorula glutinis	20
Rhodotorula glutinis	322
Rhodotorula glutinis	2367
Rhodotorula glutinis var. *dairenensis*	4406
Rhodotorula graminis	3043
Rhodotorula minuta var. *texensis*	2177
Rhodotorula pallida	320
Rhodotorula pilimanae	5804
Rhodotorula rubra	17
Saccharomyces cerevisiae	1171
Saccharomyces cerevisiae	1172
Saccharomyces cerevisiae	1539
Saccharomyces cerevisiae	1907
Saccharomyces uvarum	395
Saccharomyces uvarum	1513[d]
Saccharomyces uvarum	6751
Turolopsis candida	940

[a] Identical with the strain called *Candida beverwijkii* by Deák and Kotyk (1968)

[b] Mating type a. The strain *Rhodotorula gracilis* of Misra and Höfer (1975)

[c] Mating type α

[d] The former type strains of *Saccharomyces carlsbergensis*

MATERIALS AND METHODS

The yeast strains studied are listed in Table 1. They were obtained from the collection of Centraalbureau voor Schimmelcultures, Yeast Division, Delft.

Two media were used for their propagation and their composition was as follows (ingredients in %): *Medium Y*, KH_2PO_4 0.1, $MgSO_4 \cdot 7\,H_2O$ 0.07, NaCl 0.1, $(NH_4)_2HPO_4$ 0.4, $FeSO_4 \cdot H_2O$ 0.003, glucose 4.0, yeast extract 0.4, pH 5.0 (Deák and Kotyk, 1968); *Medium R*, K_2HPO_4 0.1, $MgSO_4 \cdot 7\,H_2O$ 0.1, NaCl 0.05, NH_4NO_3 0.066, $FeCl_3 \cdot 2\,H_2O$ 0.005, $CaCl_2 \cdot 2\,H_2O$ 0.025, glucose 2.0, yeast extract 0.03, pH 5.4 (Klöppel and Höfer, 1976).

Cells were cultivated on an orbital shaker at 28° C for 30 h. After harvesting the cells were washed twice with distilled water and starved by aerating their water suspension for 3 h. The aerated suspension was washed once more and fresh distilled water suspensions of about 5–10 mg/ml dry weight were prepared for the experiments.

For pH measurements 1 mM $CaCl_2$ was added to the unbuffered cell suspension to adjust its ionic strength. The pH was measured by Metrohm pH-meter (Type E561/3, Switzerland) using Ingold-type combined electrode (I6205, Switzerland). For the amplification of pH scale Servogor S Kompensationsschreiber (RE541, Goerz Electro, Germany) was used.

The responses of unbuffered cell suspension to the addition of alkali or acid, transportable substrates (D-glucose, D-xylose and in some cases L-arabinose) as well as uncouplers (2,4-dinitrophenole, DNP, carbonyl cyanide m-chlorophenyl hydrazone, CCCP) were studied as described by Misra and Höfer (1975).

The endogenous rate of respiration was determined by a biological oxygen monitor (Yellow Springs Instruments, Ohio, Model 53) with a Clark-type oxygen sensor. The intracellular pH of yeast cells was taken 5.8–6.1 (Kotyk, 1963; Kotyk and Höfer, 1965).

Transport measurements were performed as described earlier (Deák and Kotyk, 1968). Sartorius SM 11305 membrane filters were used for the separation of cells, and their intracellular sugar content was determined chemically (Kotyk, 1967).

RESULTS

In order to make the data comparable it was intended to cultivate all strains used in the same medium. In our earlier comparative study (Novák and Deák, 1972) the medium Y proved to be satisfactory for the propagation of various *Saccharomyces* and *Candida* species. In preliminary experiments, however, *Rhodosporidium toruloides* when cultivated in medium Y grew poorly and its cell suspensions after only 2 h starvation were unable to maintain a pH value below 5 or to respond to the addition of alkali, sugars or uncouplers. Moreover, the pH of the cell suspension increased and the rate of endogenous respiration decreased with the time of starvation (Table 2). When 20 mM glucose was added to the suspension starved for 24 h and it was further aerated for 3 h the cells, after being washed and resuspended in distilled water, created a low pH and acquired the ability of building up a proton gradient across the membrane (Fig. 1, triangles).

Rhodosporidium toruloides cultivated in medium R with 1, 2 or 4% glucose for 16–36 h and starved for 9–24 h invariably was capable of extruding protons. The pH of the cell suspensions was always less than 5, it increased only little with the time of starvation, and was the lower the more glucose was included into the growth medium. Cells harvested in their earlier growth phase maintained a somewhat lower pH in suspension than those harvested in later growth phase (Fig. 1). The cells were able to regain the original pH of their suspension even after several times of their suspension even after several times of repeated alkalinization (Fig. 2).

Table 2. The change of pH of unbuffered cell suspension and of the rate endogenous respiration (expressed as Q_{O_2}) with the time of starvation

Growth medium	Starvation time (h)	*Rhodosporidium toruloides 6681*		*Hansenula anomala 5702*		*Saccharomyces cerevisiae 1907*	
		Q_{O_2}	pH	Q_{O_2}	pH	Q_{O_2}	pH
R	0	96.5	4.41	165.8	4.73	21.2	4.70
	2	69.3	4.36	76.6	4.63	15.4	5.63
	6	33.1	4.61	47.3	4.69	13.8	6.05
	10	23.1	4.82	28.2	5.10	9.8	5.95
	24	22.3	4.98	21.2	5.57	6.7	6.17
Y	0	74.1	4.95	112.5	5.07	100.2	5.25
	2	31.5	5.48	68.3	5.42	50.6	5.81
	6	22.8	6.10	52.3	5.58	43.7	5.98
	10	14.8	6.62	36.5	6.16	41.6	6.20
	24	9.1	7.25	29.3	6.40	16.9	7.14

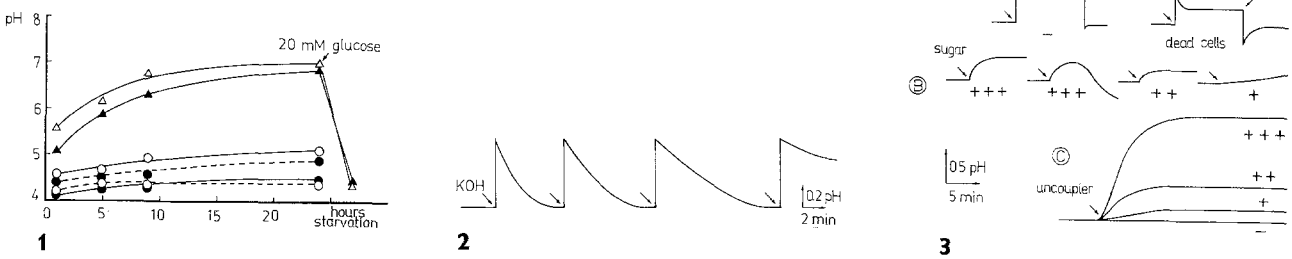

Fig. 1. Effect of growth conditions and starvation on the pH of the cell suspension of *Rhodosporidium toruloides Empty marks:* cells cultivated for 16 h; *full marks:* cells cultivated for 36 h; *dotted lines:* medium R with 1% glucose; *full lines:* medium R with 4% glucose; *triangles:* cells cultivated in medium Y with 1% glucose

Fig. 2. Recovery of the original pH of the cell suspension of *Rhodosporidium toruloides* after repeated alkalinization

Fig. 3A–C. Various responses of cell suspensions to the addition of alkali and acid (A), sugars (B) and uncouplers (C). The extent of responses is arbitrarily graded and marked by the sign as shown

In preliminary experiments *Hansenula anomala* although growing well in both media maintained a low pH and showed a proton extrusion in suspension when the cells were cultivated in medium R (Table 2). Hence this medium was chosen to test the other strains too. With the exception of *Saccharomyces* strains all others grew satisfactorily in medium R. The cell yield was 1.1–5.3 g wet weight per 100 ml medium after 30 h cultivation, and the endogenous rate of respiration was 15–64 µl O_2/h/mg dry weight after 3 h starvation. *Saccharomyces* strains, however, grew poorly in medium R. Their cell yield was only 1/3–1/4 of that in medium Y. In preliminary experiments *Saccharomyces cerevisiae* 1907 showed hardly any capacity of proton extrusion when it was grown in either medium (Table 2). In the course of further investigations both media were applied for the cultivation of *Saccharomyces* strains.

The responses of cell suspensions to the addition of alkali, acid, sugars or uncouplers were expressed in arbitrary units according to their extent as shown in Figure 3. In Tables 3–6 the results are summarized by using the signs of Figure 3. In the tables the data represent averages of 2–6 experiments.

Table 3 shows the results obtained with *Rhodosporidium* and *Rhodotorula* species. Except the lack of pH recovery after alkalinization in some cases, all strains gave positive responses to all tests chosen for screening the existence of proton extrusion and proton-sugar symport. In some selected strains the intracellular concentration of D-xylose was also determined. 3–8-fold accumulation of the pentose was found showing the active transport ability of the strains investigated.

The species listed in Table 4 were shown to transport monosaccharides actively (Novák and Deák,

Table 3. Characteristics of *Rhodosporidium* and *Rhodotorula* strains

Species and strain	pH[a]	KOH	Xyl	Glc	DNP	CCCP
R. toruloides 6681	4.5–5.0	+++	+++	+++	+++	+++
R. toruloides 14	4.2–4.8	+++	+++	+++	+++	+++
R. glutinis 20	4.3–4.7	–	++	+++	+++	+++
R. glutinis 322	4.5–5.7	–	+	++	+++	+++
R. glutinis 2367	4.4–4.5	–	++	++	+++	+++
R. glutinis var. *dair.* 4406	4.1–4.9	–	+++	+++	+++	+++
R. graminis 3043	5.2–5.7	–	+++	+++	+++	+++
R. minuta var. *texenis* 2177	4.4–4.9	++	+++	+++	+++	+++
R. pallida 320	4.5–5.0	++	++	++	+++	+++
R. pilimanae 5804	4.0–4.6	++	+++	+++	+++	+++
R. rubra 17	4.1–4.7	+++	+++	+++	+++	+++

[a] pH = original pH of fresh cell suspension; KOH = recovery of the original pH after the addition of alkali; Xyl = increase of pH after the addition of D-xylose; Glc = increase of pH after the addition of D-glucose; DNP = increase of pH after the addition of 2,4-dinitrophenole; CCCP = increase of pH after the addition of carbonylcyanide m-chlorophenyl hydrazone
See Figure 3 for the explanation of signs

Table 4. Characteristics of yeast possessing active transport ability

Species and strain	pH[a]	KOH	Xyl	Glc	DNP	CCCP
C. claussenii 4908	4.1–4.2	++	+++	+++	+++	+++
C. guilliermondii 566	3.6–3.8	–	–	–	+++	+++
C. pseudotropicalis 607	4.0–4.1	–	–	+++	+++	++
C. rugosa 613	4.5–5.1	–	–	–	++	++
M. pulcherrima 5833	4.4–4.5	++	+	+	+++	+++
T. candida 940	3.9–4.2	–	–	–	+++	+++

[a] See legend of Table 3

Table 5. Characteristics of yeast possessing inductively active transport ability

Species and strain	pH[a]	KOH	Xyl	Glc	DNP	CCCP
C. albicans 562	4.1–4.5	++	–	+++	+++	+++
C. stellatoidea 1905	4.2–4.7	++	–	+++	+++	+++
C. tropicalis 94	3.9–4.1	+	–	–	+++	++
H. anomala 605	4.2–4.4	–	–	–	++	–
H. anomala 5702	4.0–4.5	+	+	++	+++	+++
H. anomala 5795	4.1–4.4	–	–	+	+++	+++
M. reukauffii 5834	4.5–5.1	–	+++	+++	+++	+++

[a] See legend of Table 3

1972). All of them maintained a pH value equal to or less than 5.1 in unbuffered water suspensions, and an increase of pH after the addition of uncouplers was also recorded. However, only a few strains were able to recover the original pH after alkalization and to respond to the addition of sugars.

Included in Table 5 are those species which also transport monosaccharides actively, some of them, however, like D-galactose and L-arabinose, only after induction (Novák and Deák, 1972). The characteristics of these strains were similar to those listed in Table 4, although they showed positive responses in more cases.

The effect of L-arabinose on the pH of cell suspensions was tested in some representative strains before and after induction. When glucose-grown cells were aerated for 3 h in the presence of 33 mM L-arabinose, and thereafter washed and resuspended, they showed no response to the addition of L-arabinose although the active transport had been induced in 90 min (Fig. 4). However, galactose-grown cells (cultivated in medium R containing D-galactose instead of glucose)

showed either an increase or an immediate decrease in the pH of suspension after the addition of L-arabinose. The inductive character of L-arabinose transport was maintained by galactose grown cells, too (Fig. 4).

Table 6 summarizes the results obtained with *Saccharomyces* strains cultivated in both media. The pH of the suspensions was lower when the cells were grown in medium R but only some strains grown in medium Y responded to uncouplers. The behaviour of the strains was rather variable in this respect, some responded either to DNP or CCCP while other to both or neither. The addition of D-glucose resulted in a rapid decrease of pH in most cases.

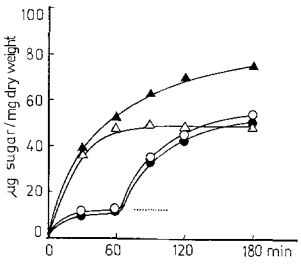

Fig. 4
Uptake of L-arabinose (○) and D-xylose (△) by *Candida albicans* grown on D-glucose (*full marks*) or on D-galactose (*open marks*). *Dotted line* shows the intracellular concentration corresponding to the outer one at diffusion equilibrium. Initial external concentration of pentose was 33 mM

The different characteristics of cells grown in media R and Y cannot be due to the lack of Ca^{2+} ions in the latter medium as no significant difference in characteristics was found when the cells were cultivated in media with or without calcium chloride (Table 7).

The other three species listed in Table 6 were characterized before (Novák and Deák, 1972) as transporting monosaccharides by facilitated diffusion similarly to *Saccharomyces cerevisiae* strains. Unlike those, however, they did not only strongly respond to the addition of uncouplers but also showed a tendency to pH recovery after the alkalinization of the suspension.

DISCUSSION

The changes of characteristics tested with the circumstances of growth conditions (the composition of growth medium, the growth phase, the starvation of cells) reflect the energy dependence of proton extrusion by yeast cells. It was shown, that *Rhodosporidium toruloides* could store a great amount of lipids as energy source (Kotyk and Höfer, 1965). This yeast,

Table 6. Characteristics of *Saccharomyces* strains and some other yeasts transporting sugars by facilitated diffusion

Species and strain	Medium	pH[a]	KOH	Xyl	Glc	DNP	CCCP
S. cerevisiae 1171	R	4.7–5.3	–	–	/[b]	–	–
S. cerevisiae 1171	Y	5.1–6.0	–	–	/	+	–
S. cerevisiae 1907	R	4.6–4.9	–	–	–	–	–
S. cerevisiae 1907	Y	5.0–5.4	++	–	/	++	++
S. cerevisiae 1172	Y	5.1–5.7	–	–	/	++	++
S. cerevisiae 1539	Y	5.3–6.3	–	–	/	++	+
S. uvarum 395	R	5.0–5.4	–	–	–	–	–
S. uvarum 395	Y	6.3–6.5	–	–	/	+	++
S. uvarum 1513	R	5.1–5.3	–	–	–	+	–
S. uvarum 1513	Y	5.3–6.4	–	–	/	+++	+++
S. uvarum 6751	Y	5.9–6.1	–	–	/	+++	+++
C. curvata 570	R	4.5–4.6	+	–	–	+++	+++
C. krusei 573	R	4.7–5.4	+	+	/	+++	+
C. utilis 621	R	3.9–4.1	++	–	/	+++	++

[a] See legend of Table 3
[b] / means the immediate decrease of pH after the addition of D-glucose

Table 7. The pH of unbuffered suspension of cells propagated in the presence or absence of Ca^{2+} ions. Cultivation for 30 h, starvation for 8 h

Growth medium	*Rhodosporidium toruloides* 6681		*Hansenula anomala* 5702		*Saccharomyces cerevisiae* 1907	
	pH	pH recovery[a]	pH	pH recovery	pH	pH recovery
R with Ca^{2+}	4.30–4.51	+	4.63–4.82	+	6.17–6.58	–
R without Ca^{2+}	4.27–4.53	+	4.50–4.78	+	6.08–6.24	–
Y with Ca^{2+}	6.48–6.50	–	5.32–5.50	–	7.16–7.45	–
Y without Ca^{2+}	6.45–6.62	–	5.17–5.35	–	6.92–7.20	–

[a] + means recovery of the original pH of suspension in 5 min after alkalinization

however, when grown in medium Y apparently could not synthesise and store enough source of energy as was revealed by the endogenous rate of respiration. The more it decreased with the time of starvation the lower pH gradient was maintained by the cells in suspension. On the other hand, *Rhodosporidium toruloides* if propagated under conditions enabling the cells to reserve enough endogeneous source of energy became rather insensitive to starvation and maintained the proton extrusion capacity for a long period of time.

No attempt was made to find the best growing conditions for each strain investigated but it was intended to use the same medium for enabling comparison. Considering the observed effect of growth conditions on the ability of cells to create and maintain a proton gradient across the plasma membrane one should be cautious in evaluating the differences obtained with the various yeast species. While positive response can be considered as evidence, the lack of response is not unequivocal. The differences observed in strains belonging to the same species when they were cultivated and prepared under the same conditions can, however, be attributed to the real differences in the properties of strains. These were rather pronounced among *Saccharomyces* strains. This fact together with the different cultivation conditions in laboratories may account for the controversy among workers in interpreting the transport properties of *Saccharomyces cerevisiae* (cf. Kotyk and Michaljanicová, 1968; Kuo and Cirillo, 1970; versus van Steveninck, 1969, 1972).

Disregarding the questioned suggestion of glucose transport by vectorial phosphorylation in *Saccharomyces cerevisiae* (van Steveninck, 1969) no one has ever been able to find free glucose accumulated in the cells of this yeast. Hence it is generally assumed that the transport of glucose proceeds by facilitated diffusion (cf. Kotyk, 1973). This process can easily be studied by using non-metabolized analogues e.g. D-xylose (Kotyk and Kleinzeller, 1963; Kotyk, 1967). It is also generally held that energy is required only for accumulation but not for facilitated diffusion, and that energy coupling converts a facilitated diffusion system into an active accumulating one. There are strong supports for this view in bacteria (cf. Harold, 1972) and in the case of oligosaccharides in yeasts as well (Okada and Halvorson, 1964). However, the energy coupling for active transport of oligosaccharides appeared to be inducible in *Saccharomyces cerevisiae*, while the active transport of amino acids was found constitutive (cf. Grenson et al., 1970; Kotyk and Rihová, 1972). Moreover it has also been shown that a proton gradient is involved in the active transport of amino acids by *Saccharomyces* species. In view all these it is not surprising that, depending on the growth conditions, the existence of a proton gradient may be demonstrated by uncouplers in some *Saccharomyces* strains, but no response was found to sugars. The results obtained with the other yeast strains listed in Table 6 can be interpreted similarly as no active transport of monosaccharides has been found in these cases either (Novák and Deák, 1972).

There are a few exceptions among the results in Tables 4 and 5 concerning the lack of response to the addition of sugars. However, according to the criteria chosen for screening and comparison, which were mainly based on the results of Misra and Höfer (1975), most of the yeasts investigated here showed more or less positive signs of proton extrusion and proton-sugar symport. Here again it should be noted, that any positive response is of significance. One should be aware of the fact, that the cells maintain a very delicate steady state in which the rate of the formation of a proton gradient e.g. by an H^+-ATPase, may be counterbalanced by the rate of its discharge, e.g. in the H^+-sugar symport. On the addition of a transportable solute a new steady state sets in which need not necessarily results in a large pH change measurable experimentally.

The most convincing evidence for H^+-sugar symport came from the investigation of red yeast related to *Rhodotorula gracilis*. The strain called *Rhodotorula gracilis* (CBS 6681 = *Rhodosporidium toruloides*, mating type *a*) is one of the best characterized yeast for transport properties (cf. Höfer, 1971). No similar work has yet been done on other red yeasts. In the present investigation not only the other mating type α of *Rhodosporidium toruloides* but also its imperfect form, *Rhodotorula glutinis*, as well as other *Rhodotorulas* proved to be identical with, or similar to, *Rhodosporidium toruloides (Rhodotorula gracilis)* CBS 6681. The red basidiomycetous yeasts are, however, not unique in their transport properties. Several other yeasts investigated here also showed evidence for proton extrusion and proton-sugar symport. They are either ascospore forming species or are related to ascomycetous perfect forms.

The results presented here together with the data of our earlier comparative study (Novák and Deák, 1972) and other data of the literature (Seaston et al., 1973; Misra and Höfer, 1975; Harris and Thompson, 1961; Okada and Halvorson, 1964; Kotyk and Höfer, 1965; Deák and Kotyk, 1968) show, that not only the active character of the sugar transport can be considered as rather widespread among yeasts but it is also probable that the generation of a proton gradient may be involved in the energization of this active transport. In a few cases an energy independent facilitated diffusion may also function in the transport of monosaccharides by yeasts.

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