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PROTON PUMPS OF THE PLASMALEMMA OF THE YEAST *RHODOTORULA GRACILIS*

THEIR COUPLING TO FLUXES OF POTASSIUM AND OTHER IONS

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(1) Intact cells of the obligatory aerobic yeast *Rhodotorula gracilis* (glutinis) generate a difference of the electrochemical proton potential ($\Delta\mu_{H^+}$) across the plasmalemma. In the range from pH 4.0 to 7.0 its value remains close to 12 kJ/mol. At pH 4.0 it is composed of the pH difference (inside alkaline) alone, at pH 7.0 of the membrane potential alone. (2) Both components of $\Delta\mu_{H^+}$ are generated by an active process, as shown by their rapid dissipation under anaerobic conditions. (3) In order to find out by which type of mechanism $\Delta\mu_{H^+}$ is generated the effect of a number of inhibitors of transport-ATPases (among them ouabain, triphenyltin chloride, quercetin, oligomycin, venturicidin, dicyclohexylcarbodiimide, Dio-9) were tested both on the generation of the membrane potential and on the extrusion of protons either in the absence or the presence of potassium ions. We found that all three processes were inhibited by Dio-9 and dicyclohexylcarbodiimide, which are specific for H^+-ATPases. Triphenyltin chloride inhibited the K^+/H^+-exchange without having any effect either on the extrusion of H^+ alone or on the membrane potential. (4) Dicyclohexylcarbodiimide and Dio-9, but not triphenyltin chloride inhibited at pH 4.0 the active transport of sugars. This class of substrates has been shown earlier to be transported by an electrogenic H^+ symport driven by $\Delta\mu_{H^+}$ across the cell membrane. (5) Neither the rate of respiration nor the intracellular level of ATP were significantly decreased by any of these inhibitors (except for venturicidin). (6) We conclude that in *Rhodotorula gracilis* the difference of the electrochemical potential of H^+ is created by an electrogenic proton pump, presumably in ATPase. The extrusion of protons in exchange against potassium is catalyzed by a different energy-dependent but electroneutral system. This conclusion is based on the observation that the H^+/K^+ exchange does not work under conditions where the membrane potential is large, and vica versa.

Introduction

Many investigators have observed an uptake of protons concomitant to the accumulation of sugars and amino acids across plasma membranes (for reviews, see Harold [1]; Eddy [2]). It is generally accepted that the cotransport of H^+ serves as a mechanism for driving the movements of substrates uphill. However, it is less clear by which mechanism metabolic energy is transduced into this ionic gradient. Many investigators have suggested that this process is mediated by a plasmalemma-bound H^+-ATPase physiologically similar to those of other energy transducing membranes. These enzymes are capable of coupling the hydrolysis of ATP to the electrogenic transport of ions.

In plasma membranes isolated from a number of fungi ATPase-activities were found which can be inhibited by some of the agents known to impair the function of the H^+-ATPases from mitochondria and bacteria (Fuhrmann et al. [3]; Scarborough [4];

Abbreviations: DCCD, dicyclohexylcarbodiimide; TPP^+, triphenylphosphonium ion.

Bowman and Slayman [5]; Scarborough [6]; Delhez et al. [7]; Dufour and Goffeau [8]; Serrano [9]). However, it could be demonstrated so far only for *Neurospora crassa* that the hydrolysis of ATP is used to transport protons electrogenically out of the cell (Slayman et al. [10]; Scarborough [4], [12]). In the case of *Saccharomyces* (Fuhrmann [13]; Malpartida and Serrano [14]), *Neurospora* (Dame and Scarboroough [15]), and *Schizosaccharomyces* (Amory et al. [16]) phosphoprotein-intermediates were found.

For these reasons it was of interest to study the generation of the difference of the electrochemical H^+-potentials ($\Delta\mu_{H^+}$) across the plasmalemma in another microorganism known to accumulate substrates by a symport with H^+. *Rhodotorula gracilis* (glutinis) accumulates sugars, amino sugars and polyalcohols by coupling their movements to the protonmotive force (Höfer and Misra [17]; Hauer and Höfer [18]; Niemietz et al. [19]; Klöppel and Höfer [20]). In the case of sugars and amino sugars this transport has been shown to be electrogenic. This communication presents indirect evidence obtained with intact cells that the electrochemical proton gradient ($\Delta\mu_{H^+}$) used to drive the mentioned transport system is generated by at least two plasmalemma-bound proton pumps. One of them operates electrogenically, the other one catalyzed an electroneutral H^+/K^+ exchange.

Experimental

Growth. Growth, harvest and aeration of the obligatory aerobic yeast *Rhodotorula gracilis* (glutinis), taxonomically *Rhodosporidium toruloides* Banno, mating type a, listed at the American Type Culture Collection, Rockville, MD, USA, (No. 26194) and at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, (No. 6681) have been described before (Kotyk and Höfer [21]).

Transport. Uptake of lipophilic cations and D-xylose as well as the extrusion of protons have been measured as described before (Hauer and Höfer [18]; Heller and Höfer [22]; Misra and Höfer [23]; respectively). Experiments dealing with the transport of potassium were started by adding 100 μM KCl to an unbuffered yeast suspension (2.5% wet wt./vol.). Samples of 1.0 ml, two before and 6 after the addition of KCl were withdrawn every 30 s and centrifuged for 10 s at 15 000 $\times g$ in an ECCO-Quick centrifuge (Collatz, Berlin, Germany). 0.7 ml of the clear supernatant were diluted 1 : 5 with distilled water, and the potassium content was measured in a Type 360 Perkin-Elmer atomic absorption spectrophotometer (Perkin-Elmer, Bodenseewerk, Überlingen, F.R.G.) employing a combined Na-K cathode lamp as a light source. The instrument was calibrated with standard solutions. In all experiments the pH of the cell suspension was recorded simultaneously. In order to stabilize the glass electrode $CaCl_2$ was added to give an 0.1 mM solution. Control experiments have shown that divalent cations at this concentration do not elicit an exchange with protons.

Determination of the pH-gradient. ΔpH was determined by measuring the intracellular pH at a known H^+-activity of the suspension. The intracellular pH was estimated by three different methods: (a) By following the uptake of an indicator dye which distributes according to the pH difference. This method, using bromophenol blue, has been described before (Höfer and Misra [17]); (b) By freezing and thawing of cells. This method was used for measuring the intracellular pH of baker's yeast (Borst-Pauwels and Dobbelmann [24]). A 2.5% cell suspension of *Rhodotorula* in distilled water was frozen in liquid nitrogen and thawed hereafter. This cycle of freezing and thawing was repeated (usually thrice) until the pH of the cell free extract remained constant. The pH measured after bringing the temperature to 28°C was taken as intracellular pH. (c) By using the polyene antibiotic nystatin. To an unbuffered 2.5% cell suspension 10 μM nystatin (solved in 1,3-propanediol) was added and the pH of the suspension was followed until a constant value was reached. Nystatin reacts with the sterols of the plasmalemma to give small pores which permit the equilibration of protons across the membrane. Because the inner mitochondrial membrane contains very little sterol it is not affected by nystatin (Von Hedenström and Höfer [25]) and this method should, therefore, give a value which is closer to the true cytoplasmic pH than those obtained by any other methods.

ATP determination. A vigourously aerated cell suspension (10% wet wt./vol.) was incubated with the appropriate inhibitor (dissolved in ethanol) or with 1% ethanol in the control without inhibitors. After 60 min samples of 1 ml were rapidly mixed with 1 ml 10% perchloric acid, frozen and thawed in order to

guarantee maximal extraction of ATP (Gancedo and Gancedo [26] and neutralized to pH 7.0 with a solution of 1 M triethanolamine and 1 M KOH. Aliquots of this extract were used to determine ATP by the hexokinase/glucose-6-phosphate dehydrogenase method (Bergmeyer et al. [27]). The NADH produced in this test was determined in a fluorimeter (Eppendorf 1101 M, Hamburg, F.R.G.).

Gas chromatography was used to determine the organic acids extruded from the cell during the spontaneous acidification of unbuffered cell suspensions. The cell suspension was centrifuged for 5 min at 5000 × *g*. 1.5 ml of the clear supernatant were withdrawn, mixed with 3 ml methanol and 0.6 ml of 50% sulfuric acid and kept at 55°C for 30 min. The methyl esters of organic acids were extracted by mixing vigourously with 1 ml water and 0.75 ml chloroform. 1–5 μl of the chloroform phase were used to measure the esters in a Perkin-Elmer, type 900 gas chromatograph (Perkins-Elmer, Bodenseewerk, Überlingen, F.R.G.) equipped with a column of 20–25% diethylhexylsebacinic acid on Chromosorb. The column temperature was 120°C.

Chemicals. Oligomycin and ouabain were from Serva, Heidelberg, F.R.G. An extract of heart-active glycosides from *Scilla bifolia* was a kind gift from Dr. K.W. Glombitza, Institut für Pharmazeutische Biologie, Bonn, F.R.G. Venturicidin was product of the B.D.H., Poole, U.K. Dio-9 was kindly supplied by the Nederlandse Gist- und Spiritusfabriek, Delft, The Netherlands. Tritiated tetraphenylphosphonium chloride (4.33 Ci/mol) was a custom synthesis from Hoechst, Frankfurt, F.R.G. All other reagents were of analytical grade purity purchased from Merck, Darmstadt, F.R.G.

Results

The pH gradient

Table I shows that the pH-difference across the membrane is a function of the H^+ activity of the medium. At pH 4.5, a difference of 1.7–2.1 units (inside alkaline) was indicated by three different methods, whereas at neutral pH the gradient was almost equilibrated across the membrane. This observation agrees with earlier results of Höfer and Misra [17].

TABLE I

INTRACELLULAR pH AS A FUNCTION OF THE EXTRACELLULAR pH

10 ml of a 5% (wet wt./vol.) yeast suspension were incubated at 28°C in 0.3 M Tris/citric acid buffer, pH 4.5 and 7.0. After 15 min portions of 3 ml were centrifuged at 5000 × *g* for 5 min and resuspended in 3 ml of distilled water. In the case of pH_{out} = 7.0, the pH of the suspending water was adjusted to 7 with small amounts of a diluted Tris solution. These suspensions were subjected to freezing and thawing or to the treatment with nystatin. For the measurement of the bromophenol-blue distribution the cells were incubated in Tris/citric acid buffer (pH 4.5) in the presence of 10 μM indicator dye for 15 min. The numbers in brackets give the maximal and minimal values obtained. *n*, number of experiments. n.m., not measured.

pH_{out}	pH_{in}, method		
	Bromophenol blue	Freezing and thawing	Nystatin
4.5	6.11 (*n* = 5) (5.91–6.44)	6.32 (*n* = 7) (5.94–6.60)	6.66 (*n* = 7) (6.36–6.88)
7.0	n.m.	6.63 (*n* = 7) (6.49–6.70)	6.78 (*n* = 7) (6.40–7.04)

The membrane potantial

$\Delta\psi$ was estimated by the accumulation of the lipophilic cation tetraphenylphosphonium (TPP^+). This ion is able to cross the membrane without mediation by a carrier or a channel. Consequently, its steady-state distribution depends entirely on the membrane potential in the way predicted by the Nernst equation. It has been shown earlier that this ion is suitable for estimating the membrane potential in a semi-quantitative fashion in this species of yeast (Hauer and Höfer [18]). Fig. 1 shows the dependence of the membrane potential on the extracellular pH. It can be seen that the relationship is complementary to that for ΔpH; at pH 4.5 the value of $\Delta\psi$ is negligible whereas it is high at pH 7.0 (ΔpH nearly zero). The values of $\Delta\psi$ obtained may vary considerably on subsequent experiments. However, we observed in all experiments a similar dependency of the membrane potential on the external pH. Under anaerobic conditions no accumulation of TPP^+ was observed indicating that in the absence of oxygen the membrane potential could not be generated (not

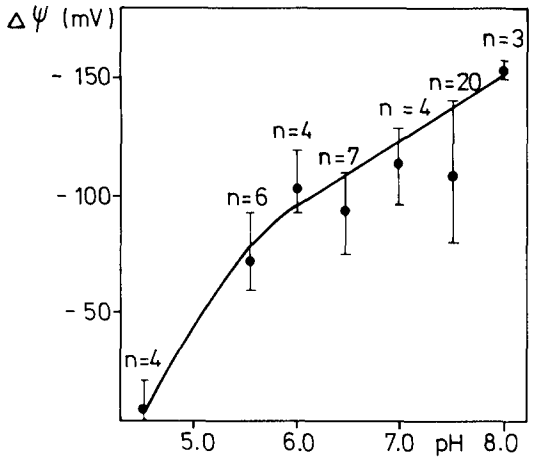

Fig. 1. Dependence of the membrane potential $\Delta\psi$ on the external pH. 15 ml aqueous cell suspension (5% wet wt./vol.) were incubated with 3 ml Tris/citric acid buffer (0.3 M) at 28°C. The experiment was started by adding 360 μl mM tetraphenylphosphonium chloride (20 μM final concentration). 500 μl samples were withdrawn at intervals of 4 min, centrifuged for 10 s at 15 000 × g and 200 μl of the supernatant were counted in 10 ml scintillation fluid. The amount of radioactivity which disappeared from the supernatant after reaching equilibrium was used to calculate the accumulation ratio of the cation assuming a value of 2 μl cell water/mg dry wt. (Höfer and Misra [17]). The value obtained was used to calculate the membrane potential by means of the Nernst equation. The vertical bars indicate the highest and lowest value observed.

shown). Table II summarizes the pH dependency of ΔpH and of $\Delta\psi$. It demonstrates that in the range of pH_{out} from 4.0 to 7.0 the energy stored in the electrochemical proton gradient is maintained close to 12 kJ/mol, the proton motive force being about −130 mV.

Generation of the pH gradient

Two sets of experimental conditions have been described (Misra and Höfer [23]) which lead to the formation of a pH difference across the plasmalemma: (a) the acidification of the medium after transferring cells to water weakly buffered at neutral pH with Tris (Fig. 2, curve a); (b) the extrusion of H^+ after adding potassium ions (Fig. 2, curve c). The stochiometry of this H^+/K^+ exchange was 1 : 1, as demonstrated in Fig. 3. This value was also indicated by the observation that the initial pH was exactly

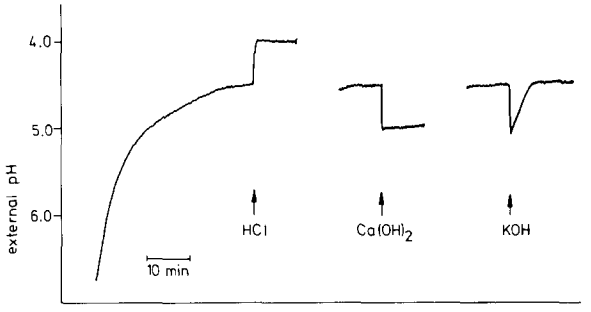

Fig. 2. Generation and manipulation of the pH-gradient. Yeast cells were washed in distilled water and resuspended in 0.1 mM $CaCl_2$ adjusted to a pH of 7.0 with small amounts of Tris hydroxide (final suspension density 2.5% wet wt./vol.). The cells started spontaneously to extrude protons until a steady-state outside pH of 4.0–4.5 was reached (see Misra and Höfer [23]), curve a. After reaching the steady-state value (a) HCl, (b) $Ca(OH)_2$ or (c) KOH were added to cause an initial pH-change of 0.5 units.

restored (cf. Fig. 2, curve c). The H^+/K^+ exchange catalyzes H^+ exchange also for other alkali ions (H^+/Na^+ stoichiometry was 1 : 1, as well) and for NH_4^+ (not shown). On the other hand, alkalinization by

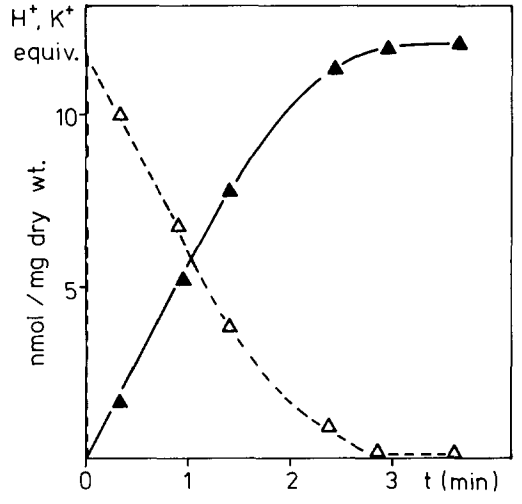

Fig. 3. Extrusion of protons in exchange against potassium. KOH was added to an unbuffered yeast suspension to give a 100 μM final concentration. The change in pH was followed and calibrated as described by Höfer and Misra [17]. Concomitantly, samples were withdrawn and analyzed for potassium as described in Materials and Methods. A stoichiometry of 1.0 ± 0.05 was obtained in 15 experiments. The initial pH was 4.5. The same stocichiometry was obtained also with NaOH or the chlorides of both alkali metals.

$Ca(OH)_2$ or acidification by HCl were not compensated (Fig. 2, curve a and b). The H^+/cation exchange could be shown, as a rule, only in cells aerated for 24 h, whereas all other activities were observed under quite variable conditions of starvation.

Generally, cells suspended in water adjusted the pH of the medium to a value around 4. This value could be lowered still further (by 0.5 pH unit or more) by adding KCl.

Identification of the anion extruded together with H^+

Also the spontaneous export of protons by the cells after resuspension in water weakly buffered with Tris (pH 7.0) (cf. Fig. 2, curve a) can occur only when electrically compensated. The most probable candidates for such compensation are anions produced by the metabolism. This assumption was tested by measuring organic acids by gas chromatography in the supernatant of cell suspensions after acidification. As shown in Fig. 4 pyruvate and either succinate or fumarate or both have been indicated by this method. The participation of CO_2 (or HCO_3^-) was excluded since bubbling of the incubation medium, acidified by the cells, by pure nitrogen did not rise its pH.

The pH dependence of the H^+/K^+ exchange

Because of the strict stoichiometry of the H^+/K^+ exchange observed in a large number of experiments under different physiological conditions, the pH dependence of this process could be measured by the determination of the movement of K^+ alone. Consequently, the measurements could be conveniently carried out in buffered suspensions. This is important because in unbuffered suspensions at pH values higher than 6 the cells rapidly extruded H^+ and thus interfered with the measurements of protons specifically exchanged against K^+. Fig. 5 shows that the initial velocity of transport at saturation (half-saturation constant = 20 μM at pH 4.0, results not shown) was maximal at pH 4. At values higher than 8 and lower than 2 no uptake of K^+ was observed. The same results were obtained when 500 μM KCl was used. This excludes the possibility that the observed pH dependence was due to a change of the affinity for potassium as a function of pH.

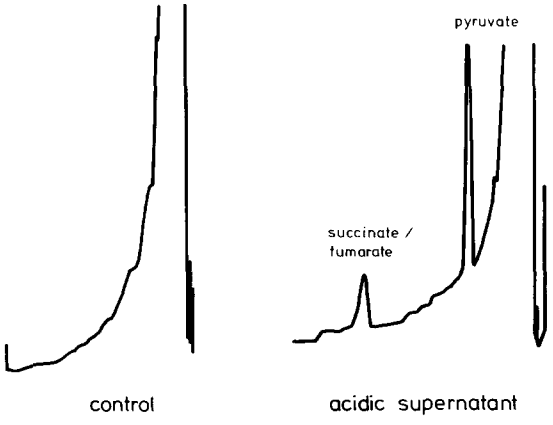

Fig. 4. Gas chromatogram of the methyl esters of organic acids exported by cells into the supernatant. A sample of 1 μl was injected. The peaks were identified as pyruvate and succinate or fumarate by comparison with chromatograms run with the methyl esters of pure substances. For experimental conditions see Material and Methods.

Effect of specific inhibitors of ion-translocating ATPases

In order to find out whether the pH gradient and the electrical membrane potential are generated by the same process the effect of a number of inhibitors of ion-translocating ATPases on the formation of both gradients was studied *. Ouabain (10 μM) and an extract of heart-active glycosides from *Scilla* (5 μg/ml) were selected as inhibitors of the ($Na^+ + K^+$)-ATPase (Glick [28]), triphenyltin chloride (10 μM), Dio-9 (70 μg/ml), DCCD (1 mM), oligomycin (10 μg/ml), venturicidin (10 μg/ml), vanadate (1 mM) and quercetin (100 μg/ml) as specific inhibi-

* In addition, 13 other compounds known to interact either with specific amino acid residues or with sulfhydryl groups were investigated. Sulfhydryl-group-specific reagents: *N*-ethylmaleimide; diethylstilbestrol; *P*-chloromercuribenzoate; 7-chloro-4-nitrobenzofurazan; trimethyltin chloride. Amino-group-specifc reagents: tetranitromethane; *N*-acetylimidazole; 1,2-naphthoquinone-4-sulfonic acid; diethylpyrocarbonate; diacetyl; 2-hydroxy-5-nitro-benzyl bromide; phenylmethanesulfonyl fluoride; picric acid. None of these compounds fulfilled, however, the criteria for an inhibitor acting specifically at the level of the plasmalemma. Those which inhibited transport of sugars across the plasmamembrane also decreased the cell respiration. For this reason these compounds were not used for further experiments. 2-hydroxy-5-nitrobenzylbromide turned out to act as an uncoupler whilst it stimulated the cell respiration, decreased the intracellular ATP level, increased the cell membrane permeability to H^+ and dissipated the membrane potential.

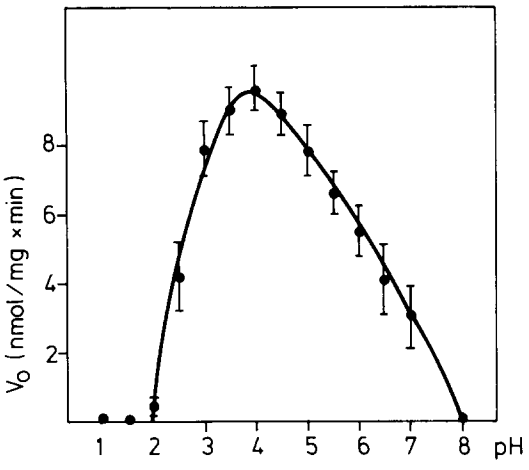

Fig. 5. pH dependence of the H^+/K^+-exchange. The experimental conditions were as described in the legend to Fig. 3 except that potassium was added as KCl (100 μM) and the cell suspension was buffered with 0.3 M citric acid titrated with $Ca(OH)_2$ to the desired pH. Tris was found to be inhibitory to the K^+ uptake. The vertical bars indicate the highest and lowest values observed in the course of 14 experiments.

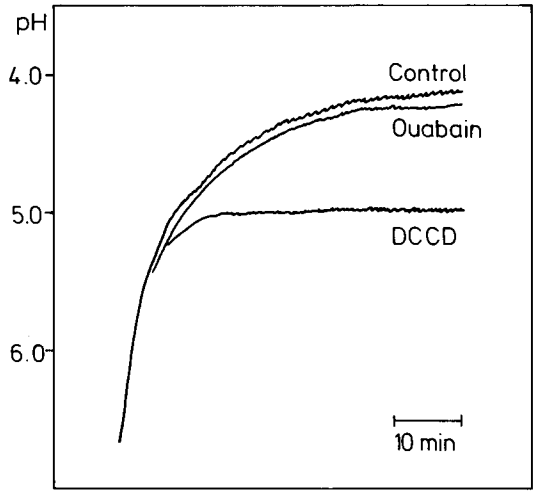

Fig. 6. Influence of ATPase-inhibitors on the extrusion of protons in the absence of potassium. 20 ml of a 2.5% yeast suspension in distilled water was preincubated with inhibitor (dissolved in ethanol) or with ethanol (final concentration in all runs 1%) for 30 min at room temperature. The suspension was then centrifuged at 5000 × g for 5 min and immediately resuspended in the same volume of 0.1 mM $CaCl_2$ containing the same amount of inhibitor and/or ethanol as before. The pH of this solution was adjusted to 7.0 by adding small amounts of Tris. The pH of the inhibitor-treated suspensions and of the control one were continuously recorded by a two-channel strip recorder. Two typical results are shown: ouabain (an equal lack of inhibitory effect showed also *Scilla*-glycosides, triphenyltin chloride and quercetin) and DCCD (the same inhibition as with DCCD was observed with Dio-9, as well). An inhibition by oligomycin was significant only in one out of eight experiments. The effect of venturicidin and vanadate was not tested. For the concentration of the inhibitors see the text. The inhibitory effect on proton pumping was tested at least four times in the presence of each inhibitor.

TABLE II

THE FREE ENTHALPY OF THE ELECTROCHEMICAL GRADIENT OF H^+ ACROSS THE PLASMA MEMBRANE AS A FUNCTION OF THE EXTRACELLULAR pH_{out}

Experimental conditions were as in Table I and Fig. 1. ($\Delta\mu_{H^+} = (\Delta\psi + Z\Delta\ pH)\ F$ with Z = 60 mV at 30°C)

pH_{out}	Δ pH	$\Delta\psi$ (mV)	$\Delta\mu_{H^+}$ (kJ/mol)
4.0	−2.2	± 0	−12.8
5.5	−1.0	− 75	−13.0
7.0	+0.3	−130	−10.8

TABLE III

INFLUENCE OF ATPase-INHIBITORS ON THE RESPIRATION AND ON THE ATP LEVEL OF INTACT CELLS

For O_2 measurements, a 1% (wet wt./vol.) unbuffered suspension was preincubated for 30 min with inhibitor. In all runs, including the controls, the pH_{out} was 4.5 and the final ethanol concentration 1%. Subsequently the rate of respiration was determined polarographically with a Clarke-type oxygen electrode (Beckman Instruments). For ATP determination see Materials and Methods. The rate of respiration and the ATP-level are given in percent of the control. n, number of experiments. n.m., not measured.

Inhibitor	% Respiration	% ATP
Ouabain (10 μM)	87 (n = 5)	n.m.
Scilla-glycosides (5 μg/ml)	94 (n = 4)	n.m.
Quercetin (100 μM)	97 (n = 4)	n.m.
Triphenyltin-chloride (10 μM)	100 (n = 4)	96 (n = 5)
DCCD (1 mM)	92 (n = 6)	85 (n = 5)
Dio-9 (100 μg/ml)	120 (n = 6)	102 (n = 5)
Oligomycin (10 μg/ml)	128 (n = 7)	100 (n = 5)
Venturicidin (10 μg/ml)	201 (n = 5)	53 (n = 5)
Vanadate (1 mM)	122 (n = 6)	n.m.

tors of the H^+-ATPase (Racker [29]; Bowman and Slayman [5]; Aldridge et al., [31]). In these experiments the effect of inhibitors on the membrane potential was measured at pH 7.5 whereas that on ΔpH was followed by monitoring the spontaneous acidification of weakly buffered cell suspension (cf. legend to Figs. 2 or 6). This was necessary because each gradient is maximal at a value unfavourable to the other one (cf. Table II and Fig. 1). The results are summarized as follows (Fig. 6, Table III and IV): ouabain, *Scilla*-glycosides vanadate and quercetin were not effective.

Triphenyltin chloride appeared to be a specific inhibitor of the H^+/K^+ exchange. DCCD, and Dio-9 inhibited significantly the extrusion of protons both in the presence and in the absence of potassium ions as well as the membrane potential. Oligomycin prevented the accumulation of TPP^+ and the H^+/K^+ exchange; the extrusion of protons in the absence of K^+ was inhibited only in 1 out of 8 experiments. The three processes investigated, H^+-extrusion in the presence and absence of K^+ and the membrane potential, are known to react very sensitively to a reduction of respiration. Therefore the action of these inhibitors might be conceived as being due to an impaired mitochondrial activity and not to an interaction with the plasmalemma. However, their failure to inhibit the cellular respiration or to decrease the ATP level (Table III) rules out this explanation.

Effect of the inhibitors of ion-translocating ATPases on active sugar transport

Because the active transport of monosaccharides in this strain of yeast is driven by $\Delta\mu_{H^+}$ this process may be regarded as an indicator of the difference in electrochemical potential difference of H^+ across the plasma membrane. In addition, this process has the advantage of being measurable in a broad pH-range

TABLE IV

INFLUENCE OF ATPase-INHIBITORS ON THE MEMBRANE POTENTIAL, ON THE ACTIVE TRANSPORT OF D-XYLOSE AND ON THE H^+/K^+ EXCHANGE

In all experiments samples preincubated for 30 min with the inhibitor (for the concentrations used, see Table III) were compared with controls containing the same final concentration of ethanol (1%). The accumulation of TPP^+ was followed as described in the legend to Fig. 1. The measurement of the K^+/H^+ exchange has been described in the legend to Fig. 3. For sugar transport, 5 ml yeast suspension (5% wet wt./vol.) were incubated in 5 ml 0.3 M potassium phosphate buffer, pH 4.5 or 7.5. The experiment was started by adding 0.1 ml D-xylose (1 M). Samples of 1 ml were withdrawn at intervals of 6 min for 54 min, filtered and analyzed for internal sugar concentration as described by Heller and Höfer [22]. For each experiment where the maximal or minimal value deviated by more than 10% of the mean, both values are given in brackets. All values are given in percent of the control. *n* is the number of experiments. n.m., not measured.

Inhibitor	TPP^+-accumulation [a] (pH = 7.5)	D-Xylose accumulation		H^+/K^+ exchange (pH = 4.5)
		pH 4.5	pH 7.5	
Ouabain	100 (n = 3)	99 (n = 2)	100 (n = 2)	100 (n = 2)
Scilla-glycosides	100 (n = 3)	111 (n =2)	93 (n = 2)	100 (n = 2)
Quercetin	n.m.	91 (n = 2)	92 (n = 2)	100 (n = 2)
Triphenyltin chloride	100 (n = 5)	99 (n = 5)	0 (n = 3)	0 (n = 5)
Vanadate	100 (n = 5)	100 (n = 2)	120 (n = 2)	n.m.
Oligomycin	40 (n = 5) (35–50)	20 (n = 2)	5 (n = 5)	0 (n = 5)
DCCD	5 (n = 5) (0–30)	0 (n = 5)	0 (n = 5)	0 (n = 5)
Dio-9	24 (n = 6) (0–40)	77 (n = 3)	15 (n = 2)	0 (n = 5)
Venturicidin	63 (n = 7)	20 (n = 4)	20 (n = 4)	7 (n = 4)

[a] In this table the TPP^+ accumulation has been chosen as a measure of the membrane potential because thus a direct comparison with the D-xylose accumulation is facilitated.

(Kotyk and Höfer [21]). Thus, measurement of the inhibition of this active transport by the compounds introduced above may be used as an independent indicator of their effect on the entire $\Delta\mu_{H^+}$. Table IV shows that only Dio-9, DCCD and oligomycin were effective whereas triphenyltin chloride at pH 4.5 had no effect even at a concentration which inhibited the H^+/K^+ exchange at this pH (10 μM). The inhibition of sugar transport by triphenyltin chloride at pH 7.5 occurred under conditions where H^+/K^+ exchange was not operating. Therefore, in our opinion the two effects are independent of each other.

Discussion

It was the aim of our work to establish the nature of the process by which $\Delta\mu_H{}^+$ across the plasma membrane of the yeast *Rhodotorula gracilis,* the source of energy for driving monosaccharides and their derivates uphill, is generated and maintained. At first the influence of several inhibitors of ion-translocating ATPase on both the driving force ($\Delta\mu_H{}^+$) and the driven process (accumulation of D-xylose) in intact cells was studied. The results, summarized in Table III, IV and Fig. 6, point to the following features:

(a) Inhibitors of the $(Na^+ + K^+)$-ATPase of the animal plasma membrane had no influence on cells of *R. gracilis.*

(b) DCCD, oligomycin, and Dio-9 suspended both the H^+/K^+ exchange and the membrane potential as well as the active transport of sugar. DCCD and Dio-9 inhibited, in addition, the acidification of the medium in the absence of K^+. Recent experiments have shown (Hauer and Höfer, unpublished data) that the concentration dependencies of the degree of inhibition were very similar both for the membrane potential and for the sugar transport.

(c) Triphenyltin chloride specifically inhibited the H^+/K^+ exchange without affecting the other processes except for sugar transport at pH 7.5.

(d) In isolated mitochondria of *Rhodotorula gracilis* the rate of oxygen consumption was decreased by ATPase-inhibitors, e.g. by oligomycin (Von Hedenström and Höfer [25]), as well as by venturicidin, DCCD, Dio-9 and triphenyltin chloride (65, 100, 100 and 32% inhibition, respectively). On the contrary, none of the inhibitors, except venturicidin, affected the cellular respiration or the ATP level in intact cells sufficiently enough to account for the observed effects. Thus, the inhibitors cannot act via the mitochondrial membrane.

(e) The inhibitors used did not induce an increase in the passive permeability of protons (not shown).

These controls rule out inhibition of mitochondrial energy transduction or an increase of passive ion fluxes across the plasmalemma as possible explanations for our results. We conclude that the target of the compounds exhibiting an inhibitory effect on $\Delta\mu_H{}^+$ and on active transport in *R. gracilis* are the active ion pumps located in the plasmalemma itself. The only exception is venturicidin, which decreased the intracellular ATP-level by one half and stimulated the rate of respiration by a factor of 2. Therefore, the effects of this oligomycin-analogue might be explained by its action on the mitochondria in this yeast. The pattern of effects of the other inhibitors is consistent with an electrogenic extrusion of protons by a plasmalemma-bound pump physiologically similar to the enzymes known to function in other fungi, e.g. *S. pombe* (Dufour and Goffeau [32]) or *N. crassa* (Scarborough [12]). We suggest that this type of system is responsible for the difference of the electrical potentials and for ΔpH across the plasma membrane of *Rhodotorula gracilis.* Consequently, this system generates the energy source for the secondary active transport of sugars.

The fact that all inhibitors effective in our strain are well known to interact with proton-translocating ATPases as well as the analogy to the work done with other fungi make it plausible that ATP is the energy source for the proton pump.

The extrusion of protons in *R. gracilis* is by up to one order of magnitude less sensitive to DCCD and Dio-9 than the hydrolysis of ATP in plasma membrane fractions isolated from other fungi (Dufour and Goffeau [8]; Serrano [9]; Bowman et al. [33]). This is probably due to the fact that in isolated membranes the ATPase is easier accessible than in intact cells which are surrounded by a thick cell wall. A similar explanation is obviously true also for vanadate and quercetin. Both vanadate (in isolated plasma membrane fractions) and quercetin (in mitochondria) interact with the active site of the ATP-hydrolyzing moiety which is localized on the inside. In *R. gracilis* cells these water-soluble compounds may be inactive

because they cannot cross the membrane. However, we cannot explain why the lipophilic triphenyltin chloride has no effect on $\Delta\mu_{H^+}$. We were surprised to find that even low concentrations of oligomycin (10 μg/ml) completely inhibited the membrane potential and the active transport. In *Neurospora* (Bowman et al. [33]), *Saccharomyces* (Serrano [9]) and *Schizosaccharomyces* (Delhez et al. [7]) oligomycin did not impair the function of the plasmalemma-bound ATPase. It can even be used to differentiate this enzyme from the mitochondrial one. We cannot rule out the possibility that oligomycin acts in *R. gracilis* by some mechanisms specific for our yeast. Multiple effects of this compound are indicated by its effects on the erythrocyte membrane (Porzig [34]). Such an explanation may also be valid for *Rhodotorula* since oligomycin inhibited the extrusion of protons in the absence of potassium ions very poorly even at concentrations very effective on the membrane potential and on the transport of sugars.

It is well known (see e.g. Nobel [35]) that a mass flow of an electrogenically transported ion can occur only if it is macroscopically compensated by the electrogenic movement of another ion. In our experiments (Fig. 2 and Fig. 6) the cells were suspended in some experiments in pure distilled water. Consequently, the extrusion of protons could be electrically compensated only by symport of an anion. An antiport with potassium ions leaking from the glass electrode has been ruled out since triphenyltin chloride did not inhibit the acidification at a 10 μM concentration which was sufficient to block the H^+/K^+ exchange. Gas chromatography of the supernatant after such acidification revealed that the H^+ efflux was electrically compensated by pyruvate, succinate and/or fumarate released by cell metabolism. An anion extrusion by an electroneutral outflow of undissociated acid is much less probable since in that case one had to postulate two independent effects of both DCCD and Dio-9 on the membrane potential, on one hand, and on the electroneutral outflow of organic acids, on the other hand. In a recent paper Janda [36], working with the same strain of *Rhodotorula*, has described the influence of a number of uncouplers, inhibitors of respiration as well as oligomycin and DCCD on cell respiration and on the transport of D-xylose, α-methyl-D-glucoside and alanine. His observations of the influence of DCCD and oligomycin agree very well with ours except that he found an almost 2-fold stimulation of endogenous oxygen consumption by DCCD. However, the author's conclusion that uncouplers, inhibitors of the respiratory enzymes and ATPase inhibitors act on the mitochondrial level is inconsistent with the following observations: (1) Uncouplers inhibit active transport directly by abolishing ΔpH and $\Delta\psi$ (Misra and Höfer [23]; Hauer and Höfer [18]). (2) Inhibitors of ATPase do not significantly interfere with respiration in intact cells with the exception of venturicidin. This is shown by the rather small changes of respiration and ATP-levels after incubation with these compounds. If these inhibitors acted via the mitochondria one would expect a decrease of respiration and ATP, which is indeed found in coupled mitochondria isolated from this strain of yeast (Von Hedenström and Höfer [25]). The author's interpretation appears to conflict with his own observation, e.g. that DCCD and oligomycin stimulate oxygen consumption.

The pumping of H^+ out of the cells in the presence of K^+, the H^+/K^+ exchange, is catalyzed by a process which is on the molecular level at least in part different from the postulated electrogenic H^+-ATPase. This is shown by its specific inhibition by triphenyltin chloride and by a distinctly different pH-optimum as compared with $\Delta\psi$ (Figs. 1 and 5). In the pH range from 4 to 7 the intracellular pH_{in} remains fairly constant (Table I; Höfer and Misra [17]). Hence, the pH dependence of Fig. 5 cannot be due to an indirect effect caused by a change of pH_{in}. Two interpretations are possible: (1) The exchange is catalyzed by a distinct noneelectrogenic H^+/K^+ exchanger such as the ATPase in gastric mucosa (Forte and Lee [37]). This system is sensitive to DCCD. The inhibition of both H^+-pumps in *Rhodotorula* by Dio-9 and oligomycin would be coincidental. (2) The H^+/K^+ exchange may also be explained by a mechanism which is common in many bacteria: Protons are electrogenically pumped out of the cells by a H^+-ATPase. Alkali ions compensate this movement electrically by depolarizing the generated membrane potential. Inhibition of the exchange by DCCD, Dio-9 and oligomycin would result as an inhibition of the H^+-ATPase. The effect of triphenyltin chloride would be limited to the compensating transport system for K^+.

However, the latter alternative conflicts with other

observations: (a) The exchange velocity is maximal at a pH value of 4 where a negative membrane potential cannot be detected (Fig. 1). At this pH potassium is taken up against gradients of the order of 1 : 10 000. If this occurred by an electrogenic process a membrane potential of at least −240 mV would be necessary. (b) At alkaline pH the membrane potential is high whereas the exchange mechanism is inactive. (c) If *Rhodotorula* had an electrogenic transport system for potassium ions similar to valinomycin, anaerobic conditions could not lead to an immediate halt of TPP^+ uptake. Under these conditions the potassium diffusion along its concentration gradient should maintain a highly negative membrane potential. For these reasons we prefer the nonelectrogenic model for the H^+/K^+-exchange. This process differs distinctly from the electrogenic transport of K^+ in *Saccharomyces*, for which the arguments have been summarized by Peña [38].

The membrane potential exhibits a remarkable pH dependence. However, this is not unusual and has been observed in quite different types of cells, e.g. in *Chlorella* (Komor and Tanner [39]) and *Halobacterium* (Michel and Oesterhelt [40]). At the present time we cannot decide whether this is due either to a change in activity of the pump or to the passive permeability of the membrane to protons.

On the basis of the present experiments we conclude that the difference of the electrochemical H^+ potentials across the cell membrane of *Rhodotorula gracilis*, $\Delta\mu_H{}^+$, which is the driving force for the secondary active transport of substrates (monosaccharides, polyalcohols, aminosugars), is generated by an electrogenic plasmalemma-bound H^+ pump. In the presence of potassium ions the pH gradient across the cell membrane is created by another pump, which catalyzes an electrically neutral exchange of H^+ against K^+ (or other alkali metal ions).

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