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PASSIVE FLUXES OF K^+ AND H^+ IN WILD STRAIN AND NYSTATIN-RESISTANT MUTANT OF *RHODOTORULA GRACILIS* (ATCC 26194)

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The passive fluxes of protons and potassium ions have been studied in the obligatory aerobic yeast *Rhodotorula gracilis*. The cellular energy metabolism was suspended by introducing anaerobic conditions. The H^+-permeability of the plasma membrane was modified by adding an uncoupler under both aerobic and anaerobic conditions. Unfortunately, the plasma membrane of *R. gracilis* was insensitive to K^+-ionophores. The passive flows of H^+ and K^+ under anaerobic and/or uncoupled conditions were electrically coupled and exhibited a constant stoicheiometry of 1:1. The H^+ permeability of the plasmalemma was shown to determine the velocity of the passive K^+-H^+ exchange. The nystatin-resistant mutant M 67 displayed distinctly lower permeability for both H^+ and K^+, which can explain the observed differences in some transport characteristics of the two strains. In order to account for the properties of passive K^+ flows, a membrane-potential-gated channel for K^+ has been proposed. Evidence is presented that the inhibitor of the plasmalemma-bound H^+-ATPase, *N,N'*-dicyclohexylcarbodiimide (DCCD), reduced at first the permeability for both K^+ and H^+ and only upon prolonged incubation the ATPase itself. Since DCCD effected an immediate hyperpolarization of the membrane potential, it has been concluded that the H^+ does not slip through the H^+-ATPase under deenergized conditions.

Introduction

In most bacteria and eukaryotic cells, sugars and amino acids are accumulated by an electrogenic H^+ or Na^+ symport [1,2]. In *Rhodotorula gracilis*, an H^+/monosaccharide symport depending on the electrochemical potential difference of protons, $\Delta\mu H^+$, was found. A plasmalemma-bound H^+-translocating ATPase very probably provides the appropriate form of energy for this process [3,4]. Upon addition of D-xylose to an unbuffered cell suspension, a transient alkalinization of the medium was observed. Moreover, a concommitant flow of K^+ out of the cells was measured with the stoicheiometry H^+ : D-xylose : K^+ of 1 : 1 : 1, as has been postulated on a basis of macroscopic electroneutrality [12].

Recently, a nystatin-resistant mutant of *R. gracilis* has been isolated [5]. Its transport properties were characterized and it was found that in the mutant an electrogenic H^+/monosaccharide symport was also operating [6]. The transport led to an accumulation of substrate and depolarized the membrane potential. The uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was able to collapse both the membrane potential and the pH gradient and consequently also the sugar transport [6]. One difference as compared with the wild strain was the lack of the transient alkaliniza-

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

tion of the unbuffered cell suspension upon adding D-xylose to the mutant cells. Thus, the idea has been put forward that a reduced permeability for the charge-compensating cation (K^+) might be responsible for this phenomenon. It is the purpose of this communication to provide experimental evidence for the postulated reduced cation permeability of the mutant plasma membrane. A preliminary report on this work has appeared [7].

Material and Methods

Growth. Growth, harvesting and aeration of *R. gracilis* (taxonomically *Rhodosporidium toruloides*, mating type a, ATCC 26194 and CBS 6681) and of the mutant M 67 proceeded as described before by Hauer and Höfer [4] and by Höfer et al. [5].

Transport. Experiments under anaerobic conditions were carried out in a closed acrylic glass cuvette with 30 ml unbuffered yeast suspension (50 g fresh weight/litre) kept at constant temperature under a flow of purified nitrogen (Messer Griesheim, Düsseldorf, F.R.G.). pH and potassium concentration in the medium were measured by a glass electrode and by atomic absorption spectrophotometry (AAS Type 360 Perkin-Elmer Bodenseewerk, Überlingen, F.R.G.), respectively, as described by Hauer et al. [8]. Intracellular potassium and D-xylose concentration was determined in hot-water extracts of cell pellets separated by a membrane filtration according to Heller and Höfer [9]. K^+ was measured by atomic absorption spectroscopy as above and D-xylose by the orcin method [9].

Membrane potential. Tritiated tetraphenylphosphonium ($[^3H]TPP^+$) was used as indicator of the membrane potential. Its accumulation was determined by counting the radioactivity in the supernatant after centrifugation of samples of cell suspensions as described by Hauer and Höfer [4].

Ion fluxes were calculated in nmol/mg dry wt. per min. For the calculation of H^+ fluxes, the pH records were calibrated by adding known amounts of HCl to a given volume of the cell suspension. When intracellular concentrations were calculated, the ratio 2 μl intracellular water volume per mg dry wt. was used (cf. Ref. 3).

Chemicals. ^3H labelled tetraphenylphosphonium chloride (11.8 μCi/mg) was a custom synthesis from Hoechst, Frankfurt, F.R.G. Valinomycin, gramicidin S and nonactin were purchased from Sigma, München, F.R.G. All other reagents were of analytical grade purity available commercially.

Results

Passive ion fluxes in *Rhodotorula gracilis* can be measured easily because it is an obligatory aerobic yeast and, hence, introducing anaerobic conditions suspends energy metabolism completely. Unbuffered yeast suspensions acidify the medium pH to values around 4.0 [10]. Since the intracellular pH is estimated to be about 7.3 (values obtained by nuclear magnetic resonance of the intracellular P_i, unpublished data), there is a chemical gradient of protons across the plasmalemma of over 10^3. In addition, there is an even larger gradient of K^+ in the opposite direction ($[K^+]_o = 3$–10 μmol/l; $[K^+]_i$ about 150 mmol/l). Upon blowing nitrogen into a closed cuvette with the yeast suspension, the ion gradients across the plasmalemma cannot be maintained, and consequently potassium ions flow out of, and hydrogen ions into, the cells as a passive K^+-H^+ exchange with a stoicheiometry $K^+ : H^+$ of 1 : 1 (Fig. 1). The mutant showed similar behaviour, but with half the velocity as compared to the wild strain.

In order to find out which one of the two ion permeabilities concerned determined the velocity of the whole process, CCCP as a specific protonophore was used to enhance artificially the passive membrane permeability for protons. Proportionally to the rise in concentration of CCCP (1–80 μmol/l), the flux of H^+ was stimulated (Fig. 2). Concomitantly measured K^+ efflux revealed, as expected, a constant $K^+ : H^+$ stoicheiometry of 1.01 ± 0.04 (S.E., $n = 18$) in the range 5–60 μmol/l CCCP. Moreover, the $K^+ : H^+$ stoicheiometry was maintained under both aerobic and anaerobic conditions. Because the driving force of potassium flux, viz. the concentration gradient of K^+, was lower in the mutant (cf. Table II), it was necessary to calculate the permeability coefficient, P_K (flux $= P_K([K^+]_o - [K^+]_i)$. Still, there was a significant difference between the two strains (Table I).

To avoid a possible difference of the primary effect of CCCP upon the two strains, the following

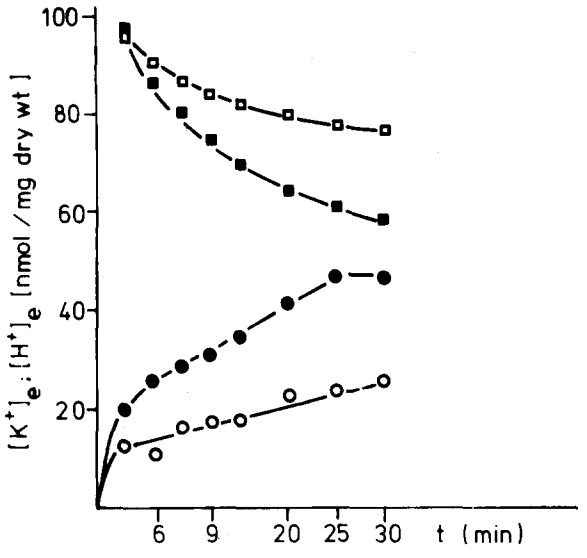

Fig. 1. Passive anaerobic flows of K^+ and H^+ in the wild strain and in the mutant M 67. Decrease in proton concentration in cell suspensions of the wild strain (■) and of M 67 (□). Increase in potassium concentration in the same suspensions: wild strain (●), M 67 (○). Experimental: unbuffered yeast suspensions (50 g fresh wt./l) were incubated at 30°C. Potassium was measured by sampling at the given intervals (see Material and Methods) and the pH was recorded continuously in the suspensions.

characteristic properties of the plasma membrane have been checked. First, the action of CCCP on the electrogenic H^+/D-xylose symport exhibited

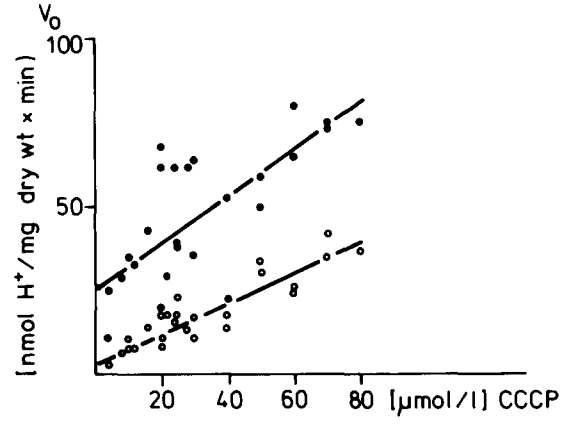

Fig. 2. Proton influx under anaerobic conditions induced by CCCP in the wild strain (●) and in the mutant M 67 (○). Unbuffered yeast suspensions (50 g fresh wt./l) were incubated at 30°C. Values are calculated from continuous pH recording (see Material and Methods). The experiments were started by adding various concentrations of CCCP.

equal concentration dependencies (Fig. 3). Second, the permeability coefficients of CCCP-induced [^{3}H]TPP^+ efflux were comparable both in the mutant (1.0 ± 0.1 min^{-1}) and the wild strain (1.1 ± 0.1 min^{-1}) at pH 7.5 and 5 µmol/l CCCP (mean value of six experiments ± S.E.). In *R. gracilis*, tetraphenylphosphonium ions (TPP^+) serve as an indicator of the membrane potential [4].

After the passive flows of H^+ and K^+ were characterized thus far, the question arose as to whether the passive flow of protons was mediated by the H^+-ATPase of the plasma membrane or (an)other membrane component(s), e.g., the postulated energy-dependent H^+-K^+ exchange [8]. The latter possibility was excluded, since the passive K^+-H^+ exchange was not sensitive, but stimulated by 10 µmol/l triphenyltin chloride.

N,N'-Dicyclohexylcarbodiimide (DCCD), which has been shown to reduce the energization of the plasmalemma without affecting either the cell respiration or the intracellular ATP level in *R. gracilis*, is very likely an inhibitor of the plasma-membrane ATPase [8]. DCCD did indeed diminish the passive anaerobic K^+-H^+ exchange, but by only about 50% (Fig. 4). Assuming, that DCCD reduced the passive anaerobic proton flow by inhibiting the H^+-ATPase, CCCP should cancel the inhibition, since uncoupler act independently of the ATPase. Fig. 5 demonstrates unambiguously that different uncouplers failed to abolish the inhibition by DCCD. Thus, the plasmalemma H^+-ATPase cannot function as an H^+ leak. On the other hand, the action of CCCP in the membrane lipids should also be insensitive to DCCD. To explain this, a multiple action of DCCD on the H^+-ATPase and on some other membrane protein(s) mediating the passive ion flows, at least on that for K^+ as the compensating cation, has to be postulated. Ionophores (valinomycin, gramicidin, nonactin, up to 100 µmol/l each) did not effect any movement of H^+ or K^+ in the presence of DCCD (not shown). However, this was not unexpected, since it has been frequently observed that ionophores other than uncouplers fail to affect ion permeability of yeast plasma membrane (personal communication of several authors; for *R. gracilis* see Ref. 6).

To support the above postulate, the action of

TABLE I

THE PASSIVE ANAEROBIC FLUXES OF K^+ AND H^+ IN THE WILD STRAIN AND IN THE MUTANT M 67

The equation for the calculation of the permeability coefficients is given in the text. All values are means of 13 experiments ± S.E. Unbuffered cell suspensions (50 g fresh wt./l) were incubated at 30°C. The experiments were started by blowing purified nitrogen into a closed cuvette. When used, CCCP was added at 25 μmol/l concentration about 2 min after N_2 exposure. V_0 is the initial velocity of ion flow, expressed in nmol/mg dry wt. per min; P, the permeability coefficient, in min^{-1}.

	$[K^+]_i$ (mmol/l)	N_2		CCCP	
		V_0	P	V_0	P
Wild strain	156 ± 4	1.55 ± 0.2	$-3.48 \cdot 10^{-3}$ $\pm 0.33 \cdot 10^{-3}$	43.6 ± 9	$-1.24 \cdot 10^{-2}$ $\pm 0.24 \cdot 10^{-2}$
M 67	112 ± 4	0.90 ± 0.1	$-2.44 \cdot 10^{-3}$ $\pm 0.32 \cdot 10^{-3}$	18.5 ± 3	$-0.80 \cdot 10^{-2}$ $\pm 0.11 \cdot 10^{-2}$

DCCD on [^{3}H]TPP^+ accumulation as an indicator of the membrane potential and on its depolarization by K^+ and CCCP was studied (Fig. 6). The following should be pointed out: (a) as expected, CCCP was more effective after preincubation of the cells with DCCD, because the reduced permeability for K^+ by DCCD obviously does not allow effective charge compensation by the outflow of K^+. (b) For the same reason, the depolarization by K^+ was less effective after DCCD treatment. (c) The addition of DCCD resulted in an immediate hyperpolarization of the membrane potential, as indicated by the enhanced [^{3}H]TPP^+ accumulation. This last effect cannot be associated simply with altered K^+ permeability. Obviously, an assumption must be made that DCCD reduced also the passive flow of protons. In fact, in the wild strain, the membrane potential showed a distinct pH dependence [8]. Addition of hydrochloric acid to a final pH of 3.5 led to a complete and rapid depolarization of the membrane potential [4]. Indeed, cells preincubated with DCCD displayed distinctly lower sensitivity of the membrane potential to its depolarization by added HCl (Fig. 7). Hence, DCCD reduced the passive flow of both K^+ and H^+. An unspecific binding of TPP^+ following the addition of DCCD was excluded by the even quicker and complete outflow of the previ-

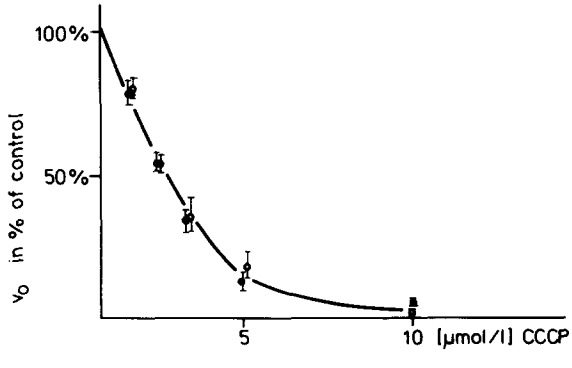

Fig. 3. Relative inhibition by CCCP of D-xylose transport in the wild strain (●) and in the mutant M 67 (○). Initial velocities of transport are expressed as percentage of the respective control without CCCP. The uncoupler was added 1 min prior to D-xylose. The yeast suspensions (50 g fresh wt./l) were incubated in 0.15 mol/l potassium phosphate buffer (pH 4.5). The points given are means of four experiments ± S.E. (vertical bars).

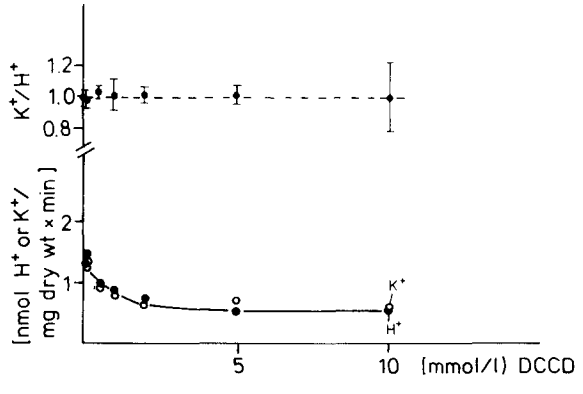

Fig. 4. Inhibition by increasing DCCD concentrations of the initial velocity of anaerobic K^+-H^+ exchange in cell suspensions preincubated 30 min with inhibitor. The increase in K^+ concentration and the decrease in proton concentration in the medium were measured as described in the legend to Fig. 1. The points represent mean values of six experiments. Upper half: the stoicheiometry of K^+-H^+ fluxes, the bars show S.E.

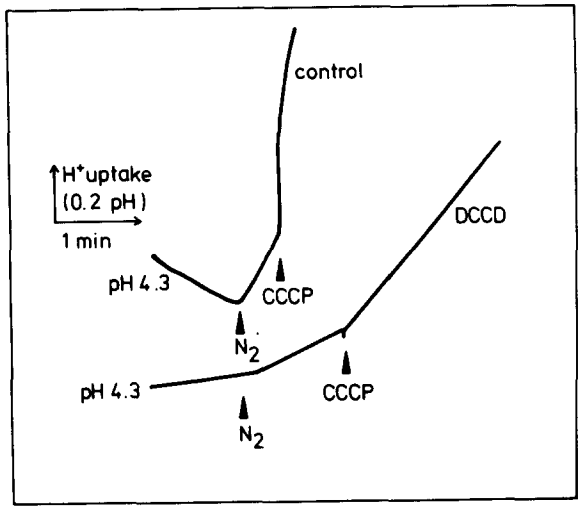

Fig. 5. Inhibition of anaerobic and CCCP- (25 μmol/l) induced K^+-H^+ exchange by 30 min preincubation with 1 mmol DCCD/l. The curves represent one of eight experiments. Similar results were also obtained with other uncouplers: 50 μmol/l 2,4-dinitrophenol, 50 μmol/l 3,3',4',5'-tetrachlorosalicylanilide. Continuous pH recording of the cells suspensions (50 g fresh wt./l) at 30°C.

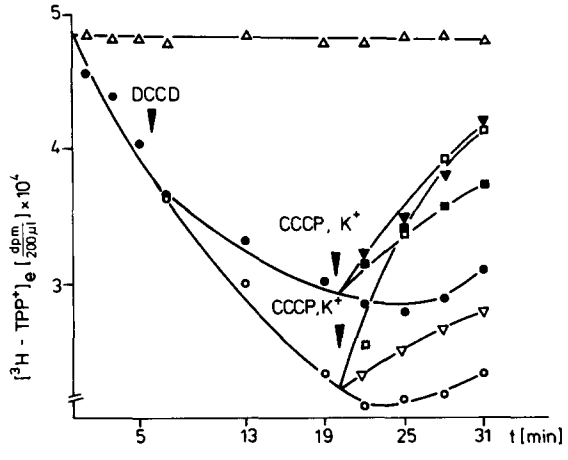

Fig. 6. Hyperpolarization of the membrane potential by DCCD. Effect of DCCD on the depolarization of the membrane potential by K^+ or CCCP. The membrane potential was indicated by [^{3}H]TPP^+ accumulation, measured by the decrease in [^{3}H]radioactivity in the supernatant. Arrows: addition of 1 mmol/l DCCD (○) or ethanol (●) to the control suspension; addition of 5 μmol/l CCCP (□,■) or 50 mmol/l KCl (▽,▼) (each later; second arrows) to the suspensions with and without DCCD. Effect of 1 mmol/l DCCD after 30 min preincubation (△). Closed symbols: control suspension; open symbols: suspensions containing DCCD. The data given represent one of four experiments.

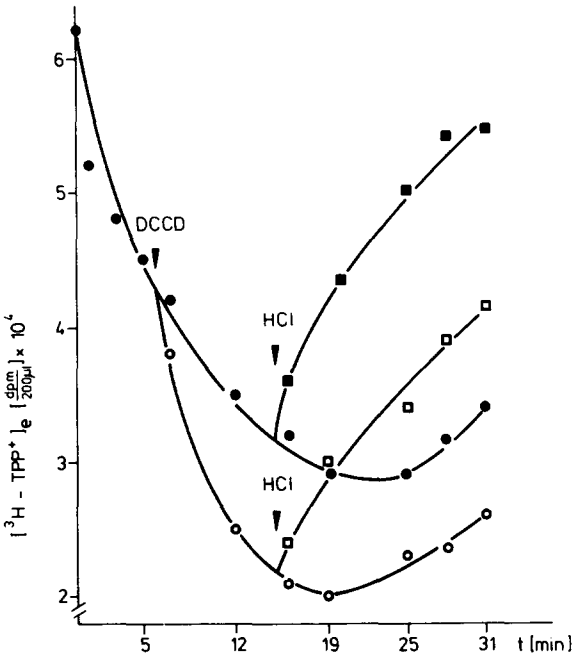

Fig. 7. Depolarization of the membrane potential by H^+ and the effect of DCCD thereon. The times of additions are indicated by arrows: first 1 mmol DCCD/l, then HCl, was added to give a final pH of 4.5. Open symbols: suspensions with DCCD; closed symbols: control suspensions without DCCD. Membrane potential was measured as in Fig. 6.

ously accumulated TPP^+ induced by CCCP (see above).

As already mentioned, the passive K^+-H^+ exchange was induced by inhibiting the membrane energization through anaerobiosis or uncoupling. The polyene antibiotic nystatin has been shown to produce unspecific ion-permeable pores in plasma membranes of eukaryotic cells. Its action in *R. gracilis* was restricted to the plasma membrane [13]. In Table II, the effect of anaerobiosis, CCCP and nystatin on the steady-state K^+ gradient across the plasmalemma is compared. Whereas under both anaerobic and uncoupled conditions considerable K^+ gradients persisted, nystatin permitted complete K^+ equilibration. The residual cellular K^+ content after incubation with nystatin has been ascribed to the mitochondria. The proton gradient was equilibrated both under uncoupled conditions and in the presence of nystatin (cf. Ref. 8).

TABLE II

STEADY-STATE INTRACELLULAR AND EXTRACELLULAR POTASSIUM CONCENTRATIONS AFTER 2 h INCUBATION OF THE WILD-STRAIN CELLS WITH N_2, CCCP AND NYSTATIN

Unbuffered cell suspensions (50 g fresh wt/l), incubated at 30°C. Potassium determinations as described in the Materials and Methods section. The values given are means of four experiments ± S.E.

K^+	Initial concentration (mmol/l)	Addition	Concentrations after 120 min treatment (mmol/l)
External concentration	0.008 ± 0.001	N_2 (anaerobiosis)	0.197 ± 0.008
		25 μmol CCCP/l	0.399 ± 0.003
		10 μmol nystatin/l	1.538 ± 0.024
Internal concentration	168 ± 7	N_2 (anaerobiosis)	165 ± 6
		25 μmol CCCP/l	151 ± 6
		10 μmol nystatin/l	18.3 ± 0.7

Discussion

Mass flows of ions across biological membranes can occur only if there is a compensating flow of other ions allowing for the overall electroneutrality [11]. This has been demonstrated in the present paper for the passive flows of protons and potassium ions. The passive ion flows, both anaerobic and uncoupled, proceeded with a $K^+ : H^+$ stoicheiometry of 1 : 1.

A question of great importance was whether the passive K^+-H^+ exchange occurs through a single electroneutral system in the plasma membrane or through two independent systems coupled electrically. K^+ is the counterion for at least three independent processes: H^+/monosaccharide symport (a), and passive K^+-H^+ exchange under anaerobic conditions (b) or induced by CCCP (c). For system (a), an electrogenic K^+ channel has already been postulated [12] which is electrically coupled to H^+ symport. In the latter processes (b,c) the flows of protons and potassium are also very probably coupled electrically. The difficulty herewith consists in the lack of an independent generation of diffusion potentials across the plasmalemma by the existing concentration gradients of K^+, e.g., under anaerobic conditions [4]. As a matter of fact, the membrane potential was the first parameter dissipated by introducing anaerobic conditions. The plasma membrane is obviously impermeable for electrogenic potassium leak under anaerobic conditions. Only electroneutral K^+-H^+ exchange can occur. An intracellular binding or compartmentation of K^+, for example, in mitochondria, appears to be excluded, since the addition of the polyene antibiotic nystatin, interacting with the plasma membrane only, induced a rapid and extensive outflow of K^+ from the cells (Table II). The most simple and hence most reasonable explanation for this apparent discrepancy is the postulate of an electrically gated K^+ channel, which is self-regulated, closing whenever electrogenic K^+ flux would generate an electrical potential difference.

The rate of passive ion exchange, either anaerobic or induced by CCCP, was obviously determined by the low permeability for protons in both the wild strain and the mutant as compared with that for potassium. This conclusion is based on the fact that CCCP, a specific ionophore for protons, was able to increase the fluxes of both H^+ and K^+. Up to 80 μmol CCCP/l there was no saturation of H^+ flux by a limiting K^+ permeability. The K^+ efflux was always high enough to compensate for the CCCP-induced proton influx.

It has been conjectured already by Huh [6] and Höfer et al. [5], that the plasma membrane of the mutant posesses lower cation permeability compared with the wild strain. The presented experiments proved the assumption to be right (Table I). Even the anaerobic leak of protons into mutant cells stimulated by CCCP and the correspondingly enhanced compensating outflow of K^+ were slower in the mutant. The dissimilarity cannot be brought

about by a difference in the primary action of CCCP upon the respective plasma membrane. This has been tested by comparing the effect of CCCP upon D-xylose transport in the two strains. Under the conditions used, the driving force of H^+ symport consists predominantly of the chemical gradient of H^+; CCCP had equal effect thereupon (Fig. 3). Moreover, CCCP effected equal depolarization of the membrane potential in both the wild strain and the mutant as demonstrated by the almost identical permeability coefficients of TPP^+ outflow induced by CCCP. The lack of the transient alkalinization of unbuffered cell suspensions of the mutant by the onset of monosaccharide uptake can also be explained by the decreased plasmalemma permeability for K^+. Assuming, that both the electrogenic H^+ symport and K^+-H^+ exchange induced by CCCP are electrically compensated by K^+ flux through the same electrogenic channel, the conclusion can be drawn that the mutant is not able to compensate for the charges taken up in H^+ symport by an opposite flow of potassium, but the cells have to pump out H^+ by recycling through the plasmalemma ATPase. Hence, no alkalinization occurs.

The failure of CCCP to abolish the inhibition by DCCD of the anaerobic H^+ leak (Fig. 5) ruled out the plasmalemma-bound H^+-ATPase as a mediator of the passive H^+ movement. The hyperpolarization of the membrane potential confirmed this conclusion (Fig. 6). However, if DCCD acted for a longer period of time, about 30 min, the membrane potential became depolarized [8]. Obviously, DCCD exerts its inhibitory effect in two steps. First, it reduces the cation permeability (both for H^+ and K^+), bringing about the hyperpolarization of the membrane potential by the decrease of passive ion fluxes. Secondly, upon longer action it inhibits the plasma membrane ATPase as demonstrated by the depolarization of the membrane potential (cf. also Ref. 8). Under the latter conditions, the H^+ symport and the acidification of unbuffered cells suspensions were also inhibited [8]. A similar hyperpolarization by DCCD of the membrane potential in *Saccharomyces cerevisiae* was observed by De la Pena et al. [14]. An inhibition of passive proton movement was reported by Mukohata and Kaji [15] in *Halobacterium halobium* and by Casey et al. [16] and Beattie and Villalobo [17] in vesicles containing reconstituted cytochrome *c* oxidase and cytochrome bc_1 complex, respectively. Together with the results presented above, there is increasing evidence that DCCD is able to act on various stages of energy transduction in biological membranes.

In recent literature, the capability of various uncouplers to associate with membrane-bound proteins has been discussed. In *Saccharomyces fragilis* the interaction of CCCP, 3,3′,4′,5′-tetrachlorosalicylanilide, and dinitrophenol with the plasma-membrane sugar translocator has been proposed [18]. A binding of several uncouplers to proteins of energy-transducting membranes and their uncoupling has been compared in Ref. 19. On the basis of the present experiments, we cannot exclude an interaction of the different uncouplers with plasma-membrane proteins in *R. gracilis* (Fig. 5). In this case, one should postulate that both CCCP and DCCD act upon the same protein(s). However, this could not explain the reduced effect of external K^+ and the enhanced effect of CCCP in depolarizing the membrane potential on DCCD-treated cells as compared with untreated cells (Fig. 6).

Höfer et al. [5] reported that the mutant M 67 was defective in ergosterol biosynthesis. In the present paper, evidence is presented that the mutant plasma membrane displays a distinctly lower permeability for cations as compared to the wild strain. This indicates strongly that the sterol content of the plasmalemma plays an important role for the permeability properties of biological membranes. The lower permeability for H^+ and K^+ contributes substantially to the explanation of the reported insensitivity of the membrane potential in the mutant cells over a wide range of H^+ and K^+ concentrations [20].

Acknowledgements

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