AN ENERGY-LINKED PROTON-EXTRUSION ACROSS THE CELL MEMBRANE *RHODOTORULA GRACILIS*

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Received 16 December 1974
Revised version received 23 January 1975

1. Introduction

It has been observed in recent years that the specific translocation of non-electrolytes across the cell membrane of certain microorganisms is associated with a coupled translocation of H^+ [1–3]. Mitchell [4] proposed that the active transport of non-electrolytes can be directly energized by a cell metabolism regulated proton gradient. It has been suggested by West and Mitchell [1] from their studies with *E. coli*, and later by Slayman and Slayman [5] with *Neurospora crassa*, that there are two systems operating in the microbial cell membranes; the one, actively pumping out H^+, and the other, using the built up electrochemical gradient of H^+ for uphill uptake of substrate molecules in symport with H^+.

In bacteria (cf. e.g. [6]), the system transporting out H^+ might be either Mg^{2+}-dependent membrane-bound ATPase or a part of the respiratory chain since, according to the chemiosmotic theory of Mitchell [7], the energy of oxidative phosphorylation is primarily conserved in the form of electrochemical gradient of H^+ across the membrane. On the other hand, eukaryotic cell membrane must have a specific system for proton-extrusion because in their case the system of oxidative phosphorylation is localized in mitochondria.

It is the purpose of this communication to report experiments showing the existence of an energy-linked proton-extrusion assembly in the cell membrane of *Rhodotorula gracilis*. This obligatory aerobic yeast has been shown to accumulate monosaccharides inside the cells against a considerable concentration gradient strictly coupled to the available metabolic energy [8]. The data obtained indicate further that the sugar transport in *Rh. gracilis* is linked to H^+-movement across the cell membrane by a symport mechanism.

2. Materials and methods

The collection strain used, *Rhodotorula gracilis* (taxonomically *Rhodosporidium toruloides* Banno, mating type a), deposited at ATCC 26 194 and CBS 6681, was cultivated as Kotyk and Höfer [9] described. Cells were harvested after 24 hr, washed thrice with distilled water and aerated in water suspension overnight. The aerated suspension was centrifuged and a fresh 5% (w.wt./v) water suspension was used for the experiments which were done at room temperature. The pH of the suspension was measured by the Radiometer pH-meter 26, Copenhagen. The pH changes were standardized by the addition of a known amount of acid or alkali to the cell suspension. For anaerobic conditions, the yeast suspension was supplied with purified N_2 (Messer Griesheim, Germany) continuously.

In the experiments no substrate was added to the cell suspension. The cells used their endogenous material as a source of energy. *Rh. gracilis* stores great amount of lipids during growth on glucose and

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its endogenous Q_{O_2}, after overnight aeration at room temperature, amounted to −15. 1±1.5 [10].

Since unbuffered aqueous yeast suspensions were used for the experiments, parallel controls with dead-cell suspensions were run to assure that the pH changes observed were not due to the unspecific responses of the glass electrode to the sudden changes of the ionic strength of the suspension. For this, the cell suspension was treated at 80°C about 3–5 min. The suspension was then washed twice with distilled water and finally suspended in water (5% w.wt./v). The pH of such suspensions, exhibiting no metabolic activity (Q_{O_2} =O), was always around 6.5. At the beginning of each control run it was brought to about 4.2 (the pH of an active yeast suspension) by the addition of HCl. In all runs, the sudden drop of pH after HCl addition was followed by a gradual pH increase of about 0.2 unit, presumably, due to absorption of protons by cell wall.

In experiments where the amount of CO_2 produced was to be estimated, the rate of O_2-consumption simultaneous to the pH-change was determined by O_2-electrode (Beckman Instruments, GmbH, München). Parallel experiments with conventional manometry confirmed that the respiratory quotient, RQ, remained unchanged during the experiment.

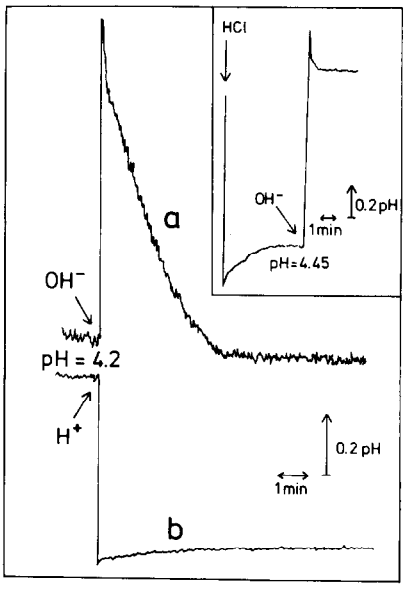

Fig.1. a) Recovery of extracellular pH by suspension of *Rh. gracilis* in water following the change effected by addition of OH^- (either as KOH or NaOH). b) Insignificant recovery of pH when the change is effected by addition of H^+ (as HCl). Inset: Control run with dead-cell suspension (see Materials and methods).

3. Results

The pH of unbuffered water suspension of *Rh. gracilis*, independent of the batch used, was between 4.2 and 4.5. This was more acidic than the intracellular pH of about 6.1 as determined by Kotyk and Höfer [8]. The pH of such suspensions, even after several washings, did not appreciably change. A sudden alkalinization of the yeast suspension, about one pH, unit, effected by the addition of OH^-(both as NaOH and KOH) was compensated rapidly and the original pH was restored in about 3 min (fig.1a). Unlike the addition of an alkali, the pH of yeast suspension was not regained after acidification (fig.1b). The above observation indicate that the cell membrane of *Rh. gracilis* is equipped with an assembly that extrudes protons unidirectionally and thus participates in maintaining the given pH-gradient across it.

The energy requirement for the function of the assembly was demonstrated in experiments with

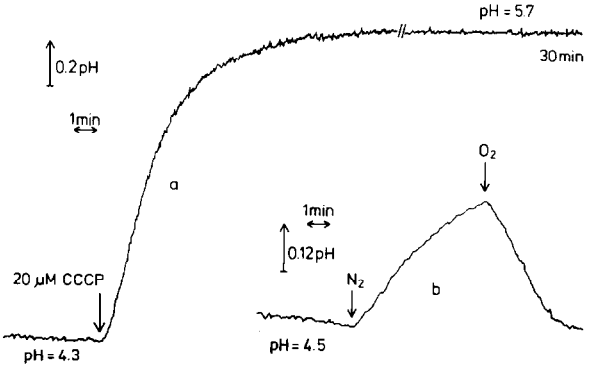

Fig.2. Evidence of the energy requirement of H^+-extruding assembly as a result of pH increase of the unbuffered suspensions of *Rh. gracilis* in water after (a) addition of 2×10^{-5} M carbonylcyanide *m*-chlorophenylhydrazone (CCCP), b) introduction of anaerobic conditions; recovery of pH on change to aerobic conditions. The same results as in a) were obtained also with 5×10^{-5} M dinitrophenol or 40×10^{-5} M NaN_3.

uncouplers or under anaerobic conditions. The addition of an uncoupler to a yeast suspension in water resulted in a rapid increase of the external pH to a value approaching the intracellular pH (fig.2a), without showing signs of its decrease (as recorded up to 30 min) though at the same time the rate of CO_2 production was doubled. The transfer of yeast suspension to anaerobic conditions also brought about a gradual increase of the external pH of the suspension. Furthermore, return of aerobic conditions was followed by a fast recovery of the pH (fig.2b). The above experiments indicate that the removal of metabolic energy supply and/or the enhancement of the passive membrane permeability for H^+ (uncouplers' effect) bring about suspension of the function of this assembly, with a consequent equilibration of intra- and extracellular pH. The recovery of external pH on resuming aerobic conditions further supports this conclusion.

To test whether the extrusion of H^+ by the yeast cells is linked to the function of a membrane-bound ATPase, the effect of dicyclohexylcarbodiimide, DCCD, (see e.g. [6]) has been tested. The effect of DCCD is time dependent, probably due to its low rate of penetration through the cell membrane. DCCD was added to *Rhodotorula* suspensions in a concentration of 0.7 mM and was allowed to react for various intervals before the ability of the yeast to recover the original pH after its alkalinization by the added alkali (cf. fig.1a) was measured. Simultaneously also the cell respiration was recorded. Fig.3 demonstrates that DCCD treatment gradually reduced the ability of yeast cells to recover the pH of the suspension. At the same time the cell respiration was slightly stimulated by DCCD. The sugar uptake tested in the presence of DCCD showed that after 60 min preincubation of the suspension with the inhibitor the cells were capable of taking up D-xylose only to the diffusion equilibrium (table 1).

Since the alkalinization of the suspensions was always combined with the addition of either Na^+ or K^+, the possible requirement of a particular cation to support the process of acidification was tested. Fig.4 shows that in the presence of Li^+ the H^+-extrusion was considerably slowed down. The usual velocity of acidification (compare fig.1a) was restored by subse-

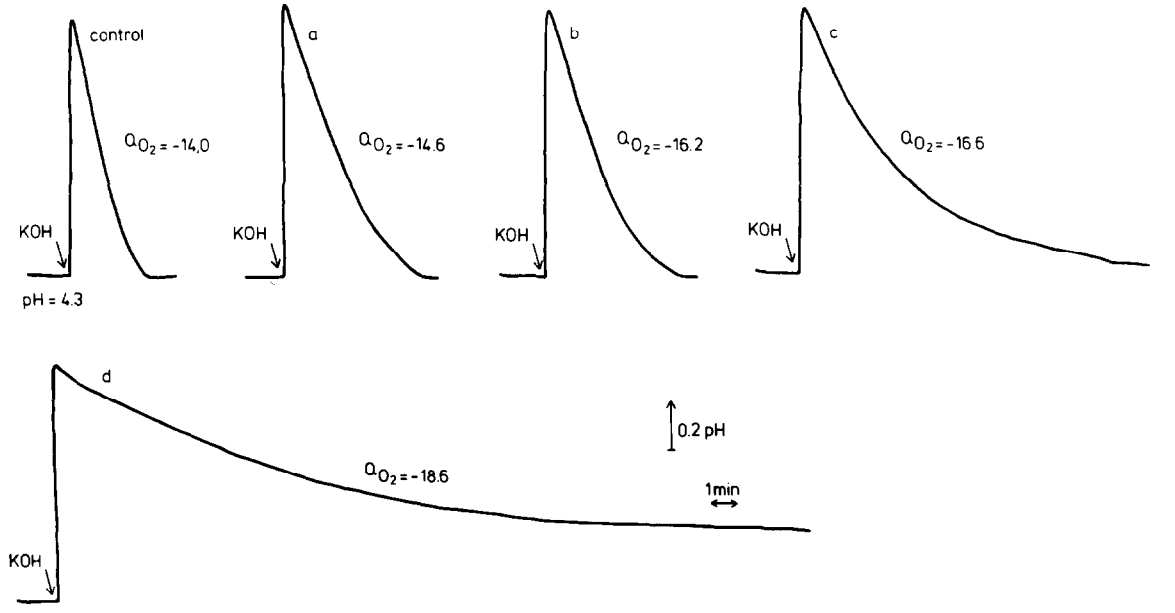

Fig.3. The rate of pH recovery by yeast suspension after its alkalinization by KOH in a control run and after treatment of yeast cells with 0.7 mM DCCD for various intervals: a) 15 min, b) 30 min, c) 45 min and d) 60 min. The given Q_{O_2} expresses the corresponding rate of cell respiration during each run as measured continuously with O_2-electrode.

Table 1

Conc. of D-xylose added	Intracellular conc. of D-xylose	
	with DCCD	without DCCD
13.0 mM	12.7 mM	103.7 mM

Rh. gracilis cells were treated with 7×10^{-4} M DCCD for 60 min. D-Xylose accumulation, 30 min after its addition, was measured both in treated and control cells. Intracellular water volume was taken from [9].

quent addition of K^+ in the form of KCl. Thus, the proper function of the H^+-extruding membrane assembly requires either the presence of a suitable counter cation (or a simultaneous efflux of an anion, probably of metabolic origin, in the case of pH-adjustment after washing the suspensions).

As with other microorganisms [1–3], a H^+-sugar symport in *Rh. gracilis* was indicated by a distinct alkalinization of the unbuffered yeast suspensions following sugar addition, as shown in fig.5. Both, the metabolizable (D-glucose) and non-metabolizable (D-xylose) sugars affected transiently the steady state distribution of H^+ across the cell membrane. The addition of D-ribose, which was reported not to be transported actively in *Rh. gracilis* [11], does not cause any measurable change in the pH of the yeast suspension.

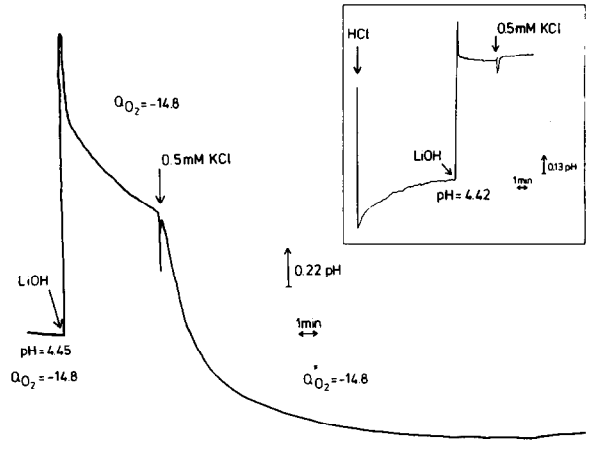

Fig.4. Acceleration by K^+ of the rate of acidification of yeast suspension compensating for the pH-change caused by LiOH. The simultaneously measured cell respiration is given as Q_{O_2} for each stage of the experiment. Inset: Control run with dead-cell suspension (see Materials and methods).

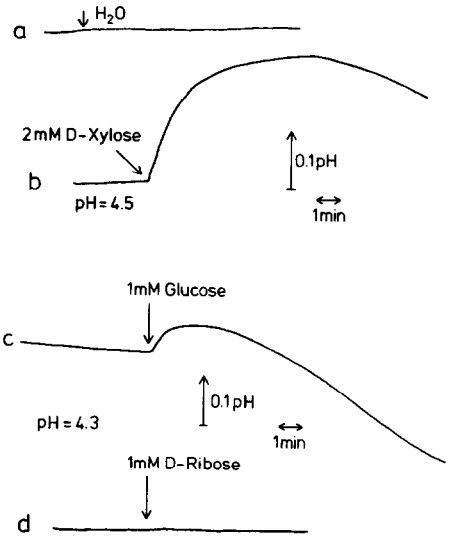

Fig.5. Transient alkalinization of *Rh. gracilis* suspension in water due to H^+-sugar symport (b,c). Addition of water a) or a non-transportable sugar, D-ribose, d) did not effect any pH change.

4. Discussion

The above results give experimental support for the postulate that an active proton-extrusion assembly operates in the cell membrane of eukaryotic microorganisms [2,3,5]. Though it is difficult, at this stage, to specify the exact nature of this assembly as to which ion species is transported primarily and how the transport is energized, nevertheless, it has been shown that eventually H^+ are translocated to the outside of the cells. The transport requires metabolic energy. The suspension of the energy supply (under anaerobic conditions or in the presence of uncouplers) paralyzes the functioning of this assembly. In addition, uncouplers increase the passive membrane permeability for H^+. This effect is seen in the higher rate of increase of extracellular pH after the addition of an uncoupler, than after a change to anaerobic conditions (cf. figs.2a

and b). The unidirectional working of the assembly is revealed by the failure of the micro-organism to compensate for the increase of extracellular H^+-concentration (cf. fig.1b).

The possibility that the adjustment of the pH of *Rh. gracilis* suspensions could be caused by retention of metabolic CO_2 is excluded by results of figs.3 and 4. DCCD does not influence the cell respiration, whereas H^+-extrusion is markedly inhibited. Similarly, CO_2 was produced with constant velocity even during abrupt acceleration of H^+ extrusion due to addition of K^+ to yeast suspension treated with LiOH.

The experimental evidence obtained with the yeast *Rh. gracilis* is consistent with the concept of two transport systems in eukaryotic microbial cell membranes for the uphill uptake of non-electrolytes, suggested by West and Mitchell [1], Eddy et al. [2,12], and Slayman and Slayman [5]. The first system, primarily requiring supply of metabolic energy, creates an electrochemical gradient of H^+ across the plasmalemma. The second system may use the existing electrochemical gradient of H^+ to effect substrate transport and accumulation inside the cells by means of an H^+-substrate symport.

Acknowledgements

The work was supported by the Deutsche Forschungsgemeinschaft, Grant No. Ho 555/4. One of us (P.C.M.) is recipient of the DAAD fellowship.

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