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Two systems mediate rubidium uptake in *Neurospora crassa*: one exhibits the dual-uptake isotherm

Alonso Rodríguez-Navarro * and José Ramos

Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, E-14071 Córdoba (Spain)

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(1) The temperature- and pH-dependence of Rb^+ uptake in low-K^+ and normal-K^+ cells were studied to accumulate information on the systems involved. Arrhenius plots of V_{max} values in the two phases of low K^+ cells were identical, and different from the Arrhenius plot of normal-K^+ cells' V_{max}. The pH-dependences of Rb^+ uptake in the two phases of low-K^+ cells and in normal-K^+ cells were all different. (2) The Rb^+ uptake in low-K^+ cells was not affected by a rapid exchange of external medium, ruling out the idea of the dual-uptake isotherm being an artifact due to unstirred layers. (3) Metabolic blockage induced net K^+ loss in normal-K^+ cells but not in low-K^+ cells. That loss had the characteristics of a K^+ diffusion. It is concluded that in low-K^+ cells, an Rb^+-H^+ cotransport occurs at low Rb^+ and an Rb^+-Rb^+ cotransport at high Rb^+. In normal-K^+ cells, Rb^+ uptake may be mediated by the system which mediates the K^+ loss induced by metabolic blockage.

Introduction

Many secondary transports in cell-walled eukaryotes are coupled to the transmembrane difference of either the electrical potential or the electrochemical potential of H^+ [1]. In some circumstances some secondary transports may be coupled to the driving force of Na^+, as in the case of phosphate in *Saccharomyces cerevisiae* [2]. For all these secondary transports the primary pump is an H^+-ATPase different in nature to the F_0F_1-ATPase of prokaryotes [3–5].

K^+ is the most abundant cation in living cells and its transport has been the subject of extensive studies in many organisms. Among cell-walled eukaryotes, the transport of K^+ has been the subject of extensive work in plants [6] and also in fungi [1,7], but a clear picture of the mechanism involved in K^+ uptake either in plants or fungi has not yet emerged. The early observation in yeast of the K^+ uptake being coupled to the H^+ efflux [8,9] was later also confirmed in plants (see Refs. 4, 6 for references). In plants, it was also found that the kinetics of a K^+stimulated ATPase was similar to that of the K^+ influx [10,11]. These observations led to the hypothesis, by analogy with the Na^+-pump of animal cells, that the K^+-H^+ exchange was the function of a K^+-stimulated ATPase [12]. This hypothesis was further supported by experiments with purified yeast ATPase [13]. The hypothesis of the K^+-ATPase, however, has not yet been accepted by many authors, in part because the K^+ stimulation of the plasma membrane ATPase seems to be a non-specific salt

* To whom correspondence should be addressed.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

effect [14] and in part because most of the work with the reconstituted enzyme of plants and fungi accommodates the notion that the ATPase is an H^+-pump [5]. In the absence of a primary K^+-pump, the driving force of H^+ could explain 'active' K^+ uptake (i.e., uphill to the total electrochemical potential of K^+) [15], and, in fact, a H^+-K^+ symport was identified in K^+-starved cells of *Neurospora crassa* [16].

Another controversial discussion on K^+ transport in plants and plant cells arose from the complexity of the concentration-dependence of K^+ and Rb^+ uptake, which presents a biphasic pattern [17]. To explain these complex kinetics, several hypotheses have been advanced: a dual system of uptake in the plasma membrane, one with high and the other with low affinity [17]; two systems in series, plasmalemma-tonoplast [18]; a system undergoing conformational changes that give rise to multiphasic kinetics [19]; a physical artifact due to the slow diffusion of K^+, which affects the actual concentration of K^+ on the external side of the plasma membrane [20]. All these hypotheses can explain the observed uptake kinetics, but their experimental support is slight. The dual system had, however, a teleological support, and energetic considerations led to the proposition that the system with high affinity is the K^+-ATPase and the system with the low affinity is a K^+ uniporter [21].

In fungi, the kinetics of K^+ uptake have been studied by an approach different to that used in plants and, therefore, the current available information on K^+ uptake in fungi cannot be easily compared to that in plants. K^+ and Rb^+ uptake in fungi have been explained by a multisite carrier which gives rise to complex concentration dependences at different pH values [22–24]. Recent works have shown that both *S.* cerevisiae [25] and *N. crassa* [26] have two systems (high- and low-affinity systems) for K^+ and Rb^+ uptake, or one system with two modes of operation. These systems do not operate simultaneously, but alternatively: the high-affinity in low-K^+ cells and the low-affinity in normal-K^+ cells. In *S. cerevisiae* the two systems (or modes of operation) have been distinguished on the basis of their temperature and pH dependences [25], and a mutant lacking the high-affinity Rb^+ uptake has also been described [27].

Unlike *S. cerevisiae*, in low-K^+ cells of *N. crassa* the concentration dependence of Rb^+ uptake presents a biphasic pattern similar to that found in plants. This pattern can be explained by the existence of two processes, both following Michaelis-Menten kinetics, with K_m values three orders of magnitude different, the actual values depending on the K^+ content of the cells. In contrast, normal-K^+ cells take up Rb^+ exhibiting standard Michaelis-Menten kinetics whose K_m depends on the external K^+ in which the cells are growing [26], but the relationship between this process and that or those taking place in low-K^+ cells has not been established.

In the present paper we propose that normal-K^+ and low-K^+ cells of *N. crassa* have different Rb^+ uptake systems, and that in low-K^+ cells the two phases of Rb^+ uptake are a consequence of the uptake mechanism.

Materials and Methods

Preparation of cells

Wild-type RL21a of *N. crassa* was used throughout this work. The methods of handling the cells and growth media were described previously [26,28], except that growth temperature was 28°C. To prepare K^+-starved cells, $3 \cdot 10^6$ conidia were inoculated per ml into the ammonium-phosphate medium [26] with 0.25 mM K^+ and the cells were grown until the external K^+ dropped to 10–15 μM, at this moment the K^+ content of the cells was 250–290 nmol $\cdot$ mg^{-1}. For normal-K^+ cells, $3 \cdot 10^6$ conidia $\cdot$ ml^{-1} were grown for 10 h in the ammonium-phosphate medium with 37 mM K^+.

Results were usually referred to dry weight. To refer the results to cell water, the factor of 2.5 ml water per g dry weight was used [28].

Rb^+ uptake

Cells removed by filtration from the growth medium were suspended in the appropriate buffer and kept in agitation for several minutes (see below) before adding Rb^+. K^+-starved cells were suspended in K^+-free buffers and normal-K^+ cells in 0.5 mM K^+ buffers. Before starting the experiment or at the end of the experiment, one sample with 12–14 mg (dry weight) of cells was removed

from the buffer, washed three times with high-quality deionized water, dried, weighed and analyzed for K^+ content. These results were used to calculate the cell density in each experiment (approx. 0.5 mg · ml^{-1}) and the K^+ content of the cells.

Unless otherwise stated, experiments were performed at 28°C and the cells were preincubated in buffer 5 min before Rb^+ addition. At other temperatures (cf. Fig. 1), the cells were preincubated 3 min at 28°C and an additional period in a bath at the chosen temperature. In our conditions, 250 ml flasks with 40 ml of medium and vigorous shaking, 5 min of preincubation equilibrated the temperature. Once the cells had been preincubated in the buffer and, when required, the temperature had been equilibrated, Rb^+ was added and four or five samples (usually alternating 8 and 4 ml) were removed in 3–5 min (shorter times were used for higher Rb^+ additions, in which the rates of uptake were higher). The samples were filtered and washed with deionized water and the cells were transferred to a new filter and washed again (the transfer to a new filter was omitted for Rb^+ concentrations lower than 0.5 mM). The filters with the cells were acid-extracted and analyzed by atomic absorption spectrophotometry. The results of the cell analyses were referred to cell density in each experiment, and plotted versus time to determine the initial rates of uptake.

K^+ loss

To determine the K^+ loss induced by the addition of different drugs to normal-K^+ cells, the cells were suspended in buffer as for Rb^+ uptake, except that the K^+ content of the buffer was 0.1, 1.0 or 10 mM. After 5 min of preincubation the drug (or drugs) was added, samples were removed (usually 6–10 in 4 min, alternating 1.5 and 3.0 ml), filtered, washed and extracted as before.

Cell-column technique

This was a small modification of the technique described for yeast [29], and it was used to test Rb^+ uptake under rapid flow of buffer, or K^+ efflux at very low external K^+ concentrations. In the first case, the experiments were designed to increase the exchange of the medium in the unstirred layers forced by the rapid flow of fresh medium (in these experiments the flow per second was up to 5-fold the extracellular volume of the column, see below). In the second case, the rapid removal of the external medium allows the maintenance of the cells exposed at a very low external K^+, even in the putative case of a significant K^+ loss.

To prepare the cell-column, two Millipore filters (Cat. No. AAWP02500) were placed together in a filter holder of 25 mm of diameter. A suspension of 10 mg (dry weight) of cells previously aerated in buffer (5 min) was introduced into the filter and the vacuum was applied. The cells set on the filters and more buffer was added before the cells became dry. Once all the cells had set, the test solution was added continuously and aeration was kept in the solution by bubbling air through a Pasteur pipette. At the required time, addition of more solution was discontinued and the cells were washed and extracted as described before for flask experiments.

By using two filters, the flow of medium was limited by the filters and not by the cells (addition of a much higher amount of cells than that used in the experiments did not decrease the flow established by the filters alone), situating the air/liquid interphase at the filter level. Under these conditions, a cell-column with 10 mg (dry weight) of cells had 25 mg of intracellular water and 90 mg of extracellular water.

Cellular pH

pH was determined by the [^{14}C]propionic acid distribution as in [30]. The experiments were performed at 53 μM propionic-propionate (7 kBq · $nmol^{-1}$) with a cell density of approx. 2.5 mg (dry wt.) · ml^{-1}.

Buffers

The standard buffer was 10 mM Mes/0.1 mM $MgCl_2$/1% glucose brought to pH 5.8 with $Ca(OH)_2$. The same acid was used for pH 6.0, 6.2, 6.3 and 6.5; Hepes for pH 7.0, 7.5 and 7.8; glycylglycine for pH 8.0 and 8.2, and tartaric acid for pH 4.5. These compounds were always used at 10 mM and brought to pH with $Ca(OH)_2$, and the buffers were completed with Mg^{2+} and glucose as in the standard one. Under these conditions, the concentration of Ca^{2+} varied from 1.5 to 3.2 mM, but preliminary experiments showed that this variation did not affect the results.

Results

Temperature-dependence of Rb^+ uptake

By analogy with higher plants, the biphasic kinetics of Rb^+ uptake in K^+-starved cells of *N. crassa* could be accounted for by two systems working in parallel [17,26]. However, one system could also give rise to biphasic kinetics, either due to the slow diffusion of the cation through the outer layers [20] or by the random binding of Rb^+ and H^+ for their cotransport [31]. In addition to the two alternatives in K^+-starved cells (one system versus two systems), the system mediating Rb^+ uptake in normal-K^+ cells could be different from that or those present in K^+-starved cells. Thus, according to current information, the number of Rb^+ uptake systems in *N. crassa* can be any from one to three.

To investigate the number of Rb^+ uptake systems, the temperature-dependence of Rb^+ uptake proved to be a good approach, as previously shown in yeast [25]. Fig. 1 shows the plot of the initial rate of Rb^+ uptake versus $1/T$ for 0.5 mM Rb^+ (saturation of first phase) and 50 mM Rb^+ (near saturation of the second phase) in K^+-starved cells (see Fig. 2), and 50 mM Rb^+ in normal-K^+ cells. The data for the plot of uptake in the second phase of K^+-starved cells were initially obtained at 150 mM Rb^+, in order to plot the V_{max} unequivocally (the K_m of the second phase in K^+-starved cells is 12.5 mM [26]). However, over 50 mM RbCl, the cells showed significant signs of osmotic shock and the results of repetitions varied too much. In normal-K^+ cells, even at 50 mM Rb^+ we found significant variations in the repetitions of the analyses.

In Fig. 1, the plot of normal-K^+ cells was linear between 15°C and 50°C, whereas the two plots of K^+-starved cells showed breakpoints at approx. 26°C, with decreasing rates above that temperature, and another breakpoint between 45°C and 50°C. The great differences between the plots of normal-K^+ cells and K^+-starved cells strongly suggest two different systems in these two kinds of cells. On the other hand, the many coincidences in the plots in the two phases of K^+-starved cells (breakpoints and positive and negative slopes of the straight lines) can be reasonably explained only if the same transport system mediates the

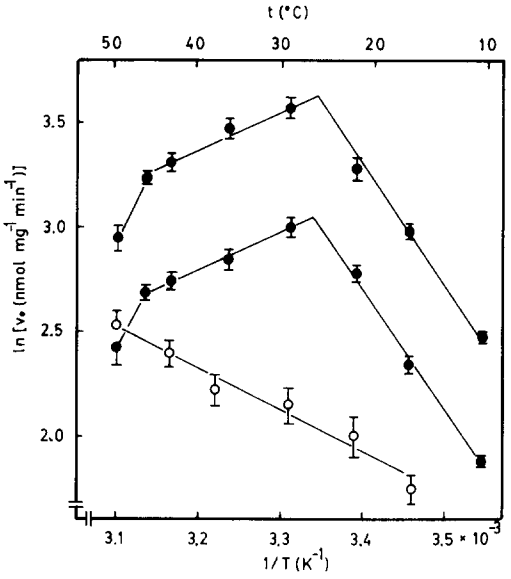

Fig. 1. Arrhenius plot of the initial rates of Rb^+ uptake. ●, K^+-starved cells assayed at 50 mM Rb^+ (upper plot) and 0.5 mM Rb^+; ○, normal-K^+ cells assayed at 50 mM Rb^+. Means of four independent experiments; bars show S.E. Assays under standard conditions at pH 5.8 (see text).

uptake in the two phases. In any case, the results rule out the possibility that uptake at 50 mM Rb^+ in K^+-starved cells is mediated, in parallel, partially by the system in normal-K^+ cells (linear plot) and partially by the system mediating uptake at 0.5 mM Rb^+ in K^+-starved cells. The temperature-dependence of such a composite rate would be completely different from that found here. It is worth mentioning that the plots in Fig. 1 of K^+-starved cells might not be Arrhenius plots in the strict sense [32], because the mechanism and kinetics of the uptake in these cells is not known (see Discussion section) and the plotted rate might not be proportional to a constant. This observation, however, does not affect the previous conclusions, because the breakpoints in the plots of Fig. 1 can be better explained by changes in protein structure than by variations of constants with temperature. In fact, in *S. cerevisiae* it has been shown that the breakpoint in the Arrhenius plot occurs plotting both the V_{max} and the rate at a concentration approximate to the K_m [25].

pH dependence of Rb^+ uptake

In K^+-starved cells, K^+ uptake at low K^+ (up

to 0.2 mM) is a K^+-H^+ cotransport sensitive to pH (Ref. 16 and unpublished results of the same authors). Since a different mechanism may be involved in normal-K^+ cells, it was interesting to compare the pH dependence of Rb^+ uptake on the two phases of K^+-starved cells and on normal-K^+ cells. Decreasing the pH below the standard 5.8, the response was similar in all cases: very slight inhibition up to pH 5.5 and then an increasing inhibition (not shown, but see pH 5.8 and pH 4.5 in Fig. 2 and 3). Increasing the pH from 6.0 to 8.2 had interesting differential effects on the three kinds of uptake process (uptake at pH 6.0 was much the same as at pH 5.8 in the three cases; Figs. 2 and 3 show pH 5.8 instead of pH 6.0 because the 5.8 pH was the standard for all the other experiments). In the first phase of K^+-starved cells and in normal-K^+ cells, the increase in pH from 6.0 to 7.5 decreased uptake, increasing K_m and decreasing V_{max} (Fig. 2, 3 and 4). However, increasing pH from 7.5 to 8.2 decreased the V_{max} abruptly in K^+-starved cells, while it abruptly increased the V_{max} in normal-K^+ cells. Between pH 6.0 and 7.5, the plot of the V_{max} versus $[H^+]_o$ in K^+-starved cells gave a straight line. In contrast to these results, Rb^+ uptake in the second phase of K^+-starved cells was stimulated by the increase of pH_o. In this case we did not try to distinguish between effects on K_m and V_{max} because we cannot assume Michaelis-Menten kinetics (in Fig. 2, the data between 1 and 50 mM at pH 6.0, 6.5 and 7.5 could be fitted to a Michaelis-Menten equation but the data at pH 8.2 deviated very much from a rectangular hyperbola). Unfortunately, the V_{max} could not be obtained by using saturating concentrations of substrate because above 50 mM RbCl the cells were affected (see above). However, a qualitative inspection of the concentration-dependence plots (Fig. 2) revealed the important point that the total V_{max} of Rb^+ uptake in K^+-starved cells was almost independent of pH_o in the interval 5.8 to 8.2. As a consequence, the V_{max} in the second phase increased with the increase of pH_o, compensating for the decrease of the V_{max} in the first phase.

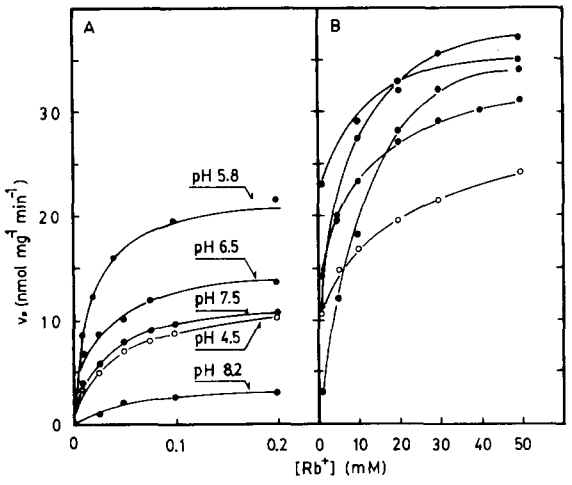

Fig. 2. Concentration dependence of the initial rates of Rb^+ uptake in K^+-starved cells at several pH_o values. (A) From 0.01 to 0.20 mM Rb^+ (first phase); (B) from 1 to 50 mM Rb^+ (second phase). Observe that at every pH the rate in the first point in (B) is approximately the same as that in the last point in (A). Curves drawn in A are Michaelis curves fitted by least squares, with the following parameters (in mM and nmol · $mg^{-1} \cdot min^{-1}$); pH 4.5, $K_m = 0.033$, $V_{max} = 12$; pH 5.8, $K_m = 0.014$, $V_{max} = 22$; pH 6.5, $K_m = 0.020$, $V_{max} = 15$; pH 7.5, $K_m = 0.027$, $V_{max} = 12$; pH 8.2, $K_m = 0.033$, $V_{max} = 3.5$. The K^+ present in the experiments was approx. 4 μM; the loss of K^+ from the cells was insignificant.

The dual isotherm is not a consequence of unstirred layers

In higher plants it has been proposed that the dual isotherm of K^+ uptake is a physical artifact due to the slow diffusion of K^+ through the cell wall and its boundary layer [20]. By using the cell-column technique, we performed Rb^+ uptake experiments under a rapid flow of medium (0.10 $ml \cdot cm^{-2} \cdot s^{-1}$ with 2 mg dry wt. per cm^2, equivalent to a renovation rate of 5.5 volumes per s), conditions under which most of the boundary layer must be removed and the diffusion in the cell wall increased. In these experiments, the rates of Rb^+ uptake at 0.01, 0.06, 0.2, 1.0, 5.0, 10 and 20 mM Rb^+ amounted to the same as in shaking-flask experiments.

K^+ efflux induced by metabolic blockage

In yeast it has been shown that ATP depletion or treatment with uncouplers produces loss of K^+ [33,34,35], in a process probably similar to that taking place during the uptake of H^+-cotransported substrates [34]. Those results have been taken as the support for the existence of a K^+

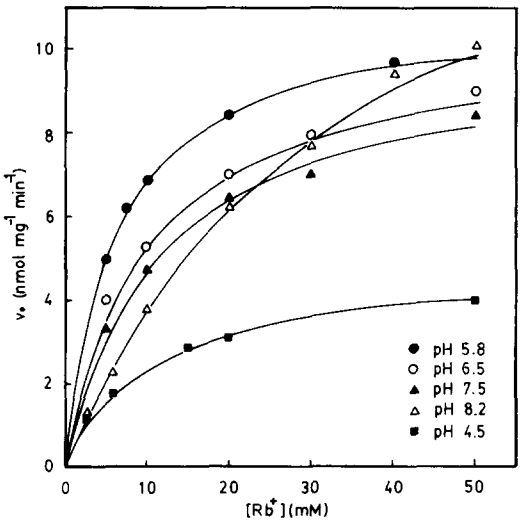

Fig. 3. Concentration dependence of the initial rate of Rb^+ uptake in normal-K^+ cells in 0.5 mM K^+, at several pH_o values. Data-points are of one typical experiment, but drawn Michaelis curves were fitted by least-square to the data-points of three or four experiments. Parameters in mM and nmol·mg^{-1}·min^{-1}: pH 4.5, $K_m = 11$, $V_{max} = 5$; pH 5.8, $K_m = 6$, $V_{max} = 11$; pH 6.5, $K_m = 10$, $V_{max} = 10.5$; pH 7.5, $K_m = 11$, $V_{max} = 10$; pH 8.2, $K_m = 33$, $V_{max} = 16.5$.

uniporter in yeast. A similar approach in *N. crassa* has the advantage that the membrane potentials are known and can be compared to the transport

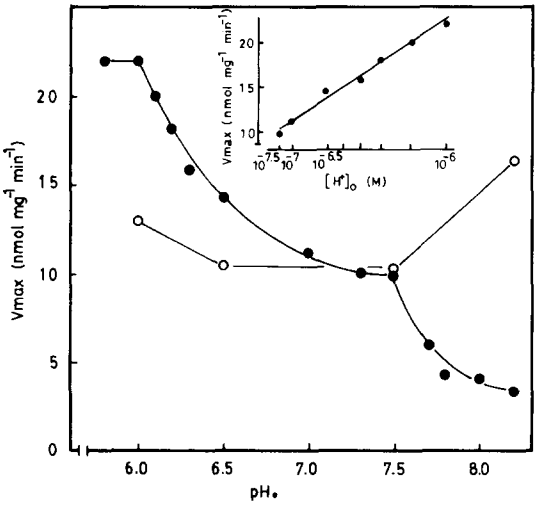

Fig. 4. pH-dependence of the maximum rate of Rb^+ uptake in the first phase of K^+-starved cells, and in normal-K^+ cells. ●, data for K^+-starved cells were taken as the rate at 0.5 mM Rb^+; ○, data for normal-K^+ cells were taken from Fig. 3. Inset, plot of the values in K^+-starved cells versus $[H^+]_o$.

reversal potentials of K^+. As previously shown in yeast [35,36], normal-K^+ cells of *N. crassa* treated with cyanide or azide lost a certain amount of K^+ and then the loss stopped (Fig. 5). The rate and extension of the loss was independent of pH_o but dependent on $[K^+]_o$. With cyanide, K^+ loss was very small at 1 mM $[K^+]_o$ and was inhibited at higher concentrations. With azide the loss at 1 mM K^+ did not differ from that at 0.1 mM, but was inhibited at 10 mM K^+. Addition of CCCP together with cyanide increased the loss greatly, well above the loss observed with azide. These results suggest that a protonophore is required for rapid loss and that azide is apparently not a good protonophore in *N. crassa*, as previously suggested [37], although in yeast the loss with azide is similar to that with CCCP [35].

To consider the mechanism involved in the K^+ loss, the membrane potential in the presence of

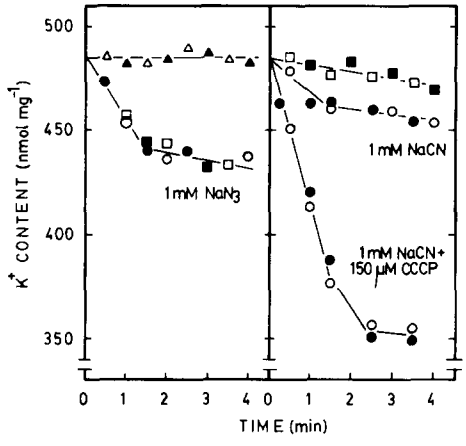

Fig. 5. Time-course of the K^+ loss from normal-K^+ cells following treatment with azide, cyanide or cyanide plus CCCP. Normal-K^+ cells were suspended at pH 5.8 (open symbols) or 7.0 (closed symbols) in the presence of 0.1 (○, ●), 1 (□, ■) and 10 mM K^+ (△, ▲); after 5 min of preincubation the drugs were added (time zero), and samples taken every 30 s. The figure presents data-points from typical experiments, from which some data-points have been omitted for clarity. At 1 mM K^+ with cyanide, both pH levels, in some experiments the cells did not lose K^+, but in other, as those shown, the loss was significant. At 10 mM K^+ with cyanide, both pH levels, there was not loss and the data-points are not shown for clarity of the figure. The results of the K^+ content of the cells at time zero varied from 470 to 490 K^+ nmol·mg^{-1}, but all the results were standardized by fixing the initial content to the mean of the K^+ content (484 nmol·mg^{-1}).

azide and cyanide (−15 mV [38] and −100 mV [39] respectively) must be compared to the transport reversal potential of the putative system at the given conditions of $[K^+]_o/[K^+]_i$ and $[H^+]_o/[H^+]_i$. A net loss will take place only if the transport reversal potential is more negative than the membrane potential. Table I gives the values of the transport reversal potentials for K^+ diffusion (E_K) and H^+-K^+ cotransport (E_{KH}) in cyanide treated cells (after cyanide treatment the pH_i values, 6.36 ± 0.06 in normal-K^+ cells and 5.8 ± 0.11 in K^+-starved cells, were not significantly different at pH 7.0 and 5.8, although this was not the case before treatment), the K^+ loss observed in normal-K^+ cells can be explained by a K^+ diffusion but not for the backward reaction of a K^+-H^+ cotransport.

In K^+-starved cells, in which K^+ uptake takes place as a K^+-H^+ cotransport [16], K^+ loss was investigated with the cell-column technique to keep the cells exposed to a very low K^+ concentration. With that technique, K^+-starved cells (either 140 or 118 mM K^+ in cell water) kept at less than 2 μM $[K^+]_o$ at pH 7.0 or 5.8, and exposed either to 1 mM NaCN or 1 mM NaN_3, did not lose any significant amount of the internal K^+ (in these experiments, a rate loss of 0.5–1.0 nmol · mg^{-1} · min^{-1} should have been detected). In these conditions the transport reversal potentials are more negative than the membrane potential of cyanide or azide treated cells (pH_i in azide-treated cells was found to be slightly lower than that in cyanide-treated cells; therefore, $E_{K\text{-}H}$ is slightly more negative than the values shown in Table I). That suggests that the K^+-H^+ symport does not work in reverse, at least in ATP-depleted cells.

Rapid change in the mode of Rb^+ uptake

The observation that, after a limited amount of K^+ loss, normal-K^+ cells did not lose more K^+, posed a question about whether these cells took up Rb^+ also like K^+-starved cells. In a previous work [26] we had observed biphasic transport in cells which had been washed with water; therefore, we tested now the temperature-dependence of the process. As expected, in these cells, the temperature-dependence of Rb^+ uptake was the same as in K^+-starved cells, suggesting that the change from one form of uptake to the other was rapid when the cell lost K^+.

Discussion

The existence of two uptake systems

Differences in the temperature-dependence and

TABLE I

OBSERVED K^+ LOSS IN CELLS TREATED WITH CYANIDE, AT SEVERAL $[K^+]_o$ AND $[H^+]_o$, AND THE CORRESPONDING EQUILIBRIUM TRANSPORT POTENTIALS FOR K^+ DIFFUSION (E_K) AND K^+-H^+ COTRANSPORT (E_{KH})

Summary of experiments in Fig. 5 and others with K^+-starved cells. K^+-starved cells with 350 K^+ nmol · mg^{-1} were prepared harvesting the cells when the K^+ concentration in growth medium was 35 μM; in these cells K^+ loss was tested by the cell-column technique (see text). Values for E_K and E_{KH} calculations: $[K^+]_i$, 200 mM in normal-K^+ cells and 140 mM for K^+-starved cells; pH_i, 6.4 in normal-K^+ cells and 5.8 in K^+-starved cells, both after cyanide treatment (see text). Membrane potential of cyanide-treated cells is approx. −100 mV [39]. ±, slow in some experiments and negative in others.

$[K^+]_o$ (mM)	pH_o	E_K (mV)	E_{KH} (mV)	K^+ loss
Normal-K^+ cells				
10	5.8	−77	−21	no
10	7.0	−77	−56	no
1.0	5.8	−135	−50	±
1.0	7.0	−135	−85	±
0.1	5.8	−194	−79	yes
0.1	7.0	−194	−114	yes
K^+-starved cells				
0.002	5.8	−286	−143	no
0.002	7.0	−286	−178	no

pH-dependence of Rb^+ uptake between normal-K^+ cells and K^+-starved cells support the notion that Rb^+ uptake in these two kinds of cell follows different pathways. This conclusion is similar to that reached in *S. cerevisiae* [25], the remarkable thing being the coincidence in the breakpoints in the Arrhenius plots (approx. 27°C and 26°C), though the slopes of the straight segments are much higher in *S. cerevisiae* than in *N. crassa*.

Another difference between normal-K^+ cells and K^+-starved cells is the tightness of K^+-starved cells as far as losing K^+ is concerned. The latter did not lose K^+ under any condition, but normal-K^+ cells lost a certain amount before being transformed into a state equivalent to that of K^+-starved cells. The faster the loss took place, the higher it was, demonstrating that the cells require a certain time to be transformed. This time may be necessary to switch off the K^+-diffusion pathway. This K^+-diffusion pathway would become non-functional as soon as the cell had lost a certain amount of K^+, and the K^+-H^+ symport [16] had taken on the function of K^+ uptake.

The important point now is whether the K^+ efflux observed in normal-K^+ cells under cyanide or azide treatment is the backward reaction through the system that normally mediates K^+ uptake. Proposing the loss as a consequence of the change in the direction of the driving force is a simple and attractive hypothesis, but it is necessary to consider that the net loss of K^+ may play a physiological role, and that the process may take place through a specially designed system. The function of that system would be to mediate the utilization of the energy stored in the form of a K^+ gradient, as proposed in bacteria [40]. In fact, both in metabolizing cells of *N. crassa* [41] and *S. cerevisiae* [42], the entrance of H^+ cotransported substrates can be followed by a pH meter, indicating that H^+ circulation through the proton pump is not the way in which the entrance of the positive charge is being neutralized. In metabolic-blocked *S. cerevisiae* cells, there is an extensive literature showing the role of K^+ efflux to compensate the charge of H^+-substrate uptake [43]. In these cells, if no substrate for uptake is present, K^+ leaks slowly in exchange for H^+, and the process is accelerated by uncouplers. This H^+ uptake can account for most of the acidification observed in metabolic-blocked cells (compare data in Refs. 35 and 44).

The dual-uptake isotherm

Temperature dependence of Rb^+ uptake in the two phases of the process in K^+-starved cells supports the idea that only one uptake system is involved, ruling out the two systems hypothesis advanced for higher plants [17]. From the other hypotheses proposed for higher plants, to explain the dual isotherm with only one system, that based on the slow diffusion of K^+ or Rb^+ through the cell wall and boundary layer [20] is also ruled out by present results. Therefore, the biphasic pattern of Rb^+ uptake must be explained by the intrinsic behavior of the uptake system. At least at low Rb^+, the system must function as a Rb^+-H^+ cotransport, as can be deduced by comparison with K^+ uptake and, therefore, biphasic kinetics could be the result of the random binding of H^+ and Rb^+ [31]. However, in this model both Michaelis constants and maximal velocities should be sensitive to external pH [31], which is not the case. In fact, the second phase probably does not follow Michaelis-Menten kinetics (this is clear at pH 8.2) and, in any case, the V_{max} increased when $[H^+]_o$ decreased, the contrary of the response in the first phase.

A model which explains the kinetic behavior observed is the transformation of the Rb^+-H^+ cotransport in a Rb^+-Rb^+ cotransport at high Rb^+. In a general formulation, the two sites of the carrier would have a significant affinity for K^+, Rb^+, H^+, and probably Li^+ and Na^+. At low K^+ or Rb^+, the carrier binds K^+ or Rb^+ and H^+, but at very high Rb^+ it binds two Rb^+ (with K^+ it would probably be the same). Kinetic analysis of the model proved to be complex because it has a twelve-state carrier cycle (there is no reason to rule out that two H^+ also bind). However, the model predicts that the total V_{max} will probably be independent of pH, because at high Rb^+, the system does not bind H^+. The model predicts as well that the second phase does not follow a Michaelis-Menten equation but a third-grade equation, all of this consistent with our findings. It must be noted that the binding of two Rb^+ to the carrier makes uptake at high Rb^+ 'non active'; that is, energetically equivalent to a Rb^+ diffusion.

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