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Potassium Fluxes in *Chlamydomonas reinhardtii*[1]

I. Kinetics and Electrical Potentials

Bhupinder Malhotra and Anthony D. M. Glass*

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

Potassium influx and cellular [K^+] were measured in the unicellular green alga *Chlamydomonas reinhardtii* after pretreatment in either 10 or 0 mM external K^+ ($[K]_o$). K^+ ($^{42}K^+$ or $^{86}Rb^+$) influx was mediated by a saturable, high-affinity transport system (HATS) at low $[K^+]_o$ and a linear, low-affinity transport system at high $[K^+]_o$. The HATS was typically more sensitive to metabolic inhibition (and darkness) than the low-affinity transport system. Membrane electrical potentials were determined by measuring the equilibrium distribution of tetraphenylphosphonium. These values, together with estimates of cytoplasmic [K^+] (B. Malhotra and A.D.M. Glass [1995] Plant Physiol 108: 1537–1545), demonstrated that at 0.1 mM $[K^+]_o$ K^+ uptake must be active. At higher $[K^+]_o$ (>0.3 mM) K^+ influx appeared to be passive and possibly channel mediated. When cells were deprived of K^+ for 24 h, the V_{max} for the HATS increased from 50×10^{-6} to 85×10^{-6} nmol h^{-1} $cell^{-1}$ and the K_m value decreased from 0.25 to 0.162 mM. Meanwhile, cellular [K^+] declined from 24×10^{-6} to 9×10^{-6} nmol $cell^{-1}$. During this period influx increased exponentially, reaching its peak value after 18 h of K^+ deprivation. This increase of K^+ influx was not expressed when cells were exposed to inhibitors of protein synthesis. The use of $^{42}K^+$ and $^{86}Rb^+$ in parallel experiments demonstrated that *Chlamydomonas* discriminated in favor of K^+ over Rb^+, and this effect increased with the duration of K^+ deprivation.

K^+ has been demonstrated to serve both biophysical and biochemical functions in plant cells. It is a major contributor to osmotic potential (Haschke and Lüttge, 1975), although this particular function can be replaced to varying extents by other solutes (Pitman et al., 1971b; Kirkby and Mengel, 1976). It is responsible for stomatal opening (Humble and Hsiao, 1970) and many rapid movements of plant parts (Satter et al., 1974). K^+ also exerts a dominating influence on the diffusion potential component of transmembrane electrical potential differences by virtue of the relatively high permeability to K^+ of biological membranes. Within the cytoplasmic phase, K^+ is maintained at high concentration (approximately 150 mM) as a specific requirement for protein synthesis (Leigh and Wyn Jones, 1984; Memon et al., 1985). In addition, K^+ serves as an activator for a large number of enzymes (Evans and Wildes, 1971). However, the low K_m values for K^+ activation of these enzymes (e.g. <5 mM for pyruvate kinase and Suc synthetase) indicate that the maintenance of cytoplasmic [K^+] at such high values is a requirement for protein synthesis rather than enzyme activation (Memon et al., 1985).

When K^+ is withdrawn from external media most plants respond rapidly by increasing the capacity for influx of K^+ across plasma membranes as well as mobilizing vacuolar reserves (Leigh and Wyn Jones, 1984; Glass and Fernando, 1992). Indeed, recent studies in which barley roots were used have demonstrated that within 6 to 12 h of withdrawing exogenous K^+ the synthesis of several polypeptides, including a 43-kD intrinsic plasma membrane polypeptide, is increased severalfold (Fernando et al., 1992).

Despite extensive kinetics studies of the absorption of K^+ in higher plants, beginning with the pioneering investigations of Epstein and co-workers in the 1950s, mechanisms of K^+ transport based on kinetics data remain controversial (Bange, 1973; Epstein, 1976; Borstlap, 1981; Nissen, 1989). Details of energy coupling are uncertain (Poole, 1974; Cheeseman and Hanson, 1980; Behl and Raschke, 1987; Glass and Fernando, 1992) and the biochemistry of high-affinity K^+ transport constitutes virtually unexplored territory. Two separate groups (Anderson et al., 1992; Sentenac et al., 1992) have cloned K^+ channels from Arabidopsis. These are considered to mediate low-affinity K^+ transport. In addition, Schachtman and Schroeder (1994) recently reported the structure and transport mechanisms of a high-affinity uptake system from wheat roots. Contributing factors to the difficulties experienced in the higher plant systems include the tissue heterogeneity, which is characteristic of plant roots, as well as the complications arising from the subsequent translocation of K^+ to the xylem.

To avoid these difficulties we have selected a unicellular alga, *Chlamydomonas*, as the experimental organism for both physiological and biochemical studies of membrane

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* Corresponding author; e-mail aglass@unixg.ubc.ca; fax 1–604–822–6089.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\mu_{K^+}$, electrochemical potential difference for K^+; $\Delta\psi$, membrane electrical potential difference; HATS, high-affinity transport system; 0K, TAPM medium with no K^+ added; 10K, TAPM medium with 10 mM K^+ added; $[K^+]_o$, external K^+ concentration; LATS, low-affinity transport system; pCMBS, *p*-chloromercuribenzene sulfonic acid; TAPM, Tris acetate phosphate modified medium; TEA^+, tetraethylammonium chloride; TPP^+, tetraphenylphosphonium.

transport of K^+. This organism has been the subject of extensive genetic studies and has provided a convenient system for the isolation of transport mutants (Polley and Doctor, 1985). The studies reported here make use of $^{86}Rb^+$ and $^{42}K^+$ to examine the kinetics of K^+ transport over a wide range of $[K^+]_o$s and to examine changes of K^+ influx and cellular $[K^+]$ associated with K^+ deprivation. Metabolic inhibitors and specific inhibitors of protein synthesis were used to characterize the observed transport systems and their responses to K^+ deprivation. To study the energy dependence of K^+ uptake, $\Delta\psi$ was determined from TPP^+ distribution across the plasma membrane at several values of $[K^+]_o$.

MATERIALS AND METHODS

Strain and Culture Conditions

Chlamydomonas reinhardtii Dang. strain CC125 (mt^+) was used for all experiments. Cells were grown in 6-L culture flasks in TAPM at pH 7.0 (Polley and Doctor, 1985) and synchronized by using a 16-h light/8-h dark regimen. Irradiance was maintained at 200 mE m^{-2} s^{-1} at 25°C within a controlled environment room. Flux measurements were undertaken between the mid-log and late-log phases of growth at a cell density of 1.5 to 2.5 × 10^6 cells mL^{-1}. Cultures were grown mixotropically and aerated with filtered air throughout the study period. Cell growth was measured by counting the cell number (Coulter Counter model TA II; Coulter, Hialeah, FL), Chl content (Harris, 1988), or fluorescence (Fluorometer, model 10–000R; Turner Designs Inc., Sunnyvale, CA).

Determination of Internal K^+ Content of the Cells

Cells were grown to mid-log phase in 10 mM K^+ and centrifuged in 250-mL bottles in a GSA rotor (2000 rpm for 2.5 min). The pellet was washed with 0K (0 mM K^+) and resuspended in 0K or 10K at equal cell densities to evaluate the effect of the duration of K^+ deprivation on cell $[K^+]$. Cultures were stirred and aerated during their subsequent growth. Samples were withdrawn axenically from the flasks at hourly intervals and 1-mL samples (four replicates for each period) were layered on top of a small volume (200 μL) of silicone oil (1:1 AR 20/AR 200; Wacker Chemie, Münich, Germany) in a 1.5-mL microcentrifuge tube (Moroney et al., 1987). These subsamples were centrifuged (Eppendorf centrifuge 5415, Brinkmann) through the silicone oil for 20 s at 10,000 rpm. Previous analyses had established that this method effectively separated cells from the original suspension medium with minimal contamination of ions from the suspension medium. The pellets were dissolved in 1 M perchloric acid and diluted with distilled water before aspiration into the flame photometer (Instrumentation Laboratory [Lexington, MA] model 443). Corrections for extracellular K^+ carried by the cells through the silicone oil were made as described below.

K^+ content of the cells was also determined by using larger volumes of cell cultures (25 mL; 3 × 10^6 cells mL^{-1}) with 10 replicates. Cells were harvested at 2000 rpm for 5 min and washed twice with 0K. This washed pellet was dissolved in 1 M perchloric acid, and an aliquot of this solution was removed and diluted before K^+ was analyzed in the flame photometer.

Influx Determinations

Log-phase cells were centrifuged and washed with and resuspended into 0K before each influx experiment. These cells were allowed to recover from possible centrifugation trauma by allowing an adaptation period of 30 min with aeration and stirring. One milliliter of resuspended cells (four replicates) was layered on silicone oil in each of the microcentrifuge tubes and, depending on the experiment, either K^+ or Rb^+ was added to the cells, followed immediately by $^{86}Rb^+$ (0.25 μCi mL^{-1}) or $^{42}K^+$ (0.25 μCi mL^{-1}). The microcentrifuge tubes were incubated on a reciprocating shaker (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL) at 60 rpm for 10-min influx periods under the same conditions of temperature and irradiance as had prevailed during the prior growth periods. After the influx period the cells were centrifuged for 20 s at 10,000 rpm through the silicone oil layer, and the resulting pellets were dissolved in 1 M perchloric acid. Radioactivities of the pellets were estimated by Cerenkov counting in a scintillation counter (Beckman, LS 60001C). Values for V_{max} and K_m for all the experiments were obtained from Eadie Hofstee plots.

To study the effect of external pH on K^+($^{86}Rb^+$) influx, cells were washed with 0K as described above. These cells were resuspended in 10 mL of 0K (adjusted to different pH values with 20 mM Tris-acetate buffer) in 20-mL glass vials. These vials were placed horizontally on a shaker for an adaptation period of 30 min under the same conditions as described in the previous section. K^+($^{86}Rb^+$) influx was measured using the same procedure as described above.

To measure $^{86}Rb^+$ or $^{42}K^+$ influx in cells starved for different durations, cells were transferred to 0K at intervals prior to the influx period and influx was measured in all samples at the same time. This method avoided any problems associated with diurnal variation in uptake rates. Correction for tracers carried through the silicone oil in the apparent free space during centrifugation was achieved by first solubilizing cell membranes of intact cells using 0.1% SDS or heating the cells at 70°C for 10 min. $^{86}Rb^+$ was added to these preparations at the same activities as present in suspensions of intact cells. The SDS or heat-treated cells were incubated for 10 min and then filtered through silicone oil as in the influx experiments. The radioactivity remaining in the pelleted material was usually small, approximately 10% of the activity associated with intact cells. Alternatively, cells were incubated with [^{3}H]H_2O and [^{14}C]inulin for a 10 min. Cells were then centrifuged through silicone oil and the radioactivities associated with the pellet were determined. The radiolabeled water was used to give a measure of volume of cells plus extracellular solution carried down in the pellet, and the [^{14}C]inulin gave a measure of extracellular volume. This method also gave a value of 12% for the extracellular volume. This value was used to correct for apparent free space. In the experiments in which metabolic inhibitors

(KCN, CCCP), a sulfhydryl modifier (pCMBS), a K^+ channel blocker (TEA^+), and inhibitors of cytoplasmic protein synthesis (cycloheximide and anisomycin) were used, cell suspensions were pretreated with different concentrations of the inhibitors prior to the uptake experiments. Concentrations of inhibitors used are as given in the appropriate tables. After the inhibitor pretreatment, the cell suspension was transferred to the microcentrifuge tubes and K^+($^{86}Rb^+$) influx was measured as described above. The inhibitors were also present in the cell suspensions during the 10-min influx periods.

Calculation of $\Delta\psi$ across the Plasma Membrane from TPP^+ Accumulation

The $\Delta\psi$ across the plasma membrane of *Chlamydomonas* was estimated by means of the equilibrium distribution of TPP^+ (Komor and Tanner, 1976). The cell suspensions were incubated with constant stirring and aeration under the same temperature and light conditions as had prevailed during the prior growth period. [^{3}H]TPP^+ (final radioactivity, 0.5 μCi mL^{-1}) and TPPBr (14.4 μM) were added to this cell suspension in a flask. To study the effects of $[K^+]_o$ in the range from 0 to 200 mM on the membrane potential, cells were resuspended in 0K and allowed to adapt for 30 min. Ten-milliliter cell suspensions were transferred to 20-mL glass vials. [^{3}H]TPP^+, TPPBr, and K^+ were added to the cell suspensions to generate final concentrations in the range from 0 to 200 mM. K^+ was added as K^+ citrate to achieve large depolarizations; the citrate anion is expected to permeate very slowly in contrast to the K^+ ion (Komor and Tanner, 1976). The glass vials were incubated horizontally on a shaker for 3 h. The incubation time of 3 h was derived from the time course of TPP^+ accumulation described above; by 3 h the accumulation of TPP^+ appeared to have reached equilibrium. At the end of the 3-h incubation period, 1-mL subsamples (four replicates) were withdrawn from the glass vials and layered on top of the silicone oil layers. The tubes were then centrifuged. The silicone oil was a mixture of AR 20 and AR 200 at a ratio of 1:1 for all $[K^+]$ except 100 and 200 mM K^+. In the case of the latter, a mixture of the ratio 1:3 was used because a 1:1 mixture of silicone oil was lighter than the TAPM (plus cells) with 100 and 200 mM K^+ and thus stayed on top of the medium after centrifugation. Cell pellets were first dissolved in 1 M perchloric acid and then mixed with 10 mL of the scintillation fluid (ACS, Amersham). Radioactivities were determined by scintillation counting in a scintillation counter (Beckman LS 60001C).

To study effects of external pH on membrane potential, 0K was prepared at a range of pH values (3.5–9.0) using 20 mM Tris acetate as a buffer. Cells growing at 10 mM K^+ were washed and resuspended in 10-mL volumes of buffered 0K (in 20-mL glass vials). After an adaptation period of 30 min at these pH values, [^{3}H]TPP (0.5 μCi mL^{-1}) and TPPBr (14.4 μM) were added to all cell suspensions in the glass vials. These were incubated on a shaker in a horizontal position for 3 h. At the end of 3 h, radioactivities associated with the cells were determined as described above using the silicone oil-filtration method.

It is assumed that at equilibrium the distribution of TPP^+ will be in accord with the electrical potential difference across the plasma membrane, as given by the following equation:

$$\Delta\psi = \frac{RT}{zF} \ln \frac{[TPP]_o}{[TPP]_i}$$

where R is the gas constant T is temperature (291 K), z is the charge (+1), and F is the Faraday constant, and $[TPP]_o$ and $[TPP]_i$ are the external and internal concentrations of TPP^+, respectively. This is the Nernst potential for TPP^+ distribution. $\Delta\psi$ is then calculated from the equilibrium distribution of TPP^+. With this value of $\Delta\psi$, together with the intracellular $[K^+]$ and $[K^+]_o$, it is possible to estimate $\Delta\mu_{K^+}$ and hence ascertain whether K^+ is moving into the cell actively or passively. Values for cytoplasmic $[K^+]$ were determined by compartmental analysis (Malhotra and Glass, 1995).

RESULTS

K^+ influx into cells of *C. reinhardtii* was studied in the concentration range from 0.005 to 30 mM. It is clear from the influx data (Figs. 1 and 2) that at low $[K^+]_o$, $^{42}K^+$ influx demonstrated saturation. At high $[K^+]_o$ (>1 mm), K^+ influx showed no saturation and continued to increase in apparently linear fashion up to $[K^+]$ as high as 30 mm (Fig. 2).

HATS

In the low concentration range from 0.005 to 0.75 mM K^+, K^+ influx was mediated by a saturable system (Fig. 1B), which conformed to Michaelis Menten kinetics. Influx saturated at about 0.25 to 0.75 mM $[K^+]_o$ in both starved and unstarved cells. A comparison of unstarved and starved cells revealed that K^+($^{42}K^+$) influx was considerably higher in starved cells (V_{max} 85 × 10^{-6} nmol h^{-1} $cell^{-1}$) than in unstarved cells (V_{max} 50 × 10^{-6} nmol h^{-1} $cell^{-1}$). The K_m values (determined by Eadie Hofstee linear transforma-

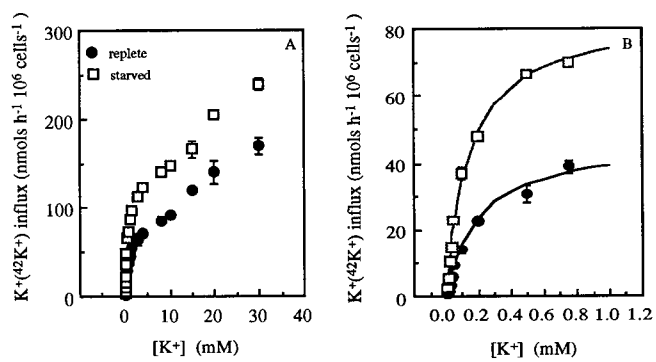

Figure 1. A, $^{42}K^+$ influx (nmol h^{-1} 10^6 $cells^{-1}$) from $[K^+]_o$ in the range 0 to 30 mM in *C. reinhardtii* cells grown in K^+-replete medium (10K; ●) and K^+-deficient medium (0K; □). B, $^{42}K^+$ influx (nmol h^{-1} 10^6 $cells^{-1}$) from $[K^+]_o$ in the range 0 to 0.75 mM showing the saturable high-affinity system in cells grown in K^+-replete and K^+-deficient media. Symbols are the same as were used in A. Results are means ± SE of four replicates.

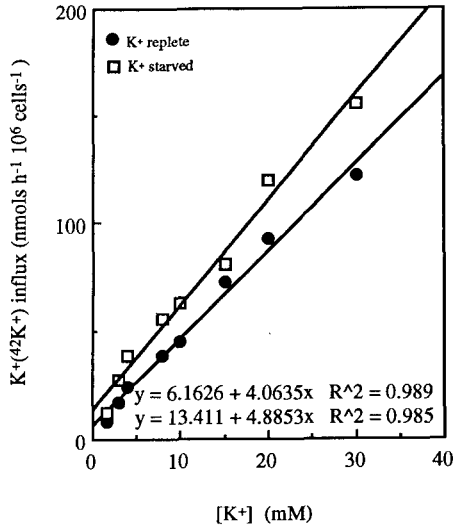

Figure 2. $42K^+$ influx ($\times 10^{-6}$ nmol h^{-1} $cell^{-1}$) showing the LATS obtained by subtracting the V_{max} of the HATS from observed fluxes in the range 1.5 to 30 mM $[K^+]_o$ (symbols are the same as used in Fig. 1).

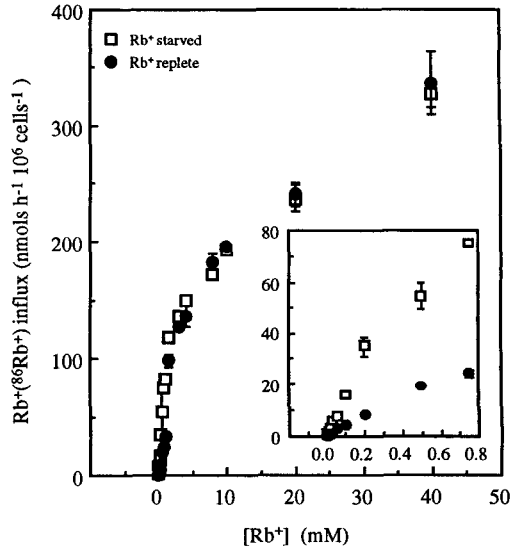

Figure 3. $86Rb^+$ influx ($\times 10^{-6}$ nmol h^{-1} $cell^{-1}$) from external $[Rb^+]$ in the range 0 to 40 mM showing the saturable pattern in cells grown in K^+-replete and K^+-deficient media. The inset shows the plot at low external $[Rb^+]$. Symbols are the same as used in Figure 1.

tions) for $K^+(^{42}K^+)$ influx in unstarved cells was determined to be 0.26 mM as compared to 0.16 mM in the starved cells (Table I). Similar trends were observed when $^{86}Rb^+$ was used as a tracer for Rb^+ or K^+. The fluxes were much lower when $^{86}Rb^+$ was used to label K^+(data not shown) than those determined with $^{42}K^+$. This was not the case, however, when $^{86}Rb^+$ was used as a tracer of Rb^+ (cf. Figs. 1A and 3, respectively).

LATS

When $[K^+]$ was increased beyond 1.0 mM, a second system for $K^+(^{42}K^+)$ influx became apparent that was linearly dependent on $[K^+]_o$ (Fig. 2). Such a linear trend was also evident in experiments in which $^{86}Rb^+$ was used to label K^+ solutions. This same linearity became apparent only at higher external Rb^+ concentrations in experiments in which $^{86}Rb^+$ was used to label RbCl (Fig. 3), because of the higher K_m value for $^{86}Rb^+$ (Rb^+) influx by the HATS. K^+-replete cells appeared to attain the same influx as K^+-starved cells at high external concentrations.

Characterization of the Transport Systems

Metabolic dependence of the transport systems was examined by exposing cells to CCCP or KCN (Table II). CCCP (10 mM) inhibited $K^+(^{86}Rb^+)$ uptake by the HATS (37%) more than uptake by the LATS (8%). KCN (0.5 mM) also demonstrated greater inhibition (55%) of the HATS than the LATS (28%). Influx experiments performed in the dark also showed a greater inhibition of the HATS (47%) than the LATS (16%). TEA^+, which has been shown to be a specific K^+ channel blocker in *Chara* (Tester, 1988) and in corn (Kochian et al., 1985), caused a relatively small inhibition (18–21%) of $K^+(^{86}Rb^+)$ influx throughout the whole range of $[K^+]$ (0.005–30 mM). The sulfhydryl reagent pCMBS was found to inhibit the HATS only slightly, whereas the LATS was unaffected.

Effect of External pH on $K^+(^{86}Rb^+)$ Influx

$K^+(^{86}Rb^+)$ influx demonstrated a strong dependence on external pH with a clear optimum at pH 6.0 (Fig. 4). There was a 54 and 38% inhibition of K^+ influx at pH 5.0 and 7.0, respectively, as compared with pH 6.0.

Time-Course Studies

To determine the decline of internal $[K^+]$ content with increasing duration of K^+ deprivation, cells were first

Table I. *V_{max} ($\times 10^{-6}$ nmol h^{-1} $cell^{-1}$), K_m (mM), and r^2 values for linear regressions (Hoffstee plots) for K^+-deplete (0 mM K^+ for 24 h) and -replete (10 mM K^+ for 24 h) cells*

$K^+(^{86}Rb^+$ or $^{42}K^+)$ and Rb^+ ($^{86}Rb^+$) influx was determined from $[K^+]_o$ in the range 0 to 0.75 mM.

Tracer	Treatment					
	K^+ replete			K^+ deplete		
	V_{max}	K_m	r^2	V_{max}	K_m	r^2
$^{86}Rb^+ + K^+$	10.7 ± 0.91	0.17 ± 0.02	0.99	16.7 ± 1.57	0.16 ± 0.01	0.93
$^{42}K^+ + K^+$	50.6 ± 4.42	0.26 ± 0.04	0.94	85 ± 0.92	0.16 ± 0.02	0.96
$^{86}Rb^+ + Rb^+$	33.5 ± 10	1.0 ± 0.24	0.81	82.6 ± 11.9	0.69 ± 0.1	0.85

Table II. *Effects of various inhibitors on K^+($^{86}Rb^+$) influx from TAPM with $[K^+]_o$ at 0.1 mM (HATS) and at 10.0 mM (LATS)*

Values given for percentage inhibition were calculated using influx in the absence of the inhibitor as control. Cells were pretreated with the inhibitors for the times shown and were also exposed to the inhibitors during the 10-min uptake period.

Inhibitor	Concentration	Pretreatment	Percentage Inhibition	
			HATS	LATS
		min		
KCN	500 μM	20	55	28
CCCP	10 μM	20	37	8
TEA^+	20mM	120	18	21
*p*CMBS	1mM	20	22	0
Darkness			47	16

grown to mid-log phase in 10 mM $[K^+]$. After the cells were resuspended in 0K, their K^+ content declined from 24 to 9.2 nmol 10^6 cells^{-1} by 24 h (Fig. 5). In the next 24 h there was only a slight decline in $[K^+]$, so that by 48 h after removing K^+, cell $[K^+]$ was 6.2 nmol 10^6 cells^{-1}. Under control conditions (cells grown at 10 mM K^+ throughout) K^+ content fluctuated between 24 and 18 × 10^{-6} nmol cell^{-1} during the 48 h of the experiment, according to the stage of the growth cycle. The control cells moved and divided normally for 48 h but cells deprived of K^+ grew and moved normally for the first 24 h. After 24 h they stopped growing and their movement slowed until no movement was evident by 48 h. K^+ influx was measured at various intervals during the 48 h of K^+ deprivation using both $^{42}K^+$ and $^{86}Rb^+$ as tracers. A negative correlation was observed between internal K^+ content and K^+($^{42}K^+$) influx until influx peaked in cells starved for 18 h, after which K^+ influx declined significantly. Similar patterns of K^+ or Rb^+ uptake were observed when $^{86}Rb^+$ was used as a tracer (data not shown). Influx values were much lower in the case of $K^+/^{86}Rb^+$ than those estimated by use of $Rb^+/^{86}Rb^+$ or $K^+/^{42}K^+$.

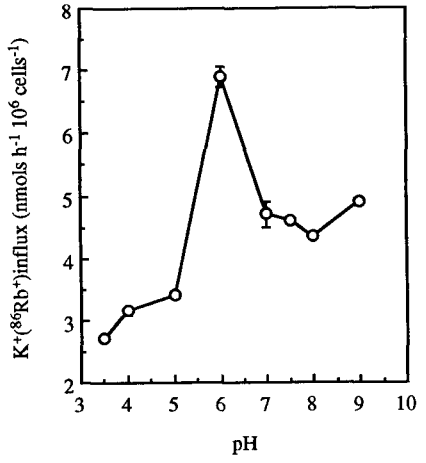

Figure 4. Effect of external pH in the range 3.5 to 9.0 on K^+($^{86}Rb^+$) influx (nmol h^{-1} 10^6 cells^{-1}). Each symbol represents the average of two separate experiments (four replicates for each treatment). $[K^+]_o$ was 100 μM.

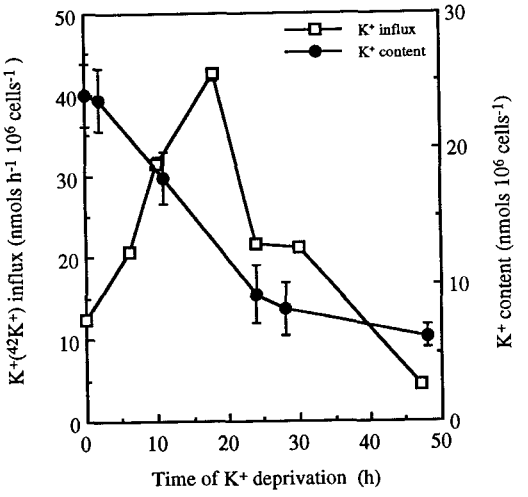

Figure 5. K^+ content (nmol 10^6 cells^{-1}) and K^+ influx (nmol h^{-1} 10^6 cells^{-1}) from 0.1 mM $[K^+]_o$ in cells deprived of K^+ for different durations. Results are means ± SE of four replicates.

Inhibitors of protein synthesis, cycloheximide and anisomycin, prevented the increase of K^+ influx normally associated with K^+ deprivation (Fig. 6). For example, 3 h of K^+ deprivation increased K^+($^{86}Rb^+$) influx by 43% compared to the flux in control cells (no K^+ deprivation), whereas K^+ influx in cells exposed to cycloheximide or anisomycin for 3 h decreased by 34 and 30%, respectively. By 6 h, K^+ deprivation had caused K^+ influx to increase by 56% compared to control cells, whereas influx in cycloheximide- or anisomycin-treated cells decreased by 60 and 54%, respectively. The lack of effect of the inhibitors of protein synthesis on K^+ influx at 0 time (during the 10-min influx

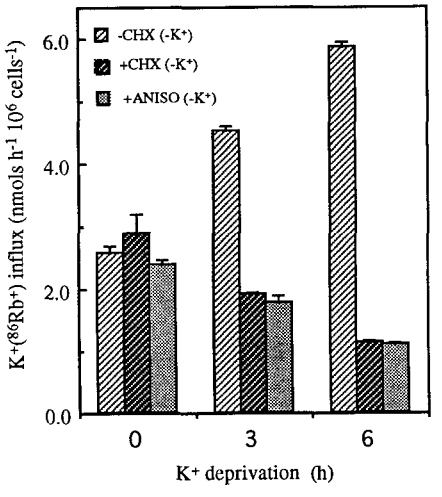

Figure 6. Effect of inhibitors of protein synthesis cycloheximide (CHX, 10 mg mL^{-1}) and anisomycin (ANISO, 3.5 mg mL^{-1}) on development of increased K^+($^{86}Rb^+$) influx during K^+ deprivation. Cells were grown at 10 mM K^+ and fluxes were determined at 0.1 mM K^+. Influx values are in nmol h^{-1} 10^6 cells^{-1}. Results are means ± SE of four replicates.

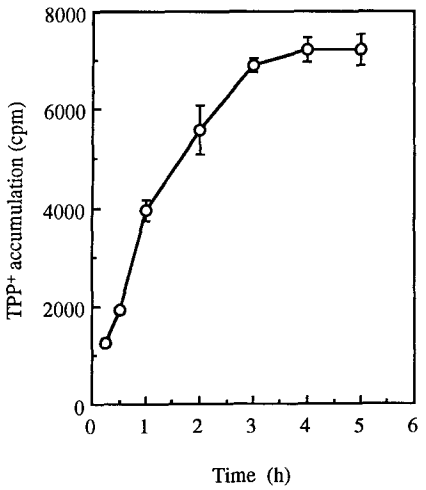

Figure 7. Time course for TPP^+ accumulation over 5 h. Counts accumulated are for 1-mL cell suspensions containing about 3 million cells.

period) indicated that there were no side effects of these inhibitors on transport per se.

Time Course of TPP^+ Distribution

It is clear from Figure 7 that equilibration of TPP^+ distribution between the cells and the external medium was not achieved until 3 to 4 h had elapsed. This ion was taken up by the cells until, at equilibrium, TPP^+ was concentrated about 150 to 200 times the external concentration. For all subsequent experiments in which this agent was used to measure $\Delta\psi$, therefore, a 3-h incubation of cells with TPP^+ was used.

Effects of pH and $[K^+]_o$ on Membrane Potential

As external pH increased, membrane potential hyperpolarized from −69 mV at pH 3.5 to −135 mV at pH 9 (Fig. 8A). Membrane potential failed to respond to $[K^+]_o$ in the range from 0 to 0.1 mM and the values remained at about −135 mV (Fig. 8C). Between 0.1 and 1 mM K^+, respectively, membrane potential hyperpolarized from −135 to −156 mV. At $[K^+]_o$ higher than 1 mM, membrane potential demonstrated a linear dependence on log $[K^+]_o$. Thus, membrane potential depolarized from a value of −156 mV at 1 mM $[K^+]$ to −70 mV at 200 mM $[K^+]$ (Fig. 8B). Membrane potential did not change greatly between 0.025 and 10 mM $[K^+]_o$ because of the hyperpolarizations observed at 0.5 and 1.0 mM $[K^+]_o$. $\Delta\mu_{K^+}$ was calculated for cells grown at 0.1 mM K^+ and briefly exposed to differing concentrations of $[K^+]_o$ using the value of $\Delta\psi$ for that $[K^+]$ across the plasma membrane (Fig. 9). $\Delta\mu_{K^+}$ had a positive value at low $[K^+]_o$ (<0.3 mM) and a negative value at higher $[K^+]_o$ (>0.3 mM).

DISCUSSION

In a previous study (Polley and Doctor, 1985), $K^+(^{86}Rb^+)$ influx in *C. reindardtii* over the range from 0 to 6 mM was described by a single Michaelis-Menten curve. In the present study, the influx isotherm for $K^+(^{42}K^+$ and $^{86}Rb^+)$ influx was resolved into a biphasic pattern, with saturable and nonsaturable components. This corresponds to earlier observations describing the patterns of K^+ uptake by barley roots (Epstein and Hagen, 1952) and corn roots (Kochian and Lucas, 1982) and more recent studies of NO_3^- uptake in barley roots (Siddiqi et al., 1990) and NH_4^+ uptake by rice roots (Wang et al., 1993). The saturable HATS corresponds to Epstein's mechanism 1 (Epstein and Hagen, 1952). It dominates in the low K^+ concentration range (0–1 mM) and shows Michaelis-Menten kinetics, saturating in the range from 0.20 to 0.75 mM $[K^+]_o$. When $K^+(^{42}K^+)$ influx was measured after cells were deprived of K^+ for 24 h, V_{max} for this influx system increased from 50 $\times 10^{-6}$ to 85 $\times 10^{-6}$ nmol h^{-1} $cell^{-1}$, and the K_m for influx decreased from 0.26 to 0.16 mM. The increased V_{max} values may be interpreted as a result of increased synthesis of carriers for high-affinity K^+ uptake. In roots of barley and corn, K^+ deprivation was also associated with decreased K_m values and increased V_{max} values (Glass, 1976; Kochian and Lucas, 1982). However, the equivalent period of K^+ deprivation in barley caused much larger increases of V_{max} and reduction of K_m than was evident in *Chlamydomonas*.

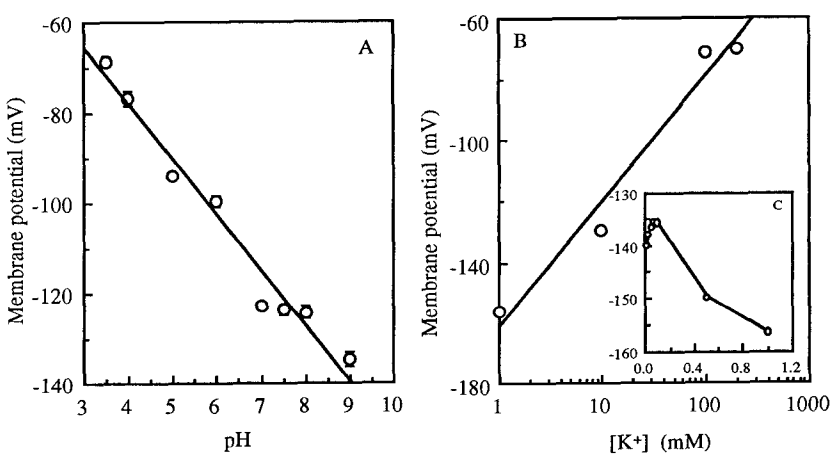

Figure 8. A, Effect of external pH in the range from 3.5 to 9.0 on $\Delta\psi$. Cells were grown at 0.1 mM K^+ and exposed to pH values shown during TPP^+ accumulation. Each symbol represents the average of two separate experiments. B, Effects of $[K^+]_o$ in the range from 1 to 200 mM on the membrane potential. Cells were grown at 0.1 mM K^+ and briefly exposed to differing concentrations of $[K^+]_o$. The regression line was calculated to be $y = -160 + 40(\log x)$; $r^2 = 0.97$. C, The membrane potential at low (0–1 mM) $[K^+]_o$.

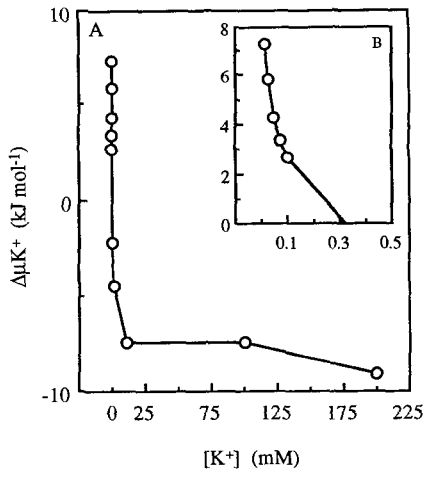

Figure 9. $\Delta\mu_{K^+}$ was calculated for cells grown at 0.1 mM K^+, using the measured values of $\Delta\psi$ at the corresponding values of $[K^+]_o$ and cytoplasmic $[K^+]$ obtained from compartmental analysis (Malhotra and Glass, 1995). B, $\Delta\mu_{K^+}$ at low $[K^+]_o$.

For example, V_{max} and K_m values in the study by Glass (1976) increased from 0.4 to 7.6 μmol g^{-1} fresh weight h^{-1} and decreased from 0.25 to 0.03 mM, respectively.

The most direct method for the determination of membrane potential is the use of microelectrodes but their use in small cells is problematic. According to Raven (1980, 1989), the two main problems with the microelectrode technique are "tip potentials" of the intracellular pipette in contact with the cytoplasmic polyelectrolytes and current leakage around the point of microelectrode insertion where the plasma membrane has not resealed properly. These will have a significant effect on the measured potential difference, tending to lower it. A further problem is the large size of the chloroplast in *Chlamydomonas*. It is very difficult to make sure that the tip is in the cytoplasm rather than the chloroplast. Despite these problems, microelectrodes have been used to measure membrane potentials in small cells such as *Chlorella* and *Mougeotia* (Barber, 1968; Wagner and Bentrup, 1973).

An alternative method of measuring $\Delta\psi$ is based on the equilibrium distribution of lipophilic cations such as TPP^+. This method has been used in studies of $\Delta\psi$ in *Chlorella vulgaris* (Komor and Tanner, 1976) and *Chlorella emersonii* (Beardall and Raven, 1981). The use of TPP^+ and similar probes in eukaryotes has been criticized by several workers (Gimmler and Greenway, 1983; Ritchie, 1982). Nevertheless, in *Porphyra purpurea* and *Ulva lactuca* TPP^+ accumulation gave similar values of membrane potentials to those measured with microelectrodes (Reed and Collins, 1981). Likewise, Remis et al. (1992) found that the results from the microelectrode technique and from the equilibrium distribution of the lipophilic ions such as TPP^+ were in agreement.

In the present study $\Delta\psi$ responded to $[K^+]_o$ in a manner consistent with expectation for a plasma membrane $\Delta\psi$, i.e. depolarization was 40 mV per decade of $[K^+]_o$ (Fig. 8B). In a similar study of barley root $\Delta\psi$ values, Pitman et al. (1971a) observed depolarizations of 43, 28, and 53 mV per decade of $[K^+]_o$ between 1 and 2.5, 2.5 to 10, and 10 to 25 mM $[K^+]_o$, respectively. Furthermore, our measurements of $\Delta\psi$ in *Chlamydomonas* by the use of TPP^+ revealed that $\Delta\psi$ was hyperpolarized as external pH increased (Fig. 8A). Such a response was noted by Poole (1974) in beet root slices. It is unlikely that pH changes in the range from 5 to 8 would have affected any other membrane than the plasma membrane. These observations lead us to conclude that in the present system TPP^+ accumulation provides a valid method of estimating $\Delta\psi$.

Estimates of $\Delta\mu_{K^+}$ obtained in the present study (Fig. 9) indicate that the high-affinity, saturable system for K^+ influx is mediated by an active transport step, accumulating K^+ against its electrochemical potential gradient from low $[K^+]_o$s as in earlier studies of corn, barley, and Arabidopsis, respectively (Kochian et al., 1989; Glass and Fernando, 1992; Maathius and Sanders, 1993). Consistent with this expectation, $K^+(^{86}Rb^+)$ influx in the HATS range of $[K^+]_o$ demonstrated a greater inhibition by CCCP and KCN and thus revealed its greater metabolic dependence than the LATS. Transferring the cells to dark caused a 50% reduction of $K^+(^{86}Rb^+)$ influx in the HATS (compared to 18% inhibition of the LATS), which again points to greater metabolic dependence of the HATS than the LATS. Similar findings with respect to the metabolic dependence of HATS have been reported by other workers (Cheeseman and Hanson, 1980; Kochian et al., 1989). By contrast, it is generally assumed that K^+ influx at high $[K^+]_o$ is thermodynamically "downhill" (Glass and Fernando, 1992; Maathius and Sanders, 1993) possibly via K^+ channels. Nevertheless, because of the contribution of metabolism to the activity of the proton pump and hence to $\Delta\psi$, it is to be expected that influx through the LATS system would also demonstrate metabolic dependence.

The nonsaturable kinetics observed at high $[K^+]_o$ conform to the linear high concentration kinetics for K^+ absorption in corn roots reported by Kochian and Lucas (1982). These authors interpreted the linear kinetics as the result of channel-mediated transport. Similar uptake kinetics have been observed for NO_3^- influx in barley (Siddiqi et al., 1990) and in the diatom *Skeletonema costatum* (Serra et al., 1978). Likewise, NH_4^+ influx in rice is mediated by saturable and linear transport systems (Wang et al., 1993). A dual pattern of K^+ influx was also reported in *Chlorella* (Kannan, 1971), but influx at high $[K^+]_o$ appeared to occur via a second saturable system.

Although TEA^+ inhibits the majority of known plant K^+ channels (Bentrup, 1990), some TEA^+-insensitive K^+ channels have been reported, e.g. the Ca^{2+}-dependent K^+ channel found in *Haemanthus* and *Clivia* endosperm (Stoeckel and Takeda, 1989) and the plasma membrane 49 pS channel from rye roots (White and Tester, 1992). The failure of the LATS to respond to TEA^+ in *Chlamydomonas* may be related to the structure and characteristics of this low-affinity transporter. First, it is possible that the 2-h treatment period may not have been long enough for TEA^+ to enter the cells in sufficient quantity to block the channel pores. Second, the pore structure of this channel responsible for transporting K^+ may be different and thus have a different

active site from the channels in corn and *Chara*. Third, this transporter may not be a channel and the linear kinetics we observed in this alga could represent the linear part of a saturable system with a very high K_m value.

In *Chlamydomonas*, pCMBS inhibited the saturable component by 28%, whereas the linear component was unaffected. In high-salt corn roots the linear component of K^+ influx remained unaffected, but in low-salt roots it was stimulated by 40% in the presence of pCMBS. Nevertheless, in *Chlamydomonas* it is evident that the saturable and the linear components respond differently to this nonpenetrating sulfhydryl reagent and probably indicate the operation of two independent mechanisms for K^+ transport. The failure of the saturable component to respond to pCMBS may be explained by the absence of sufficient sulfhydryl groups on this transporter exposed to the extracellular surface. The detection of the dual transport system for K^+ in a single-celled organism suggests that it is unnecessary to invoke spatially separate tissue systems (e.g. epidermis and cortex) as the locations of the saturable and linear transport processes as suggested by Ehwald et al. (1973) and Bange (1973).

$K^+(^{86}Rb^+)$ influx showed considerable sensitivity to external pH with an optimum at pH 6.0. Clearly, when H^+ serves as the driver ion, effects on transport due to changes of external pH might result from conformational changes in the transporter or from effects on the proton motive force. Such a response to pH was also demonstrated in *Neurospora crassa* (Rodriguez-Navarro et al., 1986; Blatt et al., 1987). The HATS in K^+-deficient *N. cells* is highly electrogenic and thought to be active. Current-voltage analysis of *Neurospora* cells indicated that the current associated with inward K^+ movement was twice that of the net K^+ flux. Thus, one additional positive charge entered with every K^+ entering the cell. The charge accompanying K^+ was suggested to be H^+, indicating that the HATS may operate as a K^+/H^+ symport. There is evidence from heterologous expression of the wheat *HKT1* gene in yeast that the higher plant K^+ transporter is also a K^+/H^+ symporter (Schachtman and Schroeder, 1994). Such a coupling mechanism would be expected to demonstrate considerable sensitivity to changes of extracellular and cytoplasmic pH values (Kochian and Lucas, 1988). The HATS in *Neurospora* showed an optimum for K^+ influx at pH 6.0, which was consistent with the proposed K^+/H^+ symport at low $[K^+]_o$. In *Chlamydomonas* also, the pH optimum for K^+ influx was at pH 6.0 (Fig. 4), which is quite different from that reported for red beet slices, in which K^+ uptake increased as external pH was increased (Poole, 1974), or of very low pH sensitivity in barley (Glass and Siddiqi, 1982) and in corn (Kochian et al., 1989). This insensitivity of K^+ uptake to external pH led the latter authors to question the obligate direct coupling of the two fluxes.

Trans-plasma membrane $\Delta\mu_{K^+}$ at 12.5 and 100 μM $[K^+]_o$ had positive values (7.3 and 2.7 kJ mol^{-1}, respectively), which means that K^+ is at a higher electrochemical potential inside the cell. Thus, K^+ would move actively into such a cell, against the electrochemical potential gradient. Glass and Fernando (1992) and Maathius and Sanders (1993) obtained essentially similar findings based on measured values of $\Delta\psi$ and cytoplasmic $[K^+]$ in barley and *A. thaliana*, respectively. From their $\Delta\mu_{K^+}$ values, they argued that at low $[K^+]_o$ K^+ is actively absorbed and could not be channel mediated as suggested earlier (Hedrich and Schroeder, 1989; Sentenac et al., 1992). The failure of membrane potential to respond to $[K^+]_o$ in the lower range (0–100 μM K^+) is similar to that observed in Arabidopsis (Maathius and Sanders, 1993) and is quite typical of algal cells (Vorobiev, 1980; L.N. Vorobiev, personal communication) as opposed to the situation in corn (Kochian et al., 1989), in which large depolarizations occur with the addition of micromolar concentrations of K^+ to the external medium. At 1.0 and 10 mM $[K^+]_o$, K^+ is at a lower electrochemical potential inside the cell (−4.5 and −7.5 kJ mol^{-1}, respectively) than outside and could move into such a cell passively, down its electrochemical potential. Above 1 mM $[K^+]_o$, membrane potential depolarized from −156 to −70 mV in *Chlamydomonas*. This may have been due to K^+ entry via K^+-specific channels, down the electrochemical potential gradient. These large depolarizations observed at high $[K^+]_o$ confirm similar observations of Komor and Tanner (1976) in *Chlorella* and Maathius and Sanders (1993) in Arabidopsis.

The time-course studies demonstrated that the K^+ content of cells declined significantly during growth in the absence of exogenous K^+. Their K^+ content decreased from 24×10^{-6} nmol $cell^{-1}$ in unstarved cells to 9×10^{-6} nmol $cell^{-1}$ in cells starved of K^+ for 24 h. This decline of internal K^+ content was accompanied by an increased $K^+(^{42}K^+)$ influx (Fig. 5) from 14×10^{-6} nmol h^{-1} $cell^{-1}$ (in K^+-replete cells) to 45×10^{-6} nmol h^{-1} $cell^{-1}$ (in cells starved of K^+ for 18 h from solutions containing 100 μM K^+). Nevertheless, at high $[K^+]_o$ there were no significant differences in K^+ influx between K^+-replete and K^+-starved cells when the flux due to the HATS was subtracted from the observed flux. Such observations were recorded previously by Glass and Dunlop (1978) for K^+ fluxes in roots of ryegrass and clover and by Kochian and Lucas (1982) for corn root segments. Glass and Dunlop (1978) interpreted this result as being due to a lack of regulation of the LATS by internal K^+. Similar negative feedback relationships between cell $[K^+]$ and K^+ influx have been reported in *Lemna minor* (Young and Sims, 1972) and barley roots (Fernando et al., 1992) in their time-course deprivation experiments. In *Chlamydomonas*, beyond 18 h of K^+ deprivation, $[K^+]$ of the cells remained low. Likewise, there was no further increase of $K^+(^{42}K^+)$ influx. Light microscopy revealed that cell motility was significantly reduced when K^+ deprivation exceeded 24 h.

Inhibitors of protein synthesis, cycloheximide and anisomycin, prevented the increase of K^+ influx associated with K^+ deprivation (Fig. 6). Thus, protein synthesis is necessary for the enhanced K^+ influx of the cells under conditions of K^+ deprivation. A similar response of $K^+(^{86}Rb^+)$ infux to protein synthesis inhibitors was reported for barley (Fernando et al., 1992).

Recently, K^+ channels have been isolated from Arabidopsis by transforming a K^+-transport mutant of yeast

(Anderson et al., 1992; Sentenac et al., 1992). The genes coding for these polypeptides appear to share considerable homology with the Shaker K^+ channels of *Drosophila*. Surprisingly, transformation of the yeast mutant by the *ATK1* gene appeared to confer on the mutant the capacity to absorb $^{86}Rb^+$ from both low (micromolar) and high (millimolar) concentrations of K^+. This observation has provoked consideration of the possibility that a single K^+ channel might be responsible for biphasic K^+ uptake in plants (Gassmann et al., 1993; Kochian and Lucas, 1993). Consideration of the electrochemical potential gradient for K^+ from low external K^+, however, led the above authors to conclude that in plants inwardly directed K^+ channels could function in K^+ absorption only at elevated K^+ concentrations (0.3–1.4 mM, respectively). Similar conclusions were recently reported by Maathius and Sanders (1993) for Arabidopsis and earlier by Glass and Fernando (1992) for barley. Gassmann et al. (1993) suggested that inward K^+ channels might serve as major components of low-affinity K^+ uptake and/or as a backup system when the high-affinity system is disabled. Thus, in addition to channel-mediated transport, it is generally held that a discrete HATS is also necessary to account for biphasic K^+ transport. The recent cloning of a discrete gene coding for the HATS in wheat (Schachtman and Schroeder, 1994) provides concrete evidence for this point.

To evaluate the effectiveness of $^{86}Rb^+$ as a tracer for K^+, parallel experiments were undertaken with $^{86}Rb^+$ and $^{42}K^+$ as tracers for K^+. The present study has established that $^{86}Rb^+$ gives a good qualitative correspondence to the uptake by $^{42}K^+$, but fluxes were reduced quantitatively. Such discrimination against Rb^+ has been reported for some higher plants: *Phaseolus* (Jacoby, 1975); algae: *U. lactuca* and *Chaetomorpha darwinii* (West and Pitman, 1967), *Chara* (Keifer and Spanswick, 1978); and bacteria: *E. coli* (Rhoads et al., 1977). This discrimination against $^{86}Rb^+$ in *Chlamydomonas* was reported earlier by Polley and Doctor (1985). These authors also observed that the extent of the K^+/Rb^+ selectivity increased with K^+ starvation. Thus, $^{86}Rb^+$ may serve as a satisfactory tracer for K^+ fluxes under certain conditions, specifically when K^+ status is unchanged. Nevertheless, even when K^+ status remains unchanged, K^+ fluxes estimated using $^{86}Rb^+$ as a tracer for K^+ may be subject to error.

CONCLUSIONS

On the basis of the concentration dependence of K^+ influx into *Chlamydomonas*, K^+ transport appears to be mediated by two distinct transport systems, an HATS and an LATS. K^+ enters the cell against its electrochemical potential gradient at low $[K^+]_o$ (<0.3 mM), and above this concentration it moves down its electrochemical potential gradient, possibly via K^+ channels. K^+ uptake is subject to negative feedback from internal K^+. Whether this is exerted by direct effects on the transporter or via transcriptional effects at the genetic level is not clear. As internal $[K^+]$ decreases during deprivation, influx increases until internal $[K^+]$ decreases to about 10×10^{-6} nmol cell^{-1} (after 18 h of K^+ deprivation). Beyond this critical value, influx declines and cells cease to swim.

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