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Active H^+ Efflux from Cells of Low-salt Barley Roots during Salt Accumulation

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ABSTRACT

Measurements were made of net H^+ loss from low-salt barley roots accumulating salt. Comparison of rates of loss from roots in different concentrations of KCl showed that H^+ loss increased in the same way as the Mechanism II component of salt uptake. This H^+ loss appeared to be coupled to salt uptake and was not due to increased respiration or metabolic breakdown of sugars. In view of the large negative potential of the cells (−60 millivolts), it is suggested that the H^+ loss is due to an outward proton transport process.

When low-salt barley roots grown on diluted $CaSO_4$ are transferred to salt solutions there can be a release of H^+ ions from the roots as salt is accumulated. This property of low-salt plants is well known and has been much studied (4, 5).

H^+ release occurs in other plant tissues, and in some appears to be an active process. From measurements of salt uptake by beet root cells and the intracellular potential, Poole (11) suggested that metabolic processes controlled HCO_3^- uptake and H^+ release. In *Hydrodictyon africanum*, HCO_3^- also appears to be transported by a metabolically controlled process into the cell (13), and, in *Nitella*, Kitosato has shown that H^+ is actively secreted (7). Spear, Barr and Barr (14) have also shown that this H^+ release is coupled with Cl^- influx.

The purpose of this paper is to show that in barley roots H^+ release is brought about by a metabolically controlled process operating against the electrochemical activity gradient of H^+. The net rate of release of H^+ appears to be correlated with Mechanism II uptake and with organic acid synthesis. It is suggested that H^+ release may be underestimated due to diffusion of HCO_3^- out of the cells, which would dissociate to OH^- and CO_2 in the solution.

MATERIALS AND METHODS

Seeds of barley (*Hordeum vulgare*, cv. Compana) were germinated on aerated 0.5 mM $CaSO_4$ at 23 C. When the seedlings were 5 days old the roots were excised for use in experiments. The pH of the $CaSO_4$ solution was 5.3 ± 0.2.

The excised roots were kept in aerated 0.5 mM $CaSO_4$ for 1 to 2 hr. The roots were then transferred to salt solutions; between 100 and 500 ml of solution were used with 1 g of roots. The pH of the solution was initially 5.3. After the roots had been added, 5-ml samples were taken at intervals to measure pH, and 5 ml of solution were replaced. When the pH reached 5.1 it, was titrated back to a value between 5.3 and 5.5 with KOH or NaOH in salt solutions having the same cation concentrations as those used in the experiment. A similar procedure with the use of salt-HCl solution was followed when pH became more alkaline. From the amount of KOH or HCl needed to adjust the pH, the H^+ equivalent to small pH changes within 5.3 ± 0.2 could be estimated.

The limit of measurement was 0.05 pH. For measurements made over 1 hr or longer, this pH adjustment was equivalent to approximately 0.05 to 0.25 μeq g^{-1} hr^{-1} of H^+ at pH 5.3 (depending upon the ratio of tissue to solution) and 0.6 μeq g^{-1} hr^{-1} at pH 4.2.

Contents of K^+ and Na^+ were determined by atomic absorption spectroscopy on acid solutions of ashed roots. Cl^- was determined by coulometric titration. All amounts of H^+, K^+, Na^+, and Cl^- are expressed on a fresh weight basis. Salt solutions contained 0.5 mM $CaSO_4$ in addition to the salt concentration given below. Respiration rates were measured by Warburg manometers, and sugar levels were determined by copper reduction as measured by arsenomolybdate color change.

RESULTS

Figure 1 shows the time course for H^+ release from low-salt barley roots transferred from 0.5 mM $CaSO_4$ to 10 mM KCl, 5 mM K_2SO_4, or kept in 0.5 mM $CaSO_4$. The pH of the solution was maintained at 5.3 ± 0.2, which was the pH of the solution in which the roots had been growing. After an initial period of 5 to 10 min the rate of H^+ release was constant. Previous work showed that this constant rate usually continued for up to 4 hr, when H^+ release became very small (8). Rates of H^+ release given in the present paper were calculated from the slope after the initial pH adjustment and were made during the first 1 to 2 hr of salt uptake. For example, in Figure 1 the rates of release into KCl, K_2SO_4 and $CaSO_4$ were, respectively, 4.5, 8.1, and 0.0 μeq g^{-1} hr^{-1}.

Similar measurements of H^+ release were made for low-salt roots transferred to KCl solutions of different concentrations. Results of several experiments are combined in Figure 2 by plotting the rate of H^+ release relative to that into 10 mM KCl. The rate of H^+ release into 10 mM KCl varied between 2 and 4 μeq g^{-1} hr^{-1}. Measurements were also made of Cl^- uptake from 10 mM KCl, which varied with H^+ release. The ratio of Cl^- uptake to H^+ loss was 2.6 ± 0.2 (standard error of the mean, 12 measurements).

In solutions below 0.5 mM, H^+ release was small, but not zero. Above 0.5 mM it increased proportionately to the logarithm of KCl concentration. Figure 2 includes estimates of Mechanism II uptake taken from a paper by Rains and Epstein (12), again plotted relative to the rate at 10 mM Cl^-. Mechanism II uptake at this concentration was 13 μeq g^{-1} hr^{-1} and Mechanism I uptake

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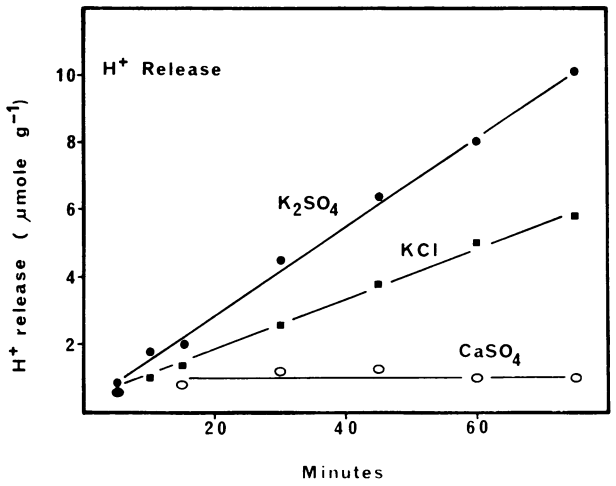

FIG. 1. H^+ release from excised low-salt roots grown in 0.5 mM $CaSO_4$ and transferred to 10 mM KCl (■), 5 mM K_2SO_4 (●), or 0.5 mM $CaSO_4$ (○), all at pH 5.3.

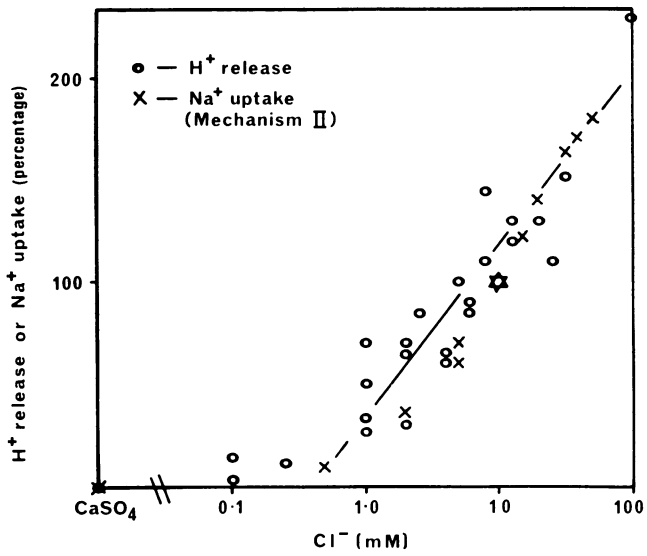

FIG. 2. Rate of H^+ release relative to that into 10 mM KCl plotted against Cl^- concentration on a logarithmic scale. Values of Mechanism II uptake (X) estimated from data of Rains and Epstein (12).

was about 7 μeq g^{-1} hr^{-1}. The data given in Figure 2 are for Na^+ uptake; nevertheless, there is good reason to think that Mechanism II uptake is the same for mixtures of KCl and NaCl or KCl alone. For example, total uptake of (K^+ + Na^+) or of Cl^- from 10 mM Cl^- solutions is independent of the K^+:Na^+ ratio (10). At this concentration Mechanism II should be dominant.

Both H^+ release and the Mechanism II uptake increase with concentration of the solution. At low concentrations when uptake was dominated by Mechanism I, net H^+ release was small. Even so, there may have been an H^+ efflux balanced by an OH^- (or HCO_3^-) efflux. At higher concentrations when Mechanism II was the dominant process, H^+ release increased proportionately to this component.

Figure 3 illustrates that the rate of H^+ loss from the tissue was independent of the ratio of K^+:Na^+ in 10 mM Cl^- solutions. Previous experiments have shown that Cl^- uptake at this concentration is also independent of the K^+:Na^+ ratio (10). H^+ release thus appears to be related to total salt uptake [*i.e.*, to (K^+ + Na^+) or to Cl^- uptake] and not specifically to K^+ uptake. On the basis of these results and those of Figure 2, it does not seem likely that H^+

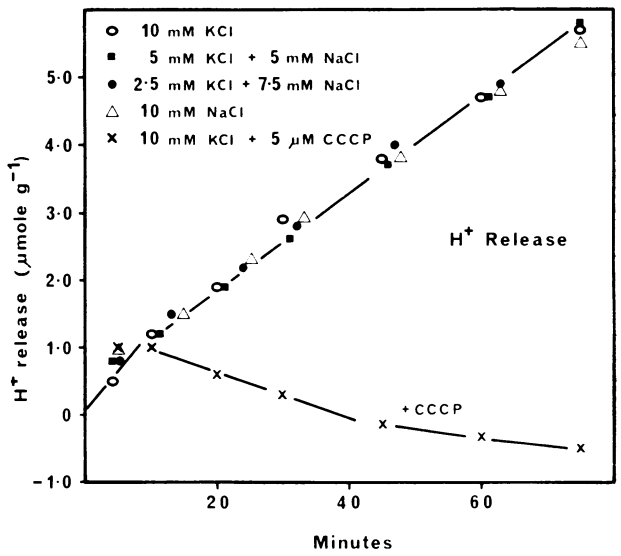

FIG. 3. H^+ release from excised low-salt roots into 10 mM KCl (○), 5 mM KCl + 5 mM NaCl (■), 2.5 mM KCl + 7.5 mM NaCl (●), 10 mM NaCl (△), and 10 mM KCl + 5 μM CCCP (X) all at pH 5.3, and containing 0.5mM $CaSO_4$.

Table I. *Effects of Inhibitors on Salt Uptake, H^+ Release, O_2 Uptake and Sugar Loss in Low-Salt Barley Roots*

Cl^- level initially 4 μeq g^{-1}. The estimated minimal rates detectable are shown in the same units as the rates of loss or uptake.

Solution	Cl^- Uptake	H^+ Release	O_2 Uptake	Sugar Loss
	μeq g^{-1} hr^{-1}		μmole g^{-1} hr^{-1}	
A. 0.5 mM $CaSO_4$	0	−0.1		
+ 10 mM KCl	6.1	2.85		
+ 2 μg/ml oligomycin	3.6	1.10		
+ 5 μM CCCP	−0.7	−0.7		
+ 100 μM arsenite	0.9	0.2		
+ 200 μM arsenite	0.6	−0.8		
B. 0.5 mM $CaSO_4$	0	−0.2		
+ 5 μM CCCP	−1.1	−0.9		
+ 0.1 mM KCl	2.2	+1.1		
+ 5 μM CCCP	−1.3	−0.8		
C. 0.5 mM $CaSO_4$	0		16.5	
+ 10 mM KCl	6.7		23.0	
+ 1 μM CCCP	1.2		23.5	
+ 3 μM CCCP	−0.2		22.0	
+ 5 μM CCCP	−0.5		29.0	
+ 100 μM arsenite	0.4		13.5	
D.[1] 0.5 mM $CaSO_4$	0			0.5
+ 10 mM KCl	6.4			5.5
+ 10 μM CCCP	0.3			4.5
+ 100 μM arsenite	0.6			0
+ 2 μg/ml oligomycin	1.1			1.5
Limit of detection (±)	0.1	0.1	1.0	0.5

[1] Data are from Pitman and Nair, unpublished results.

release is coupled to the selective K^+ uptake that takes place by Mechanism I.

Figure 3 and Table I show the effects of various inhibitors on H^+ release, and on salt uptake, O_2 uptake and sugar loss. In

Table II. *H^+ release and Discrepancy between Uptake of Cations ($K^+ + Na^+$) and Anions (Cl^-) during 4-Hr Period*

Solution	H^+ Release	Uptake Discrepancy ($K^+ + Na^+ - Cl^-$)
	$\mu eq\ g^{-1}$	
10 mM KCl, pH 5.3	14.0	14.5
2 mM KCl, pH 5.3	8.8	9.1
1 mM KCl, pH 5.3	6.3	5.9
0.1 mM KCl, pH 5.3	1.8	2.7
5 mM K_2SO_4, pH 5.3	29.0	35.5
10 mM KCl, pH 4.2	0.0	8.9

general, there is a parallel between H^+ release and Cl^- uptake which is not found between H^+ release and respiration. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)[2] which acts as an uncoupler of oxidative phosphorylation, did not reduce sugar consumption or respiration and so presumably did not reduce the flux of CO_2 out of the cell. However, CCCP was a potent inhibitor of H^+ release and salt uptake, and there was a loss of OH^- (or uptake of H^+) when salt uptake was inhibited. Conversely, sodium arsenite reduced respiration as well as salt uptake. This inhibitor blocks the tricarboxylic acid cycle at α-ketoglutarate and so reduces the substrate for oxidative phosphorylation. Both sodium azide and oligomycin are inhibitors of oxidative phosphorylation and of sugar breakdown. Thus H^+ release does not appear to be a product of sugar breakdown or loss of CO_2 from the cell, but like salt uptake, is controlled by oxidative phosphorylation.

Hiatt (2) has shown that the discrepancy between cation and anion uptakes by low-salt barley roots is equal to the increase in organic acid level in the sap. For solutions of pH 5.3, this discrepancy was also equal to the H^+ released from the tissue, as shown in Table II. Uptake of SO_4^{2-} from K_2SO_4 solution is very small compared with K^+ uptake, and organic acid synthesis provides the balancing anion (2). Both in KCl and K_2SO_4 solutions, H^+ release appears to be equivalent to organic acid formation. In order to maintain charge balance in the cell, the organic acid anion must have been produced at the expense of another anion. The simplest system would be that HCO_3^- ions are converted to organic acid anions instead of diffusing out of the cell.

At pH 4.2, there was some difference between cation and anion uptakes even though H^+ release appeared to be zero. The relatively large uncertainty in measuring H^+ release at this pH prevents accurate assessment of charge balance. Similar differences between K^+ and Br^- uptake at low pH can be seen in data from Hoagland and Broyer (4) and from Jacobson *et al.* (6). At pH over 6.0 to 6.5, H^+ release was difficult to estimate because titration included dissolved CO_2 as well as H^+ and so overestimated H^+ release.

The potential difference between vacuoles of cortical cells and the solution was measured with microelectrodes. The potential of the vacuole was negative to the solution by 110 $\pm$ 3 mv in 0.5 mM $CaSO_4$ (37 measurements) and by 55 $\pm$ 2 mv in 10 mM KCl (12 measurements).

DISCUSSION

The H^+ release measured during salt accumulation can be best interpreted as an active process. First, inhibitors of oxidative phosphorylation inhibit both H^+ release and salt transport. Second, the movement of H^+ appears to be against the electrochemical gradient. Roots in $CaSO_4$ solution are in H^+ equilibrium with a solution of pH 5.3 in spite of the potential difference of -110 mv between cell and solution. If this were a passive flux equilibrium the pH of the cytoplasm would have to be about 3.4. Roots in 10 mM KCl lose H^+ rapidly to the solution, again at pH 5.3, and similar calculations show that cytoplasmic pH would have to be at least 4.3 if this loss represented diffusion in response to the electrochemical gradient. These estimates of cytoplasmic pH seem ridiculously low, as many cytoplasmic enzymes would not operate at pH 3 to 4. In particular, the enzyme thought to be responsible for organic acid synthesis during salt uptake, phosphoenolpyruvate carboxykinase, requires a pH of 7 to 7.5 (1). This enzyme is in the soluble protein fraction and so should be controlled by cytoplasmic and not organelle pH levels.

Estimation of the rate of active H^+ transport is complicated because it is only one of several processes contributing to H^+ release. For example, efflux of organic acids, OH^- ions or HCO_3^- ions will all add to or subtract from apparent H^+ release. Figure 3 and Table I show that in 5 μM CCCP, when salt uptake is inhibited, there is a loss of OH^- from the roots. It is suggested that active H^+ efflux is also inhibited in CCCP and that this OH^- loss is due to diffusion of HCO_3^- ions from the cells (HCO_3^- dissociates to OH^- and CO_2 at pH 5.3). The HCO_3^- efflux during salt uptake is not known, but the OH^- loss from inhibited roots can be used as an approximation inasmuch as CCCP does not reduce breakdown of sugar to CO_2. Active H^+ loss, therefore, can be expected to be 1 to 2 $\mu eq\ g^{-1}\ hr^{-1}$ larger than net H^+ loss from the tissue.

An HCO_3^- efflux of 1 $\mu eq\ g^{-1}\ hr^{-1}$ is not unreasonably large. It is only 5 to 10% of the over-all loss of $[HCO_3^- + CO_2]$ and about the magnitude expected in comparison with fluxes of other univalent anions. For example, at pH 7.5, HCO_3^- concentration in equilibrium with atmospheric CO_2 at 20 C is 1.6 mM. As there is a large flux of CO_2 out of the cell the CO_2 concentration must be above atmospheric and HCO_3^- could well be larger than 1.6 mM. For a concentration of 2 mM HCO_3^- and permeability of 2×10^{-8} cm sec^{-1} (Cl^- permeability is 1.2×10^{-8} cm sec^{-1} (9)) it can be calculated with the use of the Goldman equation that the flux of HCO_3^- would be 0.4 $\mu eq\ g^{-1}\ hr^{-1}$.

On this interpretation of active H^+ efflux, cells of roots in $CaSO_4$ solution may have an active H^+ efflux balanced by a diffusive loss of HCO_3^-, each of about 0.5 to 1.0 $\mu eq\ g^{-1}\ hr^{-1}$. In solutions of higher concentrations of KCl (*e.g.*, 10 mM) the active H^+ efflux increases to about 3 to 5 $\mu eq\ g^{-1}\ hr^{-1}$, whereas HCO_3^- loss stays at about 1 $\mu eq\ g^{-1}\ hr^{-1}$ (*cf.* Table I).

The loss of H^+ is not an artifact of cutting as it is shown by roots of whole plants transferred from $CaSO_4$ to salt solution.

It has been suggested (5) that H^+ efflux may be coupled with K^+ uptake. The present results do not contradict this view, but do set certain limits on coupling between salt uptake and H^+ transport.

First, H^+ release is much smaller than cation uptake from KCl solutions by a factor of 2 to 3 but, very nearly the same as cation uptake from K_2SO_4 solutions. One-to-one coupling of H^+ and cation requires a one-to-one coupling of OH^- (or HCO_3^-) efflux with Cl^- uptake, which is suppressed in SO_4^{2-} solution. An alternative would be that H^+ release is related to organic acid synthesis, which is high during K^+ uptake from K_2SO_4 and equal to ($K^+ - Cl^-$) uptake from KCl solution.

Second, H^+ release does not seem to be coupled to the K^+-selective Mechanism I uptake of cations. H^+ release increased with increasing Mechanism II uptake (Fig. 2) and was the same into mixtures of KCl and NaCl (Fig. 3), and into NaCl alone when Mechanism I should have been low. Moreover, H^+ release is not a general property of barley roots in salt solutions, but is most evident when there is a large net uptake of salt by low-salt

[2] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone.

roots. Salt-saturated roots show a low rate of H^+ release even though the active transport of cation into the roots is continuing (8, 10).

Whatever the relation between H^+, K^+, and Cl^- transport, it is suggested that the large H^+ release from low-salt roots in KCl solution is a sign of a temporary disturbance of the regulation of cell processes. Cells of low-salt roots in $CaSO_4$ solution are considered to be in a balanced state, with a steady low-salt content and high sugar level, and with H^+ active efflux balancing HCO_3^- diffusion out of the cell. After a period in salt solution the cells again reach a state of balance, but now with a higher level of salt in the cell at a flux equilibrium and again with H^+ efflux balancing HCO_3^- loss in respiration. Low-salt roots in salt solution can be thought of as in transition between two different states of control. It has been shown (8) that this transition is marked by stimulated salt uptake, respiration and sugar breakdown, altered selectivity for K^+ *versus* Na^+ and a less negative cell potential. The present results show that an increased active efflux of H^+ is also a property of this transitional phase, and raise the question that Mechanism II behavior may be induced by the disruption of a state of balance in low-salt roots, and may not be an intrinsic property of all roots.

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