Plant Physiol. (1976) 58, 210-213

Kinetics of Hormone-induced H^+ Excretion[1]

Received for publication October 23, 1975 and in revised form March 12, 1976

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ABSTRACT

A study has been made of the kinetics of hormone-induced H^+ excretion from peeled *Avena* coleoptile sections using a new, simple technique involving direct application of the pH electrode to the surface of the section. Hormone-induced H^+ excretion begins after lags and occurs at rates which are consistent with a role of H^+ in regulating cell elongation. With fusicoccin, H^+ excretion begins within the 1st minute, and an external pH of 5 (optimal for wall loosening) is reached in 5 to 8 minutes, while with auxin the lag averages 14 minutes and pH 5 is reached in 20 to 30 minutes. KCN, which inhibits cell elongation in 3 to 5 minutes, stops H^+ excretion in less than 1 minute, whereupon the external pH rises sharply. Cycloheximide stops auxin-induced H^+ excretion in 3 to 8 minutes, and the pH then rises slowly. In the absence of hormones, the pH of the extracellular solution comes to equilibrium at 5.6, but the actual pH of the wall solution is probably about 0.3 unit below this due to Donnan effects.

The acid growth theory (7, 12, 19, 20) states that cell wall loosening, and thus cell elongation, is controlled by the pH of the wall solution. The hormones auxin and FC[2] induce rapid cell elongation by causing cells to excrete protons, thereby lowering the pH of the wall solution and permitting cell wall loosening to occur. There is considerable evidence to support this theory (3, 7, 12, 18-20, 25), but recently questions have been raised concerning the kinetics of hormone-induced H^+ excretion (14, 16, 21, 22). If the H^+ excretion is the cause of the cell elongation the hormone-mediated H^+ excretion must be demonstrated to start at the same time or before the onset of rapid growth. In the original reports the lags prior to the onset of H^+ excretion were 5 and 20 min, respectively, for FC (4) and IAA (3, 18), while the lags prior to growth were 2 and 10 min (4, 17).

Because H^+ excretion can be detected only after the protons have diffused to the electrode, and only after sufficient protons have been excreted to cause a measurable decrease in pH, the time needed to measure H^+ excretion will depend, in part, on the technique used. In the original reports (3, 12, 18) the glass pH electrode was located some distance from the excreting surface, and the volume of the external solution was large as compared with the extracellular, intratissue solution (at least 100 times larger). In order to assess better the rapidity of H^+ excretion the extracellular volume must be kept at a minimum, and the electrode must be positioned as close to the excreting surface as possible. Microelectrodes implanted into the extracellular spaces can be used (*e.g.* see ref. 10), but they are difficult to use. Instead, a simple technique has been developed which allows minimal solution volumes to be used, and places the electrode directly in contact with the tissue. It will be shown that the kinetics of H^+ excretion measured with this technique are those expected if cell elongation is regulated by the pH of the cell wall solution.

MATERIALS AND METHODS

The plant material consisted of 15 to 20 mm sections cut from 25 to 32 mm coleoptiles of *Avena sativa* L. cv. Victory. Seedlings were grown as described earlier (2). In most cases the leaves were not removed. The sections were peeled by removal of epidermal strips with fine forceps (18) and were then preincubated for at least 1 hr under conditions which permit optimal H^+ excretion; 10 mM K_2SO_4 for FC, and 1 mM K_2SO_4 + 1 mM $CaSO_4$ for IAA (unpublished data). Then seven sections were lined up on a glass slide, the ends were anchored with small pieces of glass and rubber bands, and a drop of the preincubation solution was placed on the sections (Fig. 1). An Ingold 6020 flat surface combination pH electrode, connected to a Beckman Zeromatic pH meter and a recorder, was allowed to rest by its own weight on top of the sections and the recording was started. When a constant pH was obtained in the absence of hormones, the solution was drawn off with a Kimwipe and replaced by a drop of hormone-containing solution whose pH had been adjusted to approximate the pH of the removed solution. Inhibitors were added in a similar fashion. Solutions used were 10 mM K_2SO_4 ± 10 μM FC, or 1 mM K_2SO_4 + 1 mM $CaSO_4$ ± 10 μM IAA. The inhibitors KCN (1 mM), CHI (10 μg/ml), and CCCP (10 μM) were used at concentrations that cause maximal inhibition of growth. The experiments were carried out at a temperature of 22 ± 1 C.

Because of the geometry of coleoptile tissues almost all of the protons appearing in the external solution must be excreted from the outer side of the outermost layer of parenchyma cells. This surface has been prepared by tearing the epidermal cells away from it, and the ability of this surface (but not necessarily the rest of the tissue) to excrete protons must depend upon the amount of damage it sustains in the peeling process. We have found that excretion is enhanced if the sections are fully turgid before being peeled, and if care is exercised to make certain that a complete peel is removed from each side of the coleoptile. The apparent rate of H^+ excretion is also a function of the amount of external solution. If this volume is too low the electrode gives erratic readings, and if too high the speed of the pH drop is reduced. For each electrode one must experiment until the optimum solution size is determined. Even with these precautions there have been runs in which no H^+ excretion could be detected; the reasons for this variability are still under study.

In this paper the term H^+ excretion is used to indicate any energy-requiring acidification of the external solution. This acidification could be due to excretion of protons or uptake of hydroxyl ions, but not to respired CO_2 (23) as the acidification persists after the solutions have been bubbled with N_2 or boiled (data not shown).

RESULTS

The direct contact method has been used to assess the kinetics of H^+ excretion following addition of hormones and metabolic

[1] This research was supported by Contract AT(45-1)2225-T19 from the United States Energy Research and Development Administration.

[2] Abbreviations: FC: fusicoccin; CHI: cycloheximide; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone.

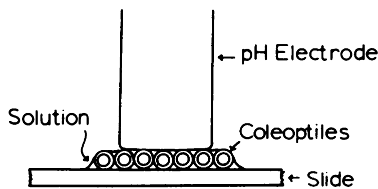

FIG. 1. Technique for measuring H^+ excretion using the direct application of a combination pH electrode to the surface of peeled *Avena* coleoptiles. Seven peeled sections are placed on a glass slide with a drop of 10 mM K_2SO_4 or 1 mM K_2SO_4 + $CaSO_4$. An Ingold 6020 flat surface combination pH electrode is allowed to rest on the upper surface of the sections under its own weight, and the pH readings are recorded.

inhibitors. Typical recordings showing the effect of auxin and FC on the external pH are presented in Figure 2, and the average values obtained from a series of pH recordings are shown in Table I. The pH of the external solution before addition of hormones stabilized at an average value of 5.6. Upon addition of FC the pH began to drop after a lag that averaged 1 min, and within 10 min the pH had fallen a full pH unit. A pH of 5, optimal for *in vitro* cell wall loosening of *Avena* coleoptile walls (18), was reached in only 5 to 7 min, and a new equilibrium pH below 4.5 was achieved within 30 min.

Auxin caused detectable H^+ excretion with a lag that averaged 14 min, but was often as short as 10 to 11 min. The pH then dropped more slowly than with FC, reaching pH 5 after 20 to 30 min, and a new equilibrium at about pH 4.8 took nearly an hr to achieve.

In contrast to the effect of hormones, metabolic inhibitors caused an immediate rise in the external pH. The effect of KCN is illustrated in Figure 3. Sections were pretreated with either IAA or FC, then the recording was started and when the external pH had fallen to 4.8 to 5 the solution was replaced with one containing 1 mM KCN, preadjusted to the same pH. In both cases the pH began to rise sharply, with a delay of at most 30 sec. The pH rise was more rapid in the presence of IAA, with a pH of 5.5 being reached in 5 to 8 min and the final equilibrium pH being 6.2 to 6.8. With FC, on the other hand, the equilibrium pH was only 5.2 to 5.5. The pH rise was not simply due to volatilization of KCN, as the pH of a drop of solution without sections hardly changed over the same time period. An immediate pH rise was also produced by 10 μM CCCP (data not shown).

The results with the protein synthesis inhibitor CHI were somewhat different. CHI at 10 μg/ml was added to auxin-treated sections as soon as the pH had dropped to 5 (20–30 min). The pH continued to drop for several min, then leveled out, and finally began to rise (Fig. 4). The time between addition of CHI and the start of the pH rise averaged 4.8 min with a range of 3 to 8 min. However, if the sections had been pretreated with IAA for longer periods (2–5 hr) it took closer to 10 min before the CHI-induced pH rise began (Table II). As shown earlier (4) FC is still active in inducing H^+ excretion even in the presence of CHI (Fig. 4).

One surprising aspect of these results was that in about 35% of the runs the equilibrium pH, in the absence of hormones, was around 5.3 rather than 5.6 (Fig. 5). The *in vitro* acid growth studies (18) would predict that these sections would have a more rapid endogenous growth rate than they actually show. A possible explanation is provided by Rubery and Sheldrake (21) who have obtained evidence from auxin uptake studies that the pH of the wall solution is up to 0.5 pH unit lower than that of the external solution due to the zeta potential (8); *i.e.* the Donnan effects of the wall carboxyl groups. Thus in those cases where the equilibrium pH of the 5.3 was found we may have been measuring the pH of the wall solution rather than that of the external solution. If this is correct, a decrease in pH should be recorded whenever the electrode, immersed in a weak pH 6 solution, is lowered into contact with the walls. Such a pH drop has been detected both with live sections and with frozen-thawed, pronase-treated cell walls, but does not occur with Whatman No. 1 filter paper which should have no zeta potential (Fig. 6). This

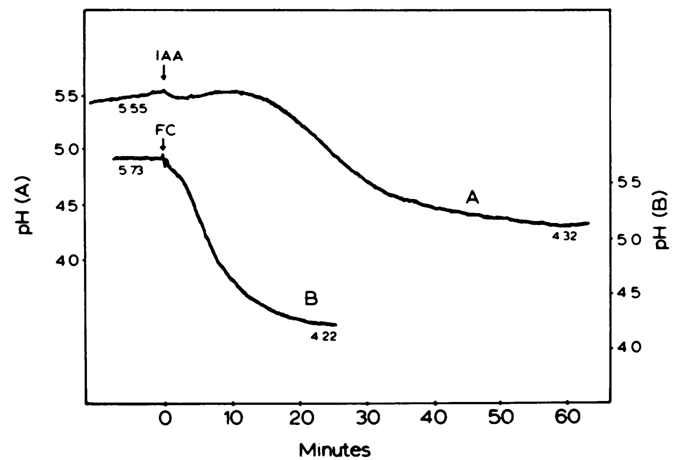

FIG. 2. Rapidity of H^+ excretion following application of IAA or FC as measured by the direct contact technique. Peeled sections were preincubated for at least 1 hr in water then placed on a slide in 1 mM K_2SO_4 + 1 mM $CaSO_4$ (curve A) or 10 mM K_2SO_4 (curve B) and the pH was continuously recorded. When the pH had come to equilibrium the solution was replaced with solutions containing 10 μM IAA (curve A) or 10 μM FC (curve B) and the pH recording was continued. In these examples the lag prior to the pH drop was 12 min for IAA and less than 1 min for FC. Initial and final pH values for each curve are marked on the graph.

Table I. *Parameters of IAA- and FC-induced H^+ Excretion and Growth*

Sections were pretreated at least 1 hr in 10 mM K_2SO_4 (FC) or 1 mM K_2SO_4 and $CaSO_4$, then direct contact pH reading was started, and when the pH had reached equilibrium, the solution was replaced with one containing 10 μm FC or IAA. Values are averages for 12 IAA and 8 FC runs. The range of values is given in parentheses.

Parameter	IAA	FC
Lag, pH drop, min	14 (10–21)	1 (0–2)
Lag, elongation, min	14 (12–17)[1]	2 (1–3)[2]
pH decrease, pH units/10 min	−0.37 (0.08–0.60)	−1.05 (0.85–1.25)
Initial pH	5.64 (5.35–5.90)	5.79 (5.55–6.00)
Final pH	4.79 (4.30–5.23)	4.46 (4.20–4.69)

[1] Unpublished data, M. Hoyt and R. Price.
[2] Ref. 4.

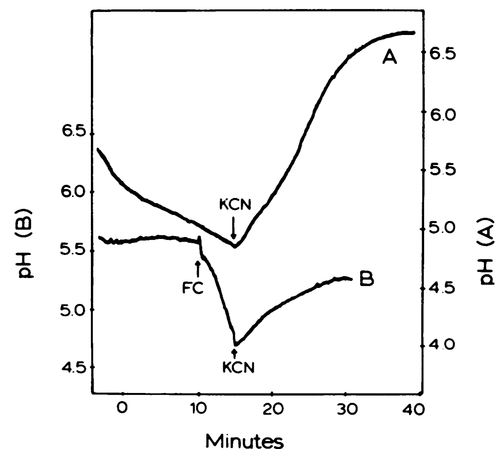

FIG. 3. Effect of KCN on H^+ excretion from *Avena* coleoptile sections. Curve A: sections pretreated 2 hr in 1 mM K_2SO_4, 1 mM $CaSO_4$ + 10 μM IAA, then recording started. Curve B: sections pretreated 1 hr in 10 mM K_2SO_4, recording started and 10 μM FC added when pH was constant. Solutions were exchanged for identical ones containing 1 mM KCN, preadjusted just prior to use to nearly the same pH as the previous solution. Note that both the rate and magnitude of the pH rise are greater for the IAA-pretreated sections.

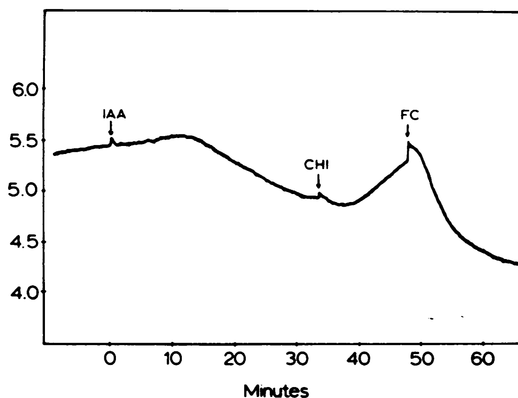

FIG. 4. Effect of CHI on H^+ excretion. Sections were pretreated 1 hr in 1 mM $CaSO_4$ + 1 mM K_2SO_4, recording was started, and IAA (10 μM) added when pH constant. When the pH had dropped to 5 the solution was changed for one containing 10 μg/ml CHI, preadjusted to 5. FC (10 μM) was added when pH had risen to 5.3. Note that CHI stops auxin-induced H^+ excretion after a lag of about 5 min in this case, and that it does not prevent FC-induced H^+ excretion.

Table II. *Effect of Preincubation Time in IAA on Speed of CHI Inhibition of H^+ Excretion*

Sections were pretreated 20 to 300 min in 10 mM KCl + 1 mM $CaCl_2$ + 10 μM IAA, then direct contact pH reading was started and when pH had fallen to 5, the solution was changed for one containing 10 μg/ml CHI. Times given are for leveling off of pH prior to pH rise.

Pretreatment Time	Inhibition Time	
	Average	Range
	min	
20-30	4.8	3-8
120-300	9.4	5-17

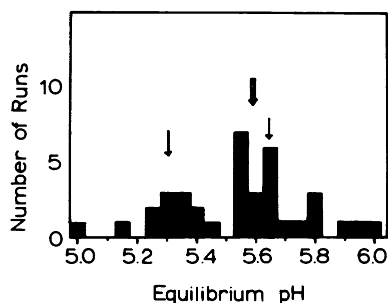

FIG. 5. Distribution of 37 equilibrium pH values for nonhormone-treated sections using the direct contact method to measure pH. Note that the pH values cluster into two groups: a major one averaging 5.65, and a smaller group at 5.3 (single arrows). The average for all values (double arrow) is just below 5.6.

suggests that at any external pH above about 4.5 the wall solution pH will be about 0.3 pH unit below that of the external solution with which it is in equilibrium.

DISCUSSION

Knowledge of the kinetics of hormone-induced H^+ excretion could help to confirm or reject the acid growth theory for the control of cell elongation. To obtain this information a technique is needed which combines placement of the pH electrodes next to the excreting surface plus a minimal volume of extracellular solution, with ease and reproducibility. Unfortunately, no such technique is currently available. Microelectrodes implanted within the tissue can be used (10, 14, 22) but are not easy to use, and one must be careful to eliminate artifacts. The technique used here is particularly simple, and with any care gives reproducible results. Its disadvantage is that the electrode is still removed from the excreting surface by a significant and apparently variable distance. In most cases the pH measured is that of the external solution just outside of the cell wall, but in some cases it appears that the pH of the wall solution is actually measured, and in these cases the pH readings are about 0.3 pH unit lower due to the zeta potential of the walls. In any case, the distance between the excreting surface and the electrode will mean that a lag can occur between the start of H^+ excretion and its detection by the electrode, and this lag can be significant when the rate of H^+ excretion is low.

How do the kinetics of H^+ excretion as measured by this technique compare with the kinetics of cell elongation? Studies on the pH-dependence of cell wall loosening (18) in isolated *Avena* coleoptile walls indicate that minimal wall loosening occurs at solution pH values of 5.8 or above, and maximal wall loosening occurs at solution pH values below 5 (in each case the actual wall solution pH will be 0.3 pH unit lower). The observed equilibrium pH of 5.6 in the absence of hormones should permit only the low endogenous elongation rate which is observed. Upon addition of FC both H^+ excretion (Fig. 2) and cell elongation (4) increased with a lag of about 1 min. The external pH dropped to 5 in 5 to 8 min (Fig. 2), a time that corresponds well to the time needed to reach maximal growth rate (4). With IAA the lag prior to the onset of both H^+ excretion (Table I) and cell elongation (M. Hoyt and R. Price, unpublished data) averaged 14 min for peeled coleoptiles. This lag is longer than the usually reported 10 min (5, 6, 17), at least in part, because these experiments were conducted at 22 C rather than 25 C (15, 17). Under these conditions an additional 10 to 15 min was needed before the maximum growth rate was attained (unpublished data). A similar time interval was needed before the external pH dropped to 5.

KCN inhibited auxin-induced cell elongation after a lag of only 1 to 2 min, and totally blocked growth after 5 to 7 min (Cleland, unpublished data; ref. 17). H^+ excretion was blocked with at most a 30-sec lag, and a pH of 5.5 was reached in 5 to 8 min (Fig. 3). The apparent absorption of H^+ into the tissue in the presence of KCN would have caused the wall pH to rise more rapidly than that of the external solution, which would account for the fact that the growth ceased just prior to the external solution reaching a pH of about 5.7. It is interesting to consider why the pH rises in the presence of KCN. The plasma mem-

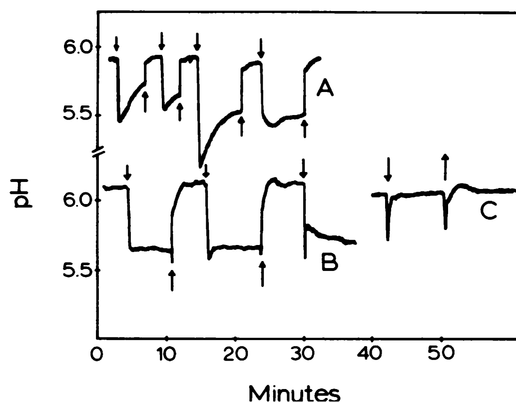

FIG. 6. Demonstration that the pH of the wall, measured by direct contact method, is lower than that of the bulk solution with which it is in equilibrium. Curve A: live sections immersed in 0.1 mM KCl, pH 5.9. Electrodes placed directly on sections at ↓ and raised off the sections, but still in the solution at ↑. Similar results are obtained for frozen-thawed, pronase-treated walls (curve B) but not for Whatman No. 1 filter paper (curve C). Conditions were the same for all three curves.

brane, which normally appears to be relatively impermeable to protons (9), might be rendered permeable by KCN with the result that the protons are taken up by the cell. Alternatively, KCN may increase the efflux of OH^- or other basic substances. Further studies will be needed to settle this point.

The mode of action of CHI in *Avena* coleoptiles is somewhat of an enigma. CHI blocked protein synthesis in this tissue within 3 min (1), but had no rapid effect on any other process tested to date with coleoptiles, including respiration or K^+ uptake (unpublished data); this suggests that CHI is a specific inhibitor of protein synthesis in coleoptiles. Auxin-induced growth was inhibited by CHI after a lag of about 10 min, with maximum inhibition occurring in 40 to 60 min (1). This led to the suggestion that a labile protein, called a growth-limiting protein (1, 13), is required for some aspect of auxin-induced cell elongation, and Rayle (18) and Cleland (3) have demonstrated that it is the H^+ excretion step which is sensitive to CHI. It was shown here that inhibition of proton excretion can be detected within 2 to 3 min after addition of CHI; *i.e.* at virtually the same time that protein synthesis is blocked. This presents a dilemma. Auxin-induced H^+ excretion might require a protein which is used up immediately upon synthesis; such a rapidly labile protein is required for DNA synthesis in chick red blood cells, for example (24). But it is difficult to fit such a protein into the plasma membrane ATPase scheme which is currently favored as the mechanism for auxin-induced H^+ excretion (7, 11). Thus the possibility that CHI exerts its inhibitory effects in some fashion unrelated to protein synthesis must still be considered.

Why have some other investigators failed to find H^+ excretion which correlates with cell elongation (14, 16, 22)? The fact that similar results were obtained in this study with a direct contact, macroelectrode technique and by Jacobs and Ray (10) with microelectrodes implanted in the tissue makes it unlikely that our results are due to some peculiarity of the experimental setup. The volume of solution in contact with the pH electrode directly affects the results. As Penny *et al.* (14) placed their microelectrode only 20 μm into the xylem vessel of an oat coleoptile it is not clear that the electrode was not simply measuring the pH of the flowing external solution rather than any free space pH. The condition of the outer, peeled surface is also important if the pH electrode is located in the external solution. The failure of Pope *et al.* (16) to find auxin-induced H^+ excretion may have been due to damage to this surface during the peeling of their coleoptiles. In any case, the close correlations between the timing and amount of hormone-induced proton excretion recorded here and by Jacobs and Ray (10) provide additional evidence to support the acid growth theory for the control of cell elongation in *Avena* coleoptiles.

Acknowledgments – The technical assistance of T. Lomax is gratefully acknowledged. The fusicoccin was kindly provided by E. Marrè.

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