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Auxin Has No Effect on Modification of External pH by Soybean Hypocotyl Cells[1]

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LARRY N. VANDERHOEF,[2] JANICE S. FINDLEY, JOHN J. BURKE, AND WAYNE E. BLIZZARD
Department of Botany, University of Illinois, Urbana, Illinois 61801

ABSTRACT

The cellular adjustment of the pH of the external environment of soybean (*Glycine max*) hypocotyl elongating cells, frequently assumed to be hydrogen ion secretion when the pH is lowered, is unaffected by auxin. These elongating cells actively adjust the external hydrogen ion concentration (from any pH in the range of 4–8) to pH 5.4 + 0.2. This pH adjustment occurs in a medium which does not contain potassium. Growth-optimum auxin concentrations have no effect on cellular pH adjustment of the external medium, whether added at the beginning of the experiment or after the equilibrium pH is attained. The pH adjustment by the cells occurs rapidly and in spite of the presence of a cuticle.

The acid growth hypothesis (3, 9, 19) is clearly supported by evidence that coleoptile (2, 7, 8, 18), pea (1, 6, 14, 15), and soybean segments (20) are induced to increase their elongation rate when the pH is decreased, *e.g.* from pH 6 to pH 4. Data comparing the kinetics of auxin-induced medium acidification to the kinetics of auxin-induced elongation, also described in support of the hypothesis, come from experiments with corn (12), *Avena* (3-5, 19), and pea (12).

These experiments characterize medium pH adjustment by segments from the elongating region of soybean hypocotyl, and the effect of auxin on this process. It was determined that pH adjustment is not affected by the hormone. These data and the determination of the accompanying report (20) do not support the acid growth hypothesis of auxin action.

MATERIALS AND METHODS

Soybean seedlings (*Glycine max* L. Merr. var. Wayne) were germinated in the dark and the elongating segment excised as previously described (23).

Medium pH was measured with a Beckman pHasar I pH meter. Hypocotyl segments were incubated (one segment/2 ml) for 1 hr at various pH values in distilled H_2O continually adjusted to the desired pH with HCl or KOH. Segments were then transferred to KOH- or HCl-adjusted distilled H_2O of identical pH (10 1-cm segments/ml) and the pH recorded every 2 min (see Fig. 1). In other experiments (Figs. 2–4), for easier comparison to previous work, segments were preincubated (one segment/2ml) in 1 mM K-phosphate (pH 6) for 1 hr (preincubation periods of 0.5–3 hr gave identical results) then transferred to 1 mM K-phosphate (pH 6) (10 segments/ml). The pH was recorded at 2-min intervals.

Figures 2 to 4 are average plots of five or more experiments. Figure 1 is a representative experiment. Additional statistical information is given in the figure legends.

The auxin used in these experiments was 45 μM 2,4-D. This compound, at this concentration, has effects identical to IAA, including induction of medium acidification in *Avena* (19), and the dual elongation response (21, 22, 24) in soybean hypocotyl (Lu and Vanderhoef, unpublished).

Electron microscopy was used to determine the presence of a cuticle on these elongating segments (see Fig. 5). One-mm segments were excised from the elongating region of light-grown (900 ft-c) and dark-grown soybean seedlings. The segments were fixed (2 hr at 4 C) in 1% glutaraldehyde (Polysciences, Warrington, Pa.) buffered with 85 mM Na-phosphate (pH 6.8), washed with 100 mM Na-phosphate (pH 6.8) and postfixed (1.5 hr at 25 C) with 2% OsO_4 in 50 mM Na-phosphate (pH 6.8). The segments were dehydrated in a graded acetone series and embedded in a mixture of low viscosity resins. Sections were cut with a diamond knife on a LKB-Huxley Mark 2 ultramicrotome, poststained with 2% aqueous uranyl acetate followed by lead citrate, and observed with a Jeol Jem 100 C electron microscope.

RESULTS AND DISCUSSION

Excised elongating soybean hypocotyl segments adjusted the external hydrogen ion concentration from any pH in the range 4 to 8 to pH 5.4 ± 0.2 (Fig. 1). This occurred rapidly (about 90% complete in 20 min, 10 1-cm segments/ml), and in spite of the presence of a cuticle (Fig. 5, see discussion below).

This adjustment of the external pH, specifically reduction from pH 6.5–8 to pH 5.5, is not entirely because of the action of the plant cells. The simple dissolution of atmospheric CO_2 in stirring water will cause the pH of water alone, adjusted to pH 8 with 10 mM KOH, to drop, via carbonic acid formation, to about 6.7 in 60 min. The plant cells are very much involved, however, because first, the pH drops from pH 8.03 to pH 5.58 (not 6.7) when plant cells are present (Fig. 1); and second, the pH of rapidly stirring water, adjusted to pH 4 with 0.01 M HCl, will not change significantly in 60 min. (In the presence of plant cells [10 1-cm hypocotyl segments/per ml], the pH will go from 3.99 to 5.17 in 60 min [Fig. 1].)

The following experiments which characterized this cellular activity (altering the external pH) were modified slightly from the above experiments so that they could be more easily compared with those of previous workers (3, 19), *i.e.* alteration of the external pH was measured in the presence of 1 mM K-phosphate. Experiments were performed to determine if there were complicating effects of potassium and/or phosphate ions on cellular adjustment of the external pH. Adjustment of pH in 1 mM K-phosphate was compared to pH adjustment in 1 mM tris buffer, adjusted to pH 6 with crystalline MES (Fig. 2). Although in this group of experiments the pH decline (from about 6.04 to about 5.68 in 25 min) was slower than usual, there was no

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[2] To whom correspondence should be addressed.

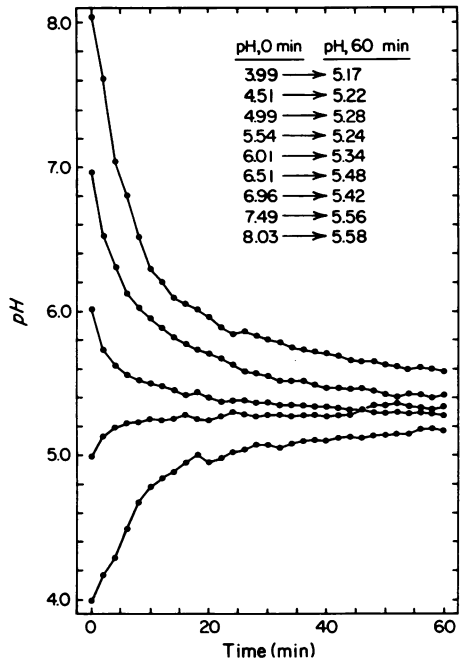

FIG. 1. Hypocotyl cells adjust the pH of their external medium. One-cm elongating hypocotyl segments were preincubated for 1 hr in distilled H_2O which had been adjusted to, and was maintained at, the beginning pH. The segments were then transferred to an identical solution and the pH was measured at 2-min intervals.

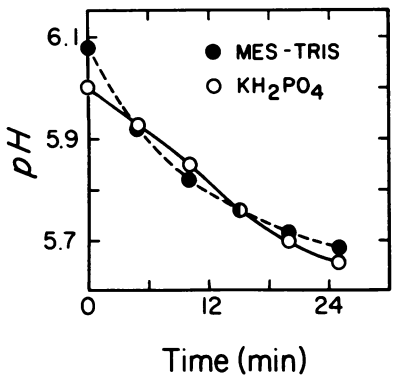

FIG. 2. Comparison of cellular adjustment of the external medium in the presence and absence of K. One-cm elongating hypocotyl segments were incubated (one segment/2 ml) for 1 hr in 1 mM buffer (K-free MES-tris, or K-phosphate) then transferred to an identical solution (10 segments/ml.) The pH was monitored at 2-min intervals. The experiment was repeated six times. The range for the zero time determinations was ± 0.06. Standard errors for all other data points ranged from 0.02 to 0.05.

difference between the tris-MES and K-phosphate treatments. These data and those of Figure 1, although not conclusive (there may be a significant amount of K^+ in the tissue free space, see ref. 10), allowed us to proceed with the following auxin experiments, with some assurance that the external pH adjustment was probably not simply a secondary effect of, or strongly coupled to, K^+ uptake.

The acid growth hypothesis asserts that auxin induces acidification of the cell wall. There were no circumstances in which auxin affected pH adjustment in soybean hypocotyl. For example, when the cells adjusted the external pH from 6.04, the final 60 min pH was 5.47 in the absence of auxin, and 5.44 in the presence of auxin (Fig. 3). Similarly, adding auxin after an equilibrium pH (pH 5.46 at 30 min, down from about pH 5.98 at 0 min) was attained had no effect on the subsequent pH; it was 5.51 at 70 min when auxin was added, and 5.48 at 70 min when water was added (Fig. 4).

It has been assumed that the cuticle is a significant barrier in experiments which attempt to measure the pH of the cell wall by measuring the pH of the surrounding medium (4, 6, 19). The major evidence for this conclusion is the correlation between cuticle removal or fraction and the enhanced effect of external protons on cell elongation (*e.g.* 6, 19). Soybean hypocotyl segments, with no cuticle removal, readily adjust the pH of the medium (Figs. 1-4), and readily respond to low pH by showing an increased rate of elongation (20, 21). These results suggest that perhaps these segments have no cuticle, not an unexpected finding for tissue grown in high humidity. In fact, however, they have an easily recognizable cuticle (Fig. 5). No one has yet published direct evidence that the cuticle alone restricts proton passage. In fact, the evidence of Figures 1 to 4 indicates that it may not be a significant barrier to the mechanism by which cells adjust the external pH.

The mechanism by which cells adjust their external pH has not been established, although active proton secretion is frequently assumed. Whatever the mechanism, measuring the pH in the surrounding medium is not an accurate measure of H^+ ion

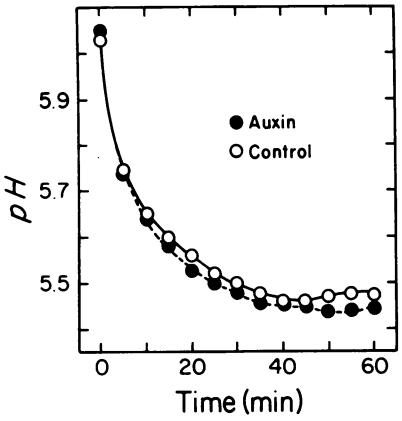

FIG. 3. Comparison of cellular adjustment of the external medium in the presence and absence of auxin. One-cm elongating hypocotyl segments were preincubated for 1 hr (one segment/2 ml) in 1 mM K-phosphate (pH 6), then transferred to the identical solution (10 segments/ml) with and without 45 μM auxin. The pH was then monitored for the next 60 min. The experiment was repeated five times. Standard errors for all data points ranged from 0.04 to 0.07.

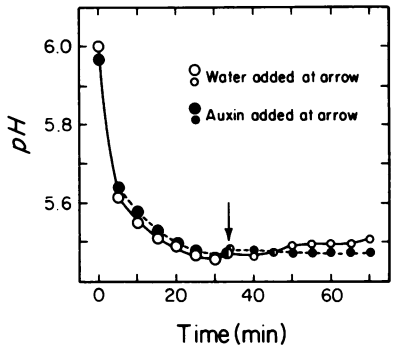

FIG. 4. Effect of the addition of auxin after cellular adjustment of the external pH to the equilibrium pH. One-cm elongating hypocotyl segments were preincubated for 1 hr (one segment/2 ml) in 1 mM K-phosphate (pH 6), then transferred to an identical solution (10 segments/ml). After the equilibrium pH was attained, 1 ml of the solution was withdrawn and 1 ml of auxin in K-phosphate (pH 5.5) was added to give final auxin and K-phosphate concentrations of 45 μM and 1 mM, respectively. The experiment was repeated six times. Standard errors for all data points ranged from 0.03 to 0.06.

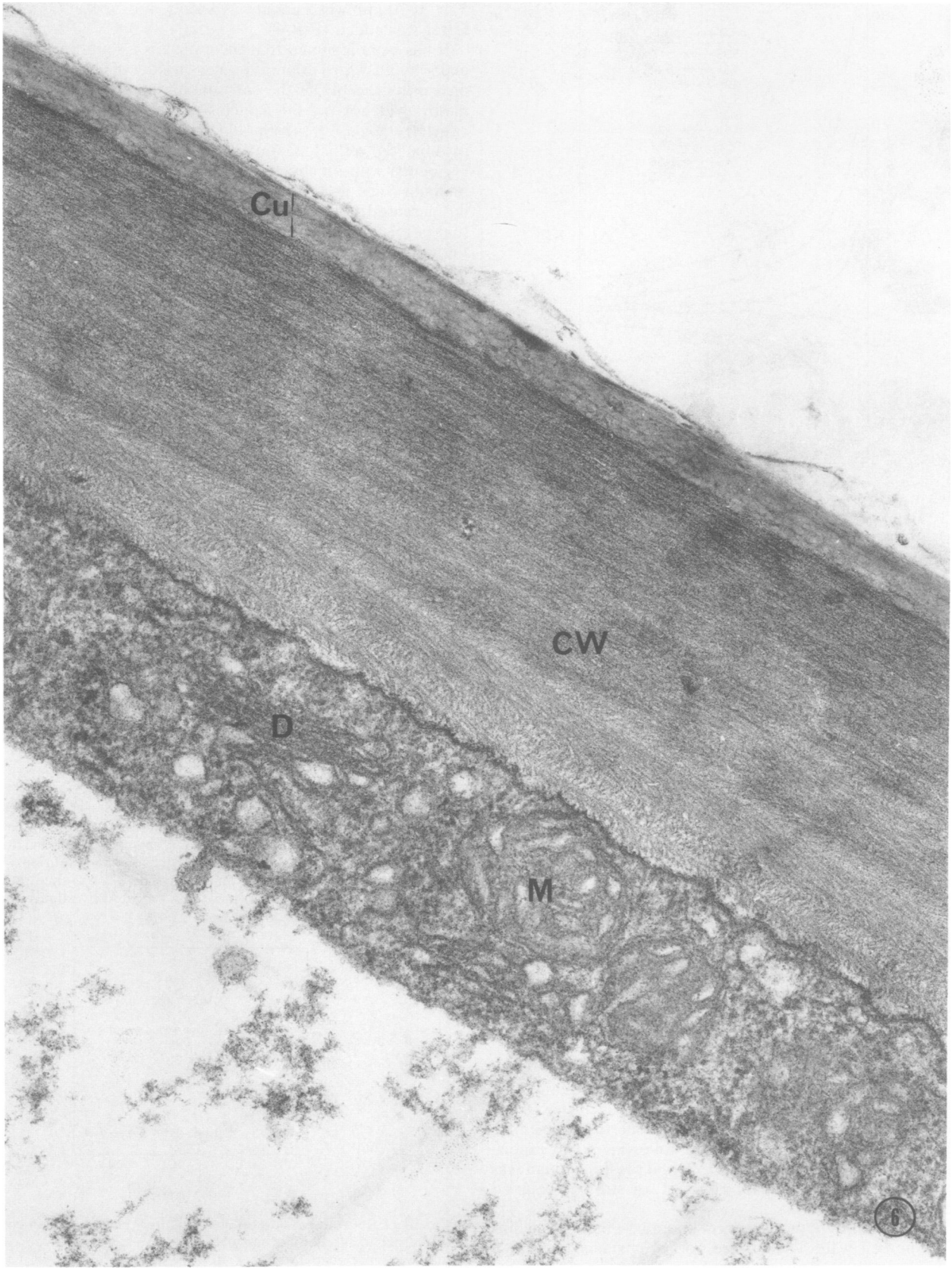

FIG. 5. Cuticle of light- and dark-grown soybean hypocotyl epidermal cells. An amorphous cuticular layer (Cu) approximately 140 nm thick can be observed on light-grown hypocotyl. Electron-dense fibers extend from the cell wall (CW) to the outermost regions of the cuticle. Mitochondria (M) and a Golgi stack (G) are also present in this view. ×83,800. An electron micrograph of the epidermal cell wall from the hypocotyl of a 3-day-old dark-grown soybean (not shown) showed a cuticle which was 145 nm in thickness. High resolution micrographs revealed identical cell wall and cuticle substructures in these light- and dark-grown soybean seedlings.

Table I. Back Titration of Tissue-adjusted pH of Incubating Solutions

Tissue (10 segments/ml 1 mM K-phosphate) was allowed to adjust the pH from about 6.09 to about 5.63 (in 10 min), and from about 6.77 to about 5.83 (in 10 min). The medium, containing the tissue, was then back titrated to the original pH with KOH. The four experiments were each repeated five times. Standard errors are given in parentheses.

Auxin Present	pH Change	H^+ Change	H^+ in 10 ml	KOH Required
		μM	nmol	μmol
No	6.08(0.02) → 5.67(0.13)	0.83 → 2.14	13.1	2.66(0.14)
Yes	6.20(0.02) → 5.59(0.10)	0.79 → 2.57	17.8	2.86(0.08)
No	6.75(0.04) → 5.84(0.17)	0.18 → 1.45	12.7	15.33(0.19)
Yes	6.79(0.01) → 5.81(0.09)	0.16 → 1.55	13.9	15.57(0.11)

production, since the cells themselves, certainly including the proteins of the cell wall, can act as buffers. This conclusion is best demonstrated by comparing H^+ ion concentration change, as measured by pH, to back titration with KOH. For example, in one experiment, a change in pH from 6.08 to 5.67 represented an increase of only 13.1 nmol of H^+ ions in the 10 ml medium, but 2.66 μmol KOH were required to back titrate from pH 5.67 to pH 6.08 (Table I). Using KOH for back titration, it was confirmed that while proton secretion certainly occurs when soybean hypocotyl segments are excised and immersed in liquid medium, it is unaffected by auxin (Table I).

Finally, it should be stated that the hypocotyl segments were, under all conditions used in these experiments, actively growing and capable of increasing their elongation rate in response to auxin. For example, elongation rates in water (unpublished) and 1 mM K-phosphate (pH 6) (21) were 0.2 mm/hr, increasing to 0.55 mm/hr upon addition of auxin.

CONCLUSIONS

The acid growth theory of auxin action has been previously challenged (1, 11, 16, 17). In one case (1), methodology was a problem (6). In others (11, 16), interpretation has been questioned (4, 18, 19). The two unique aspects of our data, however, are these: (*a*) Elongating soybean hypocotyl segments depress the pH of their immediate environment despite the presence of a cuticle; (*b*) this pH adjustment activity is unaffected by auxin. Furthermore, as seen in the following report, this pH adjustment by the cells has no effect on the elongation rate of the segments (20).

Measuring external pH or free space pH does not accurately measure cell wall pH (5, 12). Although predictions can be made (5), accurate direct measurement of cell wall pH awaits the development of new electrode(s). Another possible complication in interpreting these results is the fact that the mechanism of pH adjustment is unknown. Auxin-induced acidification does not appear to be due to respired CO_2 in *Avena* (5). Certainly respired CO_2 is not responsible for alkalinization, *e.g.* medium pH adjustment from pH 4 to pH 5.1 (Fig. 1). Whatever the mechanism of medium pH adjustment, however, the conclusions of these experiments are unaltered, *i.e.* medium pH adjustment by elongating cells is not affected by auxin.

Hence, these data do not support the acid growth hypothesis. While it is possible that there are alternative explanations for our experiments, *e.g.* measurement of medium pH (Figs. 1-4; refs. 3-5, 14, 15, 19) may not give a clear indication of cell wall pH, we must tentatively conclude that the acid growth hypothesis is not universally applicable for auxin-induced elongation. However, two biochemically separable (22) elongation responses to auxin occur in excised hypocotyl segments (13, 21, 24). As seen in the following report (20), the first response has certain similarities to the low pH-induced elongation. If this first response, which is transient, is not an artifact of the excised system (a "fast response," as such, may not exist in the intact seedling), then the acid growth hypothesis may yet explain this first transient portion of auxin-induced elongation in soybean hypocotyl. It may be that the first transient response induces the long lasting second response.

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