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Evidence that Auxin-induced Growth of Soybean Hypocotyls Involves Proton Excretion[1]

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ABSTRACT

The role of H^+ excretion in auxin-induced growth of soybean hypocotyl tissues has been investigated, using tissues whose cuticle was rendered permeable to protons or buffers by scarification (scrubbing). Indoleacetic acid induces both elongation and H^+ excretion after a lag of 10 to 12 minutes. Cycloheximide inhibits growth and causes the tissues to remove protons from the medium. Neutral buffers (pH 7.0) inhibit auxin-induced growth of scrubbed but not intact sections; the inhibition increases as the buffer strength is increased. Both live and frozen-thawed sections, in the absence of auxin, extend in response to exogenously supplied protons. Fusicoccin induces both elongation and H^+ excretion at rates greater than does auxin. These results indicate that H^+ excretion is involved in the initiation of auxin-induced elongation in soybean hypocotyl tissue.

The acid-growth theory (18) has been proposed to explain how auxin induces coleoptiles and stem cells to elongate. According to this theory, auxin causes cells to excrete protons into the wall solution. The lowered wall pH activates one or more enzymes which cleave load-bearing bonds in the cell walls, thus allowing for accelerated turgor-driven cell extension. If one is to apply this theory to a given auxin-sensitive tissue, three conditions should be met: (*a*) H^+ should be capable of substituting for auxin in causing cell-wall loosening; (*b*) tissues should excrete protons in response to auxin, and (*c*) neutral buffers infiltrated into the walls should prevent auxin-induced cell elongation. All three conditions hold for coleoptiles (2, 7, 18), indicating that the acid-growth theory is likely to be correct for this tissue. The situation for dicotyledonous stems, however, is less clear. Although auxin-induced H^+ excretion has been recorded for several dicot stem tissues (9, 11, 13), it could not be detected in other cases (14, 15, 19, 20). In addition, Vanderhoef *et al.* (19, 20) have reported that neutral buffers are unable to block auxin-induced growth in soybean hypocotyl sections. Vanderhoef *et al.* (19) employed sections whose cuticles were intact because they believed that the cuticle would not be a significant barrier to the movement of protons. Inasmuch as we have demonstrated that the soybean cuticular layer is, in fact, a significant barrier to both protons and buffers (S. A. Dreyer, unpublished data), we felt that we should reinvestigate the role of proton excretion in the auxin-induced growth of soybean hypocotyls, using material in which the cuticle was rendered more permeable to protons and buffers by scarification (scrubbing). It will be shown that the three conditions required to validate the acid-growth are confirmed for soybean hypocotyl sections.

MATERIALS AND METHODS

Seeds of *Glycine max* L., var. Hawkeye (Illinois Foundation Seeds, Champaign, Ill.), were soaked 0.5 to 1 h in water, planted in moist Vermiculite, and germinated at 25 C in a room illuminated with dim red light ($<10^{-2}$ $\mu E/m^2 \cdot s$). When 5 to 6 days old, seedlings with hypocotyls 3 to 4 cm long were selected. Scrubbed hypocotyls were produced by rubbing the hypocotyl with two strokes of a mixture of emory (American Optical Co., Grade M180) and water, rotating the hypocotyl 90°, and giving it two more strokes. The hypocotyls were rinsed to remove the emory. A 14-mm section was cut from each hypocotyl, starting 2 to 3 mm below the node, and preincubated for 30 to 60 min in water or buffer before experimentation began.

For growth studies, sections were incubated in 1 mM KCl + 0.1 mM $CaCl_2$ (pH 6.5), with or without addition of a buffer (Mes-Tris, Hepes-NaOH, or K-phosphate). IAA (10 μM) was added to some sections and growth was measured every 2 h with a dissecting microscope fitted with a stage micrometer (Figs. 5 and 6) or measured continuously by means of our constant stress extension apparatus (Figs. 1 and 7; for details, see ref. 17). In some experiments, after scrubbing the hypocotyls were frozen, then thawed, and placed in the constant stress apparatus under 15 g load. In such cases the segments were first incubated in 10 mM K-phosphate buffer, pH 7.0. After 30 to 60 min of incubation, the extension rate was recorded, and the solution was changed to a K-phosphate-citrate buffer at a lower pH. The extension rate was recorded continuously (Fig. 2) or recorded after 20 min, followed by a change to a new solution at a lower pH (Fig. 3).

H^+ excretion was determined using the direct-recording method of Cleland (3). A group of six to eight sections was placed on a slide so that the sections were parallel to one another. A drop of 1 mM KCl-0.1 mM $CaCl_2$ then was placed on the sections and an Ingold No. 6020 combination pH electrode was lowered directly onto the surface of the sections. The pH on the surface of the segments was recorded continuously and, when stabilized, the solution was replaced with a new drop at the same pH, containing auxin or FC[2]. Experiments designed to test the ability of non-scrubbed segments to adjust the pH of the medium (Figs. 9 and 10) followed the methods of Vanderhoef *et al.* (19, 20) and details are presented in the figure legends. FC was kindly provided by Prof. E. Marrè.

RESULTS

In our initial experiments, we ascertained that the scarification of soybean hypocotyl sections does not interfere with their ability

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[2] Abbreviations: FC: fusicoccin; CHI: cycloheximide.

to grow in response to auxin. Rapid elongation commences 10 to 12 min after addition of auxin (Fig. 1) and persists for hours (Fig. 7). The growth response of scrubbed sections appears to be very similar to that of intact sections; therefore, in our opinion, information obtained about the initiation of auxin-induced elongation of scrubbed soybean hypocotyl sections should be applicable to unscrubbed tissues as well.

Next, we tested the three conditions which must be met if the acid-growth theory is to be applied to soybean hypocotyl tissue. Acidic solutions induce rapid elongation of both live and frozen-thawed sections; we show only the data for frozen-thawed sections here (Fig. 2), however, since Vanderhoef *et al.* (20) have already reported on the acid-induced extension of live soybean hypocotyl sections. Any solution with a pH below 6.5 induces some elongation in scrubbed sections with the extension rate proportional to the log of the hydrogen ion concentration down to a pH of 3.0 (Fig. 3). If sections with intact cuticles are used instead, the acid-extension rate at any pH is reduced as compared with scrubbed

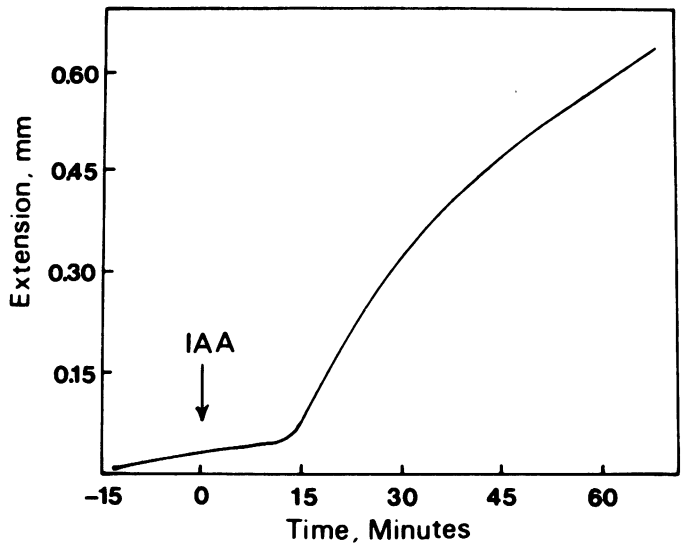

FIG. 1. Ability of scrubbed soybean hypocotyl sections to elongate in response to auxin. A single scrubbed section was incubated in 1 mM KCl + 0.1 mM $CaCl_2$, elongation was monitored continuously with a linear position transducer (17), and then 10 μM IAA was added at the arrow.

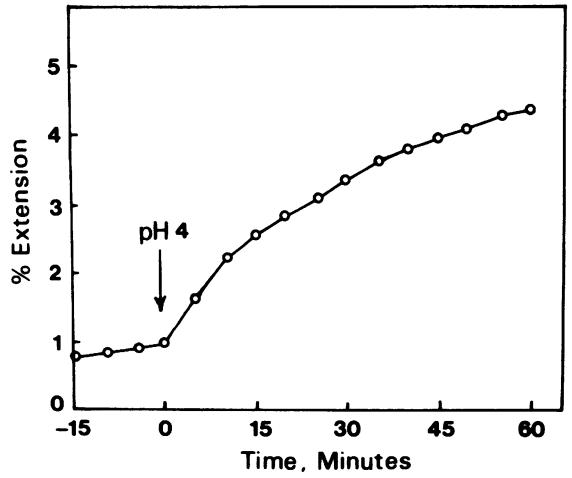

FIG. 2. Acid-induced extension of scrubbed soybean hypocotyl sections. Frozen-thawed section was incubated in the constant-stress apparatus (17) under 15 g load and in K-phosphate buffer, pH 7.0. At the arrow, the solution was changed to a K-citrate-phosphate buffer at pH 4.0.

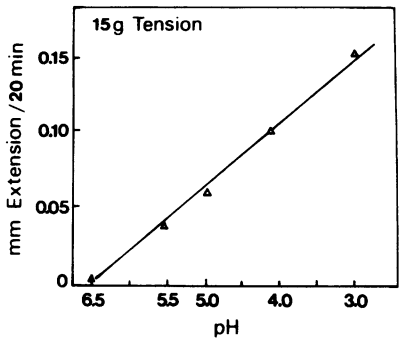

FIG. 3. Relation between pH of the external solution and the rate of extension of frozen-thawed scrubbed soybean hypocotyl sections. A section was incubated under 15 g load in the constant-stress apparatus in a K-phosphate buffer at pH 6.5. The extension rate was determined after 20 min, the solution was changed to one at a lower pH, and the extension during the next 20 min was determined. The solution then was replaced with one of even lower pH and the measurement was repeated with progressively lower pH solutions.

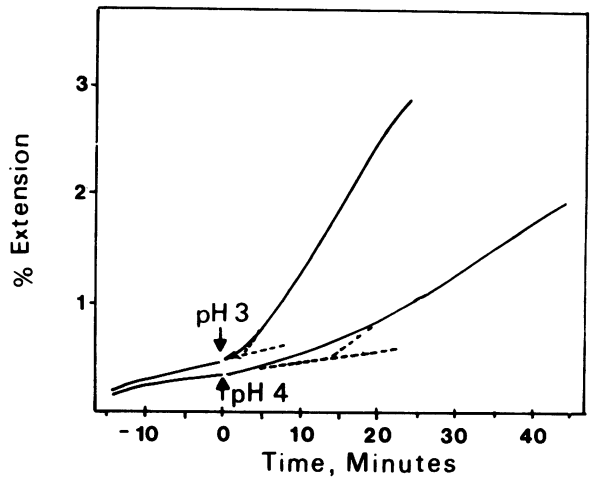

FIG. 4. Time course of acid-induced extension of unscrubbed, frozen-thawed soybean hypocotyl. Intact sections were frozen-thawed, then incubated in our constant stress apparatus under 15 g load in K-phosphate buffer, pH 7.0. At the arrow, the solution was changed to K-phosphate-citrate buffers at pH 3.0 or 4.0. Note that pH 4 shows a longer lag relative to pH 3 (7 *versus* 1.5 min) and a longer time to reach maximum rate (33 *versus* 6 min), as expected if H^+ entry is primarily through the cut ends, except at low pH values.

sections, and there is a distinct lag prior to the onset of the extension, especially at suboptimal pH values (Fig. 4). These results are consistent with the cuticle being a barrier to proton movement.

Auxin causes scrubbed soybean hypocotyl sections to excrete protons after a lag of not more than 10 min (Fig. 5). Within an additional 30 min, the pH of the external solution has dropped to pH 5.0 or below. Thus, the kinetics of auxin-induced H^+ excretion for soybean hypocotyls are similar to those reported for the *Avena* coleoptile (3), in terms of both the length of the initial lag and the rate at which the external pH decreases in response to auxin. As with coleoptiles, addition of the protein synthesis inhibitor CHI causes a disappearance of protons from the external solution until the external pH reaches 6.2 or above (Fig. 5).

The presence of neutral buffers in the external solution inhibits auxin-induced growth of scrubbed but not unscrubbed sections (Fig. 6). The amount of inhibition increases as the buffer strength increases (Figs. 6 and 7); the inhibition is not due to the increased osmotic strength of the solution, as a mannitol solution at the

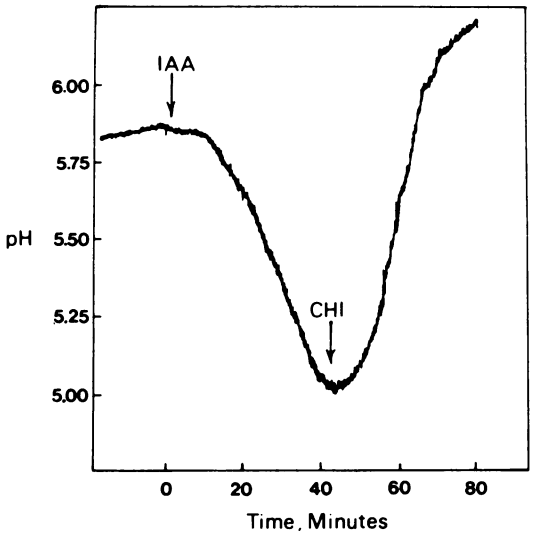

FIG. 5. Auxin-induced H^+ excretion from scrubbed soybean hypocotyl sections. The pH was measured using the direct contact method of Cleland (3) and the bathing solution was 1 mM KCl + 0.1 mM $CaCl_2$. At the first arrow, the solution was replaced with one containing 10 μM IAA and, at the second arrow, it was replaced with one containing IAA and 1 mg/l cycloheximide at pH 5.0.

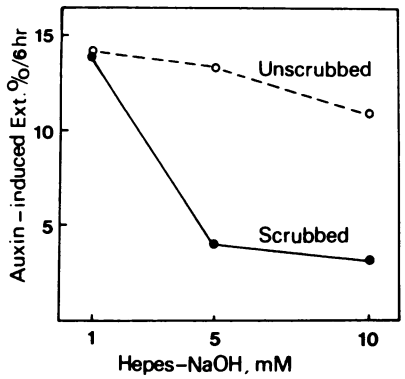

FIG. 6. Demonstration that neutral buffers inhibit the auxin-induced elongation of scrubbed but not unscrubbed soybean hypocotyl sections. Groups of 10 14-mm scrubbed or unscrubbed sections were measured, incubated for 6 h in Hepes-NaOH buffer (pH 6.5) at 1, 5, or 10 mM, ± 10 μM IAA, and remeasured. The difference in growth between + and − auxin is plotted as a junction of buffer strength.

same osmotic concentration has no effect on auxin-induced growth (Fig. 7). The inhibition is not restricted to a single buffer; Mes-Tris, and Hepes-NaOH are equally inhibitory. K-phosphate is less effective but still causes substantial inhibition of auxin-induced growth (data not shown). The inhibitory effects of the buffer are not restricted to the "early" phase of auxin-induced growth, as suggested by Vanderhoef *et al.* (20), but occur equally throughout at least the first 6 h of auxin-induced growth.

Another piece of evidence favoring acid-mediated elongation in coleoptile tissues is the fact that the fungal toxin FC, which induces elongation at rates greater than that induced by auxin, causes coleoptile tissues to excrete protons at a rate greater than that induced by auxin (4). A similar situation exists in soybean hypocotyl sections (Fig. 8). FC induces both growth and H^+ excretion after a lag of 1 to 2 min at most, and both growth and H^+ excretion occur at rates greater than those induced by auxin (compare with Figs. 1 and 4).

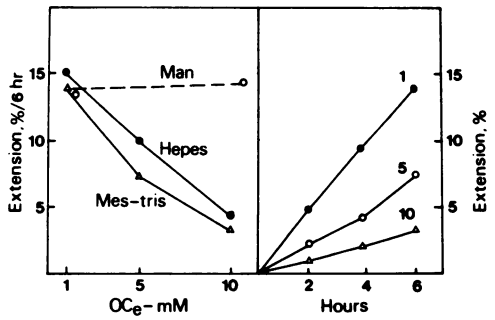

FIG. 7. Left, Demonstration that inhibition of auxin-induced growth of scrubbed soybean hypocotyl sections by neutral buffers is not due to the buffer type or to osmotic effects. Groups of 10 sections were incubated for 6 h in 10 μM IAA containing 1 to 10 mM mannitol (man), Hepes-NaOH buffer, or Mes-Tris buffer, all at pH 6.5. OC_e, osmotic concentration of the external solution. Right, Demonstration that neutral buffers inhibit auxin-induced growth throughout a 6-h period. Groups of 10 sections incubated for up to 6 h, in 1, 5, or 10 mM Mes-Tris buffer (pH 6.5) + 10 μM IAA.

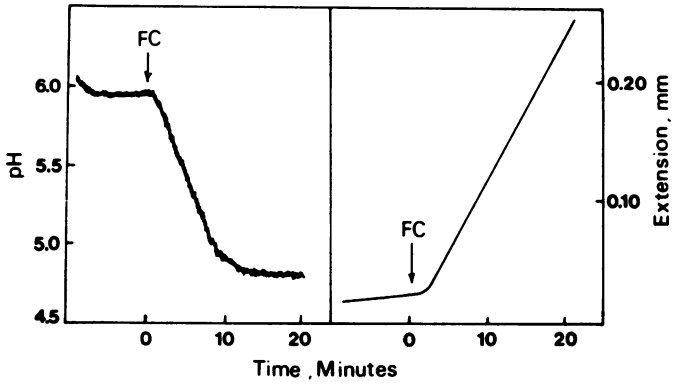

FIG. 8. Effect of fusicoccin on H^+ excretion and growth of scrubbed soybean hypocotyl sections. Left, H^+ excretion; conditions same as in Fig. 4. Right, elongation; conditions same as in Fig. 1. FC was used at 10 μM.

DISCUSSION

The role of hydrogen ions as a mediator in auxin-induced growth of coleoptile tissues is supported by a substantial amount of evidence (7, 18, and references therein), but there is less information for dicot stem tissues. Here, we have demonstrated that three predictions based on the acid-growth theory are valid for scrubbed soybean hypocotyl sections; namely, that added protons will substitute for auxin in initiating growth, that auxin will cause the tissue to excrete protons, and that neutral buffers can block auxin-induced growth.

Before concluding that the acid-growth theory is valid for soybeans, we must explain why Vanderhoef *et al.* (19, 20) obtained such different results using unscrubbed sections. For example, they reported that soybean sections rapidly adjust the external solution to about pH 5.2 from initial pH values between 4 and 8, but this pH adjustment is insensitive to auxin. In addition, they found that neutral buffers were not effective in inhibiting auxin-induced growth.

We believe that the differences between the Vanderhoef *et al.* data and ours can be explained in a way that is compatible with the acid growth theory. First, we believe that the soybean cuticle is an effective barrier to the entry or exit of protons or buffers (S. A. Dreyer, unpublished data). As a result, when nonscrubbed segments are used, buffers can infiltrate and inhibit the growth of only those cells at or near the cut surface and, therefore, should not be expected to inhibit auxin-induced growth substantially in

the entire section. Further, the apparent proton secretion observed by Vanderhoef *et al.* (19, 20) in nonscrubbed sections can be shown to arise from respiration. Figure 9 shows that nonscrubbed segments rapidly adjust the pH of the external medium surrounding them from pH 6.5 to about 5.3. However, if one vigorously bubbles the acidified solution with N_2 or CO_2-free air, the pH returns to the original starting value. These data are best explained by arguing that the relatively large tissue volume, compared to the volume of the external medium used by Vanderhoef *et al.* (19) results in a substantial contribution of CO_2 to acidification of the medium. Indeed, the rate of the downward pH adjustment as reported by Vanderhoef and shown in Figure 9, is independent of the number of cut surfaces and dependent on the tissue volume. Thus, it seems unlikely that the downward adjustment of the pH of the medium reported by Vanderhoef *et al.* (19) is due to direct proton excretion through the cuticle; rather, it arises from the ability of respiratory CO_2 to diffuse through the cuticle.

Although the release and subsequent hydration of respiratory CO_2 effectively explains the downward adjustment of the medium pH, it was also observed (19) that nonscrubbed segments could rapidly adjust the medium from pH 4.0 to about 5.3. We repeated these data and found (Fig. 10) that this phenomenon is related to the number of cut surfaces present in the solution rather than to the tissue volume. It seems that this adjustment is not due to a flux of protons through the cuticle but rather to the absorption of protons by cells at the cut surfaces.

We feel the data presented in Figures 9 and 10 explain the cellular adjustment of the medium observed by Vanderhoef but, one might still ask, why does one not observe auxin-induced H^+ excretion from nonscrubbed segments due to the activity of the cells at the cut surfaces? Unfortunately, we cannot answer this question with any certainty. However, there are two points which seem to pertain to this problem. First, we cannot be sure the cells at the cut surface are still auxin-sensitive. If they are not sensitive, the question is trivial; however, if they are sensitive, a question remains as to whether our methods of detecting H^+ excretion are sensitive enough to monitor this activity. This latter possibility may indeed be the case, as the growth of the dicot stem tissues is controlled primarily by the outer several layers of cells (1, 8, 12). Only these cells respond significantly to auxin (9; D. L. Rayle, unpublished data). In nonscrubbed sections, only those auxin-sensitive outer cells at the cut surfaces, a minority of the cells present, would be expected to excrete protons into the surrounding medium in response to auxin, and the magnitude of this excretion could be too slight to be detected by standard methods. Increasing the permeability of the soybean cuticle to protons will change this situation. Now protons excreted from the longitudinal surface of the auxin-sensitive epidermal cells can escape into the external medium and, as we have shown, are readily detectable by our surface measurements. In addition, neutral buffers can penetrate into the walls of all the epidermal and subepidermal cells, inhibiting cell elongation effectively. Thus, the results we have obtained with scrubbed sections and the results obtained by Vanderhoef *et al.* (19, 20) with unscrubbed sections are both consistent with what one would expect to obtain if hydrogen ions are acting to mediate auxin-induced growth of soybean hypocotyls and if the cuticle is a barrier to proton flux.

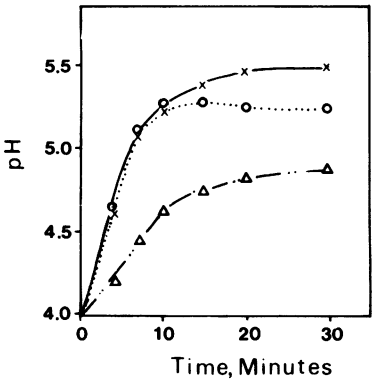

FIG. 10. Nonscrubbed soybean segments were placed in 30-ml beakers containing 4 ml H_2O (+1 mM KCl) which had been adjusted to pH 4.0 with dilute HCl. The pH of the medium was monitored for 30 min. The lower curve (△) represents data obtained when 10 10-mm segments (20 cut surfaces) were used. The upper curves represent data obtained when either 40 10-mm segments (○) or 40 2.5-mm segments (×) were used. In both cases, there were 80 cut surfaces, although the tissue volumes were different.

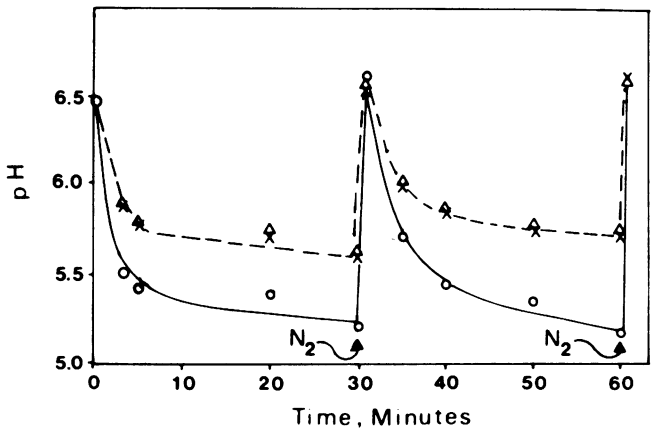

FIG. 9. Nonscrubbed soybean segments were placed in 30-ml beakers containing 4 ml H_2O (+1 mM KCl) which had been adjusted to pH 6.5. The pH of the medium then was monitored for 60 min. After the 30- and 60-min readings, the solution surrounding the segments was vigorously bubbled with a stream of nitrogen for approximately 1 min. The lower curve (○) represents data obtained when 40 10-mm segments (80 cut surfaces) were used. This is equivalent to the tissue to solution ratio used by Vanderhoef *et al.* (17). In the upper curves, the beakers contained either 10 10-mm segments (△) and, therefore, 20 cut surfaces or 40 2.5-mm segments (×) with 80 cut surfaces.

Studies with scrubbed sections are valid only if it can be shown that the scarification process has not damaged the tissue in a way which alters its response to auxin. Visual examination of the epidermis by optical microscopy or by scanning electron microscopy (S. A. Dreyer, unpublished data) has shown that most epidermal cells are intact. Furthermore, the scrubbed sections show normal growth responses to auxin and FC, both in terms of timing and magnitude. Although the scrubbing may cause some damage to the tissue, we think it unlikely that it generates any new auxin responses and, therefore, conclude that the relationship between H^+ excretion and auxin-induced growth has not been altered by scarification.

Can the results we have obtained with soybean hypocotyls be extrapolated to other dicot stem tissues? The impermeability of the cuticle to protons has been demonstrated for sunflower and pea stems (S. A. Dreyer, unpublished data) and would appear to be a general phenomenon. The restriction of auxin sensitivity to the outer cell layers would appear to be a common feature of most dicot stems (1, 8, 12). Thus, one should not expect to find substantial auxin-induced H^+ excretion from either intact or peeled dicot stem sections. But if the cuticle is scarified, auxin-induced H^+ excretion should be detectable; indeed, Mentze *et al.* (13) were able to demonstrate such proton excretion from all scrubbed dicot stem sections which they tested. Marrè *et al.* (11) have reported detectable auxin-induced proton excretion from pea stem sections without scarification of the cuticle; whether their tissue possessed a particularly permeable cuticle or the auxin-sensitive cells are not restricted to the outer layers is not known. In any case, we

speculate that auxin-induced proton excretion is involved in the initiation of elongation in dicot stem tissues.

It must be pointed out that, although proton excretion may be sufficient to initiate elongation, it is certainly not sufficient to maintain rapid elongation. Continued elongation requires osmoregulation and the maintenance of the capacity of the wall to be loosened (5). These processes may not require proton excretion. Vanderhoef *et al.* (19) and Penny *et al.* (16) have divided auxin-induced growth into "early" and "late" phases. Although the elongation rate in the first phase may be regulated by the wall pH, the rate of the second phase may well be controlled by the rate of osmoregulation or the rate at which the wall-loosening capacity is restored. But the ability of buffers to inhibit growth during both phases is difficult to explain any way other than by assuming that proton excretion is required whenever rapid elongation is occurring in the soybean hypocotyl.

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