An Improved Method for Detecting Auxin-induced Hydrogen Ion Efflux from Corn Coleoptile Segments

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ABSTRACT

Conditions necessary to detect maximal auxin-induced H⁺ secretion using a macroelectrode have been investigated using corn coleoptile segments. Auxin-induced H⁺ secretion is strongly dependent upon oxygenation or aeration when the tissue to volume ratio is high. Cuticle disruption or removal is also necessary to detect substantial auxin-induced H⁺ secretion. The auxin-induced decrease in pH of the external medium is stronger when the hormone is applied to tissue in which the cuticle has been disrupted with an abrasive than when the hormone is applied to tissue from which the cuticle and epidermis have been removed by peeling. The lower detectable acidification of the external medium when using peeled segments appears to be due in part to the leakage of buffers into the medium and in part to the removal of the auxin-sensitive epidermal cells.

The sensitivity of corn coleoptile segments to auxin, as measured by H⁺ secretion, increases about 2-fold during the first 2 hours after excision. This change in apparent sensitivity to auxin as reflected by H⁺ secretion is paralleled by a time-dependent change in the growth response to auxin. Under optimal conditions for detecting H⁺ efflux (oxygenation, abrasion, hormone application 2 hours after excision), the latent period in auxin-induced H⁺ efflux (about 7 or 8 minutes) is only half as great as the latent period in auxin-induced growth (about 18 to 20 minutes). These observations are consistent with the acid growth hypothesis of auxin action.

According to the acid growth hypothesis of auxin action, auxin enhances growth by stimulating the secretion of H⁺ ions from the cytoplasm into the cell wall. The resultant acidification of the cell wall is thought to lead to wall loosening and accelerated growth (5, 8, 13, 14).

If auxin-induced H⁺ secretion acts as a mediator of auxin-induced growth, one would expect hormone-induced H⁺ efflux to precede hormone-induced acceleration of growth. The most commonly used method of measuring H⁺ efflux is to place a large number (e.g. 40) of 1-cm long tissue segments in a small volume (e.g. 5 ml) of stirred weak buffer and monitor the change in pH of the medium in the presence and absence of hormone (12, 14). Since the cuticle is said to offer substantial resistance to the entry and exit of H⁺ ions (3), it is common practice to remove the cuticle either by peeling the tissue segments with a pair of fine-tipped forceps or abrading them with emery powder. Using this method, auxin-induced H⁺ efflux appears to begin about 20 min or more after application of hormone (1, 12, 14) even in those tissues in which growth is maximally promoted within 15 min. This rather long apparent latent period in auxin-induced H⁺ secretion seems to be due, at least in part, to the large volume of the incubation solution compared with the volume of the cell wall fluid in which H⁺ would normally be accumulating. When the volume of the external medium is minimized, shorter latent periods are observed. Cleland (2), using a flat-tipped electrode resting on a row of peeled Avena coleoptile sections, reported an average latent period of 14 min in induction of H⁺ secretion by auxin, whereas Jacobs and Ray (9), using a pH microelectrode inserted into the free space, reported latent periods of 12 min in corn coleoptile segments and an average of 15 to 18 min in pea stem segments. Inasmuch as the latent periods observed for auxin-induced H⁺ secretion in these tissues were less than the corresponding latent periods for auxin-induced growth, these data may be viewed as consistent with the acid-growth hypothesis. However, in both studies, those authors (2, 9) reported considerable variability in the latent periods for H⁺ efflux and, in both studies, there were instances in which no auxin-induced H⁺ efflux was detected (personal communications).

Through slight modifications of previously reported methodology, we have developed a simple procedure for measuring hormone-induced H⁺ efflux in corn coleoptile segments. Using this procedure, we can reproducibly detect auxin-induced H⁺ secretion in corn coleoptiles with a latent period of about 7 min. This is approximately half the latent period associated with auxin-induced growth in this tissue.

In this report, some observations on improving methodology for detecting H⁺ efflux are presented and the use of the method to detect rapid auxin-induced H⁺ efflux is described. The system is also used to examine time-dependent changes in the magnitude of auxin-induced H⁺ efflux which closely parallel changes in tissue sensitivity to auxin as measured by enhancement of growth.

MATERIALS AND METHODS

Plant Material. Experiments were done using coleoptiles from 4- to 5-day-old corn (Zea mays L., hybrid WF 9×38, Customaize, Inc., Momence, Ill.). Growing conditions were as described previously (6).

Scanning Electron Microscopy. Scanning electron microscope observations were made of the surface of undisturbed, abraded, and peeled coleoptiles. Segments were fixed for 5 min in 1.5% (v/v) phosphate-buffered glutaraldehyde for 30 s followed by the addition of aqueous OsO₄ to a final concentration of 1% (v/v). The total fixation time was 10 min. The segments were dehydrated in a graded ethanol series and subjected to critical point drying using liquid CO₂. The segments then were coated with gold and observed at 20 kev using an Hitachi S-500 scanning electron microscope.

Cuticle Removal. To facilitate H⁺ efflux from coleoptile segments, the cuticle was removed either by peeling (12) or by rubbing the segments with an abrasive (4). Peeling was done by physically removing strips of material from the surface of the coleoptile before excising segments. This was done over an esti-
mated 80% of the surface of the coleoptile. For abrasion, a 1:1 mixture of emery No. 305 (Edmund Scientific Company, Barrington, N. J.) in water was used. The coleoptile was stroked gently three times from base toward the tip with the emery paste between thumb and forefinger. The coleoptile was rotated approximately 60° between strokes. After the abrasion procedure, the coleoptiles were rinsed thoroughly with distilled H2O and floated on a large volume of buffer until segments were cut and the leaves were removed. Although the purpose of the abrasion procedure is merely to disrupt the cuticle, A. M. Jones and L. N. Vanderhoef (personal communication) found that the procedure causes cellular damage in localized regions of abraded materials (Fig. 1). This is in large measure unavoidable since they determined that the emery particles are on the average some 35 times larger in diameter than the thickness of the cuticle.

Measurement of H+ Efflux. Forty coleoptile segments 1 cm in length were placed in a glass vial containing 4 ml 1 mM K-phosphate (initial pH 6.2-6.3). The vial was 63 mm in height and 17 mm in diameter with a total volume of 14 ml. A small Teflon magnetic spin bar was used to stir the incubation medium. The spin bar was separated from the coleoptile segments by a disc of plastic screen mounted 5 mm above the bottom of the vial. A Markson semimicro combination pH electrode (No. 2885) with a Corning model 7 pH meter was used to measure pH. Output for the pH meter was recorded on a Sargent-Welch model SRLG recorder with a full scale sensitivity of 0.1 mv using a chart speed of 15.24 cm/h. The output from the pH meter was attenuated with a back voltage to allow adjustment of the recorder to a full scale displacement over the pH range of 5.7 to 6.4. In those experiments in which oxygenation was used in addition to stirring, O2 was bubbled at the rate of 26 liters/h through the incubation medium using a 22-gauge (0.4 mm i.d.) syringe needle.

At the end of some experiments, the total amount of H+ released after the addition of auxin was determined by titrating the medium back to the pH at the time of hormone addition. This was done using the same experimental set-up and titrating with 8 mm NaOH with the segments remaining in the medium.

The sensitivity of corn coleoptile segments to auxin has been shown to increase with time after excision (15). Therefore, in those H+ secretion experiments involving application of auxin, the hormone was added 90 min after excision of the tissue, unless otherwise noted.

Measurement of Buffering Capacity of Incubation Medium. To determine the extent to which buffering substances were released into the medium during the course of an experiment, the segments were removed from the medium and the medium was adjusted to pH 4.0 using HCl. The medium then was titrated with 8 mm NaOH over the pH range 4.0 to 6.3. The titration was recorded using the experimental setup for measuring H+ secretion, except that the recorder sensitivity was reduced to 0.5 mv full scale and a chart speed of 152.4 cm/h was used.

Measurement of Growth. Growth experiments with corn coleoptile segments were done using the shadowgraphic growth recording device described previously (7). Preparation of the segments was as described by Vesper and Evans (15).

RESULTS

Surface Effects of Peeling and Abrasion. Figure 1 shows scanning electron micrographs of the surface of undisturbed, abraded, and peeled corn coleoptile segments. In abraded specimens there was occasionally an area where obvious cellular damage had occurred (Fig. 1C), but the great majority of the epidermal cells remained intact (Fig. 1D). In peeled segments, large sheets of epidermal cells were removed, revealing the underlying cells (Fig. 1B). There were numerous tears in underlying cells, but the damage at any one location was not as great as the isolated areas of damage found in abraded tissue.

Oxygen Dependence of Auxin-induced H+ Secretion. In most of the published studies of hormone-induced H+ secretion using submerged segments, the segments have been stirred or shaken, but no additional precautions have been taken to assure adequate aeration, (1, 10, 12). Parrish and Davies (11), in a study of H+ efflux in pea stem segments, used aeration to agitate the segments and noted a strong dependence of the apparent rate of H+ secretion on the rate of aeration. We have compared auxin-induced H+ secretion in peeled corn coleoptile segments using stirring alone versus stirring supplemented with oxygenation (Fig. 2). In the absence of oxygenation, the pH of the medium equilibrates at about 6.0, as compared with 6.3 to 6.4 when oxygenation is used. The lower equilibration pH in the absence of oxygenation is most likely due to the accumulation of respiratory CO2. There is a major difference in the kinetics of auxin-induced H+ secretion in oxygenated versus nonoxygenated peeled tissue. Using oxygenated tissue, the latent period is about 20 min, whereas, with nonoxygenated tissue, the latent period is nearly 30 min. There is also a difference in the apparent rate of H+ secretion. With oxygenation, the pH drops from about 6.3 to 5.8 over an 80-min period after the initiation of auxin-induced H+ secretion, whereas, in nonoxygenated tissue, the pH drop over a comparable time period is from about 6.0 to 5.8. Over that portion of the pH range traversed in both experiments (i.e. below about 6.0), the rate of drop in pH is about twice as great in oxygenated tissue as compared with nonoxygenated tissue. In similar experiments, it was noted that aeration was as effective as oxygenation in maximizing auxin-induced H+ secretion.

Auxin-induced H+ Secretion in Peeled versus Abrided Tissue. Figure 3 shows a comparison of apparent auxin-induced H+ secretion in peeled versus abraded coleoptile tissue as well as in tissue which has been neither abraded nor peeled. In all cases, the solutions were oxygenated. Using intact tissue, no auxin-induced H+ secretion is detectable during the first 90 min after addition of hormone. After 90 min, weak acidification of the medium can be detected in some experiments (not shown in Fig. 3). The lack of significant auxin-induced H+ secretion in intact segments supports the conclusion drawn by others (3, 14) that abrasion or peeling is a prerequisite for detecting auxin-stimulated acidification.

Of the two common methods for removing or disrupting the cuticle, the abrasion method allows considerably stronger and more rapid auxin-induced H+ secretion (Fig. 3). Using abraded tissue, auxin-induced H+ secretion is consistently detectable with a lag of about 7 min, whereas, in peeled tissue, the lag is about 15 min. Similarly, the rate of auxin-induced H+ efflux appears to be greater in abraded tissue, the pH dropping from about 6.4 to 5.7 in 50 min as compared with about 100 min in peeled tissue.

Release of Buffering Substances by Peeled and Abrided Tissue. Although the data of Figure 3 indicate that auxin-induced H+ efflux is stronger in abraded than in peeled tissue, the indication is an indirect one. The data show that the rate at which the pH of the medium drops is greater when using auxin-treated abraded tissue than when using auxin-treated peeled tissue, but they provide no indication of the absolute amount of H+ released. We have noticed an accumulation of buffering substances in media containing peeled or abraded tissue, with considerably more buffering capacity accumulating in media containing peeled tissue (Fig. 4). Using the titration technique described under "Material and Methods," it was found that approximately 1 μeq OH- was required to titrate 4 ml 1 mM K-phosphate incubation buffer itself from pH 4.0 to 6.3 and that this medium tended to act as a buffer near pH 6 as expected. After the medium had held peeled coleoptile segments for 2.5 h, approximately 3 μeq OH- were required to adjust the pH over the same range, and the shape of the titration curve was indicative of accumulation of substances adding buffering capacity over the more acid range (pH 4 to 5.5), as might be expected if organic acids were released into the medium from

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wounded cells. The buffering capacity of medium containing abraded tissue was intermediate between that of medium without segments and medium containing peeled segments, whereas medium which had held intact segments had only slightly greater buffering capacity than the phosphate buffer alone. This differential release of buffering substances from peeled versus abraded tissue may arise from differences in the extent of tissue wounding during peeling versus abrasion. In any event, the differential leakage of buffering substances into the medium makes direct comparison of pH shifts an invalid measure of relative net H⁺ efflux using these two methods.

The data suggest the possibility that the apparent weaker induction of H⁺ secretion by auxin in peeled tissue as compared with abraded tissue is due, at least in part, to the greater leakage of buffering substances from peeled tissue. To test this possibility further, peeled coleoptile segments were placed in a large volume (1 liter) of 1 mM K-phosphate buffer which was oxygenated and stirred rapidly for 1 h. The segments then were placed in the specially constructed vial for H⁺ secretion measurement. The washing of the segments was included to disperse buffering substances leaking from the wounded segments. Washing led to a measurable increase in apparent auxin-induced H⁺ secretion in peeled segments, but the rate remained less than that shown by nonwashed abraded segments (data not shown). This indicates that the apparent reduced rate of H⁺ secretion in peeled segments is only partly due to the greater leakage of buffering materials from peeled tissue.

That the differential release of buffering substances from peeled versus abraded segments does not account totally for differences in apparent H⁺ secretion is not surprising. According to Figure 4,

**FIG. 1.** Scanning electron micrographs of undisturbed, abraded, and peeled corn coleoptile segments. A, Undisturbed; B, peeled; the epidermis has been peeled from the right hand portion, causing some cellular damage in underlying cells (arrows). A smaller strip of epidermis has been peeled near the left hand portion of the field of view; C, abraded. The field was chosen to include the area where obvious cellular disruption had occurred; D, abraded segment at lower magnification to show the isolated nature of cellular damage. A, B, and C, × 100; D, × 40.
the major buffering activity of the released material is from about pH 4 to about pH 5.5. The pH shift resulting from auxin treatment ranges from about 6.3 to 5.7 where the influence of these buffers should be greatly reduced. Another factor which might be involved in the weakening of apparent auxin-induced H+ secretion in peeled tissue is that peeling removes cells which may contribute to total H+ efflux. To assess the relative contribution of tissue removal to the apparent weakening of auxin-induced H+ efflux from peeled tissue, total auxin-induced H+ efflux from peeled versus abraded coleoptiles was determined using back titration to eliminate differences due to differential release of buffers (Table I). Clearly, the net amount of H+ secreted by abraded tissue substantially exceeds that by peeled tissue. These results suggest that lesser apparent auxin-induced H+ efflux in peeled tissue is due in part to the release of buffering substances from the tissue and in part to the removal of the auxin-sensitive epidermal layer.

**Time-dependent Changes in H\(^+\) Secretion Response to Auxin.**

In an earlier report (15), it was noted that the sensitivity of the growth response to auxin in corn coleoptile segments changes as a function of time after excision. It was found that, during the first 3 h following excision, the magnitude of the auxin response increases 2- to 3-fold, whereas the latent period associated with the response decreases by about 3-fold. If auxin-induced H+ secretion is closely tied to growth, one might expect similar changes in the magnitude and latent period of auxin-induced H+ secretion in the hours following excision. This was tested by measuring auxin-induced H+ efflux when the hormone was added to abraded segments 30 min versus 2 h after excision (Fig. 5). The rate of drop in pH when auxin is added to tissue 2 h after excision is about 3 times the rate of drop in pH when auxin is added 30 min after excision. Total H+ secreted as measured by back titration 1 h after exposure to auxin was found to be 2.04 μeq in the tissue used 2 h after excision as compared to 0.6 μeq in the tissue used 0.5 h after excision. Similarly, the latent period in auxin-induced H+ secretion is about 3 times as long as when the hormone is added to tissue 0.5 h after excision versus 2 h after excision. These time-dependent changes in auxin-induced H+ secretion are paralleled closely by time-dependent changes in the growth response as shown in the upper portion of Figure 5. Although the latent period in the growth response using tissue 0.5 h after excision is about 60 min in the example shown in Figure 5, the average value for all experiments was about 48 min.

**Table I. Total Auxin-induced H\(^+\) Secretion during First 60 Min After Addition of 10 μM IAA to Intact, Peeled, or Abraded Corn Coleoptile Tissue**

<table>
<thead>
<tr>
<th>Tissue Preparation</th>
<th>Total H(^+) Secreted during First 60 min (μeq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0</td>
</tr>
<tr>
<td>Peeled</td>
<td>0.79</td>
</tr>
<tr>
<td>Abraded</td>
<td>2.04</td>
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</tbody>
</table>

**FIG. 2.** Effect of oxygenation on auxin-induced hydrogen ion secretion. Segments were placed in the measuring vial with moderate stirring of the medium. Upper curve: Stirring supplemented with oxygenation; lower curve: no oxygenation. IAA (10 μM) was added at the arrow in each curve. Curves are from representative experiments. Each experiment was repeated at least 12 times.

**FIG. 3.** Influence of the method of cuticle disruption on apparent auxin-induced H\(^+\) secretion. Segments were prepared by abrasion, peeling, or neither treatment ("intact"). IAA (10 μM) was added at the arrow in each curve. Medium was both oxygenated and stirred in all cases. Curves are from representative experiments. Each experiment was repeated at least 12 times except for the "intact" sample (three times).

**FIG. 4.** Titration curves of incubation medium after containing tissue segments prepared by various methods. In each case, 40 1-cm segments were placed in 4 ml stirred oxygenated buffer for 2.5 h. The segments were then removed and the medium was adjusted to pH 4.0 with HCl before titrating to pH 6.3 with 8 mM NaOH. Curves are from representative experiments. Each experiment was repeated three times.
DISCUSSION

The magnitude and latent period of auxin-induced H⁺ secretion in corn coleoptile tissue is strongly dependent on the extent of oxygenation, the method of cuticle disruption, and the time of hormone application relative to the time of excision of the segments. Oxygenation (or aeration) is crucial for maximum hormone-induced H⁺ efflux. Stirring the medium is, by itself, insufficient. Auxin-induced H⁺ efflux is stronger and more rapid in segments which have been abraded as compared with segments which have been peeled. This appears to be due in part to the greater release of buffering substances by peeled segments and in part to the removal or disruption of the auxin-responsive epidermal cells during peeling.

The sensitivity of corn coleoptile segments to auxin, as measured by auxin-induced H⁺ efflux, increases about 3-fold during the first 2 h following excision. This is reflected both by a strong increase in the magnitude of auxin-induced H⁺ secretion and by a large reduction in the latent period preceding auxin-induced H⁺ efflux. These time-dependent changes in the magnitude and latent period of auxin-induced H⁺ efflux are closely paralleled by time-dependent changes in the growth response to auxin. Although this indicates a close correlation between growth and H⁺ efflux, it does not address the question of whether or not the rate of H⁺ efflux occurring after auxin treatment is sufficient to account for the observed growth response (16).

Under optimal conditions, i.e. abraded tissue, stirring supplemented with oxygenation, and hormone addition 90 to 120 min following tissue excision, strong auxin-induced H⁺ secretion can consistently be observed beginning about 7 or 8 min after addition of hormone. The latent period of the growth response at a corresponding time after excision is nearly 20 min. Using the methodology described here, we can consistently detect auxin-induced H⁺ secretion with a latent period only half as great as that associated with growth. This observation as well as the closely parallel time-dependent changes in sensitivity to auxin as measured by both H⁺ secretion and growth is consistent with the H⁺ secretion hypothesis of auxin action.

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LITERATURE CITED

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