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Rapid Auxin-induced Decrease in Free Space pH and Its Relationship to Auxin-induced Growth in Maize and Pea[1]

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ABSTRACT

A pH microelectrode has been used to investigate the auxin effect on free space pH and its correlation with auxin-stimulated elongation in segments of pea (*Pisum sativum*) stem and maize (*Zea mays* var. Bear Hybrid) coleoptile tissue. Auxin induces a decrease in free space pH in both tissues. In maize coleoptiles, free space pH begins to fall within about 12 minutes of exposure to auxin and decreases by about 1 pH unit by approximately 30 minutes. In pea, pH begins to decrease within an average of 15 to 18 minutes of exposure to auxin and falls by about 0.9 pH unit by approximately 40 minutes. Auxin-stimulated elongation, measured in the same two tissues similarly prepared, appears in maize at the earliest 18 minutes after auxin application, while in pea it appears at the earliest 21 to 24 minutes after auxin application. The auxin analogs *p*-chlorophenoxyisobutyric acid and phenylacetic acid do not stimulate elongation above control levels in maize or pea tissue segments and do not cause a decrease in free space pH in either tissue. These findings are consistent with the acid secretion theory of auxin action.

According to the currently popular acid secretion hypothesis of auxin action (2, 7), auxins stimulate growth by causing acidification of the cell walls, which at low pH undergo an increase in extensibility that leads to rapid cell enlargement. Several lines of evidence are consistent with this hypothesis (7, 9, 19, 24), but published measurements of auxin-induced release of acid from tissue into an external bathing medium (3, 10, 11, 18) have not shown that auxin stimulates H^+ secretion quickly enough to account for the rapid response (17) of elongation to auxin. If the acid secretion theory is correct it must be demonstrable that following exposure to an auxin the pH in the cell wall space falls to an elongation-stimulating value by the time that observable elongation in response to auxin actually commences, *i.e.* within the latent period for auxin action on elongation. A pH microelectrode that permits this type of measurement (13) has recently been used to deny the validity of the acid secretion hypothesis (14). Employing here the same type of electrode to examine more critically the pH in the free space of maize coleoptile and pea stem segments we are able to confirm the predictions of the acid secretion hypothesis.

MATERIALS AND METHODS

Plant Material. Seeds of *Pisum sativum* (var. Alaska) were soaked for 6 hr in tap H_2O, planted in Vermiculite, and germinated for 6 to 7 days at 25 C in the dark, except for brief exposures to dim red light every other day during watering. Segments approximately 8 mm long were cut from the third internode beginning 3 mm below the apical hook.

Seeds of maize (*Zea mays* var. Bear Hybrid) were soaked for 5 hr in tap H_2O, planted in Vermiculite, and kept in the dark at 25 C for 4.5 days, except for brief exposures to dim red light every other day during watering. A segment 8 mm long was cut from the coleoptile beginning 3 mm below its tip.

Free Space pH Measurements. Free space pH was measured using a Beckman Expandomatic pH/millivolt meter (model 76 A) equipped with a pH microelectrode (antimony film on stainless steel core, model 814, Transidyne General Corp., Ann Arbor, Mich.) of exposed tip length of 5 μm and tip diameter of 2 μm. The reference electrode was a segment of glass tubing (80 $\times$ 1 mm) the end of which was drawn into a capillary with a tip diameter of a few μm. This tube was filled with 3 M KCl, in which was immersed an AgCl-coated silver wire that connected to the reference terminal of the pH meter. The electrode was read using the mv mode of operation of the meter and was calibrated against phosphate-citrate buffers (50 mM in K-phosphate and Na-citrate) of pH 4 and 6 before and after each experiment.

The relation between pH of a buffer solution and mv reading by the pH microelectrodes was checked for several microelectrodes with a series of solutions varying in pH from 4 to 7. Because this relation was always found to be linear over the pH range, mv readings from experiments were converted to pH values by linear interpolation using the mv values measured for the calibration buffers of pH 4 and 6.

The sensitivity of a pH microelectrode varied, for different experiments, from 50 to 58 mv/pH unit, but it was always constant through a given experiment. Between the beginning and the end of a typical experiment, however, the absolute mv values recorded by the microelectrode for the two calibration buffers would often change by a few mv. This drift in value was always of the same magnitude and in the same direction for both calibration buffers. Thus, the sensitivity of the microelectrode did not drift. Although calibration of the microelectrode against buffers of known pH before and after each experiment allowed us to monitor microelectrode drift, we used only the results of the postexperiment calibration for conversion of microelectrode mv readings to pH values.

During use a gradual aging of the antimony microelectrode occurred, as evidenced by its speed of response. During its first 10 to 15 hr of use an electrode responded fully, within about 2 sec, to replacement of one buffer with another differing by 2 pH units. After 10 to 15 hr of use, however, an electrode came to require 30 to 60 sec to respond fully to transfer between different buffers. Such an "aged" electrode often needed to be presoaked for 2 to 3 hr in a tissue macerate or implanted into a tissue segment for that length of time, before it would read pH changes in experimental tissue in response to IAA, whereas a

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"young" electrode would register pH changes in response to IAA in the first tissue segment into which it was implanted on a given day, as well as in subsequent segments. Interestingly, once an aged electrode finally began to respond, it would continue to do so, repeatedly, for the remainder of the given day. It is nevertheless recommended that for faster, more reliable readings, these electrodes be used for only 10 to 15 hr.

For pH measurement a tissue segment was rinsed briefly in phosphate-citrate buffer (1 mM in both K-phosphate and Na-citrate), pH 6, then suspended horizontally from its ends approximately 3 mm above the floor of a 5-cm Petri dish, using two pieces of dental wax (Fig. 1A). About 8 ml of 1 mM phosphate-citrate buffer, pH 6, were added until the liquid level was such that the lower surface, cut ends, and parts of the sides of the segment, but not its upper surface, were bathed by the buffer.

The pH microelectrode and reference electrode, mounted together on a micromanipulator, were put into place while being viewed through a dissecting microscope (7×) on the stage of which the Petri dish rested. The microelectrode was always implanted in the unsubmerged upper surface of the maize or pea segment, out of contact with the bathing medium. For pea experiments, the microelectrode tip was inserted approximately 50 μm into the tissue, about 1.5 mm from the apical end of the segment, immediately withdrawn part way, and reinserted not quite as far as the first time, before readings were taken. For maize experiments, the microelectrode tip was placed approximately 50 μm into tissue near to, but not part of, one of the vascular bundles, again 1.5 mm from the apical end of the segment, in the same fashion as with pea. The reference electrode was placed in the bathing solution near the apical end of the segment (Fig. 1A).

By examination of freehand paradermal sections prepared after the pH measurements were completed, we found that the pH microelectrode so placed would usually kill one epidermal cell that it punctured and one subepidermal parenchyma cell just beneath the first cell, but leave adjacent cells in both cell layers intact.

Once the electrodes were in place, pH readings were taken every 3 min in room light. After four or five readings (about 15 min), the indicated pH had usually become steady (pH 5.7 to 6 for maize, pH 6 to 6.2 for pea). At this time we added to the bathing medium 1/10 its volume of 1 mM phosphate-citrate buffer, pH 6, either plain (control runs), or containing IAA or another test compound to give a final concentration normally of 20 μM. Stirring of the medium around the segments during this addition was accomplished by injecting the added solution into the dish using a syringe and 25 gauge needle, the tip of which was held below the surface of the solution and its stream directed horizontally, parallel to the wall of the dish. The rapid injection of the treatment solution caused an obvious stirring to take place. The solutions were not stirred further; pH readings were taken every 3 min for the next 40 to 50 min.

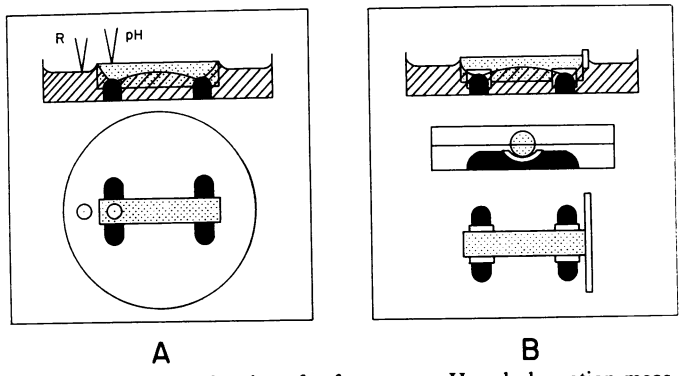


FIG. 1. Segment chambers for free space pH and elongation measurements. A: Free space pH measurement: segment was positioned horizontally, partially submerged in treatment solution, in a 5-cm Petri dish. Each end was supported by a piece of dental wax, and electrodes were positioned as shown during readings. B: Elongation measurement: segment was positioned horizontally as in A, but rested on two U-shaped pieces of glass which were fixed to the dental wax, and one end abutted against a flat piece of glass fixed perpendicularly to the segment.

Experiments on pea and maize with IAA and buffer treatments were repeated more than ten times. Experiments with other treatments were repeated at least three times.

Elongation Experiments. Segments of pea or maize were rinsed briefly in 1 mM phosphate-citrate buffer, pH 6, then placed in a 5-cm Petri dish as for free space pH measurement, but with the following differences. (*a*) Instead of resting directly on the dental wax, the segment ends rested on U-shaped half-tubes of thin glass that were fixed to the dental wax. This provided a more friction-free surface for segment elongation (Fig. 1B). (*b*) The top half of one end of the segment was pushed up against a small, vertical, rectangular glass plate, cemented at its ends to the walls of the Petri dish and placed across the dish in a direction perpendicular to that of the segment's long axis.

Phosphate-citrate buffer (1 mM), pH 6, was again added so that the lower surface of the segment, one cut end, and the lower half of the other cut end were covered by buffer. The dish was then mounted on a compound microscope stage with the light beam coming up from below past the free end of the segment. Segment elongation was followed by observing the free end of the segment against a calibrated ocular micrometer using a 40× objective and a 10× eyepiece.

Readings were taken every 3 min for 15 min, then buffer (control) or buffered solutions of test compounds were added in the same manner as in the free space pH measurements. Elongation was followed for 50 to 60 min.

Tests of segment elongation as a function of external pH were made using segments 8 mm long cut from maize coleoptiles or pea third internodes that had been abraded by stroking with emery paste, as previously described (5), to render the cuticle permeable to water and solutes. Elongation of pea segments was measured by determining the aggregate length of groups of 10 segments each, as described (9). After preliminary experiments on maize using the same method, elongation of maize coleoptile segments was measured using the apparatus of Green and Cummins (6), with the modification that only the external surface (not the central cavity) was perfused. For both maize and peas the growth media were 12 mM in K_2HPO_4 and 12 mM in Na-citrate plus citric acid, to give the indicated pH values. The elongation rate of each maize coleoptile segment placed in the perfusion chamber of the elongation recording apparatus was measured first in pH 7 buffer, then the perfusion medium was changed to a test buffer of lower pH, and measurement was continued until completion of the transient rise and subsequent decline of the elongation rate (usually about 20 min). The acid-stimulated rate was taken as the maximum rate reached within 1 to 3 min after transfer to the more acidic medium.

RESULTS

The pH microelectrode measurements to be presented were taken within the cavity made by puncturing a single epidermal cell and the subepidermal parenchyma cell located interior to it. The technique of electrode insertion, withdrawal, and partial reinsertion (see "Materials and Methods") ensures that the penetrated cells are killed and their cytoplasmic membranes thoroughly disrupted, so that the penetrated cell cavities become part of the free space of the tissue. Because of the distances of only a few μm involved in diffusion between these cell cavities and the adjacent uninjured free space, the cell sap contents of the penetrated cells must diffuse away into the surrounding free space within a very small fraction of a min (15). By the same

token, and because the penetration is made in a position where the penetrated cell chambers cannot exchange solutes directly with the medium that bathes the tissue segment (Fig. 1A), the pH within the penetrated cell cavities must equilibrate rapidly with the pH of the adjacent cell wall space and is therefore employed as a measure of the latter.

From Donnan principles, the pH in the cell wall itself cannot be exactly the same as that in a dilute liquid free space with which it is in equilibrium (20). Therefore, the pH values in these experiments do not strictly represent cell wall pH and will be referred to as free space pH values. For comparison with the effects of external pH on segment elongation, the liquid free space pH rather than cell wall pH is really the pertinent parameter. A system more suited to the measurement of cell wall pH itself is the pH microelectrode being developed by Saftner and Hollander (21).

Effect of IAA on Free Space pH. In Figure 2, eight separate experiments are shown in which the free space pH of maize coleoptiles was monitored following addition of either IAA or buffer alone to the dilute phosphate-citrate medium bathing the segments. The treatment and control experiments in each graph were done consecutively, so that conditions were as comparable as possible for each pair of experiments. IAA initiated a decrease in free space pH after a lag that varied, in different experiments, from apparently less than 3 min (Fig. 2B) to as long as 18 min (Fig. 2D). The decrease in pH continued for 18 to 21 min, then ceased at a value that varied from pH 4.4 to 5. The free space pH of control segments (buffer alone added at time zero) appeared to decrease very gradually during the same time from initial values of about 5.8 to 5.9 to final values of 5.7 to 5.8.

In Figure 3, eight different experiments are shown in which the free space pH of pea stem segments was similarly monitored following addition of IAA or buffer alone to the medium. As with maize coleoptiles, IAA and control experiments were done consecutively. IAA brought about a decrease in free space pH after a lag that varied from 12 min (Fig. 3A) to 15 min (Fig. 3B). The decrease continued for 28 to 30 min, then ceased at a value that varied from pH 5 to 5.2. The free space pH of pea control segments decreased gradually during the same time from initial values of 6.2 to 6.6 to final values of 5.8 to 6.3. In several control experiments that were extended to 1-hr duration, those control readings which had not ceased to decrease at 45 min had become constant by 1 hr at values never less than pH 5.6 (data not presented).

The apparent variations from segment to segment measured in the response of pea and maize free space pH to auxin application undoubtedly have components other than biological variation. The occurrence and direction of the drift in values recorded for the calibration buffers during an experiment could not be controlled. The maximum calibration drift encountered during these experiments was about 5 mv, equivalent to a deviation of about 0.1 pH unit from the real pH value. It is also possible that differences in the age of the microelectrode from experiment to experiment, even within the 15 hr maximum age limit that we considered advisable (see "Materials and Methods"), could have affected the apparent length of the lag between auxin application and onset of decrease in free space pH. However, although new electrodes responded more rapidly than older ones to changes in calibration buffers, no strong correlation between young electrodes and short lag periods in response to IAA was noticed.

Figures 2, 3, 6B, and 7B also show that sudden changes in the

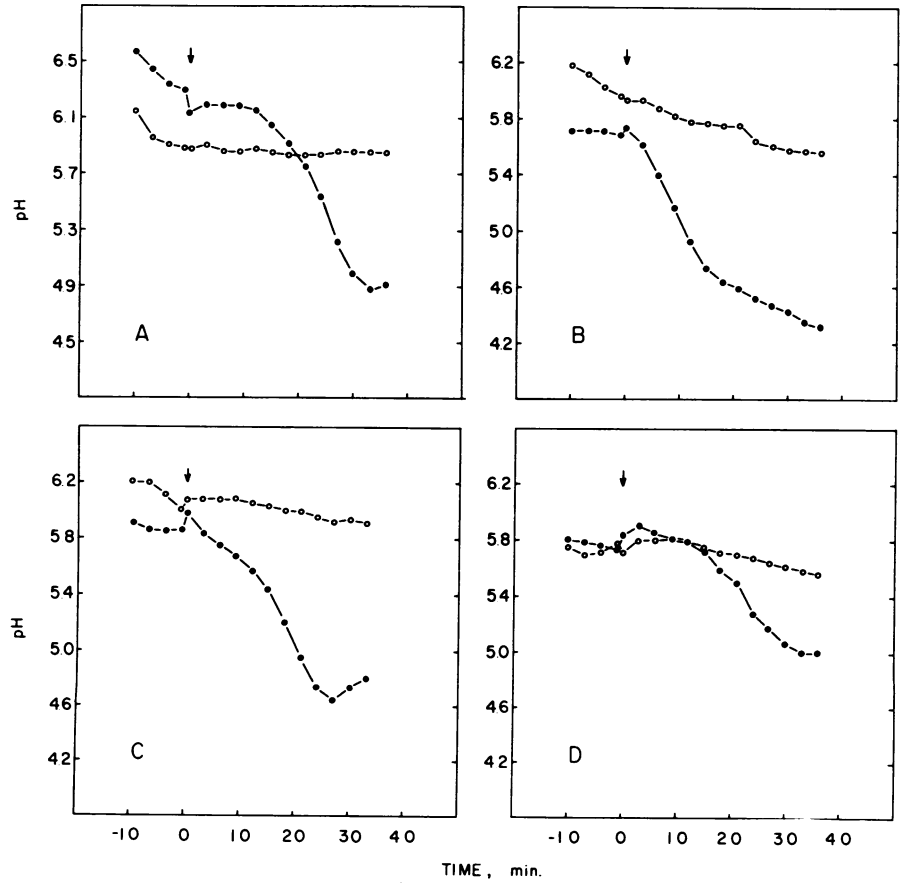


FIG. 2. IAA-induced decrease in free space pH in maize. A, B, C, and D show eight different experiments in which 20 μM IAA in 1 mM phosphate-citrate buffer, pH 6, or buffer alone was added at the arrow. Change in pH was monitored with a pH microelectrode positioned as described under "Materials and Methods." ●: IAA; ○: buffer control.

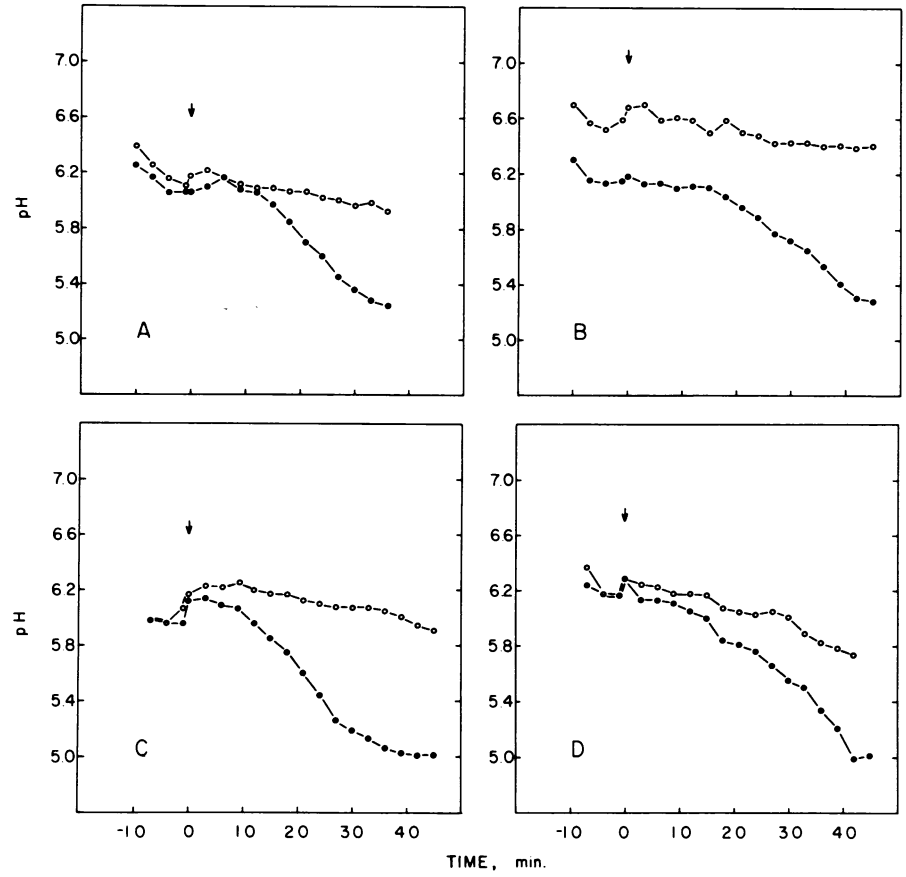


FIG. 3. IAA-induced decrease in free space pH in pea. A, B, C, and D show eight different experiments in which 20 μM IAA in 1 mM phosphate-citrate buffer, pH 6, or buffer alone was added at the arrow. ●: IAA; ○: buffer control.

pH reading of 0.1 to 0.2 pH unit were sometimes observed upon addition of test solutions. The changes were not predictable in occurrence and were presumably instrumental artifacts. Several procedural variations were tried in an effort to eliminate such artifacts but no modification eliminated them altogether.

The free space pH measurements for control and IAA treatments of maize (Fig. 2) and pea (Fig. 3) were averaged with the results of three other similar control and IAA experiments on each tissue and these means are plotted in Figures 4B and 5B, respectively. In maize coleoptiles (Fig. 4B) the mean free space pH declines from pH 5.8 to 4.8 in IAA; the decrease appears to begin immediately after exposure to IAA, but because of variance among the experiments 12 min is the earliest that the decrease in pH becomes statistically conclusive. In pea segments (Fig. 5B) the mean pH decreases from pH 6.2 to 5.1 in IAA, apparently beginning 12 min after exposure to IAA and becoming statistically conclusive by about 18 min considering the variance among experiments.

Timing of Auxin Elongation Response. Figures 4A and 5A show the average time course of elongation response of maize coleoptile and pea stem segments, respectively, to IAA. The segments were mounted and the treatments applied in a manner closely corresponding to that used in the free space pH experiments. Experiments from which these averages were computed, showing individual variations in timing of the elongation response, are included in Figures 6A and 7A. Auxin-induced increase in the elongation rate of both maize coleoptile and pea stem segments under these conditions began after an average latent period of 21 min. Thus, for both pea and maize, the decrease in pH of the free space induced by IAA began earlier than the auxin-induced elongation response.

Effects of Auxin Analogs. The effects on elongation and on free space pH of 20 μM PAA[3] and PCIB are shown for maize coleoptile and pea stem segments in Figures 6 and 7, respectively. The auxin analogs were tested in a series of experiments that included IAA and control treatments which are also shown.

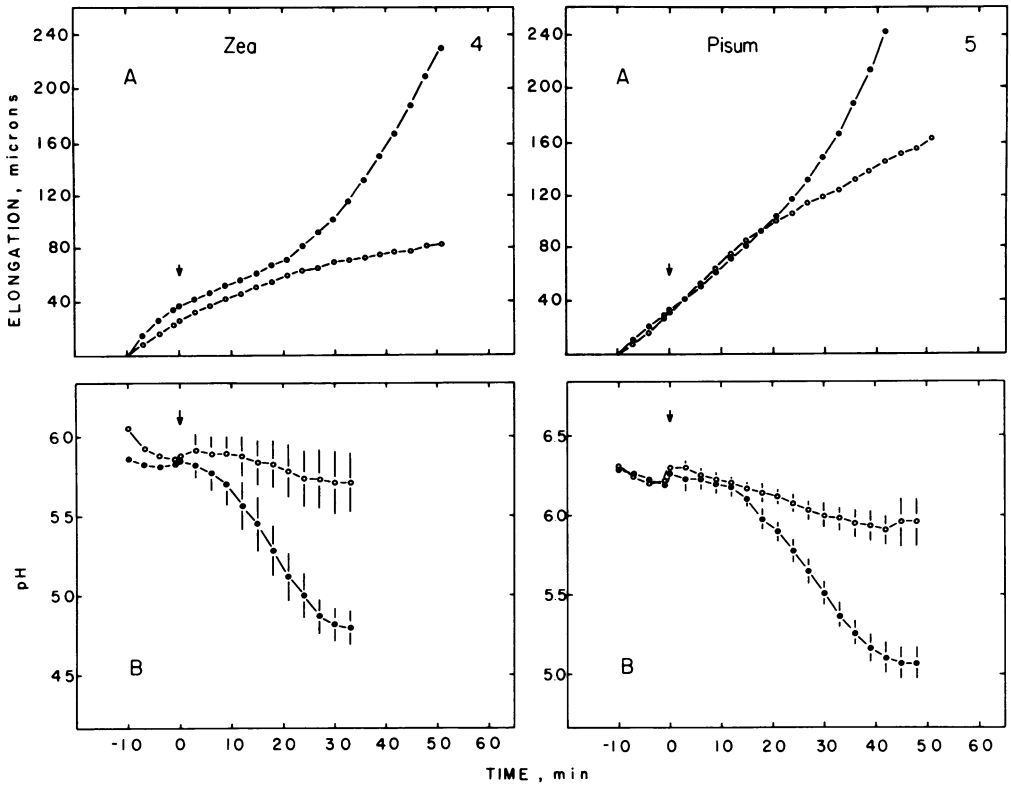
PCIB or PAA did not have any effect on pH or on elongation significantly different from the results with buffer alone. Similar results have been obtained previously with certain IAA analogs in pH measurements on external bathing media (10, 18).

Elongation in Response to pH. To determine the dependence of elongation rate on free space pH we treated maize coleoptile and pea stem segments with phosphate-citrate buffers of different pH. Neither of these tissues gave a substantial response to acidic media unless the segments had been scrubbed with emery paste to make the cuticle permeable (5). If the cuticle was abraded, both maize coleoptile and pea stem segments under our conditions elongated in response to acidic media but this elongation rate, unlike that caused by IAA, declined rather rapidly with time during exposure to an acidic medium.

As illustrated previously (9), the elongation response of pea segments to acidic pH lasted almost 2 hr, so a 2-hr treatment was used to determine the elongation response as a function of pH (Fig. 8). The results are similar to those previously given for peeled segments from light-grown pea seedlings (25). Allowing for the decline in elongation rate with time in acidic media (9), the early elongation rate at pH values around 5 appears to be similar to the elongation rate of segments treated with IAA.

In the case of maize coleoptiles, rapid elongation of scrubbed segments in response to acidic media declined within about 20 min, as illustrated in Figure 9. Therefore, the elongation rate

[3] Abbreviations: PAA: phenylacetic acid; PCIB: *p*-chlorophenoxyisobutyric acid.



FIGS. 4 AND 5. Comparison of auxin-caused decrease in free space pH with auxin-caused increase in elongation rate in maize coleoptiles (left) and in pea stem segments (right). A: Average elongation in response to 20 μM IAA or buffer control added at the arrow. B: Changes in free space pH in response to 20 μM IAA or a buffer control added at the arrow. Each curve is the mean of seven different experiments with the corresponding treatment. Vertical bars are the standard error of the mean for each point. ●: IAA; ○: buffer control.

response of maize coleoptile tissue to pH was measured in short term experiments like that of Figure 9, using the apparatus of Green and Cummins (6), with results given in Figure 10. The initial elongation rates observed after transfer to pH 4.5 to 5 were similar to the steady elongation rate of maize coleoptile segments treated with IAA. A pH curve very similar to that shown in Figure 10 was obtained by measuring the elongation of scrubbed coleoptile segments over 2 hr by the same technique used for peas in Figure 8 (data not presented). But because of the rapid decline of elongation rate with time (Fig. 9), the total elongation of scrubbed coleoptile segments during 2 hr at pH 4 to 5 was much less than that of unscrubbed coleoptile segments kept for 2 hr in IAA.

These results indicate that the steady elongation rate attained by pea stem or maize coleoptile segments when treated with IAA is similar to the initial elongation rate that these tissues exhibit when they are exposed to a phosphate-citrate buffer of about the same pH as that which develops in their free space under treatment with IAA, according to our measurements (Figs. 4B and 5B).

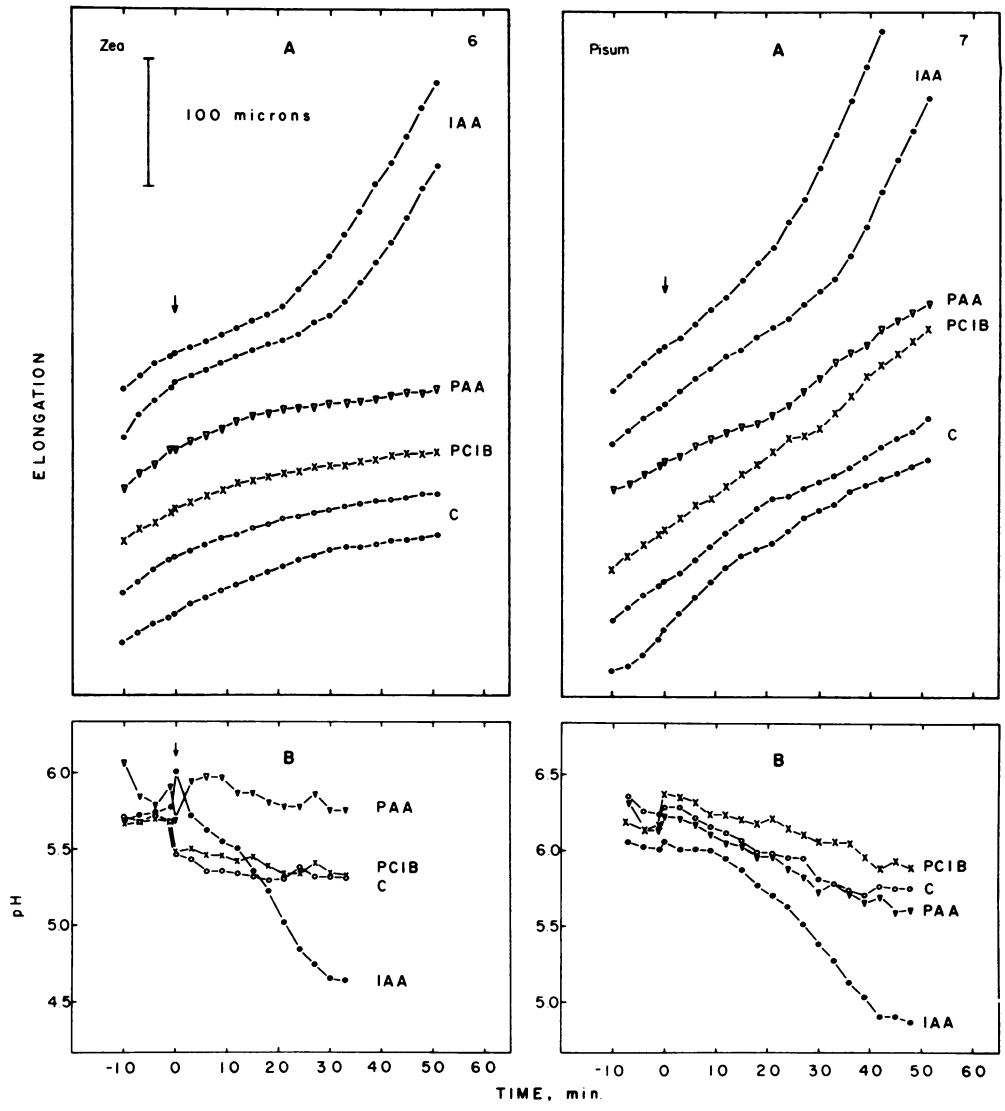
DISCUSSION

The reported experiments show that IAA can cause a decrease in free space pH in tissue of both a dicotyledon, pea, and a monocotyledon, maize. The decrease induced is, in both tissues, to a pH value that of itself is capable of stimulating segment elongation to a rate similar to that obtained by treatment with IAA. In both tissues, the pH decrease occurs in a time range short enough to qualify it as causative of the early elongation that is induced by IAA under these conditions. The analogs PCIB and PAA, which at the same low concentration as that employed with IAA do not cause significant stimulation of elongation in either tissue, also do not cause any substantial change in free space pH. Since PCIB and PAA are physically similar to IAA and each is at least as strong an acid as IAA, this result shows that the decrease in pH seen in IAA is not due to diffusion of undissociated IAA into the free space.

Penny *et al.* (14) recently reported attempts to detect auxin-caused decrease in extracellular pH in oat coleoptile and lupine hypocotyl using the same type of pH microelectrode that was employed in the present experiments. They detected no marked drop in pH in response to 30 μM IAA. However, the microelectrode tip was placed only 20 μm into the open end of a xylem vessel exposed at one cut end of a tissue segment mounted horizontally in a flow-through solution chamber. Since the segment's cut end was apparently submerged, diffusion of protons past the inserted electrode over the short distance between xylem vessel and bathing medium would have had a half-time of about 5 sec (15). This would effectively prevent any appreciable pH changes from being recorded at the site of measurement even if substantial pH changes occurred in the free space deeper within the tissue segment.

Auxin can induce changes in external bioelectric potentials of young plant tissues (1, 12) and the microelectrode used here may be sensitive to these potentials. However, any major interference in the pH readings reported here by hormone-induced potential changes can be discounted since reported values for these changes have been in the range of 5 to 6 mv (1, 12), whereas we observe IAA-induced changes in pH microelectrode potential of 50 to 80 mv. By the same token, the artifacts of ± 5 mv often introduced by the addition of test solutions, or by drifts in the calibration potential against standard buffers, were also too small to constitute major sources of interference.

The results demonstrate, in both maize and pea, that the free space pH begins to drop significantly before the onset of increased elongation in response to IAA. Cleland's recent work (4) agrees with this finding: using a more sensitive technique for



FIGS. 6 AND 7. Effects of auxin analogs on elongation and free space pH in maize coleoptiles (left) and in pea stem segments (right). A: Elongation responses; B: effects on free space pH. At the arrows, 10 μM IAA, PAA, PCIB, or buffer alone (C) was added.

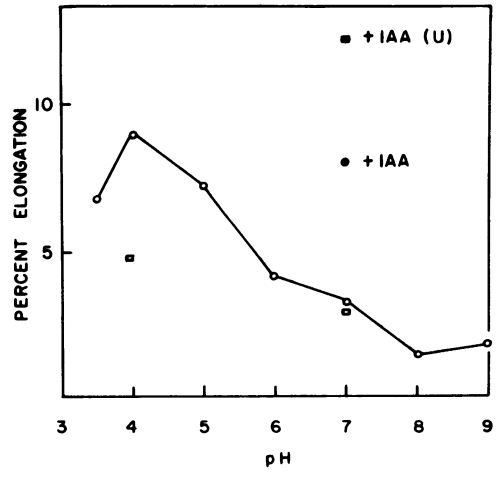


FIG. 8. Elongation of pea stem segments during 2 hr in phosphate-citrate buffers of indicated pH. Circles show segments with abraded epidermis. Squares indicate unscrubbed segments that possess an intact cuticle. IAA (17 μM) was added to samples shown by closed symbols. Each point is the mean for duplicate samples of 10 segments each.

measuring proton secretion by *Avena* coleoptile segments, he has now found an IAA-induced decrease in pH of the segments' bathing medium that begins early enough to explain IAA-induced elongation in that tissue.

The evidence that rapid acidification of the free space occurs in response to auxin does not necessarily mean that auxins activate an H^+ pump as often assumed (7, 11, 16). Sloane and Sadava (22) recently claimed that the slight acidification of an external medium that they obtained with auxin-treated pea stem segments was due entirely to CO_2. Retention of respiratory CO_2 could contribute to free space acidification either directly or through formation of organic acids (8). Whatever its mechanism, the phenomenon of rapid auxin-induced acidification of the free space now seems firmly established for at least one dicotyledonous (*Pisum*) and two monocotyledonous species (*Zea* and *Avena;* present work and ref. 4). In view of the ability of acid pH to directly cause wall extension, the auxin-induced acidification appears sufficient to account for the rapid cell enlargement response in these tissues to IAA.

Although it occurs rapidly, auxin-induced acidification of the free space is not short-lived but persists for many hours, as shown by previous long term measurements with external bathing media (3, 8, 10, 11, 18). However, the transient nature of

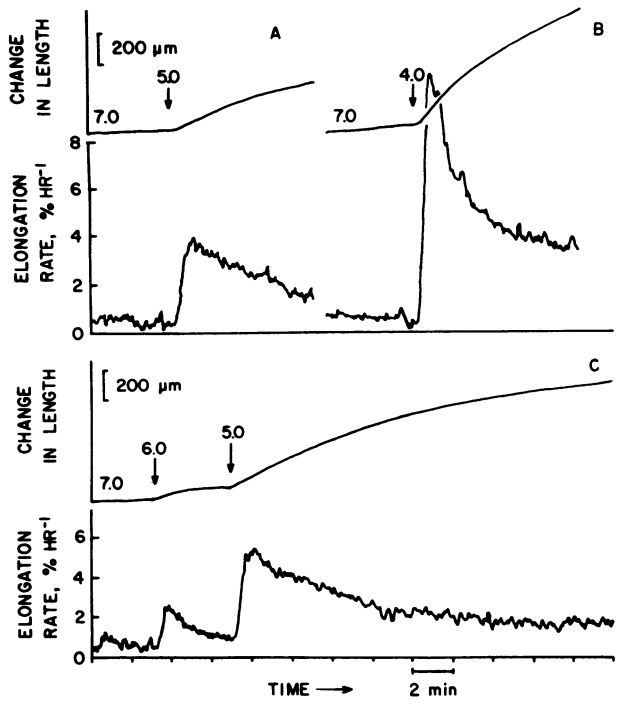


FIG. 9. Elongation and elongation rate of maize coleoptile segments as recorded by transducer measurement during transfer from phosphate-citrate buffer, pH 7, to similar buffers of lower pH.

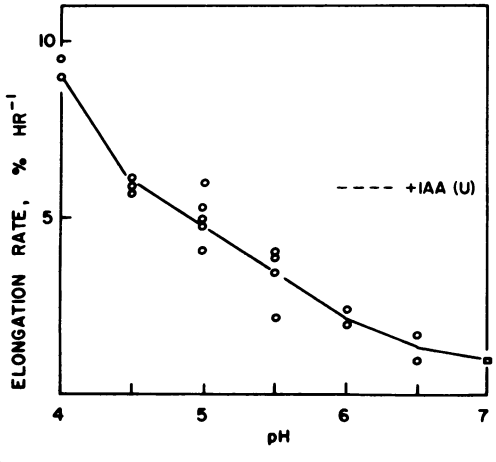


FIG. 10. Initial elongation rate response of maize coleoptile segments to pH. Elongation rate of each segment was measured first at pH 7 (mean value given by square) prior to transfer to a lower pH, for which each circle shows the results with a different coleoptile segment. Measurements were made as in Fig. 9 A and B. Dashed line shows elongation rate of unscrubbed coleoptile segments after their response to 20 μM IAA (Fig. 4).

the elongation response to acidic pH values in pea (9) and maize (data given here) gives reason to think that the longer term, continuous elongation of stem or coleoptile segments in auxin requires some kind of performance additional to acidification of the cell wall space. A similar suggestion regarding soybean hypocotyl segment elongation was recently advanced on other grounds (23).

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