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On the Relationship between Extracellular pH and the Growth of Excised Pea Stem Segments

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ABSTRACT

Studies with stem segments of peas (*Pisum sativum* L. var. Alaska) suggest that the pH of the medium bathing elongating tissue does not always reflect intramural (cell wall) conditions or that pH is not a controlling factor in elongation. Peeled, green segments, and peeled or nonpeeled etiolated segments appear to regulate the pH of their bathing medium causing it to become acidified with or without the addition of auxin. The growth rates of segments are greatest during a period before acidification is evident and slow during the time in which the medium becomes acidified. We cannot reproduce the dramatic auxin-induced pH shifts reported in the literature because the control segments are becoming more acid also; but there is some evidence that acidification may occur in response to auxin treatments. K^+ additions mimic the acidifying tendency of auxin but are without growth-promoting effect. Emergent growth (an extremely rapid burst of growth following anaerobic treatments) is not accompanied by a drop in pH of the bathing medium. Proper aeration of the bathing medium in extracellular pH studies is crucial and may explain differences between our results and other published accounts. The data suggest that the techniques used for most extracellular pH studies may not very closely approximate *in vivo* conditions or properly reflect intramural H^+ concentration fluxes.

In 1932, Strugger (29) reported that acidic media promoted the growth of sunflower roots. Bonner (2) found that oat coleoptiles were also responsive to lowered pH and suggested that "there might be some relation between growth substance [auxin] and the 'acid growth' of Strugger." Most of the early workers concluded, along with Went and Thimann (32), that acid growth was probably due to the dissociation of auxin from the salt to free IAA (which was presumed to be the growth-active form). In the next 30 years, a number of workers alluded to or reported without comment pH effects on growth and auxin action (see 19). In 1970, the topic was revived by at least three new reports of the effect of pH on growth (*e.g.* 26). These were followed by a lengthy and well supported paper by Hager *et al.* (11) who proposed that auxin acted by promoting extrusion of protons, acidifying the cell wall with consequent loosening and elongation. Several more reports appeared which documented the acid growth effect and lent indirect support to an active acidification mechanism (*e.g.* 7). Hager's proton pump model received direct support when several workers reported that auxin induced a pH drop in the solution's bathing treated tissues (3, 16, 33). Marré and co-workers proposed that the proton extrusion was partially electrogenic (17) and that K^+ acted as a counterion (18). That auxins hyperpolarize cells (8) and promote ion uptake (6, 12) had been known for some time.

There have been reports which appear to contradict the acidification model for cell wall extension. Barkley and Leopold (1) could not evoke an acid growth response in green peas. Cleland and Rayle (5) duplicated the work but used segments which had been stripped of their epidermis and found the peeled segments to be fully responsive to lowered pH. A number of investigators have reported that they find no rapid auxin-induced pH drop (22, 24, 25) or that there are basic differences between auxin-induced and acid-induced growth (9, 23). Sloan and Sadava (28) have suggested recently that the auxin-induced pH drop may be due to increased production of respiratory CO_2.

We report here that pH changes in the medium bathing pea stem segments show no consistent correlation with growth rates (3). The evidence suggests that although a pH drop may be associated with increased growth, the most commonly used techniques for measuring pH change may not reflect intramural conditions as closely as has been assumed.

MATERIALS AND METHODS

Expanding internodes were excised from 7- to 10-day-old glasshouse-grown or 6-day-old etiolated peas (*Pisum sativum* L. var. Alaska). The epidermis was removed with forceps and the stem segments trimmed to 1 cm. In some experiments, the segments were left unpeeled or the epidermis was slit with four shallow, longitudinal incisions. Segments were washed in 1 mM Na or K-phosphate (pH 6.2-6.4) and then placed in fresh, vigorously aerated buffer with or without 100 μg/ml penicillin G.

For pH measurements, a number of segments (usually 30) were placed in a 10-ml beaker with 5 ml continuously aerated buffer. A Corning semimicro combination glass electrode was inserted into the solution. For continuous recording, the Corning model 12 pH meter was joined to a Leeds and Northrup Speedomax XL680 potentiometric recorder. During anaerobic treatments, the beaker was covered with Parafilm and 100% N_2 bubbled in the buffer.

Growth measurements of a column of segments using an angular position transducer and production of anaerobic conditions for growth measurement were carried out as previously described (21).

RESULTS

Green stem segments which have had their epidermis slit or removed cause a distinctive pattern of endogenous pH changes in their bathing medium. ("Endogenous" is used in the sense that the changes are regulated by the segments themselves). Under continuous aeration, the pH of a weakly buffered bathing medium rises gradually to about 6.5 and begins to fall after 1 to 3 hr (Fig. 1). The pH normally decreases during the next 3 to 5 hr

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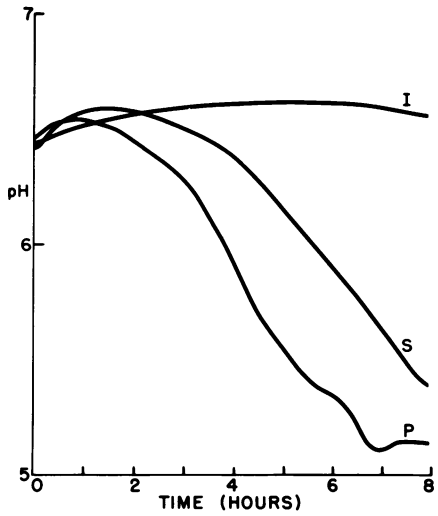

FIG. 1. Changes in pH of media containing green stem segments. Freshly harvested segments (30/treatment) were placed in 5 ml 1 mM Na phosphate buffer and bubbled with air. Segments were intact (I), peeled (P), or slit with four shallow incisions (S).

to about 5 and stabilizes. Some variation in inflection times but not pH values is a result of varying the number of segments. The endogenous pH drop depends partially upon removal of the epidermis, as intact, green segments show a much reduced acidifying tendency, and slit segments respond more slowly than peeled ones. The buffering capacity of the 1 mM bathing medium does modify the pH changes somewhat: following the addition of peeled green segments, the pH of aerated distilled H_2O rises within 1 hr to about 6.5 and then drops within 2 to 3 hr to about 5 and stabilizes (data not shown). Although light-grown pea stems exhibit large and relatively rapid pH shifts only if peeled, etiolated peas show the same pattern of a pH rise and subsequent acidification whether peeled or not (Fig. 2).

Removal of segments from the endogenously acidified solution does not cause the pH to rise, suggesting that respiratory CO_2 is not contributing to acidification in the air-bubbled system. The pattern of pH changes is the same in the presence or absence of 100 μg/ml penicillin G. DNP[2] blocks acidification but not the initial pH increase (Fig. 3). Anaerobic treatments delay the endogenous pH drop by the amount of time the segments are without O_2 (Fig. 4). Vigorous aeration appears to be crucial for observation of the pH drop: if air is not bubbled continuously, the acidification is markedly reduced or even reversed (data not shown).

In about half of more than 20 trials, auxin treatments of up to 60 peeled segments in 5 ml buffer caused the medium to become slightly more acid than auxin-free controls (Figs. 2, 5, and 6). (In those trials where no additional auxin-induced acidification developed, the pH values for the control never dropped below the auxin-treated segments.) A lag of about 15 to 20 min is observed, and the maximum differences between auxin-treated and control segments occurs by 3 to 4 hr after addition of IAA. Thereafter, the declining pH of the medium in the control treatment brings the values together. The inability to detect large differences in pH after addition of IAA is not due simply to endogenous acidification obscuring the auxin effect. When IAA is added immediately after peeling (2 to 3 hr before the endogenous pH drop), the initial alkalinization is not prevented or reversed (data not shown). The concentration of auxin used in the pH studies (10 μM) was growth-promotive, enhancing the growth of peeled green stems 2- to 4-fold with a lag time of 5 to 10 min (Fig. 5). The growth rate of freshly peeled segments was initially much higher than the unpeeled, but those which were peeled underwent a rapid loss of vigor (as expressed by growth rate) (Fig. 5). Nonpeeled stems, in contrast, grow more slowly, remain viable longer, and are more responsive to IAA (21).

Potassium causes acidification of the bathing medium when segments are present (Fig. 6). When added to K^+-free buffer, K^+

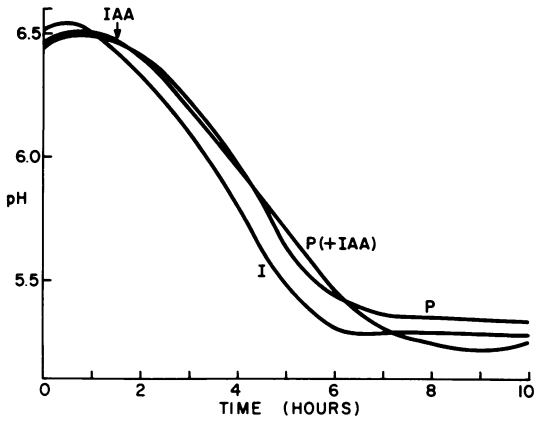

FIG. 2. Changes in pH of media containing etiolated stem segments. Thirty segments were added to 5 ml 1 mM phosphate buffer at 0 time. Segments were intact (I) or peeled (P). IAA (10 μM) was added to one set of peeled segments (P + IAA) after 1 hr. IAA likewise produced no distinct pH effect when added to nonpeeled etiolated segments.

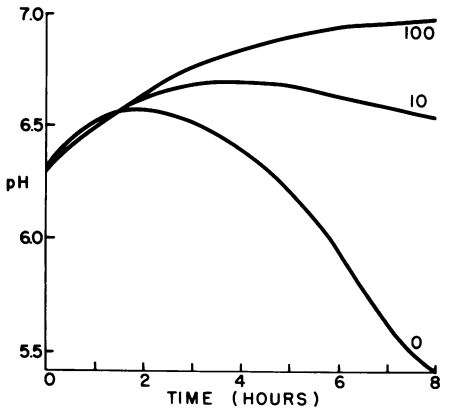

FIG. 3. Effect of DNP on endogenous pH changes. Freshly harvested and peeled segments (20/treatment) were aerated in buffer to which had been added 0, 10, or 100 μM DNP.

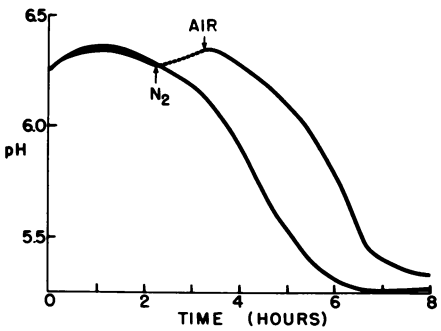

FIG. 4. Effect of anoxia on endogenous pH changes. Peeled segments (27/treatment) were bubbled with air except for the period indicated by the dashed line (during which the beaker was covered with Parafilm and bubbled with N_2). No pH measurements were made during the period of N_2 bubbling.

[2] Abbreviations: DNP: 2,4-dinitrophenol.

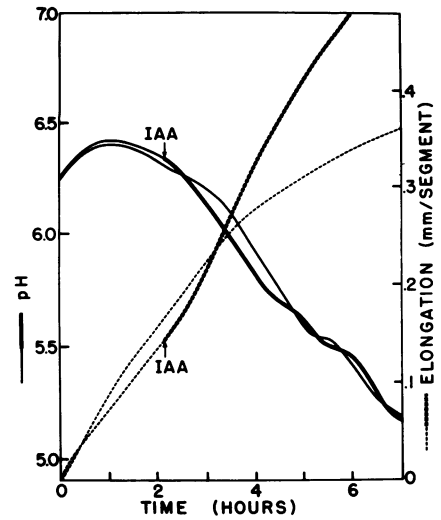

FIG. 5. Effect of IAA on elongation and endogenous pH changes. Peeled stem sections were placed in the auxanometer in buffer, bubbled with O_2, and their growth recorded (- - -). Twenty-five peeled segments were simultaneously placed in buffer for the pH studies (——). IAA (10 μM) was added at the arrows. Elongation and pH changes in auxin-treated segments are indicated by bolder lines.

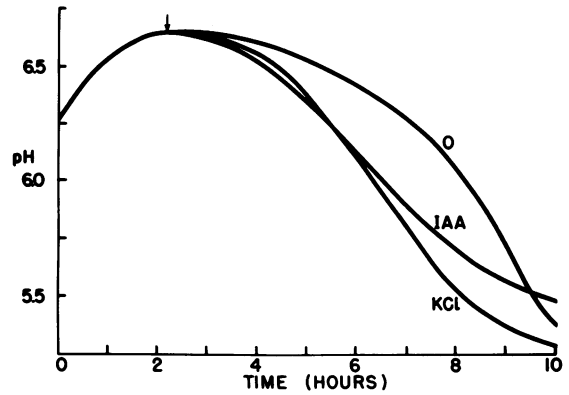

FIG. 6. pH response to auxin and potassium. Freshly peeled segments (22/treatment) were added to aerated K^+-free buffer. At the arrow, 10 μM IAA, 5 mM KCl, or nothing was added.

causes the pH to drop slightly below control values within 15 to 20 min. KCl-treated segments stay at a pH below that of the control segments throughout the course of the measurements. The stabilized pH values are 4.7 to 5 for KCl, and 5 to 5.3 for control or IAA-treated segments. The acidifying phenomenon seems to be specific for K^+, as Na^+ and Ca^{2+} were without similar effect. Despite its acidifying tendency, the effect of K^+ addition on growth is negligible (Fig. 7). The 5 mM solution of KCl does not osmotically restrict growth as larger osmotic shifts fail to affect IAA-induced growth (unpublished results).

While anaerobic treatments delay the endogenous acidification (Fig. 4), restoration of O_2 after 15 min to 2 hr of anoxia results in a burst of elongation such that growth "lost" during the inhibitory treatment is recovered and often exceeded by 100 to 200% (Fig. 8). This phenomenon, which we have named "emergent growth" (20), will be reported in some detail elsewhere. For the present, we wish only to note that the extremely rapid period of growth following anaerobic treatments does not coincide with an acidification of the bathing medium. We reported earlier (20) that acidification of the medium followed anoxia but now find that the pH drop was due to reintroduction of CO_2 (19).

One other relationship to be noted with regard to growth rates and pH of the bathing solution is that the most rapid phases of growth in freshly peeled segments (Figs. 5 and 7) coincide with the time when alkalinization of the medium is occurring (Figs. 1–6). The slowing down of elongation after 2 to 3 hr coincides with

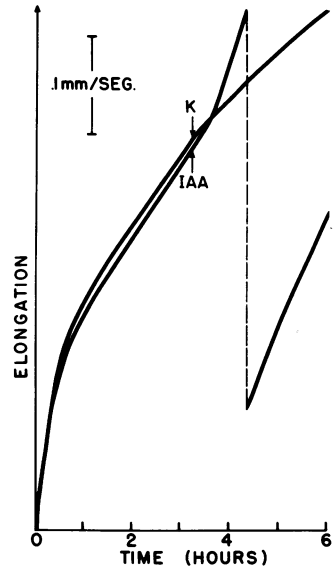

FIG. 7. Growth response to auxin and potassium. Freshly peeled segments were placed in the auxanometer in 1 mM K^+-free buffer. At the arrows, 10 μM IAA or 5 mM KCl was added.

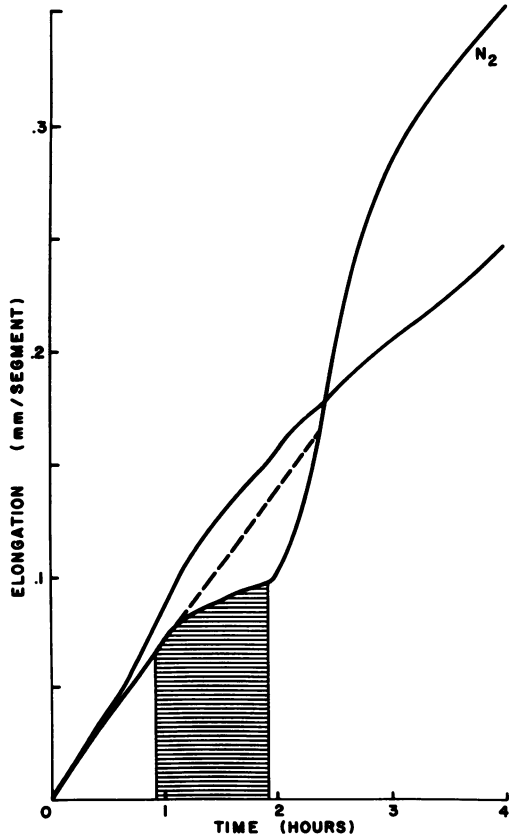

FIG. 8. Emergent growth following anoxia. Unpeeled segments were placed in the auxanometer in O_2-bubbled buffer. One column of segments (N_2) was bubbled with N_2 during the period indicated by ⊟; (- - -): continuation of the pre-N_2 growth rate.

an increasing acidification of the medium. Similar growth patterns occur with nonpeeled etiolated and green segments, although the rates are usually lower in the initial stages.

DISCUSSION

The epidermis represents a barrier to proton diffusion in extracellular pH studies, especially in light-grown stems, on which the cuticle may be 2.5 times thicker than on etiolated plants (10). Workers 40 years ago recognized and overcame the problem of penetration and elution by stripping away the epidermis (2, 31). The problem and technique were uncovered again only recently (5, 7). Some recent workers have also slit, abraded, or punctured the epidermis (4, 5, 33) and/or used shorter segments to increase cut surface to volume ratios (18).

The usual assumptions made in studies such as these are that the pH of the medium bathing segments reflects the pH within the cell walls (of the outermost cells) and that peeled or abraded segments respond as they would *in vivo*. The identical response by peeled or nonpeeled etiolated stem segments suggests that endogenous pH changes are not a response to the wounding which occurs during peeling. Extrapolations from peeled to nonpeeled segments should obviously be made as cautiously as extrapolations from isolated segments to intact plants. It is possible that excision of stem segments may induce "aging" or "washing" phenomena such as the well known case with potato tuber slices. The endogenous pH shift demonstrated here might be due to aging processes and not reflect *in vivo* conditions or physiology *per se*. The submersion and partial perfusion of segments certainly present a nonphysiological condition for the tissue. Our earlier studies (21) as well as the present results suggest that without proper aeration, the segments may become O_2-limited. Results under such conditions must be interpreted with care.

The other assumption, that pH of the bathing medium reflects intramural pH, has come under scrutiny in some recent studies. Rubery and Sheldrake (27) have presented theoretical considerations and data which suggest that the pH within and immediately around the negatively charged cell wall (a Donnon phase) will be up to half a pH unit more acid than the external medium. The discontinuity of pH values between cell wall and the adjacent free space is quite sharp as suggested by microelectrode studies (4, 14). The values obtained in extramural pH studies (which include essentially all of the studies done) are, therefore, probably higher than intramural pH, but the direction and magnitude of pH changes within the wall can still be assumed to be reflected in the external measurements. However, under certain conditions, the assumption might be invalidated by other principles involving ionic relationships within an electrical double layer. For example, we have seen an extremely rapid pH transition when K^+ is added to segments in a K^+-free medium after it has been endogenously acidified and stabilized around pH 5: the pH drops 0.3 to 0.4 units within 5 min. Rather than being an active acidification process, we suggest that the sudden increase in H^+ activity might be the result of cation exchange: K^+ replacing H^+ on carboxyl or other negatively charged wall components. In such a case, acidification of the medium would actually be caused by a partial deacidification of the cell wall. Under most conditions, we would expect the pH of the bathing medium to reflect, albeit somewhat incorrectly, the intramural pH.

The alkalinization of the medium during the initial period of incubation probably represents an equilibration between the buffer and the tissue. Ions may be exchanged altering the buffering capacity and balance of dissociated buffer components, or alkaline materials may leach out of the newly exposed cells. We do not have any reason to think that the alkalinization is an active process since DNP does not prevent the pH rise: in the presence of the uncoupler at 100 μM, the pH rises to about neutrality (Fig. 3).

On the other hand, acidification is inhibited completely by 100 μM DNP and anaerobiosis. The pH decrease appears to be an energy-requiring process, probably driven directly or indirectly by ATP. The exact nature or cause of the acidity is unknown, but we presume that it reflects net efflux of protons. A very real possibility exists that acidification is due to a cation absorption process in which protons are exchanged. Ion uptake in excised stem segments has been reported by a number of workers (12, 13), and coupled proton extrusion is a commonly suggested mode for nonelectrogenic cation absorption (18).

Endogenous acidification in auxin-free media is a feature of pea stem segment physiology not observed in most accounts (16, 33). Higinbotham *et al.* (13), Thimann (30), and Cleland (4) have found that nonpeeled etiolated peas or coleoptiles not only acidify their medium but tend to regulate the pH at a particular value: exactly the response observed here. (In our studies, additions of acid or base to shift the pH up to 2 units appeared to prompt a homeostatic response which returned the medium to a stabilized pH of about 5.) We suspect that the absence of an acidification response in some reports may stem from inadequate aeration: vigorous bubbling of the bathing medium with air or O_2 is crucial in keeping submerged segments viable and responsive (21), and interruption of aeration reduced or reversed the pH drop in our studies. The possible significance of proper aeration and support for the cation/proton exchange mechanism for endogenous acidification comes from a report by Jacobson *et al.* (15) that rapid bubbling of root tissue in a buffer medium promoted K^+ influx more than slow bubbling.

Acidification occurred after the addition of auxin: pH changes as large as those reported in the literature were observed. However, the auxin-free peeled segments were also becoming more acid, so that the auxin treatments *per se* could be seen to have only a slight effect. Respiratory CO_2 does not alter the pH in our studies; because the buffer, being bubbled with air and open to the atmosphere, is in continuous equilibrium with atmospheric CO_2. Respiratory CO_2 does lower the pH in nonaerated solutions and solutions bubbled with O_2 in closed systems where atmospheric CO_2 is excluded (19). If the procedure used in published pH studies is to place stem or coleoptile segments in stirred, but not vigorously aerated solutions, bicarbonate will accumulate. Under such circumstances, the auxin-induced pH drop might be due partially to increased respiration and carbonation of the medium (14, 28). Our method eliminates the respiratory CO_2 contribution to pH but still shows a slight auxin-induced acidification in most trials. We conclude that a decline in intramural pH due to proton extrusion may occur in response to auxin applications.

It has been suggested that the absence of a consistent and appreciable auxin-induced acidification may be due to damage incurred during peeling. However, nonpeeled, etiolated segments, which have been reported to acidify their medium in response to auxin (16), did not do so in our studies. The etiolated epidermis apparently was fully permeable to protons since endogenous acidification paralleled that of the peeled segments. We conclude that absence of auxin-induced acidification is not due to removal of the epidermis. It is probable, however, that damage does occur in the peeling process and that such will alter the physiology of the segment in some way. The majority of this work (Figs. 1, 3–6) was done with peeled green segments rather than nonpeeled etiolated material because it is part of a larger study on light-grown pea stems. When treated in an auxanometer with a constant flow of the treating solution, these peeled segments still respond to either auxin or lowered pH by increasing their rate of growth.

The K^+-induced acceleration of acidification with no consequent growth promotion implies that acidification, as measured by extracellular studies, may not be sufficient for growth of pea stems. The pH drop in the presence of K^+ may be due to a

coupled proton exchange during K^+ uptake by the tissue. Alternatively, it may be a cation exchange process involving net movement of H^+ out of the Donnon phase, not the protoplast. Whatever its etiology, in our laboratory the acidification due to K^+ was more consistently reproducible than that due to auxin. It has been suggested that IAA promotes uptake of K^+ and that the uptake might be related to the growth-promoting mechanisms of the cation and the hormone (6, 12, 18). (Our studies failed to show a K^+-enhanced growth of auxin-free, peeled segments.)

The correlations between pH of the bathing medium and growth of segments are the opposite of those expected if cell wall acidity is the factor controlling elongation rates and extracellular pH (3): the rapid growth of freshly harvested, peeled or non-peeled segments coincides with a period of rising pH in the bathing medium, and the growth rate declines as the pH begins to fall. We are not suggesting that this direct relationship is causal, only that the correlation between extracellular pH and growth is not always an inverse one. The evidence suggests that either pH is not a controlling factor or the technique used in most studies does not truly reflect intramural pH. The well known acid growth effect on the extension of live and dead cell walls argues in favor of the latter.

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