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THE AGAR-DYE METHOD FOR VISUALIZING ACID EFFLUX PATTERNS DURING TROPIC CURVATURES

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There is considerable evidence supporting the hypothesis that H^+ efflux mediates the action of auxin on growth (11). In stems or stem-like tissues, growth promotive concentrations of auxin promote H^+ efflux, and the enhancement of H^+ efflux precedes the enhancement of growth (3, and references cited therein). These findings along with the well established growth-promoting ability of applied acid (10) indicate that auxin-induced acid efflux plays a causal role in the enhancement of stem elongation by the hormone.

Recent evidence indicates that H^+ movement also might be involved in the regulation of root growth. Acid promotes root elongation (1,2) and acid efflux occurs from the elongation zone of intact maize roots but not from the non-growing regions on either side of the elongation zone (7). Furthermore, concentrations of auxin which inhibit the growth of intact maize roots also inhibit or reverse H^+ efflux from the roots. The latent period for auxin-induced inhibition of H^+ efflux is shorter than the latent period for inhibition of growth (4).

If acid efflux patterns are, in fact, correlated with growth patterns in developing plant organs, one would expect environmentally-induced modification of growth (eg, tropic responses) to be accompanied by modification of H^+ efflux patterns. To test this idea it is necessary to have a means of detecting H^+ efflux in growing intact plant organs. Weissenfeld et al (13) described a technique for accomplishing this. The method involves placing a growing seedling on a layer of agar containing a pH indicator dye (see below for details). Regions of acid influx or efflux can then be visualized by observing color changes at the seedling-agar interface. Using this method we have found that in geotropically-stimulated maize roots there is enhanced H^+ efflux from the elongation zone on the upper side of the root and reduced efflux from the elongation zone on the lower side of the root (7). Also, we have noted that in shoots, negative geotropism is accompanied by enhanced acid efflux from the lower side, where growth is rapid. Similarly, positive phototropism in shoots is accompanied by enhanced acid efflux from the shaded side (8). Since these pat-

terns of acid efflux begin before the tropic curvature begins, modification of acid efflux patterns may be involved in the development of the curvatures.

The purpose of this report is to give a description of the method for observing H^+ efflux patterns in seedlings, to summarize recent findings on the correlation between H^+ efflux patterns and tropisms, and to relate these findings to earlier work on tropisms.

Procedure for Visualizing H^+ Efflux in Seedlings. The agar-dye medium upon which the seedlings are placed consists of a low concentration of non-nutrient agar (0.6% for root experiments, 0.4% for hypocotyl or coleoptile experiments), bromocresol purple indicator dye (0.71 mM), and the following inorganic nutrients* (see footnote, next page): 1.5 mM $Ca(NO_3)_2$; 1 mM each $MgSO_4$, KH_2PO_4 , KNO_3 ; 20 μM H_3BO_3 ; 3.8 μM $ZnCl_2$; 0.18 μM MoO_3 ; 0.14 μM $CuCl_2$. This mixture is adjusted to pH 5.0 with 0.1N HCl. The solution is then boiled and poured to a depth of about 4 mm in 100 x 15 mm Petri plates (about 15 ml per plate). When the plates have cooled, a seedling is placed on the surface of the agar and the organ to be observed (root or shoot) is gently pressed into the agar so that half to two-thirds of the circumference is in contact with the medium. The plate is mounted vertically. The organ to be studied is oriented horizontally for geotropism studies or vertically for phototropism or straight growth studies. To observe H^+ efflux in coleoptiles or hypocotyls, the surface of the organ must be peeled or abraded (3) before placing the seedling on the agar. Abrasion is recommended because it results in less damage. We use mirror grinding abrasive #40016, Edmund Scientific, Barrington, NJ, USA. All sides of the organ are abraded and the surface is rinsed with distilled water before placing the seedling on the agar. H^+ efflux patterns can be observed in roots without abrading their surface.

Expected Color Changes. Bromocresol purple indicator dye exhibits color changes over the pH range of 3.5 to 10. It is yellow from pH 3.5 to 4.8, orange from pH 4.8 to about 5.5 and red from pH 5.5 to 6.4. As the pH increases from 6.4 to 10

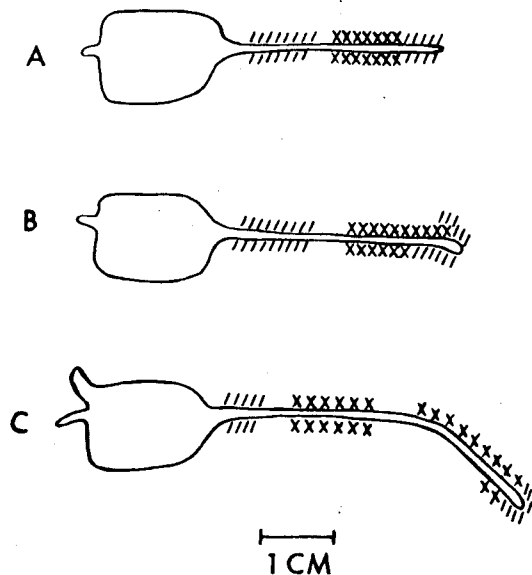


Figure 1. pH pattern developing during positive geotropism in maize roots. A, 2 min; B, 20 min; C, 120 min. Acid efflux is indicated by cross hatching (XXXX). Areas where the pH has increased are indicated by slashes (///). Absence of cross hatching or slashes indicates that the pH does not change significantly. Copyright 1981 by the American Association for the Advancement of Science.

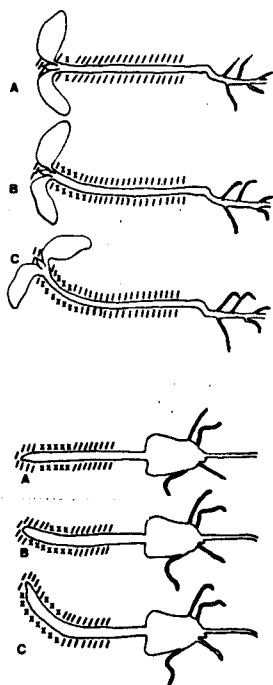


Figure 2. pH pattern developing during negative geotropism in sunflower hypocotyls and maize coleoptiles. Top: sunflower hypocotyls, A, 5 min; B, 120 min; C, 240 min. Bottom: maize coleoptiles, A, 5 min; B, 90 min; C, 180 min. For meanings of symbols, see legend to Fig 1.

*We include the inorganic nutrients in order to compare the agar-dye experiments with certain long-term root growth experiments in which the roots are immersed in an aqueous medium and growth is measured using a root auxanometer (2). For short-term experiments (< 8 h), omission of the inorganic nutrients from the agar-dye medium does not alter the results.

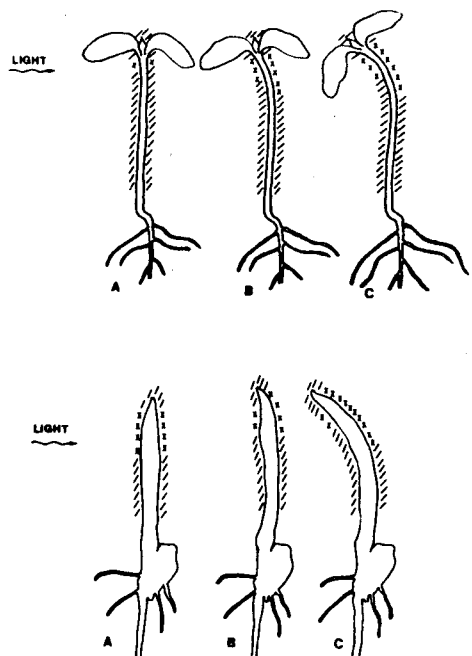


Figure 3. pH pattern developing during positive phototropism in sunflower hypocotyls and maize coleoptiles. Top: sunflower hypocotyls, A, 5 min; B, 90 min; C, 240 min. Bottom: maize coleoptiles, A, 5 min; B, 90 min; C, 240 min. For meanings of symbols, see legend to Fig 1.

the dye turns dark red and then to various shades of violet. Since the agar-dye plates are prepared at pH 5.0 their initial color is orange. In regions where H^+ efflux occurs from the tissue into the agar, the pH drops below 4.8 and the agar turns yellow. In regions where H^+ uptake (or OH^- efflux) occurs, the pH of the agar rises above 5.5 and the agar turns red. These color patterns begin to develop within minutes after placing the seedlings in contact with the agar and reach full intensity within an hour. Since the color develops rapidly, one can take seedlings which have grown enough to tear the agar, transfer them to a new agar plate and observe acid efflux patterns within minutes. The observation of color patterns is facilitated by placing the plates on a light box.

H^+ Efflux Patterns During Positive Geotropism in Primary Roots of Maize. When a 3-day-old maize (*Zea mays*, Bear hybrid WF9 \times 38MS) seedling is placed on an agar-dye plate with the root in a horizontal position, the pattern of H^+ efflux and root curvature develops as shown in Fig 1 (also see reference 7). Within 2 to 3 min, a yellow (acid) region develops where the elongation zone contacts the agar (Fig 1A). The location of the elongation zone is confirmed by marking experiments using either India ink (7) or fingerprint ink. Fingerprint ink is better since India ink tends to bleed into the medium under acidic conditions. After 20 min in the horizontal position the acid zone is clearly more intense and broader on the upper surface than on the lower surface. At this time geotropic curvature is barely detectable (Fig 1B). After 120 min (Fig 1C) curvature is well underway and there is a striking difference in acidification on the upper and lower surfaces, with the acid zone being much more extensive on the upper surface.

H^+ Efflux Patterns During Negative Geotropism in Sunflower Hypocotyls and Maize Coleoptiles. When a 6-day-old light-grown sunflower (*Helianthus annuus*, cv Russian) seedling is abraded, placed on the agar-dye surface and exposed to a geotropic stimulus, the H^+ efflux pattern develops (8) as shown in Fig 2, top. During the first 5 min a narrow zone of

acid secretion develops just below the cotyledons (Fig 2A). No curvature is evident during this time. The yellow zone intensifies with time and extends back from the cotyledonary node. Fig 2B shows the H⁺ efflux pattern at 120 min. By this time negative geotropic curvature is underway and the zone of acid efflux is broader and more intense on the lower than the upper surface of the hypocotyl. After 240 min (Fig 2C) the geotropic curvature is stronger and the region of H⁺ efflux on the lower surface of the hypocotyl is more intense and widespread. Similar results are obtained with coleoptiles of 3.5-day-old dark-grown maize seedlings (8) except that the curvature and efflux patterns develop more quickly and with greater intensity (Fig 2, bottom).

H⁺ Efflux Patterns During Phototropism in Sunflower Hypocotyls and Maize Coleoptiles. Using the agar-dye method, the H⁺ efflux patterns which accompany phototropic curvature also can be observed. Sunflower or maize seedlings are pressed into the agar with the shoot oriented in a vertical position. The plate is covered with a black box with a horizontal slit in one side. The seedling is illuminated with fluorescent light passing through the slit with an intensity of approximately 125 $\mu\text{E m}^{-2} \text{sec}^{-1}$ at the seedling surface. During the first 5 min, a yellow (acid) zone develops in the agar just below the cotyledonary node of the sunflower seedling (Fig 3, top). The yellow zone intensifies with time and extends back from the node. By 90 min, positive curvature is underway and there is more H⁺ efflux on the shaded side of the hypocotyl than on the light side (Fig 3B). After 240 min the curvature is very strong and the acid zone on the shaded side is very intense. Similar results are obtained with coleoptiles of unilaterally illuminated maize seedlings (Fig 3, bottom).

Relationship to the Cholodny-Went Model of Tropistic Curvature. The classical model for the differential growth that results in tropic curvature is the Cholodny-Went (C-W) model (5, and references cited therein). According to this model the upward curvature of horizontally placed stems occurs as a result of movement of auxin toward the lower half of the stem. The increased auxin in the lower half of the stem is thought to accelerate growth, causing the stem to curve upward. The same model is applied to roots except that, in horizontally oriented roots, the accumulation of auxin on the lower side is thought to inhibit growth, resulting in downward curvature. The C-W model also has been applied to shoot phototropism. In this case curvature toward the light is thought to occur as a result of movement of auxin toward the shaded side of the shoot.

The C-W model of geotropism and phototropism has not gone unchallenged (5). There is some question, for example, whether a sufficiently large redistribution of auxin occurs before the initiation of curvature. Alternatives to the C-W hypothesis include suggestions that: a) curvature is caused by redistribution of some growth promoter other than auxin; b) root curvature results from redistribution of a growth inhibitor other than auxin; c) curvature results from a gradient in *sensitivity* to auxin rather than a redistribution of auxin; d) the outer cell layers are the site of geoperception and georesponse, and their growth rate is directly dependent upon their orientation.

Even if one assumes that differential acid efflux is responsible for the differential growth that leads to tropic curvature, the occurrence of asymmetric acid efflux prior to curvature does not allow one to distinguish between the various tropism models. Differential acid efflux is consistent with the C-W hypothesis since the rate of H⁺ efflux has been shown to depend on auxin concentration (9). In shoot geotropism, for example, if there were an accumulation of auxin on the lower side at the expense of auxin on the upper side, one would expect greater H⁺ efflux on the lower side since auxin promotes H⁺ efflux. Similarly, in roots an accumulation of auxin on the

lower side at the expense of auxin on the upper side would lead to greater H⁺ efflux on the upper side since, in roots, moderate concentrations of auxin inhibit H⁺ efflux. In experiments with roots, we have noticed that, when inhibitors of auxin transport (eg, naphthylphthalamic acid; 2,3,5-triiodobenzoic acid) are incorporated into the agar-dye medium, neither differential acid efflux nor geotropic curvature occurs. This is indirect evidence that auxin redistribution is necessary to the development of differential acid efflux and geotropic curvature.

Although the asymmetric acid efflux associated with tropisms is consistent with the C-W hypothesis, the alternatives to the C-W hypothesis (see above and ref 5) may also be consistent with the differential acid efflux, especially since the basis of the differential acid efflux is not known.

Relationship to Other Aspects of Tropisms. Directional acid efflux might account for certain other characteristics of tropically responding organs. It is known, for example, that geotropically or phototropically stimulated maize coleoptiles develop electrical potential differences from side to side with the rapidly growing side of the coleoptile becoming positive (see review by Jaffe and Nuccitelli, 6). Jaffe and Nuccitelli suggested that this polarization might arise as a consequence of lateral movement of auxin leading to auxin accumulation and enhanced H⁺ efflux on the rapidly growing side. Our observations are consistent with this suggestion. The observation that the development of potential differences in tropically stimulated organ is dependent upon a supply of auxin in the tissue (6) indicates that lateral redistribution of auxin may play a role in the induction of differential acid efflux patterns. Differential H⁺ efflux during tropic responses might also play a role in the apparent redistribution of cations in responding organs. The rapidly growing side of tropically responding organs is reported to increase in K⁺ content and decrease in Ca²⁺ content. This might be due to enhanced K⁺ uptake along the membrane potential gradient which develops (6) and perhaps to H⁺/Ca²⁺ competition for binding sites in the cell wall (12).

Concluding Comments. The agar-dye method of Weisenseel et al provides a simple means of detecting, on a qualitative basis, the regions of acid efflux and influx in growing intact seedlings. The tropism experiments described here can be completed in 4 h or less. This makes the technique attractive for use in introductory or intermediate level class experiments.

The results obtained may be of particular significance to the question of the involvement of H⁺ efflux in determining growth patterns in intact seedlings. The findings indicate that H⁺ efflux patterns are closely related to growth patterns as would be predicted by the acid-growth hypothesis. The H⁺ efflux/growth pattern correlations are observed in relatively undisturbed seedlings (no excision, minimal handling, no exogenous buffers or hormones), particularly in the case of root experiments where no abrasion is necessary. This indicates that there is normally a correlation between regions of rapid growth and rapid H⁺ efflux and that H⁺ efflux is not an artifact induced by wounding, application of excess hormone, etc.

This method should continue to be useful for investigating the relationship between H⁺ efflux and growth in plant organs. We are working on ways to adapt the method to allow quantitative measurement of H⁺ efflux so more accurate measurements of the kinetics of shifts in H⁺ efflux patterns can be made and compared with the timing of tropic curvature.

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