Plant Physiol. (1981) 68, 664–667
0032-0889/81/68/0664/04/$00.50/0

Low Proton Conductance of Plant Cuticles and Its Relevance to the Acid-Growth Theory[1]

Received for publication November 11, 1980 and in revised form March 23, 1981

S. Ann Dreyer, Virginia Seymour, and Robert E. Cleland
Department of Botany, University of Washington, Seattle, Washington 98195

ABSTRACT

Evidence obtained on the relation between the pH of the medium and the growth of intact stem sections is compatible with the acid-growth theory only if the proton conductance of the cuticle is so low that the cuticle is an effective barrier to the entry or exit of protons from the tissue. By measuring the rate at which protons cross frozen-thawed epidermal strips of sunflower (*Helianthus annuus* L.) and soybean hypocotyls (*Glycine max* Morr.) and enzymically isolated cuticles of *Berberis aquifolium* Persh. and tomato (*Lycopersicum esculentum* Mill.) fruit, we have now demonstrated the low proton conductance of the cuticular layer. Unless the conductance is enhanced by abrasion of the cuticle or by removal of the cuticular waxes, proton movement into and out of a tissue across the cuticle will be significant only over long time periods.

The aerial portions of all higher plants are covered by a cuticle, which is believed to act as a barrier to the entry and exit of materials from tissues (8). The ability of the cuticle to limit movement of cations such as K^+ (7, 21), organic substances (4), and water (17) has been established, but the proton conductance[2] of a cuticle has been examined only after long periods (3). Knowledge about the proton conductance of the cuticle is of importance in regard to the acid-growth theory of auxin-induced growth (15).

The acid-growth theory states (3, 15) that auxin causes the cells to excrete protons into the wall solution where the resulting increased acidity activates wall-loosening enzymes. The excreted protons might be expected to acidify the external solution, too, but it has been difficult to demonstrate any auxin-induced acidification (11, 12, 19) unless the cuticle is abraded (3, 9, 16) or removed (1, 13). This led us to suggest (1, 13, 15) that proton conductance of the cuticle is so low that protons excreted into the wall solution are trapped within the tissue unless they escape through cut surfaces. A low proton conductance would also explain why, in order to achieve a maximum rate of acid-induced growth, it is necessary to add a 50-fold greater proton concentration to intact *Avena* coleoptile sections as compared with sections whose cuticle had been removed (13).

Vanderhoef *et al.* (19) have pointed out, however, that a low proton conductance of the cuticle has never been demonstrated, and concluded from their studies on intact soybean segments that the cuticle may not be a barrier to protons. In this study, we have made use of a simple assay that allows us to assess the ability of protons to cross cuticular layers, and we show for the first time that the cuticle is an effective barrier to the entry and exit of protons.

MATERIALS AND METHODS

Seedlings of sunflower (*Helianthus annuus* L., cv. Russian Mammoth; C. H. Lilly Co., Seattle, WA) were grown for 5 days at 25 C under constant illumination (400 μE m^{-2} s^{-1} from cool white fluorescent tubes). Epidermal strips, 3 to 4 × 15 to 25 mm, were then peeled from the upper, growing regions of the hypocotyl with fine forceps. The strips contained one to two layers of cells, as judged by microscopic observation. The strips were placed on glass slides, frozen on Dry Ice, and stored frozen and wrapped in foil until use. Seedlings of soybean (*Glycine max* Morr., cv Wayne, Champaign County Seed Co., St. Joseph, IL) were grown at 25 C under red light ($<10^{-2}$ μE m^{-2} s^{-1}, from red fluorescent tubes wrapped in red cellophane), or under constant white illumination (400 μE m^{-2} s^{-1}), and epidermal strips were then isolated in the same manner.

The cuticle of some seedlings was abraded by rubbing the hypocotyl five to ten times with a slurry of emory powder (American Optical, Seattle, WA, grade M180) before isolation of the epidermal strips. Other seedlings were wiped twice with a 3:1 (v/v) mixture of chloroform:ethanol to remove some of the cuticular waxes.

Leaves of *Berberis aquifolium* Dursh. were selected from a plant growing outside the laboratory. To isolate the cuticle, leaf pieces were infiltrated and incubated for at least 2 days with a solution containing 8% pectinase (No. 102533; ICN, Cleveland, OH) and 0.8% cellulase (No. 101308; ICN) in 0.1 M Na-acetate buffer (pH 3.7). The upper epidermis was then lifted off the piece with fine forceps, washed well with water, and stored frozen until use. Enzymically isolated tomato (*Lycopersicum esculentum* Mill.) fruit cuticles was a gift of Dr. M. J. Bukovac, Michigan State University.

Proton Conductance Measurements. To measure the relative conductance of the cuticle to protons we used the apparatus shown in Figure 1. The epidermal strips or isolated cuticles were thawed and then glued with Duro Super Glue 3 across a 1 × 6 mm opening cut in the bottom of a 5-ml plastic beaker cup; the inner surface of the strip was oriented against the lower surface of the cup except where noted. A 100-μl drop of unbuffered 10 mM KCl (pH 6.5), was placed over the opening against the inner surface of the strip and a combination pH electrode (No. 6020; Ingold Electrodes, Cambridge, MA) was inserted into a drop. This assembly was lowered into a beaker containing 5 ml unbuffered 10 mM KCl (pH 3.0), so that the external surface of the cuticle was in contact with the pH 3.0 solution while the internal surface was in contact with the pH 6.5 solution. The beaker cup was covered with Parafilm to reduce evaporation. The pH of the drop on the inner surface of the cuticle was measured with an Orion 701 pH

[1] Supported by United States Department of Energy Contract DE-AT06-76ER76019.

[2] The term proton conductance is used rather than proton permeability to indicate that no assumptions are being made concerning the path of proton movement through the cuticular layer or the mechanism of H^+ movement (*e.g.* whether H^+ movement is accompanied by an anion in the same direction or a different cation in the opposite direction).

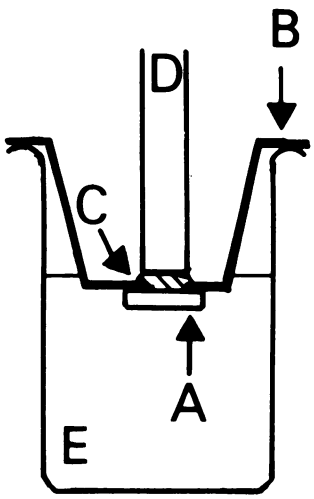

FIG. 1. Apparatus for measuring proton diffusion across cuticles. Epidermal strip or isolated cuticle (A) was glued across a 1 × 6 mm slit in a 5-ml plastic beaker cup (B). A 100-μl drop of 10 mM KCl, pH 6.5 (C) was placed on the inner side and a combination pH electrode (D) was inserted into the drop. The beaker cup was lowered into a 15-ml beaker containing 10 mM KCl (pH 3.0) (E), and the pH in C was continuously recorded.

meter and the pH was recorded continuously.

Some epidermal strips were preincubated for 3 h in a 0.2 mg/ml solution of pronase in 10 mM Tris-HCl buffer (pH 7.5), to remove proteins associated with the walls adhering to the inner cuticular surface. The strips were then washed with water before use.

Considerable care is needed to insure reproducibility. The abrasion process is difficult to quantify, with the result that the rapidity with which protons cross-abraded strips shows considerable variation. The results presented here have been selected as representative of the data; each experiment was repeated a minimum of eight times.

Thawed epidermal strips were air-dried, coated with gold-palladium, and viewed in a JEOL model JSM-U3 scanning electron microscope.

RESULTS

The speed with which protons can diffuse from the lower (pH 3.0) solution into the upper (pH 6.5) solution in our apparatus was first tested using a dialysis membrane as the barrier, inasmuch as a dialysis membrane is not expected to impede proton diffusion strongly. Rapid diffusion of protons occurred, resulting in a drop in the pH of the upper solution to 4.0 within 10 min, but complete equilibration of protons took more than 1 h (Fig. 2). When log $[(H^+)_o-(H^+)_i]$ was plotted as a function of time, a straight line was obtained as expected for a diffusion process (Fig. 2, inset).

When epidermal strips from unabraded, light-grown sunflower hypocotyls were used as the barrier, the diffusion of protons was greatly impeded (Fig. 2). After 10 min the pH of the solution on the inner side of the strip had decreased less than 0.1 pH unit, and even after 1 h the pH was still above 6. The low conductance of the cuticular layer[3] to protons is independent of the direction of proton movement, as a similar lack of proton diffusion across unabraded strips was obtained when the strip was reversed so that the acid was against the inner surface (data not shown). With longer periods of time, proton diffusion across unabraded epidermal strips was detected (Fig. 3). After a lag which varied between

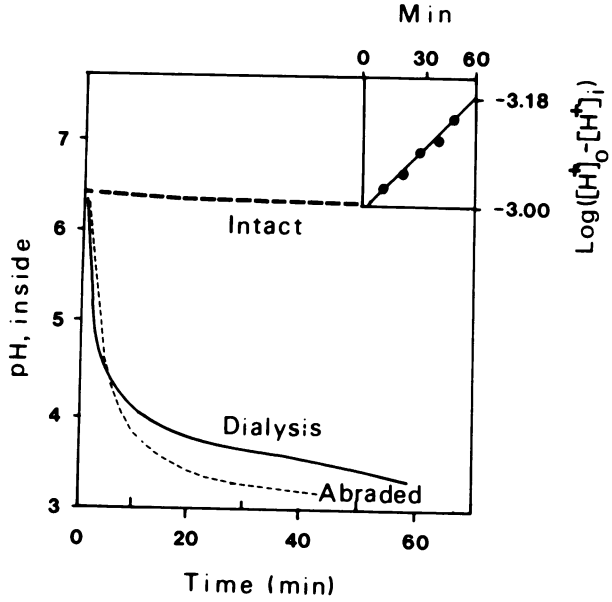

FIG. 2. Movement of protons across abraded and unabraded epidermal strips of light-grown sunflower hypocotyls. Pieces of unabraded (intact), abraded sunflower hypocotyl epidermis, or dialysis tubing were tested for proton permeability, using the apparatus in Figure 1. Insert shows the curve for the dialysis tubing plotted as log $[(H^+)_o-(H^+)_i]$ *versus* time and shows the straight line expected for a diffusion process.

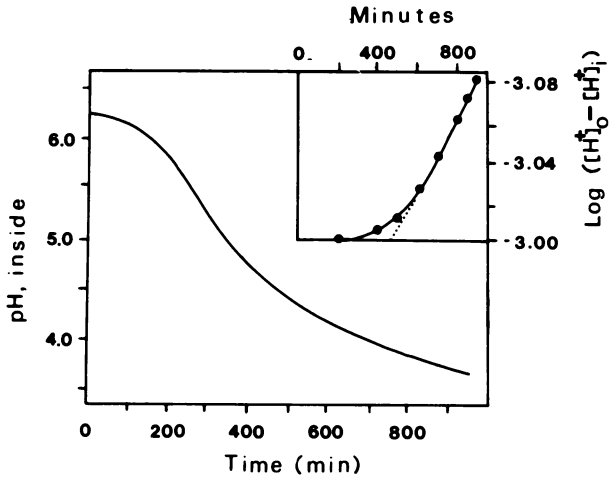

FIG. 3. Movement of protons across unabraded sunflower epidermal strip over long time periods. Epidermal strip was treated with pronase prior to use. Insert shows the same curve plotted as log $[(H^+)_o-(H^+)_i]$ *versus* time, and shows the long lag before a linear diffusion curve is obtained.

50 and 150 min, the pH of the upper solution began to drop, and a pH of 4.0 was reached only after 10 to 15 h. When log $[(H^+)_o-(H^+)_i]$ was plotted as a function of time, a straight line was obtained, but only after a lag which usually exceeded 300 min. The ability of protons to cross the cuticular layer was greatly increased when abraded epidermal strips were used; in this case proton movement was as rapid as that across the dialysis membrane (Fig. 2).

Cuticular layers from other plant tissues showed a similar, low proton conductance. Epidermal strips from hypocotyls of dark-grown soybean seedlings, taken from material identical with that used in our proton excretion studies (16), were only slightly permeable to protons unless the cuticle was abraded, whereupon the conductance was greatly enhanced (Table I). Similar results were obtained with light-grown soybean hypocotyls (data not

[3] Cuticular layer is used here to denote the cuticle with underlying epidermal walls still attached, while the term cuticle is used only when the cuticle has been separated from most of the wall materials.

Table I. *Proton Movement across Soybean Hypocotyl Epidermal Strips*

Epidermal strips were peeled from dark-grown soybean hypocotyls, frozen-thawed, and mounted in apparatus shown in Figure 1. Five ml 10 mM KCl (pH 3.0) was placed against the outside surface and 100 μl 10 mM KCl, pH 6.5, was placed against the inner surface. The pH was monitored continuously.

Material	Inside pH after 10 min	
	Unabraded	Abraded
Dialysis tubing	3.85 ± 0.09	
Soybean hypocotyl	6.12 ± 0.15	3.85 ± 0.05

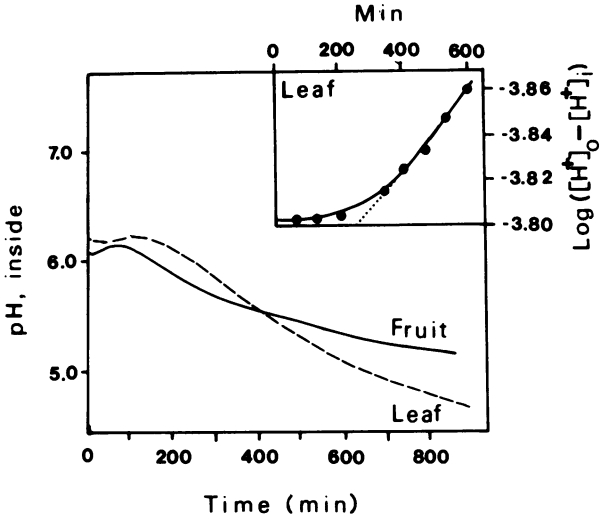

FIG. 4. Proton conductance of enzymically isolated cuticles from tomato fruit and *B. aquifolium* leaves. Conditions were the same as in Figure 1 except that the external (lower solution) was pH 3.0 for the fruit and 3.8 for the leaf cuticle. Insert shows the curve for the leaf cuticle replotted as log $[(H^+)_o-(H^+)_i]$ *versus* time in min.

shown). Isolated leaf cuticles, obtained from the upper epidermis of *Berberis* leaves, showed a proton conductance similar to that of unabraded *Helianthus* hypocotyls (Fig. 4). Tomato fruit cuticle was even less permeable to protons; in the example shown in Figure 4, the rate of proton diffusion was lower in tomato fruit cuticle than with the *Berberis* leaf cuticle, despite a greater pH gradient across the cuticle.

Epidermal strips contain, in addition to the cuticle and its adhering cell walls, considerable protein. This protein does not contribute to the low proton conductance, as indicated by the fact that pretreatment of the strips with the proteolytic enzyme pronase did not enhance proton movement (Fig. 5). Partial removal of cuticular waxes with a chloroform-ethanol solution, however, markedly increased the proton conductance (Fig. 5). Proton conductance does depend upon the acid which is present. At pH 3 the proton conductance of unabraded sunflower epidermal strips was much greater when the acid was acetic acid, compared with hydrochloric, phosphoric, or citric acid at the same pH (Fig. 6), suggesting that the cuticle is more permeable to the undissociated acetic acid than it is to protons. As a result, acetic acid should be more effective than HCl at any particular pH in causing acid-induced growth of isolated stem sections.

Abraded and unabraded epidermal strips, used in the proton conductance experiments, were examined with the SEM[4] (Fig. 7). Abrasion alters the surface by causing many small holes and tears.

[4] Abbreviations: SEM, scanning electron microscope.

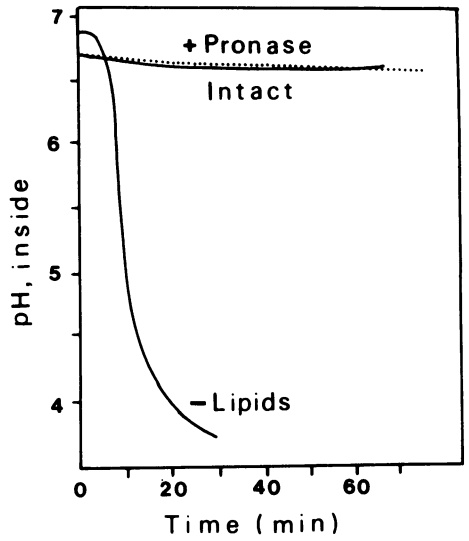

FIG. 5. Effect of removal of proteins and cuticular waxes on proton conductance of sunflower hypocotyl epidermal strips. Strips were treated for 2 h with 0.2 mg/ml of pronase, or the hypocotyls were wiped twice with a 3:1 chloroform:ethanol mixture before peeling off the epidermal strip. Conditions were the same as in Fig. 1.

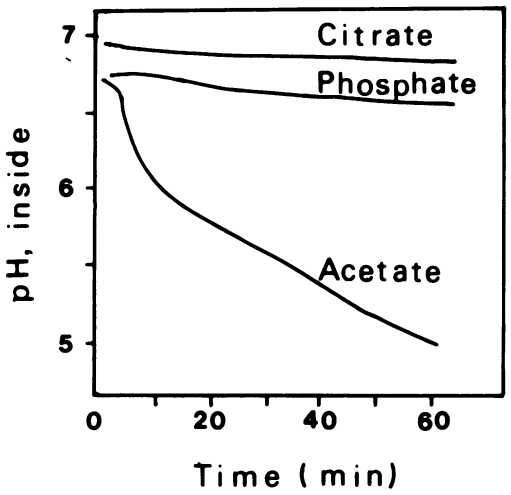

FIG. 6. Effect of acid on proton conductance of sunflower epidermal strips. Conditions were the same in each case except for the acid used to titrate the outside KCl solution to 3.0. Acids used were 10 mM acetic acid, phosphoric acid, and citric acid. The curve for HCl was similar to that for phosphate or citrate.

DISCUSSION

The ability of protons to penetrate the cuticle has apparently not been examined in any detail previously. Acidic solutions promote the growth of plant stem (16, 21) and coleoptile sections (5, 14), but it is not known whether the protons entered the tissues through the cuticle or simply through the cut ends. The inability to detect significant auxin-induced acidification of the external medium with sections of some dicot stems (12, 19) has been taken by some authors to indicate that the cuticle is impermeable to protons (*e.g.* 15) and by others, that auxin does not induce H^+-excretion (*e.g.* 19).

We have shown here for the first time that the cuticles of a leaf, of tomato fruit, and the epidermal layers of growing soybean and sunflower stems have a low proton conductance, but that some protons will diffuse across the cuticle given sufficient time and concentration gradient. Our results agree with the predictions of

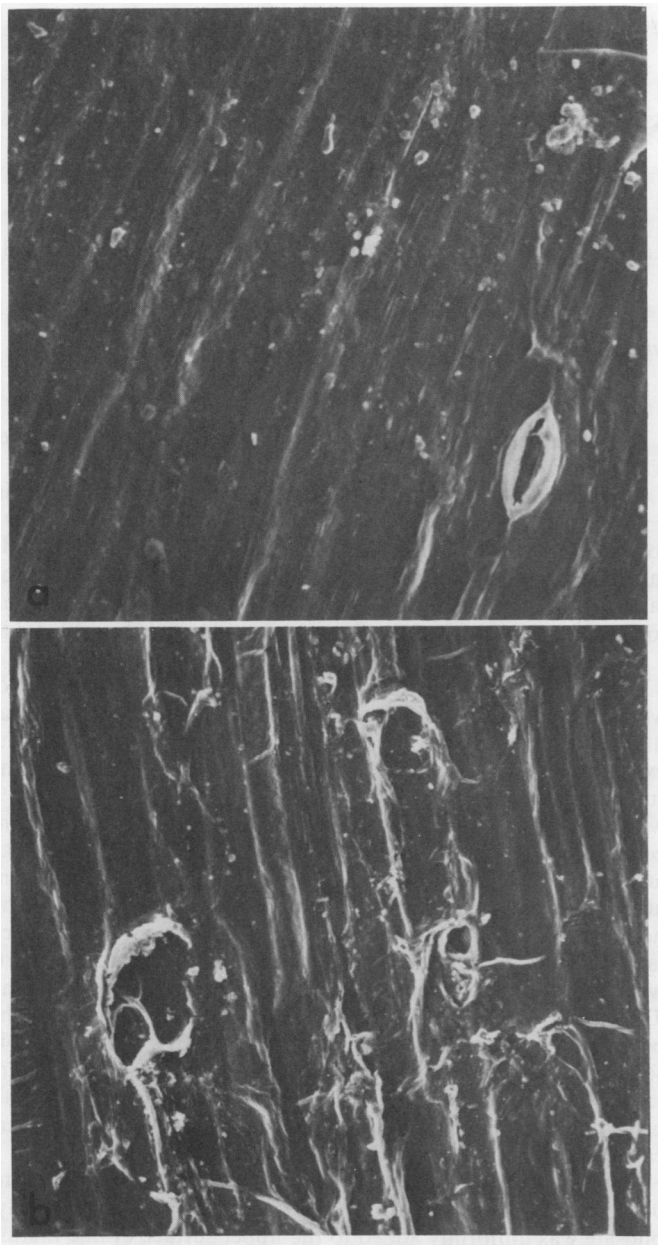

FIG. 7. Effect of abrasion on the cuticular surface of the sunflower hypocotyl as viewed in the SEM. (a) Intact, unabraded; (b) abraded. Note the disruption of the cuticular surface caused by the abrasion. × 750.

the acid-growth theory (15) which states that the cuticle insulates the wall solution from the external solution, and traps excreted protons within the tissue.

Can the relative impermeability of these cuticles to protons be explained as an artifact of the preparation of the experimental material or the techniques used here? It is known that the permeability of cuticles can be altered by some isolation procedures, especially chemical ones (10), but the effect is an increase rather than a decrease in permeability. Here the cuticles from the stem tissues were isolated by mechanical means, which might be expected to produce cracks or holes in the cuticle, but apparently this occurs only rarely, as judged by the SEM and by the low proton conductance of these strips.

Yamada *et al.* (20) found that the permeability of tomato fruit cuticle to Rb^+ and Ca^{2+} was considerably greater when the direction of movement was outside to inside as compared with inside to outside. The data presented here are all from experiments where the direction of proton movement is outside to inside, but we have conducted sufficient experiments in which the direction of proton movement was reversed so as to demonstrate that the direction of proton movement has little effect on its conductance (data not shown).

The barrier to proton movement across epidermal layers would appear to be the cuticle itself rather than the underlying cell walls, since isolated leaf and fruit cuticles have the same low proton conductance as that of epidermal layers, and since removal of cuticular waxes greatly increases the proton conductance of epidermal layers. Similar conclusions have been reached for the permeability of the cuticular layers to other substances such as water (17).

The proton conductance can be greatly enhanced by removal of cuticular waxes. While this technique has been used by Penny *et al.* (12), in our experience the dewaxing causes unacceptable damage to the underlying cells, and is therefore of limited use in studies on the proton excretion of plant tissues. Alternatively, the proton conductance can be enhanced by abrading the tissue. This technique has been widely used (*e.g.* 6, 9, 18) and, if gently applied, causes minimal damage to the tissues, but care must be exercised since abrasives of too large size or use of too great pressure can cause severe damage. The abrasion technique, while not ideal, is currently the most suitable one for increasing the proton conductance of stem sections.

Acknowledgment—We gratefully acknowledge the gift of tomato fruit cuticles from Dr. M. J. Bukovac.

LITERATURE CITED

1. CLELAND RE 1973 Auxin-induced hydrogen ion excretion from *Avena* coleoptiles. Proc Natl Acad Sci USA 70: 3092–3093
2. CLELAND RE, DL RAYLE 1975 Hydrogen ion entry as a controlling factor in the response of green pea stem sections. Plant Physiol 55: 547–549
3. CLELAND RE, DL RAYLE 1978 Auxin, H^+-excretion and cell elongation. Bot Mag Tokyo Spec Iss 1: 125–139
4. DARLINGTON WA, N CIRULIS 1963 Permeability of apricot leaf cuticle. Plant Physiol 38: 462–467
5. EVANS ML, PM RAY, L REINHOLD 1971 Induction of coleoptile elongation by CO_2. Plant Physiol 47: 335–341
6. GREEN PB, WR CUMMINS 1974 Growth rate and turgor pressure. Auxin effect studies with an automated apparatus for single coleoptiles. Plant Physiol 54: 863–869
7. MCFARLANE JC, WL BERRY 1974 Cation penetration through isolated cuticles. Plant Physiol 53: 723–727
8. MARTIN JT, BE JUNIPER 1970 The Cuticles of Plants. St. Martin's Press, New York
9. MENTZE JB, B RAYMOND, JD COHEN, DL RAYLE 1977 Auxin-induced H^+-secretion in *Helianthus* and its complications. Plant Physiol 60: 509–512
10. NORRIS WF, MJ BUKOVAC 1969 Some physical-kinetic considerations in penetration of naphthaleneacetic acid through isolated pea leaf cuticle. Physiol Plant 22: 701–712
11. PARRISH DJ, PJ DAVIES 1977 On the relationship between extracellular pH and the growth of excised pea stem segments. Plant Physiol 59: 574–578
12. PENNY P, J DUNLOP, JE PERLEY, D PENNY 1975 pH and auxin-induced growth: a causal relationship? Plant Sci Lett 4: 133–136
13. RAYLE DL 1973 Auxin-induced hydrogen-ion excretion in *Avena* coleoptiles and its implications. Planta 114: 63–73
14. RAYLE DL, RE CLELAND 1970 Enhancement of wall loosening and elongation by acid solutions. Plant Physiol 46: 250–253
15. RAYLE DL, RE CLELAND 1977 Control of plant cell enlargement by hydrogen ions. Curr Top Dev Biol 11: 187–214
16. RAYLE DL, RE CLELAND 1980 Evidence that auxin-induced growth of soybean hypocotyls involves proton excretion. Plant Physiol 66: 433–437
17. SCHÖNHERR J 1976 Water permeability of isolated cuticular membranes: the effect of cuticular waxes on diffusion of water. Planta 131: 159–164
18. STUART DA, RA JONES 1978 The role of acidification in gibberellic acid- and fusicoccin-induced elongation growth of lettuce hypocotyl sections. Planta 142: 135–145
19. VANDERHOEF LM, JS FINDLEY, JJ BURKE, WE BLIZZARD 1977 Auxin has no effect on modification of external pH by soybean hypocotyl cells. Plant Physiol 59: 1000–1003
20. YAMADA Y, SH WITTWER, MJ BUKOVAC 1964 Penetration of ions through isolated cuticles. Plant Physiol 39: 28–32
21. YAMAGATA Y, R YAMAMOTO, Y MASUDA 1974 Auxin and hydrogen ions actions on light-grown pea epicotyl segments. II. Effects of hydrogen ions on extension of the isolated epidermis. Plant Cell Physiol 15: 833–841