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# The *in-vitro* Acid-Growth Response: Relation to *in-vivo* Growth Responses and Auxin Action

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*Summary.* We have examined in detail the characteristics of the hydrogen-ion extension response in frozen-thawed *Avena* coleoptile sections (*in-vitro* acid-growth response). These data allow us to compare the *in vitro* response with the *in-vivo* extension responses initiated by auxin and hydrogen ions. The *in-vitro* response has three characteristics in common with the *in-vivo* responses: a similar $Q_{10}$ (3–4 between 15 and 25° C, but almost 1 between 25 and 35°); a minimum yield stress; and a lack of stored growth (*i.e.*, an inability to induce a potential for growth during periods of reduced wall tension). Both the *in-vivo* and *in-vitro* acid-growth responses have a threshold pH of about 4.5 and give an optimum response at pH values of 3 and below. These similarities suggest that the *in-vitro* and *in-vivo* acid-growth responses have a common wall-loosening and wall-extension mechanism. We have also examined the effects of Pronase, sodium lauryl sulfate (SLS), elevated temperatures, calcium, and potassium ions on the *in-vitro* acid-growth response. We suggest that hydrogen ions do not activate wall-associated enzymes, but act to hydrolyze non-enzymatically some acid-labile linkages in the cell wall. Furthermore, we suggest that auxin induces cell elongation either by causing the release of hydrogen ions from the protoplast or by causing the appearance in the wall of an enzyme which can hydrolyse the acid-labile linkages.

## Introduction

We have previously reported on some of the characteristics of the hydrogenion growth response in turgid *Avena* coleoptile sections (*in-vivo* acid-growth response) and have shown that this response is similar in many respects to auxin induced growth (Rayle and Cleland, 1970; Rayle and Cleland, in press). In these studies, we had made use of the rapid recording technique of Evans and Ray (1969), and therefore, our data were limited to conditions which favored normal, turgor-driven cell extension. More recently we have shown that frozen-thawed *Avena* coleoptile sections, when under tension, will undergo rapid and prolonged extension in response to hydrogen ions (Rayle *et al.*, 1970). This technique has several advantages over more conventional methods for studying

cell extension growth. For example, since turgor is replaced by a constant external load, conditions can be tested which would otherwise be impossible (e. g. high levels of osmoticum, membrane-disrupting substances, *etc.*). Another advantage is the fact that frozen-thawed sections have lost many of their biochemical activities including the ability to synthesize new wall material (Rayle *et al.*, 1970). The latter fact, in particular, has lead us to suggest that this system might have some unusual advantages for the study of the biochemistry of cell-wall extension. Of course, this system would be of rather limited interest if it did not accurately simulate normal *in-vivo* cell extension. In this paper we will show that the frozen-thawed system (*in-vitro* system) does closely mimic normal hydrogen-ion or auxin-induced extension responses and that it is likely the *in-vitro* and *in-vivo* growth responses have similar extension mechanisms. In addition, we have attempted to determine whether the hydrogen ions are acting directly to cause non-enzymatic hydrolysis of some wall component, or are acting to activate some already existing enzymatic system.

## Material and Methods

*Plant Material.* The plant material used in this work consisted of 15 mm sections cut from 25–32-mm-long coleoptiles of *Avena sativa* L., cv. Victory. Detailed instructions for growing the *Avena* seedlings and harvesting the sections can be found elsewhere (Cleland, 1972). Frozen-thawed sections were prepared by rapidly freezing coleoptile sections with Freon (Freeze Up, Mann Research Lab., New York) and then thawing them rapidly in distilled water. This treatment was repeated at least twice.

*Extension Measurements.* Three methods were used to monitor cell extension growth. A shadowgraphic recording devise (Evans and Ray, 1969) was used to follow the growth of sections the extension of which was driven by their turgor (Method A). For live sections subjected to an applied force and for frozen-thawed sections, the apparatus shown in Fig. 1 was most often used (Method B). The sample (A) is inserted between a lower, immobile clamp (B) and an upper clamp (C) attached to a pivoting beam (D). The initial distance between the clamps is adjusted by a setscrew (E). Force is applied to the sample by stretching a spring (F) which is attached at one end to the beam and at the other end to a rack-and-pinion (G). Extension of the sample causes rotation of the beam. This displacement is detected (H) by a Hewlett-Packard (Palo Alto, Calif.) linear displacement transducer (585DT-500). The sample is immersed in the incubation medium contained in a water-jacketed chamber (J). The temperature of the incubation medium is mainained by circulation of water through the jacket (K). The incubation chamber has an outlet (L) to facilitate solution changes. Aeration is accomplished by insertion of a needle (M) through the outlet plug. The output from the transducer (I) is rectified by a demodulator unit. The rectified signal is passed without filtering to a 10-mV multipoint recorded (Honeywell Ft. Washington, Pa.) which records the average value of the signal once every 30 seconds. The AC excitation is provided by a Hewlett-Packard oscillator via an independent-matching transformer. We are currently using five demodulator units so that five records can be obtained simultaneously. Each demodulator unit is isolated from its companions by a 1:1 trans-

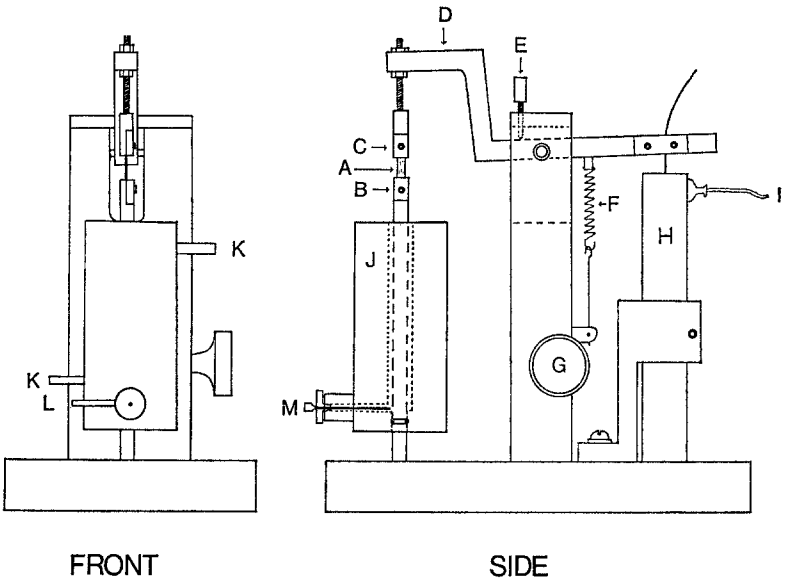


Fig. 1. Apparatus for measuring extension of cell walls under constant applied stress. Details described in Material and Methods

former. An alternative method for following the extension of frozen-theawd segments under constant force made use of an Instron extensometer as described by Rayle *et al.* (1970 (Method C).

*Solutions.* Incubation solutions contained potassium phosphate (0.01 M, pH 7.0) citric acid-sodium citrate (0.01 M, pH 2.7–4.5), or potassium maleate buffer (0.0025 M, pH 4.7). When pH values below 2.7 were needed, dilute HCl was employed. Solutions also contained, where indicated, indole-3-acetic acid (IAA; 5 μg/ml) and mannitol (0.2M). Pronase (Cal Biochem, Los Angeles, Calif.) (200 μg/ml) was dissolved in 0.01 M Tris buffer (pH 7.5).

## Results

### *Characteristics of the Growth Responses*

First, the pH dependence of extension was determined for both the *in-vivo* and *in-vitro* acid-growth system (Fig. 2). In both cases a maximum extension response is achieved at about pH 3 and the extension rate decreases rapidly as the pH increases until a basal value is reached at about pH 4.5 The two curves differ, however, at pH values below 3. This apparent difference between the *in-vitro* and *in-vivo* responses might have a trivial explanation, however, since live sections lose turgor at very low pH values and hence there is no driving force for cell elongation. This would obviously not be a concern with frozen-thawed sections since the driving force for extension is a constant external load.

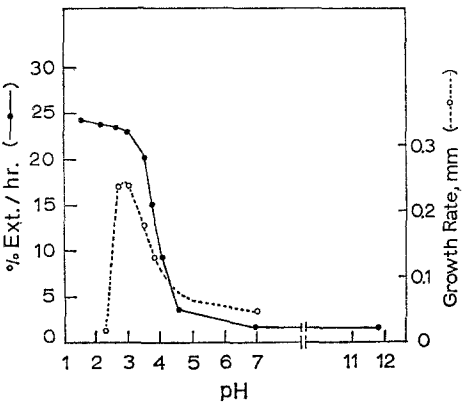


Fig. 2. Dependence of the *in-vivo* and *in-vitro* growth responses on pH. *In-vivo* response measured by Method A, *in-vitro* response by Method B (see Material and Methods). In each case extension rate measure 10–20 min after addition of solution of indicated pH or when the rate became linear. Applied force 25 g in the *in-vitro* response

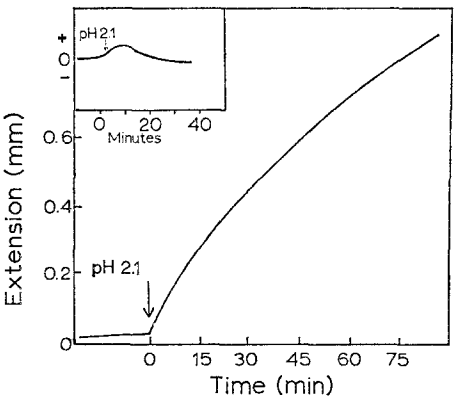


Fig. 3. Demonstration that continued acid-induced wall loosening can occur in intact sections at pH 2.1 as long as turgor is supplemented by an applied force. Sections incubated at pH 7 without an applied force (insert) or with an applied force of 15 g, then pH of solutions was changed to 2.1 ((dilute HCl) and extension was measured (Method B)

If this explanation is correct, live sections should show a strong and prolonged response at pH values below 3 when an external force is applied to supplant the normal driving force of turgor. Such an experiment is shown in Fig. 3; rapid and prolonged elongation of live coleoptile sections is induced at pH 2.1 when an external force is applied, but not without the external load (Fig. 3, insert).

Table 1. Comparison of $Q_{10}$ values for different growth responses.

Growth rate was determined sequentially at 15, 25, 35, 25 and 15° C. $Q_{10}$ values are calculated from steady-state rates at each temperature rather than from initial rates.

| Growth response | $Q_{10}$ | |
|---|---|---|
| | 15—25° C | 25—35° C |
| pH 3.0, fresh | 4—5 | 1.1 |
| pH 3.0, frozen-thawed | 5 | 1.2 |
| IAA, $3 \times 10^{-5}$ M, fresh | 5 | 1.2 |

It has been suggested (Ryle *et. al*, 1970) that one difference between the *in-vivo* and *in-vitro* acid-growth responses is the duration of the response. While the *in-vitro* response persists for 6–12, the *in-vivo* response at pH 3 persists for only 1–2 h. This difference also appears to be related to a loss of turgor in acid-treated live sections, since the hydrogen-ion response will persist for at least 5–6 h in live sections if an external force is applied (data not shown).

A second similarity between the responses is their temperature dependence (Table 1). $Q_{10}$ values for the *in-vivo* and *in-vitro* acid-growth-responses as well as the auxin response were determined by measuring the steady-state growth rate for sections successively at 15, 25, 35, 25 and 15° C. Our procedure differs from that of Ray and Ruesink (1962) in that we measure steady-state growth rates rather than initial growth rates after a change in temperature Similar $Q_{10}$ values were obtained for any particular temperature interval whether the temperature was increased or decreased across the interval. Ray and Ruesink (1962) have shown that auxin-induced growth has an unusual $Q_{10}$ in that it is higher at lower temperatures than it is at elevated temperatures. This has been confirmed, and it is shown here that both the *in-vivo* and *in-vitro* acid-growth-responses show a similar $Q_{10}$.

A third similarity is the presence of a yield stress. Auxin-induced growth only occurs when the driving force (the turgor pressure) exceeds a critical value or yield stress (Cleland, 1959). Similarly, the *in-vivo* acid-growth response also shows a yield stress (data not shown). The relationship between extension and external stress for frozen-thawed sections is shown in Fig. 4. Note that extension is proportional to stress only above a certain applied force, and thus the respnose would appear to have a yield stress. While the absolute value of the yield stress in the *in-vitro* system varied somewhat with different batches of coleoptiles, it was always apparent.

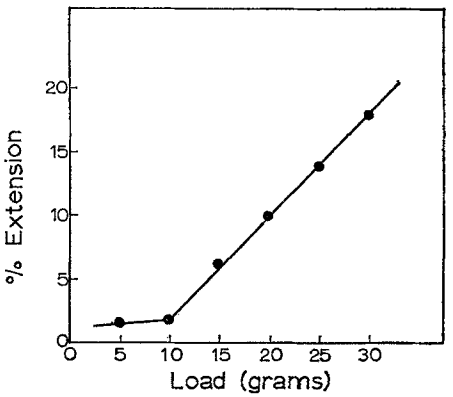


Fig. 4. Demonstration of a yield stress in the *in-vitro* acid-growth response. Frozen-thawed sections were subjected to the indicated force at pH 7.0 for about 30 min. The pH of the solution was then changed to 3.0 and the subsequent extension rate monitored. Rates at each load were calculated after response became linear (generally about 10–15 min) and converted to percent extension. Extension monitored by Method B

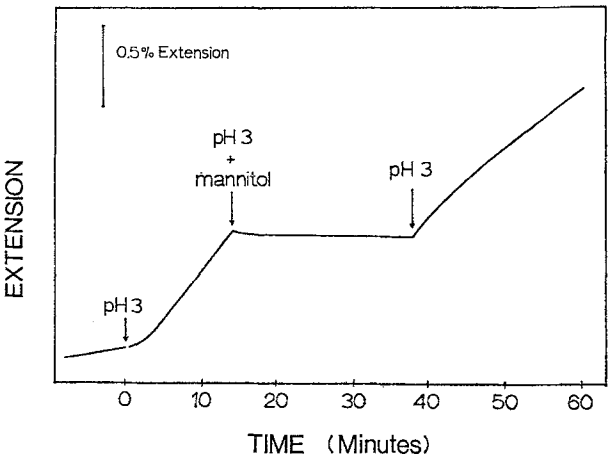


Fig. 5. Inability of hydrogen ions to act when tension in the walls of live sections is reduced. Sections were first treated with pH 3 buffer, then 0.2 M mannitol (pH 3.0) was added after 15 min to reduce wall tension. After 25 min sections returned to mannitol-free pH 3 buffer. Note absence of stored growth. Growth monitored by Method A

A final similarity is the requirement in each response for the wall to be under tension in order for the growth-promoting agent, auxin or hydrogen ions, to cause wall loosening. In live sections this is shown by the fact that if the tension is temporarily reduced with mannitol,

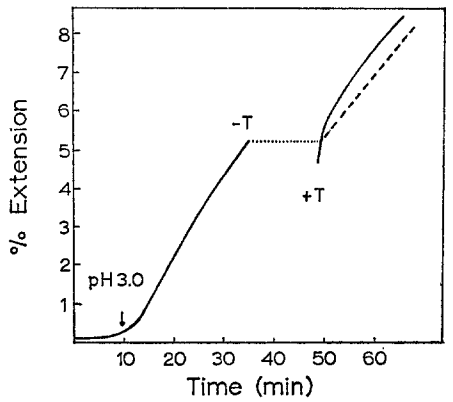


Fig. 6. Lack of stored growth in the *in-vitro* acid-growth response. Frozen-thawed sections subjected to applied force of 20 g at pH 7, then solution changed to pH 3. After 35 min the force was removed entirely and restored 15 min later. Note that extension resumes at a constant rate after only a small growth burst, indicating that only a small amount of stored growth has occurred. Dashed line indicates expected growth curve if no stored growth had occured

the growth rate upon resumption of normal turgor is no higher than the original growth rate (Cleland and Rayle, 1972; Fig. 5). In other words, there has been little or no potential for growth "stored" during the period of reduced turgor. A similar phenomenon can be seen with frozen-thawed sections. If the external load is removed completely, then reapplied at a later time, extension resumes at approximately the same rate as initially seen with only a small extension burst (Fig. 6). Likewise, if one simply drops the tension to a lower level (but above the threshold level for extension) a new rate is quickly established after an initial shrinkage (elastic). Upon reapplication of the original load a new, higher rate is obtained but again only a little stored growth is evident (Fig. 7).

### *Mode of Action of Hydrogen Ions*

It has been pointed out earlier (Rayle *et al.*, 1970) that hydrogen ions might induce wall loosening in two different ways; either by activating some wall-loosening enzyme already present in the walls, or by directly hydrolyzing some acid-labile cell wall bonds. The following experiments were carried out in an attempt to clarify this situation.

First we have attempted to eliminate the possibility that the hypothetical acid-activated enzymes are identical with the unstable "growth-limiting proteins", the continued synthesis of which is necessary for auxin-induced growth (Cleland, 1971). Sections were pretreated for 30 min

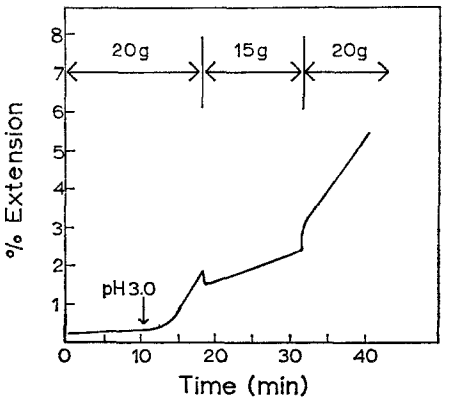


Fig. 7. The behavior of frozen-thawed sections when subjected to a period at slightly reduced applied force. Frozen-thawed sections incubated at pH 7 under 20 g force, then pH changed to 3.0. Force reduced to 15 for 15 min, then returned to 20 g. Upon resumption of the original force the section extends at nearly the original extension rate with little indication of any stored growth.

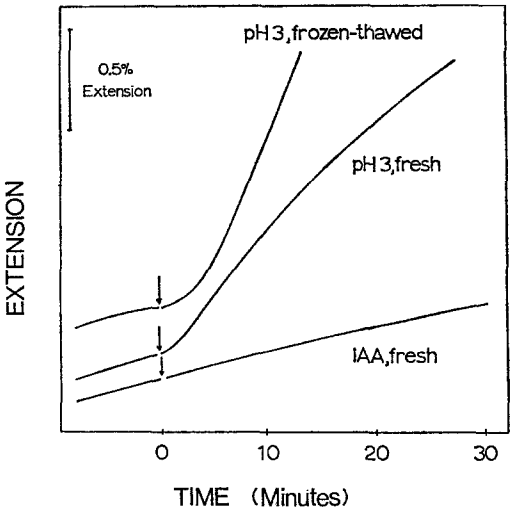


Fig. 8. Difference in auxin and acid-growth responses in their requirement for protein synthesis. Live sections pretreated 30 min or 2 h at pH 7 with 6 μg/ml cycloheximide. Fresh sections (30 min cycloheximide treatment) then given IAA at pH 7, or pH 3 buffer, and growth monitored by Method B. Other sections (2 h cycloheximide pretreatment) frozen-thawed, then subjected to 20 g force and given a pH 3 buffer

with 6 μg/ml cycloheximide, then treated with auxin or hydrogen ions, or first frozen and thawed and then treated with acid. After the cycloheximide pretreatment, auxin is unable to induce any cell elongation

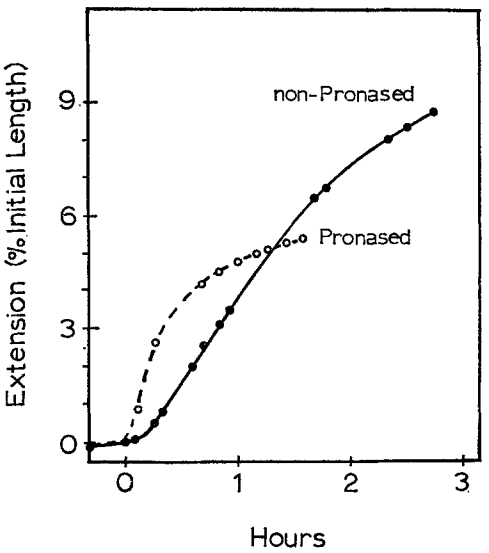


Fig. 9. Ability of frozen-thawed sections to extend in response to hydrogen ions after extensive proteolysis with Pronase. Frozen-thawed sections treated overnight with 200 μg/ml Pronase or used without further treatment. Extension monitored by Method B, applied force 20 g

(Fig. 8), but the live, turgid sections will still respond to hydrogen ions. The frozen-thawed walls are likewise capable of undergoing an acid-growth response. If hydrogen ions activate wall-loosening enzymes, these proteins must be stable for more than 30 min.

Secondly, frozen-thawed walls were subjected to extensive proteolysis with Pronase prior to treatment with hydrogen ions in an attempt to inhibit the growth response. These sections are still capable of showing an acid-growth response, although the response is slightly abnormal-less linear and of slight shorter duration (Fig. 9). These results would seem to make an enzyme-mediated response unlikely. It must be remembered, however, that Pronase does not remove all cell protein, even though it removes the bulk of it (e.g., Cleland, 1967, showed that over 90% of the cell-wall proline is removed by such a treatment).

Attempts were then made to denature cell-wall proteins under conditions that do not extract substantial amounts of cell-wall polysaccharides. Frozen-thawed sections were pretreated at pH 7 for up to 90 min at 42° C; this treatment did not affect their ability to undergo the acid-induced extension. Likewise, the sections still underwent a normal acid growth response if the pretreatment at 42° was carried out at pH 3, as long as the sections were not under tension. But if the effect of hydrogen

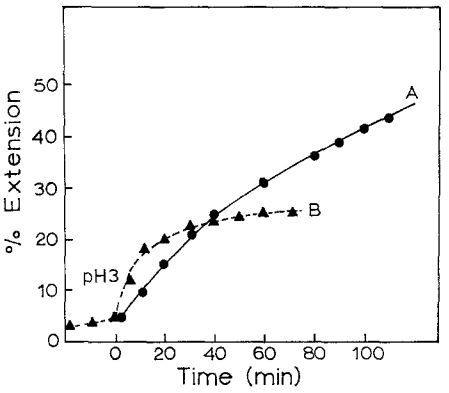


Fig. 10 A and B. Effect of a treatment at 42° C on the *in-vitro* acid-growth response. Frozen-thawed sections subjected to a force of 15 g and treated as follows. A Pretreated for 90 min at 42° C at pH 7.0, then incubated at pH 3.0 and 22° C. B Preincubated 90 min at pH 7.0 and 22° C, then incubated at pH 3.0 and at 42° C. Note that the extension response is normal in A, but is more rapid than normal and of short duration in (B). Extension monitored by Method B

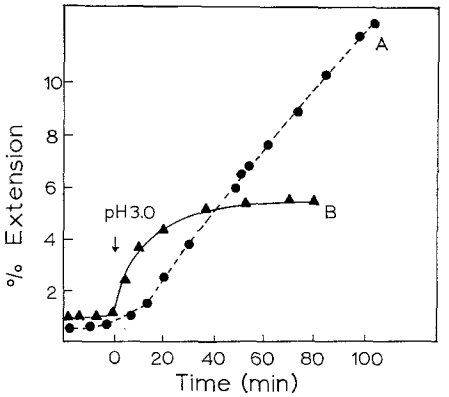


Fig. 11. Effect of SLS on the *in vitro* acid-growth response. Frozen-thawed sections subjected to a force of 15 g and treated as follows: A Pretreated 90 min with 1% SLS at pH 7.0 then incubated at pH 3.0 without SLS. B Preincubated 90 min at pH 7.0 without SLS, then incubated at pH 3.0 with 1% SLS. Note that the extension response is normal in (A) but is more rapid than normal and of short duration in (B). Extension monitored by Method B

ions is tested at 42° on walls under tension, the resulting response is distinctly abnormal and of short duration (Fig. 10). Furthermore, the sections will not respond to pH 3 if the temperature is lowered to 25°.

The results obtained with SLS (Fig. 11) are similar; this agent alters the ability of frozen-thawed sections to respond to acid only if given

to walls under tension and in the presence of the hydrogen ions. These results are difficult to reconcile with the hypothesis that hydrogen ions activate a cell-wall-bound, wall-loosening enzyme.

Finally, the possibility has been considered that hydrogen ions induce elongation simply by extracting calcium from the wall. If so, the response should be antagonized by calcium ions and enhanced by potassium ions. Neither calcium (up to 0.2 M) or potassium ions (up to 0.2 M) have any effect on the acid-growth response of frozen-thawed sections.

## Discussion

The ability of hydrogen ions to induce rapid cell elongation is well established (Strugger, 1932; Bonner, 1934; Rayle and Cleland, 1970; Ganot and Reinhold, 1970; Evans *et al.*, 1971; Hager *et al.*, 1971). We have recently reported (Rayle *et al.*, 1970) that frozen-thawed walls, under applied tension, also elongate in response to hydrogen ions, and have suggested that this *in vitro* acid-growth response would make an ideal test system for studying the mechanism of cell wall extension. Obvious advantages of this system include the fact that an applied force is used in place of turgor with the result that the response is not affected by the molarity of the incubating solutions or by materials which affect membrane permeability. Likewise, the biochemistry of wall extension can be easily studied in this system because of the elimination of such biochemical processes as wall synthesis and protein synthesis. However, results obtained with this system would be of only limited importance if the *in-vitro* acid-growth response did not have a wall loosening mechanism similar to the *in-vivo* mechanism initiated by auxin and hydrogen ions. In this paper we have attempted to point out the similarities and differences between the *in vitro* acid-growth response and the *in vivo* responses to auxin and hydrogen ions.

We have shown earlier (Rayle and Cleland, 1970) that the two *in-vivo* responses under considerations are similar in that the maximum growth rate is the same whether the inducing agent is auxin or hydrogen ions. Furthermore, in both cases the plastic extensibility (DP) increases to the same extent, reaching a maximum only 60—90 min after addition of the growth promoting agent. Yet the two responses are clearly different in that neither protein synthesis (Fig. 8) nor oxygen (Hager *et al.*, 1971; Rayle and Cleland, unpubl.) is needed for the acid-growth response while both are needed for the response to auxin. This must mean that hydrogen ions are, themselves, growth-promoting agents, and do not simply act by activating endogenous auxin as has been suggested by Bonner (1934).

In this paper we show that the two *in-vivo* responses and the *in-vitro* acid-growth response share three common characteristics. First, all show

the same rather unusual $Q_{10}$ for growth, higher between 15° and 25° than between 25° and 35°C (Table 1; Ray and Ruesink, 1963; Zenk, in press). It should be pointed out that the low $Q_{10}$ for the 25—35° interval cannot be explained by thermal denaturation of the wall-loosening enzymes at 35° since the same growth rate prevails at 25° before and after an interval at 35°.

A second common characteristic is the absence of stored growth, i.e., the apparent inactivity of the inducing agents whenever wall tension is reduced below a critical level. In theory, if rapid elongation is temporarily interrupted by a period of reduced turgor, and if the induced agent is active during this period, the growth rate upon resumption of turgor will be greater than normal for at least a limited period. In fact, the sections resume extending at the original rate, indicating that the inducing agents were not active during the period of reduced turgor (Cleland and Rayle, 1972; Figs. 5—7).

Finally all three responses show a minimum yield stress, i.e., a stress which must be exceeded if rapid extension is to occur. While the occurence of a yield stress is well established for auxin-induced growth (Ordin *et al.*, 1956; Cleland, 1959, 1967) we believe that the present report constitutes the first report of a yield stress in an isolated cell wall system.

The two acid-growth responses show, in addition, similar pH profiles within the range where comparisons are possible (pH values of 3 and above). At pH values below 3 the *in-vivo* response is obscurred by the adverse effects of hydrogen ions on turgor, but if turgor is supplemented by an applied force, the acid-growth response can still be demonstrated. Earlier reports suggested that the two acid-growth responses differed in their duration (Rayle and Cleland, 1970) as the *in-vivo* response rarely exceeded 2 h while the *in-vitro* extension may persist for 6—8 h. The short duration of the *in-vivo* response appears to be another artifact caused by an acid-induced loss of turgor, since the *in-vivo* response will persist for at least 6 h if turgor is supplemented by an applied force.

The above facts have led us to conclude that the three responses considered herein have a common mechanism for wall loosening and wall extension, even though the events leading up to wall loosening may be different. We conclude that the *in-vitro* response may be used to study the mechanism of cell-wall extension.

What, then, can the studies on the *in-vitro* acid-growth response tell us about the mechanism of cell wall extension? First, it is obvious that wall synthesis cannot be involved in the loosening process since there is no wall synthesis in the *in-vitro* extension system (Rayle *et al.*, 1970). Rather, it seems more likely that the role of wall synthesis must be in maintaining the normal organization of the wall so continued

wall-loosening can occur. Secondly, this system would seem to provide the best direct evidence that cell-wall bonds are broken in extension growth, and eliminate any mechanism involving elastic extension and subsequent fixation by wall synthesis (Burstrom *et al.*, 1967; Masuda, 1968).

The action of hydrogen ions must be to cause the cleavage of some cell-wall crosslink, either by directly participating in the hydrolysis of some acid-labile cell-wall bond or by activating some wall-associated enzyme which catalyzes the breakage of cell-wall bonds. Hager *et al.* (1971) have supported the latter possibility, but the evidence for this seems to consist primarily of the fact that the acid-growth response is inhibited by copper ions. We have proposed a mechanism involving direct hydrolysis by hydrogen ions (Rayle and Cleland, in Press; Rayle *et al.*, 1970), and the evidence presented here seems to support this idea. Walls which have been deproteinized with Pronase or have been treated under conditions which should denature proteins (incubations with urea or SLS or at 42°) are still capable of extending in response to hydrogen ions. Either the wall-loosening enzymes are remarkably resistant, or hydrogen ions must be acting directly on the wall. One piece of evidence which might seem inconsistent with a mechanism involving an acid-mediated hydrolysis is the plateau seen between pH 2 and 3 in the dose-response curve of the *in vitro* acid-growth response; the acid-mediated hydrolysis of glycosidic linkages usually shows no such plateau but increases in rate whenever the pH is lowered. However, such a plateau has been recorded for at least one glycosidic bond, the glycosyl-uronic acid link (Smidsrød *et al.*, 1966).

If acid acts directly in the wall to hydrolyze some load-bearing acid-labile bond, how does auxin induce wall loosening? At least two possibilities deserve consideration. Auxin may facilitate the appearence in the wall of an enzyme which can cleave these acid-labile bonds. This could be accomplished either by stimulating the synthesis of these proteins or by potentiating their transfer through the plasma membrane. These proteins could be identical to the "growth-limiting proteins" (Cleland, 1971; Penny, 1971), although this seems unlikely in view of the data presented in Fig. 8. Alternatively, auxin may cause the release of hydrogen ions from the protoplast into the wall, where they hydrolyse acid-labile cell-wall bonds. Hager *et al.* (1971) has suggested that the action of auxin is to active a membrane-bound ATPase which accomplishes this transfer of protons across the plasma membrane.

The possibility that the wall-loosening agent activated by auxin is actually hydrogen ions may seem unlikely at first in view of the fact that auxin stimulates the elongation of sections incubated in buffered solutions. Nevertheless it is possible to imagine situations where the buffer

does not penetrate the cuticle and thus does not modify the pH of the cell wall solution or specific areas in the wall which are somehow shielded from the influence of particular buffers. We are currently testing the possibility that auxin does change the pH in the immediate vicinity of the cell wall.

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