THE ROLE OF POLYSACCHARIDASES IN ACID WALL LOOSENING OF
EPIDERMAL TISSUE FROM YOUNG *PHASEOLUS VULGARIS* L. HYPOCOTYLS

By

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ABSTRACT

The extension of frozen-thawed epidermal strips prepared from the first centimetre below the hypocotyl hook of six day old dark grown *Phaseolus vulgaris* seedlings while immersed in various buffers and under various tensions was characterized. This was done in an attempt to determine if the acid wall loosening phenomenon, which according to the Acid-growth theory (Taiz, 1984) is thought to mimic part of the auxin mechanism of action, is mediated by unspecified wall loosening enzymes.

Epidermal strips were found to be significantly loosened by media pH 6.0 to pH 2.6 (0.05M citric acid–0.10M disodium phosphate) relative to pH 7.5. A minimum stress between 1.6 and 7.6 grams was required for the acid-extension of strips 4.5±0.5 mm wide. Regardless of tension, extension by tissues in an acid medium was largely transient. For example, tissues tensioned by a 16.0 gram load reached a maximum extension rate of 6.18±1.37% of initial length per hour (L°/hr) between 4 and 6 minutes after immersion in pH 4.8. The rate was 1.29±0.17% L°/hr between 55 and 60 minutes and 1.05±0.14% L°/hr between 220 and 240 minutes. Total acid-extension over four hours was 4.24±0.57% L°. The extension response was found to be stable; newly harvested tissues whether frozen or not performed similarly to strips aged up to 15 days at -12°C before being extended. The performance of strips immersed in unbuffered solutions indicated that tissues were self-buffering at an acid pH probably because of the fixed carboxyls within the wall. The capacity for acid-extension by epidermal strips was lost in mature tissues harvested 4–5 cm below the hypocotyl hook.

Temperature coefficients from extension rates were determined at several pHs. The results were highly variable. The acid-extension of strips boiled 15 minutes in ethanol or extracted in 3M NaCl for 4 hours at 4°C or 6M LiCl for 8 hours was determined in several pHs. The impact of the treatments was largely a suppression of the initial burst of acceleration. Extension rates following the initial surge were relatively unaffected.
Glycosidase activities in untreated, ethanol-boiled, or salt extracted strips were determined. \(\beta\)-glucosidase was found to be most active in untreated strips with lesser levels of \(\beta\)-galactosidase and \(\beta\)-xylosidase and a trace of \(\alpha\)-galactosidase being detected. Ethanol-boiling and LiCl-extraction removed or deactivated all four activities from the strips and NaCl-extraction lowered all four activities 70–80%. NaCl proved to have solubilized most of the missing \(\beta\)-glucosidase and \(\beta\)-galactosidase when the extraction solution was assayed following desalting and concentration. LiCl solubilized most of \(\beta\)-xylosidase. It was concluded that glycosidases and any other similarly soluble enzyme cannot be responsible for long term acid wall loosening in bean epidermis. If an enzyme is involved, it must be extremely stable and tightly bound to the wall. The acid-extension performance of frozen-thawed longitudinally halved hypocotyl sections in comparison to epidermal strips, as well as other evidence was considered support for another hypothesized mechanism of acid wall loosening, the displacement of calcium bridges.
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INTRODUCTION

Auxin and Cell Extension

Primary tissues in the growing shoots and roots of higher plants are derived from meristems of rapidly dividing, relatively small, isodiametric cells. Cell division is followed by considerable cell expansion. For example, the average volume of pea root cells increases roughly fifteen times in the one centimetre behind the organ tip (Brown, 1952). This pattern of plant growth presumably arises from the life history of plant cells which is conventionally divided into three phases: division, elongation, and maturation. Indeed, moving basipetally in the stems of higher plants, the rate of cell division peaks and declines more rapidly than that of elongation and cells become progressively more differentiated (Sachs, 1965).

We have an extremely limited understanding of how the events of growth are directed in individual cells. The extent of predetermination in the final size of growing cells is unclear (Wareing, 1978) and few ideas exist to explain the differentiation of different cell and tissue types (Mohr, 1983). It is known however, that plant growth substances play a role in the primary growth of higher plants. For example, the growth phases of cell division and cell extension in excised tissues are paralleled by peaks of sensitivity to cytokinin and auxins (Trewavas, 1981). Progress towards understanding how plant growth substances actually stimulate growth has been most successful with auxins (Penny and Penny, 1978; Zeroni and Hall, 1980). The natural auxin, indole acetic acid (IAA) appears almost universally to be a controller of cell extension (Zeroni and Hall, 1980).

The cell elongation mediated by IAA is largely the result of extension of the semi-rigid envelope surrounding the cell, the cell wall. Since it is the turgidity of elongating cells that drives their growth, the rate of cell elongation can be expressed in
terms of a single equation: \( \dot{v} = L \phi \frac{\Delta \pi - Y}{L + \phi} \) where \( \dot{v} \) is the rate of change in cell volume, \( \phi \) is the yielding compliance or wall extensibility, \( \Delta \pi \) is the osmotic potential difference between the cell and its environment, \( Y \) is the yield threshold, and \( L \) is the hydraulic conductivity or permeability of the cell (Ray et al, 1972). For the growth rate to increase, either \( L \), \( \phi \), or \( \Delta \pi \) must increase or \( Y \) decrease. If \( L \) is relatively large, the water potential difference between the cell and its environment, \( \Delta \psi \), is near zero. Since \( \Delta \pi - P = \Delta \psi \) and \( \Delta \psi = P \) where \( P \) is the cell turgor pressure. In this case, \( \dot{v} = \phi \left( P - Y \right) \), the most familiar form of this equation. Doubts have been raised as to whether \( L \) can be entirely ignored in this fashion (Boyer, 1985), but this is disputed (Cosgrove, 1986).

IAA does not appear to influence the magnitude of \( Y \) (Cleland, 1971a). Most recently Cosgrove (1985) measured the growth rate of pea epicotyls as well as the turgidity of individual cells within the elongating organ. He found that, when the epicotyls were excised and growth rate eventually stopped, turgidity stabilized at less than half of its previous value. This was interpreted to be \( Y \). Pre-incubation of the excised epicotyls in IAA increased the initial growth rate. Growth and turgidity declined more quickly: growth to zero and turgidity to the same value of \( Y \) as untreated tissues.

Direct pressure probe measurements of the turgidity of elongating cells indicate that \( P \) is not altered by application of IAA (Cosgrove, 1985; Cosgrove and Cleland, 1983; Cosgrove et al, 1984).

Measurement of Wall Extensibility

Treatment with IAA has consistently been shown to increase the wall extensibility (cause cell wall loosening) in those tissues in which it can elicit a growth response (Cleland, 1981). Wall extensibility is defined as the ability of cell walls to undergo irreversible extension when under constant stress (Cleland, 1981). There is no universally agreed method to measure wall extensibility (Taiz, 1984). Three methods are popular: stress
relaxation, the instron technique, and the creep test. Each of these mechanical tests are performed on isolated tissues. Traditionally the undefined effects of enzymes have been eliminated by boiling the tissue samples in methanol and later rehydrating them. The most popular method is the instron technique in which tissues are extended twice successively at a constant rate. The stress required to maintain the continuous strain is recorded as a function of extension. Measures of elastic extensibility (DE) and plastic extensibility (DP) are derived from these results (Cleland, 1967a; 1984). This method offers the advantage that a large number of samples can be rapidly assessed. In stress relaxation, the walls are extended and held at a constant strain while the stress along the walls is measured as a function of time (Masuda, 1978). Two values are computed in this case, the minimum stress relaxation time \( T_{o} \) and the maximum stress relaxation time \( T_{m} \). The simplest test and that most closely approximating extension \( \text{in vivo} \) is the creep test. In this case walls are extended by a constant stress. Extension is usually recorded against log time (Cleland, 1981). The three methods appear to provide qualitatively similar information since the treatment of any tissues with IAA that results in increased extension rate also increases DP and creep test extension, and decreases the \( T_{o} \) of these tissues when compared to untreated tissues.

Acid Wall Loosening and the Acid Growth Hypothesis

With methanol-boiled samples, the constant stress of the creep test yields a straight line, termed creep, when extension is plotted against log time. This is the result of viscoelastic extension: part instantaneous elastic extension typical of heavily cross-linked polymeric substances like rubber, and part irreversible plastic extension typical of infrequently linked substances like oil (Cleland, 1971a). The heteropolymeric network that is the cell wall falls somewhere in between. The exponential slowing of creep results from the resistance of polymer linkages, as chemical agents that cleave linkages can convert creep into continuous extension. Such extension is called chemical creep (Cleland, 1971a).
Constant stress has been applied to tissues not boiled in methanol (Rayle et al, 1970; Rayle and Cleland, 1972) in the investigation of one form of chemical creep. It had been known for some time that low pH induces rapid cell elongation somewhat similar to auxin (Cleland, 1971a). Tissues from elongating coleoptiles of the monocot *Avena*, frozen and thawed to eliminate the effects of quantitative or qualitative changes in wall synthesis on the measure of wall extensibility, were extended by a force of 15.0 or 20.0 grams. To separate viscoelastic extension from any chemical creep or wall loosening effects of high proton concentration, the coleoptile tissues were first stressed while immersed in a neutral buffer. After forty minutes and the completion of all measurable extension that would have been detected by the creep test, the bathing solution was changed to one of pH 3.6 and rapid continuous extension resulted, suggesting that low pH increased wall extensibility.

This discovery led to the proposal of the acid–growth hypothesis (Cleland, 1971a; Hager et al, 1971). Since it was already known that the dramatic growth response to auxin by excised living tissues was preceded by a lag of some minutes (Penny and Penny, 1978), it appeared likely that the primary action of auxin occurred not in the cell wall but at the plasmalemma or within the cell (Evans and Ray, 1969). The acid–growth hypothesis proposed that auxin stimulated a plasma membrane proton pump, probably an \( \text{H}^+ \text{--ATPase} \), which acidified the cell wall and caused the cell wall loosening so universally a feature in auxin–stimulated growth. It was soon shown that *Avena* coleoptile tissues did indeed excrete protons in response to IAA. This was demonstrated by floating the tissues on very weak buffers with and without auxin and monitoring changes in the pH of the medium (Cleland, 1973; Rayle, 1973). In the absence of IAA, the pH stabilized at pH 5.7–6.0; with an optimal IAA concentration, the pH decreased to 4.8. Since proton pumping was not directly measured however, other explanations for the acidification of the medium are possible (Penny and Penny, 1978).
Problems with the Acid Growth Hypothesis

There have been several reports of failure to detect acidification by dicotyledonous tissues treated with IAA (reviewed by Cleland and Rayle, 1978; Taiz, 1984). In many cases growing stem segments were stripped of their epidermal layer before the acidification response was tested. This was done because the epidermal cuticle is a significant barrier to protons (Dreyer et al, 1981). In dicots however, the epidermal region appears to be of critical importance to elongation. Peeling the epidermis removed the growth response to both IAA and H+ from stem segments of peas (Brummel and Hall, 1980; Tanimoto and Masuda, 1971; Yamamoto et al, 1974), cucumbers (Kazami and Katsumi, 1976; Pearce and Penny, 1983), and sunflowers (Mentze et al, 1977). Tanimoto and Masuda (1971) found that treatment of pea segments with the artificial auxin 2,4-D decreased the minimum stress relaxation time ($T_0$) of the epidermis once it was peeled off, while $T_0$ of the underlying tissue was unaltered. Peeling also removed acidification response to IAA by sunflower tissues (Mentze et al, 1977), though not apparently by pea epicotyls (Marrè et al, 1973; Yamamoto et al, 1974). Though soybean hypocotyl segments did not acidify the medium if unpeeled (Vanderhoef et al, 1977), they did if the cuticle was lightly abraded to permeabilize it to protons (Rayle and Cleland, 1980). Inclusion of K+ in the medium was also required to detect H+ excretion from tissues, presumably because K+ uptake was driven by H+ efflux at the plasmalemma (Cleland, 1976).

Evidence that cells do indeed pump protons into their cell walls in response to auxin comes from work with the ATPase inhibitor vanadate. In pea and oat segments, auxin-induced proton extrusion was rapidly and completely inhibited by vanadate (Jacobs and Taiz, 1980). This was closely paralleled by suppression of elongation. In cucumber, where vanadate also inhibited auxin-induced growth and acidification, acid-induced growth was not affected (Brummel, 1986). Jacobs and Taiz (1980) also found that vanadate did not inhibit protein synthesis or respiration though it did inhibit amino acid uptake.
A consensus now exists that, under optimal conditions, which include permeabilizing the cuticle and including K\(^+\) in the medium, rapid auxin–induced proton secretion can be detected readily in those tissues that show an acid–growth response.

There remains some question, however, whether the protonation of cell walls apparently stimulated by auxin actually causes growth in response to auxin. As is frequently noted, acid–induced growth in excised tissues is comparatively short–lived (1–2 hours) compared to auxin–induced growth (6–24 hours) (Taiz, 1984). Exogenous acid application may cause acid wall loosening, but it does not generate the membrane potential produced by an electrogenic proton pump. Since this membrane potential is essential for solute uptake by the expanding cells (Spanswick, 1981), the \(\Delta \pi\) between them and the medium will decline and they will lose turgidity. Cleland and Rayle (1978) have shown that if this lost turgidity is replaced by applied tension, *Avena* tissues will continue to extend for more than six hours in acid. It has also been suggested that auxin–induced wall acidification, as measured with a microelectrode placed 20\(\mu\)m inside the cut end of a xylem vessel of a stem segment, does not become detectable until long after the elongation response begins (Penny et al, 1975). Kutschera and Schopfer (1985) reached a similar conclusion after comparing the pH of an auxin containing medium surrounding corn tissues to the elongation of the tissues. Other authors however, reported a rapid decrease in pH (Jacobs and Ray, 1976; Cleland, 1976). The acid–growth hypothesis does not offer a ready explanation for the biphasic extension response of auxin treated tissues. In tissue that responds to auxin, extension rates typically accelerate after a lag of ten to fifteen minutes until a first maximum is reached after which the growth rate declines somewhat to a minimum after about one hour. The growth rate then increases and stabilizes at a second maximum, usually not as great as the first (Penny and Penny, 1978). Cleland and Rayle (1978) suggested that, in the auxin response, increased solute uptake may lag behind wall loosening and water uptake to produce a brief dip in turgor pressure. Other authors
suggested the acid effect accounts only for the first maximum while some other effect produces the second (Vanderhoef and Stahl, 1975; Masuda and Yamamoto, 1985).

What Does Acidity Loosen?

Determining whether auxin–induced wall protonation actually causes auxin–induced growth might be easier if another long standing question is answered: by what mechanism do protons create acid wall loosening? \textit{In vitro} acid–extension is evidence for the acid–growth hypothesis if this wall loosening by acid buffers occurs by a mechanism that is feasible to occur \textit{in vivo}. The first demonstrations of wall loosening in frozen–thawed \textit{Avena} coleoptiles by protons were not achieved until the pH was below 4.0 (Rayle and Cleland, 1970). This suggested that wall loosening could only occur by some non–enzymatic mechanism. Rayle (1973) subsequently showed that the requirement for such a low pH was an artifact of the impermeability of the cuticle to protons, and that maximum wall loosening of \textit{Avena} coleoptiles occurred at 4.7, a pH more compatible with an enzymatic mechanism. It is now assumed that the wall contains "polysaccharide–degrading enzymes which possess acid pH optima" and these are activated by the lower pH resulting from auxin treatment (Cleland and Rayle, 1978). Given the considerable complexity of plant cell walls however, it is not clear what specific degradative activities must function as wall loosening enzymes.

The primary cell wall of dicotyledons is comprised of 90–95\% polysaccharides by dry weight (Preston, 1974). Roughly one–fourth of this consists of cellulose polymers hydrogen–bonded together into microfibrils (Albersheim, 1976). These in turn appear to be coated by an electrostatically bound layer of xyloglucans (molecules consisting of $\beta-1,4$–linked glucan backbone bearing numerous sidechains largely composed of xylose) (McNeil et al, 1979), although in the monocotyledonous grasses, xyloglucan is replaced by arabinoxylan (McNeil et al, 1975). The pectic matrix between microfibrils constitutes up to
one half of the wall polysaccharides. Like cellulose and xyloglucan, the monomers of the pectic polymers are glycosidically linked in either $\alpha$ or $\beta$ configuration. A number of different pectins have been purified and characterized following solubilization by the action of endo-$\alpha$-1,4-galacturonase on primary cell walls of dicotyledons. They have been identified as rhamnogalacturonans, homogalacturonan, arabinan, galactan and arabinogalactan (McNeil et al, 1979). Isolation of a number of partially digested fragments composed of more than one polymer type suggested that the various polymers of the cell wall are extensively interconnected. A preliminary model based on these results was presented in which all but one of the wall polysaccharides are joined in a covalently linked network, the exception being xyloglucan hydrogen-bonded to the cellulose microfibrils (Albersheim, 1976). The 5-10% of the dry weight of the cell wall that is not polysaccharide consists largely of a hydroxyproline-rich glycoprotein, extensin. Although extensin is extensively polymerized, it is not believed to be linked to other wall polymers (Kato, 1981). Since the Albersheim model was first proposed, it has been suggested by Albersheim and his coworkers that covalent polymer cross-linking was over emphasized (McNeil et al, 1979). In particular, such interconnections between xyloglucan and the pectins are infrequent. Recently, McNeil et al (1984) stated that "only in rare cases can we present evidence for specific interactions or connections between cell wall polymers".

Regardless of the degree of covalent polymer cross-linking, pectic polysaccharides probably do interact through non-covalent chemical bonding which confers strength to the cell wall. One non-covalent binding possibility is calcium bridging. In higher plants, the largest calcium concentrations are always found in cell walls (Demarty et al, 1984). At least 60% of the total calcium is associated with the cell wall in lupins (Rossingnol et al, 1977). The inhibiting effect of exogenous calcium on elongation is well established (Cooil and Bonner, 1957). It has been suggested that calcium may gel pectins within cell walls by chelating to uronic acid residues in separate pectic polymers (Grant et al, 1973). Tepfer and Taylor (1981) however, found that the relative ability of different divalent cations to
antagonize acid-induced growth was unrelated to their relative abilities to bind pectins and form pectate gels. Other non-pectin calcium binding sites may exist in the cell wall which might explain this observation (Bates and Ray, 1981). In any case, the carboxyl groups of the uronic acid residues of cell wall pectic polysaccharides are known to be highly methylated (McNeil et al, 1979). Pectin methyl-esterase has been isolated and purified from mung bean cell walls (Goldberg, 1984), but when pectin was purified from soybean hypocotyls (Yamaoka and Chiba, 1983), the pectin of the growing region was found to be rich in neutral sidechains and tended to remain soluble in the presence of pectin methyl-esterase and Ca\(^{++}\), while pectin of the mature non-growing zone was readily precipitated. Soll and Böttger (1982) found that frozen-thawed hypocotyl tissues from *Helianthus* seedlings under constant stress extended when irrigated with an EDTA solution. This response was short-lived however when compared to acid-induced extension of the same tissues.

Besides covalent cross-linking and calcium bridging, other types of interactions between pectic polymers and other cell wall polymers that contribute to wall extension resistance are possible. For example, hydrogen bonding may be important elsewhere besides among cellulose and xylomalcan. Such possibilities have not been investigated however (McNeil et al, 1979).

Do Enzymes Mediate Acid Wall Loosening?

The presumed mechanism of polysaccharide-degrading enzymes has always been hydrolysis of the glycosidic bonds between polysaccharide monomers. Two possibilities have been suggested: simple hydrolysis may be involved or a transglycosylase may reversibly break polysaccharide cross-links reforming them with new partners (Albersheim, 1976). This second mechanism would offer an explanation why tension is required for wall loosening (Cleland and Rayle, 1972; Rayle and Cleland, 1972). In the absence of turgor, bonds
would be reformed in their original positions.

Many glycosyl hydrolases and transferases are known to be present in plant cell walls (Lamport and Catt, 1981). Some of these are exo-glycosidases including $\beta$-glucosidase (E.C.3.2.1.21), $\beta$-galactosidase (E.C.3.2.1.23), $\beta$-xylosidase (E.C.3.2.1.37), $\alpha$-galactosidase (E.C.3.2.1.22) and acid invertase (E.C.3.2.1.26); all of which have been isolated from the cell walls of *Phaseolus vulgaris* hypocotyls (Nevins, 1970; Pierrot et al, 1982). A number of endoglycanases have been documented to exist in walls as well. The best studied is endo-$\beta$-$1,4$-$D$-glucanase (E.C.3.2.1.4) from peas where two isozymes are known (Wong and Maclachlan, 1979). Endo-$\beta$-$1,3$-$D$-glucanase activity has been detected in mung bean walls (Goldberg, 1977) as well as in corn (Huber and Nevins, 1981).

Attempts to correlate various hydrolytic activities to growth rates in intact stems, auxin-induced growth, or acid-induced growth have been generally unsuccessful (Taiz, 1984). Although Nevins (1970) suggested that $\beta$-glucosidase levels correlated with growth in light grown *P. vulgaris*, others found little (Murray and Bandurski, 1975) or no correlation (Datko and Maclachlan, 1970; Pierrot et al, 1982) working with peas and dark grown beans. Specific aldolactone inhibitors of $\beta$-glycosidase activities did not inhibit auxin-induced growth even though the activities of the enzymes were much reduced (Evans, 1974; Goldberg, 1977). A role for $\alpha$-galactosidases in cell wall extension is still a possibility however (Lamport and Catt, 1981; Labrador and Nicolas, 1984). Glycosidases may have some role other than in cell wall extension. They might permit cellular uptake of either complex extracellular carbohydrates (McNeil et al, 1984) or transported oligosaccharides (Thomas and Webb, 1979).

Auxin has been shown to induce increased $\beta$-$1,4$-glucanase activity in pea stems (Fan and Maclachlan, 1967). This activity did not begin to increase for at least an hour and was not complete until the third hour (Datko and Maclachlan, 1968), and as such did not correlate well with auxin-induced growth. A much faster $\beta$-$1,3$-glucanase activity
increase was stimulated by auxin in *Avena* (Masuda and Yamamoto, 1970). Detectable increases in the activity levels solubilized from coleoptile cell walls were found after as little as a ten minute pre-treatment of tissues with IAA. $\beta-1,3$-glucanase isolated from corn coleoptile walls was found to be inactive however in promoting the growth of corn coleoptiles when the cuticle had been abraded. $\beta-1,3$-glucanase has also been hypothesized to have a non-growth role in cell walls. Abeles et al (1970) suggested that this enzyme may function in dicots as a defense against pathogenic fungi which contain $\beta-1,3$-glucans in their cell walls. An indirect argument supporting this idea was the finding that a bean pathogen, *Colletotrichum lindenmuthianum* produced an inhibitor that blocked the enzymatic activity of bean $\beta-1,3$-glucanase (Albersheim and Valent, 1974).

Though no obvious glycosyl hydrolase activity has so far been convincingly implicated in auxin-induced growth, there is evidence to suggest the involvement of enzymes in wall loosening. Deproteinization of frozen-thawed *Avena* coleoptile segments with pronase or other proteolytic enzymes greatly reduced the capacity of these tissues to undergo acid-extension (Cleland and Rayle, 1978). In peas, pronase treatment also rendered the epidermis of stem segments unresponsive to acid (Yamagata et al, 1974). Yamagata and coworkers also found that once frozen-thawed pea epidermis was boiled in methanol, it remained responsive to only extreme acid under constant stress. They interpreted this as indication that a wall loosening enzyme had been denatured.

Pronase treatment and methanol-boiling, though they undoubtedly have a profound impact on cell wall enzymes, are so brutal to the wall itself that conclusions drawn from these experiments are questionable. Pronase, which has very broad substrate specificity (Nomoto et al, 1960), digests cell wall structural protein (extensin) as well as wall enzymes. Treatment with pronase of *Avena* tissues which were first boiled in methanol removed 85–95% of wall proline as well as 45% of the hydroxyproline (Cleland, 1967b). Proteolysis also altered the viscoelastic properties of these tissues, increasing the plastic
extensibility (DP) relative to tissues boiled in methanol only (Cleland, 1967a). Boiling in methanol also alters the native gel structure of the cell wall. This treatment of Avena coleoptiles removed over 50% of the dry weight of these tissues (Olson et al, 1965). DP of methanol–boiled tissues was also greater than that of unboiled tissues (Cleland, 1967a).

Investigation of the Role of Enzymes

Unambiguous determination of the role of unspecified enzymes in wall loosening appears to require modulation of their activities by less aggressive means minimally disruptive of the wall itself. This may be possible through solubilization of proteins that are non–covalently bound to cell walls. Ideally this would permit correlation estimates between degree of solubilization of cell wall protein as well as individual enzymatic activities and acid wall loosening.

Salt solutions have been used to solubilize cell wall proteins. Huber and Nevins (1980) extracted 8–17% of the protein of pelleted cell walls from corn coleoptile homogenates using a solution of 3M LiCl. Their extract contained both endo- and exo–glucanases. Labrador and Nicolas (1984) used 3M LiCl to separate glycosidases from isolated pea epicotyl cell walls. Other workers solubilized phosphatases from sycamore (Crasnier et al, 1980) and β−1,4–glucanase from soybean hypocotyl (Koyama et al, 1981) using 1M NaCl.

Yamagata et al (1974) appear to have made the only attempt to estimate the impact of enzyme solubilization on wall loosening. They found that treating pea epidermis with a detergent (0.1% deoxycholate) did not suppress the acid–extension response of these tissues. They did not, however, attempt to determine the extent of protein solubilization, though incubation of homogenized Avena cell walls in the same concentration of deoxycholate solubilized approximately two-thirds of wall bound β−1,3–glucanase activity (Masuda and Yamamoto, 1970).
It appears that epidermal strips of dicots represent an ideal system to investigate the impact of protein solubilization on acid-extension in vitro. As described earlier, the epidermis, and the underlying tissue layer typically removed with it in the preparation of epidermal strips (Mentze et al, 1977), are particularly responsive to auxin-induced growth as well as acid-extension. In addition, such strips are only a few cells wide and present a large surface to volume ratio and that would permit extraction of non-covalently bound proteins from them as efficiently as as have been achieved from crude cell wall homogenates.

Because of its simple and well characterized growth, the best model system for investigating various aspects of the role of IAA in cell extension in dicotyledons is probably the hypocotyl of etiolated seedlings of *Phaseolus vulgaris*, the common bean. When bean seeds are germinated and grown in the dark, almost all growth is restricted to the hypocotyl. Six days following imbibition, a seedling has typically developed an upright hypocotyl with a length of approximately ten centimetres that bears several secondary roots towards its base (Horemans et al, 1986). Cell division is restricted to the hypocotyl hook located atop the hypocotyl axis and proximal to the cotelydons (van Holst et al, 1980). The rate of elongation is maximal in the first centimetre below the hook; the axis extending 5–8% of initial length per hour in this region (Horemans et al, 1986; van Holst et al, 1980). The elongation rate of the hypocotyl is progressively less below this point with tissues more than four centimetres below the hook no longer growing. Elongation of cells following the cessation of cell division appears to result in a five fold increase in average cell length. IAA levels along the length of the hypocotyl have been determined (Horemans et al, 1986) as have the changing pattern of cell wall sugar composition (van Holst et al, 1980), and the activity of wall bound glycosides (Pierrot et al, 1982). It has also been shown that incorporation of arabinose and uronic acids into the pectic polysaccharides of hypocotyl cell walls from myo-inositol (Sasaki and Taylor, 1984) and from reserve sugars (Sasaki and Taylor, 1986) is active only in the hook region.
and in the mature non-growing portion of the axis.

**Objectives**

This study was undertaken to examine the possibility that some undetermined cell wall associated enzyme is responsible for *in vitro* acid-extension of cell walls isolated from elongating regions of dicotyledonous plants. Specific objectives of the study were:

(a) To characterize acid-extension of epidermal strips prepared from the hypocotyl of *Phaseolus vulgaris*. Specifically, I chose to determine at which pHs, and under how much tension, strips prepared from the rapidly elongating portion of the hypocotyl are loosened and at which they are not. I also chose to determine how acid-extension was affected by buffer concentration and by prolonged pre-extension storage at -12°C. Attempts were also made to determine how acid-extensibility changed with tissue maturation and how acid-extensibility of epidermal strips compared to that of the hypocotyl as a whole.

(b) To estimate the role of enzymes in the characterized acid-extensibility of epidermal strips. I chose to determine the impact of extraction with concentrated LiCl or NaCl on the acid extensibility of strips and correlate this with estimates of protein and enzyme activity extraction. Other approaches to this objective included estimation of the temperature coefficient of acid-extensibility and re-examination of the impact of alcohol-boiling on *in vitro* acid-extension.
MATERIALS AND METHODS

Chemicals

Sodium carbonate, sodium bicarbonate, sodium EDTA, sodium azide, and sodium citrate were purchased from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey. $p$-nitrophenol, $p$-nitrophenyl-$\beta$-$D$-glucopyranoside, $p$-nitrophenyl-$\beta$-$D$-galactopyranoside, $p$-nitrophenyl-$\beta$-$D$-xylopyranoside, $p$-nitrophenyl-$\alpha$-$D$-galactopyranoside, and citric acid were acquired from Sigma Chemicals, St. Louis, Missouri. Lithium chloride and disodium hydrogen orthophosphate were from BDH Chemicals of Toronto. The sodium chloride was purchased from American Scientific and Chemical of Seattle, Washington. Commercial bleach (5.25% sodium hypochlorite when packed) was purchased locally from Sunbrite Bleach.

Plant Material

Dwarf bean (Phaseolus vulgaris L. var. Top Crop Green Pod) seeds were obtained locally (Buckerfields, Vancouver, B.C., Lot numbers 077–01–210 and 01–023–286). In one experiment, peas (Pisum sativum L. var. Alaska) were used and these were obtained from Alberta Nurseries and Seeds, Bowden, Alberta (Lot number 1228). Both beans and peas, were first surface sterilized in 10% (v/v) commercial bleach (0.5% sodium hypochlorite) for 10 minutes, rinsed 3 times in sterilized deionized and distilled (purified) water and finally planted using flamed or bleach sterilized forceps. They were germinated and grown in closed storage dishes (Corning no. 3250, Pyrex brand), twelve seeds per dish, containing approximately 150 ml of vermiculite (W. R. Grace and Company, Ajax, Ontario), and moistened with approximately 100 ml of purified water. The storage dishes containing vermiculite and water were autoclaved and allowed to cool before seeding. The beans were grown in complete darkness at room temperature (21–25°C) for 6 days at which time
they were harvested. Only those beans whose hypocotyls measured 6 to 8 cm from the top of the hook to the first secondary root were selected for experimentation, approximately 35% of the beans being rejected as too tall or too short. Also rejected were beans from any dishes containing signs of fungal contamination as well as any individuals with discoloured or collapsed hypocotyls symptomatic of calcium deficiency (Helms, 1971). A short section including the last 2–3 mm of the apical hook and the first centimetre below the hook were excised from the hypocotyls of the selected plants. In one experiment, a hypocotyl segment 4–5 cm below the hook was used. In the preparation of epidermal strips, the newly harvested segments were sliced in half longitudinally. They were then lightly squashed while lying pith side up. This had the effect of breaking the pith and vascular tissue free of the epidermis and its immediately adjacent cell layers. The epidermal strips, still lying epidermis side down, were gently scraped twice to remove any loosely adhering tissues, trimmed when necessary to 4.5±0.5 mm wide, and washed briefly in purified water. In one experiment some of the strips were used immediately following preparation, otherwise they were frozen at -12.0°C in a small quantity of purified water. For some other experiments, hypocotyl segments, some of which had been very gently stroked twice in each quarter with emory paper (3M wet or dry Tri-M-ite A wt 600), were longitudinally split and then rinsed and frozen at -12.0°C. The epidermal strips and halved hypocotyl segments were stored frozen until required for periods up to one week except in one experiment when the strips were deliberately aged for periods up to 15 days. Before use tissues were thawed to room temperature.

Peas were sown in the same manner as beans but, in an attempt to duplicate the growing conditions described by Yamagata et al (1974), were grown under continuous fluorescent (cool white) light at 27–28°C for ten days. As reported by Yamagata et al, the upper 1.5 cm of the fifth internode was excised from those plants in which the internode measured between 2–3 cm in length. Epidermal strips were prepared and stored as they were when prepared from beans.
In some cases, frozen-thawed specimens were treated following freezing and thawing and before acid-extension testing. The ethanol-boiled epidermal strips were prepared by placing them in a large volume of boiling ethanol for 15 minutes. They were then rehydrated in purified water at room temperature for 15 minutes. Extraction with NaCl involved placing approximately 60 epidermal strips into 200 ml of rapidly stirring 3M NaCl at 4.0°C. After 4 hours, the strips were rinsed twice for 15 minutes in 200 ml of purified water also at 4.0°C. The strips were then refrozen until required. The LiCl-extraction procedure differed from the NaCl-extraction only in the salt concentration (6M LiCl) and in the length of the extraction period (8 hours).

Microscopy

Light microscope sections of cross-sections from the first millimetre of tissue below the hypocotyl hook were prepared from three bean seedlings grown for six days in the dark. Cross-sections were also made of three epidermal strips prepared as for acid-extension experiments. The plant material was fixed with 2% glutaraldehyde buffered to pH 7.0 (0.05M citric acid-0.10M disodium phosphate), dehydrated in an ethanol series to 95% ethanol, and finally embedded in JB-4 plastic (J.B. EM Services Inc., Pointe Claire, Quebec). Sections were cut 1 μm thick with a glass knife and stained with toluidine blue.

Measurement of Acid-Extension

The in vitro acid-extension of tissues was produced and measured with an apparatus similar to ones described in the literature (Rayle and Cleland, 1972; Yamagata et al, 1974). With this system (figure 1), specimens were clamped between a lower immovable clamp and an upper movable clamp which were initially held 7 mm apart by a set screw. Once secured, the specimen was immersed in a buffer (0.05M citric
Figure 1. Horizontal view of apparatus for measuring \textit{in vitro} acid-extension of isolated tissues. Specimen (A); immovable clamp (B); movable clamp (C); clamp set screw (D); resevoir (E); weight (F); linear displacement transducer (G); transducer slug (H); voltage attenuator (I); chart recorder (J); transducer positioning screw (K); micrometre scale (L); stopcock (M). Operation of apparatus is outlined in materials and methods. The voltage attenuator and chart recorder are not drawn to scale.
acid–0.10M disodium phosphate pH 7.5 unless specified otherwise), contained within a
resevoir which could be moved up the post on which the lower clamp was mounted.
Tension was provided by the weight (16.0 grams in most experiments) attached to the
opposite lever arm from the movable clamp and was applied to the tissue by backing off
the set screw. Extension was detected by a linear displacement transducer DC–DC (Trans.
Tek Inc., Ellington, Connecticut, Series 240 3–30 volt excitation) through the movement of
the transducer slug which was suspended from the lever a distance from the fulcrum
equal to that from the fulcrum to the clamp on the opposite side. The DC signal from
the transducer was amplified by a homemade voltage attenuator with adjustable gain and
recorded on a chart recorder (Honeywell model no. Y15303846–(03)–02–0–000–(003)–23).
Once tension was applied, the recorder trace was always initially off scale due to the
high sensitivity of the amplified signal to extension movement. The trace was positioned
towards the right or maximum mV side of the chart paper by adjusting the position of
the transducer relative to the slug through manipulating the transducer positioning screw.
This process was delicate and required as much as 30 seconds to complete which meant
that early extension could not be recorded. Prior to experimentation, the amplification was
adjusted to produce a 10 cm displacement of the recorder trace for every 0.1 mm
movement of the slug in the transducer. This was done by turning the transducer position
screw enough to produce small changes in the distance of the transducer as measured at
the micrometer scale. To separate viscoelastic extension from the wall loosening effects of
the test pH, the specimens were first placed under tension while in a neutral or near
neutral buffer for 60 minutes in every experiment. At the end of that hour, the buffer in
the reser voir was replaced with that of the test pH by draining through the stopcock and
refilling from above by pipette. The reser voir was always rinsed once at the new pH. In
my experiments, three separate acid–extension devices were monitored simultaneously, the
voltage attenuator and chart recorder having the capacity to amplify and record three
separate signals.
The acid-extension by individual tissues was quantified by measuring the total leftward displacement of the trace over a given time interval. The time increments were 2 minutes for the first 20 minutes both in the initial near neutral pH and again once the pH was changed to the test pH. Subsequently, the extension was measured for 5 minute increments. There was one exception to this format; since it was not possible to measure very early extension (described earlier), the first time increment was only 1 minute long, lasting from 1 minute after application of tension to 2 minutes. In longer term experiments, extension was measured over even longer time intervals than 5 minutes once the tissues had been extending in the test pHs for an hour. The measured displacement of the trace for each time interval was converted to a % of the initial length (7 mm) of the tissue; a 10 cm displacement at the chart recorder equaled a 0.1 mm tissue extension or 1.4% of the initial length. The data was expressed in two ways: 1) as cumulative extension, i.e. the extension from each time period being added to those before to give a running total of extension against time 2) as extension rate, i.e. the total extension for each time period being divided by the length in minutes of that time period and then multiplied by 60 to give % of initial length per hour as extension rate for that period.

The acid-extension of similarly prepared and treated tissues predictably did not produce identical traces. To estimate typical performance, the cumulative extension and extension rates for each time period for several tissues (usually N=6) were averaged and the 95% confidence interval calculated. For example, six frozen-thawed bean epidermal strips, having been first extended one hour in a buffer medium at pH 7.5, and then immersed in a buffer at pH 4.8, were found to have extended a total of 0.89±0.36% of their initial length during 59 minutes in pH 7.5 and 6 minutes in pH 4.8. Two minutes later, this total was 1.03±0.40% initial length and during that two minute period the average extension rate was 4.08±1.50% initial length per hour.
In one experiment, the temperature was either raised to 37.0±0.5°C or lowered to 2.0±0.5°C by placing the extension apparatus excluding the voltage attenuator and the chart recorder into an appropriately controlled environment chamber (Controlled Environments, Winnipeg, Manitoba, Model number T18L). Six tissues were acid-extended by a 16.0 gram load for one hour at each of pHs 2.6, 3.6, 4.8, 6.0, and 7.5 after having been extended in pH 7.6 for one hour. Average total extension and extension rates were determined as described above. Temperature coefficients were determined from the formula \[ \log Q_{10} = \frac{10}{(T_2 - T_1)} \log \left( \frac{E_2}{E_1} \right) \], where \( T_2 \) is the higher temperature, \( T_1 \) is the lower temperature, \( E_2 \) is the total extension or the extension rate at the higher temperature, and \( E_1 \) is the total extension or the extension rate at the lower temperature. Maximum extension rates, extension rates over the last 5 minutes of acid-extension (55–60 min) and total acid-extension over one hour were compared between 2° and 23° C and between 23° and 37° C. Upper and lower estimates for \( Q_{10} \) were calculated using the 95% confidence limits estimated for the total extension and extension rates determined at each temperature and at each pH.

Assay Method for Glycosidase Activity

The glycosidase activity of bean epidermal strips was estimated following simple freeze–thawing, or with additional treatment by boiling in ethanol or salt extraction in NaCl or LiCl as described earlier. In addition, strips which had been extended one hour in the pH 7.5 buffer and then one hour pH 4.8 buffer were assayed. The activities extracted into salt solutions were also determined.

Approximately 60 epidermal strips were extracted in each 200 ml salt solution. Once the strips had been removed, each solution was filtered through Whatman glass microfibre filters to remove tissue particles. Each was then dialyzed three times against 2 litres of purified water for 1, 15, and 1 hours, at 4° C. The dialysis tubing (Fischer
Scientific, Pittsburg, Pennsylvania, 08-667-E) was prepared according to McPhie (1971). It was simmered for 1 hour in 2 litres of 50% ethanol and then immersed sequentially for periods of 1 hour each into one volume of 50% ethanol, two changes of 10mM sodium bicarbonate, 1mM EDTA solution, and two changes of distilled water. The tubing was then stored in 3mM sodium azide and rinsed thoroughly with distilled water before use. Following dialysis, the solutions still in their dialysis tubing were concentrated to less than 20 ml by placing them on a bed of Aquacide (Calbiochem) for 72 hours at 4.0°C.

Enzyme activities were estimated with p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-α-D-galactopyranoside, and p-nitrophenyl-β-D-xylopyranoside. In the assay, single epidermal strips were first patted dry and weighed; each invariably weighed between 0.01 and 0.02 grams. They were then incubated in a rapidly stirring reaction mixture at 23.0°C consisting of 1.2 ml 50mM sodium citrate buffer (pH 4.8), 0.6 ml of a given 25mM nitrophenyl glycoside, and 0.28 ml of distilled deionized water. After 10 minutes, or 2 minutes in the case of β-glucoside, the reaction was stopped with 3.90 ml of 200mM sodium carbonate. The glycoside activity extracted by the NaCl and LiCl solutions was determined with 0.02 ml aliquots of the filtered, dialyzed, and concentrated solutions assayed in the same fashion as for epidermal strips. Distilled water was the control. The amount of nitrophenol released was calculated from the increase in absorbance at 400 nm with reference to a nitrophenol standard curve. The calculated moles of nitrophenol released was divided by the weight of assayed tissues and the number of assay minutes or, in the case of the extraction solutions computed in terms of aliquot and concentrate volumes as well as the total weight of tissues extracted and the number of assay minutes, so that all results were reported in terms of μmoles of nitrophenol released per gram of tissue per minute. Total protein extracted by NaCl and LiCl was determined by the method of Lowry et al (1951).
RESULTS

The Anatomy of Epidermal Strips

Dark grown *P. vulgaris* seedlings were harvested six days after sowing on moist vermiculite. The cross-sectional anatomy of the hypocotyl immediately below the hook (figure 2a), includes a wide central parenchymous pith, surrounded by a ring of vascular bundles, a zone of cortical parenchyma and finally a single layer of epidermal cells. Cross-sections of typically prepared epidermal strips from the first centimetre below the hypocotyl hook (figure 2b) show the epidermal cell and also a band of cortical parenchyma seven to ten cells wide. The accordionated walls of many broken parenchymatous cells suggests that scraping the strips during preparation caused cell rupture. No vascular tissue appeared attached to any of the strips.

The "Non-wall Loosening" pH

Preliminary constant-stress tests of frozen-thawed epidermal strips indicated that these tissues continued to extend at a significant rate even after immersion in an incubation medium at pH 6.0 for one hour. Figure 3 shows the extension response of epidermal strips prepared from the first centimetre below the hypocotyl hook of six day old dark grown beans to incubation in four different pHs (4.8, 6.0, 7.0, 7.5) while under 16.0 grams of tension. The results demonstrated that the tissues in pH 6.0 medium extend significantly more than those in a medium at pH 7.5 over the course of one hour. By the end of one hour the rate of extension by tissues in both pH 7.0 and 7.5 was slower than those in pH 6.0 (table I). At the end of the sixty minutes, the medium surrounding each tissue was replaced by one at pH 4.8 resulting in a dramatic increase in extension by those tissues previously incubating in pH 6.0, 7.0, or 7.5. Those previously in pH 4.8 were not affected by the change. The rates of extension increased after a lag of about
Figure 2. Cross-sectional anatomy of bean hypocotyl and prepared epidermal strip.
2a. Cross-sectional view of the hypocotyl immediately below the hook of 6 day old bean seedling.
2b. Cross-section of typically prepared bean epidermal strip.
Legend: pith (Pi); vascular tissue (V); outer parenchymous layer (Pa); epidermis (E).
Figure 3. Effect of pH on initial extension of frozen-thawed bean epidermal strips. Plots represent the average performance of six tissues under 16.0 grams of tension in a medium at pH 7.5, 7.0, 6.0, or 4.8. After 60 minutes, the medium surrounding the tissues was replaced with one at pH 4.8. Error bars represent 95% confidence limits.
Table I. Rate of extension of epidermal strips at neutral or near neutral pH. Extension of the tissues reported in figure 1 are reported here as rates (increase in length as percent of initial length per hour) for selected time periods. Each rate represents the average performance of 6 tissues each extending for 60 minutes in media of the pHs indicated below. After 60 minutes, all media were replaced with solutions of pH 4.8.

<table>
<thead>
<tr>
<th>pH (0–60 min)</th>
<th>TIME PERIOD (MIN)</th>
<th>55–60</th>
<th>62–64</th>
<th>140–160</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td></td>
<td>1.12±0.21</td>
<td>1.07±0.32</td>
<td>0.73±0.25</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td>0.51±0.19</td>
<td>5.61±1.32</td>
<td>0.64±0.34</td>
</tr>
<tr>
<td>7.0</td>
<td></td>
<td>0.19±0.05</td>
<td>11.25±4.40</td>
<td>0.80±0.32</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td>0.18±0.05</td>
<td>6.57±4.30</td>
<td>0.88±0.30</td>
</tr>
</tbody>
</table>
one minute to reach a maximum after only about two minutes (table I: 62–64 minutes). From this point extension rates declined steadily until by the completion of the experiment, after two hours and forty minutes, both total extension (figure 3) and extension rates were not significantly different between treatments. Subsequent experimental constant-stress tests of the plastic response of bean epidermal strips to acidity were preceeded by one hour of tension in pH 7.5. This permitted a maximum separation of limited viscoelastic (non-pH dependant) extension, a property of all cell walls under tension (Cleland, 1971a), from continued (pH-dependent) extension resultant from wall loosening events (Cleland, 1981).

Yield Stress of Acid-Extension

(a) bean epidermal strips

One curious aspect of the above described experimental results was the speed with which extension rates of tissues incubated at pH 4.8 slowed once they had reached a maximum extension rate, regardless of previous treatment (figure 3). Other researchers (Rayle and Cleland, 1972; Cleland and Rayle, 1978) reported a much more gradual decline in the response of frozen-thawed Avena coleoptiles to acid. Yamagata et al (1974) reported that pea epidermis responded to acidity by extending at a constant rate for two hours in vitro. A possible explanation for my contrary results is that insufficient tension was applied to the tissues to exceed the wall yield stress and the response that I measured was some irrelevant transient phenomenon, perhaps the net result of widespread pH-dependant conformational changes within the wall. To test this possibility, six epidermal strips were constant-stress tested at each of five different tensions (40.0, 25.0, 16.0, 7.6, 1.6 grams). The extension of tissues under tension was first monitored for one hour while they were immersed in pH 7.5 buffered medium. The medium was replaced with one of pH 4.8 and tissue extension was monitored for four more hours. My hypothesis was that,
if the originally applied tension of 16.0 grams was less than the yield stress then, at some increased tension, epidermal strips in acid would extend at rates much more linear with time. The amount of tension applied to tissues for one hour at pH 7.5 had almost no impact on their performance over this period. The only significant difference was between those tissues under 25.0 grams of tension, which extended an average of 0.71±0.13% of their initial length, and those under 1.6 grams of tension which extended 0.37±0.13% of their initial length (figure 4). Changing the medium bathing the epidermal strips to a pH 4.8 solution resulted in acid-extension at all weights except 1.6 grams which did not respond to acidification. In general, those tissues that were under greater tension extended much more than those under less tension. Five of the six tissues that were subjected to 40.0 grams of tension ruptured in the course of acid extension; the first broke more than an hour after the pH was changed. The tissues under 40.0 grams of tension were also the most variable in their response. Superficial examination of figure 4 suggests that increasing the tension resulted in a more constant rate of acid-induced extension. Human perception of slope change however, is poor (Silk, 1984). Slope change, or change of extension rate, is obvious when data is presented as extension rate vs. time. From figure 5 it is clear that acidification of the tissues does not yield a constant rate. Changing the medium from pH 7.5 to 4.8 was followed by a dramatic increase in the rate of extension by all but those tissues under the least stress, 1.6 grams, which were not significantly affected. A loosely proportional relationship was apparent: the greater the degree of tension, the greater the initial rate increase following the pH change. The large and rapid increase in the extension rates following medium change was immediately followed by a slow decrease in those rates. This deceleration of rates was nearly as pronounced as the preceding acceleration. Essentially within one hour, the decrease in extension rates by tissues under 25.0 grams of tension amounted to at least 90% of the initial increase in the rate of extension following the change in pH (figure 5). The average rate of extension among tissues under 16.0 and 7.6 grams of tension continued to
Figure 4. Effect of applied tension on the total extension of frozen-thawed bean epidermal strips. Each plot represents the average performance of six tissues tensioned with 40.0, 25.0, 16.0, 7.6, or 1.6 grams. With 40.0 g of tension, all but one of the tissues broke during the experiment. The medium bathing the tissues was pH 7.5 for the first 60 minutes; then it was changed to one of pH 4.8. Error bars represent 95% confidence limits for time more than 60 minutes. In all cases for time less than 60 minutes, error was smaller than symbol size.
Figure 5. Effect of applied tension on the rate of extension of frozen-thawed bean epidermal strips. Data presented is the same as that of figure 4 but expressed in rates (% of initial length per hour).
decline significantly for two hours, though this decline was at least 75% complete one hour after the change in pH (figure 5). With these treatments at least 95% of the extension rate increase following the pH change was reversed in the following decline in extension rates, although after four hours in acid, the extension rates were still significantly greater than they had been immediately before the pH was changed from 7.5 to 4.8. The extension rate of tissues under 40.0 grams of tension also slowed markedly after an initial burst of acceleration; the extent and duration of this deceleration is unclear however, due to both the large variability of measured responses among these tissues and to a tendency of these tissues to rupture. The extension rate of those tissues under the least tension, 1.6 grams, showed no significant decline through four hours of immersion in the pH 4.8 medium. It was concluded from this experiment that the pronounced deceleration of the acid-induced extension rate of tissues under 16.0 grams of tension was not simply an artifact resulting from insufficient tension because it occurred with 25.0 grams and, to a lesser extent, with 40.0 grams of tension. However, application of only 1.6 grams of tension was not sufficient to exceed the yield stress of bean epidermal strips that I prepared. At this tension, there was no significant response to acidity.

(b) pea epidermal strips

As mentioned previously, Yamagata et al (1974) reported that frozen-thawed pea epidermis under tension extended at a constant rate when acidified with a pH 4.5 buffer. Because bean epidermal strips that I prepared performed so differently, I also constant-stress tested the response of pea epidermis to an acid medium. I prepared strips of epidermis from the fifth internode of ten day old light grown Pisum sativum L., cv. Alaska (the same variety as used by the Japanese researchers) in a similar manner as strips were prepared from P. vulgaris hypocotyls. From three to six tissue pieces were subjected to a tension of 13.6, 9.8, 4.0 or 1.6 grams in pH 7.5 for one hour and then in pH 4.8 for an additional 100 minutes. Figure 6 shows the results of this experiment.
Figure 6. Effect of applied tension on the total extension of frozen-thawed epidermal strips of *Pisum sativum* L. c.v. Alaska. Strips were extended with a 13.6, 9.8, 4.0, or 1.6 gram load for one hour in a buffer medium at pH 7.5 (0.05M citric acid–0.10M sodium phosphate) and then for an additional 100 minutes in pH 4.8. The plot labelled 13.6 grams represents the average performance of three tissues. Six tissues were stressed with 9.8 grams and four each at 4.0 and 1.6 grams. Error bars represent 95% confidence intervals.
As with bean epidermal strips, pea epidermis under tension responded rapidly to acid by acceleration of the rate of extension. This increase in the extension rate was followed immediately by a slower but very significant decrease. All three of the tissues under the greatest tension, 13.6 grams, ruptured during treatment with pH 4.8 medium. These results appear analogous to those with beans (figure 5) and clearly conflict with those reported by Yamagata et al. Explanations for this discrepancy will be discussed later.

Stability of the Acid-extension Response

The constant-stress test experiments described above were conducted using freshly prepared epidermal strips prepared from bean hypocotyls that were stored frozen at $-12^\circ$C for up to one week before use. I became concerned that the unusually transient nature of acid-induced extension in these tissues was an artifact resulting from the length of time spent frozen. To test this possibility, I constant-stress tested three epidermal strips after each of ten periods of storage at $-12^\circ$C ranging from thirty minutes up to fifteen days. I also extended epidermal strips immediately following preparation without freezing and thawing them. The extension of strips under tension was first monitored for one hour while the strips were immersed in pH 7.5; the buffer was then exchanged for one of pH 4.8. As seen previously, there was little extension in pH 7.5 by any of the epidermal strips. Once the pH was changed, all tissues responded to acidification with a pronounced increase in the rate of extension. This acceleration was inevitably followed by deceleration. There did not appear to be any clear difference between the acid extension of frozen-thawed and in unfrozen strips, nor did there appear to be any impact of prolonged frozen storage at $-12^\circ$C. Figure 7 shows the total extension by the variously frozen and unfrozen tissues during immersion in pH 4.8 for one hour. The slightly higher average extension by unfrozen tissues compared to those frozen-thawed appears to be the result of a slightly higher peak in average extension rate immediately following acidification; this
Figure 7. Effect of frozen-aging on total extension of bean epidermal strips. Following preparation, strips were frozen at 12°C for indicated periods before they were thawed and acid-extended in pH 4.8 buffer (0.05M citric acid-0.10M sodium phosphate) under 16.0 g of tension. Each plot represents average performance of three strips except N=6 for unfrozen strips. Error bars represent 95% confidence limits.
apparent difference however was insignificant.

Effect of Buffer Concentration

It has been suggested (Kutschera and Schopfer, 1985) that pretreatment of tissue segments in strong, neutral buffers promotes the subsequent extension response to weakly acidic buffers by these tissues. My next experiment tested the possibility that the rapid deterioration of the acid response was an artifact arising from the buffer concentrations employed (0.05M citric acid–0.10M disodium phosphate). My hypothesis was that if the strength of the buffer resulted in the transient response, then decreasing the strength of the buffer should result in a more sustained period of acid extension. Extension by tissues under 16.0 grams of tension was monitored for one hour while they were bathed in a solution of pH 7.5 and for an additional 100 minutes at pH 4.8. The solutions employed for the first six tissues were the same as those of the buffer system employed previously, i.e. 0.05M citric acid–0.10M disodium phosphate. Six more tissues were bathed in pH 7.5 and pH 4.8 buffers of one tenth of this concentration, i.e. 0.005M citric acid–0.01 disodium phosphate. Another six tissues were extended in unbuffered distilled water which had been carefully calibrated to pHs 7.5 and 4.8 using 0.1M HCl and 0.1M NaOH. These solutions were repeatedly changed with freshly calibrated water to prevent the pH of the medium from drifting substantially. A last set of six tissues were bathed only in water adjusted to pH 4.8. The results of this experiment appear in figure 8. Reducing the strength of the buffer to one-tenth of its previous strength did not appear to alter the response. Unbuffered water at pH 7.5 resulted in substantial extension. Subsequent acidification with water at pH 4.8 did not result in the usual pronounced increase in the rate of extension. Tissues in unbuffered water at pH 4.8 from time zero extended in a very similar way to those immersed in 0.05M citric acid–0.10M disodium phosphate at pH 4.8 (figure 3). Regardless of treatment, total extension was not significantly different by the
Figure 8. Effect of buffer strength on acid-extension of frozen-thawed bean epidermal strips. Strips were acid-extended in full strength buffer (0.05M citric acid-0.10M disodium phosphate), one-tenth strength buffer, or unbuffered water at pH 7.5 for one hour and then 100 minutes at pH 4.8 except for one set of tissues which were immersed in unbuffered pH 4.8 from time zero. All were tensioned with a 16.0 g load. Each plot represents average performance of 6 tissues. Error bars represent 95% confidence limits.
completion of the experiment. These results suggested that the concentration of the buffer
did not influence the transient nature of acid-extension, as seen by the similarity between
the extension of strips treated with full and one-tenth concentration buffer solutions and
the total extension by the end of the experiment by all tissues regardless of treatment.
The considerable extension by strips immersed in unbuffered water adjusted to pH 7.5
probably stems from the self-buffering nature of the cell walls of the epidermal strips
and will be discussed later.

Optimal pH for Acid-extension

In the experiments described to this point I had arbitrarily used pH 4.8 as an
acid-extending medium. I next attempted to determine the acid-extension response of
epidermal strips over a range of acidic pHs. Epidermal strip extension was monitored with
the tissues in pH 7.5 under 16.0 grams of tension for sixty minutes; the pH was then
changed to one of the following: 2.6, 3.0, 3.6, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.6, 7.0, 7.5, with
three tissues for each pH; the extension was then monitored for a further sixty minutes.
The entire experiment was then repeated with tissues prepared from a new harvest of
etiolated bean seedlings. The data was pooled with the earlier results so that six tissues
were extended at each pH. Figure 9 shows the response of the tested strips in the
various acidic buffers following the initial hour in pH 7.5. The total extension of strips
over one hour appeared to be roughly proportional to acidity of the buffer: the more
acidic the buffer, the greater the extension. Although this relationship appeared to plateau
at pH 4.4, there did not appear to be a clear indication of an optimal pH for extension.

After the sixty minutes of acid extension, the buffer bathing those strips shown in
figure 9 was changed again to pH 7.5. The extension rate of all tissues slowed within
twenty minutes. However, those tissues that were extended in the most acidic solutions
prior to the reapplication of pH 7.5 initially responded to the near neutral conditions with
Figure 9. Effect of increasing buffer acidity on the total extension of frozen-thawed bean epidermal strips. Strips were extended by 16.0 grams of tension for one hour at pH 7.5, then the buffer was changed to one of pH 2.6, 3.0, 3.6, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.6, 7.0, or 7.5. Total extension over the subsequent 60 minutes is shown above. Each plot shows average performance of six tissues. Error bars represent 95% confidence limits.
a burst of accelerated extension which rapidly faded as seen in figure 10. It was also found that if the pH of the medium around the strips under tension was shifted repeatedly between pH 2.6 and pH 7.5, each solution change from acidic to basic or vice versa was followed by a pronounced but short lived acceleration in extension (data not illustrated). Shifting the pH of the medium around the strips incubated in pH 4.8 to pH 7.5 was followed by a tiny but significant increase in extension rate that faded within four minutes (figure 10). Shifting the pH back to 4.8 following thirty minutes in pH 7.5 resulted in an increase of extension rate close to that immediately preceding the pH shift applied thirty minutes earlier (data not illustrated).

Acid-extension of Longitudinally Halved Hypocotyl Segments

Working with peas, Tanimoto and Masuda (1971) found that auxin treatment of epicotyl segments seemed to change the plasticity of the epidermis but not underlying tissues. Others working with sunflowers (Mentze et al, 1977), cucumbers (Pearce and Penny, 1983; Kazami and Katsumi, 1976), and pea seedlings (Yamamoto et al, 1974; Brummel and Hall, 1980) found that peeling the epidermis from organ segments removed the growth response of auxin. To this point in my experiments I assumed that organ elongation in *P. vulgaris* was determined by the plasticity of the cell walls of the epidermal and near epidermal cells. Application of 16.0 grams of tension to frozen-thawed hypocotyl segments from which the epidermal strip was removed resulted invariably in near instantaneous rupture of the tissue. Although the epidermal strip forms only a small part of the cross-sectional area of the hypocotyl, it is vastly more resistant to longitudinal stress than the remainder of the organ. I investigated further the relationship between acid-extension in epidermal strips to that of the hypocotyl as a whole by constant-stress testing frozen-thawed hypocotyl segments that had been cut in half longitudinally but were otherwise intact. I expected that if acid-extension by the hypocotyls as a whole was largely a product of acid-extension of the epidermal region, then the extension response
Figure 10. Effect of reimmersion in pH 7.5 on the total extension of frozen-thawed bean epidermal strips following one hour in a range of acid buffers. Strips were extended by 16.0 grams of tension for one hour at pH 7.5 and then for another hour at indicated pHs. Finally, the pH was returned to 7.5. Total extension over the subsequent 20 minutes is shown above. Each trace represents the average performance of six tissues. Error bars represent 95% confidence limits.
of these halved hypocotyls would be very nearly as great as the extension of the epidermal strip from a hypocotyl half of similar dimensions tensioned with the same weight in the same buffer.

Preliminary experimentation with frozen-thawed halved hypocotyls indicated that they were much slower to respond to acidity than epidermal strips, maximum extension rates not occurring for fifteen to thirty minutes following the change of pH from 7.5 to pH 2.6, 3.6 or 4.8. Maximum extension rates were relatively slow but were sustained for a longer period so that total extension after sixty minutes was not significantly different from the extension of acidified epidermal strips. There was considerable variability between the performance of individual halved hypocotyls. I suspected that the relatively slow response was a result of slow infiltration of H\(^+\) through the thickness of tissue to the epidermal area. Since infiltration by H\(^+\) should reach those epidermal and near epidermal cells near the cut surface faster than others, the response of halved hypocotyl segments to acid would be expected to be slower to begin, not as great when maximal, and sustained longer. Halved hypocotyls proved difficult to constant-stress test due to the tendency for the organ segment ends to become crushed and distorted by clamping in the constant stress apparatus. This may have increased the variability between replicates because crushing opened channels allowing access of buffer to subepidermal regions. To maximize understanding of the extension of these organs, I increased to thirty the number extended in pH 4.8. Extension of these frozen-thawed half hypocotyl segments was then compared to the extension by the thirty-three frozen-thawed epidermal strips creep tested in the examination of the effect of freezing for periods up to two weeks. The results of this comparison are shown in figure 11. Also included is the extension response of frozen-thawed halved hypocotyl segments which had been lightly abraded with fine emery paper. The cuticle of higher plants is highly impermeable to protons (Dreyer et al, 1981). Lightly abrading the waxy cuticle should allow passage of protons and provide shorter path length from the acid buffer to the cell walls of epidermal cells. The results
Figure 11. Rate of extension of frozen-thawed epidermal strips, halved hypocotyl segments, and abraded halved hypocotyl segments. Strips and segments were tensioned by 16.0 grams in pH 7.5 for one hour and then in pH 4.8 for two hours. The extension response of the tissues over the two hours at pH 4.8 is shown above and expressed as extension rates. Error bars indicate 95% confidence limits. N = 33 for epidermal strips, 20 for halved hypocotyls, and 12 for abraded halved hypocotyls.
illustrated the much more delayed, and then sustained establishment of, maximal extension rate of halved hypocotyls. Total extension by these tissue samples was $2.11 \pm 0.31\%$ their initial length over two hours at pH 4.8; not significantly different from the total extension of epidermal strips, $2.40 \pm 0.17\%$. Abrasion of the hypocotyl cuticle resulted in an earlier and more pronounced increase in the extension rate of the hypocotyl organ halves following the medium change to pH 4.8. This result suggested that the infiltration rate of buffer to the epidermal region of a halved hypocotyl did indeed slow the onset and maximal amplitude of the acid-extension rate in hypocotyl halves compared to epidermal strips. The rate of acid-extension of abraded hypocotyl halves remained relatively high following the maximum. Total extension by these samples was therefore substantially more than either epidermal strips or unabraded hypocotyl halves, reaching $4.47 \pm 0.72\%$ of initial length at two hours. Why a very light abrasion of the cuticle made the frozen-thawed hypocotyl so much more acid-extendible appears mysterious and will be discussed later.

**Acid-extension of Mature Hypocotyl Strips**

In all experiments described to this point using the *P. vulgaris* hypocotyl, acid-extension has been characterized for frozen-thawed tissue from the first centimetre of hypocotyl below the hook of six day old dark grown seedlings. This was because *in vivo* this portion of the plant extends rapidly at this time (see Introduction). It was of interest to compare the acid-extension of tissue from the growing portion of the hypocotyl to that of tissue from the more distal non-growing, or mature portion of the hypocotyl. Figure 12 shows the extension of epidermal strips prepared from just below the hypocotyl hook and from four to five centimetres below the hook. As usual the tissues were allowed to extend for sixty minutes under 16.0 grams of tension in pH 7.5. The buffer was then replaced with one of pH 4.8. There was no difference between extension of the two types of epidermal strips while at pH 7.5. Acid treatment of mature strips did not significantly increase their length over 100 minutes although the average extension of these tissues
Figure 12. Effect of tissue maturation on total extension of frozen-thawed bean epidermal strips. Extension is shown of tissues prepared from immediately below the hypocotyl hook and of tissues prepared from 4.5 cm below the hook. 16.0 grams of tension was applied to tissues at pH 7.5 for one hour and then for 100 minutes at pH 4.8. Plots represent average response of six strips in each case. Error bars represent 95% confidence limits.
increased. Apical tissues extended significantly as in previous experiments.

**Temperature Coefficient of Acid-extension**

The major objective of the work presented in this thesis was to determine if cell wall loosening as evidenced by acid-extension in bean epidermal strips, is mediated by enzyme catalysis. One simple test of this possibility is to determine a $Q_{10}$ or temperature coefficient value for acid extension. $Q_{10}$ is defined as the factor by which the velocity of a reaction is raised with a $10^\circ$C increase in temperature. Enzymatic reactions typically have $Q_{10}$s between one and two. Accordingly, epidermal strips were creep tested at $2^\circ$C and at $37^\circ$C. Extension was monitored as always for one hour in pH 7.5 and then for one hour in pH 2.6, 3.6, 4.8, 6.0 or 7.5. Six epidermal strips were tested at each pH for each temperature. Since acid-extension as monitored at room temperature occurred across a range of acid pHs from pH 6.6 to at least as low as pH 2.6, it is possible that only a portion of this response range is the result of enzymatic (hydrolytic) action. Observation of different temperature coefficients at different pHs would suggest that the wall loosening that produces extension is the result of different mechanisms at different acidities. The results from constant-stress tests at $2^\circ$C and $37^\circ$C were compared to the relevant room temperature ($23^\circ$C) results presented in figure 9. $Q_{10}$s were calculated three ways. First, maximal extension rates achieved in the hour following the hour in pH 7.5 were compared between the same pH treatments at the three different temperatures. Second, the extension rates over the last five minutes of the experiment were compared. Last, the total extension over the test hour was compared.

Lowering the experimental temperature generally reduced acid-extension rates. The calculated temperature coefficients for acid-extension between $2^\circ$C and $23^\circ$C appear in table II. Due to variability between strip replicants, both at room temperature and $2^\circ$C, it was not possible to determine precise $Q_{10}$ values. Note that in some instances extension
Table II. $Q_{10}$ values for different acid-extension (pH) treatments. $Q_{10}$s were calculated for maximum extension rates achieved, extension rate from 55–60 minutes following immersion in test pH, and total extension over 60 minutes of acid-extension of the frozen-thawed epidermal strips. Values are presented as estimates of upper and lower limits.

<table>
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<tr>
<th>2–23°C</th>
<th>Medium pH</th>
<th>Maximum extension rate</th>
<th>Extension rate at 55–60 minutes</th>
<th>Total extension 0–60 minutes</th>
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</thead>
<tbody>
<tr>
<td>2.6</td>
<td>1.15–2.24</td>
<td>1.15–1.98</td>
<td>1.37–2.16</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>1.14–2.61</td>
<td>1.39–1.91</td>
<td>1.36–1.74</td>
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<tr>
<td>4.8</td>
<td>1.40–2.09</td>
<td>1.31–2.27</td>
<td>1.26–1.83</td>
<td></td>
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<tr>
<td>6.0</td>
<td>1.10–2.78</td>
<td>0.93–1.91</td>
<td>1.21–2.37</td>
<td></td>
</tr>
<tr>
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<td>0.45–</td>
<td>0.84–4.16</td>
<td>1.03–2.47</td>
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</table>

<table>
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<tr>
<th>23–37°C</th>
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<th>0.85–1.76</th>
<th>0.63–1.85</th>
<th>0.63–1.31</th>
</tr>
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<td>3.6</td>
<td>1.10–2.19</td>
<td>0.90–1.79</td>
<td>1.15–1.60</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>0.67–1.33</td>
<td>0.88–2.60</td>
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</tr>
<tr>
<td>6.0</td>
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<td>0.95–2.79</td>
<td>0.81–2.14</td>
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<tr>
<td>7.5</td>
<td>0.06–4.89</td>
<td>0.34–2.74</td>
<td>0.69–2.08</td>
<td></td>
</tr>
</tbody>
</table>
rates were not significantly different between 2°C and 23°C. In these cases the lower limit estimate of the \( Q_{10} \) value will be less than one. No value appears for the upper limit estimate of the \( Q_{10} \) for maximal extension rate of strips extended in pH 7.5 because the maximum rate of strips at 2°C was not significantly different from zero extension per hour. Increasing the temperature to 37°C did increase average rates and total extension above those at 23°C but the major effect was an increase in the variability between replicates so that most lower limit estimates of \( Q_{10} \) were less than one (table II).

The results of the estimation of temperature coefficients suggested that enzymes mediate acid-extension in epidermal strips as I have prepared them because few values greatly exceeded two. This conclusion required that some important assumptions be made which will be discussed later. It is interesting that acid-extension at different pHs appeared to have similar \( Q_{10} \)s. This suggested that the extension mechanism is similar at different pHs.

**Impact of Enzyme Inactivation or Extraction on Acid-extension**

A more direct test of the role of enzymes in cell wall loosening is to observe the impact of attempts to denature or remove proteins from the cell walls on their acid extension. I chose two methods to attempt this experiment. In the first, epidermal strips prepared and frozen-thawed as usual were boiled in ethanol for fifteen minutes. At one atmosphere, ethanol boils at 78.5°C. Although examples of extremely heat stable enzymes are known (e.g. adenylate kinase (E.C.2.7.4.3) which will withstand prolonged heating at 100°C), most plant and animal enzymes are inactivated very rapidly at temperatures well below 100°C and in a majority of cases below 70°C (Dixon and Webb, 1979). After boiling in ethanol, epidermal strips were rehydrated in distilled water for ten minutes. In the second experimental approach, I attempted to extract enzymes from epidermal strips using concentrated salt solutions. A number of authors have used either concentrated NaCl
(Crasnier et al, 1980; Koyama et al, 1981), or concentrated LiCl (Huber and Nevins, 1980; Labrador et al, 1984), to extract hydrolytic activities from cell wall preparations. Two salt extraction methods were attempted. In the first, frozen–thawed strips were rapidly stirred in 3M NaCl for four hours at 4°C. They were subsequently stirred in distilled water twice for fifteen minutes. In a second more severe method, tissues were extracted with 6M LiCl for eight hours at 4°C; they were then washed twice in distilled water for a total of thirty minutes. The boiled or salt extracted tissues were stored frozen for up to five days.

To test the acid extensibility of the ethanol-boiled, NaCl-extracted and LiCl-extracted tissues, all strips were extended first in pH 7.5 for one hour under 16.0 grams of tension. For a second hour, the pH was shifted to pH 2.6, 3.6, 4.8, 6.0 or 7.5 for ethanol-boiled and NaCl-extracted strips, and to pH 2.6, 4.8 or 7.5 for LiCl-extracted strips. Three strips were tested at each acidity from each of the three treatments. Later, from a second harvest of bean seedlings, more strips were ethanol-boiled or extracted in NaCl or LiCl. These tissues were also constant-stress tested as just described and the data pooled with the earlier results.

The total extension of the variously boiled and extracted epidermal strips during the hour in the range of pHs is shown in figure 13 together with the response of similarly tested control tissues which were neither boiled nor extracted; there were no significant differences in extension between the tissues while at pH 7.5. Total acid-extension by ethanol-boiled strips after one hour in pH 2.6 was not significantly different from unboiled strips. The initial surge in the extension rate by ethanol-boiled tissues in response to pH 2.6 medium was much less than by untreated tissues however, reaching a maximum extension rate of only 5.68±1.51% of initial length per hour compared to 21.26±6.87%. Because the extension rate of the boiled strips declined very slowly after the post pH change increase, by twelve minutes after being immersed in pH
Figure 13. Effect of ethanol-boiling or salt extraction on the total extension of frozen-thawed bean epidermal strips. Strips were either A) untreated following preparation and frozen-thawing B) boiled in ethanol for 15 minutes C) incubated in 3M NaCl for 4 hours at 4°C or D) incubated in 6M LiCl for 8 hours at 4°C. Treated tissues were subsequently rehydrated or desalted in distilled water. Each plot represents the average extension of six strips under 16.0 grams of tension at the indicated pH following one hour at pH 7.5. Error bars represent 95% confidence limits. No errors are shown for treatments in pH 7.5 as all were smaller than symbol size.
2.6 these tissues were extending faster than were the untreated strips at that time (figure 14A). The difference between extension rates after twenty-five minutes was not significant. Total acid-extension by strips in less acidic media over one hour was more profoundly affected by boiling in ethanol (figure 13). Again maximum extension rates were much lower than similarly acidified untreated strips but subsequent extension rates were also lower throughout the hour (figure 14). Strips extended at pH 7.5 appeared unaffected by ethanol-boiling.

Total acid-extension by strips previously extracted with 3M NaCl for four hours was not significantly different after one hour from that of unextracted strips except in the case of strips at pH 3.6, which only extended 1.52±0.20% of their initial length compared to 2.34±0.25% (figure 13C). Maximum extension rates achieved with these tissues were lower than those of untreated tissues but only significantly different at pH 3.6 by the end of an hour (figure 14). Total acid-extension by LiCl-extracted tissues was significantly less than untreated tissues at pH 4.8 but not at pHs 2.6 and 7.5 (figure 13). Maximum extension rates at pHs 2.6 and 4.8 were also low though extension rates were not significantly less than the control through most of the experiment (figure 14).

In general the impact of both ethanol-boiling and salt extraction on acid-extension was suppression of the initial burst of extension in response to acidification. Extension rates following the initial surge were relatively unaffected by the treatments except in the case of strips boiled in ethanol and tested in more weakly acidic conditions, and possibly NaCl-extracted strips extended at pH 3.6 and LiCl-extracted tissues extended at pH 4.8.

Impact of Enzyme Inactivation or Extraction on Glycosidase Activities

Having assessed the effect of ethanol-boiling and salt extraction on acid-extension, I chose to determine the impact of these treatments on acid polysaccharidases within these tissues, presumably within the cell walls. Accordingly, preweighed epidermal strips were
Figure 14. Effect of ethanol-boiling or salt extraction on the extension rate of frozen-thawed bean epidermal strips. Data presented is the same as that of figure 13 but expressed in rates (% initial length per hour). Strips were either untreated, ethanol-boiled, NaCl-extracted, or LiCl-extracted and then acid-extended in pH A) 2.6, B) 3.6, C) 4.8, or D) 6.0.
assayed for glycosidase activities (β-glucosidase, β-galactosidase, β-xylosidase and α-galactosidase) by placing individual strips into rapidly stirring reaction mixtures containing 25mM nitrophenol glycoside buffered to pH 4.8 with 0.033M citrate-sodium citrate. After ten minutes, or two in the case of β-glucoside, the reaction was stopped with sodium carbonate and the amount of nitrophenol released was determined colorimetrically. Six strips were individually assayed for activity for each of the four hydrolytic activities. Epidermal strips that had been boiled in ethanol or extracted with NaCl or LiCl were assayed as well. The β-glucosidase activity remaining in epidermal strips that had been acid-extended one hour in pH 7.5 (0.05M citric acid–0.10M disodium phosphate) and one hour in pH 4.8 (same concentrations) under 16.0 grams of tension was also determined. To insure that only tissues fully exposed to the buffered medium were assayed, the clamped tissue ends were trimmed from the strips after acid-extension. I also determined the levels of glycosidases extracted into the NaCl and LiCl solutions from preweighed samples of epidermal strips. The salt solutions were first dialysed against distilled water and concentrated using Aquacide. Aliquots were assayed for both glycosidase activity and protein concentration.

All results were computed in terms of the weight of tissue assayed and are presented in figure 15. Most of the acid glycosidase activity in the untreated strips was determined to be β-glucosidase and in descending order levels of β-galactosidase and β-xylosidase, and a trace of α-galactosidase were found. Pierrot et al (1982) found the most common glycosidase activity in cell wall preparations from P. vulgaris hypocotyls was β-glucosidase but the next greatest was β-xylosidase followed by β-galactosidase. Boiling in ethanol eliminated all four glycosidase activities. Extraction with NaCl removed or inactivated seventy to eighty percent of each of the activities. Most of the β-glucosidase and β-galactosidase activities were subsequently detected in the dialysed and concentrated salt extraction solution. The solubilized activities were probably overestimated relative to the same activities bound to the strips because it was assumed that infiltration of the
Figure 15. Effect of various treatments on the glycosidase activity of frozen-thawed bean epidermal strips. Tissues were either untreated, boiled in ethanol for 15 minutes, extracted 4 hours in 3M NaCl at 4°C, extracted 8 hours in 6M LiCl at 4°C, or acid-extended for 1 hour in pH 7.5 and 1 hour in pH 4.8. Activities were determined from the release of nitrophenol when preweighed tissues were incubated in one of β-glucoside, β-galactoside, β-xyloside, α-galactoside, buffered to pH 4.8. Extracted activities were also determined in aliquots of the salt solution following desalting and concentrating. Error bars present indicate 95% confidence limits, others too small to indicate. N=6 for each bar.
nitrophenol glycoside substrate into strips was not rate limiting. The acid-extension of halved hypocotyl sections suggests this may not have been true. LiCl treated strips were inactive against all four substrates; in this case most of the β-xylosidase and none of the β-glucosidase or β-galactosidase activity were traced to the extraction solution. Interestingly, more protein was extracted with NaCl (9.10±0.08 mg/gram of tissue) than with LiCl (6.27±0.15 mg/gram of tissue), though Li⁺ is the more electropositive cation. The acid-extension of bean epidermal strips solubilized approximately 50% of β-glucosidase activity.

Some conclusions were apparent from the results of the glycosidase assays. Ethanol-boiling denatured, inactivated or removed at least some of the acidic polysaccharidase activities found in epidermal strips. Though less effective in the case of NaCl, salt extraction had a similar action. The detection of substantial portions of the glycosidase activities in the salt solutions suggested that the proteinaceous hydrolases were electrostatically bound to the epidermal strips, presumably in the cell walls, and that these interactions were disrupted by high salt concentrations and by buffer to a lesser extent. That different glycosidase activities were variously solubilized and stable in NaCl and LiCl suggested that more than a single hydrolytic enzyme with different affinities for the different substrates was detected.

Assay results and the results of acid-extension experiments with ethanol-boiled and salt-extracted epidermal strips did not suggest that acid-extension in bean epidermal strips is highly dependent on the assayed glycosidase activities or any other enzyme activity inactivated or extracted with them. The only feature of acid-extension that was highly correlated with the presence of ethanol-unstable, salt extractable enzymes is a high initial burst of extension. As will be discussed later, this accelerated acid-extension may not be related to steady state growth in living bean hypocotyls.
DISCUSSION

Anatomy

Peeling away the epidermis of elongating organs of young dicots removes the underlying tissue as well (Mentze et al., 1977). An exception appears to be *Pisum* in which the epidermal cell layer of the epicotyl can be removed alone (Masuda and Yamamota, 1972). My method of epidermal strip preparation also isolated epidermis with underlying tissue. That the epidermis always separated cleanly from vascular tissue may be due to a concentric line of weakness at the outer edge of the stelar tissue.

The "Non-wall Loosening" pH

Epidermal strips continued to extend at a detectable rate at the end of one hour regardless of the pH in which they were incubated. In pH 6.0, this rate was still between 0.7 and 0.3% of initial length per hour after 55–60 minutes (Table I). The rate was slower however in the more basic pHs, less than 0.25% per hour at pH 7.5. These results suggest that acid wall loosening occurs even when the external medium is at least as basic as pH 6.0.

Experiments with frozen–thawed peeled coleoptile segments of the monocot *Avena* suggested that an incubation medium of pH 6.0 is basic enough to prevent significant wall loosening (Cleland and Rayle, 1978). Under 20.0 grams of tension, these tissues extended no more rapidly (less than 2% per hour) immersed in pH 6.0 than they did in pH 6.8 (Rayle, 1973). The viscoelastic extension rate of methanol-boiled, pronase-digested *Avena* coleoptiles after one hour of creep test was still about 1.0% per hour (Cleland, 1971b), though this result was achieved with the extreme tension provided by a 36.0 gram load. Direct measurement of the free space pH with a microelectrode 2 μm in diameter inserted 50 μm into young tissue segments detected a pH of between 5.7 and 6.2 for
corn and 5.8 and 6.2 for peas over the course of one hour in the absence of exogenous IAA (Jacobs and Ray, 1976). Interestingly, the monocot corn tissues extended only about 0.75% of their initial length in the course of an hour without IAA while the dicot pea segments grew twice as much. It is possible that wall loosening continues at higher pHs in dicots in general than in monocots.

**Yield Stress of Acid-extension**

(a) *bean epidermal strips*

Rayle and Cleland (1972), working with *Avena* coleoptiles, and Yamagata et al (1974), working with *Pisum*, found that the acid-extension of frozen-thawed segments required that these tissues be under tension in excess of some minimum stress: a 10.0 gram load in the case of oats, and 4.8 grams in the case of pea epidermis. My results (figures 4 and 5) suggest that a similar minimum tension requirement exists for bean epidermal strips. This yield stress must be in excess of 1.6 grams but less than 7.6 grams as there was no extension response to acidification with a 1.6 gram load but there was with 7.6 grams.

Cleland et al (1983) analyzed mathematically the acid-extension of frozen-thawed *Avena* coleoptiles. At a 20.0 gram load, an exponential phase predominated over the first hour but resulted in only a 5% increase in length. Subsequently, extension became linear with time and continued for 10 hours. This analysis was recently extended to acid-extension of young frozen-thawed bean leaves (Van Volkenburgh et al, 1985) and cucumber hypocotyls (Cleland et al, 1987). With the abraded hypocotyl of the dicot cucumber under a 20.0 gram load, exponential extension was less pronounced than with *Avena* while the linear extension phase persisted at a high rate (apparently greater than 5% per hour) for up to 48 hours. In bean leaves, the linear extension rate exceeded 3% per hour.
The ability of applied acid to cause this linear wall extension with time is major supporting evidence that the acid-growth hypothesis as it mimics steady state growth (Taiz, 1984). In my experiment however, the acid-extension response of bean epidermal strips was highly transitory at any tension load tested in excess of 1.6 grams. Only those tissues under the most extreme load (40.0 grams) were still extending after an hour in pH 4.8 at a rate greater than half the 5–8% per hour growth rate sustained by the first centimetre below the hook from which these strips were prepared. These same highly stressed strips also had a great tendency to break suggesting that 40.0 grams exceeded the longitudinal stress born by these tissues in vivo. Direct determination of the longitudinal stress born by these cell walls in vivo is not possible at this time. For a cylindrical cell, longitudinal stress ($SI$) is determined by $SI = P \cdot r / (2d)$, where $P$ is pressure, $r$ is cell radius, and $d$ is radius of the load bearing portion of the wall (Cosgrove, 1986). Accurate determination of $d$ causes the difficulty in calculating $SI$. Disruption of cell wall synthesis in the unicellular algae Nitella indicated only orientation of cellulose microfibrils of the inner 25% of the wall diameter determined directionality of cell extension, suggesting that only a small portion of wall thickness is load bearing (Richmond et al, 1980). The situation in higher plants is unknown (Taiz, 1984). The uniaxial stress applied to isolated epidermal strips cannot be assumed to reproduce the multiaxial stress applied to the epidermal region of the extending hypocotyl by the turgidity of growing cells (Taiz, 1984). Unless uniaxial stress is significantly more disruptive than multiaxial stress however, growing cells of the hypocotyl under multiaxial stress equivalent to that in epidermal strips under 40.0 grams of uniaxial stress, would need to lay down the entire stress bearing region of their wall every few hours, to avoid bursting. I conclude therefore that 40.0 grams of tension represents more stress than the epidermal region is subjected to in vivo. Possible reasons as to why epidermal strips do not sustain an appreciable rate of linear extension in acid solution unlike other dicotyledonous tissues will be presented later.
(b) pea epidermal strips

I also found that acid-extension of pea epidermal strips was largely exponential figure 6), although the average extension rate of 6 tissues under a 9.8 gram load was still 2.98 ± 1.10 % per hour at 100 minutes following pH change. These tissues showed no tendency to break in that time.

My results obviously contradict those published by Yamagata et al (1974) in which they reported that frozen-thawed pea epidermis extended at a constant rate in response to immersion in a pH 4.5 buffer. My experiment was not conducted under exactly the same conditions as they reported. They separated the epidermis from the epicotyl by peeling as opposed to my scraping the internal cell layers from the epidermis of the longitudinally halved epicotyl segments and they acidified their tensioned tissues with a buffer of pH 4.5 (0.01M citric acid–0.02M potassium phosphate) while I used pH 4.8 (0.05M citric acid–0.10 disodium phosphate). Although these and perhaps other differences may account for the differences between our results, I suspect the origin of much of the discrepancy lies in the presentation of their results. Yamagata and coworkers reported only a single trace showing the total extension over time of what is either a typical example or an idealized impression of the performance of a number of tissues. It may be that these workers extrapolated their results from the initial response of a number of tissues, failing to notice that the response which may have appeared linear when recorded as total extension over time, was actually a curve. Yamagata et al recorded their results using tissues of similar dimensions as mine but tensioned by a 13.7 gram load. Since the three tissues I extended with a 13.6 gram load had all broken within 80 minutes of immersion in acid buffer, it seems likely that the line extending at a constant slope for two hours in their paper is only an extrapolation.
Stability of the Acid-extension Response

The long term acid-extension experiments of Cleland et al (1983; 1987) and Van Volkenburgh et al (1985) suggested that if acid-extension is mediated by enzymes then these activities remain stable for long periods of time in vitro. Cline (1979) pretreated Avena coleoptile segments with cyclohexamide then tested their acid growth response and showed that any existing wall loosening enzyme must have a half-life of at least 7–8 hours. A possible explanation for the transient acid-extension response that I observed in both bean and pea epidermal strips is that it arose from instability of an enzyme activity responsible for wall loosening. However, the results of testing the acid-extension response of unfrozen as well as various frozen-aged tissues (figure 7) does not support that possibility. There appeared to be no change in extension performance after the freeze-aging treatment. Should an acid activated enzyme be responsible for wall loosening in bean epidermal strips, it must either be extremely stable or so unstable that its activity is negligible after one hour in the pH 7.5 buffer as was the case of freshly harvested tissues. This latter possibility seems doubtful as enzymes secreted to function outside the cell are unlikely to be effective if they have very short half-lives.

Effect of Buffer Concentration

Reducing the buffer concentration by 90% had no impact on the extension behavior of strips in pH 7.5 and subsequently in pH 4.8 (figure 8). However, tissues immersed in unbuffered water at pH 7.5 for one hour extended much more over the course of that hour than those in water buffered at pH 7.5. The extension of these strips was at least as much as those in buffer at pH 6.0 (figure 1), suggesting that the strips are themselves self-buffering at some acidic pH. Indeed, the presence of fixed ionized charge in primary cell walls, largely the carboxyl groups of uronic acids, means that isolated cell walls are electrically more negative than the bulk of any bathing medium with which they are
equilibrated (Demarty et al, 1984). The walls thus behave as a Donnan system. As a consequence, the concentrations of mobile cations, including H⁺, are larger in the wall than in the medium and vice versa for mobile anions. Sentenac and Grignon (1981) found that when cell walls isolated from the roots of lupin seedlings were equilibrated with 1 mM KCl medium at pH 5.0 and then transferred to 1mM CaCl₂, the medium pH fell. Titrations of the medium showed that at pH 5.0 about 50% of fixed anionic charges in the wall were protonated. Since the pK of purified galacturonic acid and pectin from these walls was determined to be approximately 3.2, the local pH within the walls was lowered more than 1.5 pH units by the Donnan effect. As the results of calcium addition in the above experiment might suggest, the lowering of the cell wall pH relative to the surrounding medium is dependant on both the density of fixed anionic charges within the wall and also on the degree to which these charges are masked or neutralized by non-proton cations. This makes prediction of actual cell wall pH complex (Sentenac and Grignon, 1981).

In my experiment, the average total extension over 60 minutes by the tissues in unbuffered water at pH 4.8 was much greater than by those in unbuffered water at pH 7.5, however this difference was not quite significant at the 95% confidence level. This suggests that, although it is unclear what the cell wall pH is in either case, equilibration with water at pH 7.5 may raise the wall pH relative to what it is when in equilibrium with water at pH 4.8. Buffering the bathing solution at pH 7.5 with either 0.005M citric acid-0.01M disodium phosphate or at 10 times those concentrations pulls still more protons from the cell walls.

Kutschera and Schopfer (1985) measured the short term acid-growth response of corn coleoptile segments that had been pretreated with either water or phosphate buffer (0.01M) at pH 6.8 for one hour. They found that preincubation in the neutral buffer inexplicably increased the subsequent response to acidity relative to pretreatment with water.
My results appear to offer an explanation for their findings. Regardless of treatment, I found that total extension after 160 minutes was very similar and that all extension rates slowed to less than 1.5% of initial length per hour. The effect of water treatment was to move much of the total extension to the first 60 minutes. It may be that Kutschera and Schopfer, by preincubating their corn tissues in water were effectively acid-growing them before the experiment began because the cell walls of their tissues would be more acidic incubated in water than in buffer. Their measure of the acid-growth response of the water incubated tissues may have only included the tail end of this transient response.

Optimal pH for Acid-extension

Rayle (1973) reported that the acid-growth response of peeled *Avena* coleoptiles and the acid-extension of frozen-thawed tissues with 20.0 gram loads was maximal with external buffers of pH 5.0–4.7 although buffers as low as pH 3.0 produced responses of equal magnitude. Tepfer and Cleland (1979) since reported that initial rates of acid-extension in *Avena* tissues under a 10.0 gram load do not plateau but instead increase with decreasing pH to pH 3.0. Among dicots, Yamagata et al (1974) found that the maximum acid-extension response for frozen-thawed pea epidermal strips was at pH 3.5 though lower pHs were not tested. With frozen-thawed epidermal strips from *Helianthus*, no optimal pH was reached even as low as pH 1.0 (Soll and Böttger, 1982). Rayle and Cleland (1980) found increasing acid-extension by frozen-thawed soybean hypocotyls right down to pH 3.0. In my experiment, total extension rose steadily as increasingly acidic pHs were tested below pH 6.6 (figure 9). This confirms my earlier discussed conclusion that cell wall loosening occurs in these tissues when the medium pH is as basic as pH 6.0. The response to acidity plateaued at about pH 4.4 as even lower pHs did not result in significantly different total extension (figure 9).
Whatever the mechanism(s) responsible for acid wall loosening in bean epidermal strips, it functions over a wide range of pHs. Yamagata et al (1974) argued that at least two separate mechanisms must operate in acid-extension of pea epidermis. They found that while frozen-thawed epidermis extended appreciably at pH 4.5 and lower, epidermis boiled in methanol did not extend at pH 4.5 but did at pH 3.0, though less than the frozen-thawed tissues did at that pH. This, they felt, indicated that acid wall loosening at pH 4.5 was mediated by an enzyme denaturable by methanol-boiling while at pH 3.0, a non-enzymatic process must have been involved. I have some evidence to support the idea that extension in very low pHs is non-enzymatic. When epidermal strips extending in extreme acid were bathed in a neutral medium, their extension rate first rapidly increased before slowing (figure 10). Switching the pH of the medium repeatedly between pH 2.6 and 7.6 produced a short lived burst of acceleration after each change of medium. This phenomena does not appear to be explainable in terms of an enzymatic mechanism. A possible explanation is that extreme acid buffers produce a large scale conformational shift of wall polymers which in turn rupture load-bearing bonds or reduce steric hindrances enough to create wall loosening. Eventually the sliding of polymers past one another puts stress on other bonds and/or creates new steric interactions. These are broken or relieved when the pH of the medium is returned to neutrality and the wall polymers return to their previous confirmations. No attempt was made to test this hypothesis.

Acid-extension of Longitudinally Halved Hypocotyl Segments

The same 16.0 gram load that epidermal strips sustained for hours, instantaneously ruptured halved hypocotyls from which the epidermis had been peeled, in spite of their comparatively large cross-sectional area. This indicates that most longitudinal stress resistance of bean hypocotyls rests in the outer cell layers. Theoretical considerations favor such a role for the organ surface though no formal analysis exists (Taiz, 1984). Turgor stresses generated in interior cells produce largely compressive stresses of the interior cell
walls, most transversal and longitudinal stress being nullified (Lockhart, 1965). At the organ surface large non-compressive stresses must be born however, so it is not surprising that the overall yielding properties of the epidermal region determine whole organ extension (Green, 1980).

In spite of the importance of the epidermal region, frozen-thawed halved hypocotyls still bearing their epidermis did not extend like isolated epidermis in pH 4.8 buffer (figure 11). When the cuticle was left unabraded, maximum extension rates were not achieved until about 30 minutes after immersion in acid, though total acid-extension over two hours was not significantly different from that of frozen-thawed epidermal strips over the same period. The delayed onset of acid-extension was probably the result of the proton impermeable nature of the cuticle because very light abrasion of this waxy coating accelerated the onset of acid-extension (figure 11).

Abraded half hypocotyls continued to extend at a faster rate in pH 4.8 than did epidermal strips or unabraded hypocotyls so that total acid-extension over two hours was much greater (4.47±0.72% vs. 2.40±0.17% and 2.11±0.31 %) in these tissues. It is possible that this phenomenon indicates that wall loosening enzymes or their cofactors are rapidly leached from epidermal strips in acid buffers so that acid-extension fades rapidly in these tissues. This would not explain however why extension of strips in unbuffered solution was transient (figure 8) nor would it explain the low extension rates of acid-extension in unabraded hypocotyls. It is possible that the pH of walls of epidermal and near epidermal cells in unabraded hypocotyl, unlike epidermal and abraded hypocotyls, never equilibrated with the pH of the surrounding medium. This seems unlikely as it is difficult to imagine that the additional 1.0–1.5 mm pathlength from some epidermal cells to the buffered medium prevented equilibration for two hours and, if the pH had not yet completed equilibration, that the rate of extension should mount steadily throughout the experiment. Another possible explanation is that Ca++ leaching is involved. In epidermal strips, some of
the tissue Ca**, much of it cytoplasmic in origin, must be removed from the strips by scraping and rinsing during preparation. The cell walls of the frozen-thawed halved hypocotyls on the other hand, can be expected to contain elevated levels of Ca** because these ions are apparently sequestered in higher plant vacuoles (Macklon, 1984). The added calcium ions should inhibit acid-extension in these tissues as exogenous application of CaCl_2 inhibited acid-extension of frozen-thawed *Avena* coleoptiles (Cleland and Rayle, 1977). It has been suggested that protons may function non-enzymatically in cell wall loosening by competitive displacement of calcium (Métraux and Taiz, 1977; Tepfer and Cleland, 1979; Soll and Böttger, 1982). Removal of calcium with a strong chelating agent does create some wall loosening (Soll and Böttger, 1982). There is a strong preference by cell walls to bind Ca** over H+ at exchangeable sites (Demarty et al, 1984), however there is some question whether the acidification of frozen-thawed tissues by a pH 4.8 buffer would displace much ionic calcium (Sentenac and Grignon, 1981; Jarvis, 1984). Nevertheless, a histochemical study indicated a redistribution of extracellular calcium ions occurs during geocurvature in oat coleoptiles, more calcium appearing on the upper side (Slocum and Roux, 1983). In gravity stimulated curvature of these organs, growth slows on the upper side and increases on the lower side (Firn and Digby, 1980). In preliminary histochemical experiments, IAA treatment of soybean hypocotyl sections appeared to move much of the calcium adjacent to the epidermal cell plasmalemma to the outer regions of the outer wall of the epidermal cell (R. E. Cleland, personal communication). In bean hypocotyl epidermal strips, any calcium ion displacement by acid buffers should be relatively rapid due to their large surface to volume ratios. In abraded hypocotyls, the rate at which Ca** is displaced from these tissues by buffers and thus the rate that the tissues extend should be faster than the rates from unabraded hypocotyls due to the cuticular barrier. This scenario does not explain why epidermal strips extended less in total than abraded halved hypocotyls. It is possible however, that the strips were partially calcium leached by the pH 7.5 buffer (0.05M citric acid–0.10M disodium phosphate) before
their acid extension was measured. Sodium, unlike other monovalent cations, shows a degree of specific binding to wall carboxylic groups and appears to displace some of the wall calcium (Demarty et al, 1978). This may be due to the similar size of sodium and calcium ions (Jarvis, 1984). Tissues to be acid-extended were routinely immersed in the pH 7.5 buffer for up to 5 minutes in my experiments once they were fastened to the clamps of the extension apparatus (figure 1) during which time significant Ca\(^{2+}\) displacement may have occurred. Because Soll and Böttger (1982) found that the extension response of frozen-thawed epidermal strips from *Helianthus* hypocotyls to addition of EDTA to the medium was very short lived, early wall loosening mediated by sodium displacement of calcium may have gone undetected during the first minute under tension before the chart recorder recorded extension. During the 59 recorded minutes at pH 7.5, extension by epidermal strips was actually less (0.52±0.06% initial length) than either unabraded (0.98±0.18%) or abraded halved hypocotyls (1.28±0.26%). Presumably the hypocotyl halves underwent Na\(^+\)/Ca\(^{2+}\) exchange during the recorded period. No attempt was made to test this possible role of Na\(^+\)/Ca\(^{2+}\) exchange in wall extension.

**Acid-extension of Mature Hypocotyl Strips**

As stem tissues mature, they lose the ability to grow in response to exogenous applications of auxin or acid. For example, with the hypocotyl of 4 day old dark grown mung bean hypocotyls, only the first 1.5–2.0 mm below the hook elongates when incubated in IAA or acid (Goldberg and Prat, 1981). Strong acidification of the medium however, occurs not just with tissues from this region but in the mature hypocotyl as well (Goldberg and Prat, 1981; Goldberg, 1980). Maturation has also been shown to correlate with a loss of the capacity for acid-induced wall loosening (CAWL). The extension response of frozen-thawed leaf strips prepared from leaves of *P. vulgaris* over the course of leaf growth and maturation was found to peak and decline in parallel with the growth rate of the tissues before harvest (Van Volkenburgh et al, 1985). My results comparing
the acid-extension of strips prepared from the first centimetre below the hook to that of strips from 4–5 cm below the hook indicate that CAWL is lost with maturation in the *P. vulgaris* hypocotyl as well.

It is generally assumed that it is the laying down of secondary walls during maturation that ultimately limits primary growth in higher plants (Sellen, 1980). Measures of wall extensibility and chemical analysis suggest that maturation fundamentally changes the nature of the cell wall. In lupin hypocotyls, both creep rate and plastic extensibility (PE) declined in methanol-boiled tissues harvested down the length of the organ (Monro et al, 1972). Analysis of cell walls isolated along the length of the hypocotyl of six day old dark grown *P. vulgaris* seedlings revealed that declining growth rate was correlated with a declining proportion of protein in the wall though its hydroxyproline content and degree of glycosylation increased (Van Holst et al, 1980). The percent of the wall determined to be cellulose and uronic acids did not change while non-cellulosic glucose increased. Absolute levels of wall cellulose and pectins apparently do increase however, because incorporation of radio labelled arabinose and uronic acids into wall polymers of the hypocotyl is active in the hook region and in the post elongating maturing hypocotyl (Sasaki and Taylor, 1984; 1986). At the crudest level, the product of this renewed wall synthesis is different from earlier wall synthesis in the hook as demonstrated by a simple experiment not described in the results. Cell walls were isolated from each centimetre along the *P. vulgaris* hypocotyl by pulverizing and rinsing the sections. The pelleted walls were weighed and then lyophilized and reweighed. A measure of cell wall density was achieved by calculating the dry weight as a percent of the wet pellet weight. This was found to decline in the elongation zone from 4.31±0.12% just below the hook to 2.64±0.11% at 2–3 cm below the hook. Beyond this point, where Sasaki and Taylor found active wall synthesis, a further slight decline in wall density occurs to 2.25±0.16%. In mung beans, Goldberg and Prat (1982) found that the amount of cell wall material per section declined through the elongation zone. They also found that total cellulose
increased during maturation while pectic compounds declined. The viscosity of extracted pectins also increased with maturation. This was apparently due to increasing molecular weight and uronic acid content coupled with increasing Ca$^{++}$ content. In soybeans, the pectic polymers of the mature hypocotyl have fewer neutral sidechains than do the pectins of the elongating region (Yamaoka and Chiba, 1983).

Changes to wall structure described above undoubtedly alter the wall's mechanical properties and possibly CAWL. Other proposals suggest that addition of new polymeric material to the walls does not render them inextensible per se. Roland et al (1982) have proposed that the cell walls of mung bean hypocotyl cells are laid down as ordered structures, appearing like twisted plywood under microscopic examination. Wall synthesis ceases with the commencement of exponential elongation with growth being at the expense of wall thickness and order. The cessation of growth occurs when and because wall order has been completely lost. However, it is difficult to see how the disordering of the wall evident in micrographs could effectively prevent further extension (Taiz, 1984). It has also been proposed that the decreasing wall extensibility of maturing tissues results from increased polymeric cross-linking. Since the hydroxyproline rich protein, extensin becomes increasingly more difficult to chemically extract from cell walls of tissues as they mature, extensin may account for decreasing CAWL (Lamport and Catt, 1981). The molecular basis of insolubility of extensin appears to be the formation of isodityrosine cross-links through the action of peroxidase and H$_2$O$_2$ within the cell wall (Cooper et al, 1987). A second possibility is the oxidative coupling of phenolic groups sometimes carried on pectic molecules to form diferulate cross-links (Fry, 1986).

It has also been suggested that loss of the capacity for acid-induced wall extension seen in mature tissue results from the failure of continued synthesis of the necessary wall loosening enzymes (Van Volkenburgh et al, 1985). Cleland (1983) showed pre-incubation of *Avena* coleoptiles in IAA for several hours before freeze-thawing enhanced subsequent
acid-extension of these tissues. This increased CAWL was thought to have resulted from IAA stimulated production of wall loosening enzymes because CAWL declined slowly in the absence of IAA, and this decline was hastened with cycloheximide but was unaffected by the presence or absence of sugars. Estimations of specific hydrolytic activity have not shown them to decline substantially with tissue age however (Koyama et al, 1981; Murray and Bandurski, 1975; Pierrot et al, 1982).

Temperature Coefficient of Acid-extension

Ray and Ruesink (1962) monitored the impact of temperature change on the steady state elongation rate of *Avena* coleoptile segments incubated in auxin containing medium. They calculated $Q_{10}$ values to be 3.5–3.7 from the changes in the elongation rates between 2° and 20° C, and to be 2.0 between 20° and 30° C. This evidence as well as the rapid response of growth rate to temperature change convinced them that extension of the cell wall was controlled directly by the rate of metabolic reactions rather than mechanical properties of the wall such as viscosity. The results with lupin hypocotyl sections growing in auxin were $Q_{10} = 2.4$ between 4° and 30° C, for endogenous growth (without auxin), $Q_{10} = 2.3$ between 4° and 30° C (Penny et al, 1972). These authors reached a conclusion similar to that of Ray and Ruesink (1962).

The viscoelastic extension of methanol-boiled tissues is undoubtedly not an enzymatically mediated process. Indeed, the creep rate of *Avena* coleoptiles so prepared was remarkably unresponsive to extreme temperature shifts. Between 3° and 23° C, $Q_{10}$ was only 1.05 (Cleland, 1971). Similar values were calculated between 23° and 60° C although tissues at 60° C tended to extend relatively rapidly after 10 minutes. Penny et al (1974) found that the $Q_{10}$ calculated from the creep rate of methanol-boiled lupin hypocotyls was less than 1.1 up to 45° C.
Rayle and Cleland (1972) calculated $Q_{10}$ values for the acid-extension of unpeeled frozen-thawed *Avena* coleoptiles in pH 3.0. These were determined to be 5.0 between 15° and 25°C and 1.2 between 2.5° and 35°C. They also found that growing coleoptile sections in auxin solutions at 15°, 25° and 35°C produced the same values. These values for the temperature coefficient of auxin-induced growth were somewhat different from those of Ray and Ruesink (1962) who determined their values from the short term impact of temperature change upon coleoptile sections growing in an auxin solution at room temperature while impaled upon a thermocouple. Nevertheless, the values of Rayle and Cleland exhibited the same peculiarity of being higher at lower temperatures than at elevated temperatures. It was felt that the similarity of $Q_{10}$ values for acid-extension of frozen-thawed tissues to those of auxin-induced growth of living tissue suggested that wall loosening must be by similar means in each case.

In my experiment, I re-examined the role of temperature on acid-extension. Regardless of how the values were determined, i.e. from maximum extension rates, total extensions, or extension rates after 55 minutes in acid, the $Q_{10}$s fell in the range of 1.1 to 2.8 between 2° and 23°C and 0.6 to 2.8 between 23° and 37°C (Table II). Although these values are imprecise, they do suggest that an enzymatic activity might be involved in the acid-extension response of the frozen-thawed bean epidermal strips because purified enzymes typically have temperature coefficients between 1 and 2 (Dixon and Webb, 1979). Values for purified cell wall hydrolases are non-existent in the literature although a report exists of both a $\beta$-galactosidase activity with a pH optimum of 3.8–4.0 and a $Q_{10}$ of 2.7 between 15° and 25°C and 1.6 between 25° and 35°C, and a $\beta$-glucosidase with a pH optimum of 4.8–5.0 and a $Q_{10}$ of 1.7 between 15° and 25°C and 1.8 between 25° and 35°C (Agrawal and Bahl, 1968). However, as these enzymes were isolated from whole *P. vulgaris* cotyledons, it is not clear that they are of cell wall origin.
The results of my experiment do not show conclusively that enzymes are involved in acid-extension. This was not just because of the imprecise calculation of $Q_{10}$ but also because I assumed that the rate of extension correlated directly with the rate of bond breakage and that a purely physical process would be either insensitive to temperature as is the case with viscoelastic extension or, if some uncatalysed reaction was involved, that the $Q_{10}$ would greatly exceed that of an enzymatic reaction.

Impact of Enzyme Inactivation or Extraction on Acid-extension

Yamagata et al (1974) found that dipping pea epidermal strips in boiling methanol for 5 minutes reduced the rate of acid-extension of these tissues by about 95% at pH 4.5. Approximately 30% of the acid-extension response remained at pH 3.0. In my experiment, bean epidermal strips were subjected to a more severe treatment. They were boiled for 15 minutes, instead of 5 minutes, and in ethanol which boils at 78.5°C instead of methanol which boils at 65.5°C. Once boiled in ethanol however, the bean tissues extended in pH 4.8 for one hour extended an average of 22% of the total extension by control tissues at the same pH. The initial extension response to acidity was more suppressed by ethanol-boiling however, as ethanol-boiled tissues did not reach their maximum rate of extension until at least 10 minutes after they were immersed in pH 4.8 (figure 14C). It may have been that similar phenomena occurred when pea epidermis was boiled in methanol and Yamagata and his coworkers determined the rate of acid-extension of these tissues shortly after immersion in pH 4.5. I also found that no significant difference existed between the total acid-extension of ethanol-boiled strips and that of control tissue when they were extended in pH 2.6. Once again the initial response was inhibited by ethanol-boiling. In this case, the treated tissues reached a peak extension rate of about 25% of the control.
Yamagata et al (1974) used the results of acid-extension of ethanol-boiled pea epidermis to argue that two mechanisms might be involved in acid-extension. The first was the action of a methanol denaturable hydrolase active about pH 4.5; the second was a non-enzymatic process, presumably the cleavage of acid labile bonds, responsible for extension in buffers below pH 4.5. Excluding the possibility that an extremely stable enzymatic wall loosener exists in bean epidermal strips, and keeping in mind that alcohol-boiled tissues have had a considerable proportion of their sugar polymers extracted (Olson et al, 1965), my results suggest that at least a part of acid-extension at pH 4.8 is the result of a non-enzymatic mechanism. Since the principal impact of ethanol-boiling is to reduce the initial burst of extension after tissues are immersed in acid both at pH 4.8 and 2.6, early extension at both pHs may be largely enzymatic.

Yamagata et al (1974) also reported that treatment of pea epidermal strips with 0.1% deoxycholate which has been shown to solubilize β-1,3-glucanase from *Avena* cell walls did not suppress acid-extension at pH 4.5. My attempts to solubilize proteins with either NaCl or LiCl only suppressed total acid-extension relative to the untreated control tissues when the strips were immersed in pH 3.6 after they had been extracted with NaCl, and at pH 4.8 after extraction with LiCl. In these two cases, the extension rates of the tissues were not significantly different from that of the control tissues by the end of 6 minutes with the NaCl-extracted tissues, and by the end of 18 minutes with the LiCl-extracted tissues. In fact, salt extraction suppressed the initial acceleration burst after the tissues were immersed in acid regardless of pH (figure 14). In general, the results of salt extraction suggest that if proteins capable of digesting wall polymers are solubilized, these enzymes must only have a brief wall loosening action, the mechanism of long term acid-extension being left intact. No attempt was made to determine to what extent salt extraction solubilizes wall polymers or otherwise disrupts the native gel structure.
That epidermal strips that had been soaked in 3M NaCl for four hours subsequently extended when immersed in a pH 4.8 buffer would seem to argue forcefully against the earlier suggestion that sodium–calcium exchange may be involved in the wall loosening! It appears however, that the competitive ability of monovalent cations for calcium binding sites within isolated cell walls increases as the pH of the external medium and presumably that within the walls is lowered (Jarvis, 1984; Wuytack and Gillet, 1978). Perhaps the calcium ions displaced by 3M NaCl are the same ones that would have been displaced by sodium ions of the pH 7.5 buffer and a further population of load–bearing calcium bridges remains undisturbed until the pH is lowered.

**Impact of Enzyme Inactivation or Extraction Treatments on Glycosidase Activities**

Evidence suggests that the four glycosidase activities assayed (β-glucosidase, β-galactosidase, β-xylosidase, and α-galactosidase) are not involved in the cell wall loosening associated with cell elongation (see introduction). These enzymes however have been demonstrated in the cell walls of *P. vulgaris* hypocotyls (Nevins, 1970; Pierrot et al, 1982) and as such may be considered markers for the solubilization of wall proteins.

The results clearly demonstrated (figure 15) that NaCl-extraction removed or inactivated most of the activities while both ethanol–boiling and LiCl treatment rendered the strips inactive. Comparison of these results to the acid-extension tests of similarly treated tissues suggests that the four glycosidase activities and any enzymes solubilized with them are not responsible for long term extension. They could play a role in the initial extension burst. It is possible however that the initial acid-extension burst is also the result of non−enzymatic processes because the dramatic slowing of the acid–extension rate following the initial burst probably is not the result of acid buffer solubilization of enzymes, 50% of β-glucosidase activity remaining in acid−extended strips.
Cleland and Rayle (1978) suggested that the failure of someone to solubilize or demonstrate involvement of an enzyme in wall loosening was because these enzymes must be extremely tightly bound to the cell wall. There has been one suggestion that a specific activity is covalently linked to cell walls. The cell walls of horseradish roots were shown to contain malate dehydrogenase activity, only 1% of which was liberated when the roots were incubated 2.5 hours at 4°C in 2M NaCl (Gross, 1977). Other reports suggested MDH was readily soluble in the cell walls of both sugar cane leaves (Terry et al, 1983) and corn roots (Nagahashi et al, 1985). My results indicated that if wall loosening enzymes are responsible for long term acid-extension in bean epidermal strips, they must indeed be resistant to ethanol-boiling and also tightly bound to the cell wall. If they are tightly bound however, how do they move within the wall to degrade polymers? It seems much more probable that long term extension is the result of a non-enzymatic mechanism such as the displacement of calcium ions.
CONCLUSIONS AND SPECULATIONS

The characterization of *in vitro* acid-extension of frozen-thawed epidermal strips from bean hypocotyls indicated that these tissues are loosened by exposure to medium pHs of at least as high as 6.0 and at least as low as 2.6. Greatest extension occurred in response to media between pH 4.4 and 2.6. The minimum stress required for extension of strips approximately 4.5 mm wide appeared to lie between 1.6 and 7.6 grams. The acid-extension was largely transitory regardless of tension. The response was similar between newly harvested tissues unfrozen and frozen-thawed and tissues aged up to 15 days at −12°C. Acidifying strips with unbuffered water indicated the cell walls of epidermal strips are self-buffering and are probably much more acidic than the surrounding medium. No capacity for acid-extension was observed in strips harvested from the mature hypocotyl.

The results of the determination of temperature coefficients for acid-extension suggested that an enzymatic mechanism is involved in wall loosening but, due to experimental design and variability, the results cannot be considered conclusive. Treatment of epidermal strips with boiling ethanol or concentrated salts clearly indicated that long term acid-extension in a pH 4.8 buffer was not dependant on an enzyme that is denaturable or extractable in these conditions. The early burst of acceleration may be mediated in part by hydrolytic activity, perhaps there are a limited number of enzymatically cleavable sites. It is also possible that the ethanol and salt treatments may rearrange the wall polymers in some way so that most immediate wall loosening potential is lost regardless of loosening mechanism.

The speculation that acid-extension involves the displacement of load-bearing calcium cross-links by protons and sodium ions derived from a large volume of external solution is not well founded. It has not even been established whether calcium is removed from isolated walls into buffer solutions. One idiosyncrasy of acid-extension of
frozen-thawed tissues, the requirement for a minimum tension, is unexplained with such a mechanism. It is possible however, that calcium ions are somehow more displaceable if the polymers they are cross-linking are being pulled apart.

If the acid-extension mechanism is fully or partially by means of calcium ion displacement, the feasibility of the mechanism is unclear for auxin-induced growth or in growing tissues in vivo. A better understanding of the pH of the walls of living tissue as well as the concentration of other monovalent ions is necessary. In acid-extension, calcium ions are presumably leached from the frozen-thawed tissues once they have been displaced, making them unavailable to form new cross-links. In living systems however, calcium ions are retained within the tissue and might form new cross-links by displacing monovalent ions. Brummell and Hall (1981) suggested that auxin application leads to active calcium ion uptake by the cell. Although calcium ions are actively sequestered into the tonoplast, it appears that they are actively expelled from the cytoplasm into the wall (Macklon, 1984).

Calcium displacement may not account for all wall loosening in living cells. In the living bean hypocotyl, cell length increases approximately five-fold as cells pass through the elongation zone to maturation (Horemans et al, 1986). Though total extension of bean epidermal strips was not determined in my experiments, it is clear that these tissues did not extend a great deal; very much less than doubling their length before their acid-extension rates had slowed to very low rates. It is possible that in this system some important population of calcium bridges which are displaced in living hypocotyl growth is somehow rendered undispplaceable in frozen-thawed bean epidermal strips, though it is difficult to imagine how.

It has been suggested that new wall synthesis might be a requirement of long term wall loosening, new wall polymers released into the wall somehow helping to weaken existing stress-bearing regions (Taiz, 1984). In Avena, galactose inhibited auxin-induced
growth after two hours (Yamamoto and Masuda, 1985). Incorporation of C$^{14}$-labelled glucose into cell walls was shown to be completely inhibited by galactose, while IAA uptake, respiration, water permeability, and the IAA induced decrease in the $T_o$ value are unaffected (Yamamoto and Masuda, 1985), suggesting that continued cell wall synthesis is needed for long term growth. Cleland et al (1987) however have reported that frozen-thawed Avena coleoptile sections will extend for 10 hours and frozen-thawed cucumber hypocotyls for up to 48 hours at a rapid rate when immersed in pH 4.5 under a 20.0 gram load. It would be interesting to determine the full acid-extension potential of frozen-thawed bean hypocotyl tissue, both epidermal strips and halved hypocotyl sections, and how this correlates with the auxin-induced growth potential of the tissues. This may be possible using a recently designed constant stress apparatus (Kutschera and Schopfer, 1986a; 1986b) in which extension from time zero can be determined.
REFERENCES


