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Characterization of long-term extension of isolated cell walls from growing cucumber hypocotyls

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Abstract. Walls from frozen-thawed cucumber (*Cucumis sativus* L.) hypocotyls extend for many hours when placed in tension under acidic conditions. This study examined whether such "creep " is a purely physical process dependent on wall viscoelasticity alone or whether enzymatic activities are needed to maintain wall extension. Chemical denaturants inhibited wall creep, some acting reversibly and others irreversibly. Brief (15 s) boiling in water irreversibly inhibited creep, as did pre-incubation with proteases. Creep exhibited a high Q_{10} (3.8) between 20° and 30° C, with slow inactivation at higher temperatures, whereas the viscous flow of pectin solutions exhibited a much lower Q_{10} (1.35). On the basis of its temperature sensitivity, involvement of pectic gel-sol transitions was judged to be of little importance in creep. Pre-incubation of walls in neutral pH irreversibly inactivated their ability to creep, with a half-time of about 40 min. At 1 mM, Cu^{2+}, Hg^{2+} and Al^{3+} were strongly inhibitory whereas most other cations, including Ca^{2+}, had little effect. Sulfhydryl-reducing agents strongly stimulated creep, apparently by stabilizing wall enzyme(s). The physical effects of these treatments on polymer interactions were examined by Instron and stress-relaxation analyses. Some treatments, such as pH and Cu^{2+}, had significant effects on wall viscoelasticity, but others had little or no apparent effect, thus implicating an enzymatic creep mechanism. The results indicate that creep depends on relatively rugged enzymes that are firmly attached to or entangled in the wall. The sensitivity of creep to SH-reducing agents indicates that thiol reduction of wall enzymes might provide a control mechanism for endogenous cell growth.

Abbreviations: DTT = dithiothreitol; EDTA = ethylenediaminetetraacetic acid ; EGTA = ethyleneglycol-bis-(b-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid

Key words: Cell wall (viscoelastic properties) Cell wall enzymes Cell wall extension – Creep of cell walls – *Cucumis* – Hypocotyl (growth)

Introduction

The growth of plant cells entails irreversible extension of the pre-existing wall. Such extension is a major control point for action by auxin, gibberellin, light and other agents (see reviews by Taiz 1984; Jones 1986; Ray 1987); however, the molecular mechanism of wall extension remains obscure. Isolated walls extend transiently when placed under tension in acidic pH (Rayle and Cleland 1970; Hager et al. 1971; Metraux and Taiz 1977; Tepfer and Cleland 1979) and more recently walls of *Avena*, *Cucumis*, and *Nitella* were shown to extend for up to 2 d under acid conditions (Taiz and Richmond 1984; Cleland et al. 1987). A key feature of such wall extension is that synthetic processes do not contribute to extension. Cleland et al. (1987) have suggested that this long-term creep is likely to be similar to the type of extension that walls undergo during normal growth. Plant walls contain numerous enzymes capable of cross-link breakage and formation (see Cassab and Varner 1988 for review), but their role in wall extension remains conjectural. The present study was aimed at deciding whether long-term extension of isolated walls requires action by endogenous wall enzymes.

Material and methods

Cucumber *(Cucumis sativus* L. cv. Burpee Pickler, from A.W. Burpee, Westminster, Penn., USA) seeds were grown in wet vermiculite at 27–29° C in darkness for 3 d. In some cases hypocotyl growth was inhibited with continuous red light (150 μmol photons $\cdot m^{-2} \cdot s^{-1}$) obtained from a 150-W flood lamp filtered through red plastic (CBS 650; Caroline Biological Supply, Burl-

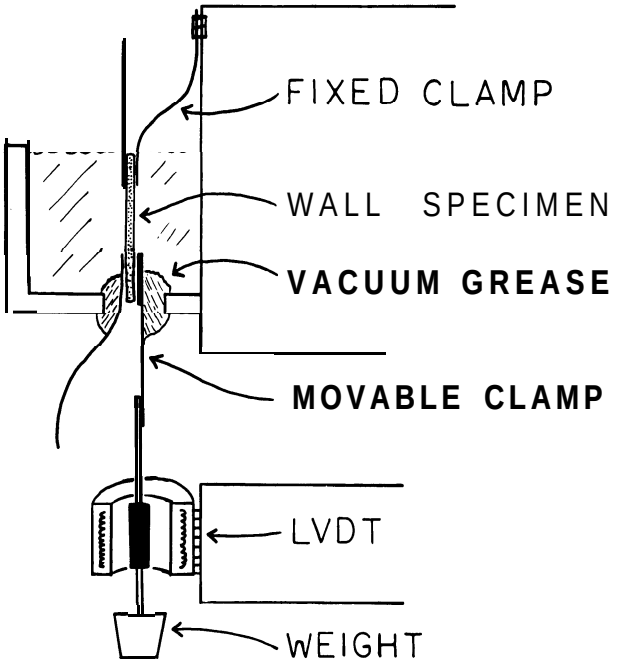

Fig. 1. Apparatus to clamp a wall specimen under constant tension and measure long-term extension (creep). The lower clamp was weighted to provide a net 20-g force on the wall. Wall extension was detected by a displacement transducer (LVDT) as movement of the transducer core (solid cylinder attached to the lower clamp). The wall was enclosed in a plastic cuvette (1 ml volume), which was sealed on the side and bottom with vacuum grease to prevent leakage

ington, N.C., USA) or with the gibberellin-synthesis inhibitor S-3307 (synonym: uniconozol; see Izuma et al. 1985) added to the rooting medium (5 mg of a 10% commercial formulation [XE-1019, gift of Chevron Chemical Co., Richmond, Cal., USA] applied in 100 ml to a 100 cm^2 area of vermiculite). Hypocotyls were excised and stored frozen at -20° C for no more than 5 d. The cuticle was abraded by rubbing the surface with a thick slurry of 300-mesh (45pm) Carborundum, then washed with water. The most apical 12 mm of the abraded hypocotyl was cut, placed between two glass slides, and 10 specimens were pressed beneath a 400-g weight for 5 min to squeeze out excess fluid. Such flattened specimens were used in the creep, Instron, and stress-relaxation measurements.

Creep measurements. Wall specimens were secured with two clamps as shown in Fig. 1, with about 5 mm between clamps and a constant tension of 20-g force. Plastic cuvettes were fitted around the clamped walls and filled with buffer solution. Except where otherwise noted, the first buffer was 50 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid), pH 6.8, for 15 min, followed by 50 mM potassium-acetate buffer, pH 4.5, for 2.25 h. Wall extension was measured with a position transducer (model HR250 with SPM/GPM 108 signal conditioner from Schaevitz, Pennsauken, N.J., USA; calibrated to 8 $V \cdot mm^{-1}$) attached to the lower clamp. Extension data from eight such apparatuses were recorded and analyzed by a microcomputer and custom software. At the end of each experiment, the pH of the incubation buffer was measured to verify pH.

Instron measurements. Wall specimens were extended to a tension of 30-g force in two cycles at 3 mm $\cdot mm^{-1}$ in a custom-made extensiometer, following the usual technique (Cleland 1984). The extensiometer consisted of two clamps (5 mm separation between jaws), the lower one attached to a force transducer (BG-100; Kulite, Ridgefield, N.J., USA), the upper one attached to a sliding stage driven by a micrometer screw connected to a digital stepper motor (K82401-P2; Airpax, Cheshire, Conn., USA). Movement of the stage was controlled by a microcomputer which measured the signals from the force transducer and a displacement transducer attached to the stage. For each of the two extension cycles, the strain data were fitted to the force data with a second-degree polynomial by the least-squares method, and the slope at the end of the cycle was computed from the fitted polynomial. Slopes are expressed as percent extension per 100-g force, and are corrected for the extension that occurs during the measurement.

Stress-relaxation measurements. Wall specimens were clamped in a custom extensiometer similar to that described above, except the upper clamp was directly driven by a linear actuator (Airpax L92121-P2) with a 50-μm step size. The 5-mm wall segment was extended at a rate of 170 $mm \cdot min^{-1}$ until 22-24-g force was reached, then held constant. Force was recorded over a 5-min interval by a microcomputer, with a minimum sampling rate of 2 ms, gradually increasing to 2 s. The first approximation of the relaxation spectrum was computed by taking the derivative of the stress with respect to log(time) (Catsiff and Tobolsky 1954; Yamamoto and Masuda 1971). Control and analysis programs for these and the Instron measurements were written in Asyst (Asyst Software Technology, Rochester, N.Y., USA).

Viscometry. The viscosity of pectin solutions was measured with a custom-made rolling-ball viscometer (Block and Spudich 1987), with time measured by a hand-held stop watch. Citrus pectin (United States Biochemicals, Cleveland, Oh., USA) was dissolved to a concentration of 2% by weight in 50 mM potassium-acetate buffer at pH 4.5. The viscometer was calibrated with sucrose solutions of known viscosity at 24° C.

Enzymes. Pronase was obtained from Calbiochem, La Jolla, Cal., USA. Sigma Chemical (St. Louis, Mo., USA) supplied catalase, trypsin (T-8253, from bovine pancreas), papain (2X crystallized, P-4762) and protease from ***Streptomyces griseus*** (P-5147, similar to Pronase). The pH sensitivity of these proteinases was assayed by the hydrolysis of BAPNA (Na-benzoyl-DL-arginine p-nitroanilide) in 50 mM potassium acetate or phosphate at various pH values, with calorimetric detection at 430 nm.

For trypsin digestion, wall specimens were pre-incubated in 50 mM Hepes buffer (1 ml/segment, pH 7.6) with 1000 units ml^{-1} trypsin, 1 mM $CaCl_2$, for 2 h at 30° C on a shaker revolving at 60 rpm. Papain and Pronase digestions were by the same procedure, except the solution was 50 mM acetate buffer, pH 5.0, containing 1 mM dithiothreitol and 2 mg *ml- ' of papain or Pronase; 4-h incubations were used.

Peroxidase activity was assayed by the formation of colored reaction product from guaiacol (Chance and Maehly 1955). For each assay a 2-cm apical region of a frozen-thawed cucumber hypocotyl was diced into l-mm pieces with a razor blade and dropped into 3 ml of 50 mM K acetate, pH 4.5, containing 20 mM guaiacol and 1.7 mM hydrogen peroxide. Absorbance at 470 nm was measured at 1-min intervals for 4 min, with intermittent shaking.

Results

Time course and pH response of wall creep. When wall specimens were placed in tension in neutral buffer or in distilled water, extension quickly ceased (Fig. 2). Change to acidic buffer induced a rapid extension which deayed over the course of l-2 h to a lower, but still substantial, rate. Under appropriate conditions, this long-term extension continued for more than 24 h and extensions of 40% were often attained before the wall specimens broke.

Figure 3 shows the broad pH optimum (2.5-4.0) for long-term creep. Several buffers were tested, including phosphate, phosphate-citrate, acetate and dimethylglutarate, with little or no difference in pH response. The one exception, formate buffer, evoked higher rates of extension, most likely because of its action as a reducing agent (see below).

For further studies the standard assay consisted of 15 min incubation in neutral buffer (50 mM Hepes, pH 6.8), followed by 2.25 h incubation in acid buffer (50 mM K acetate, pH 4.5), with pretreatments or additions to the acidic buffer as described. To compensate for the mechanical weakening or rigidification caused by some pretreatments, data are expressed as the extension rate in acidic buffer (at 2.5 h) minus the rate in neutral buffer (at 0.25 h), divided by initial length (units are % $\cdot h^{-1}$).

Biological correlates. As shown in Fig. 4A, several treatments known to inhibit hypocotyl elongation were found to inhibit creep. Hypocotyls from red-light-grown seedlings exhibited reduced creep. In dark-grown seedlings, basal (nongrowing) regions of the hypocotyl showed almost no long-term creep. Treatment with the gibberellin-synthesis inhibitor S-3307 inhibited both growth rate and creep rate of the hypocotyls.

The epidermis is thought to restrict stem growth in cucumber and other species (Iwami and Masuda 1974; Kutschera et al. 1987). To test whether creep was likewise restricted by the epidermis, hypocotyls were either peeled to remove the epidermis or bored with a small drill to remove the inner tissue (about half of the cross-sectional area was removed). Removing the inner tissue did not alter creep rate, whereas removal of the epidermis doubled the creep rate (Fig. 4A). Thus, the epidermis appears to restrict creep more than the inner tissue.

Molecular nature of creep. To test whether wall creep is enzymatic, walls were treated in various ways to denature, inactivate, or accelerate the activity of endogenous wall enzymes. Several denaturing treatments were examined, including boiling in water and addition of denaturants to the acidic buffer. As shown in Fig. 4B, such treatments eliminated long-term creep. High concentrations of chaotropic agents (urea, guanidine . HCl, sodium perchlorate) inhibited creep activity irreversibly. In contrast, the inhibitory effect of high NaCl and 50% methanol was reversed when these agents were removed (data not shown).

Proteases inhibited long-term creep, although a transient extension still occurred in the first 30 min. Preincubation at pH 5.0 with papain and Pronase destroyed creep activity, while autoclaved proteases were ineffective (Fig. 4 B). Preincubation

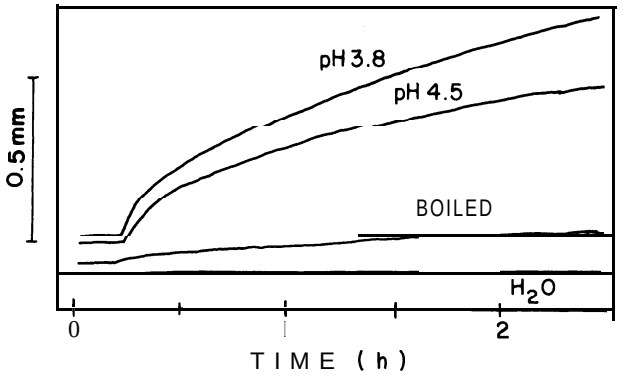

Fig. 2. Time course for wall creep in isolated cucumber-hypocotyl cell walls. In the trace marked "H_2O", the wall was placed in distilled water (equivalent results were obtained with pH 6.8 Hepes buffer). In the other three traces, the walls were placed in pH 6.8 Hepes, then at 15 min switched to acidic buffer. For the trace marked " BOILED", the wall was pretreated with boiling water for 15 s, then hung in pH 6.8 buffer followed by pH 4.5 buffer

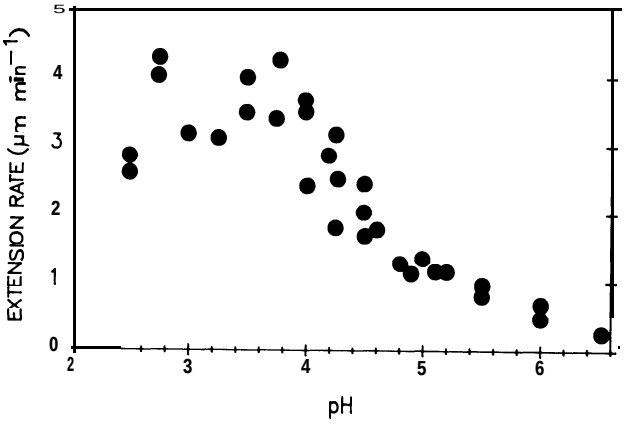

Fig. 3. Creep rate in isolated cucumber-hypocotyl cell walls as a function of pH. The pH range was obtained with 50 mM potassium acetate, phosphate, and phosphate-citrate buffers

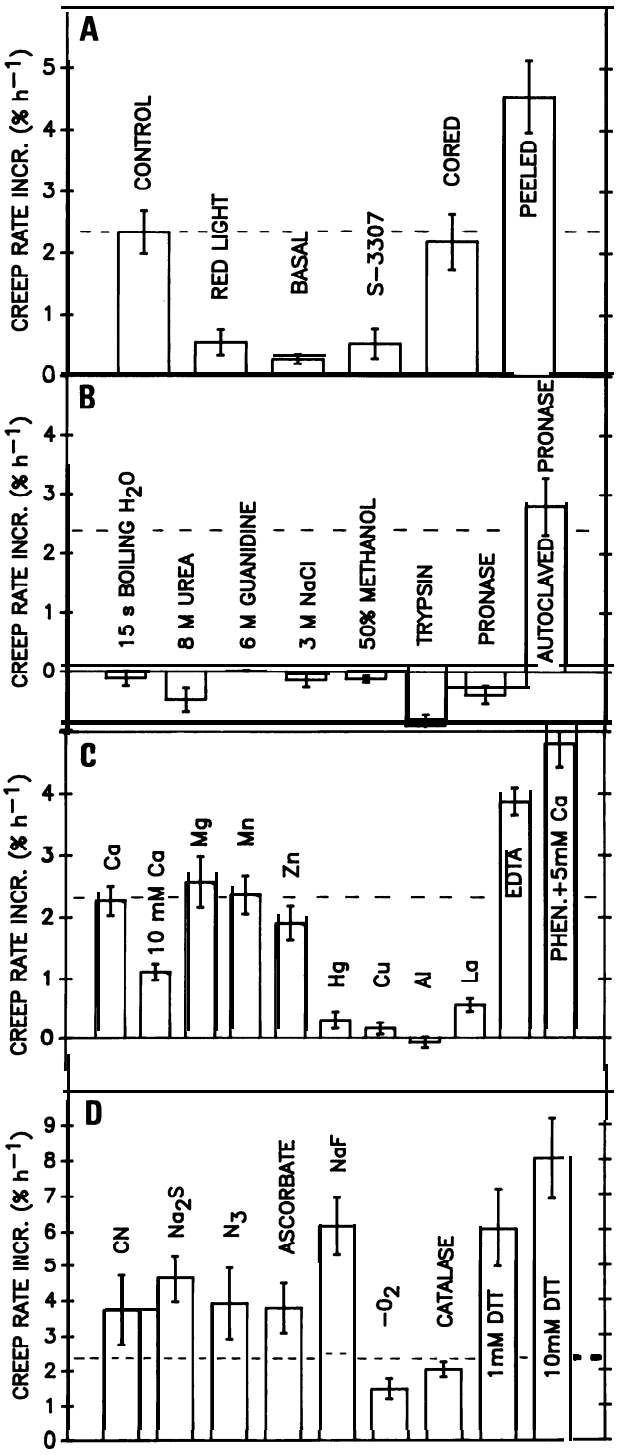

Fig. 4A-D. Creep rate in isolated cucumber-hypocotyl cell walls as a function of different treatments. Dotted line in the panels shows the value for untreated wall specimens. **A** Cucumber plants were untreated (CONTROL), or treated with red light or the gibberellin-synthesis inhibitor S-3307. For the BASAL group, hypocotyl sections were cut 5 cm below the apical hook, whereas for the CORED and PEELED treatments, the inner or outer tissues of apical segments were removed. **B.** Effect of protein denaturation or digestion on creep rate. Chaotropic agents were added to the pH 4.5 buffer; boiling and protease digestions were carried out before clamping the specimens. The negative values arise in some cases because of mechanical weakening of the wall, which leads to higher initial extension rates (in pH 6.8 buffer at 15 min). C Effect of cations (1 mM except where noted) and chelators (5 mM) added to the acid buffer. D Effect of peroxidase inhibitors and sulfhydryl reagents on creep rate. NaCN, Na_2S, NaN_3 and ascorbic acid were supplied at 1 mM in the pH 4.5 buffer. NaF was supplied at 10 mM (1 mM was not effective). Anaerobic condition (– 0,) was attained by vigorous bubbling of the buffers with argon. Catalase (50 units-ml- [1]) was supplied in the acidic buffer. Note the difference in scale relative to A-C. Means are plotted ± SE of eight or 16 samples

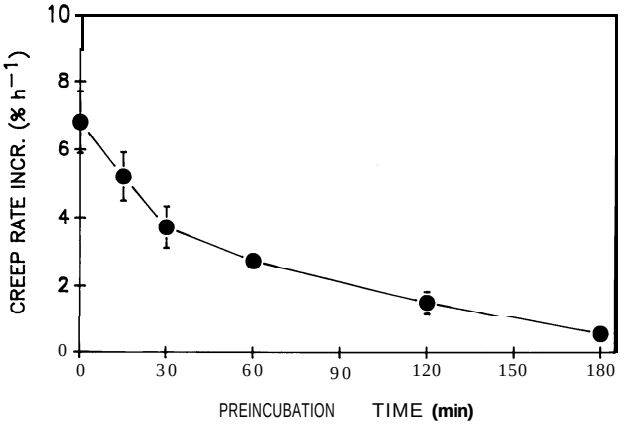

Fig. 5. Effect of pre-incubation at pH 6.8 on subsequent creep rate of isolated cucumber-hypocotyl cell walls at pH 4.5. For this experiment, Hepes was used as the pH 6.8 buffer, but in other experiments phosphate and Mes (2-(N-morpholino)ethanesulfonic acid) were used, with similar effect. Creep activity was assayed in the usual way, with 15 min of pH 6.8 buffer followed by pH 4.5 buffer, except in this experiment 1 mM dithiothreitol (DTT) was added to the acidic buffer (see Fig. 6). The same loss of creep activity was noted in replicate experiments without DTT. Additions of 1 mM ascorbate, 1 mM DTT, or 1 mM CN^- did not eliminate loss of creep activity at neutral pH. Likewise, the inactivation was not affected by inclusion of a cocktail of protease inhibitors (0.5 $\mu g \cdot ml^{-1}$ leupeptin, 0.7 $\mu g \cdot ml^{-1}$ pepstatin, 20 $\mu g \cdot ml^{-1}$ phenylmethane sulfonyl fluoride, and 0.2 mg · ml [1] EDTA-Na,). Means are plotted ± SE of eight samples

with trypsin at pH 7.6 also eliminated creep (Fig. 4B), but control experiments showed that, at the neutral pH needed for trypsin activity, segments spontaneously lost their ability to undergo creep. This inactivation occurred with a half-time of about 40 min (Fig. 5). Addition of protease inhibitors and antioxidants did not prevent this inactivation (see Fig. 5 legend). Creep activity was stable below pH 5.5, but trypsin is inactive at this pH (as measured by hydrolysis of Nα-benzoyl-DL-arginine p-nitroanilide, BAPNA).

The effect of various cations on creep rate is summarized in Fig. 4C. At 1 mM, most cations,

Table 1. Influence of temperature on creep rate in cucumber-hypocotyl cell walls and viscosity of citrus pectin. Wall specimens were extended in 50 mM acetate buffer, pH 4.5, with the following temperature programs : 20 → 30 → 20° C, 30 → 20 → 30° C, 40 → 50° C, or 50 → 40° C (1.5-2 h at each temperature, with creep rate measured at the end of each interval). The data for upshifts and downshifts between 40 and 50° C were pooled. Ratios were calculated individually, with creep rate at lower temperature in the denominator. Means and SE are shown, with sample number in parentheses. Viscosity of 2% citrus pectin solutions in pH 4.5 acetate buffer was measured by the rolling-ball technique

Temperature regime (°C)	Creep rate ($\mu m \cdot min^{-1}$)	Ratio
20	2.28 ± 0.34 (28)	1.9 ± 0.12 (28)
30	3.84 ± 0.50 (28)	
30	4.50 ± 0.41 (23)	3.8 ± 0.29 (23)
20	1.35 ± 0.19 (23)	
40	7.43 ± 1.87 (5)	0.63 ± 0.06 (5)
50	4.47 ± 1.24 (5)	
	(pectin viscosity, cp)	
21	32.8	
31	24.3	1.35[a]

[a] With viscosity at 31° C in the denominator

including Ca^{2+}, exerted little effect on long-term creep (ineffective cations not shown in Fig. 4C include Cd^{2+}, Fe^{3+}, Fe^{2+}, Ni^{2+} and Ba^{2+}). Markedly inhibitory cations at 1 mM include Cu^{2+}, Al^{3+} and Hg^{2+}. The chelators EDTA and EGTA (at 5 mM) stimulated creep rate ; 5 mM 1 ,10-phenanthroline (a metal chelator with low selectivity for calcium) in combination with 5 mM $CaCl_2$ likewise stimulated creep, indicating that these chelators stimulate creep largely by chelating heavy metals, not calcium.

Since enzymatic processes typically have a high Q_{10}, the temperature sensitivity of long-term extension was investigated (Table 1). When temperature was reduced from 30° C to 20° C, the Q_{10} was 3.8 ; when temperature was raised from 20° C to 30° C, the Q_{10} was lower (1.9) because of loss of creep activity at the higher temperature. In comparison, pectin solutions exhibited a Q_{10} of only 1.35 for viscous flow at these temperatures. Thermal loss of creep activity was further examined by measuring creep rate at 40 and 50° C (Table 1). The rationale was that viscous flow and pectin gel-sol transitions would be accelerated at the higher temperature, whereas enzymes would likely show thermal inactivation. Creep rat dropped off by 37% at the higher temperature. When the temperature was raised to 60° C, creep was inhibited even more strongly (not shown). These results support the enzyme hypothesis.

Peroxidase activity and SH reagents. NaCN (1 mM) was added to the buffers to verify that wall creep did not depend on residual mitochondrial activity. Unexpectedly, CN^- was found to *enhance* creep rate (Fig. 4D). The CN^- stimulation might be explained as an inhibition of peroxidase, which is believed to catalyze phenolic cross-linking of pectins, proteins and possibly other wall polymers (see Biggs and Fry 1987). In accord with this idea, other inhibitors of peroxidase (Saunders et al. 1964) stimulated creep (Fig. 4D). Further results, however, contradicted this hypothesis. Oxygen removal should also inhibit peroxidase activity, yet it had no effect on creep rate (Fig. 4D). Addition of catalase to remove hydrogen peroxide likewise had no effect on creep. The most convincing result was that addition of 0.1 mM NaCN did not stimulate creep, but did inhibit cucumber peroxidase activity by > 95% (assayed using diced cucumber hypocotyls). These results indicate that endogenous peroxidase does not inhibit wall creep.

Another possible explanation is that CN^- reduces thiol groups of wall enzymes, as in papain activation by 1 mM CN^- (Klein and Kirsch 1969). To test this idea, dithiothreitol (DTT), a more potent thiol reducing agent, was added to the creep incubation solution. A large stimulation of creep resulted (Fig. 4D). Addition of cysteine, another SH reagent, also stimulated creep, but less effectively than DTT. It is noteworthy that DTT eliminates much of the decay in creep during seen during l-2 h after switch to acidic pH (Fig. 6). This indicates that the usual decay in creep rate was caused by thiol oxidation of wall enzymes. To test this explanation, DTT was added 1.5 h after the change to acidic buffer (Fig. 6). The resulting stimulation of creep confirms this hypothesis. However, DTT was not able to prevent or reverse the loss of creep activity at neutral pH (e.g. Fig. 5), so a different inactivation mechanism must be involved there. Also, because DTT did not stimulate creep in neutral buffer (Fig. 6), it is unlikely that DTT was acting by breaking disulfide bridges between structural proteins in the wall (such proteins are very low in sulfur amino acids, in any case). While DTT enhanced creep rate, the shape of the pH-response curve remained similar to that without DTT (not shown).

Mechanical characteristics of *the wall.* One might argue that acidic pH and the chemical treatments

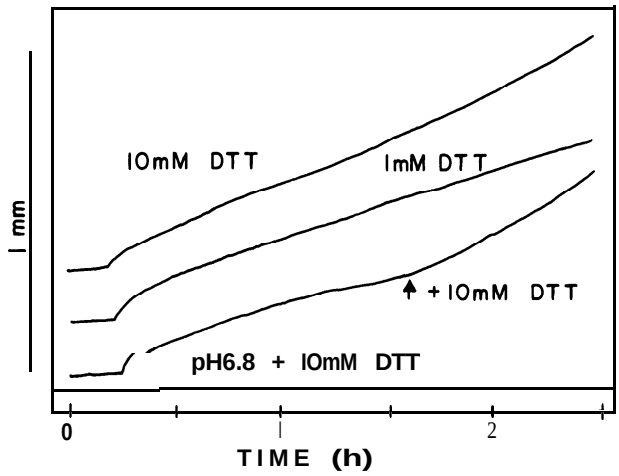

Fig. 6. Effects of DTT on creep in isolated cucumber-hypocotyl cell walls. Dithiothreitol was included in the acid buffer (1 mM and 10 mM), or was added midway through a creep assay (trace with arrow marked + 10 mM DTT), or included in the neutral buffer (held at pH 6.8 throughout assay). With 10 mM DTT, there sometimes was a slight acceleration of creep in the later half of the assay

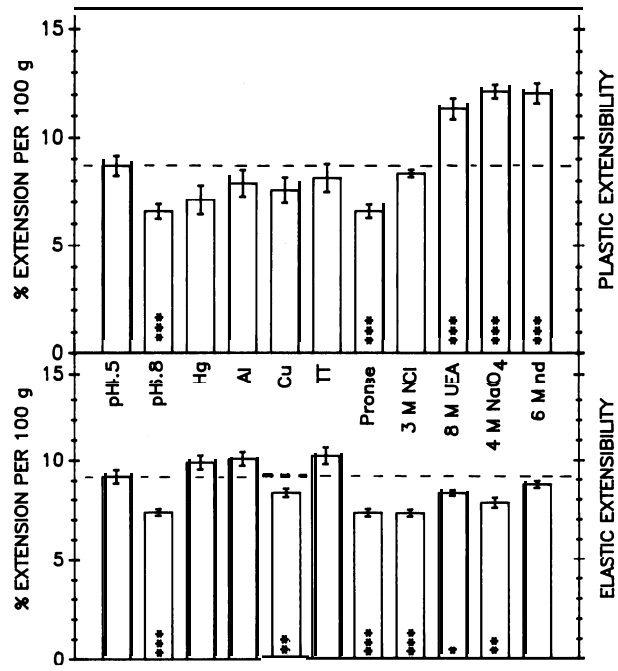

Fig. 7. Plastic and elastic extensibilities of isolated cucumber-hypocotyl cell walls, measured by Instron technique, as a function of inhibitors and stimulants of creep. Except as noted, wall specimens were pre-incubated before measurement for 30 min at 4° C in 50 mM pH 4.5 acetate buffer, with additions of 1 mM $HgCl_2$, $AlCl_3$, $CuCl_2$, 10 mM DTT, or the noted concentrations of denaturants. For the bar marked "pH 6.8", walls were incubated in 50 mM pH 6.8 Hepes buffer for 30 min at 4" C. For the bar marked " Pronase", walls were predigested in Pronase before transfer to pH 4.5 acetate for 30 min. Means are plotted $\pm$ SE of 14-18 samples, with significant differences from pH 4.5 shown at the 0.95, 0.99 and 0.999 probability level by in the order given, *, **, and ***. Unmarked bars are not statistically different from pH 4.5 (by Student's *t* test)

described in the last two sections exert their effects largely by altering the physical interactions between wall polymers, making the wall physically more rigid or more fluid. This possibility was examined by Instron and stress-relaxation measurements.

The Instron technique measures the mechanical extensibilities of a sample under a rapidly-imposed strain. Wall specimens were pre-incubated for 30 min at 4° C in buffers containing various creep inhibitors. Low temperature and short time were used to minimize possible enzymatic activity, so purely physical effects of the treatments could be examined. As shown in Fig. 7, walls incubated in pH 6.8 buffer had significantly lower elastic and plastic extensibilities (by about 20%) than walls in pH 4.5 buffer. Assuming these differences to be entirely physical, we may use them to judge whether other treatments produce equivalent changes in mechanical extensibilities. Although Al^{3+}, Cu^{2+} and Hg^{2+} strongly inhibited creep, these cations reduced plastic extensibility by a small, statistically insignificant amount; of these cations only Cu^{2+} reduced elastic extensibility. Because DTT had no effect on Instron extensibilities, it is unlikely that it acts by severing disulfide links between structural proteins in the wall. High concentrations of denaturants had mixed effects, in some cases reducing elastic extensibility (NaCl, $NaClO_4$, urea) while *increasing* plastic extensibility ($NaClO_4$, guanidine, urea). The Instron results indicate that altered wall viscoelasticity, alone, cannot account for the alteration of creep by these agents, although in some cases physical effects may be important.

Stress-relaxation spectra characterize the rate of relaxation as a function of time and provide clues about the nature of molecular rearrangements in the wall, e.g. large molecular reorderings take longer than small ones (Masuda 1978). Spectra of walls pre-incubated in acidic and neutral buffers at 4° C (Fig. 8) may be used to gauge the action of other treatments. Subtraction of the two spectra (Fig. 9A) indicates that walls in neutral buffer have reduced relaxation over most of the spectrum (compared with walls in acid buffer), with minimum effect at 60-70 ms ($-$ 1.2 in logarithmic units). The difference spectrum in Fig. 9B shows the variation typical between two sets of identical treatments.

The effects of various denaturants are shown in Fig. 9C-D. These agents might be expected to weaken the wall by disrupting hydrogen and ionic

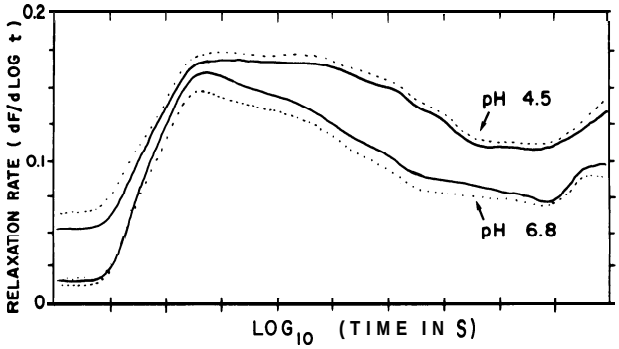

Fig. 8. Stress-relaxation spectra of specimens of isolated cucumber-hypocotyl cell walls pre-incubated for 30-min at 4" C in pH 4.5 or 6.8 buffers. Relaxation is shown as the derivative of force (in g) with respect to log,, of time (in s). The plots are the averages of eight samples. *Dotted line* indicates SE

bonds between wall polymers. At the same time they might also rigidify the wall by reducing repulsive (hydrophobic, osmotic) forces (Gould et al. 1965). Also, unfolding of wall polymers under the action of these denaturants might lead to greater steric hindrance and higher effective viscosity of the relaxing elements of the wall. Guanidine was found to enhance relaxation in the time range of about 1 s, but inhibited relaxation at times beyond 100 s. Urea, $NaClO_4$, and NaCl retarded relaxation over a wide time range (urea results are not shown, but were similar to 3 M NaCl). The results indicate (though not prove) that some of the denaturants may inhibit creep partly through a physical effect on the wall.

In contrast, DTT (Fig. 9D) hardly influenced the relaxation spectrum. Likewise, the action of the inhibitory cations on creep could not be explained through their physical effects on wall viscoelasticity (Fig. 9 E-F). Hg^{2+} had almost no effect on wall relaxation, Al^{3+} *enhanced* relaxation over a large part of the spectrum, and Cu^{2+} had a mixed effect, enhancing relaxation in the 0.01-l s phase and inhibiting relaxation beyond about 10 s.

Protein digestion with papain likewise had a mixed effect on the relaxation spectrum, enhancing relaxation in the millisecond range but inhibiting relaxation at times longer than 2 s. Pronase affected the relaxation spectrum similarly (not shown). The difference spectrum between 10 mM Ca^{2+} and 100 mM EDTA (Fig. 9 F) showed almost no effect of Ca^{2+} on relaxation.

Extraction and methanol-boiling of walls. Attempts to extract the creep activity from the wall showed that chaotropic agents such as 8 or 4 M urea and 4 M $NaClO_4$ caused irreversible loss of creep activity. This loss may be the result of extraction of

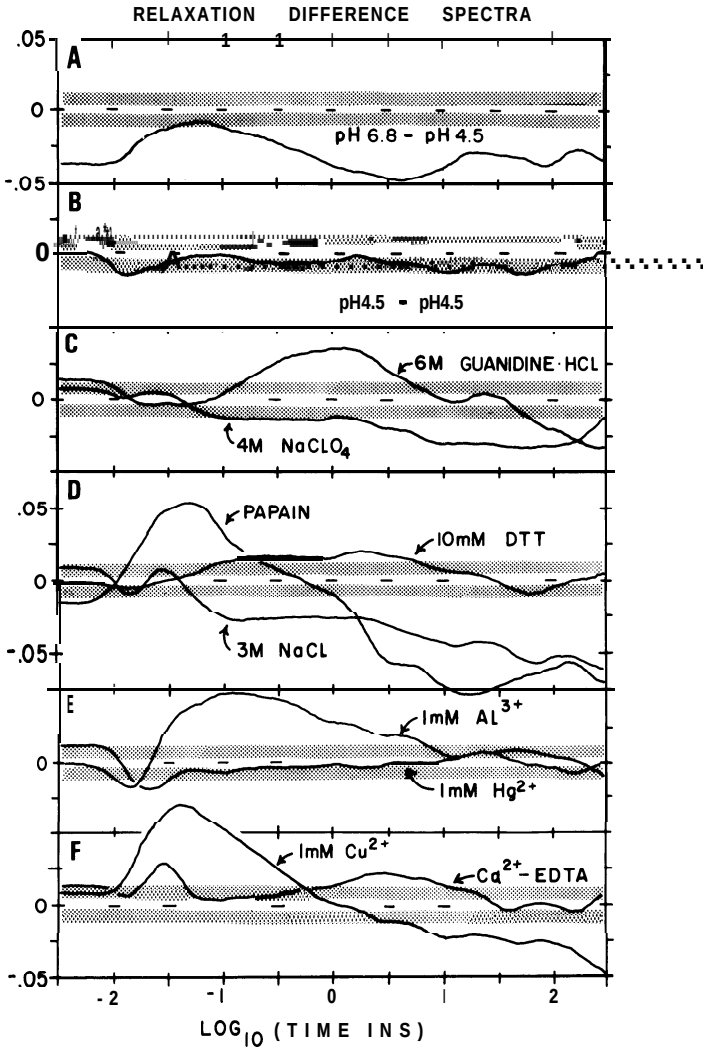

Fig. 9A-F. Relaxation difference spectra of cucumber-hypocotyl cell walls, as affected by inhibitors and stimulants of creep activity. Each curve is the difference between two spectra, which are themselves the averages of eight samples. **A** The spectra in Fig. 8 were subtracted to show physical effects of pH on wall relaxation. **B** Spectra from two replicate experiments were subtracted to show the variability between runs. The shaded zone demarks the area considered an insignificant difference. **C-F** Spectra were obtained from wall specimens in pH 4.5 acetate buffer, and subtracted from spectra of walls in identical buffer plus: C 6 M guanidine HCl or 4 M sodium perchlorate; **D** 10 mM DTT or 3 M NaCl; **E** 1 mM $AlCl_3$ or 1 mM $HgCl_2$; **F** 1 mM $CuCl_2$. For the spectrum marked "PAPAIN", walls were pre-incubated for 4 h in 50 mM potassium-acetate buffer, pH 5.0, containing 2 mg *ml- ' of papain. They were then washed with distilled water and incubated another 30 min in pH 4.5 buffer before measurement. The spectrum was subtracted from that of walls identically treated except papain was omitted. Similar results were obtained for Pronase pretreatment. For the spectrum marked "Ca^{2}-EDTA", walls were incubated in cold pH 4.5 buffer containing either 10 mM $CaCl_2$ or 100 mM EDTA, and the EDTA spectrum was subtracted from the Ca spectrum. For each difference spectrum, values above the shaded area indicate enhanced relaxation, whereas values below the shaded area indicate reduced relaxation

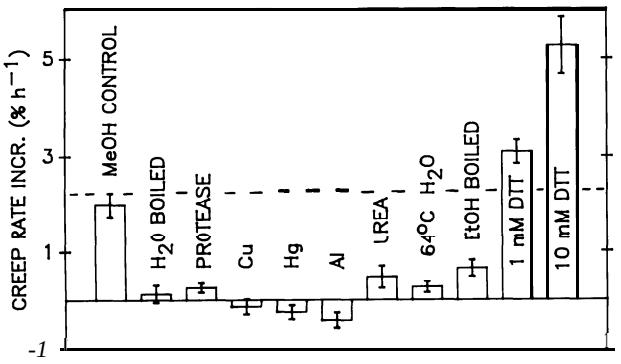

Fig. 10. Effect of various inhibitors on creep activity of isolated cucumber-hypocotyl cell walls surviving methanol boiling (5 min). *Dotted line* shows the value for untreated controls (from Fig. 4). First bar shows creep in walls boiled in methanol (MeOH) for 5 min. Subsequent treatment with boiling water (15 s) and Pronase inhibits creep (treatment with these agents prior to methanol boiling also inhibits creep). Addition of 1 mM $CuCl_2$, $HgCl_2$, $AlCl_3$ or 8 M urea to the acid buffer likewise inhibits creep of methanol-boiled walls. When untreated segments are incubated for 5 min at the boiling temperature of methanol in water (64" C H_2O) creep is inhibited. Likewise, boiling in ethanol (EtOH BOILED) inhibits creep. Addition of 1 mM or 10 mM DTT to the acid buffer of methanol-boiled walls stimulates creep. Means are plotted ± SE of eight samples

protein which is hydrogen-bonded to the wall, but is more likely caused by irreversible denaturation because even short (20 min) treatment under unstirred conditions caused irreversible loss of activity (data not shown). Interestingly, creep activity remained after extraction with 3 M NaCl or 5 M LiCl (4 h with vigorous stirring), when assayed after removal of the high salt. This result indicates either that the protein is strongly (perhaps covalently) bound to the wall, or that its escape is sterically hindered by the network of wall polymers. Extraction with detergents such as 1% Triton X-100 (a mixture of polyoxyethylene ethers) or 1% octylglucinol likewise failed to remove creep activity.

A more surprising observation was that, after cucumber walls were boiled in 100% methanol for 5 min, creep activity could be restored by returning the walls to pH 4.5 acetate buffer. This restored creep was still inhibited by proteases, metal cations, and boiling water (Fig. IO), and showed a similar pH-response curve as untreated walls (data not shown). Dithiothreitol stimulated creep in methanol-boiled walls, but less markedly than in unboiled specimens (Fig. 10). The shape of the extension curve was changed after boiling, in that it was nearly linear from the start, i.e. the early decay in extension rate was eliminated. This loss of creep decay may account for the reduced stimulation by DTT. Methanol appeared to act as a protectant, since pre-incubating wall specimens for 5 min in water at the boiling temperature of methanol (64" C) inhibited creep activity, as did boiling in ethanol (Fig. 10).

Discussion

The aim of this study was to determine whether long-term extension (creep) of cucumber hypocotyls was simply viscoelastic flow, or whether the activity of endogenous wall enzymes was necessary to maintain viscoelastic flow. The results in support of an enzymatic mechanism include: (a) inactivation by denaturants, certain metal cations, boiling water, and proteases; (b) activation by SH-reducing agents; and (c) high Q_{10} between 20 and 30° C, with inactivation at higher temperatures. Parallel measurements indicated that many of these treatments did not substantially alter viscoelastic behavior of the walls (e. g. Hg^{2+}, DTT, some denaturants), although some agents (e. g. Cu^{2+}) might well act in part through physical rigidification of the wall. Each of these results provides indirect evidence in support of an enzymatic mechanism for wall creep; in aggregate they point rather strongly and consistently toward an enzymatic mechanism, which might entail hydrolysis of wall polymers (e.g. Yamamoto and Nevins 1981; Hayashi et al. 1984; Labrador and Nicolas 1984), transfer of covalent cross-links (e.g. a transglycosylation), or perhaps displacement of noncovalent bonds between wall polymers. Definite proof of an enzymatic mechanism will require extraction and reconstitution of the relevant enzymes. The characterization of creep in this paper provides new information that may be used to search for these enzymes, by correlations between enzymatic activities and creep activity.

The results rule out some proposed mechanisms, at least for the in-vitro extension studied here. For example, one hypothesis holds that wall extension represents a balance between crosslink breakage by autolytic enzymes and formation by peroxidase (Biggs and Fry 1987). The finding that 0.1 mM CN^- inhibited cucumber peroxidases but did not stimulate creep discounts this hypothesis. Another possibility, involving gel-sol transitions of wall pectins (Gould et al. 1965 ; Yamaoka and Chiba 1983), is contradicted by the reduction of creep at temperatures that should increase pectin melting.

A surprising result was that cucumber walls showed little weakening during incubation in high concentrations of salts and chaotropic agents

(Fig. 7). This observation would seem to contradict the common view that wall integrity depends strongly on ionic and hydrogen bonds. It is posible that such noncovalent interactions are too strong to be disrupted by these treatments. If so, then they may well be too stable to be involved in endogenous wall extension. Another interpretation is that physical entanglements or covalent linkages between wall polymers influence much of the mechanical behavior of the wall. Relaxation spectra gave some indication that denaturants reduced repulsive (hydrophobic, osmotic) forces within the wall, leading to less relaxation (Fig. 9).

Also surprising is the observation that creep activity can survive methanol boiling and is not removed by salt extraction. Such ruggedness is unusual, but not unprecedented (e.g. ribonuclease is renowned for its survival of harsh treatments). Polacheck and Rosenburger (1978) and Nagahashi and Seibles (1986) found wall autolytic enzymes that were not solubilized from fungal and plant cell walls by high salt. The creep activity that remains after methanol boiling of cucumber walls is still sensitive to proteases, pH, denaturants and metal cations. The enzyme(s) responsible for creep may well be protected by close association with carbohydrates, which in other systems act as protectants (Lapanje 1978). This ruggedness may be useful in future studies to identify the creep enzyme(s).

An important, and still unresolved, question is the physiological significance of the wall extension studied here. Evidence in favor of a role during endogenous cell expansion includes the correlation between creep rate and long-term growth rate (Fig. 4A), and the relatively high rate and long duration of extension in vitro. The uniaxial 20-g force used to extend the wall specimens is small compared with the longitudinal force normally exerted on the wall by turgor (e.g. a turgor of 4 bar and stem cross-section of 1.5 . 1.5 mm^2 gives a longitudinal force equivalent to 90 g). This fact, plus the uniaxial character of the stress, makes it difficult to compare in-vitro creep with in-vivo growth in a quantitative fashion, but they appear to be similar in magnitude. The pH sensitivity of long-term creep would fit in with some of the observations underlying the acid-growth hypothesis (Cleland and Rayle 1978 ; Cleland et al. 1987), but the wall pH in cucumber hypocotyls has not yet been investigated. The results of this study indicate that irreversible enzyme inactivation at neutral pH might provide another mechanism for pH control of growth, in addition to the usual idea of pH-dependent catalytic rate.

Finally, the sensitivity of creep to SH-reducing agents leads me to suggest that such a redox process might be involved in endogenous control of wall extension, by analogy with the control of photosynthetic enzymes by thioredoxin (see Buchanan 1983 for review). Plasma-membrane electron-transport systems have been found in a number of plants (see Møller and Lin 1986) and, speculatively, one of their roles might involve control of thiol reduction of wall enzymes.

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References

Biggs, K.J., Fry, S.C. (1987) Phenolic cross-linking in the cell wall. In: Physiology of cell expansion during plant growth (Symp. in Plant Physiology, Pennsylvania State Univ.), pp. 46-57, Cosgrove, D.J., Knievel, D.P., eds. American Society of Plant Physiologists, Rockville, Md., USA

Block, S.M., Spudich, A. (1987) The rolling ball viscometer. An automated way to monitor assembly states of biopolymers. Am. Biotechnol. Lab. 5, No. 2, 38-46

Buchanan, B.B. (1983) Role of thioredoxins and related proteins in photosynthetic enzyme regulation. In : Functions of glutathione. Biochemical, physiological, toxicological and clinical aspects, pp. 231-242, Larsson, A., Orrenius, S., Holmgren, A., Mannervik, B., eds. Raven Press, New York

Cassab, G.I., Varner, J.E. (1988) Cell wall proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 321-353

Catsiff, E., Tobolsky, A.V. (1954) Relation betwen stress-relaxation studies and dynamic properties of polyisobutylene. J. Appl. Physics 25, 145-152

Chance, B., Maehly, A.C. (1955) Assay of catalases and peroxidases. Methods Enzymol. 3, 764-775

Cleland, R.E. (1984) The Instron technique as a measure of immediate-past wall extensibility. Planta 160, 514-520

Cleland, R.E., Cosgrove, D.J., Tepfer, M. (1987) Long-term acid-induced wall extension in an in vitro system. Planta 170, 379-385

Cleland, R.E., Rayle, D.L. (1978) Auxin, H^+-excretion and cell elongation. Bot. Mag. Tokyo, Spec. Issue 1, 125-139

Gould, S.E.B., Rees, D.A., Richardson, N.G., Steele, I.W. (1965) Pectic polysaccharides in the growth of plant cells: molecular structural factors and their role in the germination of white mustard. Nature 205, 876-878

Hager, A., Menzel, H., Krauss, A. (1971) Versuche und Hypothese zur Primarwirkung des Auxins beim Streckungswachsturn. Planta 100,47-75

Hayashi, T., Wong, Y.-S., Maclachlan, G. (1984) Pea xyloglucan and cellulose II. Hydrolysis by pea endo-1,4-β-glucanases. Plant Physiol. 75, 605-610

Iwami, S., Masuda, Y. (1974) Geotropic response of cucumber hypocotyls. Plant Cell Physiol. 15, 121-129

Izuma, K., Kamiya, Y., Sakura, A., Oshio, H., Takahashi, N. (1985) Studies of sites of action of a new plant growth retardant (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3307) and comparative effects of its steroisomers in a cell-free system from *Cucurbita maxima*. Plant Cell Physiol. 26, 821-827

Jones, R.L. (1986) The control of plant cell elongation by auxin

and gibberellin. In: Plant Growth Substances 1985, pp. 274-283, Bopp, M., ed. Springer, Berlin

Klein, I.B., Kirsch, J.F. (1969) The mechanism of activation of papain. Biochem. Biophys. Res Commun. 34, 575-581

Kutschera, U., Bergfeld, R., Schopfer, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coeloptiles. Planta 170, 168-180

Labrador, E., Nicolás, G. (1984) Autolysis of cell walls in pea epicotyls during growth. Enzymatic activities involved. Physiol. Plant. 64, 541-546

Lapanje, S. (1978) Physicochemical aspects of protein denaturation. John Wiley and Sons, New York

Masuda, Y. (1978) Auxin-induced cell wall loosening. Bot. Mag. Tokyo, Spec. Issue 1, 103-123

Métraux, J.-P., Taiz, L. (1977) Cell wall extension in Nitella as influenced by acids and ions. Proc. Natl. Acad. Sci. USA 74, 1565-1569

Møller, I.M., Lin, W. (1986) Membrane-bound NAD(P)H dehydrogenases in higher plant cells. Annu. Rev. Plant Physiol. 37, 309-334

Nagahashi, G., Seibles, T. (1986) Purification of plant cells walls: isoelectric focusing of $CaCl_2$ extracted enzymes. Protoplasma 134, 102-110

Olson, A.C., Bonner, J., Morré, J.D. (1965) Force extension analysis of *Avena* coleoptile cell walls. Planta 66, 126-134

Polacheck, I., Rosenburger, R.F. (1978) Distribution of autolysins in hyphae of *Aspergillus nidulans:* evidence for a lipid-mediated attachment to hyphal walls. J. Bacterial. 135,741-747

Ray, P.M. (1987) Principles of plant cell growth. In: Physiology of cell expansion during plant growth (Symp. in Plant Physiology, Pennsylvania State Univ.), pp. 1-17, Cosgrove, D.J., Knievel, D.P., eds. American Society of Plant Physiologists, Rockville, Md., USA

Rayle, D.L., Cleland, R.E. (1970) Enhancement of wall loosening and elongation by acid solutions. Plant Physiol. 46,250-253

Saunders, B.C., Holmes-Siedle, A.G., Start, B.P. (1964) Peroxidase. Butterworths, Washington, D.C., USA

Taiz, L. (1984) Plant cell expansion : regulation of cell wall mechanical properties. Annu. Rev. Plant Physiol. 35, 585-657

Taiz, L., Richmond, P.A. (1984) Regulation of cell wall mechanical properties in vivo and in vitro. In: Structure, function and biosynthesis of plant cell walls (Symp. in Botany, Univ. of Cal., Riverside), pp. 185-194, Dugger, W.M., Bartnicki-Garcia, S., eds. American Society of Plant Physiologists, Rockville, Md., USA

Tepfer, M., Cleland, R.E. (1979) A comparison of acid-induced cell wall loosening in *Valonia ventriculos* and in oat coleoptiles. Plant Physiol. 63, 898-902

Yamaoka, T., Chiba, N. (1983) Changes in the coagulating ability of pectin during growth of soybean hypocotyls. Plant Cell Physiol. 24, 1281-1290

Yamamoto, R., Masuda, Y. (1971) Stress-relaxation properties of the *Avena* coleoptile cell wall. Physiol. Plant. 25, 330-335

Yamamoto, R., Nevins, D.J. (1981) Coleoptile growth-inducing capacities of exo-β-(1 → 3)-glucanases from fungi. Physiol. Plant. 51, 118-122

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