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Control of Cell Elongation in *Nitella* by Endogenous Cell Wall pH Gradients

MULTIAXIAL EXTENSIBILITY AND GROWTH STUDIES[1]

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ABSTRACT

The multiaxial stress of turgor pressure was stimulated *in vitro* by inflating isolated *Nitella* cell walls with mercury. The initial *in vitro* extension at pH 6.5, 5 atmospheres pressure, returned the wall approximately to the *in vivo* stressed length, and did not induce any additional extension during a 15-minute period. Upon release of pressure, a plastic deformation was observed which did not correlate with cell growth rates until the final stages of cell maturation. Since wall plasticity does not correlate with growth rate, a metabolic factor(s) is implicated. Walls at all stages of development exhibited a primary yield stress between 0 and 2 atmospheres, while rapidly growing cells (1-3% per hour) exhibited a secondary yield stress of 4 to 5 atmospheres. The creep rate and plastic deformation of young walls were markedly enhanced by acid buffers (10 millimolar, pH ≤ 5.3).

***Nitella* cells produce acid and base "bands" along their length due to localized excretion of protons and hydroxyl ions. Marking experiments showed that growth is largely restricted to the acid regions. Growth in the acid bands was inhibited by alkaline buffers, and growth in the base bands was stimulated by acidic buffers. The two zones have similar mechanical properties. When the proton-binding capacity of the wall was taken into account, the pH of the solution in contact with inner wall surface in the acid band was estimated to be about 4.3, well within the threshold of acid-enhanced creep. Since the inner 25% of the wall controls extensibility, we conclude that growth in the acid band is caused by the action of protons on the wall.**

The giant internodal cells of *Nitella* provide a useful model system for studying the growth and mechanical properties of plant cell walls. Cell expansion occurs by diffuse growth in a predominantly longitudinal direction (5). Green (7), Probine and Preston (16), and Gertel and Green (4) have shown that the arrangement of cellulose microfibrils is anisotropic and conforms to the multinet growth hypothesis (19), *i.e.* the microfibrils are deposited by apposition in a transverse orientation and are passively realigned during cell elongation. In extensibility tests with isolated wall strips, Probine and Preston (17) found that the transverse elastic modulus is two to five times greater than the longitudinal modulus, with the higher value characteristic of rapidly growing cells. Longitudinal creep (time-dependent viscoelastic extension under constant stress) correlated well with *in vivo* growth rates, whereas creep in the transverse direction could not be detected (17). More recently, we have verified the anisotropy of longitudinal *versus* transverse creep (13). Thus, structural anisotropy has its correlate in the mechanical properties of the wall, which, in turn, influence the direction of cell expansion.

Although a qualitative relationship has been established between wall mechanical properties and the directionality of cell expansion, the rate of extension of isolated walls greatly exceeds growth rates, even when the longitudinal stresses are identical (13, 17). This discrepancy is believed to be due to the uniaxial nature of the applied stress in most extensibility experiments, which causes distortions in the microfibril arrangement absent in growing cells. Kamiya *et al.* (10) simulated the multiaxial stress induced by tugor pressure by inflating wall cylinders with mercury. As expected, multiaxially stressed walls were much less extensible than those stressed uniaxially. However, since only walls from nongrowing cells were tested, little information relevant to growth was obtained.

The ion exchange properties of *Nitella* walls were first described by Probine and Preston (17). Potassium ions increase wall extensibility, apparently by exchanging with calcium ions. Morikawa (15) observed that acidic solutions increased wall extensibility. We have recently shown that acid pH stimulates elongation in living internode cells, and that protons effect wall loosening through a nonenzymic mechanism (12).

Here we have reexamined the mechanical properties of *Nitella* walls from growing as well as nongrowing cells, using the mercury inflation technique of Kamiya *et al.* (10). In addition, we provide evidence that wall extensibility *in vivo* is regulated by proton extrusion into the acid band regions of the wall.

MATERIALS AND METHODS

Cultures of *Nitella axillaris* Braun were grown in autoclaved soil extract as previously described (12). The growth medium was changed every 2 weeks and the cells were kept free of visible epiphytes.

Multiaxial Extensibility Measurements. An internode cell was excised with a razor blade at one end, and the cytoplasm was brushed out with a hair loop. The tapered end of a Pasteur pipette, which had been filled with 2 ml of mercury, was inserted into the open end of the wall cylinder. By selecting a pipette of appropriate diameter a snug fitting junction was obtained. The junction was then allowed to dry in air, while the remainder of the wall was kept wet in a Petri dish filled with water. The junction was sealed with De Kothinsky cement (Central Scientific Co., Chicago) or a 1:3 mixture of beeswax and rosin (10). The tip of the pipette with

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the cell attached was then lowered into a perfusion chamber and the open end was connected to a compressed air tank equipped with a manometer (Fig. 1). The standard perfusion buffer was 1 mM Mes-Tris (pH 6.5). Anion exchange resin beads (8) were positioned on the wall and a small amount of pressure (0.5 atm) was applied to the system. Mercury replaced the water in the wall tube and did not leak out. When an intact cell was left above the node, cytoplasmic streaming continued throughout the experiment, indicating that transcellular disturbances were negligible. The distance between selected beads at 0.5 atm was taken as the initial length. The pressure was then raised and the new distance recorded with a traveling microscope (Gaertner Scientific, Chicago). A pressure of 5 atm was considered to be equivalent to turgor pressure on the basis of measurements by Green (9). This method is similar to the system of Kamiya *et al.* with the following modifications: *(a)* Extension was measured as the distance between two points rather than the displacement of a single point. This is important, since we observed elastic stretching at the glued junction. *(b)* Pressure was applied with a compressed air tank rather than a syringe. In this way the pressure remained constant regardless of small leaks in the system. *(c)* A low pressure (0.5 atm) was used to eject water from the wall tube, instead of the 2 to 3 atm employed by Kamiya *et al.* (10). This ensured that no irreversible stretching occurred before the measurements began.

The procedure for measuring contraction of a living cell after turgor loss was the following. The cell was held in place at the bottom of a Petri dish by clamping the neighboring cell with a small glass cantilever similar to that used by Green (9). Resin beads were placed along the surface, and the distance between two beads was determined with a traveling microscope. The cell was then gently cut at one end with sharp surgical scissors, and the kinetics of shrinkage determined. Afterwards, the wall was prepared for multiaxial extension as described above. Once in the perfusion chamber a new length was measured between a different pair of beads at 0 atm pressure, and extensibility was followed as usual.

The terms D_t[5], D_e, and D_p were used to denote total, elastic, and plastic deformation, respectively, for a particular constant load. These deformation values should not be confused with the related compliance (strain/stress) values, DT, DE, and DP, measured with the Instron extensometer using a constant strain rate (2). D_t, D_e, and D_p refer to the relative deformation at a constant load after an arbitrarily selected period of 15 min. Relative deformation was expressed as the per cent increase over the initial length.

Growth Measurements. Filaments grown in autoclaved soil extract were placed in distilled H_2O. Anion exchange beads were positioned at regular intervals along a selected cell and the filament was transferred to the dilute salt solution (Kb medium) of Spear *et al.* (20). After an overnight incubation, the filaments were plated onto 2% (w/v) agar in large Petri dishes containing Kb medium (pH 6.8) and 0.1 mM phenol red, without disturbing the resin beads. Best results were obtained by keeping the basal cells of the filament submerged in a well scooped out of the agar and filled with Kb solution. After a 30-min illumination with a fluorescent light source (10 w m^{-2}) the pH bands became visible as red or yellow zones in the agar. By selecting a pair of resin beads within an acid of a base region it was possible to monitor growth in the two zones using a traveling microscope. Growth was measured until the band migrated to a new location. The bands were stable for at least 30 min and for as long as 16 h. Growth rates were expressed as relative rates, (1/L) (dL/dt), where L is the distance between beads and t is time (6). This allows a direct comparison of the rates of different zones, since the relative rate is independent of the absolute distance between marking beads. In practice the relative rate is determined by $(\ln L_2 - \ln L_1)/(t_2 - t_1)$.

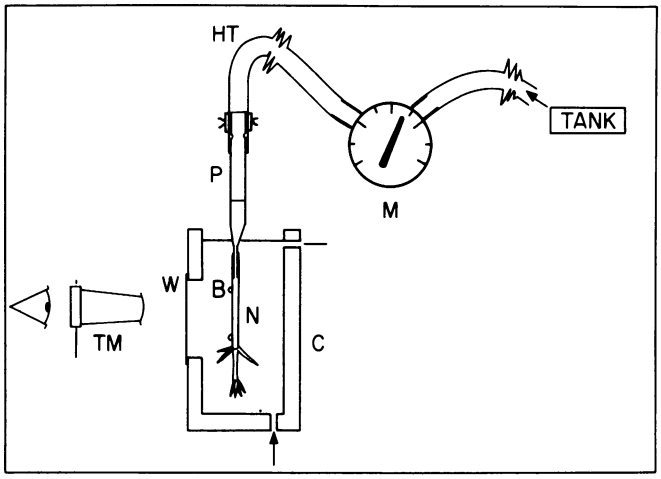

FIG. 1. Experimental set-up for measuring multiaxial extensibility of *Nitella* cell walls. The pressure source is a compressed air tank. Air passes through high pressure tubing (HT) into a manometer (M) and from there into a mercury-filled Pasteur pipette (P) fitted to the open end of a *Nitella* wall (N). A living internode is maintained at the distal end. The wall is placed in a Plexiglas perfusion chamber (C) with a glass window (W). Displacement of resin beads (B) is measured with a vertical traveling microscope (TM).

Proton-binding Experiments. A wall cylinder from a growing cell 2 cm in length was excised, and tapered capillary tubes were inserted at each end. The central wall region (1 cm) was kept moist while the two ends were dried and glued to the capillaries as described above (see Fig. 8). One capillary was connected by tubing to a flask containing solution which was peristaltically pumped through the cylinder at a rate of 1 ml/min. Enough back pressure was present to force a small amount of the solution through the wall. A standard volume (7 μl) was collected from the wall surface by means of a micropipette, spotted onto a sheet of Parafilm, and measured with a miniature pH electrode (model MI-405, Microelectrodes, Inc., Londonderry, N. H.). The calibration of the electrode was done similarly by using 7 μl of standard buffer solution.

Measurements of wall surface pH were performed by applying the miniature pH electrode to the surface of cells incubating on agar containing Kb medium. The electrode was mounted in a micromanipulator.

RESULTS

Multiaxial Extensibility. When a living internode cell is cut the wall undergoes a viscoelastic contraction due to the release of turgor pressure. The first curve in Figure 2 shows the per cent of reversible or elastic shrinkage, D_e, which occurs during cell excision. When the same cell is repressurized with mercury at 5 atm, the per cent increase in length equals the per cent shrinkage (Fig. 2, second curve). In other words, the wall returns to the maximum length attained under turgor pressure, and no further, at least during a 20-min extension. In five similar experiments it was found that the wall returned to 94.2% (± 15.5%) of the maximal length *in vivo*. Since all of the walls were extended at a standard 5 atm pressure, the variability in D_t could be due to the fact that for some walls this was slightly above the *in vivo* turgor pressure, which for others it was slightly below. When the mercury pressure is released, the wall contracts again by an amount, D_e. The difference between D_t and D_e is the irreversible D_p. Note that although the force was the same, and the maximum length of the turgor-extended wall was not exceeded, the "second" extension

[5] Abbreviations: D_t: total deformation; D_e: elastic deformation; D_p: plastic deformation; Kb medium: dilute salt solution; PEi: instantaneous plastic extensibility.

had a significant D_p. Thus, a portion of the previous elastic recovery was converted to plastic extension, without any increase in D_t. This differs from mechanical conditioning experiments, in which a second equal extension is entirely elastic (2). Subsequent mercury-induced extensions are almost completely elastic if carried out at equal pressures and the same medium (data not shown).

The relationship between the internal pressure and the relative longitudinal deformation for young and old cell walls is shown in Figure 3, A and B. After extensions at each pressure the wall was returned to 0.5 atm, and the deformation values determined. A typical young, rapidly growing wall (based on the growth rate of the previous 24 h) exhibits a marked increase in D_p between 4 and 5 atm, a value slightly below normal turgor pressure. Such a break in the strain *versus* stress curve is usually referred to as a yield stress (2). However, D_p occurs prior to this stress, indicating that a primary yield stress occurs below 2 atm. The yield stress between 4 and 5 atm can be considered a secondary yield stress. On the average, five growing ($1.5 \pm 0.2\%\ h^{-1}$) cells exhibited a secondary yield stress at 4.4 ± 0.2 atm. Old, nongrowing cells have a much reduced over-all extensibility, and show no secondary yield stress up to 5 atm (four cells tested). D_p and D_e are about equal in nongrowing walls.

Since plastic extensibility is often linked to growth (2), it is of interest to plot D_p as a function of the relative growth rate. With the exception of older cells (0–1% h^{-1}) there is no correlation between D_p and growth rate (Fig. 4A). Furthermore, there is a great deal of scatter in D_p values even for walls of the same growth rate. Again, we have assumed that all cells have a turgor pressure equivalent to 5 atm. There are probably slight variations in turgor from one cell to the next, and the applied stress may not always be equivalent to turgor, causing variations in D_t. However, since the D_t curve parallels the D_p curve (Fig. 4A), we used the D_p/D_t ratio to compensate for such differences. When D_p/D_t is plotted against growth rate (Fig. 4B) the scattering is reduced, but the D_p/D_t ratio appears to be constant over a 3-fold range of growth rates (1–3% h^{-1}). This indicates that the basic mechanical properties of growing walls are constant during much of development, and cannot account for differences in growth rates.

Time-dependent viscoelastic extension (creep), which is linear with log time, is characteristic of multiaxial as well as uniaxial extension (Fig. 5, A and B). Creep at pH 6.5 is greater in young

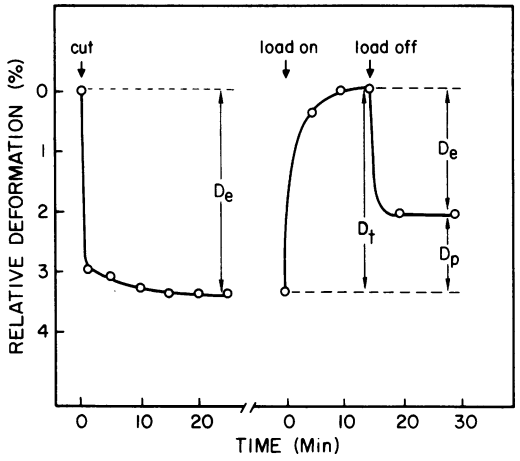

FIG. 2. Time course of the contraction which occurs when a living cell is cut (left side). The wall from the same cell is inflated with 5 atm (right curve). The pressure is released after 15 min. D_t consists of D_p and D_e.

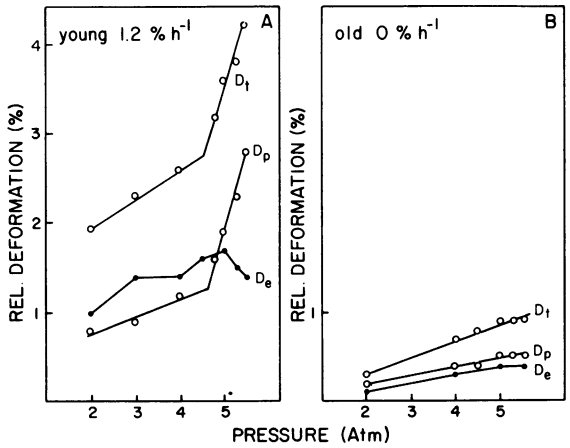

FIG. 3. Relation between the applied pressure and the deformation for multiaxially extended walls. A: wall taken from a young cell (growth rate 1.2% h^{-1}); B: wall taken from an old cell (growth rate 0% h^{-1}).

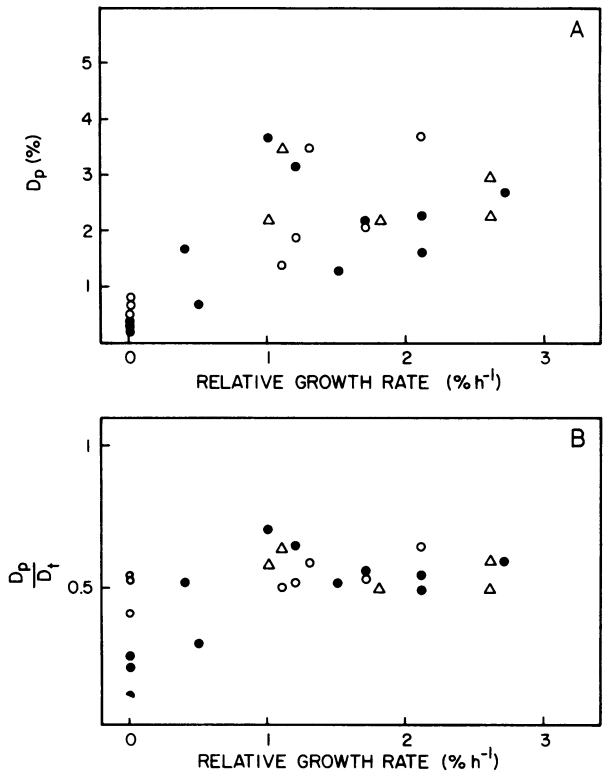

FIG. 4. A: D_p of multiaxially extended *Nitella* walls plotted against their relative growth rates *in vivo*. D_p was measured after 15 min, at which time extension was essentially complete. Pressure at 5 atm. B: ratio of D_p to D_t of *Nitella* walls plotted against their relative growth rates *in vivo*. (○): Standard buffer, wall extended directly from 0 to 5 atm; (●): standard buffer, wall extended stepwise as in Figure 3; (△): 10 mM Tris buffer, wall extended directly from 0 to 5 atm.

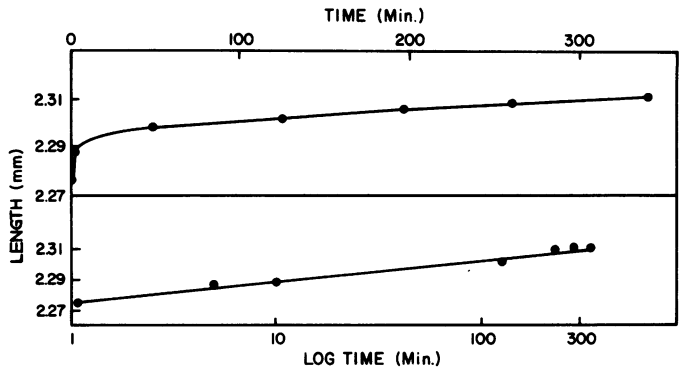

FIG. 5. Viscoelastic deformation of a *Nitella* wall under multiaxial stress plotted against time (upper curve) and log time (lower curve). Pressure at 5 atm.

cell walls than in old cell walls (Table I), as was found in uniaxial experiments (13, 17).

Role of Acid in Extension Growth. The major conclusion from the multiaxial extension studies is that wall mechanical properties do not adequately account for differences in the growth rates of *Nitella* cells until the final stages of maturation, when growth ceases. Thus, a metabolic factor is strongly implicated. In higher plants, protons have been implicated in the auxin-induced growth of stem and coleoptile tissues. We have shown previously that uniaxially stressed *Nitella* walls exhibit acid-enhanced extensibility (12, 14). The same effect was also observed under multiaxial conditions (Fig. 6). The wall was extended at pH 6.5, and the D_e and D_p were measured. The wall was then reextended at pH 4.0. This resulted in an additional extension, which was entirely due to an increase in D_p. In calculating acid-induced extension we have included the extension at pH 6.5 (Table II). Acid pH increased D_t and D_p, and decreased D_e. The response of young, growing walls was about twice that of old cell walls. The method for comparing creep rates is shown in Figure 7. Walls were extended for 15 min at pH 6.5, followed by an additional 15 min at pH 4.0 (Fig. 7A). Both parts of the curve were viscoelastic and linear when plotted against log time (Fig. 7B). The increase in vicoelastic strain (creep) caused by pH 4.0 is expressed as the per cent increase between the strain after pH 6.5 and the total strain after pH 4.0 treatment. It can be seen in Table I that there is a dramatic enhancement of the creep rate of growing walls, whereas the effects of acid on old walls was relatively small. Protons are thus extremely effective and specific regulators of extensibility in growing walls.

In *Nitella* it is possible to correlate cell wall pH and growth rates *in vivo*, due to the presence of alternating acid and alkaline bands along the length of each cell. These pH bands are caused by proton and hydroxyl ion excreting zones on the plasmalemma (20). The acid and alkaline zones can be visualized by plating the cells on agar containing phenol red. Growth on agar in the Kb medium continues for at least 48 h, although at about half the rate in liquid soil extract medium. The results of marking experiments on 15 different cells are shown in Table III. Growth is consistently greater in the acid region, although it is not always limited to the acid zone. Since it has previously been unequivocally shown by Green (5) that elongation is uniform, our results appear to be in conflict. The apparent conflict can be resolved, however, if cells grown under Green's conditions, *i.e.* autoclaved soil extract, do not produce pH bands. In our studies, cells grown in autoclaved soil extract and plated directly onto agar normally failed to produce pH bands. When regions were marked at random along the length of the cell, growth was observed to be uniform, as described by Green (data not shown). Thus, localized growth is dependent on the presence of pH bands.

From the growth rates alone one cannot discriminate between acid stimulation and alkaline inhibition of growth. In previous uniaxial studies we observed no inhibition of wall extensibility by alkaline pH (Metraux and Taiz, unpublished data). However, it is possible that precipitates of calcium carbonate, which are known

Table I. *Effect of 1 mM Mes-Tris Buffer pH 4.0 on Creep Rate of Multiaxially Extended Walls (p = 5 atm)*

Creep at pH 6.5 was measured as viscoelastic strain after 15 min. The solution was then changed to pH 4.0 and the total strain measured after an additional 15 min. Per cent change was calculated from the average strains after each treatment (±SE, N = 5).

Young Wall			Old Wall		
Strain pH 6.5	Total Strain pH 6.5 + 4.0	% Change	Strain pH 6.5	Total Strain pH 6.5 + 4.0	% Change
0.68 ± 0.2	2.08 ± 0.4	205	0.2 ± 0.03	0.3 ± 0.04	50

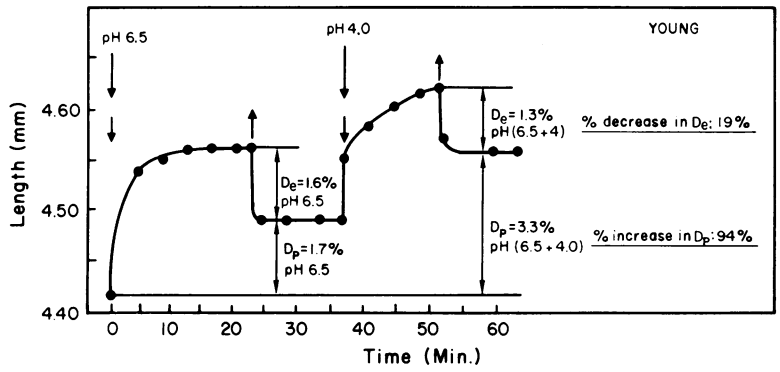

FIG. 6. Effect of 1 mM Mes-Tris buffer at pH 4.0 on D_p, D_e, and D_t of a young wall extended multiaxially at 5 atm for 15 min at each pH. Small downward arrows indicate load on; upward arrows indicate load off.

Table II. *Effect of 1 mM Mes-Tris Buffer at pH 4.0 on D_p, D_e, and D_t*

Walls were extended at 5 atm. Per cent increase in D_p, D_e, and D_t was calculated for 15-min extension at pH 6.5, followed by 15-min extension at pH 4.0. Average growth rate for the young cells: 1.3% h^{-1} ±0.4; average growth rate for old cells: 0% h^{-1} ±0. Percent change was calculated from averages indicated (±SE, N = 5).

	Young Wall ±SE			Old Wall ±SE		
	Rel. Deformation pH 6.5	Rel. Deformation pH 6.5 + 4.0	% Change	Rel. Deformation pH 6.5	Rel. Deformation pH 6.5 + 4.0	% Change
D_p	2.0 ± 0.5	3.7 ± 0.8	85	0.4 ± 0.04	0.6 ± 0.03	50
D_e	1.7 ± 0.1	1.3 ± 0.04	−24	1.1 ± 0.13	0.9 ± 0.08	−20
D_t	3.8 ± 0.4	4.1 ± 1.2	8	1.5 ± 0.08	1.6 ± 0.13	7
D_p/D_t	0.5 ± 0.04	1.2 ± 0.3	140	0.3 ± 0.04	0.4 ± 0.03	33

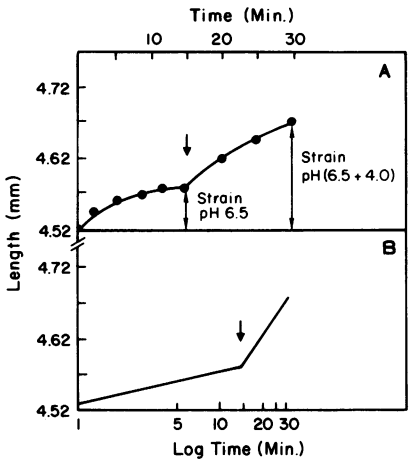

FIG. 7. A: effect of 1 mM Mes-Tris buffer at pH 4.0 on a multiaxially extended young wall under 5 atm pressure. B: replot of the same curve *versus* log time. Arrows indicate a change of solution from pH 6.5 to pH 4.0.

Table III. *Growth Rates of Acid and Base Regions of Nitella Internodes Plated on Agar*

Growth rates were measured as long as the bands were stable. Growth rates were found to be smaller in the Kb solution. Similar results were found in cells of *Nitella clavata*, a closely related species.

Cell No.	Time Measured	Growth Rate in:	
		Acid band	Base band
	h	% h^{-1}	
1	3.5	1.0	0
		0.5	0
2	16	0.32	0.05
		0.33	
		0.26	
3	4	0.4	0.2
		0.25	
4	3	0.17	0.06
5	1.2	0.2	0
6	1.3	0.9	0
7	3	0.9	0.07
8	1	0.6	0
9	0.5	0.6	0
		0.8	
10	1.5	0.2	0
11	1.2	1.5	0.13
12	1	0.7	0.5
13	1	0.6	0
14	1.2	0.41	0
		0.26	
15	1.3	0.25	0
		0.4	

to be associated with the alkaline zones of *Chara* cells (1), reduce wall extensibility in the alkaline region. Although no deposits were visible in our cells, we tested this by measuring the relative extensibilities of the acid and alkaline zones. As shown in Table IV, there were no significant differences in the mechanical properties of the two zones. Similar results were obtained using a uniaxially applied force (data not shown). Thus, enhanced growth in the acid zone is under continuous metabolic control.

If growth is regulated by proton excretion, it should be possible to inhibit growth in the acid region with alkaline buffers and to stimulate growth in the alkaline regions with acidic buffers. Addition of 50 mM Mes-Tris buffer (pH 5), promoted growth in base bands, while the same buffer at pH 9 inhibited growth in the acid bands (Fig. 8). The inhibitory action of pH 9 is not due to a loss of turgor, since readdition of pH 5 buffer restores the high growth rate (Fig. 8D). Similar, but less dramatic, results were obtained with 5 mM Mes-Tris buffer (data not shown).

It remains to be demonstrated that the pH of the acid band region is sufficiently low to enhance wall extensibility. We reported previously that the pH threshold for acid-stimulated extension is 4.5 using 1 mM buffer concentrations (14). In *Chara*, Lucas has measured pH values as low as 5.0 in the acid regions (Ref. 11 and personal communication). We have attempted similar measurements of the pH bands of *Nitella*. Using a miniature pH electrode, we measured a pH of about 9.0 in the base zones and 5.5 in the acid zones. Thus, there seems to be a discrepancy between the pH threshold for acid-enhanced growth and the actual pH of the wall. It is possible that the pH of the outer surface is higher than the pH of the inner surface due to the buffering capacity of the wall. This possibility was tested by perfusing the inside of an empty wall cylinder with unbuffered HCl at pH 4 (Fig. 9). Droplets forming on the outside of the wall were collected and the pH was determined at various time intervals. The total

Table IV. *Growth Rate and Mechanical Properties of Acid and Base Regions of Nitella Internodes*

Growth rates of acid and base regions measured in *Nitella* internodes plated on agar. The same regions were then extended at 5 atm in standard buffer for 15 min (±SE, N = 4).

	Acid Band	Base Band
Growth rate (% h^{-1})	0.45 (±0.13)	0 (±0)
D_p (%)	1.7 (±0.45)	1.9 (±0.5)
D_e (%)	1.4 (±0.13)	1.8 (±0.9)
D_t (%)	3.1 (±0.6)	3.7 (±0.9)
D_p/D_t	0.5 (±0.09)	0.45 (±0.2)

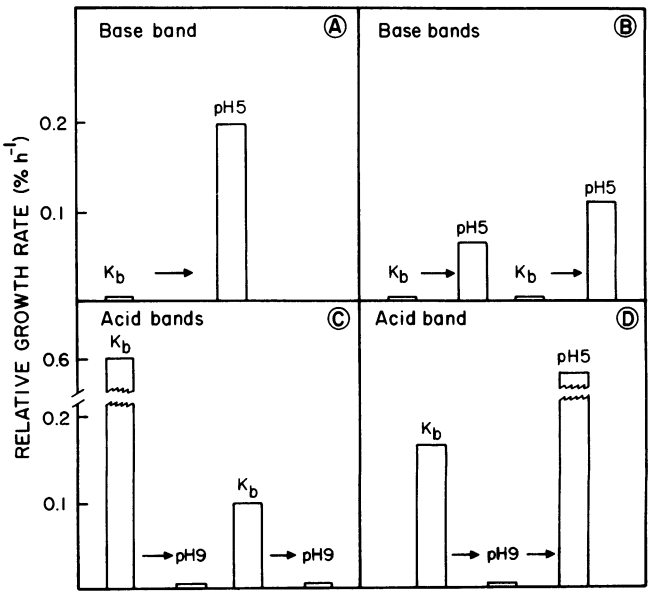

FIG. 8. Reversibility experiments on living *Nitella* cells producing acid and alkaline bands. Growth rates were measured between 1 and 4 h. Cytoplasmic streaming continued undiminished throughout all treatments. A: a nongrowing base band (cell in Kb solution) is acidified with a buffered solution at pH 5; B: two base bands (Kb) are treated with buffered pH 5 solution; C: growing acid bands (Kb) are made basic with buffer solution at pH 9; D: an acid band (Kb) is made basic (pH 9). The same region is then acidified at pH 5. Buffer solutions are 50 mM Mes-Tris in Kb solution.

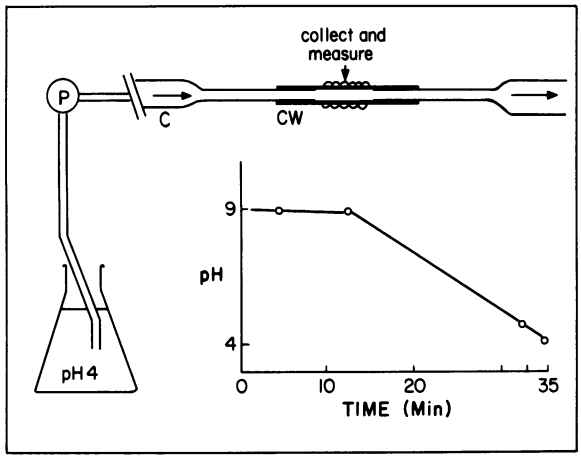

FIG. 9. Experimental set-up for the proton binding experiment. A solution of HCl at pH4 was perfused by means of a peristaltic pump (~1 ml/min) through two capillaries joined together by a *Nitella* wall cylinder (CW). Due to a slight back pressure in the system, small droplets exuded through the wall. These were quantitatively collected and their pH measured. The time course of the pH change of the droplets is shown.

volume of the droplets which crossed the wall was equal to 28 μl. As shown in Figure 7 the outer pH had risen to 9 during the first 10 min, even though the pH of the solution inside the wall was 4. Apparently, the wall completely buffered the first 14 μl of HCl. The outer surface pH took 35 min to equilibrate with the inner surface pH. Since the volume of HCl which was completely buffered with 14 μl (for simplicity the protons bound after 14 μl are ignored) and the proton concentration of the inner solution was 10^{-4} μeq/μl (pH 4), the wall (7.7 μg dry weight) was able to bind a minimum of 14×10^{-4} μeq H^+, or 0.18 μeq/mg dry weight. This value is somewhat lower than the value of 0.5 μeq/mg obtained by Morikawa (15) using a titration method.

Assuming that the buffering capacity of the wall is maintained by exchange with the ions of the external solution, and that protons rather than buffers are excreted, the pH of the inner wall surface should be lower than the pH of the outer surface by an amount equivalent to the steady-state proton-binding capacity of the wall. The proton-binding capacity we measured is only about one-sixteenth the maximum binding capacity reported by Morikawa (15) for walls at pH 3, and is therefore a conservative estimate of the wall's steady-state buffering capacity. Inasmuch as we determined that the external pH of the acid band region of the wall was 5.5, it is interesting to estimate what the pH of the solution in contact with the inner wall surface would be, assuming that 14×10^{-4} H^+ were bound by the wall during passage to the outside. In other words, $[H^+]_{inner} = [H^+]_{outer} + [H^+]_{bound}$. The $[H^+]_{outer}$ is $10^{-5.5}$ μeq/μl or 3.16×10^{-6} μeq/μl. In the perfusion experiment, 14×10^{-4} μeq H^+ were bound per 28 μl crossing the wall; hence, the $[H^+]_{bound} = 14 \times 10^{-4}$ μeq/28 μl, or 5.0×10^{-5} μeq/μl. The $[H^+]_{inner}$ is therefore $3.16 \times 10^{-6} + 5 \times 10^{-5}$ μeq/μl or 5.316×10^{-5} μeq/μl, which corresponds to a pH of 4.27 at the inner wall surface. This calculation emphasizes that the inner surface pH can be significantly lower than the outer surface pH, although the magnitude of the gradient will vary with the conditions.

In light of the wall's buffering capacity we thought it worthwhile to reexamine the pH threshold for acid-enhanced wall extension using various buffer concentrations. It was found that with 1 mM Mes-Tris buffer, acid-induced wall extension begins at pH 4.7, while with 10 mM buffer or greater, the threshold occurs at pH 5.3 (data not shown). The actual pH threshold is 5.3, which is approximately the value obtained by measuring the pH of the outer wall surface. The estimated inner solution pH (~4.3) is well within the stimulatory range of protons.

DISCUSSION

Technique of Mercury Inflation. Kamiya *et al.* (10) developed the technique of mercury inflation to study the elastic properties of mature *Nitella* cell walls. Volume changes were determined from the volume of mercury entering the wall cylinder as it passed through a graduated capillary tube. Length was measured microscopically by monitoring a single point. Diameter changes were calculated from the length and volume. In their experiments, applied forces of up to 6.45 atm resulted in volume increase curves similar to the viscoelastic extension shown in Figure 5. Little or no D_p occurred, and the D_t in length was about 1%. The absence of D_p was considered to be consistent with the nongrowing status of the mature cells from which the walls were taken. These results differ from ours (see Fig. 3B), since walls from mature cells exhibited roughly equal amounts of plasticity and elasticity, although D_t was drastically reduced compared to a growing cell wall. It is possible that irreversible extension was completed before Kamiya *et al.* began their measurements, since a relatively high pressure, 2-3 atm, was used to fill the wall with mercury.

Kamiya *et al.* (10) also found a hyperbolic relationship between strain and stress, *i.e.* strain was greater at lower pressures than at higher pressures. In contrast, we observed a linear relationship for nongrowing cells and a J-shaped curve for growing cells, due to the presence of a yield point (Fig. 3, A and B). The two experiments may not be directly comparable, since in our study the walls were returned to 0.5 atm prior to each turgor step-up while in the experiment of Kamiya *et al.* the pressure was raised without an intervening period of elastic recovery. We do not think this can account for the discrepancy, however, since we have found no effect of omitting the elastic recovery phase on the stress/strain curves of growing cell walls (Metraux, Richmond, and Taiz, unpublished data). In experiments of this kind, one possible source of error is the elasticity of the glued junction between wall and inlet tube. Using the same glue as Kamiya *et al.* (beeswax/rosin), we have observed significant elastic stretching at this region. Measuring the volume increase or length change of a total cell wall could introduce a significant error, with the mechanical properties of the glue masking those of the wall. It is necessary to measure the distance between two points on the wall to avoid such artifacts.

Significance of Extensibility Measurements. Cleland (2) has pointed out that the significance of extensibility data is problematical, since it is not at all clear what is being measured. Cleland defined "instantaneous plastic extensibility" (PEi) as "the potential of the wall at a particular moment to undergo turgor-driven plastic extension" (2). PEi values obtained by uniaxial stress techniques, such as irreversible deformation under constant load or Instron analysis, are generally about 10 times in excess of the estimated *in vivo* PEi (2). The extra extension is a consequence of microfibril reorientation in response to a unidirectional applied force (18). Cleland has suggested that the plastic compliance (strain/stress) may also represent loosening events which accumulated during the previous 60–90 min of growth (2). The example is given of a material which exhibits low plasticity when extended twice in the same direction by the same force, but which exhibits enhanced plasticity when the direction of the applied force is altered. According to the model, unused loosening events can be expressed only when the direction of stress is changed (*e.g.* from multiaxial to uniaxial). Since in *Nitella* there can be adjacent regions of the same wall with different growth rates, one would predict that the acid regions would have a higher plastic extensibility than the base regions. However, the mechanical properties of the two regions were found to be equal under multiaxial or uniaxial conditions. Thus, irreversible deformation measurements do not reflect the previous growth rate of the wall.

Under multiaxial conditions, microfibril reorientation is negligible and, as expected, D_p values are significantly less than under uniaxial conditions. Some insight into the nature of D_p was gained

when it was discovered that walls extended at 5 atm pressure (equal to turgor pressure) do not expand beyond their maximum length attained prior to cell excision, at least during the first 15 min. Nevertheless, the first *in vitro* extension contains a significant irreversible component. Since no new extension has occurred, this D_p represents a special case of wall plasticity caused by contraction of a living cell followed by reextension in the absence of metabolism. It is as if the wall underwent a slight amount of irreversible shrinkage and must break bonds simply to return to its original length. Apparently, wall polymers rearrange in such a way that D_e is converted to D_p without increasing total extensibility. The mechanism of this conversion remains obscure. It appears to be a once-only event, since further *in vitro* contraction-expansion cycles are entirely elastic, *i.e.* the wall is conditioned.

Wall Extensibility, pH, and Growth. What is the relationship between wall extensibility and growth? There is a rough correlation between the D_p and the growth rate of mature, slow growing cells. Since this is a period when a random secondary wall is being deposited (6, 7), we postulate that D_p reflects differences in wall structure or composition. Amoung young, rapidly growing cells D_p is highly variable, even for cells of the same growth rate. We feel that this variability is for the most part an artifact caused by the failure to mimic turgor pressure precisely for each cell. This causes variations in D_t which will affect D_p. This variability can be reduced by expressing the plasticity as a fraction of the total deformation (D_p/D_t). However, neither D_p nor the D_p/D_t ratio correlates with the growth rates of younger cells, suggesting that the primary wall structure is essentially the same among rapidly growing cells.

The property most often correlated with growth rate is creep (13, 14, 17), but in the case of multiaxial extension, creep beyond 15 min is too low to be readily measured. The low levels of creep *in vitro* suggest that metabolic input is required for rapid extension *in vivo*. In this paper we have focused on the role of proton extrusion.

When *Nitella* cells are transferred from soil extract to Kb medium in the light, they begin to excrete protons and hydroxyl ions in discrete pH bands (11, 20). Concurrently, growth shifts from diffuse growth to localized growth in the acidic regions. Although acid and base regions of the wall have similar mechanical properties, there is almost no growth in the alkaline region. This is consistent with the extremely low levels of creep at pH > 5.3. At pH 5.3 or lower, multiaxial creep is dramatically stimulated. The pH dependence of creep thus parallels the pH dependence of growth.

Is the pH of the acid band acidic enough to enhance the creep rate? Direct measurements yielded pH values of 9.0 in the alkaline band and about 5.5 in the acid band. However, the outer wall pH is likely to be higher than the inner wall pH. Our own experiments and those of Morikawa (15) confirmed that the wall has a considerable ability to bind protons. Dainty and Hope (3) have estimated that the concentration of indiffusible anions in *Nitella* walls is about 0.6 eq/l. When the proton-binding capacity of the wall is taken into account, the pH of the inner wall solution (the space between the plasmalemma and the inner wall surface) was estimated to be about 4.3 in the acid band. In the accompanying paper (18) we have demonstrated that the inner 25% of the wall governs the wall's mechanical properties. This information, plus the fact that growth in the acid region is reversed by alkaline buffers (and vice versa) constitutes a strong argument in favor of proton-regulated growth in *Nitella*.

It is almost certain that wall synthesis is required for linear growth. Mechanically extended walls rapidly strain-harden, and protons merely delay this process. The metabolic event which confers continuous plastic extensibility to the wall is likely to be primary wall deposition. Experiments are underway to determine whether deposition is also localized in the acid bands. Preliminary results indicate that wall deposition occurs randomly. If this is the case, the role of primary wall deposition is to maintain the plasticity of the wall, while the role of protons is to allow the plasticity to be expressed as continuous linear growth in the presence of constant turgor pressure.

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