MASTER

STUDIES ON THE CONTROL OF CELL WALL EXTENSION

Yearly Progress Report

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I. Introduction

The research in this laboratory is centered around the question as to how plant cell enlargement is controlled and regulated at the cellular level. Cell enlargement is influenced by many factors, but most of them act indirectly. For example, growth is altered by such environmental factors as light and temperature, and by internal factors such as the genetic complement of the cells, yet none of these directly control cell enlargement. The metabolism of cells, including the production of ATP and the synthesis of RNA, can have a major impact on cell enlargement, but again the effects are indirect. Theoretical considerations, and the research of Lockhart, Green and myself have shown that the growth rate (dV/dt) of cells can be defined in terms of five parameters:

$$dv/dt = \frac{L'p \cdot f(dW/dt \cdot WLC) \cdot (\pi_c - \pi_o - Y)}{L'p + f(dW/dt \cdot WLC)} \quad (1)$$

where $L'p$ is the hydraulic conductivity of the tissue, dW/dt is the rate of export from the cell to the wall of a wall loosening factor, WLC is the capacity of the wall to be loosened in response to the wall loosening factor, π_c and π_o are the osmotic potential of the cells and outside solution, respectively, and Y is the yield threshold for wall expansion. When a cell is induced to expand, the expansion must occur by a change in one of more of these five parameters. Likewise, when growth ceases it must be because of changes in these parameters. The basic goal of my research is to assess each of these parameters under conditions where growth is being enhanced

or inhibited, with the purpose of explaining at the cellular level just how growth is being controlled.

In the past most of our work has been concentrated on one single situation; the induction of cell elongation in the oat coleoptile by the hormone auxin. Addition of auxin causes the growth rate of coleoptile sections to increase by at least 5-fold after a lag of only about 10 minutes, making it one of the fastest hormonal responses known in plants. Our research during the 60's provided strong evidence that the effect of auxin is to increase the extensibility of the cell walls. All the indications were that auxin increased the export of a wall loosening factor from the cells to the walls (dW/dt) but the identity of W was not known. Then in 1970-71, Hager and coworkers in Germany and Rayle and myself proposed that the wall loosening factor was simply hydrogen ions. The "acid-growth" theory which Hager and ourselves formulated states that auxin causes the cells to excrete protons, and that the resulting lowered pH of the walls activates some polysaccharide degrading enzymes in the walls, with the result that the wall becomes loosened and cell expansion takes place. It should be noted that this mechanism is similar to the one involved in protein digestion in the stomach, where the hormone gastrin induces the excretion of protons (HCl) into the stomach, and the lowered pH activates the enzyme pepsin. In the years following the formulation of the acid-growth theory we were principally involved in obtaining evidence as to whether the theory was correct or not. We have now accumulated an impressive amount of data which seemed to indicate that protons are the wall loosening factor in auxin-induced growth of stem and coleoptile cells.

In the course of these studies it became apparent that while changes in the rate of export of the wall loosening factor (hydrogen ions) might be sufficient to explain the continuing control of long-term cell

elongation. We therefore decided to direct our attention to the other four cellular parameters in order to learn more about their physical and biochemical nature, and to learn whether changes in one or more of these parameters might control the growth rate.

Two years ago we initiated a study of the role of π_c in the control of growth. The driving force for wall extension is the turgor pressure, which is proportional to the osmotic concentration of the cell as long as $L'p$ is not limiting (which we believe to be the case). As a cell takes up water during growth, it dilutes out the OC and the turgor pressure falls, and thus the growth rate decreases unless osmoregulation takes place; i.e., unless there is the uptake or generation of new osmotic solutes so as to maintain the OC. It had already been demonstrated by Ordin et al. in 1956 that osmoregulation occurs during the auxin-induced growth of Avena coleoptiles, but it was not known what effect auxin has on this process or how it is controlled. We initiated a study of osmoregulation, using intact coleoptile sections. We demonstrated the following. First, coleoptiles can osmoregulate using sucrose, glucose, NaCl or KCl as the source of absorbable solutes. In the absence of auxin the OC slowly increases, while in the presence of auxin the OC remains nearly constant and is always below that of the control sections. Total osmotic solutes (TOS) increase with or without auxin, but the increase is about 60% greater if auxin is present. By inhibiting growth with CaCl_2 or mannitol we showed that the auxin-induced increase in TOS is, in fact, not a direct response to auxin, but is a response to the auxin-induced growth, since the auxin effect is completely eliminated whenever growth is inhibited osmotically. We concluded from these studies that the limiting factor for the uptake of osmotic solutes might well be the rate of their entry into the section through the cut surfaces (because of the impermeability of the cuticle, little uptake occurs along the longitudinal surface of the

section). Auxin, by causing increased water uptake, caused more solutes to enter the tissue and thus ultimately, enter the cells. We predicted that if the cuticle were removed from the surface of the section, uptake of osmotic solutes would be increased significantly, and that auxin would have no effect on the OC of the tissue.

During the past year we have tested these predictions by examining the change in OC and TOS which occurs in peeled sections (i.e., sections whose epidermis and cuticle has been removed) upon incubation in a sucrose solution. The results of these studies will be presented in Section IIA.

The second factor which we have been concerned with is the wall extensibility. It is now generally accepted that wall extension involves a series of steps, each of which consists of the breakage of some load-bearing bond in the wall (wall loosening). The problem was, how should we measure the wall extensibility parameter, m ? This was first done by such indirect methods as bending techniques, but in 1966 a new technique, the Instron technique, was introduced by Olson et al and by myself, and it was proposed that the plastic extensibility measured with the Instron was, indeed, a measure of m . Since then the Instron technique has been widely used as a measure of wall extensibility with a variety of tissues under a variety of conditions. We have had our doubts, however, as to whether it really measures the parameter m from equation 1, or whether it measures some other, related property of the wall. During the past year we have completed a study to learn more about the relationship between Instron extensibility and the m parameter. These results, and the conclusions which we have reached, are presented in Section IIB.

The third parameter with which we have been concerned is the wall loosening capacity (WLC), which is the ability of walls to undergo wall

loosening when given constant stress and an optimal concentration of the wall loosening factor, protons. This is measured by placing frozen-thawed sections under constant tension, giving them a pH 3 solution and measuring the rate of extension one hour later. We have shown previously that the WLC is increased by auxin, and that this effect may be a direct effect of auxin. The increase in WLC could be due to increased activity of the wall loosening enzymes, either by increased concentration of enzyme or by greater activity of existing enzymes, or it could be due to increased availability of sites for action by the enzyme. Unfortunately, our original method for measuring WLC did not allow us to distinguish between these two possibilities. Mark Tepfer, who was then a graduate student with me, attempted to fit the acid-extension curves for frozen-thawed sections to various equations, and determined that the acid-extension curve for coleoptile walls fits the first order equation:

$$l = b - ae^{-kt} \quad (2)$$

where l is the length, t the time after addition of acid, and a , b and k are constants. We would predict that the constant a would be a measure of the number of sites available for attack and the constant k would reflect the activity of the wall loosening enzymes. Since current theories state that the protons act by activating the wall loosening enzymes, we should expect a decrease in pH to increase k without affecting a . To our surprise, the effect that we got in a preliminary experiment was that lowered pH increased a rather than k . It was apparent that we must fit all our extension data to equation 2, but since this can be a very time-consuming process, we elected to develop a more automated system where the extension transducers are mated directly to a microcomputer which stores the data, and then fits it to equation 2. We have spent considerable time this year getting such a system operational; details about this are contained in Section IIC.

IIA. Studies on Osmoregulation and its Control

The procedure used in these studies is as follows. Avena coleoptile sections were first separated from their enclosed leaves, then the epidermis was peeled off with fine forceps. Sections (5 mm) were then incubated in 20 ml of 0.1 mM Mes-tris buffer, pH 6.0 + 2% (w/v) sucrose for varying periods of time before harvest. Harvest consisted of first measuring the lengths of the sections on a microscope fitted with a stage micrometer, followed by a rapid removal of any liquid in the central cavity with air, and freezing in foil packets on dry ice. Groups of 4 frozen sections were thawed on a small filter paper disc, the cell contents were pressed out, and the OC of the cell contents were measured using a Wescor Micro-osmometer. These OC values are ca 10-12% low because of apoplastic solution, but we have not corrected for this error since it would appear to remain constant. A relative value for total osmotic solutes was calculated by multiplying OC times the section length, and a Δ TOS value was calculated by the equation:

$$\Delta\text{TOS} = \frac{\text{TOS}_f - \text{TOS}_i}{\text{TOS}_i} \times 100 \quad (3)$$

where f and i stand for final and initial values, respectively.

We obtained the following results. First, we have confirmed the prediction that removal of the cuticle will result in a greater uptake of osmotic solutes. This is shown in Table 1. Note that the OC of the peeled sections has risen considerably over a 9 hr incubation period, in contrast to the situation for unpeeled sections where there has been only a slight change in OC. We followed the changes in OC and Δ TOS over a 30 hour time period, with and without auxin (Fig. 1). The OC increases for the first 8 hours at the same rate, with or without auxin. At that point the OC of auxin-treated sections reaches a plateau value, while the OC of the controls continues to increase and reach its plateau value only after 20-24 hours. Thus while auxin has no

effect on the initial rise in OC, it does result in a plateau OC which is 70-120 mOs below that of the controls. Because the auxin-treated sections are elongating more rapidly than the controls, Δ TOS is greater + auxin at all times.

We then asked whether the auxin-induced increase in Δ TOS and the auxin-induced decrease in plateau OC were direct effects of auxin or whether they were only a response to the auxin-induced growth. To test this we inhibited growth osmotically with PEG (Table 2) so that auxin-induced growth was eliminated. This removes any effect of auxin on Δ TOS, indicating that this is an indirect effect of auxin. The difference in plateau OC, on the other hand, is reduced, but not eliminated. Thus it appears that auxin exerts a direct effect on the final plateau OC level which is reached by the sections. We are planning to study possible mechanisms by which auxin might be exerting such an effect this year.

What determines the equilibrium plateau OC of coleoptile cells? In many algae the controlling factor is the turgor pressure; as the pressure increases, solute uptake is decreased and solute efflux is increased until a plateau OC is reached. We have examined the possibility that the same type of control might exist with coleoptiles, and have eliminated turgor as a controlling agent. First of all, the reduction in turgor produced by PEG does not result in any increase in plateau OC in control sections (Table 2) as would be predicted from such a theory. Secondly, when we incubated sections in a solution containing two different absorbable solutes, sucrose and NaCl, the plateau OC reached was higher than that ever reached with either solute alone (Table 3). It would appear that each absorbable solute has a maximum concentration which it can reach in cells, and that this maximum is independent of the concentrations of any other osmotic solutes. We have concluded that the control of solute uptake occurs independently for each solute and is not

under the control of turgor. It is interesting to note that auxin reduces the plateau OC by about the same amount whether the absorbable solute is NaCl or sucrose.

This work was conducted, to the largest extent, by Tom Stevenson, with the help of Ann Dreyer. A manuscript covering the work on unpeeled sections has been accepted for publication in Plant Physiology. A second manuscript on the work with peeled sections is being prepared, also for Plant Physiology.

B. The Relationship Between Instron Extensibility and Wall Extensibility

In the Instron technique, sections are incubated in the desired solution, killed in boiling methanol and extended twice to 30 g. load in an Instron extensometer. From the slope of the two load-extension curves one can calculate elastic (DE) and plastic (DP) compliance values. We originally suggested that DP was a measure of the wall extensibility parameter, m , in equation 1, but in recent years we have come to wonder about this interpretation. We decided to reinvestigate this matter.

If DP is a measure of m , it should increase and decrease in parallel or before the changes in the growth rate. To test this we have incubated Avena coleoptile sections in auxin solutions for varying lengths of time and determined the time course for changes in DP following addition of auxin (Fig. 2). From parallel experiments we knew that the growth rate begins to increase about 10 minutes after addition of auxin, and reaches a maximum 20-30 minutes later. DP, on the other hand, rises more slowly, reaching a maximum after 100 to 200 minutes. Clearly the changes in DP follow rather than precede the changes in the growth rate. We have also tested the situation where growth is inhibited by KCN. Here the growth rate falls to near zero within 5 minutes after addition of KCN, but the DP does not reach a minimum value until 45-90 minutes later (Fig. 3). Again the changes in

DP follow the changes in growth rate, rather than preceeding them as expected if DP is a measure of wall extensibility.

Another test is to do a residual growth experiment. Here sections are treated with or without auxin under conditions where growth is prevented osmotically, and are then transfered to water and allowed to expand under conditions where the further action of auxin is prevented by the presence of argon (Table 4). Sections which have been pretreated with auxin undergo greater expansion during the water-argon treatment than do control segments. If DP is equivalent to m , the expansion in water-argon should use up any auxin-induced increase in DP and DP of auxin-treated sections should fall. On the contrary, DP actually increases during the expansion period.

We have concluded that DP is not a measure of m ; i.e., it does not measure the capacity for the walls to extend during the next increment of time. We propose, instead, that DP is a measure of the rate of wall loosening events over the immediate past. In other words, it is a measure of past wall extensibility rather than future wall extensibility. We believe that it works this way for the following reason. When walls are subjected to a particular set of force vectors, they extend a certain amount, which depends upon the number of wall bonds which have been broken. If the force vectors are changed even slightly, the walls become capable of additional extension. At the moment cells are killed, the walls possess little or no capacity for extension under the force vectors of turgor, because any possible extension will have already occurred. Whenever bonds are broken by the wall loosening enzymes, the potential for new extension will be immediately converted into extension by turgor. But whenever the walls are subjected to a different set of force vectors, additional extension can occur as a result of the already broken bonds. If the force vectors are once again changed, another increment of extension can be obtained. This can be demonstrated by subjecting

sections to extension in the Instron; the first extension is both plastic and elastic, while subsequent extensions are entirely elastic. However if the walls are removed from the Instron clamps and then replaced (which results in a slight shift in the force vectors), the plastic extensibility of the walls is reestablished (Fig. 4).

We have concluded, therefore, that DP is a measure of the rate of past wall loosening events. As such it is a very useful measurement. Take the following example. You add cytokin to radish cotyledon pieces and growth is enhanced; is this due to an increase in wall extensibility? To test this you measure the DP of walls before and 2-3 hours after growth is initiated by the cytokinin. If the growth involves an increase in wall extensibility, the DP of the cytokinin treated sections should have increased, while if the increased growth was entirely due to increases in amount of osmotic solutes, there should be no difference in DP between cytokinin-treated and control segments. We have conducted such DP measurements on radish cotyledons for Dr. Cleon Ross, and have shown that cytokinin does cause a large increase in the rate of wall loosening.

A final possibility which we had to consider was that DP was not a measure of wall loosening, but only a reflection of the rate of cell elongation in the immediate past, regardless of the cause of the growth. We have tested that possibility in the following way. We know that small changes in turgor, by changing the effective driving force for wall extension cause large changes in the growth rate without affecting proton excretion to any appreciable extent. We therefore grew coleoptile sections under conditions of normal turgor (A) or slightly reduced turgor induced by 0.06 M mannitol (AM), then transferred half of each group to fresh A or AM solutions and measured the growth and DP after 6 hours (Table 5). In either case, the sections in A grew more rapidly than those in AM, and if DP was simply a reflection of the past

growth rate, we would expect the DP of these sections to be greater. On the contrary, the DP of the sections in AM was always greater than that of the A sections. We believe that this difference is due to the fact that sections in AM undergo some wall loosening which is not converted into extension because of the reduced turgor. We therefore have concluded that DP is not directly related to the growth rate, but rather to the rate of wall loosening.

This work is being written up for publication in *Planta*.

C. Studies on the Wall Loosening Capacity

In measurements of the WLC frozen-thawed sections are subjected to a constant applied force (10-20 g) in a pH 7 solution, the solution is changed to one at pH 3 and the resulting acid-extension curve is recorded. The acid-extension data are then fitted to equation 2 to obtain a and k values, which characterize the extension. These experiments are very time-consuming for two reasons. First, each acid-extension run takes about 3 hours to complete; a complete experiment normally requires at least two weeks to run as a result. But even more time-consuming was the procedure of transcribing the data from the chart records onto cards and then running the card on the computer. To eliminate this step we have developed a system in which the extension data are fed directly into a Horizon microcomputer to be stored on discs, and then the data are fitted to equation 2 using a program written for this purpose by Dr. Cosgrove.

During the past year we have been engaged in putting this system together and getting it working. It took about 6 months to obtain the necessary components and to get them assembled. We then spent about 3 months testing out the system. As a result we have only recently begun to collect data with

it. The first series of experiments concerned the effect of auxin on the WLC. Previous results suggested that auxin causes an increase in the WLC, and we wanted to know if this was an effect on the enzyme activity (k) or on the number of sites available for enzymatic attack (a). To test this coleoptile sections were incubated for 6 hours with or without auxin, and acid-extension curves were obtained from sections both before and after the incubations. As shown in Table 6, there is a slight reduction in k values during the incubations, but auxin has little effect on this. In the absence of auxin the a value falls by about 25%, while in the presence of auxin the a value increases by 20%. As a result the a value, and thus presumably the number of sites available for attack by the wall loosening enzymes, is about 65% greater in the auxin-treated walls as compared with the controls after 6 hours.

III. Personnel Connected with this Research

Dr. Robert E. Cleland, Principal Investigator

Dr. B. Rubinstein, Professor of Botany, University of Massachusetts,
summer 1980.

Thomas T. Stevenson, graduate student

Richard Stout, graduate student

Elizabeth Van Volkenburgh, graduate student

Sheryl Ann Dreyer, technician

Dr. Daniel Cosgrove, Postdoctoral Research Associate, November 1979 to
March 1980.

IV. Degrees Received

Elizabeth Van Volkenburgh, Ph.D., May 1980

Richard Stout, Ph.D., August 1980

Thomas Stevenson, M.S., August 1980

V. Publications Resulting from this Research

Bates, G.W., and R.E. Cleland. (1980). Protein patterns in the oat coleoptile as influenced by auxin and by protein turnover.

Planta 148: 429-436.

Stevenson, T.T., and R.E. Cleland. (1981). Osmoregulation in the Avena coleoptile in relation to auxin and growth. Plant Physiol. in press.

Rayle, D.L., and R.E. Cleland. (1980). Evidence that auxin-induced growth of soybean hypocotyls involves proton excretion. Plant Physiol., in press.

Cleland, R.E. (1980). Auxin and H^+ -excretion: the state of our knowledge. In: Proceedings in Life Sciences, ed. F. Skoog, Springer Verlag: Berlin. pp. 71-78.

VI. Seminars and Talks Given on this Research

Washington University, St. Louis, Physiology Dept., Nov. 1979

Washington University, St. Louis, Biology Dept., Nov. 1979

Univ. of Nebraska, Biology Dept. (2 seminars), March 1980

Symposium, Washington Section, ASPP, and USDA, Beltsville, MD, April 1980

American Soc. Plant Physiology meetings, Pullman, WA, August 1980.

Poster given by T.T. Stevenson and R.E. Cleland on the control of osmoregulation by auxin.

Table 1. Effect of removal of cuticle on the uptake of osmotic solutes by Avena coleoptile sections.

Treatment	$\Delta L, \%$	OC, mOs	$\Delta TOS, \%$
Unpeeled, initial	-	234	-
" , + IAA	46	246	53
" , - IAA	6	292	32
Peeled, initial	-	242	-
" , + IAA	27	310	63
" , - IAA	12	328	52

Oat coleoptile sections incubated in 0.1 mM Mes-tris, pH 6.0 + 2% sucrose for 9 hrs.
IAA, 5 $\mu\text{g/ml}$

Table 2. Effect of osmotic inhibition of growth on auxin-induced increase in ΔTOS and auxin-induced decrease in plateau OC.

Treatment	8 hours			25 hours	
	$\Delta L, \%$	OC, mOs	$\Delta TOS, \%$	$\Delta L, \%$	OC, mOs
Initial	-	259	-	-	259
-PEG, + IAA	14	368	63	57	379
-PEG, - IAA	4	370	48	22	475
+ PEG, + IAA	0	340	31	8	447
+ PEG, - IAA	0	345	33	3	471

Sections were incubated for 8 or 25 hours in 0.1 mM Mes-tris, pH 6.0 + 2% sucrose and + 5 $\mu\text{g/ml}$ IAA, and with or without PEG 4000 sufficient to give a solution OC of 180 mOs.

Table 3. Effect of having two absorbable solutes on plateau OC

Treatment	IAA	$\Delta L, \%$	OC, mOs	$\Delta TOS, \%$
Sucrose	+	61	387	147
Sucrose	-	8	478	104
NaCl	+	45	415	138
NaCl	-	7	533	126
Sucrose + NaCl	+	77	524	268
Sucrose + NaCl	-	12	644	185
Initial		-	252	-

Sections incubated for 25 hrs in 0.1 mM Mes-tris, pH 6.0 ± 5 $\mu\text{g/ml}$ IAA and with 20 mM NaCl, 40 mM sucrose or both. When only one solute was used, 40 mM mannitol was also present so that all solutions had the same OC.

Table 4. Demonstration that Instron extensibility is not used up during extension.

Treatments		$\Delta L, \%$	DP
1st 90 min	2nd 120 min		
Auxin + man,	- - -	-0.02	44
Auxin + man,	auxin + man	-0.03	52
Auxin + man,	water + argon	+0.23	65
Mannitol,	- - -	+0.02	40
Mannitol,	mannitol	+0.01	43
Mannitol,	water + argon	+0.13	44

Sections incubated for 90 min in 0.1 M mannitol in 25 mM K-maleate buffer, pH 4.7, ± 5 $\mu\text{g/ml}$ IAA, then transferred to fresh solutions or to water + argon. ΔL and DP determined. Units of DP are $10^{-11} \text{ cm}^2/\text{dyne}$.

Table 5. Lack of correlation between DP and growth rate when growth rate is altered by turgor shifts.

Treatment	ΔL , %	PE _x
AM pretreatment:		
Initial	---	26
6 hr AM	10.5	40
6 hr A	19.1	26
A. pretreatment:		
Initial	---	34
6 hr AM	11.7	56
6 hr A	27.8	49

Oat coleoptile sections incubated 3 hr in AM (0.01 M K-Pi, pH 6.0, + 10 μ M IAA + 0.06 M mannitol) or A (same, - mannitol), then transferred for 6 hrs to AM or A solutions. L and PE_x (% extension/100 g load) determined.

Table 6. Effect of auxin on the parameters of acid-extension.

Treatment	k (min)	a (μ)
Initial	2.07×10^{-2}	881
6 hrs, - auxin	1.73×10^{-2}	642
6 hrs, + auxin	1.84×10^{-2}	1047
+ auxin/- auxin	1.05	1.63

Sections were harvested immediately, or incubated 6 hrs + 10 μ M IAA. Acid-extension determined under 15 g tension at pH 3.0. Data fitted to equation 2.

- Figure 1. Time course of changes in OC and Δ TOS for peeled Avena coleoptile sections incubated in 0.1 mM Mes-tris, pH 6.0 + 2% sucrose and with or without 5 μ g/ml IAA. Dotted lines, OC; solid lines, Δ TOS.
- Figure 2. Change in Instron plastic extensibility with time after addition of auxin. Sections were incubated in 2.5 mM K-maleate buffer, pH 4.7, and 5 μ g/ml IAA was added at time 0. Sections were harvested and DP was determined at indicated times. Change in DP over zero time value is plotted as a function of time.
- Figure 3. Change in Instron plastic extensibility following inhibition of growth with KCN. Sections were incubated for 150 minutes in K-maleate buffer + 2% sucrose, with or without 5 μ g/ml IAA. KCN (0.3 mM) added to some, and sections harvested at indicated times for measurement of DP. Time zero is time when KCN added.
- Figure 4. Regeneration of plastic extensibility by changing force vectors. A MeOH-killed Avena coleoptile section was extended three successive times at 30 g load in the Instron, then the section was removed from the clamps, reinstated and extended three additional times. Noted that the initial extension contains both plastic and elastic extension, while the 2nd and 3rd are entirely elastic. The 4th extension also is both plastic and elastic as a consequence of the changed force vectors produced by altering the section in the clamps. The initial point on the extension axis was moved for curves 4-6 for clarity.

Fig 2.

Fig 3

Fig 4.