Auxin-Induced Cell Wall Loosening in the Presence of Actinomycin D[1, 2]

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Actinomycin D, an inhibitor of RNA synthesis (10, 12), will prevent auxin-induced cell elongation (4, 8, 12, 16). It has been assumed that it acts by inhibiting the action of auxin (8, 12, 16). If this is correct, it would indicate that RNA synthesis is required for auxin action.

Auxin-induced elongation is a complex process which requires a number of factors (5). In *Avena* coleoptile tissues the factor which normally controls elongation is the extensibility of the cell wall (2, 7, 21). Addition of auxin to a tissue causes a loosening of the cell wall which, in turn, results in rapid elongation. Any inhibitor that blocks the auxin-induced wall loosening will inhibit elongation. But a compound that inhibits elongation does not necessarily have to have any effect on wall loosening. Mannitol, for example, inhibits rapid elongation but does not affect wall loosening (2). Since other auxin-insensitive factors are needed for growth, inhibition of any of these will also lead to an inhibition of elongation. Thus a compound could inhibit elongation either by blocking the auxin-induced wall loosening or by affecting some other factor. In the past, it has not been possible to separate these 2 possibilities.

Recently Olsen et al. (18) have shown that it is possible to directly measure the extensibility of the wall by using a modification of the technique of Heyn (9). At the end of the incubation period, the bulk of the protoplasm is removed from the cells with boiling methanol followed by Pronase. Then an Instron stress-strain analyzer is used to measure the load which develops across the walls as the walls are subjected to a constant rate of strain. The strain per unit stress (WEx) is determined from the resulting load-extension curve. It should be noted that WEx is a measure of the combined elastic and plastic extensibility of the walls.

Using this technique, Olson et al. (18) have shown that auxin increases the extensibility of *Avena* coleoptile sections. This increase occurs even when elongation is osmotically inhibited (17).

This technique can be used to study the effects of inhibitors on auxin-induced wall extensibility. This investigation was undertaken in order to determine whether actinomycin D was inhibiting elongation by blocking the auxin-induced increase in wall extensibility or was acting on some other factor necessary for elongation.

Materials and Methods

The plant material consisted of *Avena* coleoptile sections. The *Avena* seedlings were grown as described earlier (3). Sections 15 mm in length were cut from the region 3 to 18 mm below the tip of 27 to 32 mm long *Avena* coleoptiles. The leaves were removed and the sections were floated on water until all were cut. The plants were grown and all manipulations and incubations were carried out under a dim red light.

Groups of 15 sections were placed in 25 x 150 mm test tubes which contained 2 to 10 ml of 2.5 mM K maleate buffer, pH 4.7. When required, the solutions also contained IAA (28.5 μM) and actinomycin D (25 μg/ml). This level of actinomycin D causes maximal inhibition of *Avena* coleoptile growth (4, 16). Sucrose (2 %, w/v) was present except in the actinomycin experiments where the inhibition was enhanced by omitting the sucrose. The test tubes were rotated at 1 rpm on a Rollardrum, and after the desired incubation period, the sections were measured with a dissecting microscope fitted with a stage micrometer. The sections were then boiled in methanol for 5 minutes. This treatment destroys the semipermeability of the cells without destroying the integrity of the sections. Olson et al. (18) have shown that this treatment has little effect on the stress-elongation properties of the walls as measured with the Instron and it greatly facilitates the subsequent WEx measurement. Proteins were not removed since it has been shown (18, unpublished results) that the presence of proteins does not influence WEx. The sections were stored in fresh methanol until used.

The extensibility of the sections was measured with a stress-strain analyzer, an Instron, using the basic procedure of Olson et al. (18). Rehydrated sections were fixed between the 2 clamps of the Instron. The distance between the clamps was 6.35 mm. The crosshead containing the lower clamp was then moved downward at a fixed rate of 1.27 mm/minute. The load which resulted across the sections was measured by an electronic load transducer attached to the upper immobile clamp. The resulting

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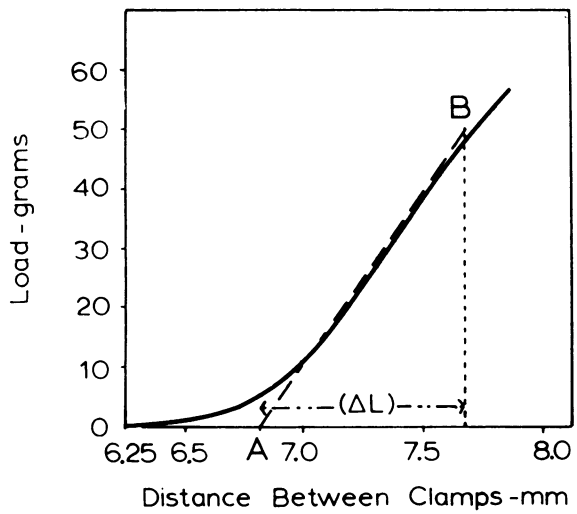

FIG. 1. Example of typical load-extension curve for *Avena* coleoptile walls obtained with Instron. Section incubated 2 hours in buffers, then boiled 5 minutes in methanol. Section then extended at 1.27 mm/minute and load recorded. For this section ΔL = 0.83 mm, A = 6.87 mm and WEx = 12.1.

load was recorded on the Y axis of graph paper which was moving at 127 mm/minute.

An example of the resulting load-extension curve is shown in figure 1. There are essentially 2 parts to the curve. At first little load was developed as the crosshead was lowered. This was largely due to the fact that the section was loosely fixed between the clamps and thus the slack had first to be taken out of the section. Thereafter load increased in a linear manner as extension occurred. The slope of the resulting line is an indication of the rigidity of the section.

A line (AB) can be drawn parallel to the slope of the curve, and WEx can be determined from this line. WEx is defined as the percent extension which must occur to produce a load of 50 g across the walls. The extension at 50 g (ΔL) is determined from the line AB. The initial length of the section is A mm. WEx is, then, $\Delta L / A \times 100$.

Treatment of tissues with auxin during the incubation period results in a change in the extensibility of the walls (17, 18). The shape of the load-extension curve is unchanged but the slope of the curve is now considerably less and thus WEx is greater. This indicates that the walls of the auxin-treated sections are more extensible. By subjecting the sections to re-extension, it is possible to separate WEx into reversible and irreversible elongation components. The effect of auxin is largely concentrated in the irreversible component (unpublished results).

Each point in figures 2 and 3 represents the average of 6 to 10 sections. Standard errors are presented for all WEx values. Each experiment was repeated at least twice.

The effect of actinomycin on RNA synthesis was measured in the following manner. Groups of twenty 10-mm coleoptile sections with leaves removed were placed in test tubes which contained 2 ml of buffer, with or without 25 μg/ml actinomycin D. After 3 hours, 1.5 μc of orotic acid-6-C^{14} (3.45 mc/mmole) was added to each tube. Sections were removed 1 to 6 hours later, washed twice for 5 minutes with 50 ml of water, blotted dry, and the water in the leaf cavity removed. The procedure of Click and Hackett (6) was used to remove soluble nucleotides and unused orotic acid. The sections were then dried, spread out on a planchet, and counted in an automatic gas flow counter (Nuclear-Chicago).

In order to test whether the radioactivity was actually in the form of RNA, the sections were incubated for 18 hours in 10 ml of 1 mM K-Phosphate buffer, pH 7.5, with or without 1 mg ribonuclease (Worthington Biochemical Corporation). The sections were then washed with ethanol, dried and recounted. Treatment with buffer alone removed less than 3 % of the C^{14} from any group of sections. In contrast, ribonuclease solubilized 70 to 80 % of the radioactivity of the actinomycin-treated sections and 85 to 95 % of the radioactivity of the control sections. It is apparent that almost all of the isotope was in the form of RNA.

Results

Before the effect of actinomycin D on WEx was examined, it seemed important to answer 2 questions about WEx. First, can inhibitors of auxin-induced elongation prevent the effect of auxin on WEx? In other words, is an effect of actinomycin on the auxin-induced WEx to be expected? This question was answered by testing the ability of cyanide to prevent an auxin-induced increase in WEx. Cyanide was selected as the inhibitor because it causes rapid (20) and total (1) inhibition of auxin-induced elongation. Sections were pretreated for 90 minutes in water or 3×10^{-4} M KCN. Some sections from each group were then harvested in order to determine the initial WEx. The remaining sections in each group were divided into 2 equal lots. IAA (28.5 μM) was added to one lot; water was added to the other. After 2 hours, all of the sections were harvested and their WEx was determined (table I).

Treatment of sections with auxin in the absence of cyanide has resulted in a 46 % increase in WEx. In the presence of cyanide, auxin has had no effect on WEx. It is apparent that inhibitors of auxin-induced elongation can prevent the effect of auxin on WEx.

The second question that was examined is what happens to the auxin-induced increase in WEx during elongation. Does it get used up in elongation or, once induced, does it persist? This question was

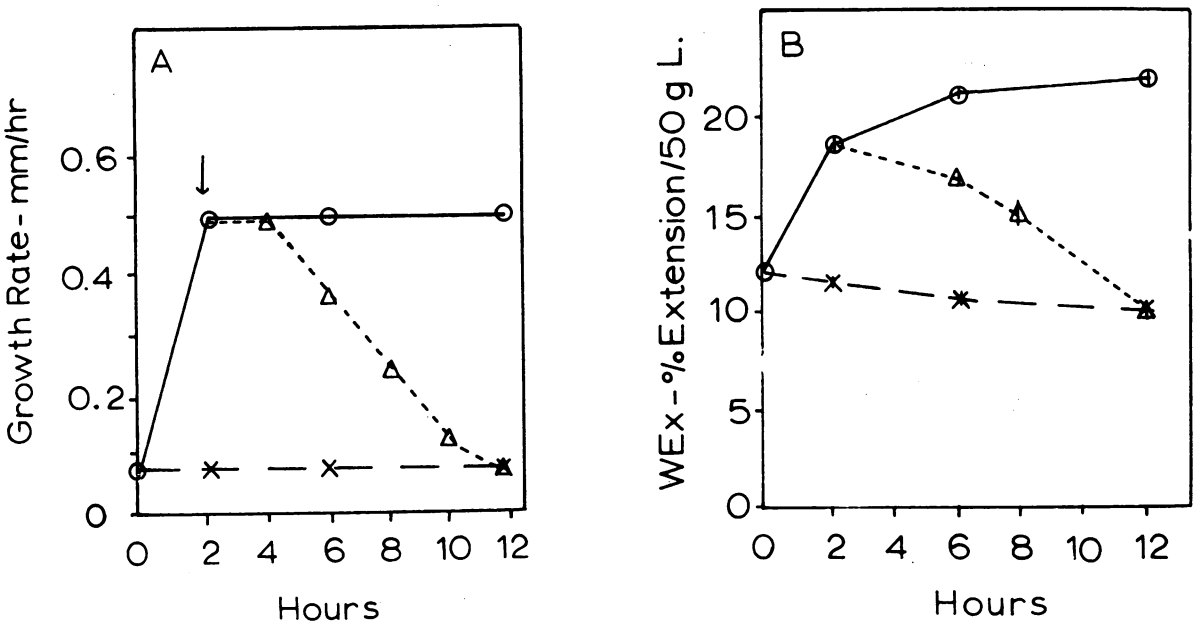

FIG. 2. Effect of removal of auxin on growth and WEx. Sections incubated with 28.5 μM IAA (—○—), no auxin (—×--) or returned from auxin to buffer (—△—). All solutions contained 2% w/v sucrose and K-Maleate buffer, pH 4.7. A, Growth rate; B, WEx.

Table I. *Prevention by Cyanide of Auxin-Induced Increase in WEx*

All solutions contained buffer, 2% (w/v) sucrose and additions as shown. All WEx values average of 10 sections.

Pretreated 90 min with:	Treated 120 min with:	WEx*	Auxin-induced WEx
Water	None	14.2	
	IAA (28.5 μM)	20.8	+6.5**
	Water	14.3	
KCN (0.3 mM)	None	13.3	
	IAA (28.5 μM) + KCN (0.3 mM)	11.4	−0.5***
	KCN (0.3 mM)	11.9	

* WEx = percent extension/50 g load.
** Significant at 1% level.
*** Not significant at 5% level.

examined by testing the effect of removal of auxin on growth rate and WEx.

Sections were pretreated for 3 hours in solutions with or without IAA. Then half of the auxin-treated sections were returned to media which was free of auxin; the remaining sections were left in their original solutions. The growth rate and extensibility of the sections were then followed for the next 10 hours (fig 2).

Sections which remained in auxin showed a high, constant growth rate and had a high WEx. Non-auxin-treated sections possessed a low, constant growth rate and had a low WEx. The growth rate of sections returned from auxin to water was rapid for 2 hours after which it dropped until at the end of 10 hours it was as the same level as the non-auxin-treated sections. The extensibility of the sections dropped in a parallel manner so that after 10 hours it, too, equalled that of the non-auxin-treated sections. Apparently the auxin-induced WEx was used up in elongation.

The effect of actinomycin D on auxin-induced wall extensibility was then examined. First, it was shown that actinomycin will effectively inhibit RNA synthesis in *Avena* coleoptile tissues. Sections were pretreated for 3 hours in buffer, with or without 25 μg/ml actinomycin D. Orotic acid-6-C^{14} was then added and the time-course for the incorporation of

Table II. *Effect of Actinomycin D on RNA Synthesis*

Sections pretreated 3 hours in actinomycin D (25 μg/ml). Orotic acid-6-C^{14} (1.5 μc) added and incubation continued 1 to 6 hours.

Hr in orotic acid-C^{14}	C^{14} Incorporation* − actinomycin	+	% Inhibition
1	215	18.5	91
3	941	62.5	93
6	2618	141	95

* cpm/20 sections.

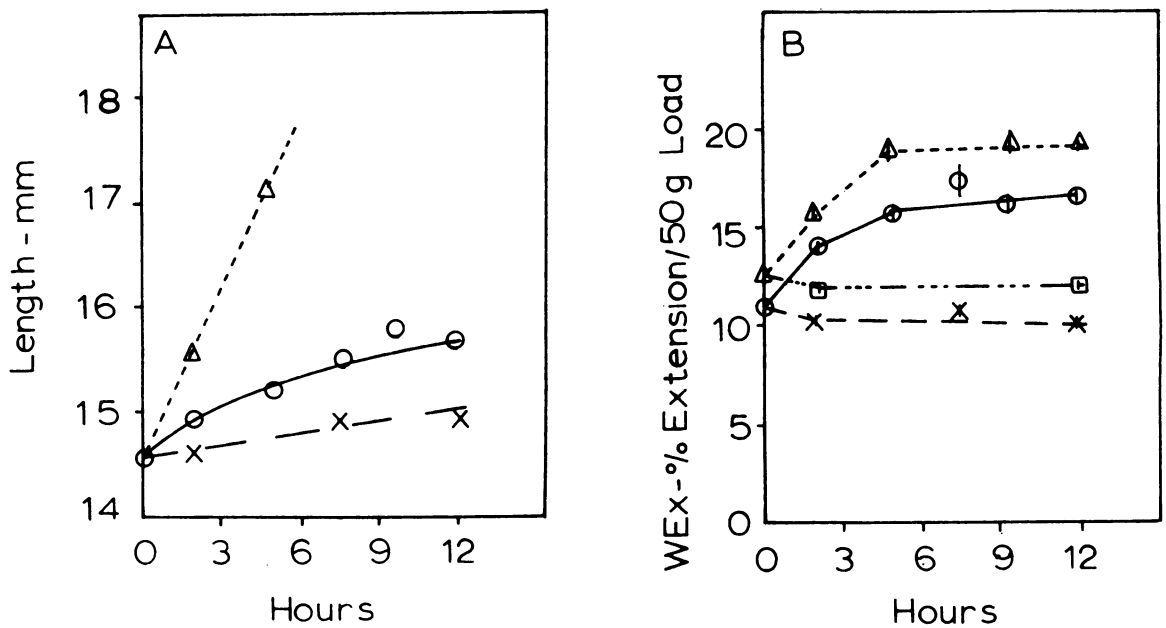

FIG. 3. Effect of actinomycin D on auxin-induced elongation and WEx. Section pretreated 3 hours in buffer ± 25 μg/ml actinomycin D. Then 28.5 μM IAA added to half of sections and length and WEx determined after 0 to 12 hours. IAA (--△--), buffer (—□--), IAA + actinomycin (—○—), actinomycin alone (—×—). A, Elongation; B, WEx.

the orotic acid into RNA was determined (table II). Actinomycin greatly inhibited the synthesis of RNA over the whole of the experimental period. The inhibition ranged from 91 % after 1 hour to 95 % after 6 hours. The presence or absence of auxin had no effect on the amount of inhibition which was obtained.

The effect of actinomycin D on elongation and WEx was then determined. Sections were pretreated for 3 hours in buffer, with or without 25 μg/ml actinomycin D. Then 28.5 μM IAA was added to half of the sections in each group. The growth and WEx were followed over a period of 12 hours.

Actinomycin severely inhibited auxin-induced elongation (fig 3a). At first some auxin-induced growth did occur, but the inhibition progressively increased until after 6 to 8 hours the growth rates of auxin-treated and control sections were identical.

The effect of actinomycin on WEx is seen in figure 3b. Three things are apparent from these results. First, even though RNA synthesis had been inhibited by the actinomycin, auxin was still able to induce a sizable increase in the extensibility of the wall. Secondly, this auxin-induced increase in WEx was not used up in elongation but persisted even after all auxin-induced elongation had been eliminated. Finally, actinomycin caused a stiffening of the walls in both auxin-treated and non-auxin-treated sections. Other experiments (unpublished) indicated that this stiffening was not sufficient to explain the actinomycin-induced inhibition of elongation.

Discussion

There can be little doubt that RNA synthesis is

required for some facet of auxin-induced elongation. This is shown by the fact that treatment of tissues with ribonuclease (14) or with actinomycin D (4, 8, 12, 16) severely inhibits elongation. Recently Key identified the fraction of RNA, which he has called D-RNA, which is required for the auxin-induced growth of soybean hypocotyl sections (13).

Elongation is a complex process which requires a number of factors. Auxin influences elongation by affecting one of these factors, the rigidity of the cell wall. Other factors, such as adequate supplies of water and osmotic solutes, are just as necessary. The question remains as to whether RNA synthesis is necessary for the auxin-induced wall loosening or for some other auxin-insensitive factor.

It has been shown in the present study that an auxin-induced increase in wall extensibility can occur even when RNA synthesis is inhibited. Treatment of *Avena* coleoptile sections with actinomycin D results in a 90 % inhibition of RNA synthesis within 1 hour. Yet 3 hours after addition of the actinomycin, auxin can still cause a sizable increase in WEx. This is in contrast to the situation with cyanide, where a 90-minute pretreatment with this metabolic inhibitor eliminates the ability of auxin to affect WEx. Apparently aerobic metabolism, but not RNA synthesis is needed for this action of auxin.

The auxin-induced increase in WEx persists for up to 14 hours after addition of actinomycin, even though auxin-induced elongation has been blocked after 6 to 8 hours. In contrast, when auxin action is terminated by removal of auxin, WEx is used up in elongation. The lack of RNA synthesis has prevented elongation even though the auxin-induced increase in WEx is still present.

It must be noted that WEx is not identical with wall loosening. WEx is a measure of the capacity of the walls to be rapidly extended by an external force; wall loosening is the capacity of the walls to be slowly deformed by turgor pressure. It has been shown for *Nitella* that the capacity of walls for extension depends upon the speed of deformation (19) and the driving force (11). Despite these differences, the close correlation between WEx and the rate of elongation in *Avena* coleoptile tissues indicates that WEx is an accurate measure of the state of wall loosening. These results show, then, that auxin-induced wall loosening can occur even when RNA synthesis is inhibited.

Similar results have been obtained independently by Morré (15) using a different technique and a different tissue. He has shown that the ability of pea-stem sections to be bent by an applied weight is increased by auxin. This effect of auxin occurs even if the sections are pretreated for 4 hours with actinomycin D.

Synthesis of RNA is not necessary for auxin-induced wall loosening. However, since RNA synthesis is necessary for continued elongation, it must be required for some auxin-insensitive factor which is also necessary for elongation. In the absence of this factor, the auxin-induced wall loosening, although present, is unable to manifest itself as elongation.

Summary

An Instron stress-strain analyzer has been used to determine the effect of various treatments on the extensibility (WEx) of *Avena* coleoptile cell walls.

Auxin causes an increase in WEx even when RNA synthesis has been inhibited by greater than 90 % with actinomycin D. Furthermore, the effect of auxin on WEx persists even after auxin-induced elongation has been completely blocked by the actinomycin. It is apparent that RNA synthesis is not necessary for auxin-induced wall loosening, but is necessary for some other factor required for elongation.

Cyanide completely prevents an effect of auxin on WEx. Aerobic metabolism is apparently necessary for the induction of wall loosening by auxin.

Removal of auxin from the sections results in a parallel decrease in the growth rate and in WEx. It would appear that wall loosening is used up in elongation.

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Literature Cited

1. BONNER, J. 1936. Growth and respiration of the *Avena* coleoptile. J. Gen. Physiol. 20: 1–11.
2. CLELAND, R. 1958. A separation of auxin-induced cell wall loosening into its plastic and elastic components. Physiol. Plantarum 11: 599–609.
3. CLELAND, R. 1963. Independence of effects of auxin on cell wall methylation and elongation. Plant Physiol. 38: 12–18.
4. CLELAND, R. 1964. The effect of hydroxyproline on the auxin-induced growth of *Avena* coleoptile tissue. Plant Physiol. 39: iv.
5. CLELAND, R. AND H. BURSTRÖM. 1961. Theories of the auxin action on cellular elongation. A summary. Encyl. Plant Physiol. 14: 807–11.
6. CLICK, R. E. AND D. P. HACKETT. 1963. The role of protein and nucleic acid synthesis in the development of respiration in potato tuber slices. Proc. Natl. Acad. Sci. 50: 243–50.
7. GALSTON, A. W. AND W. K. PURVIS. 1960. The mechanism of action of auxin. Ann. Rev. Plant Physiol. 11: 239–76.

8. GALSTON, A. W., P. JACKSON, R. KAUR-SAWHNEY, N. P. KEFFORD, AND W. J. MEUDT. 1964. Interactions of auxins with macromolecular constituents of pea seedlings. In: Regulateurs Naturels de la Croissance Vegetale, J. P. Nitsch, ed. Edit. Cent. Nat. Recherche Scien., Paris.
9. HEYN, A. N. J. 1933. Further investigations on the mechanism of cell elongation and the properties of the cell wall in connection with elongation. I. The load extension relationship. Protoplasma 19: 78–96.
10. HURWITZ, J., J. FURTH, M. MALAMY, AND M. ALEXANDER. 1962. The inhibition of the enzymatic synthesis of RNA and DNA by actinomycin D and proflavin. Proc. Natl. Acad. Sci. 48: 1222–30.
11. KAMIYA, N., M. TAZAWA, AND T. TAKATA. 1963. The relation of turgor pressure to cell volume in *Nitella* with special reference to mechanical properties of the cell wall. Protoplasma 57: 501–21.
12. KEY, J. L. 1964. Ribonucleic acid and protein synthesis as essential processes for cell elongation. Plant Physiol. 39: 365–70.
13. KEY, J. L. AND JOHN INGLE. 1964. Requirement for the synthesis of DNA-like RNA for growth of excised plant tissue. Proc. Natl. Acad. Sci. 52: 1382-88.
14. MASUDA, U. 1960. Physiological significance of ribonucleic acid in the growth promoting action of auxin. J. Inst. Polytech. Osaka City Univ. 11D: 1–23.
15. MORRÉ, D. J. 1964. Cell wall-cytoplasm interactions associated with plant growth. Plant Physiol. 39: iv-v.
16. NOODEN, L. D. AND K. V. THIMANN. 1963. Evidence for a requirement for protein synthesis for auxin-induced cell enlargement. Proc. Natl. Acad. Sci. 50: 194–200.
17. OLSON, A. C. AND R. CLELAND. 1964. Changes in the mechanical properties of tissue promoted by IAA under nonelongating conditions. Plant Physiol. 39: v.
18. OLSON, A. C., J. BONNER, AND D. J. MORRÉ. 1962. Studies on the incorporation of C^{14}-proline into cell walls. Abstr. Meeting Am. Chem. Soc., September, p 16C.
19. PROBINE, M. C. AND R. D. PRESTON. 1962. Cell growth and the structure and mechanical properties of the wall in internodal cells of *Nitella opaca*. II. Mechanical properties of the wall. J. Exptl. Botany 13: 111–27.
20. RAY, P. M., AND A. W. RUESINK. 1962. Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells. Develop. Biol. 4: 377–97.
21. SETTERFIELD, G. AND S. T. BAYLEY. 1961. Structure and physiology of cell walls. Ann. Rev. Plant Physiol. 12: 35–62.