Auxin-induced changes in Avena coleoptile cell wall composition associated with elongation

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Auxin-induced changes in *Avena* coleoptile cell wall composition associated with elongation

by

Wayne Harold Loescher

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Botany (Plant Physiology)

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LIST OF ABBREVIATIONS

TFA: trifluoroacetic acid
gl: glucose
ga: galactose
m: mannose
x: xylose
a: arabinose
IAA: indoleacetic acid
LITERATURE REVIEW

The term growth in a broad sense may connote all the activities involved in ontogenesis, i.e., division, differentiation, enlargement, and maturation. Or, in a more restricted sense, growth may be defined as an irreversible increase in volume (Lockhart, Bretz, and Kenner, 1967; Thimann, 1969). In either sense, in the growth of plants where the cells are typically surrounded by walls, enlargement or generation of new volume requires a yielding of the cell wall to the stresses within it (Green, 1969). To study growth from this aspect, one must deal with the rate at which a cell wall increases in surface area under turgor stress and cell volume consequently increases by osmotic absorption of water. To influence growth, a factor or agents must act by influencing the mechanism of cell wall expansion or its driving force, turgor pressure (Ray, 1969).

These topics, growth, cell enlargement or elongation, and cell wall expansion, the factors governing them and their importance have long been recognized, and a considerable number of papers have dealt with them directly or peripherally. A number of reviews have resulted; some dealing specifically with the subject of cell enlargement (Cleland, 1971a; Green, 1969; Heyn, 1940; Lockhart, 1965a; Preston, 1961; Ray, 1969; Setterfield and Bayley, 1961; Wilson, 1964), others with associated facets of the problem, i.e., cell wall ultrastructure (Albersheim, 1965a; Frey-Wyssling, 1959; Mühlethaler, 1967; Roelofsen, 1959; Roelofsen, 1965), cell wall metabolism (Albersheim, 1965b; Hassid, 1967; Hassid, 1969; Lamport, 1970), growth hormones and the control of enlargement (Galston and Davies, 1969; Key, 1969; Ray, 1969; Thimann, 1969), and the physical nature of cell elongation (Burström, 1961; Lockhart, 1965b). Of these, Heyn's review of 1940 best
summarizes the early investigations; Wilson's review of 1964 provides a good introduction to the structural aspects of wall extension; and the article by Ray (1969) on the action of auxin in cell enlargement and the review on cell extension by Cleland (1971a) are up-to-date and excellent.

From the literature certain information now appears to be basic and beyond dispute (Cleland, 1971a): (a) cell enlargement involves stretching of the already present wall in addition to synthesis of new wall; (b) turgor pressure is necessary for expansion; (c) enlargement is not a passive process but rather an active one normally requiring respiration; (d) synthesis of RNA and protein are needed for enlargement to continue at a constant rate; (e) in higher plants a certain portion of enlargement is regulated by auxin. How these facts may fit together to explain cell enlargement still remains a problem, but there are a number of approaches currently popular attempting to offer solutions. The approaches include kinetic studies of the growth process, studies of the physical properties of cell walls, ultrastructural studies, compositional analyses, and investigations of various enzymes and wall metabolism.

The kinetic approach deals with the enlargement process in tissues that are in the process of elongating or have the potential to do so. The material is exposed to treatments that variously promote or inhibit enlargement and the results so obtained have adduced most of the above-mentioned basic conclusions. The technique is not new (Bonner, 1933; Went and Thimann, 1937), but refinements (Evans and Ray, 1969; de la Fuente and Leopold, 1970; Green, 1968; Nissl and Zenk, 1969; Ray and Ruesink, 1962) have increased resolution of the period between treatment and onset of the effects so that changes in the rate of growth can now be detected immediately. Among the
results is evidence that in those tissues where elongation is controlled by auxin there is virtually no lag between application of auxin and change in the rate of growth. The initial, immediate effect is inhibition, followed very shortly by enhancement at a rate which may or may not continue depending on the tissue (Barkley and Evans, 1970; Evans and Hokanson, 1970; Evans and Ray, 1969; dela Fuente and Leopold, 1970). Enhancement under conditions designed to promote auxin uptake may occur within 2 minutes (Ray, 1969). Use of the methyl ester of indoleacetic acid results in enhancement in less than a minute (Rayle, Haughton, and Cleland, 1970). The upshot of such studies has been to cast doubt on the traditional gene activation hypothesis of auxin action (Evans and Ray, 1969) and has prompted proposal of several alternative modes of auxin action and control of cell enlargement (Ray, 1969).

RNA and protein synthesis still seems to be necessary for enlargement since a number of compounds known to inhibit various steps in the sequence of events involved in protein synthesis inhibit auxin-induced growth (Key, 1969; Masuda and Wada, 1966; Nooden and Thimann, 1963; Nooden and Thimann, 1965; Penny and Galston, 1966). The high-resolution studies, however, indicate that the effect of inhibitors such as these is not on the timing but rather on the magnitude of the response. These inhibitors will inhibit auxin-induced growth, provided that they are added prior to auxin treatment. If added at the same time as auxin, inhibition is evident only after a lag, within about 20 minutes for cycloheximide, within approximately an hour for actinomycin-D, suggesting that auxin-induced growth may not require protein synthesis initially but may require certain growth-limiting proteins which are unstable or have a small pool size (Evans and Ray, 1969). Nevertheless,
this aspect of the question of the mechanism of auxin action must remain open, for the possibility of rapid enhancement of RNA and protein synthesis cannot be discounted (Key, 1969). It has been reported that enhanced RNA synthesis can be detected within 10 minutes of auxin treatment (Masuda and Kamisaka, 1969); detection of enhanced cellulase activity has been reported in 15 minutes (Maclachlan, Davies, and Fan, 1968).

Further complicating the question of cell enlargement are reports that low pH or carbon dioxide have rapid promotive effects on elongation. These promotions are striking, on the order of 10-fold, and they are rapid, occurring within a minute. They are, however, of relatively short duration, lasting only several hours in spite of continued exposure to promotive conditions (Evans, Ray, and Reinhold, 1971; Rayle and Cleland, 1970; Rayle et al., 1970). At least three possibilities have been considered as explanations of these effects of carbon dioxide or low pH: (a) carbon dioxide or hydrogen ion may act on the wall in some physicochemical manner by altering interactions between wall components; (b) they may cause acid hydrolysis of acid-labile linkages or promote the action of enzymes mediating such cleavages; (c) they may affect plasma membrane permeability causing factors to be released which have an effect on the wall (Evans et al., 1971).

Kinetic studies have also yielded information on the role of turgor in growth. Its role may be complex. On a theoretical basis, if turgor is the driving force for elongation, then one could expect rate of enlargement to be directly proportional to the magnitude of turgor pressure (Lockhart, 1965a). Early studies (Cleland, 1958; Ordin, Applewhite, and Bonner, 1956), however, revealed that rate of growth is not directly proportional to turgor pressure but rather proportional to the pressure in excess of some critical
pressure below which growth does not occur. This has been confirmed for
Avena coleoptiles and a variety of other tissues (Boyer, 1968; Boyer, 1970; 
Green, 1968; Lawlor, 1969; Ray and Ruesink, 1963). But, the critical 
pressure may not be independent of turgor; in Nitella, growth rate appears 
to be proportional, not to turgor, but to the difference between turgor and 
the yielding threshold of the wall. Variations in turgor do cause momentary 
changes in rate of growth, but there is soon a change in the yielding 
threshold of the wall which re-establishes the steady state rate (Green, 
Erickson, and Buggy, 1971). Also, Evans and Ray (1969) in their high-
resolution studies report that the effect of auxin is to lower the critical 
pressure thus allowing growth at lower turgor pressures. Cleland (1971a) 
is critical of these reports of shifting critical pressures and he suggests 
that growth may be the result of several concurrent processes, each con-
tributing to enlargement, and each with a different dependence on turgor 
pressure.

In those tissues where there may be auxin-induced growth, turgor and 
kinetic studies also show that at subcritical pressures there are no auxin-
induced modifications in the wall which result in growth upon return of 
the tissue to high turgor pressures (Cleland, 1971a; Ray, 1969). Earlier 
experiments had revealed such results, but the increase in growth was 
small, less than 2 per cent (Cleland and Bonner, 1956). This requirement 
for turgor is thought to suggest that auxin-induced modifications of the 
wall which result in growth occur only when the wall is extended or under 
tension. This may indicate modifications involving reversible cleavages, 
and if cleavages are not followed by extension then the crosslinks may 
reform in the original position (Cleland, 1971a).
Investigations of another type, ultrastructural studies, have generally been limited to observations of the distribution and orientation of microfibrils of cellulose within the wall. The observations do reveal in *Avena* coleoptiles and in primary cell walls from other sources that there is a difference in orientation of microfibrils on the inner and outer surfaces. Those on the inner surface are transversely oriented in a fairly regular fashion whereas those towards the outside are more dispersed, tending to a longitudinal orientation. If the multinet growth hypothesis of Roelofsen and Houwink (1953) is correct, then these differences arise developmentally. The microfibrils are thought to be deposited by the protoplast transversely on the inner wall surface, and with extension, to be progressively reoriented longitudinally. There is little to refute this hypothesis and it has been restated by a number of investigators since first proposed (Frey-Wyssling, 1959; Green, 1969; Mühlethaler, 1967; Preston and Hepton, 1960; Probine and Barber, 1966; Setterfield and Bayley, 1961; Wilson, 1964).

Certain aspects of multinet growth, especially the realignment process which requires that the microfibrils slip relative to one another and become further apart, have suggested that wall synthesis may be a causal factor in enlargement (Setterfield and Bayley, 1961). Realignment and expansion would thus be associated with the intercalation of new material into the wall. Ray (1967), using radioautography, various extraction techniques, and electron microscopy, has found evidence which tends to support this: while cellulose is deposited at the inner surface, non-cellulosic constituents, hemicellulosic in nature, are incorporated into the cell wall interior. But, wall synthesis or intussusception of new wall material between existing
microfibrils has been considered to be a poor explanation of extension. For example, rates of wall synthesis rarely correlate well with elongation. Typically, in rapidly elongating cells, synthesis lags behind elongation (Abdul-Baki and Ray, 1971; Baker and Ray, 1965b; Wilson, 1964). Also where elongation is inhibited by calcium, there is continued auxin-promoted synthesis of some wall constituents, notably the non-cellulosic polysaccharides (Ray and Baker, 1965). There is, however, a correlation between elongation and promotion of synthesis of one wall component. Promotion of cellulose synthesis apparently requires elongation, enhanced synthesis not occurring with auxin treatment when elongation is inhibited by calcium. Still, enhancement of cellulose synthesis is not detectable until about an hour after enhancement of elongation (Ray and Baker, 1965).

An alternative explanation of extension has been that the microfibrils are crosslinked in some fashion, perhaps by some of the matrix materials which form 40 to 60 per cent of the wall, and cleavage of these crosslinks changes the mechanical properties of the wall which is then stretched by water uptake resulting from the osmotic potential of the cell sap (Lockhart, 1965a). That mechanical properties of cell walls are modified in some way that results in wall expansion is not disputed. This has been recognized since the early studies of the growth process (Heyn, 1940). These modifications were first referred to as being increases in plasticity, but more recently there have been attempts to more rigorously define the changes on a physical basis (Lockhart, 1965b; Probine and Preston, 1962; Probine and Barber, 1966). Also, there have been attempts to determine the changes in the mechanical properties by utilizing either measurements of mechanical extension properties of cell walls (Cleland, 1967a; Haughton and Sellen,
1969; Olson et al., 1965; Uhrstrom, 1969) or high-resolution elongation measurements where changes in strain rate can be measured in living tissues upon change in stress (Green, 1968; Green et al., 1971; Ray, 1969). While there is strong evidence for changes in the properties of the wall, i.e., increases in plastic and elastic components of wall extensibility that sometimes correlate well with growth (Cleland, 1971b; Cleland and Haughton, 1971; Haughton and Sellen, 1969; Rayle et al., 1970; Yamamoto, Shinozaki and Masuda 1970), the results have to a certain extent been ambiguous. The experiments have been reviewed by Ray (1969), who has been critical of these studies, and also by Cleland (1971a), who has done many of them. The consensus now is that attempts to correlate growth and the observed parameters of mechanical extensibility must await improvements in methods and technique.

Whatever the changes in the physical characteristics of plant cell walls which result in extension, to account for such changes in molecular terms as has been done for certain bacteria (Strominger and Ghysen, 1967) most investigators have sought chemical explanations, these usually involving cleavage of covalent bonds within the wall. Lamport (1970) has listed a number of the possible wall linkages and cross linkages which might be cleaved in such a process. Given the predominately polysaccharide nature of the wall, it is not surprising that most investigators have attempted to assess the changes occurring in carbohydrates. In spite of the number of reports, the nature of the linkages which may be cleaved during growth remains unknown. Nonetheless, it has been found in a variety of tissues that certain components of the wall undergo profound changes in composition during growth (Jensen and Ashton, 1960; Nelmes and Preston, 1968; Nevins, English,
and Albersheim, 1968; Roberts, 1967; Roberts and Butt, 1969; Stoddart and Northcote, 1967; Stoddart, Barrett, and Northcote, 1967; Thornber and Northcote, 1961). Without a great deal of foundation, it has been assumed that these changes in composition reflect changes in metabolism which may be involved in the enlargement process. Lamport (1970) has summarized the results of a selected number of these and other papers reporting changes in the wall.

Several papers report on the composition and the changes in composition of *Avena* coleoptile walls. The first of these which attempted to assess the contribution of various fractions to total wall composition found that the non-cellulosic fractions constituted the largest share of the wall (Bishop, Bayley, and Setterfield, 1958). Later work, using improved techniques, measured composition before and after 18 hours of elongation and found that when elongation occurred without exogenous glucose being available there was evidence of either degradation or turnover in the wall: the wall's content of non-cellulosic glucose and galactose decreased, and cellulose content increased. When glucose was present auxin caused a general increase in all cell wall components, but non-cellulosic glucose did not increase as much as did the other components (Ray, 1962; Ray, 1963).

Other approaches attempting to assess changes in the wall have utilized radioisotopes to determine if observed increases in a particular fraction of the wall are less than the increases in incorporation of a particular isotope. If so, this would indicate if turnover of certain fractions had occurred. Katz and Ordin (1967) did this with *Avena* coleoptile tissue and reported turnover of hexoses in an acid-soluble fraction of the wall. They were unable to determine if auxin had a significant effect on
turnover. Similar results showed turnover of hexoses in growing pea epicotyl tissue (Maclachlan and Duda, 1965; Maclachlan and Young, 1962).

Another approach utilizes tissue exposed to a pulse of labelled material. The tissue is then incubated in unlabelled material to determine if there is loss of label from a particular fraction, thus indicating turnover or degradation. Several papers report such results for various fractions of Avena coleoptile cell walls, i.e., a hot dilute acid fraction (Katz and Ordin, 1967a), and a 4N:KOH fraction (Wada, Tanimoto, and Masuda, 1968). Similar results have been reported for an ethanol soluble fraction in corn root tips (Roberts and Butt, 1969) and for several fractions of pea seedling stems (Matchett and Nance, 1962). In all cases the evidence of turnover of certain constituents is usually interpreted as possibly representing the cleavage of crosslinks, thus affecting the mechanical properties of the wall.

Another line of evidence suggesting a possible explanation of the enlargement process is the large number of reports of polysaccharide hydrolases and their apparent role in multinet growth. Cleland's review (1971a) provides us with an extensive compilation of these papers and it seems unnecessary to cite them all here. From the literature it is now apparent in many systems, especially in bacteria, fungal hyphae, and the tip growth of pollen tubes, that these hydrolases are essential to growth. In other systems the evidence is not so clear, but similar enzymes are closely associated with the cell wall. In oat coleoptiles specifically, β-1,3-β-1,4-, β-1,6-, and α-1,6-glucanohydrolase activities (Heyn, 1969; Heyn, 1970a), as well as unidentified hydrolase activities (Katz and Ordin, 1967b) have been found in association with wall preparations. In several systems
auxin has been reported to cause an increase in the activity of such enzymes. In oat coleoptiles dextranase (Heyn, 1970b), \(\beta\)-1,3-glucanase (Tanimoto and Masuda, 1968), and certain unidentified hydrolase activities (Katz and Ordin, 1967b) have been reported to increase with auxin treatment. Various hydrolases have also been reported to increase mechanical extensibility in coleoptile cell walls, i.e., cellulase (Olson et al., 1965; Ruesink, 1969), and \(\beta\)-1,3-glucanase (Masuda, 1968; Masuda, Oi, and Satomura, 1970; Masuda and Wada, 1967). There are also reports by Masuda and his colleagues that \(\beta\)-1,3-glucanase preparations can induce elongation in the *Avena* coleoptile (Masuda, 1968; Masuda and Wada, 1967; Tanimoto and Masuda, 1968; Wada et al., 1968).

In spite of this evidence, the idea that hydrolases are responsible for elongation is still received with considerable skepticism. The reasons given are varied. Some investigators question how the reported 5 or 10 per cent auxin-induced increases in dextranase or \(\beta\)-1,3-glucanase activity can be responsible for the observed auxin-induced increases in rate of growth that are on the order of 500 to 800 per cent (Cleland, 1971a). Another criticism arises from attempts to induce elongation in coleoptiles by treating them with various enzyme preparations. The data of Masuda and his coworkers have been questioned; confirmation has been achieved only by using coleoptiles subjected to a low turgor pretreatment (Cleland, 1971a). Attempts to induce elongation with other \(\beta\)-glucanases have been unsuccessful, but in one report (Wada et al., 1968) whole sections were used which, due to the presence of the cuticle, may have prevented penetration of the enzyme into the walls. In another report (Ruesink, 1969), in order to
facsilitate penetration, the epidermal layers of tissue were removed prior to treatment. There is evidence, however, that epidermal layers of tissue control rate of elongation more than underlying tissues (Lockhart, 1965b; Roelofsen, 1965; Schoch-Bodmer, 1939).

Further questions arise as the result of experiments where coleop­tiles are deprived of oxygen, or treated with KCN or the competitive auxin antagonist, 4-chlorophenoxyisobutyric acid. Inhibition becomes established within 20 minutes in all cases (Hertel et al., 1969; Evans and Hokanson, 1969; Evans and Ray, 1969). These results, together with the effects of inhibitors of protein synthesis, suggest that the factors affecting growth are very unstable, a condition not often found in polysaccharide hydrolases (Cleland, 1971a).

For these and other reasons, both Lamport (1970) and Ray (1969) are sharply critical of reports of turnover and degradation, and of the role of polysaccharidases in elongation. Lamport feels that the alternative explanation lies in a distinctive hydroxyproline-containing protein within the wall which may serve as a labile crosslink between wall polysaccharides. That such a protein exists is not recognized by everyone (Steward et al., 1970), but there is evidence that a certain part of the wall is proteinaceous (Lamport, 1965; Gotelli and Cleland, 1968; Chris­peels, 1969) and that there are linkages between protein and carbohydrate in the wall, i.e., a glycosidic link between L-arabinose and hydroxy­proline (Lamport, 1969). Further, treatments with 2,2'-dipyridyl, a substance which inhibits proline hydroxylation, result in increases in growth rate and cell wall extensibility. The proposed explanation is
that, by preventing hydroxylation of proline, 2,2'-dipyridyl prevents formation of crosslinks of hydroxyproline-rich proteins to polysaccharides and might therefore lead to a mechanically weaker, i.e., a more extensible, wall (Barnett, 1970). Both Ray (1969) and Cleland (1971a) consider wall protein or "extensin" to be a possibility, but Ray, in spite of his criticisms, indicates that he feels the possibilities suggested by the evidence for turnover, degradation, and polysaccharidases are the most likely explanations of the enlargement process.
PART I. AUXIN-INDUCED CHANGES IN AVENA COLEOPTILE WALL COMPOSITION RELATED TO GROWTH
INTRODUCTION

In *Avena* coleoptiles the increases in wall area which accompany cell elongation are apparently the result of two processes: (a) modifications of the existing wall (Bonner, 1935; Wilson, 1964), and (b) synthesis of new wall (Ray, 1962). The rapid stimulations of elongation by auxin and other substances (Evans et al., 1971; Evans and Ray, 1969; Rayle and Cleland, 1970; Rayle et al., 1970) and the apparent lag in auxin-induced synthesis (Baker and Ray, 1965a; Ray and Abdul-Baki, 1968) suggest that modifications of the existing wall are primarily responsible for establishing the enhanced rate of elongation. Although the nature of these modifications remains unknown, it is likely that these entail changes on a molecular level, perhaps involving cleavages of chemical bonds.

Although a very large number of papers deal with the effects of conditions that variously promote or inhibit *Avena* coleoptile growth, relatively few are concerned with wall composition and the possible compositional changes occurring in the wall which may be associated with the modifications affecting rate of elongation. Nonetheless, from these reports, there is evidence that turnover or degradation occurs in certain wall fractions (Katz and Ordin, 1967a; Wada et al., 1968), and that, in at least one case (Ray, 1963), there is an auxin-induced decrease in content of a component of one wall fraction. Although such reports are of interest because they suggest a possible mechanism controlling elongation, these reports have been received with skepticism. There is doubt that the techniques used are reliable or precise enough to detect turnover, especially auxin-enhanced turnover at the levels reported. Where the changes are more striking, i.e., decreases in glucose content of certain wall fractions, it
has been proposed that the changes reflect either decreases in food reserves, i.e., starch, or decreases unrelated to elongation that are the result of differentiation of vascular tissues (Ray, 1969).

In an effort to determine what modifications of the wall are correlated with growth and possibly involved directly in the elongation process, this study investigates changes in wall composition which occur as *Avena* coleoptile tissue elongates. Particular attention has been given to the changes that occur under conditions designed to separate the processes of elongation and wall synthesis, the intent of the separation being to enable detection of those changes associated with elongation while the changes resulting from wall synthesis are at a minimum.
METHODS AND MATERIALS

Plant material

Oats (Avena sativa, var. Victory, obtained from Allmanna Svenska Utsadesaktifolget, Svalof, Sweden) were grown following a regimen modified from that of Wiegand and Schrank (1959). Seeds were dehulled, placed in a petri plate with distilled water at room temperature for 4 hr, then spread on the surface of 2.5 cm of distilled water-saturated vermiculite in plastic vegetable crispers with lids. Crispers with seeds were placed in dim red light (Figure 31, Appendix) for 44 hr, then transferred to darkness for 24 hr. Temperature ranged from 22 to 24 C. After a total of 73 to 76 hr, 25 to 30 mm coleoptiles were selected and the primary leaf removed. Beginning 3 mm from the tip, 10 mm sections were cut using a double-bladed cutting device. All manipulations were done in dim green light (Figure 32, Appendix). After cutting, sections were floated on distilled water for 30 minutes prior to treatment.

Treatment of sections

Treatments involved incubating coleoptile sections in 43 x 20 mm Stender dishes containing various solutions. The rim of the Stender dish was coated with petrolatum (Standard Oil) to prevent evaporation. Incubations were done using a Dubnoff shaker at 26 C, 60 oscillations per minute. After treatment, sections were measured to the nearest 0.1 mm using a 10 X dissecting microscope fitted with an ocular micrometer, wrapped in aluminum foil, and frozen at -24 C for subsequent analyses.

Preparation of wall samples

Wall samples were prepared using a technique adapted from that of Ray (1962). Frozen sections were placed between two 10 x 10 cm pieces of plate
glass, thawed, then crushed using a force of approximately 0.5 kg per
\text{cm}^2 applied manually. The crushed material was washed into a medium
fritted glass funnel using 10 ml of distilled water at room temperature.
The water was rapidly drawn off with suction and the water wash repeated
twice more. After the water wash, 5 ml of acetone was added, allowed to
stand with occasional swirling for 5 minutes, then removed by suction.
This acetone extraction was repeated twice more. The residue was then
similarly extracted three times, 5 minutes each, with 5 ml of chloroform:
methanol (1:1, v:v). After extraction, samples were transferred to alumi­
um weighing pans and dried \textit{in vacuo} over phosphorous pentoxide. Sample
weights were determined twice after at least two, then four days of drying.
Sections prepared in this fashion weighed from 150 to 250 micrograms per
section, and represented about 0.3 per cent of the tissue fresh weight.

**Hydrolysis and preparation of wall samples for gas chromatography**

To analyze non-cellulosic neutral sugars the technique of Nevins,
English, and Albersheim (1967) was utilized with only slight modification.
This technique involves a 1 hour hydrolysis with 2 N trifluoroacetic acid
(Eastman Organic Chemicals) in a sealed tube at 121 C. The sugars liberated
by hydrolysis are subsequently prepared for analysis by gas liquid chroma­
tography by converting them to their corresponding alditol acetates.
The conversion involves reduction of the sugars to the alditols using
sodium borohydride, and acetylation of the alditols to the volatile
acetate esters using acetic anhydride. To serve as an internal standard
throughout the procedure, myo-inositol (Calbiochem) is added with the
hydrolysis reagent. In these experiments the amount of inositol added,
0.5 to 2.0 mg per sample, was dependent on the size of the sample, 3.0 to
10.0 mg, representing wall fractions from 15 to 50 coleoptile sections. Using this technique, variations in sugar content of duplicate wall samples were small. For duplicate samples, standard deviations for glucose, arabinose, and xylose ranged up to 2.4 per cent, with an average between one and two per cent. For mannose and rhamnose, standard deviations ranged up to 9.0 per cent, with an average of about 6 per cent. For galactose the standard deviation ranged up to 4.0 per cent, with an average of 2.6 per cent.

Analyses of the total neutral sugar composition of the wall were done using methods adapted from those of Saeman et al. (1954). Approximately 10 mg samples were hydrolyzed in 1 ml of 72 per cent (w:w) sulfuric acid at 30 C for 1 hour. At 5 minute intervals during this period the tubes containing the samples were agitated with a vortex mixer. Following this primary hydrolysis, 28 ml of water containing 1 mg of inositol were added. The samples were transferred to 50 ml test tubes, covered, and autoclaved 1 hour at 121 C. Upon cooling, Dowex 1-X4, 20-50 mesh, carbonate form, was added to each sample until the pH was slightly above 4. The samples were then decanted, and the resin washed twice with 5 ml of water. The combined extracts in 100 ml beakers were frozen at -24 C, then lyophilized. The first time this technique was used the samples were dried at 40 C using a rotary evaporator, but all subsequent samples were freeze-dried. After drying, samples were taken up in 5 ml of water, transferred to test tubes, dried, and converted to the alditol acetates for gas chromatography in the same fashion as sugars resulting from 2 N trifluoroacetic acid hydrolysis. A comparison of the hydrolytic procedure with that using trifluoroacetic acid is summarized in Table 1. With this procedure, variations in sugar
content of duplicate wall samples were larger than those obtained in 2 N trifluoroacetic acid analyses. For duplicate samples, standard deviations ranged up to 6.4 per cent for glucose, 4.5 per cent for arabinose, 2.9 per cent for xylose, 3.7 per cent for galactose, 11.8 per cent for mannose, and 6.0 per cent for rhamnose. Average standard deviations were: glucose, 4.2 per cent; arabinose, 3.8 per cent; xylose, 2.7 per cent; galactose, 2.1 per cent; mannose, 9.1 per cent; and rhamnose, 5.3 per cent.

Analyses of the uronic acid content of wall samples followed a technique proposed by Thomas Jones and Peter Albersheim (personal communication) and adapted here for Avena coleoptile walls with several modifications. Figure 1 summarizes the procedure employed to analyze the uronic acid content of wall samples.

Walls of 25 to 50 coleoptile sections, representing approximately 5 to 10 mg of wall material, were placed in a 13 x 100 mm test tube. To each tube was added 2 ml of 0.2 N trifluoroacetic acid containing 1.0 mg of myo-inositol. The tubes were sealed and hydrolysis carried out at 121 °C for 1 hour. Following hydrolysis, the tubes were opened, and the trifluoroacetic acid and water removed by evaporation at 50 °C with a stream of air. Samples were stored overnight in vacuo over potassium hydroxide pellets to remove any residual trifluoroacetic acid.

Samples were next hydrolyzed with a mixture of enzymes isolated from a culture of the fungus Sclerotium rolfsii Sac. A culture, isolate 14, of this organism was obtained from Dr. D. F. Bateman, Cornell University, and was maintained on potato dextrose agar (Difco). Preparation of the enzyme extract was similar to the method of Van Etten and Bateman (1969). The fungus was grown on 100 g of autoclaved 8-day-old bean (Phaseolus
Table 1. Comparison of the yields of three different hydrolytic procedures

<table>
<thead>
<tr>
<th>Constituent</th>
<th>72% Sulfuric Acid$^a$</th>
<th>2 N Trifluoroacetic Acid$^b$</th>
<th>0.2 N Trifluoroacetic Acid plus Enzymic Hydrolysis$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>62.7 (30.8)$^d$</td>
<td>21.5 (10.5)$^d$</td>
<td>22.5 (11.3)$^d$</td>
</tr>
<tr>
<td>Xylose</td>
<td>22.1 (10.7)</td>
<td>21.1 (10.1)</td>
<td>22.5 (11.1)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>16.7 (8.0)</td>
<td>15.9 (7.7)</td>
<td>18.1 (8.7)</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.7 (2.3)</td>
<td>4.6 (2.3)</td>
<td>5.5 (2.7)</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.3 (1.2)</td>
<td>0.6 (0.3)</td>
<td>1.0 (0.5)</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.4 (0.2)</td>
<td>0.5 (0.2)</td>
<td>1.6 (0.8)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>109.0 (53.2)</strong></td>
<td><strong>64.2 (31.1)</strong></td>
<td><strong>71.3 (35.1)</strong></td>
</tr>
<tr>
<td>Galacturonic Acid</td>
<td></td>
<td></td>
<td><strong>10.0 (5.5)</strong></td>
</tr>
<tr>
<td>Glucuronic Acid</td>
<td></td>
<td></td>
<td><strong>1.7 (0.9)</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>11.7 (6.4)</strong></td>
</tr>
</tbody>
</table>

$^a$Average of six determinations.

$^b$Average of fourteen determinations.

$^c$Average of two determinations.

$^d$The first number represents the amount of each constituent in micrograms per section; the number in parentheses represents the percentage contribution to wall dry weight.
vulgaris L. var. Red Kidney) hypocotyls for 10 days at room temperature. Enzymes were extracted by adding 100 ml water to the culture, then blending 60 sec in a Waring blender. The mixture obtained was filtered through 4 layers of cheesecloth and centrifuged at 10,000 g for 30 minutes at 2 C. The supernatant was dialyzed overnight against distilled water at 4 C, lyophilized, and stored at 2 C. Prior to use, a 0.1 per cent (w:v) solution of lyophilized extract in 10 mM sodium acetate, pH 4.5, was prepared and centrifuged at 10,000 g for 30 minutes at 2 C. The supernatant, used for enzyme assays and for wall treatments, contained from 50 to 65 micrograms of protein per ml as determined by the Folin technique (Lowry et al., 1951) using crystalline bovine serum albumin (Armour) as a standard. Activities of this enzyme preparation towards various substrates are listed in Table 2.

A 2 ml portion of the preparation was added to each wall sample, and incubated for 6 hours at 30 C in a reciprocating water bath. At the end of this period the hemiacetal groups of the liberated sugars and uronic acids were reduced by adding 0.5 ml of 1.0 N ammonium hydroxide containing 3.0 mg of sodium borohydride and 1.0 mg of L-mannonic acid to each sample. The L-mannonic acid was added to serve as an internal standard for the determination of the uronic acids in the sample. It was prepared by base hydrolysis of L-mannono-1,4-lactone. This was done by evaporating to dryness at 45 C in a rotary evaporator a solution of 1.0 N ammonium hydroxide containing 1.816 mg of l-mannono-1,4-lactone (Pfanstiehl Laboratories) per ml of solution. The residue was taken up in an equal volume of 1.0 N ammonium hydroxide containing 6.0 mg per ml of sodium borohydride, giving the reducing reagent containing 1.0 mg of L-mannonic acid and 3.0 mg of sodium
Figure 1. Flow sheet of the procedure employed to analyze the uronic acid content of wall samples
Wall sample

\[ \text{Hydrolysis (0.2N TFA)} \]

\[ \text{Hydrolysis (} \text{Sclerotium rolfsii} \text{ enzyme prep.)} \]

\[ \text{Reduction of hemiacetal groups} \]

\[ \text{Separation of alditols and aldonic acids} \]

\[ \text{Supernatant with alditols} \]

\[ \text{Dowex resin pellets with aldonic acids} \]

\[ \text{Acetylation} \]

\[ \text{Wash with 1.0N HCl to remove aldonic acids} \]

\[ \text{Dry to form aldonolactones} \]

\[ \text{Reduction to alditols} \]

\[ \text{Acetylation} \]
Table 2. A comparison of some enzyme activities of an extract isolated from a culture of *Sclerotium rolfsii*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl-β-galactoside</td>
<td>6.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-nitrophenyl-β-glucoside</td>
<td>2.30</td>
</tr>
<tr>
<td>p-nitrophenyl-β-xyloside</td>
<td>0.06</td>
</tr>
<tr>
<td>p-nitrophenyl-α-galactoside</td>
<td>14.90</td>
</tr>
<tr>
<td>p-nitrophenyl-α-glucoside</td>
<td>0.87</td>
</tr>
<tr>
<td>p-nitrophenyl-α-xyloside</td>
<td>0.00</td>
</tr>
<tr>
<td>araban</td>
<td>2.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>xylan</td>
<td>1.70</td>
</tr>
<tr>
<td>laminarin</td>
<td>0.90</td>
</tr>
<tr>
<td>carboxymethylcellulose</td>
<td>0.70</td>
</tr>
<tr>
<td>dextran</td>
<td>0.00</td>
</tr>
<tr>
<td>pectin</td>
<td>5.50</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activities expressed as micromoles of product (see b and c) released per hour per ml of enzyme extract.

<sup>b</sup>Reaction mixture consisted of 1 ml of 50 mM Na-citrate (pH 4.6) of which 0.8 ml contained 0.1 % (w/v) enzyme extract and 0.2 ml contained 5 mM of substrate. Activities expressed as micromoles of nitrophenol released.

<sup>c</sup>Reaction mixture consisted of 1 ml of 50 mM Na-citrate (pH 4.6) of which 0.5 ml contained 0.1 % (w/v) enzyme extract and 0.5 ml contained a 1.0 % solution of substrate. Activities are reducing groups liberated expressed as micromoles of glucose.
borohydride per 0.5 ml.

Reduction of the liberated hemiacetal groups was continued for 1 hour at room temperature. The reaction was stopped and excess borohydride decomposed by adding glacial acetic acid until effervescence ceased. One ml of methanol was added and the samples mixed with a vortex mixer. Samples were centrifuged in a clinical centrifuge for 5 minutes at high speed, the supernatant drawn off with a capillary pipette, and the residue washed twice with 0.5 ml of 70 per cent ethanol. The ethanol washings and the supernatant were combined and evaporated to dryness at 50°C with a stream of air. To remove borate which interferes with acetylation, the samples were taken up in 1 ml of 10 per cent (w:v) acetic acid in methanol and evaporated to dryness. This process was repeated five times to remove borate as the volatile trimethyl ester (Nevins et al., 1967).

To separate the alditols and aldonic acids which resulted from the borohydride treatment the dried samples were taken up in 3.0 ml of distilled water and transferred to tubes containing 0.3 g (air-dried weight) of Dowex 1-X4 anion exchange resin, 20-50 mesh, acetate form. The samples were agitated with a vortex mixer at 5 minute intervals for 1 hour after which the resin was sedimented by centrifugation in a clinical centrifuge. The supernatant was transferred with a capillary pipette to a second tube containing approximately 0.2 g of the resin where it was again agitated at 5 minute intervals for 1 hour. Following a second centrifugation the supernatant was drawn off with a capillary pipette and transferred to a 13 x 100 mm test tube. The alditols in this tube constituted the neutral sugar fraction of the wall. See Table 1 for a comparison of this neutral
sugar fraction with those from other hydrolytic procedures.
The resin pellets were each washed twice with 5 ml of distilled water
and the washings discarded. To remove the aldonic acids which constituted
the uronic acid fraction of the wall, the two resin pellets from each sam­
ple were each suspended in 2.0 ml of 1.0 N HCl and agitated with a vortex
mixer at 5 minute intervals for 30 minutes at room temperature. Samples
and resin were then centrifuged and the supernatants from the two tubes re­
presenting a sample were transferred to a 13 x 100 mm test tube with a
capillary pipette. The solutions were evaporated to dryness at 50 C in a
stream of air. This evaporation of the acidic solution converted the
aldonic acids to aldonolactones. The dried residue was then stored over­
night in vacuo over potassium hydroxide pellets to remove residual HCl.

Reduction of the aldonolactones to the alditols involved dissolving
the dried samples in several drops of 10 mM boric acid and then adding
10.0 mg sodium borohydride in 0.5 ml of the boric acid solution. Reduction
was continued for 1 hour whereupon the reaction was stopped and the excess
borohydride decomposed with the addition of glacial acetic acid until
effervescence ceased. Samples were then evaporated to dryness at 50 C in
a stream of air. To remove borate, samples were redissolved in 1 ml of
10 per cent (w:v) acetic acid in methanol and evaporated to dryness. This
process was repeated 5 times. The alditols in the dried residue constitu­
tuted the uronic acid fraction of the wall.

The alditols from the neutral sugar fraction and from the uronic acid
fraction were converted to the volatile acetate esters for gas chromatog­
raphy using acetic anhydride in the same fashion as was done for the sugars
resulting from 2 N trifluoroacetic acid hydrolysis.
Several aspects of the uronic acid determination caused some concern. One of these was the possibility of contamination of the sample with uronic acids from the enzyme preparation. Contamination, however, proved negligible, dialysis apparently removing interfering substances.

A second concern was the effect of primary hydrolysis with 0.2 N trifluoroacetic acid. Although it has been reported that treatment with dilute acid increases yield of uronic acids from the wall with subsequent enzymic hydrolysis (Ray and Rottenberg, 1964), other reports indicate that nearly any acidic hydrolysis may destroy uronic acids (Setterfield and Bayley, 1961). My results confirm this: 2 N trifluoroacetic acid for 1 hour at 121 C results in nearly total degradation of galacturonic and glucuronic acid. To test the effect of 0.2 N trifluoroacetic acid under the same conditions employed for the uronic acid analysis, appropriate standards were prepared and subjected to acidic and enzymic treatment, enzymic treatment alone, or no treatment prior to reduction and the subsequent steps in the uronic acid procedure. Comparisons of the areas on chromatograms of the products indicate that 0.2 N trifluoroacetic acid results in losses of 55 to 40 per cent for galacturonic and glucuronic acid respectively. Enzymic treatment alone had negligible effects. Table 3 summarizes the results.

Choice of a suitable anion exchange resin was a third concern. Because of reports (Blake and Richards, 1971) that weakly basic resins such as Amberlite IR-45 are slow to release neutral sugars, and that strongly basic resins such as Dowex 1-X4 may cause degradation or rearrangement of sugars, the recovery of galacturonic acid from these resins was measured using different extraction procedures. Table 4 summarizes the results.
Table 3. Recovery of galacturonic and glucuronic acid after treatment with 0.2N trifluoroacetic acid and a *Sclerotium rolfsii* enzyme extract, or enzymic treatment alone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uronic Acid</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2N trifluoroacetic acid followed by enzymic treatment</td>
<td>galacturonic acid</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>glucuronic acid</td>
<td>60%</td>
</tr>
<tr>
<td>enzymic treatment alone</td>
<td>galacturonic acid</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>glucuronic acid</td>
<td>101%</td>
</tr>
<tr>
<td>control (neither enzymic or acid treatment)</td>
<td>galacturonic acid</td>
<td>101%</td>
</tr>
<tr>
<td></td>
<td>glucuronic acid</td>
<td>101%</td>
</tr>
</tbody>
</table>

Table 4. Recovery of galacturonic acid from Amberlite IR-45 and Dowex 1-X4 anion exchange resins using either 1.0N HCl or 6N acetic acid

Resins were in the acetate form.

<table>
<thead>
<tr>
<th>Resin</th>
<th>Extraction</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amberlite IR-45</td>
<td>1.0N HCl</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td>6.0N Acetic acid</td>
<td>15%</td>
</tr>
<tr>
<td>Dowex 1-X4</td>
<td>1.0N HCl</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>6.0N Acetic acid</td>
<td>52%</td>
</tr>
</tbody>
</table>
Dowex extracted with 1.0 N HCl gave the highest recovery and was used in the uronic acid procedure. But, even this extraction procedure did not give complete recovery. Because of this, and also because of the extensive degradation resulting from acid hydrolysis, yields for all wall were multiplied by appropriate recovery factors calculated from standards run concurrently with the wall samples. With this technique, standard deviations in uronic acid content of duplicate wall samples were 2.7 per cent for galacturonic acid and 10.0 per cent for glucuronic acid.

Gas chromatography

Whatever the means of hydrolysis, all samples following acetylation were stored in sealed tubes at - 24 C until analysis with gas chromatography. Just prior to analysis the tubes were opened, and the contents transferred with a capillary pipette to 2 ml serum vials. The acetic anhydride was evaporated nearly to dryness at room temperature with a stream of air, and the residue redissolved in 0.5 or 1.0 ml of methylene dichloride (Baker Analyzed Reagent). It has been found that use of methylene dichloride as solvent significantly reduces tailing of the solvent peak (Crowell and Burnett, 1967), and here it also appeared to prolong column life.

Separation of the alditol acetates employed conditions similar to those of Nevins et al. (1967). Column material consisted of 0.2 per cent polyethylene glycol adipate, 0.2 per cent polyethylene glycol succinate, and 0.4 per cent XF-1150 silicone oil coated on Gas-Chrom P (100-200 mesh). All components of the column material were obtained from Applied Science. Slightly over 1.2 g of this material was packed with vibration into 4 feet of washed 1/8 inch O.D. copper refrigeration tubing, .030 inch wall. After
preparation, columns were conditioned at 180°C for 2 to 4 hours.

Chromatography was done using a Packard 7311 dual column instrument with flame ionization detectors. Helium was used as the carrier gas. Hydrogen and compressed air were used for the detectors. Output of the electrometer was recorded either on a Honeywell Electronik 19 or a Beckman 10-inch recorder.

To separate the neutral sugar fractions of wall samples, temperature programing was employed. The instrument was equipped with a Model 847 analog type programmer which permitted non-linear programing of column oven temperature. Figure 2 depicts the program used. Starting temperature was 147°C; final temperature was 178°C. Injection port temperature was 200°C; detector temperature was 225°C. Separation of the uronic acid fractions did not require temperature programing. Oven temperature was maintained at 178°C. All other conditions were the same.

Peak heights and widths were determined by triangulation. These values for each alditol acetate and the internal standard, either mannitol hexaacetate or myo-inositol hexaacetate, for each injection were entered onto data cards. Using an Omnitab program, from the data on these cards the area of each peak was determined, the ratio of the area relative to that of the internal standard calculated, and the ratios averaged and the standard deviations calculated for the 3 replicate injections of each sample. These average ratios were, for each set of samples, compared to those of an identically treated sample run with each set which contained in addition to the internal standard, 1 mg each of seven neutral sugars i.e., rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose, or 1 mg each of the two uronic acids, galacturonic and glucuronic acid, and adjustment made
Figure 2. Column oven temperature programming employed for gas chromatographic separation of the neutral sugar fractions of wall samples.
for the specific recovery factor for each of the components for that set of samples. Data were expressed as mg of each component per sample, and also, by dividing these values by the number of coleoptile sections, mg per section.

**Determination of protein content**

Wall samples weighing from 10 to 14 mg were hydrolyzed overnight in sealed tubes at 121°C with 5 ml of a hydrolysis mixture containing 3 N HCl and glacial acetic acid (1:1, v:v). Following hydrolysis, the tubes were opened and dried at room temperature with a stream of air. Samples were re-dissolved in hot water to give approximately 10 micrograms of nitrogen per ml. To 0.5 ml of this solution 2 ml of ninhydrin reagent was added (Moore and Stein, 1954). Tubes were boiled for 15 minutes, cooled to room temperature, and 5 ml of 50 per cent ethanol added. Absorbancies were measured at 570 nm. A standard curve was prepared using threonine as a standard; absorbancies were converted to micrograms of α-amino nitrogen per sample; and protein was calculated as 6.2 x nitrogen. Protein content for all samples analyzed with this technique ranged from 16.4 to 23.8 per cent. For those wall samples isolated from coleoptiles which had been subjected to no pretreatment the average protein content was 21.2 per cent; the range was from 20.6 to 21.7 per cent.

An indirect method of measuring protein content was also employed. Wall samples, 10 mg, were incubated in a solution of Type VI Protease (Sigma). The solution contained initially 2 mg of enzyme per ml of 50 mM phosphate buffer, pH 7.5. Not all of the enzyme went into solution, however, and the particulate matter remaining was removed with centrifugation at 10,800 g for 30 minutes. To each wall sample, 2 ml of supernatant or
boiled supernatant was added and incubated at 37 C for 5 hours. Following incubation, 10 ml of water was added and the entire sample placed in a fritted glass funnel where the fluid portion was drawn off with suction. Samples were subsequently washed with acetone, then chloroform:methanol (1:1, v:v), and dried in vacuo over phosphorous pentoxide. Wall weights were determined before and after enzyme treatment. Decreases in wall weight resulting from Protease treatment averaged 20.2 per cent. There was only a trace of ninhydrin-positive material remaining in these walls.

**Starch determination**

Microscopic examination of wall preparations stained with iodine indicated the presence of a number of small pink particles primarily located in the cells surrounding the vascular elements. Examination of these particles in polarized light showed some birefringence like that associated with starch grains. To determine the amount of starch present, α-amylase was incubated with wall samples and the amount of glucose in the wall susceptible to enzyme treatment measured indirectly by hydrolysis and chromatography of wall samples before and after enzyme treatment.

The procedure employed a freshly prepared stock solution consisting of 0.1 ml of α-amylase (Sigma, Type I-A, from hog pancreas) in 100 ml of 0.02 M sodium phosphate, pH 6.9, and 6 mM sodium chloride. Activities of a 20 x dilution of this stock with 1 per cent soluble starch as substrate were determined using a dinitrosalicylate method (Luchsinger and Cornesky, 1962) to measure the reducing groups released. Maltose was used as a standard. Absorbancies were measured at 540 nm. Activities were determined before, during, and after incubations of wall material in the enzyme preparation. Initial activity was 9.2 micromoles per minute per milliliter of enzyme
stock, and final activity, after 21 hours at 30 °C, was 5.0 micromoles per minute per milliliter.

Activity of the stock towards a suspension of oat starch was also determined. Oat starch was prepared by removing the embryo and distal ends of oat caryopses and soaking the remaining central portion in 80 per cent ethanol overnight at 4 °C. The soaked material was then crushed in a mortar, filtered through several layers of cheesecloth, and the filtrate suspended in water and centrifuged at 1000 g for 2 minutes. The pellet was resuspended in 80 per cent ethanol and centrifuged again. This washing was repeated 4 times in all. The final pellet was transferred to a petri plate and dried in vacuo over phosphorous pentoxide several days. Microscopic examination of this preparation revealed only starch grains. Hydrolysis and chromatography indicated that the preparation was homogeneous, containing only glucose. A suspension of this material, 100 mg in 10 ml of buffer, was added to 10 ml of the stock enzyme preparation and incubated in a reciprocating water bath at 30 °C. Duplicate aliquots, 0.5 ml each, were withdrawn to assess the reducing groups released after varying periods of incubation. There was no increase in reducing groups in the boiled enzyme control.

Wall samples were incubated with 2 ml of the stock enzyme solution or 2 ml of boiled enzyme under the same conditions as the oat starch. Following incubation, 10 ml of water was added and the entire wall sample transferred to a fritted glass funnel where the fluid portion was removed with suction. Samples were subsequently washed with acetone and chloroform: methanol (1:1, v:v). Wall samples were hydrolyzed with 2 N trifluoroacetic acid, and the sugars released analyzed using gas chromatography.
RESULTS

In order to determine the effects of auxin on changes in *Avena* coleoptile cell wall composition, in particular those changes associated with the growth process, coleoptile cell walls were analyzed after varying periods of incubation. The incubation conditions included treatments designed to give the most rapid growth or the least amount of wall synthesis. Two major fractionation procedures were employed, one using 2 N trifluoroacetic acid and the other using 72 per cent sulfuric acid; the first was used to determine non-cellulosic sugar composition of the walls, and the second was used to determine total neutral sugar composition. A third fractionation procedure was employed to determine the contribution of uronic acids to total wall composition. In addition, analyses were run to determine the contribution of other substances, i.e., starch and protein, to the composition of the wall preparations.

**Qualitative and quantitative composition of the walls**

The changes in wall composition resulting from treatment with $\alpha$-amylase and the reducing groups released from oat starch with $\alpha$-amylase are given in Figure 3. It is apparent that most $\alpha$-amylase-susceptible glucose in the wall preparations was released within the first few hours of incubation. There was a 13 per cent decrease in glucose in the first 4 hours. Subsequent incubation, 16 hours, resulted in a further decrease of only 4 per cent for a total of 17 per cent.

In contrast to the wall preparations, 34 mg of maltose equivalents were released from the oat starch in the first 4 hours of incubation, and an additional 62 mg were released in the next 16 hours. These results indicate that the enzyme was still active against starch after 4 hours and that
Figure 3. Effect of α-amylase on (A) *Avena* coleoptile wall glucan content and (B) release of maltose equivalents from oat starch.
mg maltose released from oat starch
the failure of the enzyme to cause a similar release of glucose from the
wall preparations was due to lack of substrate. It therefore seems likely
that 17 per cent can be adopted as a reasonable maximum value representing
the contribution of starch to the glucan content of these wall preparations.

In sections cut from 73 to 76 hour-old coleoptiles and subjected to
no further treatment, the predominant wall sugars released with hydrolysis
using 2 N trifluoroacetic acid were xylose, arabinose, and glucose (Table 1).
There was a smaller amount of galactose. Rhamnose and mannose were present,
but represented less than 2 per cent of the weight of this fraction.
Hydrolysis of similar coleoptile sections with 72 per cent sulfuric acid
resulted in only slight increases in the yields of arabinose, xylose, and
galactose. Yields of mannose and glucose, however, increased markedly
(Table 1). There was not a significant change in the yield of rhamnose.
The major difference between these two hydrolytic procedures probably re­
flects the resistance of cellulose and glucomannans to hydrolysis in tri­
fluoroacetic acid.

Hydrolysis using 0.2 N trifluoroacetic coupled with an enzymic hydroly­
sis, when compared to hydrolysis in 2 N trifluoroacetic acid, resulted in
slightly higher yields of all sugars. Of the uronic acids released,
galacturonic acid was predominant, representing 85 per cent of the total.
Glucuronic acid comprised the remainder. Table 1 summarizes the results of
this procedure and compares it with the other two extraction procedures.

Protein content, as represented by ninhydrin-positive material, for
all wall samples analyzed regardless of pretreatment, ranged from 16.4 to
23.8 per cent. The amount of protein, on a per coleoptile section basis,
ranged from 34 to 51 micrograms. For those wall samples isolated from
coleoptile sections which had been subjected to no pretreatment the average protein content was 21.2 per cent; the range was from 20.6 to 21.7 per cent.

Protein content, as determined indirectly by weight loss after Protease treatment, was 20.2 per cent. Table 5 summarizes the effects of Protease treatment on wall weight and wall composition of coleoptile sections subjected to no pretreatment prior to analysis. Although Protease treatment resulted in a 20.2 per cent weight loss, it had a negligible effect on non-cellulosic neutral sugar composition.

The total of the weight of the anhydrous sugars released with hydrolysis in 72 per cent sulfuric acid and the weight of the other components determined, i.e., protein and anhydrous uronic acids, represents 81 per cent of the dry weight of the wall preparations.

Changes in composition with growth

Time course studies of auxin-induced changes in composition were determined initially under two sets of conditions. One set involved those changes occurring when coleoptile sections were exposed to the optimum conditions for growth, these conditions having been found to be 0.05 M glucose and $1.25 \times 10^{-5}$ M indoleacetic acid in 2.5 millimolar potassium citrate buffer, pH 5.4. The other set involved minimizing wall synthesis by depriving the tissue of wall precursors so that relatively little increase in total wall material could occur. This was achieved to a certain degree by using the above medium without glucose.

Figure 4 compares the effects of these conditions, with and without IAA, after various periods of incubation. The initial effect of auxin on growth, regardless of the presence or absence of glucose, is to enhance rate of elongation. The effect of glucose only becomes evident after
Table 5. Effect of Protease on wall composition and wall weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rhamnose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Wall Weight</th>
<th>Per cent change in weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled enzyme control</td>
<td>0.6</td>
<td>13.8</td>
<td>19.8</td>
<td>0.7</td>
<td>4.8</td>
<td>19.2</td>
<td>191</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>0.7</td>
<td>14.2</td>
<td>20.3</td>
<td>0.7</td>
<td>4.5</td>
<td>18.8</td>
<td>153</td>
<td>20.2</td>
</tr>
</tbody>
</table>
incubation when the rate of growth in those sections lacking exogenous glucose drops rapidly, this decrease being particularly apparent after 4 hours incubation. After 8 hours incubation the rate of elongation is very nearly that of the auxin-free controls, with or without glucose. In contrast, sections incubated with exogenous glucose maintained their auxin-enhanced rate of growth much longer, the rate being nearly constant and only diminishing toward the end of the incubation period.

Determination of weights of wall preparations from sections exposed to these same sets of conditions indicated that an effect of exogenous glucose was to increase wall weight (Figure 5). Presence of auxin enhanced this effect of glucose. In sections incubated without auxin, wall weights increased 47 per cent. If exogenous glucose was not supplied in order to minimize wall synthesis, changes in wall weight after 20 hours were almost negligible, auxin-treated sections increasing 4 per cent and the controls increasing 2 per cent. The time course studies indicated that these small increases were the result of an initial increase followed by a slow decrease (Figure 6).

Certain changes in total sugar composition paralleled the changes in wall weight. For example, exogenous glucose increased incorporation of all sugars in the wall. The effect of auxin under these conditions, however, was more specific. Synthesis of arabinose, xylose, and galactose was enhanced two-fold with auxin treatment, but there was little auxin effect on total wall glucose. After 20 hours, the glucan content of auxin-treated walls was 90.7 micrograms per section; in the controls, 88.4 micrograms per section. These changes in the five major neutral sugar components, with exogenous glucose and with or without auxin treatment, are depicted in
Figures 7 and 8 respectively. As a constituent which comprised less than 1 per cent of this fraction, rhamnose is not depicted; however, it did increase about 50 per cent with both treatments.

As the changes in wall weight indicated, synthesis of wall components was slight when exogenous glucose was not available to coleoptile sections. Changes in the total neutral sugar composition of auxin-treated and control sections when exogenous glucose was lacking are depicted in Figures 9 and 10 respectively. There were increases in arabinose and xylose, but auxin only slightly enhanced these increases. Galactose did not increase with auxin treatment; rather, after 20 hours there was less than there was in the wall initially. Glucose increased slightly in the first few hours of incubation, then changed little. The total change for both galactose and glucose was small.

Comparisons of changes occurring in the non-cellulosic fraction of the wall, that hydrolyzed with 2 N trifluoroacetic acid (Figure 11), with the changes observed in total sugar composition (Figure 7) reveal one major difference. When exogenous glucose was available to auxin-treated coleoptile sections not all neutral sugar components of this fraction increased. Non-cellulosic wall glucose content did increase initially, 25 per cent with auxin (Figure 11), 27 per cent without auxin (Figure 12), but further changes were slight. Glucose continued to increase slightly in the control, to 30 per cent after 20 hours (Figure 12), but it decreased in auxin-treated sections, for a net increase of 19 per cent after 20 hours (Figure 11). Arabinose, xylose, and galactose increased in much the same manner as they did in the 72 per cent sulfuric acid hydrolysates. Mannose and rhamnose, not depicted, represented less than 1 per cent of this fraction and changed
very little.

In those sections where wall synthesis was inhibited by not supplying exogenous glucose, auxin-induced changes in this non-cellulosic fraction were striking (Figure 13). Although there was an initial increase of 11 per cent in wall glucan content, after 2 hours it began to decrease. Between 4 and 8 hours, there was nearly a 50 per cent decrease. After 8 hours the decrease continued at a slower rate so that at 20 hours non-cellulosic wall glucan content was 25 per cent of the initial value.

There was also a decrease in the auxin-free control, but this decrease was less than half that induced by auxin: final non-cellulosic glucan content was 63 per cent of the initial value (Figure 14). Somewhat similar changes are seen in the content of wall galactose. In sections without auxin or exogenous glucose, wall galactose changed little, decreasing only 3 per cent in 20 hours, but with auxin it decreased 13 per cent. These decreases, especially those in glucan content, became apparent at the same times as did the decreases in growth rate under the same conditions.

Comparisons of changes in glucose and galactose in the non-cellulosic fraction with the changes in the same components in the 72 per cent sulfuric acid hydrolysates indicate that the decreases in the first fraction are nearly balanced by increases in the latter fraction. These changes reflect an increase in α-cellulose at the same time as non-cellulosic glucan is decreasing. These changes are depicted in Figures 15a and 15b which summarize data represented in previous graphs of the changes in wall glucose which occur in 2 N trifluoroacetic acid and 72 per cent sulfuric acid fractions when sections are treated with and without IAA in the absence of exogenous glucose. The upper curve in each graph represents total wall
Figure 4. Effects of presence and absence of 0.05 M glucose and $1.25 \times 10^{-5}$ M IAA on elongation of *Avena* coleoptile sections
Figure 5. Changes in wall weights of coleoptile sections incubated with 0.05 M glucose in the presence and absence of $1.25 \times 10^{-7}$ M IAA.
Figure 6. Changes in wall weights of coleoptile sections incubated without glucose and in the presence and absence of $1.25 \times 10^{-5}$ M IAA.
Figure 7. Changes in total sugar composition of walls from coleoptile sections incubated with 0.05 M glucose and $1.25 \times 10^{-5} \text{ M IAA}$
Glucose

+ IAA
+ Glucose

μg sugar/section vs. time (hrs)
Figure 8. Changes in total sugar composition of walls from coleoptile sections incubated with 0.05 M glucose and without IAA
- IAA
+Glucose
Figure 9. Changes in total sugar composition of walls from coleoptile sections incubated with $1.25 \times 10^{-5}$ M IAA and without glucose
Figure 10. Changes in total sugar composition of walls from coleoptile sections incubated without glucose or IAA
- IAA
- Glucose

μg sugar/section

- gl
- x
- a
- ga
- m

time (hrs)
Figure 11. Changes in non-cellulosic sugar composition of walls from coleoptile sections incubated with 0.05 M glucose and $1.25 \times 10^{-5}$ M IAA
Figure 12. Changes in non-cellulosic sugar composition of walls from coleoptile sections incubated with 0.05 M glucose and without IAA.
Figure 13. Changes in non-cellulosic sugar composition of walls from coleoptile sections incubated with $1.25 \times 10^{-5}$ M IAA and without glucose.
Figure 14. Changes in non-cellulosic sugar composition of walls from coleoptile sections incubated without IAA and without glucose
- IAA
- Glucose

μg sugar/segment

time (hrs)
Figure 15. Changes in total wall glucose (the upper curve), glucose released with 2 N trifluoroacetic acid hydrolysis (the lower curve), and the difference between the two curves (the dashed line) when sections are treated without IAA (A) and with IAA (B) in the absence of exogenous glucose.
A. 
- glucose 
- IAA

B. 
- glucose 
+ IAA

Graphs showing the changes in micrograms of total glucose, cellulose, and non-cellulosic glucose over time (0 to 20 hours) for both treatments A and B.
Figure 16. Changes in total wall glucose (the upper curve), glucose released with 2 N trifluoroacetic acid hydrolysis (the lower curve), and the difference between the two curves (the dashed line) when sections are treated without IAA (A) and with IAA (B) in the presence of 0.05 M glucose.
A.  
+glucose  
-IAA  
total glucose  
cellulose  
non-cellulosic glucose

B.  
+glucose  
+IAA  
total glucose  
cellulose  
non-cellulosic glucose

micrograms/section

0 1 2 4 8 20

time (hrs)
Figure 17. Influence of auxin on changes in wall weight and wall glucose in walls from coleoptile sections pretreated 4 hours prior to beginning auxin treatment.
Figure 18. Influence of auxin on changes in wall weight and wall glucose in walls from coleoptile sections pretreated 6 hours prior to beginning auxin treatment.
Figure 19. Influence of auxin on changes in wall weight and wall glucose in walls from coleoptile sections pretreated 8 hours prior to beginning auxin treatment
Wall Weight

Wall Glucose

Time (hrs)

μg / section

-IAA

+IAA

μg / section

-IAA

+IAA
Figure 20. Growth of *Avena* coleoptiles and changes in non-cellulosic sugar composition of 1.0 cm sections cut from the coleoptiles during the period of most rapid elongation.
μg sugar/section

Coleoptile length

hours after planting

length (mm)
glucose, and the lower curve represents the glucose released with 2 N tri-fluoroacetic acid. The difference between the two curves, the dashed line, is taken here to represent cellulose, a glucan resistant to hydrolysis by 2 N trifluoroacetic acid but hydrolyzed by 72 per cent sulfuric acid treatment. It is clear that, although there is little increase in total wall glucose, and while non-cellulosic wall glucose is decreasing, there is still synthesis of cellulose. Similar increases in cellulose also account for the increases in total wall glucose in those sections which were supplied with exogenous glucose. Figures 16a and 16b depict these latter changes.

Since the absence of exogenous glucose did not entirely inhibit wall synthesis, especially that enhanced by auxin, subsequent time course studies of auxin-induced changes in wall composition were of sections that had been pretreated for varying periods of time so that the sections would be depleted of endogenous precursors of wall synthesis. The pretreatments involved cutting sections in the usual fashion and then floating them on 2.5 millimolar potassium citrate buffer, pH 5.4, without auxin or glucose. After 4, 6, or 8 hours the buffer was removed and replaced with fresh buffer, with or without auxin. Samples were taken at the beginning of auxin treatment and at 1, 2, 4, and 6 hours thereafter. Figures 17, 18, and 19 depict the auxin-induced changes in wall weight and 2 N trifluoro-acetic acid soluble wall glucose which resulted. Other sugars are not depicted; they underwent little change in these treatments. After a 4 hour pretreatment to deplete sections of precursors auxin still enhanced synthesis of wall glucose; however, within 2 hours of auxin treatment a decrease relative to the control was apparent. Parallel changes in wall weight
occurred also, with an initial increase followed by a decrease (Figure 17). After a 6 hour pretreatment there was no auxin-induced increase in glucose incorporation; instead, a slight decrease was apparent which became greater with time. Wall weight did increase, reflecting promotive effects of auxin on incorporation of arabinose and xylose under these conditions (Figure 18). After an 8 hour pretreatment there was neither an increase in wall weight or wall glucan (Figure 19). These sections subjected to an 8 hour pretreatment did grow. In six hours, 1.16 cm sections elongated to 1.19 cm without auxin, and to 1.34 with auxin, and the only auxin-induced change detectable in the wall was a decrease in non-cellulosic glucose.

Figure 20 depicts the growth of intact coleoptiles and the neutral sugar composition of the non-cellulosic fraction of 1 cm sections cut from the coleoptiles at various times during the period of most rapid growth. This allows comparison of the changes occurring during growth of the intact tissue with the changes occurring in excised sections subjected to the various concentrations of auxin and glucose. Strict comparisons cannot be made, however, for, unlike the experiments with excised sections, the tissue sampled each time did not exactly represent the tissue sampled previously. Rather, as the coleoptiles elongated, a 1 cm section cut from them represented a smaller and smaller fraction of the cells or tissue sampled initially. With this limitation, it can be seen that neutral non-cellulosic wall constituents decreased on a per unit length basis. Wall dry weights decreased also. All decreased at approximately the same rate, about 25 per cent over the 24 hour period sampled. The decrease was not just the result of a thinning process as the walls elongated; the rate of decrease was much less than the rate of elongation
which was nearly 300 per cent over the same period. Thus, the changes observed reflected net changes resulting from thinning and wall synthesis. If adjustment is made for the decreases that resulted from taking what in some respects was a smaller sample each time, the changes in intact coleoptiles were very similar to those observed in excised sections treated with auxin and glucose.

Figure 21 depicts changes in the content of galacturonic acid and glucuronic acid over a 20 hour period when sections were treated with and without auxin in the presence and absence of exogenous glucose. The major factor regulating changes in the uronic acids appeared to be auxin. With auxin treatment galacturonic acid increased. The controls increased also, but at a progressively slower rate so that the differences were not apparent until after about 8 hours incubation. In contrast to the neutral sugars where exogenous glucose had definite effects on the changes occurring, changes in uronic acid content were more independent of exogenous glucose. With 20 hours of auxin treatment, galacturonic acid increased 50 per cent in sections without glucose, and 58 per cent in sections with glucose. Also, unlike the neutral sugar components, galacturonic acid continued to increase even after 8 hours incubation without exogenous glucose. All other wall components under these conditions after 8 hours either decreased or ceased to increase (See Figures 9 and 13 for comparison). Glucuronic acid content changed also, but only when sections were treated with both glucose and auxin. Under these conditions the content of glucuronic acid increased, as did the content of nearly all wall components (See Figures 7 and 11 for comparisons).
Figure 21. Changes in glucuronic acid and galacturonic acid content of walls from coleoptile sections treated without (A) and with (B) IAA and with (-----) and without (------) exogenous glucose
DISCUSSION

Upon considering the arbitrariness of most wall fractionation schemes and the ambiguity surrounding the susceptibility of linkages within the wall to various extraction techniques (Albersheim, 1965b), no attempts were made here to fractionate the non-cellulosic portion of the wall. In this report the fraction of the wall susceptible to hydrolysis with 2 N trifluoroacetic is considered to represent the neutral non-cellulosic polysaccharides of the wall, primarily because of the lack of additional quantities of sugars other than glucose or mannose being released upon total hydrolysis in 72 per cent sulfuric acid. Other investigators have arrived at a similar interpretation of the yield of sugars from walls treated with a 2 N trifluoroacetic acid hydrolysis (Nevins et al., 1968). Previous investigators concerned with Avena coleoptile wall composition have employed wall extraction procedures and in their reports some of the fractions obtained also have been referred to as representing the non-cellulosic polysaccharide portion of the wall. One of these groups (Bishop et al., 1958), utilizing ammonium oxalate and sodium hydroxide extractions, found that the non-cellulosic composition of the wall was 34.3 per cent glucose, 29.8 per cent xylose, 29.1 per cent arabinose, and 6.7 per cent galactose. Another study (Ray, 1962), using dilute sulfuric acid and potassium hydroxide extractions, found the non-cellulosic composition of the wall to be 29.7 per cent glucose, 33.6 per cent xylose, 27.7 per cent arabinose, and 6.7 per cent galactose. In the current studies hydrolysis with 2 N trifluoroacetic acid yielded a non-cellulosic fraction consisting of 33.5 per cent glucose, 32.8 per cent xylose, 24.9 per cent arabinose, and 7.2 per cent galactose. Considering the different extraction procedures employed,
possible differences in coleoptile age, and the different varieties of oats, the results appear similar. The other technique employed here, using 0.2 N trifluoroacetic acid and enzymic hydrolysis, resulted in a fraction very similar to that obtained with 2 N trifluoroacetic acid, i.e., 31.6 per cent glucose, 31.6 per cent xylose, 25.4 per cent arabinose, and 7.7 per cent galactose.

Several groups have attempted to assess the contribution of uronic acids to Avena coleoptile wall composition and the results have varied. One group (Bishop et al., 1958) found less than one per cent of the wall dry weight to be galacturonic acid. Another group (Albersheim and Bonner, 1959) obtained 3.5 to 4 per cent. A third group (Jansen et al., 1960) using gentler techniques, i.e., more dilute acid hydrolysis coupled with an enzymic hydrolysis, obtained 5 per cent anhydrouronic acid. Ray (1962) found 7 per cent uronic acid. Ray and Rottenberg (1964), using hot dilute acid and pectinase, found the uronic anhydride content to be about 5 per cent, of which two-thirds was galacturonic acid and one-third was glucuronic acid and 4-O-methyl glucuronic acid. Since methods of wall sample preparation varied greatly it is difficult to compare these results. The lower values, however, are thought to have resulted from hydrolytic destruction of the uronic acids (Setterfield and Bayley, 1961). The results obtained here with galacturonic acid representing 5.5 per cent of the wall dry weight and glucuronic acid representing 0.9 per cent compare more closely with the higher values.

In attempts to characterize the integrity of the wall preparations the amount of starch and protein were determined. Other reports of Avena coleoptile wall composition reported starch present as a contaminant in
their preparations on the basis of results obtained using different tech­niques, namely extractions coupled with determinations of iodine-positive material. One of these reported 3 distinct iodine-positive substances comprising a total of 22 per cent of the non-cellulosic fraction (Ray, 1962). Another reported about 7 per cent of the non-cellulosic glucose to be iodine-positive (Bishop et al., 1958). The results reported here show that 17 per cent of the non-cellulosic glucan was susceptible to α-amylase.

The total of the weight of the anhydrous sugars released with hy­drolysis in 72 per cent sulfuric acid and the weight of the other com­ponents determined, i.e., protein and anhydrous uronic acids, represented 81 per cent of the dry weight of the wall preparations. The remaining 19 per cent may represent losses due to degradation resulting from the hydrolytic procedures employed. This possibility could also account for the slight increases in yield which resulted when the wall was hydro­lyzed using the milder conditions of 0.2 N trifluoroacetic acid and en­zymic hydrolysis. Alternatively, there are several wall constituents which, although not determined here, could account for a share of the wall dry weight, these being lignin, ash representing mostly calcium, and methoxy groups and methyl esters of uronic acids. Although the procedure used to isolate wall samples, extraction with acetone and chloroform: methanol (1:1, v:v), makes it unlikely that there was any residual lipid left on the wall, it is likely that a certain share of the wall weight represented water remaining after extended drying times. Wall prepara­tions were dried in vacuo over phosphorous pentoxide, but they were quite hygroscopic, and the lag between removal from the dessicator and weighing
may have resulted in water uptake. Effort was made to minimize this lag, but changes in wall weight were observed to occur.

Although wall synthesis has been implicated as a causal factor in the mechanism of cell wall extension, the data from the pretreatment experiments indicate that auxin has little or no effect on wall synthesis when wall precursors are in short supply or are not available. For example, after an 8 hour pretreatment to remove endogenous precursors of wall synthesis, addition of auxin causes only a single wall neutral sugar to change. That component is glucose and it decreases. Wall weight decreases also, in amounts equivalent to the decreases in glucose. Yet under the same conditions auxin enhances rate of elongation. These results alone cast doubt on the dependency of growth on wall synthesis.

In the experiments where sections were not pretreated prior to adding auxin there did appear to be correlations between growth and certain increases in the wall, especially auxin-induced increases in arabinose and xylose which parallel elongation, but such correlations are apparent only when precursors are available.

It is possible, as has been suggested elsewhere (Ray, 1969), that there may be two (or more) types of auxin-enhanced wall synthesis, one not essential to growth which does not occur when the supply of precursors is limited, and another that is the "causal factor" in elongation. If so, under normal conditions, with a pool of precursors available, the contribution of growth-independent wall synthesis might obscure that component of wall synthesis upon which enlargement is dependent. It might also obscure any correlations between growth and wall synthesis.
should expect increases in that wall component or fraction any time the sections grow, even under conditions where the precursor pool was limited and where other wall synthesis was inhibited. Inspection of the data from the experiments where the sections were pretreated prior to adding auxin indicates that no non-cellulosic neutral sugar increases when those sections grow. Involvement of these components in this type of mechanism therefore seems unlikely. A more likely candidate for this type of mechanism is galacturonic acid. Galacturonic acid incorporation continues independent of the presence or absence of exogenous glucose and appears to be related to auxin-induced growth. But, if synthesis were a prerequisite for growth, then enhanced synthesis should become apparent with or before the onset of enhanced growth. This does not occur. Auxin effects on synthesis become apparent only after 4 to 8 hours incubation. Likewise after 8 hours treatment when the rate of growth diminishes in those sections lacking exogenous glucose one should expect a corresponding decrease in galacturonic acid incorporation. This also does not occur. Galacturonic acid incorporation continues undiminished. Thus, it may be that galacturonic acid synthesis is an example of a type of synthesis that is not related to growth instead of an example of a type which is a causal factor in the growth process.

Another possibility, if there are two types of wall synthesis, is elongation dependent on synthesis utilizing as precursors the products resulting from biochemical rearrangement of the existing wall. This might be difficult to detect, especially if the precursor (product) were only slightly modified upon utilization in synthesis, for example, if it were incorporated in the opposite anomeric configuration. Assuming an
appropriate fractionation scheme was adopted, such turnover might become apparent with a decrease in one fraction being matched by an increase in another. Of the results reported here, there is the decrease in non-cellulosic glucan which is auxin-enhanced, and the corresponding increase in cellulose. Whether these increases in cellulose are examples of a kind of synthesis or transition within the wall that is responsible for growth is questionable for several reasons. First, although there is more cellulose in those sections treated with auxin than in those without auxin, there does not appear to be a correlation between the extent of these increases and the amount of elongation. This is especially true in those sections incubated with and without auxin in the absence of exogenous glucose. There is only slightly more cellulose in the auxin-treated material than in the control, yet elongation is more than twice that of the control. Secondly, other studies of wall synthesis have indicated that there is a lag in auxin-induced wall synthesis, especially synthesis of cellulose (Ray and Baker, 1964) and β-glucan (Abdul-Baki and Ray, 1971). It should be pointed out, however, that the tissue in one of these experiments was incubated in labeled glucose for only one hour prior to auxin treatment, and, as the authors admit, it is easily possible that the precursors through which auxin promotes wall synthesis may not have reached isotopic equilibrium in that time (Ray and Baker, 1964). A longer incubation period would help establish the direct effect of IAA on synthesis. Somewhat similar problems were encountered in the second report cited above (Abdul-Baki and Ray, 1971). There the problem was considerable isotope dilution by endogenous substrates, and promotion by auxin was said to develop over a period of one to two hours.
A third problem that arises is that enhanced synthesis of cellulose may be an indirect effect of auxin. When coleoptile sections were incubated in appropriate concentrations of calcium ion, elongation was completely inhibited. Auxin treatment under these conditions did result in promotion of wall synthesis, but only non-cellulosic wall synthesis. Cellulose synthesis apparently did not occur. The conclusion was that cellulose synthesis was an indirect effect of auxin treatment, being stimulated by elongation and not auxin (Ray and Baker, 1964). However, the results presented here fail to support this; little or no correlation between cellulose synthesis and elongation was found.

A fourth problem arises from ultrastructural studies of the pattern of cellulose synthesis. In many primary cell walls cellulose synthesis apparently is exclusively appositional (Frey-Wyssling and Mühlethaler, 1965, Roelofsen, 1959). Cellulose is not intercalated into the existing wall, a condition thought to be necessary if synthesis does play a role in growth (Wilson, 1964). In *Avena* coleoptile cell walls Ray (1967), in his study combining radioautography, electron microscopy, and various extraction techniques, found that cellulose synthesis was appositional and that only non-cellulosic polysaccharides were incorporated into the existing wall. He thought that internal incorporation of hemicelluloses might play a role in the cell wall expansion process. This conclusion seems unlikely in view of the results here which indicate growth still occurs even when exogenous glucose is lacking and synthesis of non-cellulosic materials is inhibited.

Whatever the contribution of wall synthesis to growth, and the evidence from the growth curves indicates that it may be necessary for
sustained auxin-induced rates of elongation, the effect on elongation of other auxin-induced modifications of the wall must be considered also. The most significant of these is the auxin-induced decrease in non-cellulosic wall glucan. This decrease represents 75 per cent of the non-cellulosic glucan or 9 per cent of the initial wall dry weight.

Somewhat similar losses have been noted before (Ray, 1962; Baker and Ray, 1964a), but in those reports the losses were not as extensive as reported here. It could be argued that this decrease represents nothing more than utilization of starch present in wall preparations as a contaminant. The exhaustive enzyme extractions, however, indicate only 17 per cent of the non-cellulosic glucan (2 per cent of the wall dry weight) is susceptible to α-amylase. Thus, even if all of the starch that is in wall preparations is utilized in response to auxin treatment, this still means that a minimum decrease in glucan of 58 per cent is involved in wall modification.

Admittedly, in some respects the auxin-induced decreases in non-cellulosic glucan do not correlate well with changes in rate of elongation. For example, when the sections are given no pretreatment before being subjected to auxin, non-cellulosic glucan increases and then decreases. It may be that such changes represent the net of two concurrent processes, synthesis and degradation, and both occur at least up to the time of precursor depletion after which obvious evidence of degradation appears. Thus, before direct effects of auxin on these decreases in the wall can be demonstrated, one must pretreat the tissue to remove endogenous supplies of wall precursors. This is analogous to depletion of endogenous levels of auxin before direct effects of auxin on
elongation can be demonstrated (Went and Thimann, 1937). The results of the various pretreatment experiments tend to support this: as pretreatment time increases, the magnitude and duration of these transitory auxin-induced increases in glucan content decrease. Thus, after 8 hours pretreatment no increases in glucan are apparent; rather, there is correlated with auxin-induced growth an immediate decline in glucan content.

What bearing these changes have on the elongation mechanism is unclear. Until more information is available regarding this fraction's contribution to wall structure it is unlikely that any definite assertions can be made. One can only speculate on what effect modification of 9 per cent of a wall or 75 per cent of a specific wall fraction would have on wall extensibility and growth, but it is well established that much more minor changes than this result in profound modification of the properties of some polysaccharides (Selby, 1963).

One possible arrangement of polysaccharides in the wall thought to be involved in extensibility and growth is glycosidic linkage of polysaccharides to protein, i.e., in hydroxyproline-O-glycoside (Lamport, 1967). With regard to this possibility, one report concerning the effect of Pronase on walls found no change in extensibility even though most of the protein had been removed. Wall hydroxyproline, however, was more resistant to this treatment and less than half was removed (Olson et al., 1965). In another report, however, removal of 90 per cent of the protein in the wall preparations with similar enzyme treatment increased plastic extensibility of coleoptile walls, suggesting that proteins do act as
stiffening agents in the wall, presumably by creating a much higher
degree of crosslinking. But, the auxin effect on plastic extensibility
was not altered by removal of protein (Cleland, 1967a), which suggests
that the effect of auxin which is related to the induction of growth
must be on some other component of the wall. Results here indicate
that removal of over 95 per cent of the ninhydrin-positive material in
these wall preparations has no effect on non-cellulosic polysaccharide
composition (Table 5). Thus, the polysaccharide component of the wall
that is most modified by auxin treatment is apparently not modified by
removal of protein. These results would suggest that auxin action is
mediated, not by alteration of a protein, but by the alteration of the
basic polysaccharide structure or some glycosyl portion of a glyco­
protein which remains firmly attached to this basic structure.

In summary: it seems clear that *Avena* coleoptile cell walls can
undergo major quantitative changes in their polysaccharide composition.
The extent to which these changes are related to growth must be inter­
preted with care, but it is apparent that there is little correlation
between growth and synthesis of the wall fractions studied here. It is
also apparent that under certain conditions auxin-induced decreases in
wall glucose are correlated with growth. These later results may suggest
a mechanism by which auxin mediates the process of cell wall expansion
and growth.
PART II. EFFECTS OF TURGOR ON AUXIN-INDUCED CHANGES IN CELL WALL COMPOSITION
INTRODUCTION

In addition to the factor which regulates the mechanism of cell wall modification one other factor directly influences growth; this is the turgor pressure which provides the driving force. There is evidence that these two factors are not independent, and that the relationship between the two is complex. For example, rates of enlargement are not directly proportional to the magnitude of the turgor pressure. Although osmotic experiments on growing cells and tissues show that elongation rates drop rapidly as turgor decreases (Cleland, 1959; Green, 1968; Ray and Ruesink, 1963), these experiments also indicate that elongation rate is apparently zero at substantial turgor pressures. Elongation is only proportional to that turgor in excess of a certain value, hence there appears to be critical turgor pressure or a yield threshold of the wall which must be exceeded for growth to occur (Cleland, 1967b; Cleland and Bonner, 1956; Green, 1968; Lockhart et al., 1967). Further evidence indicates that the modifications of the wall which result in wall extension at normal turgor pressures do not occur at turgor pressures below some critical value. Thus, at subcritical pressures there are no auxin-induced modifications in the wall which result in growth upon return of the tissue to high turgor pressures. Early experiments had indicated that a potential for extension could accumulate during periods of subcritical turgor which could then mediate extension whenever the turgor was increased above the critical pressure (Carr and Ng, 1959; Cleland and Bonner, 1956), but more recent evidence suggests that such "stored growth potential" was an artifact and does not occur (Cleland, 1971a).
Other evidence, however, indicates that there does not seem to be a critical pressure required for auxin-induced changes in plastic extensibility as measured by the Instron technique. These changes are evident at all positive turgor pressures (Cleland, 1967b). Thus it seems that there are two alternatives: (1) turgor pressure above a critical value is required for wall modification and also to provide the driving force for growth; or (2) hyper-critical turgor pressure is required only for causing some increment of growth, with modification of the wall occurring at any turgor pressure.

There is ample evidence from studies of various tissues that many other aspects of metabolism are modified as a result of lowered turgor pressures. For example, respiration is enhanced then decreases as turgor is lowered (Greenway, 1970), certain hydrolytic enzyme activities are lowered (Jones and Armstrong, 1971), and photosynthesis is decreased (Boyer, 1971). In *Avena* coleoptiles it has been found that respiration rates are affected (Ordin et al., 1956), and that cell wall synthesis is inhibited (Baker and Ray, 1964b; Ordin, 1960; Ray and Baker, 1962).

The following is a report on studies of auxin-induced changes in cell wall composition and in oxygen uptake that result when *Avena* coleoptile sections are treated with various concentrations of mannitol designed to lower the water potential and thus the turgor pressures of the coleoptile sections.
METHODS AND MATERIALS

Analyses of wall composition

Plant tissue was grown and harvested as described in Part I. Cut sections were floated on distilled water a minimum of 45 minutes, then transferred to 5 ml of incubation medium in 43 x 20 mm Stender dishes. In addition to 2.5 mM potassium citrate buffer, pH 5.4, the incubation medium contained various concentrations of mannitol, and in some experiments, 0.05 M glucose. In some experiments sections were equilibrated with the medium for 1 hour prior to adding auxin, and treatments were begun by adding 5 ml of medium with and without IAA, 2.5 x 10^{-5} M. In other experiments auxin and mannitol treatments were begun at the same time. Sections were removed after various periods of treatment, measured, and frozen at -24 C for subsequent analysis. Wall samples were prepared as described in Part I. All wall samples were hydrolyzed in 2 N trifluoroacetic acid. Subsequent procedures were the same as those described in Part I for analysis of the neutral sugar composition of the 2 N trifluoroacetic acid hydrolysates.

Determination of respiration rates

Standard manometric techniques were used to measure respiration rates. Coleoptile sections were placed in Warburg flasks containing 2.9 ml of 2.5 mM potassium citrate, pH 5.4, with or without 200 mM or 400 mM mannitol. There was 0.2 ml of 20 per cent KOH in the center well, and 0.1 ml of buffer containing an appropriate concentration of mannitol with or without 3.75 x 10^{-4} M IAA in the sidearm. After an hour of equilibration at 26 C, readings were taken at 20 minute intervals for 100 minutes. Three
hours after placing the sections in the flasks, the contents of the sidearm were added. IAA concentration after addition was $1.25 \times 10^{-5}$ M. Sections were again equilibrated for one hour, and readings taken. Readings were also taken 7 and 19 hours after the sections had been placed in the flasks, or 4 and 16 hours after the contents of the sidearms had been added.

In an experiment to determine the reversibility of mannitol effects, sections were preincubated with buffer with and without 400 mM mannitol. After 2 hours the medium was removed and the sections washed several times with buffer. After fresh buffer was added and sections equilibrated for one hour, the sidearm contents with and without auxin were added, and an hour later readings were taken at 20 minute intervals for 100 minutes.
RESULTS

Earlier work has established not only that turgor is required for elongation and wall loosening (Cleland, 1967b), but also that rate of elongation is proportional to the amount by which turgor pressure exceeds the critical pressure (Lockhart et al., 1967). Thus, the effects of various turgor pressures on changes in wall composition and elongation induced by auxin are of interest. The extent to which such changes were affected by various concentrations of mannitol designed to decrease turgor are listed in Table 6. The presence of mannitol decreased not only both the basal and the auxin-induced increments of elongation but also the changes in wall glucan which are associated with growth. For example, the auxin-induced decrease in glucan was reduced from 52 per cent in the mannitol-free control to 18 per cent in 400 mM mannitol-treated sections. Growth also decreased as mannitol concentration increased, and was completely inhibited in 400 mM mannitol. Most other changes in the wall appeared unaffected with increased mannitol concentrations. There were, however, small increases in mannose in those walls from mannitol-treated tissues.

At the lower mannitol concentrations, 150 to 250 mM, the residual glucan in auxin-treated sections was much the same as the mannitol-free control, but the glucan remaining in walls from auxin-free sections decreased with increasing mannitol concentration (Table 1). At the higher mannitol concentrations, 300 to 400 mM, the level of glucan increased and the difference between auxin treatments and controls decreased.
Table 6. Effect of mannitol concentration on IAA-induced changes in wall composition and elongation

<table>
<thead>
<tr>
<th>Mannitol conc.</th>
<th>Rhamnose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Glucose change in length</th>
<th>IAA-induced change in wall glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM -IAA</td>
<td>0.7</td>
<td>18.0</td>
<td>25.5</td>
<td>0.8</td>
<td>3.6</td>
<td>13.1</td>
<td>20%</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.7</td>
<td>18.1</td>
<td>26.7</td>
<td>0.8</td>
<td>4.1</td>
<td>6.5</td>
<td>69 - 52%</td>
</tr>
<tr>
<td>150 mM -IAA</td>
<td>0.8</td>
<td>17.0</td>
<td>25.6</td>
<td>1.0</td>
<td>4.1</td>
<td>11.9</td>
<td>7</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.9</td>
<td>17.3</td>
<td>25.6</td>
<td>1.2</td>
<td>4.1</td>
<td>6.7</td>
<td>18 - 43</td>
</tr>
<tr>
<td>200 mM -IAA</td>
<td>0.8</td>
<td>15.7</td>
<td>23.7</td>
<td>0.9</td>
<td>3.6</td>
<td>9.4</td>
<td>5</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.9</td>
<td>16.5</td>
<td>24.7</td>
<td>1.7</td>
<td>3.8</td>
<td>6.7</td>
<td>14 - 29</td>
</tr>
<tr>
<td>250 mM -IAA</td>
<td>0.8</td>
<td>16.0</td>
<td>24.4</td>
<td>1.0</td>
<td>3.6</td>
<td>9.1</td>
<td>4</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>1.0</td>
<td>16.9</td>
<td>25.7</td>
<td>1.6</td>
<td>4.3</td>
<td>6.2</td>
<td>7 - 32</td>
</tr>
<tr>
<td>300 mM -IAA</td>
<td>0.9</td>
<td>17.0</td>
<td>24.9</td>
<td>0.8</td>
<td>4.7</td>
<td>11.2</td>
<td>4</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.9</td>
<td>17.7</td>
<td>26.1</td>
<td>1.3</td>
<td>4.5</td>
<td>7.5</td>
<td>6 - 33</td>
</tr>
<tr>
<td>350 mM -IAA</td>
<td>0.7</td>
<td>16.2</td>
<td>24.0</td>
<td>0.9</td>
<td>5.0</td>
<td>13.2</td>
<td>1</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.8</td>
<td>18.3</td>
<td>26.6</td>
<td>1.6</td>
<td>4.9</td>
<td>10.2</td>
<td>2 - 22</td>
</tr>
<tr>
<td>400 mM -IAA</td>
<td>0.8</td>
<td>18.1</td>
<td>26.8</td>
<td>1.0</td>
<td>5.6</td>
<td>18.6</td>
<td>0</td>
</tr>
<tr>
<td>&quot; +IAA</td>
<td>0.8</td>
<td>16.9</td>
<td>25.5</td>
<td>1.0</td>
<td>5.0</td>
<td>15.2</td>
<td>0 - 18</td>
</tr>
</tbody>
</table>
In another set of experiments time course studies were conducted to determine changes in wall composition and elongation when sections were incubated with 200 mM or 400 mM mannitol with and without $1.25 \times 10^{-5}$ M IAA. Although in the previously described experiment auxin and mannitol were added at the same time, in the experiments described below sections were preincubated in the appropriate concentration of mannitol for one hour prior to adding auxin. It was felt that auxin might have an effect prior to turgor pressure equilibration of the tissue with the external medium.

There is a report (Cleland, 1967b) that when auxin and mannitol are added at the same time a definite increase in wall extensibility is induced. Pretreatment in mannitol prior to addition of auxin lowers this response until after 60 minutes the tissues are no longer responsive to auxin.

Figures 22 and 23 depict the changes in sugar composition that occurred with and without auxin when sections were incubated in 200 mM mannitol. The magnitude of the major effect of auxin treatment, specifically, a decrease in wall glucose, was very nearly the same as that which occurred when sections were incubated under similar conditions without mannitol (Figures 13 and 14, Part I). Also, under these conditions the sections did elongate. In 20 hours, 1.07 cm sections elongated to 1.10 cm without auxin, and to 1.19 cm with auxin (Table 7). Figures 24 and 25 depict the changes in sugar composition that occurred when sections were incubated in 400 mM mannitol. Only slight differences were induced by auxin treatment. At the end of 20 hours there was slightly more arabinose and xylose with auxin treatment. These effects were similar to those observed when mannitol was absent (Figures 13 and
Figure 22. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 200 mM mannitol and $1.25 \times 10^{-5}$ M IAA in the absence of exogenous glucose.
Figure 23. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 200 mM mannitol in the absence of IAA and exogenous glucose.
+ 200mM Mannitol
- IAA
- Glucose

μg sugar/segment

time (hrs)
Table 7. Effect of mannitol and auxin on growth of coleoptile sections

The incubation medium contained, in addition to the various concentrations of mannitol with and without 1.25 x 10^{-5} M IAA and with and without 0.05 M glucose, 2.5 mM potassium citrate buffer, pH 5.4.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Mannitol Treatment without Glucose</th>
<th>Mannitol Treatment with Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM -IAA +IAA 200 mM -IAA +IAA 400 mM -IAA +IAA</td>
<td>0 mM -IAA +IAA 200 mM -IAA +IAA 400 mM -IAA +IAA</td>
</tr>
<tr>
<td>-1 hr</td>
<td>1.07 1.07</td>
<td>1.07 1.07</td>
</tr>
<tr>
<td>0</td>
<td>1.08 1.07</td>
<td>1.08 1.07</td>
</tr>
<tr>
<td>1</td>
<td>1.09 1.13</td>
<td>1.07 1.08</td>
</tr>
<tr>
<td>2</td>
<td>1.10 1.18</td>
<td>1.08 1.08</td>
</tr>
<tr>
<td>4</td>
<td>1.13 1.27</td>
<td>1.08 1.09</td>
</tr>
<tr>
<td>8</td>
<td>1.16 1.42</td>
<td>1.09 1.11</td>
</tr>
<tr>
<td>20</td>
<td>1.20 1.54</td>
<td>1.10 1.19</td>
</tr>
</tbody>
</table>

*Auxin added.*
14, Part I), and identical to those in the previous experiment where the sections were not pretreated (Table 6). Effects on wall glucan were not identical. Unlike the results of the previous experiments, auxin-induced changes in wall glucan were not apparent. Glucan content did decrease, probably reflecting loss of starch from the wall preparations, but after 20 hours the auxin-treated material contained very nearly the same amount of glucose, with 16.4 micrograms per section, as did the control, with 16.3 micrograms per section. Apparently the equilibration with mannitol prior to adding auxin prevented an auxin response. Although under these conditions sections did change length, shrinking initially from 1.07 cm to 1.03 cm, and then recovering slowly, there was no further elongation nor was there an auxin effect (Table 7). After 20 hours the lengths were the same as they were initially, 1.07 cm, regardless of the auxin treatment.

Figures 26 and 27 depict the changes that occurred when sections were incubated in 50 mM glucose and 200 mM mannitol. Auxin treatment was begun after a 1 hour pretreatment in mannitol. Exogenous glucose was added to determine the effect of mannitol on synthesis. The changes were very similar to those that occurred when mannitol was absent, i.e., with glucose available wall components increased (Figures 11 and 12, Part I). Also, in these treatments wall glucose increased initially then decreased. The effect of auxin was as apparent here as it was when mannitol was absent. The content of xylose, arabinose, and galactose in the auxin-treated material was greater and glucose content was less than in the control. Also, these sections elongated (Table 7), just as those incubated in 200 mM mannitol without exogenous glucose.
Figure 2h. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 400 mM mannitol and 1.25 x 10^{-5} M IAA in the absence of exogenous glucose
+400mM Mannitol
+ IAA
- Glucose

µg sugar/section

time (hrs)
Figure 25. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 400 mM mannitol in the absence of IAA and exogenous glucose
400mM Mannitol
- IAA
- Glucose
Figure 26. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 200 mM mannitol and $1.25 \times 10^{-5}$ M IAA in the presence of 50 mM glucose.
+ 200 mM Mannitol
+ IAA
+ Glucose
Figure 27. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 200 mM mannitol in the presence of 50 mM glucose and without IAA.
+200 mM Mannitol
- IAA
+ Glucose

μg sugar/section

time (hrs)
Figure 28. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 400 mM mannitol and $1.25 \times 10^{-5}$ M IAA in the presence of 50 mM glucose.
+ 400 mM Mannitol
+ IAA
+ Glucose
Figure 29. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 400 mM mannitol in the presence of 50 mM glucose and without IAA.
Figures 28 and 29 depict the changes that occurred when sections were incubated in 400 mM mannitol and 50 mM glucose. Differences with auxin treatment, if any, were less than those which were apparent when sections were incubated in 400 mM mannitol without exogenous glucose. Changes in length were negligible, limited to an initial shrinkage followed by a slow recovery to their initial length (Table 7).

An anomaly became apparent in these experiments where exogenous glucose was available to the sections incubated in mannitol. Without exogenous glucose the mannose content of the walls changed little, and it was not depicted on the graphs for that reason. But, when glucose was available there were striking increases in mannose. In 200 mM mannitol without auxin, mannose content increased from 0.6 micrograms per section to 1.9 micrograms per section (Figure 27). With auxin, mannose content increased to 2.1 micrograms per section (Figure 26). In the 400 mM mannitol treatments, the increases were greater, to 5.9 micrograms per section without auxin (Figure 29), and to 8.0 micrograms per section with auxin (Figure 28). It was thought that these increases may have been the result of mannitol in the incubation medium not being removed completely when the walls were prepared for analysis. But, the same procedure was used for all samples and the large increases in mannose were apparent only in those sections incubated with both mannitol and glucose. When glucose was absent only small increases were observed (Table 6). The reason for these differences is not known.

Mannitol had other effects, especially on respiration when its concentration was high. Table 8 lists rates of oxygen uptake as influenced
by auxin and 200 and 400 mM mannitol. The initial effect of mannitol was to decrease respiration. With further equilibration respiration rates recovered, but the increment of respiration enhanced by auxin was decreased by 200 mM mannitol, and was completely inhibited by 400 mM mannitol. With further incubation in 400 mM mannitol, an auxin-induced increment of respiration became apparent. Also, after extended incubation both basal and auxin-induced respiration were reduced in both control and 200 mM mannitol treatments, but in 400 mM mannitol, respiration increased and was greater in the auxin-treated material. Apparently the initial inhibitory effect was reversible. Other evidence indicating that the initial effects of 400 mM mannitol are reversible are listed in Table 9. Pretreatment in 400 mM mannitol appeared to have no effect on oxygen uptake or auxin effects on oxygen uptake after the mannitol was removed and the tissue was allowed to re-equilibrate in the basal medium for 1 hour.
Table 8. Effects of auxin and 200 mM and 400 mM mannitol on rate of oxygen uptake

Each value represents the average of duplicate determinations.

<table>
<thead>
<tr>
<th>Total Incubation</th>
<th>Rate of oxygen uptake, microliters per hour per section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0.0 M Mannitol 0.2 M Mannitol 0.4 M Mannitol</td>
</tr>
<tr>
<td></td>
<td>-IAA +IAA -IAA +IAA -IAA +IAA ---------------------</td>
</tr>
<tr>
<td>1 hr</td>
<td>2.08±.10 1.94±.09 1.61±.08</td>
</tr>
<tr>
<td>4</td>
<td>2.21±.07 2.60±.09 2.22±.03 2.45±.24 2.08±.08 2.03±.05</td>
</tr>
<tr>
<td>7</td>
<td>2.05±.20 2.25±.13 1.80±.01 2.02±.16 1.55±.01 1.81±.02</td>
</tr>
<tr>
<td>19</td>
<td>1.56±.11 1.44±.14 1.20±.06 1.21±.03 2.01±.15 2.25±.05</td>
</tr>
</tbody>
</table>

Table 9. Effect of a 2 hour pretreatment with 400 mM mannitol on oxygen uptake

Each value represents the average of duplicate determinations.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>-indoleacetic acid</th>
<th>+indoleacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sections pre-treated with buffer</td>
<td>2.39±.05</td>
<td>2.68±.06</td>
</tr>
<tr>
<td>Sections pre-treated with buffer plus 0.4M mannitol</td>
<td>2.37±.06</td>
<td>2.74±.05</td>
</tr>
</tbody>
</table>
DISCUSSION

The results obtained in this study indicate that Avena coleoptile cells must be subjected to a differential internal to external pressure for auxin-induced changes in wall composition. This observation is consistent with other studies that have indicated similar requirements for auxin-induced growth (Cleland, 1959; Cleland and Bonner, 1956; Ray and Ruesink, 1962) and auxin-induced changes in the physical properties of the walls (Cleland, 1967b).

The requirement for turgor is usually interpreted as meaning that there is a minimum force ("critical turgor") below which one or more of the processes involved in the mechanism of cell elongation cannot occur. Although earlier studies had indicated that this was not the case, that when coleoptile sections were incubated at subcritical turgor pressures the wall loosening factors could continue operation so that upon restoration of normal turgor the sections would grow (Carr and Ng, 1959; Cleland and Bonner, 1956), more recent studies indicate that the changes in the wall leading to growth occur only when the wall is extended or under tension (Cleland, 1971a).

The implication of these results has suggested several mechanisms of cell wall extension. On the basis of studies of growth rates as affected by variations in turgor and metabolic inhibitors, Ray and Ruesink (1962) concluded that the mechanism of wall extension was similar to that in some polymeric systems where when under tension a material can undergo chemical changes resulting in extension that it cannot undergo when it is not under tension. It was concluded that in Avena coleoptile tissue this
could involve reversible cleavage and reformation of some wall cross-links. When under tension, cleavage of crosslinks and displacement would be followed by reformation of new crosslinks in unstrained configurations. When not under tension, cleaved crosslinks would reform in the original configuration. In a physical sense this type of mechanism was considered to be similar to chemically induced extension under constant load, or in a rheological sense, chemical creep.

Cleland (1967b) also considered turgor requirements on the basis of his studies of changes in the mechanical properties of walls. One possibility that he suggested, in contrast to that proposed by Ray and Ruesink, was chemical or metabolic stress relaxation. This involves the decrease in tension that occurs in a material after it has been subjected to a certain amount of strain. In such a mechanism the linkages in the material must be under tension to be broken. The greater the stress the more linkages which will be under tension, and thus the more bonds susceptible to being broken. Turgor pressure or turgor above a critical value would provide a threshold stress for growth, i.e., sufficient linkages under tension to allow for irreversible displacement processes. Cleland also suggested as an alternative, like Ray and Ruesink, that it may be the reformation rather than the breakage of linkages that is affected by turgor pressure. The new configurations established by such a mechanism when the wall is under tension would alter the mechanical properties of the wall. Re-establishment of the original configurations would result in a wall with unchanged properties.

Another mechanism suggested by Cleland (1967b) was a possible
requirement for elastic extension of the wall. Turgor pressure by elastically extending the wall would allow loosening factors, presumably enzymes, to penetrate to sites of action that would otherwise be blocked. Unblocking of sites could involve removal of molecules or configurational changes in some polymer. The number of such sites available would be a function of the amount of elastic extension that had occurred, which in turn would be a function of the turgor pressure.

Since chemical creep involves rearrangements that result in extension under constant load, it, and not stress relaxation, is now thought to be more comparable to the mechanism of cell elongation where rearrangements in the wall result in extension under constant turgor. Creep, however, is only a physical description of the extension process. As such, it or any other physical parameter determining wall extensibility provides little insight into those factors influencing the rate at which it occurs. The rate-determining factors include the contribution of biochemical or metabolic processes, the agents which may be involved in reversible cleavages and reformation of crosslinks, and the loosening factors which may attack unblocked sites.

The results obtained here provide no direct evidence for or against any of these possibilities. They may in fact be explained in terms of any one of them. For example, if, during cleavage and reformation of a new crosslink, a wall component should become more resistant to acid hydrolysis, this could explain decreases in non-cellulosic glucan that may be correlated with increases in cellulose (Figures 20 and 21, Part I). This may be unlikely, however, since with appropriate pretreatment the net loss in glucose corresponds to the loss in wall weight (Figure 17, Part I).
If tension is required for cleavage of certain linkages, or if unblocking of sites is involved, then nearly any change or lack of change in composition of a wall could be explained, especially those involving hydrolytic cleavages and losses of some wall component.

Consideration should also be given to the possibility that auxin at low turgor does not effect release from the protoplast or plasmalemma of the wall loosening factors which thereafter migrate into the wall. Since the metabolic contribution to the extension process is thought to be involved in the export of wall loosening factors to the wall (Evans et al., 1971; Ray, 1969), it may be that the effect of low turgor is to inhibit this process. It is difficult to visualize how this might occur, but it is equally difficult to understand why there should be no auxin-induced increment in respiration at low turgor pressures. Lack of an auxin-induced increment in respiration was not only found here, but similar effects of high mannitol concentrations on respiration have been reported before (Ordin et al., 1956). The additional dramatic effects of mannitol shown here on the mannose content of walls when incubated with glucose have not previously been reported. These results demonstrate clearly that this presumably inert substance has rather profound effects on wall composition.

Effects of mannitol or low turgor on respiration are perhaps an indication that experiments like these should be interpreted with care. It has long been known that metabolism is required for auxin effects (Went and Thimann, 1937), and it may be that the lack of auxin effects on the wall at low turgor pressures is a secondary effect of low turgor on respiration.
Whatever the mechanism, certain aspects of the data obtained here may provide an explanation for some previous observations. When Cleland (1967b) measured the effect of auxin on plastic extensibility in coleoptile sections incubated in various concentrations of mannitol, he found that addition of auxin had no effect on plastic extensibility when turgor was zero. As turgor increased, auxin effects on extensibility increased. These auxin effects on extensibility correlate well with the changes observed in non-cellulosic glucan, i.e., lack of auxin effects on changes in non-cellulosic glucan at high mannitol concentrations or low turgor pressures, and increased auxin effects at lower mannitol concentrations or high turgor pressures. Cleland also found that in order to prevent auxin effects at high mannitol concentrations, i.e., low turgor pressures, it was necessary to preincubate in mannitol for an hour prior to adding auxin. A similar preincubation was adopted here to prevent auxin effects on changes in wall composition. Although there are discrepancies between determinations of plastic extensibility and growth (Cleland, 1971a; Ray, 1969), there is evidence that these determinations do provide a measure of total wall extensibility. As such, these correlations may suggest a relationship between the mechanical properties of the wall and the levels of non-cellulosic glucan, and perhaps also a biochemical relationship to the mechanism of growth.
PART III. POLYSACCHARIDE HYDROLASES AND AUXIN-INDUCED CHANGES IN CELL WALL COMPOSITION
INTRODUCTION

Considerable effort in the past has been expended to determine the identity of the factor(s) responsible for initiating wall loosening in various tissues when those tissues are treated with auxin. Although a number of theories have been proposed (Cleland, 1971a; Lamport, 1970; Ray, 1969), perhaps the one with the most proponents postulates that the process is mediated by polysaccharide hydrolases whose synthesis or activity is influenced by auxin. In some systems, i.e., in bacteria and in those cells which undergo tip growth such as pollen tubes (Roggen and Stanley, 1969) and fungal hyphae (Thomas and Mullins, 1969; Wessels, 1969), hydrolases do appear to be essential to wall extension, but in most higher plant tissues their role in the extension process is less clear.

Evidence for the role of hydrolases includes reports that hydrolases do exist in a number of plant cell walls (Cleland, 1971a; Lamport, 1970). In Avena coleoptiles, for example, β-1,3-glucanase (Heyn, 1969; Tanimoto and Masuda, 1968), exo-β-1,4-glucanase (Heyn, 1969), β-1,6-glucanase (Heyn, 1969; Tanimoto and Masuda, 1968), exogalactanase (Katz and Ordin, 1967b), α-1,6-glucanase (Heyn, 1970a), unspecified hydrolases (Katz and Ordin, 1967b), and β-glucosidases (Chkanikov et al., 1969) have been found. Further evidence includes reports that auxin treatment increases the activity of certain hydrolases in several systems (Cleland, 1971a). In Avena coleoptiles auxin treatment has been reported to increase the activities of β-1,3-glucanase (Masuda and Yamamoto, 1970; Tanimoto and Masuda, 1968), α-1,6-glucanase (Heyn, 1970a), and the unspecified hydrolases (Katz and Ordin, 1967b).
Other evidence for the role of hydrolases has arisen from studies of walls in vitro after various extractions attempting to remove protein. Such walls were found to be capable of autolysis. Incubations of these preparations resulted in solubilization of some of the wall substance suggesting that there were enzymes tightly bound to these preparations capable of hydrolyzing wall polysaccharides (Lee et al., 1967). In one case, pretreatment with auxin was reported to accelerate this solubilization process (Katz and Ordin, 1967b). Related to these results are compositional analyses which report that auxin treatment caused loss of non-cellulosic glucan from the walls of intact coleoptiles (Baker and Ray, 1964a; Ray, 1963).

There are also reports that some enzymes affect cell walls. Cellulase treatment, for example, resulted in increased extensibility as measured by the Instron technique (Olson et al., 1965; Ruesink, 1969). Similar effects on extensibility have been observed with β-1,3-glucanase treatment (Masuda, 1968; Masuda et al., 1970; Masuda and Wada, 1967). Treatments with β-1,3-glucanase, particularly an exo-β-1,3-glucanase, have also been reported to induce cell elongation (Masuda, 1968; Masuda and Wada, 1967; Masuda et al., 1970; Tanimoto and Masuda, 1968; Wada et al., 1968).

In spite of this evidence there are criticisms of the possible role of polysaccharide hydrolases in wall loosening. First, even though cellulase does increase wall extensibility it does not increase cell growth (Ruesink, 1969; Wada et al., 1968). Second, attempts to reproduce the stimulation of elongation by β-1,3-glucanases have either met with failure (Cleland, 1971a; Ruesink, 1969), or with only slight promotions.
that require special pretreatments for their detection (Cleland, 1971a). Third, the reported auxin-induced increases in enzyme activities are small, less than 10 per cent, yet elongation increases 500 to 800 per cent with auxin treatment.

Other criticisms of the role of polysaccharide hydrolases arise as a result of the rapid inhibition of elongation by KCN, absence of oxygen, or inhibitors of protein synthesis (Evans and Ray, 1969; Ray and Ruesink, 1962). These results suggest that the wall loosening factors are highly unstable, a condition usually not characteristic of plant polysaccharide hydrolases (Pan and Maclachlan, 1967).

In view of these ambiguities an effort was made here to investigate the extent of relationships between activities of various glycosidases, factors affecting elongation, and modification of the coleoptile cell wall composition by exogenous enzymes. As a result, there were three objectives in this study. The first was to measure how effectively a number of substrates could be degraded by enzyme preparations from Avena coleoptile tissue. The second was to determine the extent to which enzyme activities were affected by auxin and other treatments which variously promote or inhibit elongation. The third was to measure the degree to which Avena coleoptile wall composition would be modified by treatment with enzyme preparations similar to those reported to increase in activity with auxin treatment, i.e., a β-1,3-glucanase and dextranase (α-1,6-glucanase).
METHODS AND MATERIALS

Plant material

Plant tissue was grown and harvested as described in Part I. Sections after cutting and removal of the primary leaf were floated on distilled water 45 to 60 minutes, then transferred to the appropriate treatment medium.

Enzyme preparation and assay

Most of the analyses of enzyme activities utilizing various p-nitrophenyl-glucosides as substrates involved placing five 1.0 cm coleoptile sections in 15 cm test tubes along with 1 ml of treatment medium. After incubation the medium was drawn off with a capillary pipette and replaced with 0.8 ml of ice-cold 50 mM sodium citrate buffer, pH 4.6. Sections were then macerated with a glass rod. After temperature equilibration for 5 minutes at 30°C in a reciprocating water bath, 0.2 ml of 25 mM p-nitrophenyl-glycoside (Pierce Chemical Co. or Koch-Light Laboratories, Ltd.) was added. After incubation, reactions were stopped with the addition of 2 ml of 200 mM sodium carbonate. The p-nitrophenol concentration was determined by measuring the absorbance at 400 nm utilizing p-nitrophenol (Eastman) as a standard (range, 0 to 0.05 micromoles per ml).

Other analyses of enzyme activities from Avena coleoptile tissue utilized different enzyme preparation procedures. To obtain these preparations, approximately 150 coleoptile sections were macerated in an ice-cold mortar and pestle with 1 ml of 50 mM sodium citrate, pH 4.6, per 30 coleoptile sections for 3 minutes. The total homogenate was then centrifuged for 10 minutes at 3000 g. Following centrifugation, the
supernatant fluid was decanted and the soluble enzyme dialyzed overnight against distilled water at 4 °C. This fraction was stored in the refrigerator until its enzyme activity was assayed as indicated below. The pellet obtained from the 3000 g centrifugation was resuspended in a 5 ml of 500 mM sodium citrate buffer, pH 4.6, and stirred in an ice bath for 15 minutes and again centrifuged for 10 minutes at 3000 g. The supernatant fluid was decanted and dialyzed overnight against distilled water. The pellet was resuspended in 5 ml of 50 mM sodium citrate, pH 4.6, and dialyzed overnight. This procedure yielded 3 enzyme preparations: one extracted by 50 mM sodium citrate, one extracted by 500 mM sodium citrate, and one associated with wall material.

Enzyme assays involved incubating 0.5 ml of the various enzyme preparations or the boiled enzyme controls with 0.5 ml of the substrate in 50 mM sodium citrate, pH 4.6. Unless otherwise mentioned, all incubations were done at 30 °C. Substrate concentrations in the reaction mixtures were 5 mg per ml except for laminarin which was 2.5 mg per ml. Substrates included soluble starch (Mallinckrodt), dextran (Type 100C, Sigma), maltose (Fisher), cellobiose (Sigma), sucrose (Baker), carboxymethylcellulose (Type 7MF, Hercules), and laminarin (Pierce). Enzyme assays were based on the liberation of reducing sugars from the polymeric substrates. Reducing sugars were estimated using a dinitrosalicylic acid reagent utilizing glucose as a standard (Luchsinger and Cornesky, 1962). Maltose was used as a standard in the evaluation of starch hydrolysis. Reactions were stopped by adding the reagent to the reaction mixture and boiling for 5 minutes. Absorbancies were determined at 540 nm. Protein determinations
utilized the Folin technique (Lowry et al., 1951) with crystalline bovine serum albumin (Armour) as a standard.

**Enzyme treatment of wall preparations**

Wall samples were prepared as previously described (Part I) from coleoptile sections which had been subjected to various pretreatments. Treatment involved incubating the walls with 2 ml of either a dextranase (Worthington, 150 units per mg protein) or a β-glucosidase preparation. The dextranase was in 100 mM potassium phosphate buffer, pH 6.0, with 0.02 per cent sodium azide. The β-glucosidase was prepared from 5.5 day-old hypocotyls of bean (*Phaseolus vulgaris* L. var. Red Kidney) following the procedure of Nevins (1970) and was in 50 mM sodium citrate, pH 4.6.

Following incubation of the wall samples with the enzymes 10 ml of distilled water was added and the samples were decanted onto a fritted glass funnel. Samples were washed twice with 5 ml of distilled water, twice with 5 ml of acetone, and twice with 5 ml of chloroform:methanol (1:1, v:v). The samples were then dried in vacuo over phosphorous pentoxide, weighed, and the sugar composition analyzed following the procedure utilizing 2 N trifluoroacetic acid for hydrolysis followed by conversion of the liberated sugars to alditol acetates as outlined in Part I.
RESULTS

For the initial objective of this study, p-nitrophenyl sugar derivatives were employed to determine the effectiveness of coleoptile tissue extracts in degrading various glycosidic bonds. These substrates provided both a sensitive assay and a model substrate of known anomeric configuration and specific sugar moiety. Table 10 lists the activity of tissue extracts towards these substrates. Of these activities, β-glucosidase was highest, followed by β-galactosidase and α-mannosidase. Intermediate amounts of α-galactosidase and β-xylosidase activities were detected. There were only trace amounts of α-xylosidase, β-mannosidase, and α-glucosidase.

Table 10. Activity of Avena coleoptile tissue homogenates towards various p-nitrophenyl-glycosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (micromoles per hr per section)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
<td>0.74</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-galactoside</td>
<td>0.26</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-mannoside</td>
<td>0.11</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-galactoside</td>
<td>0.052</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-xyloside</td>
<td>0.035</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-xyloside</td>
<td>0.006</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-mannoside</td>
<td>0.006</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-glucoside</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Using these substrates, attempts were made to determine what effect various pretreatments would have on the subsequent activity of the tissue extracts. Coleoptile sections were treated with and without auxin for varying periods of time prior to estimating enzyme activities. This was repeated at least three times for all substrates, and a greater number of times for the three substrates most effectively degraded by the tissue extracts, i.e., β-glucoside, β-galactoside, and α-mannoside. In all cases no consistent nor significant differences could be found with auxin treatment. Coleoptile sections were also treated with various concentrations of auxin and these too had no effect in promoting different levels of enzyme.

Coleoptiles were treated with the inhibitors cycloheximide (25 micrograms per ml), actinomycin-D (25 micrograms per ml), and potassium cyanide (3 x 10^-4 M) in amounts sufficient to inhibit auxin-induced growth. Potassium cyanide and cycloheximide had only a slight effect, a 4 per cent reduction of β-glucosidase and β-galactosidase activities after one hour. Prolonged incubation further reduced activities, but the effects were still slight. After 3 hours cycloheximide and potassium cyanide reduced β-glucosidase and β-galactosidase activities 8 per cent. Actinomycin-D had no significant effects on either enzyme activity, even after 4 hours. Other substrates and inhibitors were not tested.

Preliminary experiments indicated that a considerable portion of certain enzyme activities was associated with insoluble portion of the tissue homogenates. Because it was thought that these affinities might provide some information as to which enzymes were most closely associated
with the walls and perhaps involved in their modification, an attempt was made to measure the extent of the affinities. Table 11 lists the degree to which various enzymes were associated with the insoluble residue. Extraction with 50 mM sodium citrate removed nearly all of some enzymes, i.e., those degrading β-xyloside and α-galactoside, but other enzyme activities were not so easily removed. The specific activities of enzymes degrading sucrose, β-glucoside, β-galactoside and α-mannoside were higher in the residue than in the 50 mM extract. The enzymes degrading laminarin and amylose were intermediate in their affinity, with the enzyme degrading laminarin having a slightly greater affinity for the walls. In view of the insoluble nature of the walls the specific activities of those fractions must be interpreted with care. It is possible that with the insoluble material the protein determination underestimated the amount of protein present.

In addition to 50 mM and 500 mM buffer other experiments utilized 1.0 M buffer to extract the enzymes, but that concentration of buffer was found to remove very little additional protein or enzyme activity from the walls. Although these results do not offer clear evidence that these enzymes are bound to the wall, or that the enzymes are normally associated with the wall, they do indicate that a certain share of the activity is not easily removed.

Other evidence which may indicate that these enzymes are associated with the surfaces of coleoptile cells is listed in Table 12. The results show the degree to which various substrates were degraded when incubated with intact coleoptile sections. It is apparent that both β-glucoside
Table 11. Activities of the various enzyme preparations derived from *Avena* coleoptile sections

<table>
<thead>
<tr>
<th>Substrate</th>
<th>50 mM</th>
<th>Extract</th>
<th>&quot;Walls&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>4.5(^a)</td>
<td>3.5(^a)</td>
<td>0.8(^a)</td>
</tr>
<tr>
<td>laminarin</td>
<td>2.7</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>sucrose</td>
<td>7.2</td>
<td>23.7</td>
<td>12.6</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
<td>25.5</td>
<td>80.4</td>
<td>57.4</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-galactoside</td>
<td>12.2</td>
<td>20.4</td>
<td>13.5</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-mannoside</td>
<td>5.8</td>
<td>4.4</td>
<td>11.9</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-xyloside</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-galactoside</td>
<td>2.8</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Percent of total protein</td>
<td>65%</td>
<td>17%</td>
<td>18%</td>
</tr>
</tbody>
</table>

\(^a\) Specific activities - micromoles released per hour per mg protein.

and β-galactoside, and to varying degrees the other substrates, were degraded under these conditions. Presumably these cleavages occurred within the wall or at the cell surface, especially in view of the short incubation times which reduced the possibility of uptake of the substrates into the cells and subsequent loss of nitrophenol into the medium.

A number of other substrates, not depicted in Tables 10 or 11, were incubated in similar enzyme preparations, but no significant activities were detected. The substrates were dextran, carboxymethyl cellulose, maltose, and cellobiose. To insure that pH was not a factor, although the β-glucosidase and laminarinase activities had shown a pH optimum
between 4.5 and 5.0, analyses for activities against these other substrates were also run at pH 4.1, 5.2, 6.0, and 7.2. Enhanced activities were not detected at any other pH. Since dextranase activity has been reported to increase with auxin treatment of coleoptile tissue (Heyn, 1970a), a number of attempts were made here to detect dextranase activity. But, neither an extended incubation, 6 hours, nor a higher temperature, 37°C, resulted in detectable increases in reducing groups.

Table 12. Activity of whole *Avena* coleoptile sections towards various *p*-nitrophenyl-glycosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation Time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td><em>p</em>-nitrophenyl-β-D-glucoside</td>
<td>.010&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>p</em>-nitrophenyl-β-D-galactoside</td>
<td>--</td>
</tr>
<tr>
<td><em>p</em>-nitrophenyl-α-D-galactoside</td>
<td>--</td>
</tr>
<tr>
<td><em>p</em>-nitrophenyl-β-D-xyloside</td>
<td>--</td>
</tr>
<tr>
<td><em>p</em>-nitrophenyl-α-D-glucoside</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activity - micromoles released per section.

In view of reports that β-1,3-glucanase increased elongation of *Avena* coleoptile sections (Masuda and Wada, 1967; Masuda et al., 1970) and the report that dextranase activity increased with auxin treatment, the effect of enzymes of similar specificities on isolated walls was of interest. A comparison was made of the effectiveness of these enzymes in degrading 3 different types of wall preparations. The wall preparations were different in that they were prepared from coleoptile sections subjected to different
pretreatments prior to isolation. In the one case pretreatment consisted of a 20 hour incubation in $1.25 \times 10^{-5}$ M IAA without exogenous glucose. These sections elongated 52 per cent and the non-cellulosic glucan content of the wall was at a minimum. In the second case pretreatment consisted of a 20 hour incubation in 50 mM glucose without IAA. These sections elongated only 17 per cent and the non-cellulosic glucan content was at a maximum. The third set of walls was isolated from sections which had been subjected to no pretreatment. These three types of sections were selected because they represented three different levels of non-cellulosic glucan (Figures 12 and 13, Part I), and also because they represented walls before and after elongation with and without auxin.

The specificity of the dextranase preparation was evaluated by assay using a number of substrates and prolonged incubations, 6 hours. Although it was very effective against dextran, no increase in reducing groups was detected using starch, carboxymethyl cellulose, maltose, and sucrose as substrates. It also did not attack the α- or β-linked p-nitrophenyl glucosides.

The other enzyme preparation was isolated from bean hypocotyls, 5.5 days-old, the time at which the most rapid phase of hypocotyl elongation was being initiated (Nevins, 1970). The activities of this enzyme preparation toward several different substrates are listed in Table 13. The enzyme preparation showed distinct specificity in its effectiveness in breaking down the various substrates. Although the enzyme preparation did attack the β-glucoside, it did not attack carboxymethyl cellulose with β-1,4 linkages. It did attack laminarin with β-1,3 and β-1,6 linkages,
and lichenin with β-1,3 and β-1,4 linkages. It may be that differences in activity towards these latter two substrates reflected the differences in the number of β-1,3 linkages in each substrate. In one report laminarin was found to contain 67 per cent β-1,3 linkages, while lichenin was found to contain 30 per cent β-1,3 linkages (Clarke and Stone, 1963). Dextran, with α-1,3 and α-1,6 linkages, and nigerin, with α-1,3 and α-1,4 linkages, were not degraded.

Table 13. Activity of an enzyme preparation from 5.5 day-old bean hypocotyls

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
<td>266</td>
</tr>
<tr>
<td>laminarin</td>
<td>11.3</td>
</tr>
<tr>
<td>dextran</td>
<td>0.0</td>
</tr>
<tr>
<td>carboxymethyl cellulose</td>
<td>0.0</td>
</tr>
<tr>
<td>lichenin</td>
<td>2.5</td>
</tr>
<tr>
<td>nigerin</td>
<td>0.0</td>
</tr>
</tbody>
</table>

aMicromoles per hour mg protein.

The effects of incubating these enzymes with the different wall preparations for 20 hours are depicted in Figure 30. It is clear that both of these enzymes can effectively degrade oat coleoptile cell walls. This evidence indicates the likelihood that both β-1,3 and α-1,6 linkages occur in the non-cellulosic glucan of oat coleoptile cell walls. It is equally
Figure 30. The susceptibility of three different wall preparations to (A) a β-1,3-glucanase preparation (See Table 13 for activities) and (B) a dextranase (activity = 240 micromoles of glucose equivalents released per hour per milliliter enzyme preparation). The open bars represent the effects of the boiled enzyme controls. The number in parentheses after the sugar abbreviation represents the per cent change in that sugar with enzyme treatment.
clear that these enzymes differ in their effects on the wall preparations. Dextranase treatment, for example, resulted generally in losses of all sugars from the wall samples. The "β-1,3-glucanase," however, resulted in losses of glucose and galactose primarily. Arabinose and xylose were affected only slightly.

It was also found that the effectiveness of the β-1,3-glucanase was markedly affected by the type of wall preparation. Wall preparations which had undergone IAA treatment and the most elongation, i.e., those from sections preincubated in IAA without glucose, were affected only slightly by the enzyme. Wall preparations from sections not treated with IAA or subjected to no pretreatment underwent striking changes with enzyme treatment. These differences in susceptibility to the enzyme indicate that those wall components susceptible to β-1,3-glucanase are modified with auxin treatment and growth. In contrast to the results obtained with the β-1,3-glucanase, dextranase showed no such specificity in its effectiveness towards the various wall preparations. All preparations showed similar changes with dextranase treatment.
DISCUSSION

Results here clearly show that two polysaccharide hydrolases, a dextranase and an enzyme preparation with β-1,3-glucanase activity, can degrade oat coleoptile cell walls. These results support other reports of the possible existence of α-1,6-glucosidic linkages (Heyn, 1970b) and the evidence for β-1,3-glucans in oat tissues (Clarke and Stone, 1963; Fraser and Wilkie, 1971a; Fraser and Wilkie, 1971b). The results also show a clear correlation between the in vitro effects of β-1,3-glucanase on purified wall material and the in vivo changes that occur in walls when Avena coleoptile sections are incubated with auxin. Dextranase, although effective in degrading wall preparations, does not show such correlations between its effects and the changes occurring in the wall with growth. In contrast to the report of Heyn (1970b) in which mainly isomaltose with some glucose and isomaltotriose were found as a product of dextranase treatment a general degradation of most wall polysaccharides was observed here. The fact that these two enzymes do affect the wall, together with the reports that both of these enzymes increase in activity with auxin treatment (Heyn, 1970a; Masuda and Yamamoto, 1970), may indicate a role for one or both in the mechanism of growth.

Of the two enzymes, dextranase and β-1,3-glucanase, it seems more probable that β-1,3-glucanase, and not dextranase, is involved in the growth process. There are several reasons for this conclusion. First, the β-1,3-glucanase preparation was one which has been shown to have high activity during the period of most rapid elongation in bean hypocotyls (Nevins, 1970). This correlation with growth may indicate a causal
relationship to the growth process in that tissue. Second, if that is the case, then the effects of β-1,3-glucanase on oat coleoptile cell walls could parallel those changes associated with growth. Not only was there evidence for degradation, but the degree of degradation was nearly identical to that which occurred during growth. Third, there are appreciable levels of β-1,3-glucanase activity in coleoptile tissue. Dextranase activity, with the techniques employed in this study, could not be detected.

The results presented here do not constitute the only evidence for the possible role of polysaccharide hydrolases in the growth process. There are a number of reports, showing either in vivo turnover of some wall constituents (Katz and Ordin, 1967a; Maclachlan and Duda, 1965; Matchett and Nance, 1962; Nelmes and Preston, 1968; Wada et al., 1968), loss of some wall constituents (Baker and Ray, 1965a; Ray, 1963), or autolysis of isolated cell walls (Katz and Ordin, 1967b; Lee et al., 1967) which, although not implicating specific enzymes, have either indicated that polysaccharide hydrolases are present in tissues or that they are presumably active in vivo. It has been concluded that these results may also indicate a role for polysaccharidases in growth.

The acceptance of the possible involvement of polysaccharide hydrolases in the elongation process has, however, met with resistance (Cleland, 1971a; Lamport, 1969; Ruesink, 1969). The arguments against their involvement include the fact that only slight increases in enzyme activities, if any, can be detected with auxin treatment. Masuda (1968), for example, reported increases of only 10 per cent in β-1,3-glucanase activity with
auxin treatment. Heyn (1970a) reported a 5 per cent increase in dextranase activity. The results here show no increases in laminarinase (β-1,3-glucanase) activities with auxin treatment, and no dextranase activities regardless of auxin treatment.

Another argument against the role of polysaccharidases cites the effects of inhibitors on elongation. The rapid inhibitory effects of these substances suggest that the factors promoting elongation are unstable, and instability is not considered typical of polysaccharide hydrolases (Cleland, 1971a). But, if there should be an unstable hydrolase involved in the mechanism of growth, then there may be an explanation to account for these observations. Ray (1969) suggested several possible mechanisms of auxin action. One of these was that the action of auxin in relation to growth might be promotion of efflux of an enzyme, a structural polymer, or a metabolite through the plasma membrane into the cell wall, where it is used in the process of wall expansion. The biosynthesis of the metabolite or polymer within the cell would be accelerated in turn by mechanisms of feedback control, and this would be the part of the mechanism that depends on RNA and protein synthesis. This mechanism would explain the rapid effects of inhibitors of metabolism and also the effects of inhibitors of RNA and protein synthesis. It might explain, if transport were affected, the apparent requirement for turgor pressure, assuming an appressed plasmalemma:cell wall interface is required for efficient transport. It would also explain why little or no increase can be detected in polysaccharide hydrolase activities after auxin treatment. If the effect of auxin is on efflux, the initial effect of the hormone
would result in no increase in total activity in the tissue, but it would increase activity in the walls. If the enzyme were appreciably unstable, then even after considerable incubation auxin might still have little effect on total enzyme activity in the tissue; the additional enzyme being synthesized as a result of feedback would replace essentially only that enzyme in the wall which had become inactivated. Evans and Ray (1969) developed a somewhat similar hypothesis to explain their observations of the kinetics of the growth response. Instead of an enzyme, however, they postulated that the factor being exported was some sort of a growth-active protein, one which was functional only at the time of its export. This to them implied a structural protein without catalytic activity.

As attractive as this efflux of hydrolases mechanism may be, it does not serve to explain another facet of the argument against the role of polysaccharide hydrolases in the growth process. If polysaccharide hydrolases are the wall loosening factors in the growth process, then the presumption is that addition of such factors to intact coleoptiles should result in growth. With the exception of Masuda's group, experiments attempting to demonstrate such growth have generally failed. Cleland (1971a), however, using one of Masuda's enzyme preparations (Masuda et al., 1970) was able to duplicate Masuda's results under special conditions. Masuda offers an explanation for the lack of results by other workers by pointing out that there are differences in the chemical nature and structure of cell walls among different plant species (Masuda et al., 1970). These differences imply that unless an enzyme of the correct
specificity is used, no growth will result. As support for this hypothe-
sis, there is a report that although an exo-β-1,3-glucanase will induce
growth, an endo-β-1,3-glucanase will not (Masuda et al., 1970). Further,
although the exo-β-1,3-glucanase will induce enlargement in sections from
oat coleoptiles, barley coleoptiles, and pea stems, it will not induce
elongation in wheat coleoptile sections and Jerusalem artichoke tissue
(Masuda et al., 1970). This indicates Masuda's selection of a fungal
enzyme (the exo-β-1,3-glucanase), of the circumscribed specificity to
promote oat coleoptile elongation, was indeed fortuitous.

Another explanation for lack of growth in some of these experiments
has been that the enzyme fails to penetrate the tissue to the site of
action where it can affect elongation. Efforts to overcome this by re-
moving the epidermal layers of coleoptiles resulted in no promotions of
growth when a crude cellulase or a crude β-1,3-glucanase preparation was
used (Ruesink, 1969). It could be argued that specificity requirements
were not met, or that there was no growth because the epidermal layers
of the coleoptile control rate of elongation more than underlying tissues
(Lockhart, 1965b; Roelofsen, 1965; Shoch-Bodmer, 1939), but even peeled
sections will respond to auxin by elongating (Ruesink, 1969).

In spite of the explanations, until the inconsistencies in the re-
ports of the effects of enzymes on elongation can be resolved, results
showing no elongation with enzyme treatment will be considered a serious
barrier to any explanations of auxin action involving polysaccharide
hydrolases. But, it seems difficult to invoke any alternative explanations
of auxin action and still account for the changes that occur in wall poly-
saccharide composition when cells enlarge.
SUMMARY

Time-course studies of changes in *Avena* coleoptile cell wall composition were conducted under conditions that promoted or inhibited elongation. The intent of these studies was to determine if there were correlations between changes in some cell wall component(s) and elongation. Such correlations, if they did exist, would suggest a mechanism by which wall extensibility could be increased and growth could take place. The results indicate that when exogenous glucose was available increases in certain wall constituents paralleled increases in length. However, under conditions where exogenous glucose was not available, and consequently, wall synthesis was severely limited, such correlations were not apparent: total wall weight initially increased slightly then decreased, reflecting net increases in cellulose and some non-cellulosic constituents and striking decreases in non-cellulosic glucose. When coleoptile sections were preincubated without exogenous wall precursors for 8 hrs to reduce the supply of endogenous wall precursors and subsequently treated with auxin, growth was promoted but there were no detectable increases in wall weight. There was instead an auxin-promoted decrease in wall weight, and this decrease paralleled a decrease in non-cellulosic glucose. The auxin-promoted decreases in non-cellulosic glucose are interpreted as contributing to a possible mechanism whereby wall extensibility is increased and growth takes place.

Since it has been demonstrated that turgor pressure influences both rates of growth and changes in the mechanical properties of walls
the effect of turgor on the changes in wall composition associated with growth was also determined. Lowered turgor pressures resulted in partial or complete inhibition of growth and auxin-promoted decreases in non-cellulosic glucose. These results indicate that there is a certain level of turgor required for these auxin-promoted decreases. These results also suggest a relationship between the mechanical properties of the wall and the decrease in non-cellulosic glucan, and perhaps also a biochemical relationship to the mechanism of growth.

Investigations of various enzyme activities of *Avena* coleoptiles and the effects of certain enzymes on coleoptile cell walls were conducted to determine if any of these enzymes could be implicated in the elongation process. The results indicate that either dextranase or β-1,3-glucanase can cause extensive degradation of cell walls, but of the two, only β-1,3-glucanase activity was found associated with coleoptile preparations, and only β-1,3-glucanase caused changes in the wall similar to those changes observed to occur *in vivo* in coleoptile sections treated with auxin. These results, as do the changes in wall composition, suggest a possible mechanism for increased wall extensibility and growth involving hydrolysis of glucan components in the wall.
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Figure 31. Emission spectrum of the red light source in which the oat seedlings were grown the first 48 hours
Emission Spectrum
Rohm and Haas No. 2423 Red Plexiglas
7.5 watt Tungsten Source at 30 cm.
Figure 32. Emission spectrum of the green light source in which all manipulations of the coleoptile tissue were performed.
Emission Spectrum
Carolina Biological Supply No. 68-6610 "Green 545" (without neutral density filter)
7.5 watt Tungsten Source at 30cm.
Figure 33. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 0.1 mM KCN in the presence of 50 mM glucose and $1.25 \times 10^{-5}$ M IAA.
Figure 34. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 0.1 mM KCN in the presence of 50 mM glucose and without IAA.
+ KCN
- IAA
+ Glucose

μg sugar/section vs time (hrs)
Figure 35. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 5 μg/ml cycloheximide in the presence of 50 mM glucose and 1.25 x 10^-5 M IAA
Figure 36. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 5 µg/ml cycloheximide in the presence of 50 mM glucose and without IAA
+Cycloheximide
- IAA
+Glucose

μg sugar/section vs time (hrs)
Figure 37. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 25 μg/ml actinomycin-D in the presence of 50 mM glucose and 1.25 x 10^{-5} M IAA
+ Actinomycin-D
+ IAA
+ Glucose

µg sugar/section

time (hrs)
Figure 38. Changes in non-cellulosic sugar composition of walls from coleoptile sections treated with 25 μg/ml actinomycin-D in the presence of 50 mM glucose and without IAA.
+Actinomycin-D
-IAA
+Glucose