THE HORMONAL CONTROL OF CELL WALL PROPERTIES

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ABSTRACT

A technique has been developed for the separation of auxin action and cell elongation. Avena coleoptile sections are treated with auxin under conditions of non-expansion. This is followed by expansion of the sections in water containing an inhibitor which blocks all auxin action. Under aerobic conditions, auxin initiates a loosening of the cell wall. The loosening can then persist over short periods of non-expansion under anaerobic conditions. The loosening can be abolished by general metabolic inhibitors such as KCN or counteracted by an auxin-independent stiffening process. The loosening is due to an increase in the plasticity of the cell wall. Ethionine administered during either phase of the process inhibits the residual auxin effect, suggesting that transmethylation is involved in the increase in plasticity.

The metabolism of the cell wall components has been investigated by the use of labeled glucose and labeled methionine. The only effect of auxin which was found was an increased rate of incorporation of label from both glucose and methionine into the methyl ester groups of pectin. This increase is abolished by the same inhibitors which abolish the auxin-induced cell wall loosening. It is proposed that the increase in rate of pectin methyl ester incorporation is an integral part of the cell wall loosening process.

Incorporation of label from methionine C-14 into the pectin fraction has also been achieved with homogenates of Avena coleoptile sections although the rate is markedly decreased by homogenization. A procedure has been developed for obtaining reproducible results with such homogenates. Dialysis of the homogenate supernatant after solubilization of the pectin by boiling has proven satisfactory.

About 85 percent of the label from methionine C-14 incorporated into the pectin of intact tissues is in the form of esterified methyl groups. Upon homogenization of the tissues, however, only 20 percent of the label is located in the methyl groups.

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INTRODUCTION

Growth is an integrated combination of cell divisions and cell elongation. Cellular elongation of many plant tissues is accelerated by the addition of small amounts of the hormone auxin, and it is possible that elongation is absolutely dependent upon the presence of this material. Since the demonstration of the existence of auxin by Went (1), many investigators have attempted to determine the mechanism of auxin-induced elongation; that is, to discover what component or property of the cell is so affected by auxin as to cause elongation to take place. Although several proposals have been made and strongly defended, no one of them has yet been demonstrated to be correct.

The first part of this work is devoted to a reinvestigation of the mechanism of auxin action along the lines of previous work, but with the use of improved experimental techniques. It is shown unambiguously that auxin promotes cell elongation through an effect upon the cell wall. The second part of the work is devoted to a biochemical study of the metabolism of cell walls in living tissue. In the third part an attempt is made to study in vitro the system which is affected by auxin.

Avena coleoptile sections have been selected as the experimental material because of their large response in growth to added auxin, because the uniformity of the mate-

rial allows good reproducibility to be achieved, and because of the extensive background of previous work with this material.

Part I

CHANGES IN THE PHYSIOLOGY OF INTACT TISSUES IN RESPONSE TO ADDED AUXIN

INTRODUCTION

Several commonly but often loosely used terms which will be frequently employed in this work will be defined. In general, the definitions are those of Heyn (2).

Elongation: any increase in length of a tissue.

Expansion: an increase in length of a tissue when it is placed in a solution whose DPD is lower than the DPD_i of the tissue. This is not synonymous with growth; e.g., the elongation in water of a plasmolyzed tissue is expansion but not growth.

Elasticity: reversible deformability of a tissue, measured, for example, by its reversible elongation.

Plasticity: irreversible deformability, measured, for example, by its irreversible elongation.

Expansion of a plant cell requires the uptake of water into the cell. Since the plant cell is surrounded by a

relatively rigid wall, one must discuss water movement in terms of diffusion pressure gradients rather than in terms of osmotic pressures. Using the terminology of Meyer and Anderson (3) and Crafts et al. (4), the forces which determine water uptake by the cell may be summarized by the following equation.

$$DPD_i = OP_i + A - WP \qquad (1)$$

where DPD_i is the diffusion pressure deficit, OP_i the osmotic pressure of the cell contents, A any force exerted by an active water uptake system, and WP the wall pressure. The DPD gradient between the cell and the outside medium can be expressed by the equation,

$$\Delta DPD = DPD_i - DPD_e \quad (DPDe = OP_e) \qquad (2)$$

where i = internal, e = external.

For water to enter the cell, the ΔDPD must be positive. Since in a non-expanding cell $DPD_i = DPD_e$, a positive ΔDPD is achieved by an increase in DPD_i. It can be seen from equation 1 that this increase in DPD_i can have three origins. It may result from an increase in osmotic pressure (OP_i), an increase in active water uptake (A), or from a decrease in wall pressure (WP).

The relationship between auxin and these three possible ways of increasing DPD_i can be formulated as follows.

I. An increase in osmotic pressure could be produced by an auxin-induced increased concentration of solutes in the cell contents.

II. An auxin-actuated water pump could cause active uptake of water. Expansion would be achieved by a passive stretching of the cell wall and would require an active mechanism to prevent loss of the water pumped in against a gradient.

There are three ways by which wall pressure could be reduced by auxin.

III. An auxin-induced deposition of new cell wall material might increase the area of the cell wall and thus decrease wall pressure.

IV. The elasticity of the cell wall might be increased by auxin. The resulting increased expansion would be reversible but could then be made irreversible by intussusception of new cellulose fibers into the expanded net.

V. The plasticity of the cell wall might be increased by auxin. Turgor pressure would then produce irreversible expansion.

All of the above possibilities, as well as minor variations of each have been proposed by one or more investigators as the cause of auxin induced growth.

Review of Previous Investigations

The first theory of the mechanism of cell elongation was proposed by Sachs in 1873 (5). He proposed that the cell wall is extended by turgor pressure and that this extension is fixed by the intussusception of new cellulose. With this theory, Sachs focused attention upon the importance of turgor pressure in cell expansion.

Since the work of Sachs, and especially since the demonstration of the importance of auxin in the process of cell elongation, extensive investigations have been conducted to determine the validity of the various proposed growth mechanisms. To facilitate the selection of the most promising line for further investigation of the problem, it is desirable to determine which theory is best supported by the evidence at hand. For this purpose, a short summary of the evidence bearing upon each of the proposed mechanisms is presented. The literature published before 1931 has been most thoroughly reviewed by Heyn (2), and the subsequent literature has been reviewed by Heyn (6), Audus (7), and Ketellapper (8). Therefore only the more pertinent investigations will be mentioned.

Osmotic Concentration of the Cell

The possibility that growth is due to an increase in osmotically active solutes in the cell was considered by several investigators before the discovery of auxin (2). In 1935, Czaja (9) proposed that auxin causes such an

increase in the osmotic concentration of the cell on the basis of some doubtful experiments with root hairs. Burström (10), finding that the osmotic concentration of wheat roots remains constant with growth, suggested that the second phase of root growth depends upon an increase in osmotically active solutes in the cell. On the other hand, both Levitt (11) and Hackett (12), who used potato discs which expand in auxin solutions, found that auxin-treated discs have a lower osmotic concentration after four days in water than do the non-auxin treated controls. Hackett showed that the percent decrease in osmotic concentration was roughly equal to the percent increase in fresh weight, suggesting that there is no change in amount of osmotically active solutes during this period. Both Ketellapper (8) and Ordin et al. (13) found a depression in osmotic concentration in Avena coleoptiles which were growing as a result of auxin treatment.

An increase in osmotically active solutes is undoubtedly important for continued expansion of a cell. Nevertheless, the weight of evidence suggests that osmotic concentration does not increase in the presence of auxin but may actually slightly decrease, due to dilution of the cell contents by the water taken up during the expansion.

Active Water Uptake

The possibility of an active uptake of water has been

considered by several authors including Thimann (14). Failure to find a residual loosening of the cell wall by auxin and the fact that metabolism is required for growth induced him to suggest active water uptake as the cause of growth. He rejected this idea in 1954, however (15). Levitt (11) carefully investigated this possibility and found no evidence to support such an idea. Both Ketellapper (8) and Ordin and Bonner (16) showed that auxin has no effect upon the rate of movement of heavy water into or out of Avena coleoptile tissue.

At the present time there is no evidence to suggest that active water uptake occurs in plants or that it could be a cause of growth. Nevertheless, since the evidence is all of a negative nature, it cannot be dismissed as a possibility.

Active Growth of Cell Wall Area

"Active intussusception," that is an auxin-dependent increase in cell wall area by the laying down of new cellulose has been supported as the mechanism of growth by several modern investigators such as Söding (17), Frey-Wyssling (18), Ruge (19), and Ketellapper (8). This support is based upon the following facts. There is usually an increase in cell wall substance during growth, and this increase is usually higher in the presence of auxin. Inhibition of metabolism causes an inhibition of growth. Orientation of the fibers does not change during the growth

of some tissues. None of these facts indicates that active intussusception of new cell wall material is a cause of growth rather than just an accompanying process.

There is some evidence, however, that the thickness of the cell wall decreases during growth. Ruge (20) found a decrease in cell wall thickness in the first stages of growth of Helianthus hypocotyls. Bonner (21) attacked this problem by growing Avena coleoptile sections in auxin at different temperatures. He found that at 25°C. the amount of wall material increases by an amount proportional to the length of the tissue but that at 4°C. auxin causes no increase in cell wall weight although it causes an increase in length.

The laying down of new cell wall must occur if continued growth of a cell is to take place. Nevertheless, the facts suggest that the increase in cell wall material is just an accompanying process or a result of growth and not a cause of growth or directly affected by auxin.

Auxin-induced Increase in Elasticity

Several early investigators observed an increased elasticity in the zone of greatest growth rate and suggested that an increase in elasticity is the cause of growth (2). In none of the investigations, however, was it possible to determine whether the increased elasticity was a cause or a result of growth. Heyn (2) almost completely suppressed the elongation of Avena coleoptiles by

excising them from their bases and placing them in a saturated atmosphere. Under these conditions he found that auxin causes no increase in elasticity and concluded that the increased elasticity is a consequence of the elongation. Ketellapper (8) determined the time curves of both growth and elasticity for Avena coleoptiles after addition of auxin. While the growth curve showed essentially no lag before increasing in rate in response to auxin, there was a lag of almost an hour before any increase in elasticity could be measured. Growth could hardly be a consequence of increased elasticity in this case. Burström (22), working with wheat roots, found an increase in elasticity which occurred before an increase in growth rate and concluded that the elasticity cannot be an effect of growth but is more probably a cause of growth in roots.

The present evidence indicates that increased elasticity is a result rather than a cause of growth in Avena coleoptiles.

Auxin-induced Increase in Plasticity

In 1928, Went (1) suggested that auxin might be causing the increase in plasticity or over-stretching which several investigators had suggested as the cause of growth. It was left to Heyn (2) to present rather striking evidence for this view. Excised and therefore almost non-expanding Avena coleoptiles, after decapitation, were placed upright in a dark, moist chamber and given either plain agar blocks

or blocks containing auxin. After one hour, the coleoptiles were fixed in a horizontal position by inserting a pin into the empty leaf chamber at the basal end. A 250 mg. weight was placed on the apical end and after removal of the weight the irreversible (plastic) angle of bending was determined. The auxin-treated coleoptiles showed a much higher plastic bending than did the non-auxin-treated controls. This seemed a clear indication that the increased plastic bending was a direct result of the applied auxin. This conclusion, however, has been attacked on three counts.

It has been suggested, although apparently not in print, that since growth was not completely suppressed, the increased plasticity was rather a result of a small auxin-actuated increase in volume of the cell contents with a resulting rupturing of the cell wall bonds and consequently an increased plasticity. In other words, the action of auxin would be on some mechanism responsible for active water uptake rather than on a mechanism concerned with the loosening of the cell wall.

Söding (23) objected to the use of weights as heavy as those used by Heyn. Using very light weights for only five seconds, he was not able to repeat Heyn's results, although he could do so using heavier weights (112 mg.).

It has been pointed out by Pohl (24) that an irreversible bending is not only an indication of the plasticity of the upper side of the tissue but also a measure of the compressibility of the lower side. Compressibility, how-

ever, unless accompanied by a change in permeability to water by these cells, is still a measure of cell wall properties.

More recently, Gessner (25) found, on studying factors which influence plasticity and growth, that those factors which influence plasticity also influence growth but that the reverse is not true. He concluded that increased plasticity must be a cause of growth. Ruge (26) detected an increase in plasticity during the first periods of growth of Helianthus hypocotyls. Thimann (15) approached this problem with potato discs which were given auxin in the presence of a sufficient concentration of mannitol to partially inhibit water uptake. On return to water these discs showed a greater rate of water uptake than auxin-treated discs which had not been subjected to inhibition. A residual increased plasticity of the cell wall was suggested as the cause of the increased water uptake.

Some authors, such as Söding, Ruge, and Ketellapper agree that auxin causes an increase in plasticity but are not convinced that such plasticity is important for the expansion of the plant cell. Söding (17) determined the plasticity of both sides of bending organs. He found an increased plasticity on the convex side but found that the increased plasticity continued even after all bending had stopped. Ruge (20) also found that the increase in plasticity due to auxin persists after the growth rate has fallen. He concludes that there are two phases of the auxin action.

The first and least important phase is an increase in plasticity. The second phase involves an active growth of the cell wall. Burström (10, 22) presents evidence that the action of auxin upon wheat roots is also a two-phase process with the first phase, an influence on the elasticity, being of secondary importance.

Ketellapper (8) dismissed the importance of plasticity by pointing to the work of Frey-Wyssling (27), Bonner (28), and others who found that growing cells of various organs retain an annular or "tubular" orientation of the cellulose throughout the elongation. This argument is based upon the work of Bonner (28) and Frey-Wyssling (18) which points to the cell wall as being a network of cellulose fibers oriented in the same general direction. The strength of the wall would lie in the bonds between cellulose fibers which occur wherever two fibers touch or "Hauftpunkte." With annularly oriented fibers a loosening of the Hauftpunkte and a stretching of the wall should change the orientation of the fibers to a longitudinal or "fiber" structure. Such a reorientation has been reported by Frey-Wyssling (29) and Preston (30). But other investigators, notably Frey-Wyssling et al. (27) and Bonner (28) could find no change in orientation. Thus it has been concluded that in the latter cases some other mechanism of growth must take place. Wardrop (31), however, has evidence to indicate that the orientation of any given cellulose framework of the expanding Avena coleoptiles actually changes from an annular to a longitudinal orienta-

tion but that new annularly oriented lattices are continuously deposited and thus mask the reorientation.

Despite some evidence to indicate that an increase in plasticity is not the cause of growth in some other organisms, the evidence at hand favors the contention that such an increase in plasticity does occur in Avena coleoptiles and that this could be a cause of growth.

Other Mechanisms

Several other theories of the growth mechanism have been proposed which are not easily related to equations 1 and 2. In the pre-auxin era, attempts were made with little success to relate the cause of growth to an increase in hydration of the protoplasm, inhibition of the protoplasm, or permeability of the cell membrane.

Strugger (32) found indications that auxin causes an increase in viscosity of the protoplasm but attempts to implicate this as a cause of growth are counteracted by the finding of Ruge (26) that the increased viscosity is first detectable 18 hours after addition of auxin.

Veldstra (33) suggested that auxin causes a turgidity of the lipophilic membrane with a resultant increase in the permeability of the membrane to water. Using model membrane systems, he demonstrated that auxins do cause increased turgidity. In a later work, Booij and Veldstra (34) found that for a series of auxins the relative abilities to promote turgidity is the reverse of the relative abilities

to promote growth. Thus they concluded that while auxin can affect the membrane, this can hardly be the cause of growth. An apparent effect of pH on the permeability of the membrane to water, reported by Pohl (24), is rendered questionable by the use of toxic acetate buffers. Ketellapper (8) and Ordin and Bonner (16) have both shown that auxin has no effect upon the permeability of tissues to water and they point out that the permeability of the membrane to water is very great even under conditions in which no auxin is present.

Plan of Procedure

The weight of evidence seems to favor an auxin-induced loosening of the cell wall as the cause of growth. This loosening might be due to an increase in plasticity or an active growth in cell wall area. The most profitable line of inquiry, then, would seem to be a reinvestigation of the effects of auxin upon cell wall loosening, using improved techniques not subject to the objections raised against Heyn's work.

Thimann (14), in 1941, says,

> One method of distinguishing (a loosening of the cell wall) would be to allow the auxin to act for a time without allowing any growth to result. If growth were then permitted afterwards, the modified cell wall (if it had been modified) should allow an unusually rapid uptake of water, at least at first.

Not even Thimann has done this, though.

Suppose we give auxin to a piece of plant tissue but inhibit expansion osmotically. Now let us allow the expansion to take place in a medium which contains an inhibitor of auxin action so as to prevent any action of auxin during the expansion. Any increase in expansion due to the previously applied auxin cannot be ascribed to active water uptake. Such a technique overcomes the objections to Heyn's work since not only does it allow for the exclusion of active water uptake but it also uses as a test of the cell wall loosening the physiological process of expansion in water rather than the non-physiological process of stretching or bending of the cell wall by weights.

The present technique consists, then, of treating Avena coleoptile sections with auxin while preventing any expansion by the use of a 0.3 M mannitol solution. In order to eliminate all auxin action before the expansion, the sections are placed for a short time in another 0.3 M mannitol solution lacking auxin and containing some inhibitor of auxin action. The sections are then allowed to expand in water which contains the inhibitor of auxin action. An increased expansion of the auxin treated sections over the non-auxin-treated controls can only be due to an effect of auxin during the period of non-expansion. Since the work of Ordin (13), Ketellapper (8), Hackett (12), and others shows that auxin does not cause an increase in osmotically active solutes in the cell sap, this auxin effect can only be due to a loosening of the cell wall. Since any effect

must persist over at least a short period of time in which neither auxin action or expansion could take place, any effect would be a residual effect of auxin upon the cell wall and will be referred to as such.

METHODS AND MATERIALS

Avena seedlings were grown in the standard manner of McRae and Bonner (35). Seeds were soaked for one hour in distilled water and then planted in moist vermiculite contained in stainless steel flats. The flats were placed in a room which was kept at 25°C. and about 85% humidity and which contained a weak red light to inhibit mesocotyl growth. When the seedlings were 90-92 hours old, those with a coleoptile length of 2.75-3.25 cm. were selected and a 5 mm. section cut from each, the apical 2 mm. being discarded. The sections were pooled and randomized. For most experiments, lots of twenty sections were used for each portion of each experiment. In others lots of 30 or 40 were used.

Each lot of sections was then subjected to four successive treatments. During an auxin pretreatment or auxin-action period the sections were floated for 45 minutes in 10 ml. of 0.3 M mannitol buffered with 0.0025 N potassium maleate and either with or without added indoleacetic acid (IAA), 5 mg./l. This concentration of mannitol is such that at the end of the 45 minutes the length of the sections (measured under a binocular microscope with stage micrometer) is slightly less than the initial length. With

a second batch of seeds, the mannitol concentration could be reduced to 0.25 M without any expansion taking place. Plasmolysis does not occur as shown by microscopic examination.

In order to assure that the action of auxin was completely blocked before the sections were allowed to expand, the auxin pretreatment was followed by a transition treatment of 30 minutes. This consisted of 10 ml. of solution containing 0.3 M mannitol to prevent expansion and, in addition, an inhibitor of auxin action. The sections were then allowed to expand in 10 ml. of buffered water containing the inhibitor. After an expansion period of 90-120 minutes, the sections were removed and measured. The sections were next tested for possible damage by the earlier treatments. They were placed in 10 ml. of solution containing IAA, 5 mg./l., and sucrose, 2.25%, and their growth response determined. If the sections maintained their turgor for 6-12 hours, they were assumed to have been not significantly damaged by the earlier treatments.

The experimental results are expressed in terms of ΔL values.

ΔL = Average length of auxin treated sections after expansion--average length of non-auxin treated sections after expansion.

A ΔL value of 0.06 mm. or greater is considered as indicating an effect of auxin which is significant at the 5% level. This is evident from the data of Table 1. Standard errors

Table 1

Uniformity trial and demonstration of residual effect of auxin on expansion of Avena coleoptile sections. Pre-treatment with IAA, 5 mg./l., and 0.3 M mannitol for 45 min. Argon used as inhibitor during transition (30 min.) and expansion period (90 min.) treatments. Initial length of sections 4.75 mm.

	Final lengths-mm.	
Replicate #	+ auxin	- auxin
1.	5.15 ± 0.023 mm.	5.03 ± 0.025 mm.
2.	5.14 ± 0.021	5.02 ± 0.022
3.	5.14 ± 0.024	5.02 ± 0.015
4.	5.14 ± 0.023	5.02 ± 0.017
5.	5.14 ± 0.021	5.02 ± 0.014
6.	5.14 ± 0.027	5.01 ± 0.017
7.	5.13 ± 0.026	5.01 ± 0.020
8.	5.11 ± 0.024	4.98 ± 0.018
Average	5.14 ± 0.024	5.01 ± 0.018
ΔL	+ 0.13 mm. ± 0.030	

were computed for all lots. The average standard error for the auxin treated lots was 0.024 mm. while the average standard error for the non-auxin treated lots was 0.018 mm. The standard error for the difference between the means of auxin and non-auxin treated lots was 0.03 mm. A ΔL value of 0.06 mm. is therefore significant at the 5% level.

RESULTS

Separation of Auxin Action and Cell Expansion

Expansion of coleoptile sections as a result of an earlier auxin pretreatment can be achieved when anaerobic conditions are used as an inhibitor during the expansion period. Auxin-induced growth is inhibited by anaerobic conditions (36). Anaerobic conditions were obtained in the present experiments by the use of argon. After an aerobic auxin pretreatment, the sections were placed in stainless steel wire baskets and immersed in solutions through which argon had been bubbled for at least twenty minutes. This was done for both the transition and expansion period solutions. The anaerobic conditions thus produced should prevent any direct auxin action during the expansion period but should permit any residual auxin effect which is not dependent upon aerobic conditions to express itself as water uptake. The data of Table 1 show that under these conditions the auxin treated sections show a significantly greater expansion than do the non-auxin treated sections.

The use of argon as the inhibitor permits expression of a residual auxin effect.

Is the Apparent Separation an Artifact?

The possibility has been considered that the apparent residual effect of auxin may be due to some artifact of the system and some effort has been made to examine and eliminate such possibilities.

During the transfer of the sections from the transition period solution to the expansion period solution, they are exposed to air for 15-30 seconds. During this time it might be possible for the sections to absorb enough oxygen to allow the auxin remaining in the tissues to cause some active growth. This was tested by diluting the 10 ml. of transition period solution with one liter of argonated water to constitute the expansion period solution. The results, given in Table 2, indicate that with this method in which the sections are not exposed even momentarily to air, the residual auxin effect still persists.

Since auxin is known to cause active accumulation of salts (37), the possibility exists that auxin is causing an uptake of potassium ions from the buffer, thus raising the osmotic pressure of the sections. The experiment was repeated without the presence of the buffer and the residual auxin effect was still present (Table 3). Thus the accumulation of ions from the buffer can hardly be the cause of the increased expansion.

Table 2

Effect of method of transference of sections from transition to expansion period solutions on the residual auxin effect. Argon used as inhibitor of auxin action after auxin pretreatment. (IAA, 5 mg./l., in 0.3 M mannitol for 45 min.)

Method of transference	ΔL - mm.	Residual auxin effect
in air	+0.13	+
by dilution	+0.17	+

Table 3

Expansion of sections after pretreatment with auxin in the presence or absence of buffer (0.0025 M potassium maleate, pH 4.5). Argon used during expansion period.

Buffer in pretreatment	Expansion - mm.
yes	0.29
no	0.27

If argon is administered during the auxin pretreatment as well as during the period of expansion, no residual auxin effect can be detected (Table 4). This is in agreement with the work of Bonner (36) which indicated that aerobic conditions are necessary for auxin action.

The possibility must be considered, however, that argon does not completely inhibit the action of auxin during the expansion period. If this were the case, the sections should grow when auxin is added during the anaerobic transition and expansion period treatments. The results of such an experiment, shown in Figure 1, indicate that the effect of auxin during the anaerobic periods is negligible. When the time course of expansion is measured, as in Figure 1, it appears that essentially all the expansion which takes place as a result of the residual auxin effect is consummated within one-half hour.

The Three Types of Expansion

The expansion of the sections in the expansion period solution appears to consist of three processes. Due to the lowering of the external DPD, the tissues undergo a rapid osmotic uptake of water until the sections are once more turgid. This results in a return to the initial length. The auxin treated sections undergo a further increase in length due to their lowered wall pressure. Then the sections undergo an endogenous expansion which is of the same

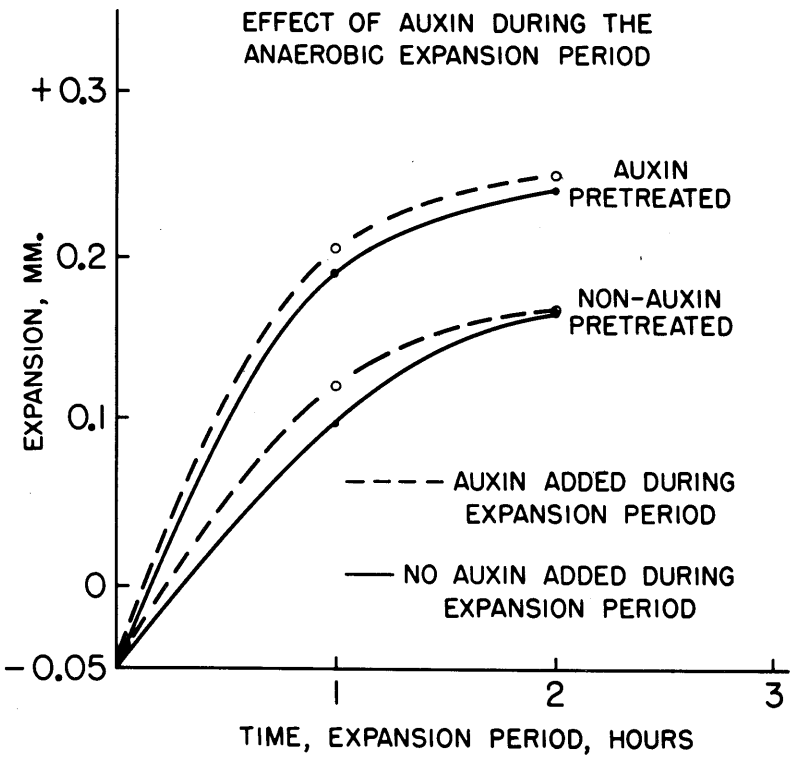

Figure 1. Time course of elongation of Avena coleoptile sections in water and under anaerobic conditions. Sections pretreated in 0.3 M mannitol for 45 min. under aerobic conditions either with or without auxin (IAA, 5 mg./l.), and either treated or not treated with auxin during the anaerobic expansion period.

Table 4

Effect of anaerobic conditions during auxin pretreatment of coleoptile sections. Argon used as inhibitor of auxin action after pretreatment (IAA, 5 mg./l. in 0.3 M mannitol for 45 min.)

Argon during auxin pretreatment	ΔL - mm.	Residual auxin effect
No	+ 0.15	+
Yes	- 0.05	-

Table 5

Effect of argon and auxin upon endogenous expansion. Sections pretreated with 0.3 M mannitol for 45 min. with or without auxin (IAA, 5 mg./l.). Expansion in water with or without argon. Expansion rate measured after expansion has progressed for 90 minutes to eliminate the residual auxin effect.

Auxin in pretreatment	Argon in expansion p.	Expansion mm./hr.	Δ
-	+	0.06	0.01
-	-	0.07	
+	+	0.07	0
-	+	0.07	

magnitude for both auxin and non-auxin treated sections.

The endogenous expansion is independent both of aerobic metabolism and of the presence of auxin (Table 5). Its magnitude varies from one experiment to another but is usually close to 0.07 mm. per hour per section. Since the endogenous expansion appears to be non-auxiniferous, only the second phase of the expansion is auxin dependent.

Evidence for a Cell Wall Stiffening Process

It is necessary that the transition period be sufficiently long to permit suppression of oxidative metabolism. The length of the transition period has been arbitrarily set at 30 minutes in the above experiments. This appeared to be sufficient since the addition of auxin during the expansion period does not affect the expansion. Various transition period durations were tried, nevertheless, to confirm this conclusion. It appears that the residual auxin effect dies away if the transition period is excessively prolonged. Thus although a residual auxin effect was found to persist through a transition period of up to one hour, it did not persist through one of 2-1/2 hours (Table 6). Heyn's work (2) has already indicated that when sections are kept in a non-expanding condition for a sufficient length of time, they lose their ability to respond to added auxin by expansion. This Heyn interpreted as due to a continuing stiffening of the cell wall.

Table 6

Relation of residual auxin effect to total time of sections in mannitol. Argon used as inhibitor of auxin action after auxin pretreatment, (IAA, 5 mg./l., 0.3 M mannitol). Expansion period 90 minutes, argon used as inhibitor.

Pretreatment (no auxin)	Auxin pretreatment	Transition period	Total time in mannitol	ΔL	Residual auxin effect
None	45 min	30 min.	75 min.	+ 0.09	+
None	45 min.	150 min.	195 min.	- 0.03	-
120 min.	45 min.	30 min.	195 min.	- 0.04	-

Heyn's hypothesis is supported by the results of the experiment of Table 6. For this experiment sections were treated for two hours in 0.3 M mannitol in the absence of auxin before being subjected to the usual treatments (45 minute auxin incubation period). No residual effect of auxin could then be detected. Some sort of stiffening of the tissue apparently takes place during prolonged periods of non-expansion. This stiffening is evidently independent of the presence of auxin in the medium and occurs both during prolonged pretreatment in 0.3 M mannitol and during prolonged transition period treatment.

Length of Auxin Pretreatment and Subsequent Expansion

The length of the auxin pretreatment has been arbitrarily set at 45 minutes in the above experiments. Variation in the length of the auxin treatment time, however, produces a proportional variation in the residual auxin effect (Δ L values) as is shown in Figure 2. The magnitude of the residual response to auxin is directly proportional to the length of the auxin treatment.

Further Evidence for a Residual Auxin Effect

Examination of possible artifacts, then, has failed to disclose any artifact that would be the true cause of the apparent residual auxin effect. A further test of the residual auxin effect can be made. Sections are treated with auxin either in the presence or in the absence of 0.3 M

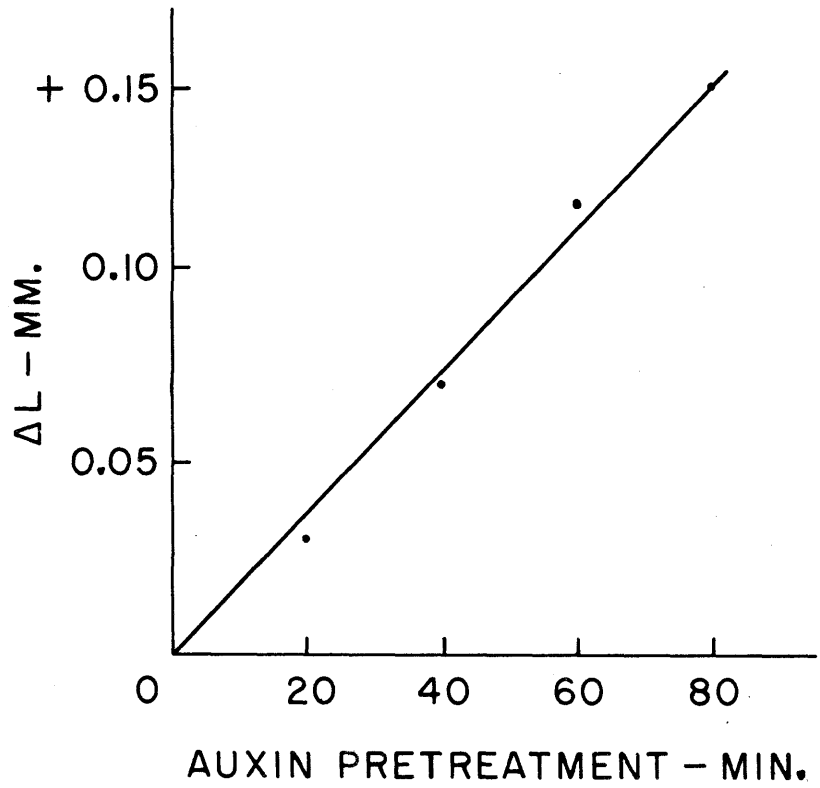

Figure 2. Relation between the magnitude of the residual auxin effect and length of auxin pretreatment. Expansion period 90 minutes. Argon used as inhibitor during transition and expansion periods.

mannitol. After an anaerobic transition period, once again with or without mannitol, the sections are allowed to expand in argonated water. Since, as shown in Figure 2, the residual effect can be stored up in the absence of expansion, the sections treated with mannitol should show an increased expansion over the endogenous expansion while the sections pretreated with auxin in water alone will have dissipated this potential as expansion during the pretreatment and thus will only undergo the endogenous expansion when under anaerobic conditions. The results of Table 7 show that this is indeed correct.

The Effect of Plasmolysis

There has been some controversy in the past as to whether the action of auxin upon expansion requires participation of the protoplasm (19, 36). Some evidence concerning this question can be obtained by determining the effect of plasmolysis upon the residual auxin effect. Sections incubated with auxin in the presence of 0.6 M mannitol, thus under conditions in which the protoplasm is not in contact with the wall, show no residual auxin effect (Table 8). This suppression of the auxin effect is not due to any irreversible damage to the auxin-sensitive mechanism by plasmolysis. This is shown by first plasmolyzing the cells in 0.6 M mannitol for 30 minutes and then conducting a standard test with 0.3 M mannitol in the auxin pretreatment.

Table 7

Expansion of sections in the presence of argon after pretreatment for 45 min. with IAA, 5 mg./l. either in the presence or absence of 0.25 M mannitol.

Mannitol in pretreatment	Expansion mm. - hour	Δ
+	+ 0.16	+ 0.12 mm./hr.
-	+ 0.04	

Table 8

Effect of plasmolysis with 0.6 M mannitol upon the residual auxin effect. Sections pretreated with 0.6 M mannitol (no auxin) for 30 minutes or not at all. Then treated for 45 min., ± auxin, with either 0.6 or 0.3 M mannitol. Transition period in 0.3 M mannitol. Expansion in water and argon, 90 minutes.

Mannitol concentration Pretreatment (no auxin)	Auxin treatment	Auxin	Final length	ΔL	Residual auxin effect
None	0.3 M	+	5.16 mm.	+ 0.15	+
		-	5.01		
0.6 M	0.3 M	+	4.99	+ 0.11	+
		-	4.88		
None	0.6 M	+	-	- 0.01	-
		-	-		

Under these conditions a residual auxin effect is obtained.

It is conceivable that the suppression of the residual auxin effect by plasmolysis is caused by lack of uptake of auxin from such a solution. This possibility was tested by incubation sections in either 0.6 or 0.3 M mannitol for 20 minutes with the C-14 labeled auxin, 2,4-dichlorophenoxy-acetic acid. At the end of this time the sections were carefully washed, 20 sections were placed on a planchet and dried under a heat lamp. The planchets were counted in an automatic scaler using flow gas. The results, in Table 9, show that while less auxin is taken up under conditions of plasmolysis, the suppression of the residual effect cannot be due to a lack of auxin uptake. These results are consistent with the supposition that intimate contact of protoplasm and the cell wall is necessary for auxin to be effective in promoting expansion.

Auxin Requirement after Pretreatment

Need auxin be present during the expansion phase or is its role completed during the pretreatment period? This may be determined by the use of antiauxins as inhibitors. If the presence of auxin is required during the period of expansion, then the presence of a sufficiently high anti-auxin concentration during the expansion period should suppress the residual effect due to auxin pretreatment. Anti-auxin was therefore added to the transition and expansion period solutions. Dichloroanisole (DCA) was initially used

Table 9

Uptake of C-14 2,4-dichlorophenoxyacetic acid by coleoptile sections in 20 minutes from 0.3 and 0.6 M mannitol solutions.

Mannitol concentration	CPM/20 min. incubation
0.3 M	88
0.6 M	69

Table 10

Inhibition of residual effect produced by various antiauxin concentrations during the expansion period treatment. DCA and 2,4,6-T used as antiauxins. Pretreatment with 0.3 M mannitol for 45 minutes, with or without IAA. Expansion in both air and argon.

Inhibitor	Auxin conc.	Expansion period in	ΔL-mm.	Residual auxin c.
2.8×10^{-4} M DCA	5 mg./l	air	- 0.01	-
None	0.1 mg./l	air	+ 0.09	+
2×10^{-4} M 2,4,6-T	"	air	+ 0.05	ca. 50%
None	"	argon	+ 0.11	+
2×10^{-4} M 2,4,6-T	"	argon	+ 0.05	ca. 50%

as the antiauxin. A concentration of 2.8×10^{-4} M DCA, applied during the transition and expansion periods, was found to completely inhibit the residual auxin effect (Table 10). However, since DCA has been reported to cause non-competitive inhibition as well as competitive (38), it was thought that it would be advisable to repeat the experiments using an antiauxin which is known to only produce competitive inhibition. The antiauxin 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) was selected since it is known to exert a strictly competitive inhibition of auxin-induced growth in Avena coleoptile section (39). The IAA concentration of the pretreatment solution was decreased to 0.1 mg./l. in order to make it possible to maintain as high as possible an antiauxin-auxin ratio in the tissues during the expansion period. 2,4,6-T can be applied in concentrations up to 2×10^{-4} M without producing toxicity. At this concentration it inhibits aerobic auxin-induced growth almost completely (39).

The data of Table 10 show that this concentration inhibits the residual auxin-induced growth by about 50%. It is apparent, therefore, that the auxin action, accomplished during the pretreatment, can be only partially undone by the presence of antiauxin during the expansion period. Auxin must evidently be present in the tissue in some form produced aerobically in order for the residual effect to persist.

The antiauxin 2,4,6-T is able to exert its effect during the transition and expansion periods even under anaerobic conditions as is shown by the data of Table 10. Thus antiauxin can inhibit the residual effect of auxin under conditions in which added auxin is itself ineffective.

Effect of Metabolic Inhibitors other than Argon

Respiratory metabolism is necessary for auxin action, and inhibitors of such metabolism other than anaerobiosis might therefore be investigated for ability to prevent auxin-induced growth during the expansion period. Three inhibitors with different modes of action were selected: KCN, 2,4-dinitrophenol (DNP), and p-chloromercuribenzoate (PCMB).

If KCN in a concentration of 3×10^{-4} M is used as the inhibitor during the expansion period, the auxin pretreated sections expand no more than do the control sections (Table 11). The sections are alive and capable of response to auxin if removed from KCN at the end of the experiment. If KCN is supplied at a concentration of 10^{-4} M, the auxin treated sections expand appreciably more than do the control sections. This is due to the fact that KCN at a concentration of 10^{-4} M is only slightly inhibitory to auxin-induced growth (40). The sections are able to grow during the expansion period since they contain auxin taken up during the pretreatment. The important fact is that it is possible to select a KCN concentration which is non-toxic

Table 11

Effect of the presence of various inhibitors during the transition and expansion periods on the residual auxin effect. Pretreatment with IAA, 5 mg./l., in 0.3 M mannitol for 45 minutes in all cases.

Inhibitor	ΔL - mm.	Damage by treatment	Residual auxin effect
KCN, 10^{-4} M	+ 0.07	-	+
KCN, $3x10^{-4}$ M	- 0.04	-	-
DNP, 2 mg./l.	+ 0.14	-	+
DNP, 4.5 mg./l.	+ 0.03	-	-
DNP, 8 mg./l.	-	+ (Dead)	
PCMB, $3x10^{-4}$ M	+ 0.07	-	+
PCMB, $5x10^{-4}$ M	- 0.02	-	-
PCMB, $6x10^{-4}$ M	-	+ (Dead)	
Cold (4^{o}C)	- 0.02	-	-

(over the time period used) and which completely eliminates any residual effect of the auxin pretreatment.

The inhibitor DNP, like KCN, is capable in appropriate concentration (4.5 mg./l.) of suppressing any residual effect of the auxin pretreatment (Table 11).

Rapidity of Inhibition by KCN and DNP

To determine the rapidity with which these inhibitors work, the effect of the length of the transition period upon the expansion was studied (Table 12). The data show that the action of both KCN and DNP is so rapid that no transition period is needed at all. Upon placing the tissues in the expansion period solution, the changes elicited in the cell by the auxin pretreatment are counteracted so rapidly by the inhibitor that the increased expansion cannot take place.

The Effect of KCN and Auxin upon the Osmotic Concentration of the Cell

The possibility exists that changes in the cell induced by the auxin pretreatment are not affected by the presence of the inhibitors, but rather that the inhibitors might cause a depression of the osmotic concentration of the auxin treated sections which would be equal in magnitude to the decrease in wall pressure caused by the auxin. Thus the auxin treated sections would not show an increased ex-

Table 12

Relation of the duration of the transition period to the residual auxin effect using KCN and DNP as inhibitors of auxin action. Pretreatment in 0.3 M mannitol, 45 min., ± auxin. Expansion period 90 minutes.

Duration of transition p.	Inhibitor	ΔL - mm.	Residual auxin effect
0	$3x10^{-4}$ M KCN	+ 0.01	-
60 min.	"	+ 0.04	-
0	4.5 mg./l. DNP	+ 0.01	-
30 min.	"	+ 0.04	-
60 min.	"	- 0.03	-

Table 13

Effect of $3x10^{-4}$ M KCN administered during transition period upon expansion in absence of KCN. After pretreatment (45 min., ± auxin, in 0.25 M mannitol), $3x10^{-4}$ M KCN applied in 0.25 M mannitol for 30 minutes. Sections then allowed to expand in water ± argon, 90 minutes.

KCN in trans. p.	Conditions-expansion p.	ΔL - mm.	Residual auxin effect
-	argon	+ 0.11	+
+	KCN	- 0.01	-
+	argon	- 0.07	-
+	air	+ 0.07	+

pansion although the effect of auxin upon the cell wall was still operative. This possibility can be investigated by determining the osmotic concentration of auxin and non-auxin treated sections which have been inhibited by KCN. Sections are pretreated in 0.25 M mannitol for 45 minutes, with or without auxin, and are then placed in solutions of various osmotic concentrations containing KCN, 3×10^{-4} M. After two hours the sections are measured. The curves in Figure 3 show that auxin has a negligible effect upon the shape of the curve, especially in the region of incipient plasmolysis, which determines the osmotic concentration of the tissue. Thus the inhibitor does not seem to be affecting the osmotic concentration of the tissues, and this possibility is eliminated.

Evidence on the Mechanism of Inhibition

As has been indicated, the presence of 3×10^{-4} M KCN during the expansion and transition periods inhibits the residual auxin effect. If the KCN is only administered during the transition period and argon is used during the expansion period instead, no residual auxin effect is observed (Table 13). But if the expansion takes place in water in which there is no inhibitor of auxin action, auxin-induced expansion takes place. Thus KCN must be counteracting the effects of the previously applied auxin without affecting the auxin itself which is in the tissues. The auxin can cause increased expansion when not inhibited by

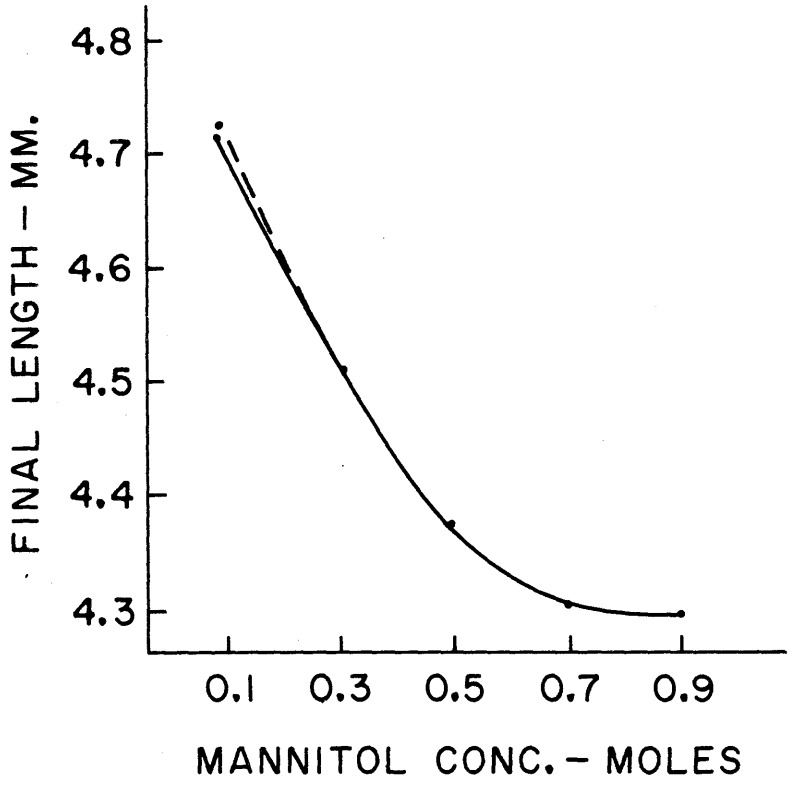

Figure 3. Change in length of coleoptile sections as a function of external mannitol concentration. Sections pretreated for 45 min. in 0.25 M mannitol, ± auxin, then incubated for two hours in solutions of various osmotic concentrations containing KCN. Solid line, - auxin; dotted line, + auxin.

KCN or argon.

A similar series of experiments has been performed using DNP as the inhibitor. Completely analogous results were obtained.

It is tempting to suppose that the action of DNP involves a lowering of the ATP level, since the usual biological effect of DNP is uncoupling of oxidative phosphorylation. Addition of 10^{-3} M ATP (K salt) to the expansion and transition period solutions in the presence of 4.5 mg./l. DNP produced no discernible effect. It is not known, however, whether the ATP can penetrate into the part of the cell where the auxin mechanism is located.

KCN and Endogenous Expansion

When the time curve of expansion is determined for sections expanding in the presence of 3×10^{-4} M KCN, it appears that there is an endogenous growth, independent of the presence of auxin. The magnitude of this growth is about 0.07 mm./hour. This is identical with that found for the endogenous growth in the presence of argon. Inhibitors thus appear to have no effect upon the endogenous growth.

PCMB

When PCMB was used as the inhibitor, it was found necessary to decrease the buffer concentration to 0.001 M

(with an accompanying rise in pH of the medium from 4.8 to 6.4) in order to get sufficient PCMB into solution to affect inhibition. Under these conditions, however, a PCMB concentration of 5×10^{-4} M does completely inhibit any residual effect (Table 11). This inhibition is not due to the effect of pH (Table 14).

It is apparent, then, that a variety of metabolic inhibitors are able to completely block the action of auxin. Under these conditions of inhibition, no residual effect of auxin on elongation can be detected.

Low Temperature

Low temperature was also used as an inhibitor of auxin action since it is known that auxin-induced section growth at, for example, $4^{\circ}C$ is slow (2). After an auxin pretreatment at $25^{\circ}C$, sections were subjected to transition and expansion period treatments at $4^{\circ}C$. The data of Table 11 show that when low temperature is used as the inhibitor of auxin action, the auxin treated sections do not expand more than the controls over the experimental period of 90 minutes. It may be concluded that the expression of the residual auxin effect is greatly delayed at low temperature. This result is not in accord with the reports of Heyn (2) and Heyn and Van Overbeek (41) who found some residual auxin effect at $1^{\circ}C$, at least over periods of 2.5 hours.

Table 14

Effect of pH upon inhibition of residual auxin effect. Sections pretreated with 0.3 M mannitol, 45 min., ± auxin, then allowed to expand in water containing various concentrations of PCMB. All expansion period solutions at pH 6.4.

PCMB Concentration	pH	ΔL - mm.	Residual auxin effect
5×10^{-4} M	6.4	+ 0.01	-
1.5×10^{-4} M	6.4	+ 0.10	+
0	6.4	+ 0.09	+

Inhibition by Calcium Ions

The growth of Avena coleoptiles is inhibited by various ions, especially divalent cations such as those of calcium (42). A thorough study of the inhibition by calcium has been made by Cooil and Bonner (43). Using an experimental procedure similar to that used in the present investigation, they were able to show that the calcium exerts a two-fold inhibition. Calcium inhibits that process which takes place aerobically in the presence of auxin and the absence of expansion, and also inhibits the residual effect of auxin if administered during the expansion period. These conclusions have been confirmed by the following experiments, done concurrently with those of Cooil. 0.01 M $CaCl_2$ is added to the auxin pretreatment period (0.3 M mannitol, 45 min., $\pm$ IAA, 5 mg./l.) or to the transition and expansion periods. The results, given in Table 15, confirm the conclusion that calcium is effective in inhibiting both the action of auxin and expansion itself.

Inhibition by Ethioinine

Certain compounds, closely related to amino acids, inhibit the incorporation of the amino acids into proteins. Such an inhibition of protein synthesis might inhibit growth. Canavanine, an antagonist of arginine, inhibits the growth of Avena coleoptiles (38). This inhibition is partially reversible by arginine. Schrank (44) reports inhibition of

Table 15

Inhibition of residual effect by calcium ions. 0.01 M $CaCl_2$ added either during auxin pretreatment (0.3 M mannitol, 45 min., ± auxin) or during transition and expansion periods (+ argon). Initial length 4.83 mm.

Calcium in Auxin pretr.	Trans. and exp. per.	Auxin	Final length	ΔL - mm.	Residual auxin effect
-	-	+	5.30 mm.	+0.18	+
		-	5.12		
+	-	+	5.10	+0.01	-
		-	5.09		
-	+	+	4.86	+0.02	-
		-	4.84		

Table 16

Inhibition of residual auxin effect by ethionine. 0.03 M ethionine added either during auxin pretreatment (0.25 M mannitol, 45 min. ± auxin), or during transition and expansion periods (+ argon). Initial length 4.75 mm.

Ethionine in Auxin pret.p.	Exp. and trans. p.	Auxin	Final length	ΔL - mm	Residual auxin effect
-	-	+	5.10 mm.	+0.12	+
		-	4.98		
+	-	+	4.97	-0.01	-
		-	4.98		
-	+	+	4.92	0	-
		-	4.92		

coleoptile sections with ethionine, an antagonist of methionine. Ethionine, however, can block transmethylation from methionine (45) as well as incorporation of methionine into proteins (46). It is of interest to determine the effect of ethionine upon the residual auxin effect. To test this, 0.03 M ethionine was added to the pretreatment solution (0.25 M mannitol, ± auxin, 45 min.). The transition and expansion periods were conducted in the presence of argon. Under these conditions a complete inhibition of the residual auxin effect was obtained (Table 16). When the ethionine was administered during the transition and expansion periods (in the presence of argon) inhibition was also obtained. It should also be noted that the actual amount of expansion for both auxin and non-auxin treated sections was smaller when the ethionine was given during the latter periods than when given during the auxin pretreatment.

Inhibition by 2-Thienylalanine

2-Thienylalanine (Th-Al), an antagonist of phenylalanine, was also tried. The original sample used was over four years old. Later two fresh samples were obtained from Arapahoe Chemicals. In these experiments, 0.01 M Th-Al was added to the standard auxin pretreatment solution and the transition and expansion phases were conducted in the presence of argon. When the old supply of Th-Al was used, the residual effect could be completely inhibited (Table 17).

Table 17

Effect of old and new samples of 2-thienylalanine upon the residual auxin effect. 0.01 M Th-Al administered during auxin pretreatment (0.25 M mannitol, ± auxin, 45 min.). Argon used during transition and expansion periods.

Th-Al sample	ΔL - mm.	Residual auxin effect
none	+0.10	+
old	0	-
new	+0.14	+

Table 18

Effect of 2-thienylalanine and phenylalanine upon growth of sections incubated in buffered water, ± auxin, ± Th-Al or phenylalanine for 5-1/2 hours. New samples of Th-Al used.

Inhibitor	ΔL - mm.
none	+0.70
0.01 M Th-Al	+0.39
0.02 M phenylalanine	+0.41

But under the same conditions, the new samples were found to be ineffective. Sections were then allowed to grow for 5-1/2 hours in buffered water, ± auxin and either with or without 0.01 M Th-Al (new samples). The Th-Al treated sections responded less to auxin than did the non-treated controls. Replacement of the Th-Al with phenylalanine produced a similar inhibition (Table 18). Thus the inhibition of straight growth by Th-Al appears not to be due to antagonism of the amino acid phenylalanine but to some non-specific type of inhibition. The inhibition due to the old sample must be attributed to some impurity arising from the age of the sample.

DISCUSSION

Treatment of Avena coleoptile sections with auxin under conditions in which no expansion occurs results in some change in the tissues such that the tissues possess an increased ability to expand when placed in water even though the action of auxin is blocked during the expansion by argon. Since the period of auxin action is separated from the period of expansion by a short period in which neither process occurs, the effect of auxin is a residual effect. The possibility that this effect is only an artifact of the experimental system has been examined, and no such artifact has been detected.

As further evidence of the reality of the residual effect, the following pieces of evidence may be adduced. First, the length of the auxin pretreatment is directly proportional to the amount of increased expansion elicited by the treatment. And secondly, pretreatment with auxin under conditions in which the expansion can occur concurrently with the auxin action fails to elicit any response to the auxin in a subsequent anaerobic expansion period. Both of these facts suggest that a potential for expansion is accumulated during the auxin pretreatment which can be expressed as increased expansion either in a subsequent period of expansion or at the actual time of auxin action. Thimann (10) has found a similar conservation of expansion potentiality in studies on the water uptake of potato discs.

The net result of the auxin action must be an increase in the DPD_i of the tissue. From equation 1 (page 2) it can be seen that there are three possible ways in which this can be achieved: an increase in active water uptake, an increase in the osmotic concentration of the cell, and a loosening of the cell wall.

The experimental conditions of these experiments are such that no expansion can take place during the period of auxin-action and no auxin-action can take place during the subsequent period of expansion. This eliminates the possibility that the expansion is entirely due to active water uptake or even that during the pretreatment period auxin might cause a small increase in cell volume by some sort of active water uptake with a resultant rupturing of cell wall bonds and a loosening of the cell wall. The lack of indication of active water uptake is in agreement with the conclusions of Ordin (16) that the movement of water into Avena coleoptile cells is entirely a passive process.

These experiments do not afford a direct demonstration that auxin does not affect the osmotic concentration of the cell. The experiments of Ordin, Ketellapper, and others, however, leave little room for doubt that auxin does not cause an increase in the osmotic concentration of Avena coleoptile cells. This possibility can apparently be ruled out.

The present experimental results are, however, compatible with the third possibility; namely, that auxin, by

acting upon the cell wall, causes a relaxation of cell wall pressure. As a result of such relaxation, water will then be taken up osmotically and the cell expands. The loosening of the cell wall could be due to an increase in plasticity, an increase in elasticity, or intussusception of new cell wall material. The present experimental procedure does not allow a differentiation to be made between these possibilities.

The first significant attempt to demonstrate auxin-induced loosening of the cell wall was carried out by A. N. J. Heyn in 1931. In Heyn's and other early experiments, however, a varying amount of auxin-induced cellular expansion was allowed to take place during the auxin pretreatment which was given in a saturated atmosphere but in the absence of liquid water. Active water uptake as the cause of cell wall loosening was thus not excluded. The separation of auxin action from expansion in the present experiments eliminates this possibility. By using as a test for cell wall loosening the reversible and irreversible bending of the coleoptile as a function of applied weight, Heyn separated the cell wall loosening into its two components; plasticity and elasticity. He concluded that cell wall loosening is entirely due to an increase in plasticity of the cell wall.

The present data also support Heyn's suggestion that a second process, a cell wall stiffening one, goes on in

the coleoptile. This process is not the reverse of auxin-induced loosening but appears to be different and independent of the latter.

Since the present experimental procedure allows a separation of the auxin action from the expansion, it is possible to study the requirements of the processes which go on in each of the two periods.

The present data reaffirm the conclusion of Bonner (36) that the auxin process requires aerobic conditions. The suggestion has been made by Thimann (personal communication) that oxygen may be required during the pretreatment only for the absorption of auxin into the tissues. He suggests that this is the process which actually goes on during the auxin pretreatment. Reinhold (47) and Johnson and Bonner (48) both show that the uptake of auxin consists of two processes: a non-metabolic diffusion of auxin into the cell and a metabolic accumulation of auxin into some part of the cell, presumably the vacuole. The latter is inhibited by inhibitors such as KCN. Neither paper shows directly that oxygen is required for this process although such a requirement seems probable. The fact that growth starts almost immediately after addition of auxin and that the external rather than the internal concentration of auxin limits growth rate strongly suggests that it is the auxin which diffuses into the tissue which is effective in promoting growth. Thus the oxygen requirement during the pretreatment appears to be connected with some action of the auxin after it has reached its site of action rather

than the uptake of auxin from the medium.

An intimate association between cell wall and cytoplasm seems to be necessary during the period of auxin-action. This does not imply that the action of auxin takes place in the cytoplasm rather than on the walls, but rather, as seems likely in view of the necessity for aerobic metabolism, that there is some substance produced in the cytoplasm which is necessary for cell wall loosening. This product would not be able to simply diffuse between the cytoplasm and the wall but could only be transfered through physical contact of the two. Other alternatives can, however, be formulated. There is, for example, the possibility that the action of auxin requires that at least a small amount of pressure be exerted upon the cell walls in order to stretch them sufficiently for some process such as intussusception to take place. This seems less probable. At any rate, the present results are at variance with the results of Ruge (26) who concluded that the cytoplasm is of no consequence in the action of auxin upon the cell wall.

Since the period of auxin action can be separated from the act of expansion, the cell wall effect can evidently persist over at least short periods of time in the absence of further oxidative metabolism. The work with metabolic inhibitors shows, however, that for the effect to persist, a particular kind of metabolism is necessary. The processes which conserve the auxin-induced cell wall expansibility are abolished by KCN, DNP, or PCMB.

The action of the KCN is not upon the osmotic concentration of the tissues but rather upon the cell wall loosening process. It has been seen that the KCN does not affect the auxin in the tissue. After sections are removed from KCN-auxin solutions, the auxin which remains in the tissue can result in increased growth. The inhibition is rather directed at the potential for expansion. This potential is not merely suppressed in the presence of the inhibitor but appears to be completely abolished. Oxidative conditions, in the absence of the inhibitor are then required for a renewal of the auxin-induced potential.

The present evidence suggests that the conserving process is suppressed by low temperatures. Heyn found, on the other hand, that auxin treated coleoptiles expanded more than non-auxin treated ones when placed in water at 1°C. Cooil has evidence (unpublished) to indicate that some increase in expansion in response to added auxin is found only when the expansion period is of sufficient duration. Thus Heyn's results are probably due to his use of longer expansion periods.

The auxin taken up from the solution by the tissue during the auxin pretreatment remains in the tissue in some form such that it can be used to promote growth during a subsequent aerobic expansion period. It is of interest to know if this auxin is necessary in some form for the persistence of the expansion potential. The fact that addition of

2,4,6-T during the expansion period causes at least some inhibition of expansion in both air and argon indicates that in order for the cell wall effect to persist, auxin must remain in the cell in some form in which it can be exchanged for antiauxin. But the fact must be explained that antiauxin supplied anaerobically is effective in inhibiting expansion due to aerobic auxin pretreatment while auxin is unable to promote expansion under the same circumstances.

It appears, then, that the effect of auxin upon expansion can be divided into at least two distinct processes: an aerobic and an anaerobic process. Auxin, under aerobic conditions, initiates a loosening of the cell wall. This loosening, achieved under aerobic conditions, can be preserved until used up as expansion or counteracted by the stiffening process as long as at least the anaerobic metabolism is present. The loss of all metabolic activity, as results in the presence of sufficient concentrations of metabolic inhibitors such as KCN, DNP, and PCMB, results in a complete negation of the expansion potential although the auxin itself appears to be unaffected. Auxin, in some form, seems to be required for the maintenance of the residual effect.

The magnitude of the incremental expansion which results from the auxin pretreatment is about 0.11 mm./section/hour of auxin treatment (for one particular experiment). Sections grown aerobically in the presence of auxin under simi-

lar conditions expand at the rate of about 0.15 mm./section/ hour (above the no-auxin control rate). The induced expansion elicited by auxin pretreatment, therefore, is a substantial fraction of the expansion which takes place simultaneously with auxin action and which is called growth. Auxin-induced growth, then, may be thought of as a continuing process of simultaneous cell wall loosenings and expansions.

There are two inhibitors whose actions are not readily fitted into the previous scheme. Calcium ions appear to inhibit both the auxin action and the expansion. The nature of the two calcium inhibitions, whether they are the same or different, cannot be deduced at present. It is tempting, however, to consider the possibility that during the expansion phase the calcium ions act simply as a glue: that the divalent calcium forms bridges between the carboxyl groups of pectic acid chains with the result that the chains are cross linked more firmly and the tissue thus stiffened. Cooil (43) shows that the inhibition by calcium of the auxin pretreatment process is not due simply to the binding by the tissue of calcium ions which then remain to inhibit the subsequent elongation. The calcium ions bound during the pretreatment can be washed out of the tissue during the transition period without a restoration of the auxin-induced potential. Calcium ions appear to directly inhibit the auxin induced pretreatment processes.

The inhibition of the residual auxin effect by amino acid antagonists does not appear to be an inhibition of protein synthesis. The lack of inhibition by 2-thienylalanine suggests that the inhibition by canavanine and ethionine are due to effects other than a blocking of protein synthesis. At the present time, canavanine is not known to inhibit any metabolic process other than protein synthesis. Ethionine, however, inhibits the transfer of methyl groups from methionine to other substances.

Ethionine inhibits the residual auxin effect when administered during the auxin pretreatment, suggesting that transmethylation is an integral part of the action of auxin. Administration of ethionine during the expansion period, however, also causes inhibition. This would mean that methionine is necessary for the expansion phase as well as the auxin initiation phase. Transmethylation, then, seems to play an integral part in the process of auxin-induced loosening of the cell wall by being necessary for the conservation of the expansion potential. Since transmethylation in animal systems required the presence of ATP, suppression of the expansion potential by KCN and DNP might be due to inhibition of formation of the ATP necessary for transmethylation. Conservation of expansion potential under anaerobic conditions would not, then, be expected. It might be postulated, however, that sufficient ATP is produced under anaerobic conditions to

allow transmethylation to occur. Either higher ATP levels or molecular oxygen should be necessary for the initial auxin reaction. Thus the conservation of potential could occur under conditions which would not allow the initiation of the potential to occur.

Some insight is obtained from the present experiments upon the nature of the endogenous expansion. This expansion appears to be independent of all metabolic activity. It is independent of auxin, oxygen, or the presence of metabolic inhibitors such as KCN. It would appear that this expansion is simply a passive mechanical stretching of the wall due to the constant pressure of the cell contents.

RESOLUTION OF THE RESIDUAL AUXIN EFFECT INTO ELASTICITY AND PLASTICITY

The experiments of the preceding section have shown that the action of auxin upon Avena coleoptile tissues results in a loosening of the cell walls. This loosening could be due to an increase in elasticity, plasticity, or intussusception of new cell wall material. Since the preceding experimental procedure is incapable of resolving the loosening into the different components, a different procedure must be used.

Heyn developed the technique of measuring and differentiating elasticity and plasticity by determining the reversible and irreversible angles of bending which are

induced in a horizontally held coleoptile by addition of a static load to the end opposite that by which the coleoptile is held. The amount of reversible bending is a measure of the elasticity and the residual bending is a measure of the plasticity. Intussusception of new cell wall, since it would occur uniformly around the cell, would be expected to appear as an increase in elasticity rather than in plasticity of the cell.

Heyn concluded that the auxin produced an increase in the plasticity of the cell wall without directly affecting the elasticity. The method and conclusions of Heyn have been attacked from various sides (see page 6). Nevertheless this method of differentiating elasticity and plasticity appears to be the best available, especially since the technique has been perfected by Tagawa (49) in this laboratory until the results are quite reproducible.

By means of this technique, then, a determination has been made of the plasticity and elasticity of coleoptiles which have been given auxin in conditions under which no expansion can occur.

Method

The procedure is identical with that of Tagawa (49) and makes use of the Tagawa bending machine (Figure 4). Coleoptiles of 3.3 cm. length are selected. The central leaf is removed and one 2.0 cm. section is then cut 3 mm. from the tip. The sections are floated for one hour in

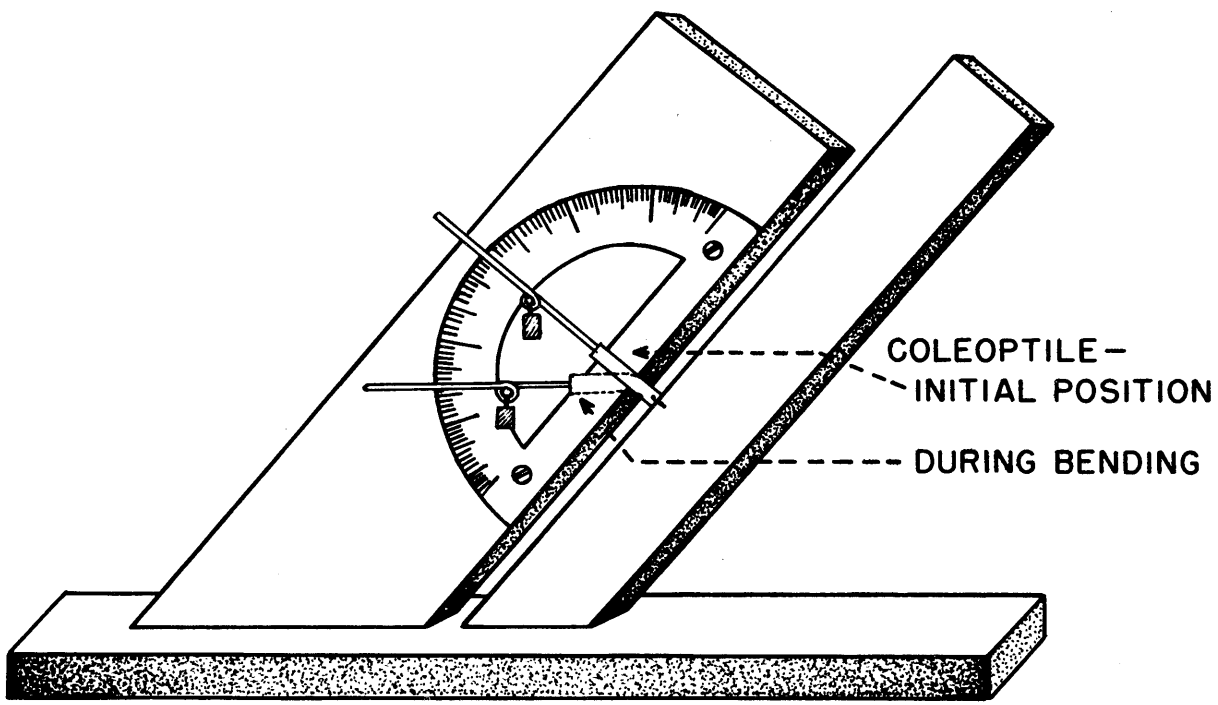

Figure 4. The Tagawa bending machine. Procedure for use described in text.

10 ml. of 0.25 or 0.28 M mannitol solution either containing or lacking IAA (5 mg./l.). The coleoptiles are then secured to the bending machine by insertion of a stainless steel pin of about 1 mm. diameter and 5 mm. length into the empty leaf chamber at the basal end of the coleoptile section. A fine glass rod, weighing 75 mg. and 5 cm. in length, bearing a central loop, is then inserted into the tip end of the coleoptile to a depth of 5 mm. The tip of this glass rod projects upon a protractor. After 5 minutes, the initial angle is read. A weight is then suspended from the loop (225 or 100 mg.). After 15 minutes the angle is again read. The weight is then removed and after 5 minutes the final angle is determined. The difference between the initial angle and the final angle is the plasticity. The difference between the final angle and the total angle induced by the weight is the elasticity.

All measurements are carried out in a dark room under a dim red light, at 25°C, and at almost 100% humidity. The time periods used for the various operations have been determined experimentally by Tagawa (49) as suitable for the purpose.

Results

Five series of experiments were performed using, respectively, 3,6,5,6, and 2 coleoptiles treated with auxin and an equal number of non-auxin treated control coleoptiles. The results are summarized in Table 19. It can

Table 19

Average elasticity and plasticity of Avena coleoptile sections after incubation for one hour in 0.25 or 0.28 M mannitol, ± auxin.

Expt. #	# of col. pairs	Mannitol conc.	Weight	Elasticity + auxin	-	Δ	Plasticity + Auxin	-	Δ
1	3	0.25 M	225 Mg.	28°	27°	+1°	40°	27°	+13°
2	6	0.25	100 mg.	19°	15°	+4°	32°	27°	+8°
3	5	0.25	100	14°	14°	0	26°	12°	+14°
4	6	0.28	100	17°	14°	+3°	30°	21°	+9°
5	2	0.28	100	18°	17°	+1°	36°	26°	+10°

be seen that the values are in reasonable agreement and certainly indicate a definite effect of auxin upon the plasticity and probably a lesser effect upon the elasticity. Table 20 gives the actual angles involved for one pair of coleoptiles from Expt. 3. Table 21 gives the values for the elasticity and plasticity for the five sets of coleoptiles in Expt. 3. This gives some indication of the reproducibility of the system. It is apparent that variations of $\pm 5^{\circ}$ can be expected. Nevertheless the differences in plasticity between the auxin and non-auxin treated tissues are statistically highly significant even in individual experiments as indicated in Table 21.

Discussion

It appears that Avena coleoptiles, incubated with auxin under conditions in which no expansion can take place, possess a greater plasticity than do non-auxin treated coleoptiles. The elasticity may also be increased, but, at most, this effect is small. It is now possible to describe the loosening of the cell wall as due to an increase in its plasticity. The increased plasticity can only be a result of the auxin action since no expansion has occurred. Thus the conclusions of Heyn are confirmed.

Table 20

Angles involved with one pair of coleoptiles from Expt. 3.

	+ Auxin	-
Angle: initial	68°	73°
+ weight	23°	40°
final	41°	57°
Total bending	45°	33°
Plasticity	27°	16°
Elasticity	18°	17°

Table 21

Elasticity and plasticity for the individual coleoptiles of Expt. 3.

Auxin	Total bending	Elasticity	Plasticity
+	42°	13°	29°
+	45°	16°	29°
+	45°	18°	27°
+	40°	14°	26°
+	31°	11°	20°
-	33°	17°	16°
-	32°	19°	13°
-	21°	10°	11°
-	25°	14°	10°
-	20°	11°	9°
Average: +	40°	14 ± 1.2°	26 ± 1.7°
-	26°	14 ± 1.7°	12 ± 1.2°
Difference	+14°	0 ± 2.10°	+14 ± 2.06°

Part II

CHEMICAL CHANGES IN THE CELL WALLS OF INTACT TISSUES IN RESPONSE TO AUXIN

INTRODUCTION

The studies of the physiology of auxin-induced expansion described in Part I have indicated that the direct result of auxin action is a loosening of the cell wall. Auxin might bring about this loosening either by merely changing the physical properties of some cell wall component or alternatively by influencing some chemical reaction which directly or indirectly involves the cell wall constituents. Support for the latter view is found in the fact that metabolism is necessary for auxin action.

The final result of auxin action is a change in mechanical properties of the cell wall. It would be possible that the action of auxin is in the cytoplasm and that the product of the reaction is transported to the wall where it causes loosening. In this case, it would be expected that the action of auxin should be upon some specific enzyme system in the cytoplasm. Attempts have therefore been made to detect any component of the cytoplasm which changes in response to the auxin status of the tissue.

Cytoplasmic Auxin Effects

The dependence of growth upon respiration was initially shown by Bonner in 1933 (36) who found both respiration and

growth to be inhibited by cyanide ions. The addition of pure auxin b, in solution, to Avena coleoptiles was found by Bonner (40) to have no effect upon respiration rate. Burstrom (10) found this to be true, also, for wheat roots. Bonner (50), using Avena coleoptiles, and Christianson and Thimann (51), using pea stems found that IAA causes an increase in respiration in the absence of other substrates. Greater increases in respiration rate in response to added auxin were found in the presence of various substrates such as sucrose and the plant acids (52,53). The mechanism of this increase in respiration has been examined by Bonner (50). He found that the rate limiting step in Avena coleoptile respiration is phosphorylative turnover and that the effect of IAA may be simulated by DNP. Subsequent work by Bonner et al. (54) and Ordin et al. (13) showed that the increase in respiration is not in response to auxin but rather in response to elongation. The effect of IAA in increasing respiration is abolished by inhibition of growth with mannitol.

Commoner and Thimann (52) found that while iodoacetate inhibits growth by 100 percent, respiration is only inhibited 10 percent by the same iodoacetate concentration. They concluded that only a part of the metabolism is necessary for growth. This has been confirmed by subsequent work (50).

Analysis of the amounts of various substances in the cytoplasm as affected by the presence of auxin has so far failed to produce any changes which can be correlated with

the changes in growth. Christianson and Thimann (55) suggest that a 10% increase in protein synthesis which they found in pea stems in the presence of auxin is significant, but neither Bonner (38) nor Burström (56) could find any correlation between growth and protein synthesis. Burrows and Bonner (57) investigated the metabolism of C-14 glycine, acetate, and sucrose in the Avena coleoptile under the influence of added auxin. Only in the metabolism of the cell wall did auxin appear to exert any effect.

Bonner and Bandurski (58) have summarized the effects of auxin upon a number of in vitro enzyme systems. Only IAA oxidase has been found to show enhanced activity in the presence of auxin. Berger and Avery (59) found an increase in alcohol and malic dehydrogenase activity when auxin is added to intact tissue but auxin has no effect upon the partially purified enzymes in vitro.

Up to the present time, then, all attempts to identify the effect of auxin with any enzyme system or metabolic function of the cytoplasm have ended in failure.

Effects of Auxin upon Cell Wall Metabolism

The site of the auxin action may be located on the cell wall. In this case some substance would be required for the auxin action which would have to be produced metabolically in the cytoplasm. Attempts have been made to find a component or enzyme connected with the cell wall fraction

that is auxin-affected.

It has long been known that during the course of coleoptile growth cell wall formation keeps pace with cell elongation (21). This relation is not an obligatory one, however, since cell wall formation can be suppressed by low temperatures which do not completely suppress cell elongation. No correlation between cell wall synthesis and growth can be found in wheat roots (56). Christianson and Thimann (60), however, have stressed the relation between cell wall formation and expansion of pea stems. Wilson and Skoog (61) find a direct relation between auxin-induced increase in fresh weight in tobacco pith and the increase in soluble and insoluble uronides.

Various enzymes located in the cell wall fraction have been investigated for response to added auxin. Ascorbic acid oxidase was found by Newcombe (62) to double in activity when auxin was applied to living tobacco pith tissue. Since the increase in enzyme activity preceded the increase in fresh weight, the effect cannot be a result of expansion. Bryan and Newcombe (63) report an increase and Neely et al. (64) a decrease in pectin methylesterase activity after addition of auxin to intact tissue. Bryan finds an inhibition of the purified enzyme by auxin and Glasziou (65) finds no effect. Ruge (66) has found that addition of pectin methylesterase to Avena coleoptiles causes an increase in the amount of growth that an application of

auxin can produce. While the evidence does not indicate that this enzyme is the one directly affected by auxin, it appears possible that pectin methylesterase activity is in some way connected with the loosening of the cell wall.

The investigations so far have failed to demonstrate that any one component of the cytoplasm or cell wall is directly changed as a result of auxin action. The site of auxin action is also undetermined. Whether the auxin action occurs in the cytoplasm or on the cell wall, the final result must be some change in the cell wall. Thus it seems advisable to concentrate upon the possible changes in the cell wall induced by auxin.

Plan of Procedure

The cell wall is composed of three main classes of materials: cellulose, pectic substances, and the other polysaccharides (polyuronide hemicelluloses and non-cellulosic polysaccharides). It is proposed to study first the incorporation of C-14 glucose into the various cell wall fractions in the presence or absence of auxin and under conditions of cell expansion and non-expansion. It can thus be determined if the synthesis or breakdown of cell wall materials is auxin dependent.

It is then proposed that the effect of auxin upon the methyl ester groups of pectin be investigated. Work with amino acid antagonists in Part I has suggested that the

inhibition of growth by ethionine might be due to an inhibition of transmethylations involving methionine. Sato (67) has shown that the methyl group of pectin can indeed arise from methionine. Therefore it is proposed that the effect of auxin upon the incorporation of C-14 labeled methyl groups from methionine into the pectins of Avena coleoptiles be investigated.

The experiments shown in Tables 23 to 34 were done jointly with Dr. L. Ordin.

MATERIALS AND METHODS

The experimental material consisted of 5 mm. sections cut 3 mm. below the tip of Avena (variety Siegeshafer) coleoptiles grown in the conventional manner (35). Primary leaves were removed from all sections. Incorporation of the carbon of glucose and methionine into the cell wall of sections was allowed to occur by incubating sections in various solutions containing these substrates. The radioactive glucose and methionine were obtained from the California Foundation for Biochemical Research.

The specific activity of the glucose used varied from experiment to experiment due to dilution of the radioactive glucose with unlabeled glucose. The methionine had a specific activity of 310×10^6 cpm/mM, and was used undiluted in all cases. Unless otherwise stated, the initial activity was 350,000 cpm/treatment.

Two hundred sections floating on 4.5 ml. of solution were used for each treatment of each experiment. Solutions were buffered with 0.0025 M potassium maleate, pH 4.8; IAA, 5 mg. per liter, was used as the auxin where indicated. At the end of the desired incubation period, the sections were rinsed with water and ground rapidly in a mortar, and the water-soluble and protoplasmic materials were separated from the cell wall debris by repeated washing and filtration with cold water on a sintered glass funnel. The washed cell wall material was then separated into constituent groups of components by the procedure outlined in Figure 5, which is adapted from Sinclar and Crandall (68) and Bonner (69). The names classically applied to the cell wall fractions of different solubilities are included in Figure 5, but there is no rigorous evidence that the fractions prepared from our material are, in fact, the classical cell wall materials. Nevertheless, for the sake of convenience, the fractions will be referred to by their classical names.

Aliquots of the hot water soluble, hot dilute acid soluble, and hot ammonium oxalate soluble extracts of the wall material were counted on copper planchets. The alkali soluble fractions were neutralized with dilute sulfuric acid before plating. All planchets were counted on an automatic sample changer using a micromil window tube and Q gas. All counts are corrected to infinite thinness.

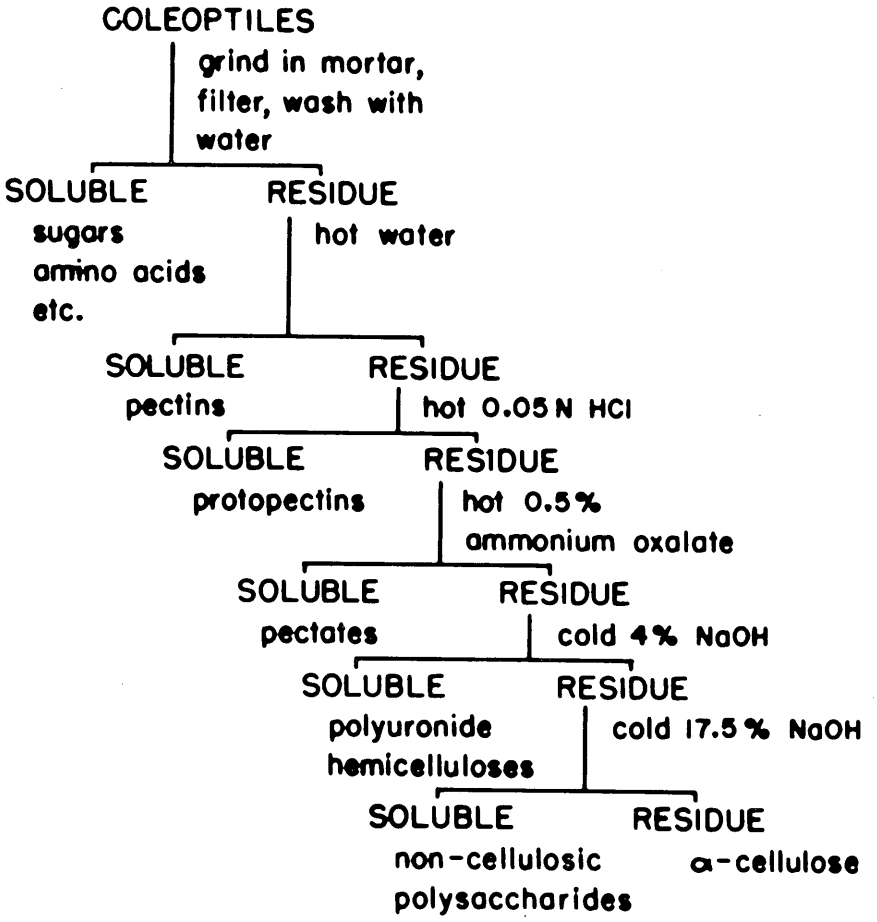

Figure 5. Fractionation procedure used for the separation of cell wall components into their various fractions on the basis of solubility.

The methyl ester content of each fraction was determined by saponifying the fraction, determining the residual activity, and then obtaining the difference between this figure and the initial activity. Saponification is achieved by incubation of the fractions with 0.1 N NaOH for 2 hours at room temperature followed by neutralization to phenolphthalein or by heating with concentrated ammonia for two hours followed by simply drying the sample on a planchet.

The existence of methyl ester has not been shown, yet, by isolation of labeled methanol after saponification, but investigations on the nature of the labeled material of the pectin fraction after incubation with methionine C-14 indicate that the bulk of the label is in the form of methyl ester.

Ordin precipitated the pectic acid which arises as a result of saponification of pectin and found that it contains only about 5 percent of the label. Thus only a very small portion of the methyl carbon of methionine becomes incorporated into galacturonic acid residues by devious metabolic pathways.

Saponification, followed by heating the sample to dryness under alkaline conditions results in a loss of from 65 to 85 percent of the label. Since 95 percent of the label is saponifiable, the saponifiable label must consists of two fractions; one, comprising 65-85 percent of the label is volatile from alkaline solution, and the other, comprising only 10-30 percent of the label, is not volatile. The pro-

portion of non-volatile component can be reduced to below 10 percent by dialysing the pectin at 0°C against water for 18 hours.

The volatile saponifiable fraction has been suspected to be methyl ester, since only a small neutral or basic molecule should be volatile from alkaline solution. Confirmation of this hypothesis has been obtained by incubating the pectin with pectin methylesterase, specific for the removal of methyl ester groups, for ten hours at 25°C. At the end of this time, the pectin had lost 80 percent of the label removable by alkaline saponification and volatilization (Table 22). Since the rate of deesterification by PME falls off rapidly after two-thirds of the ester groups have been removed (70), the loss of 80 percent of the label strongly suggests that all of the volatile saponifiable material is methyl ester.

With the exception of Tables 32 and 34, all data are expressed as cpm per 10 mg. dry cell wall. In the case of the exceptions, since the inhibitors used inhibit uptake of methionine into the tissue, the data are corrected for equal uptake of methionine so that a comparison of the actual incorporation of the methionine can be made.

Table 22

Effect of hot ammonia and pectin methylesterase upon removal of label from pectin fraction.

Treatment	cpm	Percent of total	Percent of saponifiable
Total pectin fraction	880	100	--
Saponifiable with hot conc. NH_3	748	85	100
Removed with pectin methylesterase	598	68	80

Table 23

Incorporation of C-14 from glucose C-14 into cell wall fractions. 3 hour incubation, 0.00004 M glucose C-14, 0.001 M unlabeled glucose. Initial specific activity 117×10^6 cpm/mM.

Cell wall fraction	+ IAA	- IAA
Pectin	624 cpm	575 cpm
Protopectin	11,050	13,300
Pectic acid	169	252
Polyuronide hemicellulose	1,560	2,370
Non-cellulosic polysaccharide	321	295
Cellulose	1,375	1,360

RESULTS

The Incorporation of C-14 Glucose into Cell Walls

The incorporation of C-14 glucose into the constituents of the cell wall has been achieved by incubating Avena coleoptile sections for three hours in a solution 0.00004 M in glucose C-14 and 0.001 M in cold glucose. The data from a typical experiment are shown in Table 23. The presence of auxin appears to increase incorporation into the pectin fraction and decrease incorporation into the polyuronide hemicellulose fraction. In order to determine if the differences are a result of the applied auxin or are only related to expansion, the experiment was repeated in the presence of 0.3 M mannitol. Similar results are obtained (Table 24) showing that the differences are a result of the auxin action rather than of the expansion.

It is known that glucose can give rise to one carbon units (71). It is necessary, therefore, to determine whether the differences in the incorporation into pectin as caused by auxin represent a difference in incorporation of glucose into galacturonic acid residues or a difference in incorporation into methyl ester. When the pectin fraction from the experiment shown in Table 23 was separated into the two components, it was found that the difference lay almost entirely in the methyl ester fraction and that the incorporation of glucose into the galacturonic acid is not affected by auxin within three hours (Table 25).

Table 24

Incorporation of C-14 from glucose C-14 into the cell wall in the presence of 0.3 M mannitol. 3 hour incubation, 0.00004 M glucose C-14. Initial specific activity, $2,470 \times 10^6$ cpm/mM.

	cpm/10 mg. dry cell wall	
Cell wall fraction	+ IAA	- IAA
Pectin	1,380	1,300
Protopectin	11,700	11,900
Pectic acid	94	86
Polyuronide hemicellulose	1,760	1,910
Non-cellulosic polysacharride	473	700
Cellulose	538	527

Table 25

Separation of pectin fraction from experiment shown in Table 23 into pectic acid and methyl ester fractions.

	cpm/10 mg. dry cell wall/80,000 cpm absorbed	
Fraction	+ IAA	- IAA
Total pectin	624	575
Pectin ester	181	114
Pectin non-ester	443	461

The turnover of cell wall components was measured by incubating sections for 17 hours in an excess of unlabeled glucose after a three hour pretreatment in C-14 labeled glucose. The data of Table 26 indicate that there is some turnover in the pectin and non-cellulosic polysaccharide fractions and that this turnover is suppressed in the presence of auxin. The fractions were not analyzed, however, for the amount of label in the galacturonic acid as compared with the label in the methyl ester.

Incorporation of C-14 Methionine into Cell Walls

The studies with C-14 glucose have indicated that auxin does not affect the incorporation of glucose into galacturonic acid but that an auxin-induced increase in the methyl ester content of pectin does occur. To study further the latter process, then, Avena coleoptile sections were incubated for four hours with labeled methionine. At the end of this time, the pectin, protopectin, pectic acid, and polyuronide fractions had become labeled. Little label was found in the non-cellulosic polysaccharides or in the cellulose (Table 27).

The significant feature of the data of Table 27 is the increase in incorporation of label into the pectin fraction in the presence of auxin. This is not true for any other fraction. This increase, with methionine as substrate, is comparable to the one found when glucose was used as substrate.

Table 26

Turnover of cell wall constituents. Sections pretreated 3 hours, 0.000167 M glucose C-14, - IAA, then incubated 17 hours, 0.09 M unlabeled glucose, ± IAA. Initial specific activity 580×10^6 cpm/mM

Cell wall fraction	cpm/10 mg. dry cell wall After 3 hr. pretreatment	After 17 hr. incubation + IAA	- IAA
Pectin	1,370	1,070	800
Protopectin	17,500	15,800	19,700
Pectic acid	483	395	650
Polyuronide hemicelluloses	5,270	4,170	6,750
Non-cellulosic polysaccharides	1,690	2,190	1,170
Cellulose	1,900	1,910	2,420

Table 27

Incorporation of label from methionine C-14 into cell wall fractions. 4 hour incubation with 0.00133 M methionine C-14, ± IAA. Initial activity 450,000 cpm/treatment.

Cell wall fraction	cpm/10 mg. dry cell wall + IAA	- IAA
Pectin	1,960	1,410
Protopectin	7,100	6,740
Pectic acid	650	227
Polyuronide hemicelluloses	507	701
Non-cellulosic polysaccharides	160	155
Cellulose	45	40

The time course of incorporation of saponifiable methyl carbon into pectin and protopectin is given in Figure 6. The data show little effect of IAA upon the amount of methionine absorbed. This situation is in marked contrast to that reported by Reinhold and Powell (72). These authors found that IAA increases glycine and glutamic acid absorption by sunflower hypocotyl sections 40 percent or more. The presence of IAA does, however, cause an increase in rate of incorporation of methionine methyl carbon. There is an initial lag in incorporation related perhaps to the time required to equilibrate the tissue with substrate. The effect of auxin in increasing rate of incorporation into pectin ester is, however, clearly apparent within 15-30 minutes after the initial lag. There is no appreciable effect of IAA upon incorporation into protopectin.

It is possible, however, that the increase in methylation of pectin is a result of growth and not a response to the added auxin. To determine this, the experiment was repeated in the presence of 0.3 M mannitol. The data given in Table 28 show that the increase in methylation of pectin occurs in the absence of expansion and thus must be a response to the added auxin.

The rate of turnover of methyl groups has been determined by means of a procedure identical with that used in the case of glucose. Sections were incubated for 17 hours in cold methionine after a three hour incubation in C-14 methionine. The data shown in Table 29 indicate that only

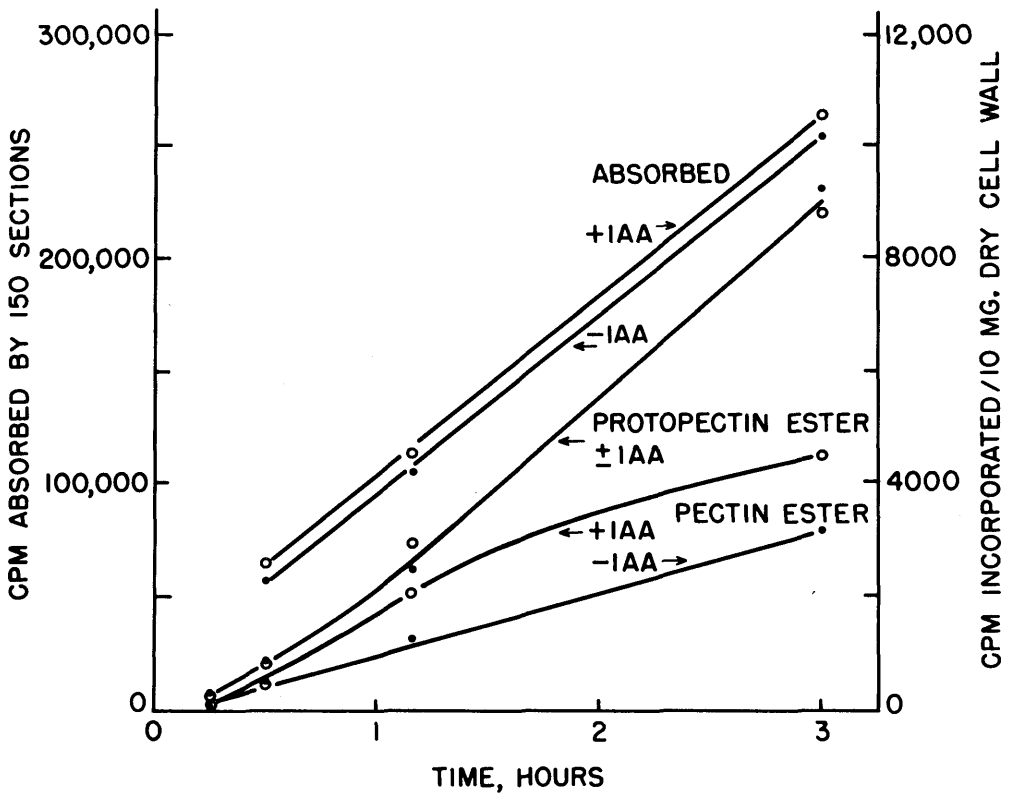

Figure 6. Effect of IAA on absorption and incorporation into esters of cell wall pectic substances of C-14 derived from methionine as a function of time. Initial activity 525,000 cpm/treatment.

Table 28

Incorporation of label from methionine C-14 into cell wall constituents in the presence of 0.3 M mannitol. One hour pretreatment, 0.3 M mannitol, - IAA, 3 hour incubation, 0.00133 M methionine C-14, 0.3 M mannitol, ± IAA. Initial activity 300,000 cpm/treatment.

Cell wall fractions	cpm/10 mg. dry cell wall + IAA	- IAA
Pectin	837	558
Protopectin	3,310	4,350
Pectic acid	85	106
Polyuronide hemicelluloses	518	681
Non-cellulosic polysaccharides	23	58

Table 29

Turnover of methyl groups in the cell wall fractions as influenced by the presence or absence of IAA. Sections pretreated with methionine C-14, initial activity 400,000 cpm/treatment, three hours. Sections then incubated 17 hours, 0.04 M unlabeled methionine, ± IAA.

Cell wall fractions	cpm/10 mg. dry cell wall After 3 hr. pretreatment	After 17 hr. incubation + IAA	- IAA
Pectin	940	938	663
Protopectin	6,430	7,530	7,330
Pectic acid	378	573	358
Polyuronide hemicelluloses	992	1,410	697
Non-cellulosic polysaccharides	400	365	230
Cellulose	26	28	15

in the pectin and polyuronide fractions does any considerable turnover occur. And, as was the case with glucose, the turnover is almost completely suppressed in the presence of auxin.

Inhibition of the Auxin-induced Increase in Pectin Methyl Ester

If the increase in methylation of pectin in response to added auxin is actually an integral part of the process of cell wall loosening, it should be possible to inhibit the increase in methylation with the same agents which inhibit growth. Therefore various growth inhibiting agents were tested for their ability to inhibit the methylation of pectin.

While growth is promoted by low concentrations of auxin, the presence of excess auxin results in inhibition of growth. Sections were therefore incubated with methionine in the presence of both 5 mg./l. IAA, which increases growth, and 500 mg./l. IAA, which inhibits growth. 500 mg./l. IAA inhibits the incorporation of label into pectin to a level below that found for the non-auxin treated control (Table 30). Thus both growth and methyl incorporation are inhibited by high concentrations of auxin.

In Part I it was shown that no residual auxin effect could be detected if the auxin pretreatment was conducted under conditions in which the tissues were plasmolyzed. The

Table 30

Incorporation of C-14 from methionine C-14 into cell wall constituents as affected by growth-promoting and growth-inhibiting concentrations of IAA. 3 hour incubation, initial activity of methionine C-14 130,000 cpm/treatment, ± IAA.

Cell wall fractions	cpm/10 mg. dry cell wall IAA concentration - mg./l. 0	5	500
Pectin	599	880	305
Protopectin	10,986	7,705	933
Pectin methyl ester	449	748	134
Pectin non-ester	150	132	171

Table 31

Incorporation of C-14 from methionine C-14 into cell wall constituents in the presence of 0.6 M mannitol. One hour pretreatment, 0.6 M mannitol, - IAA. Three hour incubation, initial activity methionine C-14 300,000 cpm/treatment, ± IAA

Cell wall fractions	cpm/10 mg. dry cell wall + IAA	- IAA
Pectin ester	491	538
Protopectin ester	975	1,480

effect of plasmolysis upon the auxin-induced increase in pectin methyl ester was determined by incubating sections with methionine in the presence of 0.6 M mannitol. Under these conditions, there is a marked decrease in amount of incorporation into both pectin and protopectin ester (Table 31). There is no increase in incorporation in the presence of auxin.

It has been shown that cell wall loosening is inhibited by both DNP and by anaerobic conditions. These inhibitors were then tested for their effect upon methyl incorporation. The data of Table 32 show that these inhibitors inhibit both the endogenous and the auxin-induced increase in incorporation into pectin methyl ester. The sections were pretreated with 2.5×10^{-5} M DNP or argon for one hour, after which time radioactive methionine was added and incorporation continued for an additional three hours. The data show that DNP reduces incorporation (corrected to equal methionine absorption) by over 90 percent and completely erases the auxin-induced increase. Argon treatment represses incorporation into pectin ester as much as does DNP but inhibits incorporation into the protopectin ester to an even greater extent. In fact, in the argon treated sections, contrary to the usual situation, there is less incorporation into the protopectin ester than into the pectin ester. The absorption of methionine is inhibited by about 70 percent by the DNP treatment and by about 60 percent by argon treatment.

Table 32

Incorporation of C-14 from methionine C-14 into esters of cell wall pectic substances as affected by 2.5×10^{-5} M DNP and argon. 3 hour incubation, initial activity methionine C-14 300,000 cpm/treatment.

Treatment		cpm/10 mg. dry cell wall/100,000 cpm absorbed Pectin ester	Protopectin ester
Control	+ IAA	3,470	8,550
	- IAA	2,121	6,250
DNP 2.5×10^{-5} M	+ IAA	225	493
	- IAA	249	535
Argon	+ IAA	215	138
	- IAA	206	107

The possible relation between methyl ester content of the cell wall and growth was originally suggested by the fact that growth is inhibited by ethionine. If the loosening of the cell wall is indeed related to this increase in the methyl ester content of the pectin, and if ethionine inhibits transmethylation, then ethionine should inhibit the auxin-induced increase in methyl ester incorporation. Such incorporation was determined in the presence of 0.05 M ethionine, a concentration sufficient to almost completely inhibit growth. The data of Table 33 show that the ethionine not only causes a decrease in the total incorporation of label into the pectin, but, of greater importance, eliminates the auxin-induced increase in the methylation of pectin.

It is clear that there is a substantial rate of methyl carbon incorporation into pectin in the absence of added IAA and that IAA merely increases this basal rate. The following experiment was done to find out to what extent the basal rate might be due to endogenous auxin. For this purpose sections were incubated with the antiauxin 2,4,6-T in the presence of C-14 labeled methionine. The data of Table 34 show that while the antiauxin causes a decrease in absorption of methionine from the medium, the incorporation of methyl ester groups is unaffected by the presence of antiauxin when corrected for equal uptake of methionine. The basal rate is apparently not auxin controlled and in this is similar to the endogenous (no added IAA) rate of section elongation which is also not inhibited by 2,4,6-T.

Table 33

Incorporation of C-14 from methionine C-14 into cell wall pectin in the presence and absence of 0.05 M ethionine. 30 min. pretreatment, ± 0.05 M ethionine, - IAA. 3 hour incubation, initial activity of methionine C-14 500,000 cpm/treatment, ± 0.05 M ethionine, ± IAA.

Treatment		cpm/10 mg. dry cell wall Pectin	
Control	+ IAA	3,064	+ 1,147
	- IAA	1,917	
Ethionine, 0.05 M	+ IAA	867	- 7
	- IAA	874	

Table 34

Incorporation of label from methionine C-14 into esters of cell wall pectic substances in the presence or absence of 2,4,6-T. One hour pretreatment, ± 10^{-4} M 2,4,6-T, - IAA. 3 hour incubation, ± 10^{-4} M 2,4,6-T, - IAA, initial activity methionine C-14 500,000 cpm/treatment. Data for esters corrected for equal uptake of methionine C-14.

	cpm/10 mg. dry cell wall 2,4,6-T concentration 0	10^{-4} M
Methionine uptake	244,000 cpm	153,000 cpm
Pectin ester	2,520	2,440
Protopectin ester	10,400	6,950

The Effect of Calcium Pretreatment on Incorporation

The presence of calcium ions causes an inhibition of auxin-induced growth. It has been shown that at least two effects are involved (43), and these two effects may be separated by the residual auxin effect experiment described in Part I. On the one hand, the presence of calcium ions inhibits the actual act of elongation. In this function calcium ions cause the cell wall to possess more resistance to elongation. On the other hand, calcium ions act to depress the cell wall loosening effect of auxin in non-growing tissues. Thus calcium ions appear to inhibit the direct cell wall loosening process. Experiments were therefore carried out to determine the effect of calcium ions on the transfer of methionine methyl to pectin.

The experiment was first done by incubating Avena coleoptile sections for two hours in 0.01 M $CaCl_2$ solutions and then transfering them to solutions containing methionine either with or without IAA. After three hours, the sections were ground up, the cell walls filtered off, and the whole cell wall counted without extraction. The results shown in Table 35 indicate that pretreatment with calcium results in an increase in the methylation of the non-auxin treated tissues. Auxin, however, causes a distinct decrease in the incorporation. In order to determine in what fraction of the cell wall this auxin-induced decrease principally occurs, the experiment was repeated and the fractions were separated.

Table 35

Incorporation of label from methionine C-14 into whole cell walls after pretreatment with calcium ions. One hour pretreatment, 0.01 M $CaCl_2$, - IAA. Three hour incubation, - $CaCl_2$, ± IAA, + methionine C-14.

$CaCl_2$ concentration	cpm/mg. dry cell wall + IAA	- IAA
0	524	-
0.01 M	436	700

Table 36

Incorporation of label from methionine C-14 into cell wall pectic substances after pretreatment with calcium ions. Pretreatment, one hour, 0.01 M $CaCl_2$. Three hour incubation, - $CaCl_2$, ± IAA, + methionine C-14.

Cell wall fraction	cpm/10 mg. dry cell wall - $CaCl_2$ - IAA	+ $CaCl_2$ + IAA	+ $CaCl_2$ - IAA
Pectin	4,240	1,203	5,670
Protopectin	7,511	7,255	10,323
Total	9,751	8,458	15,993

The principal inhibition occurs in the incorporation of label into the pectin fraction (Table 36).

Thus pretreatment with calcium results in a complete reversal of the effect of auxin upon incorporation of methyl ester into pectin; namely, auxin causes a repression of incorporation in this case.

Possible Intermediates in the Transmethylation Process

Before work is done to obtain an in vitro system which will perform the transfer of methionine methyl groups to pectic acid, it would be helpful to gain some information about whether there may be intermediates in the process.

To determine whether a particular compound might be such an intermediate, use was made of the isotope dilution technique. Sections were incubated with labeled methionine either in the presence or absence of the suspected intermediate given as the unlabeled compound. Sections were then ground, the cell walls filtered off, and the whole cell wall counted. If the compound is actually an intermediate, it will cause a decrease in the amount of label incorporated into the pectin. Since the compound may also influence growth, the effect of these compounds on growth has also been determined. The results of such studies are shown in Table 37.

The methyl group of methionine ultimately becomes a methyl alcohol esterified to the pectic acid. Free methanol might be suspected as a possible intermediate. Methanol,

Table 37

Incorporation of label from methionine C-14 into whole cell walls as affected by the presence of various unlabeled substrates. Pretreatment, one hour, + substrate, - methionine C-14. Incubation, 3 hours, + methionine C-14, + substrate, + IAA.

Substrate	cpm/mg. control	cpm/mg. + substrate	% of cont.	% growth
Methanol, 1%	2,281	2,089	92	95
Choline, 0.01 M	430	428	100	100
Betaine, 0.01 M	430	496	100	95
DL-homocysteine, 0.01 M	472	26	5	0
DL-homocysteine, $5x10^{-3}$ M	494	50	10	0
DL-homocysteine, 10^{-3} M	494	266	54	22
DL-homocysteine, $5x10^{-4}$ M	494	387	78	90
DL-homocysteine, 0.01 M + 0.01 M choline.	430	47	11	-
+ 0.01 M betaine	430	28	6	0
CH_2O, $5x10^{-3}$ M	472	126	27	18

however, has no effect upon the methylation process, and thus it is unlikely that the process ever involves free methanol.

Choline in animals (73) and betaine in both plants (74) and animals (75) can serve as a precursor of methyl groups for transmethylation. These compounds were tried to ascertain their effect upon the present system. Neither compound produces any effect upon the C-14 incorporation.

The formation of methionine in animal systems requires both a methyl donor such as betaine and homocysteine (73). DL-homocysteine was tried in this system both in the presence and absence of choline and betaine. 0.01 M DL-homocysteine almost completely inhibited both growth and incorporation of label into the cell wall. Neither choline nor betaine had any visible effect in the presence of homocysteine. When a series of concentrations of homocysteine was used, the percent inhibition of growth and methylation was quite similar. The inhibitions may be due to the trapping of methyl groups from L-methionine in the form of D-methionine, arising from methylation of D-homocysteine.

5×10^{-3} M formaldehyde was also tried. This concentration depresses incorporation to about 20 percent of the control, but growth is also suppressed by an approximately equal amount. It is quite possible that the inhibition of methyl incorporation in this case is due to an inhibition of some other process which in turn causes inhibition of both the

methylation process and growth, and that formaldehyde itself is not an intermediate.

DISCUSSION

The studies of the physiology of auxin-induced expansion described in Part I have indicated that the direct result of auxin action is a loosening of the cell wall. Some change in cell wall properties must take place, then, in the presence of auxin. To detect this change, the metabolism of C-14 labeled glucose and methionine has been investigated in the presence and absence of auxin.

The label from uniformly labeled glucose C-14 is incorporated into all fractions of the cell wall. In the presence of auxin, the amount of incorporation into the hot water soluble or pectin fraction is increased. This small increase, however, is entirely due to an increase in incorporation of label into the methyl ester groups, and incorporation of label into the galacturonic acid residues is not affected by the presence of auxin. It may be concluded, then, that at least over short time periods auxin exerts little effect on net synthesis of pectin and that the effect of auxin on pectin esterification is the more important reaction.

When sections elongate in optimal osmotic concentration the presence of auxin induces an increase in cellulose synthesis as has been shown by Boroughs and Bonner (57)

with sucrose as substrate and by Ordin and Bonner (76) with galactose as substrate. Under the present conditions, however, no increase in cellulose synthesis in the presence of auxin can be detected. This confirms the conclusion that intussusception of new cellulose does not play a significant role in the initiation of auxin-induced cell wall loosening. Increases in cellulose synthesis accompany normal growth, but this would appear to be a response to growth rather than to auxin.

Inhibition of the residual auxin effect by ethionine has suggested that the process of transmethylation may be intimately connected with the action of auxin. The formation of methyl ester groups from methionine has been shown to occur in radishes by Sato (67). The formation of methyl ester groups in Avena coleoptile section cell walls was therefore studied using $C^{14}H_3$-methionine as the source of the methyl groups.

The label is incorporated in significant amounts into the pectin, protopectin, pectic acid, and polyuronide hemicellulose fractions. The presence of auxin has no effect upon the incorporation of label into any fraction except the pectin fraction. The pectin, however, incorporates 25-50 percent more label in the presence of auxin than in its absence. It has been shown that this is not due to any difference in uptake in methionine from the external solution. The increased methylation is also obtained in the presence of 0.3 M mannitol. Thus the increase is not a

result of expansion but is a result of the applied auxin.

A certain amount of methylation occurs in the tissue in the absence of auxin and even in the absence of expansion. There is non-auxin-dependent synthesis of cell wall materials as is shown by the incorporation of glucose C-14 into walls even in the absence of expansion. The non-auxin-induced methyl esterification undoubtedly accompanies this synthesis.

As was the case with glucose C-14, the incorporated label can be fractionated into a saponifiable and a non-saponifiable fraction. The saponifiable fraction, comprising from 60 to 85 percent of the label, has been indirectly shown to be in the form of esterified methyl alcohol. The increase in incorporation due to the presence of auxin is found almost entirely in this methyl ester fraction.

Nance (77) has shown that IAA accelerates incorporation of acetate-1-C^{14} into the pectic substances of the pea stem and that part of this increase is due to incorporation of acetyl groups. Acetyl groups may function in pea stems as do methyl groups in the Avena coleoptile.

Auxin, then, causes an increase in the incorporation of methyl groups from methionine into pectin. This phenomenon may be a part of the cell wall loosening process or it may simply be an extraneous process which happens to also be auxin-dependent. Some information about this can be obtained by examining the effects of various agents which abolish the residual auxin effect. If the two processes are

identical, these agents should also abolish the increase in methylation.

Excess auxin results in an inhibition of growth. Incorporation of label into both pectin and protopectin is decreased in the presence of auxin at growth-inhibiting concentrations to a level below that obtained with non-auxin treated controls.

Addition of the antiauxin 2,4,6-trichlorophenoxyacetic acid, which has no effect upon the incorporation of label into pectin methyl ester, also markedly decreases incorporation into protopectin. It might be considered that the incorporation of label into protopectin follows a classical auxin concentration curve. Antiauxin, in the presence of added auxin, results in a decrease in the effective concentration of the auxin. Although the addition of 2,4,6-T does not inhibit the endogenous growth of sections, it might be considered that the 2,4,6-T is actually lowering the effective concentration of residual auxin in the tissue until it is below that found in non-treated sections. At this lowered auxin concentration incorporation into protopectin is repressed. The normal level of auxin in the tissue is apparently the optimum auxin concentration for incorporation of methyl groups into protopectin. The maximum is broad enough so that addition of low levels of auxin has no appreciable effect. Increased auxin concentrations result in a decrease in incorporation. Thus the typical bell-shaped auxin concentration curve is obtained.

A residual effect is not induced by auxin in the presence of either anaerobic conditions or DNP. These same agents cause a decrease in incorporation of labeled methyl groups into the pectic substances. As would be expected, these conditions inhibit absorption of the substrate, but when the data are calculated on the basis of amount absorbed, it becomes apparent that both auxin and non-auxin controlled incorporation of methionine methyl carbon are much more sensitive to metabolic conditions than is methionine absorption. Auxin, moreover, has no effect upon amount of incorporation in the presence of anaerobic conditions or of DNP.

No residual cell wall loosening occurs when auxin is administered to sections which are in a state of plasmolysis. Under these conditions, incorporation of labeled methyl groups into pectin is greatly decreased.

It has been surmised from the results of Part I that the inhibition of growth by ethionine is due to an inhibition of transmethylation. If this is indeed correct, and if the loosening of the cell wall is related to the increase in methyl ester content of the pectin, then ethionine should inhibit the auxin-induced increase in methyl ester incorporation. This it was found to do.

Thus all of the agents which cause an inhibition of the residual loosening of the cell wall also result in the abolition of the auxin-induced increase in pectin methyl

ester. This is not direct proof that this process is actually involved in the process of cell wall loosening. No other cell wall property, however, has been found which is auxin-sensitive. Thus it appears reasonable to suggest that this increase in pectin methyl ester is, indeed, related to the cell wall loosening.

How, then, might such loosening occur? The loosening might be due to a conversion of water insoluble protopectin to the more soluble pectin or it might simply involve making the pectin less rigid without affecting the protopectin.

A breakdown of protopectin to pectin appears to be unlikely. If such an auxin-mediated process does occur, there should be a significant increase in incorporation of label of glucose C-14 into the galacturonic acid of pectin as a result of breakdown of labeled protopectin. No such increase is found.

That pectin may possibly be a precursor of protopectin in the synthetic process (with no direct relation to the auxin mechanism) is suggested by progress curves of incorporation in which pectin is as highly labeled as is protopectin in the early periods. Only over longer times does protopectin become the more labeled. Since protopectin constitutes most of the cold water insoluble pectic substances of the cell wall, it may behave as a sink for pectin. When glucose is used as the substrate, a similar relation between pectin and protopectin is apparent over short time periods. Thus conversion of small pectic units to larger

ones appears to be possible. Alternatively, of course, it is possible that pectin is first labeled and that protopectin is labeled later merely because of differential reaction rates.

The rigidity of pectin is due, in large amount, to the presence of polyvalent cations such as calcium. Both auxin and non-auxin-induced growth are inhibited by calcium. Bennet-Clark (78) has found that chelating agents which sequester such ions increase endogenous expansion of Avena coleoptile sections. The suggestion has been made (79), therefore, that methyl esterification may be the method by which polyvalent cation bridges between adjacent carboxyl groups of cell wall pectin are broken under the influence of auxin giving rise to increased cell wall plasticity. Calcium bridges between pectin chains are undoubtedly those of greatest importance but there is a possibility that pectin may be cross linked and that auxin may split anhydride bridges, these bridges being of the nature suggested by Kertesz (70). The bond splitting may either require methyl esterification as a primary part of the reaction or may require it to stabilize the split once made.

All the evidence presented in Part II is in agreement with this hypothesis except the experiments involving calcium pretreatments. It should be possible to increase the number of calcium bridges by pretreatment of the tissue with calcium. Then on incubation of the tissue with methionine in the absence of calcium and in the presence or absence of

auxin, there should be a greater increase in the amount of methylation over the non-auxin treated controls than occurs with non-calcium pretreated sections. On the contrary, after pretreatment with calcium, auxin caused a depression in the uptake of label into the cell wall. This decrease is almost entirely located in the pectin fraction. A not unexpected increase in label in the non-auxin treated tissues was found. The calcium appears to protect sites which would normally undergo methylation. Transfer of methionine methyl to the carboxyl group of pectin is apparently inhibited if the carboxylate ion is already bound as the calcium salt. Removal of the calcium by return of the tissues to a non-calcium-containing medium unmasks these extra sites for methylation.

Even though auxin increases the apparent rate of pectin esterification, it does not necessarily follow that transmethylation is the auxin-influenced process. Recent work by Glasziou (65) suggests that, indeed, it is not. He solubilized the enzyme pectin methylesterase (PME) from tobacco pith tissue. Upon addition of the soluble PME to washed cell walls, some of the PME was adsorbed onto the walls. Addition of auxin to the medium resulted in an increase in the amount of enzyme adsorbed. Glasziou suggests that auxin acts by removing PME from the cell. If the bound PME can no longer demethylate pectin, such a removal would result in an apparent increase in incorporation of label into pectin since less of the incorporated

label would be removed by the PME. Studies on the decorporation of label from pectin show that auxin depresses the decorporation as would be expected on the basis of this theory. The actual loosening of the wall, once again, would be a function of the number of cross bridges broken by methyl groups.

In this system, the actual incorporation of methyl groups would be non-auxin-dependent. Ethionine, then, should cause an equal depression in incorporation of methyl groups in the presence and absence of auxin. Auxin would still cause a decrease in PME activity and thus a difference in the decorporation of label. Thus ethionine should result in a decrease in amount of label found in the pectin but the increase in label due to the presence of auxin should still persist. This was not found to be true.

The present evidence, then, is not completely compatible with any one of the theories. The results do suggest, however, that breakage of cross links between pectin chains by methylation of the carboxyl groups involved may be a cause of cell wall loosening. Auxin may either be affecting the methylation reaction itself or may be preventing demethylation from occurring. At the present time, the former possibility seems most likely.

It would appear that the most profitable line of further inquiry would involve attempts to isolate the transmethylation enzymes from homogenates. The actual auxin dependence of the system can then be more exactly determined. The beginnings of such an effort are described in Part III.

PART III

THE OCCURRENCE OF TRANSMETHYLATION IN HOMOGENATES

INTRODUCTION

Incubation of intact Avena coleoptile tissues with methionine results in the formation of methyl ester groups of the cell wall pectin from the methyl carbon of methionine. This transfer of methyl carbon proceeds in the absence of added auxin but its rate is considerably enhanced by the addition of auxin. It is proposed that the nature of this reaction should be studied in an in vitro system for two reasons.

The purpose of the investigation as a whole has been to determine the nature of the specific reaction which is sensitive to auxin. Since it appears that auxin causes an increase in rate of pectin methyl esterification, detection and isolation of the actual enzyme systems involved in this reaction may permit a determination of the exact process which is auxin sensitive. Secondly, even if the actual transmethylation process is completely non-auxin-sensitive, it appears worthwhile to determine the nature of the reaction and to attempt to isolate the enzymes involved. This transmethylation results in a transfer of the methyl carbon to a carboxyl group. No analogous reaction is known.

Transmethylation, the transfer of intact methyl groups, was first demonstrated in animals by Du Vigneaud et al. (80).

The transfer of methyl groups from choline (80), betaine (75), and thetins (81) to homocysteine to form methionine has been conclusively demonstrated in animals, but not, as yet, in plants. The methyl group of methionine has been shown to be utilized to form the N-methyl groups of choline, betaine, creatine in animals (75), and the N-methyl groups of choline (82), nicotine (83), hordenine (84), and ricinine (85) in plants. The transfer of the methyl group to form methoxyl groups has been demonstrated for lignins (86) and ricinine (84). The formation of methyl ester groups from methionine was first demonstrated by Sato (67). He found that the methyl group of $C^{14}H_3$-methionine is incorporated almost entirely into the methyl ester groups of radish cell wall pectin. When C^{14}deuterio-methionine was used, there was equal incorporation of the C-14 and deuterium labels, indicating that the methyl group is transferred intact. This is then a true transmethylation.

The transmethylation from methionine to pectin is the reaction which is to be studied. Deuterium labeled methionine has not been used with Avena coleoptiles to prove that the transfer is actually a transmethylation since the work of Sato (67) has already shown that this is probable.

The chemical nature of the transmethylation reaction has been studied in detail only in animals. Cantoni and coworkers (87) have shown that the formation of creatine in liver homogenates involves two distinct enzymatic reactions. The first step is the formation of an "active

methionine" in the presence of ATP, magnesium ions, and glutathione. Active methionine then donates its methyl group to guanidinoacetic acid in the presence of a second enzyme. There is no evidence, however, to suggest whether all transmethylations are mediated by the same series of reactions.

It is proposed, then, that attempts be made to obtain the methylation of cell wall pectin in Avena coleoptile homogenates. Once this has been achieved, the sensitivity of the system to auxin can be determined and the fractionation of the system into the various enzymes involved can be attempted.

MATERIALS AND METHODS

The experimental material consisted of both Avena coleoptile and pea stem sections. The Avena coleoptile sections were prepared as described in Parts I and II. The pea stem sections were obtained by selecting and cutting the youngest internode from five day old pea seedlings, grown under the same conditions used for Avenas.

Experiments using intact tissues were conducted in the manner detailed in Part II.

Experiments involving homogenates were performed as follows. The freshly cut sections were placed in an Omni-mixer with 2.5 ml. cold 0.1 M THAM buffer, pH 7.0, per gram fresh weight pea sections or per 50 coleoptile sections. The sections were then blended for two minutes in the cold.

The homogenate was transferred to centrifuge tubes and centrifuged for 15 minutes at 15,000 g. The supernatant was discarded and the pellet resuspended in 1 ml. THAM per gram fresh weight for peas or 1 ml. buffer per 40 coleoptile sections. Three ml. of the slurry were then placed in a 10 ml. erlenmeyer flask which contained methionine C-14, initial activity 500,000 cpm, in 2 ml. 0.1 M THAM buffer. The specific activity of the labeled methionine was 360×10^6 cpm/mM. The flasks were incubated for three hours at 30°C. with constant shaking. The flasks were then removed from the shaker, brought to a boil on a hot plate to inactivate enzymatic activity and then cooled. Since various methods of preparation of the pectin were used, these will be outlined in the next section.

In the experiments of Tables 44 and 45, the homogenates were prepared by either grinding the sections in a morter or by blending in the Omni-mixer with 4 ml. buffer per gram fresh weight of tissue. The homogenates were then used without removal of the supernatant by centrifugation. Work by Sato has indicated that incorporation is slightly enhanced in preparations made by this latter method.

The experiments of Tables 40 and 41 were done in cooperation with Dr. Sato.

RESULTS

The Effect of Washing Procedures on the Isolation of Pectin

Classical pectin is a completely water soluble compound. It goes into solution rather slowly in cold water but much more rapidly in hot water. Since the cell wall samples are washed on a funnel several times with cold water before extraction with hot water, the question arises as to how much of the pectin might have been solubilized by the washings. Pectin is usually thought of as being insoluble in 70 percent ethanol; and sections, after incubation in methionine C-14, were therefore ground in alcohol and then washed with ethanol before extraction with hot water. Under these conditions, the amount of label recovered in the pectin fraction is greatly increased (Table 38). The increase in label due to the addition of auxin is still present under these conditions (Table 39). Thus it appears that much of the pectin is lost by washing of the samples with cold water prior to extraction.

If washing with water can remove so much pectin from the sample, incubation of a homogenate for three hours should result in solubilization of most of the cell wall pectin. Separation of the pectin from the unused methionine is then important. Use of ethanol to precipitate the pectin might overcome this problem. 2 ml. aliquots of an incubation mixture were therefore placed in two centriguge tubes. Ethanol was added to one and water to the other sufficient

Table 38

Effect of washing sample with water or alcohol upon amount of label in pectin fractions from Avena coleoptile cell walls. Incubation 3 hours, methionine C-14, - IAA

Condition of tissues	cpm in pectin/10 mg. dry cell wall Walls washed with ethanol	water
Intact	6,550	1,819
Homogenate	742	174

Table 39

Incorporation of label from methionine C-14 into Avena cell wall pectin in the presence or absence of IAA. 3 hour incubation, 0.00133 M methionine C-14, ± IAA. Sections ground and washed with 70% ethanol.

Fraction	cpm/10 mg. dry cell wall + IAA	- IAA
Pectin	11,030	9,000

to bring the volumes of each to 10 ml. The samples were then stirred and centrifuged. The supernatant was removed and its activity determined. The washing procedure was repeated three more times. The samples were then extracted as usual and their ester contents determined. The data for pea sections, shown in Table 40, indicate that use of alcohol for washing results in an increased recovery of the label.

The effect of varying the number of washings with ethanol was then determined. The procedure used for washing the samples was as above. It is apparent that although pectin is not very soluble in ethanol, it is removed by washing with ethanol (Table 41). Thus it does not appear that it is possible to wash the wall free of impurities with ethanol without extensive removal of the pectin.

Pectin is partially soluble in ethanol; it should be less soluble in longer chain alcohols. Butanol, therefore, might be a more suitable washing agent for separation of pectin from methionine C-14. To test this possibility, 0.2 ml. of methionine C-14 stock solution, containing 500,000 cpm, was added to 15 ml. of 2 percent pectin solution. To a 5 ml. aliquot, 15 ml. of butanol was added, the contents of the tube mixed, and the butanol layer removed and discarded. Since water is partially soluble in butanol, after two such washings, all of the water had been dissolved in the butanol layer, leaving a precipitate of pectin and methionine C-14. One ml. of water was then added

Table 40

Removal of radioactivity from pea stem homogenate by washing with water or alcohol. Label in each wash and in pectin fraction after four washes. Washing procedure given in text. Incubation, 3 hours, methionine C-14, - IAA, at 30°C.

Wash number	Wash with - 70% ethanol	water
1	520,680 cpm	478,920 cpm
2	52,884	51,960
3	5,940	4,668
4	118	774
Pectin, total	1,512	371
ester	1,050	152

Table 41

Effect of number of washings with 70% ethanol upon label in pectin fraction from pea stem homogenate. Incubation, 3 hours, methionine C-14, - IAA, 30°C.

Wash number	cpm/removed per wash	
1	554,750	539,800
2	61,340	73,900
3	10,480	11,100
4	1,730	1,840
5	310	37
6	0	not done
Pectin, total	412	1,000
ester	209	638

and the washings were continued. After five washings the water layer was finally removed, plated out, and counted. There were still 12,200 counts left in the water layer at the end of five washings. Butanol failed to remove all the methionine. Determination of the partition coefficients of methionine between butanol and water indicate the reason. A partition of 5:1 between butanol and water is found. Determination of the partition coefficient for pectin suggests that it is about 1:10. Thus washing of the sample with butanol is not feasible since the solubility of the methionine in butanol is not sufficiently great to assure its removal without loss of pectin.

How many washings should be utilized in preparation of the sample if washing is indeed to be used at all? The incorporation of methionine C-14 into intact tissue and homogenates of Avena coleoptiles and pea stems has been determined using 12 washings as an arbitrary number. The intact tissues were ground in ethanol and washed 12 times with 3 ml. of ethanol. The homogenates were washed 12 times with 3 ml. of ethanol using the procedure outlined above. The results are shown in Table 42. Use of homogenates decreases the incorporation into the hot water soluble fraction to one-tenth the value for intact Avena coleoptiles. Incorporation into the methyl ester is decreased even more. Incorporation into intact pea sections is considerably less than with intact Avena sections on a 10 mg. dry cell wall basis, and incorporation in pea homo-

Table 42

Comparison of incorporation label into pectin of Avena coleoptile and pea stem sections, intact and homogenates. Incubation 3 hours, + methionine C-14, - IAA, 30°C.

Material	Pectin fraction	cpm/10 mg. dry cell wall intact	homogenate
Pea stem	Total	649	273
	Ester	449	63
	Non-ester	200	210
Avena coleoptile	Total	6,923	572
	Ester	4,568	213
	Non-ester	1,361	359

Table 43

Use of ion exchange columns to separate pectins from unused methionine C-14.

	Column	Source of label	cpm - initial	recovered in eluate
a	Dowex-50	Methionine C-14 stock	500,000	1,292
b	Dowex-50	Label recovered from a	1,169	873
c	Dowex-50	Avena homogenate	400,000	2,664
d	Dowex-50 and IR-4	Methionine C-14 stock	700,000	298
e	same	Avena incubate	260,000	270
f	Dowex-50	Avena incubate	455,000	6,905
g	IR-4	Label recovered from f	2,500	482

genates is lower than in Avena homogenates.

It is apparent that use of washing procedures to separate the unused methionine C-14 from the pectin has serious disadvantages. The amount of pectin recovered is too dependent on the amount of washing. It would therefore appear profitable to search for other methods of pectin separation.

The Use of Ion Exchange Resins to Isolate Pectin

Williams and Johnson (88) have reported that ion exchange resins can be used to remove ions from a solution without absorption of pectins. They used a double column which contained both a cation and an anion resin. Since methionine should be removed from a solution by a cation resin, Dowex-50 resin was first tried. The column was constructed in a glass tube, 1.2 cm. in diameter, fitted with a stopcock at the bottom. About 6 cm. of washed resin was packed into the tube on top of a glass wool plug. The column was thoroughly washed with water before use. Avena homogenates were prepared and incubated in the usual manner. At the end of the incubation, the flask was placed in a boiling water bath for one hour to extract the rest of the pectin. The mixture was then filtered on a sintered glass filter and the filtrate adjusted to pH 2 with HCl. Aliquots were next removed and placed on the column. The column was eluted with 50 ml. of water, fractions were collected, and aliquots plated out and counted. The data obtained from such experiments are given in Table 43.

When a solution which contains only the stock methionine C-14 is run through a Dowex-50 column, most of the radioactivity is removed but about 1,300 cpm per 500,000 cpm administered are passed through. If the material which passes through is then placed on a fresh column, most of the label can be recovered by elution of the column with water. If the Dowex-50 column is coupled with a column which contains IR-4, an anion resin, the number of counts which pass through is diminished to around 300 cpm per 500,000 cpm administered. Thus it appears that the methionine contains both an anionic and a neutral impurity.

When Avena homogeantes are placed on a Dowex-50 column, approximately twice as much label can be recovered as with methionine solution alone. When the double column is used, however, the number of counts recovered is depressed to the level of the methionine alone. That this is due to absorption of pectin on the IR-4 can be shown by placing on an IR-4 column the solution recovered from the Dowex-50 column. The IR-4 column removes almost all of the radioactivity.

Dowex-50, then, will separate the pectin from the methionine but the pectin is contaminated with some impurity found in the original methionine. Anion resins remove this impurity but also remove the pectin. Thus use of ion exchange resins has not proved feasible for isolation of pectin. The difference between these results and those of Williams and Johnson is probably due to their use of pectin

solutions of much higher concentration. Large amounts of pectin can probably be passed through anion resin columns where small amounts would only be trapped and lost.

Use of Dialysis to Isolate Pectins

Dialysis has been recommended by Fellenberg (89) as a suitable method for the separation of pectins from other components of a solution. To test the possibilities of this technique, a large batch of intact Avena coleoptile sections was incubated with methionine C-14. The sections were ground and washed with ethanol and the walls were extracted first with hot water and then with hot dilute acid in the usual manner. Three 3 ml. aliquots from each solution were placed in sacks of cellophane dialysis tubing and dialyzed against one liter of water at room temperature for 36 hours. Four changes of water were made. At the end of this time, the contents were removed from the sacks, made up to 3 ml. again, and 1 ml. aliquots were plated out and counted. The results are shown in Table 44. It can be seen that reproducibility is good. Dialysis of a stock methionine C-14 solution under identical conditions resulted in almost complete loss of the radioactivity from the solution. Dialysis of the pectin and protopectin fractions, however, resulted in a uniform amount of label being retained. Saponification of these dialyzed fractions caused loss of about 85 percent of the activity from the pectin fraction and 65 percent from the protopectin fraction. Dialysis

Table 44

Preparation of pectin and protopectin fractions from intact Avena coleoptiles by dialysis. Sections incubated 3 hours, 0.00133 M methionine C-14, - IAA. Ground and washed with 70% ethanol. Dialyzed 36 hours, room temp., against 1 liter water, four changes of water

Fraction	Cpm/treatment Before	After dialysis
Pectin	15,000	1,854 1,932 1,752
Protopectin	5,500	1,865 1,880 1,955
Methionine C-14 stock	113,000	214

Table 45

Preparation of pectin and protopectin fractions from Avena coleoptile homogenates by dialysis. Homogenate incubated 3 hours, 30°C., methionine C-14 initial activity 300,000 cpm per treatment. Dialysis 18 hours, room temp., against 1 liter water, four changes of water

Fraction	Cpm - before dialysis	after	Cpm per 10 mg. wall	% ester
Pectin	280,000	316	1050	18
Protopectin	593	319	1060	5

appears to be a suitable method for the separation of pectin from the unused methionine.

It can be seen in Table 44 that dialysis results, however, in a considerable loss of material. Small molecular weight pectins are probably lost while those pectins of a certain molecular weight or above are retained within the sack. Dialysis for only 18 hours, which still removes essentially all of the methionine C-14, considerably reduces the loss of label.

The experiment was then repeated with an Avena coleoptile homogenate. After incubation, the homogenate flask was placed in a boiling water bath for one hour to extract the pectin. The cell walls were removed by filtration on a sintered glass filter and extracted with dilute acid in the usual manner. The hot water and hot dilute acid solutions were then dialyzed as above, except that dialysis lasted only 18 hours, with four changes of water. While considerable label is present in both solutions after dialysis, the amount of saponifiable label is quite low (Table 45).

Differences in amount of activity retained are caused by slight differences in procedure. Thus if the solutions are dialyzed in the cold instead of at room temperature or if the cell walls are removed from the hot water extract by centrifugation rather than by filtration, an increase in retention of activity is achieved (Table 46). This is not attended by any increase in the percentage of methyl ester.

Table 46

Dialysis at room temp. vs. cold and removal of cell walls by centrifugation vs. filtration as affecting incorporation of label from methionine C-14 into Avena homogenate pectic substances. Conditions same as in Table 45.

Dialysis-cold or warm	Filtration or centrifugation	Cpm per treatment	% ester
Warm	Filtration	337	0
Cold	Filtration	505	23
Cold	Filtration	570	8
Cold	Centrifugation	998	14

Table 47

Effect of various treatments upon the activity in the pectin fraction of Avena coleoptile homogenates after dialysis.

Treatment	Cpm
After initial dialysis	3,500
After alkaline saponification	2,980
After precipitation of pectic acid	2,240
After redialysis	1,280
Counts in pectic acid	6
Count in TCA precipitate	11

Incorporation of label into the pectin of homogenates results in a decrease, as compared with intact tissues, in the percentage of label found in methyl ester. An effort has been made to learn something about the nature of this non-saponifiable label (Table 47). A pectin solution was selected which contained 3500 cpm after dialysis. Alkaline saponification decreased the activity by 15 percent, to about 2980 cpm. Unlabeled pectic acid was then added to the solution and precipitated with $CaCl_2$. The precipitated pectic acid was carefully washed, plated out and counted. It was unlabeled. The remaining solution now contained 2240 cpm. This solution was then dialyzed for 18 hours in the cold against water. At the end of this time, 1280 cpm remained. Treatment of the solution with 12 percent TCA gave a precipitate which was unlabeled. Thus the label has not been incorporated into proteins or into any portion of the pectic acid molecule other than the ester group.

DISCUSSION

Classical pectin is a completely water soluble compound. The procedure used in Part II involved grinding the intact Avena coleoptile tissues in water and washing them three times with water on a sintered glass filter before extraction with hot water. This washing with water might be expected to solubilize some of the pectin. By grinding and washing the cell walls with ethanol instead of water, it was shown that about three times as much label could be

recovered. Much pectin is lost, apparently, by the usual washing procedure. The auxin-induced increase in methylation is, however, obtained with both washing procedures.

If washing with water can remove so much pectin from the sample, incubation of a homogenate for three hours should result in solubilization of most of the cell wall pectin. The initial problem, then, is to separate, in some manner, the soluble pectin from the unreacted methionine C-14. Since pectin is generally considered to be insoluble in 70 percent ethanol, homogenates, after incubation, were made up to 70 percent in ethanol. After filtering off the cell walls, the walls were washed with ethanol. Some of the pectin, however, is actually solubilized by the ethanol. As a result, the amount of label remaining in the pectin fraction after washing is a direct function of the amount and duration of the washings. Reproducibility is difficult to achieve since small differences can easily occur due to unavoidable differences in the amount of washing. Butanol, in which pectin is less soluble than in ethanol, was tried in place of ethanol but was also unsuitable because of the relative insolubility of methionine in this solvent.

Further efforts have therefore been made to find some system for removing the methionine from the pectin without loss of a variable amount of the pectin. Williams and Johnson (88) have successfully used a combination cation-anion resin column to isolate pectins from impure solutions.

The use of ion exchange resin columns was therefore tried. It was found that the cation resin Dowex-50 removes the methionine C-14 but allows an unknown labeled impurity to pass through with the pectin causing excessive contamination. Allowing this eluate to then pass through an anion resin column results in removal of most of the impurity but also results in loss of the pectin on the column. Thus attempts to make use of ion exchange resin columns have so far proved unsatisfactory.

The possible use of dialysis to separate the methionine from the pectin has been investigated. Dialysis results in a loss of a large amount of the label from the pectin fraction. The amount of label which remains, however, does not vary between replicates. Thus, although much of the small molecular weight pectins are lost through the dialysis tubing, reproducibility is good since all pectins of a certain size or greater are apparently retained. Dialysis of the boiled homogenate for 18 hours in the cold against water appears to be a practical way of separating pectin from the unreacted methionine.

The incorporation of label from methionine C-14 into the pectin of intact tissues and homogenates of pea stem and Avena coleoptile sections has been compared. Ethanol washing before extraction with hot water was used as the means of isolation of pectin. Intact Avena coleoptile sections incorporate ten times as much label as do intact pea stem sections. About 65 percent of the label is

saponifiable in each case. Use of homogenates instead of intact tissues decreases incorporation into Avena coleoptile pectin by 90 percent. Only 35 percent of this label is in the methyl ester. Pea homogenates incorporate about one-half the activity of Avena coleoptile homogenates and the methyl ester content is decreased to about 20 percent. Homogenizing of the tissue results in a considerable decrease in incorporation of label into the pectin. With pea stems, this decrease is almost entirely in the methyl ester incorporation. When the pectin from Avena coleoptile homogenates is isolated by dialysis, it is found that some incorporation of label has occurred. Only a very small percentage of this label, however, is in the form of the methyl ester. The nature of the non-saponifiable label has not been determined but it does not seem to be located in proteins or in pectic acid.

At this time, then, a suitable system has been determined for obtaining reproducible fractions of pectin from homogenates. Incorporation into methyl ester is, at present, greatly decreased by homogenizing the tissue.

Future work will be directed towards attempting to increase the incorporation of label into methyl ester to a degree comparable to that found in intact tissues. The response of this system to auxin will then be examined. When these aims have been achieved, attempts can be made to fractionate the system into the actual enzyme systems involved in the transmethylation.

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