METHYL ESTERIFICATION OF CELL WALL CONSTITUENTS UNDER THE INFLUENCE OF AUXIN

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It has been shown in an earlier communication (11) that auxin increases the amount of incorporation of carbon-14 derived from methyl labeled methionine into a fraction of the cell wall of Avena coleoptile sections which possesses the properties classically associated with pectin. Incorporation into this material represents but a small part of the total, the main part being into the protopectin fraction. Such incorporation is not significantly affected by the presence of auxin. The auxin effect on incorporation into the pectin fraction persists in solutions which contain sufficient mannitol to prevent elongation during the course of the experiment. C14 from glucose was also found to be incorporated into pectin more rapidly under the influence of auxin. Bennet-Clark (1) and others have also suggested that auxin causes an increase in the methyl content of pectic substances of cell walls of elongating tissue. The hypothesis may be formulated that methylation of the carboxyl groups of adjacent pectin molecules under the influence of auxin may be involved in the splitting of anhydride or calcium bridges which contribute to the mechanical properties of the cell wall.

The present investigation was undertaken to determine to what extent general metabolic inhibitors interfere with the incorporation of methionine methyl into the pectin fraction and to answer definitively the question of whether methyl carbon incorporation accompanies only net pectin synthesis or whether such incorporation occurs at a more rapid rate than net synthesis and is therefore to be regarded as methyl ester turnover. It will also be shown below that esterification of pectin by methyl derived carbon is an auxin controlled reaction.

MATERIALS AND METHODS

The material for the experiments described below consisted of 5 mm sections cut 3 mm below the tip of Avena (var. Siegshafer) coleoptiles grown in the conventional manner (10). Primary leaves were removed from all sections. Incorporation of the carbon of methionine or glucose into the cell wall of sections was allowed to occur by incubating sections in various solutions containing these substrates. One hundred fifty sections floating in 3.5 ml of solution were used for each treatment of each experiment. Solutions were buffered with 0.0025 M potassium maleate, pH 4.8; indoleacetic acid (IAA) was used at a concentration of 5 mg/l where indicated. Aliquots of the medium were counted before and after incubation to determine the amount of substrate taken up by the tissue.

After incubation for 3 hours at 25°C, sections were rinsed, dried on filter paper and ground with cold water in a mortar. The ground debris was handled and analyzed as described earlier (11). The varied cell wall fractions are separated on the basis of their solubilities and do not necessarily represent specific chemical entities. The names classically ascribed to each fraction will however be used in this paper for convenience.

All planchets were counted on an automatic sample changer using a micromil window tube and Q gas. All counts are corrected to infinite thiness.

The counts incorporated are expressed on a 10-mg dry cell wall basis. The amount of radioactive substrate taken up by the tissue varied somewhat from experiment to experiment. A calibration experiment was therefore done in which the extent of incorporation into each tissue component was determined as a function of amount of labeled substrate supplied and taken up. The calibration curves so obtained have been used to convert all of the data to incorporation per unit absorption of substrate by 150 sections (equivalent to 8 to 9 mg dry weight of cell wall). The data are expressed as cpm incorporated per 80,000 cpm absorbed for glucose; per 100,000 cpm absorbed for methionine.

Saponification of the methyl ester groups of the pectic fractions was accomplished by treatment with 0.1 N NaOH or NH4OH at room temperature for one hour. No difference in the effectiveness of the two treatments was noted. Neither does it appear to be of importance whether the alkaline reaction mixture is neutralized before being taken to dryness for determination of radioactivity. As a check on the specificity of the alkaline saponification procedure, samples of the present pectin preparations were also demethylated by use of a highly purified pectin methyl esterase preparation. It was found that there is general agreement between the two methods so far as amount of radioactive material liberated by them in volatile form is concerned, although alkali always yielded somewhat more.

Incorporation of homogenates with labeled substrate followed by analysis indicated little contamination of cell wall fractions by adsorption or occlusion of substrate.

RESULTS

The time course of incorporation of saponifiable methyl carbon into pectin and protopectin is given in
figure 1. Unlike the remaining data this is expressed on a 10-mg dry wt basis without corrections for amount of substrate absorbed. The data show little effect of IAA upon amount of methionine absorbed. This situation is in marked contrast to that reported by Reinhold and Powell (14). These authors found that IAA increases glycine and glutamic acid absorption by sunflower hypocotyl sections by 40% 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 to 30 minutes of the initial lag. There is no appreciable effect of IAA upon incorporation into protopectin.

The radioactive pectin extracted from sections incubated with labeled methionine may be saponified for removal of the methyl ester groups. The residue from the saponification contains only 20 to 30% of the original radioactivity, 70 to 80% having been lost as a volatile material. The peptic acid liberated by the saponification may also be precipitated with alcohol as calcium pectate. The calcium pectate thus prepared was found to contain about 10% of the radioactivity present in the original pectin fraction. This behavior is in contrast to that of pectin prepared from sections incubated with glucose-C\(^14\). Such pectin loses only about 20% of its radioactivity on saponification and about 80% is recoverable in the purified calcium pectate. These observations confirm those reported earlier (11).

It was previously reported that auxin exerts an effect in increasing rate of methyl carbon incorporation into pectin even in sections prevented from elongating by incubation in 0.3 M mannitol solution. The data of figure 2 concern the rate of methyl carbon incorporation into pectin over the range of external osmotic concentration from very dilute buffer to just above incipient plasmolysis. It may be seen that increasing external osmotic pressure results in reduced incorporation into pectin esters over a 3-hour incubation. The absorption of methionine is not depressed by the increasing osmotic pressure. The effect of auxin on incorporation into pectin ester decreases slightly (percentagewise remains constant) as mannitol concentration increases, suggesting that a small part of the auxin induced incorporation may be an artifact of elongation. However, as Ordin, Applewhite and Bonner (10) have shown, elongation under these circumstances drops much more rapidly with increasing external concentration.

Although in the particular experiment of figure 2 the incorporation into protopectin ester is slightly less in the presence of auxin than in its absence, the data of figure 1 and of numerous other experiments indicate that there is no significant difference between the treatments.

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 auxin 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) in the presence of \(^{14}\)C-labeled methionine. The data of figure 3 show that absorption of methionine was decreased by the higher 2,4,6-T concentrations. The incorporation of methyl carbon into protopectin ester was decreased even more. Incor-

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**Fig. 1.** Effect of IAA on absorption by Avena coleoptile sections and incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C\(^4\)-methionine as a function of time. Potassium maleate pH 4.8, 0.0025 M, l-methionine, 0.00036 M, 5 mg L IAA where indicated. The average specific activity of solution was \(320 \times 10^6\) cpm/mM.

**Fig. 2A (left).** Effect of external osmotic concentration on absorption by Avena coleoptile sections of carbon-14 derived from methyl-C\(^4\)-methionine in presence and absence of IAA. Conditions same as those of figure 1 except for addition of mannitol.

**Fig. 2B (right).** Effect of external osmotic concentration on incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C\(^4\)-methionine in presence and absence of IAA. Conditions same as in figure 2A.
Fig. 3. Effect of various concentrations of 2,4,6-T on absorption by Avena coleoptile sections and incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C'<sup>14</sup>-methionine. Conditions same as those of figure 1 except for absence of IAA.

incorporation into pectin ester however was essentially unaffected by 2,4,6-T. The antiauxin has then no effect on basal rate of incorporation into pectin. 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.

It has been shown by Bonner (2, 3), Hackett and Thimmann (6, 7) and others that 2,4-dinitrophenol (DNP) and anaerobic conditions inhibit auxin-induced cell elongation. These inhibitors also inhibit both the continuing and the auxin-induced increase in methyl carbon incorporation into pectin. The data of table I concerning the effects of 2.5 x 10<sup>-5</sup> M DNP or of argon on incorporation of methyl carbon into pectin and pectin esters. The sections were pretreated with the inhibitors for one hour, after which time radioactive methionine was added and incubation continued for an additional 3 hours. The argon was supplied as described by Ordin, Appllewhite, and Bonner (11). Table I shows that DNP reduces incorporation (corrected to equal methionine absorption) by over 90% and completely erases the auxin-induced increase. Argon treatment represses incorporation into pectin ester as much as does DNP but inhibits incorporation into pectin ester to an even greater extent. In fact, in the argon treated sections, contrary to the usual situation, there is less incorporation into the pectin ester than into the pectin ester. The absorption of methionine is inhibited by about 70% by the DNP treatment and by about 60% by argon treatment.

Earlier experiments (5, 11) have shown that the carbon of glucose-C'4 is incorporated into cell wall constituents in the Avena coleoptile and that this incorporation is influenced by auxin. The data of table II show that glucose carbon is incorporated both into the saponifiable (ester) group of pectin and into the non-saponifiable residue, presumably into the polyuronide residues. That glucose can give rise to active one carbon units is known (15). The data of table II show in addition that the increased incorporation of glucose carbon into pectin in the presence of auxin, is due completely to the effect on incorporation into ester.

**Discussion**

It is known that certain polyvalent cations, particularly calcium, inhibit both endogenous (no added IAA) and IAA-induced growth. Bennet-Clark (1) has found in addition that chelating agents which sequester such ions increase endogenous elongation of Avena coleoptile sections. The suggestion may be made 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 (8). The exact compounds or enzymes to which IAA attaches itself to split such bonds are unknown. 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.

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**Table I**

<table>
<thead>
<tr>
<th>Cell wall fraction</th>
<th>Control</th>
<th>DNP **</th>
<th>Argon †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-IAA + IAA</td>
<td>-IAA + IAA</td>
<td>-IAA + IAA</td>
</tr>
<tr>
<td>Pectin</td>
<td>2020</td>
<td>2470</td>
<td>249</td>
</tr>
<tr>
<td>Protopectin</td>
<td>6250</td>
<td>8550</td>
<td>535</td>
</tr>
</tbody>
</table>

* Incubation, 3 hrs; methionine, 0.00036 M.
** Bubbled 1 hr before and during incubation.
† Bubbled 1 hr before and during incubation.
Average initial specific activity of culture solutions: 300 x 10<sup>6</sup> cpm/mM.

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**Table II**

<table>
<thead>
<tr>
<th>Cell wall fraction</th>
<th>-IAA</th>
<th>+IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpm/10 mg dry cell wall x 10,000 cpm absorbed by 150 sections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectin</td>
<td>575</td>
<td>624</td>
</tr>
<tr>
<td>Protopectin</td>
<td>13,300</td>
<td>11,050</td>
</tr>
<tr>
<td>Pectin ester</td>
<td>114</td>
<td>181</td>
</tr>
<tr>
<td>Pectin non-ester</td>
<td>457</td>
<td>436</td>
</tr>
</tbody>
</table>

* Incubation, 3 hrs; glucose, 0.001 M.
Initial specific activity of culture solution: 117 x 10<sup>6</sup> cpm/mM.
It seems clear that in the Avena coleoptile, bond splitting between pectin chains occurs as the result of methyl esterification with methionine as a methyl donor. Nance (9), on the other hand, has shown that IAA accelerates incorporation of acetate-1-\textsuperscript{14}C into the pectic substances of the pea stem and that part of this increase is due to incorporation of acetyl groups. Although acetyl groups may be involved in the cell wall metabolism of the coleoptile, their role has yet to be assessed.

It has been shown that a steady rate of methyl esterification is maintained in the coleoptile in the absence of auxin even when no elongation is taking place. Some synthesis of cell wall constituents is also maintained in the absence of added auxin. This can be detected by use of labeled glucose and such synthesis also occurs in the absence of elongation. The non-auxin induced esterification appears to accompany such synthesis. This basal esterification rate declines as external osmotic pressure is increased but the auxin induced increment in rate remains almost constant in absolute terms up to external osmotic concentrations near incipient plasmolysis. At osmotic concentrations higher than this the auxin effect disappears.

The small increase in rate of pectin synthesis under the influence of IAA which is indicated by the use of glucose-\textsuperscript{14}C as substrate is due to an effect of the auxin on a saponifiable fraction. It may be concluded that, in short term periods at least, 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 (5) with sucrose as substrate and by Ordin and Bonner (12) with galactose as substrate.

Perl's (13) also found that in elongating pea stems IAA enhances incorporation of acetate carbon into cellulose, protopectins and hemicellulose.

The antiauxin, 2,4,6-T, in the absence of IAA, and in the antiauxin concentration range, causes a slight depression of absorption of methionine and also causes a marked inhibition of incorporation of radioactivity into protopectin ester but does not inhibit incorporation into pectin ester. The cause of this inhibition is not known but it apparently has little to do with the endogenous elongation which may occur under these circumstances and which is not sensitive to antiauxin.

Anaerobic conditions and DNP both decrease incorporation as would be expected of an auxin sensitive reaction. 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.

A question that remains unanswered is whether the increase in esterification mediated by auxin accompanies a conversion of protopectin to pectin which thereby loosens the cell wall, or whether the pectin fraction itself becomes less rigid, the protopectin remaining unaffected. That pectin ester may possibly be a precursor of protopectin ester in the synthetic process (with no direct relation to the auxin mechanism) is suggested by progress curves of incorporation. Pectin is as highly labeled as in 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 substance 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, it is possible that pectin is first labeled and that protopectin is labeled later merely because of differential reaction rates.

Efforts to demonstrate a direct conversion of pectin to protopectin have yielded negative results. As indicated by Ordin, Cleland and Bonner (11) the presence of auxin slows loss of radioactive ester from pectin which has been previously ester labeled, but causes no significant change in protopectin. It is therefore virtually impossible to demonstrate a conversion of pectin to protopectin in vivo, if this does indeed occur. The retardation of loss of radioactive label from pectin caused by IAA is not in agreement with the findings of Bryan and Newcomb (4) who showed that IAA causes pectin methyllesterase (PM) activity to increase in cultured tobacco pith before and during the cell enlargement phase. The action of PM should liberate carboxyl groups which would then be available for esterification by radioactive methionine. The present findings do not suggest that PM activity is increased by auxin in the Avena coleoptile.

**Summary**

IAA treatment of Avena coleoptile sections causes the methyl carbon of \textsuperscript{14}C-labeled methyl methionine to be incorporated into the pectin ester of the cell wall more rapidly than is the case in the absence of IAA. The effect is detectable within 30 to 60 minutes. IAA has no apparent effect on incorporation of methyl carbon into protopectin ester.

As the osmotic pressure external to the sections is increased, incorporation of methionine methyl into pectic esters decreases. The auxin induced increase in incorporation into pectin ester is however essentially unaffected up to external mannitol concentrations of 0.3 M.

The antiauxin 2,4,6-T, given in the absence of added auxin, has no effect on methyl carbon incorporation into pectin ester but depressed incorporation into protopectin ester. Incorporation of methyl carbon into pectin apparently possesses a basal rate which is not auxin controlled.

2,4-DNP and argon treatment both inhibit incorporation of methyl carbon into all cell wall fractions.
This inhibition is apparent even when corrections are made for effects on the amount of substrate absorbed.

The increased incorporation of glucose-C\(^{14}\) into pectin caused by auxin can be shown to be principally due to incorporation into saponifiable substituents, presumably methyl ester groups.

It is concluded that the data support the hypothesis that esterification of carboxyl groups of pectin is involved in the auxin mechanism of cell expansion.

The authors are indebted to Dr. Eugene Jansen, Western Regional Laboratory, U. S. Department of Agriculture, Albany, California for the gift of a highly purified pectin methyl esterase preparation.

LITERATURE CITED

THE FORMATION OF STARCH AND THE BEHAVIOR OF ISOCITRIC ACID IN EXCISED LEAVES OF BRYOPHYLLUM CALYCINUM CULTURED IN DARKNESS

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In an experiment carried out in 1949 (1), confirmation was obtained of an earlier observation of the late Dr. G. W. Pucher that the starch content of leaves of Bryophyllum calycinum excised at daybreak may undergo a moderate increase during prolonged culture in water in darkness. It was noted at the same time that the isocitric acid content of the leaves decreased during the first 24 hours of the treatment by about 30% of the amount initially present. This last observation has since been found to be unique. Isocitric acid is notably stable under most of the conditions, including long periods of culture in darkness, to which Bryophyllum leaves have been exposed in a variety of studies of the behavior of this substance. Only when the leaves are cultured in water for many hours under continuous illumination is there any indication that isocitric acid becomes involved in the metabolic reactions. In a recent experiment (2), for example, about 9% of the isocitric acid disappeared after the leaves had been held for 74 hours under artificial lights. The rate of the reaction was notably sluggish and the total effect was small. Accordingly, it seemed essential to reexamine the behavior of starch and of isocitric acid under the conditions that were employed in the previous experiment.

In the early work of Dr. Pucher, the leaves had been supported in wire baskets in complete darkness in a sterilized metal cabinet where they were subjected to a continuous fine spray of water at 24° C. The surfaces of the leaves were thus continuously washed with water, the object being to minimize the possibility of contamination with microorganisms the growth of which might confuse the experimental results. Under these circumstances, the initiation of

\(^1\) Received January 22, 1957.