Phosphorylated and Nucleotide Sugar Metabolism in Relation to Cell Wall Production in Avena Coleoptiles Treated with Fluoride and Peroxyacetyl Nitrate

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ABSTRACT

Coleoptile sections of Avena sativa L. were pretreated with sodium fluoride or peroxycetyl nitrate at levels which inhibit auxin-induced growth but did not affect glucose uptake or CO₂ production when postincubated for 30 minutes in a ¹⁴C-glucose medium without auxin. Labeling of metabolites involved in cell wall synthesis was measured. Peroxyacetyl nitrate decreased labeling, and it was concluded that the pool size of uridine diphosphoglucose, sucrose, and cell wall polysaccharides decreased compared to control. The changes suggest that peroxycetyl nitrate inactivated sucrose and cell wall synthesizing enzymes including cellulose synthetase and decreased cell growth by inhibiting production of cell wall constituents. Fluoride treatment had no effect on production of cell wall polysaccharides, with or without indoleacetic acid stimulation of growth. The only change after fluoride treatment was a decrease in uridine diphosphoglucose during incubation without indoleacetic acid, a decrease that disappeared when indoleacetic acid was present. It was concluded that some other aspect of cell wall metabolism, not determined here, was involved in fluoride-induced inhibition of growth.

Growth inhibition by fluoride was shown to occur in plant tissue where there was no decrease in respiration. Under these conditions, the growth of the tissue was shown to be inhibited to a greater degree than was total cell wall synthesis (2, 3, 9, 13). Inhibition of growth and of cell wall metabolism by PAN¹ was demonstrated at levels that do not affect respiration in oat coleoptiles (8, 10, 12). It was suggested that the inhibitory effect of both agents might be directly on some part of the cell wall synthetic pathway. A likely mode of inhibition would be direct inactivation of one or more of the enzymes involved in this synthesis, which includes those converting hexose phosphates to nucleotide sugars and polymers. Several of these enzymes have been shown to be sensitive to fluoride or PAN in vitro (6, 11, 12, 14, 16) while in vivo, measurements have shown a number of them to be decreased in level after PAN treatment (6, 11). It is not known, however, which of the enzymes is rate-limiting in inhibition of either growth or cell wall formation. Such an inhibitor-sensitive step might very well produce changes in pool sizes of metabolites the enzyme utilizes or produces. The purpose of the present work was to determine the effect of growth-inhibiting levels of fluoride and PAN on the labeling pattern of metabolites involved in cell wall synthesis after short time incubation in ¹³C-glucose, and to relate any change to reported inhibitor-induced retardation of certain enzymes involved in regulation of these metabolites.

MATERIALS AND METHODS

Plant Material. Oat seedlings (Avena sativa L. var. Segre-havre) were grown for 88 hr on vermiculite at 26°C under red-orange light and were watered daily with distilled water. Harvesting of sections was carried out in diffuse daylight, and all incubations were done in darkness. Coleoptiles of 25 to 35 mm length were selected, defoliated, and the 3-mm apical tip was removed. The next two 5-mm sections were used in the ¹³C-glucose incorporation studies, whereas only the first section was used in growth experiments.

Groups of 20 or 100 sections each in stainless steel mesh baskets were placed in 2-liter gas washing bottles containing 1.9 liters of 25 mm KCl at pH 4.8 through which PAN or carbon-filtered air was bubbled for 4 hr. Oxidant level was determined as described earlier (12). Sections for fluoride treatment, 20 or 100, were placed in 20 ml of 0.005 N NaF or Na₂SO₄ at pH 6 for 2 hr.

Growth Measurements. After PAN or fluoride pretreatment, sections to be measured for growth were rinsed with 20 mm glucose, blotted, and initial length was measured. Each 20 sec bath was then transferred to 20 ml containing 20 mm glucose and 2.5 mm phosphate buffer, pH 4.8 or 6, and 28 μM potassium indoleacete and incubated for 4 hr. The final length was then measured.

Short time growth of fluoride pretreated sections was measured using a modification of the method of Evans and Ray (4). Batches of 15 sections each were strung on a cotton thread and tightly suspended inside a glass tube stoppered at both ends. The tube was filled with 0.005 N NaF or Na₂SO₄, pH 6, and air was bubbled through the solution while the sections were incubated in the dark for 2 hr. The solution was then drained, and the sections were rinsed with 20 mm glucose. The tube was filled with a solution containing 20 mm glucose and 2.5 mm phosphate buffer, pH 6, and 28 μM KIAA and the length of the coleoptile column was measured. Subsequent measurements were made after incubation for 30 to 60 min.

Metabolism Studies. Uniformly labeled ¹³C-glucose was used to study glucose utilization by inhibitor-pretreated sections. These sections were rinsed with deionized H₂O, and each batch of 100 sections was placed in a Warburg flask contain-
ing 5 ml of 20 mm glucose and 2.5 mm phosphate buffer, pH 4.8 or 6, and 35 μC 14C-glucose, with or without 28 μM potassium indoleacacetate. A KOH-saturated filter paper wick was taped to the side of the vessel to trap CO2. Incubation proceeded for 30 min in a 25 C shaking water bath. The labeled sections were removed, rinsed, and extracted with 80% (v/v) ethanol under reflux for 2 min and allowed to stand for 1 hr at room temperature. A similar extraction was done with 20% ethanol, and the extracts were combined and made up to an ethanol-chloroform H2O mixture of 12:5:8 (v/v). The aqeous phase was stored at −15 C and later used as the labeled cell extract. As a measure of 14CO2 the wicks were dried and counted by liquid scintillation.

The pellet was extracted at 70 C with a benzene-ethanol mixture (2:1, v/v) to remove lipids. The remaining residue, which consists of cell wall and precipitated macromolecules such as protein, was then fractionated by sequential solvent extraction into: (a) a hot 0.05 N HCl soluble fraction, (b) a 4.6 N NaOH-soluble fraction (room temperature), and (c) cellulose. Aliquots of each fraction were measured for radioactive activity by liquid scintillation.

**Chromatographic Methods.** The various metabolites in the labeled cell extract were measured after separation and isolation by paper chromatography in various solvent systems. The total extract of each batch (100 sections) was chromatographed on Whatman No. 3 MM paper in an ascending manner in the following series of solvents, each in the same direction. In sequence, each solvent running the given distance after the previous solvent had evaporated from the paper: (a) isopropanol-acetic acid-H2O (3:1:1), 8 cm; (b) methanol-formic acid-H2O (80:15:5), 8 cm; and (c) isopropanol-acetic acid-H2O (3:1:1), 20 cm. Autoradiography showed four bands of activity which included the following: (a) containing nucleoside diphosphate sugars, (b) hexose phosphates, (c) containing free sugars and amino acids, and (d) containing organic acids. All bands were cut out and counted by liquid scintillation as a measure of ethanol-H2O soluble activity. Band a was eluted and rechromatographed on Whatman No. 1 paper in a descending manner in isopropanol-0.05 N HCl (3:2) to separate UDP-glucose on the paper from other compounds. Band b was eluted and chromatographed on Whatman No. 3 MM in an ascending manner in 1-propanol-NH4OH-H2O (6:3:1) to separate the hexose phosphates from other compounds. This band was then eluted, hydrolyzed in 1 N HCl for 10 min at 100 C and rechromatographed on Whatman No. 1 paper in a descending manner in the same solvent system to isolate G6P and G1P (which was hydrolyzed to glucose). Band c was eluted and rechromatographed on Whatman No. 3 MM in a descending manner in acetone-1-butanol-H2O (7:2:1) to separate the free sugars. All compounds were located via autoradiography and characterized by cochromatography with known standards and by analysis of hydrolysis products.

**RESULTS**

The growth of the coleoptile sections after inhibitor pretreatment was measured as extension over a 4-hr period in which the sections were incubated in a glucose medium containing IAA at a concentration which produces optimum growth. The effect of the inhibitors on 14C-glucose utilization, however, was measured after a 30-min incubation in a glucose medium without IAA. The time period of 14C-glucose incubation was shortened from 4 hr to 30 min, because the tissue showed indications of recovery from inhibition after 4 hr of post-treatment. The 30-min incubation period was brief enough, however, to observe any change in metabolite labeling due to growth inhibition. Unpublished data indicate that the pools of the intermediate metabolites were probably saturated with respect to specific radioactivity by 30 min and that the size of the pools increased with time. IAA was omitted from the post-treatment incubation in order to eliminate the complicating situation of the control tissue increasing in mass by a different amount from the inhibitor-treated tissue. It was assumed in this case that inhibition of a reaction in cell wall biosynthesis would occur whether auxin was stimulating the synthesis or not. There was essentially no cell extension of coleoptile sections during the 30-min incubation in the glucose solution without auxin.

**Inhibition by Peroxyacetyl Nitrate.** The growth of sections was inhibited by PAN to a greater extent than was respiration over a range of concentrations (Fig. 1). The greater sensitivity of cell extension over general cell metabolism allows the determination of the effect of PAN on 14C-glucose utilization at levels of inhibition where limited energy production is not a factor. Such an effect was measured at PAN levels where growth was inhibited from 23 to 64%, while respiration, as measured by 14CO2 production, decreased less than 12%. The measurements of the pool sizes of metabolites involved in cell wall synthesis are shown in Table I and show that soluble activity, which is a measure of glucose uptake, was not affected by PAN treatment. The labeling of G6P and G1P also showed no significant change due to PAN. However, UDP-glucose showed a significant decrease in pool size of 19%, and the cell wall-containing residue and sucrose decreased by 31% and 21%, respectively. All three fractions of the residue showed similar inhibition in labeling due to PAN treatment, with cellulose being reduced most. These results clearly indicate that PAN treatment at this level markedly reduced the production of cell wall material as was shown earlier (8, 10, 12).

**Inhibition by Fluoride.** Inhibition of the growth of sections by fluoride over a range of concentrations also occurred to a greater extent than did inhibition of respiration, as shown in Figure 2. When sections were pretreated for 2 hr in 5 mM NaF, growth was inhibited 40%, while respiration showed no effect. The effect of fluoride on 14C-glucose utilization was de-
Table I. Effect of PAN Pretreatment of Coleoptile Sections on $^{14}C$-Glucose (UL) Utilization

Batches of coleoptile sections (100) were pretreated for 4 hr with 12-14 μl/liter PAN or air and then incubated for 30 min with 35 μC $^{14}C$-glucose in a 20 mM glucose medium at pH 4.8. The cells were then killed and radioactive metabolites were measured after separation procedures described in "Materials and Methods." Measurements represent the average of three sets of triplicate batches.

<table>
<thead>
<tr>
<th>Radioactivity in Coleoptile Sections</th>
<th>Control</th>
<th>PAN</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mean dpm × 10⁴/100 section</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol-H₂O soluble</td>
<td>990.0</td>
<td>920.0</td>
<td>93</td>
</tr>
<tr>
<td>CO₂</td>
<td>29.9</td>
<td>30.6</td>
<td>102</td>
</tr>
<tr>
<td>Sucrose</td>
<td>479.0</td>
<td>380.0</td>
<td>79</td>
</tr>
<tr>
<td>Residue</td>
<td>104.0</td>
<td>72.4</td>
<td>69</td>
</tr>
<tr>
<td>0.05 N HCl soluble</td>
<td>74.5</td>
<td>52.3</td>
<td>70</td>
</tr>
<tr>
<td>4.6 N NaOH soluble</td>
<td>8.4</td>
<td>6.8</td>
<td>80</td>
</tr>
<tr>
<td>Cellulose</td>
<td>21.1</td>
<td>13.0</td>
<td>62</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>73.2</td>
<td>77.0</td>
<td>105</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>8.1</td>
<td>7.7</td>
<td>95</td>
</tr>
<tr>
<td>UDP-Glucose</td>
<td>38.1</td>
<td>31.0</td>
<td>81</td>
</tr>
</tbody>
</table>

¹ NS: mean difference not significant at 0.05 level of probability.  
² Mean difference significant at 0.01 level.

There existed the possibility that the effect of fluoride on $^{14}C$-glucose utilization might occur only during auxin-stimulated growth. This might be the case if auxin directly activated one of the enzymes involved in the regulation of an intermediate on the cell wall pathway, or if the synthesis of such an enzyme were stimulated by auxin. Therefore, the effect of fluoride on $^{14}C$-glucose utilization was determined after post-incubation for 30 min in the $^{14}C$-glucose medium which included the growth-optimum concentration of 28 μM IAA. All metabolites measured were unaffected by fluoride, including UDP-glucose. While there was a difference between the incubation with and without IAA with respect to UDP-glucose, the absence of any change in cell wall labeling still indicates that fluoride had no obvious effect on the synthesis of cell wall material. It was apparent from the measurements of the labeled metabolites that the general pattern of $^{14}C$-glucose utilization after 30-min incubation was the same with or without auxin.

**DISCUSSION**

The biosynthesis of the cell wall in oat coleoptiles may be quite complex and involves intermediates that are also utilized in other metabolic pathways in the cell. There has been evidence presented for all factions of the scheme shown in Figure 3, and these reactions must be considered when analyzing the labeling of intermediates during $^{14}C$-glucose utilization. The inhibition of cell growth by PAN occurred at a level that had no effect on respiration or glucose uptake. The measured decrease of incorporation of labeled glucose into cell wall polysaccharides shown here and earlier (8, 10, 12) clearly indicates inhibition of enzymes on these pathways. Previous work in vivo showed that cellulose synthetase and phospho-

Table II. Effect of NaF Pretreatment on the Short Time Growth of Oat Coleoptile Sections

Groups of 5-mm coleoptile sections (15) stacked end-to-end on a cotton thread were pretreated for 2 hr with 5 mM NaF or 2.5 mM Na₂SO₄ and incubated in a 20 mM glucose medium. Measurements represent the average change in length of quadruplicate groups of sections.

<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Growth in the presence of Na₂SO₄</th>
<th>NaF</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30</td>
<td>0.85</td>
<td>0.32</td>
<td>62</td>
</tr>
<tr>
<td>30-60</td>
<td>1.05</td>
<td>0.70</td>
<td>33</td>
</tr>
<tr>
<td>0-60</td>
<td>1.90</td>
<td>1.02</td>
<td>46</td>
</tr>
</tbody>
</table>

In order to show that growth was indeed reduced by fluoride after only 30 min of postincubation, short term growth measurements were run. Results shown in Table II show that after 30 min, cell extension was inhibited by 62% with the effect decreasing with time, indicating a substantial fluoride effect.

glucomutase levels, but not UDPG pyrophosphorylase, were decreased in the oat after PAN treatment (6, 11, 12). The effect of PAN on the labeling patterns of metabolites in the present work showed no change in the G6P and G1P pools. This indicates that if phosphoglucomutase was inhibited by PAN, it was not a great enough inhibition for the normal conversion of G6P and G1P to be affected during cell growth. The decrease by PAN of UDP-glucose, sucrose and cell wall constituents indicates the possibility that one or all of the following enzymes may be inhibited: sucrose and/or sucrose-P synthetase, one or more of the cell wall synthetases, UDPG dehydrogenase and even possibly myoinositol oxygenase. All have been shown to be sulfhydryl enzymes (5, 6, 11, 14), and it has been suggested that the mechanism of inactivation of enzymes by PAN is by oxidation of sulfhydryl groups (7).

The best explanation of PAN inhibition of cell wall growth is an inactivation of cell wall synthetases, including cellulose synthetase. A decrease in the UDP-glucose pool could occur in the absence of a direct PAN inhibition of UDPG pyrophosphorylase if the size of the pool were regulated by feed-back inhibition. In this type of situation, the inhibitor would be a metabolite located at a step in the pathway near an end-product, which would build up when conversion of the metabolite to the end-product was slowed down. The increase in concentration of this “feed-back inhibitor” would lower the activity of its target enzyme—in this case, UDPG pyrophosphorylase (15).

It was shown in various plant tissues with treatment in vitro that two enzymes probably involved in cell wall precursor production, phosphoglucomutase (16) and UDPG dehydrogenase (14), are quite sensitive to fluoride. The effect of fluoride on 14C-glucose utilization, with or without auxin, did not reflect any inhibition of phosphoglucomutase. If this enzyme was affected, the labeling of G1P would be decreased and G6P might be increased; however, neither showed a significant change. The only change in 14C-glucose utilization occurring after fluoride treatment was a decrease in UDP-glucose after post-treatment without auxin. Inhibition by fluoride of UDPG dehydrogenase or of some other enzyme leading to end products not measured here, might lead to reduced UDPG as discussed above. No effect on UDPG in the presence of IAA despite inhibition of growth suggests that UDPG utilization for some reaction not critical for growth also may be auxin-controlled. The inhibition of cell growth by fluoride appears to be quite different from inhibition by PAN with respect to the manner in which growth is impeded. PAN obstructs the conversion of precursors into wall polymers, even in short time periods, thereby preventing cell growth through the reduction of the needed quantity of cell wall material. It is apparent from this work, however, that fluoride did not reduce the quantity of cell wall material when used at levels where respiration was not affected and during short incubations. The inhibition appeared to be a prevention of some other aspect of the cell wall expansion process during auxin-stimulated growth.

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LITERATURE CITED