Inhibition of Metabolism in Avena Coleoptile Tissue by Fluoride

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Fluoride continues to be a major problem of air pollution. One of the aspects that is most insidious is the effect on growth or on respiration without visible tissue injury (2). Over a decade ago Bonner and Wildman (3) found that fluoride-induced inhibition of phosphatase in Avena coleoptiles was about the same as the inhibition of growth by fluoride. Respiration, on the other hand, was much less affected. Bonner and Thimann (4) concluded that inhibition of enolase was not involved in the inhibition of growth of excised plant sections.

Fluoride inhibition of growth of yeast cells has been linked to interference with cell wall polysaccharide synthesis, particularly by inhibiting the conversion of hexose-6-P to hexose-1-P (6). Christiansen and Thimann (5) found a small inhibition in net synthesis of cellulose and hemicellulose in the presence of IAA and fluoride in pea stem segments. Since partial growth occurred during these observations it is not clear whether the effect was a consequence of inhibited growth or not. Earlier reports (12, 13) have described the effect of some growth inhibitors on cell wall polysaccharides in the absence of growth hormones.

Because of the possibility that inhibition of metabolism of specific cell wall constituents might be the cause of the inhibition of growth, an investigation of the influence of fluoride on incorporation of C^{14} from glucose-U-C^{14} into the cell wall was undertaken. The present report shows that, even in the absence of IAA-induced elongation, growth inhibiting concentrations of fluoride markedly affect metabolism of certain components of the cell wall.

Materials & Methods

Oat seedlings (*Avena sativa*, L. var. Siegessähe) were grown and harvested as described earlier (12). Coleoptiles were defoliated and 3 mm apical sections were discarded. The next two successive 5 mm sections were used in C^{14} experiments. For growth experiments, similar 5 mm decapitated sections with primary leaves in situ were used.

In each replicate, sections were pretreated in 20 ml of NaF or Na_{2}SO_{4} solution for 6 hours, unless noted otherwise. The number of sections per ml of pretreatment solution varied from 5 for C^{14} experiments to 1 for growth experiments. Preliminary experiments indicated that the use of 5 sections per ml during pretreatment did not affect subsequent growth results when 20 sections were incubated in 20 ml of growth solution.

Since the amount of external fluoride carried over from a pretreatment solution was found to be insufficient to affect the growth results, the sections were blotted without rinsing before transfer to fluoride-free or sulfate-free solutions containing either glucose-C^{14} or IAA. All pretreatments and sub-
sequent incubations were conducted at pH 6 at 25° in darkness. Unpublished data in this laboratory show that if the fluoride-free growth solution is at pH 4.8, not only is inhibition of growth enhanced but inhibition of oxygen uptake is also quite marked following 6 hours pretreatment in 0.01 mM NaF at pH 6. Growth solutions contained 2.9 × 10⁻⁵ M IAA and the measurements of growth were carried out as described previously (12).

Glucose-U-C¹⁴ was obtained from Dr. R. C. Bean. Glucose-1-C¹⁴, glucose-2-C¹⁴, and glucose-6-C¹⁴ were purchased from Calbiochem or Volk Radiochemical Company. For cell wall studies, triplicates of 100 sections each (half of which were from the younger region & half from the adjacent older region unless noted otherwise) were incubated in 5 ml of radioactive solution contained in a 100 ml Warburg flask with 1 ml of 1 N NaOH in the sidearm. The flasks were shaken at 100 strokes per minute. Oxygen uptake was determined during the 4 hour incubation. The sections were then removed, rinsed, and stored as described earlier (12).

Studies of catabolism of specifically labeled glucose were carried out by incubating 30 decapitated and defoliated sections in 1.5 ml of glucose-C¹⁴ contained in 15 ml 2-arm Warburg flasks. The C¹⁴O₂ was trapped in 0.2 ml of 1 N NaOH in one arm of the flask. The reaction was stopped by tipping in 0.2 ml of 10 N H₂SO₄ at the end of the desired incubation period, usually 4 hours, and letting the flasks shake an additional 15 minutes. Each value is the average of duplicate flasks.

Cell walls were isolated and the fractions were extracted and counted by methods described in an earlier publication (12). Thus, the wall was fractionated into: A. A cold water-soluble fraction containing some of the pectic substances as well as glucon, galactan, araban, and xylan; B. A hot watersoluble fraction (classical pectin), similar in sugar composition to the first fraction; C. A hot 0.05 N HCl soluble fraction (classical propectin) containing mostly hemicellulose; D. A 1 N NaOH-soluble fraction (polyuronide hemicellulose); E. A 4.6 N NaOH-soluble fraction (the remaining non-cellulosic polysaccharides), and F. Cellulose. In some cases cellulose was extracted by suspending the alkali-extracted and dried cell wall material in 2 ml of 10% aqueous tetraethyl ammonium hydroxide. The volume was reduced to 0.5 ml under vacuum and the dissolved cellulose was reacted with an excess of aqueous tetraethyl ammonium monochloracetate. The carboxymethyl cellulose formed was precipitated in 80% ethanol and redissolved in water. Aliquots of this material plus any unprecipitated material in the 80% ethanol were plated out and counted as cellulose. The gross results are the same as extraction by acetylizing reagents (12).

The cell wall extracts were hydrolyzed for 4 to 5 hours with 1 N H₂SO₄ in sealed ampules at 100°. The BaCO₃ neutralized hydrolysates were chromatographed on 2.5 cm wide strips of S & S No. 589 White Ribbon filter paper for 20 hours in ethyl acetate: pyridine: water (10:4:3). The region containing galacturonic acid from the aqueous-soluble and acid-soluble extracts was rechromatographed in ethyl acetate: water: acetic acid (10:6:5) for 8 hours or in pyridine: ethyl acetate: acetic acid: water (5:5:1:3) in a chamber saturated with pyridine: ethyl acetate: water (11:40:6) for 48 hours. The strips were scanned with a Vanguard 880 Autoscan-ner. The radioactive areas were measured and the data are presented as radioactivity in relative units for each fraction. For each fraction, the xylene of the sulfate treated tissue was set equal to 1.0 and all other components of both fluoride and sulfate treated tissues were calculated relative to this xylene. The radioactivity is corrected for aliquot size but not for self absorption on paper.

Results

In a preliminary experiment, groups of 100 sections were pretreated for 14 hours in 0.003 N NaF or Na₂SO₄ and were transferred to 0.003 N fluoride or sulfate plus 10⁻⁵ M glucose-U-C¹⁴ for an additional 4 hours. Oxygen uptake was unaffected. A 10% inhibition of glucose uptake, as measured by C¹⁴O₂ evolution or cytoplasmic C¹⁴ was found. There was also little difference in incorporation into any wall fraction except for a 30% inhibition of incorporation into the aqueous-soluble components and cellulose. Growth, measured in the presence of IAA, was inhibited 40% under such conditions. Chromatograms of hydrolysates of the aqueous and acid-soluble fractions showed little differential inhibition of incorporation of radioactivity into sugar components except for a slightly greater decline of glucose and galacturonic acid of the cold water and hot water-soluble fractions and of the acid-soluble fraction.

In order to decrease the preliminary time before C¹⁴ was administered, further experiments were carried out in which higher concentrations of fluoride or sulfate were used during the pretreatment only. Incubation of sections in 10⁻⁵ M glucose-U-C¹⁴ following 6 hour fluoride pretreatments resulted in only slight repression of both absorption of glucose and of incorporation into the cell wall. The main effect on C¹⁴ utilization was a 16% inhibition of incorporation into cellulose. Under such conditions, however, growth was repressed 70%.

Unpublished data from this laboratory indicated that low concentrations of glucose might limit rate of incorporation. If uptake were inhibited, it might mask effects on the incorporation into the cell wall under some conditions. At high concentrations of glucose, rate of incorporation was less governed by amounts present in the external medium. Therefore, the use of a high concentration of glucose-U-C¹⁴ offered a means of separating effects on incorporation from effects on uptake.

Sections were pretreated for various periods of time in 0.01 N NaF or Na₂SO₄ at pH 6 and were
then transferred to 1 nAA + 0.05 M glucose + 0.0025 M potassium phosphate at pH 6. The 2 hour sulfate curve and all of the fluoride curves are presented in figure 1. The other sulfate curves showed slopes equal to the 2 hour sulfate curve.

Leafless sections were pretreated with 0.01 n NaF for 6 hours followed by 4 hours in 0.05 M glucose-U-C14. According to data in table 1, uptake of glucose was barely affected but incorporation, particularly into cellulose, was inhibited.

An approximately 5000 fold increase in external glucose concentration resulted in a several hundred fold increased incorporation of glucose. This greater amount of incorporated exogenous glucose could cause variations in the labeling patterns since metabolic pools which had possibly been limiting could operate closer to saturation. As was expected, incorporation into the cell wall was affected by the inhibitor much more than was absorption. The metabolism of the alkali-soluble fractions also was inhibited here.

![Figure 1](image)

**Table 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Sulfate cpm x 10^-3</th>
<th>Fluoride C14% of control</th>
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<tr>
<td>Cytoplasm</td>
<td>120.1</td>
<td>100</td>
</tr>
<tr>
<td>C14O2</td>
<td>131</td>
<td>92</td>
</tr>
<tr>
<td>Cell wall fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cold water-soluble</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>hot water-soluble</td>
<td>8.8</td>
<td>74</td>
</tr>
<tr>
<td>0.05 n HCl-soluble</td>
<td>42.0</td>
<td>70</td>
</tr>
<tr>
<td>1 n NaOH-soluble</td>
<td>9.4</td>
<td>62</td>
</tr>
<tr>
<td>4.6 n NaOH-soluble</td>
<td>6.8</td>
<td>75</td>
</tr>
<tr>
<td>cellulose</td>
<td>12.0</td>
<td>53</td>
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Detailed examination of hydrolysates of the cell wall fractions confirmed marked inhibition of glucose components of the hot water-soluble and acid-soluble wall fractions as shown in figure 2. No differential effects on individual sugars in the alkali-soluble fractions were noted.

To see if the effect on respiration of exogenous glucose was a consequence of uptake or whether it was a result of direct interference with respiration, specifically labeled glucose-C14 was utilized. Sections were pretreated 6.5 to 7 hours in 0.01 n NaF and were then incubated for 4 hours in 0.05 n specifically labeled glucose-C14 to determine if there was an alteration in catabolic pathways. According to
Table II

Effect of Pretreatment with NaF or Na₂SO₄ on C₁⁴O₂ Yields from Specifically Labeled Glucose in Avena Coleoptile Sections

The sections (30) were pretreated 6.5 to 7 hours in 20 ml of 0.01 n NaF or Na₂SO₄ followed by a 4 hour incubation in 0.0025 m potassium phosphate pH 6 containing 0.05 m glucose-C¹⁴. The initial activity (cpm × 10⁻³) per vessel was glucose-1-C¹⁴ 686, glucose-2-C¹⁴ 620, glucose-6-C¹⁴ 684, and glucose-U-C¹⁴ 7650 (calculated from table I for 30 sections). The total volume was 1.5 ml.

<table>
<thead>
<tr>
<th>Position</th>
<th>Ratio</th>
<th>Sulfate % yield*</th>
<th>**</th>
<th>Ratio</th>
<th>Fluoride % yield*</th>
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<tbody>
<tr>
<td>C₀</td>
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<td>0.47</td>
<td>36.0</td>
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<tr>
<td>C₁</td>
<td>0.50</td>
<td>6.4</td>
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<td>0.43</td>
<td>5.5</td>
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<tr>
<td>C₂</td>
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<td>4.0</td>
<td></td>
<td>0.29</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>C₆</td>
<td>0.46</td>
<td>5.9</td>
<td></td>
<td>0.33</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>C₆ + C₄</td>
<td>0.92</td>
<td>18.7</td>
<td></td>
<td>0.77</td>
<td>18.9</td>
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<td>C₆/C₁</td>
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<tr>
<td>C₆/C₂</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* % of initial C¹⁴ in Warburg vessel.
** cpm × 10⁻³ per 30 sections, calculated on basis of each labeled carbon having activity of 1275 × 10³ cpm per vessel.
*** Estimated minimum contribution = C₀ – (C₁ + 2C₂ + C₆).

Discussion

Although overall oxygen uptake was essentially neither inhibited nor stimulated by fluoride, the effects on C¹⁴O₂ from specifically labeled glucose indicated an apparent shift in respiratory pathway. Ross et al. (17) recently suggested that fluoride induces a shift from the Embden-Meyerhof-Parnas (EMP) pathway to the pentose phosphate pathway on the basis of results with specifically labeled glucose. The finding that C₆/C₁ ratios were near unity in control tissue here agrees with the results of Gibbs and Beevers (10) suggesting that the EMP pathway is operative. The high C₆/C₁ ratios reminiscent of the works of Humphreys and Dugger (11) and of Ramsey and Wang (15) indicate, however, that C₆ may be lost more readily by paths other than the tricarboxylic acid cycle.

Evidence presented by Finkle et al. (9) suggests that the glucuronic acid-xylulose cycle may be operative in higher plants. Catabolism of glucose partly via the pentose phosphate pathway and partly via the glucuronic acid cycle could lead to a higher production of C¹⁴O₂ from both C₆ and C₁ than from C₂ of specifically labeled glucose. Since it is likely that glucose-1-P is involved as a precursor for the glucuronic acid cycle, inhibition of phosphoglucomutase by fluoride would limit production of C¹⁴O₂ from glucose-6-C¹⁴. Similarly, inhibition of uridine diphosphoglucose dehydrogenase by fluoride (19) could also account for the observed effects because formation of the required uridine diphosphoglucuronic acid would be blocked.

The small fluoride induced inhibition of C¹⁴O₂ production from glucose-1-C¹⁴ suggests that additional sensitive catabolic pathways were operative. Possibly, both C₆ and C₁ in a glucose-6-P pool may...
have been lost via the pentose phosphate pathway after prior randomization of the glucose-6-P by reversible functioning of part of the glycolytic pathway involving recombination of the isomerized triose phosphates. Evidence for partial randomization in saccharides has been found by Edelman et al. (8). Such reversal is more likely to occur, according to Couri and Racker (7), via a transaldolase catalyzed reaction between glyceraldehyde-3-P and sedoheptulose-7-P yielding erythrose-4-P and fructose-6-P, and an aldolase catalyzed reaction between erythrose-4-P and dihydroxyacetone phosphate yielding sedoheptulose-1,7-diP. Sedoheptulose diphosphatase catalyzes the reformation of sedoheptulose-7-P, the net effect being conversion of two triose phosphates to fructose-6-P. Both fructose diphosphatase, which catalyzes conversion of fructose-1,6-diP to fructose-6-P after recombination of the triose phosphates, and sedoheptulose diphosphatase are sensitive to fluoride (14). Hence, fluoride inhibition of either enzyme would account for decreased C¼O₂ production from both glucose-1-C¹⁴ and glucose-6-C¹⁴. The decreased C₄/ C₃ ratios could be the result of C¹⁴O₂ production from non-randomized C₃, becoming relatively more prominent when randomization is reduced by fluoride. If such “reversal” of glycolysis were occurring, the absence of radioactivity in pentosan when glucose-6-C¹⁴ was used as substrate, as shown below, would indicate separate glucose-6-P pools for catabolism and anabolism respectively.

Another possibility is that some C₄ was lost during formation of xylan and arabin from glucose (1, 18). Fluoride or sulfate pretreated coleoptile sections which had been incubated in specifically labeled glucose solutions were extracted with hot 0.05 N HCl and the extracts were hydrolyzed. The chromatograms showed essentially no radioactivity in xylose and arabinose when glucose-6-C¹⁴ was used as substrate. The pentoses were radioactive, however, when either glucose-1-C¹⁴ or glucose-2-C¹⁴ was used. Inhibition of formation of these pentosans by fluoride would be reflected in less C¹⁴O₂ from C₄. Since inhibition both of the major part of polysaccharide pentose metabolism and of production of C¹⁴O₂ from C₄ disappeared simultaneously, this latter alternative probably occurs to a greater or less extent. It is unlikely that this inhibition of C¹⁴O₂ production from glucose-6-C¹⁴, whatever its mechanism, is related to restraint of growth since conditions could be adjusted to eliminate this interference with catabolism without eliminating effects on growth or on certain components of the cell wall.

Because no inhibition was observed on the estimated C₃ + C₄-derived C¹⁴O₂, it is concluded that, under the conditions of fluoride treatment used here, endolase was not inhibited.

Unlike previous studies of cell wall metabolism under the influence of various inhibitors, incorporation into the wall as a whole was found to be inhibited by fluoride much less than was growth. This phenomenon was also observed by others (5, 16) with reference to net cell wall synthesis. Incorporation into the glucose components of the aqueous and acid-soluble wall fractions is apparently quite sensitive to fluoride. Cellulose was also sensitive to fluoride although not as markedly as in other cases of inhibition (12, 13).

Recent studies by Yang and Miller (20) suggest that phosphoglucomutase is the most fluoride-sensitive enzyme in the synthetic pathway leading to sucrose formation in higher plants. Since, according to present theories on carbohydrate interconversions, all incoming glucose would have to pass through a phosphoglucomutase-catalyzed step before it could be converted to various polysaccharides, it is most likely that the inhibition of polysaccharide synthesis in this investigation is due to the effect of fluoride on phosphoglucomutase. The differential sensitivity of polysaccharide metabolism to fluoride raises a problem. Phosphoglucomutase is found in the non-particulate portion of the cytoplasm. If the enzyme were bound within a membrane so that glucose-6-P could enter readily but glucose-1-P were quickly used in a series of wall synthetic reactions, a series of differentially sensitive phosphoglucomutases could be postulated. On the other hand, if the same enzyme were being inhibited, competition for glucose-1-P would have to be postulated, with the synthesis of xylans and arabans being more efficient than the synthesis of glucans. The similarity of response to fluoride of the more soluble glucans and of cellulose suggests that these substances are produced from common intermediates.

The effects on cellulose relative to growth in the presence of dilute glucose solutions requires some explanation. Possibly, synthesis of cellulose potentially important to growth was actually inhibited but the route from an endogenous pool was more dominant when dilute exogenous glucose was used shortly after harvest of sections. After a long pretreatment, however, the endogenous pools may have been sufficiently depleted so that inhibition of utilization of dilute exogenous substrate could be manifested. On the other hand, when exogenous substrate was present in excess, relatively more exogenous glucose might be utilized in the potential growth associated reaction and inhibition under such conditions would be more apparent. In any case, it appears that only a small portion of cellulose synthesis could be crucial to growth.

Similarly, inhibition of metabolism of the alkali soluble fractions became relatively more manifest when high concentrations of exogenous glucose were used. Whether the inhibition of incorporation into the alkali-soluble fractions or the inhibition of incorporation into the glucose components of the more soluble wall fractions has a direct role in the fluoride inhibition of growth is unknown at present.

**Summary**

Following treatment of Avena coleoptile sections with 0.01 N NaF for 6 hours, an 80% inhibition of...
growth in the presence of 0.05 M glucose and 2.9 \times 10^{-2} M indoleacetic acid ensued, followed by partial recovery in fluoride-free solutions. Studies of glucose utilization in the absence of indoleacetic acid showed that absorption of uniformly labeled C^{14} glucose was barely affected but inhibition of incorporation of C^{14} into cellulose and the glucose components of the aqueous and acid-soluble wall fractions was particularly notable.

Possible causes of an apparent effect of 0.01 N NaF pretreatment for 6.5 to 7 hours on respiratory pathways, detected by the use of specifically labeled glucose, are discussed.

At lower levels of inhibition of growth, the effect on catabolism disappeared but certain effects on the cell wall were still apparent.

Interference with metabolism of specific components of the wall including cellulose is considered to be a factor in fluoride inhibition of growth.

**Literature Cited**

14. **RACKER, E. & E.A.R. SCHROEDER.** 1958. The reductive pentose phosphate cycle. II. Specific C-1 phosphates for fructose 1,6-diP and sedoheptulose 1,7-diP. Arch. Biochem. Biophys. 74: 326-44.