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The Ohio State University, Ph.D., 1975
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STUDIES ON THE MECHANISM OF ACTION
OF AUXIN AND FUNGAL TOXINS IN THE
MODIFICATION OF CELL ELONGATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Robert Allen Saftner, B.A., M.S.

The Ohio State University

1975

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SPECIFIC BINDING OF VICTORIN AND CALCIUM: EVIDENCE FOR CALCIUM BINDING AS A MEDIATOR OF VICTORIN ACTIVITY

INTRODUCTION

Helminthosporium victoriae Meehan and Murphy and its host-specific toxin (victorin) induce the same disease symptoms and show the same host specificity on various susceptible cultivars of oats. The primary effect of the toxin has been postulated to be at the plasma membrane (41) since the toxin causes rapid loss of electrolytes from tissues of susceptible plants (45, 46, 47, 51). Other membrane-related toxin effects include depolarization of membrane potentials (15, 39, Saftner unpublished), formation of blister-like bodies on plasma membranes (18, 31), loss of ability of cells to plasmolyze, increased apparent free space in roots, bursting of protoplasts isolated from coleoptile cells, and loss of active absorption of solutes in root cells (46, 47). None of these effects is observed with low concentrations of victorin in resistant cultivars of oats.

A number of workers have recently provided evidence that calcium may play an important role in victorin-induced disease. Hanchey et al. (18) were the first to indicate a possible relationship between calcium and victorin by showing that many of the ultrastructural (and macroscopic) effects of victorin in susceptible oat cells resemble those
of calcium deficiency reported by Marinos (33) in barley cells. Other
evidence suggesting a relationship between calcium levels and victorin
action include reports that victorin toxicity in susceptible tissue
may be suppressed by Ca\(^{2+}\) (5), that victorin-induced loss of electro-
lytes from susceptible but not resistant tissue (51) is similar to that
associated with calcium deficiency (30), that susceptible tissue is
more sensitive to calcium deficiency than resistant tissue (5, 30),
and that the calcium content of resistant tissue is higher than that
of susceptible tissue (30). In addition, calcium has been correlated
with disease resistance in several other plant diseases (3, 24, 25,
51). The role of the calcium ion in plants is not clearly understood
but certainly is involved with the maintenance of membrane integrity
(33) and differential permeability (7, 24).

In this study, I have investigated the relationship between victorin
toxicity and calcium binding in susceptible and resistant cultivars
of Avena. These data reveal a difference in the calcium binding
properties of membrane preparations of the two cultivars. This, in
conjunction with evidence that victorin itself strongly binds calcium,
suggests that the toxic effects of victorin in susceptible tissue may
be mediated by calcium.
MATERIALS AND METHODS

Plant Material

Oat (Avena sativa L.) cultivars susceptible (cv. Park) and resistant (cv. Victory) to H. victoriae and to its toxin were used in all experiments. Seedlings were obtained by soaking unhusked seeds in deionized water for 2 hr and planting them in covered plastic trays containing vermiculite wetted with double-distilled, demineralized water. The trays were placed in a darkroom at room temperature and exposed to red light from a 25W bulb enclosed in a Kodak No. 1-A safelight filter and positioned about 30 cm above the surface. After 24 hr, the red light was turned off, and the seedlings were allowed to grow in darkness to an age of 108 to 120 hr at which time coleoptiles greater than 3 cm long were harvested for experimentation. All subsequent experimental operations were performed under subdued or normal laboratory lighting.

Toxin Preparation

Victorin was partially-purified as described previously (45) except that the toxin was desalted by column chromatography in all cases before use. This preparation, at a dilution of 1:1000 (v/v), completely inhibited root elongation of susceptible oats while having no effect on root elongation in resistant oats even at a dilution of 1:10. In all experiments reported here, we used a dilution of 1:25 to 1:50, diluting the toxin with double-distilled, deionized water and adjusting
the pH to 5.5 if necessary with 1.0 mM NaOH.

To prepare samples of inactive victorin for control experiments, victorin was extracted as described above but adjusted with 1 M NaOH to pH 10.5 for storage. Based on the root elongation assay (41), victorin samples are completely inactivated when the pH is raised to about 10.5 and stored at room temperature for 4 days (32).

VICTO R IN samples containing $^{45}$Ca$^{+2}$ (as $^{45}$CaCl$_2$) were obtained by first mixing 2 ml of partially-purified victorin with sufficient $^{45}$Ca$^{+2}$ (initial specific activity of 0.9 mCi/mmole) to bring the final concentration of calcium in the victorin sample to 7.5 x 10$^{-7}$ M and then desalting on a 1.5 x 20 cm Sephadex G-15 column developed with 0.02 M NaCl solution. Toxin, along with a large peak of $^{45}$Ca$^{+2}$ activity, came off the column between 1.5 and 2.0 void volumes. The eluate at 1.7 void volumes contained maximal victorin activity as well as maximal $^{45}$Ca$^{+2}$ radioactivity.

Homogenation and Precentrifugation

Homogenates from oat coleoptiles were obtained using a modification of the procedure of Hertel et al. (19) and Hertel and Ray (unpublished). Oat coleoptiles were chopped in a cold room over ice for 5 min with 2 razor blades in a volume in ml of oat-grinding medium (OGM: 50 mM Tris buffer, pH 8.0; 250 mM sucrose; 1 mM disodium dihydrogen ethylenediamine-tetraacetate dihydrate) equal to the fresh weight in g of tissue used. The slurry was then gently crushed for 2 min using a mortar and pestle and filtered through a single layer of nylon mesh (10 μm pore diameter). The residue was again crushed in a volume of
OGM equal to that used above and filtered. The filtrates were combined and centrifuged at 12,100 x g in a Sorvall RC2-B centrifuge for 20 min to remove nuclei, plastids, mitochondria, and large membrane fragments. The supernatant was dispensed into 3.5 ml polypropylene tubes and centrifuged at 48,200 x g for 40 min. These supernatants which contained microsomes and some membrane fragments were discarded and the pellets were dislodged and dispersed in a volume of oat-suspension medium (OSM: 5 mM Tris-maleate buffer; 250 mM sucrose) at pH 6.0 equal to the volume of discarded supernatant. The tubes were again centrifuged at 48,200 x g for 40 min and the supernatants discarded. The pellets were combined and suspended (Potter-type homogenizer) in OSM at pH 5.5 to give a final level of membrane preparation (final homogenate) from 0.5 g fresh tissue per ml. In some binding experiments with 3-indoleacetic acid (IAA), 10 mM citrate buffer was used in the OSM instead of 5 mM Tris-maleate buffer. All of the above procedures were carried out at 0 to 4° C.

**Binding Experiments and Evaluation**

In order to determine binding of victorin to membrane preparations from *Avena*, a \( ^{45} \text{Ca}^{+2} \)-victorin complex was added to the resuspended pellets to give a dilution of the radioactive victorin sample of 1:50. The homogenate was then divided into equal fractions. To one fraction was added sufficient victorin to bring the concentration of unlabeled victorin to a 1:25 dilution. To another fraction was added sufficient inactive victorin to bring the final concentration of inactivated victorin to a dilution of 1:25. \( \text{CaCl}_2 \) was added to the other fractions to bring the concentration of unlabeled \( \text{Ca}^{+2} \) to from 0 to \( 10^{-4} \text{ M} \) as specified.
The suspensions were mixed and distributed in 1 ml aliquots into 1.5 ml nitrocellulose tubes. These were centrifuged at 39,100 x g for 40 min. A 200 μl portion of each supernatant and the pellet from each tube were placed in separate scintillation vials containing 10.5 ml of Bray's solution for determination of radioactivity. Radioactivity was measured using the open channel of a Beckman Model LS-230 liquid scintillation spectrometer. Percent binding of radioactive victorin samples was calculated as CPM in the pellet x 100/total CPM (pellet and supernatant). Percent total binding was taken as percent binding in the absence of unlabeled Ca\(^{2+}\) or victorin. Percent specific binding was estimated as percent total binding minus percent binding in the presence of a high (1:25 dilution) concentration of unlabeled victorin.

Determination of calcium binding to membrane preparations from Avena was done in a similar manner. \(^{45}\)Ca\(^{2+}\) (initial specific activity 0.9 mCi/m mole) was added to the final suspension to give a concentration of 10\(^{-7}\) M. CaCl\(_2\) was added to bring the concentration of unlabeled calcium to from 0 to 10\(^{-2}\) M as specified. These suspensions were then centrifuged and radioactivity measured by the same techniques used in the victorin-binding experiments. Percent total binding was taken as percent binding in the absence of unlabeled Ca\(^{2+}\). Percent specific binding was estimated as percent total binding minus percent binding in the presence of a high (10\(^{-3}\) M) or near-saturating (10\(^{-2}\) M) concentration of unlabeled Ca\(^{2+}\).

For determination of IAA binding, \(^{14}C\)-labeled IAA (initial specific activity 55 mCi/m mole) was added to the membrane preparation to give a concentration of 10\(^{-7}\) M. IAA was added to bring the concentration
of unlabeled IAA to from 0 to $10^{-4}$ as specified. In some experiments, victorin was added to give a final dilution of unlabeled victorin of 1:25. Percent total binding of IAA was taken as percent binding in the absence of unlabeled IAA or victorin. Percent specific binding was taken as percent total binding minus percent binding in the presence of a saturating ($10^{-4}$ M) concentration of unlabeled IAA.

**Protein Determination**

The total protein content of pellets from binding experiments was determined using the Lowry method (29) with bovine serum albumin as standard.

**Calcium Determination**

The calcium level of various solutions was measured by atomic absorption spectrophotometry using a Perkin-Elmer 303 Atomic Absorption Spectrophotometer connected to a Sargent-Welch Model SRG Recorder. The determinations were carried out in 10% trichloracetic acid (TCA) for TCA-soluble calcium measurements, in 1 M nitric acid for total and TCA-insoluble calcium measurements, and in 0.02 M NaCl for calcium measurements of column eluants. Standards and blanks for each type of experiment were treated similarly. To extract TCA-soluble calcium from coleoptile tissue, freshly harvested tissue was chopped over ice for 5 min with 2 razor blades in a volume in ml of 10% TCA equal to the fresh weight in g of tissue used. The slurry was then gently crushed for 2 min in a mortar and pestle and centrifuged at 0 to 4° C in a Sorexall RC2-B centrifuge at 27,000 x g for 15 min. The pellet was
resuspended in a volume of 10% TCA equal to that used above and recentrifuged at 27,000 x g for 15 min; this step was repeated on the resulting pellet. Calcium determinations were done on the combined supernatants. To extract calcium from oven-dried tissue or TCA-insoluble pellets, the material was suspended in concentrated nitric acid and the suspension was held in a boiling water bath for 10 min. The solutions were then diluted with double-distilled, deionized water so that the final concentration of nitric acid was 1 M.

**Glassware**

All glassware was acid washed, rinsed 4 times each in tap, deionized, and double-distilled, deionized water, respectively before air drying.

**RESULTS**

**Binding of $^{14}$C-labeled IAA to Membrane Preparations from Avena Coleoptiles**

Table 1 summarizes data from experiments on the binding of IAA to membrane preparations from Avena coleoptile tissue. When a low concentration (e.g. $10^{-7}$ M) of labeled IAA is added *in vitro* to homogenates of oat coleoptiles, radioactivity is reversibly bound to pelletable particles, i.e. the addition of a saturating concentration (e.g. $10^{-4}$ M) of unlabeled IAA significantly ($P \leq 0.01$) reduces the percent binding of labeled IAA. In a representative experiment, the GFM in pellets were about 290 and 210 in the absence and presence of $10^{-4}$ M IAA. Similar results using corn coleoptiles have been reported by Hertel *et al.* (19). The percent total and specific binding varies.
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>% Total Binding</th>
<th>% Specific Binding with $10^{-6}$ M IAA</th>
<th>% Specific Binding with $10^{-6}$ M IAA Plus 1:25 Victorin</th>
<th>Test Buffer</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>3.51 ± .08</td>
<td>0.79 ± 0.06</td>
<td>0.85 ± 0.10</td>
<td>Citrate</td>
</tr>
<tr>
<td>2</td>
<td>2.98 ± .10</td>
<td>0.44 ± 0.03</td>
<td>0.56 ± 0.06</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>3.45 ± .11</td>
<td>1.00 ± 0.04</td>
<td>0.91 ± 0.10</td>
<td>&quot;</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td>3.31 ± .29</td>
<td>0.74 ± 0.28</td>
<td>0.77 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.07 ± .09</td>
<td>0.50 ± 0.07</td>
<td>0.54 ± 0.08</td>
<td>Tris-Maleate</td>
</tr>
<tr>
<td>2</td>
<td>3.03 ± .04</td>
<td>0.55 ± 0.05</td>
<td>0.59 ± 0.04</td>
<td>&quot;</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td>3.05 ± .03</td>
<td>0.53 ± 0.04</td>
<td>0.57 ± 0.04</td>
<td></td>
</tr>
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somewhat depending on the buffer used in the OSM. However, the overall significance of the results using two different buffers (Tris-maleate or citrate) buffering near pH 5.5 is the same. The percent specific binding of IAA in the final homogenate ranges from 0.53 to 0.74 depending on the buffer used. Victorin was found to have no significant ($P \geq 0.10$) effect on the specific binding of IAA in the final homogenate from either susceptible (see Table 1) or resistant (data not shown) tissue. In addition, Table 2 shows that the percent total binding of IAA is not significantly ($P \geq 0.10$) influenced by the addition of victorin to the final homogenate from either susceptible or resistant tissue.

**Binding of $^{45}$Ca$^{2+}$ to Victorin**

When a $7.5 \times 10^{-7}$ M solution of $^{45}$CaCl$_2$ is applied to a 1.5 x 20 cm Sephadex G-15 column, $^{45}$Ca$^{2+}$ elutes between 8 to 16 void volumes (see Figure 1). However, when the same concentration of $^{45}$CaCl$_2$ is mixed with undiluted victorin and chromatographed on a Sephadex column, at least 80 percent of the $^{45}$Ca$^{2+}$ elutes at 1.5 to 2.0 void volumes, i.e., at the elution point for victorin (see Figure 2). The calcium content at the peak of victorin activity is about 35 to 45 PPM. Part of the calcium eluting with victorin is unlabeled and comes from calcium present in partially-purified victorin samples. When a solution of inactivated victorin containing $^{45}$Ca$^{2+}$ is chromatographed, less than 4 percent of the radioactivity applied to the column is associated with the victorin elution point (i.e., 1.5 to 2.0 void volumes).
Table 2: Influence of Victorin on $^{14}$C-IAA Binding to Membrane Preparations from Oat Coleoptiles

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>% Total Binding$^a$</th>
<th>% Specific Binding with 1:25 Victorin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible Tissue</td>
<td>1</td>
<td>4.84 ± 0.08</td>
<td>0.15 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.76 ± 0.10</td>
<td>0.06 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.92 ± 0.06</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>m ± S.D.</td>
<td>4.84 ± 0.08</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.06 ± 0.06</td>
<td>-0.01 ± 0.10</td>
</tr>
<tr>
<td>Resistant Tissue</td>
<td>2</td>
<td>4.20 ± 0.08</td>
<td>0.03 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.14 ± 0.22</td>
<td>-0.25 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>m ± S.D.</td>
<td>4.13 ± 0.07</td>
<td>-0.08 ± 0.15</td>
</tr>
</tbody>
</table>

$^a$Total binding was higher in these experiments than in those of Table 1 since the initial centrifugation in these experiments was 2000 x g instead of 12,100 x g.
Figure 1. Activity of Samples Collected from a Sephadex Column Loaded with $^{45}\text{Ca}^{2+}$. Two ml of $7.5 \times 10^{-7}$ M $^{45}\text{CaCl}_2$ solution was applied to a 20 x 1.5 cm Sephadex G-15 column and developed with 0.02 M NaCl. The bar represents the elution point for victorin.
Figure 2. Analysis of Samples Collected from a Sephadex Column Loaded with $^{45}$Ca$^{+2}$ with Either Active or Inactivated Victorin.

Two ml of active or inactivated victorin containing $7.5 \times 10^{-7}$ M $^{45}$CaCl$_2$ was applied to a 20 x 1.5 cm Sephadex G-15 column and developed with 0.02 M NaCl. The bar represents the point at which victorin elutes from the column.

\(-\times-\times-\) = radioactivity of active victorin samples;
\(-\times-\times-\) = radioactivity of inactivated victorin samples;
\(-\bullet-\bullet-\) = PMM Ca$^{+2}$ associated with active victorin samples;
\(-\circ-\circ-\) = PMM Ca$^{+2}$ associated with inactivated victorin samples.
Figure 2: Activity (CPM/ML) × 10^-4 vs. Void Volumes
binding of $^{45}$Ca$^{+2}$-victorin complex to membrane preparations from Avena coleoptiles.

The peak radioactive sample from a Sephadex column loaded with victorin and $^{45}$Ca$^{+2}$ contained high victorin activity and was used in all victorin-binding experiments. Table 3 shows that the total binding of radioactive victorin does not differ significantly ($P \geq 0.10$) in the final homogenates of susceptible (17.08%) and resistant (17.21%) tissue. Unlabeled CaCl$_2$ (10$^{-4}$ M) reduces the binding of radioactive victorin by 4.30 and 4.43 percent in membrane preparations from susceptible and resistant tissue respectively. In a representative experiment, the CFM in the pellets were about 600 and 450 in the absence and presence of 10$^{-4}$ M CaCl$_2$. This effect is significant, $P \leq 0.05$. However, the percent binding of radioactive victorin in the presence of 10$^{-4}$ M CaCl$_2$ did not differ significantly ($P \geq 0.10$) between susceptible and resistant tissue, i.e., Ca$^{+2}$ has no selective effect on the binding of labeled victorin in the two cultivars.

Table 4 shows that a high concentration of unlabeled victorin reduces the binding of radioactive victorin in susceptible and resistant tissue homogenates by 7.19 and 7.73 percent, respectively. These effects are significant ($P \leq 0.01$). However, the percent binding of radioactive victorin in the presence of high concentrations of victorin is about the same in susceptible and resistant tissue homogenates.

Since unlabeled victorin samples contain relatively large concentrations of calcium even after desalting by column chromatography, it seemed possible that the reduction in percent binding of the $^{45}$Ca$^{+2}$-victorin complex by unlabeled victorin was due to the calcium content.
Table 3: $^{45}$Ca$^{2+}$-Victorin Binding to Membrane Preparations from Oat Coleoptiles

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>% Total Binding$^a$</th>
<th>% Specific Binding$^a$ with $10^{-4}$ M Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>1</td>
<td>17.72 ± 1.79</td>
<td>3.86 ± 0.25</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>18.93 ± 1.61</td>
<td>5.14 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.59 ± 0.74</td>
<td>4.14 ± 0.36</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>17.08 ± 2.24</td>
<td>4.38 ± 0.67</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>15.82 ± 1.79</td>
<td>3.76 ± 0.57</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>17.95 ± 0.82</td>
<td>5.01 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.87 ± 1.48</td>
<td>4.51 ± 0.87</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>17.21 ± 1.21</td>
<td>4.43 ± 0.63</td>
</tr>
</tbody>
</table>

$^a$ Binding percents based on tissue fresh weight is the standard method used to present binding data and is the one used in this paper (see Materials and Methods). The large standard deviations for any set of binding experiments presented in this paper are considerably reduced when the binding data is based on the pelletable protein content of the final homogenate.
Table 4: $^{45}\text{Ca}^{2+}$-Victorin Binding to Membrane Preparations from Oat Coleoptiles.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>% Total Binding</th>
<th>% Specific Binding with 1:25 Victorin</th>
<th>Expected Specific Binding % Based on Ca$^{2+}$ Content of Victorin Samples</th>
<th>% Specific Binding with Deactivated 1:25 Victorin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>1</td>
<td>17.72 ± 1.79</td>
<td>7.33 ± 0.47</td>
<td>2.6</td>
<td>1.42 ± 0.40</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>18.93 ± 1.61</td>
<td>8.16 ± 0.28</td>
<td>2.7</td>
<td>1.46 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.59 ± 0.74</td>
<td>6.09 ± 0.64</td>
<td>1.4</td>
<td>1.48 ± 0.92</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>17.08 ± 2.24</td>
<td>7.19 ± 1.04</td>
<td>2.2 ± 0.7</td>
<td>1.45 ± 0.03</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>15.82 ± 1.79</td>
<td>6.68 ± 0.79</td>
<td>2.8</td>
<td>1.35 ± 0.15</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>17.95 ± 0.82</td>
<td>8.75 ± 0.49</td>
<td>3.5</td>
<td>1.42 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.87 ± 1.48</td>
<td>7.76 ± 0.11</td>
<td>3.1</td>
<td>1.69 ± 0.84</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>17.22 ± 1.20</td>
<td>7.73 ± 1.03</td>
<td>3.1 ± 0.4</td>
<td>1.49 ± 0.18</td>
</tr>
</tbody>
</table>
of the partially-purified victorin samples. Therefore, binding experiments using a constant concentration of $^{45}\text{Ca}^{+2}$-victorin but varying concentrations of calcium ($0, 10^{-5}, 5 \times 10^{-5}, 10^{-4}$ M) were performed to determine the dependence of $^{45}\text{Ca}^{+2}$-victorin binding on the concentration of unlabeled Ca$^{+2}$. Results of such tests (data not shown) indicated that the concentration of calcium (between 6.3 and 6.8 x $10^{-5}$ M) that would be established in the membrane preparation by the addition of unlabeled victorin should reduce the binding of the $^{45}\text{Ca}^{+2}$-victorin complex by only 2.2 and 3.1 percent in susceptible and resistant tissue homogenates, respectively, i.e., the inhibition of binding of the $^{45}\text{Ca}^{+2}$-victorin complex by unlabeled victorin cannot be accounted for totally on the basis of calcium contamination of the unlabeled victorin preparations. It was also observed that neither inactivated victorin (see Table 4) nor IAA (data not shown) significantly reduces $^{45}\text{Ca}^{+2}$-victorin binding in susceptible or resistant tissue homogenates.

### Binding of $^{45}\text{Ca}^{+2}$ to Membrane Preparations from Avena Coleoptiles

The total binding of $^{45}\text{Ca}^{+2}$ when supplied at $10^{-7}$ M was found to be 31.58 and 29.16 percent in the final homogenates of susceptible and resistant tissue, respectively (Table 5). In the presence of either a high ($10^{-3}$ M) or a near-saturating ($10^{-2}$ M) concentration of unlabeled CaCl$_2$, the binding of $^{45}\text{Ca}^{+2}$ was reduced to about 1.1 to 4.2 percent in preparations from both susceptible and resistant tissues. Thus, neither the percent total binding of $^{45}\text{Ca}^{+2}$ nor the effect of unlabeled calcium on the binding of $^{45}\text{Ca}^{+2}$ differ significantly in membrane preparations from susceptible as compared to resistant tissue. However,
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>% Total Ca$^{+2}$ Binding</th>
<th>% Specific Binding with $10^{-3}$ M Ca$^{+2}$</th>
<th>% Specific Binding with $10^{-2}$ M Ca$^{+2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>1</td>
<td>35.97 ± 2.32</td>
<td>31.63 ± 0.12</td>
<td>34.84 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32.16 ± 1.65</td>
<td>27.07 ± 0.17</td>
<td>30.96 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.60 ± 2.24</td>
<td>23.24 ± 0.13</td>
<td>25.50 ± 0.06</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>31.58 ± 4.71</td>
<td>27.31 ± 4.20</td>
<td>30.43 ± 4.69</td>
</tr>
<tr>
<td>Resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>1</td>
<td>25.86 ± 4.25</td>
<td>21.50 ± 0.17</td>
<td>24.73 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31.11 ± 1.13</td>
<td>26.93 ± 0.01</td>
<td>30.01 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30.51 ± 1.28</td>
<td>26.11 ± 0.14</td>
<td>29.39 ± 0.05</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>29.16 ± 2.87</td>
<td>24.85 ± 2.93</td>
<td>28.04 ± 2.89</td>
</tr>
</tbody>
</table>
this observation does not necessarily indicate that susceptible and resistant tissue have the same number or type of calcium binding sites. In order to determine the number of calcium binding sites as well as the \( K_m \) and concentration of each type of site, data from experiments in which the amount of labeled calcium was held constant while the concentration of unlabeled calcium was varied were plotted on a Scatchard plot (19, 48). This involves plotting the data for specific binding as moles bound/molarity free vs moles bound as shown in Figure 3 for resistant tissue. In such graphs, each linear phase represents a separate class of calcium binding site with the slope of the straight line representing the \( K_m \) value for that binding site and the intercept on the abscissa representing the number of moles of each class of calcium binding site per mg pelletable protein in the membrane preparation. Results from Scatchard plots indicate that there are at least 2 distinct classes of binding site for calcium in both susceptible and resistant Avena coleoptile tissue. The value for the \( K_m \)'s and the number of binding sites for each class of calcium binding site are presented in Table 6 and 7. Preparations from susceptible and resistant tissue show a small number of high affinity calcium binding sites with half maximal saturation at 2.09 to 0.68 \( \times 10^{-6} \) M respectively (Table 6), and a larger number of low affinity calcium binding sites with half maximal saturation at 7.20 to 7.71 \( \times 10^{-5} \) M, respectively (Table 7). The affinity for Ca\(^{+2}\) and the number of binding sites for Ca\(^{+2}\) for the high affinity binding site are significantly different (\( P \leq 0.01 \)) in susceptible and resistant homogenates. The high affinity binding site in the final homogenate of susceptible tissue has about a 3.1-fold lower affinity
Figure 3. Scatchard Plot of Specific Binding Data for Calcium in Resistant Tissue. The curve has 2 linear phases indicating 2 classes of Ca\textsuperscript{2+}-binding site. The slope of each straight line represents the $K_m$ value for that binding site and the intercept on the abscissa represents the number of moles per mg pelletable protein of each class of Ca\textsuperscript{2+}-binding site.
Table 6: Scatchard Plot Data for High Affinity Ca\(^{2+}\) Binding Sites

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>(K_m \times 10^{16} , (M))</th>
<th>No. of Binding sites (\times 10^{19}) (Moles/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>1</td>
<td>1.76</td>
<td>5.4</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>2.40</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.10</td>
<td>5.4</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>2.09 ± 0.32</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>0.87</td>
<td>1.4</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>0.76</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.40</td>
<td>2.2</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>0.68 ± 0.25</td>
<td>2.1 ± 0.6</td>
</tr>
</tbody>
</table>
### Table 7: Scatchard Plot Data for Low Affinity Ca$^{+2}$ Binding Sites

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>$K_m \times 10^{+5}$ (M)</th>
<th>No. of Binding Sites $\times 10^{+7}$ (Moles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>1</td>
<td>7.17</td>
<td>1.92</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>7.25</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.19</td>
<td>1.60</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>7.20 ± 0.04</td>
<td>1.66 ± 0.23</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>6.24</td>
<td>1.87</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>9.27</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.61</td>
<td>1.61</td>
</tr>
<tr>
<td>m ± S.D.</td>
<td></td>
<td>7.71 ± 1.52</td>
<td>1.86 ± 0.25</td>
</tr>
</tbody>
</table>
for Ca^{+2} and is present at about a 2.5-fold greater concentration than for Ca^{+2} and is present at about a 2.5-fold greater concentration than the high affinity site in the final homogenate of resistant tissue binding site, on the other hand, do not differ significantly (P \geq 0.10) between susceptible and resistant tissue homogenates (Table 7). In some experiments, there was evidence of a second low affinity Ca^{+2} binding site with a K_m value of about 2 \times 10^{-4} M.

**Calcium Content of Susceptible and Resistant Avena Coleoptiles**

Table 8 contains data on the calcium content of oat coleoptiles of susceptible and resistant seedlings grown under calcium-deficient conditions. Resistant tissue was found to consistently contain significantly (P \leq 0.01) higher total, TCA-soluble, and TCA-insoluble levels of calcium than susceptible tissue.

**DISCUSSION**

Radioactive victorin, calcium, and IAA bind reversibly and specifically to membrane preparations from both victorin-susceptible and victorin-resistant oat coleoptiles. Physiological evidence, such as rapidity of effects (7, 11, 46), electrical potential changes (9, 15, 20, 39), and ultrastructural changes in the appearance of the plasma membrane (18, 33, 38) strongly suggest that the sites of action of IAA, calcium, and victorin may be at or near the cell surface (e.g., the plasma membrane). Further evidence concerning the location of IAA, calcium, and victorin receptors must await the development of procedures that allow experimentation with highly-purified membrane preparations.
Table 8: Calcium Content of Oat Coleoptile Tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Expt. No.</th>
<th>Total Ca(^{+2}) (PPM Ca(^{+2})/g Dry Wt.)</th>
<th>TCA-soluble Ca(^{+2}) (PPM Ca(^{+2})/g Dry Wt.)</th>
<th>TCA-insoluble Ca(^{+2}) (PPM Ca(^{+2})/g Dry Wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible Tissue</td>
<td>1</td>
<td>776.9 ± 13.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>679.3 ± 36.9</td>
<td>548.5 ± 11.8</td>
<td>130.6 ± 31.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>830.1 ± 37.6</td>
<td>605.5 ± 12.0</td>
<td>224.6 ± 32.5</td>
</tr>
<tr>
<td></td>
<td>m ± S.D.</td>
<td>762.1 ± 76.5</td>
<td>577.0 ± 40.3</td>
<td>177.7 ± 66.3</td>
</tr>
<tr>
<td>Resistant Tissue</td>
<td>1</td>
<td>895.6 ± 12.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1117.8 ± 64.4</td>
<td>767.2 ± 13.6</td>
<td>350.6 ± 55.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1061.3 ± 11.3</td>
<td>728.2 ± 33.6</td>
<td>313.1 ± 37.5</td>
</tr>
<tr>
<td></td>
<td>m ± S.D.</td>
<td>1024.9 ± 115.5</td>
<td>747.7 ± 27.6</td>
<td>331.9 ± 26.5</td>
</tr>
</tbody>
</table>
Data presented in this study show that victorin does not interfere with auxin binding or vice versa (i.e., victorin probably binds to a site different from the IAA receptor). Other work in this laboratory (Saftnner, Vesper, unpublished) has shown that auxin does not interfere with calcium binding, suggesting that the calcium and auxin receptors are also different.

Since $^{45}\text{Ca}^{+2}$ elutes with victorin, partially-purified victorin samples most probably bind $\text{Ca}^{+2}$. Inactivated victorin samples do not substantially bind $\text{Ca}^{+2}$. Since inactivated victorin samples may contain the same contaminants as active victorin samples, and since it seems unlikely that partially-purified victorin samples would contain a $\text{Ca}^{+2}$-binding agent other than victorin which would elute at the same point as victorin, we conclude that victorin binds $\text{Ca}^{+2}$.

One might suggest that the apparent binding of $^{45}\text{Ca}^{+2}$-labeled victorin in these experiments is in fact simply the binding of $^{45}\text{Ca}^{+2}$ which has dissociated from the $^{45}\text{Ca}^{+2}$-victorin complex. If this hypothesis were correct, one would expect to find the same relative percent total binding using $^{45}\text{Ca}^{+2}$ as is observed using $^{45}\text{Ca}^{+2}$-victorin samples. However, the total binding of $^{45}\text{Ca}^{+2}$ is nearly double the total binding of $^{45}\text{Ca}^{+2}$-victorin samples in the final homogenates of both susceptible and resistant tissue. These results would better support the interpretation that the calcium-victorin complex specifically and reversibly binds to membranes from both susceptible and resistant oat coleoptiles. This interpretation, based on the binding data of this paper, is contrary to the hypothesis that resistance is based on the lack of a receptor (50) or on the presence of a receptor with a lower affinity for the toxin (14).
Physiological evidence shows that victorin has a drastic and almost immediate effect on the plasma membranes of susceptible but not resistant tissues (15, 18, 31, 39, 41, 45, 46, 47, 51). If a calcium-victorin complex does bind equally well to membranes from susceptible and resistant tissue, why does victorin cause drastic effects only in susceptible tissue? Doupnik (5) suggested that calcium may play an important role in victorin-induced disease by showing that victorin-induced permeability changes, leaf discoloration, and wilting were suppressed by 0.1 M CaCl₂ treatment. Earlier Hanchey et al. (18) noted similarities between victorin-induced ultrastructural changes in root cells of oats and the submicroscopic changes reported by Marinos (33) in calcium-deficient cells of barley. Furthermore, Luke and Barnett (30) reported that calcium deficiency causes electrolyte leakage in susceptible tissue but not in resistant tissue. Based on this evidence, it seems likely that the selective effects of victorin may be mediated by calcium.

Our calcium-binding experiments indicate that there are at least 2 classes of calcium-binding site in both susceptible and resistant tissue. At the low affinity calcium-binding site, the Kₘ and the total number of binding sites for Ca²⁺ are not significantly (P ≥ 0.10) different in the final homogenate from susceptible and resistant tissue. The Kₘ value for this low affinity, calcium-binding site in the final homogenate from susceptible and resistant tissue agrees quite well with the Kₘ value of 10⁻⁴ M reported by Epstein (7) for the effect of Ca²⁺ in maintaining the selectivity of the plasma membrane for various cations. At the high-affinity, calcium-binding site, the Kₘ value and the total number of binding sites for calcium are significantly higher in homogenates
of susceptible tissue than in resistant tissue. Although the point at which calcium becomes deficient in various plant tissues is not clearly defined (8), a general approximation of $10^{-7}$ to $10^{-6}$ M can be estimated. The high affinity class of calcium binding sites might, therefore, be associated with maintenance of conformation and functional integrity of the plasma membrane.

Based on physiological evidence and the binding data presented here, the following working hypothesis for the selective effect of victorin on susceptible and resistant cultivars of *Avena* is suggested. With the high affinity of victorin for Ca$^{+2}$, one would expect the toxin to bind Ca$^{+2}$ *in vivo*. My data indicate that the Ca$^{+2}$-victorin complex would then bind to membranes of both susceptible and resistant plants. Since resistant plants contain more calcium than susceptible plants and have a calcium-binding site of a higher affinity than susceptible plants, one might expect the majority of these sites to remain occupied in spite of calcium complexing by victorin so that normal membrane conformation and integrity could be maintained, and the toxic effects of victorin avoided. In susceptible tissue, which has a lower initial level of Ca$^{+2}$, sequestering of Ca$^{+2}$ by victorin may reduce the level of free Ca$^{+2}$ to the extent that there would be insufficient calcium to occupy a substantial fraction of the relatively large number of high-affinity, calcium-binding sites on the plasma membrane. Hence, normal membrane function would be impaired and the effects of the toxin would be manifested. Alternatively, if the level of free Ca$^{+2}$ in toxin-treated, susceptible tissue were great
enough to saturate the high affinity sites in that tissue, it is possible that the calcium-victorin complex may be more effective in displacing Ca$^{+2}$ from binding sites in susceptible than in resistant tissue since the binding site in susceptible tissue has a lower affinity for Ca$^{+2}$. In either case, the binding of Ca$^{+2}$ to the high-affinity binding site in susceptible tissue may be insufficient to maintain plasma membrane conformation and integrity, resulting in electrolyte leakage, decrease in plasma membrane potential, and other membrane-related toxic effects attributed to victorin.
USE OF pH MICROELECTRODES IN THE STUDY OF AUXIN- AND FUSICOCCIN-INDUCED H⁺-ION SECRETION INTO THE CELL WALLS OF OAT COLEOPTILE TISSUE.

INTRODUCTION

During the past several years, there has been considerable interest in a possible relationship between auxin, low pH, and growth (11). When coleoptile or stem segments are treated with acidic buffers, there is a latent period of a minute or less and then the rate of elongation increases suddenly to a value comparable to that induced by optimal levels of auxin (1, 2, 10, 11, 12, 17, 42, 43). The acid and auxin response are similar in terms of the growth rate (43), and effects on wall extensibility (1, 37, 43), but dissimilar in that various metabolic inhibitors prevent the auxin response but not the acid response (10, 12). It has also been found that acid solutions, but not auxin, will induce increased cell wall extensibility in frozen-thawed tissue segments (1, 44). Furthermore, acid-induced and auxin-induced elongation differ in that acid-promoted elongation declines after about an hour while auxin-promoted elongation continues at an optimal rate for 4 hr or longer, i.e., the acid response is short-lived relative to the auxin response (11, Evans, personal communication).

Hager et al. (17) showed that basic solutions inhibit auxin-induced growth and that carbonylcyanide m-chlorophenylhydrazone (CCCP),
which increases membrane permeability to protons, inhibits auxin-promoted growth rapidly (2 to 3 min) with no effect on respiration. They also demonstrated that 2 mM ATP acts to stimulate elongation under anaerobic conditions in which auxin itself is ineffective; this effect is enhanced by K⁺ and Mg²⁺ ions. Based on this and other evidence, these investigators suggested that auxin stimulates growth by activating an ATPase or proton pump driven by respiratory energy. Acidification of the wall resulting from the outward pumping of H⁺ ions was viewed as activating or increasing the activity of one or more cell wall loosening enzymes with a pH optimum in the acid range. There is corroborating evidence to suggest that ion pumps exist in plant cell membranes and that auxin can influence their activity (11). Furthermore, auxin treatment of coleoptile segments in an aqueous medium leads to acidification of the medium (4, 34, 35, 40, 42) from about 6.5 to about 5.0 during a subsequent 2 hr period. Since, in peeled coleoptile segments, a pH of 5.0 has been found to be optimal for growth promotion (23, 42), it has been suggested that auxin-induced H⁺-ion secretion may mediate the stimulation of elongation by auxin. However, published data indicate that the pH drop in the medium begins about 20 min after the addition of auxin while auxin action on growth begins within 15 min. If wall acidification is to be viewed as a possible mediator of auxin action on growth, it must be shown that acidification precedes growth stimulation, not vice versa. It has been pointed out (42) that if acidification of the medium begins in 20 min, it is likely that acidification of the cell wall region, which is immediately adjacent
to the source of protons, should occur earlier, perhaps well within the latent period of the auxin effect on elongation. Hence, published data on the timing of \( H^+ \)-ion secretion do not rule out acidification of the cell wall prior to auxin action on growth.

Commercially-available, pH microelectrodes have been used to measure pH in the cell wall and lumen of vessels of plant tissues treated with auxin. With the pH microelectrode (pH uB) positioned within a vessel, Penny et al. (40) were unable to detect any drop in the pH of auxin-induced oat coleoptiles until well after the increase in the elongation rate. In fact, after 60 min in auxin, they found only a very small drop (\(< 0.2 \) of a decade change in pH) in the pH of the vessel and thus suggested that an auxin-induced drop in pH was not the cause of accelerated growth. However, in their system the coleoptile segment was immersed in a large volume (600 ml) of aqueous medium which would be expected to dilute the \( H^+ \) ions secreted from the tissue so that no substantial pH drop in the medium would be expected. Furthermore, localized reductions in pH in the vicinity of the electrode tip would be resisted since the medium was continually circulated around the segment.

Hay and Jacobs (personal communication) have found that when a pH uB is positioned in the cell wall of coleoptile tissue being bathed in a small volume of aqueous medium, a pH drop sometimes occurs within 4 min after auxin treatment of the coleoptile, well within the latent period for auxin action on growth. However, the outer diameter of the tips of commercially-available pH uB is rather large (1 to 5 u) and since coleoptile segments tend to grow onto the uB
placed in the cell wall, cell damage may readily occur leading to
leakage of the acidic cytoplasmic and vacuolar fluids into the cell
wall which could influence pH measurements.

Cleland (personal communication) has positioned a pH uB immediately
adjacent to the cell walls of a pooled coleoptile segment. He found
that an auxin-induced pH drop in the vicinity of the cell wall occurred
after 11 to 14 min, i.e., at the same approximate time that auxin
begins to promote growth. This observation would seem to support
the theory that auxin-induced growth and pH drop may be closely
correlated.

In this investigation, a highly sensitive pH uB was used that
was suitable for measuring pH in the cell wall of auxin-treated tissue,
i.e., a pH uB strong enough to penetrate between cell walls without
breaking, yet small enough (tip diameter < 600 Å) to penetrate growing
tissue without causing any apparent cellular damage. This pH uB
could then be used to study the possible relationship between auxin
[and other growth regulators such as victorin (23) and fusicoccin
(28, 34, 35)], low pH, and growth.

MATERIALS AND METHODS

Plant Material

Oat coleoptiles were obtained as described in Chapter 1. To
prepare coleoptile tissue for pH measurements, coleoptile segments
in the region 0.5 to 2.0 cm below the coleoptile tip were excised
from oat seedlings with a razor blade. Since the epidermis is a
major barrier to the diffusion of $H^+$ ions in *Avena* coleoptiles (4, 40, 42), part of the outer epidermis was removed from the coleoptile segment. After removal of the primary leaf from within the coleoptile, the basal end of each coleoptile segment was fastened with a silastic band to a 1.2 x 1.2 x 1.0 cm Lucite block. These blocks were then submerged in a volume (about 60 ml) of aqueous tissue medium (ATM: 1 mM KCl; a known concentration of NaCl at about 0.9 mM and a known concentration of NaOH at about 0.1 mM such that the final Na$^+$ ion concentration was 1 mM and the pH was 6.5) or double-distilled, deionized water. After a 1 hr equilibration period, one of the coleoptile segments was positioned horizontally in a 6 x 4 x 4 cm Lucite chamber (see Figure 4). Either ATM or double-distilled, deionized water was added to the chamber to submerge all but the upper horizontal surface of the coleoptile segment. Control tissue characteristics were then recorded for 20 min before the addition of $10^{-5}$ M 3-indoleacetic acid (IAA), $2.0 \times 10^{-5}$ M fusicoccin, or a 1:25 dilution of victorin. The pH in the cell wall vicinity was estimated by subtracting 5 mV from the total potential to account for the cell wall potential (5, Saftner, unpublished), then reading the observed pH from the standard pH curve for the pH uB used.

**Preparation of pH Microelectrodes**

Microelectrodes with $H^+$-ion sensitivity were prepared from Corning 0150 glass capillary tubing with an outer diameter of 0.99 mm. The glass tubing was made into uB with a microelectrode puller designed
Figure 4. Lucite Chamber Assembly Used in All pH Studies. The coleoptile segment was fastened at the basipetal end by a silastic band to the Lucite block. All pH uE penetrations were made at the acropetal end of the segment, which was in contact only with the ambient solution.
OUTLET

COLEOPTILE SEGMENT

SILASTIC BAND

LUCITE EXTENSION

LUCITE BLOCK

INLET

FIGURE 4
and made by Dr. P. B. Hollander. The pH were filled at room temperature by the following method also suggested by Dr. P. B. Hollander:
The pH were mounted on a Lucite holder (21) and placed vertically, tip downward, in a glass container connected to a vacuum source. The pH were immersed in absolute methanol and filled by evacuation before being transferred to double-distilled, deionized millipore-filtered water saturated with glass powder. After 2 days, the pH were transferred to a 0.25 M KCl solution. The pH pH were stored in this solution for at least 2 days before use. This method for filling pH avoids prolonged exposure to heat and vigorous shaking associated with other filling methods (13, 15, 20, 36) and thus reduces the possibility of tip damage.

Open-tipped microelectrodes have in theory at least 2 parallel channels determining the resulting potential difference. One is a passway through the pore at the pH tip and the other goes through the glass wall of the pH. Since the pH pH were made of low resistivity glass and filled with a 0.25 M KCl solution, the leak resistance through the glass wall of the pH is not negligible and thus cannot be neglected. Moreover, because this glass is pH sensitive and behaves as a cation-exchange membrane, the emf generated across the wall of the pH will fluctuate depending, in part, on the pH, the composition, and the activity of the solutions inside and outside the pH. Even if these 2 solutions are held constant, the wall potential, and thus the pH potential, would fluctuate depending on the geometry of the pH wall as well as the depth to which the pH is immersed in the outside solution: the wall potential is a function
of both the thickness and asymmetries of the glass wall and the surface area of the uB exposed to the outside solution (see reference 6). If all but the tip (perpendicular to the longitudinal axis of the uB) of the uB could be insulated from the outside solution, the uB potential would, in theory, depend primarily on the potential difference generated at the uB tip.

Although the uB have very small dimensions and a high surface tension, insulation of the outer glass surface of pH uB was established in several ways. One method was to dip the tip of the uB in Dow Corning 200 Silicone Fluid or in General Electric RTV-11 Fluid, a liquid silicone rubber. The Dow Corning 200-treated pH uB were used immediately while uB treated with RTV-11 were dipped in a solution of Nuodex, a catalyst for silicone resins (Tenneco Chemicals, Inc.), and held, tip upward, until the silicone rubber solidified (1 to 2 min). Another method tested to insulate the outer glass surface of the uB was to place all but the blunt end of the uB into a 1 percent (v/v) solution of Siliclad (pH about 8.5), a water-soluble silicone from Becton Dickinson and Co., at 40°C for either 2 or 60 min, and then allow to air dry for 24 hr. The uB were insulated twice in this manner before being filled. In some cases, the uB were washed with a 2 percent solution of either NaOH or HNO₃ for 2 min before being insulated with Siliclad. In other cases, a surfactant (2% Tween 80, 0.5% Photoflo or 0.1% Triton X-100 from Sigma Chemical Co.) was added to the Siliclad solution. In one set of experiments, the Siliclad treatment was performed on pH uB already filled with a 0.25 M KCl solution after which the uB were allowed to air dry for 24 hr.
before being placed in a sealed beaker containing a few drops of distilled water in order to maintain a water-saturated atmosphere. This method was utilized to prevent the Silicolad on the glass surface from gradually dissolving while in the uE storage solution. Micro-electrodes were also electroplated with alumina using conventional techniques common in thin-film transistor technology.

The uE were calibrated for pH measurements by attaching the electrode as described below to an oscilloscope and lowering the uE with the aid of a micro-manipulator until the tip of the electrode made contact with various buffer solutions (pH of the standard buffers: 9.18; 7.0; 6.47; 6.0; 5.0; 4.63; 4.0; and 3.0) and recording the potential directly from the oscilloscope. In order to determine what effect, if any, the wall potential has on the uE potential, the pH uE was then lowered 1 mm into the solution and the potential again recorded from the oscilloscope. From these data, a standard pH curve was plotted for each pH uE. The calibration of pH uE was done before and after each experiment. With the pH uE just making contact with a solution, the resistance of the pH uE varied between about 20 and 1400 meg ohms (M-Ω-) depending primarily upon the method by which the uE were fabricated. The data from uE prepared as stated above were compared to a random sampling of commercially-available, pH electrodes (Beckman, Fisher and Corning).

Recording Assembly

The recording assembly for making pH measurements was similar to the one described by Hollander and Sakai (22). Briefly, a pH uE
was connected in series with a Corning calomel electrode (475017) assembly, an Instrumentation Laboratory 181 impedance matching device and one channel of a Tektronix 565 dual-beam oscilloscope. Another calomel cell, placed in contact with the aqueous medium, was connected in series with a Bricelectric CA5 voltage-duration calibration unit and completed the ground return of the system.

**Location of the pH Microelectrode**

The general location of the pH uB was observed visually through a Bausch and Lomb dissecting scope with 33x oculars and adjustable to a magnification of about 20 to 400x. For a more detailed location of the pH uB within the plant tissue, pH uB were filled with 4 percent Niagara Sky Blue 6B (C.I. 24410) solution instead of 0.25 M KCl. With the tip of the uB in the plant tissue, a positive current (about 1 uA in 100 m sec pulses) was passed through the uB causing the ejection of the dye into the plant tissue. Immediately after ejection, the location of the dye within the plant tissue was observed through a Spencer microscope at about 400x magnification (see references 16; 26).

**RESULTS**

**Characteristics of pH Microelectrodes**

The sensitivity of pH uB to H⁺ ions seems to depend on the resistance of the uB, i.e., the sensitivity of the pH uB generally increases with increased resistance of the uB up to about 1000 kΩ.
Figure 5. $H^+$-Ion Sensitivity vs. Resistance of pH Microelectrodes Used in This Investigation. The curve was plotted using the least square regression technique. Each point indicates the result of one electrode.
Figure 6. Standard pH Curve for a pH Microelectrode Insulated with Dow Corning 200 Fluid. The resistance of this electrode was about 1280 MΩ and had a H⁺-ion sensitivity of about 60 mV per decade change in pH.
FIGURE 6
at which point uB with a higher resistance do not show any further
H\textsuperscript{+}-ion sensitivity (Figure 5).

Figure 6 shows a standard pH curve for a pH uB insulated with
Dow Corning 200 Fluid. In any one of the pH buffer solutions, the
potential measurement is generally the same whether the uB tip has
just made contact with the buffer solution or is 1 mm deeper in the
solution, i.e., the outer wall of the pH uB is insulated from the
outer solution and thus the uB potential does not fluctuate as the
uB is lowered into the solution. Also the H\textsuperscript{+}-ion sensitivity of these
pH uB was found to be nearly linear over the pH range 3 to 9. These
results verify the findings of P. B. Hollander (personal communication)
using pH uB fabricated in the same manner. In most cases, these
pH uB will not reproduce the initial calibration numbers unless
reinsulated. In some cases, potential measurements with these uB
may fluctuate after about 30 min unless they are reinsulated (see
the discussion below for a possible explanation of these findings).

Microelectrodes insulated with RTV-11 were either not sensitive
or had a low sensitivity (13 mV/pH unit) for H\textsuperscript{+} ions. When pH uB
of this type made contact with a buffer solution, the potential measure-
ment would not stabilize for at least 6 min. Subsequent exposures
to other solutions were accompanied by further fluctuations requiring
more than 6 min for stabilization. Since RTV-11-treated pH uB respond
slowly to changes in pH, they would not be suitable to study the
kinetics of an auxin-induced pH drop in plant tissue and thus were
not used in any pH experiments using coleoptile tissue.
Figure 7 shows a standard pH curve for a pH uB insulated twice for 1 hr in a 1 percent solution of Siliclad. Microelectrodes insulated with Siliclad were observed to respond linearly to $H^+$ ions over the pH range 3 to 7. However, these pH uB always showed some depth sensitivity, i.e., the output of the electrode with the tip 1 mm into a buffer solution was 10 to 25 mV greater than that of an electrode just penetrating the surface of the same solution. However, the depth sensitivity of these pH uB was relatively small and constant over the pH range 3 to 7, i.e., these uB could be used to monitor accurately relative changes in pH in plant tissue even though the measured pH value at the electrode tip may be in error by as much as 0.2 pH unit due to the depth sensitivity of these electrodes.

There was no apparent difference in pH uB treated for 2 min as opposed to 60 min with Siliclad. The addition of surfactants (2% Tween 80, 0.5% Photoflo, or 0.1% Triton X-100) to the Siliclad solution had undesirable results in that the surfactant treatment did not decrease the depth sensitivity of the pH uB insulated with Siliclad. Furthermore, pH uB treated with Tween 80-Siliclad had an unacceptably low $H^+$-ion sensitivity ($\sim 10$ mV per decade change in pH) even though their resistance was relatively high ($>750$ M$\Omega$). Microelectrodes plated with alumina for insulation also proved to be depth sensitive and difficult to use.

The pH uB used in this investigation which had a resistance $>750$ M$\Omega$, (see Figure 5, 6, 7) were comparable to certain commercial pH electrodes available for general laboratory use (see Figure
Figure 7. Standard pH Curve for a pH Microelectrode Insulated with Siliclad. The resistance of this electrode was about 760 MΩ and had a H⁺-ion sensitivity of about 56 mV/pH unit. (———) = potential when the uE had just made contact with the pH buffer; (———) = potential when the uE was positioned one mm deeper in the pH buffer.
FIGURE 7

The graph shows a relationship between pH and mV, with mV decreasing as pH increases from 3 to 9.
Figure 8. Standard pH Curves for Some Commercially-available pH Electrodes.
FIGURE 8

BECKMAN 53210
R = 480 MΩ
m = 55 mV/pH UNIT

FISHER 13-639-90
R = 660 MΩ
m = 55 mV/pH UNIT

CORNING 476022
R = 200 MΩ
m = 54 mV/pH UNIT
Both the pH uB and commercial pH electrodes tested have a high H⁺-ion sensitivity (m ≈ 55 mV per decade change in pH) and, in general, respond linearly to pH over the range 3 to 9. However, the pH uB insulated with Siliclad or Dow Corning 200 Fluid equilibrate to changes in pH much more rapidly than the commercial pH electrodes tested in this investigation, i.e., 0.1 ms vs. 1 to 3 min for pH uB and commercial pH electrodes, respectively.

Based on these and other data, I settled on pH uB insulated with either Dow Corning 200 Fluid or Siliclad for investigating the possible relationship between auxin, fusicoxin, low pH, and growth in coleoptile tissue. These pH uB have the following characteristics: (1) high H⁺-ion sensitivity when their resistance is > 750 MΩ, (2) linear response to pH over the range 3 to 9, (3) a low or zero depth sensitivity, (4) quick response time (< 0.1 ms), (5) small size (outer tip diameter < 600 Å), (6) strength to allow penetration through or between plant cell walls, and (7) simplicity in fabrication and use. The pH uB insulated with Siliclad have the additional characteristics of being more stable with time and less difficult to use than pH uB insulated with Dow Corning 200 Fluid. Dow Corning 200 Fluid tended in some experiments, to leak off the uB into the solution bathing the plant tissue. However, pH uB insulated with Dow Corning 200 Fluid were used in preference to pH uB insulated with Siliclad since the preparation of the former is much simpler and the resulting microelectrode is less depth sensitive.
Location of the pH Microelectrode in the Plant Tissue

Direct observation of pH uB penetrations into coleoptile tissue show entry between the transverse walls of parenchymal cells. The uB was withdrawn from the tissue whenever penetration of a plant cell seemed to have occurred, i.e., penetrations in which there was a collapse of the radial walls from straight to concave, a rapid movement of fluids into the tip of the uB (see reference 16), a large change in potential (\( > 10 \text{ mV} \)), and a movement of fluids away from the vicinity of the uB tip. In the initial experiments, when a pH uB filled with Niagara Sky Blue 6B and having a resistance between 60 and 280 M\( \Omega \) appeared by microscopic examination to be located intercellularly, its position was confirmed using iontophoretically-ejected dye which appeared in the cell wall vicinity and was quickly diluted by surrounding fluids. No dye was observed intracellularly except in cases where penetration of a plant cell seemed to have occurred.

Effect of IAA and Fusicoecin on Cell Wall pH

Preliminary evidence indicated that an IAA-induced acidification of the cell wall began at about 21 min after IAA treatment of the coleoptile segment (Figure 9). Earlier (\(< 8 \text{ min}\)) pH drops in the cell walls of auxin-treated tissue occurred only when there was cellular damage caused by penetration of the uB into the tissue. An auxin-induced pH drop in the cell wall was never observed between 11 and 14 min after auxin treatment of the coleoptile segment, i.e., at the time Cleland (see Introduction) first observed an auxin-induced
Figure 9. Time Course of Auxin-Induced Acidification of the Cell Walls of Partially-peeled, Coleoptile Tissue. Results are for 3 experiments, A, B, and C. In each experiment, the tip of a pH microelectrode insulated with Dow Corning 200 Fluid was positioned in the transverse cell wall of a parenchymal cell of coleoptile tissue equilibrated for 1 hr in demineralized, double-distilled water. The cell wall pH was monitored for 20 min before exposing the tissue to a $10^{-5}$ M IAA solution at the arrow.
Figure 9

Time after addition of IAA to the tissue (min)

pH of the cell wall
pH drop in the cell walls of auxin-treated coleoptiles. Figure 9 further shows that the rate of auxin-induced pH drop varied from experiment to experiment and in only 1 experiment did the pH in the cell wall drop below 6.0 after 50 min of IAA treatment. However, the auxin-treated tissue was in contact with a large volume (~60 ml) which may have diluted the H\(^+\) ions being secreted into the cell walls. Therefore, the auxin-induced pH drop measured in vitro by the method used in this investigation may be substantially less than what occurs in vivo.

Fusicoecin, on the other hand, caused an acidification of the cell wall from 6.4 to 5.0 within 10 min after the tissue was treated with the pathotoxin (Figure 10). Furthermore, the fusicoecin-induced pH drop in the cell wall was detectable within 1 min after fusicoecin was applied to the tissue.

DISCUSSION

The resistance of open-tipped uE is a function of the tip diameter and the included angle (angle of taper of the electrode tip); the surface charge of the glass of which the electrode is made; and the concentration, composition, and the pH of the solution inside the uE and surrounding the uninsulated tip region of the uE (49, Hollander, personal communication). The pH uE used in this laboratory show increased H\(^+\)-ion sensitivity with increased resistance of the uE up to about 1000 MΩ, beyond which point sensitivity does not increase with a further increase in resistance. It should be noted
Figure 10. Time Course of Fusicoccin-Induced Acidification of the Cell Walls of Partially-peeled, Coleoptile Tissue. A pH microelectrode insulated with Dow Corning 200 Fluid was positioned in the transverse cell wall of a parenchymal cell of coleoptile tissue equilibrated for 1 hr in demineralized, double-distilled water. The cell wall pH was monitored for 20 min before exposing the tissue to $2.0 \times 10^{-5}$ M fusicoccin solution at the arrow.
Figure 10

Time after addition of fusicoecin to the tissue (min)

pH of the cell wall
that in this study the pH uB, with a resistance of about 1000 Hm or higher had optimal H⁺-ion sensitivity (about 60 mV per decade change in pH) as suggested by the Nernst equation \[ V_H = \frac{RT}{F} \ln \frac{H_0}{H_1} \]
where \( V_H \) is the potential (mV); \( R \), the gas constant (8.314 joules/degree/mole); \( T \), absolute temperature; \( F \), Faraday (96,500 coulombs/mole); \( H_0 \), H⁺-ion concentration or activity outside; \( H_1 \), H⁺-ion concentration of activity inside.

The potentiometric responses associated with Corning 0150 pH glass may be explained as follows. It has been suggested that H⁺ ions permeate about 6 Å into the outer glass surface changing the crystalline structure and distorting the electric field of the glass such that the potential of the glass is changed (6) and is "seen" by the filling solution of the pH uB relative to the indifferent electrode. Based on this theory and the above data, we suggest that the range in resistance and H⁺-ion sensitivity of the pH uB may be a combination effect of tip diameter and the thickness and outer surface area of the uninsulated glass of the uB. Microelectrodes that have their outer glass surface completely insulated should have a resistance (and H⁺-ion sensitivity) dependent only on the tip and thus show a relatively constant resistance and H⁺-ion sensitivity as the uB is lowered to various depths into a buffered solution; microelectrodes insulated with Dow Corning 200 Fluid behaved in this manner. However, these electrodes became depth-sensitive with time or after tissue penetrations suggesting that the fluid was removed from the uB. On the other hand, pH uB insulated with Siliclad showed
a decrease in resistance and $H^+$-ion sensitivity as the uE was lowered about 5 mm into a buffer solution. This indicates that these pH uE were not ideally insulated. Since these electrodes did not quickly become increasingly depth-sensitive with time or after tissue penetrations, Silicolad may be more firmly attached to the electrode glass than Dow Corning 200 Fluid. Microelectrodes insulated with Silicolad-Tween 80 had a high resistance and a low $H^+$-ion sensitivity. The tip of these uE, when examined microscopically, appeared to be enveloped with a substance resembling Tween 80. The resulting occlusion of the electrode tip would explain the high resistance-low $H^+$-ion sensitivity of these electrodes. As for pH uE insulated with RTV-11, their slow response to $H^+$ ions could be due to ambient solutions leaking between the glass surface of the electrode and the RTV-11 creating a micro-solution associated with the $H^+$-ion-sensitive portion of the electrode which would gradually reach equilibrium with other solutions in which the uE was placed.

The effect of IAA and fusicoecin on cell wall pH was monitored with pH uE insulated with Dow Corning 200 Fluid and having a resistance of at least 750 MΩ. By direct observation and iontophoresis, it was confirmed that the tip of the microelectrode was in the cell wall vicinity of cells relatively undamaged by the penetration of the uE into the tissue. In Avena coleoptiles, auxin caused an acidification of the cell wall within 30 min after auxin treatment. This preliminary evidence supports the theory that auxin stimulates $H^+$-ion efflux from plant cells but does not add any evidence to suggest that auxin-induced acidification of the cell wall is essential for
auxin-induced growth. On the other hand, the cell wall region of
tissues treated with $2.0 \times 10^{-5}$ M fusicoccin becomes more acidic within
1 min after toxin treatment, i.e., before fusicoccin stimulates
growth. Furthermore, the kinetics of fusicoccin-induced pH drop
(see Figure 10) correlates quite well with the time course of
fusicoccin-induced growth promotion of coleoptile tissue. These
data are in full agreement with the hypothesis that the effect of
fusicoccin on growth is mediated by its capacity to induce acidifica-
tion in the cell wall leading to cell wall loosening and enhanced
growth.

In summary, pH uE made from Corning 0150 glass were used to
measure cell wall pH in *Avena* coleoptiles. Between pH 3 and 7, the
approximately linear output of these pH uE (with $R > 750 M \Omega$)
decreased by a mean of 55 mV per decade change in pH. These pH uE
with a tip diameter $< 600 \AA$ penetrate through or between cell walls
of growing plant tissue without causing any apparent cellular damage.
Data collected using these pH uE in coleoptile tissue confirms that
a acidification in the proximity of the cell wall was produced by
both auxin and fusicoccin treatment, but that, only in the case of
fusicoccin did acidification precede growth promotion. These results
suggest that, although $H^{+}$-ion secretion may accompany auxin-induced
growth, it cannot be viewed as the mediator of auxin-induced growth.
LIST OF REFERENCES


