Histochemical Evidence for the Occurrence of Oligomycin-sensitive Plasmalemma ATPase in Corn Roots

CARL P. MALONE, JOHN J. BURKE, AND JOHN B. HANSON
Department of Botany, University of Illinois, Urbana, Illinois 61801

ABSTRACT

A cytochemical study has been made on the localization of ATPase activity in corn (Zea mays L.) roots. Light microscopy shows washing for 4 hours to increase the general ATPase activity in the peripheral layers of the root cortex; oligomycin and N,N-dicyclohexylcarbodiimide inhibit this activity, oligomycin being more effective. Ultrastructural studies of ATPase location show oligomycin treatment to inhibit both mitochondrial and plasmalemma ATPase, but only in the epidermis and outer cortex. Studies with lipid-soluble dyes indicate that oligomycin might not penetrate very deeply into root tissue in the time span of these experiments. It is suggested that the strong inhibition of ion absorption by oligomycin without a corresponding decline in ATP content is probably due to inhibition of ion absorption in the peripheral cell layers, thus limiting the supply of ion for symplastic transport to the uninhibited tissues.

The discovery by Whittam et al. (29) that oligomycin can inhibit the [Na^+ -K^+]ATPase of animal membranes has led to a number of additional studies on the nature of the oligomycin inhibition of ion transport. In liver slices van Rossum (28) attributed the inhibition to blocking of oxidative phosphorylation and hence ATP supply. Bedsoe et al. (3) and Jacoby and Plessner (15) came to the same conclusion for plant roots. However, Atkinson et al. (1) came to slightly different conclusions. Hedges (14) initially believed that the inhibition of ion transport in oat roots was due to an effect on membrane transport ATPases, but in vitro no inhibition of the cation-stimulated ATPase of the plasmalemma fraction can be found (20, 21). Lin and Hanson (22) found that preincubation of corn root tissue for 2 hr in 1 μg/ml of oligomycin would produce 57% inhibition of phosphate uptake, but only 16% decline in tissue ATP; they suggested that this might be due to preferential binding to the cell surface, acting here as an inhibitor of phosphate absorption. We have made a histochemical study of the sites of ATPase activity in corn roots and the effect of oligomycin on this activity. Since DCCD2 has been reported to inhibit cation-stimulated plasmalemma ATPase activity (21), it was included for comparison in some experiments.

MATERIALS AND METHODS

One-cm root segments from 3-day-old etiolated corn seedlings (Zea mays L., WF9×Mo17) were cut (after removing the apical 0.5 mm) and washed in 0.2 mM CaCl2 as previously reported (19). After 4 hr, 2 μg/ml of oligomycin or 60 μM DCCD were added (maintaining a control with no additions), and the washing continued for an additional 2 hr. This treatment produced root tissue comparable to that used by Lin and Hanson (22). ATPase staining was done at this time.

Histochemical localization of ATPase activity was based on the widely used technique of precipitating the phosphate released by the dephosphorylation of ATP as an insoluble metal phosphate (5, 11–13, 24, 25). Several variations in the technique were used and the procedures are compared in Table I. The small segments used in methods 2, 3, and 5 were cut after washing (and pretreatment with oligomycin or DCCD) and stained as thin sections rather than as 1-cm segments. Washing and pretreatment were the same for all roots in methods 1 through 5. If the insoluble metal phosphate was Ca₃(PO₄)₂ then it was converted to Pb₃(PO₄)₂ by incubating the specimen in 1 mM Pb(NO₃)₂ for 10 min. If the specimens were to be observed by light microscopy, Pb₃(PO₄)₂ was converted to PbS by incubating specimens in 4% ammonium sulfide for 10 min. Specimens were rinsed in distilled H₂O (if not done previously, 250-μm free hand sections were cut at this time) and photographed. If the specimens were to be observed by electron microscopy, specimens were rinsed twice in buffer (see Table I) then postfixed in 2% OsO₄ in buffer for 1 hr. Specimens were rinsed three times in buffer and dehydrated in a graded ethyl alcohol series. Propylene oxide was used as a solvent for the Epon embedment.

Controls were maintained by incubating specimens in glucose-6-P or β-glycerophosphate-P at pH 7 in place of ATP, by completely omitting any phosphorous containing compound, or by omitting the capture agent.

RESULTS AND DISCUSSION

Light Microscopy. Freshly cut, unwashed root segments show a peripheral band of ATPase activity in the outer two to four cell layers of the cortex (Figs. 1, 5, and 9). The epidermal layer does not stain as intensely, and in some instances there appears to be little staining (e.g., Fig. 1). This staining pattern is in contrast to the high epidermal and low cortical activity reported in the root tip (11). It is possible that rinsing the tissue might have removed some of the precipitate from the surface, but we do not think this is a major factor, because the epidermal cells always show stain in washed tissue (see below). The other region of the root exhibiting dense stain was the stele. For this study we have not investigated the responses in the stele since the oligomycin does not appear to penetrate to it.

Washing consistently intensified the ATPase activity in the peripheral cortex (Figs. 2, 6, and 10). Occasionally it appeared that the region of high ATPase activity might extend deeper into the root with washing (cf. Figs. 9 and 10) but this was not consistent. From the histochemical data oligomycin pretreat-

---

1 Supported in part by Energy Research and Development Administration Contract E(11-1)-790 and National Science Foundation Grant PCM76-80886.
2 Abbreviation: DCCD: N,N-dicyclohexylcarbodiimide.
plant inhibited the ATPase activity of the outer cortex (Figs. 3, 7, and 11), as did DCCD (Figs. 4, 8, and 12).

Since the evaluation of histochemical data is subjective, an attempt was made to determine the phosphate released from formaldehyde-fixed root tissue by substituting 3 mm MgCl₂ and 50 mm KCl for 0.2 mm CaCl₂, increasing ATP to 3 mm, and determining inorganic phosphate (26). Washed roots liberated 7.2 × 10⁻⁸ g of P/mg dry wt·min while washed roots pretreated with 2 μg of oligomycin/ml liberated 5.6 × 10⁻⁸ g of P/mg dry wt·min (a 22% decrease). Thus, there is an oligomycin-sensitive component of ATPase activity detectable by this means. But activity is low, and the effect of omitting the Ca²⁺ trap is unknown.

In order to estimate any contribution of other phosphatases to the heavy peripheral staining, glucose-6-P and β-glycerol-P were substituted for ATP as substrate. Procedures were as in Figure 2. Scurcly any phosphatase activity could be detected with glucose-6-P (Fig. 13), and only a little epidermal activity with β-glycerol-P (Fig. 14, cf. Fig. 2); the epidermal β-glycerol activity has been reported by Hall (11). Comparison of Figures 2 and 14 indicates that most of the staining resulted from an oligomycin-inhibited ATPase. Although not shown here, there was no ATPase staining in the absence of ATP or the capture agent (Ca²⁺ or Pb²⁺).

To determine if the intense peripheral band of ATPase activity was an artifact due to failure of ATP to penetrate into the inner cortex (sections in Figs. 1–4 were cut from the center of 1-cm root segments after fixation and staining) 250-μm-thick sections were cut from 1-cm root segments, then fixed and stained (Figs. 5–8). Other than the difference in segment size at the time of fixation and staining, the tissue represented in Figures 1 through 4 and 5 through 8 is similar. This insured that the diffusion path length to the inner cortex was not limiting. With these thin sections the intensity of staining was reduced (possibly due to overfixation), but the peripheral localization was maintained. Clearly, there is more ATPase activity in the outer cortex than in the inner.

It has been observed that tissue fixation reduces ATPase activity. When unfixed 250-μm sections were used (Figs. 9–12) the same pattern of ATPase activity was found, but the intensity of staining appeared to be somewhat less (cf. Figs. 1–8). Our interpretation is that loss of ATPase activity due to fixation was more than offset by the improved penetration of ATP and capture agent into fixed cells.

Since oligomycin and DCCD are lipid-soluble, hydrophobic compounds, presumably they penetrate into the root by dissolving in the lipid phases of the cells they contact. In order to estimate how far lipid-soluble compounds might penetrate in the 2-hr pretreatment, we resorted to lipid-soluble dyes with differing solubilities in organic solvents (10). In 2 hr Sudan black B in aqueous solution scarcely penetrated beyond the epidermis (Fig. 15), while Sudan IV penetrated only two or three cell layers (Fig. 16). Judging from these dyes, neither oligomycin nor DCCD would be expected to penetrate very far beyond the surface of root segments.

Electron Microscopy. Although light microscopy was adequate to identify tissues with ATPase activity subject to oligomycin inhibition, ultrastructural investigations were needed for subcellular localization of the enzymes. Unlike the case with light microscopy, it was not possible to demonstrate ultrastructurally any increase in ATPase activity with washing. The electron micrographs shown here (Figs. 17–23) are from washed tissue.

Specimens prepared by method 4 (fixed in formaldehyde and incubated in ATP at 4 C for 20 min) show deposits resulting from ATPase activity to be associated with the plasmalemma, mitochondria (Fig. 17), ER, and plasmodesmata (Fig. 18). Controls incubated without ATP or without a capture agent showed no deposits (micrographs not shown). If the incubation in method 4 was increased to 45 min, the density of the deposits in the plasmalemma and mitochondria increased, but tonoplast deposits were never observed (Fig. 19). Failure to find tonoplast ATPase activity has been previously reported (6–8). The deposits formed in the mitochondria are like those previously reported (9, 17); the deposits are in or immediately inside the inner membrane, occasionally in the matrix, and nearly absent on the outer membrane. Plasma membrane deposits have also been previously reported (6–8, 12, 23).

Oligomycin-treated root segments incubated as in Figure 19 showed scant deposits along the plasmalemma or in mitochondria of outer cortical cells (Fig. 20). However, deeper in the same root ATPase activity could be shown, especially along the plasmalemma and within mitochondria (Fig. 21).

There are reports that mitochondrial ATPase is very sensitive to glutaraldehyde fixation, while formaldehyde fixation preserves more activity (5). Also, incubation at higher temperatures promotes nonenzymic dephosphorylation of nucleoside phosphates, and it is believed that plasma membrane staining can result simply from an affinity for phosphate or phosphate-containing compounds. However, it is also reported that plasma membrane staining occurs rapidly and that nonenzymic hydrolysis does not produce sufficient phosphate to account for the staining. Figures 22 and 23 are glutaraldehyde-fixed root segments incubated at 27 C. These results confirm that glutaraldehyde fixation inhibits the mitochondrial ATPase, but there are heavy wall deposits and the plasma membrane deposits can still be seen (Fig. 22). Oligomycin eliminated much of the plasma membrane deposit, but wall deposits are still present (Fig. 23).

The fact that the wall deposits are not observed when tissue is stained at 4 C (Figs. 17–21) has been used as an argument that

---

**Table 1. Comparison of Preparation Techniques**

<table>
<thead>
<tr>
<th>Method</th>
<th>Segment size</th>
<th>Fixative</th>
<th>Fixation time</th>
<th>Incubation temp</th>
<th>Incubation pH</th>
<th>Incubation time</th>
<th>Buffer</th>
<th>Capture agent</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 cm</td>
<td>Formaldehyde</td>
<td>20 min</td>
<td>27 C</td>
<td>6.8</td>
<td>45 to 123 min</td>
<td>50 mM cacodylate</td>
<td>0.2 M CaCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>2</td>
<td>250 μm</td>
<td>Formaldehyde</td>
<td>5 min</td>
<td>27 C</td>
<td>6.8</td>
<td>20 to 60 min</td>
<td>50 mM cacodylate</td>
<td>0.2 M CaCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>Formaldehyde</td>
<td>0</td>
<td>27 C</td>
<td>6.0</td>
<td>30 to 60 min</td>
<td>50 mM tris-</td>
<td>0.2 M CaCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>4</td>
<td>1 cm</td>
<td>Glutaraldehyde</td>
<td>20 min</td>
<td>4 C</td>
<td>7.4</td>
<td>25 to 60 min</td>
<td>50 mM cacodylate</td>
<td>2.4 M Pb(NO₃)₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>5</td>
<td>1 mm</td>
<td>Glutaraldehyde</td>
<td>30 min</td>
<td>27 C</td>
<td>7.4</td>
<td>45 min</td>
<td>50 mM cacodylate</td>
<td>1.0 M Pb(NO₃)₂</td>
<td>2 mM</td>
</tr>
</tbody>
</table>
Figs. 1-8. ATPase activity in 1-cm corn root segments fixed in formaldehyde and incubated 60 min for ATPase (see method 1 in Table 1). After incubation free-hand sections were made for light microscopy. Fig. 1. Fresh root. Fig. 2. Washed root. Fig. 3. Oligomycin-treated root. Fig. 4. DCCD-treated root. Figs. 5-8. ATPase activity in fixed 250-μm-thick free-hand sections of corn root (method 2 in Table 1). Note that fixation time and incubation time are reduced, but other conditions are as in Figs. 1-4. Fig. 5. Fresh root. Fig. 6. Washed root. Fig. 7. Oligomycin-treated washed root. Fig. 8. DCCD-treated washed root. All figures at same magnification; line scale in Fig. 1 represents 25 μm.
Figs. 9-16. ATPase activity in unfixed 250-μm-thick free hand sections of corn root prepared per method 3 in Table I. Fig. 9. Fresh root. Fig. 10. Washed root. Fig. 11. Oligomycin-treated washed root. Fig. 12. DCCD-treated washed root. Figs. 13 and 14. Phosphatase controls for Figs. 1-4. Fig. 13. 250-μm-thick section of washed root incubated in 2 mm of glucose-6-P and 0.2 M CaCl₂. Note lack of dense precipitate in outer cortex. Fig. 14. Same as Fig. 13 but incubated in 2 mm β-glycer-P. Figs. 15-16. Fresh roots incubated with lipid-soluble dyes in aqueous solution. Fig. 15. Root incubated with Sudan black B for 2 hr. Note surface staining. Fig. 16. Root incubated with Sudan IV for 2 hr. Note peripheral staining. All figures at same magnification; line scale in Fig. 9 represents 25 μm.
the ATP is being nonenzymically dephosphorylated (5). However, it is also possible that these deposits result from a soluble, exocellular ATPase (4). If this exocellular ATPase were oligomycin-insensitive and cold-labile, it would be consistent with the observations on cell wall deposits presented here. Thus, it appears that the plasmalemma deposits arise from an oligomycin-sensitive ATPase which is relatively resistant to glutaraldehyde fixation.

**General Discussion.** There are certain technical aspects of this work which are of importance in interpreting the microscopic observations. In order to preserve maximum ultrastructure, short incubation times (20–40 min) were needed for electron microscopy. On the other hand, the overstains needed to facilitate light microscopic observation required longer incubation (1–2 hr). Calcium was an effective capture agent for light microscopy as previously reported (25), but gave poor ultrastructural preservation, and this method was subsequently used for light microscopy only. Poststaining of thin sections for electron microscopy was omitted because both lead citrate and uranyl acetate tended to obliterate the fine deposits in and along the membranes, particularly with short incubation times.

The question of specificity of ATPase for ATP rather than other phosphodiester phosphates has been previously considered (8). Since we do not know the effect of fixation on transphosphorylases and the rate of loss of nucleoside diphosphates from fixed tissue, it is impossible to resolve adequately the question of substrate specificity. Furthermore, the nonenzymic dephosphorylation of ATP by Pb is also temperature-dependent (5). These and other difficulties with the ATPase staining technique are reviewed elsewhere (5) but it is concluded that the technique is a useful one.

Perhaps in some of these technical problems lies the answer to the question as to why the effect of washing was readily visible at the tissue level, but could never be demonstrated at the ultrastructural level. Since washing increases the cation-stimulated ATPase of the microsomal fraction up to 30% (18), it was anticipated that deposits on some cell membranes might show a corresponding increase. However, it is now our opinion that present techniques are inadequate to demonstrate ultrastructurally a change of this magnitude.

Apparently this is not true of the general ATPase activity observed by light microscopy. Visible activity is largely confined to the outer cortex and epidermis, which accumulates more precipitate in washed roots. The precipitate does not appear if ATP or trapping cations are omitted, and is far less dense if phosphatase substrates are substituted for ATP. Nothing can be said about the identity or cellular location of the enzyme, but the activity is reduced if the tissue is preincubated with oligomycin or with DCCD. Membrane preparations from corn roots (21) show oligomycin-sensitive ATPase activity with the mitochondria, but not the plasmalemma. On this basis, a reasonable explanation of the effect of oligomycin observed by Lin and Hanson (22) would be that oligomycin penetrated only into the outer cortex where it blocked ATP formation. This would not greatly lower the ATP content of the bulk tissue, but would inhibit ion absorption and thus subsequent symplastic transport.

However, oligomycin also inhibits the ATPase activity associated with the plasmalemma (Figs. 19 and 22 versus 20 and 23) and it might in addition have a direct effect on ATP-linked transport. As already indicated, the *in vitro* work with plasma membrane preparations is in opposition to this conclusion, and it is possible that our observations represent some indirect response to preincubation of the tissue with oligomycin (e.g. deterioration of the membranes). On the other hand, the possibility exists that isolated membrane preparations have lost their oligomycin sensitivity; they appear to have lost their ability to pump protons and ions, and only from certain tissues do they respond to fusocin (22).

In conclusion, the cytochemical evidence is that oligomycin directly or indirectly inhibits an ATPase of the plasmalemma as well as the mitochondrial ATPase. General oligomycin-sensitive ATPase activity is concentrated in the outer cortex, and it increases with root washing, as do ion absorption rates. It appears that the outer cortex and epidermis (16, 27) must have a primary energy-linked role in ion absorption by the root.

**LITERATURE CITED**

1. *Atkinson* MT, G* Echermann*, M* Grant*, RN* Robertson* 1966 Salt accumulation and 
adenosine triphosphate in carrot xylem tissue. *Proc Nat Acad Sci USA* 55: 360-364
60-64
Van Nostrand Reinhold Co. New York pp 44-76
6. *Gilder* J J* Cronshaw* 1973 Adenosine triphosphatase in the phloem of *Cucurbita* 
Planta 110: 189-204
7. *Gilder* J J* Cronshaw* 1973 The distribution of adenosine triphosphatase activity in 
differentiating and mature phloem cells of *Nicotiana tabacum* and its relationship to 
phloem transport. *J Ultrastruct Res* 44: 388-404
8. *Gilder* J J* Cronshaw* 1974 A biochemical and cytochemical study of adenosine 
triphosphatase activity in the phloem of *Nicotiana tabacum*. *J Cell Biol* 60: 221-235
adenosine triphosphatase activity. *J Histochem Cytochem* 16: 645-653
375-377
11. *Hall* JL 1969 A histochemical study of adenosine triphosphatase and other nucleoside 
roots. *Planta* 85: 105-107
93: 219-225
426
15. *Jacobson* B, OE* Plissner* 1970 Oligomycin effect on ion absorption by excised barley roots 
16. *La Scala* A 1967 Untersuchungen über Verteilung und Transport von Ionen in 
Pflanzenorganen mit der Röntgen-mikrosonde. 1. Versuche an vegetativen Organen von Zea 
mayes. *Planta* 75: 185-206
17. *Lazaro* SS, H* Barnes* 1964 Ultrastructural localization of mitochondrial adenosine 
18. *Leonard* RT, JB* Hanson* 1972 Increased membrane-bound adenosine triphosphatase 
activity accompanying development of enhanced solute uptake in washed corn root 
19. *Leonard* RT, JB* Hanson* 1972 Induction and development of increased ion absorption 
and cation transport in corn roots. *Plant Physiol* 58: 331-335
22. *Lin* W, JB* Hanson* 1974 Phosphate absorption rates and adenosine 5'-triphosphates 
concentrations in corn root tissue. *Plant Physiol* 45: 250-256
27. SOHAN V, G LAZAR 1965 Some data concerning the accumulation of neutral red in various tissues and regions of the maize root. Physiol Plant 18: 329-336
28. van ROSSUM GDV 1976 The effects of oligomycin on energy metabolism and cation transport in slices of rat liver. Biochim Biophys Acta 423: 111-121