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# Oligomycin Inhibition of Phosphate Uptake and ATP Labeling in Excised Maize Roots[1]

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*Abstract.* **ATP labeling by newly absorbed $^{32}P$ in excised maize roots was reduced 34% by the presence of oligomycin during a 4-min uptake period with no reduction in rate of phosphorus absorption. Longer exposure to oligomycin, during pretreatment periods or longer uptake periods, reduced phosphorus absorption and further reduced ATP synthesis. In these tissues it appears that oligomycin inhibits ATP production at the mitochondria, that ATP is the energy source for phosphorus uptake at the plasmalemma, and that a depletion in the ATP supply causes a reduced rate of uptake.**

Absorption of phosphate by plant tissues is influenced by factors that also influence oxidative phosphorylation (10, 11, 14, 16), but how these 2 processes are coupled is not understood. Although it is likely that ion uptake requires energy at a membrane, it is not known which membranes in the cell are the sites of active phosphate uptake. Hodges and Hanson (9) presented a model for accumulation of divalent ions and phosphate by corn mitochondria in which a phosphorylated high-energy intermediate of oxidative phosphorylation was the energy source for ion accumulation. If mitochondrial membranes are the sites of active phosphate uptake in intact cells, the process might be driven by a high-energy intermediate of oxidative phosphorylation. If, however, phosphate uptake by intact cells occurs at the plasmalemma, this uptake might be driven by ATP supplied by the mitochondria.

Jackson *et al.* (11) studied the effects of several respiratory substrates and inhibitors on phosphate esterification by barley root mitochondria and on phosphate uptake by excised barley roots. They found that the responses of the mitochondria and the roots to the various metabolites and inhibitors were similar. These findings linked phosphate uptake with mitochondrial phosphorylation and also supported the possibility of uptake at the mitochondrial membrane. However mitochondria might not be favorably located within the cell to explain the rapid changes in the rate of phosphate uptake with changes in the external phosphate concentration and pH (6). An alternative interpretation compatible with the results of Jackson *et al.* (11) is that ATP produced in mitochondria is used at the plasmalemma to provide energy for phosphate uptake there.

Evidences that ATP is indeed essential to drive phosphate uptake in plant tissues include observations that carbonyl cyanide-*m*-phenylhydrazone, an inhibitor of photophosphorylation, blocks the light-driven absorption of this ion in *Nitella translucens* (15), and that arsenate inhibits both ATP synthesis and phosphate uptake in maize roots (16). At an arsenate concentration which decreased ATP labeling by 24%, phosphorus uptake was reduced 12%. At higher arsenate concentrations both were inhibited more than 90%. Unpublished results in our laboratory also support this alternative interpretation. Phosphate initially accumulated in maize root cells as inorganic phosphate, and the first organic phosphate compound to be labeled was ATP, indicating that phosphate uptake and subsequent esterifications are separate processes.

Our work was undertaken to study further the energy source for phosphate uptake by plant cells. Oligomycin, an inhibitor of ATP synthesis in isolated maize mitochondria (12), was used in experiments studying phosphate uptake, ATP labeling, and phosphate esterification reactions in maize root sections. Ion uptake by isolated mitochondria may be driven by respiratory substrates or by ATP (9). Oligomycin blocks only ATP-driven ion uptake (12), and this suggests that if phosphate uptake across the plasmalemma is indeed dependent on ATP, the uptake should be inhibited by this antibiotic.

## Materials and Methods

Corn plants (*Zea mays* L., Ohio 51A × B8) were grown in solution culture in a controlled environment chamber on 16-hr photoperiod. The nutrient solution was a one-fifth Hoagland's No. 2 (7) modified by reduction of the phosphorus concentration to 0.1 mM. Cultures of 4 plants were

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grown in 2-liter plastic pots and solutions were changed weekly. Plants were harvested after 4 weeks and the roots were excised, rinsed, and placed in a basal sulfate solution (1.2 mM potassium, 0.80 mM calcium, 0.40 mM magnesium as sulfates). The root mat was gently disentangled beneath the water surface and any discolored segments and older larger roots were discarded. The selected roots were then rinsed with several portions of the basal sulfate solution and incubated in this solution with constant aeration prior to the uptake experiments. Roots prepared in this manner respired actively at a rate of 300 to 400 $\mu$l $O_2$ per g fresh wt per hr. Roots were blotted and 2- or 3-g portions were weighed and placed in polyethylene rings fitted with nylon mesh screens so that the roots could be quickly drained and moved to the next solution. Following uptake periods, root samples were drained and immediately rinsed in 6 successive portions of sulfate basal solution. The roots were frozen in 100 % ethanol and dry ice and extracted with formic acid—80 % ethanol as previously described (3).

Oligomycin (Sigma Chemical Company; 15 % oligomycin A, 85 % oligomycin B)[2] was dissolved in absolute ethanol to make a concentration of 10 mg/ml. Aliquots of this stock solution were diluted and dissolved in half of the pretreatment and uptake solutions, resulting in a final oligomycin concentration of 1, 10, or 20 $\mu$g/ml. At 20 $\mu$g/ml a suspension was formed. Control samples were prepared by adding equal amounts of ethanol (final conc. 0.2 %) to pretreatment and uptake solutions without oligomycin. The uptake solution was always $5 \times 10^{-5}$ M $KH_2PO_4$ in basal sulfate solution at a final pH of 5.5 to 5.7. The solutions also contained 2 to 4 $\mu$c $^{32}P$/ml. Pretreatment solutions consisted of the basal sulfate solution without phosphate and with or without oligomycin. The procedures for extraction, thin-layer chromatography, identification, and quantitative estimation of labeled compounds have been described (3).

## Results

In preliminary experiments, 15-min oligomycin (2.0 $\mu$g/ml) pretreatments reduced by about 20 % phosphate labeling of the alcohol-soluble fraction of excised maize roots during a subsequent 15-min $^{32}P$ uptake. The alcohol-soluble phosphate was divided into inorganic ($P_i$) and ester ($P_e$) forms by phase separation of the phosphomolybdate complex in isobutyl alcohol (3). Oligomycin caused little or no reduction in the level of labeled phosphate present in the $P_i$ form, but the $P_e$ fraction was reduced 34 to 48 %.

In further experiments, oligomycin present during a 15-min pretreatment reduced the rates of phosphate uptake during subsequent 4-min and 15-min uptake periods. Differential effects of oligomycin on phosphate incorporation into 3 fractions ($P_i$, $P_e$, and alcohol-insoluble residue) are shown in table I. Incorporation into the $P_e$ fraction was reduced to a greater extent by oligomycin treatments than was incorporation into the other 2 fractions. It is interesting that with oligomycin pretreatment, phosphate uptake into the $P_i$ fraction was reduced only 27 to 36 %, even though incorporation into $P_e$ was reduced 47 to 56 %. ATP was separated by thin-layer chromatography (3) and its radioactivity measured on spots scraped from thin-layer plates. Calculations of m$\mu$moles of newly absorbed P/g fresh wt in ATP and ADP were based on the specific activity of phosphate in the uptake solutions. When oligomycin was present only during the 4-min uptake period (no pretreatment), labeling of ATP was reduced 34 % (table I), but labeling of the $P_i$ and total $P_e$ fractions was not affected. With a 15-min pretreatment, oligomycin inhibited labeling of ATP by 50 %; total $P_e$, 47 %; and $P_i$, 27 %. The fact that ATP labeling was reduced earlier and to a greater extent than was the total $P_e$ fraction supports the hypothesis that oligomycin inhibited the formation of ATP, from which the phosphate in most other esters was presumably derived. Since $P_i$ uptake was inhibited much less than either ATP labeling or phosphate esterification, it is likely that the primary effect of oligomycin was on ATP production in oxidative phosphorylation, and that inhibition of phosphate uptake was a secondary effect due to a reduced supply of ATP.

Pretreatments with lower concentrations of oligomycin were extended to measure the influence of this inhibitor on total pool sizes of ATP. These pretreatments (65 and 100 min) were followed by 4-min, labeled phosphate uptake periods. Although there was no 100-min pretreatment control without oligomycin, previous experiments in this laboratory have shown that pretreatment with low (0.2 %) ethanol concentrations has little or no effect on phosphate uptake and esterification. The incorporation of newly absorbed phosphate into the 3 fractions (table II) resulted in patterns similar to those of previous experiments (table I). While labeling of the $P_i$ pool was inhibited 14 to 20 %, $P_e$ labeling was depressed 44 to 65 %. Incorporation into ATP and other major esters was greatly reduced, ATP being inhibited the most (85 %, table II).

Total pool sizes of 6 major phosphate esters as determined by phosphate analysis of chromatographically separated compounds also were reduced by a 100-min oligomycin treatment (table III). The concentration of ATP was reduced 68 %, more than concentrations of any other ester. The observation

[2] Trade name and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the products listed by the United States Department of Agriculture.

Table I. *The Effects of Oligomycin in Pretreatment and Uptake Solutions on Phosphate Absorption and Distribution in Various Fractions of Maize Roots*

Oligomycin concentration was 20 μg/ml. All values are means of 3 samples, and values of $P_i$ and $P_e$ followed by the same lower case letters are not significantly different at the 1 % confidence level. Values in parentheses represent percent inhibition by oligomycin.

| Pretreatment | Uptake conditions | Total P absorbed | Phosphorus incorporation | | | | |
|---|---|---|---|---|---|---|---|
| | | | $P_i$ | $P_e$ | Residue | ATP | ADP |
| | | | *mμmoles P/g fresh wt* | | | | |
| None | 4 min, control | 45.4 | 19.0 | 14.6 | 12.8 | 1.70 | 0.77 |
| None | 4 min, oligo. | 45.6 (nil) | 19.2 (nil) | 13.9 (nil) | 13.1 (nil) | 1.12 (34 %) | 0.76 (nil) |
| 15 min, control | 4 min, control | 51.2 | 21.8b | 16.3bc | 16.0 | 1.36 | 0.38 |
| 15 min, oligo. | 4 min, oligo. | 34.9 (32 %) | 15.9c (27 %) | 8.6a (47 %) | 12.3 (23 %) | 0.69 (50 %) | 0.29 (24 %) |
| 15 min, control | 15 min, control | 178 | 54.2d | 64.0f | 46.9 | ... | ... |
| 15 min, oligo. | 15 min, oligo. | 94.2 (47 %) | 34.5e (36 %) | 27.9g (56 %) | 26.0 (44 %) | ... | ... |

that oligomycin reduced ATP concentration more than half and only reduced the total phosphate uptake 35 % suggests 2 possibilities. The ATP concentrations in these roots may be sufficiently high that large reductions must be made before ATP supply limits ion uptake. Alternatively, the ATP necessary to drive ion uptake may represent only a small portion of the total ATP present in various cells in the roots.

Table II. *Effects of Extended Oligomycin Pretreatments Upon Phosphate Uptake and Distribution in Various Fractions of Maize Roots*

The 4-min phosphate uptake periods with or without oligomycin immediately followed pretreatment periods. Values are means of duplicate samples.

| | Pretreatment | | |
|---|---|---|---|
| | 65 min (no oligo.) | 65 min (1 μg/ml oligo.) | 100 min (10 μg/ml oligo.) |
| | *mμmoles P/g fresh wt* | | |
| Total P absorbed | 64.9 | 44.8 | 35.9 |
| $P_i$ | 27.4 | 23.6 | 22.0 |
| $P_e$ | 14.1 | 7.9 | 4.8 |
| Residue | 23.4 | 13.3 | 9.1 |
| ATP | 2.25 | ... | 0.41 |
| ADP | 0.22 | ... | 0.13 |
| UTP | 0.75 | ... | 0.21 |
| UDPG | 0.85 | ... | 0.24 |
| Glucose-6-P | 2.46 | ... | 0.57 |
| Glucose-1-P | 1.32 | ... | 0.27 |

Table III. *Inhibition by Oligomycin of Pool Size of Unlabeled Phosphate Esters*

Total pool sizes were determined for plants pretreated for 100 min (column 3, table II). All values are means of duplicate samples.

| | Concn of unlabeled phosphate esters | | |
|---|---|---|---|
| | Control | Oligomycin | Inhibition |
| | *mμmoles/g fresh wt* | | % |
| ATP | 97 | 31 | 68 |
| ADP | 34 | 13 | 62 |
| UTP | 38 | 19 | 50 |
| UDPG | 59 | 34 | 42 |
| Glucose-6-P | 107 | 55 | 49 |
| Glucose-1-P | 55 | 39 | 29 |

To determine the effect of oligomycin on phosphate incorporation into a number of esters, identification and quantitative estimation of these esters were made after chromatographic separations. Fig. 1 illustrates a radioautograph showing separation of 15 compounds. Table IV lists the identified compounds and shows that oligomycin reduced phosphate incorporation into most of them.

Table IV. *Inhibition by Oligomycin of Phosphate Incorporation Into Phosphate Esters in Excised Maize Roots*
The roots were pretreated for 15 min with or without oligomycin (20 μg/ml). Pretreatments were followed by 15 min $^{32}P$ uptake periods. Values are means of duplicate chromatographic analyses.

| | Phosphorus distribution | | | |
|---|---|---|---|---|
| | Experiment I | | Experiment II | |
| | Control | Oligomycin | Control | Oligomycin |
| | *% of total $^{32}P$ activity on thin-layer chromatograph* | | | |
| $P_i$ | 41.10 | 69.10 | 48.20 | 61.10 |
| Sugar phosphates | 35.40 | 15.90 | 28.20 | 19.20 |
| UDPG | 5.83 | 4.06 | 7.40 | 6.20 |
| UDP + UTP | 5.11 | 2.78 | 3.56 | 4.04 |
| ATP | 5.88 | 3.68 | 6.77 | 5.01 |
| ADP | 0.90 | 1.18 | 0.81 | 1.45 |
| AMP | 0 | 0.47 | 0 | 0.23 |
| CTP | 0.44 | 0.67 | 0.68 | 0.50 |
| GTP | 0.49 | 0.21 | 0.52 | 0.46 |
| Phosphoglyceric acid + phosphoenolpyruvate | 3.48 | 1.02 | 2.35 | 1.12 |
| Ethyl phosphate[1] | 0.59 | 0.49 | 0.73 | 0.11 |
| Origin | 0.45 | 0.21 | 0.26 | 0.27 |
| Unknowns | 0.40 | 0.26 | 0 45 | 0.30 |

[1] Probable artifact formed during killing and extracting procedures (3).

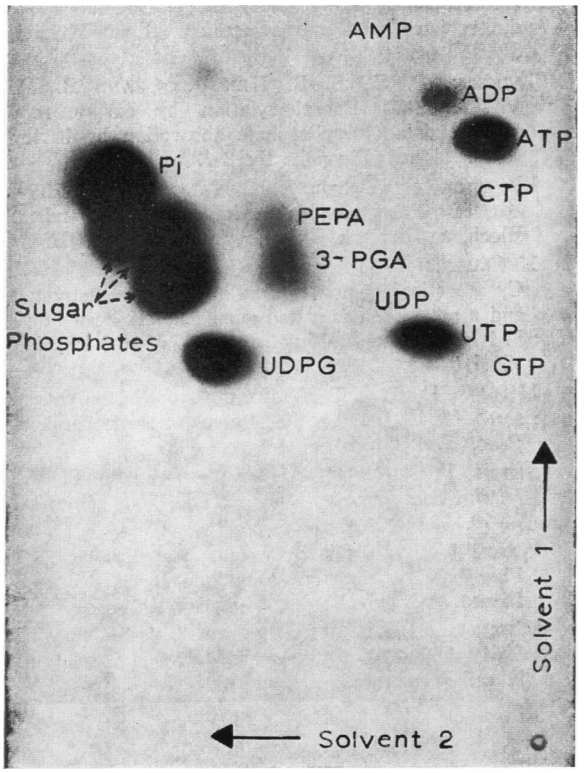


FIG. 1. Radioautograph of 2-dimensional thin layer chromatogram of phosphorus esters in corn roots labeled during a 15-minute uptake period. **Solvent 1, IBA.** Isobutyric acid/concentrated $NH_4OH/H_2O$, **57/4/39** v/v. Solvent 2, MAA. Methanol/1 M ammonium acetate, 7/3 v/v (3). PEPA, Phosphoenolpyruvate; 3 PGA, phosphoglycerate.

## Discussion

Our results are probably most easily interpreted by assuming that the site of oligomycin action is upon ATP production in the mitochondria, that ATP is the energy source for phosphate uptake at the plasmalemma, and that a depletion in the ATP supply leads to a reduced rate of uptake. Phosphate uptake was not affected by oligomycin until after ATP synthesis was inhibited. Thus a 4-min uptake period in the presence of this antibiotic (but in the absence of any pretreatment) resulted in no inhibition of total labeled phosphate entering the cells, but did cause a 34 % reduction of ATP labeling. When the time of exposure to oligomycin was extended by pretreatment periods, or longer phosphate uptake periods, ATP synthesis was inhibited even more than before and phosphate absorption was then also reduced. These results suggest that oligomycin inhibits ATP production by the mitochondria and that the reduced supply of ATP in turn depresses phosphate uptake rates. In various kinds of animal cells it is clear that oligomycin can inhibit absorption of potassium and secretion of sodium ions by blocking the utilization (hydrolysis) of ATP by ATPases located in membranes at the cell surfaces (4, 17). It thus inhibits cation transport in human erythrocytes which produce ATP only by glycolysis (17). Although such ATPases apparently also exist in plant cells (2, 5, 13), there is no evidence that they are involved in phosphate uptake. Nevertheless, it is still a possibility that oligomycin can inhibit phosphate absorption by interfering with reactions at the plasmalemma involving ATP produced in mitochondria. It should be noted that the amount of phos-

phate recovered in the roots as $P_i$ was consistently less affected by oligomycin than was total absorption or esterification. This result is consistent with interference with oxidative phosphorylation and a resultant partial accumulation of absorbed phosphate in the $P_i$ pool. It would be difficult to explain if oligomycin were only inhibiting ATP utilization by an ATPase at the plasmalemma. In the latter case $P_i$ values would probably have been inhibited more than the total phosphate uptake, since oxidative phosphorylation and subsequent esterification reactions should then utilize greater percentages of that reduced supply of phosphate which was absorbed. It is also possible that oligomycin inhibited ATP utilization at the plasmalemma as well as ATP synthesis by the mitochondria, but that the latter process was more sensitive to oligomycin.

In some tissues where the ATP supplies are limiting, ion uptake may be rapidly reduced by even small inhibitions of ATP synthesis (see Jackson *et al.*, 11). In other tissues the ATP concentrations may be nonlimiting (1) and ATP synthesis may then be reduced considerably before ion uptake is influenced. The maize roots used in these experiments apparently have relatively large ATP pools, since the total ATP pool was reduced 68 % (table III) before the 4-min uptake period, the subsequent phosphate absorption was reduced only 35 %, and accumulation in the $P_i$ pool, only 20 % (see table II).

In these experiments oligomycin clearly penetrated the root cells, since inhibition of phosphate esterification was observed. In experiments reported by Hodges (8), oligomycin inhibited total phosphate accumulation but had no effect on root respiration as measured by oxygen consumption. Hodges concluded that oligomycin had not penetrated into the cells, and that inhibition of uptake was due to effects of oligomycin on a membrane transport system. We cannot explain the difference between Hodges' results and ours, unless phosphorylation in Hodges' oat roots was sufficiently uncoupled from respiration that oligomycin inhibition of phosphorylation had no effect on oxygen consumption.

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