Loss of Organic Acids, Amino Acids, K, and Cl from Barley Roots Treated Anaerobically and with Metabolic Inhibitors

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Summary. Excised roots of barley (Hordeum vulgare, var. Campana) lost organic acids, amino acids, K+, and Cl− within 15 minutes after initiation of anaerobic treatment or treatment with NaCN and 2,4-dinitrophenol. Initial loss of organic acids when roots were placed under N2 is attributed to a decarboxylation reaction, possibly catalyzed by phosphoenolpyruvate carboxykinase. Organic and amino acids began to leak from the roots to the bathing medium after 1 to 2 hours under N2, indicating injury to cell membranes. During the first hour of anaerobic treatment, K+ loss from low-salt roots was equivalent to organic acid loss. Potassium loss from roots containing high levels of KCl was approximately equal to organic acid plus amino acid loss; and Cl− loss was approximately equal to amino acid loss. It is postulated that, within cells, organic acids may electrostatically bind an equivalent quantity of cations and that amino acids may bind an equivalent quantity of both cations and anions.

The requirement of metabolic energy for the accumulation of ions by plant roots is well documented in published literature. Anaerobiosis, uncouplers of energy transfer, and inhibitors of respiration have all been shown to inhibit ion uptake. It has generally been considered that metabolic energy is required for the transport of ions across cell membranes.

In recent years evidence that the permeability of cell membranes may be modified by unfavorable conditions has been presented. Rains et al. (15) reported that Rb+ uptake by barley roots from solutions of high acidity decreased with time. The effect of acidity was attributed to injury to the transport mechanism. Addition of Ca2+ to the treatment solution prevented this injury. Marschner et al. (13) found that the loss of K+ by sections of corn roots to the bathing medium was increased markedly when the solution pH was decreased from 7.0 to 5.5. The presence of Ca2+ in the medium reduced the loss of K+, and they suggested that H+ increases cell permeability by removal of Ca2+ from membrane sites rendering K+ bound in the cytoplasm more easily exchangeable.

Handley and Overstreet (4) suggested that Sr2+ uptake by corn roots is limited by the outer cell membrane, which is metabolically maintained and is readily destroyed under anaerobic conditions. Marschner et al. (13) reported that K+ loss from corn root sections was much greater under anaerobic conditions than under normal aeration. Electron microscopic studies indicated that the cytoplasm of roots treated anaerobically was deranged and much less dense than that of roots receiving air. Treatment of roots at pH 4.4 for 3 hours gave similar results. In both cases, Ca2+ was beneficial in preserving the fine structure of the cytoplasm.

Schadele and Jacobson (16) reported that Chlorella cells exposed to dark-N2 conditions accumulated less K+ after return to air-light conditions than did cells which did not receive the anaerobic treatment. Likewise, the photosynthetic capacity of leaves of plants is damaged irreversibly when they are kept under N2 in darkness (12). In general, aerobic metabolism appears to be required to maintain the integrity of aerobic cells.

We have been investigating the role of metabolically produced ions such as organic and amino acids in the accumulation of inorganic ions by plant roots. Roots of 6-day old barley seedlings cultured in 0.2 mM CaSO4 contain 25 to 30 μeq/g fresh weight each of organic acids and amino acids (unpublished data). These supply 50 to 60 μeq/g of negative charges and 25 to 30 μeq/g of positive charges which would be available for electrostatic binding of inorganic ions. Additional charges would be associated with proteins and other organic molecules. Under conditions of excess cation uptake, organic acids are synthesized in quantities equivalent to the quantity of excess cations accumulated (2, 5, 6, 7, 9, 10, 18), and Schadele and Jacobson (16) have suggested that the upper limit of K+ accumulation by Chlorella is determined by the capability of the cells to synthesize negative charges. Jackson and Stief (8) allowed barley roots to equilibrate with 10 mM KCl + NaCl solutions in which the Na:K ratio was 1:10, 1:1, and 10:1. Regardless of the Na:K ratio in the treatment...
solutions, the total Na + K content of the roots was 78 to 79 μeq/g fresh weight. Bear and Prince (1) found the sum of the equivalents of Ca, Mg, and K per unit weight of alfalfa to be relatively constant, although the ratio of the cations varied considerably in alfalfa grown on different soils. It appears that plant species have a characteristic upper limit of salt content which is relatively independent, within physiological ranges, of the composition of the medium in which they are grown.

Organic ions are synthesized in the cytoplasm and behave as nondiffusible ions within a semi-permeable membrane. It is possible that the upper limit of ion accumulation in higher plants is determined by the availability of charged sites supplied by organic ions. Evidence is convincing that excess cation uptake is closely interrelated with the synthesis of organic acids which may serve as electrostatic binding sites for the excess cations (5,6,7,9,10).

While conclusive proof is lacking that metabolic energy is required in the transport of ions across cell membranes, this report is not intended to refute this concept. The data in this paper, however, do indicate that respiration is required to maintain initial levels of organic ions in cells of barley roots and this phenomenon should receive careful consideration in studies of the requirement of metabolic energy for ion accumulation.

Materials and Methods

Barley seedlings (Hordeum vulgare, var. Campana) were dark-grown in continuously aerated 0.2 mM CaSO₄ essentially as described by Epstein and Hagen (3). Excised roots from 6-day old plants were rinsed several times in 0.2 mM CaSO₄ and were suspended in approximately 30 times the root volume of 0.2 mM CaSO₄ for 30 minutes before use.

For anaerobic experiments 0.2 mM CaSO₄ was boiled for 30 minutes to remove dissolved gases. One hundred ml of this solution was transferred to each of several 125 ml suction flasks and allowed to cool. Each flask was thoroughly flushed with N₂ by alternately evacuating the flask and refilling the air space with N₂. Before use the solutions were saturated with N₂. Three g of roots were placed in each flask and the flasks were gently shaken at slow speed on a mechanical shaker to provide continuous mixing of the contents. At specified time intervals flasks were removed, the substrate solution was decanted and reserved, and the roots were rinsed for 1 minute with distilled water and placed in 40 ml of 80% ethanol.

For the NaCN treatments, 2 mM NaCN was prepared using air-saturated distilled water and adjusted to pH 5.6 with HCl. Two g of roots were placed in 4 liters of this solution. The solutions were not aerated but were stirred frequently.

Solutions in treatments of longer than 2 hours were renewed after 2 hours. In treatments with 2,4-dinitrophenol (DNP), roots were placed in 4 liters of aerated 100 or 10 μM DNP. The pH of 10 μM DNP was 5.2 and the pH of 100 μM DNP solutions were adjusted to this level with tris. The final concentration of tris was approximately 150 μM. No CaSO₄ was included in the NaCN or DNP treatment solutions.

The roots were extracted with hot 80% (v/v) ethanol and hot water and the organic and amino acids were separated by the procedure described by Hiatt and Hendricks (7). Total organic acid content was determined by titration of the organic acid fraction with 0.020 N NaOH. Amino acids were determined with a Technicon autoanalyzer using a modification (17) of the Moore and Stein (14) ninhydrin reagent. Samples were mixed directly with the ninhydrin reagent without separation. Quantitative estimates were made by comparison with a mixture of amino acids (Technicon Standard Amino Acid Mixture, Technicon Chromatography Corp., Chauncey, New York).

Potassium and Cl⁻ were completely extracted by the procedure used to extract organic and amino acids. Therefore, an aliquot of this extract was used for K⁺ and Cl⁻ determination. Potassium was determined flame photometrically and Cl⁻ by means

![Figure 1](https://www.plantphysiol.org/)

**Fig. 1.** Solution pH and K⁺, organic acid (OA), and amino acid (AA) content of 6-day old barley roots treated anaerobically for various intervals of time. Three g roots were placed in 100 ml 0.2 mM CaSO₄ under N₂. Amino acids in μmoles/g.
of a Buchler-Cotlove automatic chloride titrator.
All experiments were repeated 2 or 3 times with very good reproducibility. Each figure represents data from a single experiment.

Results

Loss of K+, Organic Acids, and Amino Acids from Roots under Anaerobiosis. Six-day old barley roots were incubated anaerobically at 23° in 0.2 mM CaSO₄ for periods of 15 minutes to 4 hours. The results are shown in figure 1. Organic acid content of the roots decreased rapidly during the first 30 minutes but changed little between 30 minutes and 1 hour. After an hour, the decrease in organic acid content of the roots resumed at a rapid rate. Organic acid loss from the roots after the first hour was accompanied by appearance of organic acids in the medium but during the first hour organic acid appearance in the substrate solution was very small. Potassium loss paralleled organic acid loss during the first hour but was slower than organic acid loss after 1 hour. Amino acids were also rapidly lost from the roots. The rate of amino acid loss did not exhibit a plateau between 30 minutes and 1 hour as did the rate of K+ and organic acid loss. The pH of the medium increased during the first hour and decreased during the last 3 hours of the experiment. Changes in composition of roots maintained for periods of 1 and 3 hours in aerated 0.2 mM CaSO₄ were negligible.

 Fifty ml aliquots of the substrate solutions were aerated for 30 minutes and adjusted to pH 3.0 with HCl and then titrated to pH 10.0 with 0.020 N NaOH. The titration curves are shown in figure 2. Solutions from the 30-minute and 1-hour treatments were only slightly buffered. Solutions from 2, 3, and 4 hour treatments were highly buffered, corresponding to the appearance of organic and amino acids in the medium.

The roots used in the preceding experiment received all their K+ from the seed. The Cl⁻ content of these roots was less than 3 μeq/g. The effect of anaerobiosis on K⁺, Cl⁻, organic acid, and amino acid loss from roots pretreated for 12 hours in an aerated solution of 1 mM KCl and 0.2 mM CaSO₄ was determined. These roots approach their upper limit of K⁺ and Cl⁻ accumulation after 12 hours. After pretreatment in KCl the roots were washed for an hour in aerated 0.2 mM CaSO₄ before exposure to anaerobiosis.

The results are shown in figure 3. The pattern of K⁺, organic acid, and amino acid loss was similar to that for the low-salt roots. Chloride loss followed a similar pattern. Loss of K⁺, Cl⁻, organic acids, and amino acids from roots aerated for 3 hours in 0.2 mM CaSO₄ was 3.5, 0, 2.2, and 1.5 μeq/g respectively.

Fig. 2. Titration curves of solutions from anaerobic experiment. One-half of each solution (50 ml) was titrated to pH 3 with HCl and then titrated from pH 3 to pH 10 with NaOH.

Fig. 3. Organic acid, amino acid, K⁺, and Cl⁻ content of high-KCl barley roots treated anaerobically for various intervals of time. Roots were pretreated for 12 hours in an aerated solution containing 1 mM KCl and 0.2 mM CaSO₄ and then placed under N₂ in 0.2 mM CaSO₄. Amino acids in micromoles/g.
Table 1. Effect of Duration of Anaerobiosis on Loss of K, Cl, Organic Acids, and Amino Acids from Roots Pretreated for 12 Hours in 1 mM KCl

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>K</th>
<th>OA</th>
<th>K-OA</th>
<th>Cl</th>
<th>AA</th>
<th>OA+AA</th>
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<td>hrs</td>
<td>μeq/g roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>7</td>
</tr>
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<td>5</td>
<td>4</td>
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<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>10</td>
<td>6</td>
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<td>4</td>
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<td>10</td>
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<tr>
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<td>8</td>
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<td>6</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
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<td>14</td>
<td>15</td>
<td>17</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td>4.0</td>
<td>47</td>
<td>18</td>
<td>29</td>
<td>31</td>
<td>22</td>
<td>40</td>
</tr>
</tbody>
</table>

Table I shows the loss of K⁺, Cl⁻, organic acids, and amino acids from the roots preincubated in KCl. During the first 3 hours Cl⁻ loss was approximately equal to amino acid loss. Since cations other than K⁺ make up only a small fraction of the total diffusible cation content, it is assumed that the organic acids exist predominantly as K salts. When organic acids are lost by decarboxylation or leakage, an equivalent amount of K⁺ should be lost from the tissue. Subtraction of organic acid decrease from K⁺ decrease will give an estimate of K⁺ lost in excess of organic acid. When this calculation was made, a close relationship was observed among K⁺ loss minus organic acid loss, Cl⁻ loss, and amino acid loss during the first 3 hours of anaerobiosis. It is also noted that K⁺ loss was approximately equal to organic acid plus amino acid loss.

**Effect of NaCN.** Figure 4 shows the loss of

K and organic acids from roots treated with 2 mM NaCN. Loss of K⁺ and organic acids from roots treated with NaCN was slower than from roots under N₂. The decline in rate of loss between 15 and 30 minutes was small but reproducible. Organic acid and K⁺ changes in roots aerated 6 hours in water were negligible.

**Effect of 2,4-Dinitrophenol.** Roots in 100 μM DNP (fig 5) rapidly lost K⁺ and organic acids during the first 30 minutes. After 1 hour there was a steady decline in both K⁺ and organic acid content. Amino acid content declined steadily over the duration of the experiment. The initial rate of loss of K⁺ and organic acids from roots in 10 μM DNP (fig 6) was not as rapid as the initial rate in 100 μM DNP. The organic acid content of roots in 10 μM DNP, however, decreased more rapidly after 1 hour than did the organic acid content of roots in 100 μM DNP. This was perhaps due to the higher content of organic acids after 1 hour in the roots exposed to the lower concentration of DNP.

**Discussion**

Barley roots treated anaerobically, with an inhibitor of terminal respiration (NaCN), or with an uncoupler of phosphate transfer (DNP) rapidly lost K⁺, Cl⁻, organic acids and amino acids. When roots were placed in anaerobic solutions there was an initial rapid rate of loss of organic acids; how-
ever, during this period there was a negligible quantity of organic acids appearing in the solutions. The organic acids disappearing from the tissue during the initial period were apparently utilized in cellular reactions. Because the solutions were devoid of CO₂ and because respiration was blocked by lack of O₂, the CO₂ content of the cells was very low. It is likely that the low CO₂ content shifted the equilibrium of transcarboxylation reactions toward decarboxylation. It has been postulated that P-enolpyruvate carboxykinase catalyzes this reaction (5,7). The increase in buffering capacity of the solutions and the rapid appearance of organic and amino acids in the solutions indicate that cell membranes were injured after an hour of anaerobiosis. During the first hour K⁺ loss from low-salt roots was stoichiometric with organic acid disappearance. The pH of the medium increased during this period which may have been due to the loss of K⁺ from the cell as KHCO₃. The decline in solution pH after 1 hour was likely due to leakage of undissociated organic and amino acids since leakage of these exceeded the rate of loss of K⁺. Inhibition of respiration by NaCN also resulted in an immediate decline in K⁺ and organic acids. The lower rate of loss as compared to anaerobiosis would be expected since the movement of cyanide into cells throughout the tissue would be slow due to physical obstruction, whereas the effect of anaerobiosis on respiration should be immediate.

Dinitrophenol at a concentration of 10 μM was as effective as 2 mM NaCN in inducing K⁺ and organic acid loss from roots. The initial loss of organic acids caused by DNP treatment should not be due to increased decarboxylation caused by low CO₂ levels because CO₂ levels should be higher in DNP-treated tissues. Roots previously incubated in KH¹⁴CO₃ for 10 minutes and subsequently placed in H₂O or 10 μM DNP according to the procedure of Hiatt and Hendricks (7) release approximately 70% more ¹⁴CO₂ to 10 μM DNP than to H₂O (data not shown). It is not known whether this decarboxylation was mediated via the Krebs cycle or by direct decarboxylation of malate or oxaloacetate.

These data indicate that metabolic energy is essential for the maintenance of membrane integrity. Furthermore, there is a rapid initial decrease in organic acid content of tissue treated anaerobically or with respiratory inhibitors. This initial loss, accompanied by loss of K⁺, is apparently due to decarboxylation of organic acids since they do not appear in the bathing medium. It is probable that K⁺ is lost from the cells because of the decrease in negatively charged sites. Under anaerobiosis the initial rate of K⁺ and organic acid loss from low-salt roots is approximately 8 μeq/g/hr fresh weight. The rate of loss in 2 mM NaCN is approximately 6 μeq/g/hr initially. These rates are near the rate of uptake of K⁺ from 10 mM K⁺ salts under optimum conditions. Since inhibition of respiration results in a rapid loss of both organic and inorganic ions from barley roots, it is questionable whether the energy requirement for ion accumulation can with assurance be attributed to ion transport across cell membranes. This is particularly pertinent in cases where cations are accumulated without accompanying anions.

It has been proposed (5,6,7) that when low-salt barley roots are placed in K₂SO₄, K⁺ is absorbed in exchange for H ions which induces the synthesis of organic acids. The synthesis of negatively charged sites could create a gradient for cation accumulation. Such a mechanism would not require metabolic energy for ion transport per se; however, the maintenance of the negatively charged sites within the cell does depend on respiratory metabolism. An analogous situation occurs with Chlorella which accumulates K⁺ but not Cl⁻ from KCl. Schaedle and Jacobson (16) suggested that K⁺ accumulation by Chlorella was limited by the ability of the cell to create new negatively charged sites. The complete inhibition of K⁺ accumulation by cells under N₂ would be expected since CO₂ would not be available for the synthesis of organic acids. Leggett et al. (11) treated cation uptake by baker's yeast on the basis of simple exchange equations used with cation exchange resins. Distribution of Rb⁺ and Na⁺ into these cells at equilibrium closely followed a mass exchange equation. Compared with organic acids, little attention has been given to the role of amino acids in ion accumu-
ulation by plants. At the pH of cell sap monocarboxylic amino acids possess both a positive and a negative charge. In high-KCl roots it is possible that amino acids may exist as KCl salts. In low-salt roots the charges are perhaps intramolecularly neutralized or the amino acids may exist as dimers. Thus, amino acids could conceivably act as a reservoir of both positively and negatively charged sites if inorganic salts such as KCl could compete with the intra- or inter-molecular charge neutralization. That such a mechanism functions in ion accumulation is purely speculative; however, the relationship among K⁺ minus organic acids, Cl⁻, and amino acids in table 1 can logically be explained on the basis that the amino acids in high KCl roots exist as KCl salts. If this were the case, then the quantities of amino acids lost from the roots should be equivalent to Cl⁻ loss and K⁺ loss in excess of that lost due to organic acid decrease.

Ion transport and accumulation have generally been considered as a 1-step process mediated by carriers. Electrostatic binding of inorganic ions by organic ions such as organic acids, amino acids, and proteins is undoubtedly involved in the process of ion accumulation. The rapid response of organic acid and amino acid levels to anoxia or metabolic inhibitors emphasizes the importance of their consideration even in ion uptake studies of short duration.

Literature Cited