Effects of NaCl on Proline Synthesis and Utilization in Excised Barley Leaves

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ABSTRACT

Proline accumulation in NaCl-treated excised barley (Hordeum vulgare var Larker) leaves was studied. Leaves were treated by placing the cut end in NaCl solutions and allowing the salt to enter the leaf via the transpiration stream. Leaves treated this way maintained turgor while the sodium content increased and the osmotic potential decreased. Proline began accumulating after 12 hours and continued accumulating over the subsequent 12-hour period at an average rate of 0.6 micromoles per hour per gram fresh weight.

During the time proline was accumulating, \(^{14}\)C-glutamate was added to measure the effects of salt on proline synthesis from glutamate and \(^{14}\)C-proline was added in separate experiments to determine the effect of salt on proline utilization. Salt treatment dramatically increased proline synthesis from glutamate. Proline utilization by oxidation and for protein synthesis was decreased by 50 and 60%, respectively, by the salt treatment.

These effects are similar to the effects of drought and abscisic acid in barley leaves. The results indicate that common mechanisms cause proline to accumulate under these different stresses.

The accumulation of soluble proline in plant tissues can be induced by environmental stresses such as drought, salt, and cold (1). Proline accumulation also can be induced by ABA treatment (1, 8) in a few plants. Metabolic effects leading to proline accumulation have been studied for drought- and ABA-induced accumulation in excised barley leaves (8, 9). Stimulated synthesis of proline from glutamate has been observed in both drought- and ABA-induced leaves. Inhibition of proline utilization by both oxidation and protein synthesis was observed in wilted leaves. This inhibition does not occur in ABA-treated leaves.

Proline accumulates in response to salt stress in both halophytes and non-halophytes (1). In general, the amount of proline accumulation correlates well with the degree of salinity. In terms of water relations parameters, proline accumulates in tissue with lowered water potential in both drought and salinity stresses. However, under salt stress, the lowered water potential is due entirely to lowered osmotic potential; whereas, under drought stress, turgor potential is lowered, usually to zero. Osmotic potential also will be lowered somewhat under drought stress due to decreases in cell volume.

This paper details experiments designed to study metabolic effects causing proline accumulation in salt-stressed excised barley leaves. Excised leaves were used to eliminate complications due to translocation of proline out of leaves and for convenience of adding isotopes.

MATERIALS AND METHODS

Fully expanded second leaves from 2-week-old barley plants (Hordeum vulgare var Larker), grown as previously described (8), were excised at the base of the leaf blade. Prior to leaf excision, plants were removed from the growth chamber to room light (11 \(\mu E \: m^{-2} \: s^{-1}\)) to reduce transpiration rates. Leaves were weighed, then placed individually in 1 × 75-cm vials with the cut end of the leaf in 1 ml of solution. The control leaves were placed in 50 mM sucrose and 1 mM glutamate. Salt-treated leaves were placed in the same solution to which 0.41 M NaCl was added. Leaves were then allowed to take up the solution in 11 \(\mu E \: m^{-2} \: s^{-1}\) fluorescent light (laboratory lighting) for varying periods of time.

Radioactive precursors were added to the cut end of the leaf in 5 \(\mu\)l of \(H_2O\). Details of amount added, specific radioactivity, and length of pretreatment are given in the figure legends. At the end of incubation, leaves were immersed in 95% (v/v) ethanol/\(H_2O\). then extracted three times, a minimum of 3 h each time, without grinding, in 80% (v/v) ethanol/\(H_2O\). After drying, each extract was washed with petroleum ether or chloroform, taken up in \(H_2O\), and added to a 1 × 4.5-cm column of Dowex-50-H\(^+\). Organic acids and other nonadsorbing substances were collected by washing thoroughly with \(H_2O\). The amino acids were eluted by adding a sufficient volume of 1 N \(NH_3OH\) to make the pH of the eluate basic. The \(NH_3OH\) (containing the amino acids) was then washed from the column with \(H_2O\). The radioactivity in the organic acids was measured in the first \(H_2O\) wash. The amino acid fraction was dried, taken up in a small volume of \(H_2O\) (0.5 ml) and a 50-\(\mu l\) aliquot was used for TLC (10). The residue from alcohol extraction was air dried and then hydrolyzed with 6 N HCl in a sealed tube at 120°C for 16 h. The hydrolysates were dried, filtered, and chromatographed (10). Scintillation solution contained 4 g PPO, 500 ml Triton X-100, and 1 L toluene. Proline was determined using a modified Chinard technique as previously described (10).

Sodium content was measured by atomic absorption spectrophotometry after digestion in 10 ml of 1:1 (v/v) concentrated \(H_2SO_4: H_2O\) in Kjeldahl digestion flasks. Water potentials were measured in a Wescor C-51 sample chamber wired to a HR-33 dew point microvoltmeter. Osmotic potentials were measured similarly after freezing the samples.

RESULTS

The content of Na\(^+\) and free proline in barley leaves incubated in NaCl for varying times is shown in Figure 1. Salt was taken up into these leaves rapidly but then at a diminishing rate due to slower transpiration. The lower transpiration rate is evident from the amount of solution taken up (Table I) and probably resulted from stomatal closure as salt accumulated. It was important that the leaves be incubated in dim light so that assimilation of the salt

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of about 0.6 μmol (g fresh weight-h)-1. This rate approaches those observed previously in wilted and ABA-treated excised barley leaves.

Proline accumulation after 24 h in varying NaCl concentrations and in the presence of sucrose and glutamate was determined and the results are shown in Figure 2. In the presence of sucrose and glutamate, the amount of proline was proportional to the osmotic potential of the salt within the −7.5 to −15 bar range. The sucrose and glutamate doubled the proline levels in the −20 bar salt-treated leaves. No effect of sucrose and glutamate was observed when leaves were treated with −15 bar or lower NaCl. Subsequent labeling experiments were conducted in −20 bar NaCl in the presence of sucrose and glutamate and the time period corresponded to the 12- to 17-
h interval of Figure 1.

**Effects of Salt on Proline Synthesis and Utilization.** The incorporation of radioactivity into proline from [14C]glutamate is shown in Figure 3. The salt caused a dramatic increase in proline synthesis from glutamate as has been observed previously with drought and ABA treatments. The specific radioactivity of proline after 5 h in NaCl was 4000 dpm (μmol)−1 compared to less than 500 dpm (μmol)−1 in the control. Thus, the larger amount of radioactivity recovered was due to increased synthesis rather than a trapping of radioactivity by the accumulation of unlabeled proline from some other source. Radioactivity recovered in organic acids, glutamate, and other amino acids accounted for all that was applied to the leaf, but no effect of salt on label recovered in these compounds was observed.

The conversion of radioactivity from proline to oxidized products (Glu, Asp, Gln, Ala, γ-aminobutyrate, organic acids) is shown in Figure 4. As with previous experiments (10), low specific radioactivity proline was added to get accurate measurements of the rate of oxidation. The rate of proline oxidation was calculated by determining the slope of the lines in Figure 4 and dividing by the specific radioactivity at that time. During the 2- to 5-h time period, the rates were 0.12 μmol (g fresh weight h)−1 in the controls compared to 0.06 μmol (g fresh weight h)−1 in the salt-treated leaves. Thus salt decreased the rate of proline oxidation by 50%. Radioactivity remaining in proline plus that incorporated into protein accounted for the remainder of the radioactivity applied to the leaf.

The utilization of proline for protein synthesis was determined from the [14C]proline feeding experiment and the results are shown in Figure 5. The rate of incorporation of [14C] into protein proline was greater in the controls than in leaves treated with NaCl. Rates of utilization of proline for protein synthesis calculated from the slopes on Figure 5 and the specific radioactivities were 26 and 11 nmol (g fresh weight h)−1, respectively, for the control and salt-treated leaves. Thus, protein synthesis, as measured by incorporation of proline, was inhibited by 60% in the salt-treated leaves.

**DISCUSSION**

Our interpretation of the tissue and cellular ion relations in the salt-treated leaves is that NaCl entered the cut end of the leaf blade and moved through the leaf in the xylem. The salt was taken up into the cells rapidly enough to prevent plasmolysis due to extracellular salt accumulation. When the salt level reached a threshold value, proline accumulation was induced. It is not possible to determine what the threshold level was from these

![Graph](image)

**Table 1. Solution Uptake, Fresh Weight Changes, and Water Relations Parameters of Excised Barley Leaves**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time</th>
<th>Avg. Solution Volume Taken Up</th>
<th>Changes in Fresh Wt</th>
<th>Water Potential</th>
<th>Osmotic Potential</th>
<th>Pressure Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>h</td>
<td>ml</td>
<td>%</td>
<td>bars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>+1.0</td>
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<td>-12</td>
<td>+3</td>
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<tr>
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<td>+2.0</td>
<td>-12</td>
<td>-14</td>
<td>+2</td>
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<tr>
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<td>+2.5</td>
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<td>-25</td>
<td>+4</td>
</tr>
<tr>
<td>NaCl</td>
<td>24</td>
<td>0.18</td>
<td>+3.5</td>
<td>-29</td>
<td>-34</td>
<td>+5</td>
</tr>
</tbody>
</table>
experiments. However, a monovalent cation concentration of 200 
μmol (g fresh weight)⁻¹ has been suggested as a threshold by 
Weinberg et al. (12) for sorghum. Coughlan and Wyn Jones (3) 
suggest incremental Na⁺ increases above 200 μmol (g fresh 
weight)⁻¹ caused proline accumulation in spinach. We do not have 
measurements of K⁺ levels in these experiments, but 200 μmol (g 
fresh weight)⁻¹ of Na⁺ was reached after 8 h. The equivalent total 
monovalent cation concentration would have been reached 
sooner. Proline began accumulating at 12 h, suggesting a lag of 4 
h or more from the time the threshold level was reached and the 
time proline accumulation was observed. An approximate 2-h lag 
has been observed between rapid wilting and proline accumulation 
in wilted leaves and a similar lag for ABA-induced accumulation 
(8). It may be that once the threshold level of salt or ions is 
reached, sequestration into the vacuole begins. Cytoplasmic proline 
accumulation might then result to serve as a balance between 
the lowered osmotic potential in the vacuole compared to the 
cytosol. Further experimental tests of this interpretation are in 
progress.

The measured osmotic potential after 24 h in salt-treated leaves 
was 20 bars lower than the controls. The Na⁺ content (considering 
equal concentrations of Cl⁻) accounts for 16 bars of this potential. 
Thus, the added salt accounts for most of the lowered osmotic 
potential. Assuming it is confined to the cytoplasm, proline 
accounted for 3.5 bars osmotic potential at 24 h. However, proline 
levels were still increasing at that time.

In general, the effects of salt on proline metabolism were similar to 
drought- and ABA-induced proline accumulation with minor 
differences. Increased synthesis from glutamic acid was the 
primary effect leading to proline accumulation. Because this increase 
was observed at the same time that proline levels were accumulating, 
it follows that there was no feedback inhibition of proline 
synthesis under salt stress. Control of proline synthesis from
glutamate by proline has been demonstrated in nonstressed barley leaves (2).

Salt treatment decreased the rate of proline oxidation by 50%. This effect compares to a complete inhibition by drought and no measurable inhibition by ABA. The measured amount of inhibition of proline oxidation does not tell the whole story when considering the contribution of this effect to proline accumulation. When one considers that the capacity to oxidize proline by plant tissue is much greater than the measured rates and the rates are proportional to endogenous proline levels (9), the rates in stressed leaves should be much faster than in nonstressed leaves. Thus, the rates of oxidation in stressed leaves, when compared to the potential rate at that proline level, reflect a greater effect of stress on proline oxidation than the measured 50% inhibition. Furthermore, it has been suggested that proline-requiring corn mutants require proline because proline oxidation prevails over proline synthesis (4). If this is true, the control of proline oxidation is essential for normal growth as well as proline accumulation under stress.

The inhibition of the incorporation of proline into protein probably reflects a slower rate of protein synthesis in salt-treated leaves compared to the control. This effect was expected but the inhibition was less severe than that previously observed in the wilted leaves (10). It is well documented that water stress causes a dramatic loss of polysomes in short-term stressed seedlings (5). Salt stress also causes some loss of polysomes (7) but barley leaves do grow under NaCl stress (6). Inasmuch as the leaves in the experiments reported in this paper had a positive turgor, it is possible that they still had the capacity to grow and synthesize protein.

It appears that drought-, ABA- and salt-induced proline accumulation in barley leaves all result from common effects. It is tempting to speculate that the observed quantitative differences among the stress effects on proline oxidation and protein synthesis may be related to the capacity of the tissue to grow. Growth would be stopped in drought-stressed tissue and there is no proline oxidation or protein synthesis. In ABA-induced leaves, proline oxidation and protein synthesis were similar to the controls. Perhaps those leaves had their normal growth capacity although this has not been experimentally tested. In salt-treated leaves, the growth capacity is reduced, but not zero, and the proline oxidation and protein synthesis rates are likewise inhibited, but not zero.

**LITERATURE CITED**