Oxygen Fixation into Hydroxyproline of Plant Cell Wall Protein*

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The discovery of bound hydroxyproline in higher plants (1, 2) and the demonstration that this hydroxyproline is in peptide linkage (2, 3) and localized in the primary cell wall (3-8) shows that study of the metabolism and role of hydroxyproline can no longer be restricted to the field of collagen studies.

Previous work (3-6) indicates that the wall-bound hydroxyproline is derived from free proline but not hydroxyproline, and exists in a protein which is soluble when first synthesized, but rapidly becomes firmly associated with the wall, possibly through a covalent cellulose-protein linkage. Of several problems raised by these findings, one under current study is the general problem of proline hydroxylation and its relation to cell wall metabolism and growth. Scharpenseel and Wolf (9) found very little incorporation of tritium into hydroxyproline when fetal rat skin was incubated in tritiated water. They also found that hydroxyproline synthesis was oxygen-dependent. They concluded that the hydroxylation of proline may involve fixation of atmospheric oxygen.

This paper describes a single experiment designed to show whether or not the hydroxyproline hydroxyl oxygen in higher plants is derived via direct oxygen fixation. The experiment involved growth of sycamore (Acer pseudoplatanus L.) cell suspensions in a constant atmosphere of 0%N₂-14 followed by isolation of pure hydroxyproline. Only one batch of cells was grown under the above conditions, but hydroxyproline was isolated from two of pure hydroxyproline. The material corresponding to hydroxyproline hydroxyl oxygen in higher plants is derived via direct oxygen fixation. The experiment in- volved growth of sycamore (Acer pseudoplatanus L.) cell suspensions in a constant atmosphere of O₂¹⁸-N₂¹⁴ followed by isolation of pure hydroxyproline. Only one batch of cells was grown under the above conditions, but hydroxyproline was isolated from two cell fractions and then assayed for isotopic abundance. The paper concludes with discussion of the way in which a direct oxygenation reaction may be related to the control of cell extension growth mediated through changes in cell wall plasticity.

While this work with plant cell suspension cultures was progressing, reports of direct oxygen fixation into hydroxyproline of collagen have appeared (10, 11).

EXPERIMENTAL PROCEDURE

Culture medium—A modified White's medium was used as follows (grams per liter): Ca(NO₃)₂, 0.2; MgSO₄·7H₂O, 0.36; KCl, 0.063; KNO₃, 0.08; Na₂SO₄, 0.2; MnSO₄·4H₂O, 0.009; ZnSO₄·7H₂O, 0.003; H₂SO₄, 0.003; KI, 0.0015; NaH₂PO₄·H₂O, 0.0165; Fe₂(SO₄)₃·6H₂O, 0.0025; glycine, 0.003; cysteine-HCl, 0.001; calcium pantothenate, 0.0001; thiamine-HCl, 0.0001; 2,4-dichlorophenoxyacetic acid, 0.002; sucrose, 20.0; coconut milk, 20% by volume. Coconut milk was deproteinized by autoclaving (15 minutes at 15 psi) and filtering before addition to the culture medium, which was then sterilized by autoclaving. Also included in the medium used to grow cells in the presence of O₂¹⁸ were 5 µc of C¹⁴-proline (specific activity, 5 mc per mmole) and 20 µc of 3,4-³H₂-proline (specific activity, 371 mc per mmole) both obtained from the New England Nuclear Corporation.

Growth of Cells in Presence of O₂¹⁸—Growth medium (100 ml) was inoculated with approximately 9 ml of 30% (= 2.7 ml) packed cell volume corresponding to a total dry weight of 135 mg. The roller bottle containing the cells (Fig. 1) was then evacuated for 10 minutes at the water pump, after which oxygen-free nitrogen and 24 atom % excess O₂¹⁸ were admitted to give 80% N₂-20% O₂. The bottle was then rotated at 60 r.p.m. on a bottle-rolling apparatus in a 27°C temperature-controlled room, and O₂¹⁸ (24 atom % excess) was added each day (Fig. 2) through an attachable manometric setup to replace oxygen taken up, the CO₂ being absorbed by 66% (weight per volume) KOH in the roller bottle bulb. After 17 days of growth, the culture yielded 27.5 ml of packed cell volume total, an approximately 10-fold increase over the starting material.

Isolation of Primary Cell Wall Fraction—Cells in stationary phase were harvested by filtration through a coarsely sintered funnel, washed with distilled water and broken in an ice-water-cooled cell mill (12) (obtained from Edmund Buhler, Ltd., Tubingen) containing glass beads 3 mm in diameter. The homogenate was decanted from the beads and centrifuged for 30 seconds at 1000 X g. The pellet (cell walls) was washed with distilled water by 10 similar centrifugations, and the clean primary wall preparation was then dried under vacuum from the frozen state. The first supernatant fraction ("cell contents") was also recovered and dried from the frozen state, after dialysis against distilled water.

Acid Hydrolysis of Cell Fractions—Cell wall preparations and cell contents were hydrolyzed in sealed tubes with 0 X HCl at 105°C for 18 hours. The tube contents were then filtered, and the filtrate was evaporated to dryness in a rotary evaporator.

Isolation and Purification of Hydroxyproline—The filtered, dried acid hydrolysate was taken up in 5 ml of water and added to the top of a column (50 x 1 cm in diameter) of Dowex 50-H⁺ ion exchange resin, which was then eluted with 1.5 N HCl. Samples (0.1 ml) of each fraction (4 ml) collected were evaporated to dryness on sandblasted glass planchets in an oven and monitored for radioactivity by a Nuclear-Chicago gas flow counter. Only two radioactive peaks occurred; one corresponded to hydroxyproline, the other to proline. The material corresponding to each radioactive peak was evaporated to dryness in a rotary evaporator.
evaporator three times, water being added each time the point of dryness was reached. The two amino acids were finally purified by electrophoresis for 40 minutes on washed Whatman No. 3MM paper buffered to pH 1.9 (glacial acetic acid, 8.7%; formic acid, 2.5% by volume) at a field strength approximately of 80 volts per cm. The paper was dried in a forced air oven at 80° and hydroxyproline and proline areas were determined by radiography (non-screen Kodak Medical x-ray film), followed by elution with water and reisolation by drying from the frozen state. Previous experiments showed that the hydroxyproline isolated following this procedure was at least 90% pure. The final yield of hydroxyproline from the wall fraction was determined chemically (13) to be 1.2 mg, to which 2.4 mg of carrier hydroxyproline were added to provide enough material for mass spectrometry. The final yield of hydroxyproline from the cell contents was 0.0258 mg to which 2.8 mg of carrier hydroxyproline were added.

Analysis of Hydroxyproline for $^{18}$O—These analyses including a control sample were performed by the Analytica Corporation of New York.

![Fig. 1. Closed roller bottle culture of sycamore cell suspensions. The small bulb containing 10 ml of 66% (weight per volume) KOH is attached to the culture bottle by a standard taper joint. The cotton wool plugs prevent contamination.](image)

![Fig. 2. Uptake of 24 atom % excess $^{18}$O by a sycamore cell suspension culture.](image)

<table>
<thead>
<tr>
<th>Hydroxyproline source</th>
<th>Quantity isolated</th>
<th>Carrier added</th>
<th>Atom % excess $^{18}$O in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary cell wall fraction</td>
<td>1200</td>
<td>2400</td>
<td>2.470</td>
</tr>
<tr>
<td>Cell contents</td>
<td>28</td>
<td>2800</td>
<td>0.112 (0.071)</td>
</tr>
<tr>
<td>Laboratory reagent</td>
<td>3200</td>
<td>3200</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* The figure in parentheses was calculated on the assumption that the hydroxyproline of the cell contents turned over and was therefore not diluted by the hydroxyproline present at the beginning of the experiment.

RESULTS

The results are in Table I.

DISCUSSION

The only other system so far used to investigate the derivation of the hydroxyproline hydroxyl oxygen was chick embryo (10, 11) and this provided only a 30 to 40% increase in hydroxyproline during the experimental period. Furthermore, because the precise increase in hydroxyproline was not known, these workers were unable to conclude that formation of hydroxyproline occurred exclusively by direct fixation of atmospheric oxygen, even though their experiments with $^{18}$H$_2$O made this very likely. There is, on the other hand, somewhat more certainty about the increase of hydroxyproline in the sycamore cell suspension system which increased in packed cell volume by 1000% during the experimental period. Judging from previous experiments there is a proportional increase in dry mass and hydroxyproline. Therefore, in the experiment involving $^{18}$O$_2$ described here, those components deriving oxygen directly by fixation of atmospheric oxygen should show an abundance of $^{18}$O equal to 24 atom % excess diluted one-tenth by $^{16}$O, giving a final figure of 21.6 atom % excess $^{18}$O. For hydroxyproline isolated from the wall fraction, the final theoretical figure for isotopic abundance (21.6/9 = 2.40) makes allowance for a 3-fold dilution by carrier proline and a further 3-fold dilution by the carboxyl oxygen atoms which exchange freely with water under the hydrolytic conditions used here (14). The results found (Table I) are in good agreement with the theoretically calculated figures, and I therefore conclude that the hydroxyproline hydroxyl oxygen is derived exclusively by the fixation of atmospheric oxygen, at least in sycamore cells grown under the conditions specified here.

The occurrence of high levels of hydroxyproline in a protein specifically associated with the primary cell wall provides a further experimental basis for Bonner's (15) speculation of 1935, suggesting a physical location for the changes in primary cell wall plasticity (cf. Heyn (16)) involved in the control of cellular extension: "Die Arbeitshypothese wurde aufgestellt, dass die Zellwand- dehnbarkeit durch die Festigkeit der intermicellaren Haftpunkte reguliert wird." There is, as I have previously suggested (3, 4),
the exciting possibility that the hydroxyproline-rich protein corresponds to Bonner’s Haftpunkte. If the hydroxyproline-rich primary cell wall protein (here I propose to name this protein “extensin”) is directly involved in controlling primary wall plasticity by providing a network of labile cross-linkages between the cellulose microfibrils, then it is imperative to know something of the mechanisms and factors controlling the synthesis and further metabolism of “extensin,” for these same factors would ultimately affect cell size and shape, the prelude to further differentiation. The $^{18}O_2$ tracer experiment described here is another step towards an understanding of these mechanisms and factors.

Because the hydroxyproline hydroxyl oxygen derives exclusively via the fixation of atmospheric oxygen, and because the $K_m$ (for oxygen) of hydroxylases is much larger than that of cytochrome oxidase (17), it now becomes important to investigate the possibility of a direct effect of oxygen tension (i.e., at concentrations which do not limit cytochrome oxidase) on protein (“extensin”) synthesis. If there is a connection between the extensibility of a primary cell wall and its hydroxyproline content, then the extensibility of a “mature” primary cell wall may be a function of the oxygen tension when the wall was first synthesized. A future report will describe the effect of oxygen tension variation on the rate of hydroxyproline synthesis. Another report discusses the mechanism of proline hydroxylation in the light of data additional to those presented here.

**SUMMARY**

1. The hydroxyproline hydroxyl oxygen of primary cell wall protein is derived exclusively by the fixation of atmospheric oxygen.

2. The provisional name of “extensin” has been given to the hydroxyproline-rich primary cell wall protein.

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**REFERENCES**

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