The cell walls of higher plants contain a large amount of protein-bound hydroxyproline (16). Lamport and Northcote (20) and Dougall and Shimbayashi (9) drew attention to the analogy between this hydroxyproline-containing protein and collagen, the structural protein found in animals. Lamport named this protein "extensin" and suggested that it had both structural and growth-regulating functions (15).

The possible growth-regulatory role of extensin has encouraged the study of the turnover and metabolism of hydroxyproline-containing proteins in plant tissue. Olson (21) studied cell wall protein, using labeled 14C-proline, in suspension-cultured tobacco cells. He found that although a large proportion of the protoplasmic 14C-hydroxyproline-containing protein turned over rapidly, that from the cell wall fractions did not. He suggested that the loss of protoplasmic proteins was related to their incorporation into the cell wall.

In a study of elongating Avena coleoptiles, Cieland (7) showed that there was a loss of 14C-hydroxyproline-containing protein from the trichloroacetic acid-insoluble cytoplasmic fraction and a concomitant rise in wall-bound 14C-hydroxyproline. In contrast to the results of Olson (21), it appeared that only a small proportion of cytoplasmic protein-bound hydroxyproline was in transit to the cell wall. These results establish clearly that hydroxyproline-containing protein is exported from the cytoplasm to the cell wall.

Some information on the mechanism of this export is available. Chrispeels (6) demonstrated that the 14C-hydroxyproline-containing protein lost from carrot-disc cytoplasm on charring with unlabeled proline is associated exclusively with membranous organelles. Dashek (8) also found that the export of hydroxyproline-containing proteins from the cytoplasm of suspension-cultured Acer pseudoplatanus cells was via membranous organelles. He concluded that these vesicles were not derived from the Golgi apparatus but might have originated from the endoplasmic reticulum. An opposite conclusion was drawn by Gardner and Chrispeels (11) who provided evidence that in carrot discs the Golgi apparatus plays an important role in the biosynthesis and secretion of the cell wall glycoproteins.

The aim of this study was to trace the path of hydroxyproline-containing proteins in suspension-cultured A. pseudoplatanus cells from the cytoplasm to the cell wall and into the nutrient medium. In addition, it was hoped that by comparing the HA1 pattern before and after incorporation into the cell wall, an insight would be gained into the mechanism of incorporation of hydroxyproline-containing wall protein.

**Materials and Methods**

Sycamore-Maple (A. pseudoplatanus L.) Suspension Culture. The cells were cultured in the modified M-6 medium described by Bauer et al. (2) except that the yeast extract polysaccharide was precipitated by 75% (v/v) ethanol, and ethanolic extract equivalent to 1.25 g of the original yeast extract was used. The suspended cells were grown at 27°C and at 100 rpm on a rotary shaker. They were subcultured every 7 days by adding 25 ml of the suspension to 100 ml of the medium in a 250-ml flask. The remaining 100 ml of the suspension was used immediately for experimental purposes. All experiments were performed on 7-day-old cultures.

**Determination of Carrier and Chase Levels of Proline to be Used in 14C-Proline Incorporation Studies.** Five μCi 14C-proline plus carrier were added to 100 ml of cultured cells. The cells were incubated for 2 hr, and two 5-ml aliquots were withdrawn at 20-min intervals. The cells were pelleted by centrifugation and washed with 10 ml of proline solution (200 μg/ml), to remove adsorbed 14C-proline. The cells were pelleted again, resuspended in 9 ml of water, and sonicated for 1 min using a Bronwell Bio-Sonic 3 at a setting of 70. The resulting brei was filtered through glass wool, and trichloroacetic acid was added to

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1 This work was supported by United States Atomic Energy Commission Contract AT(11-1)-1338.

2 Abbreviations: SEPS: sycamore extracellular polysaccharides; S.E.: salt-extractable; HA: hydroxyproline-arabinoside.
a concentration of 5% (w/v). The preparation was allowed to stand at 4 C and was then filtered by suction onto a Whatman glass-fibre filter. The filters were dried and radioactivity was measured in 5 ml of Aquasol (New England Nuclear).

One μmol of proline/100 ml of cell suspension was found to be adequate to ensure a linear incorporation of the radiolabel into the protein over 1 or 2 hr. Three μmol were used for the 2-hr incubations to give a margin of safety.

When desired, further incorporation of 14C-proline over the experimental period was strongly inhibited by the addition of 2 mM of proline to a flask.

Preparation of the Particulate Cytoplasmic Fraction. After harvest by filtration, the cells were homogenized in the medium described by Ray et al. (23) (1 mg/tissue) for 7 min, using a glass tissue homogenizer with a motorized Teflon piston. The brei was diluted with homogenization medium and was then centrifuged for 1 hr at 1,000 g. The supernatant was centrifuged for 1 hr at 37,000 g. The resultant pellet was rinsed with water and then hydrolyzed with either Ba(OH)2 to yield hydroxyproline-araninosides, or with 6 N HCl for the determination of the specific radioactivities of proline and hydroxyproline.

Label was allowed to accumulate in the particulate fraction for either 30 or 60 min to obtain labeled hydroxyproline-araninosides.

Preparation of Salt-extractable Wall and SEPS Fractions. Aliquots (10 ml) of cell suspension were pelleted using a clinical centrifuge at full speed for 3 min. The supernatant solution was filtered through glass wool, and then 5 ml of the filtrate was pipetted into 15 ml of absolute ethanol. The SEPS was precipitated by leaving the solution overnight at 2 C.

The pellets of cells were resuspended in 10 ml of water containing proline (200 μg/ml) and centrifuged. The cells were then suspended in 10 ml of water and were sonicated for 1 min as described earlier. The brei was centrifuged, and the pellet resuspended in 10 ml of water and pelleted once more. The wall material was washed twice more with water. The water-washed pellet was resuspended in 10 ml 1 M NaCl and centrifuged for 3 min. The supernatant constituted the S.E. fraction. The salt was removed by dialysis against distilled H2O overnight at 4 C. The pellet was resuspended in 10 ml 1 M NaCl and was filtered by suction on to a Whatman glass-fibre filter. The wall material retained on the filter was then washed with 20 ml of water, and dried at 105 C overnight.

An alternative method was employed to produce enough of the S.E. fraction to permit the determination of its HA profile, and 14C-HA profile. Flasks containing 100 ml of suspended cells were incubated for 2 hr with 5 μCi 14C-proline and 3 μmol of proline. The cells were filtered onto glass wool, and then washed with 10 ml of 0.5 M NaCl to obtain the S.E. fraction. This was removed by dialysis against distilled H2O at 4 C overnight. The contents of the dialysis bag were lyophilized and stored until they were sufficient for Ba(OH)2 hydrolysis.

Preparation of Hydroxyproline-X. About 350 mg of lyophilized SEPS was hydrolyzed with Ba(OH)2 as described below.

The hydrolysate was neutralized, concentrated to 1 ml, and applied to a Sephadex G-25 column (0.9 × 90 cm). The hydroxyproline-containing material eluted in the void volume of the column (hydroxyproline-X) was lyophilized, redissolved in 1 ml of 0.1 M acetic acid, and then applied to a Sephadex G-75 column (0.9 × 90 cm). The eluate was monitored for hydroxyproline.

Neutral Sugar Analysis. This was performed by the method of Albersheim et al. (1) as modified to a microscale by Lamport.

Samples containing between 20 and 300 nmol of sugar were placed in a 300-μl Kontes microflex tube and dried under a stream of N2. After placing 50 μl of trifluoroacetic acid (15%, v/v) containing 100 nmol of myo-inositol as internal standard into the microvial, the mial was sealed and heated for 1 hr at 121 C. On cooling, the trifluoroacetic acid was evaporated under a stream of N2. The sugars were reduced by 25 μl of freshly made solution of sodium borohydride (20 mg/ml in 3 M NH4OH). The solution was mixed vigorously and was then allowed to stand for 1 hr at room temperature. The reaction was stopped by the addition of glacial acetic acid until bubbling ceased. One hundred μl of methanol was then pipetted into the microvial. This mixture was agitated, and evaporated to dryness.

A further 100-μl aliquot of methanol was added and evaporated; 100 μl of a methanol-water mixture (1:1) was then pipetted into the microvial and evaporated. Finally, 100 μl of methanol was added to the vial and evaporated; this step was performed three times in all. The microvial contents were further dried by desiccation in vacuo over P2O5 and KOH overnight.

The steps from the addition of sodium borohydride were carried through without interruption. The derivatization was completed by the addition of 50 μl of acetol anhydride to the microvial which was closed and heated at 121 C for 1 hr. Samples (1-5 μl) of the cooled solution were injected into the gas chromatograph.

Acid Hydrolysis. Dried samples were hydrolyzed with 2 ml of 6 N HCl in a sealed ampoule at 105 C for 18 hr.

Separation of Hydroxyproline from Proline. A complete separation of the two amino acids was achieved by ion exchange chromatography. Dowex AG-50-X8, 200 to 400 mesh, in the H+ form was packed into columns (8 × 80 mm). The columns were developed with 0.35 N HCl. Six samples were separated simultaneously. A Technicon proportioning pump was used to draw the acid through the columns at a rate of 0.2 ml/min. The first 20 ml column was discarded. Hydroxyproline appeared within the next 30 ml, and proline within the last 50 ml. The eluate from each column was pumped into a test tube (13 × 100 mm) maintained at 70 C in a water bath. Compressed air was directed into each tube. By this means it was possible to evaporate the acid continuously without completely drying the eluted amino acid. At the appropriate time (250 min), the column outlet tubes were transferred to a fresh series of test tubes to collect the proline.

The degree of separation achieved by this method was established by collecting 2-ml fractions, hydroxyproline was eluted between fractions 12 to 22 and proline between fractions 29 to 50. The efficiency and reproducibility of the technique are shown in Table 1.

Assay for Hydroxyproline and Proline. Hydroxyproline was assayed quantitatively by means of an automatic analyzer as described by Lamport and Miller (19); the method of Chinard (5) was used to assay proline.

Hydrolysis with Ba(OH)2. Between 50 and 300 mg of the various fractions were hydrolyzed by 10 ml of saturated Ba(OH)2 (0.22 M) for 6 hr in a sealed ampoule at 105 C. Under these conditions, peptide bonds are not. The hydrolysate was neutralized with H2SO4, centrifuged, and the supernatant evaporated to about 1 ml under reduced pressure at 60 C.

Separation and Determination of Hydroxyproline-Araninosides. Samples containing 100 to 200 μg of hydroxyproline were separated on a Chromobeads B column (H+ form) as described by Lamport and Miller (19). The eluate was collected in 2-ml aliquots instead of being routed directly into the hydroxyproline analyzer. One ml was assayed for hydroxyproline content and 0.5 ml was assayed for radioactivity using a scintillation spectrometer.

Table I. Recovery of Hyp and Pro from a mixture.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Applied to column</th>
<th>Recovered from column</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp</td>
<td>3.75</td>
<td>3.16 ± 0.22</td>
<td>94</td>
</tr>
<tr>
<td>Pro</td>
<td>3.72</td>
<td>2.01 ± 0.06</td>
<td>90</td>
</tr>
</tbody>
</table>
After the appropriate corrections for volume differences, the number of counts, corrected for background, in each radioactive peak was divided by the total amount of hydroxyproline in that peak to give the specific radioactivity.

This procedure for determining specific activities was checked by taking advantage of the presence of the cis and trans forms of hydroxyproline in the alkaline hydrolysate. The Chromobeads B column separates each HA and hydroxyproline as a double peak, the cis and trans form of each molecular type. The specific radioactivity was determined separately for each peak of a pair. If the procedure was reliable, each peak of the pair should have the same specific radioactivity, as the peaks represent the cis and trans forms of one original HA, which was subsequently modified by hydrolysis. This was found to be the case.

14C-Proline. The L-U-14C-proline (290 mCi/mmol) was supplied by the Amersham/Searle Corp.

Scintillation Counting. The samples were made up to 1 ml each with water and 10 ml of Aquasol (New England Nuclear) was added. 14C was counted with 92% efficiency by a Packard Tri-Carb scintillation spectrometer.

RESULTS

Path of Hydroxyproline-containing Protein to the Cell Wall. It has been implied (4) that in carrot tissue the S.E. fraction of the cell wall represents a pool of wall protein awaiting incorporation into the cell wall. This idea was tested by following the kinetics of 14C-hydroxyproline-containing protein into the S.E. wall fraction of cultured A. pseudoplatanus cells with and without a proline chase. If the fraction were indeed a pool supplying the cell wall, it should saturate (achieve a constant specific radioactivity) when label is present continuously and is being incorporated into the cell wall at a constant rate. A precursor pool should also lose label when sufficient nonradioactive proline is added to inhibit further incorporation of 14C-labeled protein into the cell wall.

Figure 1 shows that the S.E. fraction does not saturate, even though the 14C-hydroxyproline is being incorporated into the cell wall at a constant rate after a 20-min lag. When the incorporation of 14C label into the cell wall is inhibited by the addition of unlabeled proline, the S.E. fraction does not lose label (Fig. 2). This result is in marked contrast to that found for the particulate fraction described below.

The kinetics of 14C-hydroxyproline and 14C-proline-containing proteins from the cytoplasmic particulate fraction was also followed. Label starts to disappear from the particulate fraction in less than 15 min after the addition of unlabeled proline (Fig. 3). The timing of the disappearance of 14C-hydroxyproline from the particulate fraction corresponds well with the appearance of 14C-hydroxyproline in the cell wall. This suggests that the particulate fraction contains wall protein in transit to the cell wall.

Chemical and Radiochemical Hydroxyproline-Arabinoside Profile of the Fractions. As the kinetics of hydroxyproline incorporation into the wall and SEPS fractions was so similar (Figs. 1 and 2), it appeared possible that the SEPS fraction represented wall protein that had escaped into the medium. This was checked by comparing the HA profile of the cell wall fraction with that of the SEPS. Most hydroxyproline residues in the cell wall are attached to between one and four arabinoses. These HA are easily separated on Chromobeads B and the labeled hydroxyproline detected both colorimetrically and by scintillation spectrometry, to give a HA profile. The profiles found in the various fractions are illustrated in Table II.

The radiochemical profiles were determined simultaneously so that short term HA profiles could be compared as well as the long term ones. This was necessary because differences between the wall and SEPS profiles might have arisen from the progressive degradation of SEPS in the medium. Table II shows that each fraction tested has a characteristic HA profile. There is a reasonable correspondence between the colorimetrically determined and radiochemical patterns, except in the case of the S.E. fraction. Because the wall and SEPS profiles are so dissimilar, I concluded that the hydroxyproline content of the SEPS cannot be equated with protein which has escaped from the wall.

The particulate fraction has a profile intermediate between that of the SEPS and the wall fractions. It is rich in hydroxypro-
line-X (hydroxyproline attached to a polysaccharide) and hydroxyproline, which are characteristics of SEPS, yet unlike SEPS there is more hydroxyproline-tetraarabinoside than hydroxyproline-triarabinoside, which is reminiscent of the wall profile.

The colorimetrically detected HA profile of the S.E. fraction resembles that of the extracellular material (SEPS), except that the level of hydroxyproline-tetraarabinoside in S.E. is higher. The radiochemically detected S.E. pattern has characteristics of both the wall and SEPS patterns. It is rich in hydroxyproline-X, like SEPS, but like the wall it has more hydroxyproline-tetraarabinoside than hydroxyproline-triarabinoside. The level of 14C-hydroxyproline is intermediate between the SEPS and wall profiles.

**Time Course of the 14C-Hydroxyproline-Arabinoside Pattern of the Cell Wall.** As it proved possible to obtain 14C-hydroxyproline-arabinoside profiles from the various hydroxyproline-containing fractions (Table II), this technique was used to obtain a time course of the 14C-HA profile of the cell wall over a short time period. It was intended to determine whether or not hydroxyproline or HA were engaged in linking extensin into the cell wall. Advantage was taken of the fact that hydrolysis with Ba(OH)2, which breaks peptide bonds but leaves glycosidic linkages intact, can only release hydroxyproline and HA that are not linked by glycosidic bonds to cell wall polysaccharides. If one of the HA or hydroxyproline is being linked to a polysaccharide, then its specific radioactivity should drop over the course of the experiment, as the 14C-labeled molecules are progressively rendered insoluble by becoming attached to the wall polysaccharides and therefore cannot appear in the HA profile.

The suspended cells were incubated in 14C-proline for 60 min. Aliquots were withdrawn at 30 and 60 min for 14C-HA profile determinations. At 60 min, a proline chase was initiated. The purpose of the chase was to emphasize any decline in specific radioactivity that might be occurring in one or more of the HA during incorporation of the glycoprotein by halting the supply of labeled extensin (Table III). There is indeed a change in the 14C-HA profile with time. Hydroxyproline and hydroxyproline-diarrabinoside are more prominent at 30 min than at 60 or 180 min. The same result was obtained in a repetition of the experiment. However, the change in pattern is not due to a transfer of 14C-hydroxyproline or 14C-hydroxyproline-diarrabinoside to a wall polysaccharide, because their specific radioactivities do not drop during the experiment (Fig. 4). Instead, as is seen in Table III, the absolute amount of radioactivity in these two components stays constant, while the other HA continue to gain radioactivity. There is no evidence to suggest that hydroxyproline or the HA are involved in the linkage of extensin to wall polysaccharide.

The similarity between the kinetics and specific activities of the hydroxyproline-mono, tri, and tetraarabinosides (Fig. 4) suggests that they may possibly be present in the same glycoprotein (extensin). These HA account for over 90% of the hydroxyproline in the cell wall. This result tends to strengthen the view that there is one dominant hydroxyproline-containing protein present in cell walls.

**Determination of the Specific Activities of the Hydroxyproline-Arabinosides from the Particulate and SEPS Fractions.** As the determination of the specific activities of the HA can yield information as to whether or not they are on one protein, as shown above, they were also determined for the particulate and SEPS fractions. The cells were exposed to 14C-proline for 30 min in the case of the cytoplasmic particulate fraction, and 120 min in the case of the SEPS. If there is only one hydroxyproline-containing glycoprotein present in either fraction, then similar specific activities would be expected for each of the HA composing that fraction. Table IV shows that the specific radioactivities of hydroxyproline and the HA derived from the particulate fraction are not the same. A similar result was found for the SEPS fraction.

![Fig. 3. Effect of a proline chase on the incorporation of 14C-hydroxyproline and 14C-proline into the particulate cytoplasmic fraction and the cell wall. Five µCi 14C-proline and 1 µmol proline were added to 100 ml suspension cultured cells. At 30 min, 2 mmol proline were added. Ten ml aliquots were withdrawn at the times indicated. Two flasks were run in parallel to provide enough material for analysis.](https://www.plantphysiol.org/attachment/3.png)

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**Table III.** Time course of the 14C-Hyp-arabinoside profile of the cell wall.

Five µCi 14C-Pro and 1 µmol Pro were added to 100 ml of cell suspension. Twenty ml aliquots were withdrawn at the times indicated. At 60 min 2 mmol Pro were added to chase the 14C-Pro.

<table>
<thead>
<tr>
<th>Time</th>
<th>Wall 14C-Hyp-arabinoside composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>HA-4</td>
</tr>
<tr>
<td>30</td>
<td>4035</td>
</tr>
<tr>
<td>60</td>
<td>16774</td>
</tr>
<tr>
<td>180</td>
<td>42608</td>
</tr>
</tbody>
</table>

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The finding that the volume and the proline separated by gel filtration on Sephadex G-25 were linked cosidically because this and other experiments (8) indicated that they contained hydroxyproline with only one or two sugar molecules attached. The hydroxyproline content was determined for each of the four samples and then volumes containing known amounts of hydroxyproline were assayed quantitatively for neutral sugars by GLC (Table VI). Hydroxyproline is not linked to the polysaccharide via arabinose. Instead, it appears likely that the attachment is via either galactose or glucose. This result was duplicated using a different sample of SEPS.

**DISCUSSION**

Path of Hydroxyproline-containing Protein to the Cell Wall.

The work reported here is in agreement with that of Chrispeels (6) and Dashek (8) in that protein-bound 14C-hydroxyproline can be chased from a particulate cytoplasmic fraction into the wall (Fig. 3).

Brysk and Chrispeels (4) found in carrot discs that before incorporation into the wall, much of the wall protein could be extracted by a salt solution. If the salt-extractable (S.E.) fraction represents a step on the way to incorporation of wall protein into the wall, it should behave as a pool. However, as Figures 1 and 2 show, it does not. Careful examination of Chrispeels's data (6) shows that also in carrot discs, the S.E. fraction does not chase or saturate, while in a later experiment (4), there was only a small decrease in 14C-hydroxyproline counts in this fraction.

I conclude that wall protein is transferred directly from a particulate fraction to the wall, where it is immediately incorporated without first passing through a salt-extractable pool. As the S.E. fraction shows little or no lag period in accumulating label.

**Partial Characterization of Hydroxyproline-X.** The colorimetric assay used to detect hydroxyproline can only function if the amino acid's carboxyl and secondary amine groups are free (19). The finding that hydroxyproline from material secreted into the medium by suspension-cultured *A. pseudoplatanus* cells is glycosidically linked to a polysaccharide was of great interest, because this linkage might help to explain the attachment of extensin to the cell wall.

Hydroxyproline-X was separated from the HA by gel filtration using Sephadex G-25. Hydroxyproline-X appeared in the void volume while the HA and hydroxyproline were retarded (Fig. 5). The material that appeared in the void volume was lyophilized, dissolved in approximately 1 ml of 0.1 M acetic acid, and rechromatographed on a Sephadex G-75 column. The eluate was tested for carbohydrate and hydroxyproline. Figure 6 shows that the hydroxyproline-containing material was separated from larger polysaccharide molecules. The neutral sugar composition was quantitatively determined for the material in the void volume and across the hydroxyproline peak at the positions indicated by arrows (Fig. 6). The neutral sugar composition at each point sampled is summarized in Table V. Arabinose and galactose accounted for approximately 75% of the neutral sugars. There is not a marked change in per cent molar composition across the peak which indicates that the polysaccharide is homogeneous. There is some enrichment in both mannose and arabinose in the shorter polymers.

In order to find which sugar is involved in the linkage between the polysaccharide and hydroxyproline, hydroxyproline-X purified on Sephadex G-25 and G-75 columns was hydrolyzed by 0.1 N trifluoroacetic acid at 121 C for 1 hr. The hydrolysate was separated on a Chromobeads B ion exchange column using a 0 to 0.2 N HCl gradient (Fig. 7). The contents of the tubes which gave rise to peaks 1 to 4 were lyophilized to give four samples. These samples were selected as their late elution from the column indicated that they contained hydroxyproline with only one or two sugar molecules attached. The hydroxyproline content was determined for each of the four samples and then volumes containing known amounts of hydroxyproline were assayed quantitatively for neutral sugars by GLC (Table VI).
dowed with hydroxyproline-triarabinoside and hydroxyproline and poorly so with hydroxyproline-tetraarabinoside; therefore, when mixed with wall precursor in the secretory vesicles, its presence would diminish the proportion of hydroxyproline-tetraarabinoside. The following three observations support this view. (a) The kinetics of wall and SEPS $^{14}C$-hydroxyproline content on chasing with "cold" proline (Fig. 2) suggests that both the wall and SEPS fractions are transported together. (b) Hydroxyproline-X which is very prominent in the SEPS HA profile but poorly represented in that of the cell wall is also present in the particulate fraction (Table II). (c) In experiments with radioactive proline, the varying specific radioactivities of the individual HA (Table IV) are consistent with the presence of a mixture of hydroxyproline-containing proteins present in the particulate fraction.

**Linkage of Extensin to the Cell Wall.** The time course of the $^{14}C$-HA profile of the cell wall (Fig. 4) is consistent with the idea that there is a single dominant hydroxyproline-containing glycoprotein (extensin) in the cell wall.

An experiment utilizing $^{14}C$-proline was designed to test whether or not extensin is linked to the cell wall through hydroxyproline or one of the HA. The specific radioactivities of hydroxyproline and the HA of material that had newly arrived at the cell wall were compared with those of hydroxyproline and HA derived from the wall some time later. Because no significant reductions in specific radioactivity in any of the HA or hydroxyproline were found (Fig. 4), a linkage between extensin and wall polysaccharides via hydroxyproline seems unlikely. This conclusion is in agreement with data presented by Holleman (12). In this work, $^{14}C$-hydroxyproline was shown to predominate over $^{14}C$-proline in the cell walls of *A. pseudoplatanus* cells. However, the total amount of radioactivity incorporated into the cell walls was unchanged in the presence of $\alpha\beta$-dipyridyl, even though this inhibitor completely blocked the formation of hydroxyproline from proline. This result would not

(Figs. 1 and 2), it might be argued that the S.E. fraction represents cytoplasmic contamination. This is possible, but as the S.E. fraction contains hydroxyproline and HA, both of which are in low concentration in the cytoplasm, the S.E. fraction most likely represents a small portion of wall protein mixed with SEPS that gets ionically bound during its passage through the cell wall.

**Is Extensin Present in the SEPS Fraction?** Table II shows that wall, SEPS and S.E. fractions have different HA profiles, demonstrating that hydroxyproline-containing glycoproteins in SEPS are not related to those in the cell wall. SEPS cannot therefore be regarded as wall protein that "escaped" into the medium.

The difference between the particulate cytoplasmic fraction and the cell wall HA profiles was also noted by Karr (13). He suggested that the lower proportion of hydroxyproline-tetraarabinoside in the particulate fraction was due to the fact that extensin was still in the process of being glycosylated. However, the difference can be viewed in another way. The lower proportion of hydroxyproline-tetraarabinoside could be due to a higher proportion of hydroxyproline-triarabinoside and hydroxyproline. I suggest that the particulate fraction consists of at least two components—one destined for the cell wall and the other for the medium (SEPS). The SEPS fraction is comparatively well-en

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**Fig. 6.** Separation of hydroxyproline-X from larger polysaccharides. The material that appeared in the void volume of the Sephadex G-25 column was lyophilized, redissolved in about 1 ml 0.1 M acetic acid, and chromatographed on a Sephadex G-75 column. It was eluted with 0.1 M acetic acid at a flow rate of 0.15 ml/min. Fractions of 0.8 ml were collected, 0.2-ml aliquots were assayed for hydroxyproline, and 0.1-ml aliquots were used to assay for neutral sugars. Arrows denote the fractions analyzed for the neutral sugar compositions presented in Table V.

**Table V.** Neutral sugar composition within selected fractions from the Sephadex G-75 Hyp-X peak from Fig. 6

<table>
<thead>
<tr>
<th>Tube</th>
<th>Neutral sugars in mole sugar/mole HYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Rha</td>
</tr>
<tr>
<td>25</td>
<td>1.1</td>
</tr>
<tr>
<td>35</td>
<td>2.7</td>
</tr>
<tr>
<td>40</td>
<td>2.6</td>
</tr>
<tr>
<td>50</td>
<td>1.4</td>
</tr>
<tr>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Fig. 7.** Separation of 0.1 n trifluoroacetic acid hydrolysat of hydroxyproline-X on Chromobeads B. Lyophilized hydroxyproline-X purified by Sephadex G-25 and G-75 gel filtration was hydrolyzed by 0.1 n trifluoroacetic acid at 121 C for 1 hr. The hydrolytic products were subsequently separated on a Chromobeads B column using a 0 to 0.2 n HCl gradient.

**Table VI.** Neutral sugar composition of low molecular weight fragments of Hyp-X

<table>
<thead>
<tr>
<th>Peak</th>
<th>Gal</th>
<th>Glu</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>n mole</td>
<td>n mole</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.50</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.22</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.64</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>
be expected if extensin is linked to the cell wall via hydroxyproline.

The kinetic data of hydroxyproline-diarabinoside and hydroxyproline (Fig. 4) are difficult to interpret. The presence of two hydroxyproline-containing proteins in the cell wall, one turning over rapidly, the other accumulating slowly, might account for the constant specific radioactivities observed during the experiment. A short lived protein would explain the constant specific radioactivity achieved by 30 min, and the expected loss of label from it during the proline chase would be balanced by the accumulation of a longer lived protein.

**Hydroxyproline-X.** This is the most notable feature of the HA profile of the SEPS fraction (Table II) and has been described briefly already (22). The polysaccharide linked to hydroxyproline consists of an arabinogalactan with some xylose and glucose attached (Table V). Hydroxyproline appears to be attached to both galactose and glucose (Table VI). It is not attached to the polysaccharide via arabinose, because this linkage was not detected among the hydrolytic products. It could be argued that HA were not detected because the linkage is extremely labile. In that case, however, free hydroxyproline should have been seen, which was not the case.

Hydroxyproline-X is also present in the particulate fraction and a small amount has been found associated with the cell wall (Table II).

Karr (13), working with suspension-cultured A. pseudoplatanus cells, isolated a molecule containing hydroxyproline, arabinose, galactose, and glucose from a Ba(OH)₂-hydrolyzed particulate cytoplasmic fraction. This molecule was found to be larger than hydroxyproline-tetraarabinoside and is undoubtedly hydroxyproline-X.

The presence of hydroxyproline-X does not appear to be restricted to wall-related glycoproteins only, because it was also found in a cytoplasmic, trichloroacetic acid-soluble hydroxyproline-rich glycoprotein. This glycoprotein has a HA profile very different from that of the other fractions (Table II). It is 95% carbohydrate by weight, with arabinose and galactose as the major sugar components (17, 18).

An arabinogalactan attached to a hydroxyproline-containing peptide has been isolated from wheat endosperm (10). Its localization is unknown. The peptide was deemed to be unrelated to extensin as it could be extracted by nondegradative means. The authors suggested that their arabinogalactan peptide could be a component of the middle lamella in mature endosperm.

Any suggestion as to the function of the arabinogalactan attached to proteins via hydroxyproline must be highly speculative. Nevertheless, among possibilities to be considered is that it is a method to render proteins more soluble. Alternatively, the arabinogalactan may function as an adaptor linking proteins to polysaccharides (16).

**Is the SEPS Fraction a Model for the Cell Wall?** Keegstra et al. (14) say that “the glycoprotein of SEPS presents an interesting model for the connection between the polysaccharides and the structural protein of the cell wall.” They found an arabinogalactan associated with a hydroxyproline-containing protein from SEPS. They concluded, but without direct evidence, that it was attached via galactose to a serine residue, however from the work presented here it might in fact be linked to hydroxyproline instead. Information derived from the SEPS fraction was used exclusively for the extensin-polysaccharide relationship in their model of the cell wall. The assumption that SEPS could be used as a model for the cell wall seemed eminently reasonable, as the carbohydrate composition of SEPS is very similar to that of the noncellulosic portion of the cell wall (3). In addition, the xylan-lucan present in the SEPS appears to be identical with that found in the wall (2).

However, hydroxyproline accounts for 1.7% of the weight of the wall, but for only 0.3% of the SEPS. The HA profiles in Table II show that the hydroxyproline-containing glycoproteins in the SEPS differ markedly from those in the wall or particulate fraction. Table IV shows that in contrast to the cell wall, the medium does not contain one major hydroxyproline-containing protein. Finally, the SEPS fraction is deficient in hydroxyproline-tetraarabinoside, the dominant HA of the cell wall. Thus, it would appear that the SEPS fraction contains little or no wall-like extensin, therefore SEPS cannot be regarded as a mixture of wall protein and hemicellulose that escaped into the medium; it therefore should not be used as a model for the cell wall.

Until more data are available from work on the cell wall itself, the method by which extensin is linked to wall polysaccharide must be regarded as undefined.

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