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Distribution and Metabolism of Protein-Bound Hydroxyproline in an Elongating Tissue, the Avena Coleoptile[1]

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Abstract. A study has been made of the distribution and metabolism of protein-bound hydroxyproline in an elongating tissue, the excised *Avena* coleoptile. The hydroxyproline-containing proteins of this tissue have been separated into 3 fractions on the basis of their solubilities. The cytoplasmic, trichloroacetic acid-insoluble proteins (S-fraction) contain the bulk of the proline of the cells but only 20 % of the hydroxyproline. The cytoplasm also contains a previously unrecognized trichloroacetic acid-soluble, non-dialyzable fraction (DS-fraction) which is low in proline but contains 20 % of the hydroxyproline. The remaining 60 % of the hydroxyproline is in the wall-bound, cold alkali-soluble fraction (extensin).

Incorporation of free proline into the proline and hydroxyproline of all fractions is linear with time for at least 12 hours. The specific activity of the proline at any time is the same in all 3 fractions while the specific activity of the hydroxyproline is 4-times greater in the S-fraction than in the W-fraction. During a pulse-chase experiment the specific activity of the proline decreases 25 to 40 % in all fractions during the chase. The labeling of hydroxyproline in the wall increases during the chase while that of the DS-fraction remains constant. In the S-fraction, the labeling in hydroxyproline rapidly drops 30 to 35 % during the chase but then remains constant. It is concluded that the majority of the hydroxyproline-proteins in the cytoplasm are not transported to the wall. It is suggested that a sizeable portion of the cytoplasmic hydroxyproline may be located in enzymatic proteins.

The occurrence of protein-bound hydroxyproline in plants (13, 29, 30) and its concentration in the cell wall (7, 16, 18) are now well established. The role of these proteins is still uncertain, although Lamport has suggested (12, 13) that they are structural proteins (extensins) which are involved in the control of cell elongation. If this is so, it will be necessary to understand their properties and metabolism in elongating tissues before we can understand the process of cell elongation.

Little is known about the hydroxyproline-proteins of elongating tissues other than that they are concentrated in the cell wall (5, 13, 19) and that their level is considerably lower than that in callus tissues (5, 10, 13, 19, 29). The metabolism of hydroxyproline-proteins has been extensively studied, to date, only in callus cells (13, 18, 21). The assumption has been made (13) that although callus cells and cells of elongating tissues differ in many ways, they have the same hydroxyproline metabolism.

This investigation into the hydroxyproline metabolism of an elongating tissue, the *Avena* coleoptile, was undertaken for 2 reasons. The primary reason was to provide the information concerning the metabolism and distribution of hydroxyproline-proteins which is necessary for the author's study of the growth-inhibiting effects of free hydroxyproline (3, 4, 6). The second reason was to determine whether the hydroxyproline metabolism of an elongating tissue is necessarily the same as that of callus tissues; *i.e.*, whether conclusions concerning the hydroxyproline-proteins which have been reached with callus tissues can be assumed also to hold for all other tissues. It will be shown that such an assumption is not valid.

Materials and Methods

The experimental material consisted of 14 mm sections cut from 25 to 32 mm long coleoptiles of *Avena sativa*, var. Victory. Seedlings were grown and sections were prepared as detailed earlier (2). Leaves were removed from all sections.

Unless otherwise stated, groups of 100 sections were incubated for 0 to 24 hours in 12 to 20 ml of basal medium that contained K-maleate buffer (2.5 mM, pH 4.7), sucrose (2 % w/v), indoleacetic acid (IAA, 5 μg/ml) and penicillin G (0.1 mM). In some experiments proline-μ-^{14}C (200 μc/μmole) was also present. Incubations were carried out in the dark in beakers which were rotated at 30 rpm on a gyrotory shaker. One of the following 3 methods was then used to prepare the material for analysis.

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Method A (Whole Tissue). Sections were rinsed with water, extracted 8 times for 5 minutes with 10 ml of boiling 80 % ethanol, washed with 100 % ethanol and dried at 90°.

Method B (Ground Tissue). After rinsing with water, groups of 30 sections were ground in 2 ml of tris buffer (0.05 M, pH 7.5) in an all-glass homogenizer, 4 volumes of absolute ethanol were added and the homogenate was boiled for 5 minutes. The cooled homogenate was then centrifuged and the pellet was extracted 7 times for 5 minutes with 5 ml of boiling 80 % ethanol and then dried. The alternative procedure of precipitating the proteins with 20 % trichloroacetic acid (TCA) at 4°, followed by the TCA wash procedure of Peterson and Greenberg (20) was found to give comparable results.

Method C (Fractionated Tissue). Washed sections were homogenized in 6 ml of tris buffer with 200 μ glass beads in a Virtis "45" homogenizer (11). Walls were separated by filtration through a bed of glass beads and washed by resuspension in water followed by filtration through a new bed of beads. The walls were freed of beads, washed with the TCA series and dried (W-fraction).

The 2 filtrates were combined and TCA-insoluble proteins (S-fraction) were precipitated with 5 % TCA at 4° for 18 hours. After collection by centrifugation, the S-proteins were washed with the TCA series and dried. The supernatant from the centrifugation was dialyzed overnight against 3 changes of distilled water and concentrated. This fraction contained the TCA-soluble proteins (DS-fraction).

Comparable results were obtained when the homogenization was carried out with glycerol instead of tris buffer and when the sections were prehomogenized in an all-glass homogenizer. Omission of the hot TCA wash step, which could be expected to solubilize any collagen-like protein, did not affect the results.

Each of the protein fractions was then hydrolyzed and the proline and hydroxyproline were separated chromatographically and assayed by methods which have already been described in detail (4, 5, 6). Protein was determined by the Kjeldahl technique of Miller and Miller (17).

All experiments were carried out at least 3 times and in most experiments replicates were run.

Results

The presence of protein-bound hydroxyproline in *Avena* coleoptiles has already been reported (4, 10, 19) and is confirmed in this study (table I). Two things should be noted about the data in table I. First, the values for the percent of hydroxyproline in protein and the ratio of hydroxyproline to proline are both considerably lower than the values which have been reported for callus tissues (13, 18). Secondly, although homogenization of the tissue leads to some loss in protein, it does not affect the relative proportion of hydroxyproline, proline, and protein.

Table I. *Properties of* Avena *Coleoptile Sections*

Sections were incubated 8 hours in basal medium, then prepared for analysis by method A (whole) or method B (ground). Values are for 1 cm sections.

	Whole	Ground
Dry wt - μg	236	...[1]
Protein - μg	58	46
Proline - μg	2.32	1.90
Proline - % of protein	4.0	4.1
Hydroxyproline - μg	0.21	0.23
H/P × 100	5.2	5.5

[1] Value not given due to contamination with glass powder.

Table II. *Distribution of Hydroxyproline and Proline among* Avena *Coleoptile Proteins*

Sections were incubated for 22 hours in basal medium, then fractions prepared by method C. Results are the average of 10 experiments.

Fraction	Proline	Hypro	H/P × 100
	μg/1 cm section		
W-fraction	0.19	0.055	29
S-fraction	1.35	0.019	1
DS-fraction	0.065	0.020	30

The hydroxyproline-proteins of the *Avena* coleoptile have been separated into 3 fractions on the basis of their solubility (table II). The cytoplasmic, TCA-insoluble proteins (S-fraction) contain over 80 % of the bound proline of the cells, but only 20 % of the hydroxyproline. The cytoplasm also contains a previously unrecognized TCA-soluble, non-dialyzable fraction (DS-fraction) which is low in proline but has an additional 20 % of the cells hydroxyproline. The remaining 60 % of the protein-bound hydroxyproline is in the wall fraction (W-fraction).

It should be noted that the wall fraction contains a low but significant amount of proline. Since King and Bayley (10) have reported that they were able to obtain an *Avena* coleoptile wall preparation by essentially identical procedures which was devoid of proline, the possibility was considered that the proline in the W-fraction was due to cytoplasmic contaminants. To test this, aliquots of the W-fraction were subjected to further homogenization and to the NaCl: sodium lauryl sulfate wash procedure of Punnett and Derrenbacker (22). These procedures lowered the protein content of the W-fraction from about 5 % to 1.25 % and lowered the content of both proline and hydroxyproline, but did not significantly alter the H/P ratio. This suggests that this proline is as indigenous to the wall as is the hydroxyproline since procedures which cause a decrease in the proline level also cause a parallel decrease in hydroxyproline.

Table III. *Removal of Wall-bound Hydroxyproline by NaOH and Formic Acid*

Sections were incubated 20 hours in basal medium + proline-^{14}C (0.5 μc/150 sections). W-fraction then were prepared by method C and aliquots extracted with NaOH (3 times, 1 N, 1 hr, 25°) or formic acid (twice, 1 hr, 100°).

	Hypro/150 sections		Dry wt
	μg	*cpm*	%
Before extraction	7.4	3200	100
After NaOH	0.4	120	55
After formic acid	0	195	51

Olson (18) has shown that the wall fraction of tobacco callus cells contain 2 hydroxyproline-protein fractions; a major fraction (85 %) that is resistant to cold, dilute alkali extraction and a minor component (15 %) which is extractable with cold alkali or hot formic acid. Dougall and Shimbayashi (7) and Lamport (13) have also demonstrated that the bulk of the wall-bound hydroxyproline in callus cells is resistant to cold alkali or formic acid extraction. In contrast, the hydroxyproline-proteins of the *Avena* coleoptile wall are almost totally extracted by both alkali and formic acid (table III). No evidence for a cold alkali-resistant hydroxyproline fraction could be found in *Avena* coleoptiles.

Olson *et al.* (19) have reported that only 40 % of the hydroxyproline of whole *Avena* coleoptile tissues is solubilized by Pronase. This might suggest that the bulk of the hydroxyproline is in a peptide which is resistant to attack by Pronase. Alternatively, the hydroxyproline-proteins of intact tissues may simply be inaccessible to the enzyme. Evidence to support this latter idea is obtained by homogenizing the *Avena* coleoptiles prior to the Pronase treatment. Under these conditions, at least 65 % of the hydroxyproline is solubilized by Pronase (table IV) and in some experiments over 85 % was removed.

We have already shown (4, 6) that free proline rather than free hydroxyproline is the normal precursor of protein-bound hydroxyproline in *Avena* coleoptiles as in other plant (13, 21) and animal systems (26). When *Avena* coleoptiles are incubated with proline-^{14}C, the proline and hydroxyproline of all 3 protein fractions becomes labeled (table V). Time-course studies show that the labeling in each case increases linearly with time for at least 12 hours at which time the proline is exhausted from the medium (fig 1). It should be noted that although the prolines of the 3 fractions differ widely at any time in total labeling (table V), their specific activities are nearly equal (fig 1). In contrast, the specific activities of the hydroxyproline varies markedly between fractions with that of the S-fraction being 4 times higher than that of the W-fraction.

Table IV. *Extraction of Hydroxyproline-Proteins by Pronase*

Sections were incubated 22 hours in basal medium, then prepared by method A (whole) or method B (ground). Tissues then were incubated 16 hours in 0.05 M tris (pH 7.5) ± Pronase (200 μg/ml).

	No Pronase	+ Pronase	% Removed
	μg/1 cm section		
Intact			
Proline	2.31	0.28	88
Hypro	0.12	0.070	42
Ground			
Proline	1.58	0.16	90
Hypro	0.075	0.025	67

Table V. *Incorporation of Proline-^{14}C into Proline and Hydroxyproline of* Avena *Coleoptile Fractions*

Groups of 100 sections incubated 20 hours in basal medium + 1 μc proline-^{14}C. Tissues prepared by method C.

Fraction	Proline	Hypro	H/P×100
	cpm/100 sections		
W-fraction	24,450	5025	21
S-fraction	180,500	5890	3
DS-fraction	4990	2855	57

The pulse-chase technique has been utilized by Olson (18) to show that most of the hydroxyproline-proteins in the cytoplasm of tobacco callus cells appear to be transferred ultimately to the cell wall. In order to determine whether a similar situation prevails in *Avena* coleoptiles, sections were incubated for 4 hours in a medium that contained proline-^{14}C, and then chased for up to 18 hours in a solution which contained an excess of unlabeled

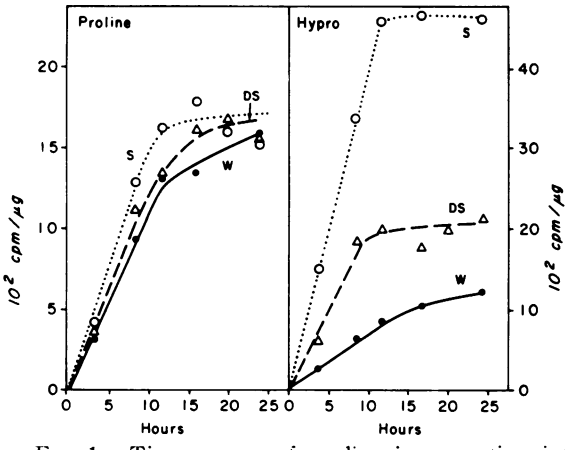

FIG. 1. Time course of proline incorporation into proline and hydroxyproline of *Avena* coleoptile protein fractions. Groups of 100 sections incubated in 13 ml of basal medium + proline-^{14}C (10 μM, 2 μc) for 3 to 24 hours. Tissues prepared by method C. In this experiment the medium was depleted of proline after 12 hours Symbols: W (—●—), S (···○···), DS (---△---).

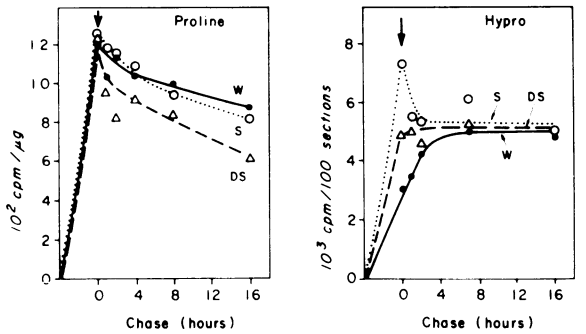

FIG. 2. Changes in labeling of *Avena* coleoptile protein fractions during a pulse-chase experiment. Groups of 150 sections incubated for 4 hours in 12 ml basal medium + proline-^{14}C (1 µM, 4 µc), washed, and placed in groups of 100 in 40 ml basal medium + 100 µM proline. Chase solution replaced after 30 minutes. Tissues prepared by method C. Arrows denote start of chase. Symbols: W (—●—), S (···○···), DS (---△---).

proline (fig 2). During the chase period the specific activity of the proline decreased in all 3 fractions until after 18 hours it was 50 to 75 % of the initial value. Since total proline remains fairly constant in all fractions, this decline would appear to indicate that some turnover of proline-containing proteins does occur in each of the fractions.

The labeling pattern of hydroxyproline presents a different picture (due to the large increase in total hydroxyproline in the W-fraction during the chase the labeling is not expressed in terms of specific activities). During the chase, the labeling of the hydroxyproline of the DS-fraction remains constant while that of the wall fraction continues to increase for 4 to 6 hours. The final labeling of the W-fraction may be 60 to 120 % greater than at the start of the chase. In the S-fraction, the label in hydroxyproline rapidly declines by 30 to 35 %, but then remains constant for the remainder of the chase period. In none of the 4 pulse-chase experiments did the hydroxyproline of the S-fraction lose more than 35 % of its label during the chase period. In each case the total increase in labeled hydroxyproline in the S-fraction was nearly matched by the increase in the W-fraction.

Discussion

It has been shown in this study that the hydroxyproline-proteins of the *Avena* coleoptile can be separated into 3 fractions on the basis of their solubility. The purity of these 3 fractions is difficult to assess and some cross-contamination may occur, but the differences in behavior of their hydroxyproline during the chase period of a pulse-chase experiment suggests that the 3 fractions are distinct. A lack of contamination of the W-fraction with cytoplasmic proteins is also indicated by the fact that while the NaCl: sodium lauryl sulfate extraction removed both proline and hydroxyproline from the W-fraction, the H/P ratio remained unaffected; if cytoplasmic proteins with their much lower H/P ratio had been present, their removal would have caused a marked increase in the H/P ratio. The finding that there was a decrease in the labeling of the proline but not of the hydroxyproline of the W-fraction during a chase period suggests that this fraction contains more than 1 protein and that the proline and hydroxyproline are located in different peptides. This is in agreement with the observation of Lamport (14) that the hydroxyproline-containing peptides of tomato callus walls contain no proline.

The location within the tissue of the S- and DS-fractions is not known, but it is unlikely that they are extracellular (*i.e.*, loosely associated with the wall) since they were always liberated upon disruption of the cells no matter how gentle was the procedure that was used. The DS-fraction (TCA soluble, non-dialyzable), which has not previously been recognized and has apparently been discarded in previous investigations (13, 18, 21), is of some particular interest since it contains 20 % of the hydroxyproline of the tissue and has a H/P ratio that is similar to that of the wall fraction. It is unlikely to consist simply of wall fragments since the labeling of its hydroxyproline remained constant during the chase period while that of the W-fraction continued to increase. Likewise, it is unlikely to be a precursor of wall protein since in that case the labeling of the hydroxyproline would have been expected to decrease during the chase period. It seems more likely that this fraction contains proteins which are chemically similar to the wall proteins but have a different location within the cell.

The metabolism of protein-bound hydroxyproline in the *Avena* coleoptile is similar to that of callus cells (13, 18) in most respects but differs from it in 2 ways. The first concerns the ability of the cytoplasmic hydroxyproline-containing proteins to be transferred to the cell wall. In callus cells the bulk of these proteins undergo such a transfer to the cell wall as judged by the pulse-chase experiments of Olson (18). This is in agreement with the suggestion of Lamport (13) that the hydroxyproline-proteins of plant cells form a single class, the extensins, whose site of action is in the cell wall, and that the hydroxyproline-proteins in the cytoplasm are simply in transit between their site of synthesis and the wall. In contrast, only a minority (30–35 %) of the cytoplasmic hydroxyproline-proteins of the *Avena* coleoptile are transferred to the wall during a pulse-chase experiment, even if it is assumed that all of the decrease in the cytoplasmic fraction and increase in the wall fraction represents transfer of hydroxyproline-proteins. This must mean that there are at least 2 classes of hydroxyproline-proteins; the wall-concentrated extensins and a second group which are located in the cytoplasm and are never

transported to the walls. In this connection it is interesting to note that hydroxyproline is reported to be concentrated in the cytoplasmic proteins of brown algae (8) and carrot callus cells (28) and concentrated in the chloroplasts of bean leaves (9). The relative abundance of the 2 classes of hydroxyproline-proteins apparently depends upon the tissue. In callus cells the level of wall-bound extensins and their cytoplasmic precursors is so high that it is difficult to detect the cytoplasmic hydroxyproline-proteins. Because the *Avena* coleoptile has a considerably lower level of wall-bound hydroxyproline it has been possible to demonstrate the existence of the cytoplasmic hydroxyproline-proteins in such a tissue.

Shannon *et al.* (25) have demonstrated that hydroxyproline is present in 3 of 5 isozymes of horse radish peroxidase. This demonstration that hydroxyproline can be present in enzymatic proteins raises the possibility that at least part of the cytoplasmic hydroxyproline in the *Avena* coleoptile is present in enzymatic proteins. It is interesting to note that when Steward and Chang (27) separated the soluble proteins of carrot callus cells by gel electrophoresis they found hydroxyproline in 8 of the 9 protein bands. Although this may simply reflect contamination of the protein bands with a single hydroxyproline-containing protein or fragments of it, it may also mean that hydroxyproline is widely spread among cytoplasmic proteins. However, such a wide distribution of hydroxyproline would not necessarily mean that hydroxyproline exists in a variety of peptides as it has been shown that the same hydroxyproline-arabinose association is present in both horse radish peroxidase (25) and tomato callus cell walls (14, 15). The possibility should be considered that a limited number of hydroxyproline-containing glycopeptides exist which when attached to other proteins or polysaccharides confer some special properties on them such as the ability to be transported across membranes.

The second difference between *Avena* coleoptile and callus cells is the lack of wall-bound, cold alkali-resistant hydroxyproline-proteins in the elongating tissue. This difference may be, in part, the cause of the different growth patterns of these 2 types of cells. Lamport has summarized the evidence (13) which suggests that alkali-resistant hydroxyproline-peptides confer rigidity on the cell wall by cross-linking to arabanogalactans. The slow growth habit of callus cells may be a consequence of the high level of these crosslinks, while the ability of *Avena* coleoptile cells to undergo rapid cell elongation may be a result of the absence of such crosslinks in this tissue.

The fact that the hydroxyproline-proteins of *Avena* coleoptile walls are extracted with cold alkali while those of callus walls are not does not, in itself, show that there is a basic difference between these hydroxyproline-proteins. The ability or lack of ability of a hydroxyproline-protein to be extracted must be due, in large part, to the hemicellulose to which it is attached and the degree of crosslinking. It is already known that there are differences in the hemicellulose components of *Avena* coleoptile (23) and callus cell walls (13, 24). Likewise, the type of polysaccharide to which the hydroxyproline-peptide is linked in tomato callus cells, an arabanogalactan (15), appears to be different from that in corn pericarp, a mucopolysaccharide (1). The chemical relationship between the alkali-extractable hydroxyproline-proteins of the *Avena* coleoptile wall and the alkali-resistant proteins of callus cells cannot be determined until an investigation into the hydroxyproline-sugar linkages in *Avena* coleoptiles is completed.

The difference in hydroxyproline-metabolism between *Avena* coleoptile and callus tissue may indicate a basic difference in hydroxyproline-metabolism between elongating and non-elongating cells, but it is just as likely that these differences are due to the differences in species and in tissues that have been used. More information is needed concerning the hydroxyproline-metabolism of elongating tissues before this can be settled. In any case, it is apparent that the hydroxyproline-metabolism of all tissues is not the same and that conclusions concerning the hydroxyproline-proteins which are reached from studies with callus cells may not be valid for all other tissues.

Acknowledgments

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