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Hydroxyproline Formation and its Relation to Auxin-induced Cell Elongation in the Avena Coleoptile[1]

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Abstract. **A study has been made of the effects on hydroxyproline formation of 4 factors that influence the rate of cell elongation in the *Avena* coleoptile; auxin, sugars, an external osmoticum, and actinomycin D. Hydroxyproline formation is increased by a combination of auxin and sucrose, but is affected to a much lesser extent by either factor alone. Its formation is inhibited by an external osmoticum but is scarcely affected by actinomycin D. The lack of correlation between the amount of hydroxyproline synthesis and the growth rate suggests that hydroxyproline formation is not involved in the actual process of wall loosening. It is suggested, instead, that if the wall is to retain its capacity for rapid extension, those hemicelluloses which are incorporated into it by intussusception rather than by apposition must be attached to a hydroxyproline-protein.**

The presence of protein-bound hydroxyproline in the *Avena* coleoptile (8, 9, 15, 22), and its synthesis from free proline (8, 9) have already been demonstrated, but the role of these hydroxyproline-proteins remains a question (17). From an analysis of the inhibitory effects of free hydroxyproline on growth (5) and on hydroxyproline formation (8), we have concluded that 1 fraction[2] of the hydroxyproline-proteins may be required in the cell wall extension process and may be synthesized only during cell elongation. If this is so, the synthesis of hydroxyproline should be sensitive to auxin and to factors which affect auxin-induced elongation. This paper reports the results of a study on the effects on hydroxyproline synthesis of 4 factors which influence the rate of cell elongation; auxin, exogenous sugars, turgor pressure, and actinomycin D.

Materials and Methods

The experimental material consisted of 14-mm sections cut from 25 to 32 mm long coleoptiles of *Avena sativa*, var. Victory. Methods for growing the seedlings and for collecting the sections have been described earlier (3, 20).

Groups of 30 to 100 sections were incubated 10 to 22 hr in potassium maleate buffer (2.5 mM, pH 4.7) which contained, where indicated, indoleacetic acid (IAA, 5 μg/ml), sucrose (2% w/v), penicillin G (0.1 mM), L-proline-μ-^{14}C (175 mc/mmole), adenine-8-^{14}C (49 mc/mmole), DL-leucine-1-^{14}C (3 mc/mmole), actinomycin D (50 μg/ml) and mannitol (0.1–0.5 M). The lengths of the sections were determined and the material was treated by procedures already described in detail (9) to give a total protein fraction (Method A) or protein which was fractionated (Method C) into wall (W-fraction), cytoplasmic, TCA-insoluble (S-fraction) or cytoplasmic, TCA-soluble, non-dialyzable (DS-fraction) fractions. Each fraction was washed with a TCA series (25) and then hydrolyzed in 6 N HCl. The amino acids were separated by descending paper chromatography in isopropyl alcohol:formic acid:water (15:2:2). The location of the proline, hydroxyproline and leucine was determined with a radio-chromatogram scanner, the amino acids were eluted and an aliquot of each was dried on an aluminum planchet and counted in an automatic gas-flow counter. Total proline (35) and hydroxyproline (29) were also determined chemically. Details of these procedures can be found in earlier papers (8, 9, 11). RNA synthesis was followed by determining the incorporation of adenine-^{14}C into RNA. The methods of Osborne (24) were used to extract the RNA and to determine its activity.

Each experiment was repeated at least 3 times with at least 2 replicates of each treatment in each experiment.

Results

Hydroxyproline synthesis and cell elongation are affected in different ways by auxin and exogenous sugars. The rate of cell elongation is stimulated by auxin whether or not sugars are present (6, 33); sugars prolong the period of auxin-induced elongation but do not themselves change the growth rate.

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[2] The term fraction is used here in a physiological rather than a biochemical sense.

Hydroxyproline synthesis, on the other hand, is increased by up to 85 % by a combination of auxin and exogenous sugars (tables I and III) but is only slightly enhanced (up to 15 %) by either auxin or sugars when administered separately.

Separation of *Avena* coleoptile proteins into wall and cytoplasmic fractions reveals that the bulk of the extra hydroxyproline which is formed in response to the combination of auxin and sugar is located in the wall fraction with smaller amounts in the 2 cytoplasmic fractions (table II). In the absence of added sugar, auxin still causes a detectable, though considerably smaller, increase in the hydroxyproline of the wall fraction.

While the reproducibility within any one experiment has usually been excellent (*e.g.*, table III), with replicates rarely varying by more than 15 %, there has been considerable variability between experiments in the magnitude of the auxin-induced increase in hydroxyproline formation. In 10 experiments, in each of which auxin caused a 65 to 80 % increase in length over 22 hr, the auxin effect on hydroxyproline synthesis varied from 2 to 85 % with an average of 41 % (table IV). It is interesting to note that regardless of the amount of the auxin effect on hydroxyproline formation, the auxin effect on proline incorporation into protein was, in each case, about one-half as great.

Auxin-induced cell elongation requires continued RNA synthesis as indicated by the ability of the RNA synthesis inhibitor actinomycin D to inhibit cell elongation (4, 14, 21). In the present experiments actinomycin D, at 50 μg/ml, caused an 85 % inhibition of the incorporation of adenine into RNA and a 65 % inhibition of cell elongation over 16 hr (table V). Incorporation of amino acids into protein was inhibited to a lesser extent and depended upon the particular amino acid; leucine incorporation was decreased by 50 % while proline incorporation was reduced by only 30 %. Hydroxyproline formation, on the other hand, is only slightly affected by actinomycin D; the 15 % decrease may simply have been due to the lowered incorporation of the precursor, proline, into protein.

Cell elongation and hydroxyproline synthesis are both inhibited by a reduction in turgor pressure (*i.e.*, by the presence of an external osmoticum such

Table III. *Variation in Amount of Hydroxyproline Formation Within a Single Experiment*

Groups of 30 sections incubated 22 hr in 5 ml basal medium with proline-μ-^{14}C (0.5 μc), sucrose (2 %) and with or without IAA (5 μg/ml). Protein prepared by method A (9).

Replicate	Hydroxyproline	
	+ IAA	— IAA
	cpm/30 sections	
1	1005	615
2	945	770
3	965	720
Avg	970	700

Table I. *The Effect of Auxin and Sucrose on Hydroxyproline Formation*

Groups of 30 sections were incubated 22 hr in 5 ml basal medium (K-maleate buffer + penicillin G) containing proline-μ-^{14}C (0.5 μc) and with IAA (5 μg/ml) and sucrose (2 %) as indicated. Tissues prepared by method A (9).

Conditions	Hydroxyproline		Proline	
	cpm/30 sections	*AE-%*[1]	*cpm/30 sections*	*AE-%*
IAA + sucrose	9750	+40 %	141,000	+21 %
Sucrose	6950		116,000	
IAA	6200	0	105,000	+14 %
Basal medium	6200		92,000	

[1] AE; Auxin effect.

Table II. *Location of Hydroxyproline Which Is Formed in Response to Auxin and Sucrose*

Groups of 80 sections incubated for 22 hr in 20 ml basal medium + proline-μ-^{14}C (1 μc, 0.1 mM) and, where indicated, IAA and sucrose. Fractions prepared by method C (9).

Fraction	IAA	Hydroxyproline			
		+ Sucrose		— Sucrose	
		cpm/80 sections	*AE-%*[1]	*cpm/80 sections*	*AE-%*
W-fraction	+	2590	+142 %	1020	+39 %
	—	1070		735	
S-fraction	+	3780	+21 %	3280	+5 %
	—	3120		3110	
DS-fraction	+	890	+53 %	590	+23 %
	—	580		480	

[1] AE; auxin effect.

Table IV. *Variation in Amount of Auxin-induced Increase in Hydroxyproline Formation and Proline Incorporation*

Groups of 30 sections incubated 22 hr. Conditions of incubation same as table III. Elongation increased 65 to 80 % by auxin in each experiment.

Experiment	Replicates	Auxin effect on: Hydroxyproline	Proline
		%	%
1	2	58	33
2	2	16	8
3	2	23	7
4	3	61	39
5	3	51	26
6	3	37	15
7	3	41	19
8	2	2	0
9	3	85	62
10	3	40	21
Avg		41	23

as mannitol). The elongation process is almost completely inhibited by a moderate drop in turgor pressure (2), while complete inhibition of auxin-induced wall loosening only occurs at zero turgor pressure (7). Wall synthesis, as judged by incorporation of glucose into wall polysaccharides (7, 23), and proline incorporation into protein (fig 1) are both progressively inhibited by a reduction in turgor pressure until a maximum inhibition of about 60 % is reached at zero turgor. Hydroxyproline synthesis is even more sensitive to turgor pressure; at all turgors below full turgor (*ca.* 9 atm) hydroxyproline

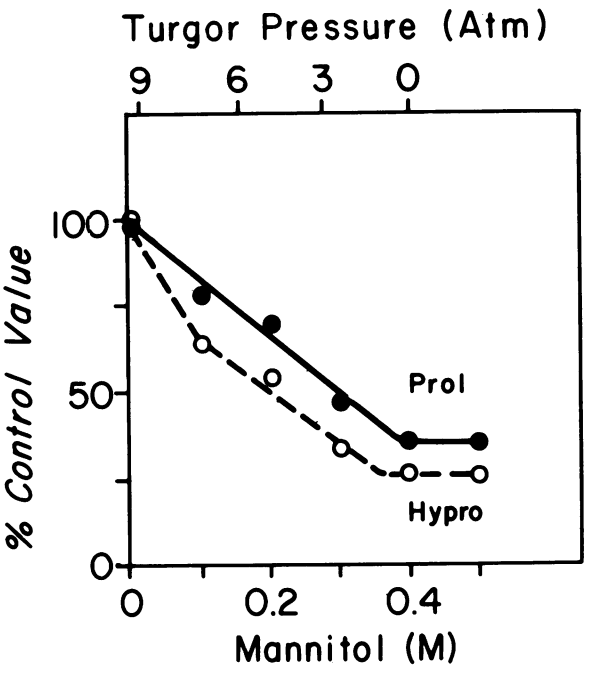

FIG. 1. Effect of turgor pressure on hydroxyproline formation and proline incorporation. Groups of 30 sections incubated 10 hr in 5 ml basal medium with addition of IAA, sucrose, proline-^{14}C (0.5 μc, 1 μM) and 0 to 0.5 M mannitol. Tissues prepared by method A (9). Values expressed as percent of control (no mannitol). Control values: Prol, 109,500 cpm/30 sections; Hypro, 6830 cpm/30 sections.

Table V. *Effect of Actinomycin D on Hydroxyproline Formation*

Groups of 30 sections incubated 16 hr in 2 ml basal medium with addition of IAA (5 μg/ml), sucrose (2 %), either proline-^{14}C (0.5 μc) and leucine-^{14}C (0.125 μc) or adenine-^{14}C (0.9 μc), and with or without actinomycin D (50 μg/ml). Protein prepared by method A (9); RNA by procedures of Osborne (23).

	—Act-D[1]	+Act-D	% inhibition
Growth - mm	6.4	2.2	65
RNA synthesis-cpm/μg RNA	4,330	660	85
Leucine incorporation-cpm/30 sections	55,300	29 600	51
Proline incorporation-cpm/30 sections	370 000	268 000	28
Hydroxyproline formation-cpm/30 sections	22,950	19 450	15

[1] Act-D; actinomycin D.

Table VI. *Comparison of Effects of Decreased Turgor on Hydroxyproline Formation in Cytoplasmic and Wall-protein Fractions*

Groups of 100 sections incubated 11 hr in 12 ml basal solution with addition of IAA, sucrose, proline-^{14}C (1 μc, 1 μM) and with or without 0.25 M mannitol. Fractions prepared by method C (9).

Fraction	Mannitol	Hydroxyproline		Proline	
		cpm/100 sections	*% Inhib.*	*cpm/100 sections*	*% Inhib.*
W-fraction	—	2350	55	13 500	35
	+	1060		8 750	
S-fraction	—	4170	32	156 000	29
	+	2820		110 000	

synthesis is inhibited to a greater extent than is proline incorporation (fig 1). Furthermore, the inhibition of hydroxyproline synthesis is considerably greater in the wall fraction than in the cytoplasm, while proline incorporation is equally inhibited in the 2 fractions by a reduction in turgor (table VI).

Discussion

Because of their concentration in the cell wall (17), the hydroxyproline-proteins seem to be likely candidates to play a role in the control of cell elongation. Lamport suggested (16, 17) that they regulate the strength of the wall by cross-linking the polysaccharides, but there is little direct evidence that the strength of the wall is due to hydroxyproline-proteins other than the observations that the extensibility of *Avena* coleoptile tissues (6) and 2 algal cells (34) was increased by treatment with Pronase. A different possibility, that the synthesis of wall hydroxyproline-proteins is involved in the actual wall loosening process, is unlikely on the basis of the present evidence. Wall loosening is induced to an equal extent by auxin in the presence and absence of added sugars (6), while hydroxyproline synthesis is enhanced by auxin only when sugars are also added. Furthermore, the correlation between the auxin effects on elongation and hydroxyproline synthesis is poor; the auxin effect on hydroxyproline synthesis is far more variable than is that on cell elongation. Finally, continued RNA synthesis is not required for hydroxyproline synthesis while it is necessary for rapid cell elongation.

An analysis of the effects of free hydroxyproline on cell elongation (5) indicated that a factor must exist in *Avena* coleoptiles whose synthesis is inhibited by free hydroxyproline and whose presence is required for auxin-induced cell elongation when exogenous sugars are present but which is not needed if sugars are absent. This factor is apparently synthesized only under the conditions in which it is used; *i.e.*, when auxin and sugars are both present. It was subsequently shown (8) that free hydroxyproline inhibits the formation of 1 fraction of protein-bound hydroxyproline. It is now shown here that 1 fraction[2] of protein-bound hydroxyproline is only synthesized when both auxin and sugars are present. These results, when considered together, suggest that the factor which is needed for auxin-induced elongation in the presence of sugars is a wall-concentrated hydroxyproline-protein fraction.

Why should this hydroxyproline-protein fraction be needed in the wall when auxin and sugars are both present but not if either factor is absent? One effect of exogenous sugars is to cause a marked increase in the rate of wall synthesis; *e.g.*, addition of 0.05 M glucose to *Avena* coleoptile sections increases the rate of wall synthesis by 20 to 100 times (1, 30). Auxin modifies this increased wall deposition in 2 ways. First, it increases the rate of deposition of the non-cellulosic polysaccharides (1, 30). Secondly, it changes the pattern of wall deposition (31). In the absence of auxin, most of the wall synthesis is by apposition (deposition on the inner face of the wall, next to the cell membrane). Auxin causes a sizable amount of the hemicelluloses to be deposited, instead, by intussusception (deposition within the wall, away from the cell membrane). The fact that this hydroxyproline-protein fraction is only needed under conditions which favor intussusception of hemicelluloses suggests that the hydroxyproline-proteins may be required in the intussusception process. In this connection it should be noted that the hydroxyproline of tomato callus walls is chemically linked to arabanogalactan, one of the hemicelluloses which can be incorporated by intussusception (18, 19).

In what way might the hydroxyproline-protein contribute to the intussusception of hemicelluloses? It is known that the strength of the wall depends on both the cellulosic and non-cellulosic polysaccharides (22, 28). Generally, both are oriented in the same direction (13, 26). When new hemicellulose is incorporated by intussusception, it is probable that it must be oriented in the same direction as the preexisting polysaccharides if the mechanical properties of the wall are not to be altered significantly (27).

A possible role of the hydroxyproline-proteins may be to assure that the hemicelluloses are incorporated into the wall with the correct orientation. Lamport (18, 19) has shown that the hydroxyproline-containing peptides of tomato callus walls contain several hydroxyprolines, each of which is linked to an arabinose. This suggests that a single peptide may be attached to several different arabanogalactan chains and may serve to align the chains in a parallel fashion. If a hydroxyproline-peptide-arabanogalactan complex with a free hydroxyproline is bonded through this hydroxyproline to a preexisting arabanogalactan chain it would result in the newly incorporated chains being oriented parallel to the preexisting one. However, if the arabanogalactan is incorporated without the hydroxyproline it might tend to be randomly oriented with the result that the wall becomes stiffened and loses its capacity for rapid extension. Wall deposition by apposition would not require the hydroxyproline-protein since, here, the orientation is presumedly imparted by some characteristic of the cell membrane such as the orientation of the particles which have been observed on the outer surface of the cell membrane (12, 32).

It should be noted that this hypothesis in no way contradicts the "extensin" hypothesis of Lamport (17) but, rather, compliments it by indicating that the hydroxyproline-proteins may have at least 2 opposite effects on wall extensibility (and thus growth), depending upon where and how they are incorporated into the wall. On one hand, intussusception of hemicellulose-hydroxyproline-protein complexes into the wall may lead to a maintenance of wall extensibility while on the other hand, apposition

of hydroxyproline-proteins may result in the formation of "extensin-crosslinks" with a concomitant decrease in wall extensibility. The relation between wall extensibility and hydroxyproline-protein synthesis will depend upon which of these processes predominates, but whenever there is a massive increase in the hydroxyproline-proteins in the wall, as there is during the cessation of growth in the pea epicotyl (10), one might expect to find a decrease in the growth rate. It is unlikely, though, that the growth rate will always be closely correlated with the hydroxyproline level in the wall since wall extensibility is influenced by a variety of factors other than the hydroxyproline-proteins.

Finally, it must be pointed out that this hypothesis, while it is in accord with all of the available information, is far from proven. However, it should be possible to test the validity of this theory by examining the pattern of hydroxyproline incorporation into the wall by autoradiography with the electron microscope.

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