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Inhibition of Formation of Protein-Bound Hydroxyproline by Free Hydroxyproline in Avena Coleoptiles[1]

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Summary. Free hydroxyproline inhibits the formation of protein-bound hydroxyproline from proline to a considerably greater extent than it does the incorporation of proline into protein of auxin-treated *Avena* coleoptiles. This inhibition is greater in the wall than in the cytoplasmic fraction. In the absence of auxin, free hydroxyproline exerts little or no inhibition of hydroxyproline formation. Furthermore free hydroxyproline has no effect on respiration, RNA synthesis or the incorporation of leucine into protein. Hydroxyproline is not a general inhibitor of metabolism or protein synthesis in *Avena* coleoptiles.

These results suggest that free hydroxyproline may inhibit auxin-induced cell elongation by blocking the formation or utilization of a particular hydroxyproline-rich protein which must be incorporated into the cell wall during auxin-induced wall extension.

Free hydroxyproline is an effective inhibitor of auxin-induced growth in *Avena* coleoptile (5, 7, 19) and a variety of callus tissues (13, 16, 29). The ability of free proline to completely reverse this inhibition (5, 13, 19, 29) suggests that hydroxyproline is acting as an antagonist of some facet of proline metabolism. Since most amino acid antagonists exert their effect on some aspect of protein synthesis (26), it was suggested (5) that free hydroxyproline inhibits growth by interfering with the synthesis of some protein which is essential for cell elongation. Available evidence was not sufficient, however, to rule out the possibility that hydroxyproline is a general metabolic inhibitor.

The purpose of this investigation was to examine the effects of hydroxyproline on several aspects of the metabolism of *Avena* coleoptile tissues and, in particular, to determine its effects on protein synthesis. Since allohydroxyproline is more effective than hydroxyproline as a growth inhibitor (7), it was also included in this study. It will be shown here that the 2 hydroxyprolines exert a specific inhibition of one facet of protein synthesis, the formation from proline of protein-bound hydroxyproline.

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Materials and Methods

The plant material consisted of 5 or 14 mm sections cut from 25 to 32 mm coleoptiles of *Avena sativa*, var. Victory. Seedlings were grown and sections prepared as detailed earlier (3). The primary leaf was removed from all sections.

L-Proline, hydroxyproline (4-*trans*-hydroxy-L-proline), and allohydroxyproline (4-*cis*-hydroxy-L-proline) were obtained from California Corporation for Biochemical Research. L-Proline-u-^{14}C (200 mc/mmole) and DL-leucine-1-^{14}C (4 mc/mmole), were from New England Nuclear Corporation. Hydroxyproline and allohydroxyproline were used at concentrations (1 and 0.5 mM, respectively) which caused maximal inhibition of auxin-induced growth (7). IAA was used at 5 μg/ml.

Oxygen uptake was determined by the standard Warburg manometric techniques with groups of thirty 5 mm sections in 2.7 ml of basal medium (2.5 mM K-malate, pH 4.7, 0.1 mM penicillin G + 2% sucrose) to which was added, after equilibration, 0.3 ml of 10 mM hydroxyproline, 5 mM allohydroxyproline or water.

To study proline incorporation into proteins, groups of thirty 14 mm sections were incubated in 5 ml of basal medium to which was added 0.5 μM proline-u-^{14}C (0.5 μc) and, where indicated, IAA, hydroxyproline

and allohydroxyproline. At the end of the incubation (usually 22 hrs) the sections were washed and then extracted 3 times for 5 minutes with 10 ml of boiling 80 % ethanol. The sections were subjected to the trichloroacetic acid wash series of Peterson and Greenberg (22), placed in ampoules with 4 ml of 6 N HCl and hydrolyzed for 18 hours at 110°. After filtration to remove humin, the filtrate was concentrated under nitrogen to near dryness and placed at 1 end of a strip of Whatman 3MM paper. The chromatograms, unless otherwise noted, were developed for 18 hours in isopropyl alcohol:formic acid:water (15:2:2, descending). Proline and hydroxyproline were located with a radiochromatogram scanner, eluted, and an aliquot of each was dried on a planchet and counted in a gas flow counter. Counts were corrected to infinite thinness; replicate counts were found to agree within 3 %. A second aliquot was used for chemical analysis of proline by the procedure of Troll and Lindsley (30) or hydroxyproline by method I of Prockop and Udenfriend (24).

Since only about 7 % of the counts in protein are in hydroxyproline and since changes in labeling of hydroxyproline are sometimes relatively small, the conclusions reached in this paper are meaningful only if one can be certain that complete separation of hydroxyproline from other radioactive compounds has been achieved. Only 2 radioactive spots can be detected after chromatography of *Avena* coleoptile hydrolysates in isopropyl alcohol:formic acid:water [an example of such a radiochromatogram is shown in fig 1 of Cleland and Olson (8)]. Each of these spots has been routinely subjected to rechromatography in phenol:water (4; 1, w/v, descending) and, in some cases, in phenol saturated with 0.1 N HCl, acending, or isopropyl alcohol:pyridine:acetic acid:water (8:8:1:4, ascending). The proline spot contains a small amount (less than 5 %) of the radioactivity in glutamic acid; this contamination has been ignored here. The hydroxyproline spot, in every case, gave a single radioactive spot on rechromatography, which cochromatographed with authentic hydroxyproline.

A separation of the tissue into cytoplasmic and wall protein fractions was accomplished by grinding the tissues in 2 ml of tris buffer (50 mM, pH 7.5) in an all-glass homogenizer, and then subjecting the mixture to further homogenization in a Virtis "45" homogenizer with 200 μ glass beads and tris buffer (15). The walls were separated by filtration through glass beads and the protein of the filtrate was precipitated with 5 % trichloroacetic acid at 4°. Both walls and cytoplasmic protein fractions were then washed with the trichloroacetic acid series and treated as indicated above.

The effect of hydroxyproline on the incorporation of leucine into proteins was checked by incubating 5 mm sections in the presence or absence of hydroxyproline in basal medium to which had been added leucine-1-^{14}C (1 μc) and IAA. After varying times, groups of 30 sections were removed, washed, and ground in 2 ml of 50 mM tris buffer (pH 7.5). An equal volume of 40 % trichloroacetic acid was added and after 2 hours at 4° the precipitated proteins were removed by centrifugation and washed with the trichloroacetic acid series as indicated above. The pellet was resuspended in water and an aliquot was dried on a planchet and counted. These counts were not corrected to infinite thinness. As a check that the radioactivity was in protein, Pronase (200 μg/ml) was added to a second aliquot; over 95 % of the label was solubilized by this treatment. By acid hydrolysis of a third aliquot and chromatography of the amino acids it was shown that over 95 % of the label was still in leucine.

RNA synthesis was followed by incubating groups of fifteen 14 mm sections in 5 ml of basal medium to which was added adenine-8-^{14}C (0.5 μc) and, where indicated, IAA and hydroxyproline. After varying periods, sections were removed and RNA extracted and radioactivity in RNA determined by the procedure of Osborne (21).

In all experiments in which radioisotopes were used, a check was made on the effect of hydroxyproline on the uptake of the isotope. Unless otherwise stated, hydroxyproline was found to have no effect.

In each experiment treatments were run in duplicate or triplicate. All experiments were carried out at least 3 times.

Results

The possibility that free hydroxyproline and allohydroxyproline might act as general metabolic inhibitors was examined and eliminated by the finding that these compounds have no effect on respiration (fig 1) or RNA synthesis (data not given).

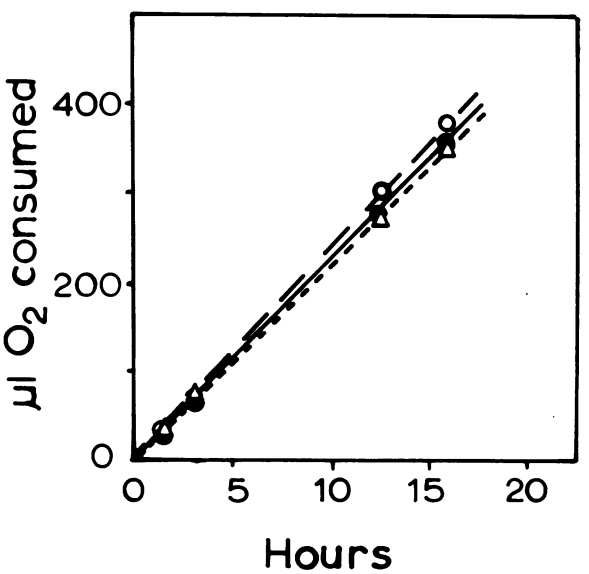

FIG. 1. Effect of hydroxyproline and allohydroxyproline on oxygen uptake. Groups of 30 5-mm sections incubated in basal medium in Warburg vessels with 1 mM hydroxyproline (— ⊖), 0.5 mM allohydroxyproline (--△--) or without inhibitors (—●—).

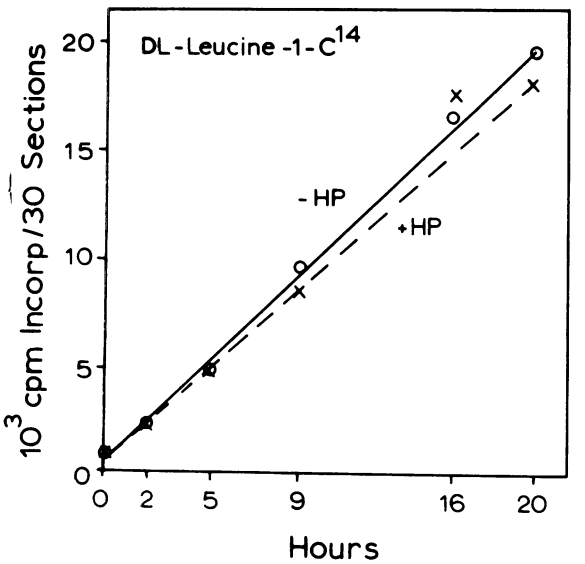

FIG. 2. Effect of hydroxyproline on incorporation of leucine-1-^{14}C into protein. Sections incubated in basal medium + IAA (5 μg/ml) and leucine-1-^{14}C (1 μc). Results corrected for small effect of hydroxyproline on leucine uptake.

Certain amino acid antagonists are known to cause a general inhibition of protein synthesis (26, 27). The possibility that the 2 hydroxyprolines might be capable of acting in this manner has been eliminated by the demonstration that the incorporation of leucine into protein is unaffected by hydroxyproline (fig 2).

A second possibility is that hydroxyproline specifically inhibits either the incorporation of its corresponding amino acid, proline, into protein or the subsequent conversion of proline to hydroxyproline. The replacement by an amino acid antagonist of the corresponding amino acid in protein with the resultant formation of inactive proteins has been demonstrated for several systems (11, 18, 26). To test this, a study was made of the effects of the hydroxyprolines on the incorporation of proline into protein. In the absence of the inhibitors free proline is incorporated into protein and a portion of it is converted into protein-bound hydroxyproline (table I). As in other plant (10, 16, 23) and animal tissues (28), essentially all of the protein-bound hydroxyproline normally arises from free proline rather than from free hydroxyproline (8). It should be noted that the bulk of the incorporated proline remains as proline; the ratio of radioactive proline to hydroxyproline in protein is 13 to 18. However, when the incorporation is expressed in terms of specific activities, the specific activity ratio for hydroxyproline to proline is 1.1 (table II) which indicates that the protein which is synthesized during the incubation has nearly the same ratio of proline to hydroxyproline as does the protein which is present at the start of the incubation. The incorporation of proline into protein-bound proline is enhanced to only a small extent by auxin. Hydroxyproline formation, on the other hand, is stimulated by an average of 40 % by IAA under these conditions (table I). A more thorough analysis of this auxin effect will be presented in a subsequent paper.

The free hydroxyprolines cause a small depression (5–15 %) in the incorporation of free proline into protein in both the presence and absence of auxin (table I). A more marked effect, however, is on the formation of protein-bound hydroxyproline. In auxin-treated tissues the formation of hydroxyproline is partially inhibited by growth-inhibiting levels of free hydroxyproline (table I). In 9 experiments the inhibition by hydroxyproline ranged from a low of 28 % to a high of 54 % with an average of 36 %. Generally, allohydroxyproline was slightly more effective than hydroxyproline. In the absence of auxin hydroxyproline formation is only slightly inhibited by free hydroxyproline. In 7 experiments the inhibition varied from 0 to 22 % with an average of 8 %.

It appears that the portion of the hydroxyproline formation which is inhibited by free hydroxyproline is the increment which is only formed in the presence of auxin. This increment may occur in response to the elongation induced by auxin rather than to auxin itself. If so, the inhibition of hydroxyproline formation might simply be due to the growth inhibition by free hydroxyproline rather than to any direct effect on the hydroxyproline-forming processes. This possibility has been eliminated in 2 ways.

Table I. *Effect of Free Hydroxyproline and Allohydroxyproline on Incorporation of Label from Proline into Protein-Bound Proline and Hydroxyproline*

Sections were incubated 22 hours in 5 ml basal medium + proline-u-^{14}C (0.5 mM, 0.5 μ) and, where indicated, IAA (5 μg/ml), hydroxyproline (1 mM) and allohydroxyproline (0.5 mM).

Conditions	Growth	Proline-^{14}C	Inhibition	Hydroxyproline-^{14}C	Inhibition
	mm	cpm/30 sections	%	cpm/30 sections	%
IAA	9.3	124,000	...	8020	...
IAA, hydroxyproline	3.0	110,000	11	4950	38
IAA, allohydroxyproline	1.9	112,000	10	4550	43
No IAA	2.3	104,000	...	5990	...
Hydroxyproline, no IAA	1.6	98,000	6	5540	8
Allohydroxyproline, no IAA	1.3	99,500	5	4570	24

Inhibition of cell elongation by hydroxyproline does not commence until 3 to 4 hours after addition of auxin (7). In contrast, maximal inhibition of hydroxyproline formation occurs during the first 3 hours and remains relatively constant thereafter (table III). Treatment of *Avena* coleoptile sections with 30 mM $CaCl_2$ results in an almost total inhibition of auxin-induced growth (9). Even under these conditions free hydroxyproline is able to cause a significant inhibition of hydroxyproline formation (table IV).

Table II. *Effect of Free Hydroxyproline and Allohydroxyproline on Specific Activity of Protein-Bound Proline and Hydroxyproline after Incubation with Proline-^{14}C*

Incubation conditions were the same as in table I, except 1 μc proline-u-^{14}C used per tube.

Inhibitor	Proline			Hydroxyproline			H/P*
	cpm	μg	cpm/μg	cpm	μg	cpm/μg	
None	259,000	126	2060	17,600	7.8	2250	1.09
Hydroxyproline	238,000	108	2200	10,750	8.0	1345	0.61
Allohydroxyproline	259,000	105	2460	9850	9.6	1025	0.42

* Ratio of specific activities of hydroxyproline to proline.

Table III. *Time Course of Hydroxyproline Inhibition of Growth and of Formation of Protein-Bound Hydroxyproline*

Conditions were the same as in table I.

Time	Free hydroxyproline	Growth	Inhibition	Protein-bound hydroxyproline-^{14}c	Inhibition
hr	1 mM	mm	%	cpm/30 sections	%
	+	1.7		3350	
3	—	1.6	5	2080	38
	+	4.6		9180	
9	—	2.5	45	6600	29
	+	10.9		15,750	
24	—	3.5	68	10,450	34

Table IV. *Inhibition of Hydroxyproline Formation by Free Hydroxyproline under Conditions where Elongation is Blocked by Calcium*

Conditions were the same as in table I. IAA present in all except controls.

$CaCl_2$	Free hydroxyproline	Growth	Inhibition	Protein-bound hydroxyproline-^{14}C	Inhibition
mM	mM	mm	%	cpm/30 sections	%
0	0	8.1	52	6220	40
0	1	3.9		3710	
30	0	2.3	26	5190	28
30	1	1.7		3760	
No IAA control		2.6		4800	

Table V. *Comparison Between Cytoplasmic and Wall Protein Fractions as to Amount of Allohydroxyproline-Induced Inhibition of Hydroxyproline Formation*

Groups of 110 sections in 20 ml basal medium containing IAA, proline-u-^{14}C (1.5 μc), allohydroxyproline (0.5 mM), for 22 hours.

Fraction	Incorporation into:	— Allohydroxyproline	+	Inhibition
		cpm	cpm	%
Cytoplasmic	Proline	136,000	121,000	11
	Hydroxyproline	4140	2540	39
Wall	Proline	18,600	14,350	23
	Hydroxyproline	4250	1320	69

Table VI. *Reversal by Proline of Hydroxyproline-Induced Inhibitions of Growth and Protein-Bound Hydroxyproline Formation*

Conditions same as in table I except that all solutions contain IAA.

Free hydroxyproline	Free proline	Growth	Hydroxyproline-^{14}C in protein:	Inhibition
mM	μM	mm	cpm/30 sections	%
0	1	9.2	8320	30
1	1	3.8	5820	
0	500	9.1	4130	0
1	500	8.6	4350	

In *Avena* coleoptiles hydroxyproline-containing proteins are about equally distributed between cell wall and cytoplasmic fractions (6, 20). The free hydroxyprolines inhibit the incorporation of proline into the protein-bound hydroxyproline of both fractions, but the greater effect is found in the wall protein factor (table V). Apparently, not only the formation of hydroxyproline but also the subsequent incorporation of the hydroxyproline-containing peptides into the wall is inhibited by free hydroxyproline.

The inhibition of growth by the hydroxyprolines can be almost completely reversed by free proline (5, 13, 29). If the inhibition of protein-bound hydroxyproline formation is related to the growth inhibition, one would expect free proline to prevent this inhibition. It has been shown that this does, in fact, occur (table VI).

Discussion

Auxin-induced elongation of *Avena* coleoptiles is inhibited by a wide variety of antimetabolites (1, 4). Each of these inhibitors appears to be able to exert its effect on metabolism in the absence of auxin. Hydroxyproline, on the other hand, is a unique inhibitor in that it appears to act only in the presence of auxin. This was first indicated by the fact that pretreatment of tissues with hydroxyproline does not shorten the lag in the growth inhibition which occurs after addition of auxin (7). This is further indicated here by the lack of effect of hydroxyproline on respiration, RNA synthesis, total protein synthesis (leucine and proline incorporation into proteins) or hydroxyproline formation in non-auxin treated tissues. This lack of effect is not due to any failure of hydroxyproline to enter the tissue since hydroxyproline uptake occurs in the absence of auxin (8).

It was suggested earlier, from an analysis of the growth inhibition, that hydroxyproline specifically inhibits the formation of some factor which is only synthesized in the presence of auxin and which is utilized in auxin-induced cell elongation (7). It has been shown here that there is an increment of protein-bound hydroxyproline which is formed in the presence of auxin and whose synthesis is inhibited by free hydroxyproline. This fraction of the hydroxyproline appears to be concentrated in the cell wall. These results suggest that the factor is a particular, hydroxyproline-rich protein which is formed in the presence of auxin and which must be incorporated into the cell wall in order for auxin-induced growth to occur.

There are several ways in which free hydroxyproline might bring about this inhibition of hydroxyproline formation. One possibility is that hydroxyproline inhibits proline hydroxylase, the enzyme which is presumed to cause the conversion of proline to hydroxyproline in plants (12) as it does in animals (25, 31). This would result in an altered protein in which proline occupied the spots which normally contain hydroxyproline. A direct competition by hydroxyproline at the active site of this enzyme seems unlikely, however, since free hydroxyproline does not inhibit the isolated proline hydroxylase from chick embryo (14) and does not affect hydroxyproline formation in sycamore cambium callus cells (13). Furthermore, 2 forms of proline hydroxylase would be required in each tissue, one of which was only active in the presence of auxin and was sensitive to free hydroxyproline while the other was sensitive to neither.

A second possibility is that it is the hydroxyproline-containing peptide rather than hydroxyproline itself whose synthesis is blocked by free hydroxyproline. This need not be due to any direct effect of free hydroxyproline on protein synthesis, but could be due to an interference with the utilization of the peptide with the result that further synthesis of the peptide is prevented by feedback inhibition. Boundy et al. (2) and Lamport (17) have shown that at least part of the wall hydroxyproline exists in a hemicellulose-peptide complex in which the hydroxyproline is covalently linked to one of the sugars. It may be the formation of this bond which is inhibited by free hydroxyproline. The resulting lack of suitable hemicellulose-peptide complexes for incorporation into the wall would be the actual cause of the growth inhibition. This possibility is currently being tested.

The third possibility is that the growth inhibition is not a consequence of the inhibition of hydroxyproline formation, but is due to a direct incorporation of hydroxyproline into some specific proteins required for wall loosening with the result that inactive proteins are formed. Holleman has shown that direct incorporation of hydroxyproline can occur in sycamore cambium callus cells (13), but similar evidence for

Avena coleoptiles is, as yet, lacking (6, 8). This possibility cannot be eliminated until the enzymes responsible for wall loosening are identified and isolated.

A final possibility is that the decrease in hydroxyproline formation is due to direct incorporation of free hydroxyproline into proteins in the place of hydroxyproline which is normally formed from proline. In order for this to occur, free hydroxyproline must replace proline rather than hydroxyproline at the time the protein is formed, since evidence from animal (31) and plant systems (12) indicates that the conversion of proline to hydroxyproline occurs only after incorporation of proline into peptides. Hydroxyproline must replace only those prolines which will subsequently be converted to hydroxyproline since if it replaces prolines at random the incorporation of free proline into protein-bound proline and hydroxyproline would be inhibited to the same extent. The fact that the conversion of proline to hydroxyproline is unaffected by free hydroxyproline in the absence of auxin makes this mechanism unlikely.

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