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THE DIRECT CONVERSION OF HYDROXYPROLINE TO PROLINE

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SUMMARY

Carrot slices convert added free trans-4-hydroxy-L-proline to L-proline by a path that brings about the loss of one half of the tritium from 5[^{3}H]trans-4-hydroxy-L-proline. The first intermediate in this path is therefore most likely 4,5-dehydro-L-proline.

INTRODUCTION

Trans-4-hydroxy-L-proline has been known as a constituent of plant proteins since the 1950's (1,2). In whole plants, in plant cells in tissue culture, and in excised plant tissues in culture hydroxyproline (formed by posttranslational hydroxylation) is most abundant as a major amino acid of a glycoprotein localized in the wall and/or secreted into the medium (3).

The conversion of free hydroxyproline to proline has already been reported in plants (2,4,5,6,7,8) but no one has studied the path for the conversion. No such conversion has been reported in animals or in bacteria (9). The most likely first step would appear to be the removal of water to form a dehydroproline.

By the use of 2,3[^{3}H]trans-4-hydroxy-L-proline and 5[^{3}H]trans-4-hydroxy-L-proline produced by biosynthesis along with U-^{14}C-hydroxyproline as an internal marker I show that the hydroxyproline-to-proline conversion in aerated carrot root slices involves loss of one half of the ^{3}H from the 5 position and no loss of ^{3}H from the 2 or the 3 position.

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MATERIALS AND METHODS

The aerated carrot slice system, as developed by Chrispeels for the study of the synthesis and secretion of hydroxyproline-rich glycoproteins (10), was used in these experiments both for the tissue for the biosynthesis of the specifically labeled hydroxyproline and for the tissue to study the conversion of free hydroxyproline to proline.

For the initial aeration of the carrot slices to allow development of the capacity to synthesize hydroxyproline-rich glycoproteins cylindrical plugs (7 x 20mm) were cut with a cork borer from the phloem parenchyma of a mature carrot (30 to 35mm in diameter, from the local supermarket). Slices (1 x 7mm) were cut from these plugs, rinsed for a few minutes in distilled water, blotted free of water droplets with filter paper, and stood on edge on a plastic support in the bottom of a petri dish lined with water saturated filter paper. The dish was covered with its top and wrapped in aluminum foil to prevent evaporation. After 24 hours at room temperature (22 to 25C) the slices were fully active in the synthesis of hydroxyproline-rich proteins.

For the biosynthesis of hydroxyproline intermolecularly labeled with ^{3}H and ^{14}C one aerated carrot slice was placed in a glass scintillation vial with [^{3}H]proline (5 to 20μCi) and [^{14}C]proline (1 to 5μCi) and potassium phosphate buffer (pH 6.0) to make a final volume of 1.0 ml 0.01 M in phosphate buffer. The vial was placed in a shaking (90 cycles/min) water bath at 25 C for 20 hours. During this incubation about 75% of the added label was taken up and 85 to 95% of the label taken up was incorporated into protein (only 5 to 15% of the label taken up was released by extraction with 70% ethanol).

After 4 hours extraction with 1.0 ml of 70% aqueous ethanol the slice was transferred to a 10 ml test tube, 1.0 ml of 6N HCl was added, the tube was sealed and heated at 105C for 20 hours. The charred insoluble material was removed by centrifugation and the clear supernatant fraction evaporated to dryness in a stream of warm air. The residue was dissolved in 0.25 ml distilled water. The labeled hydroxyproline and proline were recovered from aliquots of the hydrolysate by paper electrophoresis at pH 1.85 (75 ml of glacial acetic acid and 27.5 ml of formic acid diluted to 1 l and the pH adjusted to 1.85) and a field strength of 50 volts/cm (11), or by paper electrophoresis at pH 11.0 (50 ml ethanolamine diluted to 2 l and CO_2 bubbled into the solution until the pH reaches 11.0) (Penelope Toothman, private communication). In both buffer systems proline and hydroxyproline are well separated from each other. Unlabeled proline and hydroxyproline were added to lanes adjacent to the lanes carrying the hydrolysates. After the electrophoresis the marker lanes were cut off and the markers located with ninhydrin spray. Labeled hydroxyproline and proline were recovered from the corresponding spots in the hydrolysate lanes. The radioactive hydroxyproline and proline spots are compact (no radioactivity just ahead or just behind the main spot corresponding to the marker). No radioactivity was found in any other area of the paper.

Labeled hydroxyproline and proline were eluted from the paper by cutting the appropriate spots into 1 cm squares and adding these squares to 1.0 ml of phosphate buffer (0.01 M, pH 6.0). Suitable aliquots of the eluant of the hydroxyproline spot were 1) counted (Packard Tricarb liquid scintillation counter) to determine the $^{3}H/^{14}C$ ratio and 2) electrophoresed at pH 11.0, eluted and counted to confirm the purity of the hydroxyproline. Data on the biosynthesis of labeled hydroxyproline are shown in Table 1.

TABLE 1

SCHEME FOR THE BIOSYNTHESIS OF SPECIFICALLY LABELED trans-4-HYDROXY-L-PROLINE

peptidylHYDROXYPROLINE ↑ —6N HCl→ HYDROXYPROLINE + PROLINE

PROLINE —tissue→ peptidylPROLINE

2,3[^{3}H]Proline[a]			
^{3}H	2.29×10^6 cpm	3.84×10^5 cpm	1.29×10^6 cpm
^{14}C	4.21×10^6 cpm[c]	6.76×10^5 cpm	2.40×10^6 cpm
^{13}H/^{14}C	0.54	0.57	0.54
5[^{3}H]Proline[b]			
^{3}H	5.96×10^6 cpm	1.02×10^6 cpm	3.62×10^6 cpm
^{14}C	2.15×10^6 cpm[c]	3.41×10^5 cpm	1.14×10^6 cpm
^{3}H/^{14}C	2.77	2.99	3.17

[a]New England Nuclear, NET-323 (Lot 1302-113). The label is 40% in the 3-trans, 42% in the 3-cis and 18% in the 2 position.

[b]New England Nuclear, NET-573 (Lot 1109-173). The label is 50% in the 5-cis and 50% in the 5-trans position.

[c]New England Nuclear U[^{14}C]Proline, NET-285.

TABLE 2

CHANGE OF ^{3}H/^{14}C RATIO

DURING THE CONVERSION OF HYDROXYPROLINE TO PROLINE

peptidylHYDROXYPROLINE ↑ —6N HCl→ HYDROXYPROLINE + PROLINE

HYDROXYPROLINE → PROLINE → peptidylPROLINE

2,3[^{3}H]hydroxyproline		
^{3}H/^{14}C	I 0.57	0.58
	II 1.74	1.72
5[^{3}H]hydroxyproline		
^{3}H/^{14}C	III 2.99	1.58
	IV 2.50	1.17

RESULTS

An aliquot of the hydroxyproline purified from the hydrolysate from one slice and representing no more than 2% of the intermolecularly labeled [^{3}H, ^{14}C]hydroxyproline produced by that slice was incubated with a single carrot slice for 20 hours. In every run approximately 90% of the label added was taken up and approximately 90% of the label taken up was incorporated into protein. After the 20 hour incubation period, and a 4 hour extraction of the slice with 70% ethanol, the slice was hydrolyzed in 6N HCl and the hydroxyproline and proline separated by paper electrophoresis at pH 1.85 and eluted for counting.

The results from two different experiments each with 2,3[^{3}H]hydroxyproline and 5[^{3}H]hydroxyproline are shown in Table 2. There was no loss of ^{3}H from 2,3[^{3}H]hydroxyproline as it is converted to proline and incorporated into protein (about 35% of the peptidylproline residues were again hydroxylated). Half of the ^{3}H of 5[^{3}H]hydroxyproline was lost during its conversion to proline.

When the hydroxyproline (resulting from the conversion of hydroxyproline to proline, incorporation of the proline into protein, and subsequent hydroxylation of that peptidylproline) from the hydrolysate of run IV was incubated with another carrot slice for 20 hours and the proline isolated from the hydrolysate the $^{3}H/^{14}C$ ratio of the isolated proline was 1.2. That is, there was no additional ^{3}H lost during the second cycle of hydroxyproline-to-proline conversion. I conclude therefore that the ^{3}H is lost from the 5[^{3}H]hydroxyproline sterospecifically.

These results indicate that the most likely first intermediate in the conversion of hydroxyproline to proline is 4,5-dehydroproline.

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