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Intracellular Site of Proline Hydroxylation in Plant Cells*

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ABSTRACT: In animal and plant tissues, hydroxyproline is synthesized by the enzymatic hydroxylation of peptidylproline. However, data from several studies on collagen synthesis have not resolved the question of whether this hydroxylation occurs before or after release of the polypeptide from a ribosomal complex. We examined this problem using disks of carrot phloem parenchyma where hydroxyproline-containing proteins are synthesized abundantly. Disks which had incorporated [^{14}C]proline into proteins began to hydroxylate bound [^{14}C]proline 3–4 min after incorporation. The proline-rich polypeptides made during this lag period could be maximally hydroxylated during a 10-min incubation in nonradioactive proline; this is well beyond the time required for release of the chains from a ribosomal complex. Isolation of polyribosomes from [^{14}C]proline-incubated tissue and analysis of labeled polypeptides attached to them showed no significant [^{14}C]hydroxyproline, even though 10% of the cytoplasmic [^{14}C]proline residues had been hydroxylated. Our results indicate that proline hydroxylation in this plant system takes place on completed, released polypeptide chains.

Hydroxyproline occurs in many plant glycoproteins (Pusztai and Watt, 1969) and is especially abundant in the protein component of the plant cell wall (for a review, see Lamport, 1970). This wide distribution contrasts with animal cells, where the imino acid has been found specifically in only two proteins, collagen and elastin (Gross, 1963). However, the scheme for assembly of hydroxyproline-containing macromolecules is similar in both plant and animal cells: the imino acid is synthesized by the enzymatic hydroxylation of peptidylproline (Sadava and Chrispeels, 1971; Rosenbloom and Prockop, 1968); the completed macromolecule is then glycosylated (Chrispeels, 1970a,b; Rosenbloom *et al.*, 1968) and subsequently secreted into the intercellular matrix (Olson 1964; Takeuchi and Prockop, 1969).

A matter of some controversy from studies on collagen biosynthesis is the question of whether proline hydroxylation occurs before or after release of the macromolecule from a protein-synthesizing ribosomal complex. Several laboratories have reported that when collagen-synthesizing tissues were incubated in radioactive proline, polyribosomal complexes which contained radioactive hydroxyproline could be isolated (Manner *et al.*, 1967; Fernández-Madrid, 1967; Goldberg and Green, 1967; Miller and Udenfriend, 1970; Lazarides *et al.*, 1971); in one recent study, no hydroxyproline was found (Bachra and van der Eb, 1970). However, protocollagen-like molecules, containing little or no hydroxyproline, have been isolated after release from the ribosomal complex had occurred (Rosenbloom *et al.*, 1967), indicating that hydroxylation must take place after release. Moreover, if the proline hydroxylase were inhibited for several hours, thus allowing an accumulation of released, nonhydroxylated chains, reversal of this inhibition led to maximal hydroxylation (Juva *et al.*, 1966). These latter two experiments support the hypothesis of hydroxylation after release of a completed polypeptide.

To study this problem in plants, we designed experiments using disks of carrot root. *In vivo* experiments showed that proline hydroxylation could occur after release of the polypeptides from the ribosomal complex. Actual isolation of polyribosomes showed no significant hydroxyproline in the nascent chains.

Materials and Methods

Organism. We performed our experiments on disks (1 cm in diameter and 0.5 mm thick) of phloem parenchyma from

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TABLE I: Ratio of [^{14}C]Hydroxyproline to Total Bound ^{14}C after Incubation of Carrot Disks in [^{14}C]Proline and Subsequent Chase in Nonradioactive Proline.[a]

	% [^{14}C]hydroxyproline		
(A) Min in [^{14}C]proline	(B) After pulse	(C) Pulse plus 10-min chase	(D) Pulse plus 10-min further label
0.5	0	9.5	9.9
1.0	0	9.2	10.7
2.0	0	10.1	12.0
5.0	4.4	13.7	13.9
10.0	9.0	15.4	15.2

[a] Lots (1 g) of aged carrot disks were incubated with 1 μCi of [^{14}C]proline for various times (A) and their extents of [^{14}C]hydroxyproline formation determined (B). Duplicate lots were, after initial incubation in [^{14}C]proline, further incubated for 10 min in excess (10 mM) nonradioactive proline (chased). The extent of hydroxylation after this period was measured (C) and compared to the extent of hydroxylation if labeling had continued during the chase period (D).

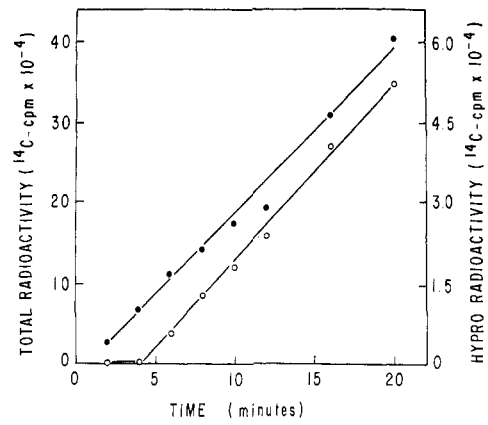

FIGURE 1: Incorporation of [^{14}C]proline into protein and the subsequent appearance of [^{14}C]hydroxyproline. At time 0, 1 μCi of [^{14}C]-proline was added to 1 g of carrot disks. At the times indicated, incorporation of [^{14}C] into trichloroacetic acid insoluble material, and the amount of [^{14}C]hydroxyproline in this material were determined: (●—●), total bound ^{14}C; (○—○), bound [^{14}C]hydroxyproline.

the storage roots of carrots (*Daucus carota, cv.*). When such disks are incubated, they undergo a large number of physiological and metabolic changes collectively termed "aging" (Thimann *et al.*, 1954). Disks were incubated with shaking at 30° for 18 hr in water containing 50 μg of chloramphenicol (Calbiochem) per ml to prevent bacterial growth (Leaver and Edelman, 1965). Generally 10 ml of liquid was used per g fresh weight of tissue. We have shown that disks aged in this way actively synthesize hydroxyproline-containing macromolecules (Chrispeels, 1969). Some experiments (indicated in Results) were repeated using liquid-cultured cells of tobacco pith (type strain XD, Filner, 1965); in these cases, 1-l. cultures 5 days after subculturing (approximately 5 g fresh weight) were used.

Incubation and Homogenization. For experiments reported in Figure 1 and Table I (see Results), duplicate 1-g lots of aged disks were preincubated at 28° in new medium (water–chloramphenicol) for 30 min. [^{14}C]Proline (uniformly labeled, specific activity 180 mCi/mmole, from New England Nuclear) was added at 1 μCi/g of tissue and shaking continued at 28°. Where a "chase" in nonradioactive proline was desired, the medium was made 10 mM using a 1 M proline stock. Experiments were terminated by quick chilling two cold water washes and then rapid freezing in acetone–Dry Ice. Homogenization and further analyses were carried out as described previously (Chrispeels, 1969).

Isolation and Analysis of Polyribosomes. A modification of the procedure of Leaver and Key (1967) was used. All operations were carried out in a cold room at 4°, using precooled glassware. Carrot disks (10 g) were incubated and chilled as described above (not frozen), homogenized in a Virtis-45 apparatus, at top speed, for 10 sec in 15 ml of 0.05 M Tris buffer, pH 7.4, containing 0.25 M sucrose–0.015 M KCl–0.2 M $MgCl_2$–0.001 M dithiothreitol (added immediately before use). The resulting homogenate was filtered through Miracloth (Chicopee Mills, Inc.), and the filtrate was centrifuged at 23,000*g* for 20 min. The clear supernatant was layered over 4.0 ml of 0.05 M Tris buffer, pH 7.4, containing 1.5 M sucrose–0.015 M $MgCl_2$, and was centrifuged for 2.5 hr in a fixed-angle rotor at 164,000*g* (average rcf). The pellet (usually a translucent yellow) of this centrifugation contained the polyribosomes and was resuspended gently in 0.7 ml of 0.05 M Tris buffer, pH 7.4, containing 0.005 M $MgCl_2$.

The ribosomal suspension was layered over a linear gradient of 10–34% sucrose in Mg^{2+} (0.005 M)–Tris (0.05 M, pH 7.4) to a total volume of 36 ml and centrifuged at 82,500*g* for 115 min in a swinging-bucket rotor. Absorbancy was monitored at 260 nm on a Gilford recording spectrophotometer equipped with a flow-through cell. Where gradient fractions were desired, they were collected after absorbancy was read in 2.5-ml amounts. Protein was precipitated by chilling, making the solution 7% in trichloroacetic acid, and adding a drop of carrier, 1 mg/ml of bovine albumin. After 30 min at 4°, the suspension was filtered on a membrane filter and washed in cold 5% trichloroacetic acid, and the filter was dried and counted in a liquid scintillation counter. The macromolecular hydroxyproline soluble in trichloroacetic acid adheres to the filter used (Chrispeels, 1969).

Ribonuclease treatment (pancreatic RNase A, from Calbiochem) was performed on ribosomal suspensions (prior to gradient analysis) at 5 μg/ml, for 10 min at 20°. Alkaline hydrolysis in nascent protein was also performed on ribosomal suspensions; the suspension was made 0.5 N in KOH and incubated 1 hr prior to gradient analysis.

Assay for Hydroxyproline. The extent of hydroxylation of [^{14}C]proline was measured as previously described (Chrispeels, 1969). Briefly, this method involved acid hydrolysis of precipitated proteins, paper chromatography in isopropyl alcohol–formic acid–water (15:2:2) to separate the amino acids, and determination of the ratio of radioactive hydroxyproline to total radioactivity. Thus: % hydroxyproline = [(cpm of hydroxyproline)/(cpm of total)] × 100. In our experiments using [^{14}C]proline, radioactivity was found only in proline and hydroxyproline. In addition to ratios, nascent protein was assayed for [^{14}C]hydroxyproline by the method of Juva *et al.* (1966). This method involves the oxidation of hydroxyproline to pyrrole, we found that the efficiency of this conversion was usually about 50%.

Sephadex Chromatography of Proteins Released by Base. A column of G-100 Sephadex (Pharmacia) that had been equilibrated in 1.0 M ammonium acetate, pH 7.2, was prepared

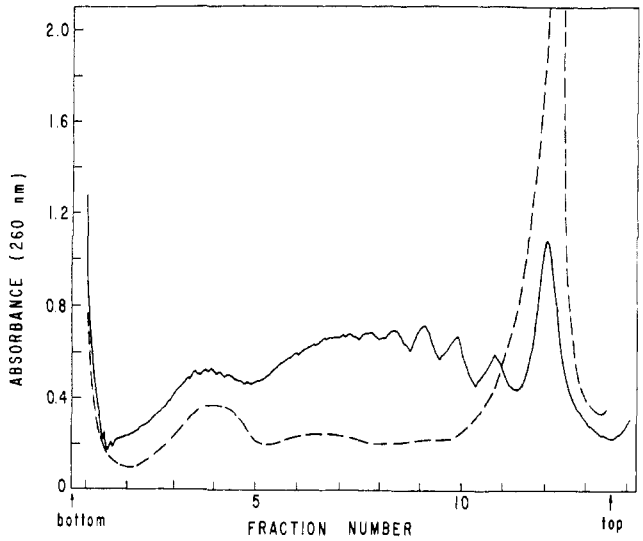

FIGURE 2: Absorption profiles of sucrose density gradient fractionations of native polyribosomes (solid line) and polyribosomes treated with ribonuclease (broken line). Each gradient represents 7.5 g of carrot disks.

and the column was washed with that buffer for 6 hr at a flow rate of 20 ml/hr. A sample after base hydrolysis (see above) was neutralized and made 1.0 M in buffer, placed on the column, and eluted with buffer at a flow rate of 20 ml/hr. Fractions were collected and their radioactivity determined on a liquid scintillation counter.

Results

When aged carrot disks are incubated in [^{14}C]proline, they rapidly take up the radioactive amino acid and incorporate it into protein at a linear rate. However, there is a lag time between the first appearance of ^{14}C radioactivity in trichloroacetic acid insoluble material and the appearance in that material of [^{14}C]hydroxyproline: in the experiment reported in Figure 1, this lag time was of the order of 3 min. Numerous replicates of this experiment showed a lag time of no less than 3 min.

The experiment reported in Table I was designed to determine whether the proline-rich proteins made during the lag period of Figure 1 could be hydroxylated during a chase in nonradioactive proline of 10 min. During this chase period,

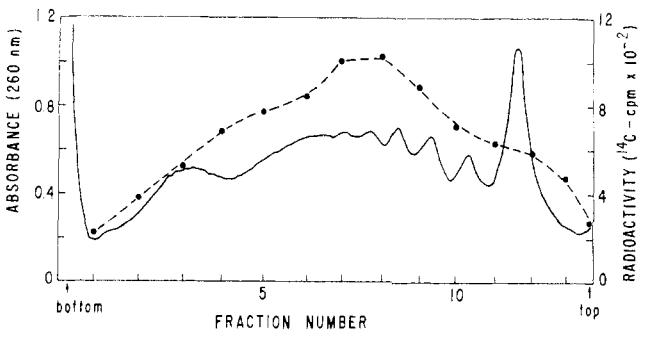

FIGURE 3: Absorption spectrum (solid line) and distribution of bound ^{14}C (broken line) of sucrose density gradient fractionation of ribosomes from 7.5 g of carrot disks which were incubated in 5 μCi of [^{14}C]proline for 10 min.

TABLE II: Ratio of [^{14}C]Hydroxyproline to Total Bound ^{14}C Associated with Ribosomes Fractionated on a Sucrose Density Gradient after Tissue Was Incubated in [^{14}C]Proline.[a]

Experiment	% [^{14}C]Hydroxyproline		
	Total Protein	Poly-ribosomes	Mono-ribosomes
1	8.9	1.5	0
2	9.5	0.3	0
3	8.8	0	0
4	9.8	0	1.3
5	9.7	0	0

[a] In experiments 1–4, carrot disks were incubated as described in legend to Figure 3; in experiment 5, cultured tobacco cells (5 g) were incubated in [^{14}C]proline for 6 min. In all cases, ribosomes were isolated, fractionated on sucrose gradients, and the radioactivities associated with polyribosomes were determined. For per cent [^{14}C]hydroxyproline determination, generally 2000 cpm of ^{14}C was on the paper chromatogram of the polyribosome hydrolysate, and 1000 cpm on that of the monoribosomes.

most of the radioactively labeled proteins would be released from the polyribosomes (see Table III). The results indicate that the radioactive nonhydroxylated proteins were indeed fully hydroxylated during the chase period.

The isolation of polyribosomes from carrot disks is shown in Figure 2; similar, although not as clean, profiles were obtained from ribosomal suspensions of cultured tobacco cells. The ribosomal pellet before gradient analysis had an ultraviolet absorption ratio at 260/280 mμ of approximately 1.4. Rough estimation based on a gradient marker (ϕX-174 bacteriophage) gave the monoribosome area a sedimentation coefficient of 80 S, characteristic of plant cytoplasmic ribosomes. As Figure 2 demonstrates, treatment of the ribosomal pellet with ribonuclease, which would disrupt stabilization of polyribosomes with RNA, shifted the heavier, ultraviolet-absorbing material to the lighter, monoribosome region. Other experiments showed that the bound radioactivity associated with the heavier regions (see below, Figure 3) was likewise shifted to the lighter region of the gradient.

Carrot disks actively incorporating [^{14}C]proline into proteins and synthesizing [^{14}C]hydroxyproline showed a considerable amount of bound radioactivity associated with the ribosomal fraction. Most of this label was in the polyribosome region upon gradient analysis (Figure 3). To check for possible nonspecific adsorption of radioactive proteins to the ribosomes, a control experiment was performed in which carrot disks were homogenized in the presence of [^{14}C]proline proteins from the cytoplasm; when ribosomes were prepared in this environment, they had no radioactivity associated with them.

When this polyribosomal-bound radioactivity was analyzed for [^{14}C]hydroxyproline, little or none was found. Two methods were used: either the relative amount of [^{14}C]hydroxyproline to total bound ^{14}C radioactivity was measured, or [^{14}C]hydroxyproline was directly assayed by a specific method. Table II gives the results of the ratio measurements: for both carrot disks and tobacco cells in culture, usually all of the bound ^{14}C radioactivity was in proline, even when analysis of the total homogenate (mostly proteins released

TABLE III: Disappearance of Bound ^{14}C Radioactivity from Ribosomes of Carrot Disks Incubated in [^{14}C]Proline and then Chased in Nonradioactive Proline.[a]

Experiment	^{14}C cpm/OD_{260}	
	Poly-ribosomes	Mono-ribosomes
Pulse, 10 min	501	395
Pulse + chase, 7 min	133	94

[a] The pulse experiment represents data from Figure 3. In the chase experiment, a duplicate 7.5-g sample of carrot disks was labeled with 5 μCi of [^{14}C]proline and then incubated a further 7 min in 10 mM nonradioactive proline. The bound ^{14}C radioactivity associated with the polyribosome (fractions 5–10) and monoribosome (fractions 11–12) was determined. Optical densities of areas were determined by relative weights of paper tracings.

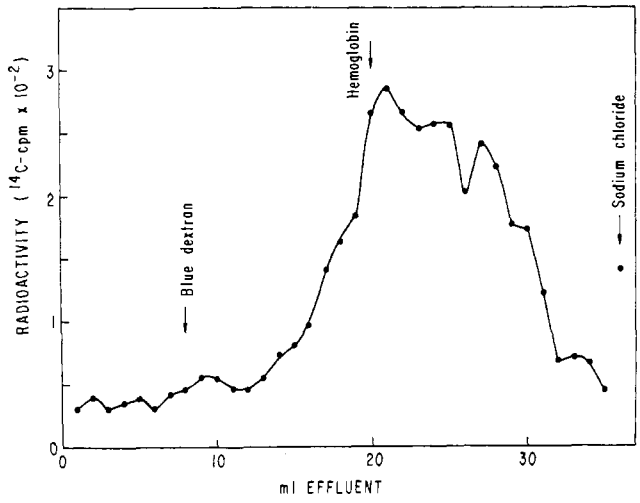

FIGURE 4: Chromatography of base-released peptides on G-100 Sephadex, as described in Materials and Methods. Blue Dextran (mol wt 2×10^6), hemoglobin (mol wt 5×10^4), and sodium chloride (mol wt 60) were used as markers.

and completed) showed 9–10% of the [^{14}C]proline residues were hydroxylated. The direct [^{14}C]hydroxyproline assays gave similar results: none was found. In some experiments, instead of pooling the radioactivity from the entire polyribosome region for assay, we analyzed fractions of the gradient similar to those shown in Figure 3. Again, both methods showed little or no [^{14}C]hydroxyproline. Ribosomes were also solubilized from the initial 23,000*g* pellet with detergent (Nicolson and Flamm, 1965); these ribosomes showed similar sedimentation and labeling characteristics to those of Figure 3 and likewise had no significant [^{14}C]hydroxyproline associated with them.

To test the validity of consideration of the bound radioactivity associated with the polyribosomes as nascent polypeptides, the experiment reported in Table III was performed. If the radioactivity was indeed in nascent chains, it should rapidly disappear from the polyribosomes during a chase in nonradioactive proline, since published times for the completion of a protein in eukaryotes are of the order of 1 min (Winslow and Ingram, 1966; Rosenbloom *et al.*, 1967). The table shows that after a short chase, most of the bound radioactivity disappeared from the ribosomes.

In the experiment shown in Figure 3, the total bound ^{14}C radioactivity associated with the ribosomes was about 9×10^3 cpm; assay for the total incorporation of ^{14}C radioactivity during the 10-min incubation showed a net incorporation to bound form of about 650×10^3 cpm. If we assume that it takes 1 min to make a protein in this system, this would mean that the disks would incorporate about 65×10^3 cpm at any moment into nascent polypeptide chains. Obviously, we did not recover all of the possible nascent protein on our gradients. It could be argued that only short chains, not yet long enough to be hydroxylated, were recovered and that the longer, hydroxylated nascent chains were removed by our isolation procedure. Nascent chains are bound to the ribosomal complex by an ester bond to transfer RNA and can be released by hydrolysis at an alkaline pH. When this hydrolysis was performed on carrot disk ribosomes, over 90% of the radioactivity was released from the polyribosomes and sedimented at the top of the sucrose gradient. When this released radioactivity was applied to a column of G-100 Sephadex, the result showed polypeptides of varying, but large molecular weight (Figure 4).

Discussion

This report presents two lines of evidence concerning the site of proline hydroxylation in plant cells: *in vivo* kinetic experiments and analysis of nascent polypeptides associated with polyribosomes. Both are consistent with the hypothesis that hydroxylation occurs after completed protein chains are released from the ribosomal complex.

There is at least a 3-min interval between the appearance of exogenous [^{14}C]proline in bound form, and the detection of bound [^{14}C]hydroxyproline in carrot disks. When the proline-rich polypeptides synthesized during this lag period are chased in nonradioactive proline for a sufficient time to allow them to be completed and released from the protein-synthesizing apparatus, they become fully hydroxylated (Table I). Thus it would appear that, *in vivo*, at least some hydroxylation can take place off the polyribosomal complex. Additional evidence comes from other experiments (Chrispeels, 1970a,b) in which the inhibition of peptidylproline hydroxylase by a chelating agent and the reversal of inhibition by ferrous ions were used to study proline hydroxylation as distinct from protein synthesis. It was shown that, after 10 min of incubation of carrot disks in [^{14}C]proline and chelator, those proline-rich proteins which had accumulated (largely off the polyribosomes, as we have shown) could be substantially hydroxylated *in vivo* by the addition of ferrous ions to the medium. In sum, the kinetic data show that proline hydroxylation can take place off the ribosomal complex.

That this is the case in the normal situation is borne out by our analyses of polyribosome-associated polypeptides. Isolated polyribosomes were sensitive to ribonuclease, which is to be expected if they are monoribosomes stabilized by messenger RNA. Moreover, the polyribosomes and not the monoribosomes contained the bulk of the radioactivity of a ribosomal preparation, which would be expected if they were the site of protein synthesis. This protein label disappeared after chase, consistent with its supposed role as nascent protein and was heterogeneous but large in size. Finally, it was suscep-

tible to release from the ribosomal complex by alkaline hydrolysis. These control experiments suggest that the bound [^{14}C]proline radioactivity associated with isolated carrot or tobacco polyribosomes was in the form of nascent polypeptide chains. Analysis by two distinct methods failed to reveal significant [^{14}C]hydroxyproline in this nascent material, even though the surrounding cytoplasm (released chains) had 9–10% of their [^{14}C]proline residues hydroxylated.

Our results partially agree, and partially disagree with data reported on hydroxylation of proline during collagen synthesis in animal cells. The *in vivo* kinetic data from plants are similar to, and agree in interpretation with, kinetic experiments from the animal system (Rosenbloom and Prokop, 1968). However, our finding no hydroxyproline in nascent protein is at variance with the bulk of reports from animal cells; only one study has found a situation similar to that reported here (Bachra and van der Eb, 1970). However, as the latter authors point out, there is considerable difficulty with nonspecific binding of synthesized collagen molecules to polyribosomes. This would not be a problem in the case of plant cells, since proline hydroxylation occurs in a wide variety of plant proteins (Pusztai and Watt, 1969). Also, a lack of hydroxyproline when the bulk protein is 10% hydroxylated would make nonspecific adsorption to the ribosomal complex unlikely. In summary, although our results do not rule out limited hydroxylation on nascent chains, they do indicate that the site of proline hydroxylation in plant cells is largely on completed proteins after release from a protein-synthesizing complex.

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