Plant Physiol. (1969) 44, 1187–1193

Synthesis and Secretion of Hydroxyproline Containing Macromolecules in Carrots. I. Kinetic Analysis

Maarten J. Chrispeels[1]

Department of Biology, John Muir College, University of California, San Diego, La Jolla, California 92037

Received April 9, 1969.

Abstract. When disks of carrot (*Daucus carota*) phloem parenchyma are incubated for 6 days there is a 10-fold increase in cell wall hydroxyproline due to the synthesis and secretion of hydroxyproline-containing macromolecules. The synthesis of these molecules and their secretion are demonstrated by measuring the kinetics of incorporation and of chase of ^{14}C-proline and hydroxyproline in different fractions of the cytoplasm and the cell wall. The hydroxyproline-containing molecules which are secreted are associated with the membranous organelles of the cytoplasm. They can be fractionated into trichloroacetic acid-soluble and trichloroacetic acid-precipitated fractions. The properties of the trichloroacetic acid-soluble fraction associated with the membranous organelles are consistent with its role as a cell wall precursor.

The secretion of carbohydrates has been studied in plant cells, especially in relation to cell wall formation (16). However, little is known about protein secretion, undoubtedly because the idea that cell walls contain proteins has only recently received experimental support [for the most recent review on cell wall proteins, see Lamport (14)]. Proteins or glycoproteins which contain hydroxyproline (hypro) are present in the cytoplasm (19), but occur more abundantly in the cell wall (5, 13). By pulsing tobacco cells in liquid culture with ^{14}C-proline, Olson (18) demonstrated that almost three-fourths of the radioactive cytoplasmic hypro was transferred to the cell wall during chase. In a similar experiment using *Avena* coleoptiles, Cleland (3) showed that 25 % of the cytoplasmic hypro was transferred. He also confirmed earlier observations (2, 10) that part of the bound cellular hypro is in a form soluble in trichloroacetic acid. Under the conditions used, the trichloroacetic acid-soluble hypro of *Avena* coleoptiles did not contribute to the observed secretion. We present here results of studies on the incorporation of ^{14}C-proline and its conversion to hypro, and the transfer of these bound radioactive amino acids to the cell wall in disks of carrot phloem parenchyma. We have identified the trichloroacetic acid-soluble proteins of the membranous organelles of the cell as an important cytoplasmic precursor of cell wall hypro.

[1] This investigation was supported by AEC Research Contract AT (11-1)-34 Project 159 and by a grant from the Cancer Research Coordinating Committee of the University of California.

Materials and Methods

Fresh carrots (*Daucus carota* cv.) were purchased in local supermarkets and stored at 5°. Disks approximately 1 mm thick were cut from a cylinder removed from the phloem with a No. 3 corkborer. Usually 20 disks were incubated in a 50 ml Erlenmeyer flask containing 10 ml of 5 mM phosphate buffer pH 6.0 and 50 μg/ml of chloramphenicol (15). The flasks were shaken at 30° on a rotary shaker for 22 to 24 hr unless otherwise indicated. Chloramphenicol did not have any effect on the processes studied, since similar results were obtained with disks cut under sterile conditions incubated with and without chloramphenicol.

Hydroxyproline in the cytoplasm and the cell wall was quantitated after hydrolysis in 6 N HCl for 18 hr at 110°. Cell wall hydrolysates were neutralized with sodium hydroxide and hydroxyproline was determined using the method of Kivirikko and Liesmaa (12). This method did not produce reliable results with hydrolysates of the cytoplasm because of an interfering yellow color. In the cytoplasmic proteins hypro was determined by the method of Juva and Prockop (11).

Two methods of homogenizing carrot disks were used. When we were interested in the incorporation of label into the cell wall 20 carrot disks were ground exhaustively in 0.5 ml of 0.5 % detergent (Nonidet P40, a nonionic detergent from Shell Chemical Company) in a large mortar in the cold. The walls were sedimented by spinning at 1000*g* for 3 min and washed 4 times with 1 mM proline. They were then

extracted with 9 ml of 0.2 M $CaCl_2$ to obtain the salt extractable cell wall (SECW) fraction. The remainder of the cell wall was washed twice with water, yielding the residual cell wall (RCW) fraction.

To keep the membranous organelles intact the tissue was homogenized in 0.6 M sucrose containing 0.01 M phosphate buffer pH 6.0 (usually 2.5 g with 2.5 ml). This was further diluted to 12 ml with 0.3 M sucrose containing the same buffer. Cell walls and starch grains were removed by spinning at 1500*g* for 3 min. The cytoplasm was fractionated into a membranous fraction (48,000*g* for 30 min in a Sorvall centrifuge) and a supernatant. The membrane fraction was resuspended in 5 % trichloroacetic acid; to the cytoplasmic supernatant we added 1:8 volume of 50 % trichloroacetic acid. Proteins were allowed to precipitate for 1 to 2 hr and then removed by centrifugation at 30,000*g* for 15 min. The trichloroacetic acid-soluble macromolecules in the resulting supernatant were collected by pouring the entire trichloroacetic acid supernatant through a membrane filter (B4 from Carl Schleicher and Schuell Company).

Radioactive proline (uniformly labeled with ^{14}C. 1.8 mc/mg) was obtained from the New England Nuclear Corporation. After the disks had been incubated with ^{14}C proline, they were rinsed 3 times with cold 1 mM proline. To determine the radioactivity in the cytoplasmic proteins and the SECW fraction, aliquots were precipitated with an equal volume of 15 % trichloroacetic acid in the presence of 1 mM proline, and collected on membrane filters. An aliquot of the resuspended cell wall was mixed with 4 volumes of alcohol in the presence of 1 mM proline and collected on a membrane filter (EHWPO2500 from Millipore Corporation). The filters were dried and the radioactivity was determined with a liquid scintillation counter. To determine the distribution of ^{14}C in pro and hypro, the radioactive proteins collected on a membrane filter were subjected to acid hydrolysis (6 N HCl for 18 hr at 110°). Insoluble humins and the cell wall residue were removed by centrifugation. The hydrolysate was taken to dryness at 80° under vacuum in a "Rotary Evapo-Mix". The residue was resuspended in 2 drops of water and applied to Whatman 3 MM chromatography paper. Proline and hydroxyproline were separated by descending chromatography for 18 hr with isopropanol:formic acid:water 15:2:2 (4). The resulting chromatogram was cut into 1 inch strips and the radioactivity determined by liquid scintillation counting.

Results

1. *Increase in Cell Wall Hypro During Incubation.* When carrot disks are incubated for a prolonged period of time (6 days) there are no significant changes in the fresh weight and the dry weight of the disks. There is (Fig. 1) a 2 and one-half-fold increase in the protein-bound hypro of the cytoplasm,

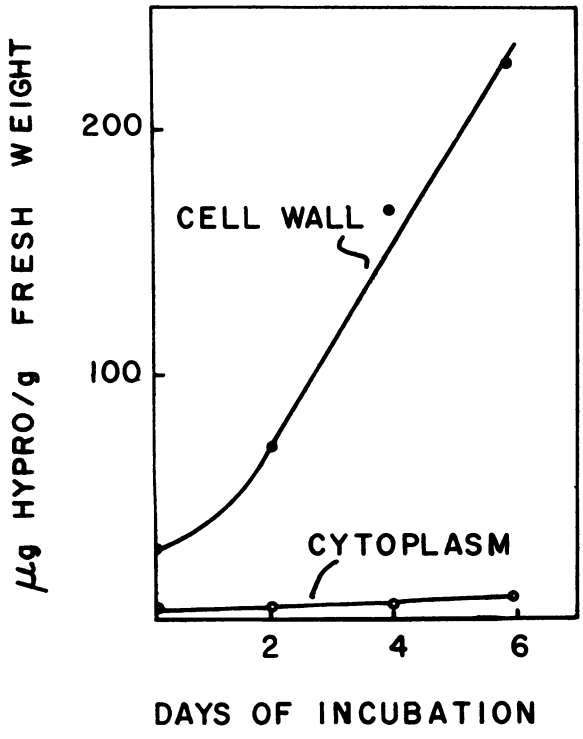

FIG. 1. Increase in the protein-bound hydroxyproline of the cytoplasm and cell wall of carrot disks incubated for 2, 4, or 6 days in buffer. Hydroxyproline was determined in the acid hydrolysates of the cell wall and the cytoplasmic proteins precipitated with 4 volumes of ethanol.

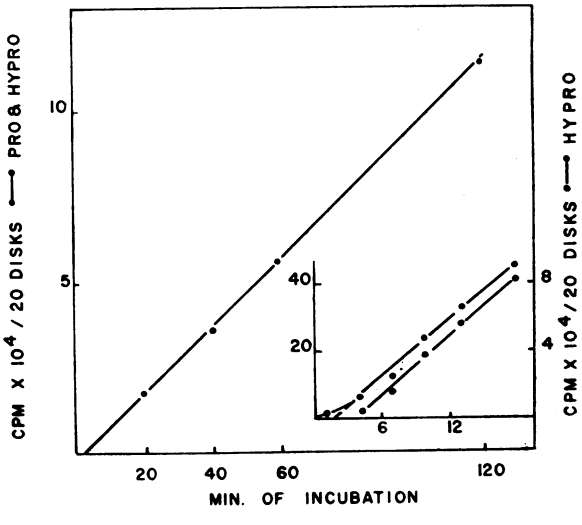

FIG. 2. Incorporation of ^{14}C proline in the cytoplasmic proteins of carrot disks. Lots of 20 carrot disks were incubated for 24 hr in buffer; 1 µc of ^{14}C proline with 0.25 µmoles of carrier proline was added to each flask and the tissue allowed to incorporate label for the times indicated. The experiment in the "inset" was done without added carrier.

and more than a 10-fold increase in the protein-bound hypro content of the wall (from 23 μg–231 μg of hypro/g fresh wt). Under the same conditions the nitrogen content of the cell wall rises 2-fold, while the amount of protein-bound proline in the wall increases from 75 μg to 153 μg/g fresh weight.

2. *Labeling of the Cytoplasm and the Cell Wall With ^{14}C Proline and Its Conversion to ^{14}C Hydroxyproline.* When carrier-free ^{14}C proline is given to disks aged for more than 2 hr the radioactive compound is taken up very rapidly and incorporated into protein. If the incorporation of ^{14}C proline is to be followed for more than 10 min it is necessary to add an appropriate amount of carrier proline (0.05 μmoles/hr of incorporation/20 disks). The incorporation of radioactivity into trichloroacetic acid-precipitated proteins collected on a membrane filter is linear with time and proceeds after a lag of about 2 min (Fig. 2) indicating that the amino acid readily penetrated into the cells of the thin disks and that the protein-synthesis-precursor-pool of proline assumes a constant specific activity in a short time. That the radioactivity is incorporated into proteins was shown in several ways. First, incorporation is inhibited more than 95 % by the addition of 10 μg/ml of cycloheximide. Second, the majority of the label is solubilized by treatment with pronase. Third. when the trichloroacetic acid-precipitate is subjected to acid hydrolysis to yield free amino acids, more than 90 % of the radioactivity is found in either proline or hydroxyproline.

In plant cells (6, 18) as in animal cells [for a review see Udenfriend (21)] proline is probably hydroxylated after it becomes incorporated into polypeptide chains. In the cytoplasm of carrot disks labeled with ^{14}C pro the appearance of ^{14}C hypro in proteins lags behind the incorporation of pro by 2 to 3 min (inset, Fig. 2).

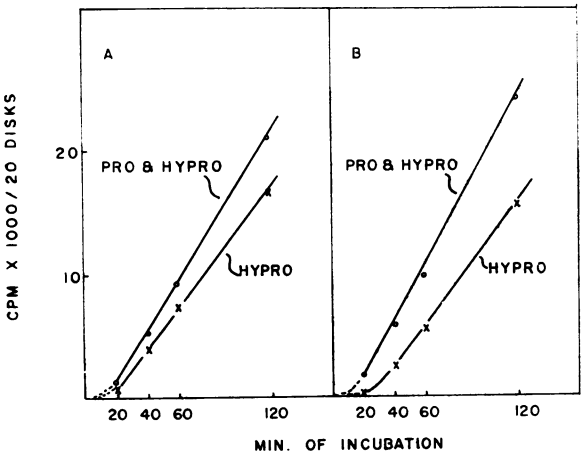

FIG. 3. Arrival of ^{14}C pro and ^{14}C hypro covalently attached to macromolecules in the cell wall. This is part of the previous experiment and the same conditions apply. A is the fraction of the cell wall which is extractable with salt (SECW) and B is the residual cell wall fraction (RCW).

Table I. *Accumulation of Protein-bound Radioactivity in the Cytoplasm and the Cell Wall*

Lots of 20 disks were incubated for varying times, then allowed to incorporate in 1 μc of ^{14}C proline with 0.1 μmole of carrier proline and collected after 30 and 90 min. This allowed us to calculate a rate of incorporation of ^{14}C proline into the cytoplasmic and cell wall proteins.

Time of incubation	Radioactivity which accumulates in fraction		
	Cytoplasm	SECW[1]	RCW[2]
hr	%	%	%
1½	91.1	7.0	1.9
10	84.9	10.7	4.4
18	77.5	13.6	8.9
24	77.0	13.5	9.5
48	79.0	12.6	8.4

[1] Salt extractable cell wall.

[2] Residual cell wall.

We have routinely fractionated the cell wall into 2 parts: a salt extractable fraction (SECW) and a residual fraction (RCW). The labeling of the SECW and RCW fractions proceeds at a linear rate, but with a time lag of 10 to 15 min (Fig. 3). Labeling of cell wall proteins is inhibited more than 90 % by 10 μg/ml of cycloheximide and acid hydrolysis of the cell walls shows that 90 % of the radioactivity is in either proline or hydroxyproline. These tests indicate that the radioactivity which is found in the cell wall is present in proteins or glycoproteins.

Experiments like the ones shown in Fig. 2 and 3 can be used to determine the rate at which protein-bound label accumulates in the cytoplasm and in the cell wall, and to calculate the percentage of newly synthesized protein which is secreted into the wall. The data in table I show that 90 min after cutting the disks, 7.0 % of the radioactivity appears in the SECW and only 1.9 % in the RCW. After 24 hr of incubation 13.5 % appears in the SECW and 9.5 % in the RCW.

The ratio of radioactive hypro to pro of the 2 cell wall fractions increases with time of labeling indicating that the arrival of ^{14}C hypro in the cell wall lags behind the arrival of ^{14}C pro. For the salt extractable fraction the lag is only about 1 or 2 min (Fig. 3a). Hypro accounts for 80 % of the radioactivity in that fraction. In the residual cell wall fraction arrival of ^{14}C hypro lags 12 to 15 min (Fig. 3b) behind the arrival of labeled proteins. Twenty min after the start of labeling only 17 % of the radioactivity is in hypro in the residual cell wall fraction. This increases to 70 % by 180 min.

3. *Secretion of Protein-bound Hydroxyproline.* Pulse-chase experiments were used to follow the secretion of ^{14}C labeled protein-bound hypro. It is first necessary to determine the time required for the chase to become effective after the carrot disks have

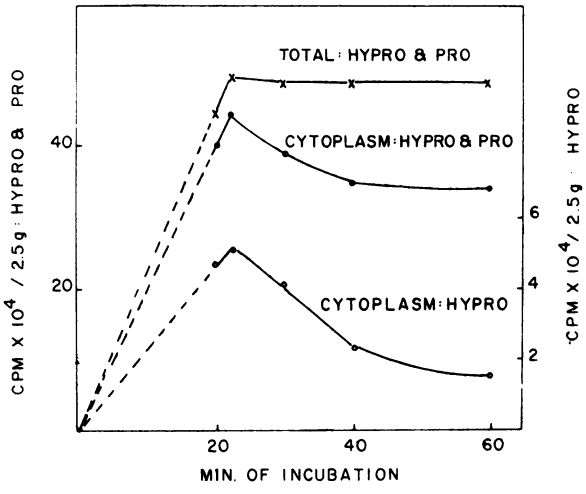

FIG. 4. Incorporation of ^{14}C proline into carrot disks and chase of the radioactivity from the cytoplasm. Lots of 2 and one-half g of tissue are incubated for 1 day, then allowed to incorporate 1 μc of ^{14}C proline with 0.05 μmoles of carrier proline. After 20 min the label is removed and 100 μmoles of carrier proline is added. Tissue is incubated further and samples are collected at times indicated.

been flooded with non-radioactive proline. Fig. 4 shows that there is continued incorporation for 2 to 3 min after the chase is applied, assuming there is no dramatic change in the rate of protein synthesis. After that time the total amount of protein-bound radioactivity (cytoplasm plus cell wall fractions) remains constant for at least 2 hr. It should be pointed out that hydroxylation probably continues for at least 5 min after chase since hydroxylation lags behind incorporation by 2 to 3 min (Fig. 2). Incorporation into the cytoplasmic proteins also continues for 2 to 3 min, but this is followed by the rapid disappearance of a minor component which constitutes 20 to 25 % of the cytoplasmic label at that time. Although more than four-fifths of the label is in proline, loss of label from hypro accounts for half the total loss.

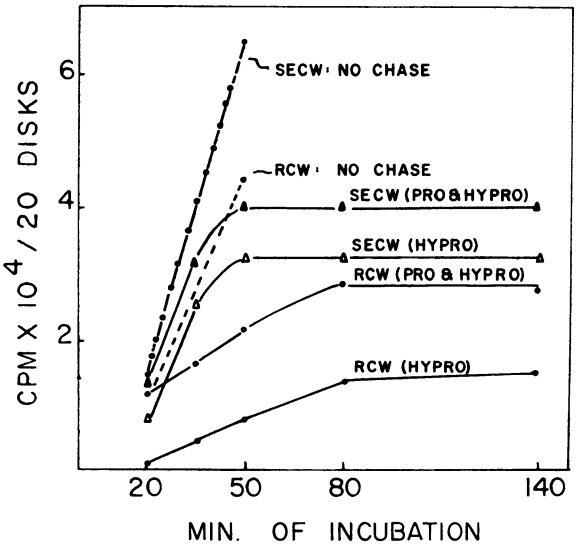

FIG. 5. Arrival of ^{14}C proline and ^{14}C hydroxyproline covalently attached to macromolecules in the cell wall. Lots of 20 disks are incubated for a day, then allowed to incorporate 1 μc of ^{14}C proline in the presence of 0.05 μmoles of carrier proline for 20 min; the label is removed and 100 μmoles of carrier proline is added as a chase. The tissue is collected at the times indicated and homogenized in 0.5 % Nonidet.

The disappearance of labeled proteins from the cytoplasm is matched by the appearance of such proteins in the cell wall (Fig. 5). Radioactivity continues to arrive in the cell wall long after the chase has become effective: up to 30 min after chase in the SECW fraction and up to 60 min after chase in the RCW fraction. In the SECW fraction radioactivity arrives at the same rate as in the absence of a chase (compare with dashed line) but in the RCW fraction the rate of arrival of radioactivity is greatly reduced by the chase. However, the total amount of radioactivity which accumulates in both fractions after chase is the equivalent of 15 min of secretion at the normal rate. The arrival of hypro in the cell wall closely parallels the arrival of total radioactivity. In both fractions 80 to 90 % of the radioactivity which arrives after chase is in hypro (Fig. 5).

4. *Secretion or Turnover?* We considered the possibility that the disappearance of radioactive hypro from the cytoplasmic proteins does not constitute secretion, but rather the metabolic turnover of these proteins. It is well known that most plant tissues do not contain detectable amounts of free hypro. This is usually cited as evidence that the hypro containing proteins are stable and that the disappearance of radioactivity in pulse-chase experiments constitutes secretion. We must bear in mind the possibility that hydroxyproline itself could be broken down rapidly and that the hypro containing macromolecules need not be broken down to individual amino acids, but rather to peptides or glycopeptides. We attempted to find breakdown products of the hypro containing macromolecules with these problems in mind.

Carrot disks were allowed to incorporate proline (1 μc/g of tissue in 10 ml of 2 mM carrier proline) for 20 min, and then chased for 30 and 60 min with 10 mM proline. The tissue was homogenized and the cell walls removed. An aliquot of the cytoplasm was precipitated with trichloroacetic acid and the proteins collected on a membrane filter. The radioactivity in hypro was determined after acid hydrolysis and chromatography. Another aliquot of the cytoplasm (containing both macromolecules and small molecules) was subjected to acid hydrolysis and the total amount of radioactive hypro was determined by the method of Juva and Prockop (11). In this method hypro is converted to pyrrol which is then

Table II. *Disappearance of Radioactive Hydroxyproline From the Cytoplasm During Chase*

Carrot disks were labeled for 20 min and then chased for 30 to 60 min. "Macromolecular hypro" was obtained by acid hydrolysis of trichloroacetic acid precipitated proteins collected on membrane filters. "Total hypro" was obtained by acid hydrolysis of the entire cytoplasm.

Time after chase	CPM in Hydroxyproline	
	Macromolecules only	Total cytoplasm
min		
0	11,360	11,400
30	4,000	3,760
60	3,600	2,940

specifically extracted from the reaction medium. Radioactivity lends increased sensitivity to this method and it becomes possible to detect 100 cpm of hypro amidst 500,000 cpm of pro.

With both methods we found the same amount of hypro (table II), indicating that the cytoplasm does not contain any hypro apart from the macromolecules which have been aged for a day contain 2.2 μg of results were obtained when the chase was carried out in the presence of 5 mM free hypro.

5. *Participation of a Trichloroacetic Acid-soluble Fraction in the Secretion of Hypro.* Carrot disks which have been aged for 1 day contain 2.2 μg of hypro/g fresh weight in the proteins precipitated with trichloroacetic acid and 2.0 μg of hypro/g fresh weight in the trichloroacetic acid-soluble fraction. This trichloroacetic acid-soluble fraction is retained by a bacterial membrane filter indicating that it consists of rather large molecules. When carrot disks are labeled with ^{14}C proline approximately 10 % of the radioactivity is found in this macromolecular trichloroacetic acid supernatant fraction. The distribution of radioactivity in pro and hypro differs sharply for the trichloroacetic acid-precipitated and trichloroacetic acid-soluble fractions. In the trichloroacetic acid-precipitated proteins about 10 % of the radioactivity is in hypro and 90 % in pro. In the trichloroacetic acid-soluble fraction on the other hand, 65 % of the radioactivity is in hypro and 35 % in pro. The trichloroacetic acid-soluble fraction therefore accounts for almost half of the radioactive hypro. In a pulse-chase experiment labeled hypro disappears at the same rate from both the trichloroacetic acid-precipitated proteins and the trichloroacetic acid-soluble fraction and the curves which are obtained are similar to the one presented in Fig. 3.

Membranous organelles have been shown to be involved in the secretion of proteins in several systems (8, 9, 17). Because it is impossible to obtain even moderately pure particulate fractions from plant tissue homogenates by differential centrifugation of the cytoplasm, we simply separated the cytoplasm in a "membrane" fraction and a "supernatant" fraction by centrifuging the tissue homogenate at 48,000*g* for 30 min. Both these cytoplasmic fractions contain trichloroacetic acid-soluble and trichloroacetic acid-precipitable hypro. The trichloroacetic acid-soluble hypro of the membrane portion differs in several ways from that of the supernatant. The trichloroacetic acid-soluble hypro from the membrane fraction represents less than 3 % of the total trichloroacetic acid-soluble hypro of the cytoplasm (0.08 μg of hypro per gram fresh wt). When carrot disks are labeled for 20 min with ^{14}C proline the distributions of radioactivity in hypro and pro in the 2 fractions are completely different. In the membrane trichloroacetic acid-soluble fraction 85 % to 90 % of the radioactivity is in hypro, while in the supernatant trichloroacetic acid-soluble fraction only 15 % is in hypro. Labeling kinetics of the 2 fractions are also entirely different. Under conditions where ^{14}C pro is continuously incorporated into protein for a period of 2 hr, incorporation into the membrane trichloroacetic acid-soluble levels off after 40 min, while labeling of the supernatant trichloroacetic acid-soluble continues for the entire period (Fig. 6). Labeling of the proteins precipitated with trichloroacetic acid also continues for the entire 2-hr period. This suggests that the membrane trichloroacetic acid-soluble has a high turnover.

When carrot disks are labeled for 20 min and subsequently chased, radioactive hypro disappears rapidly from the membrane trichloroacetic acid-soluble, but not from the supernatant trichloroacetic

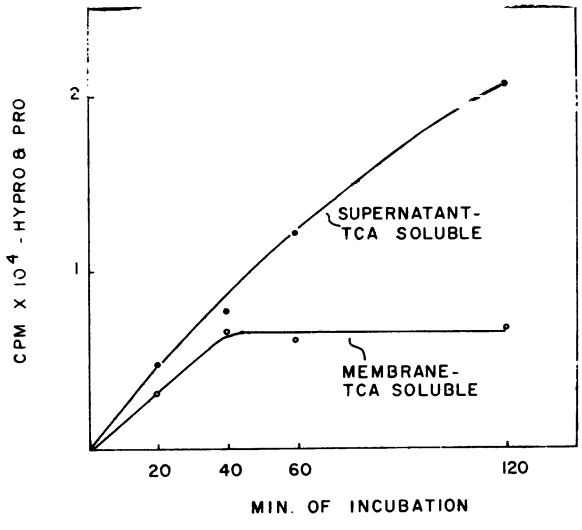

FIG. 6. Kinetics of incorporation of ^{14}C proline into the membrane trichloroacetic acid-soluble and the supernatant trichloroacetic acid-soluble fractions of the cytoplasm. Lots of 2 and one-half g of carrots are incubated for a day, then labeled with 1 μc of ^{14}C proline and 0.2 μmoles of proline. The disks are harvested at the times indicated and homogenized in 0.6 M sucrose with 0.01 M phosphate pH 6.0. The cytoplasm is centrifuged at 48,000*g* for 30 min to spin down the membrane fraction. Addition of trichloroacetic acid and subsequent centrifugation yields a membrane trichloroacetic acid-soluble and a supernatant trichloroacetic-acid-soluble.

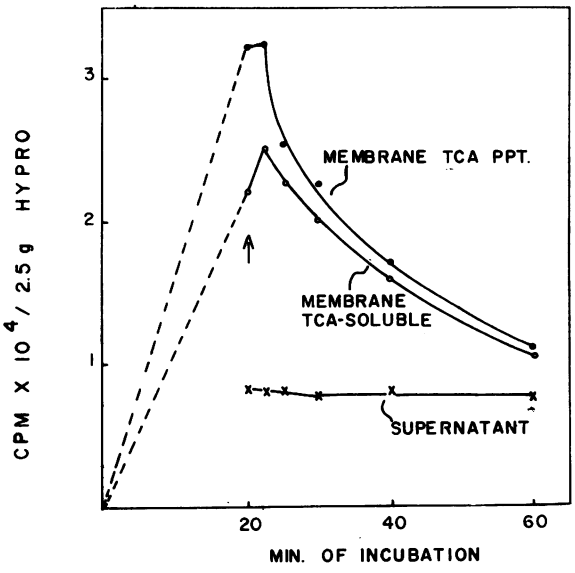

FIG. 7. Disappearance of ^{14}C hypro from the trichloroacetic acid-soluble membrane, trichloroacetic acid-precipitated membrane, and supernatant fractions. Lots of 2 and one-half g of tissue are incubated for a day, then allowed to incorporate 1 μc of ^{14}C proline in the presence of 0.05 μmole of carrier proline. After 20 min the label is removed and 100 μmoles of carrier proline is added as chase. The tissue is collected at the times indicated and homogenized in sucrose-phosphate. The membrane fraction is collected by centrifugation at 48,000*g* for 30 min and resuspended in trichloroacetic acid. Trichloroacetic acid is added to the supernatant and the proteins in the various fractions are collected on membrane filters.

acid-soluble (Fig. 7). It also disappears rapidly from the membrane trichloroacetic acid-precipitate, but not from the supernatant proteins. (In Fig. 7 the trichloroacetic acid-soluble and trichloroacetic acid-precipitate of the supernatant are combined.) The non-membrane cytoplasm does not seem to contribute to the secretion of hypro-containing proteins.

Discussion

Whenever experiments of the kind reported in this paper are performed it is necessary to consider the problem of cross-contamination between the fractions. Cross-contamination of carrot cell walls with carrot cytoplasm is readily visible with the unaided eye because contaminated walls are orange. Most methods of grinding the tissue result in contaminated walls. The contamination cannot be removed by washing the walls with water, detergents or salt. On the other hand, grinding small amounts of tissue with detergent or water in a mortar usually gives white cell walls. When non-radioactive tissue was homogenized in the presence of radioactive carrot cytoplasm containing 0.5 % Nonidet or when non-radioactive cell walls (extracted with salt or not) were mixed with labeled carrot cytoplasm, very little radioactivity remained bound to the cell walls after isolation and purification in the usual manner. Thus, cytoplasmic proteins do not become readily bound to the cell wall when they have been solubilized with detergent.

The completely different kinetics of labeling and of chase observed for the cell wall and cytoplasmic fractions is the strongest evidence for distinct fractions. Indeed, during chase, labeled hypro rapidly disappears from the cytoplasm and accumulates in the cell wall. Any cross-contamination which exists between these 2 fractions must be minimal. To dispel all doubts about the existence of proline and hydroxyproline containing proteins in the cell wall, we examined by autoradiography carrot disks which were labeled with ^{14}C proline and subsequently plasmolyzed. Our results show the incorporation of label in both the cytoplasm and the cell wall (20).

The walls of cells which are proliferating in an unorganized manner (as calluses or in cell cultures) contain more hypro than cell walls of organized tissues (14). This observation is partially explained by our finding of an increase in the hypro content of the cell wall as a function of time of incubation of tissue disks. Liquid cell cultures and calluses are obtained normally by allowing small pieces of tissue to proliferate on a solid nutrient medium containing the proper hormonal balance. Initially these cells probably undergo a wound response not unlike that observed in incubating tissue disks. Indeed, the increase in the hypro content of the cell wall is similar whether the disks are incubated in a buffer or on a solid nutrient medium. It would appear then that the hypro content of the cell wall increases as part of the wound response which excised tissues undergo.

Radioactive proline is readily incorporated into the cytoplasmic proteins and converted to hydroxyproline. There is a lag of 2 to 3 min between incorporation and hydroxylation (Fig. 2). At present there are no data concerning the cytological site of hydroxylation. The 2 min time lag could represent the time it takes to finish the protein and move it to the site of hydroxylation, although the possibility that nascent protein is hydroxylated cannot yet be excluded. Our preliminary results (1) indicate that the hydroxylation is carried out by an enzyme which has many characteristics in common with a hydroxylase [Hutton *et al.* (7)] isolated from cells which synthesize and secrete collagen.

The labeling experiments suggest that there is a small pool of cytoplasmic proteins which are continuously synthesized and secreted. Labeled proteins arrive in the cell wall after a time lag of about 15 min. In a pulse-chase experiment, labeled proteins continue to arrive in the cell wall for a similar period of time after the chase has become effective. The different lag-times of arrival in the wall and the different kinetics of arrival in the wall during chase for the various fractions which were examined sug-

gest that each fraction consists of one or more distinct proteins. The continued arrival of hypro in the wall during chase is matched by its disappearance from the cytoplasm. This confirms similar observations made by Olson (18) with cultured tobacco cells, and by Cleland (3) with *Avena* coleoptiles. We were unsuccessful in finding breakdown products of the hypro containing macromolecules indicating that their disappearance from the cytoplasm most probably does not constitute metabolic turnover, but rather secretion.

Hydroxyproline in the cell can be fractionated into trichloroacetic acid-soluble and trichloroacetic acid-precipitated fractions (2, 3, 10). The trichloroacetic acid-soluble fraction is non-dializable (3) and is retained by bacterial membrane filters suggesting that it is macromolecular. We fractionated the cytoplasm into a fraction containing membranous organelles and a supernatant fraction, and then separated trichloroacetic acid-precipitated hypro from trichloroacetic acid-soluble hypro in each fraction. This allowed us to show that the hypro containing macromolecules which are secreted are associated exclusively with the membranous organelles, and that they are evenly distributed over the trichloroacetic acid-soluble and the trichloroacetic acid-precipitated proteins. We have no evidence for a chemical difference between trichloroacetic acid-precipitated and trichloroacetic acid-soluble hypro. However, because a large measure of purification is achieved by precipitating the bulk of the proteins with trichloroacetic acid we concentrated first on the characterization of the trichloroacetic acid-soluble fraction from the membranous organelles. It has a number of properties consistent with its role as a cell wall precursor: it constitutes only a small amount of the cytoplasmic hypro; it is labeled to saturation within 40 min after the addition of ^{14}C pro to the carrot disks; this label disappears rapidly in a pulse-chase experiment and has a half-life of roughly 20 min; 85 to 90 % of the label is in hypro and 10 to 15 % is in pro; although it is soluble in trichloroacetic acid, it is retained by membrane filters indicating that it is either a large molecule or an aggregate which is not readily dissociated. The importance of this fraction as a cell wall precursor warrants its further characterization.

Acknowledgments

It is a pleasure to thank Mrs. M. Doerschug and Mr. E. Walker for their valuable assistance. Mary Ericson, Angeline Douvas, Greg Brewer, and Dave Weaver, all first year graduate students in Biology, contributed to these results during their rotation projects.

Literature Cited

1 CHRISPEELS, M. J., M. R. DOERSCHUG, AND D. SADAVA. 1969. Secretion of hydroxyproline containing macromolecules in carrot cells. Abstracts, XI Intern. Botan. Congr. p 32.

2. CLARKE, F. M. W. AND G. M. ELLINGER. 1967. Fractionation of plant material. J. Sci. Food Agr. 18: 536–40.
3. CLELAND, R. 1968. Distribution and metabolism of protein-bound hydroxyproline in an elongating tissue, the *Avena* coleoptile. Plant Physiol. 43: 865–70.
4. CLELAND, R. AND A. M. KARLSNES. 1967. A possible role of hydroxyproline containing proteins in the cessation of cell elongation. Plant Physiol. 42: 669–71.
5. DOUGALL, D. K. AND K. SHIMBAYASHI. 1960. Factors affecting growth of tobacco callus tissue and its incorporation of tyrosine. Plant Physiol. 33: 396–404.
6. HOLLEMAN, J. 1967. Direct incorporation of hydroxyproline into protein of sycamore cells incubated at growth-inhibitory levels of hydroxyproline. Proc. Natl. Acad. Sci. 57: 50–54.
7. HUTTON, J. J., JR., A. L. TAPPEL, AND S. UDENFRIEND. 1966. Cofactor and substrate requirements of collagen proline hydroxylase. Arch. Biochem. Biophys. 118: 231–40.
8. JAMIESON, J. D. AND G. E. PALADE. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell I. J. Cell Biol. 34: 577–96.
9. JAMIESON, J. D. AND G. E. PALADE. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. J. Cell Biol. 34: 597–615.
10. JENNINGS, A. C. AND W. B. WATT. 1967. Fractionation of plant material. J. Sci. Food Agr. 18: 527–35.
11. JUVA, K. AND D. J. PROCKOP. 1966. Modified procedure for the assay of ^{3}H or ^{14}C labeled hydroxyproline. Anal. Biochem. 15: 77–83.
12. KIVIRIKKO, K. I. AND M. LIESMAA. 1959. A colorimetric method for determination of hydroxyproline in tissue hydrolysates. Scan. J. Clin. Lab. Invest. 11: 128–33.
13. LAMPORT, D. T. A. AND D. H. NORTHCOTE. 1960. Hydroxyproline in primary cell walls of higher plants. Nature 188: 665–66.
14. LAMPORT, D. T. A. 1965. The protein component of primary cell walls. Advan. Botan. Res. 2: 151–218.
15. LEAVER, C. J. AND J. EDELMAN. 1965. Antibiotics as a means of control of bacterial contamination of storage tissue disks. Nature 207: 1000–01.
16. MOLLENHAUER, H. H. AND D. J. MORRÉ. 1966. Golgi apparatus and plant secretion. Ann. Rev. Plant Physiol. 17: 27–46.
17. NEUTRA, M. AND C. P. LEBLOND. 1966. Synthesis of the carbohydrate of mucus in the golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose ^{3}H. J. Cell Biol. 30: 119–36.
18. OLSON, A. C. 1964. Proteins and plant cell walls. Proline to hydroxyproline in tobacco suspension cultures. Plant Physiol. 39: 543–50.
19. POLLARD, J. K. AND F. C. STEWARD. 1959. The use of ^{14}C proline by growing cells: its conversion to protein and to hydroxyproline. J. Exptl. Botany 10: 17–32.
20. SADAVA, D. AND M. J. CHRISPEELS. Cell wall protein in plants: autoradiographic evidence. Science 165: 299–300.
21. UDENFRIEND, S. 1966. Formation of hydroxyproline in collagen. Science 152: 1335–40.