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Involvement of the Golgi Apparatus in the Synthesis and Secretion of Hydroxyproline-rich Cell Wall Glycoproteins[1, 2, 3]

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ABSTRACT

Pulse labeling of carrot root phloem parenchyma (*Daucus carota* L. cv. Nantes) tissue with ^{14}C-proline followed by fractionation of the cytoplasmic organelles on sucrose gradients was used to determine the identity of the membranous organelles involved in the secretion of the hydroxyproline-rich glycoproteins of the cell wall. Identification of the organelles was done through electron-microscopical observations and through the localization of marker enzymes on the sucrose gradients. Enrichment of the organelles involved in secretion was determined by measuring the percentage of the incorporated radioactivity present as ^{14}C-hydroxyproline. The Golgi apparatus (dictyosome) was found to be a major site of glycoprotein transport. This identification was based on the observed enrichment of dictyosomes paralleling the purification of newly synthesized cell-wall glycoproteins. A marker enzyme for the Golgi apparatus, inosinediphosphatase, banded with the newly synthesized cell wall glycoproteins on sequential isopycnic and rate zonal sucrose gradients. Marker enzymes for the endoplasmic reticulum and the plasma membrane were clearly separated from the dictyosome-rich fraction. UDP-arabinose arabinosyl transferase, an enzyme involved in the glycosylation of the peptide moiety of this glycoprotein, also banded with the dictyosomes on both kinds of gradients. The results suggest an important role of the Golgi apparatus in the biosynthesis and the secretion of the cell wall glycoproteins of higher plants.

The primary walls of plant cells contain a structural hydroxyproline-rich glycoprotein called "extensin" (for review see Lamport [12]). This protein is synthesized in the cytoplasm as a proline-rich polypeptide and the proline residues are hydroxylated by a soluble peptidyl proline hydroxylase (20). The hydroxyproline-residues formed in this way are subsequently glycosylated by a particulate UDP-arabinose arabinosyl transferase (11). Each hydroxyproline residue acquires a short side-chain consisting of several arabinose residues (13). Secretion of the finished glycoprotein to the cell wall involves its transient association with one or more cytoplasmic organelles (3, 6). The organelles involved in the glycosylation of the polypeptide and in the secretion of the glycoprotein have not been identified. Dashek (6) examined this problem several years ago but his results were inconclusive. Secretion seemed to be mediated by smooth membrane elements but he was unable to establish whether these were derived from the endoplasmic reticulum, the plasma membrane, or the Golgi apparatus. Progress in the methodology of cell fractionation and the availability of new marker enzymes prompted us to reinvestigate this question. Our results suggest that the Golgi apparatus plays a major role in the biosynthesis and secretion of the hydroxyproline-rich cell wall glycoproteins.

MATERIALS AND METHODS

Materials. Carrot roots (*Daucus carota* L. cv. Nantes) were purchased in local supermarkets and stored at 4 C. Radioactive chemicals were obtained from New England Nuclear Co.: UDP-arabinose [L-arabinose-^{14}C(U)] (183 mCi/mmole); UDP-glucose [D-glucose-^{14}C(U)] (227 mCi/mmole); GDP-mannose [D-mannose-^{14}C(U)] (167 mCi/mmole); UDP-galactose [D-galactose-^{14}C(U)] (254 mCi/mmole); and L-proline-^{14}C(U) (233 mCi/mmole) or ICN: GDP-glucose [D-glucose-^{14}C(U)] (203 mCi/mmole) and ICN isotope. Inosine diphosphate, NADH, and Cyt *c* were obtained from Sigma. Proteins precipitated with trichloroacetic acid were collected on cellulose nitrate filters obtained from Schleicher and Shuell (Type B-4).

Fractionation of Cytoplasmic Organelles. Carrot root phloem parenchyma disks were prepared and incubated for 24 hr at 30 C as described (3). The isolation and purification of the membranous organelles was based on a modification of the methods of Ray *et al.* (18). Glutaraldehyde was included in the homogenization medium to insure structural preservation of the Golgi apparatus (15). The homogenization medium consisted of ice-cold 50 mM tris-Cl buffer (pH 8.0) containing 10 mM KCl, 0.1 mM $MgCl_2$, 1 mM Na_2EDTA, 1 mM dithiothreitol, 1% dextran (mol wt 250,000), 0.1% BSA, 10% sucrose, and 0.5% glutaraldehyde. Glutaraldehyde was omitted in some experiments to determine its effect on the sedimentation behavior of the cytoplasmic organelles and the activity of marker enzymes. The tissue was usually minced with a hand-held razor blade and then thoroughly chopped with a pair of stainless steel razor blades mounted in an electrically driven

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[2] A preliminary account of this work was presented at the annual meeting of the American Association of Plant Physiologists in July 1973 (8).

[3] This is paper No. 8 in a series on the "Synthesis and Secretion of Hydroxyproline-containing Proteins in Carrot." No. 7 appears as reference 4.

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mechanical chopper. This operation was performed in a plastic Petri dish held on ice. After homogenization the brei was filtered through one layer of Miracloth to remove debris and was centrifuged in the cold at 1000*g* for 5 min. The 1000*g* supernatant was used to further fractionate the cytoplasmic organelles. Linear sucrose gradients were made by dissolving sucrose (Merck) in the homogenization medium from which glutaraldehyde had been omitted. Cytoplasmic organelles layered on linear 20 to 50% (w/v) gradients were centrifuged to equilibrium by centrifugation at 27,000 rpm for 2 hr at 3 C (Spinco Rotor SW 27). For rate zonal separations the organelles were layered on linear 20 to 35% (w/v) gradients and centrifuged for 20 min at 15,000 rpm (Spinco rotor SW 27.1). When necessary, organelles were concentrated onto a 50% sucrose cushion by centrifugation at 20,000 rpm for 20 min.

***In Vivo* Labeling of Newly Synthesized Protein and Determination of Radioactivity in Protein-bound Proline and Hydroxyproline.** The tissue was thorouhgly rinsed and then incubated with ^{14}C-proline (1 μCi/g of tissue) for 20 min at 30 C on a shaking waterbath. Chloramphenicol (50 μg/ml) was routinely included and does not affect the synthesis of cell wall proteins (3). Aliquots of the tissue homogenate or of gradient fractions were precipitated with an equal volume of 15% trichloroacetic acid and the precipitated proteins were collected on cellulose nitrate filters. The filters were washed with 5% trichloroacetic acid, dried, and the radioactivity was determined with a liquid scintillation counter after immersion of the dry filters in vials containing a toluene-based scintillation cocktail. The filters were then removed from the vials, washed with toluene, dried, and hydrolyzed in sealed glass ampules containing 6 N HCl (autoclaved for 90 min at 120 C and 22 psi). The HCl was removed by evaporation, and the proline and hydroxyproline in the residue were separated by paper chromatography. Radioactivity in each amino acid was determined and the percentage of radioactivity in proline and hydroxyproline was calculated (3).

Enzyme Assays. Gradient fractions were collected and assayed for absorbancy at 280 nm (A_{280}), for inosine diphosphatase, a marker enzyme for the Golgi apparatus (7, 9), for NADH-Cyt *c* reductase, a marker enzyme for the endoplasmic reticulum (5), UDP-glucose and GDP-glucose glucosyl transferases, marker enzymes for the plasma-membrane (23), and for UDP-arabinose arabinosyl transferase, an enzyme involved in the glycosylation of the cell wall glycoprotein (11).

IDPase activity was assayed by measuring phosphatase activity for 60 min at 35 C using 5 mM inosine-diP in 50 mM tris-Cl, pH 7.2, containing 100 mM KCl and 5 mM $MgCl_2$. Phosphate released was determined by the method of Taussky and Shorr (22) after protein precipitation with an equal volume of 15% trichloroacetic acid.

Cyt *c* reductase activity was measured spectrophotometrically at room temperature by recording the rate of reduction of oxidized Cyt *c* at 550 nm. The reaction mixture contained 50 mM potassium phosphate, pH 7.5, 0.05 mM Cyt *c*, 0.5 mM NADH, and 1.6 mM KCN.

UDP-arabinose arabinosyl transferase activity was measured by determining the radioactivity incorporated into trichloroacetic acid-insolub'e products retained on cellulose nitrate membrane filters. The reaction was performed by adding 0.04 μCi UDP-arabinose [L-arabinose-^{14}C(U)] (183 mCi/mmole) to 250 μl of membrane fraction (0.86 μM final UDP-arabinose concentration) and incubating 15 min at 35 C. The reaction was stopped by adding an equal volume of 15% trichloroacetic acid, precipitating 20 min in an ice bath, and collecting the residue on a membrane filter. UDP-glucose and GDP-glucose glucosyl transferase activity was determined similarly but the reaction was performed in 100 mM tris-Cl, pH 8, 1 mM Na_2EDTA, 1 mM dithiothreitol, 20 mM $MgCl_2$, 15 mM cellobiose, 2 mg of carrier cellulose, and 0.1 μCi UDP-glucose [glucose-^{14}C(U)] (227 mCi/mmole) or 0.1 μCi GDP-glucose [glucose-^{14}C(U)] (203 mCi/mmole). Counts incorporated into lipid soluble and base stable fractions were assayed by washing the centrifuged pellet three times with hot water, extracting the lipid soluble fraction twice with 1 ml of chloroform-methanol (2:1), and removing hot base soluble polysaccharides with 1 N NaOH, followed by two further water washes. The remaining base stable residue was collected on a glass-fibre filter. Filters and chloroform-methanol fractions were dried and counted in toluene based scintillation cocktail. Base solubilized radioactivity was neutralized with 2N HCl and counted in Aquasol (New England Nuclear Co.).

Electron Microscopy. Electron microscopy was performed by collecting an appropriate gradient fraction as a centrifuge pellet (48,000*g*, 30 min), prefixing for 15 min with 2% glutaraldehyde in 0.1 M potassium phosphate, pH 7.4, and post-fixation with 1% OsO_4 in 0.1 M potassium phosphate, pH 7.4, for 1 hr. Dehydration was through an ethanol series followed by an ethanol-propylene oxide series. The membrane pellets were infiltrated overnight with propylene oxide-Epon (1:1) in a vacuum desiccator then embedded in Epon which was cured for 48 hr at 60 C in a vacuum oven. Sections were cut with a diamond knife and stained with uranyl acetate and lead citrate, followed by observation with a Phillips 200 electron microscope.

RESULTS

Partial Purification of Membranous Organelles on Sucrose Gradients. The partial purification of the organelles involved in the secretion of cell wall glycoproteins was performed by using sequential isopycnic and rate zonal sucrose gradients. When carrot disks are incubated with ^{14}C-proline for 20 min, the newly synthesized cellular proteins become radioactively labeled (3). The conversion of ^{14}C-proline to ^{14}C-hydroxyproline is a post-translational modification mediated by a cytoplasmic peptidyl proline hydroxylase enzyme (20). In the cytoplasmic proteins, only about 5% of the proline residues are hydroxylated, but the cell wall precursor proteins are extensively hydroxylated, and up to 75 or 80% of the proline residues become hydroxyproline residues (3). The percentage of the protein-bound radioactivity in hydroxyproline residues can therefore be used as a measure of purification of the cell wall precursors (2) or the organelles containing these precursors.

Homogenates of carrot disks, pulse labeled for 20 min with ^{14}C-proline, were layered on linear 20 to 50% sucrose gradients and centrifuged to equilibrium. The resulting UV absorption profile (280 nm) and the position of the protein-bound ^{14}C-hydroxyproline are shown in Figure 1. The UV absorption profile shows a sharp peak at 1.21 g/cm^3. These organelles were identified as mitochondria by electron microscopy. The large shoulder at the top of the gradient contained not only the soluble proteins but also all the orange-colored chromoplasts characteristic of carrot roots. The newly synthesized cell wall proteins (protein-bound ^{14}C-hydroxyproline) were contained in a diffuse band of organelles with an average density of 1.15 g/cm^3. In this region of the gradient, 35% of the incorporated radioactivity was in hydroxyproline while 65% was in proline. These figures suggest that only a partial purification of the secretory organelles had been achieved and that other newly synthesized proteins were present. An electron micrograph of the membranes collected from the ^{14}C-hydroxyproline labeled fractions of the gradient is shown in Figure 2. Numerous

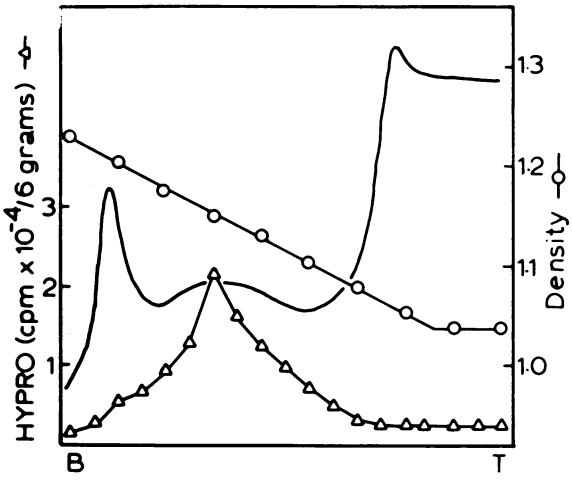

FIG. 1. Aged carrot tissue disks were labeled for 20 min with 1 μCi of proline-^{14}C, rinsed, and homogenized in the homogenization medium containing 0.5% glutaraldehyde to stabilize dictyosomes. Cytoplasmic membranes were centrifuged through a 20 to 50% sucrose gradient to isopycnic equilibrium and gradients were analyzed for UV absorbing particles (——), gradient density (○), and hydroxyproline-^{14}C in macromolecules (△). The hydroxyproline-rich membranes contain 35% of their total radioactivity in hydroxyproline, have an average density of 1.15 g/cm^3, and are separated from the more dense mitochondria (identified from electron micrographs) and the less dense orange band of plastids which float at the gradient-homogenate interface.

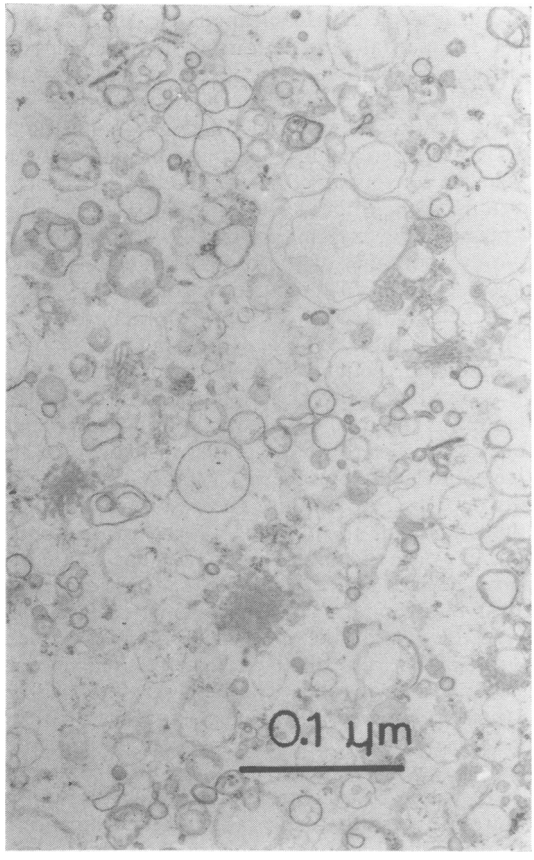

FIG. 2. Electron micrograph of membranous organelles contained in the hydroxyproline-rich isopycnic fraction. Dictyosomes, rough endoplasmic reticulum, and smooth vesicles are evident.

membranous structures are detectable, including dictyosomes, rough-endoplasmic reticulum, and smooth membranous vesicles of different sizes.

The labeling kinetics of the cytoplasmic protein-bound ^{14}C-hydroxyproline suggest that most of it is present in cytoplasmic cell wall precursors (3). To show that the ^{14}C-hydroxyproline present in these membranous organelles is also in cell wall precursors, we performed a pulse-chase experiment in which the tissue was labeled for 20 min and then chased in 1 mM proline for periods up to 30 min. This experiment showed (Fig. 3) that the ^{14}C-hydroxyproline in these organelles is transient, with a half-life of about 10 min. Similar values for cell wall glycoprotein secretion have been obtained by us (3) and by others (6, 17).

Further purification of the organelles containing the newly synthesized cell wall proteins was accomplished using rate zonal sucrose gradient centrifugation of the appropriate fractions from the isopycnic gradients. The organelles present in the diffuse UV-absorbing band of the isopycnic gradients were collected by centrifugation onto a 50% sucrose cushion. After a suitable dilution, they were transferred to the top of a linear 20 to 35% sucrose gradient and centrifuged at 15,000 rpm for 20 min (rate-zonal separation). The UV-absorbance profile and the location of the protein-bound ^{14}C-hydroxyproline is shown in Figure 4. Most of the ^{14}C-hydroxyproline is associated with organelles that sediment about halfway through the gradient. About 69% of the radioactivity associated with this peak is in hydroxyproline. This represents a considerable increase over the previous value (35% in the fractions from the isopycnic gradient), suggesting that organelles containing other newly synthesized proline-containing proteins have been removed. The figure now approaches the level normally found in cell walls at which 75 to 80% of the radioactivity is in hydroxyproline. Figure 5 is an electron micrograph of the membranous organelles present in the middle peak of the rate-zonal gradient. Most apparent is a considerable enrich-

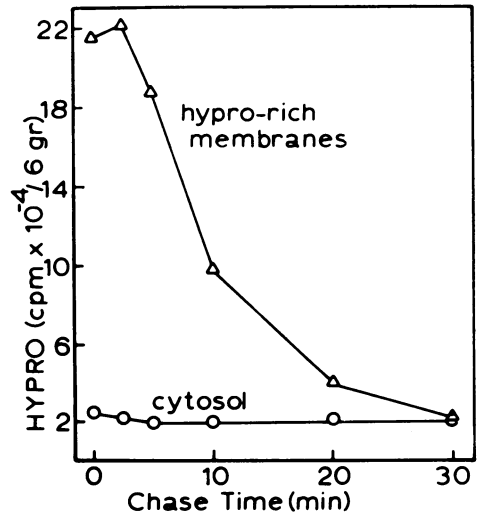

FIG. 3. Pulse-chase experiment in which aged tissue disks were labeled for 20 min with 1 μCi of proline-^{14}C followed by a chase period in 1 mM proline. Tissue disks were homogenized and cytoplasmic fractions applied to isopycnic gradients. Following centrifugation the amount of hydroxyproline-^{14}C in hydroxyproline-rich fractions was determined. Radioactivity continues to be incorporated into membrane protein (△) for 2 to 3 min after the chase but then decreases with a half-life of about 10 min, whereas that in cytosol proteins (○) remains low and only decreases slightly.

ment in structurally intact dictyosomes although numerous other membranous structures are also present in this fraction.

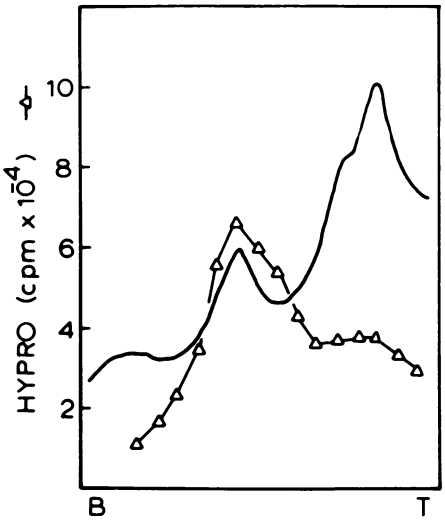

FIG. 4. Profile of UV absorbing particles (——) and membranous hydroxyproline-^{14}C (△) on a rate zonal gradient after preliminary purification on an isopycnic gradient. Radioactive hydroxyproline moves coincident with the middle UV absorbing peak and represents 69% of the total radioactivity of that band of membranes.

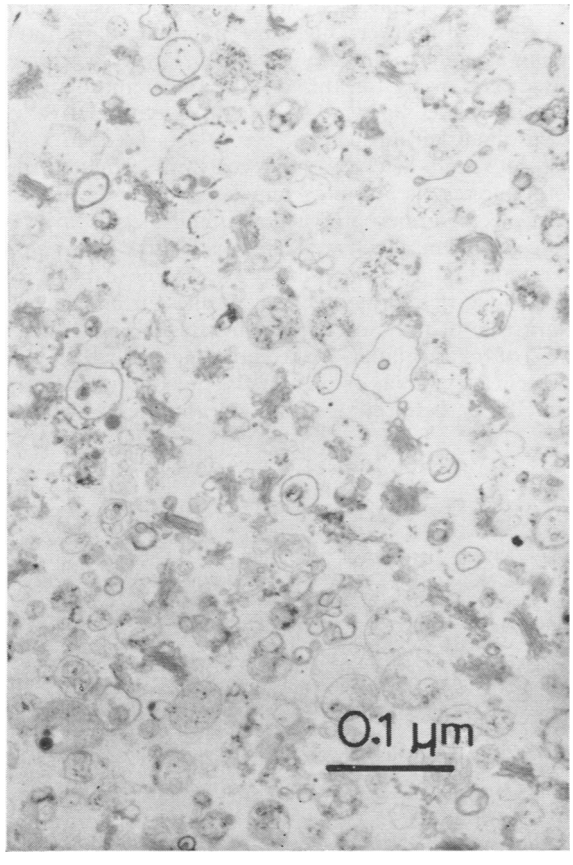

FIG. 5. Electron micrograph of purified membranes isolated from the middle hydroxyproline-rich band of a rate zonal gradient after previous partial purification by isopycnic sedimentation. Most membranes are intact dictyosomes or appear to be derived from dictyosomes by vesiculation.

Table I. *Incorporation of ^{14}C-Sugars from Various Sugar Nucleotide Precursors into Products Soluble in Chloroform-Methanol (2:1) or Hot 1 N NaOH or Insoluble in Hot Base*

The total membrane pellet of 20-g aged disks was resuspended in 2 ml of homogenization medium and 100 μl were used in the subsequent assays. Each reaction mixture (200 μl total volume) contained 100 mM tris, pH 8, 1 mM Na_2EDTA, 1 mM dithiothreitol, 20 mM $MgCl_2$, 15 mM cellobiose, 2 mg of carrier cellulose, and sugar nucleotide: 2 μM UDP-glucose [glucose-^{14}C(U)] (227 mCi/mmole), 2.5 μM GDP-glucose [glucose-^{14}C(U)] (203 mCi/mmole), 3 μM GDP-mannose [mannose-^{14}C(U)] (166 mCi/mmole), 2.5 μM UDP-arabinose [arabinose-^{14}C(U)] (183 mCi/mmole), or 2 μM UDP-galactose [galactose-^{14}C(U)] (254 mCi/mmole). All reactions were performed at 35 C for 15 min and were stopped by heating in a boiling water bath for 5 min before washing and extracting the various fractions as indicated in "Materials and Methods." The data are expressed as the total cpm incorporated into the various extractable products and the percentage of total counts incorporated into each fraction.

	Chloroform: Methanol		Base Soluble		Base Insoluble	
	cpm	*%*	*cpm*	*%*	*cpm*	*%*
UDP-glucose	22,857	92.4	1,010	4.1	862	3.5
GDP-glucose	207	10.6	403	20.6	1,346	68.8
GDP-mannose	695	7.4	1,437	15.4	7,202	77.2
UDP-arabinose	90	6.1	785	53.2	601	40.7
UDP-galactose	7,652	88.2	6.7	7.1	403	4.6

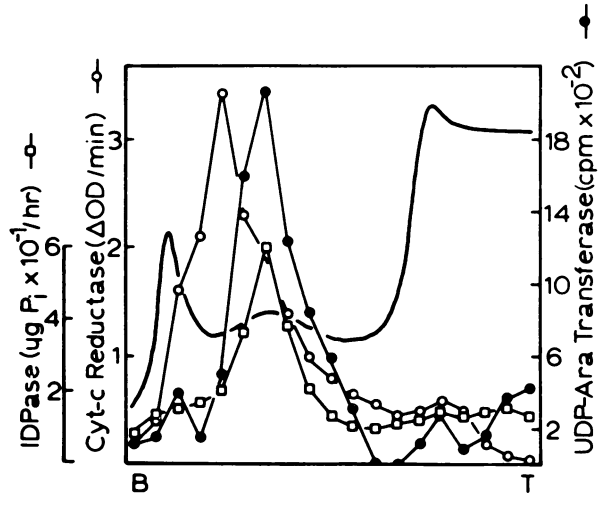

FIG. 6. Profile of marker enzymes on an isopycnic sucrose gradient: IDPase (□), Cyt-*c* reductase (○), UDP-arabinose arabinosyl transferase (●), and UV absorbance (——). IDPase and arabinosyl transferase coincide with the diffuse hydroxyproline-rich band of membranes whereas Cyt reductase is intermediate between the hypro-rich membranes and the peak identified as mitochondria.

Location of Marker Enzymes on the Sucrose Gradients. Appropriate marker enzymes were used to locate the various membranous organelles on the two types of sucrose gradients and to further identify the organelles involved in glycoprotein secretion. Inosine diphosphatase was used as a marker for dictyosomes, Cyt *c* reductase as a marker for the endoplasmic reticulum, and UDP-glucose glucosyl transferase, which transfers glucose to lipid-soluble products, as a plasma membrane marker. UDP-arabinose arabinosyl transferase, an enzyme known to be involved in the glycosylation of peptidyl-hydroxy-

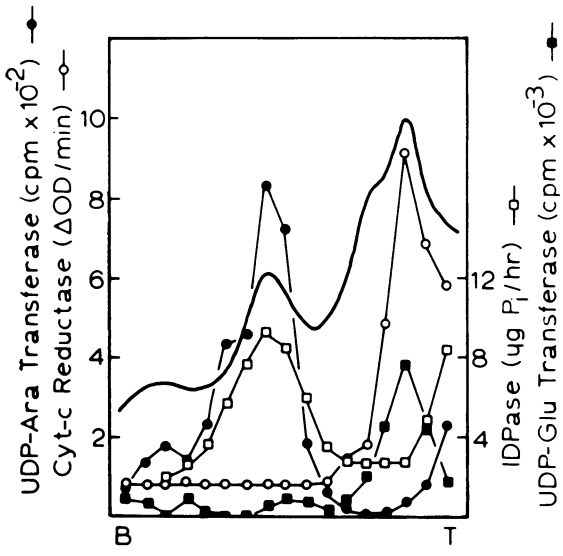

FIG. 7. Profile of marker enzymes on a rate zonal gradient: IDPase (□), Cyt *c* reductase (○), UDP-arabinose arabinosyl transferase (●), UDP-glucose glucosyl transferase (■), and A_{280} (——). IDPase and UDP-arabinose arabinosyl transferase coincide with the intermediate band found to be rich in hydroxyproline. Cyt *c* reductase and UDP-glucose glucosyl transferase are found to be associated with membranes that remain near the top of the gradient.

proline, was used as a marker for the site of glycosylation of the hydroxyproline-rich peptide. Preliminary experiments were done with total membrane fractions to check the transfer of sugars from sugar nucleotides to endogenous acceptors. We found (Table I) that most of the sugar from UDP-Glucose and UDP-galactose was transferred to chloroform-methanol (2:1) -extractable products (possible glycolipids), whereas the sugars from GDP-mannose, UDP-arabinose and GDP-glucose were transferred largely to nonlipid molecules.

The position of the marker enzymes in the isopycnic gradients is shown in Figure 6. The organelles containing the newly synthesized cell wall glycoprotein banded together with the marker for dictyosomes and with the arabinosyl transferase. The marker for the endoplasmic reticulum occurred in the same general area of the gradient but had a somewhat greater mean density. The plasma membrane was also found in this region of the gradient (data not shown). These data are consistent with the presence of rough endoplasmic reticulum, smooth membranes, and dictyosomes in the electron micrograph shown in Figure 2. Further purification on rate zonal gradients showed (Fig. 7) that the marker enzymes for the endoplasmic reticulum and the plasma membrane can be clearly separated from the protein-bound ^{14}C-hydroxyproline which remained associated with the IDPase and the arabinosyl transferase.

DISCUSSION

It has long been known that the Golgi apparatus plays an important role in the biogenesis of the plant cell wall (for a review see Mollenhauer and Morré [14]), although the precise function of this organelle is only now being elucidated, primarily as a result of improved cell fractionation techniques. Recent evidence suggests that the Golgi apparatus is involved in the biosynthesis and the secretion of the hemicellulosic component of the cell wall (1, 18) and of other extracellular polysaccharides (9). In animal cells, the Golgi apparatus functions in the secretion and terminal glycosylation of extracellular proteins and mucopolysaccharides (10, 16, 21, 24). The observation that the glycosylation of cell wall proteins (11) and the secretion of the completed glycoproteins (3, 6) is mediated by cytoplasmic membranous organelles led us to investigate the possibility that the Golgi apparatus also functions in the processing and secretion of extracellular glycoproteins in plant cells. Our results suggest that this is indeed the case.

We observed a parallel enrichment in structurally intact dictyosomes and newly synthesized cell wall protein when cytoplasmic organelles were first fractionated on isopycnic gradients and then further purified on rate-zonal gradients. In both types of gradients the newly synthesized cell wall protein banded with IDPase, a Golgi apparatus marker enzyme (7, 18), and with UDP-arabinose arabinosyl transferase, an enzyme involved in the glycosylation of the polypeptide moieties of the glycoprotein (11). The average buoyant density of the organelles involved in the cell wall glycoprotein secretion was found to be 1.15 g/cm^3, a figure which is consistent with the density of dictyosomes from pea stems (18). Such evidence suggests that dictyosomes are involved in both glycosylation of the hydroxyproline-rich protein and the subsequent secretion of the glycoprotein. Our results show that the dictyosome-rich fractions containing the hydroxyproline-rich glycoprotein can be separated from smooth membrane fractions containing endoplasmic reticulum and plasma membrane—based on marker enzyme identifications. Dashek (6) was unable to distinguish between the different types of smooth membranes and vesicles. Roberts and Northcote (19) interpret their results to show that the hydroxyproline-rich protein enters the cell wall by intussusception from smooth membranes other than Golgi vesicles. However, they are not able to differentiate between grains derived from proline or hydroxyproline and have used as a basis for this conclusion a micrograph of a forming cell plate. They also conclude that relatively little hydroxyproline-rich protein is being deposited in walls of dividing cells and newly formed cell wall. The discrepancy between their results and ours may be attributable to the fact that their electron micrographs containing dictyosome vesicles are of newly forming cell wall, in which little hydroxyproline is deposited, and in which one would not expect to find hydroxyproline-rich proteins. Our results further suggest the important role of the Golgi apparatus in the biosynthesis and secretion of extracellular macromolecules, whether they be glycoproteins of animal or plant cells, mucopolysaccharides of animal cells, or hemicellulosic polysaccharides of plant cells.

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