Plant Physiol. (1972) 50, 275–282

Isolation of an Enzyme System Which Will Catalyze the Glycosylation of Extensin[1]

Received for publication September 9, 1971

ARTHUR L. KARR, JR.[2]
Michigan State University-Atomic Energy Commission Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

Enzymes which catalyze the glycosylation of the cell wall protein extensin using uridine diphosphate L-arabinose-^{14}C as a substrate are present in a crude extract prepared from suspension cultured sycamore cells (*Acer pseudoplatanus* L.). This enzyme system sediments when the crude extract is subjected to centrifugation at 37000*g*. A base hydrolysate of the product contains a mixture of hydroxyproline-arabinosides which are electrophoretically and chromatographically identical to those obtained by hydrolysis of extensin isolated from the cell wall. The hydroxyproline-rich protein used as an acceptor in the glycosylation reactions is present in the particulate fraction. In addition, evidence is presented which indicates that hydroxyproline-rich tryptic peptides prepared from the cell wall can also be used as an acceptor by this enzyme system. The presence of Mg^{2+} or Mn^{2+} in the reaction mixture increases the enzyme-catalyzed incorporation of arabinose into extensin by about 1.4 times. About two-thirds of the product mixture is composed of arabinose-containing compounds which have not been identified. Some of these products appear to be hydroxyproline-glycosides which have not been previously reported.

The presence of hydroxyproline-containing proteins in the cell wall fraction of plants has been well established (10, 12, 14, 19). Lamport (13) has shown that at least one of these proteins is covalently bonded to an oligosaccharide of arabinose by a glycosidic bond between arabinose and C-4 of hydroxyproline. He finds that hydroxyproline accounts for as much as 30% of all of the amino acid residues in the cell wall proteins of suspension cultured tomato cells, and that about 70% of these hydroxyproline residues can be isolated covalently bonded to glycosides.

Lamport (12) has suggested that these glycoproteins serve a structural function by acting as crosslinks between cell wall polysaccharides. Since the number of these crosslinks is potentially quite large, they could make a major contribution to the strength of the cell wall. In view of the changes in cell wall strength that take place during cell growth by extension and the role the glycoproteins could play in changes in cell wall strength, these proteins have been named extensin (12).

Little is known about either the synthesis or degradation of extensin. The protein appears to be assembled on the ribosomes (4), but this has not yet been definitely proven. Proline is the precursor for hydroxyproline in the protein (4, 6), and hydroxylation of the peptide-bound proline is catalyzed by cytoplasmic enzymes (4). The protein is then transported to the cell wall by a mechanism involving smooth membranes (5). In addition, Chrispeels (3) has shown that upon centrifugation hydroxyproline-rich proteins will sediment with the particulate fraction from carrots and that this particulate fraction of extensin is rapidly transferred into the cell wall. Enzymes responsible for the glycosylation or the specific degradation of extensin have not been described.

The present paper describes the isolation and partial characterization of an enzyme system from sycamore cells which will catalyze the transfer of arabinose from UDP-arabinose to the hydroxyproline-rich protein in the particulate cell fraction (3) to produce the series of oligosaccharide side chains which are characteristic of extensin (13). The complexity of the enzyme system responsible for the assembly of the oligosaccharide side chain of extensin is dictated by the complexity of the side chain. The tetrasaccharide of arabinose commonly found attached to extensin in sycamore cells is linked together by three different types of bonds (Karr, unpublished results). The first arabinose unit in the side chain is linked to hydroxyproline by a glycosidic bond as reported by Lamport (13). The three remaining arabinose units are attached by a combination of 1,2- and 1,3-glycosidic bonds. Since it is likely that the formation of each of these different bonds will be catalyzed by a different enzyme (22), three enzymes may be necessary for the assembly of the tetrasaccharide side chain. Furthermore, even more enzymes may be required if glycolipids are necessary intermediates for the synthesis of the side chain (8) or if interconversion of ring forms (the arabinose in the side chain of extensin isolated from sycamore cells is present as L-arabinofuranoside; Karr, unpublished results) is a preliminary necessity to synthesis of the side chain.

MATERIALS AND METHODS

Substrate. Uridine diphosphate L-arabinose-^{14}C (UDP-arabinose-^{14}C) with a specific radioactivity of 183 mc/mmole was purchased from New England Nuclear, Waltham, Massachusetts. A 10 μM solution (10,000 cpm/10 μl) of UDP-arabinose-^{14}C in water is used as the substrate solution in the assays for arabinosyl-transferase activities.

Plant Material. Sycamore cells (*Acer pseudoplatanus* L.) are grown in liquid culture in 1-liter flasks containing 500 ml of the medium described by Lamport (11). The cell cultures are

[1] This work was supported by the United States Atomic Energy Commission Contract AT(11-1)-1338.

[2] Present Address: Department of Botany and Microbiology, Montana State University, Bozeman, Mont. 59715.

grown at 27 C and 40% relative humidity and are continuously agitated on a gyrotory shaker at 110 rpm. Cells are routinely harvested for enzyme preparation 7 to 12 days after transfer to fresh medium.

Preparation of Standards. A mixture of hydroxyproline-arabinoside standards is prepared from the cell wall fraction isolated from cultured sycamore cells. Cells (18–20 days after transfer to fresh medium) are collected on a coarse sintered glass funnel and washed with 10 volumes of 50 mM potassium phosphate, pH 7.0. The washed cells are broken by sonication, and the cell wall fraction is prepared as described previously (7). The cell wall material is subjected to hydrolysis for 6 hr at reflux temperature in 0.44 N barium hydroxide, and the hydroxyproline-arabinosides present in the hydrolysate are fractionated by column chromatography on Sephadex G-25 and ion-exchange chromatography with Aminex (Dowex 50 W-X2, 200–325 mesh) as described by Lamport (13). Hydroxyproline isolated from the hydrolysate is found to be covalently attached to a tetrasaccharide of arabinose (hypro-ara_4) or to a trisaccharide of arabinose (hypro-ara_3). The other hydroxyproline-arabinosides, hydroxyproline attached to a disaccharide of arabinose (hypro-ara_2) and to arabinose (hypro-ara), are prepared by subjecting 10-mg lots of the mixture of hypro-ara_4 and hypro-ara_3 to acid hydrolysis in 500 mM trifluoroacetic acid. Hydrolysis is carried out in a sealed tube at 105 C for 20 min. The solvent is removed by passing a stream of air over the hydrolysis mixture. The residue is dissolved in 10 ml of water. This standard mixture contains hypro-ara_4, hypro-ara_3, hypro-ara_2, and hypro-ara. The mixture is stored frozen at −20 C.

Preparation of Enzyme. Sycamore cells are collected on a coarse sintered glass funnel and washed at room temperature with 10 volumes of 50 mM potassium phosphate, pH 7.0. The washed cells are suspended at 4 C in 50 mM tris-HCl, pH 6.9, which contains 400 mM sucrose, 1% (w/v) albumin (bovine albumin, fraction V, Sigma Chemical Company), and 4 mM sodium metabisulfite (1.0 ml of buffer for each g fresh weight of cells). The sodium metabisulfite prevents "tanning" of proteins (20), and when it is included the arabinosyl-transferase activities observed in the final enzyme preparation are increased three to five times. Ice is added to the cell suspension, and the cells are broken by sonication for two 30-sec periods at a thrust of 6.5 g. A Bronwill Bio-Sonic 3 is commonly used for sonication. The resulting sonicate is subjected to centrifugation at 1000*g* for 15 min at 4 C to remove cell wall fragments. The pellet contains no detectable arabinosyl-transferase activities and is discarded. The supernatant liquid is subjected to centrifugation at 37,000*g* for 60 min at 4 C. The supernatant liquid contains no detectable arabinosyl-transferase activities and is discarded. The pellet (particulate fraction) is suspended in a solution consisting of 50 mM sodium acetate, pH 6.5, 400 mM sucrose, and 1% (w/v) albumin with the aid of a glass tissue homogenizer (1 ml of buffer per 20 g fresh weight of cells). This particulate fraction is the only cell fraction of the crude extract of sycamore cells which contains detectable arabinosyl-transferase activities. For the purpose of discussion, the suspended particulate fraction will be termed the particulate system.

Assay for Arabinosyl-Transferase Activities, Method I. The arabinosyl-transferase activities are assayed by mixing 10 μl of solution containing UDP-arabinose-^{14}C (10,000 cpm, 0.1 mmole) with 10 μl of enzyme preparation. When additional components such as magnesium chloride are included in the reaction mixture, these are added in 10 μl of 50 mM sodium acetate, pH 6.5. In these cases, appropriately diluted controls are included. The reaction is carried out at 25 C for the desired length of time and terminated by the addition of 20 μl of a solution of 50 mM sodium tetraborate, pH 9.5. The mixture is spotted on Whatman No. 1 chromatography paper, dried, and subjected to paper electrophoresis (45 v/cm) in 50 mM sodium tetraborate, pH 9.5, as described previously (17). The origin of the electrophoretogram is cut out, placed in 15 ml of counting solution consisting of 0.5% (w/v) PPO and 0.025% (w/v) POPOP in toluene, and the radioactivity is determined at 50% efficiency in a liquid scintillation counter. The enzyme-catalyzed incorporation of radioactivity into borate-immobile products is a measure of the incorporation of arabinose-^{14}C into polymers. It should be noted that the borate-immobile products are made up of a mixture of extensin and other arabinose-containing polymers.

Assay for Arabinosyl-Transferase Activities, Method II. Reaction mixtures are prepared by combining 200 μl of the substrate solution (200,000 cpm, 2 μmoles) and 200 μl of the enzyme solution. When additional components such as magnesium chloride are included in the reaction mixture, these are added in 200 μl of 50 mM sodium acetate, pH 6.5. Reactions are carried out at 25 C for the specified length of time and terminated by freezing at −20 C. The reaction mixtures are thawed and transferred into 13- × 150-mm test tubes containing 0.5 ml of a mixture of hydroxyproline-arabinoside standards, 1.1 ml of water, and barium hydroxide. The final concentration of barium hydroxide in the mixture is 0.44 N. The tube is sealed, and hydrolysis is carried out for 6 hr at 105 C. After hydrolysis, insoluble material is removed by centrifugation in a clinical centrifuge. The pellet is washed two times with 0.5-ml portions of 100 mM acetic acid, pH 2.9. The supernatant liquid and the washes are combined and placed on a Sephadex G-25 column (1.2 × 67 cm). The sample is eluted from the column with 100 mM acetic acid, pH 2.9, and 1.5-ml fractions are collected. The hydroxyproline-containing compounds resulting from the standard mixture are located in the eluent by a continuous flow method (16), utilizing the colorimetric procedure described previously (9). The radioactivity present in the fractions is measured at 50% efficiency on a scintillation counter by placing an aliquot of each fraction in 15 ml of counting solution. The counting solution consists of 0.5% (w/v) PPO, 0.025% (w/v) POPOP, and 14% Beckman Biosolv BBS-3 in toluene. Fractions 30 through 45 from the Sephadex G-25 column (Fig. 6) are pooled and solvent removed by lyophilization. The lyophilized material is dissolved in 1 ml of water and placed on a Chromabeads B (Technicon Corporation) column (0.6 × 65 cm). The Chromabeads B column is pumped at a flow rate of 0.75 ml/min (400 p.s.i.) with a gradient of water and 500 mM trifluoroacetic acid. Two-milliliter fractions are collected. This method of ion-exchange chromatography is used to separate the hydroxyproline-arabinosides that result from the hydrolysis of extensin (16). The presence of both hydroxyproline-containing standards and radioactivity in the fractions is determined as described above. Using method II, the total incorporation of arabinose into polymers can be calculated by adding the radioactivity present in extensin (as hydroxyproline-arabinosides) and in other arabinose-containing polymers (fractions 16 through 29, Fig. 7). The values for total arabinose transfer obtained using method II are the same as those obtained by measuring the production of borate-immobile products (method I).

pH Optimum. The particulate system for this experiment is prepared as described above in "Preparation of Enzyme," except that the 1000*g* supernatant liquid is divided into 10 equal portions. Each portion is subjected to centrifugation at 37,000*g* for 60 min at 4 C. The resulting 10 pellets are suspended in 50 mM tris-HCl, 50 mM sodium acetate, or 50 mM potassium phosphate at the appropriate pH with the aid of a glass tissue homogenizer. The enzyme-catalyzed incorporation

of arabinose-^{14}C from UDP-arabinose-C^{14} into polymers is measured by method I. The pH of a 1:1 (v/v) mixture of the enzyme preparation and water is measured (the small amount of substrate used in the authentic reaction mixture has no effect on pH values). In addition each mixture is subjected to centrifugation at 37,000*g* for 60 min to remove the particulate system. The supernatant liquid is collected, and the pH of the liquid in the absence of the particulate system is measured. Both methods of measurement given identical pH values for the enzyme suspensions.

Detergent Extraction of the Arabinosyl-Transferase Activities Present in the Particulate System. A 3-ml portion of the suspension containing the particulate system is frozen at −20 C. The suspension is thawed and mixed with an equal volume of a solution containing 50 mM sodium acetate, pH 6.5, 400 mM sucrose, 1% (w/v) albumin, and 0.5% Triton X-100 (B grade, Calbiochem) with the aid of a glass tissue homogenizer. The resulting homogenate is subjected to centrifugation at 37,000*g* for 60 min at 4 C. After detergent extraction, arabinosyl-transferase activities are detectable only in the 37,000*g* supernatant liquid.

Preparation of Tryptic Peptides of Extensin. Tryptic peptides of extensin are prepared from cultured tomato cells (*Lycopersicon esculentum* Mill.) (15). The procedure involves preparation of cell walls, removal of the carbohydrate side chains of extensin in the cell walls by acid hydrolysis, and digestion of the "stripped" protein with trypsin. The tryptic peptides are divided into two fractions by elution from a Sephadex G-25 column. There is a peak of high molecular weight peptides not retained by the column. The fractions which contain the high molecular weight peptides are pooled, and the solvent is removed by lyophilization. The residue is dissolved in 50 mM sodium acetate, pH 6.5, at a concentration of 415 μg of peptide-bound hydroxyproline per ml. This solution contains the peptides of extensin used in the study of the arabinosyl-transferase activities of sycamore cells.

Carbohydrate Analysis. Sugars are identified by descending chromatography after subjecting the sample mixtures to hydrolysis in 2 N trifluoroacetic acid in a sealed tube for 1 hr at 120 C (1). After hydrolysis the solvent is removed by passing a stream of air over the solution. The residue is dissolved in water. The hydrolyzed sample and a mixture of standards containing glucose, galactose, mannose, xylose, and arabinose are spotted on Whatman No. 1 chromatography paper. The sugars are fractionated by developing the chromatogram with ethyl acetate-pyridine-water (8:2:1, v/v). Reducing sugars are located used silver nitrate (21). Radioactivity associated with sugars is measured at 50% efficiency on a scintillation counter by cutting the chromatogram into 1-cm strips and placing the strips into 15 ml of the counting solution described above in "Assay Method I."

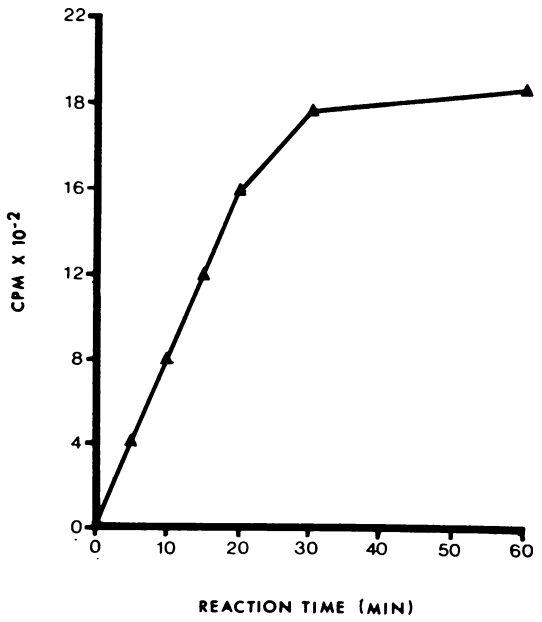

FIG. 1. Enzyme-catalyzed incorporation of arabinose was measured by Assay Method I (see "Materials and Methods"). The reaction mixture contained both 1 mM magnesium chloride and 1 mM manganous chloride.

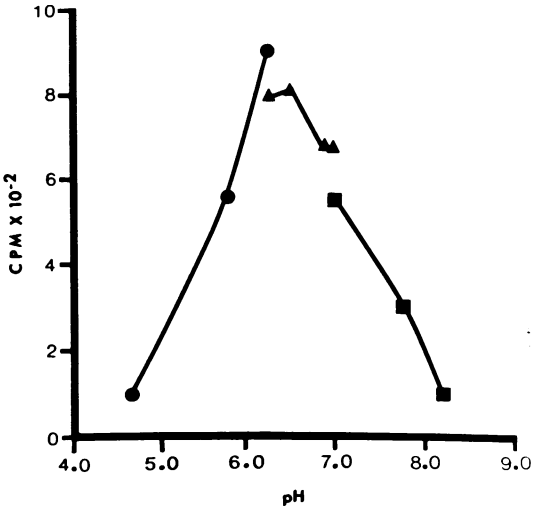

FIG. 2. The pH optimum for the arabinosyl-transferase activities was measured by Assay Method I as described in "Materials and Methods." The particulate system was suspended in 50 mM sodium acetate (●), 50 mM potassium phosphate (▲), or 50 mM tris-HCl at the appropriate pH (■). Each reaction mixture contained 1 mM magnesium chloride.

RESULTS

Enzymes which catalyze the transfer of arabinose-^{14}C from UDP-arabinose-^{14}C into borate-immobile products are present in a crude extract from sycamore cells. These enzymes, the arabinosyl-transferases, sediment during centrifugation at 37,000*g*. The activities of the arabinosyl-transferases (by "Assay Method I or Method II") in the particulate system are lost when the enzyme preparation is heated for 5 min at 100 C. The enzyme-catalyzed incorporation of arabinose-^{14}C into borate-immobile products is linear for 20 min and no further synthesis of product is observed after 60 min (Fig. 1). The particulate system may be frozen and thawed one time without detectable loss in the arabinosyl-transferase activities, whereas repeated sequences of freezing and thawing result in complete loss of enzyme activity. The particulate system can be stored frozen at −20 C for about 1 day without detectable loss of arabinosyl-transferase activities. After 1 day of storage, the enzyme activities decrease rapidly.

The arabinosyl-transferase activities present in the particulate system have a pH optimum at pH 6.5 (Fig. 2). Reactions were carried out in duplicate for 5 min, 10 min, and 20 min at each pH. The data displayed in Figure 2 are from 10-min reactions.

The arabinosyl-transferase activities in the particulate system are affected by the presence of either magnesium chloride or manganous chloride in the reaction mixture. The incorporation of arabinose into borate-immobile products increases 1.4 times

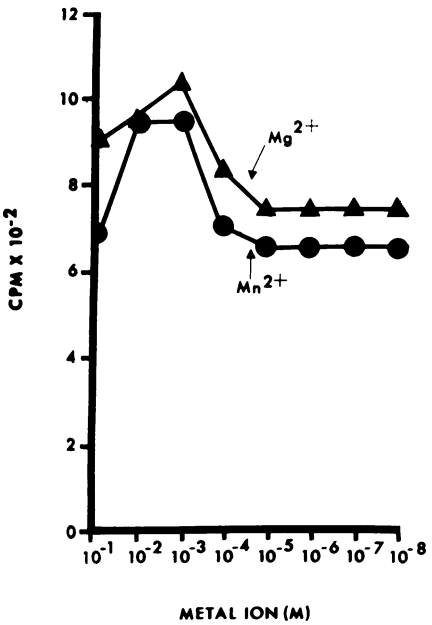

FIG. 3. Effect of metal ions (Mg^{2+} and Mn^{2+}) on the arabinosyltransferase activities was measured by Assay Method I.

Table I. *Relative Amounts of Hydroxyproline and Glycosylated Hydroxyproline Present in Hydrolysates of Cell Wall Extensin and Extensin from the Particulate Cell Fraction*

Hydroxyproline and hydroxyproline-arabinosides were prepared from cell walls and the particulate system by the method of Lamport (13). The relative amounts of each hydroxyproline-containing component was estimated by the method of Lamport and Miller (16) and is listed as percentage of total hydroxyproline present.

Origin of Extensin	Component	%
Particulate fraction	Hypro-ara$_4$	61
	Hypro-ara$_3$	17
	Hypro-ara$_2$	3
	Hypro-ara	3
	Hypro	16
Cell wall	Hypro-ara$_4$	85
	Hypro-ara$_3$	15
	Hypro-ara$_2$	trace
	Hypro-ara	trace
	Hypro	trace

over the control at the optimal concentration of each metal ion (Mg^{2+}, 1 mM; Mn^{2+}, 1 mM–10 mM; Fig. 3). Arabinose incorporation in the absence of added metal ions is in each case the same as that observed when 10 nM metal ion is present in the reaction mixture. The incorporation of arabinose is not further enhanced if both metal ions are included simultaneously at optimal concentrations in the reaction mixture.

The particulate system contains a hydroxyproline-rich protein which appears to be partially glycosylated extensin. This extensin-like glycoprotein contains a lower percentage of glycosylated hydroxyproline and a lower average oligosaccharide side chain length than does extensin isolated from the cell wall fraction of sycamore cells (Table I). The presence of this extensin-like protein in the particulate system was demonstrated by subjecting the enzyme preparation to base-catalyzed hydrolysis and fractionating the products in the hydrolysate by chromatography on a Sephadex G-25 column (13). The profile of hydroxyproline-containing products eluted from the Sephadex G-25 column is shown in Figure 4. Peak III is hydroxyproline present in the hydrolysate and results from hydroxyproline residues which were not glycosylated in the parent protein. The mixture of compounds which make up peak II was fractionated further by ion-exchange chromatography on Chromabeads B and was found to be hypro-ara$_4$, hypro-ara$_3$, hypro-ara$_2$, and hypro-ara. The relative amounts of hydroxyproline and glycosylated hydroxyproline present in the hydrolysate are listed in Table I. The components which make up peak I (Fig. 4) were not retained by the Sephadex G-25 column and represent hydroxyproline-containing compounds which are larger than hypro-ara$_4$. The structures of these compounds are not known. Sugars present in the fractions constituting peak I were identified by subjecting the pooled fractions to acid hydrolysis and fractionating the hydrolysis products by paper chromatography as described in "Materials and Methods". Glucose, galactose, and arabinose were present in the hydrolysate. No mannose or xylose was present.

The enzyme-catalyzed incorporation of arabinose into borate-immobile products is stimulated by tryptic peptides of extensin (Fig. 5). In the presence of these peptides, the period during which arabinose is incorporated is extended from 1 hr to 2 hr. The total incorporation of arabinose is increased more than two times. These tryptic peptides are added to the enzyme preparation by breaking the cells in buffer containing these peptides (see "Materials and Methods"). The incorporation of arabinose is not affected if the tryptic peptides are added directly to the final enzyme preparation (Fig. 5).

Large scale reactions were carried out to obtain quantities of the product sufficient for chromatographic analysis (see

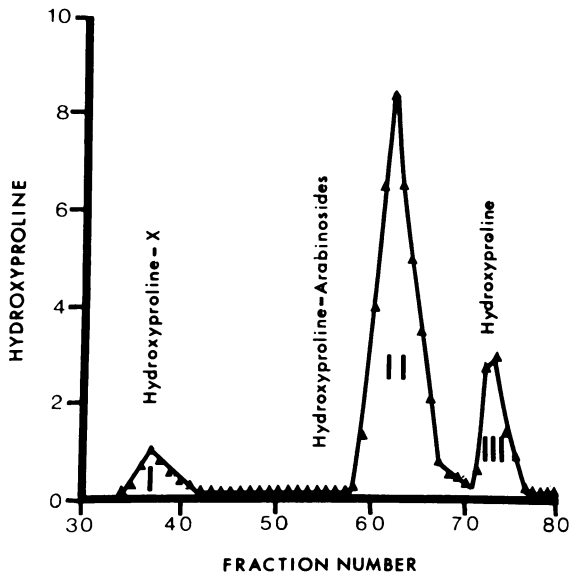

FIG. 4. The particulate system was prepared from 50 g (fresh weight) of sycamore cells. The particulate system was subjected to hydrolysis in barium hydroxide and the compounds present in the hydrolysate fractionated by elution from a Sephadex G-25 column (3.5 × 117 cm). Ten-milliliter fractions were collected, and 100-μl aliquots were removed from each fraction and diluted to 1 ml for the hydroxyproline assay. Hydroxyproline was estimated using an automated analysis system which was adjusted so a full scale pen deflection (10 units of hydroxyproline above) resulted from a 5 μg/ml solution of hydroxyproline. Peak I: hydroxyproline-X; peak II: hypro-ara$_4$, hypro-ara$_3$, hypro-ara$_2$, and hypro-ara; peak III: hydroxyproline.

"Assay Method II" for details). All of the borate-immobile products synthesized in these reactions sedimented when subjected to centrifugation at 37,000*g*. These products could not be extracted from the pellet with water, 50 mM sodium acetate, pH 6.5, 50 mM tris-HCl, pH 7.0, or 100 mM acetic acid, pH 2.9. In addition, extraction with 1% (v/v), 0.5% (v/v), or 0.25% (v/v) Triton X-100 did not release the product into the soluble fraction.

The presence of arabinose-^{14}C extensin in the product mixture was demonstrated by subjecting the mixture to base-catalyzed hydrolysis and fractionating the components in the hydrolysate by a combination of chromatography on Sephadex G-25 (Fig. 6) and ion-exchange chromatography on Chromabeads B (Fig. 7) as described in "Materials and Methods" (Assay Method II). The hydroxyproline-containing compounds assayed by the colorimetric procedure and displayed in Figures 6 and 7 result from the hydroxyproline-arabinosides standards added to the hydrolysis mixture.

The mixture of arabinose-^{14}C labeled hypro-ara$_4$, hypro-ara$_3$, hypro-ara$_2$, and hypro-ara which result from base-catalyzed hydrolysis of extensin glycosylated in the *in vitro* reaction is eluted from the Sephadex G-25 column in the same fractions as the added standards (Fig. 6). The ^{14}C-labeled hydroxyproline-arabinosides appear as a small shoulder on the leading edge of the large peak of unreacted substrate. The ^{14}C-labeled hydroxyproline-arabinosides and the unreacted substrate contained in fractions 30 through 45 (Fig. 6) were combined and fractionated using a Chromabeads B column. The fractionation pattern from the Chromabeads B column (Fig. 7) consists of a large peak of ^{14}C-labeled material which is not retained by the cation-exchange resin. This peak results from unreacted substrate present in the product mixture. It is followed by a series of ^{14}C-labeled compounds giving double peaks. These double peaks have been identified as hypro-ara$_4$, hypro-ara$_3$, hypro-ara$_2$, and hypro-ara, respectively (16). The double peaks of ^{14}C-labeled compounds are eluted from the Chromabeads B column in the same fractions as the added standards. The double peaks result from isomerization of the hydroxyproline during base-catalyzed hydrolysis to give the *cis* and *trans* forms of 4-hydroxyproline (13).

The compounds present in the hydrolysate of the *in vitro* product can also be fractionated by paper electrophoresis in acetic acid-formic acid buffer, pH 1.9, or by ion-exchange chromatography on "Aminex" using procedures described by Lamport (13). Fractionation of the products by these methods gives results identical to those obtained using chromatography on the Chromabeads B column (Fig. 7).

The radioactivity in the hydroxyproline-arabinosides from the product mixture is present as arabinose-^{14}C. This was demonstrated by combining the fractions containing the compounds which comprise each set of double peaks shown in Figure 7 to give four samples. The samples were subjected to acid hydrolysis in 2 N trifluoroacetic acid and the products in the hydrolysate were fractionated by paper chromatography as described in "Materials and Methods". All of the radioactivity in each sample moved as a single component which had an $R_{glucose}$ identical with authentic arabinose.

It appears from the data shown in Figure 7 that the enzymes present in the particulate system catalyze the transfer of no more than four monosaccharide units to the side chain of extensin. This is not the case. It must be remembered that hypro-ara$_4$ was the largest standard added to the hydrolysis mixture. When fractions from the Sephadex G-25 column (Fig. 6) were combined, the criterion for choosing the fractions was that they should contain material which had a molecular weight equal to or less than hypro-ara$_4$. This process would exclude products resulting from the transfer of more than four monosaccharides from the mixture placed on the Chromabeads B column. It was found that if the fractions preceding fraction 30 (Fig. 6) were included in the mixture fractionated by chromatography on Chromabeads B that two new sets of double peaks appeared in the fractionation pattern from the

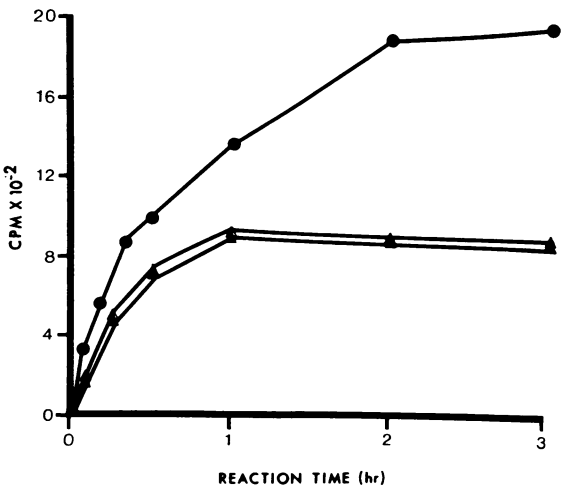

FIG. 5. Effect of tryptic peptides of extensin on the arabinosyltransferase activities as measured by Assay Method I. All reaction mixtures contained 1 mM magnesium chloride. Reaction mixtures were prepared as follows: ●: The particulate system was prepared from cells which had been ruptured in buffer containing tryptic peptides of extensin (46 μg of peptide-bound hydroxyproline/ml of buffer). ■: The particulate system was prepared as described in "Materials and Methods." ▲: The particulate system was prepared as described in "Materials and Methods," but each reaction mixture contained 10 μl of a solution of tryptic peptides of extensin (105 μg of peptide-bound hydroxyproline/ml of solution).

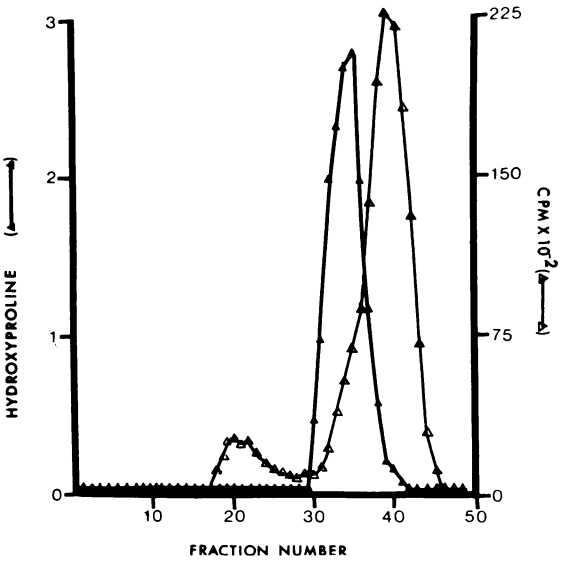

FIG. 6. Fractionation of a base hydrolysate of the *in vitro* product by elution from a Sephadex G-25 column as described in "Materials and Methods" (Assay Method II). Each reaction mixture contained 1 mM magnesium chloride. Radioactivity was measured in a 200 μl aliquot of each fraction and hydroxyproline was measured in a 100 μl aliquot of each fraction. The sensitivity of the hydroxyproline analyzer was adjusted so a full scale pen deflection on the recorder (3 units of hydroxyproline above) resulted from a solution containing 2 μg/ml of hydroxyproline. △: radioactivity; ▲: hydroxyproline.

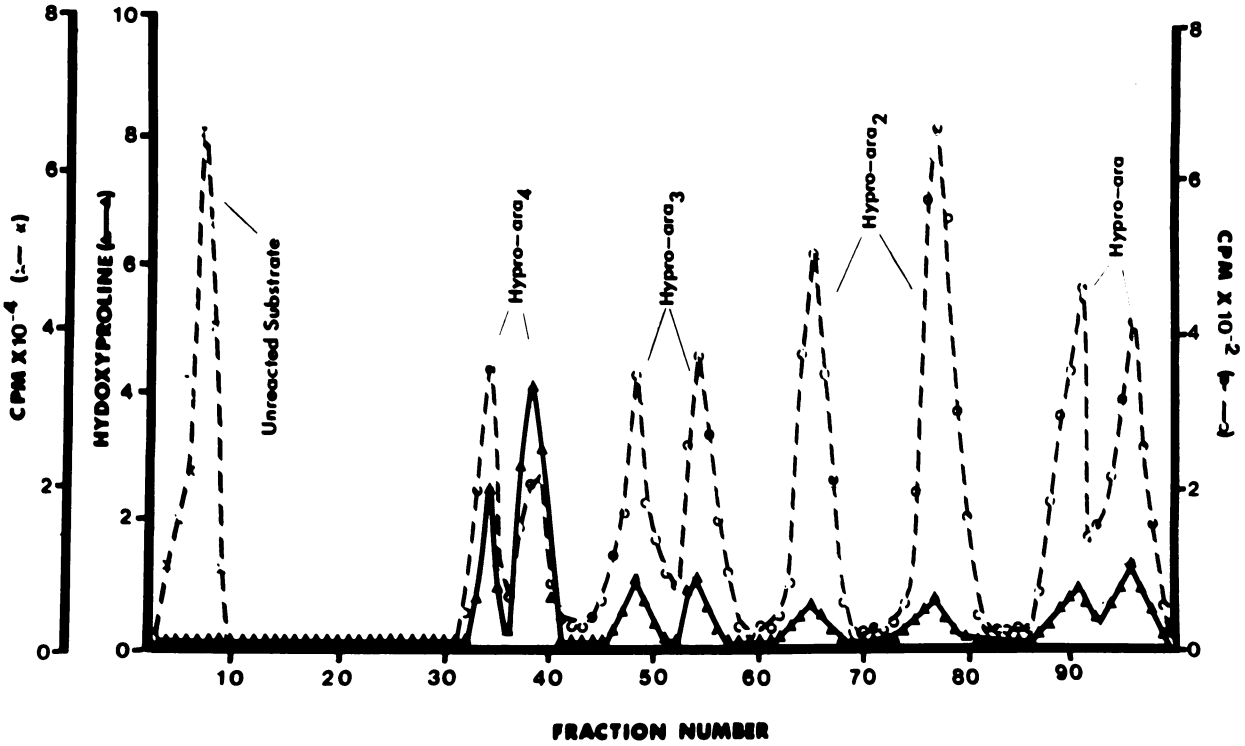

FIG. 7. Fractionation of the compounds present in fractions 30 through 45 of Figure 6 by ion-exchange chromatography on Chromabeads B column as described under Assay Method II. Radioactivity was measured in a 500-μl aliquot of each fraction, and hydroxyproline was measured in a 100-μl aliquot of each fraction. The sensitivity of the hydroxyproline analyzer was adjusted so a full scale pen deflection on the recorder (10 units of hydroxyproline above) resulted from a solution containing 5 μg/ml of hydroxyproline. ○: Radioactivity in hydroxyproline-arabinosides; ×: radioactivity in unreacted substrate; ▲: hydroxyproline from the added standards.

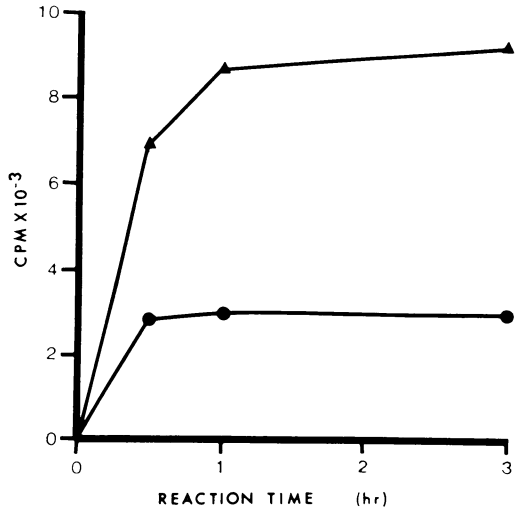

FIG. 8. The enzyme-catalyzed incorporation of arabinose as a function of reaction time was measured by Assay Method II. ▲: Total radioactivity present in fractions 16 through 29 from the Sephadex G-25 column; ●: total radioactivity present in hydroxyproline-arabinosides.

ion-exchange column. These two new components were eluted from the column in fractions preceding hypro-ara$_4$. These products were present at very low levels and the radioactivity in these products represented less than 1% of the radioactivity present in the total of the other hydroxyproline-arabinosides. It was also possible to detect these two new components in a hydrolysate of the cell wall fraction. They represent less than 0.5% of the hydroxyproline released from the cell wall fraction by base-catalyzed hydrolysis. The structure of these components is not known.

The arabinosyl-transferase activities present in the particulate system also catalyze the incorporation of arabinose from UDP-arabinose into a second kind of product. This product is present in fractions 16 through 29 (Fig. 6) and represents about two-thirds of the arabinose transferred into products (Table II). The chemical structure of this product has not been determined. The product, after removal from the particulate system by base-catalyzed hydrolysis, is water soluble. It is not degraded further by repeated base-catalyzed hydrolysis in 0.44 N barium hydroxide. Acid-catalyzed hydrolysis of this product in 2 N trifluoroacetic acid results in the release of arabinose as the only ^{14}C-labeled compound.

Using Assay Method II, it is possible to measure separately the enzyme-catalyzed incorporation of arabinose into extensin and other polymers as a function of reaction time. The results of such an experiment are displayed in Figure 8. The incorporation of arabinose into the higher molecular weight products of the hydrolysate (fractions 16 through 29; Fig. 6) is detectable for 60 min and then ceases. Incorporation into extensin has virtually ceased by 30 min. Even though total incorporation of arabinose into extensin has stopped within the first 30 min, the amount of ^{14}C-label present in the individual hydroxyproline-arabinosides is changing (Fig. 9). After 30 min the amount of hypro-ara$_3$ is decreasing while the amount of hypro-ara is still increasing. The amount of radioactivity present in hypro-ara$_2$ and hypro-ara$_4$ is constant after 30 min.

Assay Method II has also been used to study the effect of magnesium chloride on the enzyme-catalyzed incorporation of arabinose. From the results of this experiment (Table II), it can be seen that the presence of 1 mM magnesium chloride in the reaction mixture causes an approximate increase of 1.4

times in the total incorporation of arabinose. These results are identical to those obtained using Assay Method I (Fig. 3). In addition, using Assay Method II, it is possible to show that the distribution of arabinose-^{14}C between extensin and the other polymers is not affected by the presence of magnesium chloride (Table II).

The particulate system can be disrupted by extraction with detergent (see "Materials and Methods"). The total incorporation of arabinose into borate-immobile material (Assay Method I) catalyzed by the detergent-extracted enzymes never exceeds 10 to 20% of the incorporation catalyzed by the enzymes in the particulate system (Fig. 10).

DISCUSSION

Glycosylation of extensin is catalyzed by enzymes present in the particulate fraction of an extract of sycamore cells. These enzymes appear to be membrane-bound but the cytological identity of the membrane is unknown. It is conceivable that the enzymes are associated with membranes coming from either the Golgi complex or the plasmalemma, both of which have been suggested as sites of polysaccharide synthesis (18, 23). Yet, it seems unlikely that the enzymes are present in the Golgi complex, since it has been reported that the Golgi do not participate in the transport of extensin into the cell wall (5).

The extensin used as substrate in these glycosylation reactions is also present in the particulate system. This particulate fraction of extensin has been shown to turn over rapidly into the cell walls of carrots and therefore is a likely precursor of cell wall extensin (3). The extensin present in the membrane fraction is only partially glycosylated when compared to cell wall extensin, suggesting that this membrane fraction is the *in vivo* site for glycosylation with these molecules resulting from a glycosylation process which was artificially terminated upon cell breakage.

The addition of tryptic peptides of extensin stimulates the enzyme-catalyzed incorporation of arabinose into polymers. This stimulation does not result from a general protein effect since the peptides cannot be replaced by albumin and, indeed, the effect is observed in the presence of 1% (w/v) albumin.

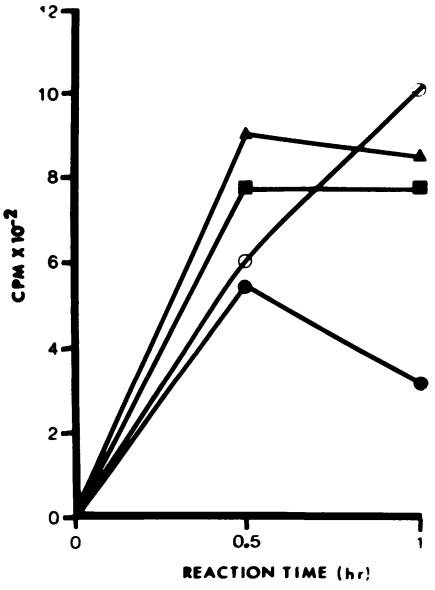

FIG. 9. The enzyme-catalyzed incorporation of arabinose into hydroxyproline-arabinosides was measured by Assay Method II. ▲: Hypro-ara$_4$; ●: hypro-ara$_3$; ■: hypro-ara$_2$; ○: hypro-ara.

Table II. *Effect of Magnesium Chloride on the Enzyme-catalyzed Incorporation of Arabinose into Products as Measured by Assay Method II*

Those reactions designated $+Mg^{2+}$ contained 1 mM $MgCl_2$.

Compound	Radioactivity		Stimulation by Mg^{2+}
	$+Mg^{2+}$	$-Mg^{2+}$	
	cpm		
Unknown product (fractions 16–29)	27,045	20,160	1.3
Hydroxyproline-arabinoside total	9,255	6,500	1.4
Hypro-ara$_4$	1,315	765	1.7
Hypro-ara$_3$	1,830	1,270	1.4
Hypro-ara$_2$	3,605	2,550	1.4
Hypro-ara	2,505	1,915	1.3

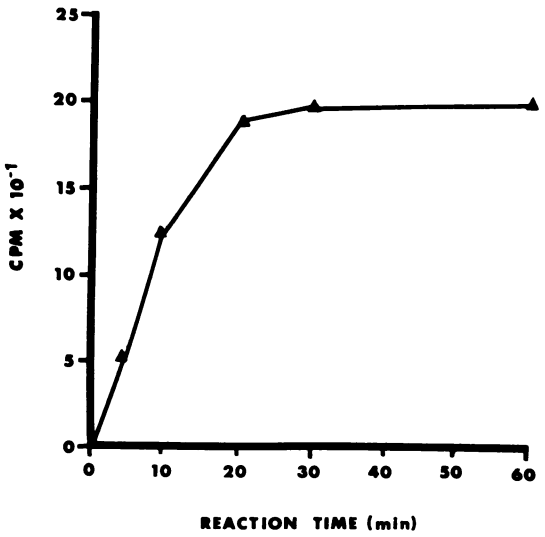

FIG. 10. Incorporation of arabinose into borate-immobile products (Assay Method I) catalyzed by the detergent-extracted enzymes.

It is curious that the stimulation is observed only when cells are broken in the presence of the extensin peptides. This could indicate that the enzymes are enclosed in membranous packets and do not have access to the extensin peptides when the peptides are added directly to the reaction mixture.

The oligosaccharide side chains produced during the *in vitro* glycosylation reaction appear to be identical to the side chains of extensin isolated from the cell wall fraction of sycamore cells. This is indicated by the fact that the hydroxyproline-arabinosides (hypro-ara$_4$, hypro-ara$_3$, hypro-ara$_2$, and hypro-ara), released by hydrolysis from extensin glycosylated *in vitro*, fractionate identically with standards prepared from cell wall extensin when both are subjected to paper electrophoresis or either of two methods of ion-exchange chromatography. In addition, the product mixture contained small amounts of two other components with the properties of hydroxyproline-glycosides. The presence of these components in the product mixture led to their discovery in a hydrolysate of the cell wall fraction. The structure of these components is not known. They may represent hypro-ara$_5$ and hypro-ara$_6$ or result from the addition of compounds other than arabinose to the tetrasaccharide side chain of extensin.

If the four hydroxyproline-arabinosides represent a related series of saccharides attached to hydroxyproline (hypro-ara +

ara gives hypro-ara$_2$; hypro-ara$_2$ + ara gives hypro-ara$_3$; *etc.*), then the assembly of the side chain must proceed by the sequential transfer of monosaccharides of arabinose. The product mixture would not be expected to contain all of the four hydroxyproline-arabinosides if any portion of the side chain were assembled before transfer to extensin. Of course, some of the hydroxyproline-arabinosides could result from the degradation which might accompany the hydrolysis and isolation of the product, but this seems unlikely since standards added to the product mixture are not degraded.

The incorporation of arabinose into extensin is increased if either magnesium chloride or manganous chloride is present in the reaction mixture. The mechanism of this metal ion effect is not clear. If the metal ion acts only as a cofactor for enzymes participating in the transfer of arabinose, it is difficult to explain the high level of synthesis in the absence of added metal ions. It can be argued that incorporation in the absence of added metal ions results from metal ions present in the cell extract. This does not seem to be the case since breaking cells in the presence of 1 M magnesium chloride, a process which should increase the level of Mg^{2+} in the cell extract, does not result in a similar 1.4 times increase in enzyme-catalyzed arabinose incorporation. If these enzymes are enclosed in membranous packets, it is possible that the added metal ion increases transport of UDP-arabinose into the packet above some minimal rate. It is also possible that a number of enzymes take part in the glycosylation of extensin and that the activities of these enzymes are affected differentially by the presence of divalent cations.

The hydrolysate of the extensin in the particulate fraction contains hydroxyproline which is not retained by a Sephadex G-25 column. The high molecular weight properties of these hydroxyproline compounds must result from substitution at the 4-hydroxyl group since the colorimetric procedure eliminates the substitution of either the carboxyl or secondary amine functional groups (2, 9). This higher molecular weight hydroxyproline may be present in the same type of molecules as the arabinose compounds in fractions 16 through 29, Figure 6. The structure of these compounds is not known, but the mixture may include hydroxyproline-arabinosides which have not been previously reported.

The sedimentation properties of the arabinosyl-transferases present in the particulate system can be altered by detergent extraction. These detergent-extracted enzymes catalyze the incorporation of arabinose from UDP-arabinose into borate-immobile products. It is not presently possible to obtain enough of the compounds synthesized by these enzymes to permit structural analysis of the product.

A number of questions remain unanswered. How many enzymes are required for the synthesis of the side chain of extensin? What role do the divalent cations play in the glycosylation reaction? Exactly how do the tryptic peptides affect the glycosylation reaction? It will be possible to approach some of these questions experimentally by using the detergent-extracted enzymes.

LITERATURE CITED

1. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340–345.
2. Bragg, P. D. and L. Hough. 1958. The oxidation of proline, hydroxyproline, and N-methylglycine with periodate. J. Chem. Soc. 4: 4050–4053.
3. Chrispeels, M. J. 1969. Synthesis and secretion of hydroxyproline-containing macromolecules in carrots. I. Kinetic analysis. Plant Physiol. 44: 1187–1193.
4. Chrispeels, M. J. 1970. Synthesis and secretion of hydroxyproline-containing macromolecules in carrots. II. *In vivo* conversion of peptidyl proline to peptidyl hydroxyproline. Plant Physiol. 45: 223–227.
5. Dashek, W. V. 1970. Synthesis and transport of hydroxyproline rich components in suspension cultures of sycamore-maple cells. Plant Physiol. 46: 831–838.
6. Holleman, J. 1967. Direct incorporation of hydroxyproline into protein of sycamore cells incubated at growth-inhibitory levels of hydroxyproline. Proc. Nat. Acad. Sci. U.S.A. 57: 50–54.
7. Karr, A. L. and P. Albersheim. 1970. Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by a "wall-modifying enzyme." Plant Physiol. 46: 69–80.
8. Kauss, H. 1969. A plant mannosyl-lipid acting in reversible transfer of mannose. FEBS Lett. 5: 81–84.
9. Kiuirikko, K. I. and M. Liesmaa. 1959. A colorimetric method for determination of hydroxyproline in tissue hydrolysates. Scand. J. Clin. Lab. Invest. 11: 128–133.
10. Lamport, D. T. A. and D. H. Northcote. 1960. Hydroxyproline in primary cell walls of higher plants. Nature 188: 665–666.
11. Lamport, D. T. A. 1964. Cell suspension cultures of higher plants: isolation and growth energetics. Exp. Cell Res. 33: 195–206.
12. Lamport, D. T. A. 1965. The protein component of primary cell walls. Adv. Bot. Res. 2: 151–218.
13. Lamport, D. T. A. 1967. Hydroxyproline-O-glycosidic linkage of the plant cell wall glycoprotein extensin. Nature 216: 1322–1324.
14. Lamport, D. T. A. 1970. Cell wall metabolism. Annu. Rev. Plant Physiol. 21: 235–270.
15. Lamport, D. T. A. 1971. Macromolecules regulating growth and development. 30th Symposium of the Society for Developmental Biology. In press.
16. Lamport, D. T. A. and D. Miller. 1971. Hydroxyproline arabinosides in the plant kingdom. Plant Physiol. 48: 454–456.
17. McNab, J. M., C. L. Villemez, and P. Albersheim. 1968. Biosynthesis of galactan by a particulate enzyme preparation from *Phaseolus aureus* seedlings. Biochem. J. 106: 355–360.
18. Ray, P. M., T. L. Shininger, and M. M. Ray. 1969. Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes. Proc. Nat. Acad. Sci. U.S.A. 64: 605–612.
19. Sadava, D. and M. J. Chrispeels. 1969. Cell wall protein in plants: autoradiographic evidence. Science 165: 299–300.
20. Stokes, D. M., J. W. Anderson, and K. S. Rowan. 1968. The isolation of mitochondria from potato-tuber tissue using sodium metabisulfite for preventing damage by phenolic compounds during extraction. Phytochemistry 7: 1509–1512.
21. Tsai, C. M. and W. Z. Hassid. 1971. Solubilization and separation of uridine diphospho-D-glucose: β-(1-4) glucan and uridine diphospho-D-glucose: β-(1-3) glucan glucosyltransferases from coleoptiles. Plant Physiol. 47: 740–744.
22. Trevelyan, W. E., D. P. Proctor, and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature 166: 444–445.
23. Villemez, C., J. M. McNab, and P. Albersheim. 1968. Formation of plant cell wall polysaccharides. Nature 218: 878–880.