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An Examination of Centrifugation as a Method of Extracting an Extracellular Solution from Peas, and Its Use for the Study of Indoleacetic Acid-induced Growth

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ABSTRACT

A technique of centrifuging pea epicotyl sections which extracts water-soluble cell wall polysaccharides with less than 1.5% cytoplasmic contamination as revealed by malate dehydrogenase activity determinations was developed. Tests for protein, hexose, pentose, and malate dehydrogenase indicate that significant damage to the cells occurs above 3,000*g*. Below this force, there is little damage, as evidenced by the similar growth rates of centrifuged and noncentrifuged sections. Centrifugation at 1,000*g* extracts polysaccharides containing rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose. An increase in xylose and glucose, presumably xyloglucan, is induced by treating sections with indoleacetic acid. Much of the alcohol-insoluble, water-soluble polysaccharide within the wall is extractable by centrifugation, since nearly as much arabinose and xylose are extractable by centrifugation as by homogenization. The utility of this method for the study of cell wall metabolism is discussed.

Reactions occurring in the cell wall are often difficult to study because of the problems in separating the solution that bathes the cell wall from the complex solution within the cell. Any process which ruptures the plasmalemma leads to a mixing of intracellular and extracellular components, making it difficult to determine whether compounds such as enzymes and water-soluble polysaccharides were localized in the cell wall or the cytoplasm.

Abeles *et al.* (1) devised a method of centrifuging pea stem sections to remove the extracellular solution. They found that pea stem sections could be centrifuged at 3,000*g*, and a solution was removed which contained cellulase, an enzyme believed to be present in the cell wall. Using a similar technique, Stafford and Bravinder-Bree (13) localized a peroxidase isozyme within the cell wall of sorghum, but some cytoplasmic isozymes were also detected. Ferrari and Arnison (6) refined this technique further and found that centrifuging pea stem sections at 500*g* released cellulase. However, some of the cells must have been broken because a cytoplasmic enzyme, malate dehydrogenase, was also found in the solution spun from the sections.

The present study describes a method of centrifuging soluble substances from the cell walls of plant stem sections and evaluates the damage to the cells and contamination of the cell wall solution by the cytosol.

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MATERIALS AND METHODS

General Procedure. Seeds of *Pisum sativum* var. Alaska were grown in Vermiculite for 7 days in darkness. Under fluorescent room lights and using a specially designed harvesting rack, 1.2-cm sections were excised approximately 1 cm below the apical hook. All subsequent operations were performed in the light. These sections were packed vertically in the barrel of a 20-ml plastic syringe (20 mm i.d.) cut off at the 6-ml mark to form a small tube (Fig. 1). The apical end of the sections rested against a 0.32-cm porous polyethylene disk in the bottom of the tube (Bel-Art Products, Pequanock, N.J.). Once packed, the sections were maintained in a vertical position, retaining the original apical end above the basal end, except during centrifugation. Each tube contained 95 to 105 sections weighing approximately 3 g. Among tubes packed for each experiment, the number of sections varied by less than four per tube and the weight varied by less than 0.1 g/tube. The packed tubes were placed in distilled H_2O covering the bottom 1 to 3 mm of tissue. One h after excising the sections, the tubes were connected to a circulating pump and 20 mM K-phosphate adjusted to pH 6.0 with HCl was passed through the tubes at 200 ml/min·tube for 30 min at 26 C to remove cytoplasmic contamination from cut surfaces. The total volume of buffer in the system was 500 ml. The sections then were washed thoroughly with ice water to remove the buffer and infiltrated for 6 min with water, 3 min with vacuum, and 3 min without vacuum. Most of the surface water was drawn off by momentary suction applied to the tip of each tube. The tubes then were placed on top of 12-ml conical centrifuge tubes cut off at the 6.8-ml mark. The conical tubes with tubes of sections on top were placed in 50-ml plastic centrifuge tubes and centrifuged for 15 min in a Sorvall HB-4 swinging bucket rotor. Unless otherwise specified, the gravitational force applied to the sections was 1,000*g*.

Light Microscopy. Sections were removed from the circulating pump system, vacuum-infiltrated, centrifuged, and removed from the syringe tubes. Sections 1 to 2 mm long were cut from the apical end of the original sections. The tips of these small pieces were tapered for identification and fixed overnight in 5% glutaraldehyde at 23 C and then rinsed several times with 0.1 M K-phosphate (pH 6.0). The sections were dehydrated in alcohol and embedded in Spurr's (4) resin. Sections (2–4 μm) were cut parallel to the long axis with an ultramicrotome and glass knives. The sections were stained with toluidine blue.

Hexose, Pentose, Protein and Malate Dehydrogenase Determinations. The sections were vacuum-infiltrated with 20 mM K-phosphate (pH 7.5), instead of water, and 1 ml of extraction buffer (20 mM K-phosphate, 0.5 μl mercaptoethanol/100 ml, and 0.2 mM EDTA) at the same pH was placed in the conical centrifuge tube before centrifuging. The solution extracted from sections centrifuged at increasing gravitational forces was assayed for hexose

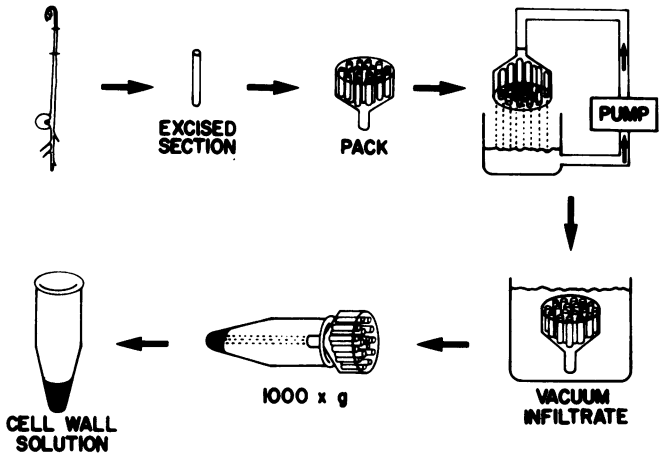

FIG. 1. Diagram of the procedure used for the isolation of cell wall or extracellular solution. Sections (1.2 cm) were cut 1 cm below the hook, packed into the barrel of a syringe, washed and treated in the pump system, vacuum-infiltrated, and centrifuged.

and anthrone (5), pentose and orcinol (5), protein (10), and malate dehydrogenase activity (14).

Gas Chromatography of Carbohydrates. The solution from two tubes of sections centrifuged at 1,000*g* was pooled and passed through Whatman GF/A glass fiber paper, boiled for 5 min, dried in a vacuum oven overnight at 47 C, and precipitated overnight with 80% ethanol, and the sugar content was determined according to Albersheim *et al.* (2), using the same gas flow rates and column packing. A Varian model 3700 gas chromatograph equipped with dual flame ionization detectors was used. Seven sugars known to be present in the primary cell walls of peas (8), L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose, were used as standards. Myoinositol was added to samples as an internal standard in order to correct for losses occurring during processing. The effects of IAA on the concentration of ethanol-insoluble polysaccharides present in the cell wall solution were measured using a treatment sequence paralleling that of Labavitch and Ray (9). Four tubes or approximately 400 sections were incubated in the circulating pump system with 170 ml of distilled H_2O for 1 h at 26 C and then for 4 h in 50 mM glucose adjusted to pH 6.0 with NaOH. The solution was replaced with fresh glucose either with or without 17 μM IAA for the final 3 h. The tubes then were vacuum-infiltrated in distilled H_2O at 0 C and centrifuged. The vacuum infiltration and centrifugation were repeated twice. The solutions extracted by centrifugation were combined, filtered, dried, redissolved in 1 ml of water, and precipitated in 80% ethanol. The precipitate was analyzed for sugar content as described.

Growth of Sections. After the original excision and vacuum infiltration with or without centrifugation, the sections were recut to exactly 1 cm before recording growth. This process required 3 h. Six sections were placed in a continuous growth-recording device similar to the one used by Reed (11) which allowed simultaneous recording of growth on four sets of sections. The sections were allowed to grow for 1 h in 20 mM K-phosphate containing 1% sucrose. The buffer then was replaced with fresh buffer plus or minus 17 μM IAA. Some centrifuged sections were also treated with 50 μg/ml cycloheximide. Growth was recorded for a total of 4 h.

RESULTS

Light Microscopy. Sections of pea epicotyl tissue centrifuged at 1,000*g* for 15 min show very little evidence of damage to the cells (Fig. 2). The mature parenchyma cells have large vacuoles which may obscure damage. The nuclei are sometimes seen at the centrifugal end of these large cells. In young developing cells, which contain more cytoplasm, the nucleus is randomly located in evenly dispersed cytoplasm. This suggests that the contents of these cells have probably been redistributed by cytoplasmic streaming after centrifugation.

In addition to the relocation of nuclei to the centrifugal end of cells, there is a folding or bending of the cell walls of the first and second tiers of cells. Among the sections examined, only a few broken cell walls were observed. The broken walls along the sides and near the tip were presumably caused when the tip was tapered for identification.

Hexose, Pentose, Protein and Malate Dehydrogenase Determinations. Figures 3 and 4 show the amounts of hexose, pentose, protein, and malate dehydrogenase released during the centrifugation of 100 segments at increasing centrifugal forces. The rates of increase are low and remain constant from 1,000 to 3,000*g*. As the force increases above 3,000*g*, the amounts of material released increase dramatically. A comparison of the levels of hexose, pentose, protein, and malate dehydrogenase released at 500 and 6,400*g* shows increases of 36-, 13-, 27-, and 65-fold, respectively. The amount of malate dehydrogenase released at 500*g* is 1.5% of that released at 6,400*g*.

The volume of solution released at 1,000*g* was carefully measured. In three experiments (24 tubes of sections), the volume of solution centrifuged from 100 vacuum-infiltrated sections was 0.4 to 0.5 ml. The amounts released without infiltration varied from 0.1 to 0.3 ml. If we estimate that this tissue has a free space of 15% and that 5% is filled with water, this would indicate that 3 g of tissue could yield 0.15 ml of extracellular solution without vacuum infiltration and 0.45 ml after vacuum infiltration. Therefore, the experimentally determined values are similar to the theoretical values.

Gas Chromatography of Carbohydrates. A chromatogram of the neutral, ethanol-insoluble sugars found in the extracellular solution collected from sections after centrifugation at 1,000*g* is presented in Figure 5. The same sugars are present in the ethanol-soluble fraction of the extracellular solution. The sugars found in the extracellular solution include rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, and an unidentified compound which partitions just after fucose. Table I shows the total ethanol-precipitable arabinose, xylose, galactose, and glucose which can be extracted by three repeated vacuum infiltrations and centrifugations after a 3-h treatment period in the presence or absence of IAA. IAA causes a 43% increase in xylose and a 33% increase in glucose. A fraction of the xylose and glucose in the nonhydrolyzed extracellular solution will bind to cellulose (unpublished data), which is a characteristic of xyloglucan (3).

For comparison with the data presented in Table I, Table II gives the amounts of four soluble, ethanol-precipitable sugars obtained by grinding 100 pea epicotyl sections in a manner similar to that used by Labavitch and Ray (8). Again, there is an increase in xylose and glucose in response to IAA. More galactose and glucose are released by grinding than by centrifugation.

Growth of Sections. Centrifuged sections (Fig. 6) grow at a rate similar to that of noncentrifuged sections (Fig. 7) and have similar biphasic growth responses to IAA. Cycloheximide inhibits the elongation of centrifuged sections.

DISCUSSION

The evidence presented here clearly shows that pea stem sections can be centrifuged at a force which releases considerable amounts of the soluble components found in the cell wall with little cytoplasmic contamination. Photomicrographs of centrifuged sections show that there is little damage to the cells at the light microscope level and that damage is limited to the first or second tier of cells whose walls sometimes fold. Although the walls are

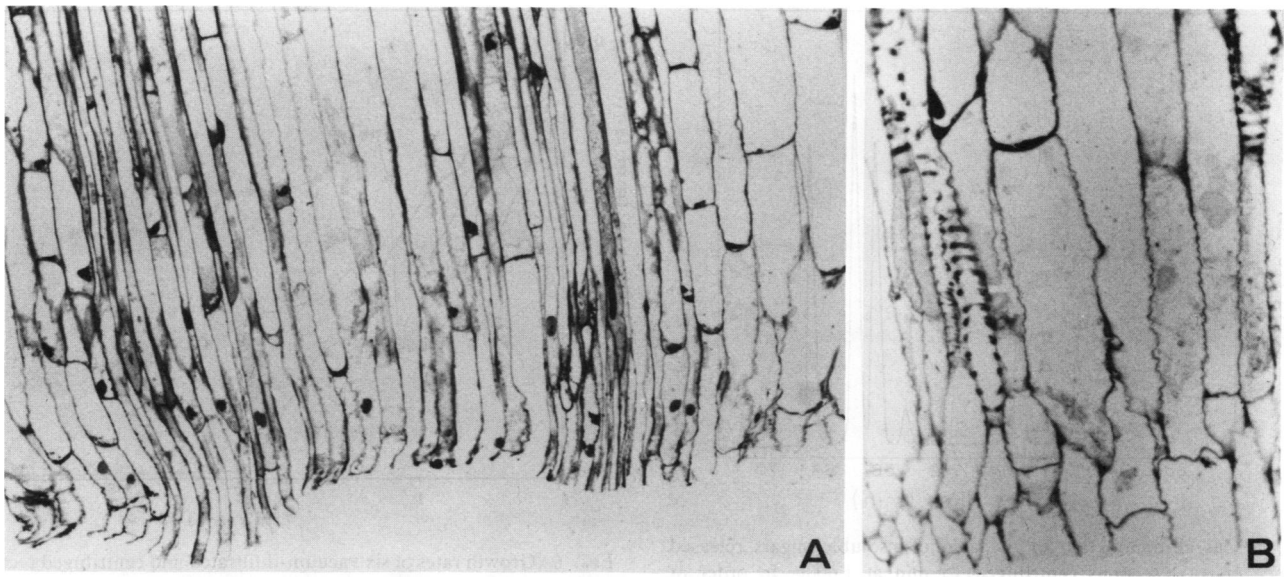

FIG. 2. Photomicrographs A (× 260) and B (× 920) of pea epicotyl tissue centrifuged 15 min at 1000*g*.

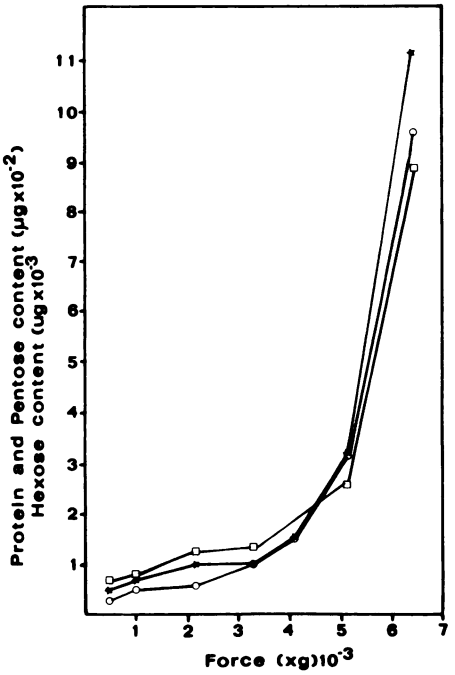

FIG. 3. Effect of centrifugation at increasing forces on the amounts of protein (★), pentose (□), and hexose (○) released from 100 pea epicotyl sections.

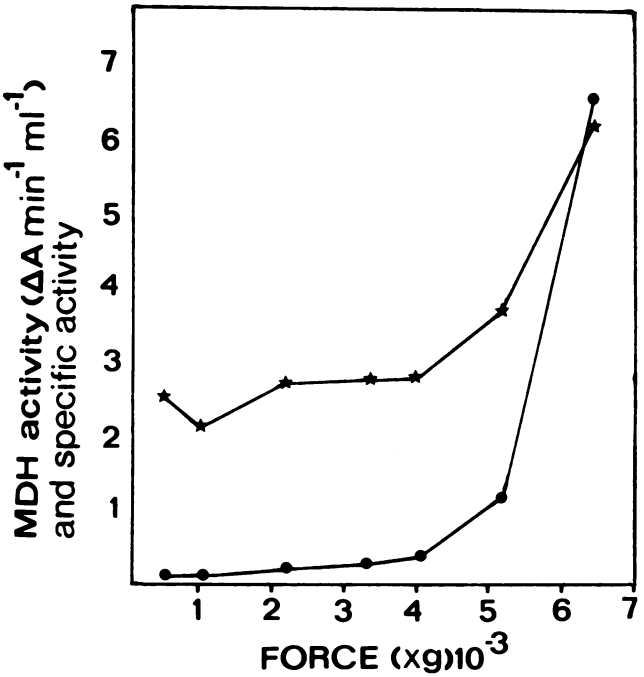

FIG. 4. Malate dehydrogenase (MDH) activity (●) and specific activity (★) detected in the solution released from 100 pea epicotyl sections centrifuged at increasing centrifugal forces.

not ruptured, there could be damage to the plasmalemma which is not evident. Goldsmith and Ray (7) have shown that the membranes of corn coleoptiles are not disrupted by centrifugation at 1,750*g* for 10 min.

When sections are centrifuged at increasing centrifugal forces up to 3,000*g* and the amounts of hexose, pentose, protein, and malate dehydrogenase released are monitored, it is clear that substances are released from the cell wall free space with only a small amount of cytoplasmic contamination. The increases in hexose, pentose, protein, and malate dehydrogenase are steady from 500 to 3,000*g* and then sharply increase with increasing force, indicating that substances are coming mostly from the cell wall at low centrifugal forces and that, above 3,000*g*, the plasmalemma no longer serves as a barrier to the contents of the cell. The presence of malate dehydrogenase reveals that there is a small amount of damage to the cells below 3,000*g*. The specific activity of malate dehydrogenase remains fairly constant from 500 to 3,000*g* and then it increases, indicating that, below 3,000*g*, much of the total protein released comes from the cell wall space. Above 3,000*g*, more protein, a greater portion of which is malate dehydrogenase, is released due to increased cell permeability and to the breakage of cells causing the specific activity to increase. A comparison of the amounts of hexose, pentose, protein, and malate dehydrogenase released at 500 and 6,400*g* shows again that most of the substances released at 500*g* come from the cell wall space. Malate dehydrogenase exhibits the largest increase in the solution centrifuged from tissue at 6,400*g*, which is expected since it is cytoplasmic in origin (6, 15). Sixty-five times as much malate

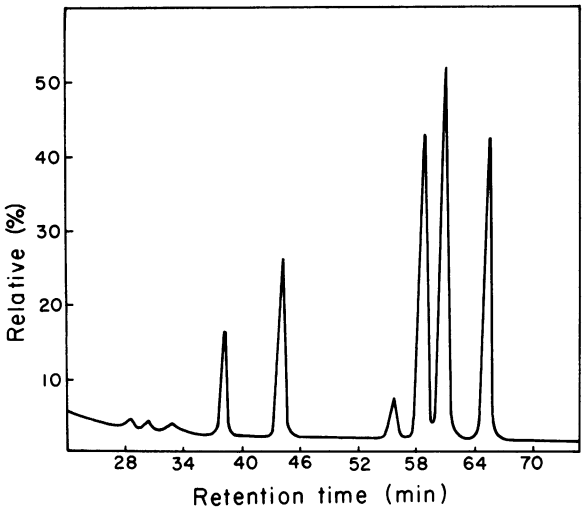

FIG. 5. Gas chromatogram of the alcohol-insoluble sugars released from pea epicotyl sections centrifuged 15 min at 1,000*g*. In order of detection, the peaks shown represent rhamnose, fucose, unknown compound, arabinose, xylose, mannose, galactose, glucose, and inositol, which was added as a standard.

Table I. *Effect of IAA on Ethanol-precipitable Sugars of the Cell Wall of Pea Stem Sections Released by Centrifugation*

Sugar	Amount of Sugar Released after Treatment		+IAA/−IAA
	+IAA	−IAA	
	μg/100 sections		*ratio*
Arabinose	36	33	1.09
Xylose	40	28	1.43
Galactose	57	83	0.69
Glucose	111	84	1.32

Table II. *Effect of IAA on Ethanol-precipitable Sugars of the Cell Wall of Pea Stem Sections Released by Grinding*

Sugar	Amount of Sugar Released after Treatment		+IAA/−IAA
	+IAA	−IAA	
	μg/100 sections		*ratio*
Arabinose	11	11	1.00
Xylose	91	48	1.89
Galactose	443	419	1.06
Glucose	223	160	1.33

dehydrogenase is released at 6,400*g* compared to that released at 500*g*, whereas only 13 times as much pentose is released at 6,400*g* compared to that at 500*g*. This indicates that the concentration of soluble pentose in the cytoplasm is low compared to that in the cell wall. Hexose and protein give intermediate values. The malate dehydrogenase released at 500*g* is 1.5% of that released at 6,400*g*. Even though this indicates a small amount of contamination at 500*g*, it is an overestimate since sections spun at 6,400*g* have their lower halves compressed and since their upper halves appear intact, indicating that all of the cells are not damaged at 6,400*g*. The amount of cytoplasmic contamination, therefore, represents less than 1.5% of that possible.

Gas chromatographic analysis of the polysaccharides released from sections spun at 1,000*g* reveals the presence of sugars which are known to be present in the cell walls of peas (8). Approximately

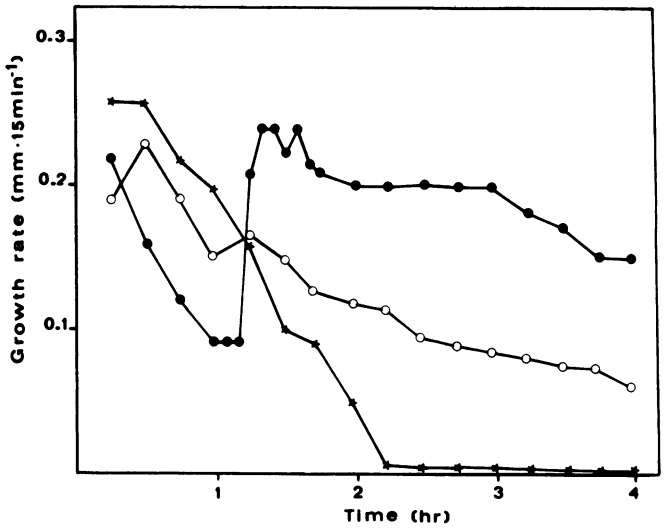

FIG. 6. Growth rates of six vacuum-infiltrated and centrifuged sections in buffer (○), 17 μM IAA (●), and 50 μg/ml cycloheximide (★).

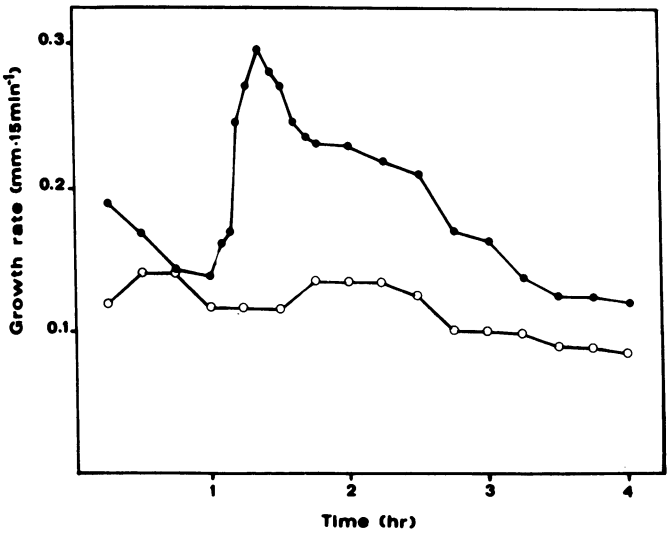

FIG. 7. Growth rates of six vacuum-infiltrated, but not centrifuged, sections in buffer (○) and 17 μM IAA (●).

80% of the soluble ethanol-precipitable sugars are released by three centrifugations when compared to the yield from five centrifugations (unpublished results). If other sugars from the cytoplasm or extracellular solution are present, they are present in very small quantities. The amounts of the sugars detected appear to be a major portion of the soluble sugars recoverable from homogenates since the amounts of xylose and arabinose released by three repeated centrifugations are similar to the amounts released by grinding the same amount of tissue. Grinding does release more glucose and galactose, however. The increase in galactose may result from the extraction of a polymer which is not released by centrifugation.

The increase in xyloglucan caused by IAA treatment points to the application of this technique to the study of physiological changes within the cell wall. Labavitch and Ray (8) showed that IAA causes an increase in soluble xyloglucan in pea cell walls. They presented evidence that IAA induces a turnover of xyloglucan which is confined to the cell wall and not the cytoplasm. Using the same treatment periods and centrifugation to release the cell wall solution, increasing amounts of a soluble polysaccharide resembling xyloglucan are recovered by centrifugation of

segments after treatment with IAA. Since the amount of xylose released by centrifugation is similar to that released by grinding, it is assumed that a similar amount of xyloglucan is extracted by centrifugation. Grinding tissue results in a mixing of intracellular and extracellular components. This makes it difficult to separate polysaccharides from other compounds, such as nucleic acids and protein, which bind to some polysaccharides (12). The amount of xyloglucan presented here is probably not the total amount of soluble xyloglucan in the cell wall of homogenized tissue.

The centrifugation technique which we have described is an improvement over previous reports utilizing centrifugation (1, 6, 13) in two respects. The cut surfaces of the sections rest on a porous polyethylene disc directing the centrifugal force in the long axis of the tissue and preventing lateral shearing forces from bending and rupturing cells, and a period of washing removes much of the cytoplasmic contamination from cut surfaces. It was found (unpublished data) that washing for 15 min removed a large fraction of malate dehydrogenase, a fraction proportional to the number of cut surfaces. The ability of centrifuged sections to grow and respond to auxin indicates the potential for removing and replacing cell wall enzymes and soluble polysaccharides during the study of cell wall metabolism as it is related to growth. The cell wall can now be examined as a separate compartment with minimal cytoplasmic contamination, thus allowing the localization of chemical reactions and metabolic pathways within the wall and the study of transport between the wall and the cytoplasm.

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