ISOLATION AND CHARACTERIZATION OF SECRETORY VESICLES IN GERMINATED POLLEN OF LILY L. LONGIFLORUM

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SUMMARY

Secretory vesicles containing polysaccharide were isolated from germinated pollen of Liliaceae longiflorum and characterized by biochemical and ultrastructural investigation. Pollen tubes exhibit a secretory pathway in which the vesicles concentrated in the tube apex are produced by the Golgi apparatus and contributed to the cell wall at the apex upon fusion of the vesicle membrane with the plasma membrane.

Secretory vesicles were isolated by a method involving the size discrimination of cytoplasmic components using Millipore filters. Cells were disrupted under conditions which minimized membrane vesiculation. Identification was made by electron-microscopic comparison of the periodic acid-silver hexamine (PASH) reactivities of in situ and isolated secretory vesicles.

The secretory vesicles contained polysaccharides which were high in galacturonic acid and similar in sugar composition to those of the hot-water-soluble fraction of pollen tube cell wall. A hot-water-insoluble, non-cellulosic glucan was the major component of the cell wall. Less than 7% of the wall was cellulosic. Chitin was absent. Similarities in the ultrastructure and PASH staining of apical secretory vesicles and an amorphous component of the cell wall support a precursor-product relationship between these two cell components. Ultrastructural investigations revealed complexes of the endoplasmic reticulum (ER) associated with electron-translucent regions of cytoplasm, suggesting a possible function of the ER in cell wall formation. Additionally, patterns of PASH staining show that changes in polysaccharides occur in secretory vesicles after vesicles have been formed by dictyosomes. Therefore, secretory vesicles may have a role in polysaccharide synthesis as well as in membrane and product transport.

INTRODUCTION

Ultrastructural and histochemical studies suggest that secretory vesicles are organelles for the transport and synthesis of materials during formation of primary cell walls in plants (Mollenhauer & Morré, 1966; Mühlethaler, 1967). However, definitive conclusions regarding the role of secretory vesicles require a direct, biochemical verification. This paper reports the results of an approach to this problem involving the characterization of isolated secretory vesicles and cell walls from germinating pollen of Lilium longiflorum. Earlier ultrastructural and histochemical studies of pollen tubes (Rosen, 1968) are consistent with a mechanism of wall formation involving a secretory pathway whereby secretory vesicles accumulate at the tube apex and, there, contribute their contents to the wall upon fusion of the vesicle membrane with the plasma membrane. Secretory vesicles were suggested to contain pectin (Dashke & Rosen, 1966) on the basis of pectinase digestion of their contents and a
positive reaction in the hydroxylamine-ferric chloride staining procedure (Albersheim, Mühlethal & Frey-Wyssling, 1960).

Secretory vesicles in pollen tubes arise from dictyosomes of the Golgi apparatus. Larson (1965) described the transition of dictyosomes during the first stages of germination from a quiescent state to a secretory, vesicle-producing state. Investigations in other plants also revealed the Golgi apparatus as a potential source of secretory vesicles in cell wall biogenesis (Mollenhauer & Morré, 1966). VanDerWoude & Morré (1968) described an extensive endoplasmic reticulum in pollen tubes of *Lilium longiflorum* as part of the membrane complex (endomembrane system) composed of endoplasmic reticulum, dictyosomes, secretory vesicles and plasma membrane. The extensive system of ER and the presence of electron-translucent regions surrounded by ER in lily pollen tubes suggest that it too may be involved in some phase of pollen tube growth.

Information concerning possible biosynthetic activities of vesicles has come from histochemical and autoradiographic studies. Dashek & Rosen (1966) described the incorporation of [Me-3H]methionine, a methyl donor, into the tip and wall components of pollen tubes. They suggested that methylation of wall polysaccharides occurred during the final stages of wall deposition. Using ultrastructural polysaccharide cytochemistry, Pickett-Heaps (1968) demonstrated a progressive increase in periodic acid-silver hexamine (PASH) staining of vesicles from the forming face to the secreting face of dictyosomes in the outer root cap cells of *Triticum vulgare*. This observation suggested a polymerization of polysaccharide contents during vesicle maturation. In autoradiographic studies performed with the same plant system, Pickett-Heaps (1967) described incorporation of derivatives of [3H]glucose as occurring initially in dictyosome vesicles and subsequently in the cell wall.

The isolation of secretory vesicles was required for a biochemical investigation of their function. In this study Millipore filtration, which has been used successfully for the isolation of small vesicles from other systems, e.g. the isolation of catecholamine storage granules from adrenal medulla (Oka, Ohuchi, Yoshida & Imaizumi, 1966), was applied to the isolation of plant secretory vesicles. PASH cytochemistry was used to establish the identity and purity of the isolated vesicle fraction.

**MATERIALS AND METHODS**

*Secretory vesicle isolation*

All procedures were at about 25 °C except where otherwise indicated. Anthers were collected at 2-day intervals from open flowers of greenhouse-grown plants of *Lilium longiflorum* Thunb. var. Ace and were air dried for 8-10 h followed by 4 h at −20 °C. Pollen was separated from anthers at the low temperature by shaking on a no. 50 sieve, and then stored at −65 °C. The percentage of viable pollen grains remained at about 70% during 8 months of storage.

Twenty to 30 mg of pollen were germinated on the surface of 30 ml of a 10%, sucrose, 10 p.p.m. boric acid solution (medium of Rosen, 1959, minus yeast extract) in each of a number of 14-cm covered glass Petri dishes. Germinated pollen was harvested by passing the liquid cultures through a no. 100 sieve which retained the germinated grains. The following operations were performed at 5 °C. Germinated grains were homogenized for 1 min with a motorized razor blade chopper (Cunningham, Morré & Mollenhauer, 1966) in 5 ml/g pollen of a homogenization medium containing 0.1M sodium phosphate buffer (pH 7.2), 0.5M sucrose and
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0.001 M EDTA. The homogenate was filtered through one layer of Miracloth (Chicopee Mills Inc., New York, N.Y.) and the crude cell wall retained by the filter was saved.

The filtrate from a 3-h culture (mean tube length about 1.4 mm) of 1 g of pollen was brought to 30 ml with the homogenization medium and passed through a Millipore prefiltro (AP 25). The filtrate was centrifuged for 5 min at 5000 g, the floating lipid layer discarded and the supernatant withdrawn. Using a syringe and filter holder, the supernatant was passed under pressure through a 25-mm diameter, 1.2-μm pore size Millipore filter and followed by 10 ml of the homogenization medium. A 10-ml portion of this filtrate was passed through a 47-mm diameter, 0.45-μm filter using a Millipore Stainless Pressure filter holder with air pressure applied to the upper liquid surface. The filter was flushed with 5 ml of homogenization medium and discarded. This procedure was repeated until the entire 1.2-μm filtrate had been passed through the 0.45-μm filter. The total 0.45-μm filtrate was centrifuged for 30 min at 58000 g. A portion of the sediment was prepared immediately for electron microscopy by resuspension to a volume of 5 ml with homogenization medium, centrifugation at 98000 g for 30 min and transfer of the pellet to fixative. Pellets for sugar analysis were washed twice by resuspension in 5 ml of water followed by centrifugation at 98000 g for 30 min and then lyophilized.

Tube cell wall isolation

The crude cell wall fraction of a 20-h culture (mean tube length about 8 mm) of 5.5 g of pollen was diluted to 300 ml with water, homogenized 2 min using a polytron 20 ST tissue homogenizer (Kinematica, Lucerne, Switzerland) at high speed, and washed with 8 centrifugation-resuspension cycles in water. Tube cell walls were isolated from grain wall by a low speed differential centrifugation, after which the tube walls were recovered from the supernatant by centrifugation and lyophilized.

Polysaccharide analyses

Replicate samples of 50 mg of tube cell wall were sequentially extracted by water (100 °C), 0.05 N H₂SO₄ (100 °C), 4 N KOH, and 72 % H₂SO₄ following Ray's (1963) procedure modified to process 10 times greater amounts of cell wall material. The extract was withdrawn after centrifugation at 10000 g for 5 min, and the residue dried and weighed after each extraction. Extracts were reduced in volume and lyophilized. The 72 % H₂SO₄ extract was acid-hydrolyzed by Ray's (1963) procedure.

Sugars in tube walls, tube wall extracts and isolated secretory vesicle fractions were identified and determined using gas-liquid chromatography (Albersheim, Nevins, English & Karr, 1967). Glucosamine in wall polysaccharide was determined spectrophotometrically (Dische & Borenfreund, 1950). Uronic acid was determined as follows. Samples were hydrolysed in 1 N H₂SO₄ for 6 h at 102–104 °C, neutralized with BaCO₃, filtered through a fibrous glass filter, passed through Dowex 50 (H⁺ form) to remove Ba⁺⁺ ions and lyophilized. A fraction of the hydrolysed polysaccharide was retained for an analysis of total reducing sugar by Somogyi's (1952) method. The remaining fraction was chromatographed in a descending system containing isopropanol, pyridine, water and acetic acid (8:8:4:1 v/v). Uronic acid was identified by parallel-chromatography with known compounds and visualization with p-anisidine-HCl (Hough, Jones & Wadman, 1950). Quantitative measurements of uronic acid by Somogyi's method were made on sugars eluted from the uronic acid zones of chromatographs. Measurements were corrected for the recovery of known amounts of uronic acid which were processed through the entire procedure and expressed as % of total hydrolysable polysaccharide.

Electron microscopy

Whole specimens from 3-h cultures were fixed for 1 h in a solution containing 0.1 M acrolein, 0.1 M glutaraldehyde and 0.1 M potassium phosphate buffer (pH 7.2). The fixed specimens were washed with buffer, post-fixed 1 h in 1 % osmium tetroxide in 0.1 M potassium phosphate buffer (pH 7.2), washed with buffer followed by water, stained overnight with aqueous saturated uranyl acetate and washed with water. Specimens were dehydrated in a graded ethanol series, transferred to acetone and embedded in Araldite 6005.
Pellets of isolated vesicle fractions were fixed overnight at 5 °C in the osmium tetroxide fixative, washed with buffer followed by water, dehydrated and embedded. Ultrathin sections of embedded material were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome. Whole specimens were sectioned in a plane parallel to their longitudinal axes. Pellets of isolated secretory fractions were sectioned in a plane parallel to the centrifugal force vector which they sustained. Sections were picked up on collodion-methacrylate membrane-covered copper grids, or for silver staining, gold grids. Sections were stained with lead citrate (Reynolds, 1963), or were treated according to the PASH procedures of either Pickett-Heaps (1968) or Swift & Saxton (1967) for the ultrastructural localization of polysaccharides. The Pickett-Heaps procedure was modified as follows: (1) Blocking of pre-existing aldehyde groups; 2% sodium bisulphate for 15 min. (2) Periodate oxidation; 1% periodic acid for 30 min. (3) Staining of silver reducing sites; 0.1% silver nitrate in borate-buffered 1% hexamine (pH 9.2) at 45 °C for 3 h. (4) Removal of unreduced silver; 2% sodium thiosulphate for 1 min. Appropriate controls were performed to certify the staining requirement for periodate oxidation.

Prepared specimens were viewed at 60 kV with a Philips EM-200 electron microscope. A 54,864 line per inch diffraction grating replica (Ladd Research Industries, U.S.A.) was used as the magnification standard.

RESULTS

Pollen tube ultrastructure

The pollen tube cytoplasm contained an extensive and complex endomembrane system (Figs. 1, 3). Secretory vesicles, dictyosomes of the Golgi apparatus, and endoplasmic reticulum (ER) were found throughout the protoplast. In addition to these components, mitochondria, lipid bodies and amyloplasts were abundant.

The apical 5 μm of the pollen tube cytoplasm contained a high concentration of secretory vesicles which ranged in diameter from 0.15 to 0.30 μm (Figs. 1, 2). Secretory vesicles in this region often had conjoined membranes. Configurations also appeared in which clear continuity of vesicle membranes was difficult to follow. In some instances, aggregations of material resembling the contents of secretory vesicles were only partially surrounded by a limiting membrane. The contents of secretory vesicles at the apex were lightly flecked with electron-dense spots (Fig. 2), and vesicles with similar contents appeared throughout the cytoplasm. However, in vesicles not at the apex, the flecked appearance was usually reduced or absent (Figs. 3, 4). Secretory vesicles which were continuous with dictyosome cisternae were not flecked (Figs. 3, 4).

A second vesicle type was found which was distinct from secretory vesicles. These vesicles were about 50 nm in diameter and had a darkly stained lumen (Fig. 3). They were present throughout the cytoplasm, often close to dictyosomes.

Dictyosomes were located near agranular and granular ER (Fig. 3). In median cross-sections, dictyosomes appeared as stacks of cisternae. Intericisternal elements (Mollenhauer, 1965) were present between adjacent cisternae (Fig. 3). In tangential views, dictyosome cisternae had a perforated central plate-like region with tubules attached at the periphery of the plate (Fig. 3) similar to dictyosome cisternae in other plant cells (Mollenhauer & Morrè, 1966).

The ER of the pollen tube cytoplasm appeared as a highly anastomosed membranous reticulum (Figs. 1, 3). ER in the apical 5 μm of the tube tip was without associated ribosomes. The lumen of the ER had a heterogeneous electron density.
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(Fig. 3). Close associations of ER and material similar to the contents of unflecked secretory vesicles were observed (Fig. 3). In these special ER configurations only one membrane was present between the lumen of the ER and the adjacent electron-translucent region. Such configurations occurred with both granular and agranular ER and bore no resemblance to the 'storage pockets' found in the ER of cotton pollen (Fisher, Jensen & Ashton, 1968).

The wall of the pollen tube was more densely flecked with the electron-dense spots than were the apical secretory vesicles (Fig. 2). A lamellar organization of electron-dense material was seen in the outer regions of the wall. At the apex of the pollen tube tip (Fig. 2), the inner portion of the wall contained membrane-bound packets of material resembling the contents of secretory vesicles of the tip. Vestiges of these packets were seen in the wall regions adjacent to the apex (Fig. 1) but not in the lateral walls.

One pollen tube, in which the ground cytoplasm stained darkly, had an unusual ultrastructure (Figs. 5, 6). Its wall was much like the walls of other specimens, having a fibrous, flecked appearance and lamellar organization. However, this unusual cell was of particular interest because the lumina of secretory vesicles and dictyosome vesicles contained wall-like regions of fibrous material flecked with minute, electron-dense particles. That wall-like material could be found in vesicles is of significance regarding the biosynthetic capabilities of dictyosomes and secretory vesicles and their contribution to the cell surface (see Discussion).

Cytochemical localization of polysaccharide

PASH staining by the Pickett-Heaps procedure varied among cell components. Ground cytoplasm was stained lightly, and membranes were delineated by finely deposited silver. Such staining could be distinguished from the larger silver deposits found over cell walls, secretory vesicles (Fig. 7) and lipid bodies (Fig. 9). Mitochondrial lumina (Fig. 8) exhibited no staining above that of background. Starch granules did not survive specimen preparation. PASH staining was either absent or very low in ER lumina (Fig. 8). Secretory vesicles displayed varied degrees of PASH staining (Fig. 9). Vesicles associated with dictyosomes were unstained. Free vesicles were either lightly stained or were stained heavily (Figs. 8, 9), in which case they resembled the vesicles at the tube apex (Fig. 7). Cell walls stained in a manner comparable to the secretory vesicles at the tube apex (Fig. 7).

Differences in the PASH staining of cytoplasmic membranes were enhanced by the procedure of Swift & Saxton (1967), although staining of polysaccharide was less intense than with the Pickett-Heaps procedure. Membranes could be placed into 2 groups according to the size of the silver grains deposited over them. The silver grains deposited over membranes of free secretory vesicles and over plasma membrane were larger than those deposited over membranes of dictyosomes, ER and mitochondria (Figs. 10-12). The larger grains were comparable in size to those deposited over the cell wall and the contents of secretory vesicles, while the smaller grains were similar to background deposition.
The isolated secretory vesicle fraction

The secretory vesicle isolation resulted in a dry weight yield of 1·1 mg/g of cultured pollen. The lower half of the pellet consisted mainly of 0·15-0·30 μm diameter vesicles, with contents of low electron density (Fig. 13). Almost all the vesicles in the lower half of the pellet stained with PASH (Fig. 15). Their staining closely resembled the PASH staining of the cell wall and in situ PASH-reactive vesicles (Fig. 7). In the upper portion of the pellet, a smaller proportion of the vesicles was PASH-reactive (Fig. 14). A few small mitochondria were present in the upper half of the pellet but absent in the lower half. The major component of the isolated fraction was, therefore, a population of vesicles which had the same size and PASH-reactivity as secretory vesicles in the pollen tube tip. Electron-microscopic examination of various fractions showed that cytoplasmic components were selectively removed from the vesicles at each step of the isolation procedure. Most vesicles in the crude homogenate which were of the same size range as in situ secretory vesicles were strongly PASH-reactive. This indicated minimum vesiculation of other membranous components during homogenization by razor blade chopping. Filtration of the crude homogenate through Miracloth and the prefilter removed cell walls. During the 500-g centrifugation, large lipid bodies and amyloplasts containing starch granules were removed, respectively, by flotation and sedimentation. This step was necessary to prevent excessive clogging of the 1·2-μm filter. The 1·2-μm filtration removed additional amyloplasts and the larger vesicles. Filtration through the 0·45-μm filter removed the remaining small starch granules and most of the mitochondria and PASH unreactive vesicles. A few remaining small lipid bodies were removed by flotation during the centrifugation of the 0·45-μm filtrate.

Pollen tube cell wall polysaccharide

The pollen tube cell wall isolation resulted in a dry weight yield of 31 mg/g of pollen cultured. The sequential extraction (Table 1) revealed that only a small portion of the cell wall polysaccharide was hot-water-soluble. Most of the remaining polysaccharides were extracted by the hot dilute acid and concentrated alkali. The small amount of KOH-insoluble polysaccharide was largely solubilized by 72% sulphuric acid. The remaining insoluble residue contained grain exine and extracellular lipid.

The gas-liquid chromatographic analysis of polysaccharides resulted in incomplete sugar recoveries for all fractions (Table 2). Incomplete hydrolysis was primarily responsible for the partial recovery. In addition, uronic acids were not hydrolysed quantitatively or detected by this procedure. The major neutral sugars of cell wall polysaccharides were, in order of decreasing abundance, glucose, arabinose and galactose. Xylose and mannose were present in small amounts, as were the deoxyhexoses, rhamnose and fucose.

The polysaccharides of the various cell wall extracts displayed sugar distributions unlike those of total cell wall polysaccharide (Table 2). Arabinose was the major sugar of the hot-water-soluble polysaccharides and was also present in substantial amounts in
Secretory vesicles of germinated pollen

the polysaccharides which were soluble in hot, dilute acid. Glucose was the main component of the alkali- and concentrated acid-soluble polysaccharides.

The uronic acid in the fractions was identified as galacturonic acid. Quantitatively, galacturonic acid was present in largest amounts in the hot-water- and alkali-soluble cell wall fractions (Table 3).

Polysaccharide content of isolated secretory vesicles

The accuracy attained in the gas-liquid chromatographic sugar analysis of the vesicle fraction (Table 2) was limited by the small amount of the fraction. Some sugars were estimated near their limits of detection. The analysis was, however, of qualitative and general quantitative value. The sugars found in the vesicle fraction were

Table 1. Sequential fractionation of pollen tube cell walls

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight (mg)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, 2 h (100 °C)</td>
<td>4.3 ± 0.2</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>0.05 N H₂SO₄, 2 h (100 °C)</td>
<td>18.6 ± 0.0</td>
<td>37.0 ± 0.0</td>
</tr>
<tr>
<td>4 N KOH, 6 h (25 °C)</td>
<td>22.6 ± 0.0</td>
<td>44.9 ± 0.0</td>
</tr>
<tr>
<td>72% H₂SO₄, 15 h (25 °C)</td>
<td>3.2 ± 0.3</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>Residue</td>
<td>1.6 ± 0.5</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Total</td>
<td>50.3</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Averages from 2 replicate samples ± average deviation.

Table 2. Composition of pollen tube cell wall and secretory vesicle polysaccharides determined by gas-liquid chromatography

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Cell wall</th>
<th>Hot water</th>
<th>0.05 N H₂SO₄</th>
<th>4 N KOH</th>
<th>72% H₂SO₄</th>
<th>Secretory vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>2.1 (0.19)</td>
<td>2.5 (0.02)</td>
<td>0.7 (0.11)</td>
<td>2.2 (0.20)</td>
<td>1.4 (0.06)</td>
<td>6.3 (0.01)</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.3 (0.03)</td>
<td>— (0.00)</td>
<td>0.3 (0.05)</td>
<td>0.2 (0.02)</td>
<td>— (0.00)</td>
<td>6.3 (0.01)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.6 (0.83)</td>
<td>5.4 (0.43)</td>
<td>20.1 (3.10)</td>
<td>0.6 (0.08)</td>
<td>1.4 (0.01)</td>
<td>12.5 (0.02)</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.4 (0.12)</td>
<td>1.3 (0.01)</td>
<td>0.3 (0.05)</td>
<td>2.0 (0.18)</td>
<td>— (0.00)</td>
<td>6.3 (0.01)</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.5 (0.04)</td>
<td>1.3 (0.01)</td>
<td>0.1 (0.01)</td>
<td>— (0.00)</td>
<td>— (0.00)</td>
<td>12.5 (0.02)</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.3 (0.55)</td>
<td>10.1 (0.08)</td>
<td>2.1 (0.32)</td>
<td>4.6 (0.42)</td>
<td>4.0 (0.03)</td>
<td>25.0 (0.04)</td>
</tr>
<tr>
<td>Glucose</td>
<td>79.8 (6.96)</td>
<td>30.4 (0.24)</td>
<td>76.4 (11.77)</td>
<td>90.1 (8.20)</td>
<td>93.2 (0.69)</td>
<td>31.4 (0.05)</td>
</tr>
<tr>
<td>Total</td>
<td>(8.73)</td>
<td>(0.79)</td>
<td>(15.41)</td>
<td>(9.10)</td>
<td>(0.74)</td>
<td>(0.16)</td>
</tr>
<tr>
<td>Sample wt (mg)</td>
<td>20.8</td>
<td>3.8</td>
<td>21.1</td>
<td>20.3</td>
<td>3.8</td>
<td>2.1</td>
</tr>
<tr>
<td>% solubilized</td>
<td>72.5</td>
<td>100.0</td>
<td>96.2</td>
<td>83.4</td>
<td>94.7</td>
<td>85.7</td>
</tr>
<tr>
<td>Recovery as % of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) amount solubilized</td>
<td>57.8</td>
<td>20.8</td>
<td>75.9</td>
<td>50.2</td>
<td>20.6</td>
<td>8.9</td>
</tr>
<tr>
<td>(b) sample</td>
<td>42.0</td>
<td>20.8</td>
<td>73.0</td>
<td>47.2</td>
<td>19.5</td>
<td>7.6</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are mg sugar recovered.
precisely those found in the cell wall. Also, the sugar distribution of the vesicle fraction resembled most closely that of the hot-water-soluble cell wall polysaccharide (Tables 2, 3).

Table 3. Analysis of galacturonic acid composition of pollen tube cell wall and secretory vesicle polysaccharides

<table>
<thead>
<tr>
<th></th>
<th>Cell wall extracts</th>
<th></th>
<th></th>
<th></th>
<th>Secretory vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment</td>
<td>Cell wall</td>
<td>Hot 0.05 N H₂SO₄</td>
<td>4 N KOH</td>
<td></td>
</tr>
<tr>
<td>Sample weight (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>3.0</td>
<td>3.7</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3.2</td>
<td>—</td>
<td>3.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Sugar recovered on hydrolysis (in mg glucose equivalents)</td>
<td></td>
<td>1.991</td>
<td>1.480</td>
<td>2.890</td>
<td>1.850</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.124</td>
<td>—</td>
<td>2.885</td>
<td>1.740</td>
</tr>
</tbody>
</table>
* Average ± average deviation.

DISCUSSION

The composition and morphology of secretory vesicles

We designate as secretory vesicles all those vesicles of the pollen tube within the size and luminar electron-density range shown by apical vesicles of the pollen tube tip at one extreme, and by vesicles associated with dictyosomes at the other extreme. Within this range, 2 classes of vesicles are distinguished. Secretory vesicles having the luminar appearance and PASH staining of vesicles in the tube apex are designated mature. Those characterized by a less-dense luminar appearance and reduced PASH staining are designated immature.

PASH staining was adequate for the identification of isolated secretory vesicles, a prerequisite for a meaningful biochemical study. The characteristic PASH staining and size range of in situ mature secretory vesicles were of great value in their identification. Isolated secretory vesicles were identified as mature secretory vesicles on the basis of their singular resemblance to mature in situ vesicles. Similar PASH staining of secretory vesicles in germinating cotton pollen has been shown by Jensen, Fisher & Ashton (1968).

In the absence of PASH staining, mature in situ secretory vesicles displayed a flecked appearance after lead citrate post-staining. Phosphotungstic acid post-staining intensified this appearance under similar conditions of fixation (Rosen & Gawlick, 1966). Comparisons of the ultrastructural luminar appearance of isolated and in situ secretory vesicles could not be made when using lead citrate post-staining because of the differences in specimen preparation.

The results provide definitive evidence for a polysaccharide content for pollen tube
secretory vesicles. The primary evidence is that the isolated secretory vesicle fraction, as identified by PASH polysaccharide cytochemistry, does contain sugars which are characteristic of the cell wall polysaccharide. The sugars found by the analysis must have been derived from polysaccharides, rather than have existed as unpolymerized sugars, since the latter would have been lost during the isolation.

The behaviour of secretory vesicles during centrifugation was consistent with a polysaccharide content. Secretory vesicles were more dense than cytoplasmic particles of similar size, as inferred from the examination of pellets of isolated vesicle fractions. Most secretory vesicles were in the bottom half of the pellets. Thus, mature secretory vesicles have a sedimentation coefficient greater than that of mitochondria and immature vesicles in the top half of the pellets. Since immature and mature vesicles were approximately the same size, the difference in sedimentation resulted from a difference in density.

The periodate oxidation of the PASH procedure is not entirely specific for polysaccharides (Pearse, 1960). However, the PASH staining, when taken together with the polysaccharide analyses and the high density of isolated vesicles, establishes that the mature secretory vesicles of pollen tubes contain polysaccharides.

The composition and morphology of the pollen tube cell wall

Determinations of the composition and morphology of the pollen tube cell wall proved useful to an understanding of the role of secretory vesicles in wall biogenesis. The fractionation of the wall polysaccharides by differential solubilization aided the chemical characterization of the cell wall. Although such a separation method is related to an artificial classification of cell wall polysaccharides (Albersheim, 1965), the fractions obtained were sufficiently different to show a heterogeneous polysaccharide composition in the pollen tube wall. Electron-microscopic images also showed that the wall is heterogeneous. The outer fibrillar and inner amorphous regions of *Lilium* pollen tube walls are similar to the 2 regions described in *Petunia* pollen by Sassen (1964).

Chemical characterization showed the cell wall to contain 6-7% cellulose (the fraction which was insoluble in 4 N KOH and solubilized by 72% H$_2$SO$_4$). Cellulose is a primary candidate for the fibrillar wall component exhibited by ethanolamine-extracted, metal-shadowed tube walls of *Petunia* by Sassen (1964) and by similarly prepared walls of *Lilium* in our studies. Also, the absence of hexosamine in the tube walls of *Lilium* rules out the presence of chitin as the fibrillar component. The presence of a non-cellulosic glucan such as callose (an amorphous, alkali-soluble $\beta$-1,3 glucan) as a major component of the cell wall is suggested by the large amount of glucose present in the alkali-soluble fraction. (See also Sassen, 1964, for cell walls of *Petunia* pollen tubes.) Cytochemically, conspicuous callose deposits are restricted to occasional plugs (Currier, 1957; Alves, Middleton & Morré, 1968), few of which were present in our tube wall preparations. Dashek & Rosen (1966) described the pectinase digestion of pollen tube wall and suggested that the amorphous phase was primarily composed of pectin. Our results show less than 8% uronic acid in tube walls. Its distribution among the extracted wall fractions (Table 3) indicates that the
galacturonic acid of the hot-water-soluble polysaccharides is contained in a polyuronide, while that in the hemicellulose fraction is linked to other sugars.

**Secretory vesicle-cell wall relationships**

The contribution of secretory vesicle contents to the cell wall has not been conclusively shown. The evidence for a polysaccharide content of secretory vesicles reported here and the images of vesicle membranes continuous with plasma membrane at the pollen tube apex shown by Rosen, Gawlick, Dashek & Siegesmund (1964) offer only static information from which conclusions regarding the kinetics of vesicle movement cannot be made. Time-course autoradiographic and chemical studies of root cap cells of wheat (Northcote & Pickett-Heaps, 1966; Pickett-Heaps, 1967) provide kinetic evidence that vesicles containing polysaccharide are produced by the Golgi apparatus and contribute their contents to the cell surface. Similar studies by Wooding (1968) support the Golgi apparatus-mediated secretion of hemicellulosic polysaccharides in sycamore seedling stems.

In pollen tubes, the contribution by secretory vesicles of material to the amorphous cell wall region is indicated by the similar appearances of these 2 components following lead or PASH post-staining. Similarities of the polysaccharides of secretory vesicles and hot-water-soluble wall material are consistent with an origination of matrix polysaccharides from secretory vesicles. The relatively large amount of galacturonic acid in the secretory vesicle fraction points to a vesicular route for the contribution of uronic acid-containing precursors to the wall. However, the composition of secretory vesicles rules out their participation as the sole contributors to the wall (i.e. insufficient glucose). Redistribution of sugars after deposition may occur and contributions to the wall from sources other than secretory vesicles are required.

**Functions of secretory vesicles**

Secretory vesicles appear to have a 2-fold function in that both wall materials and membrane are contributed to the cell surface. Regarding the first of these functions, ultrastructural and chemical observations indicate that secretory vesicles are more than transport vehicles. PASH-reactive material is absent in immature vesicles. This may be due to either limited polymerization of the polysaccharides and subsequent loss during electron-microscopic preparation (Pickett-Heaps, 1968) or the absence of polysaccharides. The apparent transitions in vesicles not associated with dictyosomes, which are seen following lead or PASH post-staining, suggest that synthetic activities occur in vesicles during their migration to the tube tip.

The hypothesis is therefore supported that secretory vesicles are synthesized, and they in turn serve as organelles of either or both polysaccharide synthesis and accumulation. Although the evidence is strong, the interpretation rests upon static images, and *in vivo* and *in vitro* kinetic studies are necessary to make definite conclusions concerning synthetic functions of secretory vesicles.

In studies of other systems, a progressive change in the appearance of secretory vesicles associated with dictyosomes was demonstrated (Mollenhauer & Whaley, 1963). Pickett-Heaps (1968) described an increase in vesicle luminar PASH staining which
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progressed from the forming to the secreting face of dictyosomes. Manton (1966, 1967) has demonstrated the development of scales within dictyosome vesicles in species of scale-forming algae. In pollen tubes, Dashek & Rosen (1966) presented cytochemical evidence for the methyl esterification of free carboxyl groups in secretory vesicles, and Bonnett & Newcomb (1966) suggested that the degree of polymerization of polysaccharides changes during vesicle maturation. These results are consistent with a biosynthetic role for secretory vesicles. In our study, the absence of progressive staining of vesicles associated with dictyosomes could be the result of inadequate staining or, alternatively, be a true expression of the polysaccharide content of the vesicles.

Vesicle production by dictyosomes of pollen tubes may be rapid. The rate in Lilium longiflorum calculated by VanDerWoude & Morrè (1968) is about 2000 vesicles per min. If vesicles are released from dictyosomes before significant polysaccharide accumulation occurs, PASH staining will not be observed until later stages of vesicle migration and maturation. This is the usual observation. However, if release of vesicles capable of polysaccharide biosynthesis or accumulation were delayed, then, under these conditions, polysaccharide synthesis could continue to final stages even in vesicles associated with dictyosomes. This type of argument provides one explanation for the appearance of the unusual pollen tube specimen of Fig. 5. Here the contents of dictyosome vesicles appear to have matured to a stage resembling that of the cell wall polysaccharide. In this specimen, the development of the fibrillar appearance in secretory vesicle contents before apparent contribution to the wall suggests that the synthetic capabilities which are required for wall maturation are present in secretory vesicles. Usually, wall polysaccharide synthesis does not appear completed until after vesicle deposition. This may be due to the rapidity with which the vesicles are released and transferred to the growing tube tip. Alternatively, vesicles may provide a mechanism for controlled synthesis and assembly of wall materials. In any event, the possibility must be considered that secretory vesicles function in cell wall synthesis as well as transport. Furthermore, biosynthetic enzymes present in the vesicles will be contributed to the cell surface during vesicle fusion. To what extent such enzymes might participate in extracytoplasmic formation of polysaccharides at the cell surface is not known.

The fibrillar component in dictyosome vesicles of the unusual specimen may be cellulose. The synthesis of cellulose within cisternae of the Golgi apparatus has been shown by Brown, Franke, Kleinig, Falk & Sitte (1970) in the marine alga, Pleurochrysis scherffeli. Thus, a specific hypothesis arises from the study of the unusual pollen tube. It is that dictyosome vesicles and free secretory vesicles contain the capability for cellulose synthesis. However, in pollen tubes this capability is not expressed until vesicles have fused with the plasma membrane.

Regarding the contribution of membrane to the cell surface by secretory vesicles, much evidence is available to support such a function. The similarity of secretory vesicle membranes and plasma membrane has been noted by Sjöstrand (1963) for animal cells, and by Grove, Bracker & Morrè (1968) for a fungus. In this study of pollen tubes the PASH-staining characteristics of the mature secretory vesicle
membrane and the plasma membrane are similar. However, increases in membrane PASH staining are correlated with an increase in the PASH reactivity of adjacent material. Thus, it is problematic whether the increased staining of mature secretory vesicle membranes and plasma membrane is related to a high PASH reactivity of these membranes, or to their proximity to PASH-reactive material.

It is possible to calculate the amount of vesicle membrane available for surface growth of the plasma membrane if it is assumed that the contribution of the vesicle contents to the amorphous cell wall region involves no radical volume changes in the contributed material. Such calculations show the possibility of a one to one stoichiometry between the amount of membrane contributed by vesicles and the increase in plasma membrane during steady-state growth (VanDerWoude & Morré, 1968).

**Origin of secretory vesicles**

The presence of secretory vesicles associated with dictyosomes shows that the Golgi apparatus is the origin of these vesicles. The wall-like contents of vesicles associated with dictyosomes in the unusual specimen support this. The Golgi apparatus appears to be the origin of vesicles in many plant and animal systems (Mollenhauer & Morré, 1966; Beams & Kessel, 1968; Grove, Bracker & Morré, 1970). However, other vesicle origins cannot be excluded. The fungi provide many examples of eucaryotic systems in which vesicle formation related to wall synthesis occurs in the absence of recognizable dictyosomes (Bracker, 1967). In some of these instances vesicles appear to originate from a Golgi apparatus consisting of a single cisterna (Girbardt, 1969; Morré, Mollenhauer & Bracker, 1970).

The nature and function of the 50-nm vesicle is unknown. Although 50-nm vesicles appear darkly stained, their lumina may be electron-transparent. One or two membrane surfaces in the plane of sectioning of a 50-nm vesicle would be within the 60–80-nm section, thus making the lumen appear electron-dense. Larson (1965) described such vesicles in pollen tubes and suggested that they functioned in the formation of dictyosome cisternae. Although no consistent associations between dictyosomes and ER have been observed in pollen tubes, the existence of such associations remains a possibility. The 50-nm vesicle may be one type of intermediate in the transfer of membrane and other materials from the ER to the Golgi apparatus in the production of secretory vesicles.

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REFERENCES


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**ABBREVIATIONS ON PLATES**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>a</td>
<td>amyloplasts</td>
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<tr>
<td>c</td>
<td>dictyosome cisterna</td>
</tr>
<tr>
<td>d</td>
<td>dictyosome</td>
</tr>
<tr>
<td>er</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>l</td>
<td>lipid body</td>
</tr>
<tr>
<td>m</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>pm</td>
<td>plasma membrane</td>
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<tr>
<td>s</td>
<td>secretory vesicle</td>
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<tr>
<td>v</td>
<td>50-nm vesicle</td>
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<tr>
<td>w</td>
<td>cell wall</td>
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Figures 1–12 are electron micrographs of ultrathin sections of pollen tubes of *Lilium longiflorum* fixed with acrolein-glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and embedded in Araldite. The sections of Figs. 1–6 were stained with lead citrate.

Fig. 1. Longitudinal section (approximately median) of the tube tip showing the presence and distribution of cytoplasmic components. Secretory vesicles (v) are concentrated at the tube apex, the region of cell wall formation. (Montage of 2 micrographs.) \( \times 5700 \).
Fig. 2. At the tube apex the contents of secretory vesicles display a flecked appearance similar to that of membrane-bounded cell wall inclusions (large arrows) and resemble closely — sometimes exactly (small arrow) — the appearance of the inner, amorphous region of the cell wall (n). Images of vesicle fusion and membrane discontinuity are characteristic of this region. × 47,000.

Fig. 3. View of the tube cytoplasm 10-14 μm from the apex. The cytoplasm contains a complex endomembrane system composed of highly anastomosed granular and agranular ER, 50-nm diameter vesicles (v), dictyosomes (d) and secretory vesicles (v). A tangentially sectioned dictyosome cisterna (lower right, c) displays a perforated, central plate-like region with tubules attached at its periphery. Portions of the ER are closely associated with electron-translucent material (arrows). × 47,000.

Fig. 4. Portion of Fig. 3 which has been photographically reproduced to intensify the very lightly flecked appearance of the secretory vesicle which is free in the cytoplasm (arrow). Vesicles attached to dictyosomes do not display the flecked appearance. × 47,000.
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3

4

0.5 μm

v

v'

er
Fig. 5. Ultrastructural appearance of an unusual pollen tube specimen. Secretory vesicles (v) of the tube apex appear coalesced and contain inclusions which resemble the cell wall (w). × 100,000.

Fig. 6. As Fig. 5 but non-apical cytoplasm. Vesicles attached to dictyosomes (d) contain material similar to the contents of free secretory vesicles (v). × 100,000.


Fig. 7. Tube apex. Secretory vesicles (v) and cell wall (w) display similar PASH staining.

Fig. 8. Section 10 µm from the tube apex. Electron-translucent regions associated with ER (large arrows) display varied degrees of PASH staining. PASH staining of the ER (small arrows) is weak or absent.

Fig. 9. Section 40 µm from the tube apex. Lipid bodies (l) stain most intensely. Staining above background is absent in vesicles which are associated with dictyosomes (d) and intermediate to intense in vesicles (v) which appear free in the cytoplasm.
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Figs. 10-12. Differential staining of cytoplasmic membranes. Sections stained using the PASH procedure of Swift & Saxton (1967). Two types of membrane staining are present: *Type 1*, membranes on which the particle size of silver deposition is very small, equivalent to that of background deposition; *Type 2*, membranes on which the deposited silver granules are more easily discerned and are of a size comparable to silver particles deposited over regions containing polysaccharide. × 57,000.

Fig. 10. Membranes of ER, dictyosomes (d) and vesicles (v) attached to dictyosomes display *Type 1* staining.

Fig. 11. Plasma membrane (pm) displays *Type 2* staining.

Fig. 12. Membranes of mitochondria are characterized by *Type 1* staining. Membranes of secretory vesicles (v) and some ER membranes which are adjacent to electron-translucent regions (arrow) display *Type 2* staining.

Figs. 13-15. Electron micrographs of ultrathin sections of secretory vesicle preparations isolated by Millipore filtration, fixed with osmium tetroxide and embedded in Araldite. × 34,000.

Fig. 13. Representative portion of the lower half of the pelleted preparation. Section stained with lead citrate.

Fig. 14. Cytochemical localization of polysaccharide in the fraction. Section stained using the PASH procedure of Pickett-Heaps (1968). Representative portion of the upper half of the fraction pellet.

Fig. 15. As in Fig. 14 but lower half of the fraction pellet. This portion of the fraction is composed almost exclusively of PASH-reactive vesicles.
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