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FUNCTION OF GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN. 1. ISOLATION OF GOLGI VESICLES

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SUMMARY

Golgi vesicles from the tips of growing pollen tubes of *Petunia* were isolated on a discontinuous sucrose gradient and compared with those in vivo by means of EM techniques. The results indicate that the isolation procedure does not alter the morphology of the Golgi vesicles. The isolation technique developed renders it possible to analyse biochemically the contents of Golgi vesicles in relation to cell wall synthesis in germinating pollen.

1. INTRODUCTION

It has been found that certain common features exist in the growth pattern of fungal hyphae (BRACKER et al. 1971; BRACKER & HALDERSON 1971; GROVE et al. 1970; GROVE & BRACKER 1970), root hairs (SIEVERS 1963; BONNET & NEWCOMB 1965, 1966), and pollen tubes (SASSEN 1964; ROSEN et al., 1964; ROSEN & GAWLIK 1965, 1966; VAN DER WOUDE & MORRÉ 1968; CRANG & MILES 1969). In each of these organs growth occurs in a region restricted to the tip of the elongating cell. In this area of cell wall extension, a variety of cell organelles has been found, including smooth and rough endoplasmic reticulum, mitochondria, ribosomes, Golgi bodies, and numerous Golgi vesicles. It has been demonstrated that the vesicles, generated at the periphery of the Golgi cisternae, are pinched off and migrate to the cell surface and are apparently essential for cell wall growth (SIEVERS 1963; SASSEN 1964; VAN DER WOUDE & MORRÉ 1968; VAN DER WOUDE et al. 1971; GROVE et al. 1970; GROVE & BRACKER 1970; BRACKER & HALDERSON 1971). The unit membrane of the Golgi vesicles has been observed to fuse with the plasma membrane, thereby increasing the surface of the latter. In this manner, the content of the Golgi vesicles is brought outside the cytoplasm; it is believed to consist of enzymes and various other substances involved in cell wall synthesis (SIEVERS 1963; SASSEN 1964; CRANG & MILES 1969; ROSEN & GAWLIK 1965; VAN DER WOUDE & MORRÉ 1968; VAN DER WOUDE et al. 1971).

In support of the above concept VAN DER WOUDE et al. (1971) reported similarities in the sugar components of the Golgi vesicles and the newly synthesized cell wall in pollen tubes of *Lilium*. Other investigators have reported the presence of pectic substances and other polysaccharides in the Golgi vesicles of *Lilium* pollen tubes (DASHEK & ROSEN 1966) and wheat seedlings (PICKETT-

HEAPS 1968). Furthermore, HEYN (1971) has shown that the Golgi vesicles of *Avena* coleoptiles are rich in synthetases. Probably the strongest evidence linking Golgi vesicles with a role in cell wall synthesis is that of BROWN et al. (1969, 1970) and BROWN & FRANKE (1971). Their papers report for the first time the presence of cellulose in Golgi vesicle-like structures of the alga *Pleurochrysis scherffelii*. This finding, however, may be an exception in the plant kingdom since this particular alga is known to have a peculiar cell wall.

Summarizing, data exist suggesting that Golgi vesicles may house the machinery necessary for the synthesis of cellulose. In spite of this evidence, all attempts to synthesize cellulose fibrils in vitro have failed so far. These failures may be due to the separation of essential components which are vital to cellulose synthesis. This could easily occur during the fractionation procedure (BARBER et al. 1964; ORDIN & HALL 1967; VILLEMEZ et al. 1967; FREY-WYSSLING 1969; MARX-FIGINI 1969; ROBINSON & PRESTON 1971, 1972).

In order to obtain further information on the synthesis of cellulose it will be necessary to isolate Golgi vesicles and analyse their contents biochemically. Particular emphasis must be given to the enzymes taking part in cellulose synthesis. The object of this work was to develop a method for isolating intact Golgi vesicles from growing pollen tubes of *Petunia*. Future work will involve the analysis of the Golgi vesicles and the newly synthesized cell wall as well as an attempt to synthesize cellulose in vitro using isolated Golgi vesicles.

2. MATERIAL AND METHODS

Pollen from *Petunia* × *hybrida*, strain W166K. were grown as previously described by SCHRAUWEN & LINSKENS (1967). After 90 min. of germination the pollen tube length was nearly twice the diameter of the pollen grain. At this stage pollen was centrifuged, washed with a 0.1 M Na-K-phosphate buffer (pH 7.2) to which was added 0.001 M FeEDTA (Abbrev: BFe), and finally homogenized for 1 min. in the same buffer with 0.3 M sucrose. The temperature during the isolating procedure was held at 0 °C, unless mentioned otherwise. After centrifugation at low speed the supernatant was removed and layered on a discontinuous sucrose gradient composed of the following concentrations: 0.5, 1.0, 1.5, and 2.0 M in BFe. This procedure is a combination of the techniques used by BLOEMENDAL et al. (1967) and by MORRÉ & MOLLENHAUER (1964). Centrifugation was carried out in an SW 27.1 for 70 min. at 27,000 rpm at 4 °C, after which clear bands appeared on the boundary of each sucrose layer. Preliminary studies revealed that all Golgi vesicles were found at the boundary separating the 0.5 and 1.0 M sucrose layers. This fraction, however, was not completely free from other cell constituents. The contaminating impurities, such as mitochondria, ER, and plastids were removed by a second centrifugation. For this purpose the first fraction containing Golgi vesicles was diluted with BFe up to the original sucrose concentration. This medium was then layered on a discontinuous sucrose gradient composed of the following concentrations: 0.5, 0.7, 0.9 and 1.1 M sucrose in BFe. The preparation was centrifuged in an SW

27.1 for 70 min. at 4 °C at 27,000 rpm after which a fine layer of material was visible on the top of the 0.9 M sucrose layer. The rest of the cell organelles was found at the bottom of the tubes. The earlier mentioned fine layer was removed with a pipette, resuspended in BFe, then centrifuged down in an SW 65 at 50,000 rpm for 60 min. at 4 °C. The resulting pellet was divided into three portions and washed with BFe and prepared for EM in the following three ways:

1. Fixation in 0.1 M Na-K-phosphate buffered (pH 7.2) 2% $KMnO_4$ and embedded in epon.
2. Fixation in 0.1 M Na-K-phosphate buffered (pH 7.2) 1% OsO_4 and embedded in epon.
3. Impregnation in 20% glycerol for 60 min. and prepared for freeze-etching, according to the method described by MOOR et al. (1961).

In order to compare isolated Golgi vesicles with those in vivo, intact germinated pollen was also prepared according to the above mentioned techniques. All preparations were studied with a Philips EM 300 electron microscope.

3. RESULTS

3.1. Golgi vesicles in intact pollen tubes

Ultrastructural examination of the pollen tube tips reveals a number of organelles such as mitochondria, endoplasmic reticulum, Golgi bodies and Golgi vesicles. The structure and organization of these organelles has been previously described by several authors (see introduction), and our results support these findings. This study, however, is restricted to the Golgi vesicles which accumulate in large numbers in the tips of the pollen tubes.

The Golgi vesicles as seen in the micrographs (*figs. 1–3*) all have the same diameter varying between 0.1–0.7 μm. The measurements of Golgi vesicles were only performed on those that showed a clear unit membrane. In freeze-etched preparations it was not always possible to distinguish between Golgi vesicles and small mitochondria (*fig. 3*). The latter have the same appearance as Golgi vesicles when they have been fractured into their outer membrane.

The appearance of the Golgi vesicles (*fig. 1–3*) varies somewhat depending on the fixative used. After $KMnO_4$ fixation the unit membrane is clearly visible around the entire circumference of the vesicle. The contents of the vesicles always appeared electron transparent (*fig. 1*). Sometimes Golgi vesicles were observed in the process of fusion (note arrow in *fig. 1*). This phenomenon, however, has not been observed in very small vesicles that are just pinched off from the Golgi bodies. In contrast to this fixation method, the unit membrane of the Golgi vesicles is difficult to observe after fixation with OsO_4 (*fig. 2*) due to the heavy staining of the surrounding cytoplasm. The Golgi vesicles contain faintly granular material and again occasionally were observed in fusion (arrow in *fig. 2*). The technique of freeze-etching (*fig. 3*) reveals Golgi vesicles with very small particles located on the visible surfaces of the unit membranes.

3.2. Isolated Golgi vesicles

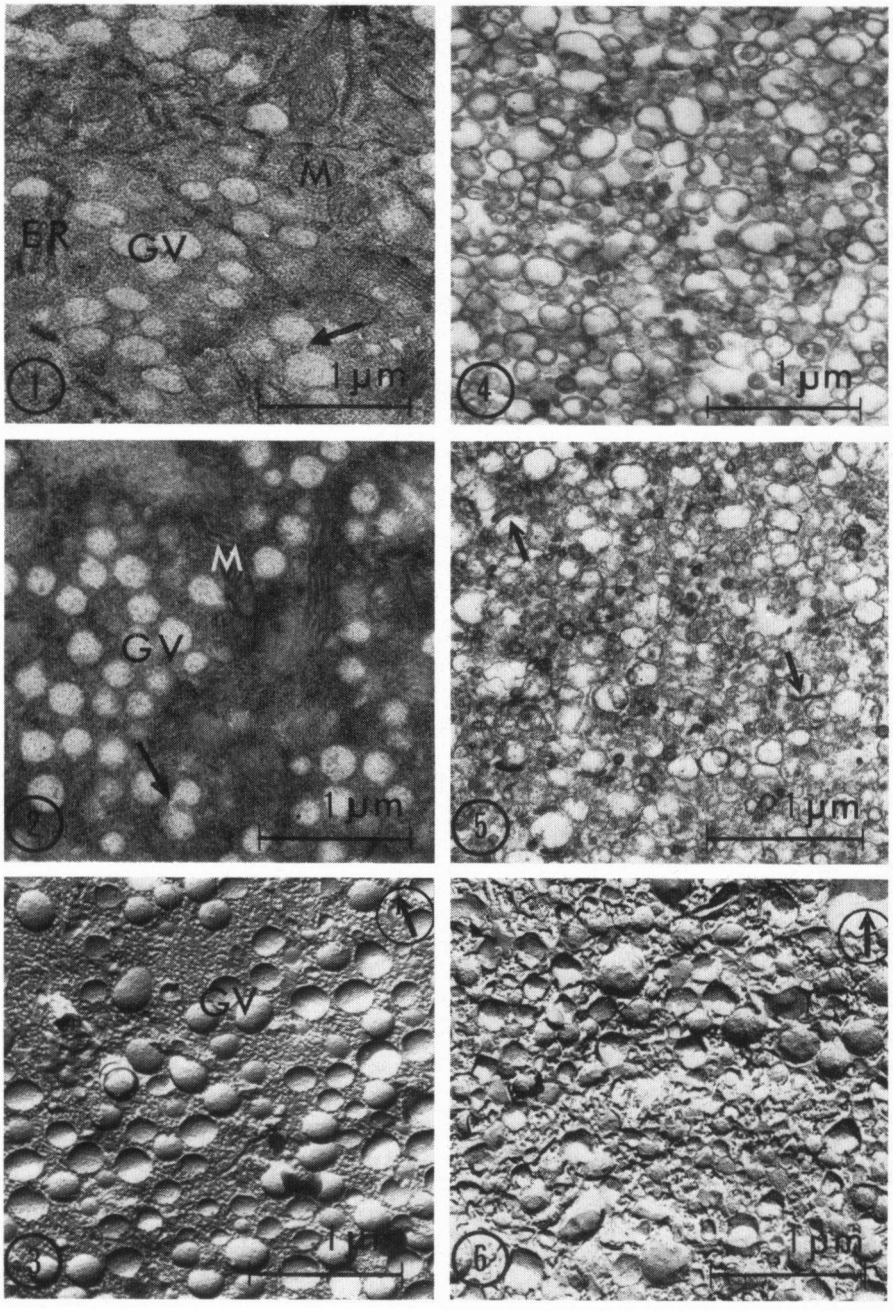
The isolation method resulted in a large quantity of vesicles free from other cell constituents (*figs. 4–6*) except some Golgi cisternae (see arrows in *fig. 5*). The size of the Golgi vesicles was found to be the same (0.1–0.7 μm) as that of the vesicles in the intact cells. Here too the same restrictions were followed when measuring vesicle diameter. As in the intact pollen tubes (*figs. 1 & 2*) after chemical fixation the Golgi vesicles are surrounded by a clearly visible unit membrane (*figs. 4 & 5*), and the contents of the vesicles are similarly electron transparent. On the visible surfaces of the unit membrane (*fig. 6*) again small particles can be found after freeze-etching.

4. DISCUSSION

Homogenization as a first step to liberate Golgi vesicles seems particularly well suited for germinating pollen, since the tip of the pollen tube is weak and is easily ruptured. Light-microscopic observations showed brief homogenization to be sufficient for breaking the tip of the pollen tube, thereby releasing its cytoplasm. The supernatant resulting from low-speed centrifugation of the homogenate was separated on two discontinuous sucrose gradients. This procedure was developed by combining the method of using a discontinuous sucrose gradient to isolate membranes (BLOEMENDAL et al. 1967) and MORRÉ & MOLLENHAUER'S (1964) method of isolating Golgi bodies by repeated centrifugations at successively higher speeds. Preliminary experiments with a continuous gradient resulted in overlapping of the different layers of organelles and the subsequent loss of vesicles by pipetting them off.

Preliminary experiments showed that one single high speed centrifugation was not sufficient for isolating the Golgi vesicles. Electron microscopic examination of material in the sucrose bands after a single centrifugation showed that the band containing Golgi vesicles also contained other organelles. For this reason the material from this band was diluted and placed on a second gradient and centrifuged again. Following the second run, the sucrose band containing the Golgi vesicles was now found to be free from other cell constituents. This method of isolating Golgi vesicles has several distinct advantages over the filtration method described by VAN DER WOUDE et al. (1971). Firstly, the method used here isolated all of the vesicles in the homogenate rather than selecting a specific size of vesicles as in the filtration method. Secondly, contaminating organelles are removed by centrifugation whereas in the filtration method organelles smaller than the sieve (0.45 μm) are also collected in the vesicle fraction. Thirdly, the yield of Golgi vesicles is greater after centrifugation and the vesicles are not subjected to mechanical disturbance.

The use of different methods of fixation showed all three techniques ($KMnO_4$, OsO_4, and freeze-etching) to produce the same results regardless of whether Golgi vesicles were isolated or left in situ in the pollen tube. Furthermore, the results show that the isolation method used does not alter the Golgi vesicles since they were found to have the same size, electron transparency, and unit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membrane characteristics as those found in the pollen tubes. Altogether, these findings indicate that cytological artefacts are minimal.

Examination of the Golgi vesicles in pollen tubes showed that small vesicles were always found adjacent to the Golgi bodies. Larger vesicles were often observed but generally not in the vicinity of the Golgi bodies themselves. Regarding the origin of the large vesicles, our analysis provided no clear evidence whether the Golgi vesicles enlarge in size or whether smaller vesicles fuse to form larger ones. Thus far smaller vesicles have never been observed to fuse whereas fusion of larger vesicles is commonly seen (VAN DER WOUDE et al. 1971).

The micrographs from the replicas reveal a number of small particles located on the fractured membranes of the Golgi vesicles. The distribution of the particles renders it easy to distinguish between the inner and outer layer of the fractured unit membranes. It has been proved that fracturing occurs in the unit membrane (STEERE & MOSELEY 1969). In freeze-etched preparations of the pollen tubes, Golgi vesicles were difficult to distinguish from small mitochondria unless cristae were visible. Therefore, not all vesicle-like structures observed in pollen tubes are Golgi vesicles, but presumably some are small mitochondria (*fig. 3*). In contrast, isolated preparations of Golgi vesicles contained no mitochondria since cristae and/or double unit membranes were never found in chemically fixed preparations.

The method of isolation of Golgi vesicles described in this study makes large quantities of vesicles available for further research. Golgi vesicles can now be collected in quantities large enough to permit biochemical analyses, particularly in relation to the process leading to cell wall synthesis. Golgi vesicle-like structures found in a certain alga seem to possess the synthetic machinery for cellulose elementary fibrils (BROWN & FRANKE 1971). The questions arise, do the Golgi vesicles from *Petunia* pollen tubes possess a similar machinery, and to what extent are they involved in the production of cell wall material? In the future biochemical analyses will be performed on Golgi vesicles and the cell wall in order to provide information on these questions.

Figs. 1–3: Parts of pollen tubes from *Petunia* with Golgi vesicle accumulation.
Fig. 1: After $KMnO_4$ fixation
Fig. 2: After OsO_4 fixation
Fig. 3: After freeze-etching
Figs. 4–6: Golgi vesicles isolated from *Petunia* pollen tubes.
Fig. 4: After $KMnO_4$ fixation
Fig. 5: After OsO_4 fixation
Fig. 6: After freeze-etching.
Magnification figs. 1–6: 23,000 ×
Encircled arrows in figs. 3, 6 indicate the shadow direction.
Abbreviations used: CW: cell wall, ER: endoplasmic reticulum, GV: Golgi vesicles, L: lamellae in the cell wall, M: mitochondria.

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