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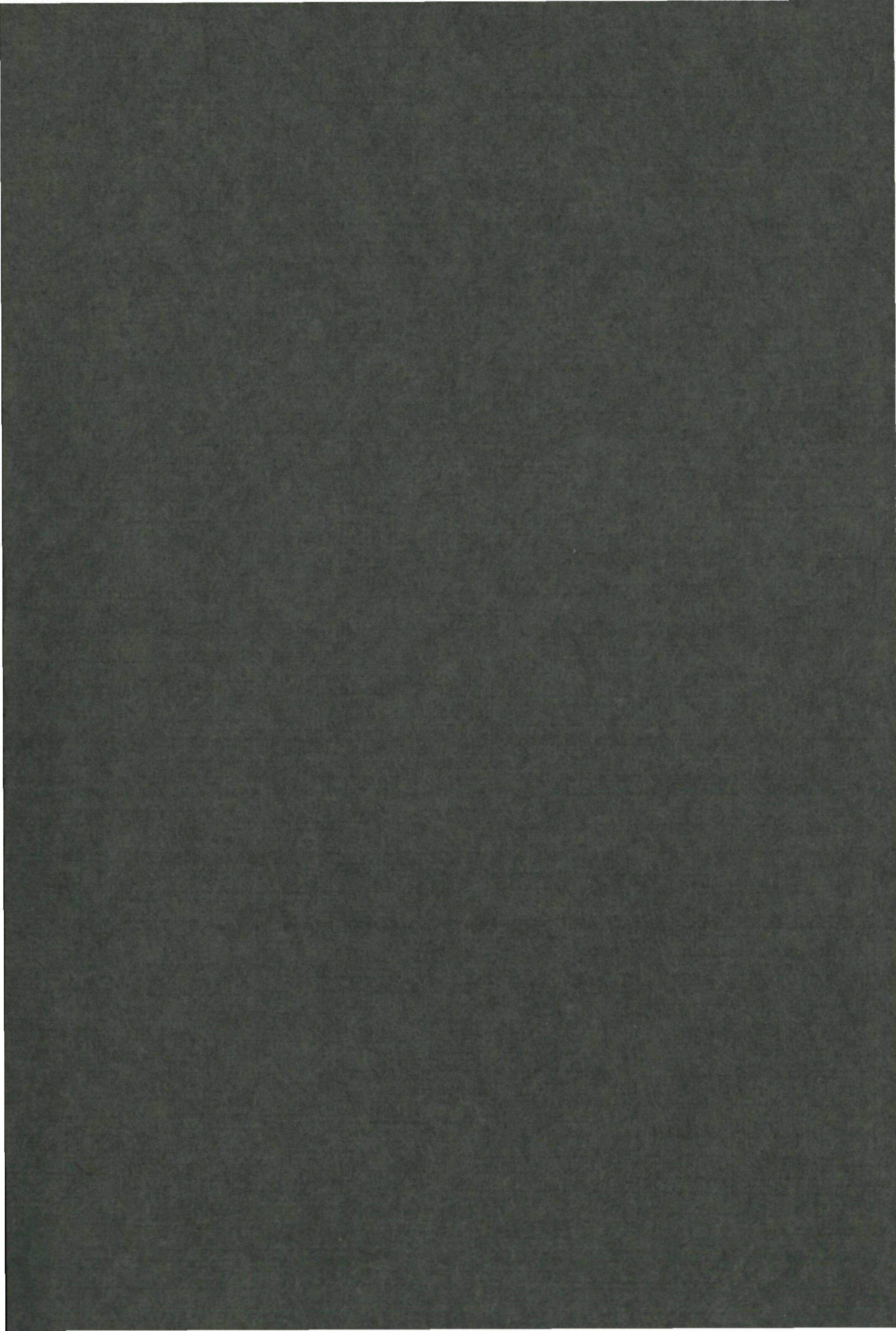
FUNCTION OF GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN

F. M. ENGELS



GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN

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GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. MR. F. J. F. M. DUYNSTEE,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
WOENSDAG 26 JUNI 1974,
DES NAMIDDAGS TE 2 UUR PRECIES
DOOR

FERDINAND MARIA ENGELS

GEBOREN TE MIERLO (N.BR)

H. VEENMAN EN ZONEN B.V. – WAGENINGEN – 1974.

Aan mijn ouders
Aan Henny en Vivienne

Reprints from:
Acta Bot. Neerl. **22**, 6–13 (1973)
Acta Bot. Neerl. **23**, 81–89 (1974)
Acta Bot. Neerl. **23**, 201–207 (1974)
Acta Bot. Neerl. **23**, 209–215 (1974)

INLEIDING

De groei van hyphen, wortelharen en pollenbuizen vindt plaats in de uiterste top van de cellen. Men vindt er een hoge activiteit van de Golgi apparaten die een zeer groot aantal Golgi blaasjes vormen, en vervolgens afsnoeren. Ieder blaasje is omgeven door een unitmembraan. De kleine, pas afgesnoerde, blaasjes worden groter door een actieve groei of door fusie van enkele kleine. De blaasjes worden door een cytoplasmatische stroming naar de celwand getransporteerd. Bij de celwand aangekomen, fuseert de unitmembraan van de blaasjes met de plasmamembraan, en de inhoud van de blaasjes komt buiten het cytoplasma te liggen (NORTHCOTE 1971, FINERAN 1973).

De inhoud van de Golgi blaasjes is met een aantal specifieke reagentia onderzocht. Radioactieve stoffen, aan de cellen toegevoegd, worden in de Golgi blaasjes ingebouwd en verschijnen vervolgens in de celwand. In de Golgi blaasjes blijken enzymatische reacties plaats te vinden. In de blaasjes zijn polysacchariden gevonden, die door enzymen in de blaasjes moeten zijn gesynthetiseerd. (DASHEK & ROSEN 1966, PICKETT-HEAPS 1968, HEYN 1971).

Tot 1970 nam men aan dat alle polysacchariden, die in de celwand voorkomen, in de blaasjes gesynthetiseerd worden; dit echter met uitzondering van cellulose. Dit β 1,4- glucose polymeer zou uitsluitend buiten het cytoplasma op de plasmamembraan of in de celwand gevormd worden. Een uitzondering hierop wordt gevonden in *Pleurochrysis scherffelii*. De wand van deze alg bestaat uit plaatjes die in het cytoplasma in de Golgi blaasjes worden gesynthetiseerd. Een belangrijk gedeelte van deze plaatjes bestaat uit cellulose (BROWN *et al.* 1970, HERTH *et al.* 1972).

Een eerste analyse van de polysaccharide samenstelling van Golgi blaasjes en pollenbuiswand is uitgevoerd by *Lilium longiflorum* door VAN DER WOUDE *et al.* (1971). De met heet water geextraheerde polysacchariden van de Golgi blaasjes vertonen een grote overeenkomst met de polysacchariden van de pollenbuiscelwand.

In dit proefschrift wordt de rol van de Golgi blaasjes in het synthese proces van de pollenbuiswand van *Petunia hybrida* onderzocht. Worden in deze blaasjes inderdaad alle polysacchariden gesynthetiseerd die ook in de celwand voorkomen, dus ook cellulose? Grote hoeveelheden pollenkorrels werden verzameld en in vitro tot kieming gebracht. De Golgi blaasjes en de pollenbuiscelwanden werden geïsoleerd. In een chemische extractie procedure werden uit de Golgi blaasjes en de celwanden de polysacchariden verwijderd, volledig afgebroken en geanalyseerd.

Door de extractie van de celwand met chemische stoffen kunnen grote veranderingen optreden in de morphologische structuur van de celwand. Om deze veranderingen te bestuderen werd na iedere stap van de extractie een monster van celwanden genomen en in het EM onderzocht. Ter vergelijking werden intacte pollenbuizen op verschillende manieren voor het EM geprepareerd.

De aanwezigheid van cellulose in de pollenbuiswand werd reeds verondersteld maar een bewijs hiervoor kon tot nu toe niet worden geleverd De aanwezigheid van cellulose kan in kleine monsters met rontgen-diffractie met zekerkeid worden vastgesteld De verschillende residuen van Golgi blaasjes en pollen buiswanden werden daarom ook met rontgen-diffractie verder onderzocht

De resultaten van het onderzoek worden met de in de literatuur bekende gegevens vergeleken en besproken

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Acta Bot. Neerl. 22(1), February 1973, p. 6–13

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

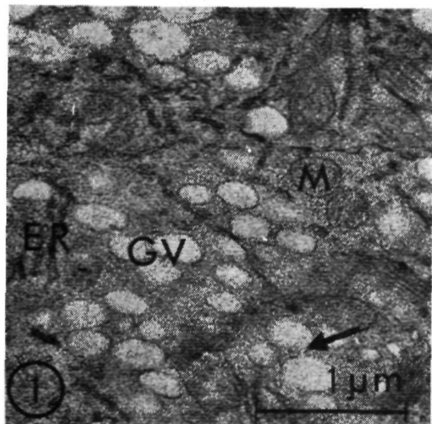
The isolation method resulted in a large quantity of vesicles free from other cell constituents (*figs. 4–6*) except some Golgi cisternae (see arrows in *fig. 5*). The size of the Golgi vesicles was found to be the same (0.1–0.7 μm) as that of the vesicles in the intact cells. Here too the same restrictions 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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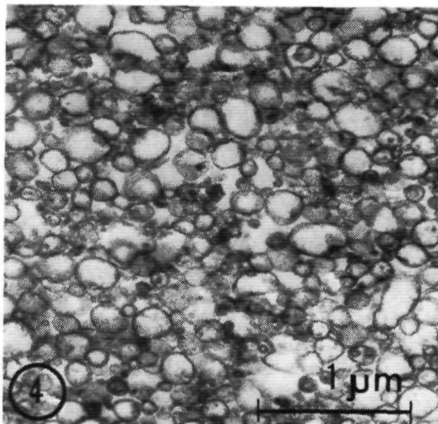
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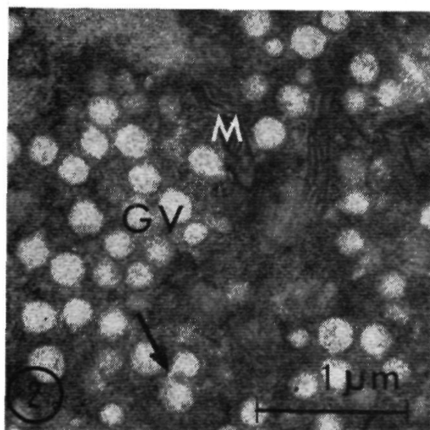
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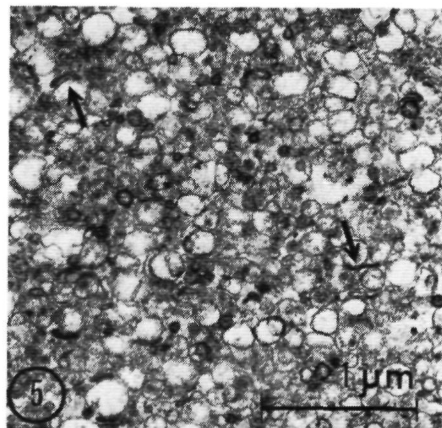
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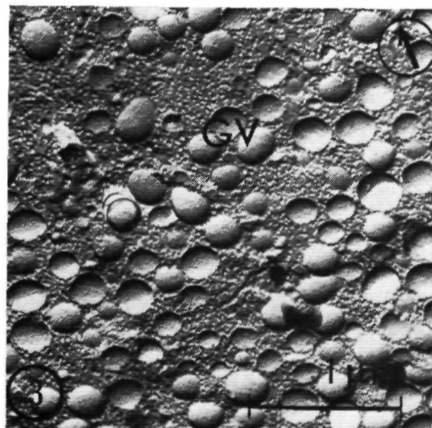
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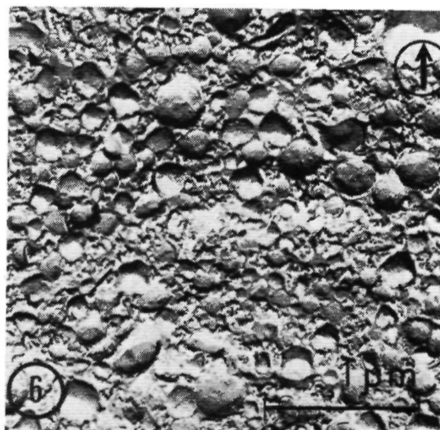
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membrane characteristics as those found in the pollen tubes. Altogether, these findings indicate that cytological artefacts are minimal.

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

ACKNOWLEDGEMENTS

The author is much indebted to Prof Dr H F Linskens for his stimulating interest, to Dr M M A Sassen for his critical discussions, and to Dr G K Livingston for reading the manuscript

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Acta Bot. Neerl. 23(2), April 1974, p. 81–89.

FUNCTION OF GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN. II. CHEMICAL COMPOSITION OF GOLGI VESICLES AND POLLEN TUBE WALL

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SUMMARY

Golgi vesicles and pollen tube walls isolated from germinating *Petunia* pollen were chemically analysed. The protein: lipid ratio of the Golgi vesicles was 70:30. The total carbohydrate contents of the Golgi vesicles and pollen tube wall were found to be 7.2% and 61 % of the dry weights respectively. The alkali insoluble material of the pollen tube wall contained 6% of the total carbohydrate; this material has been found to be cellulosic in nature. The sugar composition of the polysaccharides extracted from the Golgi vesicles and the tube wall is presented and discussed.

1. INTRODUCTION

It is possible to distuinguish three main groups of polysaccharides by means of acidic and alkaline extractions of primary cell walls viz. cellulose, hemicellulose, and pectin. Since it is questionable whether always a complete separation of these polysaccharides is obtained by these procedures (ALBERSHEIM et al. 1967, BLAKE & RICHARDS 1971, HERTH et al. 1972, NANCE 1973, VAN DER WOUDE et al. 1971), quantitative results are to be considered only as rough estimates.

In *Lilium longiflorum* Thunb. the hot water extracts of pollen tube walls and Golgi vesicles isolated from the pollen tubes have a similar carbohydrate composition (VAN DER WOUDE et al. 1971). Polysaccharides are found in the Golgi vesicles of *Lilium* pollen tubes (DASHEK & ROSEN 1966, VAN DER WOUDE et al. 1971) and of wheat seedlings (*Triticum vulgare*) (PICKETT-HEAPS 1968). The transport of polysaccharides by means of Golgi vesicles to the plasma membrane and the release of the vesicle contents outside the cytoplasm has been observed by NORTHCOTE & PICKETT-HEAPS (1966) and VAN DER WOUDE & MORRÉ (1968).

Cellulose has not been found as a component of the Golgi vesicles in plant materials, except in the alga *Pleurochrysis scherffelii* (BROWN & FRANKE 1971). The cell wall of this alga is composed of scales synthesised in the Golgi vesicles (HERTH et al. 1972, BROWN & FRANKE 1971). These results indicate an involvement of Golgi vesicles in cell wall synthesis at least in algae.

In a preceding paper (ENGELS 1973) a method was described for isolating Golgi vesicles in large quantities. In this article the carbohydrate composition of Golgi vesicles and the pollen tube wall is given. The results are discussed in

connection with the question whether the polysaccharide composition of Golgi vesicles is similar to that of the tube wall and whether the Golgi vesicles contain the machinery for the synthesis of the pollen tube wall polysaccharides.

2. MATERIALS AND METHODS

2.1 Isolation of Golgi vesicles

The isolation of Golgi vesicles (GV) has been described previously by ENGELS (1973) The GV were stored at −20 °C prior to analysis.

2.2 Isolation of the pollen tube wall

Pollen from *Petunia hybrida*, strain W 166K, were grown for 15 hr under conditions as described previously (ENGELS 1973). Germinated pollen were centrifuged and washed. Pollen grains with large tubes were separated from those with small tubes and ungerminated ones by the use of a fine screen sieve. The water adhering to the material was removed with filter paper and the germinated pollen were placed in a mortar. Liquid nitrogen was added and pollen tubes were broken at several places by light grinding while the pollen grains remained intact. After thawing the homogenate was placed on a 20 μm sieve and small tube pieces were washed through the sieve with water. The adhering cytoplasm was removed by a short ultrasonic treatment and subsequent washing The pollen tube walls (PTW) prepared in this manner were stored at −20 °C prior to analysis.

2.3 Extraction of GV and PTW

GV and PTW were pre-extracted with a series of 80, 90, and 100% ethanol (ETOH) solutions, respectively. The extracts were combined and evaporated to dryness The residues after ETOH extraction were extracted 1) twice with 100% diethylether, 2) 1 hr with boiling water, 3) 1 hr with 1N HCl at 100 °C, 4) 15 hr with 2N KOH at room temperature. The supernatants of 1) to 3) were evaporated directly to dryness. The supernatant of 4) was first passed through a Dowex-50 (H^+) column and then evaporated to dryness The residues of the GV and PTW after KOH extraction were dried. All fractions obtained by this procedure were hydrolysed in 80% (V/V) trifluoro-acetic acid for 48 hr at 120 °C. The hydrolysates were evaporated and the acid was removed by repeated addition of water and evaporation

2.4. Separation of neutral and acidic sugars

The neutral and acidic sugars present in the various extracts were separated and chromatographed according to the methods used by KROH (1973) The ETOH extracts of the GV and the PTW were either directly chromatographed or hydrolysed prior to chromatography.

2.5. Measurements

The protein content of the ETOH soluble and ETOH insoluble fractions of the

GV and PTW were measured according to LOWRY et al. (1951). The ETOH extract of the GV was therefore further separated by the chloroform-methanol-water method of BLIGH & DYER (1959).

Lipid estimations were done gravimetrically as well as colorimetrically according to RENKONEN (1961).

Carbohydrate estimations on ETOH soluble and insoluble fractions of the GV and PTW and on the alkali insoluble fraction of the PTW were carried out colorimetrically by means of the phenol-sulfuric acid method (DUBOIS et al. 1956).

3. RESULTS

3.1. Composition of the GV and PTW

The components of the GV and PTW are divided into ETOH soluble and insoluble fractions. The ETOH soluble fraction of the GV is divided into protein, lipid, and carbohydrate fractions *(table 1)*.

Table 1. The amount of carbohydrate, protein, and lipid in ETOH soluble and ETOH insoluble fractions as percentage of the original dry weights.

	ETOH soluble		ETOH insoluble	
	GV	PTW	GV	PTW
Carbohydrate	4.0	3.5	3.2	57.5
Protein	4.4	0.0	40.0	13.3
Lipid	5.0	0.0	13.5	0.0
Unrecovered	28.6	0.0	1.3	25.7
Total	42.0	3.5	58.0	96.5

The ETOH extraction removes 42.0% of the total dry weight of the purified GV. In addition to carbohydrates (4.0%) distinct amounts of proteins (4.4%) and lipids (5.0%) are present. In contrast to GV only 3.5% of the tube wall material is soluble in ETOH. This 3.5% represents exclusively carbohydrates. The ETOH insoluble material of the GV fraction contains mostly protein (40.0%) and lipid (13.5%) and only a small amount of carbohydrate (3.2%), while in the fraction of the PTW carbohydrates (57.7%) and protein (13.3%) are the main constituents. Lipid could not be found in the ETOH insoluble fraction of PTW.

3.2. Neutral sugars

Separation by thin-layer chromatography (TLC) of the neutral sugars from the hydrolysates of the various extracts gives the monosaccharides commonly found in plant cell walls *(table 2)*.

The ETOH fraction of the GV contains xylose but lacks arabinose whereas the reverse is true for the PTW. Quantitative differences are found in mannose and glucose. Both ETOH fractions reveal a reducing carbohydrate (X) on

Tabel 2. Monosaccharides detected in the different fractions after TFA hydrolysis. The presence or absence of each monosaccharide is indicated by the symbols + and −, respectively, as revealed by comparison with reference sugars. The symbol ⊗ is used when a sugar could be barely detected, the symbol ++ when a sugar gives a very intensive spot.

	Fractions									
	ETOH		H_2O		HCl		KOH		TFA	
Sugars	GV	PTW	GV	PTW	GV	PTW	GV	PTW	GV	PTW
rha	+	++	+	+	++	+	++	++	−	−
fuc	+	+	+	+	+	+	+	−	−	−
xyl	+	−	+	−	⊗	⊗	+	−	−	−
ara	−	+	−	−	+	−	+	−	−	−
man	++	+	++	−	++	+	+	+	−	−
glu	+	++	+	++	+	++	+	+	+	+
gal	−	−	⊗	+	⊗	−	⊗	+	−	−
X	+	+	−	−	−	−	−	−	−	−
glu UA	−	−	+	⊗	+		−	−	−	−
XX UA	+	−	−	−	−	−	−	−	−	−
gal UA	−	−	+	⊗	−	−	−	−	−	−

chromatographs located between the origin and galactose both before and after TFA hydrolysis. The nature of this compound remains unknown. The only difference between the unhydrolysed and hydrolysed ETOH fraction is noted at the origin which is cleaned up after hydrolysis.

The composition of the hot water soluble fraction is the same in GV and PTW, with one exception. Xylose is present only in the GV fraction. On chromatographs of the GV fraction mannose stains more intensily, i.e. is predominant over glucose. In the PTW fraction, however, glucose is predominant over mannose. Since the staining is less sensitive for mannose than for glucose, this indicates a large difference in the concentration of the two sugars. The HCl extract also shows a large difference between GV and PTW with respect to the relative concentrations of mannose and glucose. The KOH extract of the PTW lacks the sugars fucose, xylose, and arabinose that are present in the KOH extract of the GV. After TFA hydrolysis of the KOH residue of the GV and the PTW fraction a clear spot appears which corresponds to glucose.

The sugar compositions of the ETOH, H_2O, HCl, and KOH extracts of the GV differ only slightly; galactose appears as an additional sugar in the H_2O-extract and this extract lacks the unknown sugar (X). The HCl-extract reveals the presence of arabinose and galactose which are absent from the ETOH-extracts. The only distinction between the HCl and H_2O extracts is the presence of arabinose in the HCl extract. The sugar composition of the KOH extract is nearly the same as that of the HCl extract.

The differences in sugar composition of the H_2O and HCl extract of the PTW fractions are small. The HCl extract reveals no galactose and the presence of xylose is doubtful. The KOH and HCl extractions differ in the presence or absence of fucose and galactose. The KOH extract contains galactose and lacks fucose while the reverse is found in the HCl extract.

3.3. Acidic sugars

The GV fraction contains an acidic carbohydrate with a mobility between galacturonic acid (gal UA) and glucuronic acid (glu UA). In the ETOH-extract of the GV and PTW no galacturonic acid and glucuronic acid could be detected. Glucuronic acid and galacturonic acid are found in the hot water and HCl fractions of both the GV and PTW. In the hot water fraction of the PTW the uronic acids are barely detectable. In addition to these uronic acids there are traces of acidic carbohydrates with higher mobilities than galacturonic acid and glucuronic acid.

3.4. Nature of the alkali insoluble material

Analysis of the alkali insoluble material of PTW preparations reveals that the carbohydrate content of this material accounts for 6% of the total dry weight. Table 2 shows that this carbohydrate material consists exclusively of glucose. Preliminary investigation by X-ray diffraction of this material from PTW reveals its cellulosic nature. The nature of the alkali insoluble material of the GV is under study.

4. DISCUSSION

The protein and lipid content of the GV in the ETOH soluble fraction is 4.4% and 5.0% of the dry weight, respectively. Small amounts of carbohydrates are found in the lipid as well as in the protein fraction. Is is not clear whether a part of the protein and lipid materials is possibly bound to the ETOH soluble carbohydrates. Some evidence has been presented indicating that such carbohydrate complexes with proteins and lipids may be precursors in polysaccharide synthesis (ANDERSON et al. 1965, DATTA et al. 1973, KNEE 1973). TALMADGE et al. (1973) found that 2% of the dry weight of sycamore primary cell walls was soluble in chloroform-methanol. This material was found to be hydroxyproline rich glycoprotein. In the ETOH soluble fraction of the *Petunia* pollen tube no protein has been detected.

The protein: lipid ratio in the GV of *Petunia* pollen tubes is 70:30. In HeLa cells the protein:lipid ratio in plasmamembranes is 60:40 and in Golgi cisternal membranes 33:67 (BOSMANN et al. 1968). Thus it appears that the protein content of the GV of *Petunia* pollen tubes is quite high. One may, therefore, assume that not all protein isolated from GV represents structural protein of the unit membrane of the GV but that part of it derives from enzymes, possibly from those involved in polysaccharide biosynthesis. Some of these enzymes have been detected in GV (FRIEND 1969, HARRIS & NORTHCOTE 1971, HEYN 1971, NORTHCOTE & PICKETT-HEAPS 1966, YOUNG 1973).

The total carbohydrate content of the PTW is 61% and the protein content 13.5% of the dry weight. These data agree very well with the values reported for the hyphal walls of *Ceratocystis* (HARRIS & FABER 1973) and the cell walls of sycamore cell suspension cultures (TALMADGE et al. 1973). The total carbohydrates of the PTW contain 6% alkali insoluble material with glucose as the only

monosaccharide after TFA hydrolysis This alkali insoluble material proves to be cellulosic in nature as will be published in detail later The amount of this cellulosic material in the PTW is in good agreement with the cellulose content (7 %) in *Lilium* pollen tubes (VAN DER WOUDE et al 1971)

Chromatographs of hot water fractions of the GV reveal the monosaccharides rhamnose, fucose, mannose, xylose, glucose, and galactose, the PTW gives the following monosaccharides rhamnose, fucose, mannose, glucose, and galactose With the exception of arabinose in the GV and xylose and arabinose in the PTW, these sugars are the same as those found in the GV and PTW from *Lilium* (VAN DER WOUDE et al 1971)

The sugar composition of the ETOH and hot water extracts of the GV is nearly identical with the exception of galactose and sugar X *(table 2)* In the ETOH fraction galactose is absent, sugar X is present, while the reverse is the case in the hot water extract

The corresponding extracts of the PTW show the same behaviour with respect to these two sugars Arabinose is found only in the ETOH extract of the PTW Hot water extraction of the GV and the PTW, without ETOH prior to it, results in a sugar composition that is found when the separated extracts of ETOH and hot water are combined The differences in sugar composition found in the subsequent extraction with ETOH and hot water stresses the importance of ETOH as starting extraction medium

The ETOH extracts of the GV and the PTW contain probably different kinds of components Some of these are monosaccharides visible on TLC plates prior to TFA hydrolysis It is known that free monosaccharides may occur in plant material, although mostly glycosides are found (SCHAFFER 1972) After TFA hydrolysis a clean origin is obtained on TLC plates However, no additional sugars are found This could mean that ETOH has extracted besides monosaccharides oligosaccharides or carbohydrates attached to proteins and lipids These last substances were also found in the ETOH extract and could be hydrolysed by TFA

When the extracts of the GV are compared in the subsequent steps of extraction with respect to their sugar composition, it is observed that the ETOH and hot water fractions resemble one another The same observation was made with respect to the HCl and KOH extracts The differences in the monosaccharide composition of the various extracts of the PTW are more pronounced than those of the GV, especially with respect to the monosaccharides xylose, fucose, arabinose, and galactose

The ETOH fraction of the GV reveals an acidic sugar with a mobility between galacturonic acid and glucuronic acid Apart from these no other uronic acids are found in the ETOH fraction of both the GV and PTW Glucuronic acid and galacturonic acid are found in the H_2O and HCl fractions of the GV and the PTW The presence of galacturonic acid and glucuronic acid in pollen tube walls from *Lilium* was reported by LABARCA & LOEWUS (1972) However, VAN DER WOUDE et al (1971) found only galacturonic acid in Lilium pollen tube walls Galacturonic acid and glucuronic acid are commonly found in pectin and

hemicellulose respectively (ASPINALL 1970, BAUER et al 1973, DANISHEFSKY et al. 1970, TALMADGE et al 1973)

The monosaccharide composition of the polysaccharides extracted from the GV and the PTW are very similar A number of data such as the enzymes found in Golgi vesicles (FRIEND 1969, HARRIS & NORTHCOTE 1971, HEYN 1971, NORTHCOTE & PICKETT-HEAPS 1966, YOUNG 1973), the presence of polysaccharides in Golgi vesicles (DASHEK & ROSEN 1966, VAN DER WOUDE et al 1971), the increasing stainability of the Golgi vesicles on their way from Golgi cisternae up to the cell wall (VAN DER WOUDE et al 1971), the resemblance in monosaccharide composition of the polysaccharides of the GV and the PTW, the fusion of Golgi vesicles with the plasmalemma (SIEVERS 1963, SASSEN 1964, CRANG & MILES 1969, ROSEN & GAWLIK 1965, VAN DER WOUDE & MORRÉ 1968, VAN DER WOUDE et al. 1971) strongly support the idea that Golgi vesicles are involved in the synthesis of the cell wall material. It can, however, at present not be excluded that besides Golgi vesicles also other organelles contribute to the cell wall synthesis. However, it must be mentioned that differences in monosaccharide composition of the GV and the PTW are observed Pollen tube preparations contain a relatively low amount of pollen tube tips (light microscopic observation). Golgi vesicles fuse with the plasmalemma at the tip of the growing pollen tube It could be that synthesis of cell wall material is continued after this fusion process and that sugars may be brought to the cell wall not only by Golgi vesicles but perhaps also by an other system Perhaps the particles derived from the endoplasmatic reticulum, which were observed by VAN DER WOUDE et al (1971) in *Lilium* represent such a system

ACKNOWLEDGEMENTS

The author is much indebted to Prof Dr H F Linskens for his stimulating interest, to Dr M M A Sassen and Dr M Kroh for critical discussions, and to E G Kirby for reading the manuscript

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FUNCTION OF GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN. III. THE ULTRASTRUCTURE OF THE TUBE WALL

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SUMMARY

Pollen tubes from germinated *Petunia* pollen were prepared in different ways for electron microscopic investigation of their walls. The ultrastructural features of the tube walls depend strongly on the preparation methods used. The tube wall was found to be composed of two layers or appeared striated after fixation with $KMnO_4$ and OsO_4, respectively. After freeze-etching the tube wall is twice as thick as after chemical fixation and a fine striation is visible. Subsequent extractions dissolve the matrix substances and a network of microfibrils is left, which is not composed of lamellae.

1. INTRODUCTION

In literature a variety of ultrastructures have been described in cell walls of pollen tubes. The different features of tube walls are strongly dependent upon the fixatives used. After prefixation with glutaraldehyde and postfixation with OsO_4 the walls at the very tip of *Lilium* pollen tubes consist of compartments. These compartments are not observed when glutaraldehyde is washed out prior to OsO_4 fixation (ROSEN & GAWLIK 1966). OsO_4 fixation alone shows the tube wall to be composed of two layers (ROSEN et al. 1964, FRANKE et al. 1972); sometimes the outer layer appears to be lamellated (VAN DER WOUDE et al. 1971). This lamellated tube wall has also been found in pollen tube walls from *Impatiens holstii* after aceton fixation and post-staining with $CuBr_2$ (FLYNN 1968). $KMnO_4$ fixation reveals a thick, a thin, or a bilayered tube wall in Lilium pollen tubes, depending on the concentration of $KMnO_4$ (ROSEN & GAWLIK 1966). In micrographs from *Petunia* pollen tubes it has been observed that the tube wall is not homogeneous after $KMnO_4$ fixation (SASSEN 1964, KROH 1967, VAN DER PLUYM & LINSKENS 1966). Altogether, the morphological ultrastructure of pollen tube walls as revealed by chemical fixation remains obscure and difficult to interpret.

After treating pollen tubes of *Petunia* and *Brassica* with strong alkali and acid, a skeleton of microfibrils is found which is assumed to consist of cellulose (SASSEN 1964, KROH 1964). In *Brassica* it has been shown that the skeleton is composed of one lamella of microfibrils (KROH 1964). A systematic breakdown of *Impatiens* pollen tubes by enzyme and chemicals, followed by negative staining, has been carried out by FLYNN (1968). By this treatment the matrix is

dissolved and a skeleton of cellulose microfibrils remains

In a preceding experiment the pollen tube cell wall of *Petunia* pollen was chemically analysed after a series of extractions (ENGELS 1974) During the subsequent steps of the extraction procedure samples were taken from the same material to make platinum carbon replicas in order to get more information on these pollen tube walls The present report deals with the results obtained after fixation of intact pollen tubes and with the ultrastructural changes observed in the extracted pollen tube walls

2 MATERIALS AND METHODS

Germinating *Petunia* pollen was prepared for thin sectioning and freeze-etching as previously described (ENGELS 1973)

Platinum-carbon replicas were made from the surface of pollen tubes after subsequent treatments with ethanol-ether, boiling water, HCl, and KOH under conditions described previously (ENGELS 1974)

Pollen tubes treated with a mixture of acetic-acid and hydrogen peroxide (1 1) were embedded in butylmethacrylate and sectioned The butylmethacrylate in the sections was dissolved in amylacetate and the remaining parts of the tube walls were shadowed with platinum

All preparations were studied with a Philips EM 201

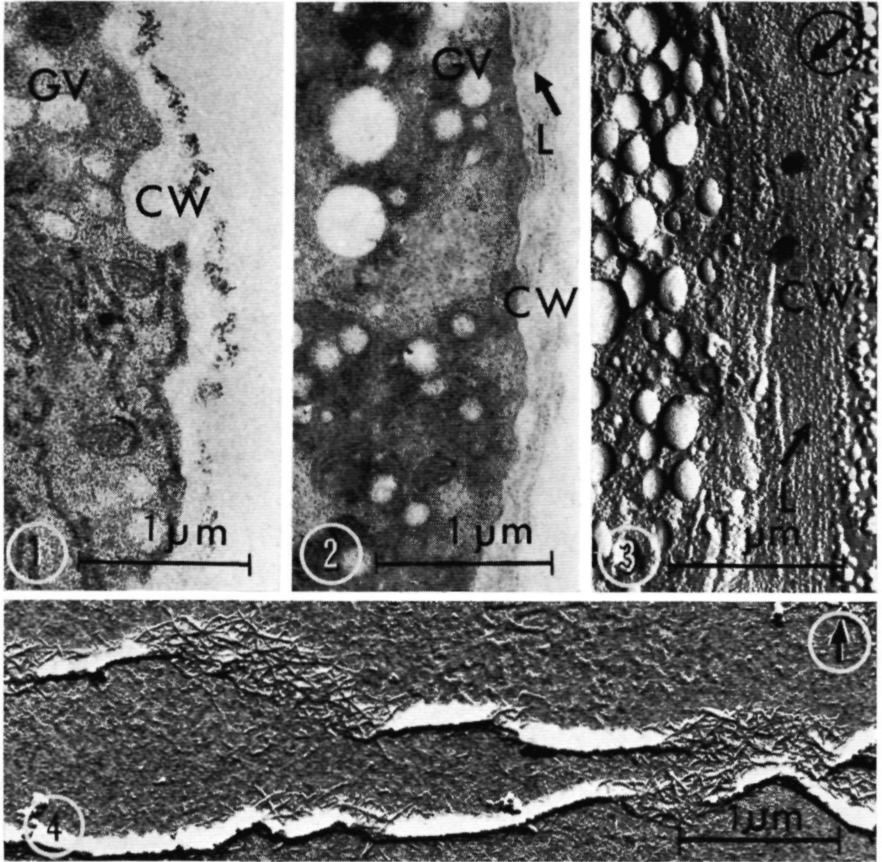
3 RESULTS

3 1 The ultrastructure of intact pollen tube walls after thin sectioning and freeze-etching

Great differences are observed in the tube wall structure, depending on the preparations used for electron microscopy After fixation with $KMnO_4$ the cell wall shows two layers The inner layer is electron-transparant, comparable to the contents of the Golgi vesicles The outer layer is electron dense and has a granular appearence (*fig 1*) In contrast, pollen tubes fixed with OsO_4 show alternating electron transparant and electron dense lamellae (*fig 2*) With both fixations undulations of the plasmalemma are clearly visible These are probably the result of fusion from Golgi vesicles with the plasmalemma However, it is striking that in freeze-etch preparations the tube wall is nearly twice as thick as a chemically fixed one Furthermore, a weak indication of lamellation is visible (*fig 3*)

3 2 Ultrastructure of tube walls treated with acetic-acid and hydrogen peroxide

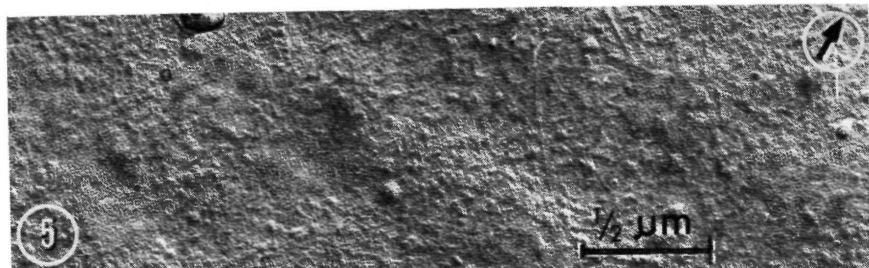
By dissolving the butylmetharylate from thin sections with pollen tubes treated with acetic-acid and hydrogen peroxide, parts of the tube wall are turned over to one side while others stay upright (*fig 4*) The skeleton of microfibrils shows the same appearence as after KOH treatment It is composed of one lamella of microfibrils



Figs. 1–4. Ultrastructure of the pollen tube wall in transverse sections:
Fig. 1. After $KMnO_4$ fixation; CW = cell wall, GV = Golgi vesicle.
Fig. 2. After OsO_4 fixation; in the cell wall (CW) a lamellation (L) is found.
Fig. 3. After freeze-etching; the thick cell wall exposes a fine lamellation (L).
Fig. 4. After treatment with acetic-acid and hydrogen peroxide; a network of microfibrils is exposed.

3.3. Morphological changes after chemical treatments

The surface of the untreated tube wall has a relatively smooth appearence (*fig. 5*). Occasionally indications of short pieces of microfibrils can be seen. Small granules are dispersed over the surface. After treatment with ethanol-ether, microfibrils embedded in amorphous matrix material become visible all over the surface (*fig. 6*). Extraction of the tube wall with boiling water exposes the uppermost microfibrils better and over longer distances (*fig. 7*). Although still more fibrils become visible after extraction with HCl, they can only be followed over shorter distances. Rough granular material is dispersed over the surface (*fig. 8*). After KOH treatment all the matrix substances are removed. The tube wall residue is composed of a skeleton of microfibrils (*fig. 9*). A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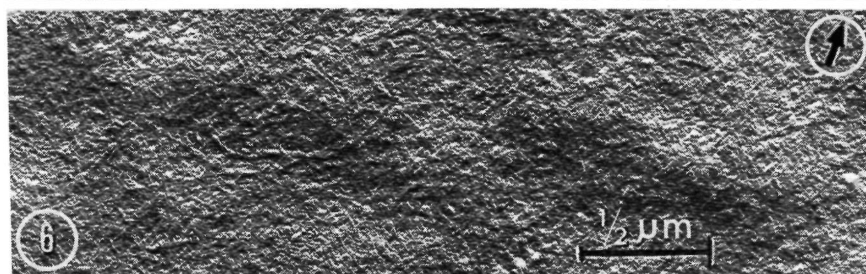
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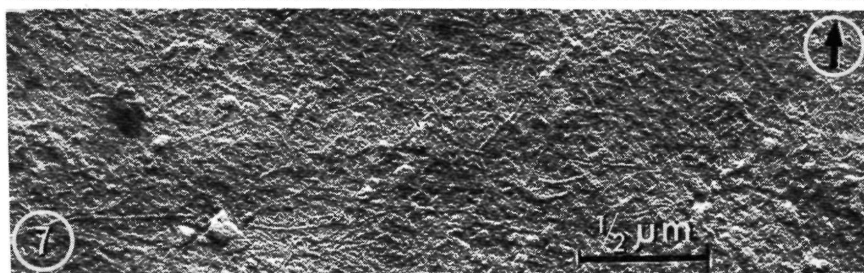
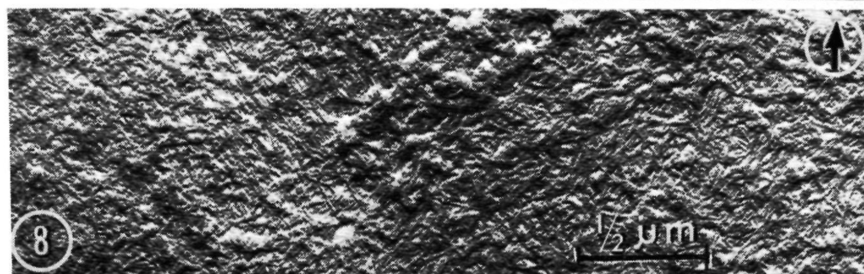
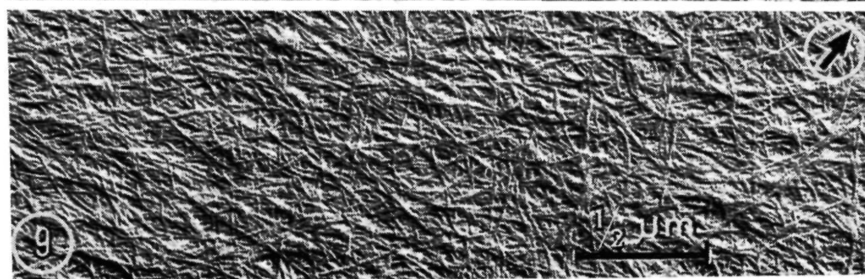

7
½ μm


8
½ μm


9
½ μm

preferential direction of the fibrils could not be observed. The diameters of the fibrils measure 15–20 nm.

4. DISCUSSION

The bilayered structure observed in pollen tube walls of *Petunia* after $KMnO_4$ fixation is in agreement with earlier findings in pollen tubes of the same plant (SASSEN 1964, KROH 1967, VAN DER PLUYM & LINSKENS 1966) and in *Lilium* (ROSEN & GAWLIK 1966). This structure is also in agreement with that found after OsO_4 fixation in *Lilium* (ROSEN et al. 1964, FRANKE et al. 1972). The lamellated appearence of the tube wall, found after OsO_4 fixation in *Petunia*, corresponds with the observations made by VAN DER WOUDE et al. (1971) on *Lilium* pollen and by FLYNN (1968) on *Impatiens* pollen. From the results it is difficult to decide which of both pictures of the tube wall is the correct one. The different fixation and staining procedures result in different features of the wall.

In tube walls of *Petunia* the lamellation observed after OsO_4 fixation is also found after freeze-etching. However, if one treats the tube walls with acetic-acid and hydrogen peroxide, only one lamella of microfibrils becomes visible. The same result is reported for tube walls of *Brassica* (KROH 1964). The pollen tube wall can be considered as a primary cell wall with randomly distributed microfibrils. By removal of the matrix the fibrils are pressed together and the skeleton therefore appears in sections as one lamella.

Remote from the tip an orientation of fibrils in tube walls has been observed by SASSEN (1964). However, this could not be confirmed in our studies.

A correlation has been made between the lamellae seen after fixation with OsO_4 in the wall of stigmatic papillae of *Brassica* after penetration of pollen tubes into the papilla wall and the cellulose lamellae left in the papilla wall after purification with acetic-acid and hydrogen peroxide (KROH 1964). This correlation may be questioned. In tube walls of *Petunia*, several lamellae are seen after OsO_4 fixation and freeze-etching; in contrast only one lamella is observed in sections after removal of the matrix. From this observation one may conclude that the lamellar structure of the tube wall after chemical fixation does not reflect the cellulose lamellae. A correlation made between EM-structures observed after chemical fixation and their chemical and physical nature "in situ" must, therefore, be handled with caution.

After extraction of the tube wall with boiling acetic-acid and hydrogen peroxide a network consisting of microfibrils remains. Substances which can resist this treatment could not be found. The treatment with acetic- acid and hydrogen

Figs. 5–9. Platinum carbon replicas of the tube walls after different treatments:
Fig. 5. Untreated; a fine granulation is visible all over the surface. Some microfibrils are visible.
Fig. 6. After treatment with ethanol-ether.
Fig. 7. After treament with boiling H_2O.
Fig. 8. After treatment with N-HCl.
Fig. 9. After treatment with 2N KOH. The encircled arrows indicate the shadow direction.

peroxide at lower temperature (SASSEN 1964) may be the cause of insufficient extraction

A systematic extraction of the pollen tube wall by different solvents has been followed by making platinum carbon replicas The presence of matrix material and microfibrils could be demonstrated A fine granulation on the surface of untreated walls which was also observed after $KMnO_4$ fixation is removed by ethanol-ether The fibrils are now observed distinctly to be embedded in matrix material After HCl treatment the matrix shows a rough granular structure partly covering the microfibrils In *Impatiens* pollen tubes a change in dimensions of granular material has been observed by negative staining after several extractions which is considered to be a manifestation of the same wall material (FLYNN 1968) In *Petunia* it is assumed that HCl dissolves the matrix substance and that a part of it precipitates during the drop of temperature preceding the removal of HCl by water washings This material is then irregularly dispersed over the surface of the tube wall A network of microfibrils is obtained after the last extraction with KOH An orientation of microfibrils could not be observed

After freeze-etching the tube wall is nearly twice as thick as after chemical fixation In this study only transverse sections of the cell wall were studied excluding the effects of sectioning on wall thickness Since it is accepted that freeze-etching does not cause artefacts we must conclude that the thin tube walls, seen after chemical fixation, are probably the result of shrinkage during fixation and dehydration This must be taken into consideration when the thickness of embedded cell walls is measured

ACKNOWLFDGEMENTS

The author is much indebted to Prof Dr H F Linskens for his stimulating interest, to Dr M M A Sassen and Dr M Kroh for critical discussions, to Dr Ir G W M Barendse for reading and to Mrs E R Tummers-Moesker for typing the manuscript

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Acta Bot Neerl 23(3), June 1974, p. 209–215

FUNCTION OF GOLGI VESICLES IN RELATION TO CELL WALL SYNTHESIS IN GERMINATING PETUNIA POLLEN. IV. IDENTIFICATION OF CELLULOSE IN POLLEN TUBE WALLS AND GOLGI VESICLES BY X-RAY DIFFRACTION

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SUMMARY

The residues of Golgi vesicles and tube walls from germinating *Petunia* pollen were studied by application of the X-ray diffraction technique after different chemical treatments In the Golgi vesicles as well as in the tube walls the presence of a cellulose component was demonstrated This result led to the assumption that the machinery for cellulose synthesis is already present in the Golgi vesicles

1 INTRODUCTION

Two alternative hypotheses have been put forward with respect to the site of cellulose synthesis The first hypothesis claims the cellulose synthesis to occur at the outside of the plasmalemma or in the cell wall itself (Preston 1963, Roelofsen 1965, Staehelin 1966, 1968, Muhlethaler 1967, Barnett 1969, Frey-Wyssling 1969, Barnett & Preston 1970, Robinson & Preston 1971, 1972) The other hypothesis stresses the likelihood of the cellulose synthesis to be located within the cytoplasm in specific organelles (Ledbetter & Porter 1963, Marx-Figini & Schultz 1966, Brown et al 1970, Herth et al 1972, Gamalei 1973)

Until 1970 the first hypothesis was generally accepted (Colvin 1972), although the evidence up to then was only obtained by morphological studies However, the evidence obtained by chemical and morphological studies performed with the alga *Pleurochrysis scherffelii* strongly supports the second hypothesis (Brown et al 1970, Herth et al 1972) In this organism a cellulosic compound was found to be present in Golgi vesicles as scales These are transported via the Golgi system to the outside of the plasma membrane

Chemical data obtained by analysing the Golgi vesicles from germinating *Petunia* pollen has led to the assumption that the alkali-resistant material found in the Golgi vesicles as well as in the tube wall material might be of cellulosic nature (Engels 1974a) Till now no evidence has been obtained which indicated that the fibrillar material observed in pollen tube walls consists of cellulose Only assumptions have been made in this direction (Sassen 1964, Kroh 1964, Flynn 1968). The present paper deals with a study of X-ray dif-

fraction patterns of material from tube walls and Golgi vesicle contents obtained from germinated pollen after different extraction procedures. This study is intended to obtain evidence about the presence of cellulose in these materials.

2. MATERIAL AND METHODS

Golgi vesicles and pollen tube walls from germinated *Petunia* pollen were isolated as described previously (ENGELS 1973, 1974a). The isolated material was treated with N-HCl at 100°C for 1 hr. The residue of the Golgi vesicles was subsequently treated with 2N KOH for 15 hrs at room temperature. In addition a chloroform extraction was carried out on the KOH residue of the Golgi vesicles during 60 hrs at room temperature. The residues from the tube wall after N-HCl and the Golgi vesicles after chloroform extraction were treated with 20% NaOH at 100°C for 1 hr. All solutions used for extraction were centrifuged at 27,000 rpm in a SW 27.1 for 1 hr before they were applied, to avoid contamination with cell wall material. Even after artificial addition of cellulose fibrils from tube walls to the isolation medium of Golgi vesicles no fibrils were found in the supernatant in which the Golgi vesicles accumulated.

Cotton hairs treated with N-HCl and 20% NaOH under conditions as described previously served as a reference for cellulose.

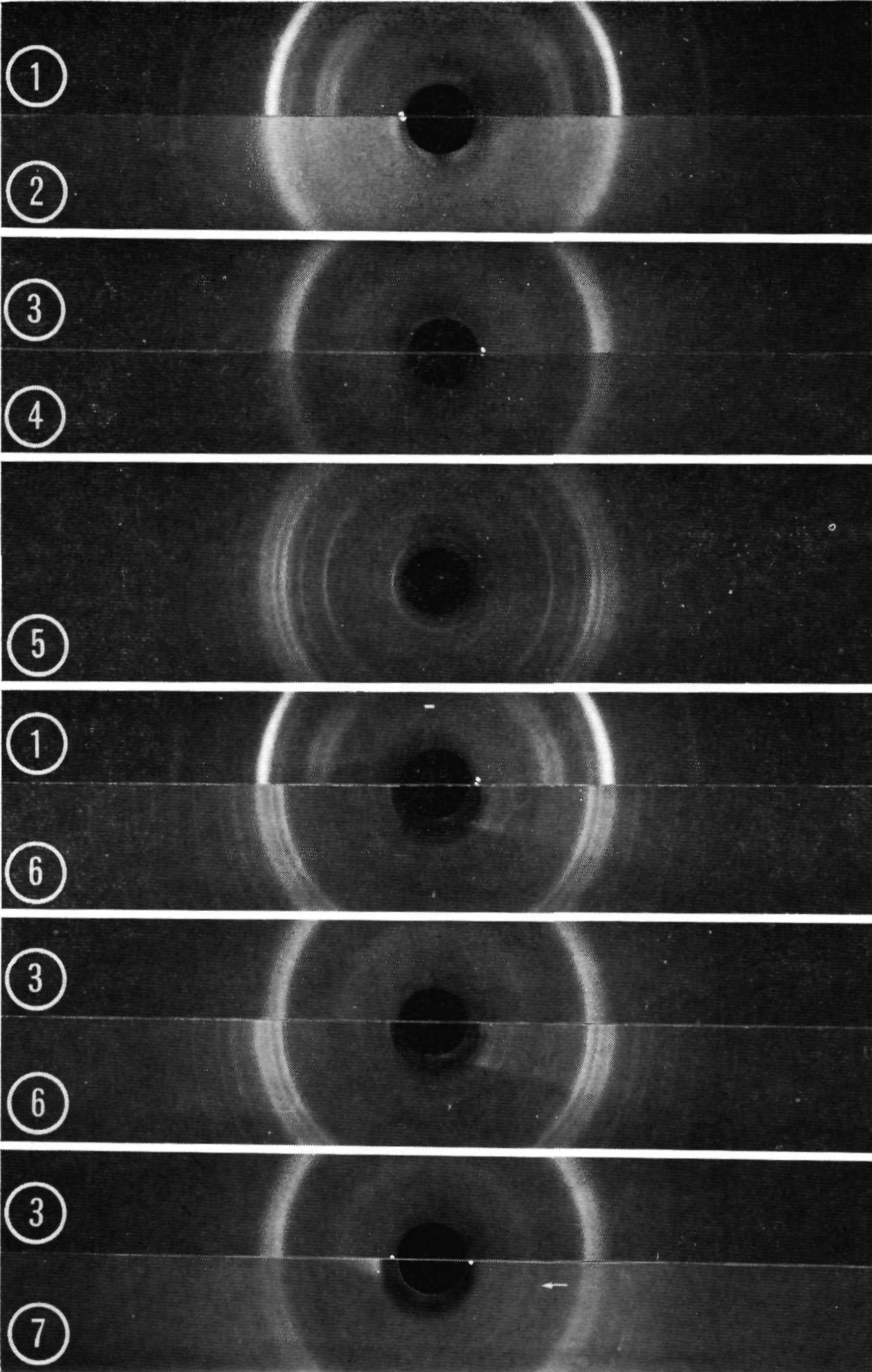
X-ray patterns obtained from the different probes were made in a Debije and Scherrer camera with 0.5 mm ⌀ collectors. The exposure time was 17 hrs.

3. RESULTS

The X-ray pattern of cotton hairs treated with N-HCl consists mainly of 4 main diffraction lines (*fig. 1*). The two inner lines are of equal intensity and correspond to angles of diffraction of 15° and 16.5°. The two outer lines are of unequal intensity and correspond to angles of diffraction of 20.5° and 22.8°, respectively. Such an X-ray pattern is characteristic for cellulose I. After the same treatment pollen tube walls give an X-ray diffraction pattern as represented in *fig. 2*, which corresponds in its essential features to that of the cellulose I pattern of cotton hairs. The two inner lines are very low in intensity and hardly visible. The two outer lines show the same relative difference in intensity as observed in the cotton sample.

Treatment of the cotton hairs with 20% NaOH results in an X-ray pattern consisting mainly of 3 lines (*fig. 3*). The inner line of the paired ones has the

Figs. 1–7. X-ray diffraction patterns obtained from purified residues of Golgi vesicles, pollen tube walls and cotton hairs.
Fig. 1. Cotton hairs after N-HCl extraction.
Fig. 2. N-HCl extracted tube walls.
Fig. 3. Cotton hairs after NaOH treatment.
Fig. 4. Tube walls after NaOH treatment.
Fig. 5. Golgi vesicles after N-HCl and 2N KOH extraction.
Fig. 6. Golgi vesicles after additional chloroform purification.
Fig. 7. Golgi vesicles after NaOH treatment.


1
2
3
4
5
1
6
3
6
3
7

highest intensity. The lines correspond to angles of diffraction of 11.6°, 20.0 and 21.0° for inner, middle, and outer line, respectively. The pattern obtained from the pollen tube walls treated with NaOH produces essentially the same diagram. Both are characteristic for cellulose II (*fig. 4*).

The residue of N-HCl purified Golgi vesicles produces a very diffuse pattern from which no further information could be obtained. After treatment with 2N KOH a pattern composed of fine lines became visible (*fig. 5*). This pattern is assumed to originate partly from the presence of fatty material. Indeed some lines disappear when the material is treated with chloroform, which results in a pattern suggesting the presence of both cellulose I and II (*fig. 6*). The pattern obtained from the residue of the Golgi vesicles treated with NaOH corresponds unambiguously with that of cotton hairs after the same treatment (*fig. 7*).

4. DISCUSSION

A number of data has been presented illustrating the presence of cellulose in pollen tube walls of *Petunia*. The X-ray patterns from the HCl resistent residue of tube walls and cotton hairs correspond with one another in their essential features. Both are characteristic for cellulose I. From the weak rings present in the tube wall pattern one may conclude that the cristallinity of the tube wall cellulose is poor and/or that impurities are still present. Chemical analysis of the HCl residue with 2N KOH reveal the presence of a polysaccharide composed of several monosaccharides (ENGELS 1974a). Tube wall residue and cotton hairs treated with NaOH produce again similar X-ray patterns, which, however, are now characteristic for cellulose II. A conversion of a cellulose I- into a cellulose II-lattice by NaOH treatment is characteristic for native cellulose. A chemical analysis of the 2N KOH residue of tube wall material has revealed the presence of glucose as the only detectable monosaccharide (ENGELS 1974a). Examination of this material by EM showed a network of microfibrils with diameters between 15 and 20 nm (ENGELS 1974b). The data obtained from the present study lead to the conclusion that the microfibrils are composed of native cellulose, thus substantiating earlier assumptions made by SASSEN (1964).

After treatment with NaOH the material of Golgi vesicles and cotton hairs show a similar X-ray pattern characteristic for cellulose II. An additional line (arrow *fig. 7*) is an indication for the presence of another substance besides cellulose. In *Pleurochrysis* it has been found that a protein is present which is strongly bound to cellulose and which could not be removed by the methods used (HERTH et al. 1972). The control experiments rule out the possibility of impurities by other compounds. Hence it must be concluded that Golgi vesicles contain a polymer chain of the cellulose type.

An extensive study of sections and freeze-etch replicas of Golgi vesicles did not reveal any indication for the presence of fibrils in Golgi vesicles (ENGELS 1973). The cellulose component found in the Golgi vesicles is, therefore, not present in the form of a crystalline structure. Therefore one has to assume that the cellulose is masked by protein and/or lipid which prevents its cristallization.

A cellulose intermediate bound to a glycolipid has been found in *Pisum* (WINTER et al. 1970). In *Phaseolus aureus* an acid labile cellulose intermediate has been reported which contains a glycolipid enzyme complex (VILLEMEZ & CLARK 1969). RAY et al. (1969) found a particulate membrane complex in Golgi membranes in *Pisum sativum* which apparently showed β, 1–4 glucan-synthetase activity.

After HCl extraction the contents of the Golgi vesicles of *Petunia* did not produce an X-ray diagram which could be interpreted. This is possibly caused by the high protein content in comparison with the amount of polysaccharides present in Golgi vesicles (ENGELS 1974a). By KOH extraction a part of the protein is removed and a pattern of fine lines is obtained which suggests the presence of cellulose. After additional extraction with chloroform an X-ray pattern is produced which is indicative for the presence of a mixture of cellulose I and II (*fig. 6*) The assumed intermediate, protected by protein and lipid, may be able to cristallize in the configuration of cellulose I after extraction of the protective substances. During prolonged treatment with KOH, cellulose I may partly transform into cellulose II. A solution of mercerated cellulose has been reported to produce a mixture of cellulose I and II when recristallization is carried out with caution (MACCHI et al. 1968, MACCHI & PALMA 1969).

Synthesis of cell wall polysaccharides with the exception of cellulose is generally accepted to take place in Golgi vesicles in higher plants (NORTHCOTE 1971). Cellulose synthesis is thought to take place exclusively at the outside of the plasmalemma or in the cell wall (PRESTON 1963, ROELOFSEN 1965, STAEHELIN 1966, 1968, MÜHLETHALER 1967, BARNETT 1969, FREY-WYSSLING 1969, BARNETT & PRESTON 1970, ROBINSON & PRESTON 1971, 1972, NORTHCOTE 1969a, b, 1971).

BROWN et al. (1970) and HERTH et al. (1972) indicated a cellulosic component in the Golgi vesicles of the alga *Pleurochrysis scherffelii*. Recently GAMALEI (1973) described structures in thin sections of Golgi vesicles of *Picea abies* interpreted as cellulose microfibrils.

Our results indicate that the Golgi vesicles in *Petunia* pollen tubes contain polysaccharides which are very similar in their composition to those of the tube wall (ENGELS 1974a). The results obtained from the X-ray studies lead us to the assumption that the Golgi vesicles also contain cellulose in addition to other polysaccharides. It is generally assumed that in connection with cell wall synthesis Golgi vesicles migrate to and fuse with the plasmalemma. This fusion implicates that the inside of the unit membrane of the Golgi vesicles resembles morphologically as well as physiologically the outside of the plasmalemma (FINERAN 1973 review). From this point of view cellulose synthesis within the Golgi vesicles needs not to be considered contrary to synthesis of cellulose at the outside of the plasmalemma.

ACKNOWLEDGEMENTS

The author is much indebted to Prof. Dr. H. F. Linskens for his stimulating interest, to Dr. M. M. A. Sassen and Dr. M. Kroh for critical discussions, to Dr. Ir. W. G. M. Barendse

for reading and to Mrs E R Tummers-Moesker for typing the manuscript The author is grateful to J M M Smits of the cristallography laboratory for taking the X-ray Debije-Scherrer photographs

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SAMENVATTING

De geisoleerde Golgi blaasjes zijn met de Golgi blaasjes in de intacte pollenbuizen vergeleken Er zijn geen morphologische veranderingen opgetreden tijdens de isolatie De toegepaste isolatie-techniek levert een groot aantal Golgi blaasjes op die niet verontreinigd zijn met andere celorganellen (1)

Het eiwit gehalte in de Golgi blaasjes blijkt erg hoog te zijn Behalve membraan eiwitten moeten er ook nog andere eiwitten voorkomen die wellicht een enzymatische functie bezitten In de Golgi blaasjes is een koolhydraat aanwezig dat geassocieerd is met eiwit en/of lipid Een aantal monosacchariden blijkt in de Golgi blaasjes voor te komen Het eiwit gehalte van de pollenbuiswand komt overeen met waarden die reeds eerder in primaire celwanden gevonden zijn Ook in de pollenbuiswand worden monosacchariden gevonden Er blijkt een grote overeenkomst te zijn tussen de samenstelling van de polysacchariden van de Golgi blaasjes en de pollenbuiswand Zowel in de Golgi blaasjes als in de pollenbuiswand wordt een glucose polymeer gevonden dat resistent is tegen alle gebruikte extractie-vloeistoffen (2)

Een inleidende studie over de structuur van de pollenbuiswand laat zien dat iedere fixatie methode een ander karakteristiek morphologisch beeld geeft in het electronen microscoop (3) Een streping in de dwarsdoorsnede van de pollenbuiswand na chemische fixatie blijkt niet veroorzaakt te worden door cellulose lamellen Bij zuivering van de pollenbuiswand met extractie-middelen wordt de matrix verwijderd en de microfibrillen komen tegen elkaar te liggen Hierdoor ontstaat één lamelachtige structuur die aan een artefact moet worden toegeschreven Hieruit blijkt dat grote voorzichtigheid bij de interpretatie is vereist wanneer men morphologische structuren wil correleren met chemische componenten

De structuurveranderingen die in de pollenbuiswand optreden door de extractie middelen zijn in het electronen microscoop bestudeerd In de opeenvolgende stappen van de extractie procedure wordt de matrix substantie in de pollenbuiswand opgelost waarna een skelet van microfibrillen overblijft Metingen van de diameters van deze fibrillen levert een waarde op die overeenkomt met de reeds gevonden waarde voor cellulose microfibrillen

Met behulp van rontgen-diffractie werden restanten van Golgi blaasjes en pollenbuiswanden na extractie onderzocht (4) De verkregen rontgen-patronen bewijzen dat in de pollenbuis natieve cellulose, en in de Golgi blaasjes een polymeer van het cellulose type voorkomt In de Golgi blaasjes is dit polymeer vermoedelijk door eiwit en/of lipid afgeschermd en in opgeloste vorm aanwezig Door extractie van dit materiaal treedt een kristallisatie op Het rontgen-patroon ervan komt overeen met dat van natieve cellulose

Met dit onderzoek wordt voor de eerste maal cellulose in Golgi blaasjes van een hogere plant aangetoond De hypothese, dat cellulose uitsluitend buiten het cytoplasma op de plasmamembraan kan worden gevormd, is niet in strijd met

de vondst van cellulose in de Golgi blaasjes. Immers, als de Golgi blaasjes bij de celwand aankomen treedt een fusie op van de unitmembraan van de Golgi blaasjes met de plasmamembraan. Men kan veronderstellen dat de physiologische en morphologische eigenschappen van beide membranen aan elkaar gelijk zijn. Een synthese van cellulose aan de binnenkant van de unitmembraan van de Golgi blaasjes zal dan mogelijk moeten zijn.

De Golgi blaasjes spelen in het proces van de celwand synthese in kiemende pollenbuizen van Petunia een zeer belangrijke rol. De overeenkomsten in de suikersamenstelling van de polysacchariden in de Golgi blaasjes en de pollenbuiswand wijst op een synthese-mechanisme dat reeds in de Golgi blaasjes actief is. Voortzetting van deze synthese na de fusie van de Golgi blaasjes met het plasmamembraan wordt zeer waarschijnlijk geacht.

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CURRICULUM VITAE

F. M. ENGELS werd op 25 april 1945 geboren in Mierlo (N.Br). De lagere school werd in Afferden (Gld.) en de openbare HBS-b in Nijmegen gevolgd. In 1963 begon hij zijn studie in de biologie aan de Katholieke Universiteit in Nijmegen en behaalde het kandidaatsexamen in 1967. De doctoraal studie bestond uit het hoofdvak Botanie en de bijvakken Chemische Cytologie en Genetica. In 1970 werd het doctoraal examen cum laude afgelegd.

Van 1970 tot 1971 was hij verbonden als wetenschappelijk medewerker op de afdeling Anatomie van het Praeklinisch Instituut van de Katholieke Universiteit in Nijmegen.

Vanaf 1971 was hij in dezelfde functie verbonden met de afdeling Botanie III van de Faculteit der Wiskunde en Natuurwetenschappen van deze universiteit.

STELLINGEN

I

Voor het model van Westergaard en Von Wettstein waarbij de rol van het 'synaptinemal complex' bij de crossing-over wordt verklaard zijn onvoldoende experimentele bewijzen.

M. WESTERGAARD & D. VON WETTSTEIN. Ann. Rev. Genet. 6, 71 (1972).

II

Hernieuwde synthese van celwand componenten is geen essentiële voorwaarde voor de celstrekking.

R. CLELAND. Ann. Rev. Plant Physiol. 22, 197 (1971).

III

De klassieke indeling van matrix polysacchariden, in pectine en hemicellulose, leidt tot verwarring omdat deze begrippen onvoldoende zijn gedefineerd.

Dit proefschrift.

IV

Bij een doelmatig beheer van wegbermen en slootkanten is het gebruik van herbiciden in het algemeen noch oecologisch noch economisch verantwoord.

P. ZONDERWIJK. Natuur en Landschap 25, 1 (1971).

V

Bij de bepaling van de mono-digalactosyl diglyceride verhouding na incorporatie van ^{14}C-galactose uit UDP-^{14}C-galactose in spinazie chloroplasten houden Ongun en Mudd geen rekening met de hoeveelheid dubbel gelabelde digalactosyl diglyceride.

A. ONGUN & J. B. MUDD. J. Biol. Chem. 243, 1558 (1968).

VI

De wetgeving ten aanzien van amfibiën, met name het Koninklijk Besluit van 6 augustus 1973, heeft slechts een educatieve waarde.

Staatsblad 488 (1973).

VII

De plasmalemmasomen in de wortelstokken van Helianthus tuberosus worden door Fowke en Setterfield ten onrechte als artefacten beschouwd.

L. C. FOWKE & G. SETTERFIELD. Can. J. Bot. 47, 1873 (1969).

VIII

Het is niet verantwoord om de biologische afbreekbaarheid van een detergens, bepaald door laboratoriumproeven, als enige maatstaf te nemen voor zijn toelaatbaarheid in het afvalwater.

R. VRIJBURG. Chem. Weekbl. 59, 179 (1963).

IX

Er zijn voldoende argumenten die er op wijzen dat de synthese van cellulose extracellulair plaats vindt.

Dit proefschrift.

X

De aanduiding 'bezet' op een vertrek betekent niet altijd dat de ruimte onrechtmatig in beslag is genomen zodat een wettelijke omschrijving en bescherming van dit begrip een 'nood'-zaak wordt.

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26 juni 1974