

# SCALE FORMATION IN CHRYSOPHYCEAN ALGAE

## I. Cellulosic and Noncellulosic Wall Components Made by the Golgi Apparatus

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### ABSTRACT

The cell wall of the marine chrysophycean alga *Pleurochrysis scherfellii* is composed of distinct wall fragments embedded in a gelatinous mass. The latter is a polysaccharide of pectic character which is rich in galactose and ribose. These wall fragments are identified as scales. They have been isolated and purified from the vegetative mother cell walls after zoospore formation. Their ultrastructure is described in an electron microscope study combining sectioning, freeze-etch, and negative staining techniques. The scales consist of a layer of concentrically arranged microfibrils (ribbons with cross-sections of  $12$  to  $25 \times 25$  to  $40$  Å) and underlying radial fibrils of similar dimensions. Such a network-plate is densely coated with particles which are assumed to be identical to the pectic component. The microfibrils are resistant to strong alkaline treatment and have been identified as cellulose by different methods, including sugar analysis after total hydrolysis, proton resonance spectroscopical examination (NMR spectroscopy) of the benzoylated product, and diverse histochemical tests. The formation and secretion of the scales can be followed along the maturing Golgi cisternae starting from a pronounced dilated "polymerization center" as a completely intracisternal process which ends in the exocytotic extrusion of the scales. The scales reveal the very same ultrastructure within the Golgi cisternae as they do in the cell wall. The present finding represents the first evidence on cellulose formation by the Golgi apparatus and is discussed in relation to a basic scheme for cellulose synthesis in plant cells in general.

### INTRODUCTION

The Golgi apparatus is recognized as a site of membrane formation and secretion. At least two different classes of biopolymers, i.e. proteins and carbohydrates, are known to be secreted by this system. An involvement of the Golgi apparatus in polymerization and secretion of plant polysaccharides was first suggested by Drawert and Mix

(10). This has been substantiated and confirmed for the case of pectins, plant slimes, and hemicellulosic compounds (37, 45, 56). Golgi-mediated synthesis of mucopolysaccharides and complex carbohydrate-proteins also has been reported for various types of animal cells (e.g., 1, 42, 43, 53).

In most reviews on polysaccharide biosynthesis,

authors have emphasized that the Golgi apparatus is unlikely to be involved in the synthesis of cellulose (37, 40, 45, 52, 56). Such belief has been based principally on the special case of exogenous synthesis of bacterial cellulose in *Acetobacter xylinum* (7, 8).

The general scheme of polysaccharide biosynthesis given by Northcote and Pickett-Heaps (45) requires two different intracellular pathways: (a) most polysaccharides such as pectins, carbohydrate slimes, and hemicelluloses are thought to be formed within the Golgi cisternae and/or vesicles by polymerization of the appropriate sugars from the membrane-surrounded pool of nucleoside phosphate sugar precursors; (b) cellulose biosynthesis should occur along a totally different, "nonmembrane flow" pathway, starting from a different pool of activated glucose. This view for the exogenous site of cellulose biosynthesis is similar to Mühlethaler's concept in which the polymerization of activated glucose monomers should occur on certain particles ("enzyme complexes") on the outer surface of the plasmalemma (40).

The separation of the cellular pathway of cellulose synthesis from that of all other kinds of polysaccharides so far investigated is somewhat puzzling. What has hindered our interpretation of the role of the Golgi apparatus in cellulose biosynthesis so far has been the lack of direct proof that a particular structural component is composed of cellulose and that it is made within the cisternae of the Golgi apparatus. Studying the marine chryso-phycean alga, *Pleurochrysis scherffellii*, Brown (5) first suggested that certain structurally definable Golgi-derived "wall fragments" might consist of cellulose, at least in part.

The present communication is a continuation of this initial observation that the Golgi cisternae produce wall fragments, now identified and equated as "scales" (Parke et al., 47; 8). We further wish to augment the evidence for the cellulosic nature of the scale component, to give a preliminary account of the chemical nature of the noncellulosic scale-associated components, and to describe the intracellular basis for synthesis and transport of these Golgi-derived components. Further papers will deal with: (a) a survey of the distribution of Golgi-produced cellulosic scale components and their taxonomic and phylogenetic

implications,<sup>1</sup> and (b) protoplast movement and its relationship to the secretory function of the Golgi apparatus in scale and wall formation.<sup>2</sup>

#### MATERIALS AND METHODS

Axenic cultures of *Pleurochrysis scherffellii*, isolated by Professor Pringsheim (51), were routinely maintained in natural seawater, 1 liter of which was enriched with the following: NaNO<sub>3</sub>—0.425 g; Na<sub>2</sub>EDTA·2 H<sub>2</sub>O—0.00372 g; MnCl<sub>2</sub>·4 H<sub>2</sub>O—0.0195 g; NaHPO<sub>4</sub>·2 H<sub>2</sub>O—0.0107 g; Difco vitamin-free casamino acids (Difco Laboratories, Detroit, Mich.)—4.0 g; biotin—0.001 mg; thiamine-HCl—0.20 mg; B<sub>12</sub>—0.10 mg; and FeSO<sub>4</sub>·7 H<sub>2</sub>O—0.0278 g.

For solidification, 16.0 g of Difco Bacto agar were added to each liter of final medium. Generally, all components of the medium were made up into stocks of 100 times the final concentration to be used, with the exception of the casamino acids which were added directly to the final medium. All stock components were dissolved in distilled water, with the exception of FeSO<sub>4</sub>·7 H<sub>2</sub>O which was stable only in acidified water (1.0 ml concentrated H<sub>2</sub>SO<sub>4</sub> per liter of distilled H<sub>2</sub>O).

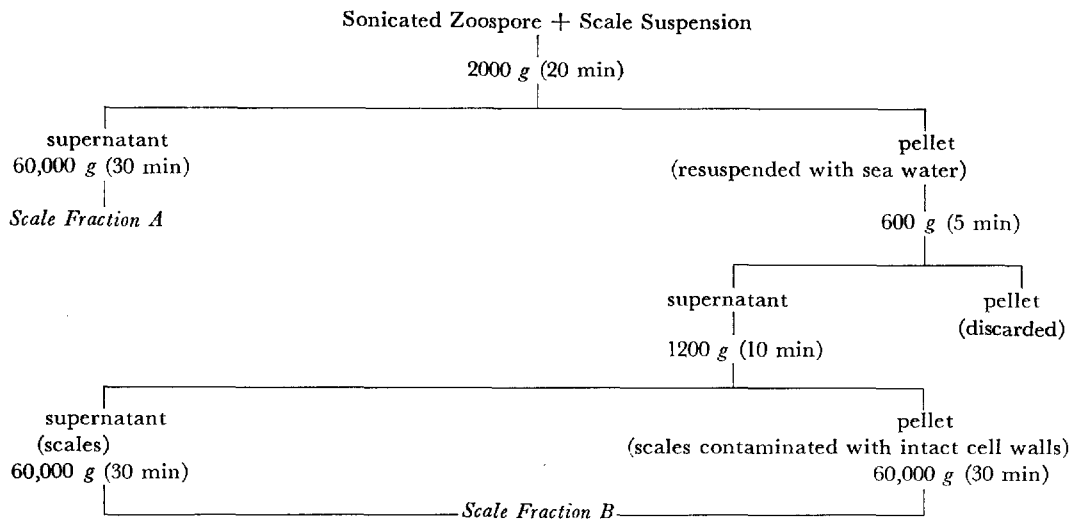
Cultures were grown in a room exposed to light from a north window (temp 18°–26°C) as well as in controlled temperature rooms (18°C) with continuous daylight fluorescent lighting (3000 lux).

For mass production of scales, 100 mm Petri plates of sterile, solidified culture media were inoculated with a zoospore suspension. The cultures were incubated for 5–6 wk and prepared for harvesting. The first step in this procedure involved flooding the cultures (which were nonmotile vegetative cells at this stage) with sterile natural seawater. Within 36 hr, the entire culture formed zoospores. A gelatinous mass of residual cell walls (scales embedded in a pectic mass) was gently scraped from the surface of the agar with the edge of a microscope slide and suspended. The scrapings from 50 Petri plates were collected, then subjected to a brief and gentle pulse of sonication (Branson sonifier [Branson Instruments, Inc., Stamford, Conn.]) to separate them from the zoospores and to suspend them. The zoospores were still intact after this sonication, and many were still swimming.

Scale purification and enrichment were achieved according to the following diagram. Temperature was kept between 5°–8°C during the entire procedure.

<sup>1</sup> R. M. Brown, Jr., and W. W. Franke. Data in preparation.

<sup>2</sup> R. M. Brown, Jr. Data in preparation.



Most of the scales appeared in scale fraction A, and this fraction was judged to be the cleanest. Consequently the following preparations were based mainly on this fraction.

Scale fractions A or B were washed three times in distilled water or seawater. When distilled water was used for the wash the scale pellet progressively lost most of its gel properties which were maintained in the seawater washes. Scale pellets obtained from both kinds of washes were used for negative staining and the various chemical tests.

For chemical analysis, the scale fractions were subjected to a pretreatment with hot 0.5 N  $\text{H}_2\text{SO}_4$  for 1 hr. Then the scales were centrifuged at 70,000 g for 30 min and the pellet was washed repeatedly with distilled water until the washings were neutral. This partially prehydrolyzed scale fraction was then treated with 24% KOH for 12 hr at 20°C including 2 hr at +100°C (native cotton was run in all experiments as a control). The resulting flocculent was washed repeatedly with distilled water and then pelleted with centrifugation (1000 g for 20 min).

The alkaline-purified scale and cotton cellulose fractions, as well as washed, untreated scale fraction A and intact vegetative cells were hydrolyzed with 5 N  $\text{H}_2\text{SO}_4$  in sealed ampoules at 60°C for 5 hr, then neutralized with  $\text{BaCO}_3$ . These fractions as well as known reagent-grade sugars were run on thin-layer chromatography as described earlier (6). Results obtained without the acid prehydrolysis, i.e. by direct alkaline purification, did not differ significantly, though the former were generally found to yield more reproducible results.

For a more quantitative determination of the ratio of glucose to nonglucose sugars, the partially hydrolyzed and alkaline-purified material was subjected to a total hydrolysis according to Saemen et al. (54) and total sugars were determined by using the

anthrone reaction (62) while glucose was determined enzymatically with glucose-oxidase (4; C. F. Boehringer, Mannheim, Germany).

Another aliquot of the partially hydrolyzed alkaline-stable scale material was washed in  $\text{H}_2\text{O}$  by repeated centrifugation, dried, resuspended in pyridine, and benzoylated with benzoyl-chloride as described by Husemann and Keilich (26); the benzoylated product (2,3,6-tri-o-benzoyl-cellulose) subsequently was precipitated with methanol, centrifuged, dissolved again in chloroform to check the successful benzoylation, and finally precipitated in methanol. This benzoyl-cellulose was used for proton resonance spectroscopical examination (NMR). Native cotton again was benzoylated in the very same way and used as a control.

An alkaline-purified fraction A (24% KOH), as well as intact vegetative cells, were treated with zinc chloride-iodine, prepared according to Behrens (2). In addition, Schweizer's cuprammonium reagent ("Cuoxam", [58], prepared according to a slightly modified Döring-method, [27]) was used to test the solubility of the alkaline-purified scale fraction A.

Untreated scale fraction A was subjected to 10% EDTA neutralized with KOH to pH 7.1 to test the solubility of the gel as well as corresponding changes in its reaction with ruthenium red, a dye commonly used for acidic polysaccharides like pectins (25).

Protein determination of scale fraction A was made by using two different methods: (a) on the basis of total nitrogen (60), and (b) by the Lowry reaction (30). To determine whether the scale fraction protein might be due to contamination from membranous fragments, in some fractions total phospholipids were also separately determined (20) after extraction in chloroform-methanol (2:1).

Three different preparative methods were used for electron microscopic investigation, namely, con-

ventional fixation and sectioning procedures, freeze-etching, and negative staining of the isolated scale material. Fixation methods consisted of glutaraldehyde-osmium tetroxide combinations which were used either sequentially with different aldehyde concentrations (0.5 to 6%), temperatures (0° to 22°C) and durations (20 min to 3 hr), or were used simultaneously with postosmication as previously communicated (16). The fixed material was sectioned on a Reichert ultramicrotome OmU<sub>2</sub> (American Optical Company, Buffalo, N. Y.), poststained with uranyl acetate and lead citrate, and examined with either the Siemens Elmiskop IA or 101. Freeze-etch preparations were made from young vegetative cells which were scraped from the agar surface (38). No glycerol or other ice-retardation methods were

necessary. For negative staining, 1, 2, or 4% phosphotungstic acid (PTA), adjusted to pH 7.2 with NaOH, was preferentially used while in some experiments uranyl acetate (1%, adjusted to pH 4.5) and ammonium molybdate (2%, adjusted to pH 7.2) were also employed. Formvar-coated grids were used either blank or hydrophilized by a glow discharge carbon layer (benzene, 1 kv, circa 2 mA, 10<sup>-2</sup> torr). For a special study carbon-coated holey Formvar films were chosen.

## RESULTS

### *Golgi Apparatus*

The general features of the cell morphology of *Pleurochrysis* have already been described (5), and



FIGURE 1 Survey micrograph of a vegetative cell of *Pleurochrysis scherffelii*. *N*, nucleus; *W*, cell wall; *C*, chloroplast; *Py*, pyrenoid; *V*, vacuole; *G*, Golgi apparatus; *M*, mitochondrion. Fixation, sequential glutaraldehyde-osmium tetroxide.  $\times 15,000$ .

only pertinent and new features will be given in this communication. The vegetative cell contains two parietal chloroplasts, each with a single pyrenoid (Fig. 1). The nucleus and the two chloroplasts are held in their relative positions by a common cytoplasmic cisterna representing either an ER equivalent or the nuclear envelope (= periplastidal cisternae [Gibbs, 21; Falk and Kleinig, 12]). A single Golgi apparatus, flanked by the ends of the two chloroplasts, is located between the nucleus and the plasma membrane (Fig. 1). The Golgi apparatus is highly polarized (5) with the proximal (= "forming") face adjacent to an extension of the nuclear envelope (= amplexus [Lang, 29]), and the distal (= "secreting") face directed toward the cell surface (Fig. 2). The proximal face consists of fenestrated cisternae which appear to be formed by vesicles which bleb

from the amplexus. The proximal cisternae have an inner width of 150–300 Å. Small vesicles also are associated with the periphery of these proximal cisternae.

Progressing towards the distal region, the most obvious feature is a central dilation in one or two adjacent cisternae (Fig. 2). The inner surface of the cisternal membrane is thickened with a dense material only in the dilated region (Figs. 3–5). The thickness of this additional layer varies from 50 to 250 Å. In those cisternae distal from these dilations, the first structural components, the concentric microfibrils, are observed. Frequently, they first appear in cross-section as double layers of periodic electron-opaque particles of about 30 Å (Fig. 7, arrow in upper right). In the next outermost cisternae, only a single layer of these particles is observed (Fig. 7, arrows). The microfibrillar

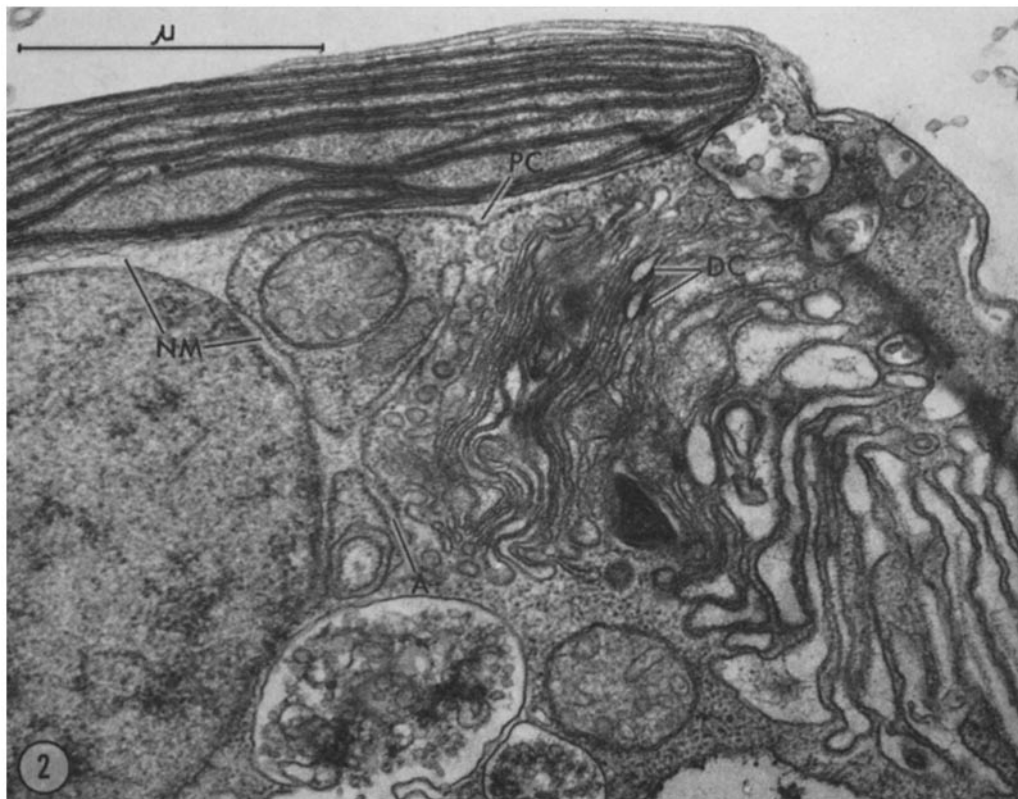
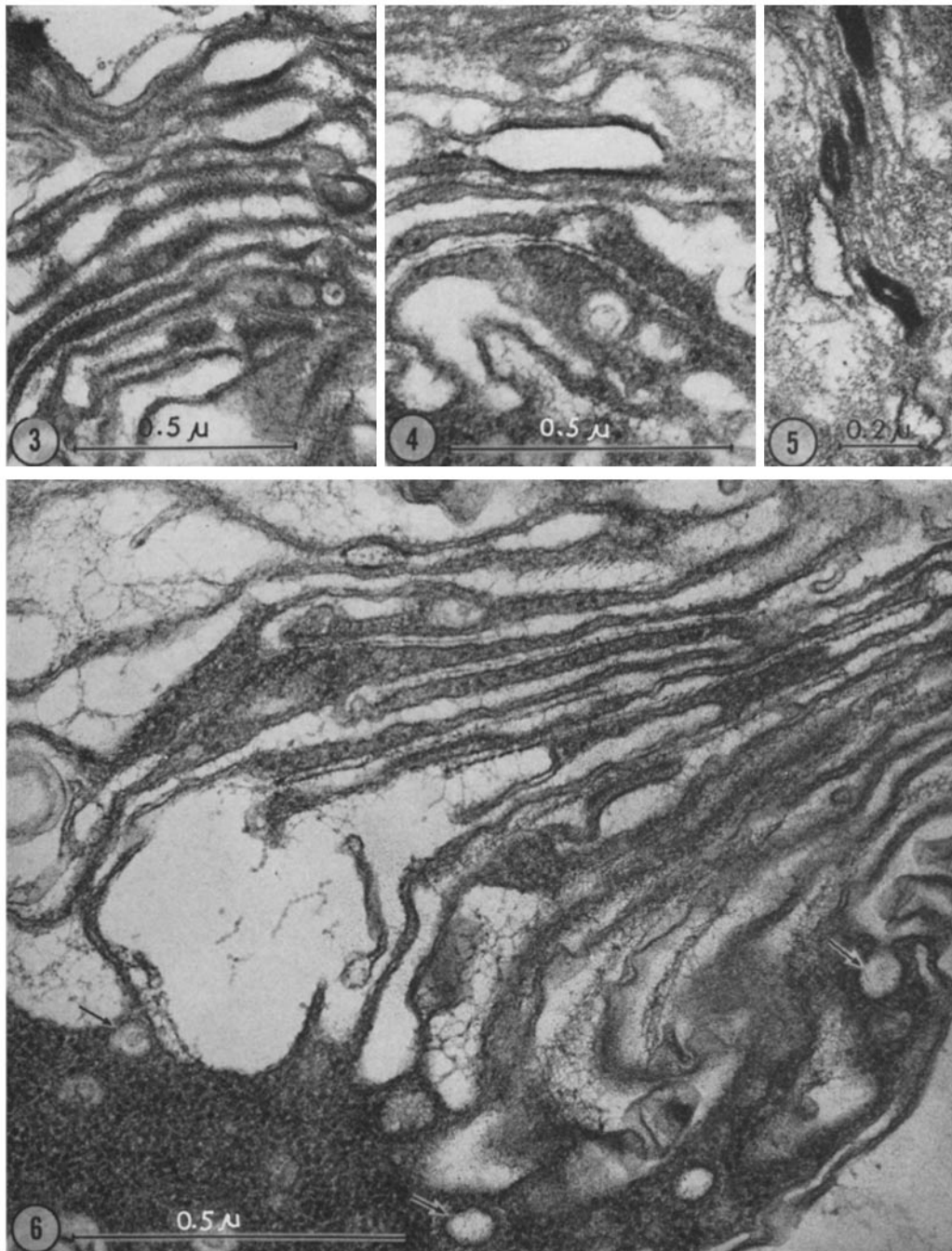


FIGURE 2 Interrelationship of the Golgi apparatus with the periplastidal cisterna and the amplexus (*A*), perinuclear cisterna (*NM*), and the periplastidal cisterna (*PC*). In between the amplexus and the proximal cisternae some of the generating vesicles of the forming face of the Golgi apparatus can be seen. Note also the polarity as demonstrated by the compact proximal and inflated distal cisternae. The central dilation (*DC*) is thought to be the polymerization center for the cellulose microfibrils. Fixation, sequential glutaraldehyde-osmium tetroxide.  $\times 40,000$ .



FIGURES 3-6. Details of the polymerization center and the peripheral inflation of the distal cisternae. Fixation, sequential glutaraldehyde-osmium tetroxide, except Fig. 5. In Fig. 3, note the electron-opaque thickening at the inner cisternal surface of the central dilation. In Fig. 4, in the cisternae distal from the polymerization center, the scale microfibrils can be recognized. Fig. 5 is an example of extreme intracisternal thickenings in the central dilation of an active Golgi apparatus. Fixation, simultaneous glutaraldehyde-osmium tetroxide. In Fig. 6, cisternae distal to the polymerization center contain scales. The first inflations occur in the peripheral region. Note the spherical vesicles (arrows) in the cytoplasm adjacent to the periphery of the Golgi apparatus. These vesicles sometimes seem to be fusing with the cisternal membranes (right arrow). Note also the nonordered strandlike material within the inflated cisternae. Fig. 3,  $\times 62,000$ ; Fig. 4,  $\times 80,000$ ; Fig. 5,  $\times 55,000$ ; Fig. 6,  $\times 95,000$ .

sheet is located equidistantly between the compact cisternal membranes (Figs. 6 and 7). In the next-most distal cisternae radial fibrils are associated with the concentric ones (Fig. 7). Grazing sections reveal the intricate network of both these types of microfibrils (Fig. 9). Thus, the entire fibrillar structure represents a compound fibrillar skeleton, recently described in preliminary form by Brown et al. (6).

Although it is not readily apparent in sectioned material, an ordered dorsoventrality of the radial and concentric microfibrils is present with the latter always oriented to the cell periphery, i.e., toward the distal face of the Golgi apparatus. This will be described below with regard to the cell wall.

The distal cisternae appear more and more inflated, and the microfibrils become coated with additional strandlike substances (Figs. 6-8), presumed to be polysaccharides of a pectic or possibly a slime type (see below). The progressive inflation

of the distal cisternae is suggested to be caused by a fusion with peripheral vesicles lying in between the Golgi apparatus and the periplastidal cisterna (Fig. 6, arrows). In Fig. 9 which shows a tangential section through a distal cisterna, the peripheral inflated portion of the cisterna as well as these vesicles can be seen.

Freeze-etch preparations (Figs. 10 and 11) support observations on the changes in the cisternal appearance and in scale formation along the maturing field. An especially interesting view of the distal Golgi cisternae is seen in Fig. 11 where part of the cisternal membrane has been splintered away thus revealing the internal structures. Although the limitation of resolution in freeze-etch shadowing is in the range of 25-30 Å, and thus interferes with the dimensions of the fibrils in question, they can, nevertheless, be detected within the cisternal membranes in cross-fractures through a Golgi apparatus (Fig. 10, arrows).

The mature scale is brought to the surface of

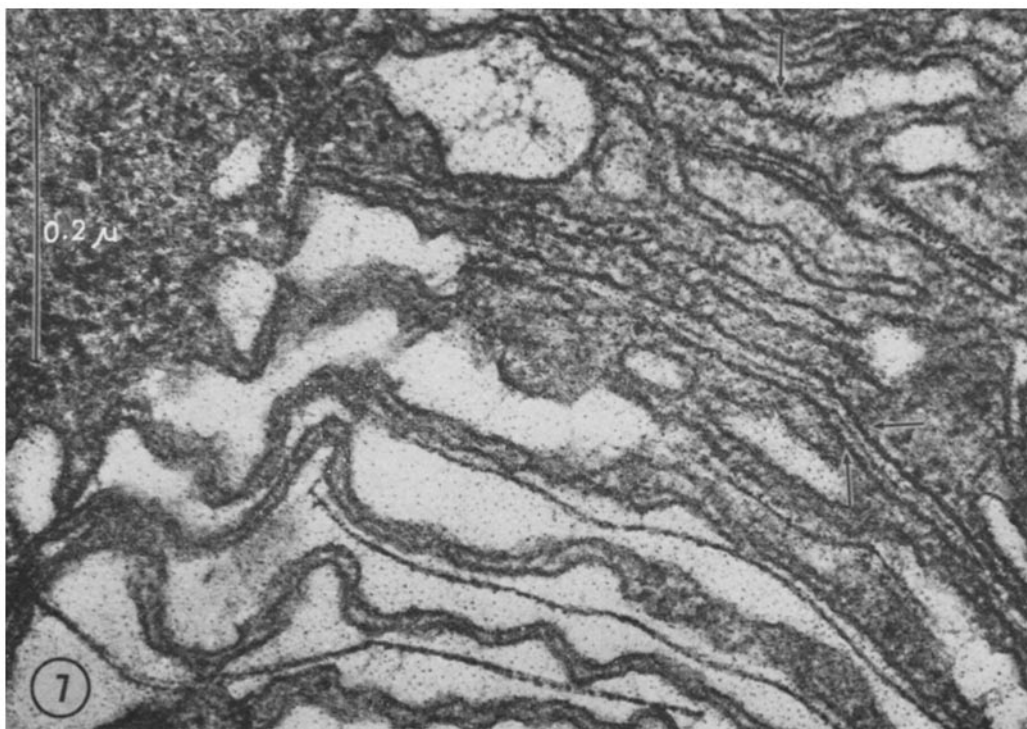


FIGURE 7 Details of the progressive maturation of scales and cisternae distal from the polymerization center. The most nascent scale microfibrils (arrow, upper right) lie close to the cisternal membranes in two layers. In the next two to four more distal cisternae they lie in a single plane equidistant within the cisternal membranes (arrows). Finally, the scales become completely separated in the distal-most inflated cisternae (lower part of figure). Fixation, sequential glutaraldehyde-osmium tetroxide.  $\times 180,000$ .

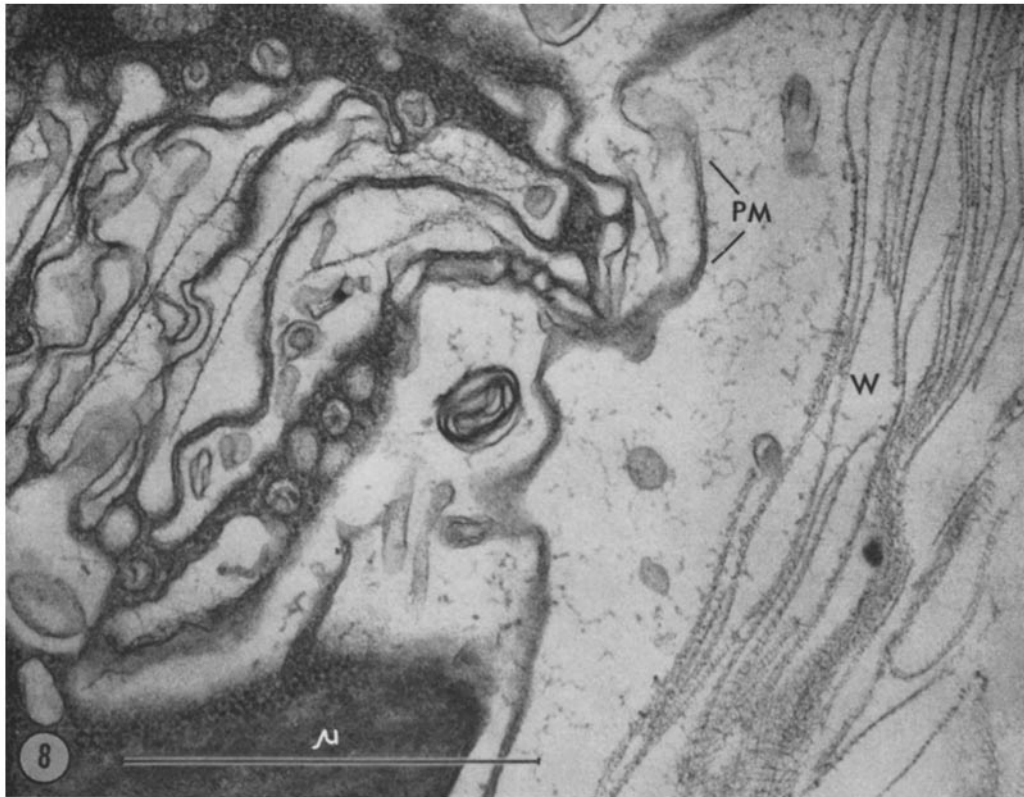


FIGURE 8 Fully mature, distal-most, inflated cisternae at the locus of exocytotic secretion of the scales. Note scales within these cisternae and as part of the cell wall (*W*). *PM*, plasma membrane. Fixation, sequential glutaraldehyde-osmium tetroxide.  $\times 55,000$ .

the cell by an exocytotic process involving the fusion of cisternal membranes with the plasma membrane. To insure a uniform distribution of the freshly produced scales over the entire cell surface, the protoplast must undergo a movement.<sup>2</sup>

#### Cell Wall

Sectioned material has demonstrated that the wall of the vegetative cell consists of closely appressed scales. The radial fibrils frequently are seen in transverse sections as dense, periodically arranged dots (Fig. 13) with the same spacing and structure as observed within the cisternae of the Golgi apparatus (Fig. 7). The concentric fibrils are best recognized in grazing sections (Fig. 12). Frequently, the radial and concentric fibrils are masked by accompanying substances of pectic nature (Fig. 13). The structure of these substances is not well preserved under the standard conditions of fixation, dehydration, and embedding. In an attempt to better resolve the pectic

constituents of the intact cell wall, the freeze-etch technique was employed; however, only amorphous material is observed in between the scales (right margin of Fig. 14).

Grazing fractures through the outer surface of the cell wall reveal a specific orientation of the scales with respect to their polarity as described above (Fig. 15). Here the concentric fibrils of the scale surface always are oriented toward the cell surface. Henceforth, the concentric scale surface will be described as the distal surface in relation to its initial geometric position in the Golgi apparatus and its later orientation in the intact cell wall. Fractures at the inner surface of the cell wall show radial fibrils as well as concentric ones (Fig. 14).

#### Isolated Scales

During zoospore formation the vegetative cell (Fig. 16) undergoes one division, giving rise to two biflagellate zoospores, each of which has a hap-





FIGURE 9 Grazing section through the distal-most region of the Golgi apparatus. Note the characteristic radial and concentric microfibrils of a scale lying within a peripherally inflated cisterna as well as numerous vesicles in the immediate vicinity of the cisternal periphery. Fixation, simultaneous glutaraldehyde-osmium tetroxide.  $\times 80,000$ .

tonema. In order for the zoospores to be released, the mother cell swells until internal pressure forces the zoospores through the "gelatinized" parent wall, i.e. the scales with the accompanying pectin-like substances (Fig. 17). As shown previously (6) these mother cell wall scales easily can be isolated and purified by differential centrifugation.

When such scale preparations are negatively

stained, a remarkable structural complexity of fibrillar and particulate matter is observed (Fig. 18). The microfibrils are arranged in radial and concentric patterns, and this array strongly resembles that described from section and freeze-etch preparations. The concentric fibrils are about 40–80 Å apart from each other. These fibrils so far could not be traced in continuity over an entire surface circumference. Thus one cannot



FIGURE 10 Fracture plane across a stack of cisternae revealing the polarity of maturation from the upper left to the lower right of the figure. Cross-fractured scales can be recognized lying within the cisternae (arrows). Note the spherical vesicles in the vicinity of the cisternal periphery. Compare with Fig. 9. Freeze-etch preparation.  $\times 56,000$ .

exclude the possibility that the concentric arrangement may be, in reality, a discontinuous array of concentrically curved microfibrils.

A typical "mature" scale (diameters from 0.7 to 1.4  $\mu$ ) consists of about 70–80 layers of concentric fibrils and about 90 radial fibrils (Fig. 20). In this connection, we should perhaps mention

that in some preparations, especially those with "older" zoospores, "minor" scales with diameters down to 0.4  $\mu$  could be found (Fig. 22). A special study will be dedicated to the question of the distribution of scale sizes in relation to the cytological and nutritional conditions.<sup>2</sup> The individual microfibrils show widths in the range of 12 to 40 Å,



FIGURE 11 Fracture along the outer surface of distal Golgi cisternae, in one of which the membrane has been splintered away producing a "window" through which intracisternal fibrils can be seen. Freeze-etch preparation.  $\times 45,000$ .

and many sites (Fig. 24, arrow) reveal their ribbon-like nature. Since surface views of the water-washed scales show a predominance of 15–20 Å widths for the concentric fibrils, it seems likely that they are oriented edge on. A preferential orientation of the radial fibrils has not been observed so far. When the scales are disrupted by sonic or chemical treatments, the ribbon-like structure of the fibrils becomes more evident because of a loss of orientation (Fig. 25).

The fibrils often have a "beaded" appearance (Fig. 23). Because the staining grain is in the same order of magnitude as the beading, this appearance may be due to an artifact rather than representing an actual structural periodicity in the microfibrillar conformation (cf. 14 and 15).

Frequently, radial fibrils fray from the periphery of the concentric fibrils, and in some instances one gains the impression that neighboring scales may be connected by these out-fraying fibrils.

In the central "eye" of the fibrillar array, the microfibrils are more elliptically arranged and reveal many cracking sites (= breakage loci) known to be characteristic for highly crystalline cellulose which is under tension (15, 39, 55; Fig. 21). The long diameter of this "eye" is identical to the dilated central cisternal region described above from thin sections. A calculation of the minimum curvature radius of the concentric fibrils in the eye region shows a value of about 700 Å. This corresponds to the value that Mühlethaler (39) has found for the 35 Å elementary fibrils of onion root cellulose.

Harsher chemical treatments of the isolated scales result in a progressive disarrangement and degradation of the fibrillar network. In a 5 min treatment with cold or boiling water, only the particulate, "non-fibrillar" scale constituents are removed, thus clearly revealing the arrangement of microfibrils (Figs. 20 and 22). Scales which have been left in sterile media for 6–8 wk after their separation during zoospore formation also have lost the particulate constituents. Treatment with 5% KOH for 5 min at room temperature results in a separation of the radial microfibrils from the concentric ones (Fig. 25), but further degradation usually does not occur. In this treatment, the ribbon structure of the microfibrils is more easily detected since the edge-on orientation is lost.

When the scales are treated with 24% KOH, a flocculent appears. Electron microscopic examination of this flocculent reveals, after negative staining, aggregations of short fibrils which appear to bond with other short fibrils in a random pattern (Fig. 26). Frequently, broader ribbons are observed, and these are obviously produced by a lateral fasciation (Frey-Wyssling, 17).

The most predominant nonfibrillar scale constituent is a particulate substance which is rather sensitive to mild alkaline and water washes. This component, presumed to be pectin, is granular and is found most abundant on the proximal surface (e.g. the surface with radial fibrils) of the fibrillar network (Fig. 18). These granules are preferentially arranged in tiers in between the radial fibrils (Fig. 19). The granules are irregularly shaped and give rise to a "spongy" appearance (Fig. 19). They can also be seen extending beyond the scale margin as masses totally separated from the scales (Fig. 18). One interesting structural feature is the rather uniform diameter of the holes that are distributed throughout these granules.

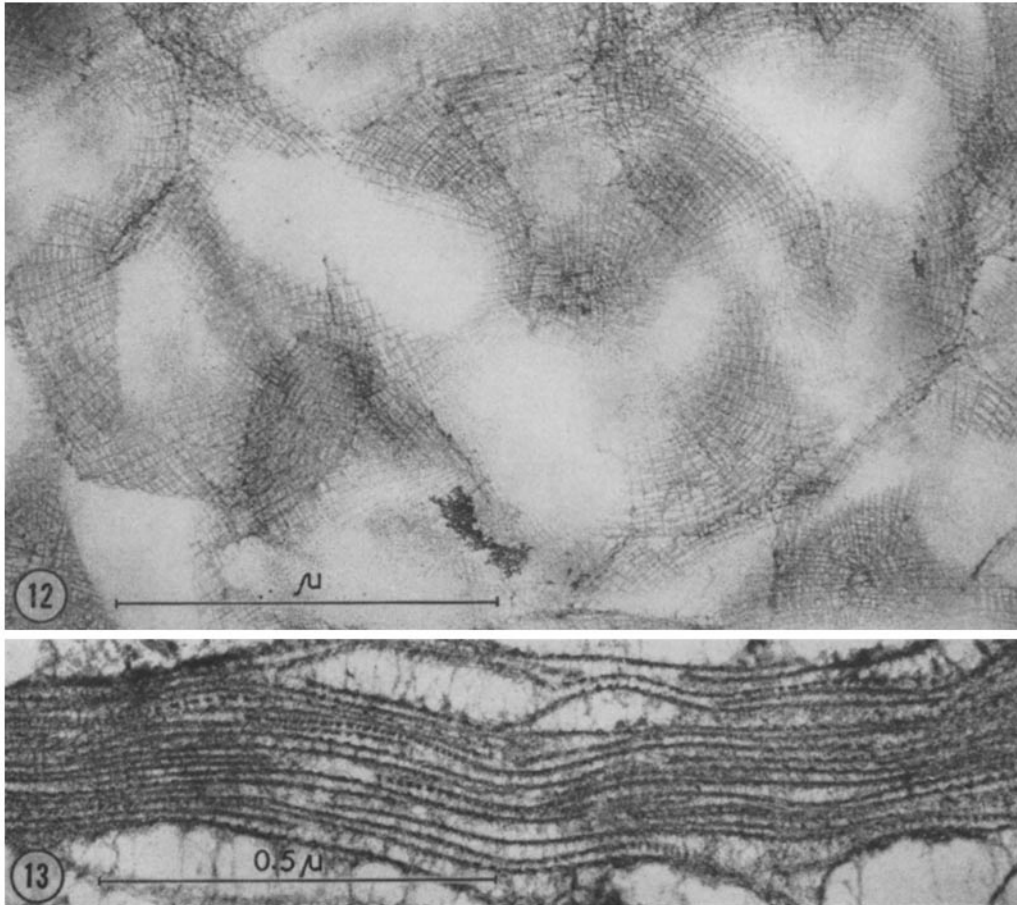


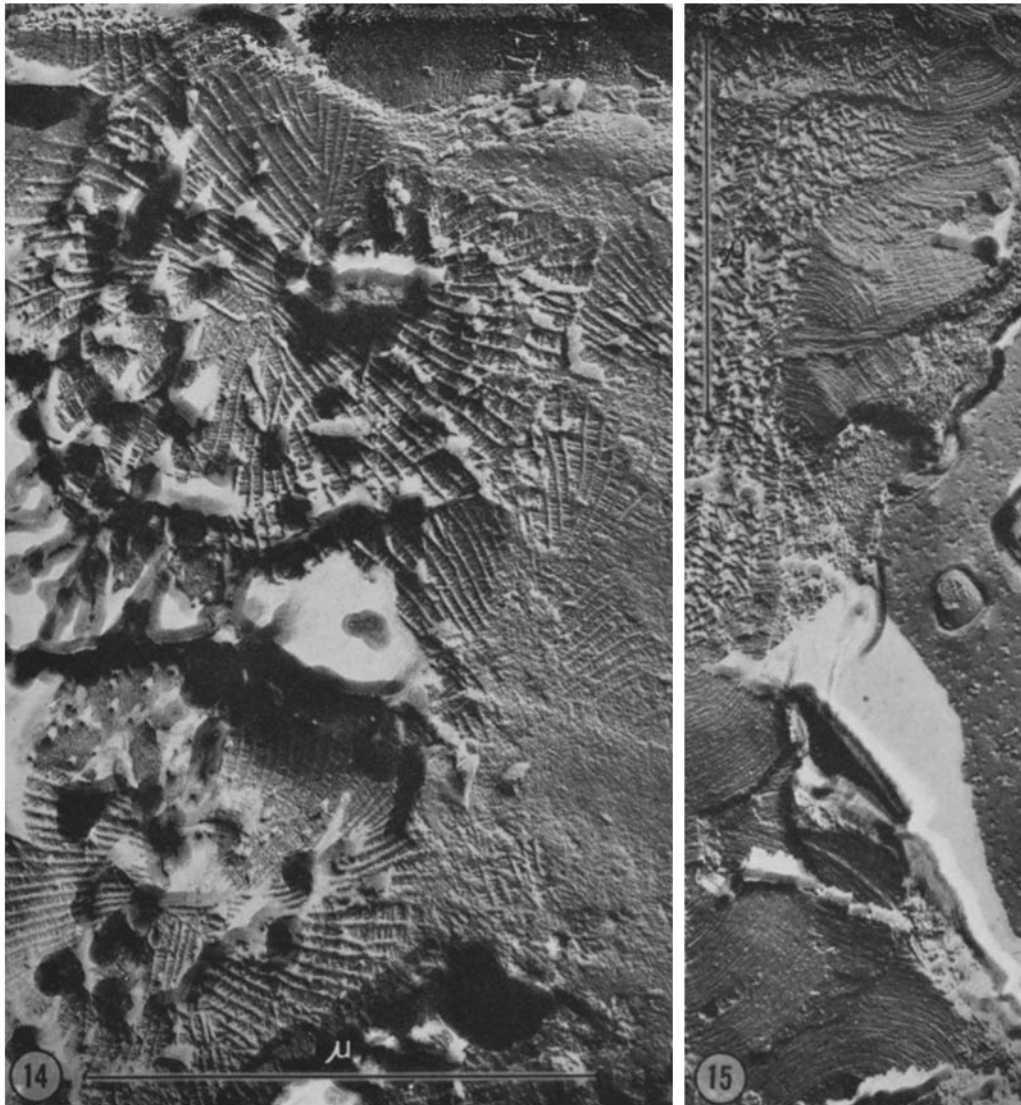
FIGURE 12 Section tangential to the intact cell wall of a mature vegetative cell revealing the concentric and radial microfibrillar network of overlapping scales. Fixation, simultaneous glutaraldehyde-osmium tetroxide.  $\times 50,000$ .

FIGURE 13 Section perpendicular to the intact cell wall. Note the stacks of scales and the thin strands of material in between them, presumed to be of a pectic nature. Fixation, sequential glutaraldehyde-osmium tetroxide.  $\times 105,000$ .

When a scale preparation is washed with distilled water, the characteristic granules disappear progressively. A comparison of Fig. 19 with Fig. 20 shows that the fibrillar constituents are more prominent in the washed scales. This difference indicates that there might be an amorphous matter in between the fibrils which acts as a "glue." At least, these observations support the finding that washing the scales with water or with mild alkali always results in a slight loss of order of the concentric fibrils as well as a frequent separation of the radial fibrils from the concentric ones.

#### Chemistry

The scale fractions contain one component which is resistant even to prolonged treatment with low and high concentrations of alkali (NaOH and KOH up to 4 N) in the cold (10°C) as well as at higher temperatures (100°C). Total hydrolysis of this alkaline-stable material and subsequent examination by thin-layer chromatography yielded glucose as the only sugar in scale fraction A or at least the predominant sugar in other preparations (Fig. 27, *a,b*). This is in contrast to the sugar pattern of a direct total

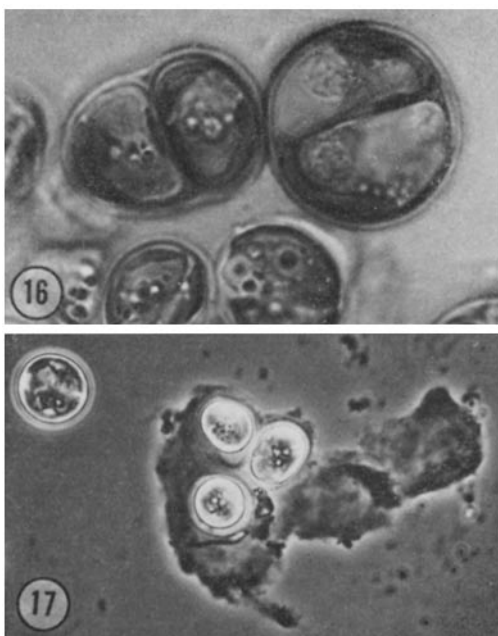


FIGURES 14 and 15 Freeze-etch preparations showing fractures along the inner surface (Fig. 14) and the outer surface (Fig. 15) of an intact cell wall. While the former fracture reveals both radial and concentric scale microfibrils, the latter shows only the concentric ones. This is an indication for the oriented dorso-ventrality of the scales. Fig. 14,  $\times 68,000$ ; Fig. 15,  $\times 50,000$ .

hydrolysis of the nontreated scale fraction (Fig. 27 *d*) and cell walls (Fig. 27 *c*) in which case galactose and ribose were the major components, accompanied by traces of glucose and arabinose. Our data are in agreement with those of Green and Jennings (22) on scales isolated from *Chrysochromulina chiton* regarding the dominance of galactose and ribose in the nontreated scales. In

all experiments it could be observed that the cleaner the scale fraction and the harsher the acid and alkaline treatment, the more pronounced was the relative enrichment in glucose vs. nonglucose sugars, especially galactose.

Using the anthrone reaction for total sugar determination, a quantitative estimation revealed that more than 95% of the sugars obtained after



FIGURES 16 and 17 Phase-contrast micrographs of living vegetative cells of *P. scherffellii*. Fig. 17 shows the liberation of the zoospores, leaving behind a gelatinous residue of the mother cell wall. Fig. 16,  $\times 1500$ ; Fig. 17,  $\times 700$ .

total hydrolysis of the partially hydrolyzed, alkaline-stable material is glucose, as measured enzymatically by the glucose-oxidase test.

The resistance of scale cellulose to acid as well as to alkaline treatment was astonishingly high, often higher than that of the cotton control, thus indicating a relatively high degree of crystallinity. The material obtained after alkaline purification was positive (intensely violet) in the zinc chloride-iodine reaction and was also soluble in Schweizer's cuprammonium reagent, though much slower than cotton cellulose.

A particularly valuable method in identifying polysaccharides is nuclear magnetic resonance spectroscopy (19). When benzoyl or acetyl derivatives of polysaccharides are studied by this method, they exhibit proton signals highly characteristic for the kind of sugar present as well as for the special type of linkage. The NMR spectrum of the tribenzoyl derivative of the purified and partially hydrolyzed scale material is compared with that of the corresponding derivative of cotton cellulose (Fig. 28). Both spectra are

virtually identical, but they differ from comparative spectra of the benzoyl derivatives of other polysaccharides, e.g. amylose, pullulan, and mannan (19).

The chemistry of the noncellulosic polysaccharide of the scale fraction is scarce and incomplete. This component can be progressively removed with distilled water, and it disappears rapidly after the addition of EDTA. The untreated whole scale pellet loses its gel properties with the addition of EDTA. After EDTA treatment, the cell walls of *P. scherffellii* do not give a further positive reaction with alcian blue (48) or with ruthenium red (25). Such behavior, as well as the dominance of galactose in the nontreated scales in contrast to its loss during acid and alkaline treatments, suggests that a pectin-like polysaccharide could be associated with the cellulosic scale.

Determinations of the total nitrogen content of the scale fraction by the Nestler and Lowry tests showed that all of the nitrogen present in the scale fraction is protein nitrogen. Therefore, *N*-containing groups in the scale fraction polysaccharides can be excluded with certainty. The protein content of the scale fraction A varied from 3 to 9% per dry weight. Since the phospholipid content of the scale fraction never exceeded 0.4% of the dry weight, it is clear that only a limited amount of the protein could be due to a membranous lipoprotein contamination. Therefore, at least half of the scale protein must represent a nonmembranous protein assuming w/w phospholipid: protein ratios in the range of 1:1 to 1:4 as typical for membranes of diverse types. Disc electrophoresis of the sodium dodecyl sulfate solubilized protein yielded four major bands and one minor band. More detailed work, including amino acid determinations, is needed to elucidate the types of protein associated with the scale fraction.

## DISCUSSION

The following results of the present and a previous study (6) indicate that the Golgi-produced microfibrillar scale moiety of *P. scherffellii* is cellulosic: (a) The microfibrils are resistant to strong alkaline solutions. (b) After total hydrolysis, the alkaline-purified microfibrillar material reveals, in thin-layer chromatography, glucose as the most dominant if not the only sugar present. (c) More than 95% of the sugar products of total hydrolysis of the alkaline-purified material have

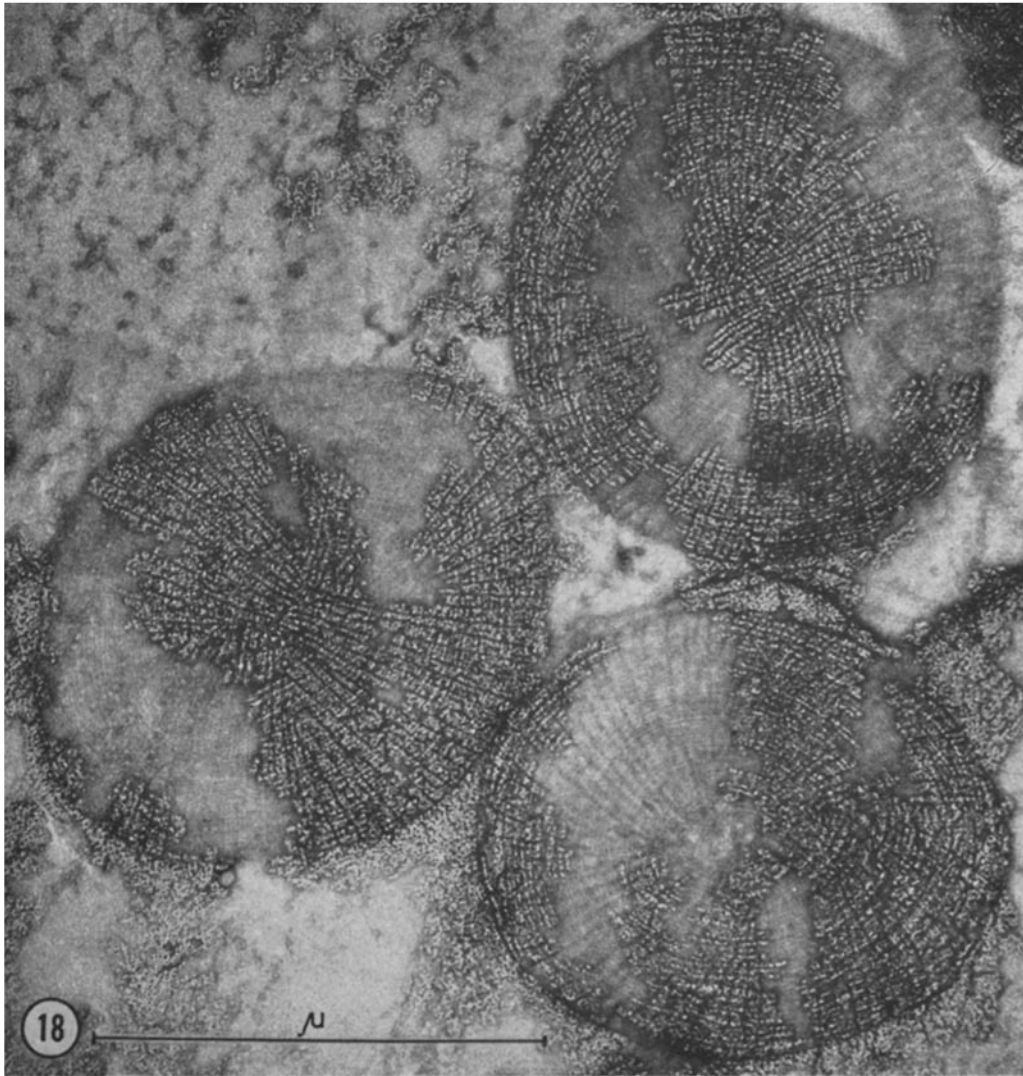


FIGURE 18 Isolated scales from fraction A. These have been washed with seawater and negatively stained with 2% PTA, pH 7.2. Note the spongy, particulate material (presumably of pectic character) which lies over the microfibrillar plate as well as separated from the scales.  $\times 60,000$ .

been determined enzymatically to be glucose. (*d*) The alkaline-purified microfibrillar material is soluble in cuprammonium reagents. (*e*) Nuclear magnetic resonance spectroscopy of the benzoylated product exhibits proton signals characteristic of cellulose and different from those of other related polysaccharides. (*f*) A positive reaction to zinc chloride-iodine is given by the alkaline-purified microfibrillar material. (*g*) The microfibrils exhibit cracking sites (= breakage loci)

which are typical for highly crystalline cellulose. (*h*) The microfibrils have a characteristic ribbon-like appearance known for various types of native cellulose. (*i*) The maximum flexibility of the microfibrils corresponds to that of other plant celluloses and is in agreement with the 2% maximal extensibility of the cellulose chain lattice calculated by Meyer and Lotmar (see 39). (*j*) The product of alkaline treatment tends to aggregate in the mode of lateral fasciation characteristic for cellulose (17).



FIGURE 19 Higher magnification of a scale similar to those shown in Fig. 18, demonstrating the frequent, oriented array of the covering pectic mass in organized tiers in between the radial microfibrils. Negatively stained with 2% PTA, pH 7.2.  $\times 160,000$ .

Although the special degree of eucellulosic character (Preston, 41) cannot be calculated, it might be emphasized that with respect to all properties thus far studied, the scale cellulose is equal to the control, cotton cellulose. The fact that strong alkaline treatment rapidly produces shorter rods but then does not further degrade the fibrillar material might be tentatively explained by the existence of sites in the longitudinal crystal which are more accessible to hydrolysis ("Lockerstellen" as defined by Schulz-Husemann-Pacsu-Ranby, see e.g. 65).

The possibility that we are dealing with a mixed polyglucan possessing  $\beta$ -1-3 linkages of considerable quantity in addition to the  $\beta$ -1-4 linkages seems to be ruled out by: (a) the extreme stability of the microfibrils to harsh alkaline

solutions; (b) their solubility in cuprammonium reagents if handled in a proper way; and (c) the proton signals H (1), H (3), H (4), and H (6) of the NMR spectrum identical to those characteristic for cotton cellulose. While it is true that  $\beta$ -1-3 linkages have been reported in other chrysophycean, phaeophycean, and dinophycean algae (e.g. 36, 44, 59), the presence of cellulose in the chrysophycean genera *Ochromonas* (59), *Dinobryon*<sup>3</sup> (13), in the xanthophyceae *Vaucheria* (28, 49), *Botrydium*, and *Botrydiopsis*<sup>4</sup> (11), and also in several brown algae (9, 28) would suggest a wider distribution than commonly believed.

Scale formation in *P. scherffelii* represents the

<sup>3</sup> W. W. Franke. Data in preparation.

<sup>4</sup> H. Falk, J. Pfisterer, and W. W. Franke. Data in preparation.



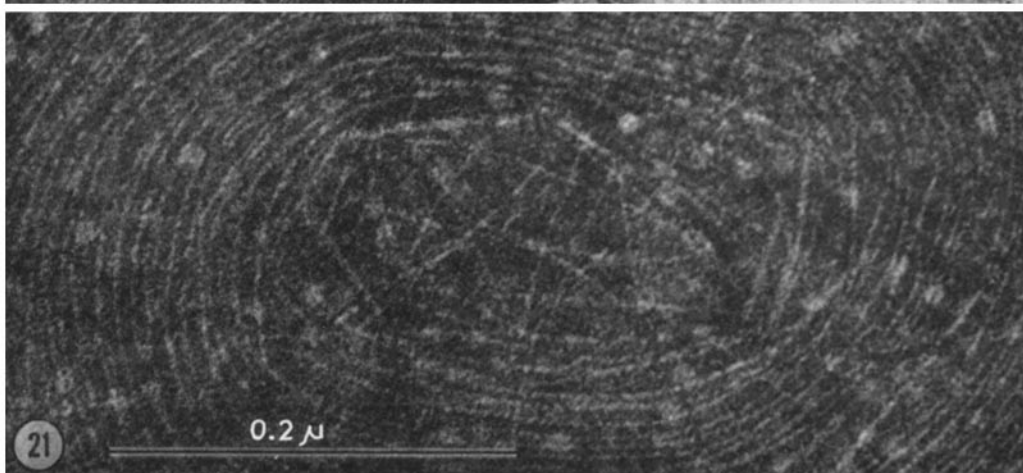
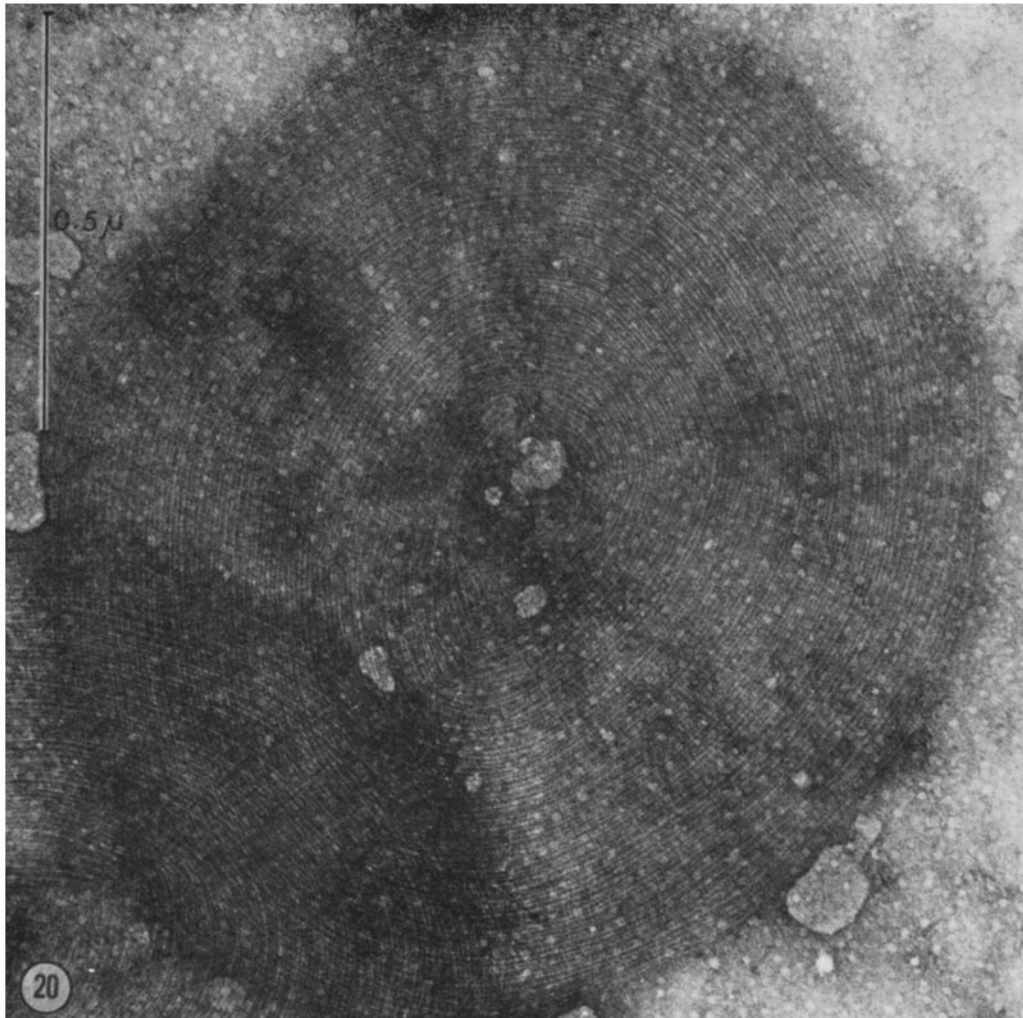


FIGURE 20 Scale preparation washed in distilled water which obviously has removed the "pectic" component, more clearly revealing the radial and concentric microfibrillar cellulose constituents. Negatively stained with 2% PTA, pH 7.2.  $\times 110,000$ .

FIGURE 21 Enlargement of the central region of the scale (the "eye"). In this region, many of the microfibrils show kinking or cracking sites (= breakage loci) characteristic for highly crystalline cellulose. Water-washed preparation, negatively stained with 2% PTA, pH 7.2.  $\times 270,000$ .

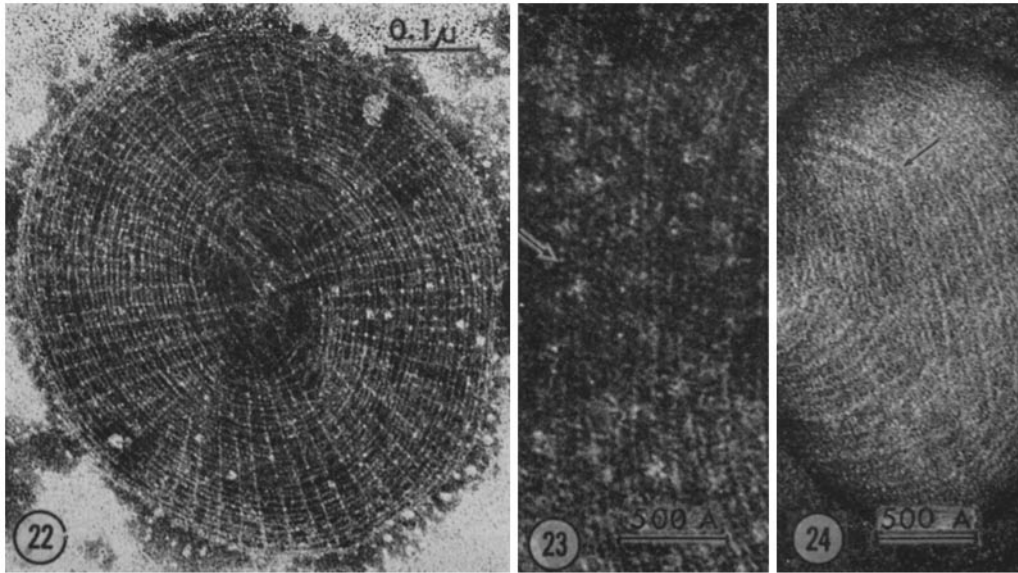


FIGURE 22 "Minor" scale washed with distilled water to demonstrate the intricate network of the cellulose microfibrils. Negatively stained with 0.5% PTA, pH 7.2.  $\times 120,000$ .

FIGURE 23 "Beaded" appearance (arrow) of the cellulosic microfibrils. Negatively stained with 2% PTA, pH 7.2.  $\times 250,000$ .

FIGURE 24 Scale microfibrils from a water-washed preparation. Arrow indicates a breakage locus where the orientation of the microfibril changes, thus revealing its ribbon shape. Scale lying over a hole in the supporting film. Negatively stained with 2% PTA, pH 7.2.  $\times 260,000$ .

first evidence that the Golgi cisternae are capable of cellulose synthesis as well as the synthesis of other polysaccharides. Thus, referring to the scheme of Northcote and Pickett-Heaps, the story of the cellular pathway of polysaccharide biosynthesis now appears to be more lucid and greatly simplified. In this new context, synthesis of polysaccharides now can be thought to occur at the inner surface of the Golgi and/or ER cisternae in which only intracisternal pools of activated sugar monomers are consumed rather than cytoplasmic, nonmembrane-surrounded ones. The kind of polysaccharide formed then could be directed and determined by the specificity of the synthesizing enzymes and/or by the relative concentrations of the activated sugars within the cisternae. The observation that newly formed scale microfibrils first can be seen directly adjacent to the inner membrane and then later situated equidistantly between the cisternal membranes leads one to the suggestion that the synthesizing enzymes are membrane-bound or, at least,

membrane-attached. This is in harmony with Mühlethaler's concept (40) that distinct particles on the surface of the plasmalemma are enzyme complexes along which the cellulose chain should be synthesized. In addition, the present finding of cellulose synthesis within the Golgi cisternae does not necessarily contradict the concept that the outer surface of the plasma membrane should be the site of cellulose polymerization since both membranes are closely related in terms of: (a) membranogenesis with respect to a continuity of the observed exocytotic membrane flow Golgi  $\rightarrow$  plasma membrane (see 23); and (b) morphological and functional similarity of the outer surface of the plasma membrane with the inner surface of the Golgi cisternal membrane.

As a consequence of our findings, one should be able to predict that the chain length determining system for cellulose molecules is membrane-attached (35), and in the case of *P. scherffelii* is located within the cisternal polymerization center.

It is interesting to note that the width of cellu-

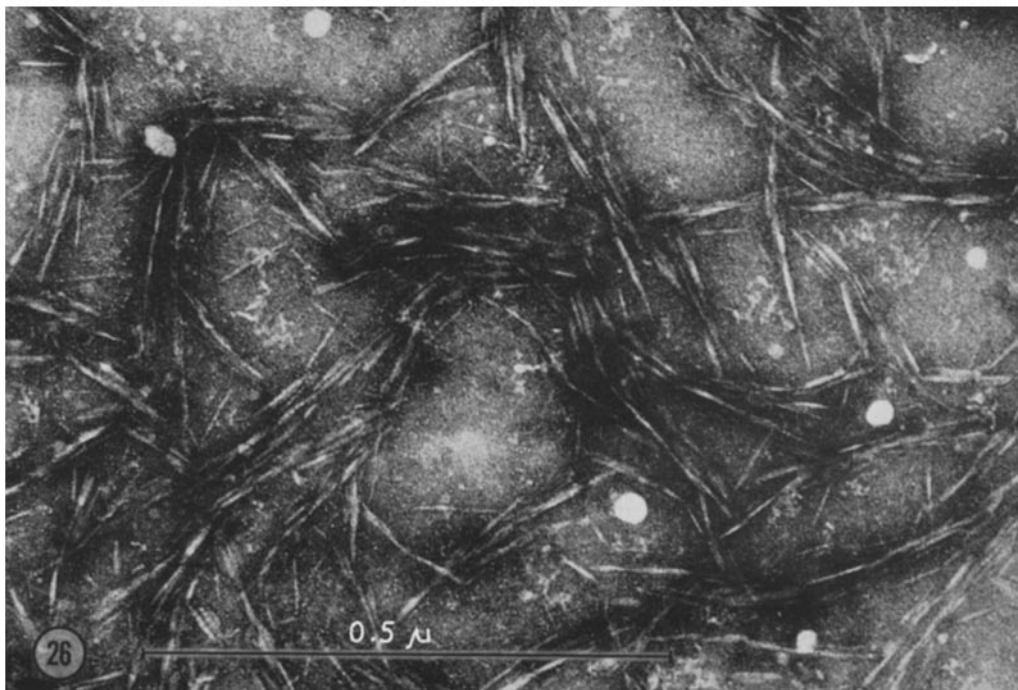
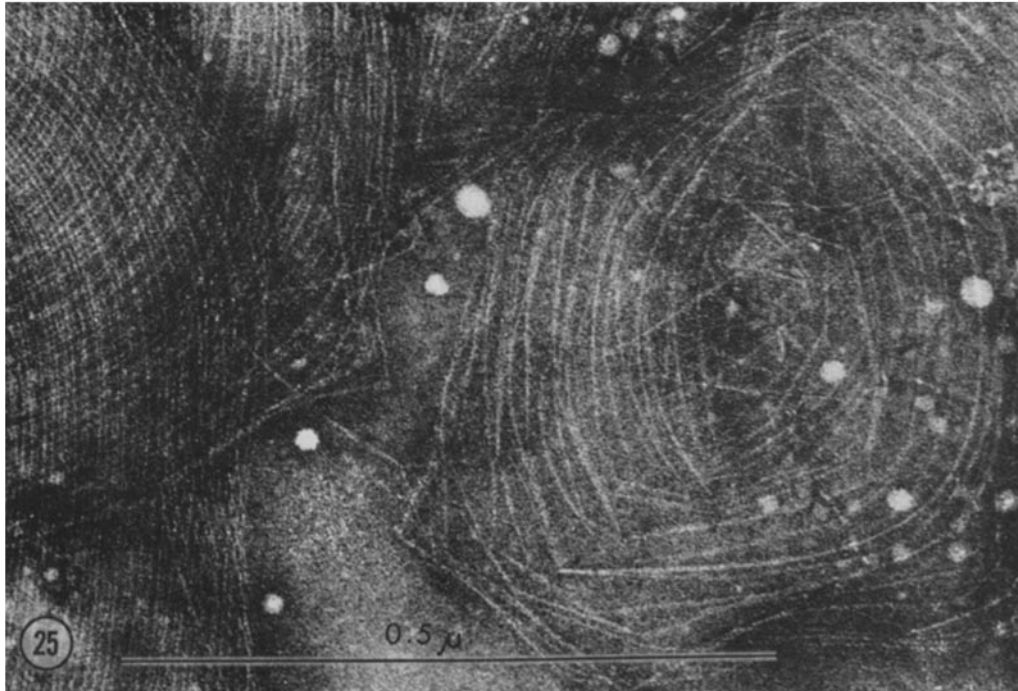


FIGURE 25 Scale preparation treated with 5% KOH for 5 min at room temperature. Note the increased disorder which reveals the ribbon structure as well as abundant breakage loci. Negatively stained with 2% PTA, pH 7.2, after neutralization with distilled water.  $\times 175,000$ .

FIGURE 26 Flocculent of the cellulose scale microfibrils resulting from 12 hr treatment with 24% KOH, including 2 hr at the boiling point. Short rods tend to aggregate by lateral fasciation. Negatively stained with 2% PTA, pH 7.2, after neutralization with distilled water.  $\times 140,000$ .

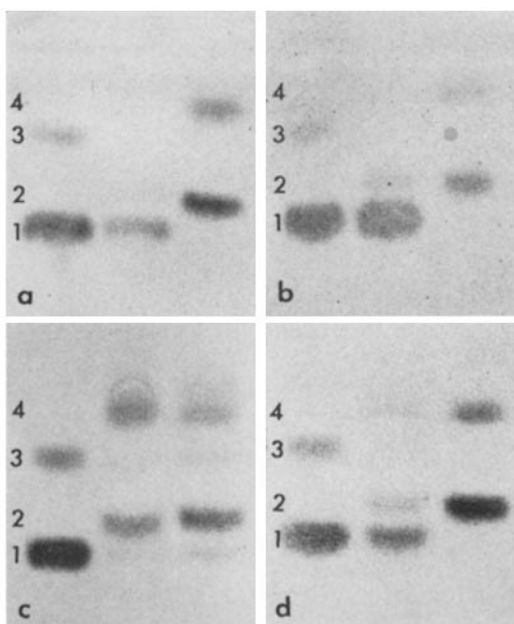


FIGURE 27 Thin-layer chromatogram of the separated sugars of cell walls and scale fractions after total hydrolysis. (a) left trace, reference sugars 1 (glucose) and 3 (arabinose); middle trace, prehydrolyzed and alkaline-purified scale fraction A; right trace, reference sugars 2 (galactose) and 4 (ribose). (b) left and right traces, reference sugars as in (a); middle trace, alkaline-purified scale fraction A. (c) left trace, reference sugars 1 and 3; middle and right trace, intact cell walls of the vegetative cells from two different preparations, without any acid and alkaline pretreatment. (d) left and right trace, reference sugars as in (a); middle trace, scale fraction A repeatedly washed with distilled water. (Xylose, mannose, and fructose were also run, but were not detected in the scale fractions and the walls.)

lose microfibrils in the scales of *P. scherffelii* is in the same order of magnitude reported for "nascent" celluloses in quince slime (14), corn coleoptiles, and *Acetobacter xylinum* (46), as well as germinating spores of *Funaria hygrometrica* (24). Moreover, the scale cellulose exists in ribbons of 12 to 25 × 25 to 40A, which is in contrast to the concept of Frey-Wyssling and Mühlethaler (18) of an isodiametric, 35 × 35 A elementary fibril as the ultimate structural unit of native cellulose.

Turning to the noncellulosic scale constituents, it is obvious that inter-scale polysaccharides of the intact cell wall exceed the quantity of cellulose as shown by the enrichment of galactose and ribose

on thin-layer chromatography. We believe that this may have been the reason why earlier workers were not able to detect the scale cellulose by histochemical methods (51), or by chemical and physical procedures (22). This polysaccharide is predominantly of pectic character as indicated by its high content of galactose and by its acid groups detected by alcian blue (48) and ruthenium reaction (25) as well as by its solubility in agents which form chelating complexes with bivalent cations. On the other hand, it should not be overlooked that a considerable amount of non-membranous proteins seems to be associated with the scales, and it is therefore possible that the scale material represents a complex protein-polysaccharide system.

The complexity of the scale chemical composition of at least two components, namely, cellulosic microfibrils and pectic granules, has hindered the detection of the cellulosic constituent in the work of Green and Jennings (22). Using shadow casting methods as a monitor for the presence and intactness of the scales, these authors did not observe a dissolution of scales of *Chrysochromulina chiton* by pectinase.

From the section studies, it seems that the most distal Golgi cisternae contain the cellulosic network in association with the amorphous material which we tentatively identify as the pectic part. On the basis of morphological evidence, we cannot decide whether this pectic material may be made within the Golgi cisternae or whether it is transported into these cisternae by a membrane-flow fusion of the peripheral vesicles which seem to be derived from the periplastidal cisternae. It is of stimulating interest in this connection to recall to mind the study by Ben-Hayyim and Ohad (3) who proposed that charged polysaccharides may play an important role in affecting the orientation and crystallization of cellulose as indicated by their studies with cell-free extracts of *Acetobacter xylinum*.

A tentative scheme for the role of the Golgi apparatus in cell wall formation in *P. scherffelii* is presented in Fig. 29. The Golgi apparatus is the organelle responsible for the synthesis and assembly of a cell wall component, the scale. The periplastidal cisternae and the amplexus are the principal sources of membrane input for the Golgi apparatus. Progressive membrane differentiation occurs along the maturing (proximal → distal) face. Those cisternae distinguished by central

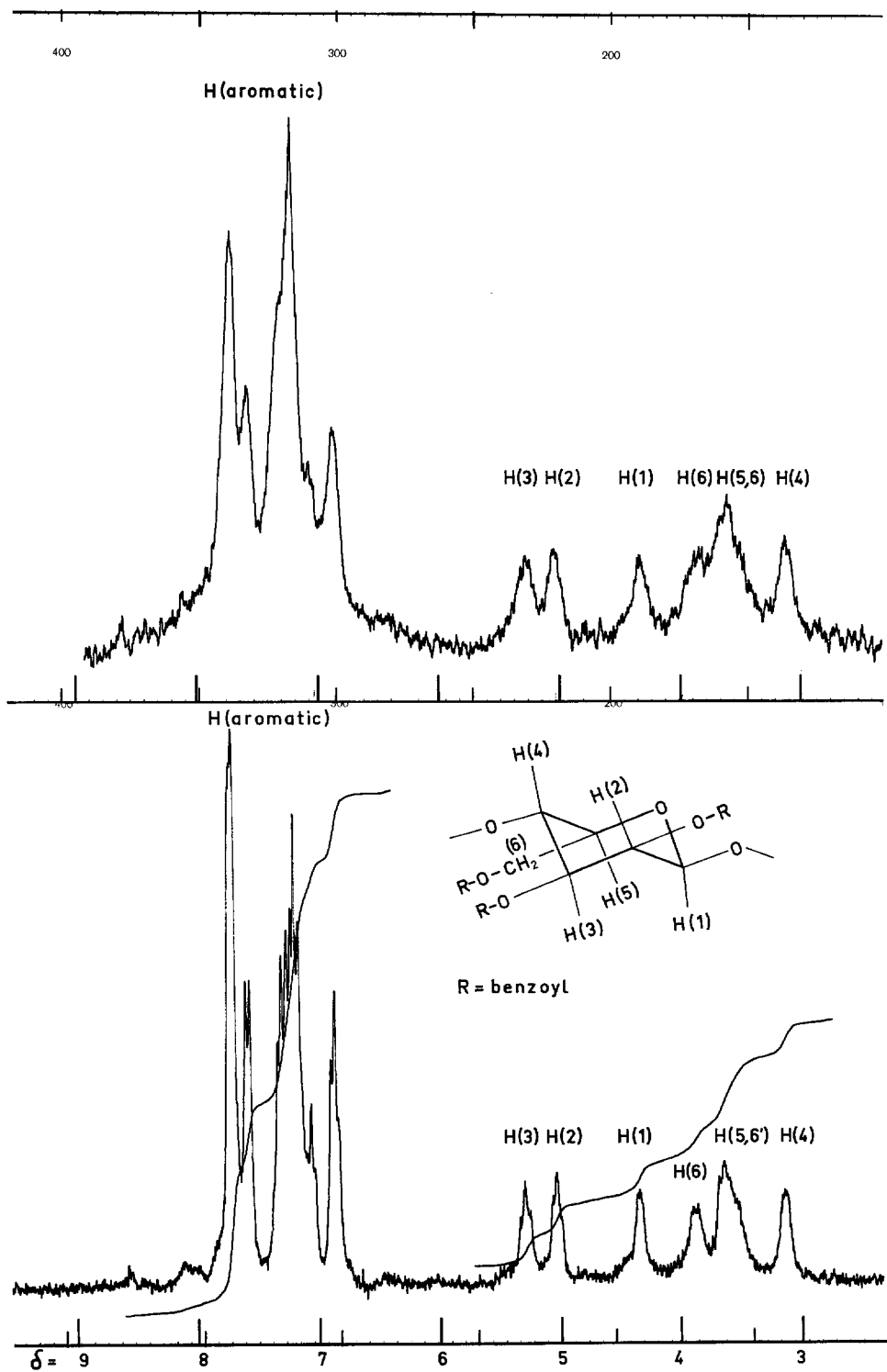


FIGURE 28 Nuclear magnetic resonance spectrum of 2,3,6-tribenzoyl-derivatives of the *Pleurochrysis* scale material (upper spectrum) and reference cotton cellulose (lower spectrum) recorded by using a Varian HR-220 spectrometer (Varian Associates, Palo Alto, Calif.).  $\delta$  = chemical shift; solvent,  $\text{CDCl}_3$ ; temperature,  $+60^\circ\text{C}$ ; frequency, 220 MHz. Positions of the proton signals are equal in both spectra. The broader peaks in the scale material might be explained due to the higher degree of polymerization (DP) of this material in comparison with the cotton cellulose used (DP  $\sim 100$ ).

dilations are interpreted to be sites of polymerization of glucose monomers into the crystalline network of the cellulose chains. At the inner thickened surface of the cisternal membranes of

the central dilations, GDP- and/or UDP-glucose is polymerized into cellulose chains of a determinate length as known from celluloses of various sources (26, 35). As the first visible result of this

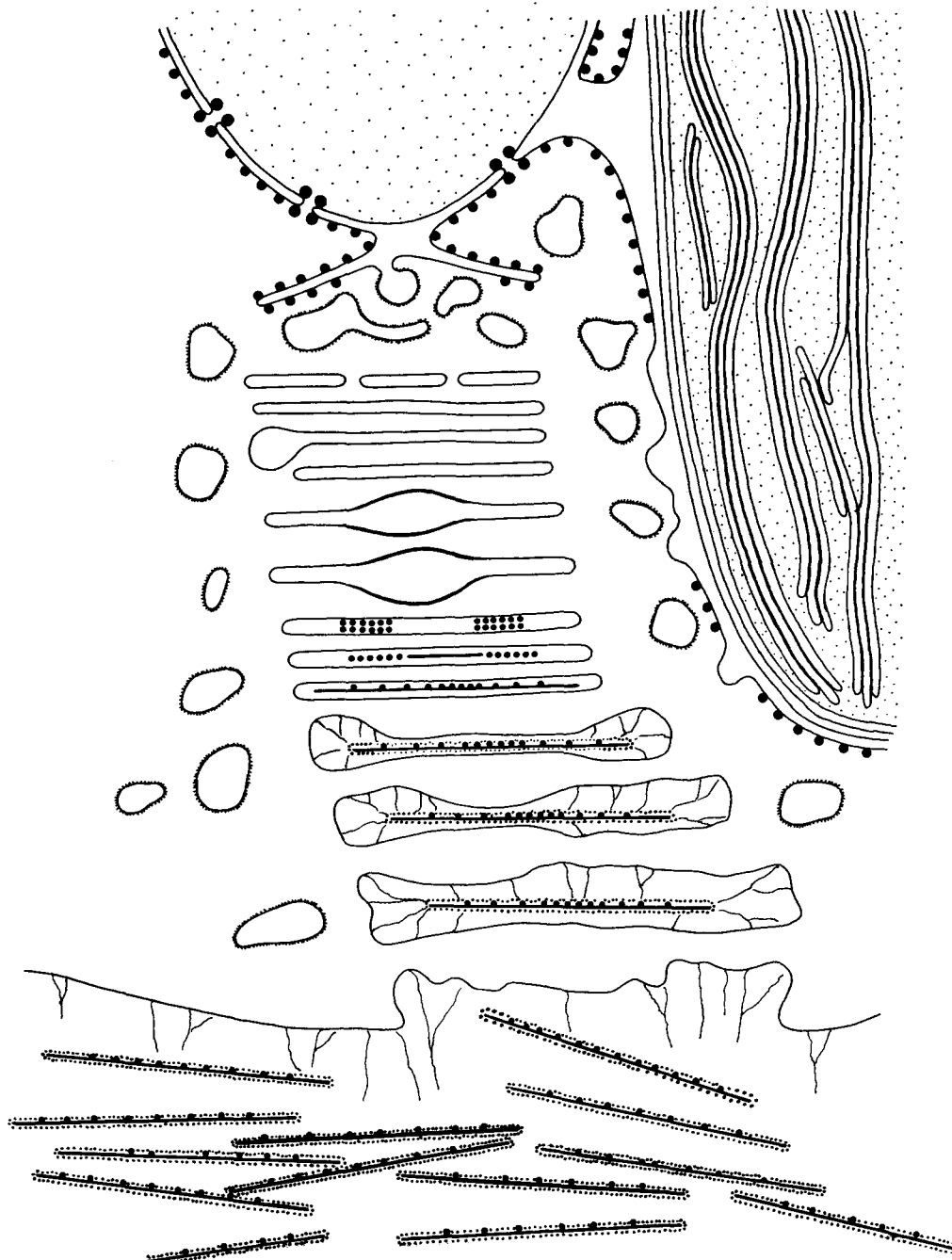


FIGURE 29 Schematic diagram for the role of the Golgi apparatus and its relation to cell wall formation. For details, see text.

polymerization, two layers of fibrils appear to lie adjacent to the inner membranes of the cisterna. These are presumed to be the first-synthesized concentric fibrils. It is possible also that some of these may be destined to become radial fibrils, but at this stage of development it is difficult to make a clear distinction. In the next-most distal cisterna, the double layers of fibrils are converted into a single layer, and shortly thereafter, the distinctive morphology of the scale complex becomes apparent. This entire process of microfibrillar formation takes place over the differentiation of three to four cisternae.

Inflations of the distal cisternae comprise the next most striking change in the Golgi apparatus. The first inflated cisternae are initiated by a more peripheral swelling, probably as a result of fusion with peripheral vesicles which may have originated from blebings of the periplastidal cisternae. In this connection, it seems to be a reasonable hypothesis that such vesicles represent a membrane-flow mechanism for further membrane input as well as for input of the noncellulosic polysaccharide constituents. Release of the scale is finally accomplished by a fusion of the cisternal membranes with the plasma membrane.

With respect to the generalized pathway of cellulose biosynthesis in the plant kingdom, we think that it is a valuable concept that the polymerization of cellulose as well as of many other polysaccharides takes place within the cisternal space of the Golgi apparatus, and possibly the ER system. The reason that structured crystalline cellulose microfibrils had not been seen previously within the Golgi cisternae of higher plants, then, could be due to the existence of a time gap between the polymerization of the glucan chain and the aggregation of many chains into the crystalline microfibril. This gap, therefore, would be extremely short in the case of *Pleurochrysis*, but could be longer in other plant cells so that the crystallization in the latter would occur extracellularly, i.e., after extrusion into the cell wall.

The observations by Brown (5) that the Golgi apparatus is involved in the synthesis of a structured wall component in *P. scherffelii* suggested that this organism might be closely related to other "scale" producing algae such as *Chrysochromulina* (32), *Prymnesium* (31), and *Hymenomonas* (33, 34, 50) inasmuch as these scales are made within the Golgi apparatus.

It is now clear that the basic morphology of the unmineralized scales of *P. scherffelii* is identical to that of other haptophycean genera as *Prymnesium* (31), *Chrysochromulina* (32), *Hymenomonas* (33, 34, 50), and *Coccolithus* (33). Furthermore, our preliminary observations strongly suggest that the fibrillar structure in these other haptophycean genera is cellulosic, and that the cellulosic network is a homologous basic structure among these algae.

In light of the confirmation for a cellulosic scale component made by the Golgi apparatus in *P. scherffelii*, it would be worthwhile to examine the wall chemistry in other Chrysophyta as well as an unrelated class of the Chlorophyta, the Prasinophyceae.

Moreover, the homology of the unmineralized scale components of the coccolithophorids with respect to those of *Chrysochromulina*, *Prymnesium*, and *Pleurochrysis* is of special interest. Our preliminary observations reveal a fibrillar network in *Hymenomonas* (formerly *Cricosphaera*, see 33), closely resembling that type described in the present study. Thus, it seems likely that the homologous basic scale constituent among all these algae is the cellulosic network, and that the observed variability, whether in the form of mineralized coccoliths or in the form of mineral as well as nonmineralized scale appendages, is due to the noncellulosic constituents that are produced on the cellulosic plate. This concept requires further proof by comparative biochemical studies of the pathway of cellulose biosynthesis among these diverse algae.

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