THE ROLE OF THE GOLGI APPARATUS IN THE BIOSYNTHESIS AND SECRETION OF A CELLULOSIC GLYCOPROTEIN IN PLEUROCHRYYSIS: A MODEL SYSTEM FOR THE SYNTHESIS OF STRUCTURAL POLYSACCHARIDES

R. Malcolm Brown, Jr., 1 Werner Herth, 2
W. Werner Franke, 3 and Dwight Romanovicz 4

1, 4 Department of Botany, University of North Carolina, Chapel Hill, 2, 3 Division of Cell Biology, University of Freiburg, Freiburg i. Br. West Germany.

Acknowledgments:
The authors would like to thank the Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, the National Science Foundation (GB 23047 to R.M.B.), Deutsche Forschungsgemeinschaft (to W.H. and W.W.F.), and the NDEA (to D.R.) for generous financial assistance.

W.H. wishes to thank Professor E. Husemann for many discussions and support of this work. For the amino acid analyses, we thank Prof. Witt from the Childrens Hospital of the University of Freiburg, Germany. We thank Professors H. Stanley Bennett, Peter Sitte, and Dr. Heinz Falk for helpful discussions.

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ABSTRACT:

The Golgi apparatus of *Pleurochrysis scherffelii* functions in the biosynthesis and transport of the polysaccharide scales. The stepwise assembly of the scale subcomponents has been followed with the silver-methenamine-periodic acid technique as well as normal poststaining procedures. The radial microfibrils are first assembled in a bilayer of parallel microfibrils, which later becomes unfolded. Spiral bands of cellulosic microfibrils are then deposited onto the distal surface. Finally, the network of radial and "concentric" microfibrils is covered with amorphous material, then the fully assembled scale is secreted. The chemical composition of certain scale subcomponents is summarized in this paper. The alkali-resistant "concentric" microfibrils consist of a cellulosic moiety intimately associated with peptides. Such an intimate association of structural polysaccharides with peptide moieties also has been found for β-(1-3)-xylan, β-(1-4)-mannan, β-(1-3)-glucan, and even cotton cellulose. This evidence suggests a general similarity of the pathways of animal glycoprotein biosynthesis and plant cell wall polysaccharides including structural polysaccharides. This general pathway derived from the *Pleurochrysis* model has been compared with the more classical pathway postulated for cellulose biosynthesis in higher plants.
SYMBOLS

PA = periodic acid
ER = endoplasmic reticulum
"Z" = a stage of cisternal membrane unfolding
GLC = Gas-liquid chromatography
TMS derivatives = trimethylsilyl-derivatives
NMR = nuclear magnetic resonance spectoscopy
DP = degree of polymerization
INTRODUCTION

In recent years, while considerable attention has been devoted to cell wall and polysaccharide biochemistry, relatively little is known about these biosynthetic pathways in terms of cellular substructure and function. In general, the plant cell wall has eluded a precise descriptive study of cytological events leading to its formation, primarily because it is composed of a rather heterogeneous and amorphous assemblage of microfibrillar and matrix substances. While certain of these constituents have been shown to be produced by the Golgi apparatus (e.g., Northcote & Pickett-Heaps) little is known about the biosynthetic pathway of structural polysaccharides, especially cellulotic products. Recent biochemical evidence by Ray (1969) has suggested that a subcellular fraction with marked \( \beta-(1,4) \)-glucan synthetase activity is confined largely to membranes of the Golgi fraction. While the role of the Golgi apparatus in cellulose biosynthesis also is suggestive for higher plants from indirect evidence, e.g., hypertrophy of Golgi vesicle cells with increased wall formation, little information concerning the secretory pathway is known with the exception of the recent work by Brown and co-workers (Brown, 1969; Brown et al., 1969, 1970). In this instance, a unicellular marine haptophycean alga has been used as a model system to investigate cellulose biosynthesis. On the other hand, more details are known about cellulose biosynthesis in certain prokaryotic systems, namely *Aerobacter* (Colvin, 1964).

It is well known that marine haptophycean algae are capable of producing an architecturally defined scale (see Manton, 1967, Pienaar, 1969, Manton & Leedale, 1969). The discovery that the Golgi-produced scales contain a cellulotic moiety came about in a somewhat strange manner. The early investigations of Green and

\[ \text{For definition of "structural polysaccharides" see Herth, et al., 1972.} \]
Jennings (1967) failed to reveal the presence of cellulose.

The investigations of Brown and co-workers (Brown, et al., 1969, 1970) showed that an alkali-stable moiety of purified scales could be hydrolyzed to glucose monomers and that by mild hydrolysis, celllobiose could be obtained (Herth, et al., 1972). In addition, a spectrum of other biochemical, biophysical and cytological techniques have confirmed that a cellulotic moiety exists in the scales of Pleurochrysis scherffeli. These tests included analysis of linkages of the sugar monomers by means of nuclear magnetic resonance spectroscopy, x-ray diffraction, and various chemical tests (see Brown, et al., 1969, 1970; Herth, et al., 1972).

Recently, it has been suggested that the harsh alkali-resistant cellulotic scale moiety is covalently linked with a peptide moiety (Herth, et al., 1972). This evidence has led to the concept that Pleurochrysis cellulose exists in a form of glycoprotein. Adding to this growing list of analyses of scale products, we are accumulating cytological and cytochemical evidence which describes the specific assembly stages of various scale subcomponents by the Golgi apparatus.

The purpose of this presentation is to consider the cytological, cytochemical and biochemical evidence for the role of the Golgi apparatus in the production of structural polysaccharides. Because Pleurochrysis is such a distinctive system in which the architecturally-defined Golgi polysaccharide products can be isolated and analyzed, there is an opportunity to correlate this background of information with data from other polysaccharide synthesizing systems, namely the structural polysaccharide components of Acetabularia, Caulerpa, lily pollen tube wall, cotton, and onion root, as they relate to growth and differentiation of the plant cell in general.
Materials and Methods

Cultures:
Axenic cultures of *Pleurochrysis scherffeli* were grown on agar plates in an enriched van Stosch sea water medium as previously described (Brown, et al., 1970).

Isolation of scales:
Scale fraction A was obtained according to the methods earlier described. (Brown, et al., 1970, and Herth, et al., 1972).

Extractions:
For the details of organic, chlorite and alkali extractions see text.

Electron microscopy:
We used the same fixation and embedding conditions as described in Brown, et al., 1970. Sectioned material to be stained with silver hexamine (Pickett-Heaps, 1967) was mounted on formvar-coated stainless steel grids which were then treated to block or reveal certain staining moieties. (For details, see text and Romanovicz, et al., in prep.) Negative staining was performed by adding 2% PTA (adjusted to pH 6.8 with 1N KOH) to specimens which had been first dried on carbon-stabilized formvar films, then briefly rinsed in distilled water.

Chemical characterization

Staining reactions
See Brown, et al., 1970 for details of the zinc-chloride-iodine staining.
Ruthenium red staining was performed with a 1% aqueous solution.
Alcian blue staining was used according to the methods of Parker and Diboll, 1966.
RESULTS

I. Cytological and Cytochemical Observations

A. Cell Wall Structure

Functionally, the cell wall of *Pleurochrysis* probably differs little from higher plant cell walls, in that it provides a structural framework which encompasses the cell protoplasm. This, in turn, probably determines ultimate cell shape and may regulate cell growth. The cell wall structure of *Pleurochrysis* however, differs significantly from higher plant cell wall systems in that it is composed of a morphological unit known as the scale. The unit scale is well known for other haptophycean cells (Manton, 1967) but in most of these algae, the scales are either released into the culture medium, or form a rather loose layer over the cell surface. In most Haptophyceae the predominant phase consists of a motile zoospore. In *Pleurochrysis*, however, the major phase is the non-motile vegetative cell which produces a very compact and definitive cell wall by aggregation or stratification of the unit scales.

Light microscopic examination of *Pleurochrysis* cell walls reveals a positive birefringence. The walls also exhibit a positive iodide dichroism (Brown, 1969). Furthermore, the walls of *Pleurochrysis* react with the periodic acid Schiff's test indicating that it is carbohydrate. This is substantiated at the ultrastructural level by silver methenamine following periodate oxidation (Fig. 8). *Pleurochrysis* cell walls also react with Alcian stains. At pH 0.5, positive Alcian staining results under conditions where carboxyl groups are fully protonated. Following methylation, the cell walls fail to react with Alcian stains at pH 0.5 or pH 2.5. Saponification with KOH reestablishes carboxyl groups which subsequently react strongly with Alcian Blue at pH 2.5, but negatively at pH 0.5. The protonation and methylation reactions suggest that at least one constituent
of the scale cell wall is a sulfated polysaccharide. These light microscopic reactions are confirmed at the ultrastructural level with the silver methenamine reaction using specific blocking reagents such as iodoacetate for sulfate groups or bisulfite for aldehyde groups (Table I). These blocking reagents have been used in combination with periodate oxidation in order to distinguish the free aldehydes present from those that were produced by the specific oxidation of 1-2 glycol groups in the polysaccharides. Treatment of vegetative cells with ruthenium red reveals a positive staining of the cell wall layers suggesting acidic constituents of the cell wall (Brown, 1970).

B. Scale Structure

When vegetative cells of Pleurochrysis undergo zoospore formation, the cell wall becomes dissociated into the unit scale structures. They can also be removed from whole vegetative cells by sonification (Herth, unpublished) and by high pressure decompression (Brown, unpublished data). Differential centrifugation methods can be used to purify and enrich scale fractions from protoplasmic and whole cell contaminants. The unit scale consists of radial and "concentric" elements of microfibrillar nature. Upon this microfibrillar base can be deposited other organic (e.g., pectic) or inorganic constituents, for example the calcium carbonate rims of the coccoliths (Franke & Brown, 1971). The radial and "concentric" microfibrils are readily observed in negatively stained isolated scale fractions (Fig. 4). The radial patterns exhibit a very precise symmetry in two planes. Amorphous acidic sulfated polysaccharide components can be deposited in between the radial microfibrils (Fig. 3).

The radial microfibrils have about the same dimensions as the "concentric" microfibrils; but they differ in their cytochemical reaction to the silver methenamine reaction and their stability in alkali washes. Furthermore, the radial
microfibrils are removed by pectinase whereas the "concentric" microfibrils are only degraded by cellulase (Herth and Brown, unpublished data). Evidence to suggest degradation of radial microfibrils rather than separation from the "concentric" microfibrils is based on two points: (1) Following 5% KOH treatment which removes the radial microfibrils (Fig. 5), the supernatants of these preparations do not contain fibrillar components that could be detected (by negative staining) in the pellet after high speed centrifugation (48,000 X G for 45 min.). Instead, the dissolved polysaccharides can be precipitated with ethanol and CaCl$_2$ ions (Herth and Romanovicz, unpublished data); (2) Different stages of radial microfibrillar dissolution could be detected with negative staining after treatment with pectinase or dilute alkali.

The so-called "concentric" microfibrils originally described to be concentrically organized (Brown et al., 1970) are now known to be organized in several continuous spiral bands of approximately 8 to 10 microfibrils each. Because these microfibrils are so closely packed, it was not possible to deduce the spiral configuration until partially alkali-degraded scales were observed (Fig. 6). The individual spiraling microfibril consists of a ribbon which is approximately 40 Å wide and approximately 20 Å in the narrow axis which lies adjacent to the radial microfibrils and parallel to the flat plane of the scale. The microfibril unit has subcomponent structures as shown in Fig. 7 in which a single spiral microfibril has split into at least three linear units which may actually be associations of several glucan polymers. In this state, the spiraling microfibrils are structurally more flexible and the scale tends to fold, or the "concentric" bands tend to separate from one another. The resulting physical damage produces consistent breakage loci in the spiral microfibrils, similar to those that occur in the central region or "eye" of the unaltered scale. These latter natural breakage loci are thought to occur during the final stages of spiral
microfibril deposition when the limited secretion space causes the bending angle to increase beyond the maximum flexibility of 2% for the crystalline product.

C. **Structure of Protoplasmic Organelles and Constituents with Particular Reference to the Golgi Apparatus**

Figures 1 and 8 depict a typical view of the protoplasmic contents of a vegetative cell of *Pleurochrysis*. The uninucleate cell contains two parietal chloroplasts. The outer nuclear membrane is continuous with and surrounds both chloroplasts. An extension of the outer nuclear membrane forms an amplexus which becomes the site of vesicle delimitation for the forming face of the Golgi apparatus (Fig. 1).

The single Golgi apparatus of *Pleurochrysis* is a most complicated organelle and is composed of a stack of differentiating cisternal membranes. The Golgi apparatus is polarized, with the secreting face of inflated cisternae always oriented toward the cell surface (Fig. 2). The forming face of the Golgi apparatus apparently becomes organized with the fusion or coalescence of vesicles which seem to originate from the budding face of the amplexus. This particular region of the amplexus is free of ribosomes. The forming face of the Golgi apparatus consists of thin, very closely appressed cisternae of rather uniform dimensions. Midway through the differentiating stack of cisternae "central dilations" appear (Fig. 2). These dilations are thought to contain the precursors and enzymes for the synthesis of polysaccharides because all cisternae on the forming side of these dilations have no visible product within them, while all cisternae on the secretion side of these dilations contain a visible secretory product. The cisternae of the secreting face of the Golgi apparatus are considerably larger than those of the forming face (Fig. 2). This may be a result of
a secondary fusion of vesicles migrating from the ER to the periphery of the
Golgi apparatus. These vesicles appear to carry additional amorphous poly-
saccharide products to the surface of the microfibrillar network which has been
produced in earlier stages.

Transport of the mature scales to the cell surface occurs when the cis-
ternal membranes fuse with the plasma membrane, releasing the scale products.
Two bundles of microtubules flank the secretory face of the Golgi apparatus
(Brown and Franke, 1971). Apparently a function of these microtubules may be
to continually orient the secreting face of the Golgi apparatus so that the
products can be transported to the cell surface. Other functions have been
postulated for these structures (Brown & Franke, 1971). When cells of Pleurochrysis
are treated with colchicine, the microtubular bundles disappear and the secre-
tory face becomes disorganized (Brown & McLean, unpublished). The continuing
intracellular production of scales results in autophagic consumption of scales
since the secretion mechanism appears to be inhibited (Fig. 18).

Adjacent and interior to the plasma membrane is a large peripheral sac
which is continuous over the cell surface except for the region immediately
opposite the secretion face of the Golgi apparatus. Through this "hole", scales
are secreted. This parietal cisterna is thought to represent the "ball-bearings"
or flexible surface upon which the protoplast could rotate to ensure a uniform

D. Developmental Pathway of Scale Biogenesis (see Fig. 15)

Fig. 15 depicts our model of the secretory pathway of scale production by
the Golgi apparatus in Pleurochrysis. This model has been developed from the
information of several thousand electron micrographs and dozens of fixations
during the past several years. Other methods used to arrive at this model include
serial sections, freeze etch replicas, negative staining, and the electron microscope tilting stage for stereoscopic analyses. Because a scale is produced about once every two minutes, the differentiation between one cisterna and the next may be quite transient (Brown, 1969, 1970). Thus, all structural variations that occur within this time usually are not present in a single electron micrograph. With these data at hand, we have been able to suggest a more precise developmental pathway than heretofore possible (for comparison with the original pathway proposal and its modifications, see Brown, et al., 1970).

Cisternal Membrane Delimitation

The immature forming face of the Golgi apparatus consists of a coalescence of vesicle membranes into a flat sac (Fig. 13). As this sac grows, presumably by continued coalescence of these budding vesicles from the ER, certain regions differentiate within the cisternal membrane. These areas include incipient organizing centers for the polymerization and/or crystallization of scale polysaccharide products. Staining with silver methenamine reveals two types of dilated organizing centers; one which is filled with product, (i.e., reactive with silver), and the other which remains non-reactive with silver under the conditions employed (Figs. 8,9). The non-staining organizing center is believed to contain the precursor monomers, enzymes, and/or noncrystalline polymers which will become organized into the "concentric" microfibrils of cellulosic glycoprotein. The silver-reacting dilated organizing center contains the precursors and enzyme machinery for the synthesis of radial microfibrils (Fig. 11).

Formation of Radial Microfibrils

The radial microfibrils of the scale exhibit a bilateral symmetry in two planes (Fig.3). The radial microfibrils are formed adjacent to the inner cisternal membranes in a folded, mirror-image pattern, i.e., the long axis of
The radial microfibril precursors of the silver reacting dilation seem to be fed mostly into a central compressed region of the cisternal membranes (Fig. 11), although the precursors can occasionally be seen feeding toward the periphery of the Golgi cisterna (Fig. 14). The folded scale axis has been observed quite frequently (see Figs. 10, 11, 12, 13). In addition, this stage has been proven with stereoscopic observation of tilted specimens. The individual radial microfibrils in the folded configuration are arranged predominantly parallel to the long axis of symmetry. Frequent cross sections perpendicular to the long axis reveal many parallel and evenly spaced microfibrils (Figs. 10, 11). To better understand the parallel compression stage of the radials along the long axis, a negatively stained mature circular scale was projected onto an enlarging easel and the radial microfibrils traced (Fig. 16A). In this instance, there are approximately 25 microfibrils per quadrant. If the individual microfibrils of one bilateral axis are compressed into an elliptical space without altering their length or order of arrangement, the earlier stages of development, especially the parallel compression stage can be visualized (Fig 16B). Similar patterns have been seen in grazing sections of early scale development in the Golgi apparatus and confirm not only the folded configuration of radials, but also the compression of folded radials along the long bilateral axis of symmetry.

One of the most remarkable features in scale development is the unfolding process that must take place in order to produce the ellipsoidal or circular scale. Fig. 7 depicts this unfolding process, in which a central compact region of the Golgi cisterna containing the folded radial microfibrils goes into a "Z" formation. The forces for this particular membrane transformation and movement are unknown. Nevertheless, such movement could account for a logical sequence for the actual unfolding of a scale shown in Fig. 8. Once the scale unfolds, the radial microfibrils at first remain in the compressed phase, the two scale halves

\footnote{Most of the evidence now speaks for a single unfolding step, but we cannot exclude the possibility of a sequential double unfolding to produce the four quadrants of radial symmetry.}
still separated and at a slight angle to one another (Fig 13, arrow 1). Presumably, shortly after this stage the radial microfibrils separate from their attached sites on the membrane and spread into a planar bi-radial configuration which produces the circular or slightly elliptical base onto which cellulosic microfibrils subsequently will be deposited.

**Development of "Concentric" Microfibrils**

Once the radial network is synthesized, unfolded and spread, the empty dilation that earlier contained the precursor of the radials, migrates to the periphery of the cisterna where it later undergoes abscission from the parent cisterna. The empty radial precursor sac is always situated on the proximal side of the cisternal membrane (Fig. 10). Corresponding, on the distal side, a very narrow constriction appears attached adjacent to the exact geometrical center of the radial network. Other tubules seem to associate with the periphery of the distal side of the cisterna. In this stage, the radial microfibrils are now enclosed by a very compact cisternal membrane (Figs. 10, 11, 13, 14). The distal associated tubules are involved in the synthesis and/or transport of the "concentric" microfibrils to the distal side of the radial network. Since the so-called "concentric" microfibrils really consist of a spiral arrangement of four bands of normally eight to ten continuous, mutually attached parallel microfibrils per band, these must be polymerized, crystallized, and cross-linked by a very well organized, rather complex process resulting in this special arrangement. We cannot yet decide between two alternative possible processes of spiral band formation and arrangement:

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This is the normal case for mature vegetative scales. Smaller scales produced by zoospores or those produced after treatment with various inhibitors have fewer bands (generally 1-2), each with fewer turns of the spiral. The number of microfibrils per band, however, remains relatively constant.
(a) Perhaps we have a Spinnerette-type functioning of a compound polymerization center in the central constriction (see Figs. 14, 15). According to this hypothesis, the four bands of eight to ten individual microfibrils are either polymerized, cross-linked by peptides and crystallized in one continuous process, or cross-linked and crystallized from previously polymerized glucan chains. Then they are fed through the central tubule into the very thin cisternal lumen onto the distal side of the radial network where the growing bands are pushed to the periphery. Final attachment of the spiral bands to the radial network occurs at the periphery of the radials where the four bands are compressed together adjacent to the peripheral cisternal membranes.

(b) The polymerization, crystallization, and cross-linking of the four bands could also start simultaneously from four starting points at the rim of the scale. The growing tips of the parallel bands would then move inwardly. In this case the polymerization center must rotate on an axis, possibly the axis of the scale.

In both cases, the cisternal membrane limitation restricts the movement of the microfibrils so that they can occur only toward the sac periphery according to possibility (a) and toward the center in case (b). In both alternatives, the bands of parallel microfibrils are synthesized continuously, laid down first at the periphery of the cisternae, and spiral inwardly until finally the space restriction causes microfibril fracture, or disordered synthesis in the central region. The movements leading to the 4-5 turns of the spiraling bands seem to be more easily explained with rotating movements of the feeding tubules from the cisternal periphery towards the center of the scale while the fibrils are formed and laid down according to hypothesis (b), thus making this possibility a little more attractive. But perhaps the tendency of the crystalline, parallel microfibrils not to be bent more than necessary in a restricted space provides enough force to ensure the spiraling, similar to that of a clockspring. That some force is used to lay down the spirals is clearly visible from the bending of all radials in the direction opposite the spiraling due to the inherent unwinding tendency of the crystalline cellulosic microfibril (Fig. 4).

4 Inward-to-outward spiraling in a counter-clockwise direction seen from the distal surface of the radials.
With the addition of the spiraling microfibrils, the basic microfibrillar skeleton of the scale is laid down and cemented together.

Addition of Other Polysaccharides to the Microfibrillar Skeleton

As the fully-formed scale microfibrillar skeleton moves in the differentiating cisternal membranes, other vesicles, or perhaps remains of either radial or cellulosic precursor vesicle membranes participate in the addition of additional polysaccharides to the surface of the microfibrillar skeleton. This begins with addition on the scale periphery, and is followed by a centripetal deposition of product, until finally the entire scale is coated (Fig. 10). Once these polysaccharides are added, the scale no longer is held in restricted space by cisternal membranes. Still, the scales cannot turn around within the membranes, and are thus secreted to the periphery in the same orientation that they were formed. In the case with Pleurochrysis, the addition of amorphous polysaccharides to the scale microfibrillar skeleton results in the adhesion of scales to form the compact vegetative cell wall. In zoospores, scales are synthesized, but without the amorphous polysaccharides and thus they tend to separate from the cell surface soon after they are secreted.

Scale Secretion Mechanisms

Scales are brought to the cell surface by a fusion of the cisternal membranes with the plasma membrane. Presumably, there is some membrane re-cycling, because the total cell surface membrane would greatly expand if total cisternal membranes were incorporated into the plasma membrane. Evidence seems to indicate that many small vesicular membrane fragments occur adjacent to the plasma membrane may be part of the re-cycling of cisternal membranes (Brown, 1969). Electron-dense autophagic vacuolar structures may be involved in this process, but at present this stage has not been thoroughly investigated. It is important
to note that on either side of the secreting face of the Golgi apparatus there exists a single bundle of approximately 200 microtubules. These microtubules exist in a hexagonal array and have been shown in detail with freeze-etching (Brown, et al., 1970) and other methods. Recent experimental data from colchicine treatments (Brown and McLean, in preparation) have shown that this drug depolymerizes the microtubular system, as expected. However, this seems to alter the direction of secretion so that scales are unable to be secreted to the exterior. Instead, they accumulate inside the cell, and the autophagic vacuolar system becomes activated and engulfs the scales as rapidly as they are produced. Sometimes, entire cisternal stacks are digested (Fig. 18). Thus, it appears that at least one parameter of the secretory regulatory mechanism resides in the microtubular bundles, and apparently the autophagic system is a regulatory mechanism for stopping scale secretion and/or re-cycling some of the components. Preliminary results of cytochalasin B treatment also suggest the involvement of microfilaments in some of the processes leading to scale formation and secretion (Herth & Brown, unpublished data). See also Herth, et al., 1972.

Protoplastic Movements and Scale Secretion

Because Pleurochrysis cells have only a single Golgi apparatus, and because the scales are evenly distributed over the cell surface, a mechanism must exist to provide a random directional movement of the secreting phase relative to the surface to insure a uniform deposition of scales. This was first hypothesized by Brown (1969) from electron micrographs and later reconfirmed by time-lapse cinematography (Brown, 1970). The remarkable cells of Pleurochrysis undergo a continuous protoplasmic rotation, which apparently insures uniform distribution of the pre-formed scale cell wall products. Not only is this peculiar movement found in Pleurochrysis, but in all members examined so far which produce a pre-formed scale product. This includes not only the Haptophyceae, but also the Prasinophyceae.
At first it was thought that the microtubular bundle might be involved in this protoplasmic movement, but it is now believed that another force is responsible for this movement, one which may reside in finger-like projections of the cisternal sac which surrounds the Golgi apparatus which surrounds the cell (Fig. 17). With freeze-etching, the surface of the finger is reminiscent of contractile protein configurations found in bacteriophages (Brown, unpublished data). Freeze-etching also shows that the fingers can exist in two states, a spherical configuration without much degree of order, and an elongated cylinder which has a high degree of order. Presumably, a transformation between spherical and cylindrical states of many of these structures may account for the gradual and very slow protoplasmic rotation observed. It should be noted that protoplasmic rotation is much slower than cyclosis and cannot be adequately observed without time-lapse techniques.
II. Scale Chemistry

A. Carbohydrate composition of the scales

Untreated scales from scale fraction A contain radial microfibrils, amorphous material, and "concentric" microfibrils. These three components can be isolated and analyzed separately for further chemical characterization.

Thin layer chromatography of the hydrolysates from scale fraction A show a predominance of galactose together with minor quantities of glucose and a pentose with the same Rf value as ribose, possible fucose (Brown, et al., 1970). The uronic acid content is unknown and needs to be analyzed. The amorphous and radial fibrillar material can be removed by a series of extractions. The scales have been first extracted with a sequential series of methanol and methanol/chloroform extractions (for details see Herth, et al., 1972). When the scales are treated with boiling water under magnetic stirring for two hours and centrifuged at 2,000 g., the supernatant contains the dissolved amorphous material while the residue contains intact scales with radial and "concentric" microfibrils.

Further degradation occurs when the scales are treated with a 1% NaClO₂/1% acetic acid solution for 24 hours at 25° C under magnetic stirring. This preparation is separated and washed by repeated centrifugation and then treated with 10% NaOH solution for 24 hours under magnetic stirring at 25° C under N₂. Under these conditions the radial fibrillar material is completely removed, leaving only short rods of the "concentric" microfibrils as the purified alkali-resistant scale material corresponding to the preparation of α-cellulose from other plant sources. This material has been further analyzed chemically while the other fractions are now being analyzed in our laboratory.
Ultrastructural, cytochemical and chemical analysis of the scale preparations give the following evidence for the cellulosic nature of the alkali-resistant scale material:

1. The *Pleurochrysis* cell wall exhibits a positive birefringence and shows a positive iodide dichroism (Brown, *et al.*, 1970).

2. Negative staining of the purified, alkali-resistant scale material reveals microfibrils with a characteristic ribbon-like appearance and similar dimensions as known from other cellulosics (Brown, *et al.*, 1969, 1970).

3. These microfibrils have a maximum flexibility of about 2% like other cellulosics (Brown, *et al.*, 1970) and show typical cracking sites (breakage loci).


5. This scale material is resistant to strong alkali solutions and quite resistant to strong acids like HCl, $\text{H}_2\text{SO}_4$ and trifluoracetic acid (Brown, *et al.*, 1969, 1970 and Herth, unpublished observations).

6. The scale material is susceptible to acetylation in a 1:9 mixture of concentrated $\text{H}_2\text{SO}_4$ and acetic acid at 100° C within less than ten minutes thus excluding the presence of a sporopollenin-like resistant material (Herth, unpublished).

7. The alkali-resistant material is soluble in cuprammonium reagents (Brown, *et al.*, 1970).

8. The alkali-resistant scale material is positive to zinc-chloride-iodine and stains intensively violet with this reagent (Brown, *et al.*, 1970).

9. Total hydrolysis of the purified alkali-resistant scale material yields glucose as the predominant sugar (besides small quantities of galactose and pentose, possibly fucose). More than 95% of the sugars present in total hydrolysates is glucose as determined by glucose oxidase (Brown, *et al.*, 1970).
(10) Cellulose is the only disaccharide present after partial hydrolysis as detected by GLC of the TMS derivatives of the sugars present in the hydrolysates. The presence of laminaribiose has been excluded by comparison with a standard (Herth, et al., 1972).

(11) The proton resonance spectrum of the benzyolated scale material is identical with the spectrum of cotton cellulose benzyolated according to the same procedure (Brown, et al., 1970).

(12) The x-ray diffraction pattern of hot water-extracted scales is identical with the cellulose I diagram of the quince slime and cotton cellulose, the line broadening in scales being due to the low crystal size. After harsh alkaline treatment, the diffraction pattern of the scales corresponds to the cellulose II pattern of mercerized cellulose (Herth, et al., 1972).

(13) The alkali-resistant scale material yields a stable nitrate with the same solubility properties as a typical cellulose nitrate (Herth, et al., 1972).

(14) The degree of polymerization (DP) as determined by fractional precipitation of the scale cellulose nitrate and viscosity measurements is 3150 with a very heterogeneous distribution of chain lengths (Herth, et al., 1972). Thus all physical, chemical and cytochemical evidence supports the cellulosic nature of scale alkali-resistant material. Of course we cannot yet exclude the presence of some linkages other than β-(1→4), namely β-(1→3) linkages. Finally, we do not know actually where the non-glucose sugars occur in the chain, but we are planning to conduct methylation analyses of the alkali resistant scale material to secure more information on this point.

B. Amino acid composition of the alkali-resistant scale material

Scale fraction A contains a variable amount of N (>1%) as determined on the basis of total nitrogen content (see Brown, et al., 1970). Contamination
from membraneous fragments is very low as revealed by electron microscopy and
determined by phospholipid analyses (Brown, et al., 1970). N-containing groups
in the scale polysaccharides can be excluded with certainty, since no amino
sugars have been detected. All nitrogen present must be protein nitrogen. The
nitrogen content of purified, alkali-resistant scales is even higher corresponding
to a value of 32% protein. This value suggests a cellulose/protein ratio of
roughly 2:1 (Herth, et al., 1972). The following values for the content of
different amino acids recovered after total acid hydrolysis is shown in Table 2,
third column (Pleurochrysis). In Pleurochrysis, about 38% of the amino acids
contain hydroxyl groups with serine as the major amino acid, and about 25% of
the amino acids contain carboxyl groups, with asparagine as major amino acid.
The original serine content might have been even higher since serine is well
known to be converted partially to glycine under the alkaline conditions used
for purification of the material. A very high content of ammonia in the hydroly-
sates and the neutral behavior of the scales in a titration assay (Herth, unpub-
lished observations) speak for asparagine and glutamine and not the corresponding
acids, to be originally present in the native scale fraction.

The microfibrils of alkali-resistant scale material do not show any loss
of N content when stirred with 8 M urea for 24 hours at 25° C or with 5 M
guanidinium chloride. This is indicative of a true covalent linkage of peptide
to the polysaccharide moieties. As far as the type of linkage is concerned, we
can only suggest that the linkage is via an ether bond between a sugar hydroxyl
group and the hydroxyl group of an amino acid or from a sugar carbon to
the nitrogen atom of asparagine or glutamine. We have not yet been able to de-
tect linked sugar-amino acid pieces with thin layer chromatography from hydroly-
sates of the alkali-resistant scale material. In addition, we have been unsuccessful
obtaining such moieties by enzymatic degradation, since no enzyme yet tested (compare Herth et al., 1972 and Green and Jennings, 1968) including cellulase and snail digestive enzyme has degraded the alkali-resistant scale microfibrils.\(^5\)

Thus, it is still an open question whether glucose or galactose or both sugars are linked to an amino acid. Future experiments include a β-elimination assay under alkaline conditions to determine the involvement of serine. In addition, we are planning to analyze the acid hydrolysates by gas chromatography to determine whether we can detect sugar-amino acid linked moieties.

III. **Peptides Linked to Other Structural Polysaccharides**

To see whether this very intimate association of peptide moieties with the structural polysaccharide in *Pleurochrysis* scales is a unique occurrence or whether it is a more general phenomenon, the investigations have been extended to include structural polysaccharides from other organisms. These polysaccharides have been purified according to the same extraction procedures described for *Pleurochrysis*. These structural polysaccharides have been identified by hydrolysis, x-ray diffraction, and NMR (Herth et al., in preparation). The alkali-resistant structural polysaccharides thus far isolated include (see Table 2):

- β-(1-3) - xylan from *Caulerpa prolifera*
- β-(1-4) - mannann from *Acetabularia mediterranea*
- β-(1-3) - glucan from pollen tube walls of *Lilium longiflorum*
- β-(1-4) - glucan (cellulose) from onion root tips
- β-(1-4) - glucan from cotton (Eucellulose standard)

\(^5\)Cellulase now has been shown to degrade concentrates (Herth, et al., in preparation).
Negative staining in all cases reveals a microfibrillar structural polysaccharide to be present in the final, alkali-resistant fraction (Herth, et al., in preparation). No amino sugars have been detected in these hydrolysates, but amino acids were detected by thin layer chromatography, gas chromatography and automatic amino acid analysis.

Table 2 summarizes the amino acids recovered from acid hydrolysates of alkali-resistant materials from *Caulerpa prolifera*, lily pollen tube wall, and cotton in comparison with the *Pleurochrysis* scale material (*Acetabularia* mannan and onion root glucan are just being analyzed now). In all cases, we see the predominance of acidic amino acids (especially with *Caulerpa* xylan) as well as hydroxy amino acids. There is an obvious similarity of the amino acid composition of the peptides linked to *Pleurochrysis* alkali-resistant β-(1-4)-glucan and the peptides linked to cotton cellulose.

The alkali-resistant pollen tube wall material can be completely dissolved in N-ethyl-pyridinium-chloride (Herth, unpublished), a potent solvent medium for cellulose and other structural polysaccharides (Husemann and Siefert, 1969). Furthermore, this material can be re-precipitated with methanol. The re-precipitated material still contains the peptide moieties, thus providing more evidence for covalent linkage of the peptides to the structural polysaccharide. (The same type of experiment will be used to test the other structural polysaccharides mentioned above). These preliminary results suggest that the peptide moieties intimately associated with the structural polysaccharide in *Pleurochrysis* are not a unique case among the plant kingdom. Thus, it is a good model for comparison with all other cases of structural polysaccharides, and we have the feeling that no really pure structural polysaccharide exists.
DISCUSSION:

While it is generally accepted that the Golgi apparatus functions as an assembly line for complex macromolecules such as mucopolysaccharides, hemicelluloses, glycoproteins, proteoglycans, and glycolipids (Whaley, 1972, Northcote and Pickett-Heaps, 1966, Northcote, 1971), the role of this organelle in cellulose biosynthesis has been excluded. The classical view of cellulose biosynthesis, based on Colvin's (1964) work with bacterial cellulose, holds that this structural polysaccharide is polymerized and crystallized extracellularly or on the surface of the plasma membrane via diffusion of non-membrane cytoplasmic pools of nucleoside diphosphate sugar compounds. On the contrary, recent evidence has suggested a role of the Golgi apparatus in the biosynthesis of cellulose. The evidence has come from two different approaches and with two different cellular systems: Ray (1969) has demonstrated a $\beta$-(1-4)-glucan synthetase to be associated with the Golgi fraction of pea seedlings. Brown and co-workers (1969, 1970) have found a cellulosic moiety in Golgi-produced scales of a marine alga. While it is now well-known that the Golgi apparatus is involved in scale formation (Manton, 1967, Manton and Peterfi, 1969) the extended observations reported here permit one to make a more precise working hypothesis about the role of this organelle in polysaccharide biosynthesis. It is possible with Pleurochrysis to differentiate the specific sites of polymerization and assembly of the morphological scale sub-components and to characterize them chemically.

At present, we think that it has been clearly demonstrated that the scales have a cellulosic sub-unit structure in the form of the so-called, "concentric" microfibrils. The microfibrils are not pure cellulose but intimately associated with peptide moieties. In addition, they are not synthesized alone but deposited
on other structured polysaccharide moieties known as radial microfibrils, and
later coated by a slimy, presumably pectic moiety known as the amorphous materi-
ral that also cross-links the scales to produce a coherent cell wall. The
different assembly steps of these three sub-components can be followed by elec-
tron microscopy, and the observations indicate that a very complex machinery is
necessary to synthesize the scale network by the stepwise addition of new com-
ponents. The entire scale assembly process requires only two minutes (Brown,
1969), a relatively short time requirement demonstrating the great efficiency
of the Golgi machinery in this instance.

Generalizing with this model we can assume that in the Golgi apparatus of
higher plants, structural polysaccharides are not synthesized alone but in associ-
ation with peptides and acidic polysaccharides. The structural polysaccharides
could thus be prevented from immediate crystallization and after secretion,
could co-crystallize ultimately producing the crystalline microfibrils of the
mature cell wall. Lysosome-like dense vesicles could secrete enzymes for removing
the coat of acidic polysaccharides, resulting in spontaneous lateral aggregation
of the "naked" structural polysaccharide polymers. The peptide moieties associ-
ated with the structural polysaccharides are thought to have two possible roles:

(a) certain peptides could be involved in cross-linking of the structural
polysaccharides, thus stabilizing special arrangements of the microfi-
brils (Lamport, 1970). This is exemplified in the Pleurochrysis model
by the lateral aggregation of six to ten cellulosic microfibrils into
a definitive band, and by the spiral arrangement of these bands to
form the scale skeleton.

(b) Other peptides could function as initiators or recognition sites for
specific sugar additions, resulting in a direct genetic control of
the type of polysaccharide being synthesized. This could be analogous
to the postulated role of specific sequences of amino acids in animal glycoprotein peptides adjacent to the amino acid-sugar linkages (Marshall and Neuberger, 1970; Spiro, 1970).

We believe that the biosynthesis of animal glycoproteins (see Weinstock and Leblond, 1971), plant glycoproteins (Chrispeels, 1970; Savada, 1972), and structural polysaccharides covalently-linked to peptide moieties (Herth, et al., 1972) follow the same general pathway even though the ratio of carbohydrate/protein content may be much higher in plants.

Another interesting question concerns the function necessary for obtaining a distinct degree of polymerization of the structural polysaccharides (compare the template hypothesis of Marx-Figini, 1969). From the Pleurochrysis model we can speculate about some possible control mechanisms:

(1) cisternal sac size; (2) membrane-attached synthetase activity and/or availability; (3) number of peptides functioning as glucan initiators; (4) precursor pool size; (5) length and number of radial microfibrils formed to function as the first ultrastructurally visible template for the deposition of the spiral bands of microfibrils; and, (6) the time-limitation imposed for any given step of the assembly line process. Experiments are now in progress to clarify the proposed hypothesis of control mechanisms in scale formation. From these data we hope to produce a model which will be directly applicable to the eventual understanding of higher plant cell wall biosynthesis.
REFERENCES


Savada, and N. J. Chrispeels, see chapter in this book.


Table 1. Staining of *Pleurochrysis* vegetative cells with silver methenamine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemical Action</th>
<th>Silver Deposition</th>
<th>Preliminary Interpretations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wall Scales</td>
<td>Intra-cisternal Scales</td>
</tr>
<tr>
<td>(a) Ag</td>
<td>reduction of Ag⁺ at cellular sites</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(b) Iodoacetate-Ag</td>
<td>blockage of reduced sulfur by acetylation</td>
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<td>-</td>
</tr>
<tr>
<td>(c) Iodoacetate-PA-Ag</td>
<td>reduced sulfur blocked by acetylation and periodic acid oxidation of 1-2 glycols to aldehydes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(d) Iodoacetate-PA-HSO₃ Ag</td>
<td>blockage of reduced sulfur by acetylation, production of free aldehydes by PA, followed by blockage of these aldehydes by HSO₃.</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

general cytoplasmic staining but more intense with wall and intra-cisternal scales.

Scale silver deposition from untreated controls due to sulfhydryl groups.

restoration of silver deposition because of PA oxidation of 1-2 glycols to free aldehydes. Sulfhydryl blockage remains.

loss of silver deposition due to blockage of PA-oxidized free aldehydes.
<table>
<thead>
<tr>
<th></th>
<th>Caulerpa prolifera</th>
<th>Lilium longiflorum</th>
<th>Pleurochrysis scherffelii</th>
<th>Cotton</th>
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<tr>
<td></td>
<td>(β-(1-3)-xylan)</td>
<td>Pollen tube wall</td>
<td>(β-(1-4)-glucan)</td>
<td>(β-(1-4)-glucan)</td>
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<td></td>
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<td>(β-(1-3)-glucan)</td>
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<td>Asp.X</td>
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LEGENDS

FIGURE 1: Survey electron micrograph of a median optical section through a mature vegetative cell (normal post staining with lead citrate and uranyl acetate). Note the prominent Golgi apparatus in the lower right hand corner surrounded by endoplasmic reticulum and many polysomes. Note the amplexus or extension of endoplasmic reticulum from the outer nuclear membrane (arrow 1), note also, prominent mitochondria associated with the Golgi region. The Golgi apparatus is polarized and the secreting face always is directed toward the cell surface. Note the parietal chloroplast with protruding pyrenoid and the wall of stratified scales. X 10000.

FIGURE 2: The Golgi apparatus of Pleurochrysis shown in median section (normal post staining with lead citrate and uranyl acetate). Note the differentiating stack of cisternae beginning with compact cisternal development adjacent to the amplexus (upper right hand corner) and progressing toward the cell surface with inflation. Two types of central dilations are seen in the proximal region of the Golgi apparatus: arrow 1 depicts the large dilation with visible product and arrow 2 shows the smaller electron transparent dilation that is surrounded by heavy electron-dense staining of material next to the membrane. The first visible scale elements (radial microfibrils) appear as a double layer in the cisterna at arrow 3. Mature scales are shown at arrow 4 where amorphous material coats the microfibrillar scale network. Scale secretion occurs at arrow 5 where the distal-most cisterna fuses with the plasma membrane and releases the scale to the cell surface where it stratifies to form the cell wall. Arrow 6 depicts a cluster of polysomes in between the cisternal stacks, probably involved in a specific stage of scale assembly. Whirls of membrane (arrow 7) appear in the forming region of the Golgi apparatus. Polysomes and
vesicles appear along the cisternae of varying stages of differentiation along the cisternal stack. X 24000 .

FIGURE 3: Scale from vegetative cell with amorphous material in between the radial and "concentric" microfibrils. Note the two axes of symmetry for the radial network. The "concentric" microfibrils really spiral and some of them end on the periphery at arrows. X 84000 .

FIGURE 4: Negatively stained scale from vegetative cell of Pleurochrysis with amorphous material removed. Note the ordered radial and "concentric" network of microfibrils. Spiral ing of the "concentric" network can be detected by occasional discontinuity of the spiral ing bands (arrows). Note the slight curvature of radials in the counterclockwise direction of the spiral which is suggestive of the force of direction of the assembly of the spiral network. X 59000 .

FIGURE 5: Scales from vegetative cells of Pleurochrysis after a short treatment with 5% KOH. Note the removal of the amorphous substances and radial microfibrils. The remaining "concentric" microfibrils have less structural stability and therefore the rims tend to fold back on one another. In addition, numerous breakage loci throughout the spiral network can be seen (arrow ). X 68000 .

FIGURE 6: A more advanced stage of degradation of scales of vegetative cells of Pleurochrysis treated with 5% KOH. Note again the complete absence of radial microfibrils. Here, the spiral ing network of microfibrils is beginning to unravel. Note the distinct bands of spiral microfibrils shown in between the brackets. Between 5 and 9 microfibrils comprise the band. X 92000 .
FIGURE 7: Details of region in Figure 6 indicated by arrow showing the subunit structure of the spiral microfibrils. A single microfibril, arrow, breaks down into two or three subcomponents. X 44000.

FIGURE 8: Actively growing vegetative cells of *Pleurochrysis* in the filamentous phase. Note the electron dense wall layers resulting from silver deposition (silver methenamine method with periodate oxidation). Each vegetative cell contains a Golgi apparatus in the section plane as well as a single parietal chloroplast with bulging pyrenoid. In the right cell the nucleus is prominent and lies adjacent to the Golgi apparatus. Note the newly formed cross wall between the two vegetative cells on the left. The central cell exhibits a large conspicuous vacuole with several electron-dense autophagic vacuoles lying between the Golgi apparatus and the large vacuole. X 8000.

FIGURE 9: Silver methenamine-stained preparation of two actively growing vegetative cells. Note the prominent Golgi apparatus in each cell and the identity of the staining properties of the Golgi scales with that of the wall material. X 13000.

FIGURE 10: Over-all view of a Golgi apparatus stained with PA-silver, showing the dilations of the radial precursor pools which react heavily with silver (arrow 1), and the cellulosic precursor pools which are electron transparent (arrow 2). The amplexus and budding ER is shown in the upper left hand corner. A stage of folded radial microfibrils is shown at arrow 3. The next distal-most cisterna contains a folded scale with radials but without added concentrics. The distal scales react more heavily with silver, indicating the addition of amorphous polysaccharide. Note cell wall in the lower left hand corner. X 41000.
FIGURE 11: Details of the radial precursor pool (arrow 1) and the folded radial microfibrillar network (arrow 2) as shown by the silver technique. Note that the folded radial microfibrils are compressed parallel to one another. The cisterna at arrow 3 contains an unfolded radial network with a central feeding tubule below it. The cisternae in the lower right hand corner contain fully assembled scales with amorphous product. X 71,000.

FIGURE 12: Detail of the "Z-stage" radial unfolding in the Golgi apparatus as shown by the silver technique. Compare with stage in schematic diagram. Also compare with Figure 13 which shows a later stage of unfolding. X 58,000.

FIGURE 13: Unfolding of radial network as shown by normal poststaining techniques. The cisterna depicted by arrow 1 shows a stage of unfolding in which the radial network is perpendicular to the normal scale orientation. Arrow 2 depicts a recently unfolded, funnel-shaped cisterna with the two, still separate radial halves within it. Possible candidates for tubules feeding the cellulosic precursors are shown by arrows 3. Fully synthesized scales are shown in the lower right hand corner. Note in the upper right hand corner the amplexus and the budding vesicles that participate in the forming face of the Golgi apparatus. X 41,000.

FIGURE 14: Details of the presumed cellulosic feeding tubule network (arrow 1) as revealed by the silver technique. Radial microfibril precursor pool shown by arrow 2. Unfolded radial stage shown by arrow 3. X 62,000.

FIGURE 15: Generalized scheme depicting the model of scale formation in Pleurochrysis. Cisternae are diagramed in median cross section to show the optimal orientation of radial and concentric microfibrils. For detailed description, see the text.
FIGURE 16: Tracings of the radial microfibril network made from a negatively stained scale preparation. 16-A depicts the exact original orientation of radial microfibrils. Note the four quadrants of microfibrils. Figure 16-B depicts the presumed compact stage of radial orientation immediately following unfolding. In this instance, the same microfibrils have been re-oriented in the compact stage. (Cross sections of this compressed stage are shown in Figure 10, 11, 12, and 14 in which the radials are folded but still lie parallel to one another.)

FIGURE 17: Section through the finger-like projections of Pleurochrysis vegetative cell showing the inner core of electron transparent material surrounded by the cytoplasmic extension of the plasma membrane. Oblique section of cell wall shown at right (normal post-staining). X 87,000.

FIGURE 18: Vegetative cell of Pleurochrysis treated with 1% colchicine for 48 hours. Note the abundance of autophagic vacuoles, the grossly disorganized cisternal network of the Golgi apparatus and profiles of scales in the autophagic vacuoles. X 20,000.