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Cellulose microfibrils: Visualization of biosynthetic and orienting complexes in association with the plasma membrane

(freeze-etching/*Oocystis*/enzyme complex/granule bands/terminal synthesis)

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ABSTRACT **Cellulose microfibril biosynthesis, assembly, and orientation in the unicellular green alga, *Oocystis*, is visualized in association with a linear enzyme complex embedded in the B face of the plasma membrane. Granule bands of the A face and complementary ridges of the B face are postulated to assist in the orientation of recently synthesized microfibrils. A model for microfibril synthesis and orientation is proposed and correlated with current hypotheses regarding cellulose biosynthesis in higher plants.**

Cellulose is the predominating wall polysaccharide of plant cells. As the most abundant macromolecule on earth, of which 10^{11} tons are produced and destroyed annually (1), it is indeed surprising that so little is known of its biosynthesis *in vivo*. Techniques in electron microscopy, and particularly the freeze etching technique, have made possible the examination not only of intracellular components and organelles, but also of elements within and on the surface of membranes.

The plasma membrane is thought to be the site for cellulose synthesis (2–4); however, convincing cytological proof for the existence of a cellulose synthesizing complex has yet to be presented. In this report, we describe the events of cellulose assembly, transport, and orientation in association with the plasma membrane of a unicellular green alga, *Oocystis*.

MATERIALS AND METHODS

One- to two-week-old axenic cultures of *Oocystis apiculata* (Indiana University Culture collection no. B418) grown on Modified Kantz Medium (5) were transferred without pretreatment to gold specimen holders, frozen in Freon 22, transferred to liquid nitrogen, and processed with a Balzers M360 freeze etch apparatus. Etching was for 2 min. Negative staining was performed with 2% phosphotungstate at pH 6.8. Cells were fixed with a 1% glutaraldehyde-tannic acid mixture followed by osmication (1%), washed, dehydrated in an ethanol series, and embedded in Epon. Material was examined with an Hitachi HU 11E electron microscope.

RESULTS

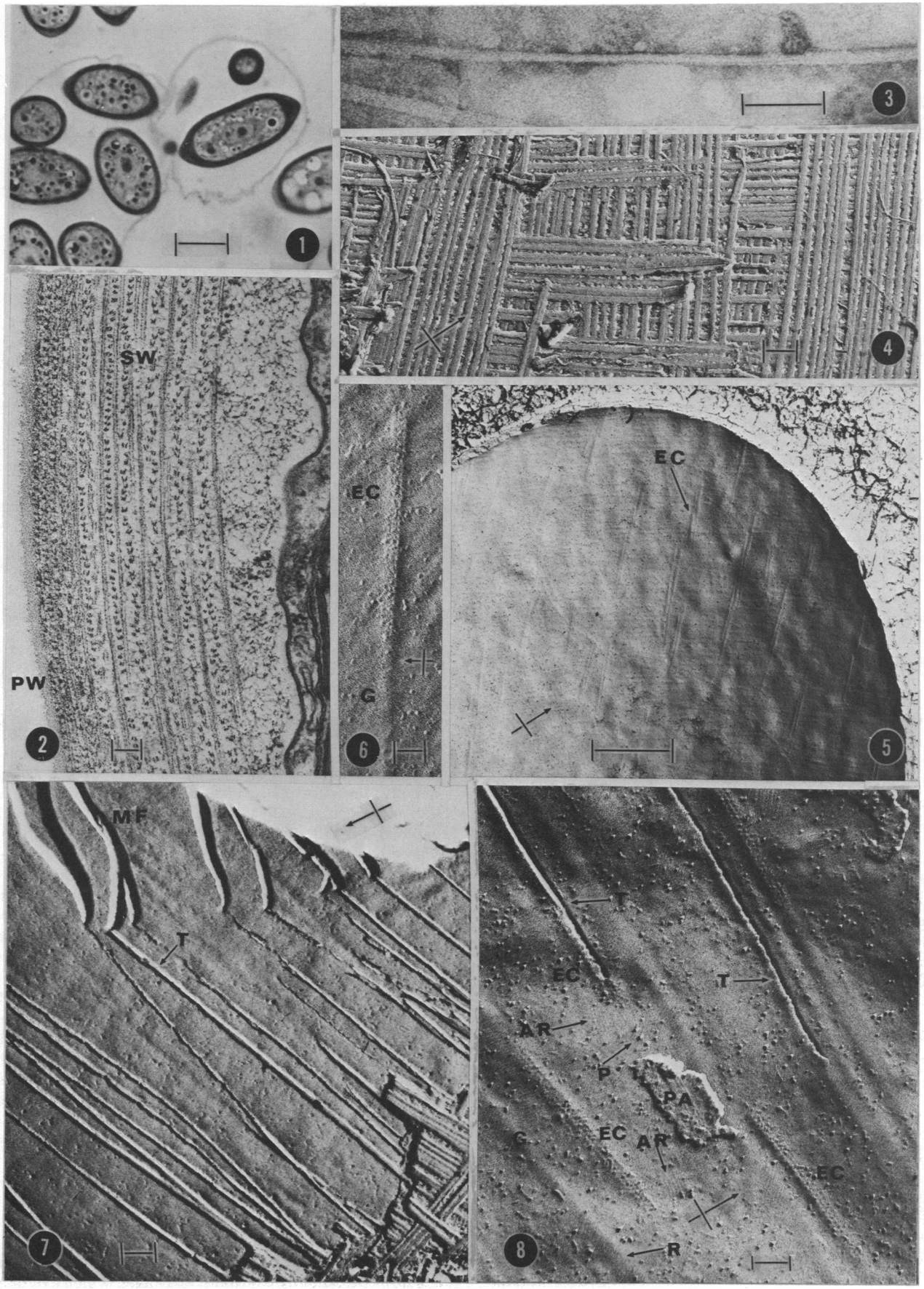
Cell Wall. Cell wall production in young autospores of *Oocystis* (Fig. 1) proceeds in two distinct phases. Immediately after cytokinesis, a thin fibrillar primary wall is deposited (Fig. 2). Abundant cortical microtubules are found adjacent to the plasma membrane. Their function is believed to be cytoskeletal rather than orientational because primary wall microfibrils are randomly distributed. Primary wall deposition maintains the ovoidal cell shape established by the cortical microtubules.

Primary wall deposition is followed by secretion of a secondary wall consisting of organized layers of cellulosic microfibrils (2, 3) (Figs. 2 and 4). These microfibrils lie in single rows, and each layer is oriented approximately 90° to the adjacent layer. In fixed-sectioned material, the central electron-translucent core of the microfibril is revealed (Fig. 2). The core is thought to consist of highly crystalline cellulose, whereas the electron-dense surface probably is composed of hemicelluloses and noncellulosic coating substances (6). The average outer dimensions of the microfibril, as demonstrated in fixed-sectioned material, are 185 Å × 221 Å. The core dimensions average 55 Å × 71 Å (Fig. 2). Negatively stained microfibrils average 187 Å × 94 Å (Fig. 3). These dimensions probably include not only the crystalline core, but also the noncellulosic coating. Freeze etching data indicate dimensions of 184 Å × 93 Å (Fig. 4).

Plasma Membrane. When cells of *Oocystis* are quick frozen in Freon 22 without fixative or cryoprotectant, the common fracture plane revealed in the plasma membrane is the inward surface of the B face [outer layer of the biomolecular leaflet or the fracture face of the E half of the membrane, designated "EF" according to the newly established nomenclature of Branton *et al.* (7)] as shown in Fig. 5. Less frequently, the outward surface of the A face [inner layer of the biomolecular leaflet, the "PF" of Branton *et al.* (7)] is exposed (Fig. 12). The cytoplasmic surface (PS) is rarely observed because of the high probability of splitting the membrane. The outer surface of the plasma membrane (ES) was never directly observed, at least during active cell wall synthesis. Apparently, the newly synthesized layer of microfibrils and the outer surface of the plasma membrane are so firmly bonded that the tendency is to split the membrane along the hydrophobic interior.

Robinson and Preston (2) have suggested that the fracturing of *Oocystis* does not split the plasma membrane; however, we have observed both surfaces with deep etching (Fig. 8). The following evidence favors our interpretation that the fracture shown in Fig. 8 is the inward exposed surface of the B face: (*i*) the shadow angle indicates in Fig. 7 that the plane of exposed membrane lies *above* the wall layer, whereby the view is from the cell interior projecting to the surface; (*ii*) evidence that the outer surface of the plasma membrane is *not* being exposed in such instances is based on views that demonstrate the innermost layer of the wall microfibrils torn back through the B face of the plasma membrane producing distinct holes (Fig. 7).

Four distinct structures are associated with the B face (Fig. 8): (*i*) randomly distributed particles, which average 95 Å in diameter; (*ii*) clusters of ridges, which have an average spacing of 85 Å and an average length of 522 Å; (*iii*)



FIGS. 1–8. *(Legend appears at bottom of following page.)*

"grooves," which are interpreted as furrows on the outer surface of the plasma membrane in which the microfibrils lie (Fig. 7); and (*iv*) distinct linear arrays of particles interpreted as the cellulosic microfibrillar enzyme complexes (Figs. 6 and 8).

The enzyme complex consists of three rows, each with about 30 particles forming a semicylinder. The complex is not revealed when glutaraldehyde or glycerol is used. The average dimension of the individual particle is 71 Å. The center-to-center spacing of these particles in a given row is constant within a complex but variable among complexes. The average length of the enzyme complex is 5100 Å, and the average number of particles per complex is 108. The arrangement of particles within a given row is linear, but the three rows are slightly staggered to produce a three-particle alignment across the complex which is approximately 71° to the long axis of the complex (Figs. 6 and 8). Note that the enzyme complexes are observed: (*i*) always parallel to one another (Fig. 5); (*ii*) only at the terminus of a groove when associated with one (Fig. 8); (*iii*) in a dimer state, which is interpreted as an inactive state as shown by the lack of an associated groove; (*iv*) in pairs with opposite ridges projecting from them, interpreted to be an early state of microfibrillar initiation and growth in opposite directions (Fig. 5); and (*v*) to have numerous fibrils projecting from the particles of the complex (Fig. 9). Careful measurements suggest that the fibril dimensions (less than 25 Å) are beyond the resolution of the platinum-carbon and could thus be in the size range for individual glucan chains. These chains could polymerize *within* the membrane and then be directed into the groove on the *surface* of the membrane where their close proximity within the enzyme complex would cause spontaneous crystallization into the microfibril. Thus, the entire complex is envisioned to move in the plane of the fluid membrane as the microfibril is generated. The force for this translational movement might come from the crystallization of the microfibril. That the microfibril may not yet be firmly crystallized while associated with the complex is suggested by Fig. 8, where the microfibril tear approaches the enzyme complex and becomes less distinct to nonexistent.

The specific orientation of the growing microfibril with its terminal enzyme complex is thought to be coordinated through the action of ridges which selectively associate with the inward surface of the B face (Fig. 8) and mirror image granule *bands* which associate with the outward surface of the A face (Fig. 12). Evidence for this interpretation comes from views like Fig. 8, in which ridges line up in linear arrays immediately under the grooves of the B face where microfibrils lie, and in the complementary A face where granule *bands* aggregate in the same linear arrays (Fig. 13). Thus, the granule band-ridge complexes could function as guide elements when arranged linearly and in direct association with the growing microfibril. Granule bands and ridges frequently are observed not only in the linear state, but as limited aggregates (Fig. 12). Typical of the aggregate state is: (*i*) an association with the enzyme complexes which are actively synthesizing microfibrils (Fig. 8); and (*ii*) diamond-shaped interconnecting networks during the periodic states of inactivity. It is suggested that through self-assembly and disassembly, the granule band-ridge complex directs the orientation for the next layer of wall microfibrils 90° to the previously synthesized layer. The dimensions of granule bands are 83 Å for individual particles, 800 Å for the band width consisting of 8 to 10 particles, and 182 Å for pair-to-pair spacings in between pairs (Fig. 13). The granule bands exist in increasing states of aggregation, first into paired bands, then into short linear segments of paired bands (Fig. 13), and ultimately into two orientations of longer linear arrays, one in which the granules are perpendicular to the longitudinal axis of the array (Fig. 12, arrow a) and the other in which the granules orient 71° to the longitudinal axis (Fig. 12, arrow b).

DISCUSSION

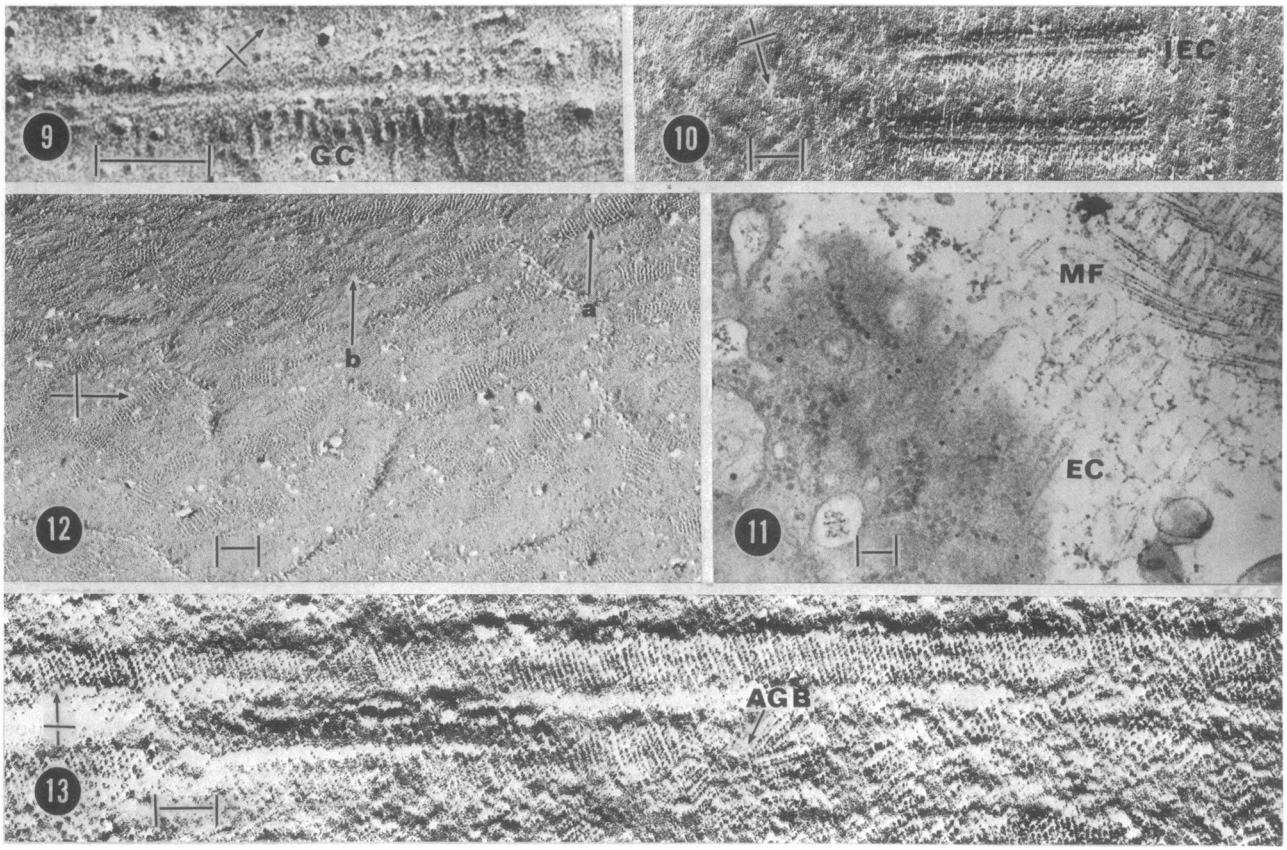
This study gives direct visual confirmation of cellulose synthesis in a eukaryotic cell. Although many points need further clarification and substantiation, the data provide a new foundation upon which to understand the synthesis of cellulose *in vivo*.

Several fortuitous circumstances made our interpretations possible: (*i*) selection of a unicellular organism having a wall of well-organized cellulosic microfibrils; (*ii*) obtaining an active state of secondary wall deposition through the use of rapidly growing cultures; (*iii*) successful preservation of the plasma membrane and its interior structures, without the need for glutaraldehyde as a stabilizing agent or glycerol as a cryoprotectant; and (*iv*) observation of microfibril tears through the inward exposure of the B face of the plasma membrane, revealing a direct association of microfibrils with enzyme complexes.

It has become more apparent recently that cellulose microfibrillar assembly might occur in association with the plasma membrane (8). Northcote and Lewis (9) observed a linear array of particles associated with the plasma membrane of pea seedlings and suggested this to be the synthesizing center for cellulose. Preston believes in the existence of an enzyme complex at the surface of the plasma membrane and has formulated on this basis the "ordered granule" hypothesis (2). Using sectioned material, Roland and Pilet (10) and Robards (11) have observed granular material in association with microfibrils at the plasma membrane surface. Recently, Willison and Cocking (12) proposed the outer leaflet of the plasma membrane to be the site of microfibrillar assembly. Biochemical investigations of glucan synthetase activity have demonstrated its active association with plasma

Key to symbols used in the figures: EC, enzyme complex; IEC, inactive enzyme complex dimer; GC, glucan chains; MF, microfibril; G, groove; R, ridge; AR, aggregating ridges; PW, primary wall; SW, secondary wall; T, tear; P, particle associating with the B face; PA, patch of remaining A layer of membrane, GB, granule band; AGB, aggregating granule band. Magnification bars in all figures represent 0.1 μm, with the exceptions of Fig. 5 = 1.0 μm and Fig. 1 = 10.0 μm. ‡ indicates the direction of shadowing in the freeze etch preparations.

FIGS. 1–8 (*on preceding page*). *Fig. 1.* Thick Epon section of young autospores. *Fig. 2.* Details of cell wall organization in section. *Fig. 3* Secondary wall microfibrils revealed by negative staining. Note the wide and narrow axis of top microfibril. *Fig. 4.* Freeze etch replica of secondary wall microfibrils, surface view. *Fig. 5.* Fractured cell showing concave inward exposure of the B face of the plasma membrane. Note polarity of enzyme complexes in various states of activity. *Fig. 6.* Detail of a single enzyme complex showing the terminus (at the top) and groove (bottom) in which the microfibril is embedded on the surface of the B face. *Fig. 7.* Fracture demonstrating exposed B face of the plasma membrane and microfibrillar tears back through the membrane. *Fig. 8.* Fracture demonstrating the inward exposure of the B face of the plasma membrane (as in Fig. 5). Note the patches of adhering A face of the plasma membrane, enzyme complexes at the termination of grooves, associated ridges with the grooves, including aggregating ridges around the enzyme complexes, and microfibrillar tears.



FIGS. 9–13. *Fig. 9.* Two enzyme complexes, the lower one demonstrating lateral fibril projections interpreted as glucan chains. *Fig. 10.* Two inactive enzyme complexes, each in the dimer state. *Fig. 11.* Grazing section through the plasma membrane revealing a cluster of enzyme complexes associating with microfibrils. *Fig. 12.* An outward exposure of the A face of the plasma membrane demonstrating granule bands in the intermediate state in between periods of activity of secondary wall synthesis. Note the pitch of the granules organized within the interconnecting diamond-shaped complex in which the angle is approximately 90° at arrow a and 71° at arrow b. *Fig. 13.* Granule bands arranged in long linear row at a 71° pitch. This is the complementary face to the ridges. Note the locus of aggregating granule bands interpreted to be in the vicinity of the enzyme complex (which remained attached to the complementary B face and therefore not shown on the A face exposure here).

membrane fractions (13, 14). Brown and coworkers (15–17) have demonstrated the capacity of Golgi membranes for polymerization and crystallization of cellulosic microfibrils. An intermediate condition has been proposed by Kiermayer and Dobberstein (18) in which the Golgi apparatus has the capacity to synthesize and transport the cellulose synthetases, but the enzymes are not activated until associated with the plasma membrane. These observations have stimulated the concept that the Golgi apparatus in higher plants may assemble then transport the cellulose-synthesizing complexes to the plasma membrane whereupon they become active.

A model of the plasma membrane of *Oocystis*, including its subcomponents responsible for the assembly, transport, and orientation of cellulosic microfibrils is presented in Fig. 14. This model takes into account the observed stimulation of glucan synthetase by so-called "lipid intermediates" (19). Inasmuch as the polymerizing face of the complex is located *within* the membrane, it would be in close proximity to lipid components of the plasma membrane, some of which could be interpreted as the intermediates.

Regarding previous work with *Oocystis*, Robinson and Preston have suggested that the "granule bands" alone are responsible for the biosynthesis of cellulose. We propose to the contrary, that granule bands and associated ridges control the orientation of the microfibril once it has become polymerized and crystallized. The linear enzyme complex we describe was never observed by Robinson and Preston, a fact which could have misled them to consider the granule bands as the synthesizing mechanism for cellulose.

The direct visualization of a linear cellulose-synthesizing complex allows one to make some interesting speculations as to why no one yet has synthesized microfibrils *in vitro* and why the yield of "alkali-insoluble" cellulose is hardly a true approximation of the product synthesized *in vivo*. We believe that an intact complex is required for microfibrillar production of any magnitude. It is true that upon cell fractionation, the subunits of the enzyme complex could become detached and still have the capacity to polymerize glucan chains. But if these subunits were not spatially situated so that the nascent glucan chains could crystallize into sufficient order, then a microfibril would not be generated. Another alternative is that the separated subunits could have the capacity to generate β 1,3- or mixed β 1,3- β 1,4-glucan linkages, thus synthesizing glucans that cannot be crystallized into sufficient order to form microfibrillar cellulose.

Since the enzyme complex is deeply embedded within the B face of the plasma membrane, and since it does not project through the cytoplasmic side of the A face, we think that it would be almost impossible to effectively isolate an intact complex without first "splitting" the plasma membrane. Furthermore, we believe that it would first be necessary to somehow deactivate cellulose synthesis in order to achieve

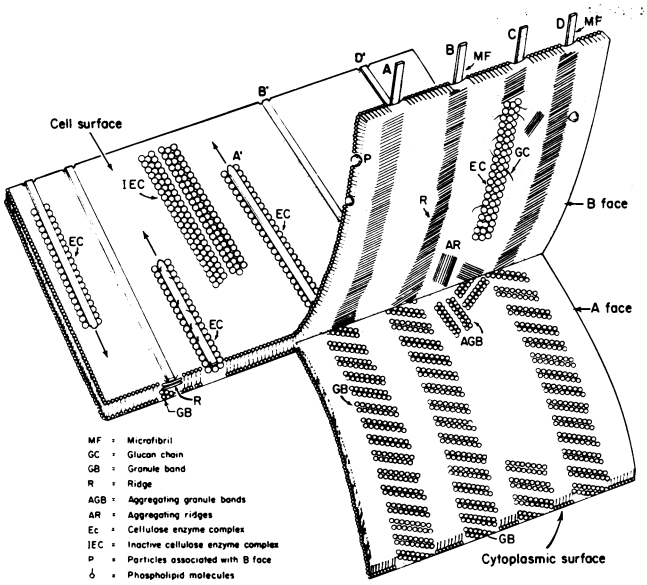


FIG. 14. Diagrammatic interpretation of cellulose synthesis in the plasma membrane of *Oocystis*. Note that the enzyme complexes do not have the stated number of subunits, and the granule bands are depicted smaller due to space limitations.

isolation since the complex is in direct contact with the microfibril during synthesis. Experiments are in progress (Montezinos and Brown) to deactivate the complex in preparation for its isolation and subsequent microfibrillar synthesis *in vitro*. How could one assay for deactivation? Since we frequently observe the dimer conditions of the complexes with which no microfibrils are associated, this would suggest the type of morphological criterion useful to assay for inactivation.

The plasma membrane of *Oocystis* typifies the fluid nature of membranes proposed in the Singer-Nicolson fluid mosaic model (20). Horizontal transport of the enzyme complex during microfibril assembly seems the more favored alternative rather than the enzyme complex remaining fixed at one point within the membrane with the microfibril "growing" from a static complex. Our concept is in harmony with the hypothesis of terminal synthesis of cellulose in the growing microfibril and argues against an antiparallel arrangement of glucan chains.

Our model provides a useful explanation of the geometry and dimensions of the enzyme complex in relation to the size and shape of the microfibrillar product. The enzyme complex has a well-defined morphology and consists of at least 100 subunit particles in the active and inactive-dimer states. If the Meyer-Misch model (21) and the dimensions of the glucan chains in the unit cell of cellulose I are considered, the number of glucan chains occupying the observed dimensions of 55 Å × 71 Å (the presumed crystalline core) is approximately equal to the number of subunits (=100) in the enzyme complex. Following such reasoning, one could infer that each subunit of the enzyme complex has the capacity to make a single glucan chain.

The specific geometry of the flat ribbon microfibril in *Oocystis* should be considered. Since the microfibril is generated by a linear complex consisting of three rows of subunits in the form of a semicylinder, it seems possible that each row of subunit particles might preferentially direct the plane of crystallization of the 30 or so nascent glucan chains into three protofibrils which subsequently aggregate into the ribbon-shaped microfibril. One could thus predict that a microfibril with a square cross section might be produced by a completely cylindrical or "microtubular" complex (22). Since the secondary wall microfibril of cotton is smaller than that of *Oocystis*, one could expect to find a less elaborate complex associated with the plasma membrane (J. M. Westafer and R. M. Brown, Jr., in preparation).

Our proposal for the active site of cellulose synthesis in *Oocystis* is in harmony with the emerging picture of cellulose biogenesis in higher plants. It is obviously difficult to postulate a universal mechanism for cellulose biosynthesis, but the knowledge gained from study of diverse organisms is helping to complete the gaps in our understanding of the biogenesis of cellulose—one of Nature's greatest enigmas.

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