ULTRASTRUCTURAL AND MOLECULAR CONCEPTS[1] OF CELL-WALL FORMATION

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ABSTRACT

Biochemical, physiological, and cytological aspects of cell-wall formation are discussed. The relationship between cell division, cell extension, and wall formation are analyzed and presented in a unified frame of reference. The cell is considered as a packet of energy—some of which is in the form of information and some of which is in the form of structure. The possibility of a self-assembly system for cell-wall synthesis is raised.

Recent work in biochemistry and genetics has elucidated the shikimic acid and chorismic acid pathways leading to the aromatic amino acids. Phenylalanine ammonia-lyase has been suggested as a key enzyme in controlling lignification, and these results, together with the Freudenberg hypothesis, have provided at least a preliminary understanding of the lignification process. The future use of auxotrophic mutants of higher plants should greatly deepen our knowledge in this area. The sugar nucleotides have been shown to be the primary agents in cellulose, hemicellulose, and pectin synthesis. Indole acetic acid directly promotes synthesis of the matrix polysaccharides of the wall but only indirectly controls the formation of the cellulose framework. Matrix polysaccharides have been shown to be intercalated throughout the pre-existing wall, while current evidence is that cellulose is added by apposition. The cytological level is probably the least known component of wall development. The role of the Golgi vesicles in the formation and orientation of the cell plate is clearly established, but whether such vesicles play a role in actual wall formation is open to question. Three types of paramural bodies found in pine and lentil seedlings are described, and their possible origins and functions in cell-wall formation are discussed. A new calculation for pore size in the hydrated and dehydrated cell walls is also presented and discussed.

Plant cell walls are certainly the most ubiquitous and abundant of natural products and perhaps the most important. They are the primary repositories of that portion of the incident solar energy available to sustain the biological world. Long ago Schrödinger (1945) pointed out that organisms, human and otherwise, feed on negative entropy. And it is the cellulose of green plants that is the major link in the world-wide food chain through which plant life passes the energy of the sun to the animal world.

Plant cell walls also assume more parochial and pedestrian importance to those of us who study them. Wood scientists and technologists are of course vitally interested in cell walls because the walls comprise their basic raw materials. From the purely basic viewpoint of developmental biology, however, the facts are that almost all higher plant cells, including meristematic cells, are encased in cell walls and if the plant cell is to grow and divide so must the wall. Moreover, current research has linked the cell wall to the initiating mechanisms of cellular growth (Baker and Ray 1965a, 1965b; Ray and Baker 1965; Ray 1967; Roberts 1969).

Before we discuss the formation of the cell wall, it is necessary to consider briefly the nature of the substance being formed.

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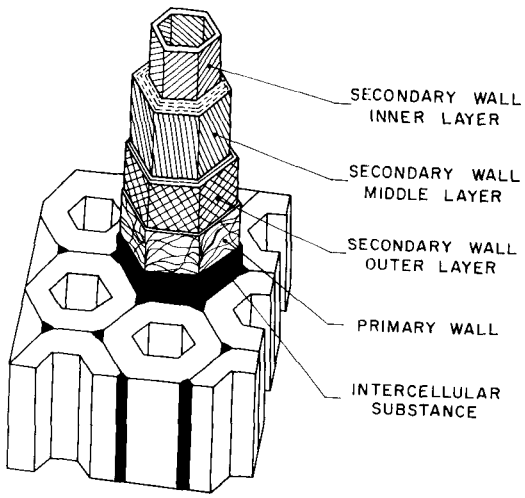

FIG. 1. Diagrammatic view of the layering in tracheid cell walls.

Figure 1 shows the wood scientist's dogma on the cell wall. Recent findings require us to modify the dogma somewhat, and in fact the structural picture of the cell wall is not nearly as clear now as it was just six years ago (cf. Berlyn 1964). According to the now traditional view, the cell walls are embedded in an isotropic amorphous middle lamella (M). The outermost part of the wall is termed the primary wall and can be defined as that part of the wall laid down at a point in space-time where cell extension or surface area expansion is occurring. The amount of surface area increase that takes place in a developing plant wall is enormous, ranging from a 10-fold to a 10^5-fold increase (Wilson 1964). It is thus not surprising that the primary wall exhibits a rather chaotic distribution of fibril orientation. Physical theories of surface area growth will be dealt with in another paper of this series (Wangaard 1970) and hence will not be considered here.

Wall layers laid down at a point in space-time where expansion is not occurring are designated secondary. The secondary wall of most normal fibers, including tracheids, fiber tracheids, and libriform fibers, is further subdivided into three concentric layers: S_1, S_2, and S_3 (Fig. 1). Vessels may have additional secondary layers: S_4, S_5, and so forth. Each sublayer, S_i, may itself consist of lamellae, i.e. 4–6 for S_1 and S_3, and 30–150 for the thicker S_2 (cf. Jutte 1969). In xylary cells that are devoid of protoplasts, there may be a warty layer and/or terminal lamella adpressed to the innermost layer of S_3 (Liese 1951). The warty layer consists of cytoplasmic debris adhered to the inner surface of the wall (Frey-Wyssling 1959; Liese 1960; Liese and Ledbetter 1963) and possibly localized thickening of S_3 (Wardrop 1963, 1964).

The walls are of course provided with pits that, together with cell lumens and intercellular spaces, comprise the first-order pore space (Berlyn 1964). Within the hydrated cell wall, there is a free space component that can be termed the second-order space (Berlyn 1964, 1968, 1969). This free space is important with respect to the movement of water, and even materials as large as proteins can move freely in this space. Distribution of chemical constituents will not be dealt with in this paper, and the reader is referred to Larson (1969) for recent information on this topic.

The structural framework of the wall is cellulose, but the question is, in what form does it exist in the intact cell wall? For a long time now, many of us have believed in the reality of the microfibril as the basic structural and functional unit of the wall. However, the advent of negative staining forces us to ask whether the microfibril actually exists in the intact cell wall or whether it is merely an artifact.

In negative staining, the stain (e.g. potassium phosphotungstate or ammonium molybdate) does not penetrate deeply into the specimen, but does fill in spaces between particles. It thus renders the particles visible by creation of steep boundary gradients of electron density. The spaces between particles will transmit fewer electrons and appear darker than the object. Figure 2 is an example of a negatively stained section from a slash pine tracheid stained with uranyl acetate. This electron micrograph was kindly provided by Dr. Anton N. J. Heyn of the University of Louisiana (New

FIG. 2. Negative stain preparation of 35-Å elementary fibrils (from Heyn 1969).

Orleans). The exciting feature is that the fibrils measure only 35 Å in diameter (cf. Heyn 1969). These structures have been termed elementary fibrils (Mühlethaler 1960, 1965, 1967), a term used by Frey-Wyssling as early as 1954 (cf. Berlyn 1964). However, the Frey-Wyssling elementary fibrils were crystalline subunits of the

microfibril and were said to measure 70 Å × 30 Å in cross-sectional dimension. Several of them were purported to be enclosed in a common paracrystalline cortex to form the microfibril. Thus these units were not homologous with the present concept of elementary fibrils.

The cable model of an elementary fibril proposed by Bonart et al. (1960) also postulated the existence of a microfibril, but the paracrystallinity was associated with the surface of the elementary fibril. Ranby (1958) concluded that there was only one crystallite per microfibril, but his fibril was 100 Å in diameter. Preston and Cronshaw (1958) modified this model to include a paracrystalline cortex with some nonglucose residues on the surface, and Preston still supports this model (cf. Nieduszynski and Preston 1970). Thode, Swanson, and Becher (1958) found 38-Å "pores" in the cell wall, and these entities were virtually unaltered by a wide variety of physical and chemical treatments, a fact which led these authors to conclude that this 38-Å unit could be an intrinsic feature of wall fine structure itself.

The problem with the negatively stained image is that it is extremely difficult to interpret (Juniper et al. 1970) and greatly different images may be produced as a consequence of very small changes in preparative techniques (Glauert and Lucy 1969). It could well be that the negative stain can penetrate the paracrystalline cortex and pore space, leaving only the crystalline core unstained (Colvin 1963). Mühlethaler (1967), however, discounts this criticism because the elementary fibrils do not change appreciably in diameter when metal-shadowed or positively stained with $K_2Cr_2O_7$. From this he concludes:

> . . .the 35 Å fibrils are ultimate structural units which are generally not aggregated into larger strands such as microfibrils.

Heyn (1966, 1969) also found the cell walls of ramie, jute, cotton, and pine to be constructed of elementary fibrils. These results were entirely consistent with data from low angle X-ray studies. In cotton secondary walls, the 35-Å unit fibrils are densely packed, and according to Mühlethaler (1967), the space between the fibrils is "only" 30–40 Å. He concluded that there is no space left for a paracrystalline sheath and that the close packing of the elementary fibrils explains why removal of noncellulosic constituents allows artifactual aggregation of the elementary fibrils into larger units such as microfibrils. This is a somewhat anomolous conclusion since 30–40-Å spacing between 38-Å elementary fibrils does not constitute close packing. It may be that Mühlethaler means that the center to center spacing is 30–40 Å (which would be close packing indeed), but if this is the case, he wrote the opposite of what he meant. In pine Heyn (1969) found the spaces between elementary fibrils were variable but in the range 20–30 Å and supported the view (Berlyn 1964) that the S_2 layer has a continuous structure, i.e. a one-phase crystalline with order defects system. It must be emphasized that these packing figures given by Mühlethaler and Heyn are for the completely dehydrated wall. The hydrated wall, as shown microspectrophotometrically by Berlyn (1969), has 25%[2] free space in the S_2 layer. However, Berlyn's spectrophotometric and compression technique did not reveal any free space in the completely dehydrated wall. The sensitivity of this system depends upon the photomultiplier tube, but the system was shown to be accurate within 2%; thus even 20-Å diameter pores between elementary fibrils would be large.

Consider a fully hydrated cell wall 5μ thick. Along this wall there is 1.25μ of free space and 3.75μ of solid space.

solid space thickness/diam. of elementary fibrils = No. elementary fibrils along a

[2] Free space $= \dfrac{M/A \text{ dehydrated} - M/A \text{ hydrated}}{M/A \text{ dehydrated}}$

$$= \frac{E_d - E_h}{E_d} = \frac{\Delta E}{E},$$

where M/A = mass of cell wall per area of photometric field, A.
E_d = optical density of area A in dehydrated cell wall.
E_h = optical density of area A in hydrated cell wall.

linear wall thickness $= 3.75 \times 10^4$ Å/36 Å $= 1.04 \times 10^3$ elementary fibrils.
No. of elementary fibrils × space between fibrils = total free space = (1.04×10^3) (20 Å) = 2.08μ.

The free space component should be 1.25μ, and the size of interelementary fibrillar space that meets this requirement is 12 Å. This value is for the hydrated state, and the spacing between elementary fibrils in the completely dehydrated wall (such as observed in the electron microscope) should be much smaller than this. Kellogg and Wangaard (1969) estimate the percentage of voids in the dry wall to be 1.64 to 4.76%. A calculation from their data indicates that the maximum spacing figure would be 1.7 Å. Of course these values assume that the diameters of the free space are relatively uniform as indicated by Mühlethaler (1967) and especially Heyn (1969). Such high packing densities for the wall are further evidence for the continuous hypothesis of wall structure.

Another controversy in the literature concerns the physical conformation of the cellulose chains. Manley (1964) has proposed a folded chain model for cellulose I, but this model has been heavily criticized by a number of workers including Mark (1967), Mark et al. (1969a, 1969b), Gillis, Mark, and Tang (1969), and Muggli (1968). Other folded chain models have also been proposed and discounted (cf. Gillis et al. 1969). The chemical composition of the wall has been reviewed previously (Berlyn 1964) and will not be dealt with here.

Cell-wall formation in woody plants is a part of the process of cellular differentiation. This process can be subdivided into a series of six continuous phases, viz: (1) karyokinetic; (2) primary cell-wall formation; (3) surface area growth; (4) cell-wall thickening; (5) lignification; and (6) senescence and death. Of course, not all cells undergo all six phases, but all are created by a division phase and all eventually die. The cell wall is thus involved in both the initiation and the termination of cell growth.

The karyokinetic phase is fundamental to wall formation and is defined broadly here to include the presynthetic state (G_1), the DNA synthesis stage (S) and the postsynthetic stage (G_2) as well as active mitosis (M). The larger process of xylogenesis is usually preceded by cell division, but the precise factors involved have not been identified (Roberts 1969). It is known that 5-fluorodeoxyuridine (FUdR) blocks thymidylate synthesis and it also inhibits xylogenesis. In addition, mitomycin C which forms cross-links between the complementary strands of DNA again has been found to block xylogenesis. Presumably some translational activity is required in order for the genes-controlling xylogenesis to be switched on.

Three critical steps in cell-wall formation can be identified. The first concerns the biochemical pathways that are involved in cell-wall biosynthesis. These biosynthetic pathways presumably lead to the formation of intermediates, which must be incorporated into the wall. The second critical step is the mechanism by which this incorporation is accomplished. The third step is the way in which each new increment of wall material is integrated into the supramolecular structure of the wall as a whole (and even the organism as a whole). Recently there has been considerable progress on step 1, but steps 2 and 3 are still largely in the black box category.

Glaser's (1958) early work with *Acetobacter xylinum* implicated sugar nucleotides (specifically uridine diphosphate glucose, UDPG) as carrier molecules in carbohydrate synthesis. However, it was not until the pioneering efforts of Hassid and his coworkers (cf. Elbein, Barber, and Hassid 1964; Hassid 1969) that this mechanism was accepted for higher plants (Fig. 8). At first Elbein et al. (1964) reported that only Guanosine diphosphate glucose (GDPG) could serve as a substrate for cellulose synthesis in *Phaseolus aureus*. However, the original particulate enzyme preparations of the Hassid group did not contain the UDPG transferase, and the synthesis of insoluble polysaccharide with

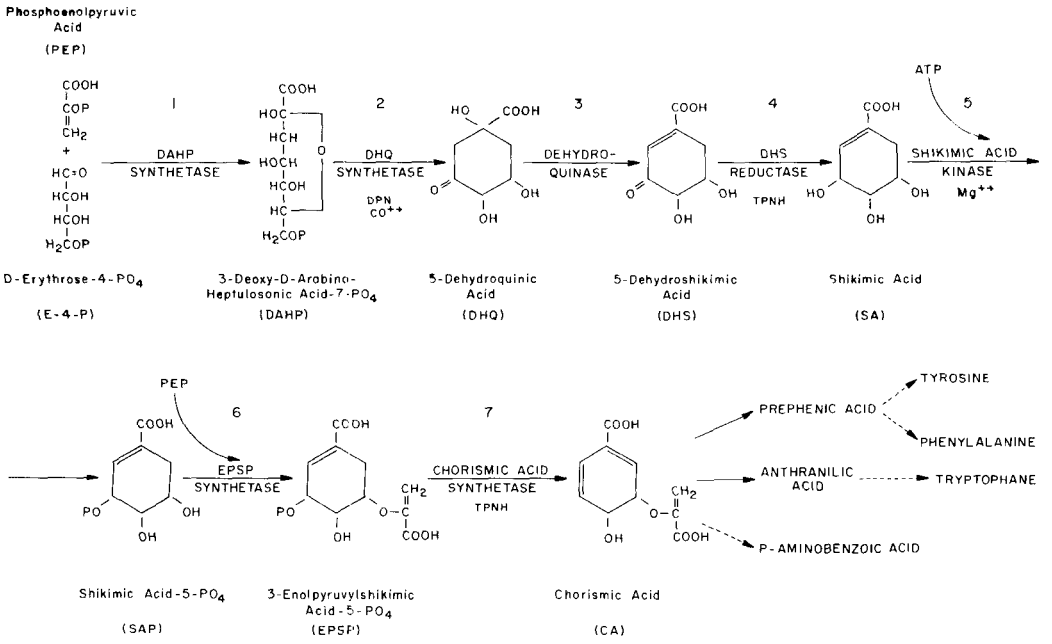

FIG. 3 Prearomatic portion of lignin pathway.

UDPG has since been demonstrated (Brummond and Gibbons 1964, 1965; Ordin and Hall 1967, 1968; Villemez, Franz, and Hassid 1967). Nevertheless Hassid (1969) presents the case that *in vivo* UDPG largely functions in hemicellulose synthesis, while GDPG is primarily concerned with cellulose synthesis. The particulate nature of the enzyme system is critical for synthetase activity, i.e. soluble preparations do not work but can be activated by the addition of boiled particles. Evidence from the synthesis of certain bacterial polysaccharides suggests that these sugars are not transferred directly to polysaccharide chains but may first become associated with lipid compounds (Colvin 1964; Robbins et al. 1967). Added complexity derives from the fact that myo-inositol represents at least an alternative pathway between hexose and uronic acid. *Myo*-inositol is an effective precursor for all uronic acid and pentose residues found in plants (Roberts, Deshusses, and Loewus 1968; Loewus and Baig 1969). D-glucuronic acid is formed from the myo-inositol ring; this eventually results in the formation of UDP-D—galacturonic acid—and the D-galacturonsyl moiety of the latter is incorporated into pectin. The gene action system that controls the switching mechanisms during the formation of wall polysaccharides has yet to be investigated.

Marx-Figini and Shultz (1966) have conducted some interesting preliminary studies on the control of cellulose synthesis in cottonseed hairs. As the primary wall is formed, the degree of polymerization of the cellulose molecules ranges from 2000–6000. As soon as secondary wall formation commences, the DP goes to 14,000 (mol wt of 2.3×10^6) and is remarkably uniform, i.e. 90% of the cellulose molecules have this DP. Marx-Figini and Schultz (1966) postulated that the cellulose biosynthetic mechanism could be regulated by either a template system or a time-dependent process. It has been suggested (Mühlethaler 1967) that the wide range of DP in the case of the primary wall makes a template mecha-

nism doubtful, but such a system could function in the biogenesis of cellulose in secondary walls. Des S. Thomas, Smith, and Stanley (1969) have proposed a stereospecific regulation of β-1-3 glucan synthetase in several species of plants. They found that glucan synthesis from UDPG (catalyzed by a particulate enzyme preparation) was stimulated by a number of sugars and glycols; however erthritol competitively inhibited glycerol stimulation of glucan synthesis. There was a marked difference in the stimulation caused by D-arabitol (651% of control)and L-arabitol (212% of control). This gave rise to the stereospecific conclusion. Magnesium promoted glucan synthesis, while calcium inhibited it.

The initial step in lignin biosynthesis is the production of aromatic compounds from sugars formed by photosynthesis (Fig. 3). The first reaction is the joining of erythrose-4-phosphate (from the pentose phosphate pathway) with phosphoenolpyruvate (from the glycolytic pathway) to form deoxyarabinoheptulosonic acid phosphate (DAHP). From here the pathway proceeds through shikimic acid to chorismic acid and thence to the prearomatic compound prephenic acid (cf. Davis 1955, 1958). There are five enzymes involved in steps 2–6 of the prechorismic acid portion of this aromatization pathway, and in eukaryotic organisms these enzymes are physically aggregated and encoded by a cluster of five genes (Berlyn and Giles, 1969; Berlyn, Ahmed, and Giles 1970). A polycistronic messenger ribonucleic acid (mRNA) derived from the five cistrons is probably involved in forming the enzyme aggregate. The physical state of the aggregate may be part of the control mechanism for this early portion of the lignification pathway.

After prephenic acid comes the transaminase reactions leading to the formation of phenylalanine and tyrosine (Fig. 4). The critical enzyme involved in switching phenylalanine directly into the lignin pathway is phenylalanine ammonia lyase (PAL), discovered in 1961 (Koukol and Conn 1961; Neish 1961). This enzyme

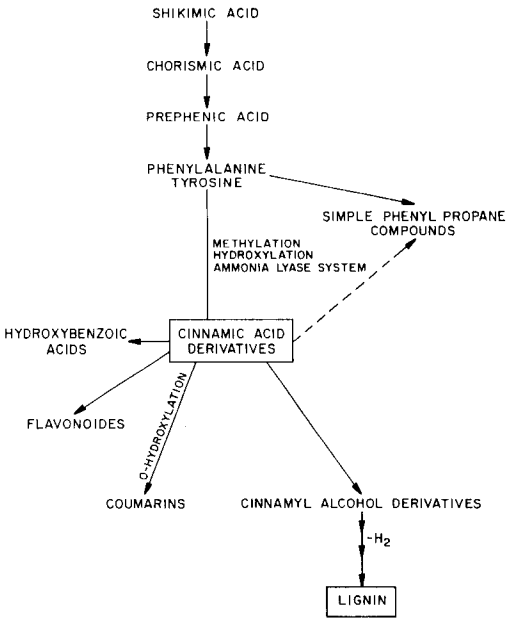

FIG. 4. Biosynthetic pathways leading to, and associated with, lignification.

catalyzes the removal of ammonia from amino acids and the formation of $\alpha-\beta$ double bonds. This results in the generation of cinnamic acid. Xylogenesis requires *de novo* protein synthesis (Fosket and Miksche 1966), and the speculation is that PAL is the protein involved because it shifts the cellular metabolic machinery from protein synthesis to the synthesis of secondary metabolites. In the formation of lignin-building stones, the cinnamic acids are reduced to primary alcohol, i.e. p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. According to the Freudenberg hypothesis (e.g. 1965), these alcohols and their glucosides are ultimate precursors of lignin. The three glucosides (glucocoumaryl alcohol, coniferin, and syringin) are found in the cambium and are transported to the differentiating xylem cells where the mediation of β-glucosidases releases the aglycons. At this point enzymes of the phenol dehydrogenase type (like the laccases and peroxidases) catalyze the removal of the phenolic hydrogen from the alcohols to form free radicals. Essentially random intercombination of these extremely un-

stable radicals leads to the formation of lignin. Up until now, work on lignin biosynthesis has been stymied because the tracer approach does not permit the identification of *obligate intermediates* (S. A. Brown 1969). Now, however, a new method has been developed that permits the rapid production of auxotrophic mutants (Carlson 1969, 1970). The use of biochemical genetical techniques to establish biochemical pathways in microorganisms has been highly successful, and use of these procedures with higher plants should greatly strengthen our knowledge of lignin —its structure and formation.

One of the major ways in which cell-wall formation is controlled is via plant hormones. The effect of IAA on cell-wall synthesis (in addition to its role in lignification and compression wood formation) is often regarded as being an indirect effect in that IAA is thought to promote elongation and the elongation is supposed directly to induce wall synthesis (Bayley and Setterfield 1957; Setterfield and Bayley 1961). However, Ray and Baker (1962) have shown that when oat coleoptile sections are inhibited by a nontoxic osmoticum such as Ca^{++}, a substantial promotion of wall synthesis by auxin may still be demonstrated. Thus IAA directly promotes wall formation. Further analysis revealed that the direct effect was limited to matrix polysaccharides such as glucuronoarobinoxylan, noncellulosic glucans, and galacturonic acid containing polymers (Baker and Ray 1965a). There is also an indirect effect of auxin in that uninhibited oat coleoptile sections also show increased cellulose synthesis in response to auxin. What happens here is that the auxin promotes elongation, which stimulates glucose uptake, and the increased glucose uptake results in increased cellulose synthesis (cf. Baker and Ray 1965a, 1965b; Ray and Baker 1965). In these studies the externally supplied glucose was directly incorporated into the wall without passing through and being diluted by the bulk of the alcohol-soluble pool. The bulk of the alcohol-soluble fraction is located in the cell vacuole, so the externally supplied glucose must be utilized as it moves along the route of uptake into the vacuole. On this basis Baker and Ray (1965b) concluded that auxin is involved only in primary wall formation (extensial synthesis) and not in secondary wall formation (intensial synthesis). This conclusion was based on their observation that in older, nonelongating oat coleoptiles, wall synthesis was still high, but it was promoted by auxin. Several lines of evidence mitigate against the generality of this conclusion. First, auxin is necessary for xylogenesis (Roberts 1969); and second, auxin induces compression wood formation in conifers (cf. Westing 1965, 1968). Third, interference with auxin transport (e.g. via triiodobenzoic acid) results in tension wood formation in woody dicots (Berlyn 1961; Kennedy and Farrar 1965a, 1965b; Cronshaw and Morey 1965; Morey and Cronshaw 1968a, 1968b, 1968c).

In the case of compression wood, there is a direct promotion of lignin and matrix polysaccharides. This has to be a direct effect since compression wood fibers are shorter than normal, i.e. elongation-induced uptake cannot account for the increased cell-wall substance incorporation. In tension wood fibers, there is an increase in the synthesis of the cellulose framework, and again this is a direct effect since these fibers are also shorter at least in the case of *Populus deltoides* (Berlyn, unpublished). Tension wood is, however, a lack of auxin effect. Obviously there is a complex metabolic regulatory system involved here (cf. Berlyn 1961). These cytogenetic pathways are the resultant of gene action systems whose intensities according to Waddington (1962) are mutually adjusted by interlocking control systems. Thus the control network is far more complicated and powerful than a simple on-off switching system. There are degrees of off and degrees of on and each degree may have secondary consequences, e.g. in feedback mechanisms and allosteric reactions.

Evidence exists that more than one hormone influences wall formation and in certain cases two or more hormones act

synergistically in the stimulation of wall formation (Roberts 1969). For example, Wareing, Hanney, and Digby (1964), working with disbudded shoots of several forest trees, found that giberellic acid (GA) plus IAA produced the maximum amount of wood formation. With IAA alone, only a small amount (discontinuous) of seemingly normal xylem was produced, while with GA only fusiform parenchyma was formed.

Peter Ray (1967) has recently provided some answers to the classic question about where the incorporation of cell-wall constituents takes place during wall formation, i.e. intussusception vs. apposition. Coupling microautoradiography at the electron microscope level with differential extraction-direct counting procedures, Ray found that matrix polysaccharides were deposited throughout the wall, while cellulose was laid down largely or entirely by apposition. This information tells us where the constituents are incorporated, but it does not reveal where the intermediates are synthesized and how they are transported to or into the wall. Various locations for the synthesis of the wall constituents have been suggested, including: (1) the outer cortex of the protoplast; (2) the surface of the plasmalemma; (3) the wall itself; and (4) a combination of the above.

It is well known that lignification begins in the cell corners and moves centripetally through the primary and then the secondary wall (cf. Wardrop 1963, 1964, 1965; Berlyn 1964; Berlyn and Mark 1965, Larson 1969). Therefore at least the final polymerization process occurs within the wall. Lignification usually begins sometime after deposition of the carbohydrate component of the wall (Wardrop 1957; Berlyn 1967), but Hepler, Fosket, and Newcomb (1970) report the simultaneity of these processes in wound vessel member formation in *Coleus*. As shown by Ray (1967), the hemicelluloses are incorporated throughout the wall, but it is quite possible that the subunits of the polymers are first synthesized and assembled in the cytoplasm before movement to and incorporation in the wall. However, except under unusual circumstances (e.g. Bouck and Galston 1967), cellulose and even hemicelluloses are not found in substantial amounts within the cytoplasm of plant cells. Normally these substances are not cytochemically detectable at the light microscope level. Therefore, cell-wall carbohydrate synthesis must occur at or near the cell-wall cytoplasm interface and subunits must be quite small.

The first part of the wall to be formed is the cell plate, and it is established that the Golgi apparatus is the primary agent of formation for this structure (Mollenhauer and Morré 1966; R. M. Brown 1969). The Golgi produce a number of small vesicles that enlarge, move to the cell equator, and at metaphase fuse to form the cell plate (Whaley and Mollenhauer 1963; Whaley, Dauwalder, and Kephart 1966). Dashek and Rosen (1966) have performed some important tracer experiments using labeled myo-inositol and methyl methionine as pectin precursors. The label first entered the Golgi vesicles and then the wall. These results find confirmation in those of Northecote and Pickett-Heaps (1966) and identify the Golgi vesicles as the sites of pectin synthesis. Final polymerization and methylation occur in the coalescing vesicles and wall.

A number of hydrolytic enzymes (e.g. esterases, lipases, and acid phosphatase) have been localized in the wall (Olszewska, Gabara, and Steplewski 1966). These enzymes probably have several roles in wall formation but one possible function, according to Mühlethaler (1967), is to decompose the mitotic apparatus at the conclusion of cytokinesis.

The formation and growth of the cell wall are intimately associated with cell-wall protein. There may be many proteins in the wall, but certainly one of the most important is the hydroxyproline-rich protein termed extensin (Dougall and Shimbayashi 1960; Lamport and Northecote 1960; Lamport 1965). This protein cross-links polysaccharides, i.e. carbon 1 of arabinose is glycosidically linked to carbon 4 of hydroxyproline (Lamport 1967). Presumably extensin changes in conformation upon the

addition of IAA and is thus the origin of the increased cell-wall plasticity that accompanies extension growth. It is postulated that the conformation change is due to reduction of disulfide bridges in the protein molecules linking the wall polysaccharides. Mühlethaler (1967) believes that plasmalemma particles (present only in freeze-etched electron micrographs) are responsible for cellulose synthesis and that these plasmalemma particles contain extensin. Under this scheme extensin is thought to have a role in both the bonding of the cellulose fibrils and in fibrillar orientation.

Microtubules were once proposed as possible organelles of cell-wall synthesis (Ledbetter and Porter 1963, 1964; Hepler and Newcomb 1964). These structures are present in both animals and plants, and the literature on microtubules has recently been reviewed by Newcomb (1969). Microtubules range in diameter from 180–300 Å, with the average diameter being 240 Å. The wall of the microtubules is ca. 70 Å thick and is composed of 13 globular subunits (each about 40 Å in diameter) equally spaced (45 Å center to center spacing) around the circumference of the tubule (Ledbetter 1965). Microtubules are proteinaceous in nature, consisting of a protein dimer with molecular weight of approximately 120,000, which can be separated into 60,000 molecular weight subunits. These monomers are thought to be the 40-Å diameter globular units observed in the electron microscope. The amino acid composition of microtubular proteins from many sources is quite similar, and the protein can bind with a nucleotide (GTP or ATP). Each dimer purportedly has two nonequivalent binding sites for the nucleotides (cf. Newcomb 1969). The similarity of microtubules and spindle fibers was duly noted and ontogenetic relationships were postulated (Green 1963; Ledbetter and Porter 1963; Berlyn and Passof 1965). These relationships have been substantiated by recent studies indicating that bundles of microtubules comprise the spindle fibers (cf. Newcomb 1969). As far as wall formation is concerned, there are those who suggest microtubules are involved in micellar orientation (e.g. Ledbetter, 1965; Cronshaw 1965; Marx-Figini and Schultz 1966; Gantt and Conti 1965), and those who see no relationship between the two (e.g. Mühlethaler 1965, 1967; Newcomb and Bonnett 1965; Preston and Goodman 1968). The problem is that in some studies the orientation of the microtubules mirrors the orientation of the cellulose microfibrils, while in other cases it does not.

Recently there have been an increasing number of papers dealing with the role of certain membrane-bound bodies in cell-wall formation (Arrigoni and Rossi 1963; Buvat 1964, 1966; Czaninski 1966; Esau, Cheadle, and Gill 1966; Halperin and Jensen 1967; Manocha and Shaw 1964; Marchant and Robards 1968; O'Brien 1967; Öpik 1966; Robards 1968; Skvarla and Larson 1966; Srivastava and O'Brien 1966, Thomson 1967; Walker and Bisalputra 1967). These membranous and vesicular bodies associated with the cell-wall cytoplasm boundary region may in some cases have their ultimate origin in the Golgi apparatus, but this is not unequivocally established. The boundary region structures have been designated paramural bodies by Marchant and Robards (1968), a name that by itself is not meant to imply any particular origin. According to Marchant and Robards (1968), paramural bodies may be divided into two classes: (1) *lomasomes*, which are derived from cytoplasmic membranes; and (2) *plasmalemmasomes*, which are formed entirely from the plasmalemma. Paramural bodies may form discrete membrane bound structures that contain vesicles, granules, tubules, and/or fibrils.

MATERIALS AND METHODS

Most of the observations were made on excised sugar pine (*Pinus lambertiana*) embryos grown *in vitro* by our usual procedures (Berlyn 1967; Berlyn and Miksche 1965); however, data were also recorded on germinating lentil roots (*Lens esculenta*).

Actively growing root tips, stem tips, and hypocotyl tissues were dissected out for electron microscopy. The material was

fixed for 9–12 hr in 3% glutaraldehyde in phosphate buffer (*p*H 6.8) at 4 C or for 2–3 hr at room temperature in the same fixative. The tissue particles were washed at least 5 times (1 hr each wash) with cold (4 C) phosphate buffer (*p*H 6.8) and then postfixed for 1–12 hr in 1% osmium tetroxide in phosphate buffer (*p*H 6.8) at 4 C. Dehydration was accomplished at 4 C with acetone or ethanol. The tissue was infiltrated and embedded in epon, sectioned on an ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, and finally observed in either an Hitachi HU-11B or a Zeiss EM9A electron microscope.

OBSERVATIONS

Three types of paramural bodies were observed in the course of these investigations. The first type is usually larger than the other two and contains a very fine fibrillar material (Figs. 5–6). These large paramural bodies are probably of cytoplasmic origin (Figs. 7–8) and have hence been designated type 1 lomosomes. They are usually present during the early stages of wall formation and might be concerned with the incorporation of matrix materials. Note the long linear double membranes of the endoplasmic reticulum with associated polysomes.

A second, usually smaller, type of lomasome is illustrated in Figs. 9–11. These membrane-bound structures usually contain tubules or granules. Type 2 lomasomes are also commonly associated with linear polysomes, and Golgi apparati are often in the vicinity (Fig. 12). Note that the Golgi vesicles are much smaller in size than the lomasomes. Rarely, these type 2 lomasomes contain myelinlike bodies (Fig. 13), and usually these membranes have the loose configuration depicted by Tamulevich and Evert (1966); however, the condensed structures shown by Robards (1968) are occasionally observed. The third type of paramural body observed was the plasmalemmasome—a structure formed by the invagination of the plasmalemma itself (Figs. 14–15). Note the microtubules present in Fig. 14 and their close association with the plasmalemmasomes at this stage.

In a number of instances, the corners of the cell walls appear to have protoplasmic contents (Figs. 16–18).

DISCUSSION

In this paper I have presented some current and interdisciplinary concepts about the process of cell-wall formation. I rest the legitimacy of such an effort on the justification provided by Schrödinger (1945):

> . . . .the spread, both in width and depth, of the multifarious branches of knowledge during the last hundred odd years has confronted us with a queer dilemma. . .I can see no escape from this dilemma (lest our true aim be lost forever) than that some of us should venture to embark on a synthesis of fact and theories, albeit with second hand and incomplete knowledge of some of them—and at the risk of making fools of ourselves.

At this stage of cell-wall formation research, we are in a position similar to that of molecular biology in the 1930's and 1940's (cf. Delbruck 1970). We have some of the basic data but are unable to mold it together into a coherent framework. Too many links are still missing in the interlocking control network. We are stymied not only by the limitations of our own science but also by the inadequacies of knowledge in supporting fields. As Delbruck (1970) has pointed out there is a tendency to underestimate greatly the things we do not know about cell biology and cell to cell interaction. Lehninger (1965) has calculated that a simple bacterial cell 2μ in diameter can replicate 10^9 binary digits of information per second. This is equivalent to the information contained in a 1000-page volume of *Encyclopaedia Britannica*! The complexity of these histogenetic systems has caused many great minds trained in physical science to sink into the Goedelian well of antonymic despair (Bohr 1933, 1963; Heitler 1967; Delbruck 1970). However, the notion of random and spontaneous generation of antonyms within living systems is totally without experimental verification, and at

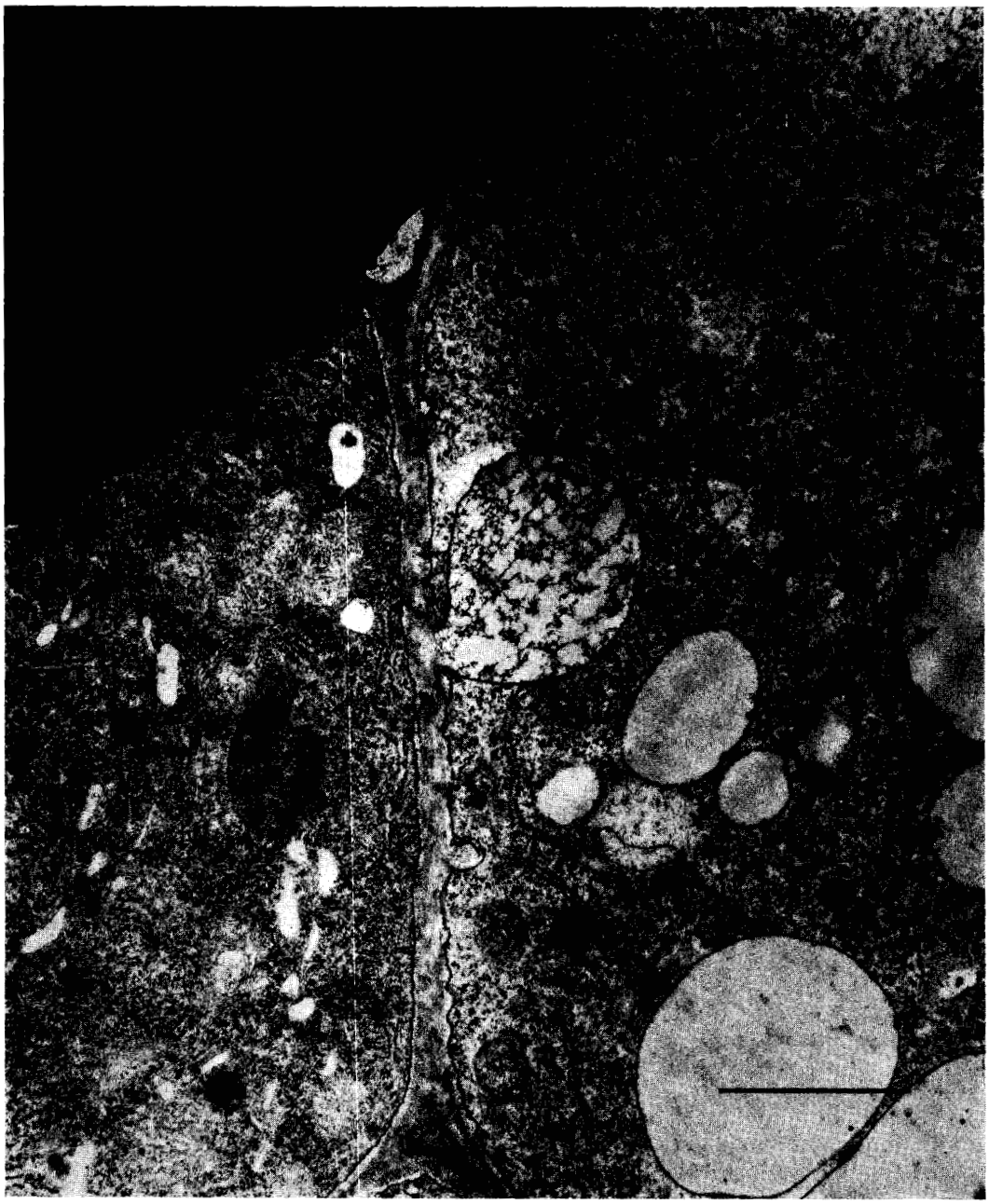

FIG. 5. Type 1 lomasome in lentil (bar on lower right corner of micrographs represents 0.5 micron).

this time its acceptance seems somewhat premature. Equally unverified but much more appealing are the ideas of Schrödinger (1945) and Elasser (1966), which suggest that some aspects of the physics of organisms may be different from, and additional to, our present concepts of physics. The latter have developed largely from the study of inanimate systems, and to Schrödinger's way of thinking, it should not be surprising if living systems reveal additional physical laws.

FIG. 6. Type 1 lomasome in *Pinus*.

FIG. 7. Type 1 lomasomes free in cytoplasm (*Pinus*).

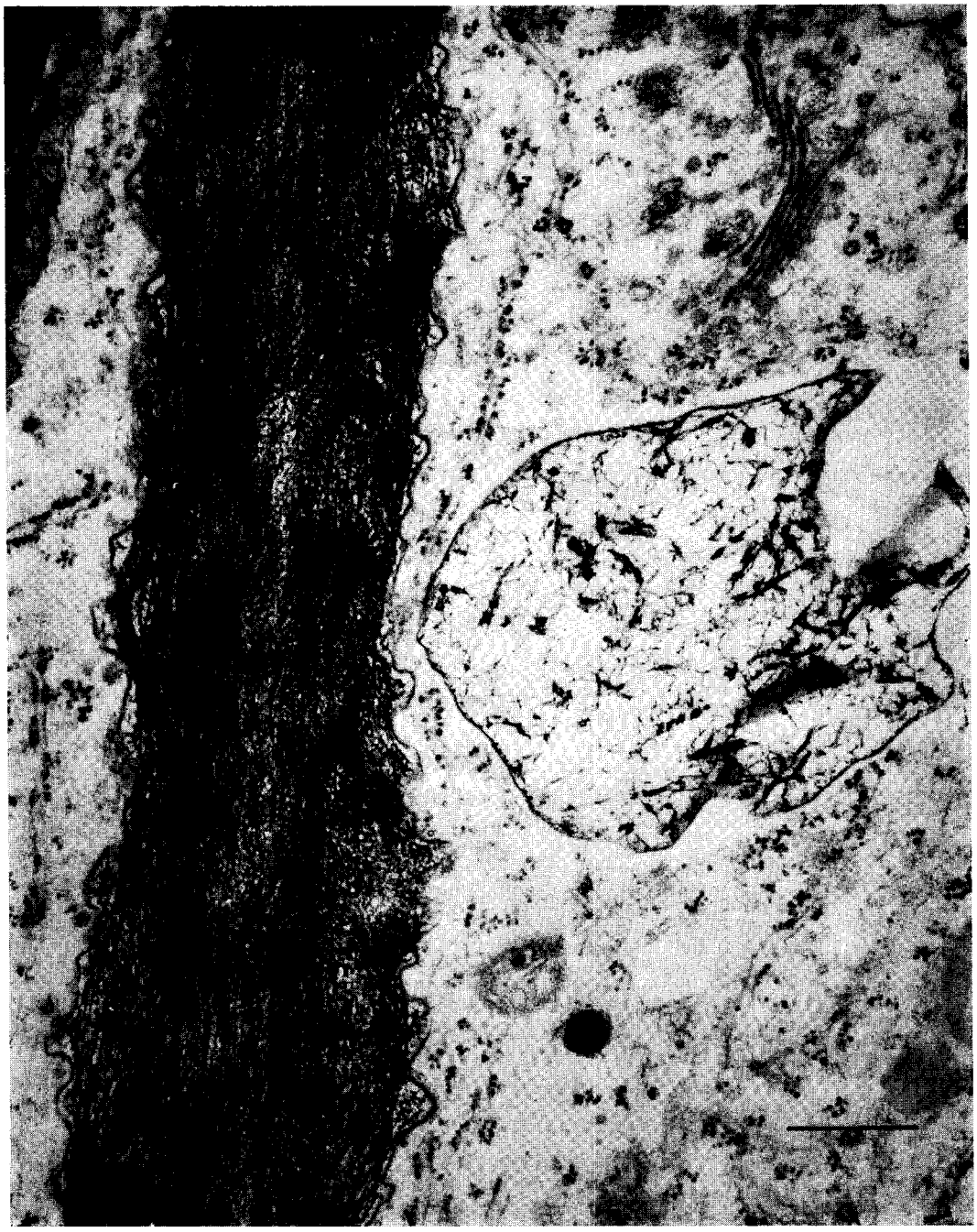

FIG. 8. Type 1 lomasomes free in cytoplasm (*Pinus*).

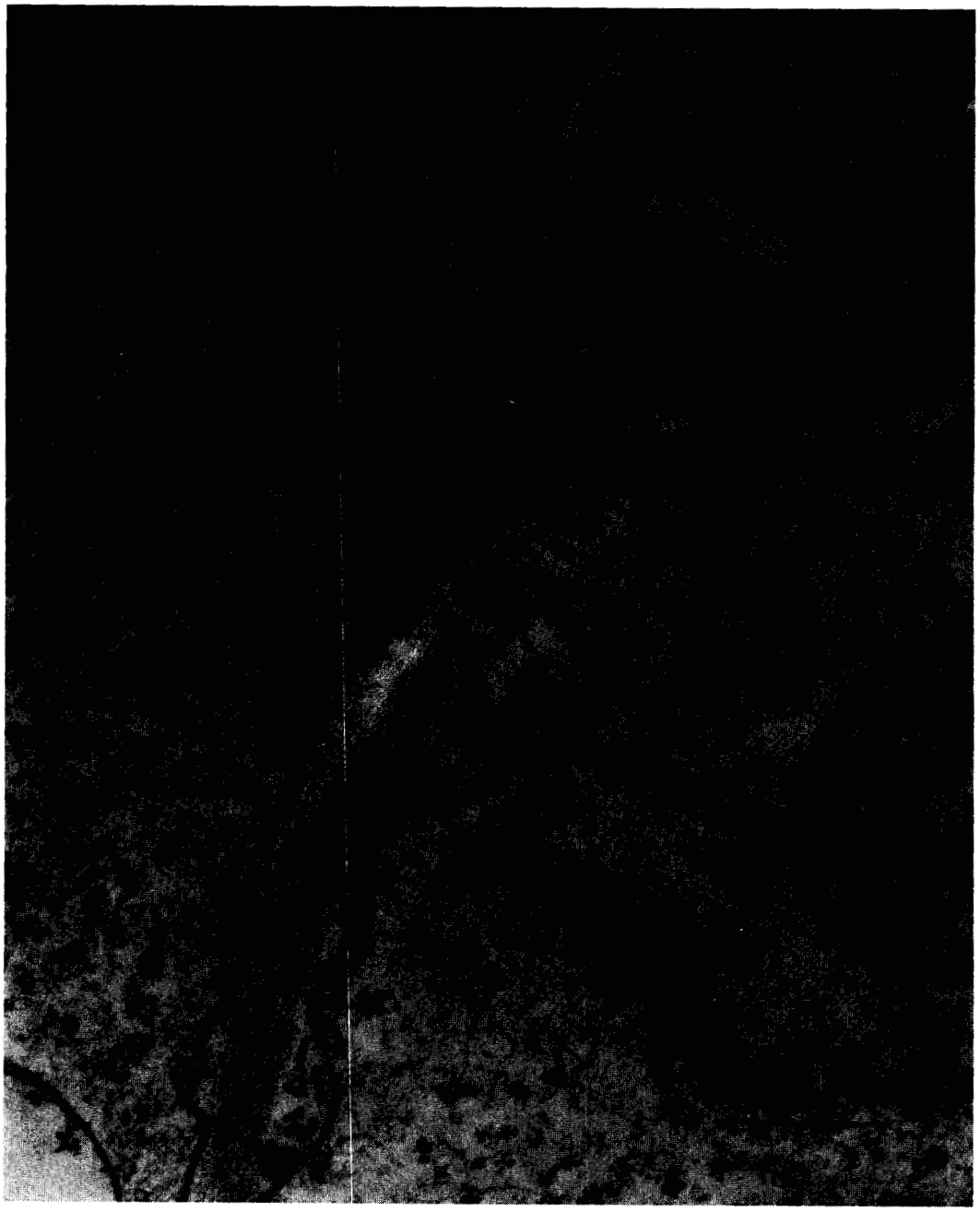

FIG. 9. Type 2 lomasome (lentil).

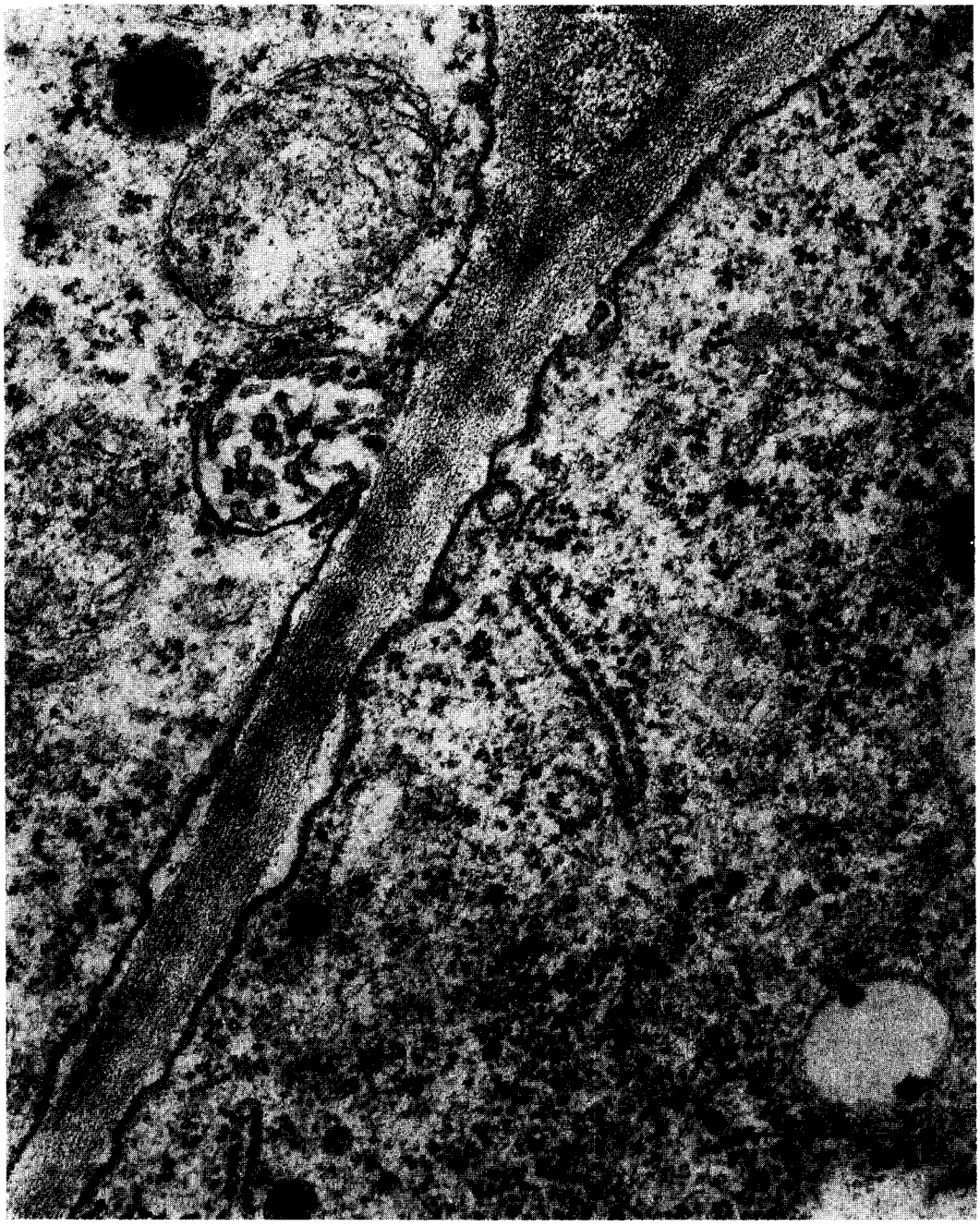

FIG. 10. Type 2 lomasome (lentil).

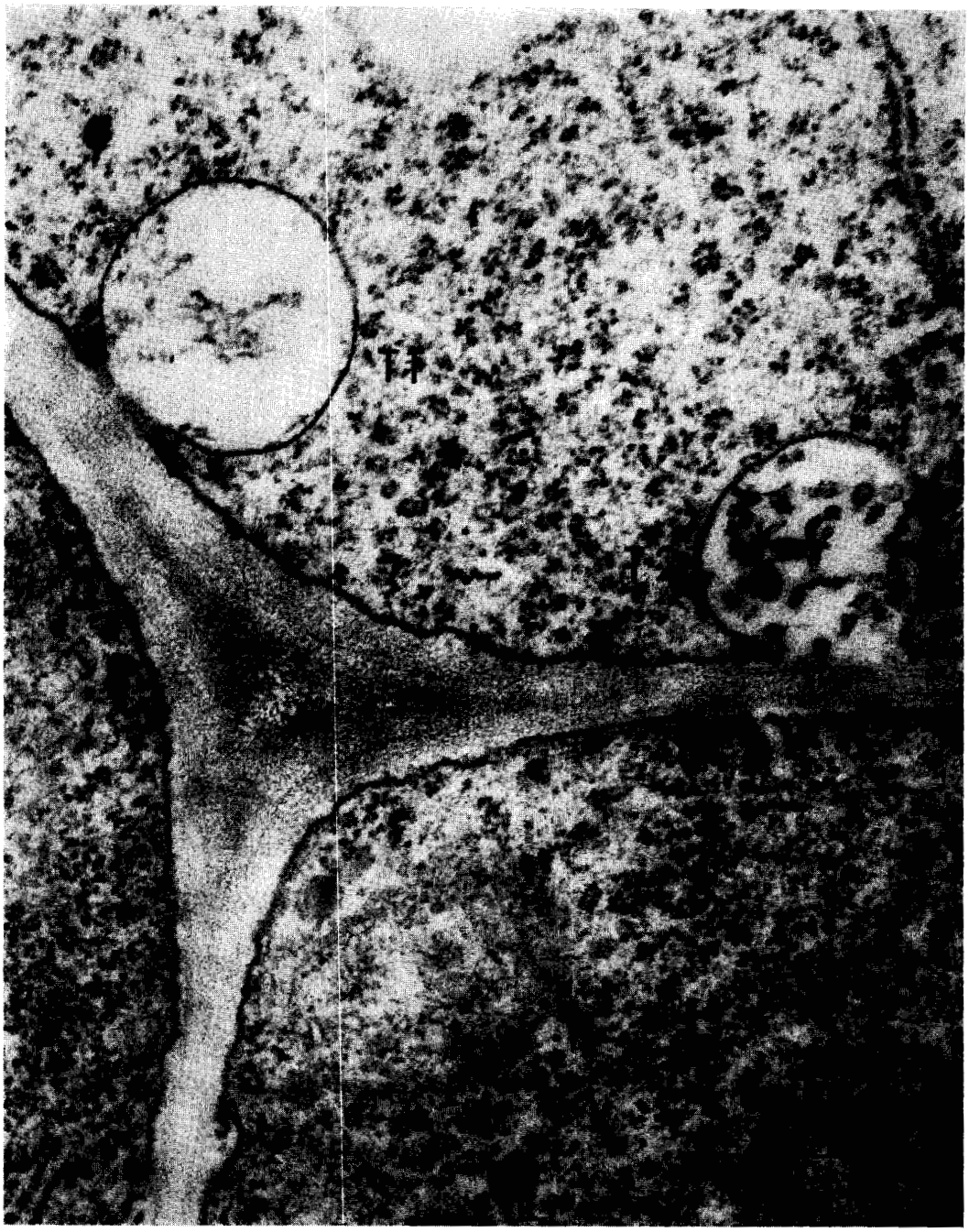

FIG. 11. Types 1 (T1) and 2 (T2) lomasomes (lentil).

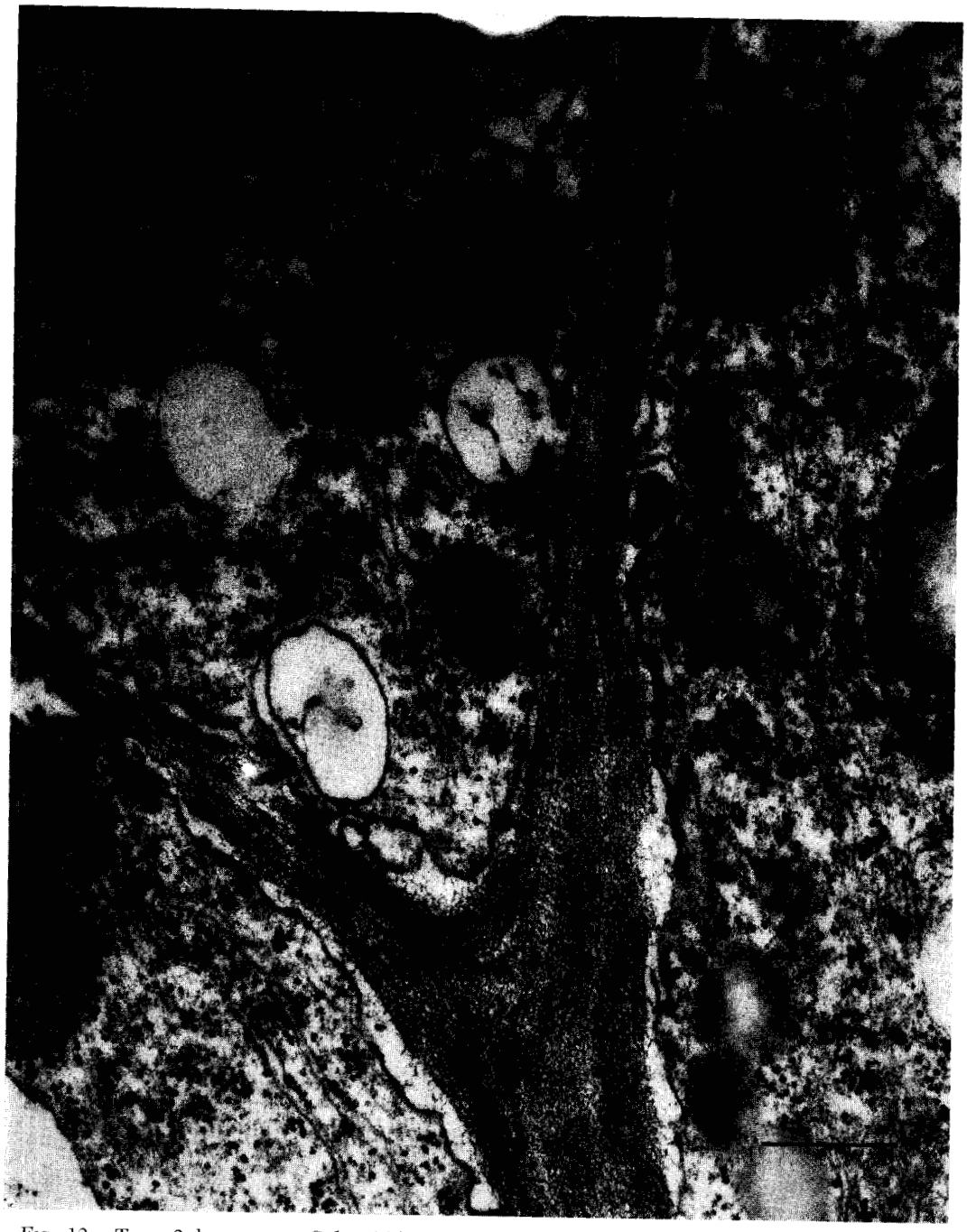

FIG. 12. Type 2 lomasomes, Golgi (G), and condensed myelin-like membranes (M) in *Pinus*.

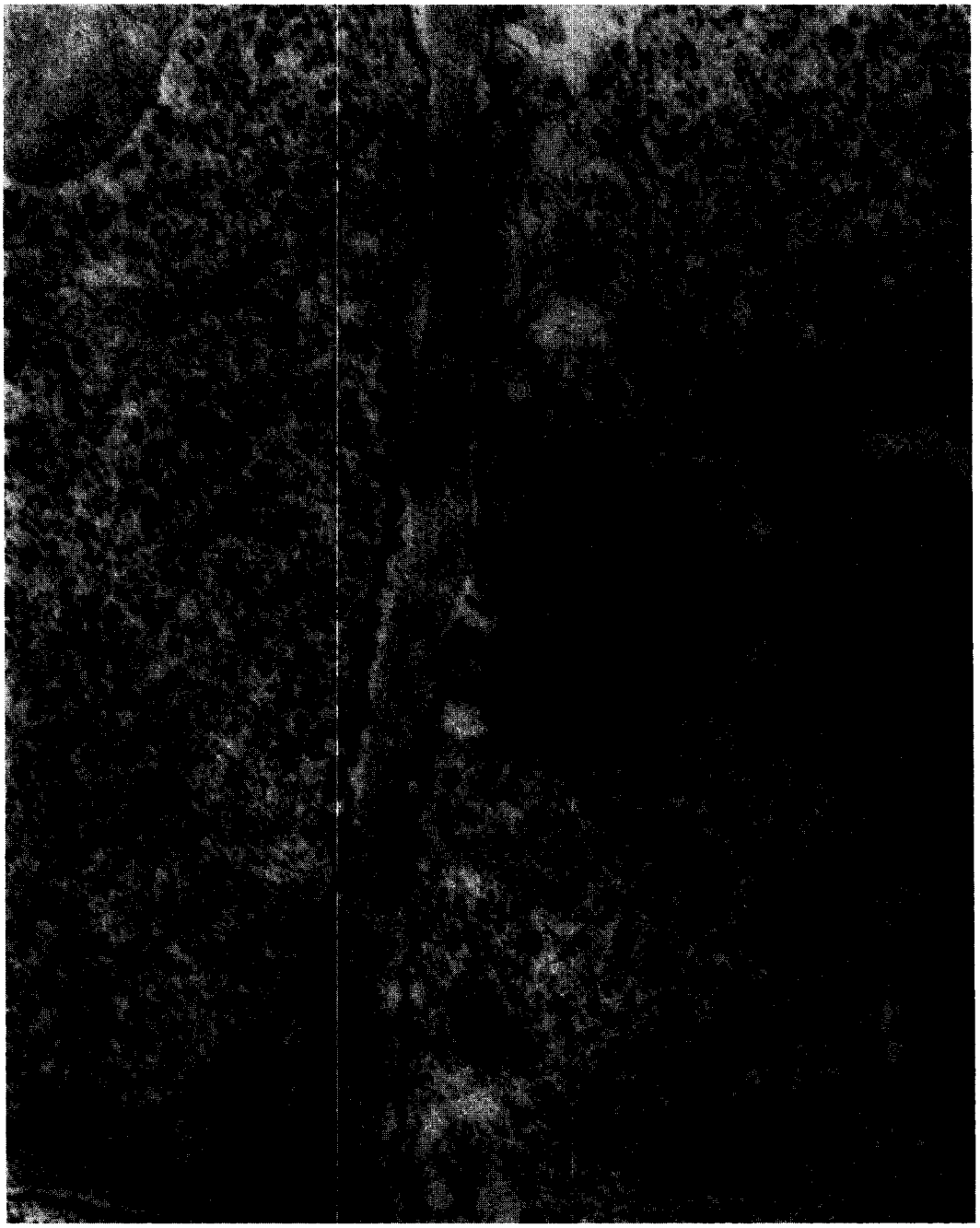

FIG. 13. Loose myelin-like membranes contained in type 2 lomasome (lentil).

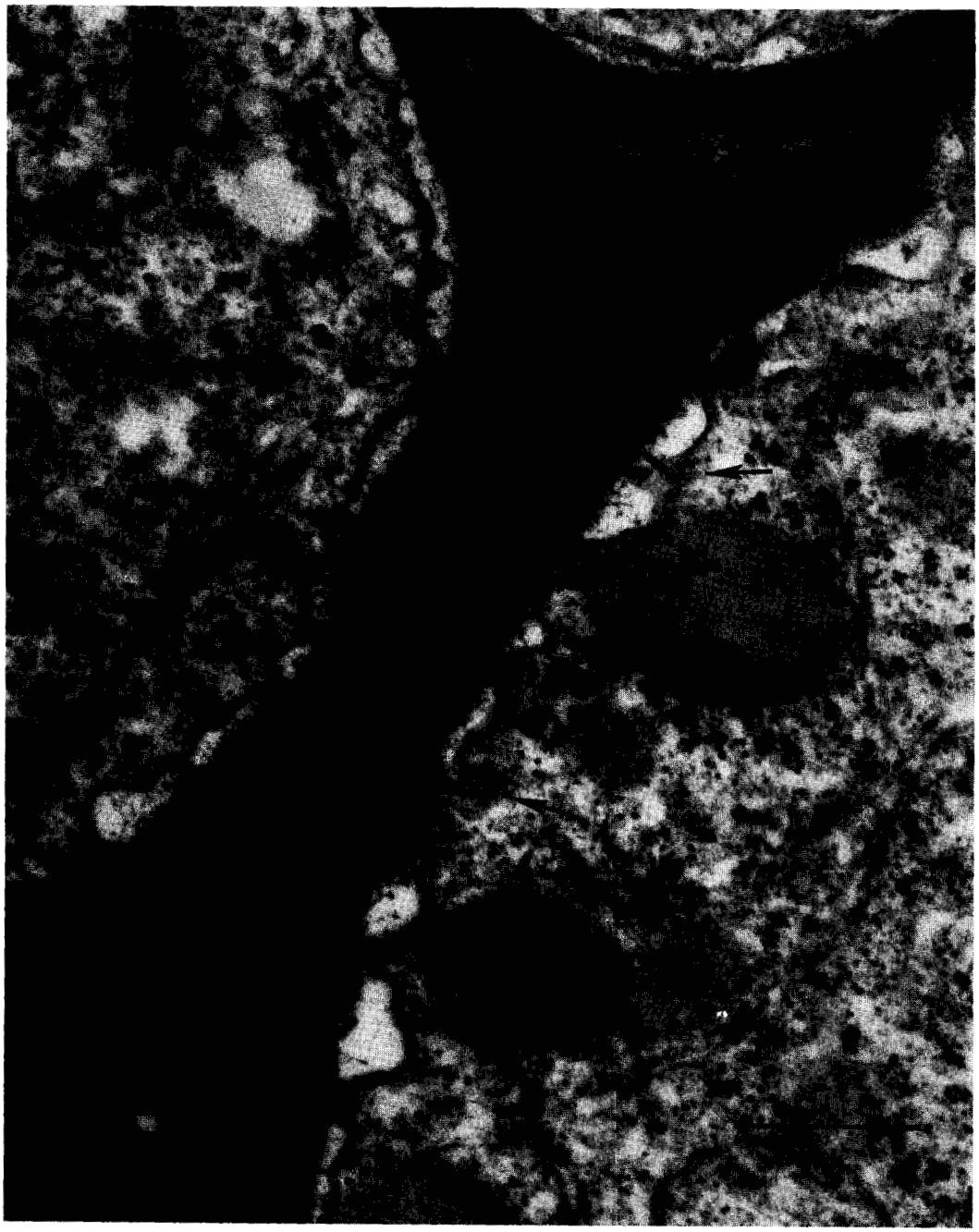

FIG. 14. Plasmalemmasome and microtubules (arrows) in *Pinus*.

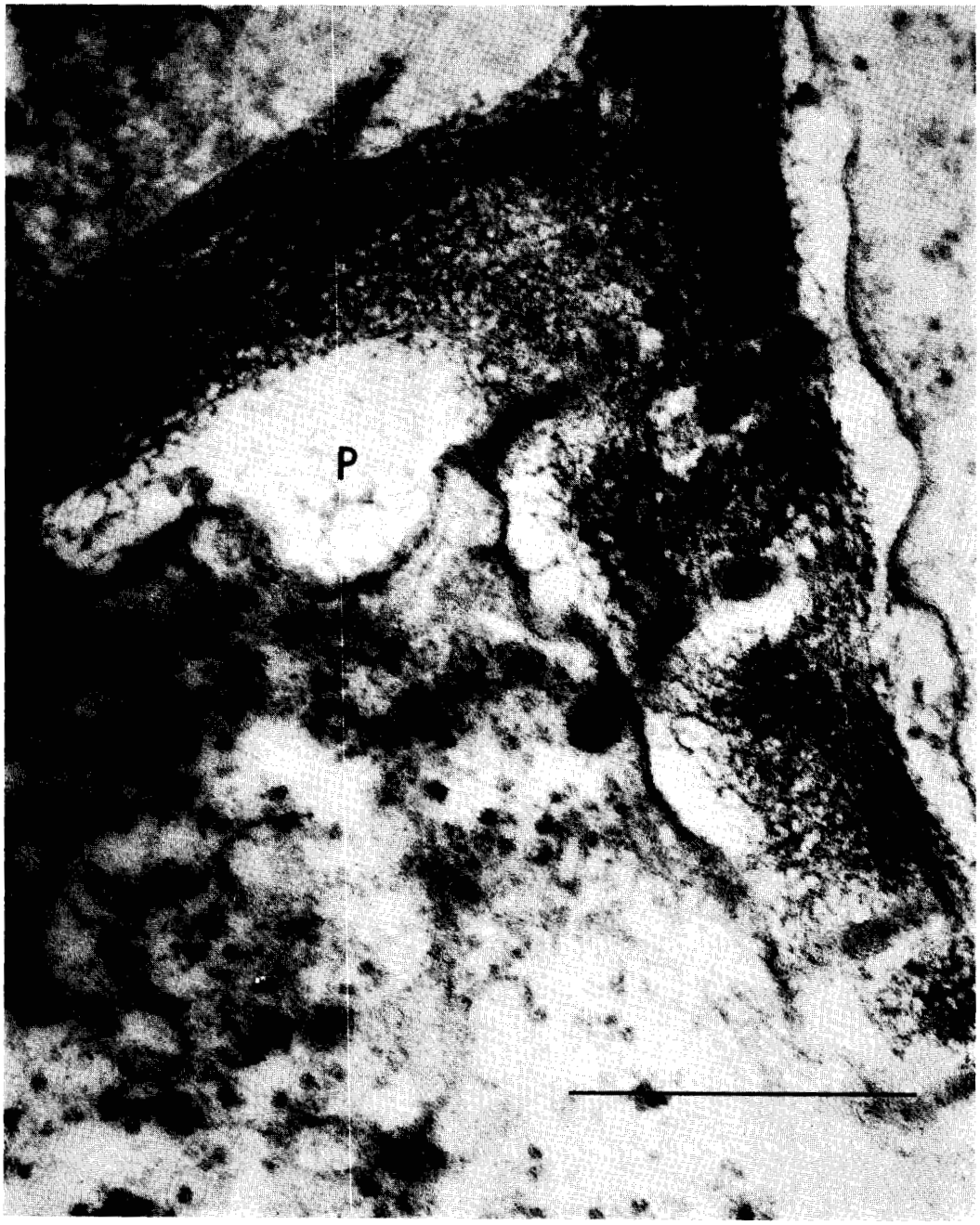

FIG. 15. Plasmalemmasome (P) in *Pinus*.

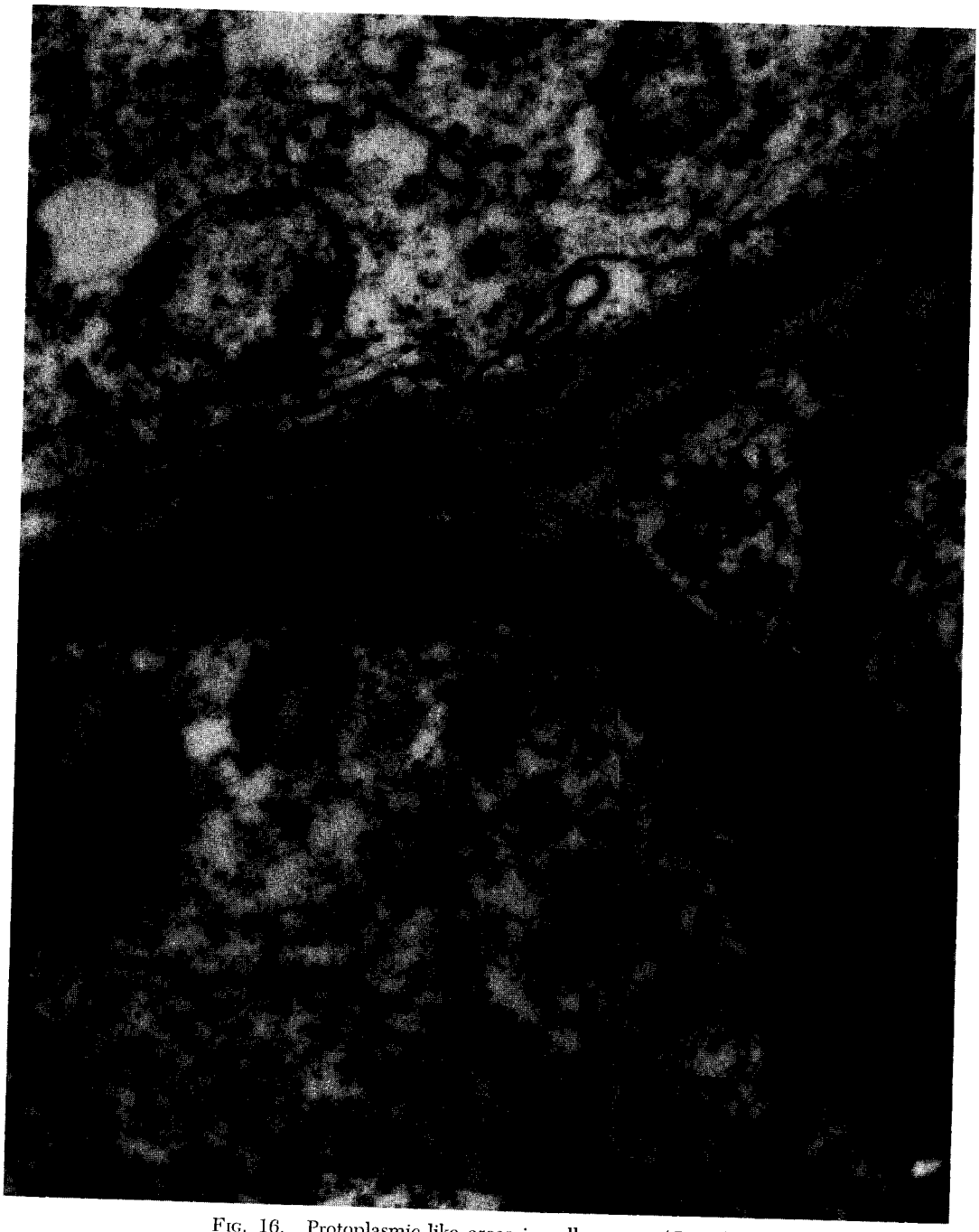

FIG. 16. Protoplasmic-like areas in cell corners (*Pinus*).

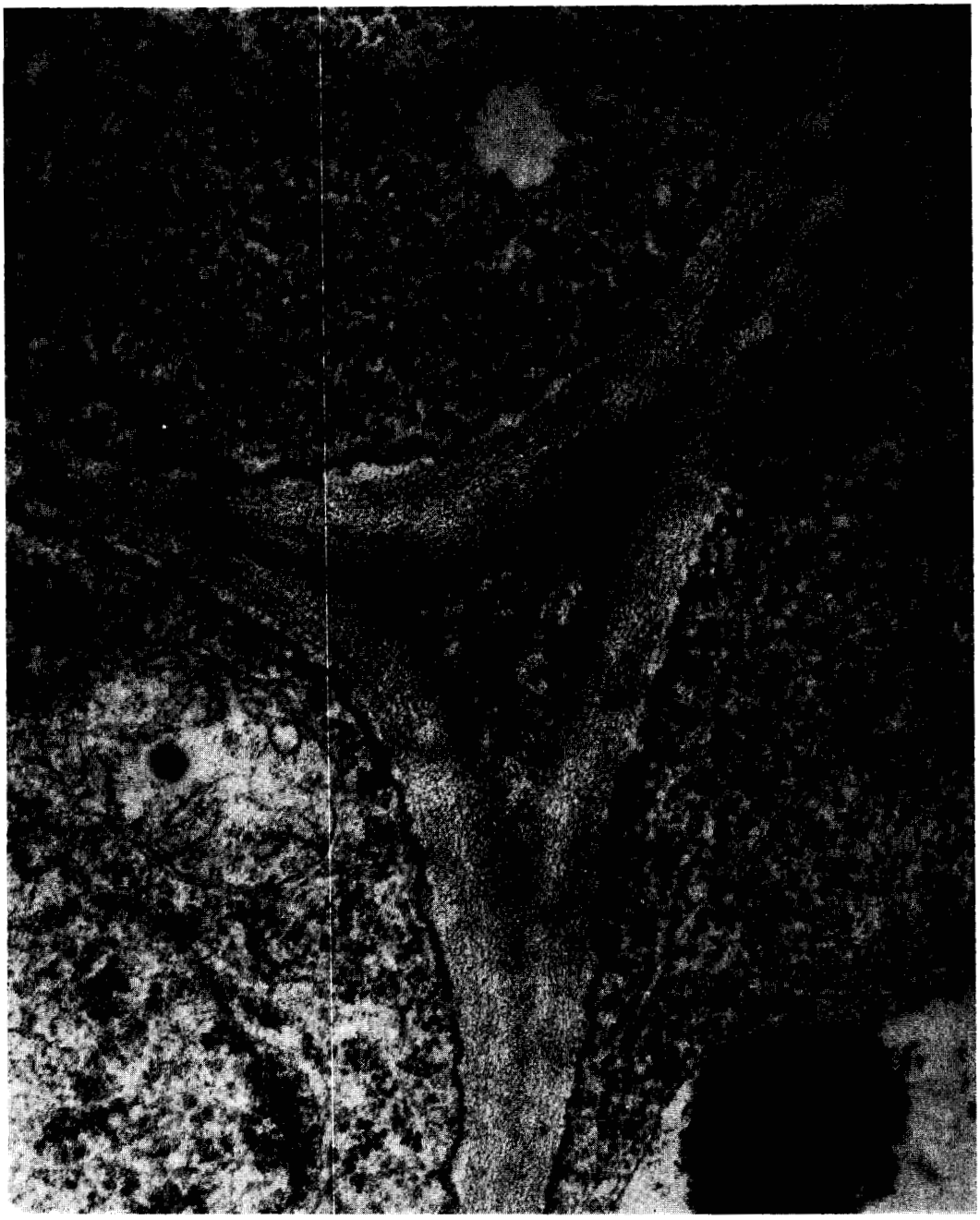

FIG. 17. Protoplasmic-like areas in cell corners (lentil).

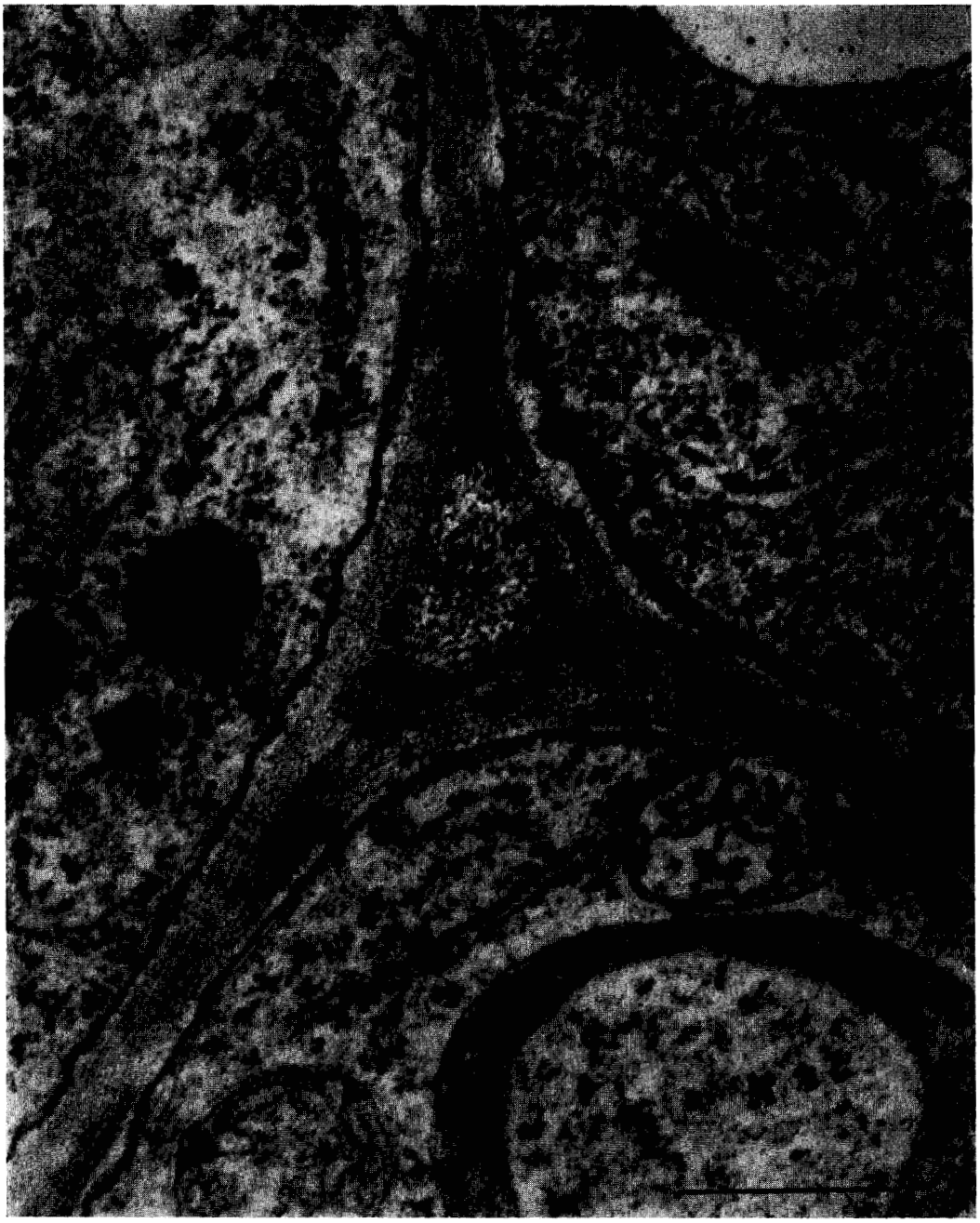

FIG. 18. Protoplasmic-like areas in cell corners, type 2 lomasomes, and Golgi apparatus (lentil).

In our present state of uncertain knowledge, electron microscope studies are of considerable value. Man's sense of vision has been his most critical faculty for understanding his environment and for stimulating creative thinking. Purely chemical and physical studies alone will not suffice to reveal the nature of cell-wall structure and formation. Such a one-sided approach is like trying to determine how a watch works by grinding it up in a mortar and pestle and analyzing the chemical constituents.

One of the interesting dilemmas about the cell wall concerns the type and size of the fibrils composing the wall. The current consensus is that 28–38-Å elementary fibrils exist, but whether these units are aggregated into larger units *in vivo* is open to question. The existence of macrofibrils is easily demonstrated at the light microscope level with freehand sections of plant cell walls mounted in water. These observations have been amplified by Emerton (1958), and if such large units (ca. 0.4μ in diam.) exist, then the reality of 100Å–250Å microfibrils is also plausible. This would be consistent with well-known principles of physics; e.g. the eminent British crystallographer and biologist J. D. Bernal (1959) has stated, "Each type of structure seems to be composed of units of fairly definite sizes which come together to form another unit on the next level." He further indicates that in general the laws of association are qualitatively different at the different levels. The mutual attachments are partly physical and partly geometric and are determined by relative sizes and shapes. However, at the present time it must be concluded that the basic framework unit of the cell wall is the 28–38Å-elementary fibril because this conclusion is the most consistent with our present electron microscope and low angle X-ray data.

The present studies, as well as those described by Hassid (1969), Mühlethaler (1967), and Roberts (1969), lead me to the conclusion that the cell-wall forming system is particulate, is performed by an organelle or organelles, and involves some type (or types) of template mechanism in which small units are added on to the macromolecules.

Experimentally we have illustrated three types of paramural bodies in the cytoplasm of lentil and pine that appear to be associated with cell-wall formation. It is suggested that a number of cell-wall substances are synthesized in the cytoplasm, assembled into subunits, enclosed in a membrane, and transported to the cell wall—cytoplasm interface. Here the cellulose is added by apposition while matrix polysaccharides move out into the wall structure for incorporation *in situ*. Preston's (1964) model of ordered three-dimensional arrays of membrane-bound enzymes synthesizing cellulose fibrils may be relevant here.

The cell corners were shown to contain protoplasmic-like material. No ribosomes were observed in these regions, but recent biochemical work by Jervis and Hallaway (1970) shows that cell walls do contain ribosomes and that these cell-wall ribosomes are identical to those of the cytoplasm. This suggests that the cell-wall corners may indeed play a biosynthetic role in cell-wall formation, especially in lignification. It is well known that lignification is initiated in the cell corners and then proceeds centripetally through the wall. Extensin and possibly other wall proteins could of course also be manufactured in the cell corners. These hypothetical staging areas are possibly interconnected with tracheid cytoplasm through previously reported channels in the tips of tracheids (Frey-Wyssling, Mühlethaler, and Wyckoff 1948; Mühlethaler 1950). Microtubules were sometimes present at the loci of active wall formation and when present were in contact with the plasmalemma and plasmalemmasomes. They did not appear to be associated with the lomasomes. The sporadic observation of these structures does not substantiate claims for a general role in elementary fibrillar orientation or formation, but they might well be important

at a particular phase of wall orientation and/or formation.

The final problem to be considered concerns the way in which the cell-wall constituents are assembled into the complex supramolecular structure of the cell wall. We know next to nothing about this problem, but I would like to pose a possible mechanism that has intuitively developed during the course of these investigations. A simple bacterial cell has been estimated to have 10^{12} binary digits of information. A complex eukaryotic cell of a woody plant (ca. 10–80μ in diam.) contains even more information and information has an energy equivalent (10^{23} binary digits equal 1.0 cal/mole-degree). Therefore it is logical to expect that evolution would select assembly systems that require a minimum of information, i.e. self-assembly systems. Such systems have been proposed for proteins, and it may be instructive to consider them in relation to the cell wall.

The basic premise is that the sequence of enzymatic and physical steps that results in the formation of the cell wall is automatically programmed so that each step of the sequence leads inexorably to the next. Thus the fully formed cell wall would be the inevitable consequence of the cell-wall formation process.

The wall is a unit and the assembly system must be based on this fact, i.e. the supramolecular structure of the wall is a consequence of the mutual arrangement of cellulose, hemicellulose, and the lignopectin complex. Proteins are in essence heteropolymers because they are composed of 20 or so different types of amino acids. Cellulose, on the other hand, is a homopolymer, and its three-dimensional configuration is dependent on the associated hetero- and homopolymers of the wall. The programming system must be capable of not only determining which polymer is to be made but also how many molecules of each. The specific time sequence for each component must also be specified. A given residue in one part of a chain molecule may have an entirely different consequence from the same residue in a different part of the chain, e.g. a given monosaccharide can function in different ways depending on its position in a polysaccharide chain. It can cause bending, branching, and/or twisting. The conformational contribution of a given residue will also be dependent on the properties of the neighboring residues in the same polymer and in associated polymers. Even quite distant residues could affect conformation by causing changes in charge. Chain molecules once formed would associate spontaneously because the free energy of association (ΔG) would be highly negative. The specific three-space shape of an elementary fibril would thus be the automatic product of the energy-minimizing steric configurations induced by the monomeric sequence of: (1) the cellulose chain molecules themselves; (2) the pectoligno-hemicellulose template, and (3) the putative carrier molecules-lipids and sugar nucleotides. The resulting interactions would give rise to a stable arrangement. Unit by unit the elementary fibril would stabilize itself inevitably into the characteristic structure observed in the electron microscope and deduced from X-ray diffraction analysis.

The lipid carrier molecules could serve the function of providing nonpolar patches on the surface of the chains. These areas would twist inward and face each other because the thermodynamic tendency of the surrounding water molecules would not permit such nonpolar surfaces to remain exposed to water. The conformation change would then be locked in place by hydrogen bonding and intermolecular forces. At neutral *p*H, the free phosphates on the nucleotides (GTP, UTP, ATP, CTP) are completely ionized, leaving the molecules heavily charged. The molecules form complexes with Ca^{++} and Mg^{++} and thereby with the pectates of these substances (possibly). The phosphate tail of nucleotides becomes coiled, perhaps creating specific configurations that can fit into the active sites of the wall-forming enzymes. Thus the sugar nucleotides might orient their passenger molecules in a way demanded by

the geometry of the enzyme-substrate relationship.

The above scheme is simply a working hypothesis, and its purpose is to serve as a base for the conception of experiments on this recondite but important and fascinating topic of cell-wall formation.

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