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NOVEL ASPECTS OF ULTRASTRUCTURE IN TWO SPECIES OF CYANOBACTERIA (AGMENELLUM QUADRUPLICATUM, MASTIGOCLADUS LAMINOSUS)

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NOVEL ASPECTS OF ULTRASTRUCTURE IN TWO SPECIES OF CYANOBACTERIA

BY

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B.A., University of New Hampshire, 1979

DISSERTATION

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ABSTRACT

NOVEL ASPECTS OF ULTRASTRUCTURE IN TWO SPECIES OF CYANOBACTERIA

by

SANDRA A. NIERZWICKI-BAUER

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Novel aspects of morphology and ultrastructure were examined in two species of cyanobacteria. A complete, three-dimensional, ultrastructural reconstruction of the unicellular cyanobacterium Agmenellum quadruplicatum was carried out by means of high-voltage electron microscopy of thick sections and computer-aided reconstruction of serial thin sections. The photosynthetic thylakoid system consisted of 3-6 membrane sheets that traversed and were parallel to the long axis of the cell. These sheets were arranged as an anastomosing network of concentric shells. They coalesced and approached the cytoplasmic membrane at three peripheral loci along the entire length of the cell. The central cytoplasm of the cell was completely surrounded by thylakoids; this appeared to be a true form of compartmentalization in a prokaryotic organism. The thylakoid membranes clearly interconnected with the cytoplasmic membrane at several locations within the cell. Some of the various intracellular inclusion bodies were always peripherally located, while others were always centrally located. The detailed three-dimensional arrangement of subcellular features was remarkably consistent from one cell to another.

The morphology and ultrastructure of the branching, filamentous (stigonematalean) cyanobacterium Mastigocladus laminosus were examined with conventional light and electron microscopy. The vegetative morphology and ultrastructure of M. laminosus were similar to those of Fischerella ambigua, the only stigonematalean cyanobacterium examined in detail to date. The ultrastructural characteristics of mature heterocysts in M. laminosus were distinctly different from those of other cyanobacteria; the former lacked certain extra wall layers, lacked cyanophycin-like plugs, contained large numbers of closely packed intracytoplasmic membranes, and contained a previously unreported type of inclusion body. The heterocyst differentiation process also differed from that seen in other cyanobacteria; the earliest events involved internal changes rather than external and bundles of stacked, lamellar membranes were formed. These results showed that heterocyst differentiation and ultrastructure in different genera of cyanobacteria vary more widely than has been thought to be the case. M. laminosus was also shown to undergo aging and morphogenetic processes analagous to some of those known to occur in eukaryotic organisms.

INTRODUCTION

Cyanobacteria (or blue-green algae) are phototrophic microorganisms that carry out an oxygenic form of photosynthesis similar to that of higher plants. The energy and reducing power generated by this process can be used to assimilate atmospheric CO_2 and, in some species, N_2. The cyanobacteria are an exceedingly diverse group of microorganisms in terms of their morphological characteristics, ranging from simple unicellular species to complex branching, filamentous forms (Rippka et al., 1979). They are fundamentally prokaryotic but both the general organization and the detailed ultrastructural features of their cells are considerably more complicated than those of most other bacteria, owing to the presence of an intricate system of photosynthetic membranes and an unusually wide variety of specialized inclusion bodies (Stanier and Cohen-Bazire, 1977). Most cyanobacteria that assimilate atmospheric nitrogen are even more complex in terms of their morphology and ultrastructure because they produce a special type of cell, called a heterocyst, in which the nitrogen fixation process takes place. Typical vegetative cyanobacterial cells undergo an intricate series of ultrastructural and biochemical changes in order to produce these heterocysts, a process that appears to be controlled by a very sophisticated and precise mechanism (Adams

and Carr, 1981). From a morphological and ultrastructural standpoint, then, the cyanobacteria are probably the most complex and diverse group of prokaryotic organisms known.

The morphological and ultrastructural characteristics of cyanobacteria have been studied extensively for many years (for reviews, see Lang, 1968; Stanier and Cohen-Bazire, 1977; Wolk, 1973), yet much remains to be learned about this subject. For example, much is known about the basic ultrastructural features of typical cyanobacterial cells, about how these features differ from one genus to another, and about how these features are affected by environmental or cultural conditions. In contrast, almost nothing is known about how these basic ultrastructural features are arranged three-dimensionally within the cyanobacterial cell. Similarly, much is known about the basic structural properties of cyanobacterial photosynthetic membranes (called thylakoids) but there is no general agreement on how these membranes are arranged to form a complete system or on whether that system is associated with the cytoplasmic membrane (Golecki and Drews, 1982). A considerable amount of information has been published regarding the ultrastructural characteristics of heterocysts, the process by which they are formed, and the mechanism by which that process is controlled (Adams and Carr, 1981), but all of this information has come from studies involving only a few species of Anabaena. Many other cyanobacterial genera produce heterocysts, but those genera have never been investigated in detail. For that matter, even basic ultrastructural studies have been confined almost entirely to the

unicellular cyanobacteria and the nonbranching filamentous forms (see Literature Review). Comparatively little is known about the ultrastructural characteristics of morphologically complex cyanobacteria such as the branching filamentous types, and virtually nothing is known about heterocysts in these organisms.

The present study was undertaken to provide information on some of the above issues by exploring selected aspects of cyanobacterial morphology and ultrastructure that have been neglected almost entirely over the past thirty years. Two independent and largely unrelated investigations were carried out in order to do this. The first of these involved the complete, three-dimensional, ultrastructural reconstruction of a unicellular cyanobacterium Agmenellum quadruplicatum. The purpose of this particular investigation was fivefold: (i) to determine for the first time the complete three-dimensional arrangement of subcellular features within a cyanobacterial cell, (ii) to determine whether specialized inclusion bodies occupy specific locations within the cell, (iii) to determine the overall arrangement of the membranes in the photosynthetic thylakoid system, (iv) to determine whether the photosynthetic membranes are in any way associated with the cytoplasmic membrane, and (v) to determine whether the detailed three-dimensional architecture of such an organism is consistent from one cell to another. The second independent portion of this study involved a detailed morphological and ultrastructural analysis of the branching, filamentous, heterocystous cyanobacterium Mastigocladus laminosus. The purpose of this particular investigation was threefold: (i) to analyze for the first time the detailed ultra-

structural characteristics of a branching, filamentous cyanobacterium so that they could be compared to those of other, more frequently studied cyanobacteria, (ii) to elucidate the detailed ultrastructural characteristics of heterocysts and the heterocyst differentiation process so that they could be compared with those of Anabaena species, and (iii) to examine the mechanism by which heterocyst differentiation is controlled in this morphologically complex organism.

The general purpose of this entire study was to better assess the diversity and complexity of morphology and ultrastructure in the cyanobacteria, and to demonstrate how such a topic can be explored effectively by using unconventional approaches. This report describes the results obtained with such approaches and indicates how those results show that cyanobacteria are considerably more structurally diverse and complicated than previously has been thought to be the case.

I. LITERATURE REVIEW

The Cyanobacteria: Classification and General Ultrastructural Characteristics

Classification

The cyanobacteria are phototrophic prokaryotes that carry out a form of oxygenic photosynthesis similar to that of green plants. They have been classified into several large subgroups, primarily on the basis of their morphological characteristics (Stanier and Cohen-Bazire, 1977). The chroococcacean cyanobacteria are unicellular rods or spheres that reproduce through binary fission or budding. Morphologically, these are the simplest cyanobacteria and the type most similar to typical nonphotosynthetic eubacteria. The pleurocapsalean cyanobacteria are also unicellular, but they excrete an extracellular mucilage that causes their cells to become cemented together into colonial masses or aggregates. The oscillatorian cyanobacteria consist of multicellular, unbranched, untapered filaments that are referred to as trichomes. Heterocysts and akinetes (see below) are not formed in these trichomes. The nostocacean cyanobacteria are morphologically similar to the oscillatorian group, except that certain cells within the filaments may differentiate into heterocysts and/or akinetes. The stigonematalean cyanobacteria differ from all other filamentous types in that they have the ability to divide in more than one plane, thereby forming true branches. All species belonging to this group can form heterocysts, while some may also form akinetes. The scytonemataceae cyanobacteria consist of

multicellular, untapered, heterocystous filaments that produce false branches. Finally, the rivulariacean cyanobacteria have multicellular, tapered, heterocystous filaments that may or may not form false branches.

The Ultrastructure of Vegetative Cells

External Features. Most cyanobacteria produce extracellular slime layers or glycocalyces that are deposited on the outer surface of the cell envelope. The precise distinction between a slime layer and a glycocalyx is not entirely clear because the two terms have not been used consistently in the literature (Stanier and Cohen-Bazire, 1977). Slime layers are generally considered to be thin, unstructured coats that surround the cells and are composed of polysaccharide (Fogg et al., 1973; Stanier et al., 1971). Their actual chemical composition is not known, however (Stanier and Cohen-Bazire, 1977). Glycocalyces clearly contain polysaccharide because they are stained by ruthenium red. They consist of an extracellular network of small, randomly arrayed fibrils. It has been suggested that, among other things, glycocalyces are involved with the attachment of microorganisms to surfaces.

Cell Membrane and Envelope. All cyanobacteria possess a Gram-negative cell envelope (Allen, 1968b; Butler and Allsopp, 1972; Edwards et al., 1968; Jost, 1965) with basic ultrastructural and chemical characteristics that are almost identical to those of cell envelopes in other Gram-negative prokaryotes (Costerton et al., 1974). The cell envelope is composed of three layers (Echlin and Morris,

1965; Ingram et al., 1972b). The innermost layer is the cytoplasmic membrane or the plasmalemma. From a functional standpoint, this may be the most important component of the envelope because it is both an osmotic barrier and a center of metabolic activity. It is estimated to be 7-10 nm thick and has a double-tracked appearance in thin sections (Echlin, 1963; Pankratz and Bowen, 1963). The cytoplasmic membrane is essentially a phospholipid bilayer that includes a large number of structural and functional proteins. The middle layer of the cell envelope surrounds the cytoplasmic membrane and is called the peptidoglycan layer. This electron-dense structure is considerably thicker in cyanobacteria than it is in most other Gram-negative bacteria, varying in width from 1-10 nm (Drews and Weckesser, 1982). Its rigidity is responsible for maintaining the shape of the cell, while its strength helps to prevent cell lysis. The outermost layer of the cell envelope is the LPS layer or the outer membrane. In thin sections, it has a double-tracked appearance and ranges from 7-10 nm in width. Its chemical composition is complex and includes proteins, phospholipids, and lipopolysaccharide oligomers. The outer membrane is usually separated from the peptidoglycan layer by an electron-transparent space (Butler and Allsopp, 1972) or periplasmic region (Costerton et al., 1974).

The cell envelopes of some cyanobacteria include an additional layer that is usually referred to as a "sheath". Sheaths are external to the outer membrane and have a fibrillar appearance in thin sections. They are composed of pectic acids and mucopolysaccharides (Dunn and Wolk, 1970). Sheaths differ from slime layers

or glycocalyces in that they have a more organized substructure and are much denser. They are thought to protect cells from drying and, in some species, may be involved in the process of gliding motility (Castenholz, 1973; Drews and Nultsch, 1962). Sheaths are known to occur in both unicellular and filamentous cyanobacteria (Lamont, 1969; Leak, 1967; Thurston and Ingram, 1971).

Thylakoids and Related Structures. Except for Gloeobacter violaceus (Guglielmi et al., 1981), all cyanobacteria examined to date contain a complex system of membranes called thylakoids. These thylakoids are probably the most important internal feature of the cyanobacterial cell, because they house the photosynthetic apparatus that allows the cell to produce energy from light. Cyanobacterial thylakoids actually consist of two complete lipid-bilayer membranes that are paired together. The two membranes are normally closely apposed to one another, but in some instances, they may split apart to produce structureless vacuole-like areas. This is usually referred to intrathylakoidal vesiculation (Lang and Whitton, 1973). Factors most frequently associated with the formation of intrathylakoidal vesicles include unfavorable growth conditions (Schiewer and Jonas, 1977; Whitton and Peat, 1969) and aging of cultures (Bousefield and Peat, 1976; Kats et al., 1979; Lang and Rae, 1967; Paran et al., 1971; Smith and Peat, 1967).

The primary function of cyanobacterial thylakoids is to serve as the location of the photosynthetic apparatus (Stanier and Cohen-Bazire, 1977). However, biochemical and cytological data have indicated that a number of important cellular components not directly

associated with photosynthetic reactions may also be located specifically on the thylakoid membranes (Bisalputra et al., 1969; Peters, 1975; Staehelin et al., 1978). These components include respiratory and/or hydrogenase enzyme systems that would be capable of sharing certain enzymatic elements with the photosynthetic pathway. Ultrastructural studies have suggested other nonphotosynthetic functions for cyanobacterial thylakoids, including compartmentalization of the central cytoplasm (Lang and Whitton, 1973), control of transport into or out of the central cytoplasm (Peat and Whitton, 1967), and distribution of nuclear material during cell division (Beams and Kessel, 1977).

The outer surfaces of the thylakoid membranes usually are coated with phycobilisomes (Edwards and Gantt, 1971; Gantt and Conti, 1966; Stanier and Cohen-Bazire, 1977). Phycobilisomes are supramolecular pigment aggregates that are composed primarily of phycobiliproteins such as phycocyanin and phycoerythrin. These structures are somewhat larger than ribosomes (about 40 nm in diameter) and serve as the primary light-gathering antannae for photosynthesis (Gantt, 1981). Detailed ultrastructural studies have shown that cyanobacterial phycobilisomes are hemispherical particles. They consist of a morphologically distinct core surrounded by a semicircular array of rods that are made up of stacked disks (Gantt, 1981; Glazer et al., 1979; Lundell et al., 1981).

Cytoplasm. Cyanobacterial cells often appear to consist of two distinct internal regions that are not actually separated by any structural boundary. These are the central cytoplasmic region and

the peripheral thylakoid region. The peripheral thylakoid region contains primarily the photosynthetic thylakoid system and its related structures. The central cytoplasmic region contains most of the nuclear material and ribosomes in the cell. The former appears in thin sections as a network of small fibrils or strands (Fogg et al., 1973), while the latter are electron-dense spherical structures approximately 10-15 nm in diameter. Nuclear strands and ribosomes are often interspersed with each other throughout the entire central cytoplasm. In most cases, neither are found in the peripheral thylakoid region (Edwards et al., 1968; Fogg et al., 1973; Ris and Singh, 1961).

Specialized Inclusion Bodies. Cyanobacterial cells may contain a variety of intracellular inclusion bodies (Shively, 1974), each of which has a specific function and chemical composition. Some of these inclusion bodies are found only in cyanobacteria, while some occur in other types of bacteria as well. The most frequently encountered cyanobacterial inclusion bodies are described below.

Carboxysomes (formerly called "polyhedral bodies") have been observed in all types of cyanobacteria (Lang, 1968; Wolk, 1973), as well as in several other groups of chemoautotrophic bacteria (Shively, 1974). They are always present in vegetative cells (Stewart and Codd, 1975), regardless of the cultural conditions (i.e., whether the cells are grown photoautotrophically, photoheterotrophically, or chemoheterotrophically). In contrast, carboxysomes are never present in heterocysts (Dohler et al., 1981; Leak and

Wilson, 1965). They are of medium electron density, have a polygonal shape, are surrounded by an electron-dense nonunit membrane (Edwards et al., 1968; Jensen, 1980; Stanier and Cohen-Bazire, 1977), and range from 100-500 nm in diameter (Lanaras and Codd, 1981; Van Eykelenburg, 1979). Their exact appearance in thin sections is dependent upon the fixation procedure used to prepare the cells for thin sectioning (Pankratz and Bowen, 1963; Wolk, 1973). Carboxysomes are homogeneously gray after fixation with OsO_4, whereas they appear to be more textured or fibrillate after fixation with $KMnO_4$. Recently, it has been determined that cyanobacterial carboxysomes are storage bodies that contain the enzyme ribulose bisphosphate carboxylase (Lanaras and Codd, 1981). Since this enzyme carries out the first step in the assimilation of atmospheric CO_2 via the Calvin Cycle, it has been suggested that carboxysomes also play a direct role in CO_2 fixation (Beudeker and Kuenen, 1981).

Polyphosphate bodies, which were originally termed metachromatic or volutin granules (Fritsch, 1945), occur frequently in cyanobacterial cells (Jensen, 1968; Lang, 1968). These electron-dense bodies are spherical or oval in shape and range from 100 to 500 nm in diameter (depending upon the cultural conditions and the species). When viewed with the electron microscope, polyphosphate bodies sometimes appear "mottled" or "holey" because they are difficult to preserve and tend to evaporate in the electron beam (Wolk, 1973). Because they are composed of long polymers of phosphate, the formation of polyphosphate bodies is dependent on the availability of exogenous phosphate (Jensen, 1968, 1969; Jensen et al., 1977; Pankratz

and Bowen, 1963). The primary function of these inclusions is believed to be phosphorous storage, although they may also serve to store energy (Shively, 1974).

Cyanophycin granules (formerly called "structured granules") have been observed in all cyanobacterial groups (Lang, 1968), but not in any other types of bacteria (Shively, 1974). They are sometimes present in vegetative cells, but are especially numerous and prominent in akinetes. Cyanophycin granules are not membrane-bound and they have an irregular, spheroidal morphology. Their diameters range from 0.5 to 1.0 μm in vegetative cells and from 0.5 to 1.7 μm in akinetes (Lang, 1968; Lang et al., 1972). Cyanophycin granules have a distinct substructure in thin sections of properly fixed cells (Allen and Weathers, 1980; Khan and Godward, 1978; Lang and Fisher, 1969; Lang et al., 1972). They are well preserved with glutaraldehyde-OsO_4 fixations, but may be dissolved and lost entirely during $KMnO_4$ fixations (Lang and Fisher, 1969; Leak and Wilson, 1965; Lefort, 1960). Simon (1977) has determined that cyanophycin granules consist of 25,000-100,000 dalton polypeptides that contain arginine and aspartic acid in a 1:1 ratio. The granules accumulate if exogenous nitrogen is abundant but protein synthesis is restricted (Simon, 1973). Hence, they store combined nitrogen and serve as a nitrogenous reserve compound.

Lipid bodies (originally called "β-granules"; Pankratz and Bowen, 1963) are small, spherical, electron-dense inclusion bodies that are presumed to contain lipid. This presumption is based solely on their reaction with OsO_4 and has never been confirmed

with chemical analyses. Lipid bodies appear in many types of cyanobacteria, usually in association with the photosynthetic thylakoid membrane system. They have been ignored by most investigators because their function (if any) is unknown (Wolk, 1973).

Ultrastructure of Specialized Cells

Heterocysts. Many of the filamentous cyanobacteria, of which the genus Anabaena is the most widely studied example, can form highly specialized cells called heterocysts when they are grown in the absence of combined nitrogen (Adams and Carr, 1981; Haselkorn, 1978). Under aerobic conditions, these specialized cells function as the exclusive sites for nitrogen fixation (Fay et al., 1968; Haselkorn, 1978). Heterocysts arise when certain vegetative cells within a filament undergo a complex series of biochemical and ultrastructural changes. As a result of these changes, heterocysts are physiologically and ultrastructurally distinct from vegetative cells. The major physiological differences include (i) the absence of a functional photosystem II and associated reactions (Bradley and Carr, 1971; Donze et al., 1972; Tel-Or and Stewart, 1977), (ii) the absence of CO_2-fixing activity, including the possible absence of ribulose bisphosphate carboxylase (Codd et al., 1980; Stewart and Codd, 1975; Winkenbach and Wolk, 1973), and (iii) the presence of an active nitrogenase system (Peterson and Wolk, 1978).

The ultrastructural characteristics of heterocysts have been elucidated with transmission electron microscopy (Kulasooriya et al., 1972; Fay, 1969; Lang and Fay, 1971; Whitton and Peat, 1967;

Wilcox et al., 1973b; Wildon and Mercer, 1963). Most heterocysts have three extra wall layers that are not found in vegetative cells. These include a laminated layer, a homogeneous layer, and a fibrous layer (Fay, 1973; Stanier and Cohen-Bazire, 1977). The function of these extra wall layers is not known, but it has been suggested that they serve to keep oxygen out of the cell in order to protect the nitrogenase system (Haselkorn, 1978). At one or both ends of the heterocysts, the cell body tapers to form a "neck" (Carr, 1979) or "pore channel" (Lang and Fay, 1971). This specialized structure leads to the adjacent vegetative cell in the filament and may somehow be responsible for controlling the transport of substances between the two cells (Haselkorn, 1978). Within the neck, there is usually a granule or "plug" that is presumed to be an accumulation of cyanophycin (Fogg, 1951; Simon, 1971). Although a specific function has not been ascribed to the plug, several researchers have suggested that it may also be involved with controlling the passage of compounds between heterocysts and adjacent vegetative cells. The heterocyst cytoplasm contains a complex system of membranes. This system includes stacks or bundles of membranes (often near the necks) and fragments of thylakoid membranes (Kulasooriya et al., 1972; Lang, 1965; Wilcox et al., 1973b). None of the inclusion bodies usually found in vegetative cells (above) are present in heterocysts (Kulasooriya et al., 1972; Lang, 1965; Lang and Fay, 1971). Ribosomal and nucleic acid contents of heterocysts are much lower than those of vegetative cells (Fogg et al., 1973). Consequently, the heterocyst is truly a specialized

cell in regard to both its structure and its biochemical functions. It is important to realize, however, that all of the detailed studies on heterocyst ultrastructure to date have involved only a few species of Anabaena and Nostoc. It is not yet known whether the ultrastructural characteristics of heterocysts formed by other cyanobacteria are identical or even similar to those seen in these two genera.

Akinetes. Akinetes, which serve as resting cells in cyanobacteria, have been observed frequently in some members of the nostocacean, rivulariacean, and stigonematalean groups (for reviews, see Adams and Carr, 1981; Nichols and Carr, 1978). The ultrastructural characteristics that distinguish these specialized cells from vegetative cells have been described in a number of electron microscopical studies (Clark and Jensen, 1969; Jensen and Clark, 1969; Leak and Wilson, 1965; Miller and Lang, 1968; Wildon and Mercer, 1963). They include (i) formation of a thick, multilayered external envelope and (ii) accumulation of large amounts of cyanophycin. Akinetes are also considerably longer and wider than vegetative cells. They usually start to form during the transition between the logarithmic and stationary growth phases of batch cultures (Stanier and Cohen-Bazire, 1977). The ultrastructural and chemical changes that take place during differentiation bestow upon these cells the ability to survive temporarily under adverse conditions. When conditions improve, akinetes can germinate to form new vegetative cells. The control mechanisms that regulate akinete formation and germination are currently under investigation.

Aspects of Three-Dimensional Ultrastructure in Cyanobacteria

Background

The ultrastructure of cyanobacteria has been studied extensively and much is known about their basic subcellular features, the structural differences among the various genera and groups, and the effects of environmental factors on internal structures (for reviews, see Lang, 1968; Stanier and Cohen-Bazire, 1977; Wolk, 1973). Little is known, however, about how the ultrastructural features of cyanobacteria are arranged three-dimensionally within the cell. Most speculations concerning the three-dimensional architecture of these organisms have come about through extrapolation of the information in randomly cut, individual thin sections. There have been no reports describing three-dimensional reconstructions of entire cyanobacterial cells by serial sectioning or any other approach. Reconstructions of this type are needed, however, because they (i) would eliminate the need for extrapolation, (ii) can resolve a number of uncertain issues in regard to cyanobacterial ultrastructure, and (iii) may lead to a better understanding of structure-function relationships in the cyanobacterial cell.

Specific Considerations

Fundamental Arrangement of the Thylakoid System. The fundamental way in which the entire system of thylakoids is arranged within the cyanobacterial cell remains unclear, despite numerous studies that have attempted to ascertain this information in different cyanobacter-

ial groups. Some investigators have reported that thylakoids occur as groups of independent disks or flattened sacs (Eschlin, 1964b; Edwards et al., 1968; Holt and Edwards, 1972; Lang and Whitton, 1973), while others have described them as concentric spheres, cylinders, or unspecified structures (Allen, 1968a; Baulina et al., 1981; Chang, 1980; Rippka et al., 1974; Stanier and Cohen-Bazire, 1977). More recently, the thylakoid system was described by Golecki and Drews (1982) as "an anastomosing network of concentric shells." It is probable that thylakoid membrane systems differ to some extent in different cyanobacterial groups, but it seems unlikely that the fundamental arrangement of these systems would vary as much as the above reports might lead one to believe. Much of the problem may stem from the fact that all of the above conclusions were based on randomly cut sections instead of on complete three-dimensional analyses of the kind that would be required to obtain definitive information on this question.

Relationship Between Thylakoids and the Cytoplasmic Membrane. One structurally and functionally important aspect of cyanobacterial ultrastructure is the nature of the relationship (if any) between the thylakoid membrane system and the cytoplasmic membrane. Specifically, it is important to know whether the thylakoids are in any way connected to or continuous with the cytoplasmic membrane. Stanier and Cohen-Bazire (1977) declared that the thylakoids and the cytoplasmic membrane are topologically distinct, despite the fact that many authors have described the occurrence of thylakoid-cytoplasmic membrane interconnections in a variety of unicellular (Allen, 1968a; Edwards et al.,

1968; Golecki, 1979) and filamentous (Ginsburg and Lazaroff, 1973; Lang and Rae, 1967; Neumann et al., 1970; Smith and Peat, 1967) cyanobacteria. Admittedly, the literature is by no means consistent on this topic; some researchers have reported interconnections in specific strains while others did not find them. Such discrepancies may result from the fact that the interconnections are just very difficult to detect and resolve with conventional electron microscopical techniques. New technical approaches will be required to obtain more definitive information on the existence of thylakoid-cytoplasmic membrane interconnections.

Assuming that thylakoid-cytoplasmic membrane interconnections do exist in cyanobacteria, many questions regarding these structures remain to be answered. It is not known whether they are permanent or temporary structures. It is not known how numerous they may be within a single cell. Furthermore, it has not been determined whether they are true invaginations of the cytoplasmic membrane (Allen, 1968b; Cox et al., 1981; Jost, 1965; Kats et al., 1979; Pankratz and Bowen, 1963; Smith and Peat, 1967) or simply a physical attachment of the thylakoids to the cytoplasmic membrane (Golecki, 1979; Lang and Rae, 1967). The functional significance (if any) of thylakoid-cytoplasmic membrane interconnections is also unknown, although several authors have suggested that they are centers for thylakoid synthesis (Allen, 1968b; Cox et al., 1981; Ginsberg and Lazaroff, 1973; Jacobs and Ahmadjian, 1973; Pankratz and Bowen, 1963; Smith and Peat, 1967). Golecki and Drews (1982) have quite rightly pointed out, however, that there has never been any direct evidence to support such a suggestion.

Intracellular Positions of Specialized Inclusion Bodies. Relatively little is known about the three-dimensional positions of specialized inclusion bodies (above) in cyanobacterial cells. Polyphosphate bodies and carboxysomes have been reported to be confined to the central cytoplasmic region of the cell in a number of unicellular cyanobacteria (Allen, 1968; Edwards et al., 1968; Gantt and Conti, 1969; Lewin et al., 1981). Jensen (1968) noted that these two inclusions were usually in direct contact with each other, while Peat and Whitton (1968) claimed that carboxysomes were always in direct contact with the thylakoid membranes. There have been no reports giving complete information on the positions of inclusion bodies throughout entire cells, nor is anything known about how consistent these positions may be from one cell to another.

Techniques for Determination of Three-Dimensional Ultrastructure

Electron Microscopical Techniques

Serial Sectioning. The most frequently used technique for obtaining information on the three-dimensional architecture of cells has been serial sectioning (Ware, 1975). Serial sections are consecutive slices or pieces through resin-embedded specimens. Serial sectioning has been used extensively in studies involving the three-dimensional architecture of eukaryotic cells (Frokjaer-Jensen, 1980; LoPresti et al., 1973; Pellegrini, 1980; White and Rock, 1980), but only occasionally in studies involving prokaryotic cells (Echlin, 1964a; Waters and Hunt, 1980). These studies have shown that randomly cut individual thin sections can give inaccurate or misleading

impressions if they are not interpreted in the context of an organism's overall three-dimensional architecture (Peat and Whitton, 1968; Ware, 1975). Serial sectioning in studies involving cyanobacterial cells has been limited to covering only small segments of cells (Echlin, 1964a; Gromov and Mamkaeva, 1976; Peat and Whitton, 1968). These limited series have provided some information on the internal structure of cyanobacterial cells, but complete series through entire cells will be needed before their three-dimensional architecture can be understood fully.

High-Voltage Electron Microscopy. Another approach that has proven useful for obtaining three-dimensional information is high-voltage electron microscopy (HVEM) of thick specimens (either thick sections or whole cells). An increased amount of three-dimensional information can be obtained directly with HVEM because of the increased thickness of the specimen. HVEM of thick samples has been used successfully with a variety of eukaryotic cell and tissue systems (Crang and Penchak, 1978; Glauert, 1974; Hawes, 1981; Kilarski et al., 1976; King et al., 1980; Paulin, 1975), but has not been applied extensively to prokaryotic specimens (King et al., 1980; Weibull, 1974).

The Computer-Aided Techniques

A number of computer-aided techniques for three-dimensional reconstruction of cells or tissues have been developed in recent years. These approaches generally involve reconstruction of tracings made from serial thin or thick sections. Several computer programs have been designed for this approach and have been used effect-

ively with a variety of eukaryotic cell systems (Crang and Pechak, 1978; Levinthal et al., 1974; LoPresti et al., 1973; Macagno et al., 1979; Moens and Moens, 1981; Sobel et al., 1980; Stevens et al., 1980; Veen and Peachey, 1977; Ware and LoPresti, 1975). The majority of these programs were designed to illustrate complex linear or branching structures such as neurons or chromosomes. To date, no one has attempted to apply computer-aided techniques to the reconstruction of prokaryotic cells.

Aspects of Heterocyst Differentiation in Cyanobacteria

Ultrastructure of the Differentiation Process

As stated earlier, heterocysts arise in cyanobacteria when vegetative cells undergo a complex series of ultrastructural and biochemical changes. The detailed ultrastructure of the differentiation process has been studied only in a few species of Anabaena (Kulasooriya et al., 1972; Lang, 1965; Wilcox et al., 1973b). These authors described the ultrastructural characteristics of proheterocysts (cells in transition between the vegetative and mature heterocyst forms) and indicated how those characteristics changed during differentiation. Such information can be useful toward developing a better understanding of structure-function relationships in mature heterocysts. Although many cyanobacteria aside from Anabaena are capable of producing heterocysts, the ultrastructure of the differentiation process has not yet been examined in any of them. Therefore, it is not known whether the sequence of changes that takes place in Anabaena and the conclusions that can be drawn therefrom

have any relevance to heterocyst differentiation in other cyanobacterial groups.

Heterocyst Spacing Patterns

Like that concerning the ultrastructure of the differentiation process, almost all of the detailed information regarding the control of this process has come from studies involving a few species of the genus Anabaena (Adams and Carr, 1981; Kulasooriya et al., 1972; Lang, 1965; Lang and Fay, 1971; Wilcox et al., 1973a, b). In Anabaena, it is thought that heterocyst differentiation is very precisely controlled. Heterocysts develop only at specific positions within the filament, thereby creating a consistent "spacing pattern". As a filament grows, new heterocysts develop from vegetative cells lying approximately in the middle of the interval between adjacent, pre-existing heterocysts. Distributions for the number of vegetative cells between heterocysts and/or proheterocysts in young A. cylindrica and A. catenula filaments have been determined by Wilcox et al. (1973a). A. cylindrica had a mean of 9.3 ± 2.8 vegetative cell between heterocysts and/or proheterocysts, and A. catenula had a mean of 10.1 ± 2.5. In both organisms, it was uncommon to have less than three vegetative cells between heterocysts, and the frequency of adjacent heterocysts was less than 0.1% (Wilcox et al., 1973a, b). This very precise spacing pattern is thought to be controlled, in part, by setting up inhibitory zones around each heterocyst or developing proheterocyst. The inhibitory zone is apparently created by an inhibitory substance (currently unidentified) that diffuses for some distance down the filament of cells.

Only the vegetative cells that lie outside of such inhibitory zones can differentiate into heterocysts. While there is now little doubt that such a mechanism functions to control heterocyst differentiation in Anabaena, there have been no attempts to determine whether it functions in other heterocystous genera as well. A first step toward making such a determination would be to examine heterocyst spacing patterns in other genera, but even that kind of preliminary investigation has yet to be described in the literature. As a consequence, nothing of real substance is known about the control of heterocyst differentiation in most of the cyanobacteria that produce these important specialized cells.

Morphology and Ultrastructure of Stigonematalean Cyanobacteria

The branching, filamentous (stigonematalean) cyanobacteria have been investigated primarily at the light microscopical level (Lee, 1972; Marcenko, 1961; Martin and Wyatt, 1974; Rippka et al., 1979; Schwabe, 1960; Spearing, 1937). From these studies, it has become apparent that stigonematalean cyanobacteria have rather complex morphological characteristics. Young filaments usually consist of cylindrical, actively dividing cells with fairly uniform morphological characteristics. As these young filaments age, their cells stop dividing but continue to grow by increasing in width. This process leads to formation of "old" filaments that contain relatively wide, spherical cells with exceedingly variable morphological characteristics. Some of the cells in old filaments undergo division parallel to the long axis of the filament. Subsequent division of either

daughter cell then gives rise to lateral branches off the main filaments. The branches sometimes elongate and taper to form new young filaments once again (Thurston and Ingram, 1971). Mature cultures consist of dense mats of intertwined young and old filaments.

Although the basic morphological characteristics of stigonematalean cyanobacteria have been described thoroughly, relatively little is known about the morphology of heterocyst differentiation in these organisms. Rippka et al. (1979) described several different types of heterocysts that developed in branching filamentous cyanobacteria, and they briefly listed some of the positions at which these heterocysts formed. They did not carry out a detailed analysis of heterocyst spacing patterns, however, and that information is still needed to study the mechanism by which differentiation in stigonematalean cyanobacteria is controlled.

Perhaps because of their complex morphological characteristics, only a few investigators have attempted to examine the ultrastructural characteristics of stigonematalean cyanobacteria (Butler and Allsopp, 1972; Ris and Singh, 1961; Thurston and Ingram, 1971; Wildon and Mercer, 1963). None of these investigations could be considered very comprehensive, and the most thorough of them (Thurston and Ingram, 1971) was limited to nitrate-grown cultures that did not form heterocysts. Thus, the detailed ultrastructural characteristics of heterocysts or of the heterocyst differentiation process in stigonematalean cyanobacteria have never been described.

Studies on Agmenellum quadruplicatum

Agmenellum quadruplicatum strain PR-6 is a unicellular cyanobacterium that is unable to fix atmospheric nitrogen. It is a marine (halotolerant) microorganism that was originally isolated and described by Van Baalen (1962).

The earliest ultrastructural studies involving A. quadruplicatum were related to cell division (Brown and Van Baalen, 1970; Ingram and Thurston, 1970; Ingram et al., 1972a) and cell separation (Brown and Van Baalen, 1970; Ingram and Aldrich, 1974). These studies examined cell division and separation in wild-type strains PR-6 and BG-1, and in various filamentous mutants of these two strains. Cell division in the various mutants differed from that in the wild-type strains either in the sequence of events that took place (Brown and Van Baalen, 1970) or ultrastructurally (Ingram and Thurston, 1970; Ingram et al., 1975). A proposed model for cell division in wild-type strain PR-6 included the following sequence of events: (i) nuclear separation, (ii) division of the thylakoid membranes, (iii) invagination of the cytoplasmic membrane, and (iv) simultaneous invagination of the peptidoglycan and outer membrane layers of the cell envelope.

A. quadruplicatum strain PR-6 recently was shown to possess an extracellular glycocalyx during all phases of growth (Balkwill and Stevens, 1980b). The glycocalyx was observed in both freeze-fractured and thin-sectioned cell preparations. It stained with ruthenium red, thereby indicating that it contained polysaccharides. Structurally, the glycocalyx consisted of a network of small fibrils that

were 8.7 - 8.8 nm in diameter. Possible functions suggested for the glycocalyx included attachment of cells to surfaces and flocculation of cells during stress.

All of the most recent ultrastructural investigations involving A. quadruplicatum strain PR-6 have dealt with the effects of nutrient limitation or starvation on intracellular ultrastructure. The effects of phosphorous (Stevens et al., 1981b), nitrogen (Stevens et al., 1981a), and iron (Hardie et al., 1983a, b) starvation have been examined to date. These studies showed that A. quadruplicatum underwent a precise sequence of intracellular, ultrastructural changes in response to nutrient starvation. These changes, which were different with each different limiting nutrient, were directed toward building up reserve materials at the expense of cell structures that had become superfluous as a result of starvation. It was assumed that this strategy helped A. quadruplicatum to survive relatively long periods of starvation and to recover from starvation quickly when nutrients are made available once again (Hardie et al., 1983a, b; Stevens et al., 1981a).

The studies cited above have provided extensive information on the basic ultrastructural features of A. quadruplicatum. However, they have not provided sufficient data to enable one to understand how these cell features are arranged with respect to each other in three-dimensional space. A full understanding of the three-dimensional architecture of this organism, then, awaits completion of a thorough three-dimensional reconstruction of entire cells.

Studies on Mastigocladus laminosus

Mastigocladus laminosus is a thermophilic, branching, filamentous cyanobacterium of the "stigonematalean" group. M. laminosus has been investigated from an ecological standpoint (Castenholz, 1976; 1977; Fagerberg and Arnott, 1979) and because of its potential for biophotolysis (Miura et al., 1980; Miyamoto et al., 1979a, b). The morphology of this organism at the light microscopical level has been described to some extent (Marcenko, 1961; Rippka et al., 1979; Schwabe, 1960), but relatively little is known about its ultrastructural characteristics (Marcenko, 1961; Ris and Singh, 1961).

Rippka et al. (1979) have suggested a new classification scheme for cyanobacteria in which the genera Fischerella and Mastigocladus were combined into the single genus, Fischerella Gomont 1895. This suggested classification scheme was based primarily on morphological similarities visible at the light microscopical level, including: (i) the ability to form true branches and (ii) a similar mechanism of cell division within the filaments. However, both the morphology of heterocyst differentiation and the detailed ultrastructural characteristics of Mastigocladus and Fischerella should be compared before any final decision regarding combination of the two genera is made.

The ecological significance of M. laminosus centers around the fact that it is the only thermophilic cyanobacterium known to fix atmospheric nitrogen, thereby converting the nitrogen into a form that can be utilized by plants. As in most other nitrogen-fixing cyanobacteria, this process takes place in heterocysts (see above);

yet, little is known about the heterocysts in this important organism. To date, there have been no reports describing the ultrastructural characteristics of heterocysts in M. laminosus, the detailed ultrastructural sequence of events that takes place during heterocyst differentiation, or the mechanism by which the differentiation process is controlled.

II. MATERIALS AND METHODS

Studies with Agmenellum quadruplicatum

Organism, Medium, and Growth Conditions

Agmenellum quadruplicatum strain PR-6 is a unicellular, halotolerant cyanobacterium that was isolated and described by Van Baalen (1962). It was grown in medium A of Provasoli et al. (1961), as modified by Van Baalen (1962) and Stevens et al. (1973). Broth cultures were housed in 38 x 300-mm glass test tubes which were incubated at 39°C in a glass water bath. Illumination for photosynthesis was provided by four (two on each side of the bath) F24T12 CW/HO fluorescent lamps producing an intensity of 380 μmol photons m^2 sec^{-1} incident on each growth tube. Continuous agitation and carbon were provided by bubbling of sterile 3% (vol/vol) CO_2 in air through the cultures.

Exponentially growing cells were used in all of the investigations described below. Growth was measured turbidimetrically by determining the absorbance of the cultures at 580 nm with a Bausch and Lomb Spectronic 20 colorimeter. A cell suspension with an optical density of 1.0 contained $4.7 \times 10^7 \pm 8 \times 10^5$ cells/ml, as determined by viable counts and a dry weight of 0.15 mg/ml.

Electron Microscopy

Preparation of Cells for Thin or Thick Sectioning. Three fixation procedures were used to prepare A. quadruplicatum cells for sub-

sequent thin or thick sectioning (see below). These were: (i) the potassium permanganate procedure, (ii) the ruthenium red procedure, and (iii) the glutaraldehyde-osmium tetroxide procedure. For the potassium permanganate fixation, cells were concentrated from their growth medium by centrifugation, fixed 1 h at 0-4°C in 1% $KMnO_4$ (aqueous) and washed twice by centrifugation in 0.1 M sodium cadodylate buffer (pH 7.0). The resulting cell pellet was resuspended in a small amount of molten, 2% Noble Agar (Difco) at 50°C. This cell-agar suspension was transferred to a glass microscope slide, allowed to solidify, and cut into small blocks (less than 1 mm on a side) with a razor blade. The samples were then prestained 2 h at room temperature in 5% uranyl acetate (aqueous). For the ruthenium red fixation, which was a variation of that described by Cagle et al. (1972), cells were concentrated from their growth medium by centrifugation and prestained 1 h at room temperature in 0.15% ruthenium red (in 0.1 M sodium cacodylate buffer, pH 7.0). The cells were then prefixed 1 h at room temperature in a mixture of 3.6% glutaraldehyde and 0.15% ruthenium red (in cacodylate buffer), washed twice in cacodylate buffer, and postfixed 1 h at 4°C in a mixture of 1% OsO_4 and 0.15% ruthenium red (in cacodylate buffer). After two further washings in cacodylate buffer, the fixed cells were embedded in agar and cut into small blocks as described above. For the glutaraldehyde-OsO_4 fixation, cells were prefixed by adding 12.5% glutaraldehyde (in cacodylate buffer) directly to the culture medium to bring the final concentration of glutaraldehyde to approximately 3%. Prefixation continued for 2 h at room temperature, after which

the cells were concentrated by centrifugation, washed with cacodylate buffer, and resuspended in two drops of tryptone-salt solution (1% tryptone, 0.5% NaCl). They were embedded in agar and cut into small blocks as described above. These blocks were postfixed overnight at room temperature in 1% OsO_4 (in cacodylate buffer). The postfixed samples were washed twice with cacodylate buffer and prestained 3 h at room temperature in 0.5% uranyl acetate (in Kellenberger buffer; Kellenberger et al., 1958). Samples from all fixation procedures were dehydrated through a graded ethanol series. Those to be used for conventional thin sectioning were embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969), while those to be used for thick sectioning were embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, PA). Both resins were cured for 15-20 h at 65-70°C.

All three fixation procedures gave equivalent results, thereby insuring that artifactual structures were not observed. The potassium permanganate technique was unsuitable for three-dimensional reconstructions, however, because (i) overall cell preservation was relatively poor and (ii) the primary fixation product (MnO_2) was apparently dissolved by acetic acid as the sections were aligned on specimen grids (see below). Micrographs from the glutaraldehyde-OsO_4 fixation were chosen for the illustrations in this report.

Preparation of Serial Thin or Thick Sections. Serial thin sections were cut with a diamond knife (M.J.O. Diatome Co., Fort Washington, PA), and serial thick (0.25-, 0.5-, and 1.0-μm) sections were cut with glass knives. An LKB Ultratome III ultramicrotome was used in both cases. The serial sections (both thin and thick) were retrieved

on formvarcoated, carbon-stabilized, multi-slot, copper specimen grids. The technique used to properly align the sections on these grids was similar to that described by Anderson and Brenner (1971). Briefly, ribbons of serial sections floating on the surface of the water in the microtome knife boat were transferred with a platinum wire loop to the surface of liquid gelatin (Difco) that was maintained at 60°C in a petri dish. The gelatin was allowed to solidify at 4°C and specimen grids were then placed carefully on top of each ribbon of sections. Each grid was placed so that the ribbon of sections was aligned parallel to the long axis of the appropriate grid slot, thereby leaving each section in the series visible through the slot. It was necessary to choose a slot that was slightly narrower than the width of the sections in the ribbon, in order to support the sections firmly enough to prevent drift and other problems with stability during subsequent electron microscopical examination of the specimens. Following proper placement of the grids, the gelatin was liquefied at 60°C. Grids were then picked up, drained, and floated section side down for 30 min on a 2% solution of acetic acid, 3-5 min on Tris buffer (pH 7.1), and 30 min on distilled water; all treatments being at 60°C. Successfully mounted series of thin sections were poststained for 15 min in 0.5% uranyl acetate (in 50% methanol) and for 2 min in 0.4% lead citrate (Reynolds, 1963). Poststaining times for 0.25-, 0.5-, and 1.0-μm thick sections were 60, 75, and 90 min, respectively, in uranyl acetate; 30, 40, and 50 min, respectively, in lead citrate. All sections (thin and thick) were stabilized by coating them with carbon after poststaining.

Conventional Transmission Electron Microscopy. The serial thin sections were examined and photographed at an accelerating potential of 80 kV with a JEOL JEM-100S transmission electron microscope. Initially, serial sections through 20 groups or clusters of cells were photographed at low magnification (2,000 X). Enlargements of these micrographs were studied, and individual cells were selected for detailed reconstruction. Over 50 fully serially sectioned cells were chosen, including representative examples of cells sectioned in different directions (i.e., some had been cross-sectioned serially, some had been sectioned longitudinally, etc.). These differing sectioning directions were selected as an internal control, since reconstructed cells would be expected to appear similar regardless of how they originally had been sectioned. Serial sections through each of the selected cells were rephotographed at magnifications sufficient to resolve the important cell features (10,000-20,000 X). These series of micrographs (50-75 sections for each cell) were then used in the computer-aided reconstruction procedures described below.

High-Voltage Electron Microscopy (HVEM). Serial thick sections (0.25-, 0.5-, and 1.0-μm) were examined and photographed at an accelerating potential of 1.0 MV with an AEI MkII high-voltage transmission electron microscope. The three section thicknesses were compared and evaluated in regard to their suitability for use in three-dimensional reconstructions. It was determined that the 0.25-μm sections were best suited for this purpose. Resolution in the thicker sections was very good, but excessive overlapping of dense cell features made it difficult to see all of the structural details clearly.

Serial thick (0.25-um) sections cut in various directions through approximately 50 complete cells were photographed as stereo pairs to facilitate interpretation of the three-dimensional information in each section. Selected sections were also tilted about two perpendicular axes with a double-tilt goniometer stage, in order to determine the effect of tilting on the clarity of certain cell features. Views which best illustrated these features and their three-dimensional characteristics were then photographed as stereo pairs. Enlargements of all HVEM micrographs were studied extensively to analyze the three-dimensional information contained therein.

Computer-Aided Reconstruction of Serial Thin Sections

Reconstructions of serially sectioned cells were done with the CARTOS (Computer-Aided Reconstruction by Tracing of Sections) Loaner System developed in the laboratory of Dr. Cyrus Levinthal (Department of Biological Science, Computer Graphics and Image Processing Facility, Columbia University, New York, NY). The CARTOS computer program for three-dimensional reconstruction has been described previously (Macagno et al., 1979), as having the specific features of the Loaner System (see "CARTOS", Research Resources Information Center, U.S. Dept. of Health and Human Services, N. I. H. Publication No. 81-2289).

Prior to reconstruction, each of the series of electron micrographs produced above was converted to a 35-mm filmstrip in which each frame depicted one section in the series. The sections were aligned optically with respect to the X and Y axes (in three-dimensional space) as these filmstrips were produced. From projected

images of each filmstrip, tracings of selected cell features in each section were entered into the computer with the aid of an automatic digitalizing device. The cell features traced for each series included: (i) the cytoplasmic membrane or cell outline, (ii) the specialized inclusion bodies (carboxysomes, polyphosphate bodies, and lipid bodies), and (iii) all of the individual thylakoid membranes. These features were all entered into separate memory files so that the computer could be programmed to reconstruct them individually or in combination. The computer was then instructed to display three-dimensional reconstructions of desired cell features or combinations thereof on a video display system. These reconstructions were studied visually while being rotated about the X, Y, and Z axes in order to obtain a variety of external views. The most effective and understandable of these views were photographed directly from the display screen with Kodak LPD-4, Tri-X, or Ektachrome 160 film. Stereo pairs of selected reconstructions were generated by rotation about the vertical axis. These were photographed to facilitate visualization of three-dimensional arrangements.

Studies with Mastigocladus laminosus

Organism

Mastigocladus laminosus was obtained from Dr. C.O. Patterson (Texas A. & M. University) who obtained it from the former Indiana University Culture Collection as IUCC No. 1931 (now UTEX 1931). It was isolated originally by Dr. R. Lewin in August, 1961, from a hot

spring at Hoeragerd, Iceland. The culture, as obtained, was contaminated with bacteria. It was isolated into axenic culture by repeated streaking of briefly sonicated cultures onto agar plates. Lack of contamination was demonstrated by lack of bacterial growth when broth suspensions were subcultured on nutrient broth-tryptone-Sabouraud Dextrose medium and on medium B of Stevens et al. (1973) supplemented with glucose, yeast extract, or Casamino acids.

Growth of Cultures

Common Incubation Conditions. All cultures were grown at 46 °C in large glass growth chambers (working volume: 1800 ml) developed by Mehta and Stevens (unpublished). The incident light intensity at the center of these chambers was 5.5×10^{17} $\mu E\ m^{-2}\ sec^{-1}$.

NO_3-Grown Cultures. Nitrate-grown (nonheterocyst-forming) cultures were grown in medium B of Stevens et al. (1973). They were bubbled continuously with sterile 5% (vol/vol) CO_2 in air to keep the filaments of cells in suspension and to provide a source of carbon for growth. Mature, exponentially growing cultures were used in the morphological and ultrastructural investigations described below.

N_2-Grown Cultures. Nitrogen-grown (heterocyst-forming) cultures were grown in medium B of Stevens et al. (1973), from which the sodium nitrate was deleted in order to stimulate heterocyst formation. Cultures were grown first while being bubbled continuously with sterile 5% (vol/vol) CO_2 in air. Exponentially growing cells from these cultures were then inoculated into fresh medium and grown while

being bubbled continuously with sterile 5% (vol/vol) CO_2 in oxygen-free N_2. Light and electron microscopical assays (see below) showed that such cultures stabilized with respect to their morphological and ultrastructural characteristics within 22 h after inoculation into fresh medium. Their morphological and ultrastructural characteristics remained relatively consistent as long as the cultures continued to grow exponentially. Samples were removed periodically between 22 and 33 h in order to (i) study the morphological and ultrastructural aspects of the heterocyst differentiation process, (ii) determine the ultrastructural characteristics of mature heterocysts, (iii) examine patterns of morphogenesis, and (iv) determine the ultrastructural characteristics of N_2-grown vegetative cells (for comparison with those of NO_3-grown cells, above).

Nitrogen-Starved Cultures. Nitrogen-starved cultures were produced by first preparing N_2-grown cultures as described above. After incubation for 27 h in the presence of 5% (vol/vol) CO_2 in oxygen-free N_2 (continuous bubbling) the cultures were switched over to continuous bubbling with 5% (vol/vol) CO_2 in argon to induce nitrogen starvation. Incubation in the presence of CO_2/argon then continued for 44 h, during which samples were withdrawn periodically for the morphological and ultrastructural analyses described below. This was done to assess the effects of nitrogen starvation on (i) the ultrastructural characteristics of vegetative cells, (ii) the ultrastructural characteristics of heterocysts, and (iii) the morphological aspects of heterocyst development.

Determination of Morphological Characteristics

Light Microscopy. Phase-contrast light microscopy of unstained wet mounts was used to monitor the general morphological characteristics of the cultures (above) and to ensure that a representative sampling of morphological types was seen in the transmission electron microscopical preparations (below). Each sample (above) was examined extensively at magnifications ranging from 400 to 1,000 X, and photomicrographs of representative fields were recorded on Kodak Technical Pan Film (TP-135-36). Photographic enlargements of these micrographs were compared to those of the electron micrographs (below) in order to assess the thoroughness and accuracy of each electron microscopical assay.

Scanning Electron Microscopy. The morphological characteristics of selected samples were examined by scanning electron microscopy of critical-point dried whole cell preparations. Mature cultures were collected on 0.45-μm Millipore filters (Millipore Corp., Bedford, MA) and washed several times with sterile growth medium to remove most of the extracellular slime that surrounds the cells. The filters (with cells) were then fixed and critical-point dried as described by Balkwill et al. (1980). Dried samples were sputter-coated with gold and viewed with an AMR-1000A scanning electron microscope. The results were compared with those obtained by phase-contrast light microscopy (above) and transmission electron microscopy (below).

Determination of Ultrastructural Characteristics

Preparation of Samples. The detailed ultrastructural characteristics of each sample were determined by transmission electron microscopy of thin-sectioned preparations. Two fixation procedures were used to prepare the samples for thin sectioning: the ruthenium red procedure and the glutaraldehyde-osmium tetroxide procedure. These were performed as in the studies with A. quadruplicatum (see above). Samples from both fixations were dehydrated through a graded ethanol series and embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969). Thin sections were cut with a Diatome diamond knife (M. J. O. Diatome, Inc., Fort Washington, PA) on an LKB Ultratome III ultramicrotome. The sections were mounted on formvar-coated, carbon-stabilized, 200-mesh, copper specimen grids. Mounted sections were poststained for 15 min with 0.5% uranyl acetate (in 50% methanol) and for 2 min with 0.4% lead citrate (Reynolds, 1963).

Both fixation procedures gave equivalent results, thereby insuring that artifactual structures were not being observed. Micrographs from the glutaraldehyde-OsO_4 fixation were chosen for the illustrations in this report.

Transmission Electron Microscopy. Thin sections were viewed and photographed with a JEOL JEM-100S transmission electron microscope, operating at an accelerating potential of 80 kV. Each sample was examined extensively to assess the variety of morphological and ultrastructural features present. More than 2,500 electron micrographs of representative cells or fields were then

taken to document these features. The results were analyzed by studying photographic enlargements of the micrographs. Quantitative data (such as the numbers of inclusion bodies per section) were taken only from longitudinal sections that appeared to traverse the center of the cell. At least 30-50 such sections were examined in each case.

III. RESULTS

The Three-Dimensional Ultrastructure of Agmenellum quadruplicatum

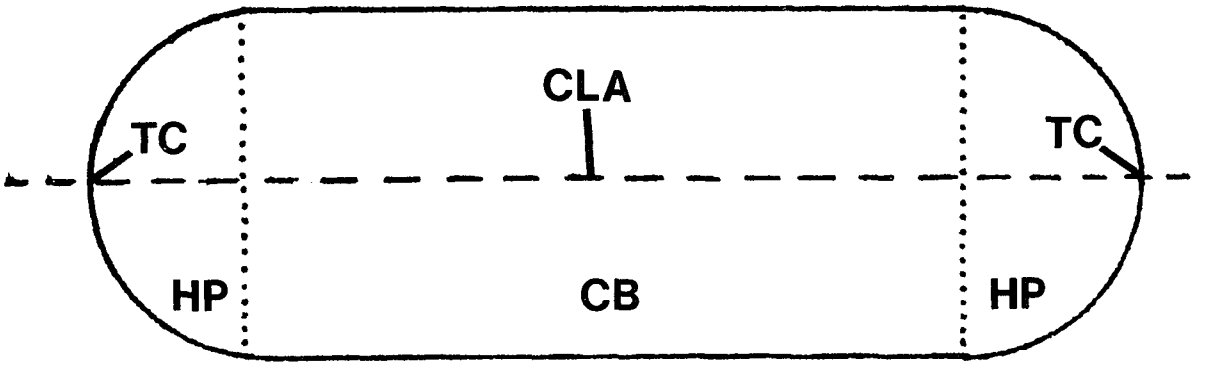
General Morphology and Basic Ultrastructural Features

The basic morphology of A. quadruplicatum, as determined by light and electron microscopy, is shown in Fig. 1. The cells were rod-shaped, ranging from 1.5 to 2.6 μm in diameter and 2.5 to 5.2 μm in length. Each cell consisted of a central, cylindrical body with two hemispherical poles. The points at which the central longitudinal axis intersected the hemispherical poles are referred to (arbitrarily) as the tips of the cell in the detailed results presented below.

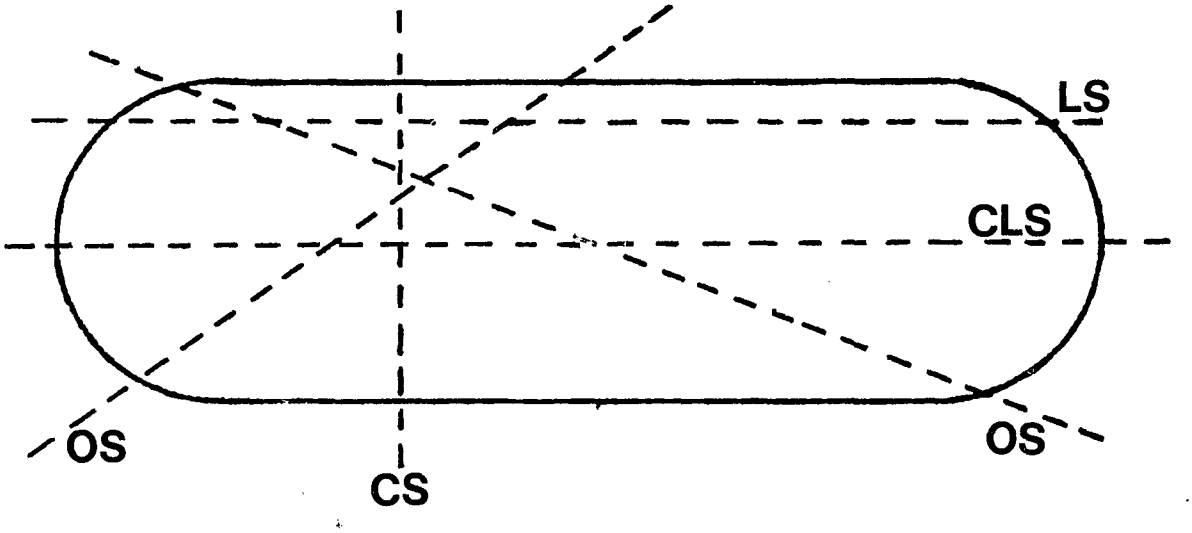
For analysis of three-dimensional ultrastructure, it was necessary to define the various ways in which thin or thick sections could pass through a typical A. quadruplicatum cell. The following types of sections were identified and are illustrated diagrammatically in Fig. 2: (i) cross sections, which run perpendicular to the central longitudinal axis; (ii) longitudinal sections, which run parallel to the central longitudinal axis; (iii) central longitudinal sections, which run both parallel to and directly through the central longitudinal axis; and (iv) oblique sections, which run neither perpendicular nor parallel to the central longitudinal axis.

Figure 1. Diagram illustrating the basic morphological features of a typical A. quadruplicatum cell. CB = central, cylindrical body; CLA = central longitudinal axis; HP = hemispherical poles; TC = tips of cell.

Figure 2. Diagram illustrating the various ways in which sections could pass through a typical A. quadruplicatum cell. CLS = central longitudinal section; CS = cross section; LS = longitudinal section; OS = oblique sections.



1



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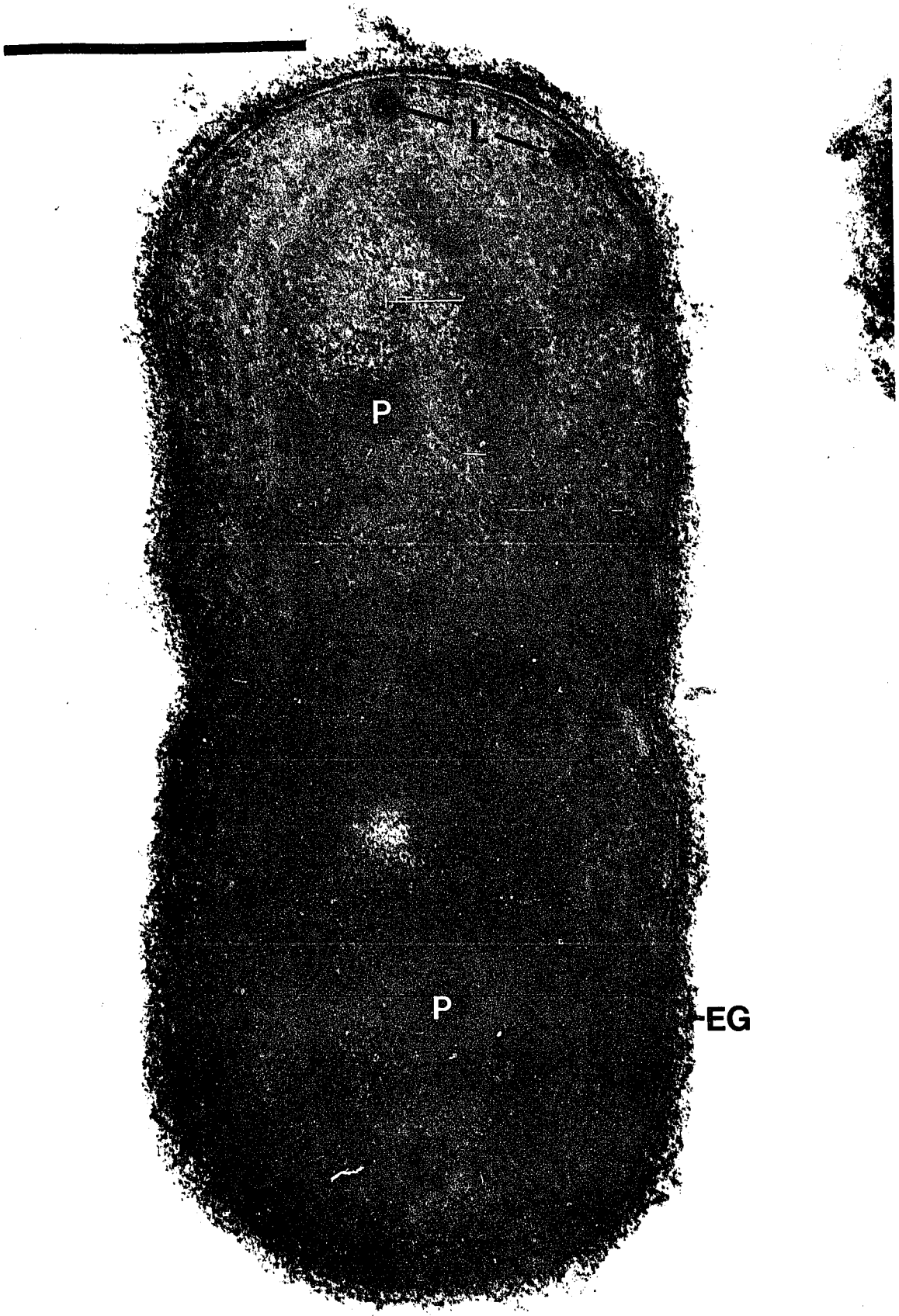
The basic ultrastructural features of A. quadruplicatum were usually visible in central longitudinal sections (Fig. 3). All cells possessed an extracellular slime layer or glycocalyx (Balkwill and Stevens, 1980a) and a Gram-negative cell envelope identical to those found in other cyanobacteria (Stanier and Cohen-Bazire, 1977; Wolk, 1973). The latter consisted of three substructures: an outer membrane, a peptidoglycan layer, and the cytoplasmic membrane or "plasmalemma." The interior of the cell included a central cytoplasmic region that appeared to be surrounded by a peripheral system of photosynthetic thylakoid membranes. Several specialized inclusion bodies were commonly present in addition to nuclear material and ribosomes. These were: polyphosphate bodies (Baxter and Jensen, 1980; Jensen, 1968, 1969; Jensen et al., 1977), carboxysomes or "polyhedral bodies" (Codd and Stewart, 1976; Shively, 1974; Stewart and Codd, 1975; Wolk, 1973), glycogen granules or "α-granules" (Chao and Bowen, 1971; Pankratz and Bowen, 1963; Wolk, 1973), and lipid bodies or "β-granules" (Edwards et al., 1968; Pankratz and Bowen, 1963; Wolk, 1973). As in previous investigations with A. quadruplicatum (Hardie et al., 1983a, b; Stevens et al., 1981a, b) and other unicellular cyanobacteria (Stanier and Cohen-Bazire, 1977), individual phycobilisomes (Gantt, 1981; Glazer, 1982) were usually not resolved.

Location of Specialized Inclusion Bodies in the Cell

Individual sections like the one shown in Fig. 3 generally allowed one to see the specialized inclusion bodies of A. quadrup-

Figure 3. Central longitudinal thick section (0.25-μm) through typical A. quadruplicatum cell. Bar = 1.0 μm. C = carboxysome; EG = extracellular glycocalyx; L = lipid body; P = polyphosphate body; T = thylakoid membranes.

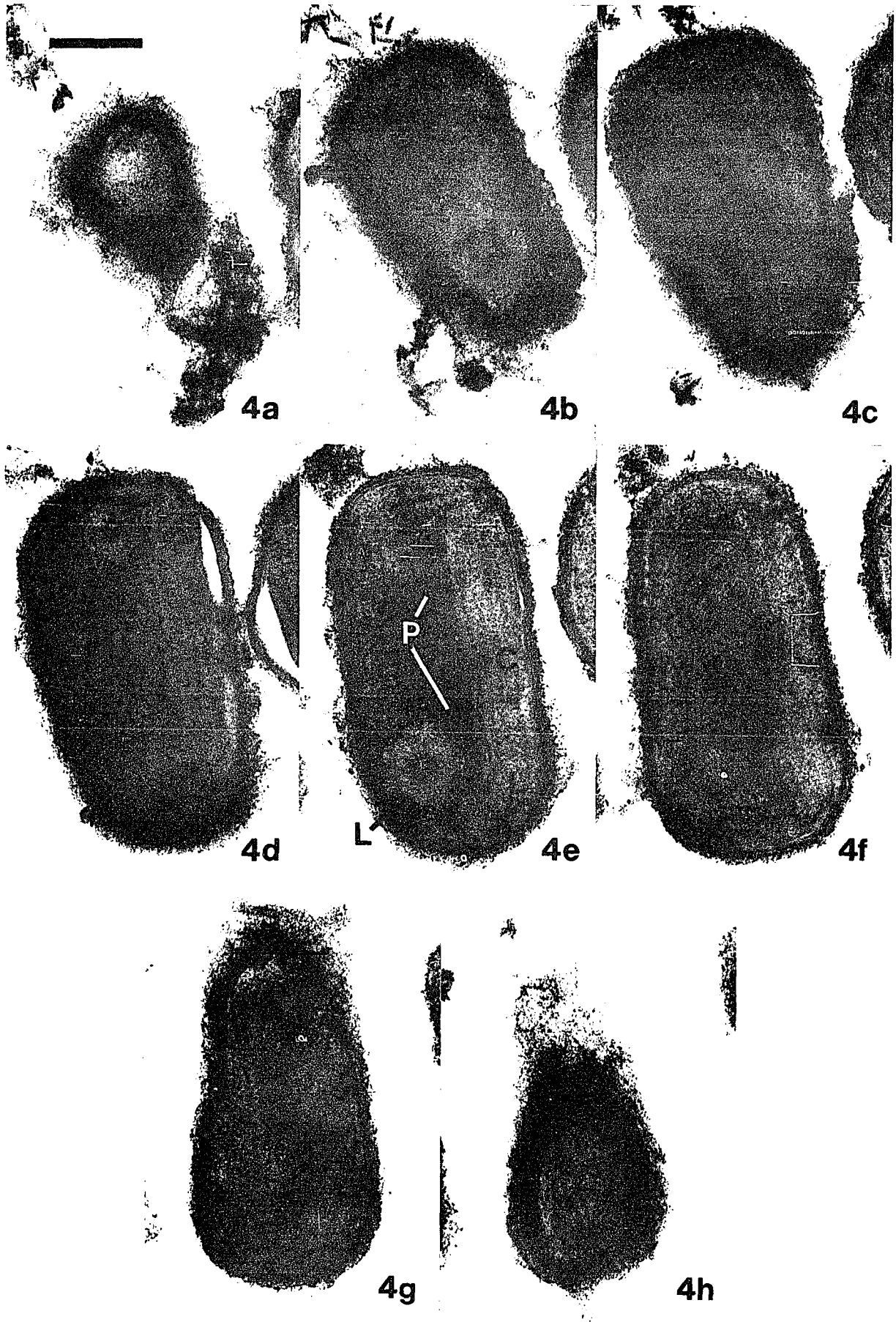


3

licatum quite clearly, but conveyed relatively little information about the three-dimensional distribution of these structures throughout the cell. By studying large numbers of randomly cut sections, it was possible to make only general inferences regarding the location of inclusion bodies; some appeared to be centrally located while others appeared to be peripheral. Definitive information about the three-dimensional distribution of inclusions could be obtained only by examining serial sections through complete cells.

The use of HVEM and 0.25-μm thick sections greatly facilitated determination of the three-dimensional locations of inclusion bodies in two ways: (i) the relatively thick sections made it possible to examine an entire cell quickly because only a few sections were required to pass through each cell and (ii) the thick sections had sufficient depth to facilitate stereo analysis of the three-dimensional information contained within each section. A typical series of 0.25-μm sections through a complete cell is shown in Fig. 4. It can be seen that the distribution of inclusion bodies within the cell is determined easily by examining such a series of sections. Examination of more than 50 cells in this way showed that nuclear material, polyphosphate bodies, and carboxysomes were located only in the central cytoplasmic portion of the cell. They were usually positioned along the central longitudinal axis and were never situated among the thylakoid membranes. Polyphosphate bodies and carboxysomes were interspersed with each other, so that direct contact between these two types of inclusions occurred frequently. Each cell contained an average of six carboxysomes and five polyphosphate

Figure 4. Series of oblique (almost longitudinal) thick (0.25-μm) sections through entire A. quadruplicatum cell, illustrating three-dimensional distribution of specialized inclusion bodies throughout the cell. Bar = 1.0 μm. C = carboxysome; L = lipid body; P = polyphosphate body.


4a
4b
4c
4d
P
L
4e
4f
4g
4h

bodies (ranges 4 - 10 and 4 - 6, respectively). Polyphosphate bodies varied in diameter, but there was no correlation between their size and the number per cell. The central cytoplasmic region containing these two inclusions was essentially an independent compartment within the cell, since it was completely surrounded by the innermost pair of membranes in the thylakoid system. Direct contact between the cytoplasmic inclusions and these thylakoids was seldom seen, however, because the inclusions were almost always surrounded by ribosomes. For the most part, the ribosomes were also confined to the central cytoplasm and filled the areas not occupied by inclusions. Glycogen granules, lipid bodies, and the thylakoids themselves were located only within the peripheral thylakoid membrane system that surrounded the central cytoplasmic region. Glycogen granules were dispersed between the membrane pairs throughout the entire thylakoid system, but lipid bodies were always positioned specifically between the cytoplasmic membrane and the outermost pair of thylakoid membranes. The average number of lipid bodies in each cell was 17 (range: 14-22). They were located about the entire periphery of the cell, including the hemispherical poles. Unlike the inclusions located in the central cytoplasm, lipid bodies were frequently in close association with the outermost pair of thylakoid membranes.

Reconstruction of serial thin sections with the CARTOS system not only confirmed the HVEM results described above, but also helped significantly to illustrate the complete three-dimensional arrangement of inclusion bodies within whole cells. This was done most effectively by displaying the inclusions against a background of dots

representing the outline of the cell surface. The difference between strictly peripheral inclusions (such as lipid bodies; Fig. 5) and strictly central inclusions (such as carboxysomes; Fig. 6) was quite obvious when this was done. Different types of inclusions could be shown in single reconstructions without confusion (Fig. 7) by illustrating each type with a specific display mode (i.e., solid lines, dotted lines, etc.). Visual understanding of intracellular arrangements could then be furthered by rotating such reconstructions about three perpendicular axes in order to provide a variety of external views (Figs. 5-7).

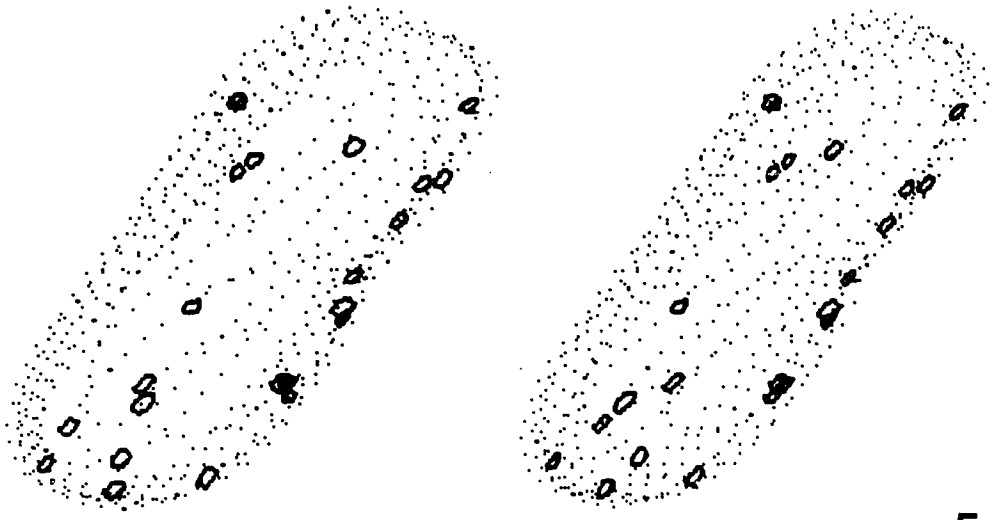
Three-Dimensional Arrangement of the Photosynthetic Thylakoid Membrane System

General Characteristics of the Thylakoid System and its Membranes.

The overall three-dimensional arrangement of the thylakoid membrane system in A. quadruplicatum was determined with CARTOS reconstructions of serial thin sections. The complete thylakoid system consisted of 4-6 pairs of membrane sheets which traversed the entire longitudinal axis of the cell (see Fig. 3). The sheet-like nature of the individual membranes and the extent to which these sheets spread throughout the cell were never apparent from individual thin or thick sections. They were seen clearly, however, in CARTOS reconstructions made by tracing a single thylakoid pair throughout the entire cell. Figure 8 illustrates several views of such a reconstruction produced by tracing the innermost thylakoid pair facing the central cytoplasm. Both the sheet-like nature of

Figure 5. Stereo pair of CARTOS reconstruction depicting the locations of lipid bodies throughout an entire A. quadruplicatum cell. Lipid bodies are represented by small circles; cell outline is represented by dots. Note peripheral location of all lipid bodies.

Figure 6. Stereo pair of CARTOS reconstruction depicting the locations of carboxysomes throughout an entire A. quadruplicatum cell (same cell as in Fig. 5). Carboxysomes are represented by circles; cell outline is represented by dots. Compare central location of these inclusions with peripheral location of lipid bodies (Fig. 5).



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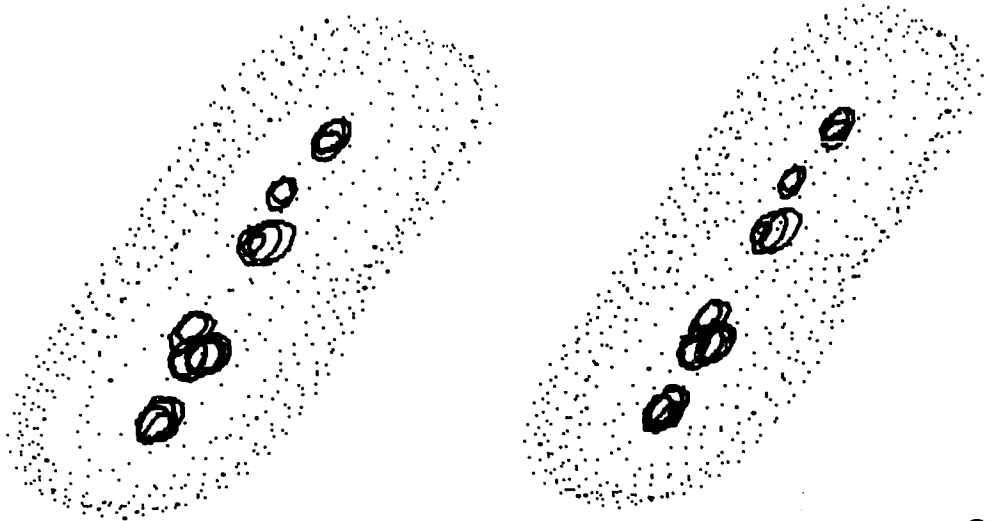

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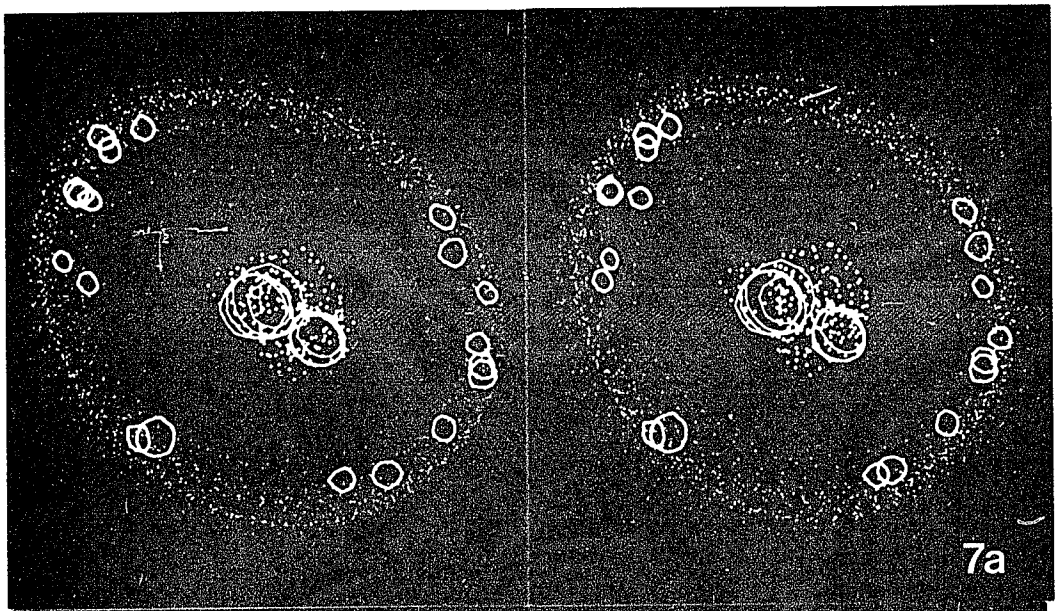
Figure 7. Stereo pairs of CARTOS reconstruction depicting the locations of three types of specialized inclusion bodies in the cylindrical portion of a typical A. quadruplicatum cell. Cell outline is represented by dots. Carboxysomes (heavy dots) and polyphosphate bodies (large solid circles) are centrally located. Lipid bodies (small solid circles) are peripherally located. Three different views of the same reconstruction are illustrated.

a. Cell viewed along its central longitudinal axis.

b. Cell viewed from the side.

c. Cell viewed at an oblique angle.



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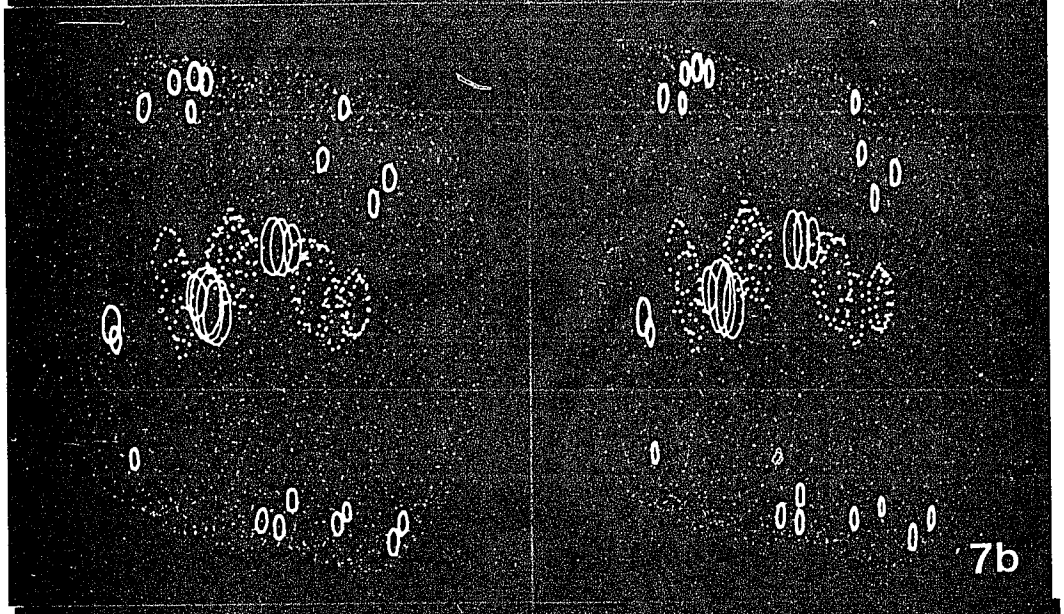

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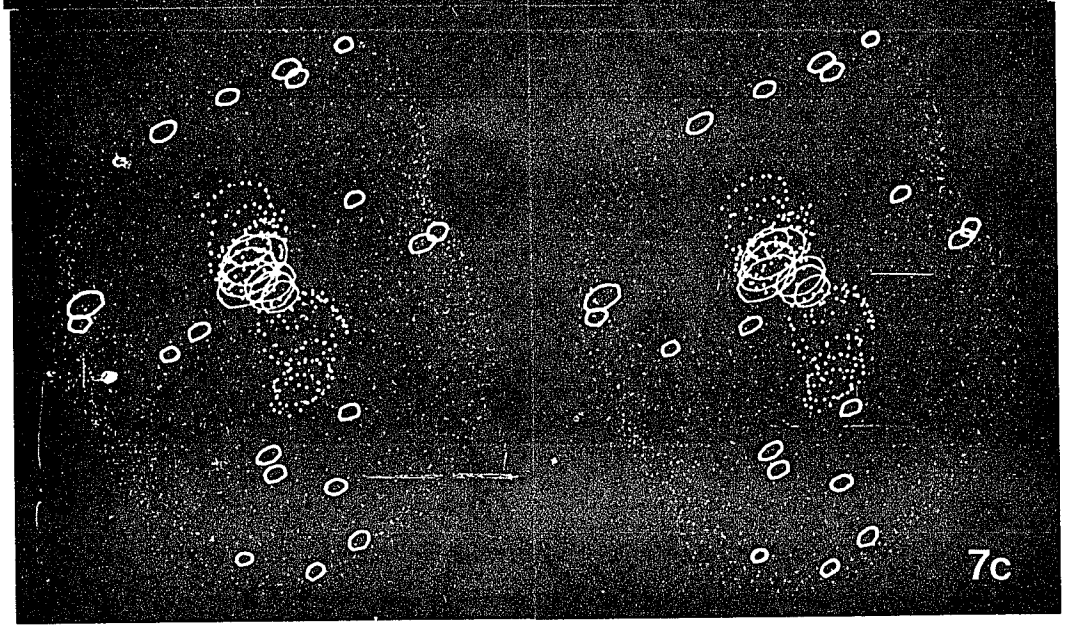


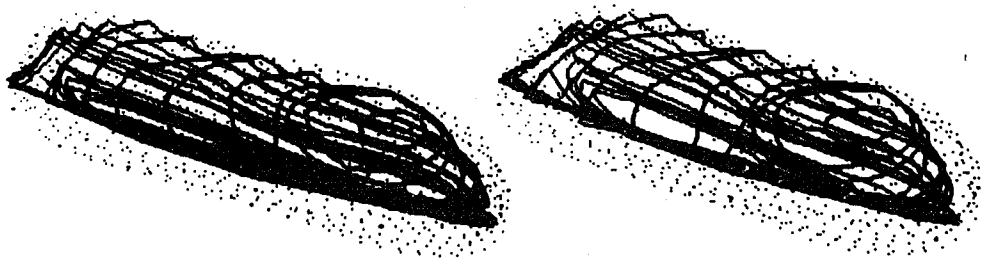
7c

Figure 8. Stereo pairs of CARTOS reconstruction depicting the arrangement of the innermost complete thylakoid membrane (solid lines) within a typical A. quadruplicatum cell (outline represented by dots). It is important to realize that the contour lines delineating the thylakoid actually represent continuous, closed sheets of membranous material. Note that thylakoid entirely surrounds central cytoplasmic region of the cell and tapers toward each cell pole. Three different views of the same reconstruction are illustrated:

a. Cell viewed from the side.

b. Cell viewed slightly off central longitudinal axis.

c. Cell viewed at oblique angle.



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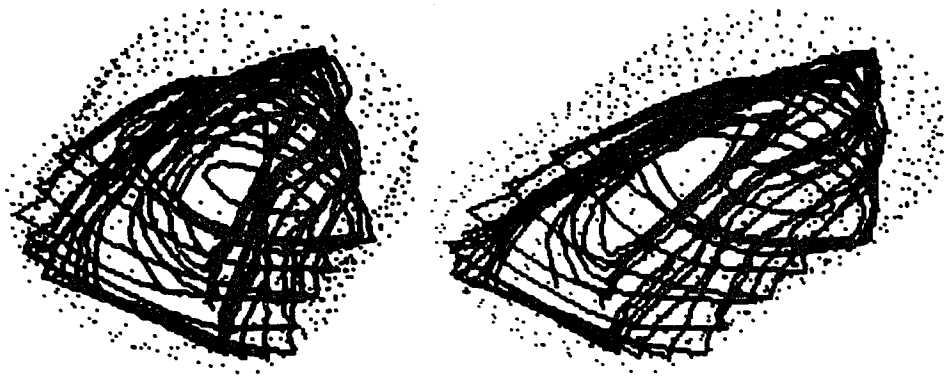

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the membranes and the fact that they surround entirely the central cytoplasmic region (as stated earlier) are evident. By individually reconstructing each of the 4-6 pairs of membranes found in a complete thylakoid system, it was shown that every one of them entirely surrounded the central cytoplasmic region as depicted in Fig. 8. The central cytoplasm, then, appeared to be an isolated and independent compartment in the center of the cell.

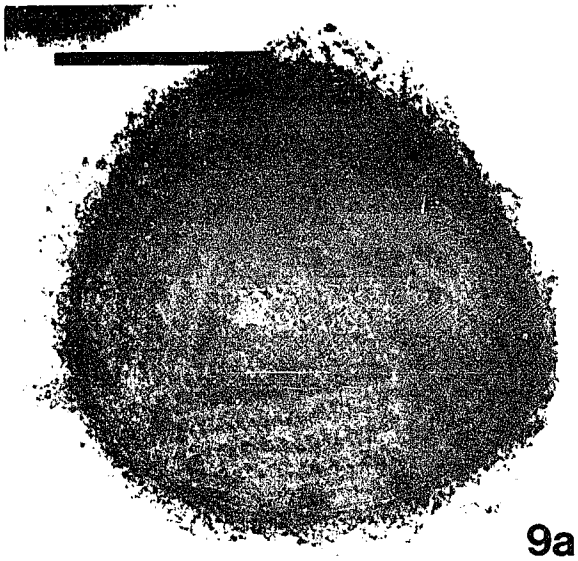
Arrangement of the Complete Thylakoid System in the Cylindrical Portion of the Cell. The three-dimensional arrangement of the complete thylakoid system in the cylindrical portion of the cell was best appreciated by studying the pattern formed by the membranes in thin or thick cross sections. Thick sections were better suited for this purpose because the thylakoids were more distinctly and completely revealed than they were in thin sections. In both cases, however, it was first necessary to determine just what the membrane pattern really was, since it appeared to vary considerably from one section to another (Fig. 9). This implied that the arrangement of the thylakoid system was not consistent from one cell to another. When cross sections were tilted about two perpendicular axes with a double-tilt goniometer stage in the HVEM, however, the appearance of the thylakoids changed drastically as the viewing angle varied (Fig. 10). It was also discovered that, by tilting sections in this way, one could always find a combination of tilt angles that made the thylakoids appear as shown in Fig. 11. After tilting, then, the arrangement of the thylakoid

Figure 9. Thick (0.25-μm) cross sections or near cross sections through A. quadruplicatum cells, depicting apparent cell-to-cell variation in the arrangement of the thylakoid membrane system. Bars = 1.0 μm.

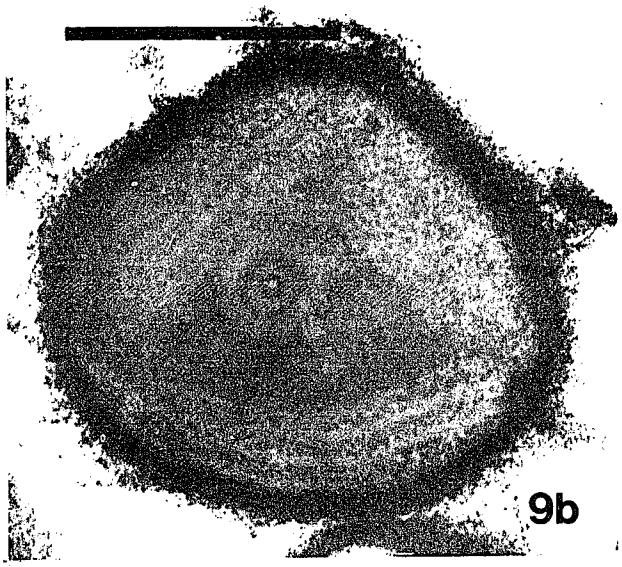
Figure 10. Thick (0.25-μm) cross section through A. quadruplicatum cell, as viewed from two different directions after tilting about two perpendicular axes with a double-tilt goniometer stage in the HVEM. Note how appearance of the thylakoid membranes changes as the viewing angle varies. Bars = 1.0 μm.

a. Tilt angles: -15° about vertical axis; 0° about horizontal axis.

b. Tilt angles: $+19^{\circ}$ about vertical axis; -20° about horizontal axis.



9a



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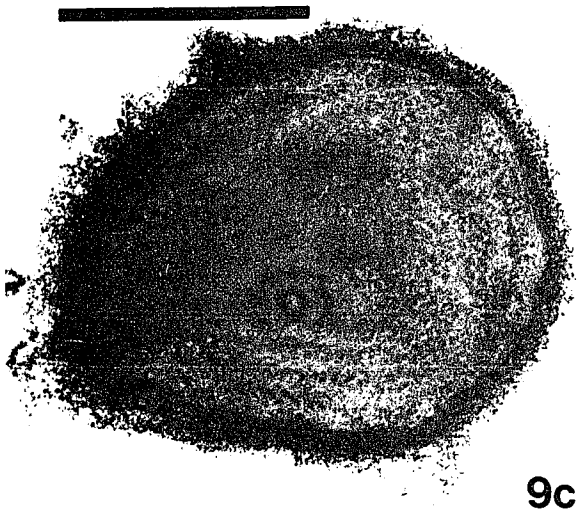

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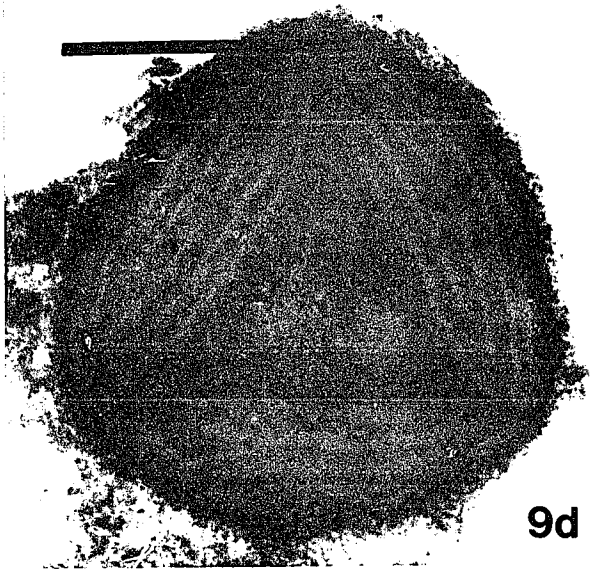

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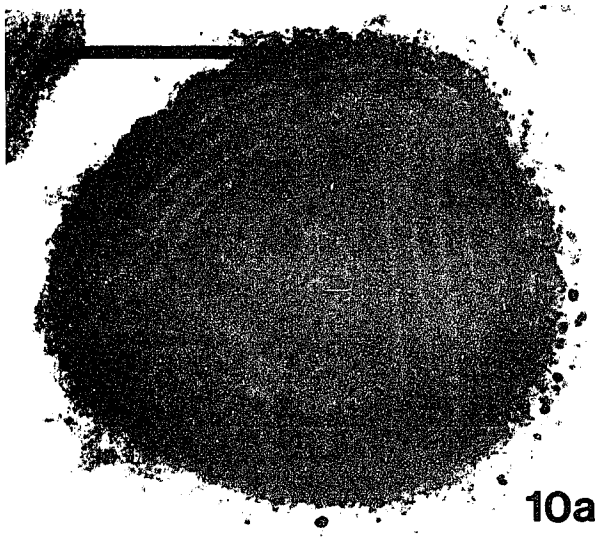

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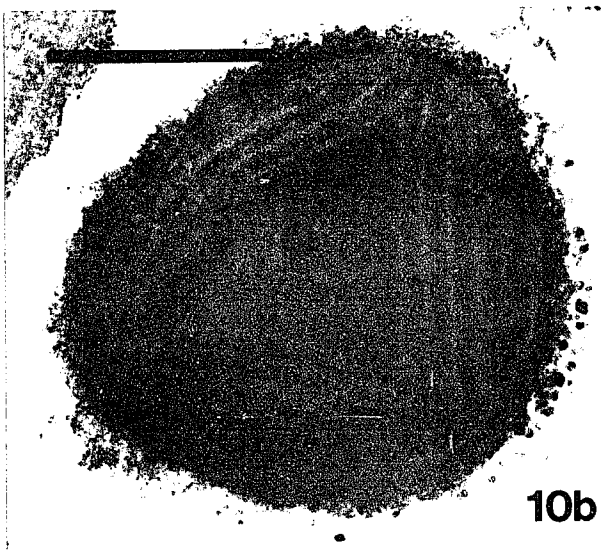
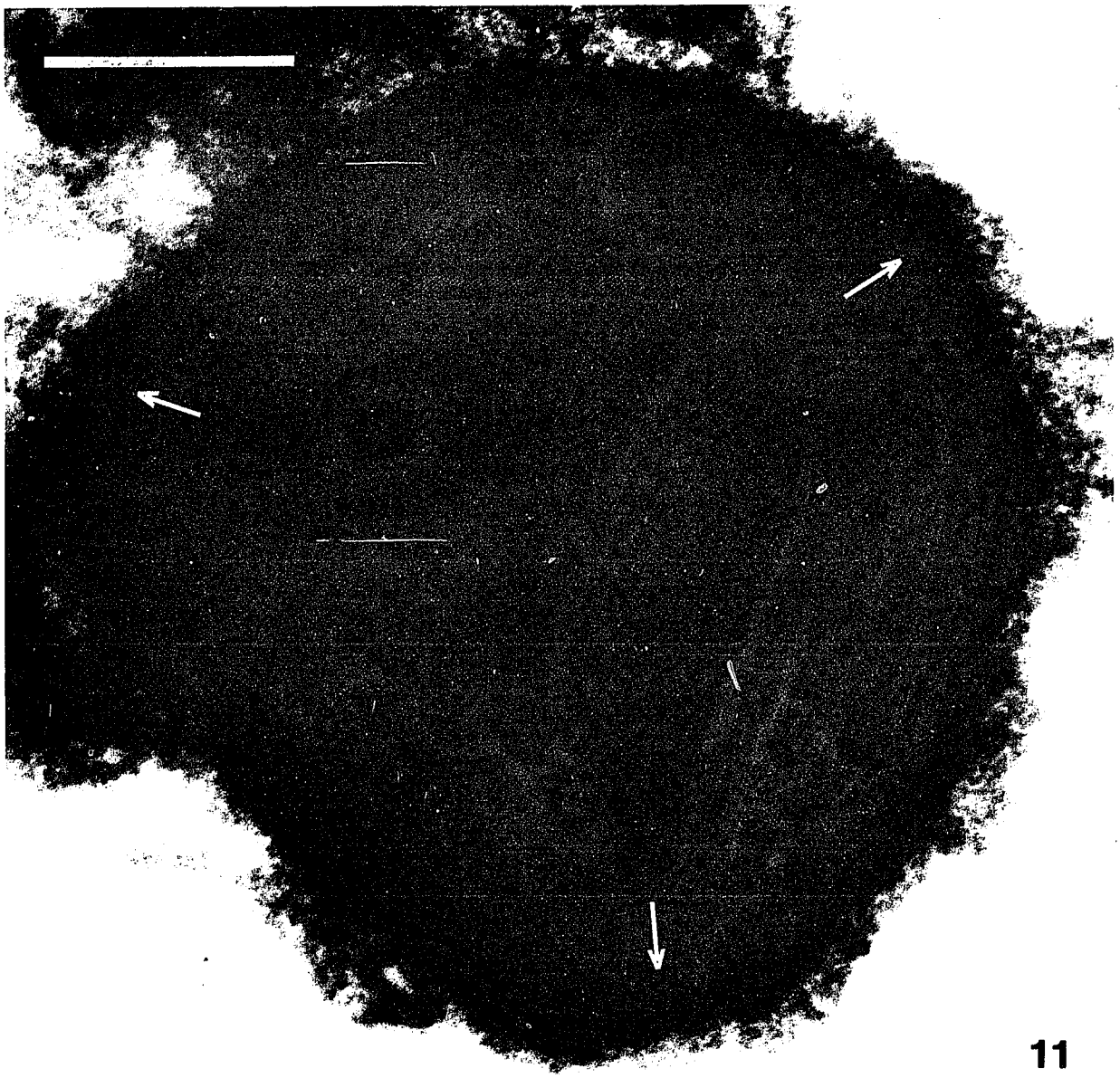


10b

Figure 11. Thick (0.25-μm) cross section through the cylindrical portion of a typical A. quadruplicatum cell, depicting the thylakoid arrangement that could be found consistently in cross sections or near cross sections after tilting these sections about two perpendicular axes. Thylakoids tend to coalesce and approach the edge of the cell at three peripheral loci (arrows), producing a pattern similar to a series of concentric triangles. Bar = 0.5 μm.



11

system did not appear to vary from one section to another. The thylakoid membranes always coalesced and approached the cytoplasmic membrane at three peripheral loci (Fig. 11). The resulting thylakoid pattern was similar to a series of concentric triangles, except that they were not truly concentric at their corners. Because this pattern was sometimes seen in untilted sections, it was assumed that it represented the true arrangement of thylakoid membranes in all cross sections. Tilting merely served to compensate for slight variations in the way in which apparent cross sections actually passed through the cell. The arrangement of the thylakoid system in the cylindrical portion of A. quadruplicatum, then, was quite consistent from one cell to another, except for one minor variation that will be described below.

The thylakoid pattern described above was seen in each section (after appropriate tilting, if necessary) when serial thick or thin sections through the entire cylindrical portion of a cell were examined. Thus, the arrangement of the thylakoid system was quite uniform throughout this part of the cell. The individual membrane sheets extended straight through the cell cylinder without changing their distance from the central longitudinal axis. Their three-dimensional arrangement could be illustrated effectively with CARTOS reconstructions of cell cylinders, provided that only one complete thylakoid was included in such reconstructions (Fig. 12). It was possible to include more than one complete thylakoid in a reconstruction by assigning different display modes to each one, but the resulting images tended to be rather confusing unless the reconstructed cell was viewed along its central longitudinal axis (Fig. 13). Even

Figure 12. Stereo pairs of CARTOS reconstruction depicting one thylakoid (solid lines), lipid bodies (small solid circles), and carboxysomes (large dashed circles) in the cylindrical portion of a typical A. quadruplicatum cell (outline represented by dots). Three different views of the same reconstruction are illustrated:

a. Cell viewed along its central longitudinal axis.

b. Cell viewed from the side.

c. Cell viewed from an oblique angle.

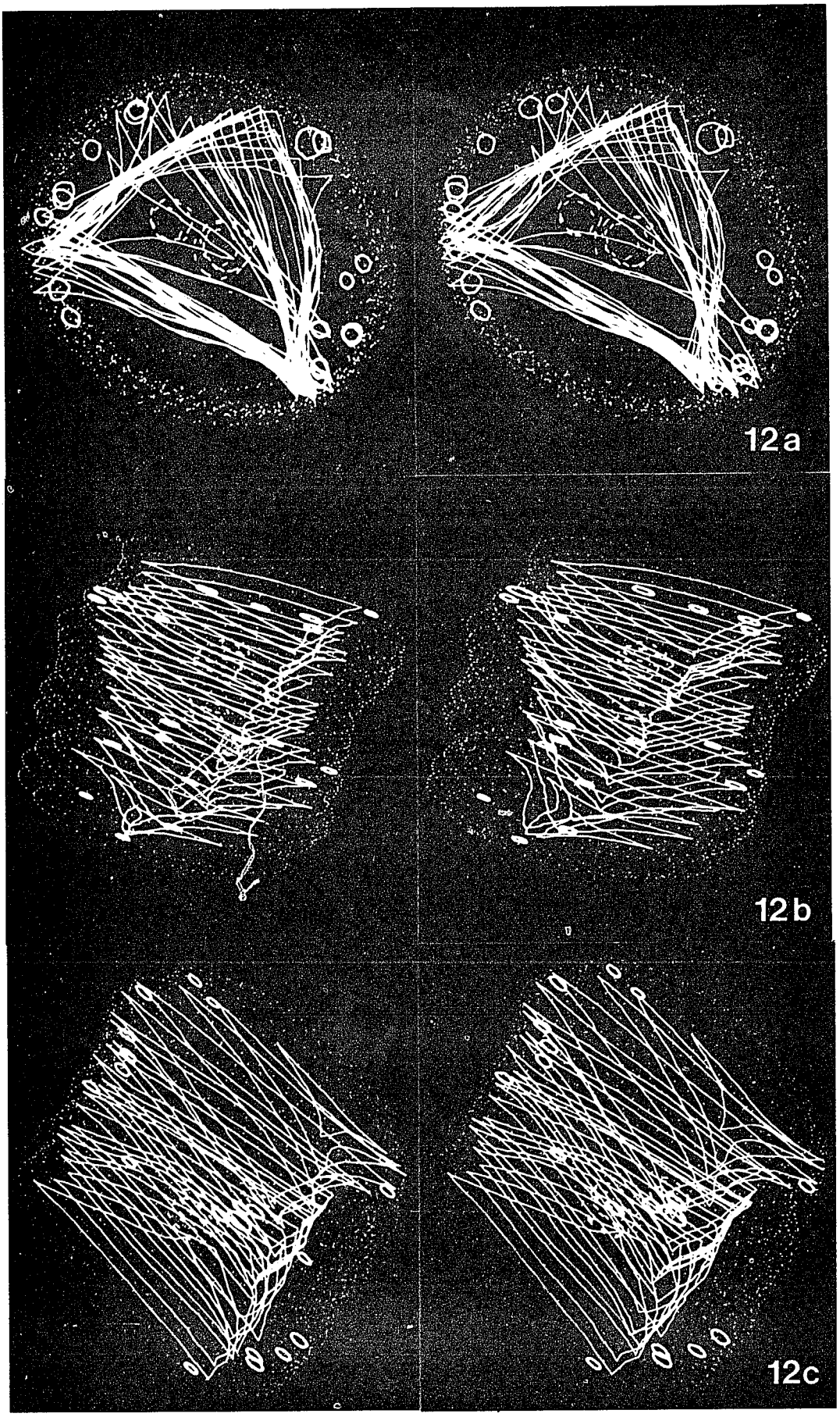
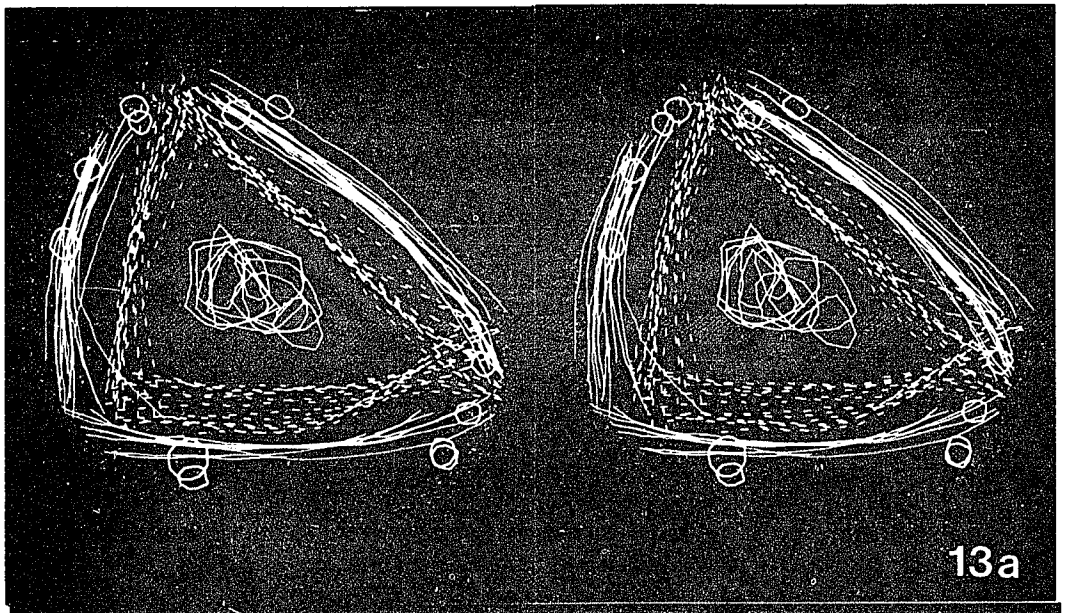

12a
12b
12c

Figure 13. Stereo pairs of CARTOS reconstruction attempting to depict more than one complete thylakoid in the cylindrical portion of a typical A. quadruplicatum cell (outline represented by dots). Thylakoids are represented by solid or dashed lines, as detailed below. Other features include lipid bodies (small, peripherally located circles) and carboxysomes (large, centrally located circles).

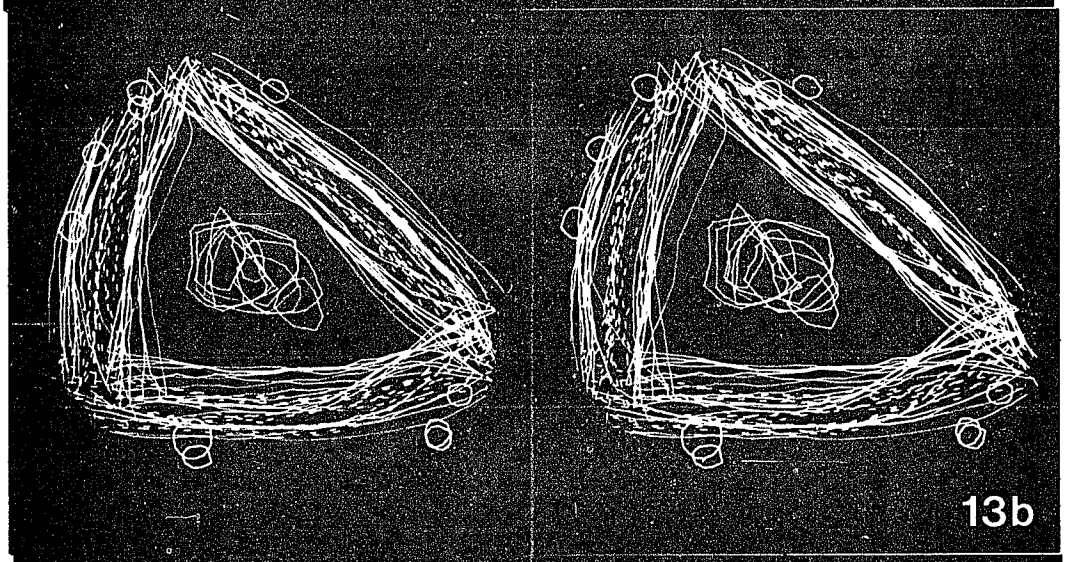
a. Reconstruction containing two thylakoids: the innermost (dashed lines) and the third innermost (solid lines). By deleting the second innermost thylakoid and rotating the cell so that it is viewed along its central longitudinal axis, it is possible to visualize the two thylakoids as clearly separate entities.

b. Same reconstruction as shown in Fig. 13a, but including the second innermost thylakoid (dashed lines) along with the innermost and third innermost (both solid lines). Note that it is more difficult to see the membranes as separate entities, although separation is still reasonable along the upper left and upper right sides of the triangle formed by the membranes.

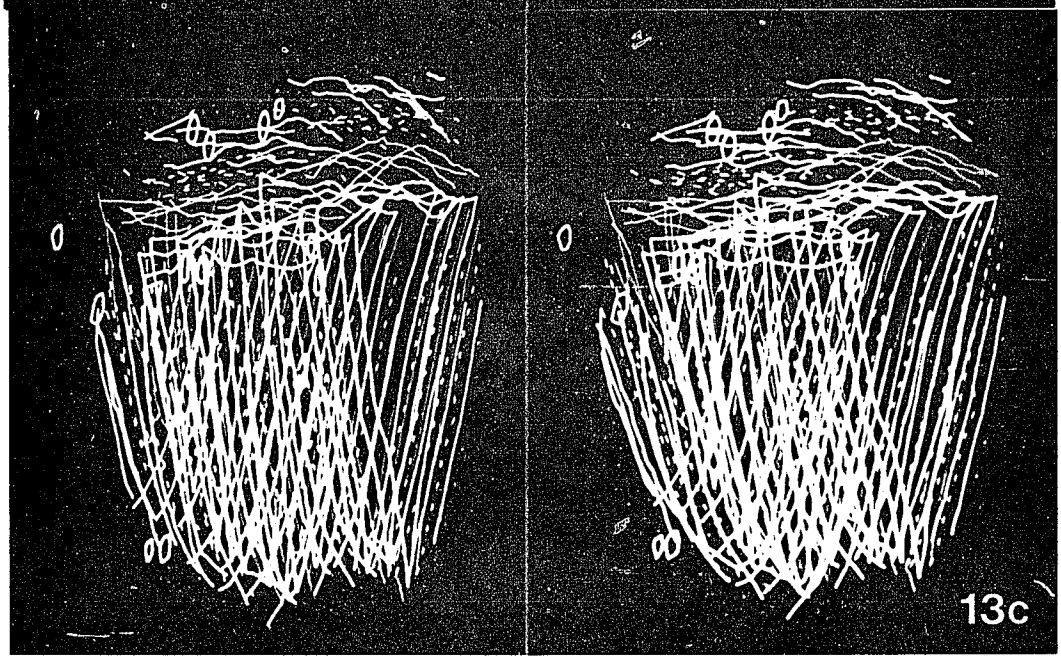
c. Reconstruction similar to that shown in Fig. 13b, but rotated so that cell is viewed from the side. Image of thylakoids is excessively confusing, due to multiple overlapping of the lines representing the thylakoid sheets.



13a



13b



13c

then, it was necessary to search for the one viewing angle that resulted in the best delineation of individual membranes. To better illustrate the three-dimensional arrangement of the thylakoid system in the cylindrical portion of A. quadruplicatum, a model was devised with the aid of CARTOS reconstructions and serial cross sections. This model is shown in Fig. 14.

The thylakoid arrangement described above was actually found to occur in approximately 70% of the cells with little or no variation. The rest of the cells possessed a slightly different arrangement that was also quite consistent from one cell to another. A cross section depicting this variation is shown in Fig. 15, and the two arrangements are compared diagrammatically in Fig. 16. In the second arrangement, the thylakoid sheets appeared to coalesce and approach the edge of the cell at four peripheral loci instead of three. Actually, they approached the outermost thylakoid membrane pair at one of these four positions. This pattern, like the first one seen in most of the cells, was consistent throughout the entire cylindrical portion of the cell. Therefore, a model of the variant thylakoid arrangement would be similar to that shown in Fig. 14, except for its appearance in cross sections. There was no correlation between thylakoid pattern and the cell division cycle of A. quadruplicatum. Cells with either pattern were found in all stages of cell division. No other patterns were observed in any of the cells, nor were any seen that could be interpreted as transition stages between the two types illustrated in Fig. 16.

Figure 14. Three-dimensional, cut-away model of the complete thylakoid membrane system in the cylindrical portion of a typical A. quadruplicatum cell. Four thylakoids (T1, T2, T3, and T4) have been included. (Based on serial cross sections and CARTOS reconstructions.)

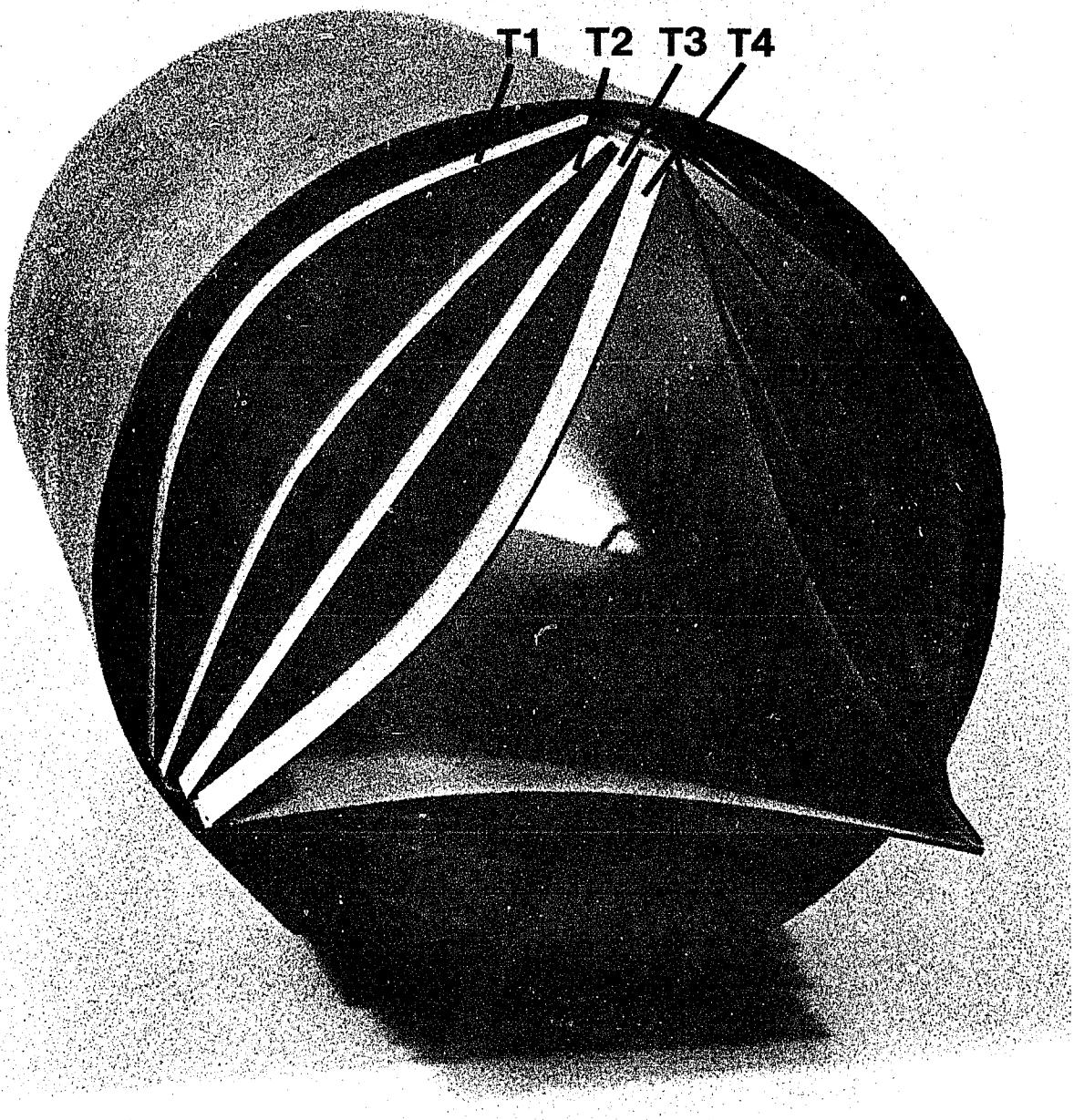


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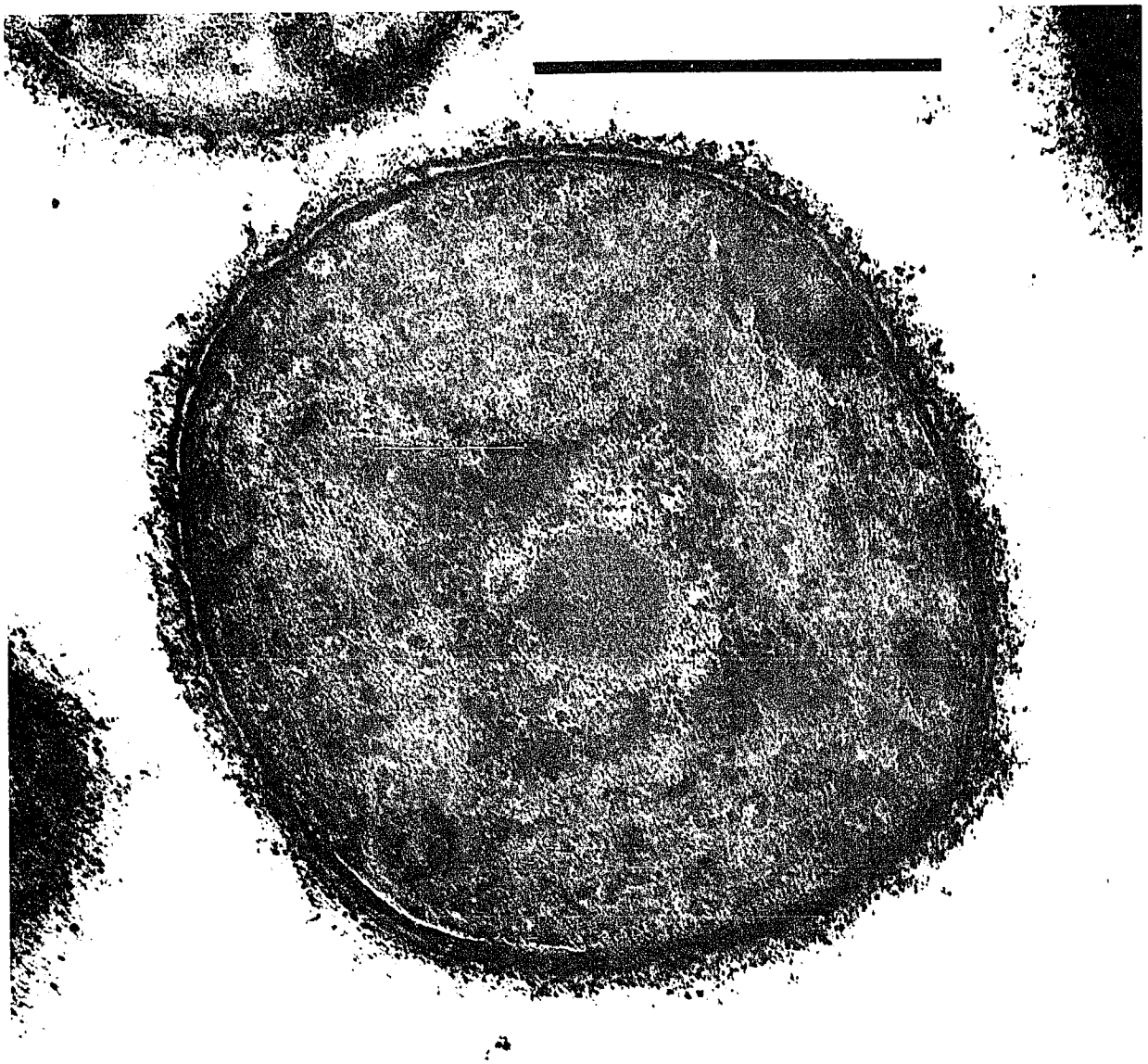
Figure 15. Thin cross section through cylindrical portion of an A. quadruplicatum cell, depicting the variant thylakoid arrangement seen approximately 30% of the cell (Fig. 16). Bar = 1.0 μm.

Figure 16. Diagrams comparing the two thylakoid patterns seen in cross sections through the cylindrical portion of A. quadruplicatum cells. At left is the pattern seen in 70% of the cells (compare with three-dimensional model in Fig. 14). At right is the variant pattern seen in remaining cells.



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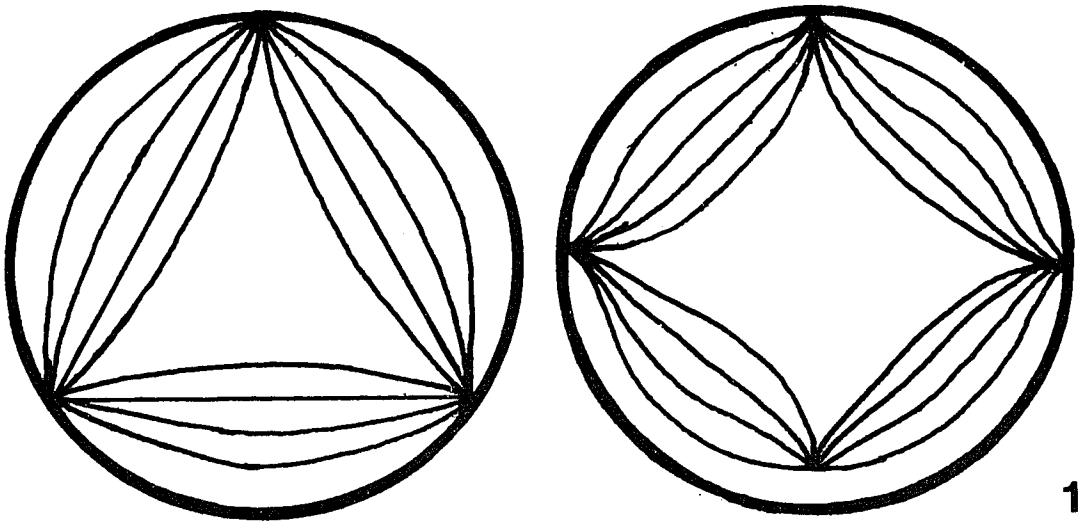


16

Arrangement of the Complete Thylakoid System in Cell Poles. The three-dimensional arrangement of the complete thylakoid system in cell poles of A. quadruplicatum was both complex and exceedingly difficult to illustrate with CARTOS reconstructions. It was apparent from central longitudinal sections that all of the thylakoid pairs tended to taper and approach the tips of the cell (Fig. 3). This tapering of thylakoids toward the cell tips was also seen in CARTOS reconstructions of cell poles that included only one or two thylakoids (Fig. 17). Attempts to include all of the thylakoids in such a reconstruction failed badly, producing only a very confusing clutter of overlapping lines (Fig. 18). As a consequence, the precise arrangement of the complete thylakoid system could be determined only by carefully studying many sets of serial thin and thick sections through cell poles.

The arrangement of thylakoids in the cell poles proved to be more complicated than that in the cylindrical portion of the cell because it was affected by cell division. The innermost thylakoid pair always terminated at a single point at or very near the tip of the cell. It was the location of the outer thylakoid sheets that varied, and the variation was best understood from an examination of pole areas in cells that had just completed cell division but had not yet separated from each other (Fig. 19). The thylakoid arrangement at the two "old" poles was always the same; all thylakoids tended to coalesce and terminate simultaneously at the tip of the cell. In the "new" poles, however, the outer thylakoids usually terminated near the cell periphery without quite reaching the cell tip. Individual cells (those no longer involved in cell division or separation) always possessed one pole with the first of the above thylakoid arrangements. The other pole exhibited

Figure 17. Stereo pair of CARTOS reconstruction depicting two thylakoids at the pole of a typical A. quadruplicatum cell (outline represented by dots). Both the inner (solid lines) and outer (dashed lines) thylakoids taper and meet at a single point on the tip of the cell.

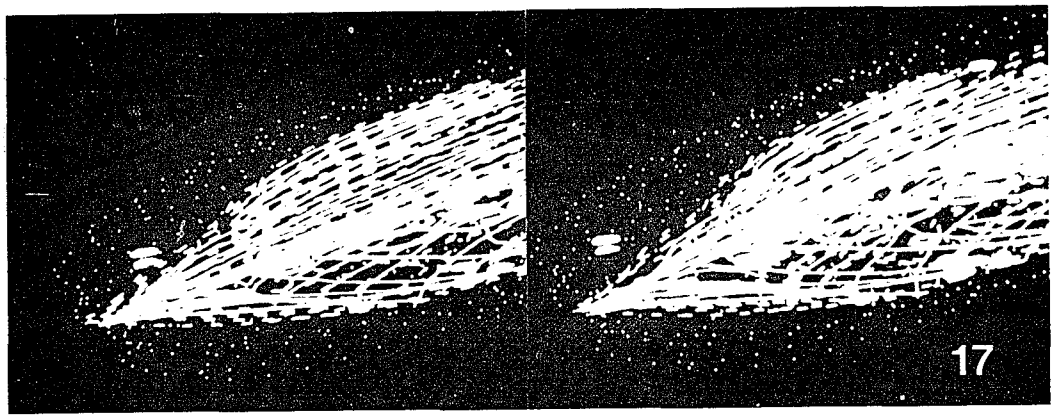
Figure 18. Stereo pair of CARTOS reconstruction depicting all of the thylakoids (solid lines) in one hemispherical pole of a typical A. quadruplicatum cell (outline represented by dots). Thylakoids taper toward tip of cell but reconstruction is visually confusing.

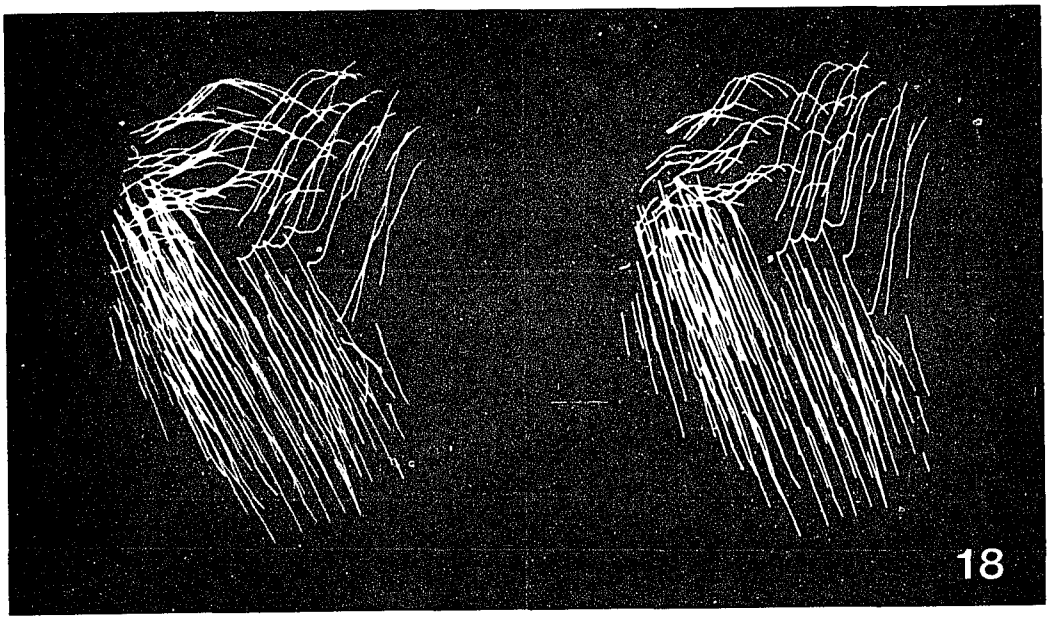
Figure 19. Diagrams illustrating arrangement of thylakoid membranes in hemispherical poles of cells that have completed cell division but have not yet separated from each other. Diagrams represent theoretical longitudinal sections passing through the central longitudinal axis of the cell.

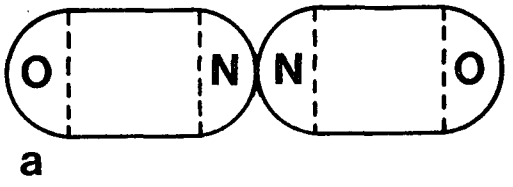
a. Outline of dividing cell pair. "Old" poles (O) are defined as those existing prior to cell elongation and division. "New" poles (N) are defined as those formed during division and which still face each other before cells separate.

b. Thylakoid arrangement in "old" poles; membranes merge at a common point near tip of cell.

c. Thylakoid arrangement in "new" poles; membranes terminate at edge of cell without merging with each other.







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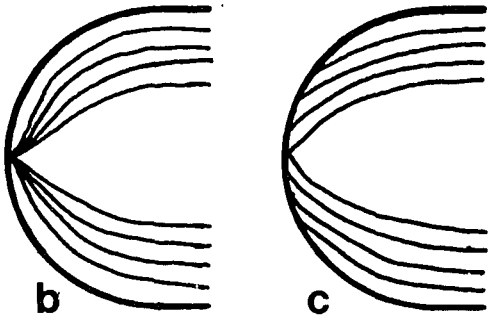


19

either arrangement or any of a variety of apparent transitions between the two.

Relationship Between Cytoplasmic and Thylakoid Membranes

CARTOS reconstructions indicated that thylakoid sheets tended to converge and approach the periphery of the cell, but these reconstructions lacked the resolution needed to see the precise details in such structurally complex areas. Examination of thick cross sections by HVEM served to indicate precisely how the individual thylakoid membranes intersected with each other at these locations. Again, the results were consistent from one cell (or section) to another only after tilting with a goniometer stage that compensated for variations in the direction of sectioning. In most untilted cross sections, the thylakoids appeared to connect with each other or to terminate at a variety of locations near the edge of the cell. By tilting, however, it was possible to show that all the thylakoids intersected with each other only at a common point at or quite near the surface of the cytoplasmic membrane (Fig. 20). The effect of tilting could be quite confusing because the clarity of the membranes varied as the viewing angle changed. Therefore, it was necessary to observe the membranes carefully while the sections were being tilted in the microscope, in order to be certain that the same membranes were seen before and after tilting (Fig. 20). When this was done, it became apparent that the thylakoids in all cells consistently intersected with each other only at common points at or near the surface of the cytoplasmic membrane.

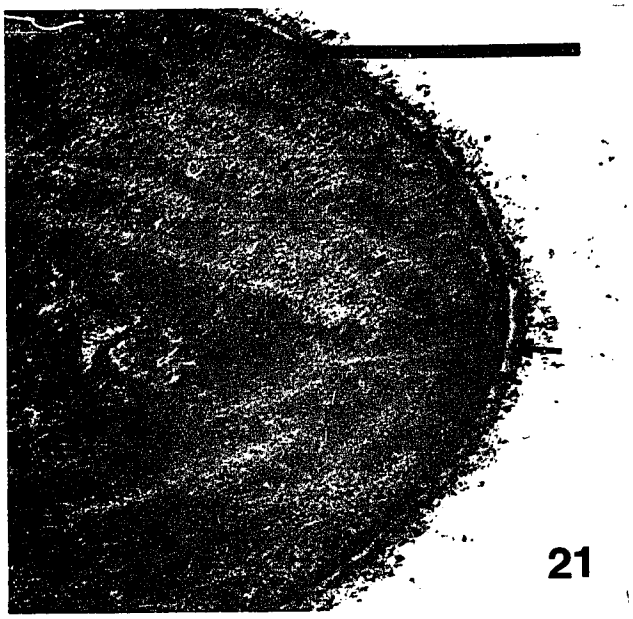
In addition to elucidating the way in which thylakoid membranes intersected with each other near the cytoplasmic membrane, HVEM of

Figure 20. Thick (0.25-μm) cross section through cylindrical portion of typical A. quadruplicatum cell before (Fig. 20a) and after (Fig. 20b) tilting about two perpendicular axes. Bars = 0.2 μm. Detail of peripheral thylakoid intersection. Tilting helped to indicate that thylakoid membranes actually intersected with other on or very near the inner surface of the cytoplasmic membrane (arrow).

Figure 21. Thin cross section through cylindrical portion of typical A. quadruplicatum cell, depicting apparent thylakoid-cytoplasmic membrane interconnections (arrow). Bar = 0.3 μm. Note that interconnection is not as well contrasted or as clearly illustrated as in thick (0.25-μm) sections (Fig. 22).

20a

20b



21

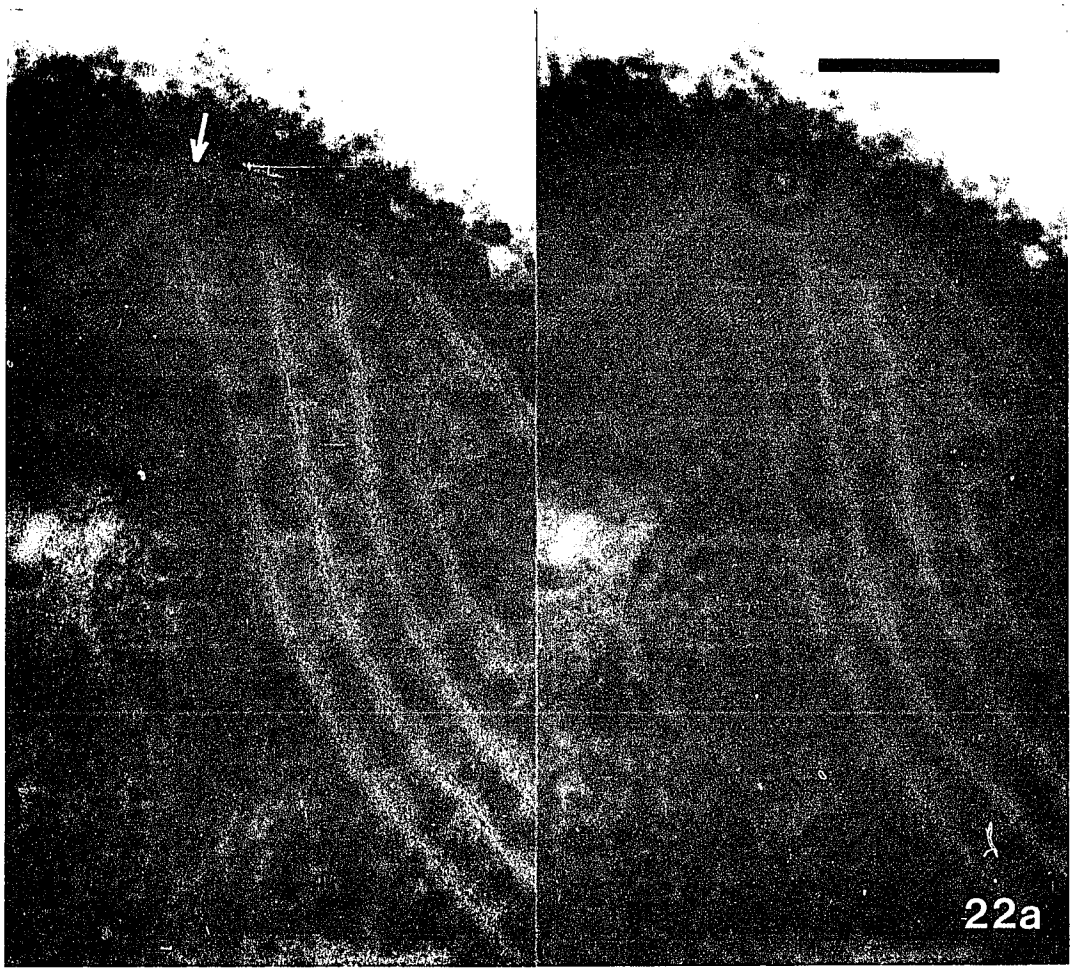
thick sections revealed that the thylakoids also interconnected with the cytoplasmic membrane at several sites throughout the cell. These interconnections were exceedingly difficult to find in randomly cut thin sections, being seen only very rarely (Fig. 21). In contrast, they were relatively easy to detect in thick sections when those sections were tilted to find the optimal viewing direction for observation of the interconnections. In the final micrographs, detailed interpretation of the thylakoid-cytoplasmic membrane interconnections was facilitated by observation of the three-dimensional information present in stereo pairs. Two examples of interconnections seen in this way are shown in Fig. 22. In some cases, the interconnections appeared to be actual invaginations of the cytoplasmic membrane itself but it was not possible to ascertain definitely that this was the case.

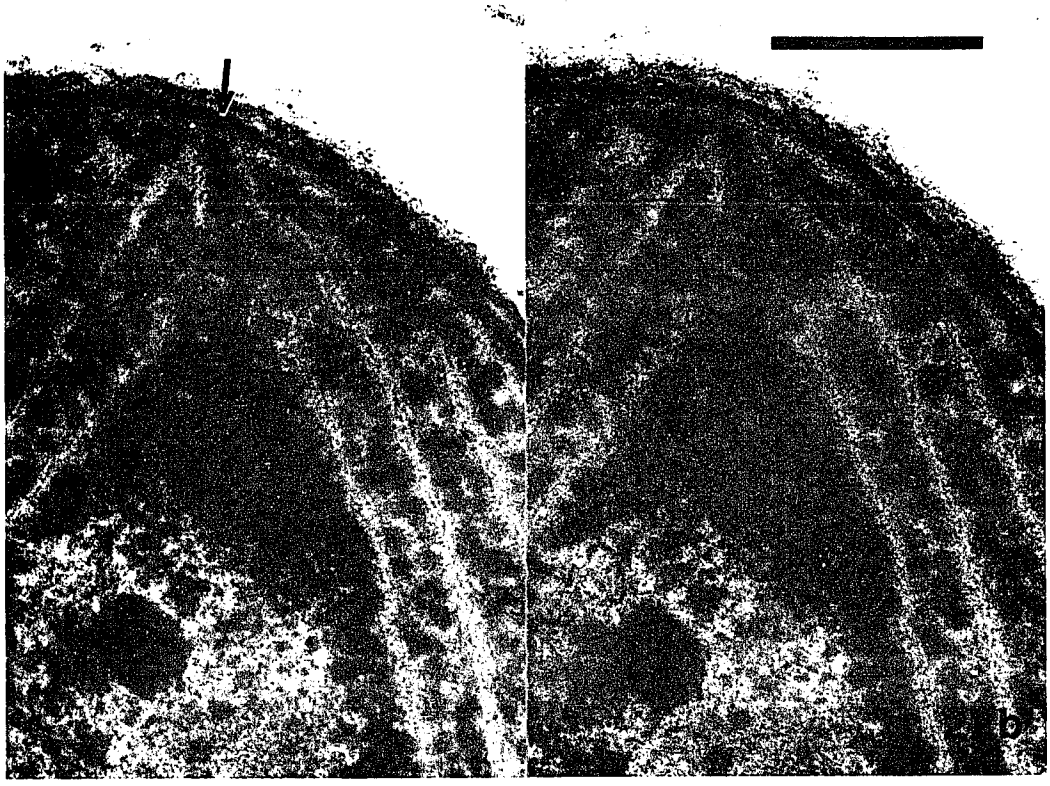
Serial thick cross sections through the cylindrical portion of the cell were examined to determine how frequently the thylakoid system interconnected with the cytoplasmic membrane in this part of the cell. In almost every section, interconnections were present at all three of the locations where the thylakoids approached the cytoplasmic membrane (as originally shown in Fig. 11) as they intersected with each other. (This was also the case for the variant pattern shown in Fig. 16 because the thylakoids approached the cytoplasmic membrane at only three of the four sites where they intersected with each other.) Serial cross sections through the cylindrical portion of the cell were also examined for thylakoid-cytoplasmic membrane interconnections, but the connections were in only 10-20% of those sections. The thylakoid-cytoplasmic membrane interconnections, then,

Figure 22. Stereo pairs of thick (0.25-μm) sections through A. quadruplicatum cells, depicting interconnections between thylakoid and cytoplasmic membranes (arrows). Bars = 0.2 μm.

a. Interconnection in an oblique section.

b. Interconnection in a near cross section.


22a


b

were intermittent rather than continuous along the length of the cylindrical portion of the cell.

HVEM of thick longitudinal sections served to determine whether thylakoid-cytoplasmic interconnections were present in the hemispherical cell poles of A. quadruplicatum. The innermost thylakoid sheet always terminated and interconnected with the cytoplasmic membrane at the tip of the cell. One or more of the outer thylakoids also terminated at this point in some cases. The thylakoid system of A. quadruplicatum, then, was in contact with the cytoplasmic membrane at both poles and at intermittent points along three lines that traversed the entire length of the cell. The data were insufficient to determine the spacing between the intermittent contact points or the consistency thereof.

Visualization of the Complete Agmenellum quadruplicatum Cell

It was decided to conclude this portion of the study by finding ways to illustrate as accurately as possible the complete three-dimensional structure of typical A. quadruplicatum cells. Consequently, CARTOS reconstructions were devised in which different colors were arbitrarily assigned to different cell features (Fig. 23). This approach facilitated the inclusion of more structures than could be shown by using different display modes (Figs. 7, 12, and 13) because the color differences were more effective in assisting the viewer to separate and identify overlapping cell structures. It was possible to include three types of inclusion bodies and at least two complete thylakoid membranes in these color reconstructions, thereby conveying a reasonably effective and accurate picture of A. quadruplicatum's overall three-dimensional architecture.

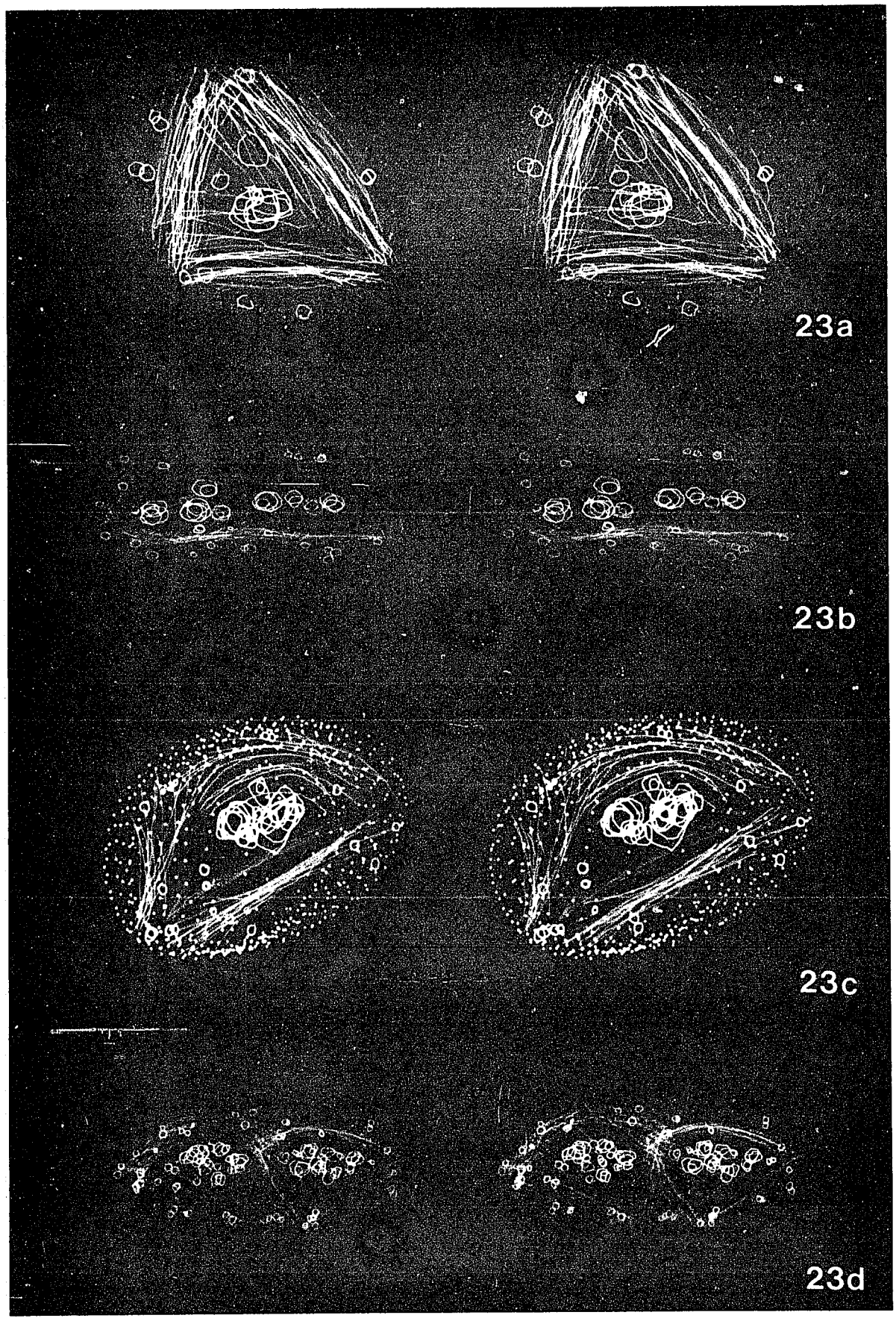
Figure 23. Stereo pairs of CARTOS reconstructions in color, illustrating a variety of features in complete cells.

a. Cell (outline in white dots) including most thylakoids (purple, yellow, green, blue, and red lines, starting with innermost membrane), carboxysomes (large white circles), and lipid bodies (small yellow circles). Cell is viewed along central longitudinal axis. Front pole of cell has been deleted to eliminate overlapping of thylakoids. Spacing between individual thylakoids is best seen along bottom edge of triangles in thylakoid pattern. Thylakoids taper toward cell tip at back of cell.

b. Side view of cell (outline in white dots) including two complete thylakoids (innermost in blue lines, outermost in green lines), carboxysomes (white circles), polyphosphate bodies (large, centrally located yellow circles), and lipid bodies (small, peripherally located yellow circles). Separation between the two thylakoids is best seen along bottom edge of cell. Tapering of thylakoids toward cell pole is especially clear at left. Note how thylakoids completely surround central cytoplasmic region of cell. Compare peripheral location of lipid bodies with central location of carboxysomes and polyphosphate bodies.

c. Different view of reconstruction shown in Fig. 23b; cell is viewed down its central longitudinal axis. Note triangular pattern formed by thylakoids in cylindrical portion of cell. Part of the outermost thylakoid has been deleted to avoid excessive overlapping of details.

d. Side view of a dividing cell (outline in white dots) including two complete thylakoids, carboxysomes, polyphosphate bodies, and lipid bodies (all depicted as in Figs. 23b and 23c). Thylakoids completely surround central cytoplasmic region in both halves of the cells and clearly taper to single points at both cell poles. Compare central location of densely clustered carboxysomes and polyphosphate bodies with peripheral location of lipid bodies.


23a
23b
23c
23d

A final summarization of the information obtained in this portion of the study was achieved by using the above reconstructions to help produce an artist's drawing of a typical cell. The result is shown in Fig. 24. The most important three-dimensional aspects of A. quadruplicatum that can be seen in this drawing include: (i) the peripheral location of the lipid bodies, (ii) the central location of the carboxysomes and polyphosphate bodies, (iii) the overall arrangement of the thylakoid membrane system, (iv) the manner in which individual thylakoids intersect with each other, and (v) the nature of the interconnections between the thylakoid and cytoplasmic membranes.

The Morphology and Ultrastructure of Mastigocladus laminosus

General Morphological Characteristics

Morphology of NO_3-Grown Cultures. The morphological characteristics of NO_3-grown M. laminosus observed by scanning electron microscopy (Fig. 25) were similar to those seen by light microscopy in earlier studies (Marcenko, 1961; Rippka et al., 1979; Schwabe, 1960) and those reported for the closely related genus Fischerella (Martin and Wyatt, 1974; Rippka et al., 1979; Thurston and Ingram, 1971). Mature liquid cultures consisted of a dense, pleomorphic mat of intertwined filaments (Fig. 25a). The older, wider filaments (Figs. 25a-25d) ranged up to 12 μm in diameter. They contained relatively large, rounded cells that varied considerably in diameter and shape (compare Figs. 25a-25c). Some of these cells gave rise to lateral branches off the main filament (Fig. 25c). Such branches

Figure 24. Artist's representation of the overall three-dimensional architecture of a typical A. quadruplicatum cell. C = carboxysomes; L = lipid body; M = cytoplasmic membrane; P = polyphosphate body; T = photosynthetic thylakoid membrane system; TC = interconnections between thylakoid and cytoplasmic membranes. Thylakoids are depicted as solid sheets, each representing a pair of closely apposed unit membranes. The spacing between thylakoid-cytoplasmic membrane interconnections is theoretical, as this was not determined precisely (see text). (Illustration does not include cell wall, ribosomes, and nuclear material.)

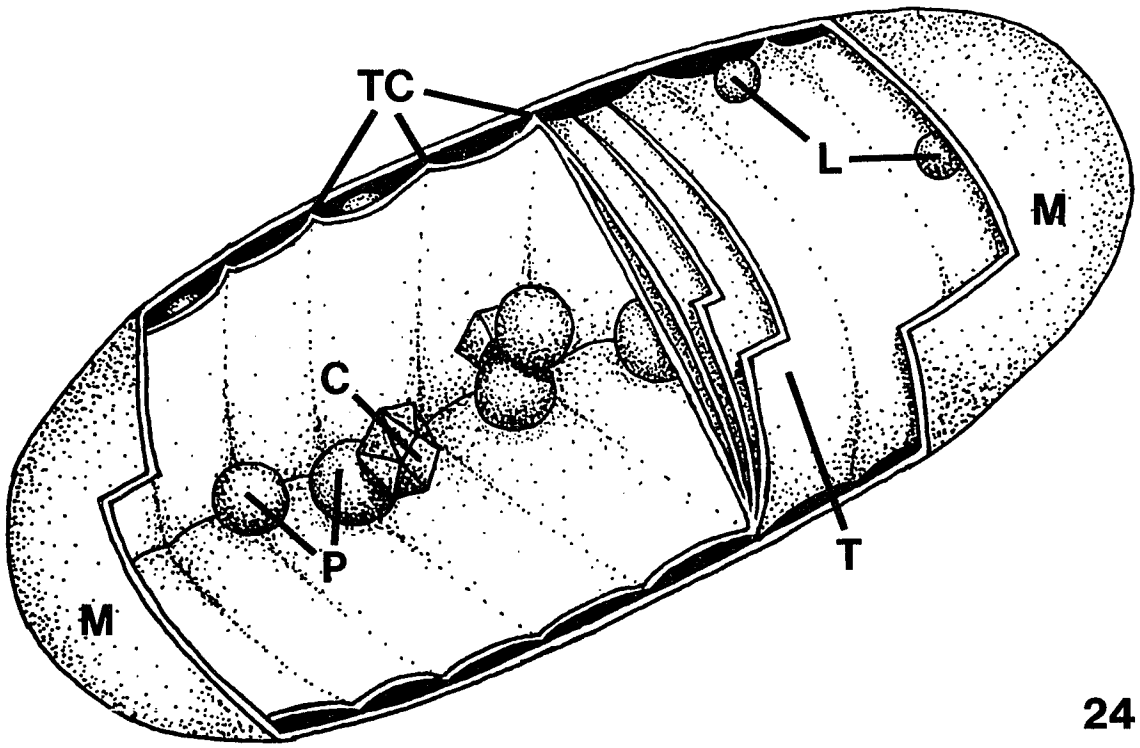


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Figure 25. Scanning electron micrographs of critical-point-dried, whole-cell preparations of M. laminosus, depicting general morphological characteristics of NO_3-grown cultures. B = lateral branch point; O = old filament; Y = young filament.

a. Dense portion of algal mat, showing a variety of filament morphologies. Bar = 15.0 μm.

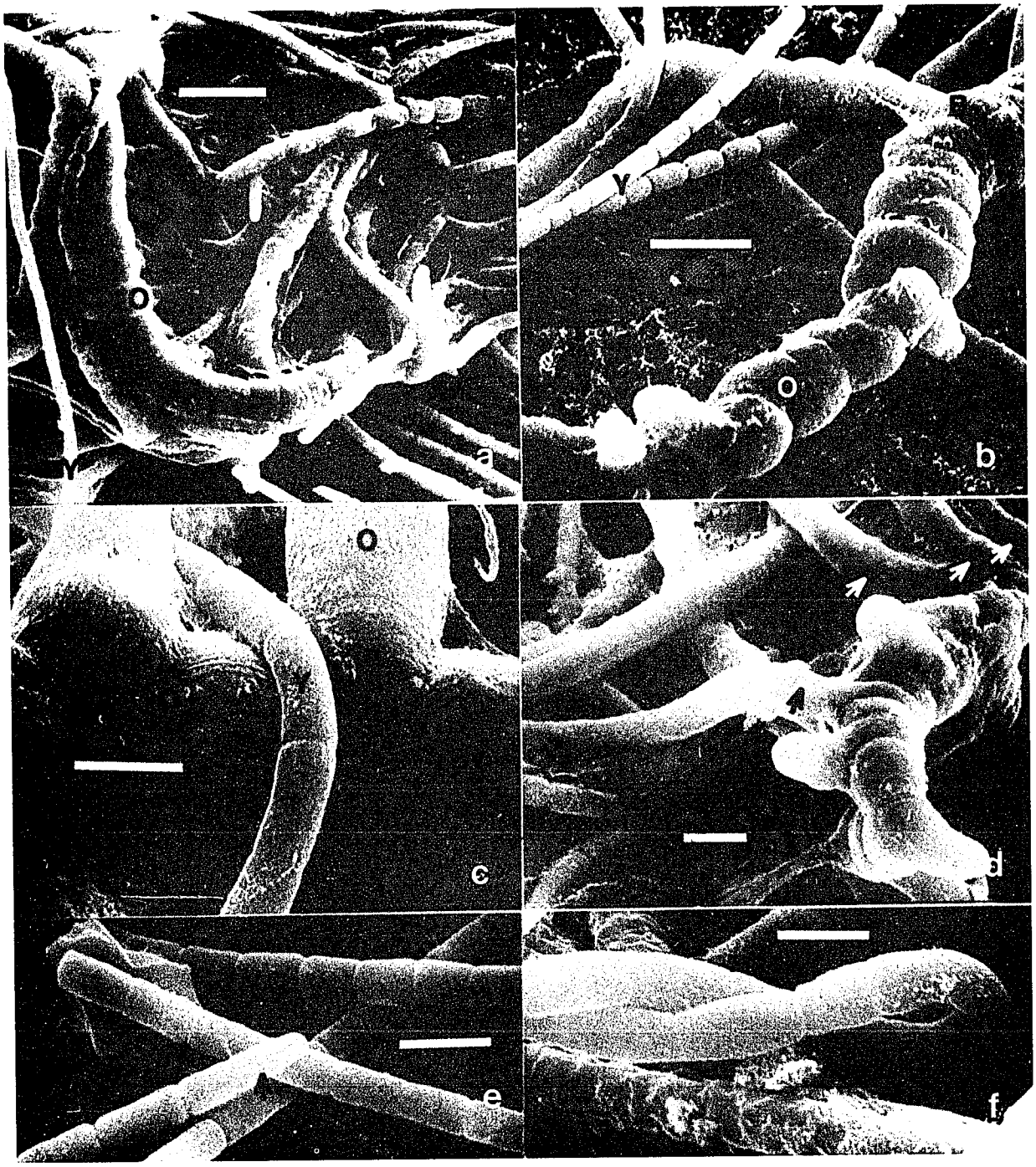
b. Pleomorphic cells in old filament contrasted with uniform cells in young filaments. Bar = 10.0 μm.

c. Detail of tapered, lateral branches off of old filament. Note difference in young and old filament diameters. Bar = 3.0 μm.

d. Multibranched portion of old filament, showing a number of branches elongated and tapered to form young filaments (arrows). Bar = 5.0 μm.

e. Detail of young filaments, showing tapered morphology of terminal cell (arrow). Bar = 5.0 μm.

f. Detail of young filament that has doubled back on itself and formed a helical braid. Bar = 3.0 μm.

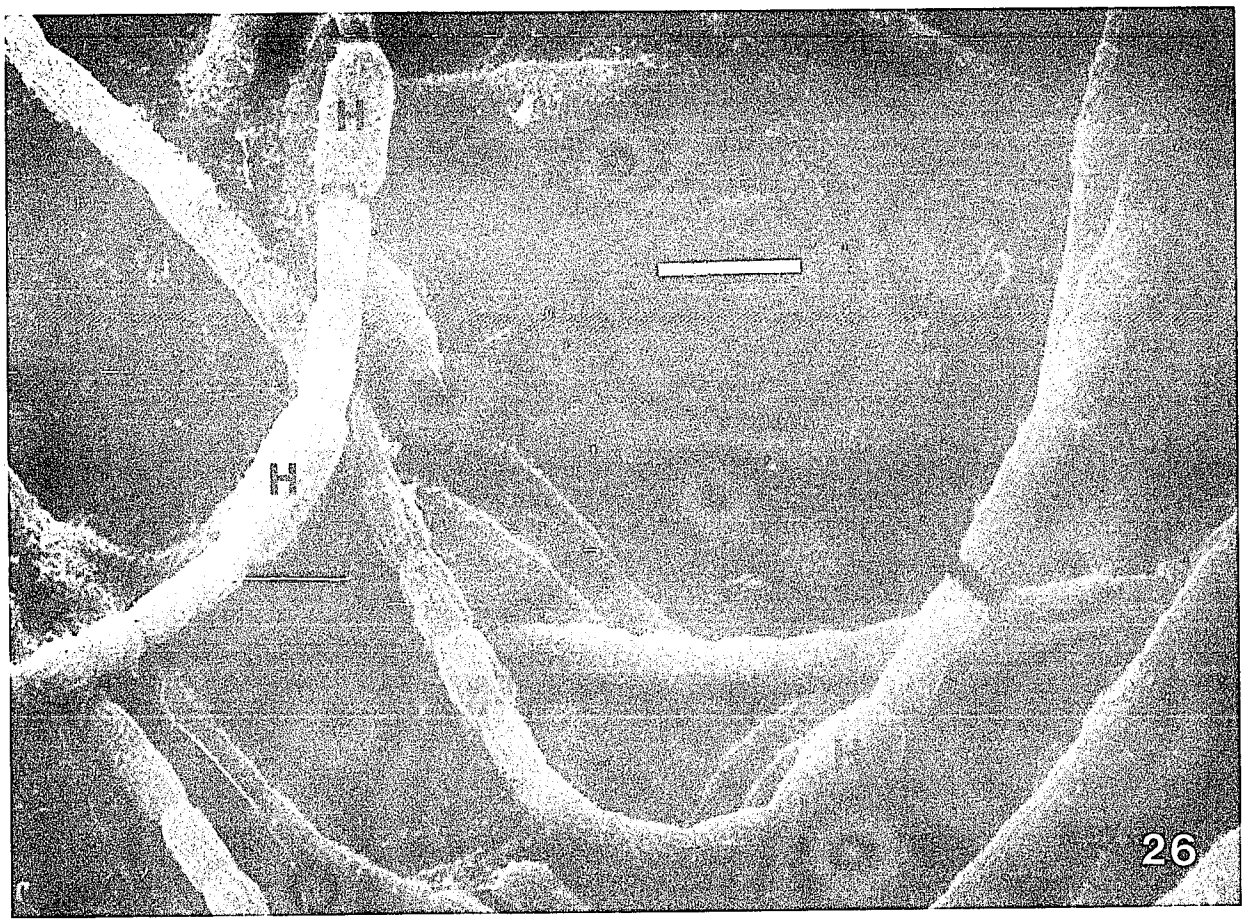

a
b
c
d
e
f

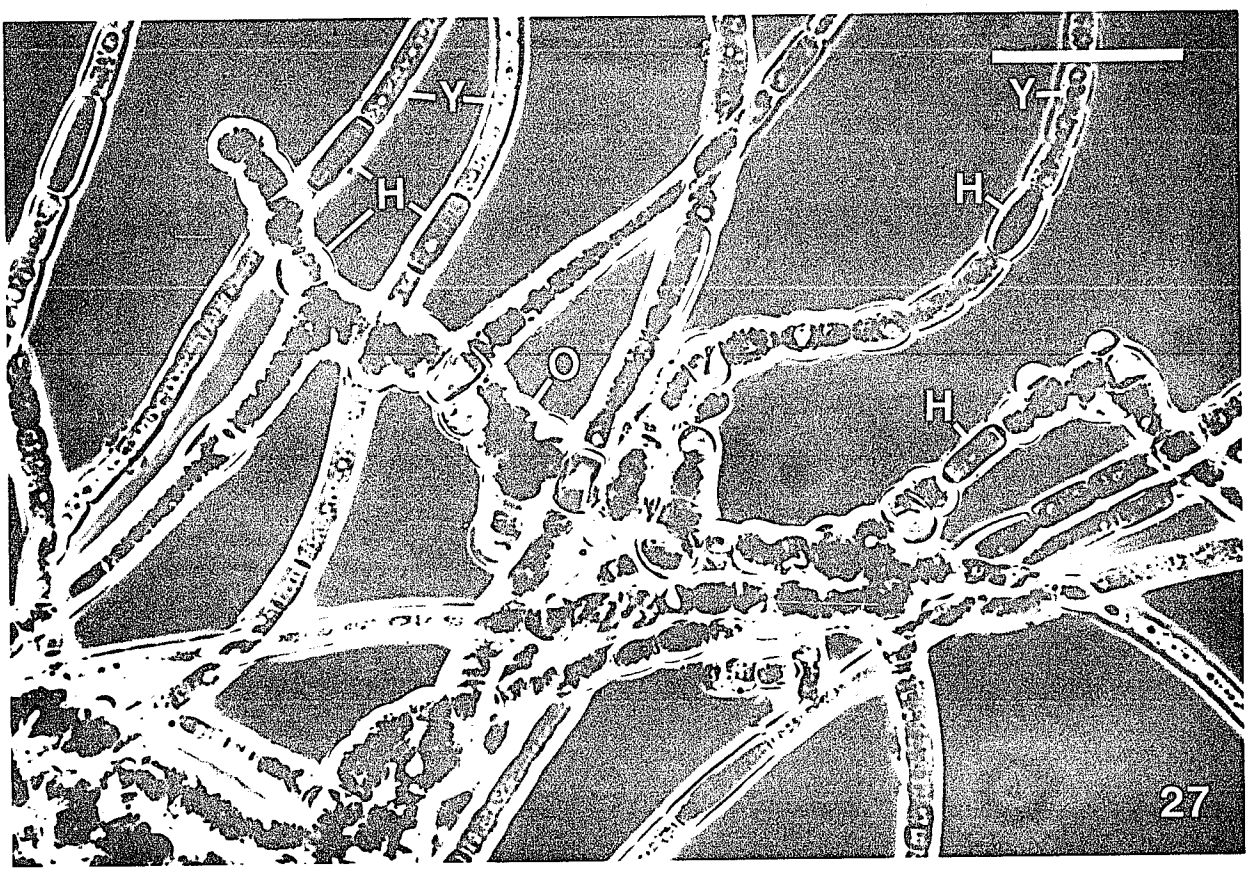
became quite numerous in the widest, most pleomorphic portions of the old filaments (Fig. 25d). Some of the lateral branches off the main filament elongated and tapered to form the younger, thinner filaments of the algal mat (Fig. 25d). The latter also arose from the growing termini of the main filaments. The young filaments differed morphologically from the older filaments in that they were considerably thinner (1.7-2.5 μm in diameter), less pleomorphic, and contained distinctly cylindrical cells (Figs. 25a, 25e, and 25f). Except for the tapered terminal cells (Fig. 25e), the individual cells of the young filaments were relatively consistent in terms of their morphological characteristics. Most of the young filaments were fairly straight, but some of them doubled back on themselves and formed helical braids (Fig. 25f).

Morphology of N_2-Grown Cultures. The general morphological characteristics of N_2-grown M. laminosus cultures, as examined by scanning electron microscopy, were almost identical to those of NO_3-grown cultures (above). The only noticeable difference was the presence of a few enlarged cells within the otherwise uniformly thin, young filaments (Fig. 26). Phase-contrast light microscopy (Fig. 27) indicated that these enlarged cells were probably heterocysts and proheterocysts, according to the descriptive criteria of Bradley and Carr (1976) and Rippka and Stanier (1978). Light microscopy indicated that heterocysts and proheterocysts were also present in old filaments, but were difficult to see in the dense mats of intertwined and branching cells (see Fig. 27). Akinetes (Adams and Carr, 1981; Stanier and Cohen-Bazire, 1977) were not detected in any

Figure 26. Scanning electron micrograph of critical-point-dried, whole-cell preparation of M. laminosus, depicting morphological characteristics of young N_2-grown filaments. Heterocysts (H) are visible as enlarged cells. Bar = 5.0 μm.

Figure 27. Phase-contrast light micrograph of unstained wet mount of M. laminosus, depicting general morphological characteristics of N_2-grown culture. H = heterocyst; O = old filament; Y = young filament. Note that it is difficult to observe cell features in old filaments because they are often intertwined with other filaments. Bar = 20.0 μm.


H
H
26


Y
H
O
Y
H
H
27

filaments with either light microscopy or scanning electron microscopy.

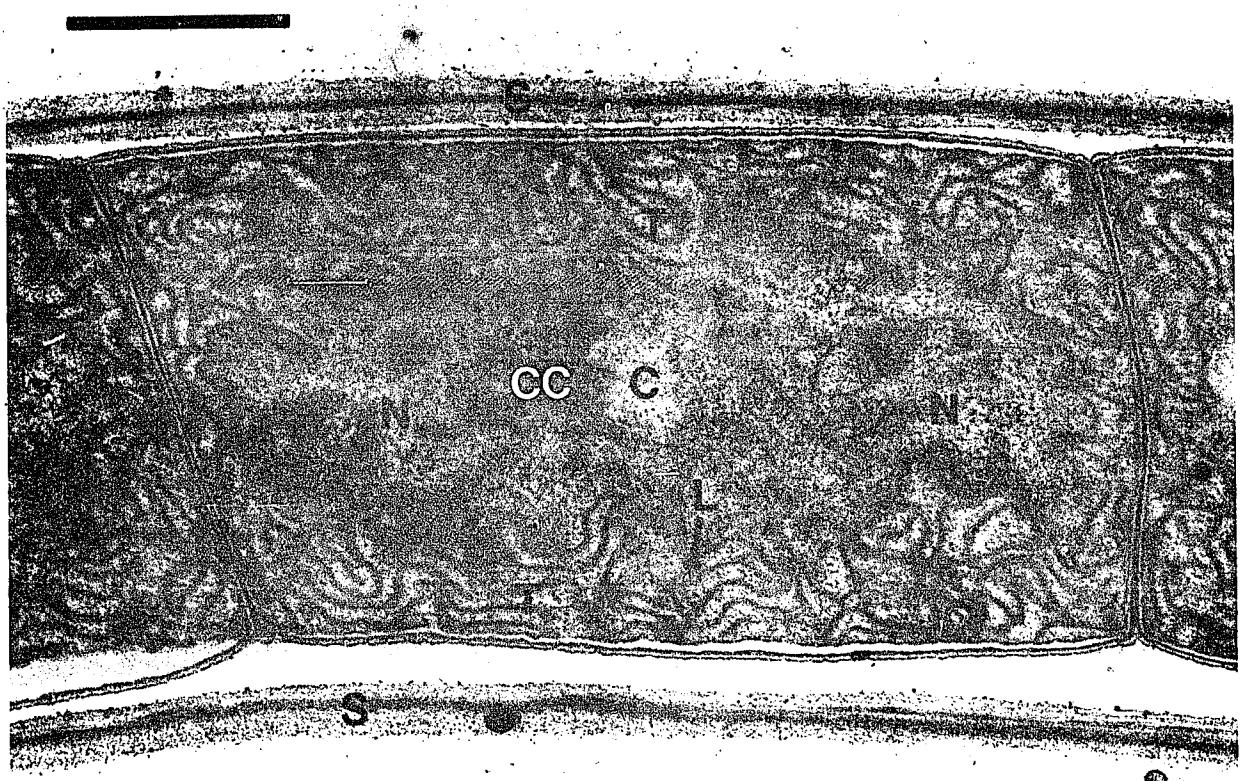
Ultrastructural Characteristics of Vegetative Cells

Ultrastructure of NO_3-Grown Cells. The cell envelope of all NO_3-grown M. laminosus cells was typical of that seen both in other cyanobacteria (Stanier and Cohen-Bazire, 1977; Wolk, 1973) and in other Gram-negative bacteria (Costerton et al., 1974). A common sheath layer surrounded all of the cells in each filament (Fig. 28) and was usually separated from the outer membrane by an electron-transparent zone (perhaps the result of cell shrinkage during dehydration).

The internal ultrastructural characteristics of a typical cell in a young, thin filament are shown in Fig. 28. The cytoplasm of these cells consisted of two reasonably distinct and separate units: a central cytoplasmic region and a peripheral thylakoid region. The central cytoplasmic portion of the cell contained nuclear material, most of the ribosomes, and all of the carboxysomes. From 1 to 3 carboxysomes were present in each longitudinal section that appeared to traverse the center of the cell (Materials and Methods). The peripheral thylakoid portion of the cell contained the photosynthetic thylakoid membranes, phycobilisomes, and lipid bodies. The lipid bodies (4-7 per section) were distributed throughout the thylakoid portion of the cell but were never located in the central cytoplasm. The thylakoid membranes themselves were arranged in tight pairs that tended to run parallel to one another. The areas between the thylakoids were somewhat less electron-dense than the central cytoplasm.

Figure 28. Longitudinal thin section through typical young NO_3-grown M. laminosus cell. C = carboxysome; CC = central cytoplasm; L = lipid body; N = nuclear material; S = external sheath layer; T = photosynthetic thylakoid membranes. Bar = 1.0 μm.

Figure 29. Longitudinal thin section through typical old NO_3-grown M. laminosus cell. C = carboxysome; L = lipid body; P = polyphosphate body. Bar = 1.0 μm.



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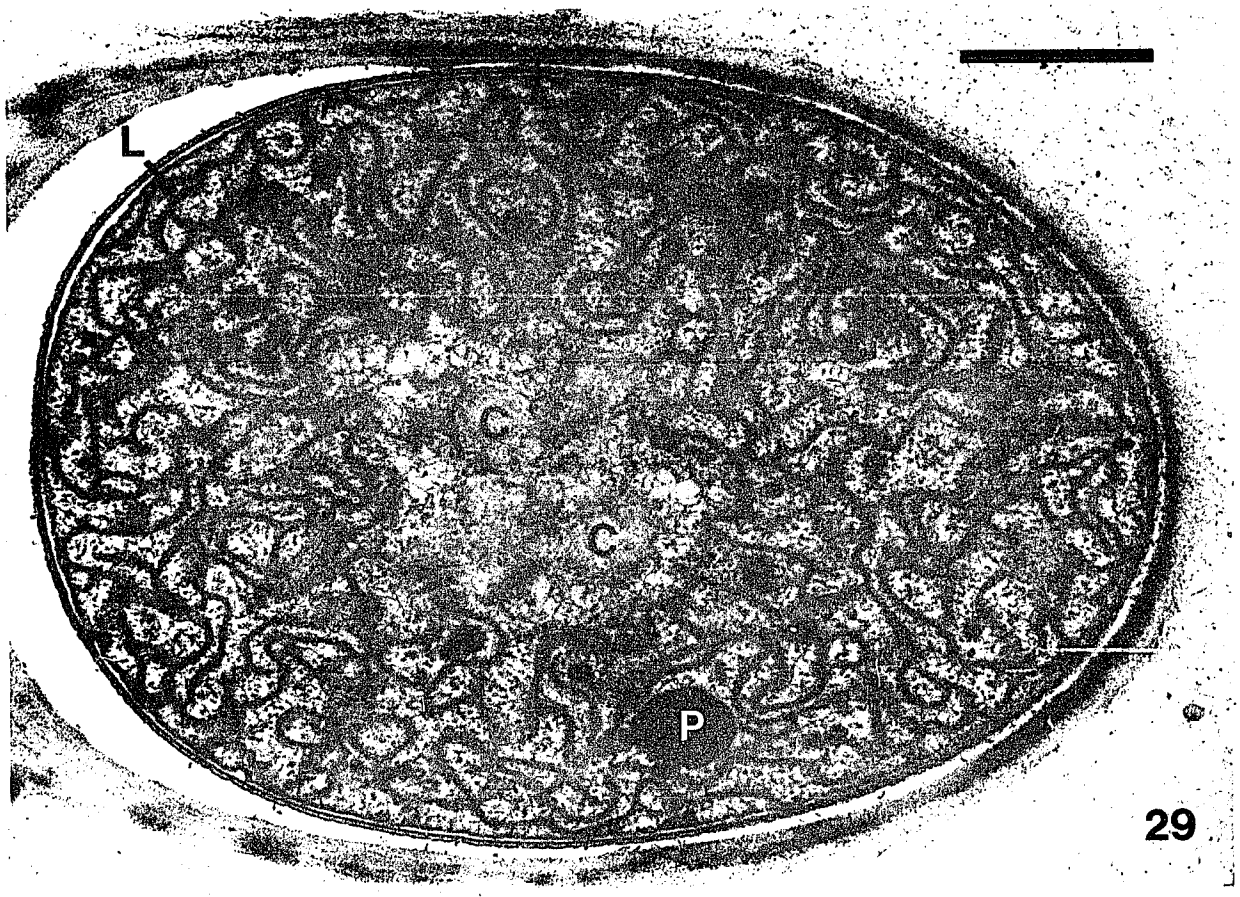

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The ultrastructural characteristics of cells in the older, wider filaments (Fig. 29) differed from those of younger cells (Fig. 28) in several ways. There was less of a clear distinction between the central cytoplasmic and peripheral thylakoid portions of the cell because the thylakoids usually extended into the central portion of the cell and became intertwined with the nuclear material. The thylakoids also had less tendency to run parallel to one another. The number of lipid bodies was higher (22-32 per section), as was the number of carboxysomes (6-10 per section). Polyphosphate bodies were also present in the older cells (1-3 per section). The ribosomal content of each old cell was reduced considerably; it was difficult to locate any ribosomes in most older cells. This led to a decrease in cytoplasmic electron density and caused the nuclear fibrils to spread out toward the peripheral portion of the cell.

Ultrastructural Characteristics of N_2-Grown Cells. The external ultrastructural features of N_2-grown M. laminosus cells were essentially identical to those of NO_3-grown cells (above). No obvious differences were detected in the ultrastructure of either the cell envelope or the common sheath layer.

The internal ultrastructural characteristics of a typical cell in a young, N_2-grown filament are shown in Fig. 30. In contrast to those of young cells in NO_3-grown filaments (above), the thylakoids in N_2-grown cells did not tend to run parallel to one another. Instead, they were randomly scattered throughout the peripheral portion of the cell. The thylakoid system consisted of many small membrane segments, rather than a few large membrane sheets. In this

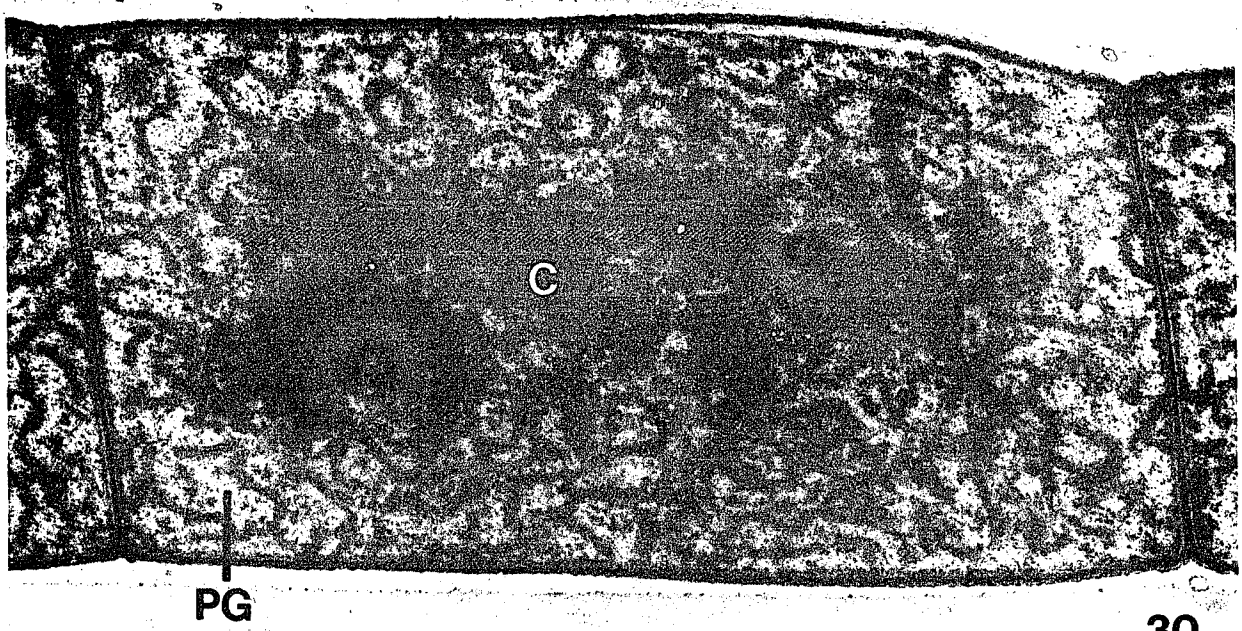
characteristic, the thylakoids were somewhat similar to those of old cells in the NO_3-grown cultures (Fig. 29). The areas between the thylakoid membranes were considerably less electron-dense than the central cytoplasm. Some phycobilisomes were present in these areas, but for the most part, they were filled with large numbers of electron-transparent polysaccharide granules or "α-granules" (Chao and Bowen, 1971; Pankratz and Bowen, 1963; Wolk, 1973). These polysaccharide granules were considerably more numerous than in young NO_3-grown cells (Fig. 28). Carboxysomes were also more numerous in N_2-grown cells (6-15 per section), while lipid bodies (0-3 per section), ribosomes, and nuclear material were present in noticeably lower amounts.

The ultrastructural characteristics of a typical cell in an old N_2-grown filament are shown in Fig. 31. As was the case in NO_3-grown cultures, the distinction between the thylakoidal and central cytoplasmic regions was less clear in old cells because the thylakoids tended to spread throughout the cytoplasm and become intertwined with nuclear material. Carboxysomes (8-22 per section) and lipid bodies (4-17 per section) were more numerous than in young N_2-grown cells.

Ultrastructure of N-Starved Cells. Nitrogen starvation did not noticeably affect the ultrastructural characteristics of either the cell envelope or the common sheath layer in M. laminosus. However, the internal, ultrastructural characteristics of both young (Fig. 32) and old (Fig. 33) cells changed markedly during nitrogen starvation. Fluctuations in the numbers of inclusion bodies were especially noticeable in both cases and are summarized in Tables 1 and 2. The

Figure 30. Longitudinal thin section through typical young N_2-grown M. laminosus cell. C = carboxysome; PG = polysaccharide granules. Bar = 1.0 μm.

Figure 31. Cross section (thin) through typical old N_2-grown M. laminosus cell. C = carboxysome; L = lipid body. Bar = 1.0 μm.



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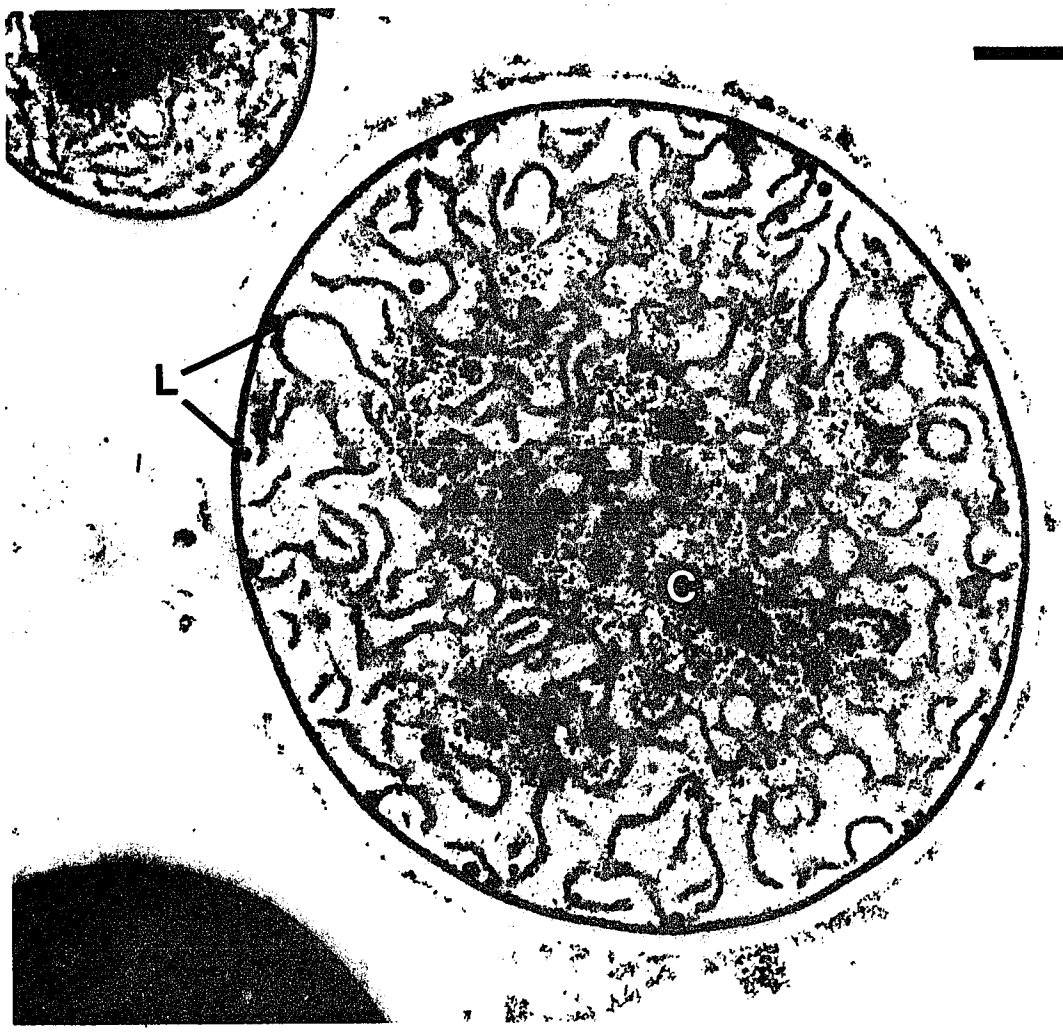

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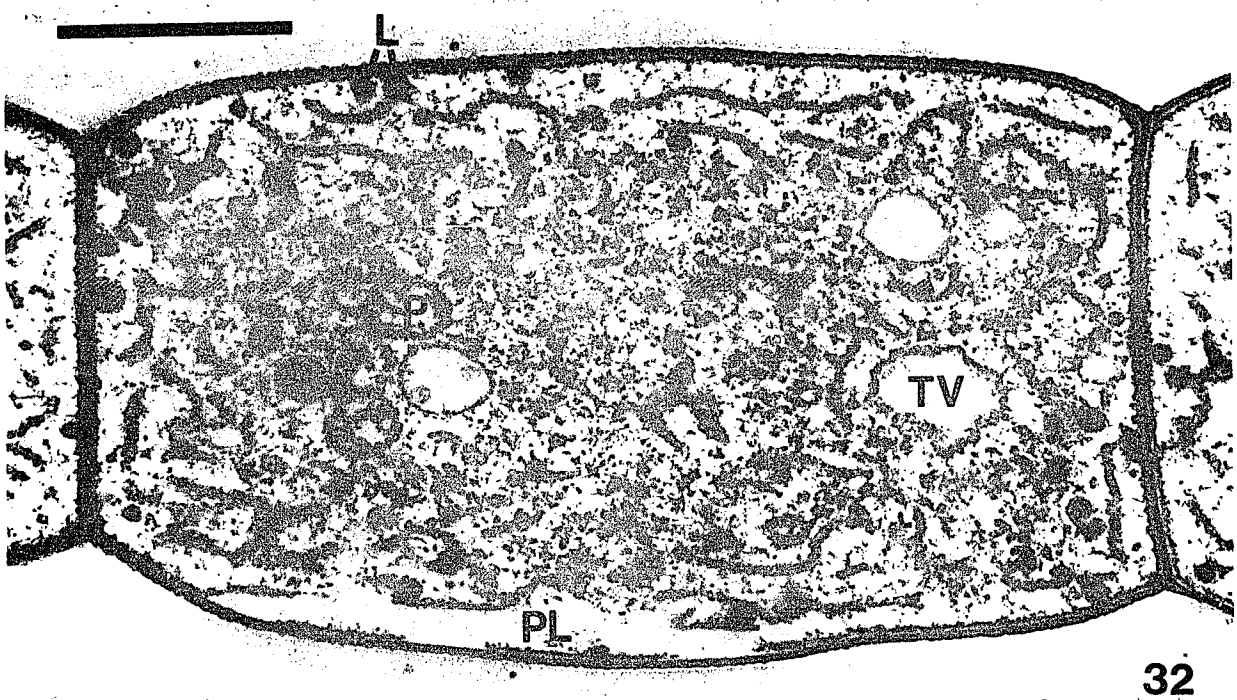
Figure 32. Longitudinal thin section through typical young N-starved M. laminosus cell. L = lipid body; P = polyphosphate body; PL = plasmolysis; TV = thylakoid vesiculation. Bar = 1.0 µm.

Figure 33. Longitudinal thin section through typical old N-starved M. laminosus cell. L = lipid body; P = polyphosphate body; PL = plasmolysis; TV = thylakoid vesiculation. Bar = 1.0 µm.



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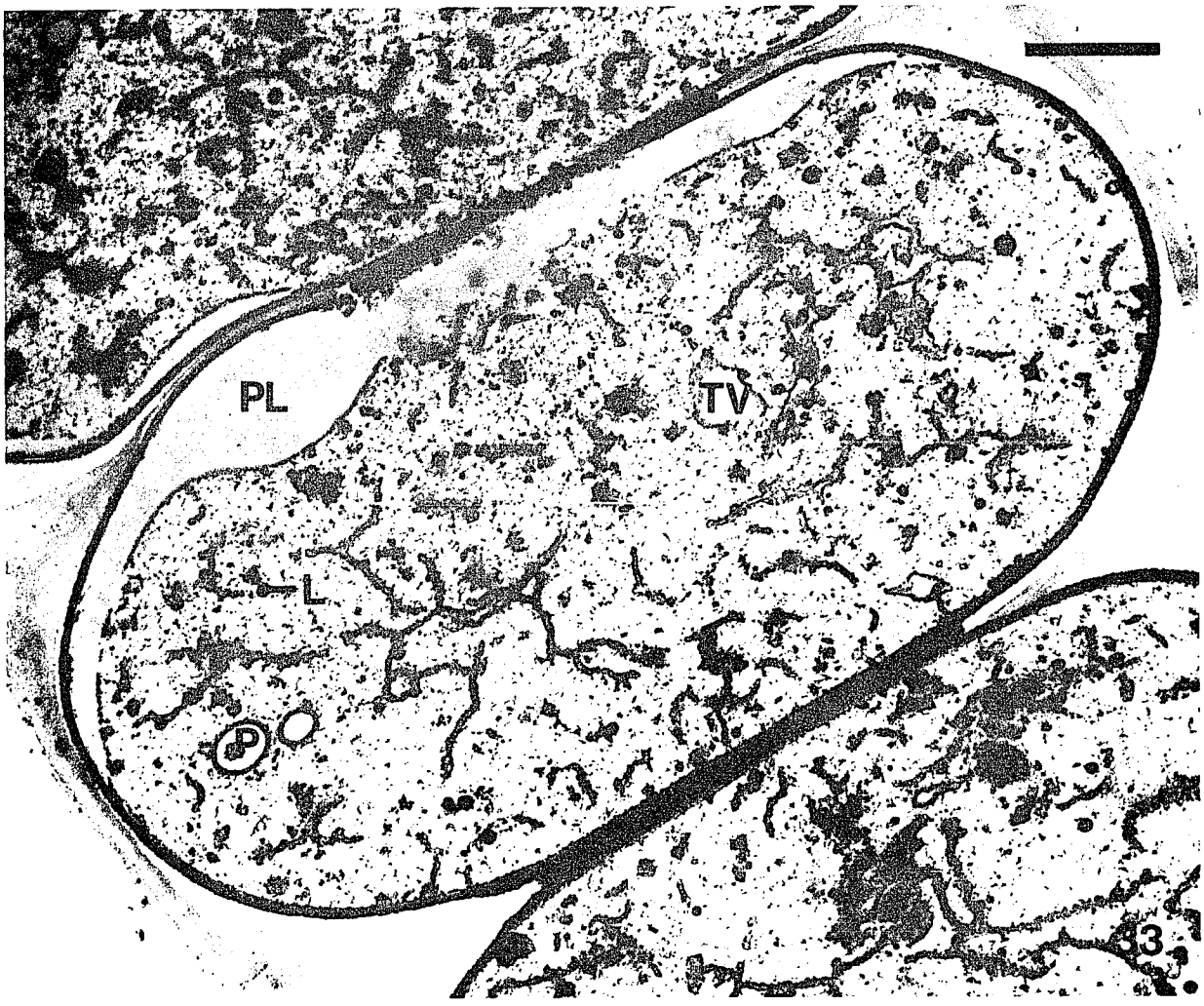

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TABLE 1. Effects of Nitrogen Starvation on the Numbers of Specialized Inclusion Bodies in Young Cells of *M. laminosus*

	Inclusion body		
	Carboxysomes	Lipid bodies	Polyphosphate bodies
Time (h)	No. per longitudinal section*		
0	6 - 15	0 - 3	Rare
6	5 - 8	6 - 13	Rare
19	0 - 1	11 - 25	0 - 1
25	0 - 1	15 - 38	0 - 1
44	0 - 1	9 - 20	0 - 3

*Data taken only from longitudinal sections that passed through the center of a cell.

TABLE 2. Effects of Nitrogen Starvation on the Numbers of Specialized Inclusion Bodies in Old Cells of *M. laminosus*

	Inclusion body		
	Carboxysomes	Lipid Bodies	Polyphosphate bodies
Time (h)	No. per longitudinal section*		
0	8 - 22	4 - 17	0
6	5 - 17	11 - 34	0
19	0 - 1	N.D.**	0 - 2
25	0 - 1	11 - 30	0 - 1
44	0 - 1	20 - 36	0 - 2

*Data taken only from longitudinal sections that passed through the center of a cell.

**N.D. = Insufficient data.

carboxysomes were depleted rapidly and extensively in response to nitrogen starvation, disappearing almost entirely within 19 h after the switchover from CO_2/N_2 to CO_2/argon (Materials and Methods). In contrast, the number of lipid bodies increased considerably. Polyphosphate bodies appeared in about 75% of the young cells and in about 60% of the old cells (up to 3 per section) between 19 and 44 h. Phycobilisomes were degraded in both cells between 0 and 19 h, leading to a reduction in the electron density of the areas between the thylakoid membranes. The thylakoids themselves fragmented into smaller pieces and tended to vesiculate, especially between 19 and 44 h (Fig. 32 and Fig. 33). Plasmolysis was evident in many cells after the phycobilisomes were degraded (within 19 h).

Ultrastructure of the Heterocyst Differentiation Process

Vegetative cells in both young and old filaments were capable of differentiating into heterocysts. The sequence of ultrastructural changes that took place during heterocyst differentiation was the same in both cases. Consequently, young and old filaments are not considered separately in the following description.

The earliest ultrastructural changes related to heterocyst differentiation was quite subtle, being evident only as minor deviations in the otherwise uniform structure of cells within a single filament. The first such change was a decrease in the number of electron-transparent polysaccharide granules between the thylakoids, thereby leading to an increase in the overall electron density of the cell. The decrease in polysaccharide was always accompanied by the appearance of electron-dense ribosomal material (Fig. 34). For

the purposes of this study, cells displaying these subtle ultrastructural changes were arbitrarily termed "differentiating cells" because (i) they differed ultrastructurally from the rest of the cells in the same filament and (ii) their ultrastructural features bore some resemblence to those of the proheterocysts described below.

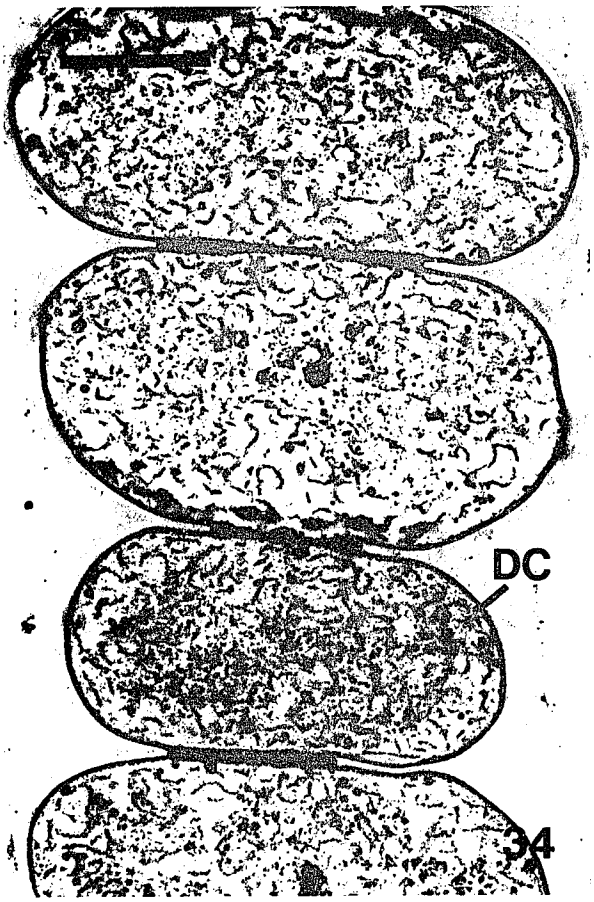
Proheterocysts (Literature Review) were defined as cells in which obvious ultrastructural changes had taken place, some of which clearly could be related to the larger formation of mature heterocysts. The earliest stages in proheterocyst development are shown in Figs. 35-37. There was a continued reduction in the number of electron-transparent polysaccharide granules between the thylakoids, resulting in the formation of structureless, light gray patches throughout the cell. The disappearance of polysaccharide was accompanied by two other events (Fig. 35): (i) the thylakoid segments tended to straighten out and elongate and (ii) the number of carboxysomes per section was greatly reduced (none in most sections, one or two small ones in the rest). As these early proheterocysts continued to develop, their overall electron density increased (Fig. 36). This was due to continued depletion of polysaccharide and an increase in the amount of thylakoid membrane. The thylakoid segments apparently were elongating and/or increasing in number. Membrane synthesis then continued (Fig. 37), leading to the production of longer or more continuous thylakoids and small bundles of closely apposed, lamellar stacks of membranes. At the same time, the poles of some heterocysts began to differentiate in order to form the "necks" (Carr, 1979) leading to the

Figure 34. Longitudinal thin section through old N_2-grown M. laminosus filament, depicting (DC) a typical "differentiating cell" (see text). Overall electron density of cytoplasm is greater than that of neighboring cells, owing to depletion of electron-transparent polysaccharide granules and accumulation of electron-dense ribosomal material. Bar = 2.0 μm.

Figure 35. Longitudinal thin section through very early proheterocyst in young N_2-grown M. laminosus filament. Small gray patches are present between thylakoids, owing to breakdown of polysaccharide granules that previously filled these areas. Note straightening thylakoids and lack of carboxysomes. Bar = 2.0 μm.

Figure 36. Longitudinal thin sections through early proheterocyst in young N_2-grown M. laminosus filament. Note increased accumulation of ribosomal material (R), compared to Fig. 35. Bar = 2.0 μm.

Figure 37. Longitudinal thin section through fully developed early proheterocyst in old N_2-grown M. laminosus filament. HL = homogeneous, extra wall layer; LS = bundles of closely apposed, lamellar stacks of membranes; NK = neck leading to adjacent cell. Bar = 2.0 μm.



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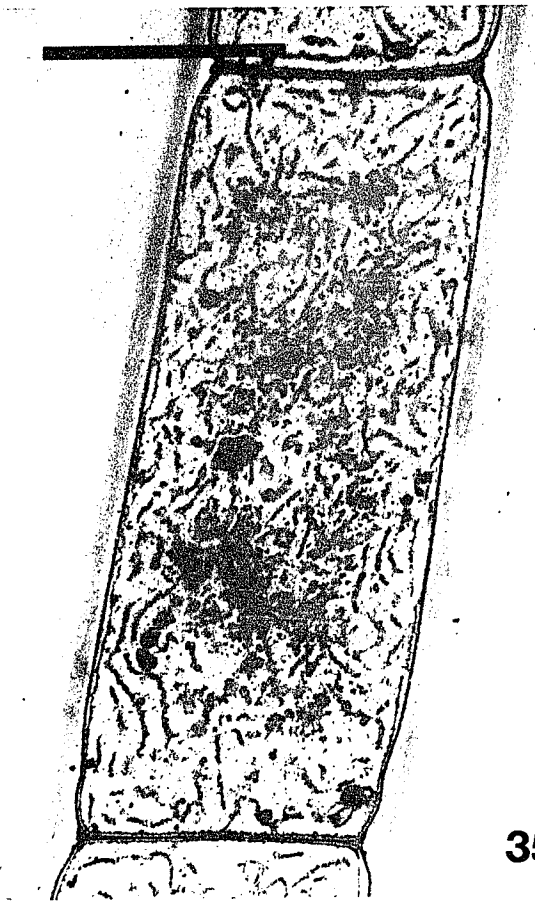

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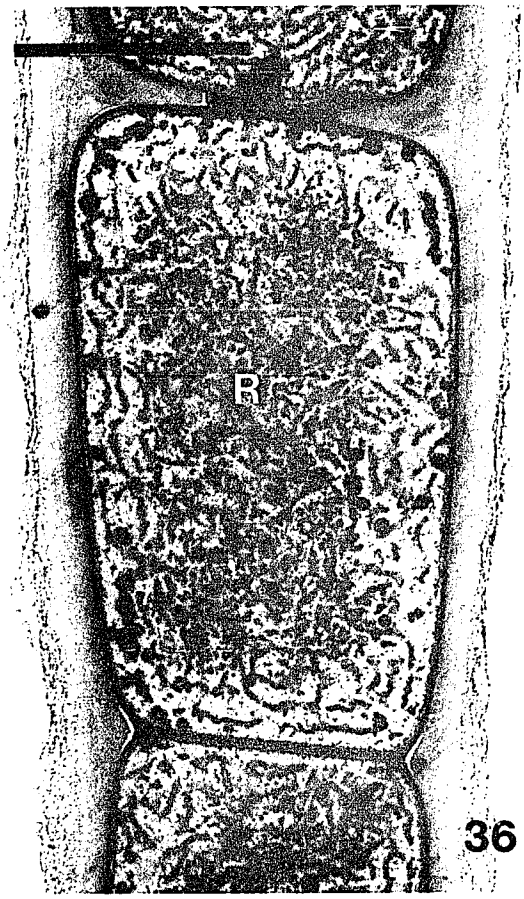

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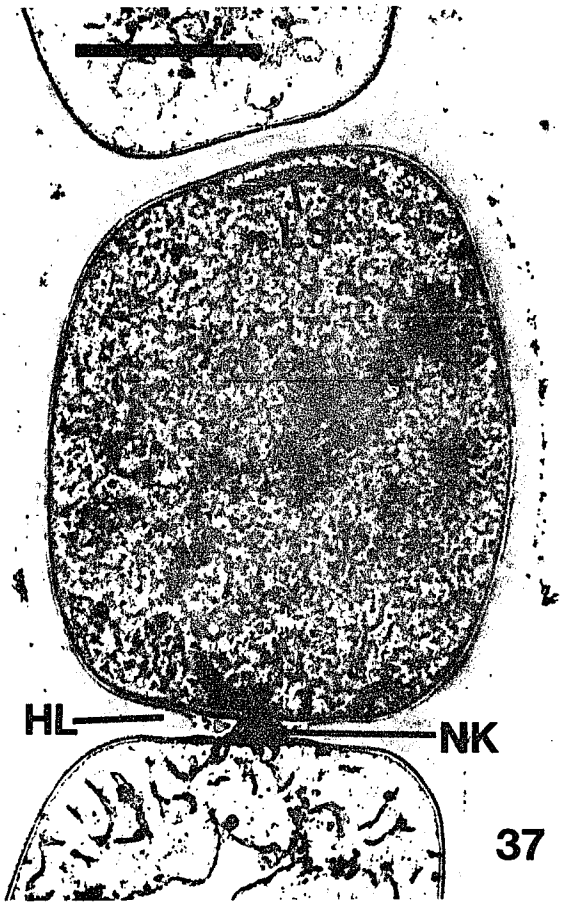

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adjacent cells in the filament, and a homogeneous, relatively electron-transparent extra wall layer began to appear at the outer surface of the outer membrane (Fig. 37).

The intermediate stages of proheterocyst development are illustrated in Figs. 38 and 39. Considerable amounts of new membrane synthesis resulted in the formation of larger and more numerous bundles of lamellar membrane stacks (Fig. 38). Most of these lamellar membrane bundles were located near the developing necks at each end of the cell, although some of the smaller bundles were located in other parts of the cell as well. Membrane synthesis also led to an increased amount of thylakoid membrane throughout the entire cell, thereby producing a marked increase in overall electron density. Intermediate proheterocysts usually had a "salt-and-pepper" appearance (Fig. 39), owing to their unusual arrangement of membranes mixed with electron-dense ribosomes and (the few remaining) electron-transparent polysaccharide granules. Lipid bodies were still numerous in the intermediate proheterocysts, but carboxysomes were not present. The homogeneous extra wall layer thickened and became more obvious, while in some cases, the terminal necks underwent further shaping and development.

The late stages in proheterocyst development are depicted in Figs. 40 and 41. Membrane synthesis continued, producing an increased number of closely packed, electron-dense membranes throughout the entire cell (Fig. 40). The distinction between thylakoids and lamellar bundles of membranes became increasingly less clear as this process continued. The necks leading to adjacent cells became fully developed during the last stages of proheterocyst development and the homogeneous extra wall layer thickened noticeably around these necks (Fig. 41).

Figure 38. Longitudinal thin section through intermediate proheterocyst in young N_2-grown *M. laminosus* filament. Numerous bundles of lamellar membrane stacks (LS) are present in cytoplasm, especially near cell poles. Note increased electron density of cytoplasm, as compared to earlier proheterocysts (Figs. 35-37). Bar = 2.0 μm.

Figure 39. Longitudinal thin section through intermediate proheterocyst in old N_2-grown *M. laminosus* filament. Note "salt-and-pepper" appearance of cytoplasm (see text) and well developed neck leading to adjacent cell. L = lipid body. Bar = 1.0 μm.

Figure 40. Longitudinal thin section through late proheterocyst in old N_2-grown *M. laminosus* filament. Note high electron density of cytoplasm, owing to accumulation of membranous material. HL = homogeneous extra wall layer. Bar = 1.0 μm.

Figure 41. Longitudinal thin section through neck area of late proheterocyst in old N_2-grown *M. laminosus* filament. Note thickening of homogeneous extra wall layer (HL) around neck area. Bar = 1.0 μm.

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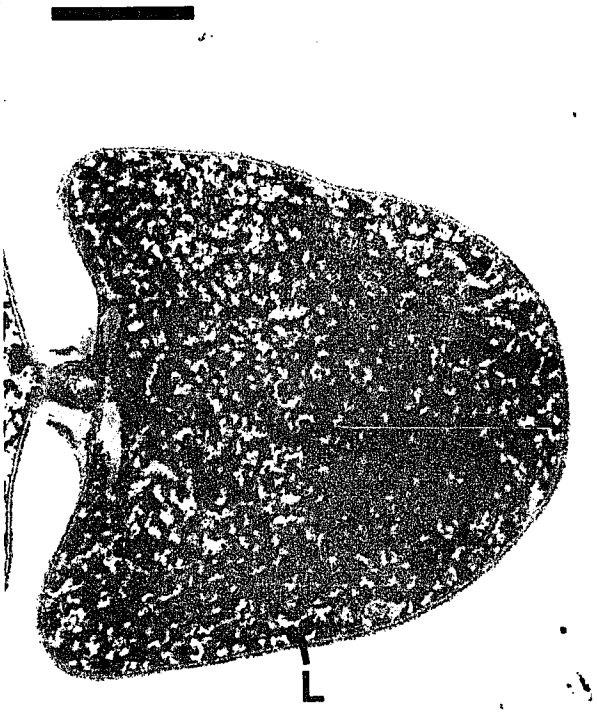

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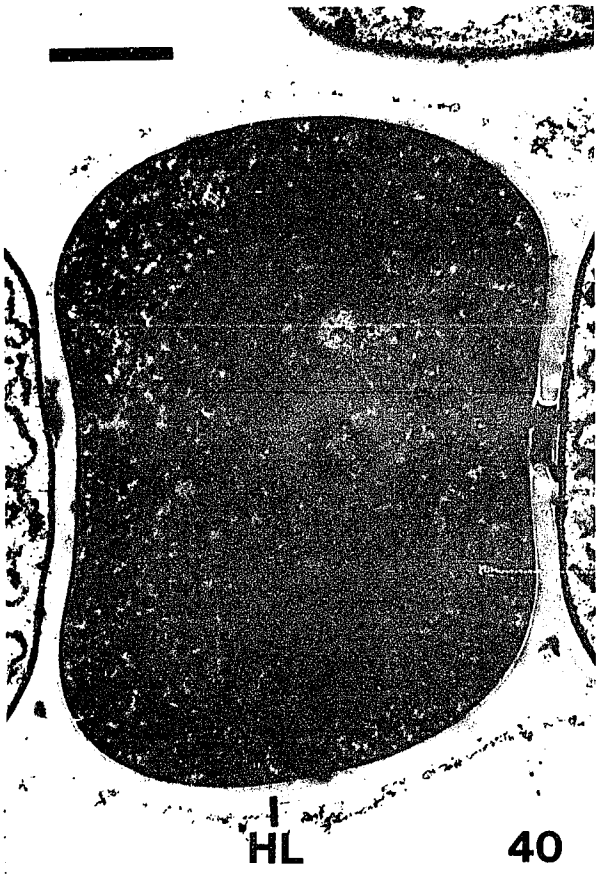

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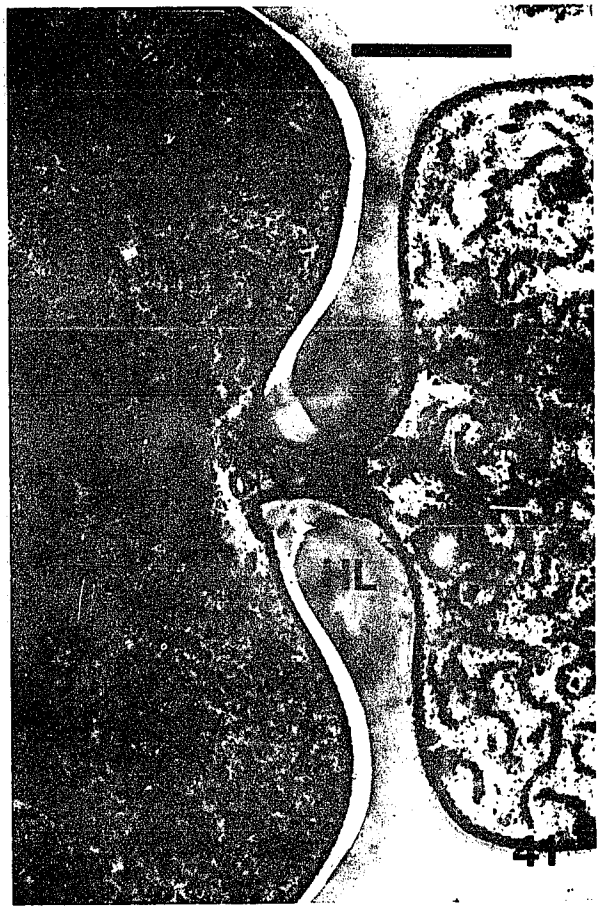

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Ultrastructural Characteristics of Mature Heterocysts

Ultrastructure of Heterocysts in N_2-Grown Cultures. Mature heterocysts (Fig. 42) in N_2-grown filaments typically possessed an electron-dense, homogeneous interior that was virtually filled with membranes. If nuclear material was present, it was obscured by the overall electron density of the cytoplasm. Polysaccharide granules, carboxysomes, and lipid bodies were absent from mature heterocysts. The only inclusions in these cells were small, spherical bodies (Fig. 43) of varying electron density. These inclusions were surrounded by a single thin, electron-dense bounding layer approximately 2 nm thick. They were about the same size as lipid bodies, but had distinctly different ultrastructural characteristics. Such inclusions have not been observed in heterocysts formed by other types of cyanobacteria. A few mature heterocysts also contained cyanophycin-like "plugs" (Lang, 1965; Lang and Fay, 1971; Wilcox et al., 1973b) near the necks leading to adjacent cells (Fig. 43), but these were not present in most heterocysts. All mature heterocysts possessed a thickened homogeneous (extra) wall layer.

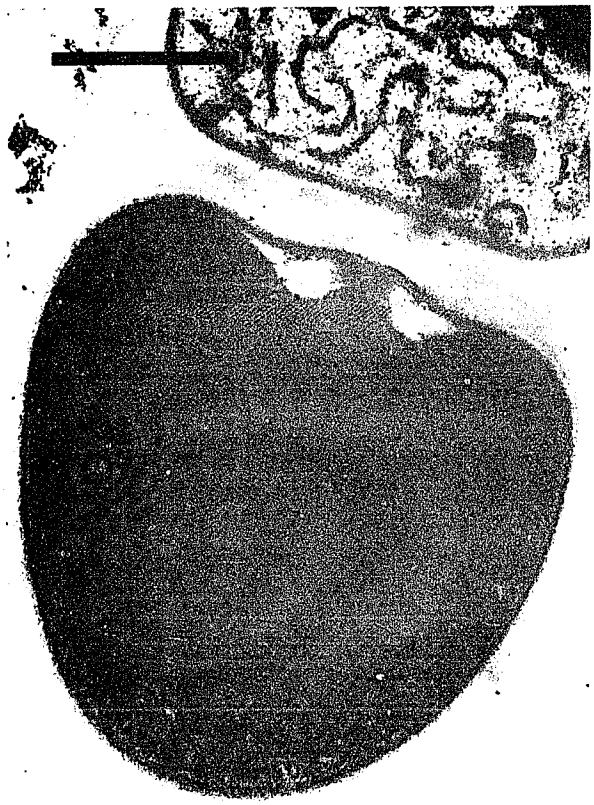
Ultrastructure of Heterocysts in N-starved Cultures. The ultrastructural characteristics of mature heterocysts in N_2-starved cultures were somewhat more variable than those of heterocysts in N_2-grown cultures. The membranes in the cytoplasm of most heterocysts tended to split apart between 19 and 25 h after the switchover to CO_2/argon, resulting in the formation of empty vesicles between them (Fig. 44). These vesicles increased in both size and frequency in some heterocysts, eventually leading to the formation of large,

Figure 42. Longitudinal thin section through mature heterocyst in old N_2-grown M. laminosus filament. Note homogeneous, electron-dense appearance of cytoplasm, which is almost entirely filled with membranes. Bar = 1.0 μm.

Figure 43. Longitudinal thin section through neck area of mature heterocyst in young N_2-grown M. laminosus filament. CP = cyanophycin-like plug; SB = spherical body of variable electron density. Bar = 0.5 μm.

Figure 44. Oblique thin section through mature heterocyst in young N-starved M. laminosus filament. Membranes have separated to form empty vesicles (V) in cytoplasm. Bar = 1.0 μm.

Figure 45. Oblique thin section through highly vesiculated mature heterocyst in old N-starved M. laminosus filament. Vesiculation has led to formation of large empty areas in the cytoplasm. SB = spherical body of variable electron density. Bar = 1.0 μm.



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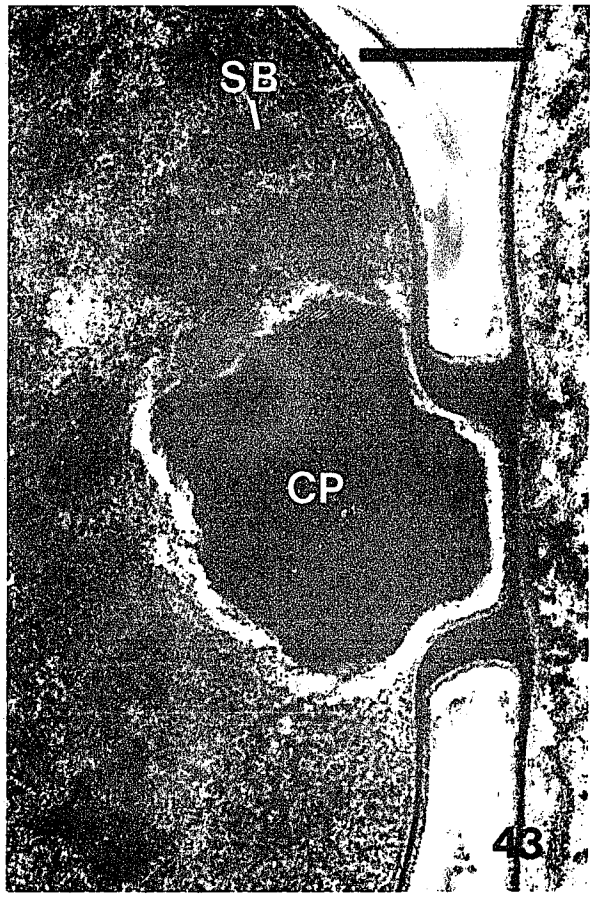

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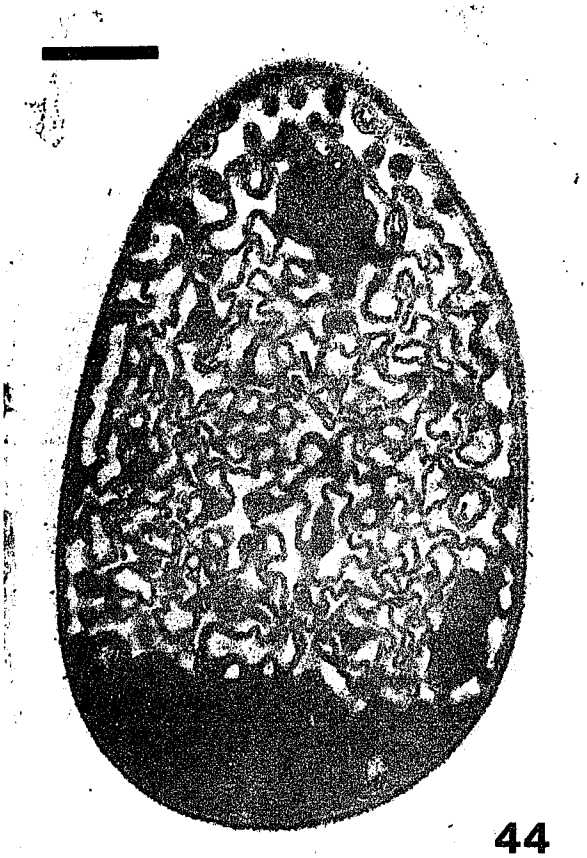

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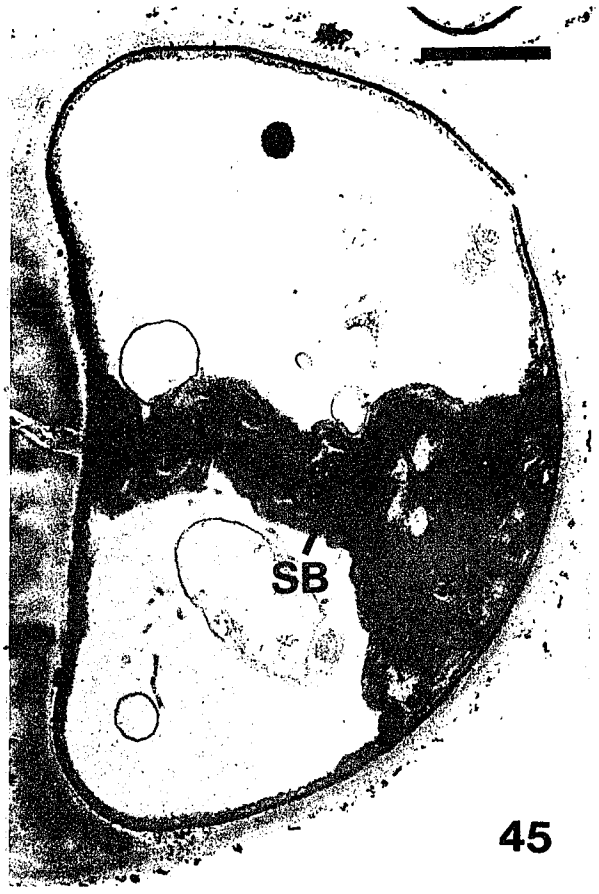

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structureless voids (Fig. 45) that sometimes filled as much as three fourths of the cell volume and usually contained vesicular fragments of membranes.

Morphological Aspects of Heterocyst Formation

Heterocyst Formation in Regard to Morphological Types of Cells.

As stated earlier, all of the morphological types of cells in M. laminosus were capable of differentiating into heterocysts. Mature heterocysts and all stages of developing heterocysts were seen in both young and old filaments. In young filaments, the heterocysts were longer and wider than the neighboring vegetative cells in the same filament (Figs. 26 and 27). Heterocyst differentiation in young filaments, then, led to a change in cellular morphology that affected size but not shape. In contrast, heterocyst differentiation in the old filaments affected neither size nor shape (Figs. 37 and 40). Since proheterocysts essentially assumed the shape of the vegetative cell from which they developed, mature heterocysts of widely varying diameters and shapes eventually appeared in older cultures of M. laminosus. Electron microscopy showed that the internal ultrastructural characteristics of these heterocysts were similar, despite the differences in their external morphology.

Positions of Heterocysts in Young Filaments.

A reproducible heterocyst spacing pattern (Literature Review) did not occur in young filaments of M. laminosus. The number of vegetative cells between consecutive heterocysts (2-20 in N_2-grown cultures; 1-9 in N-starved cultures) varied by a factor of ten, even within a single filament. In addition, heterocysts did not appear to differentiate

as a result of asymmetric cell division, as has been described for Anabaena species (Mitchison and Wilcox, 1972; Wilcox et al., 1973b).

Positions of Heterocysts in Old Filaments. Heterocysts differentiated at several locations within the branching portions of old, pleomorphic filaments. Mature heterocysts were of two types: those which had terminal necks leading to both neighboring cells (Fig. 46; "intercalary heterocysts" as defined by Rippka et al., 1979) and those which had only a single neck (Fig. 47; "terminal heterocysts" as defined by Rippka et al., 1979). Heterocysts of the second type sometimes became the terminal cell of the main filament (Fig. 48). Proheterocysts and mature heterocysts within the main filament were sometimes isolated from branch points by neighboring vegetative cells, although the majority were situated immediately adjacent to branch filaments. The heterocysts formed on one (Fig. 49) or both (Fig. 50) sides of the branches in these cases.

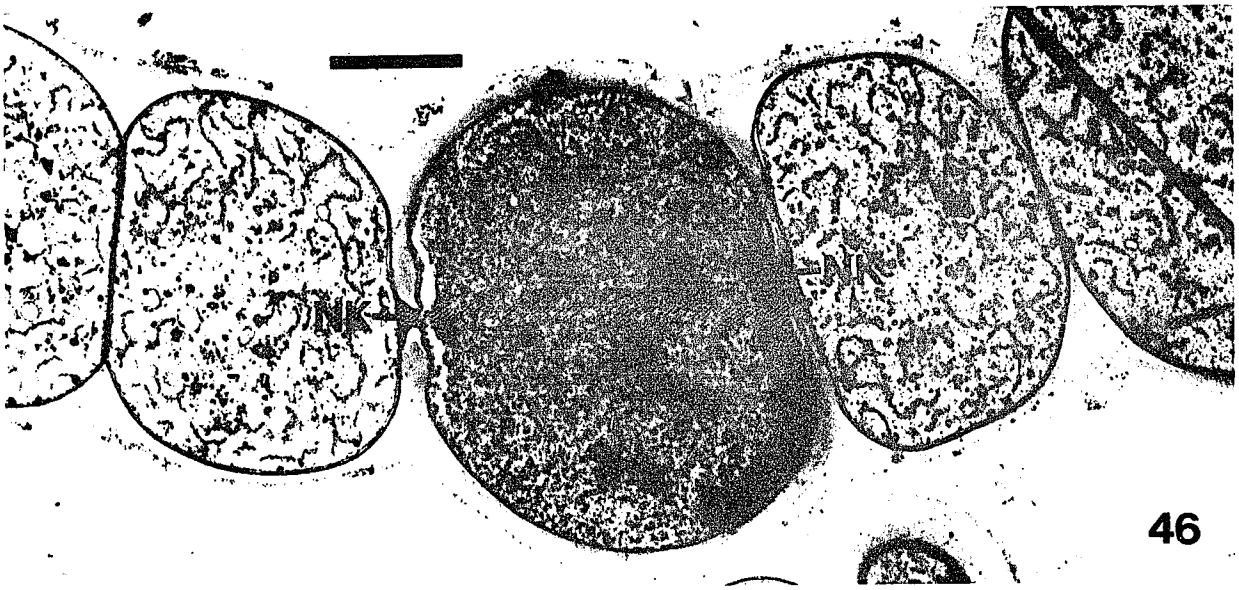
Heterocysts also developed in the lateral branches of the old, pleomorphic filaments. These heterocysts usually became terminal cells of those filaments (Fig. 51), possibly preventing further elongation of the branches. Terminal heterocysts frequently formed in place of branch filaments (Fig. 51), or in rare instances on both daughter cells produced when a cell in the old filament divided parallel to the long axis of the old filament (Fig. 49).

Numbers of Heterocysts. M. laminosus was capable of forming unusually large numbers of heterocysts in comparison to the other heterocystous cyanobacteria that have been studied to date (Adams and Carr, 1981). This was especially true in N-starved cultures.

Figure 46. Longitudinal thin section through old N_2-grown M. laminosus filament containing intercalary heterocyst (actually a developing proheterocyst). Note that necks (NK) lead to adjacent cells on both ends of heterocyst. Bar = 2.0 μm.

Figure 47. Longitudinal thin section through old N_2-grown M. laminosus filament containing terminal heterocyst. Heterocyst has only one neck (NK) leading to adjacent vegetative cell. Bar = 2.0 μm.

Figure 48. Longitudinal thin section through old N-starved M. laminosus filament in which a terminal heterocyst has become the terminal cell of the filament. Note cyanophycin-like plug (CP) and extensive vesiculation of heterocyst membranes. Bar = 2.0 μm.



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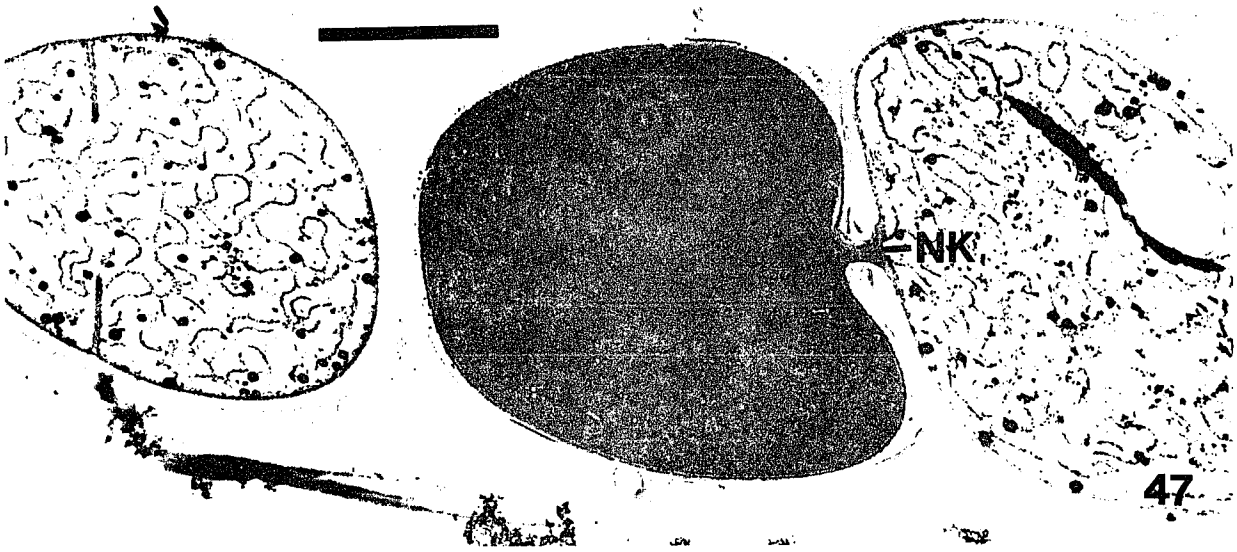

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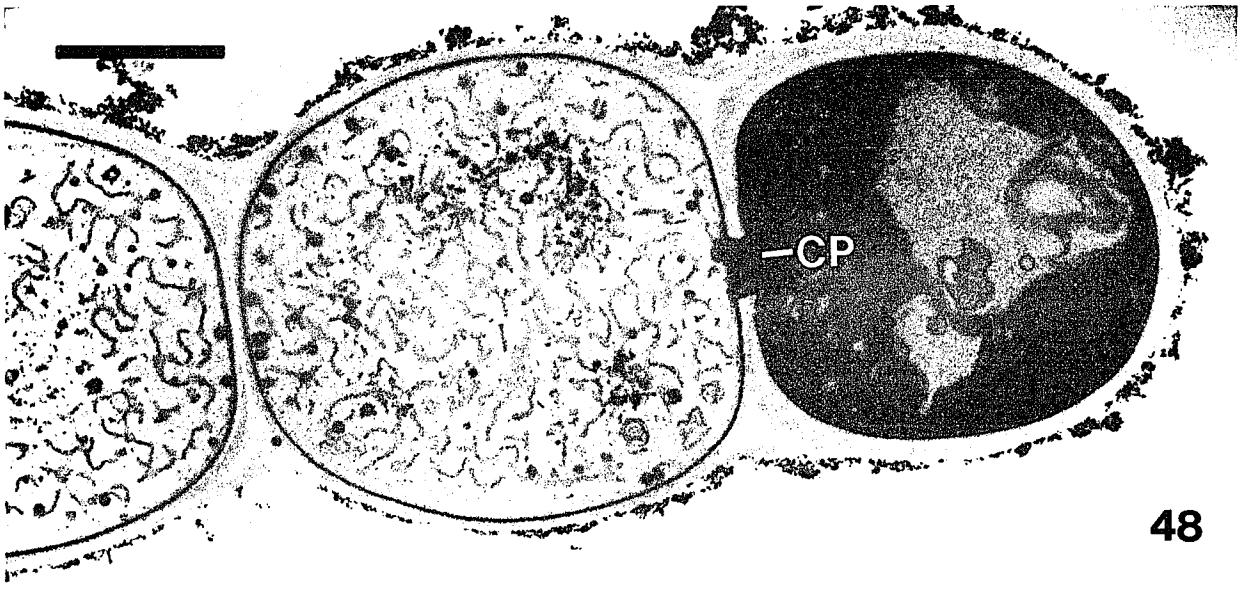

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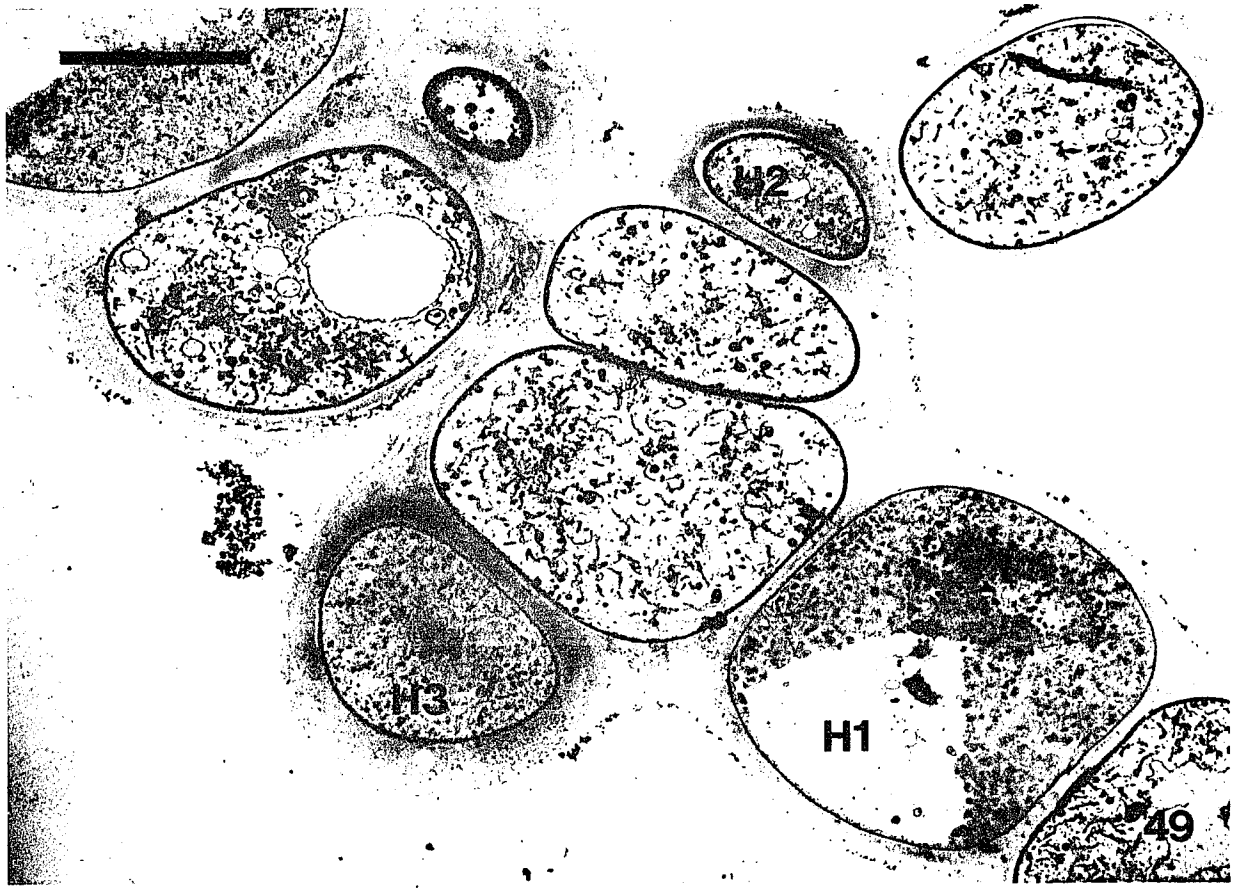
Figure 49. Longitudinal thin section through branching portion of old N-starved M. laminosus filament. Heterocyst (H1) has formed on only one side of branch point. Heterocysts (H2 and H3) have also formed on both daughter cells produced by division of old cell parallel to long axis of the main filament. Bar = 3.0 μm.

Figure 50. Longitudinal thin section through branching portion of old N_2-grown M. laminosus filament. Heterocysts (H1 and H2) have formed on both sides of branch point. Note that old cell at branch point is undergoing division diaonal to long axis of main filament. Bar = 3.0 μm.


H2
H3
H1
49

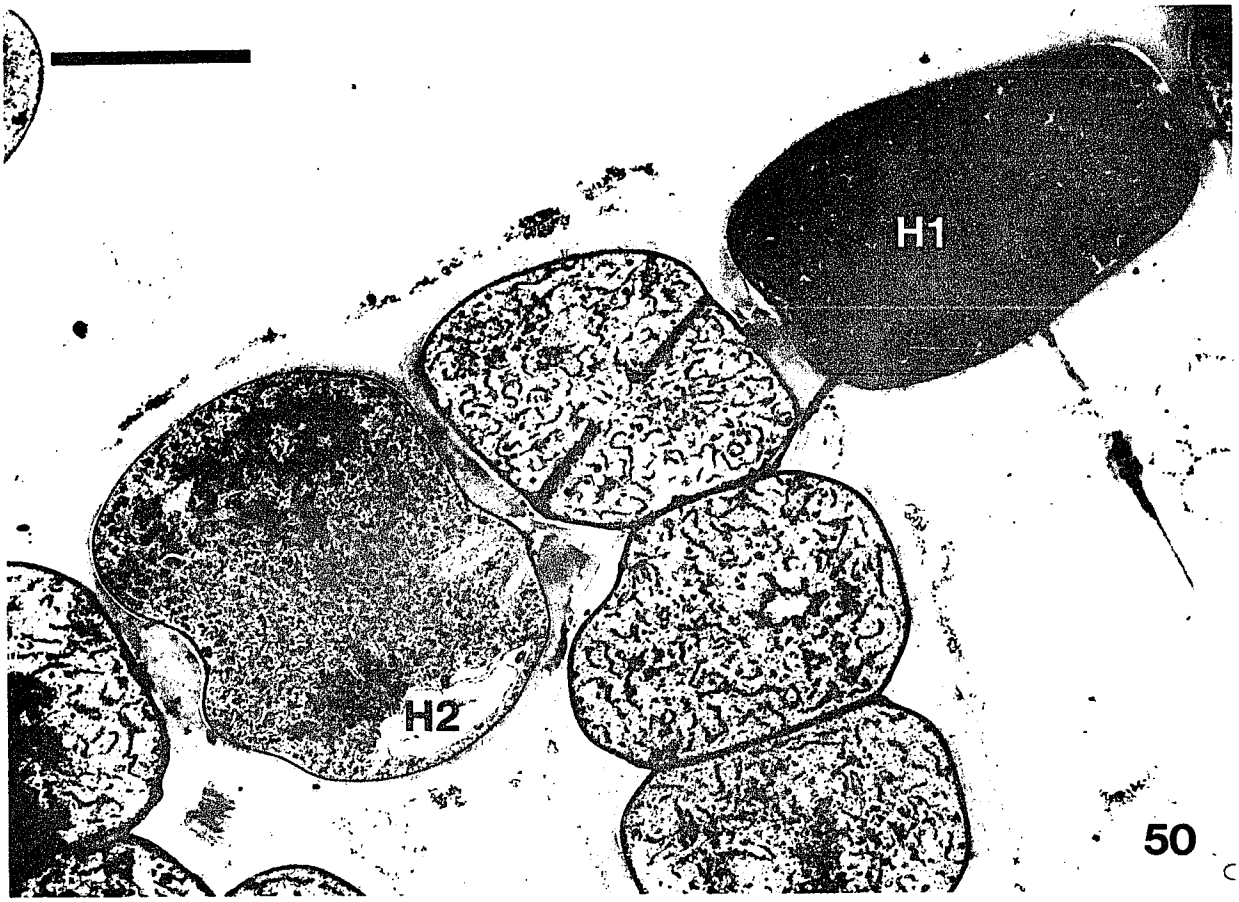

H1
H2
50

Figure 51. Longitudinal thin section through multi-branched, pleomorphic portion of old N_2-grown M. laminosus filament. Heterocysts have formed in main filament (H1), as terminal cell of branch filament (H2), and in place of branch filament (H3). Note division of old cells parallel (D1) and diagonal (D2) to long axis of main filament. Note that cells giving rise to branches do not differ ultrastructurally from other cells in main filament. Bar = 5.0 μm.

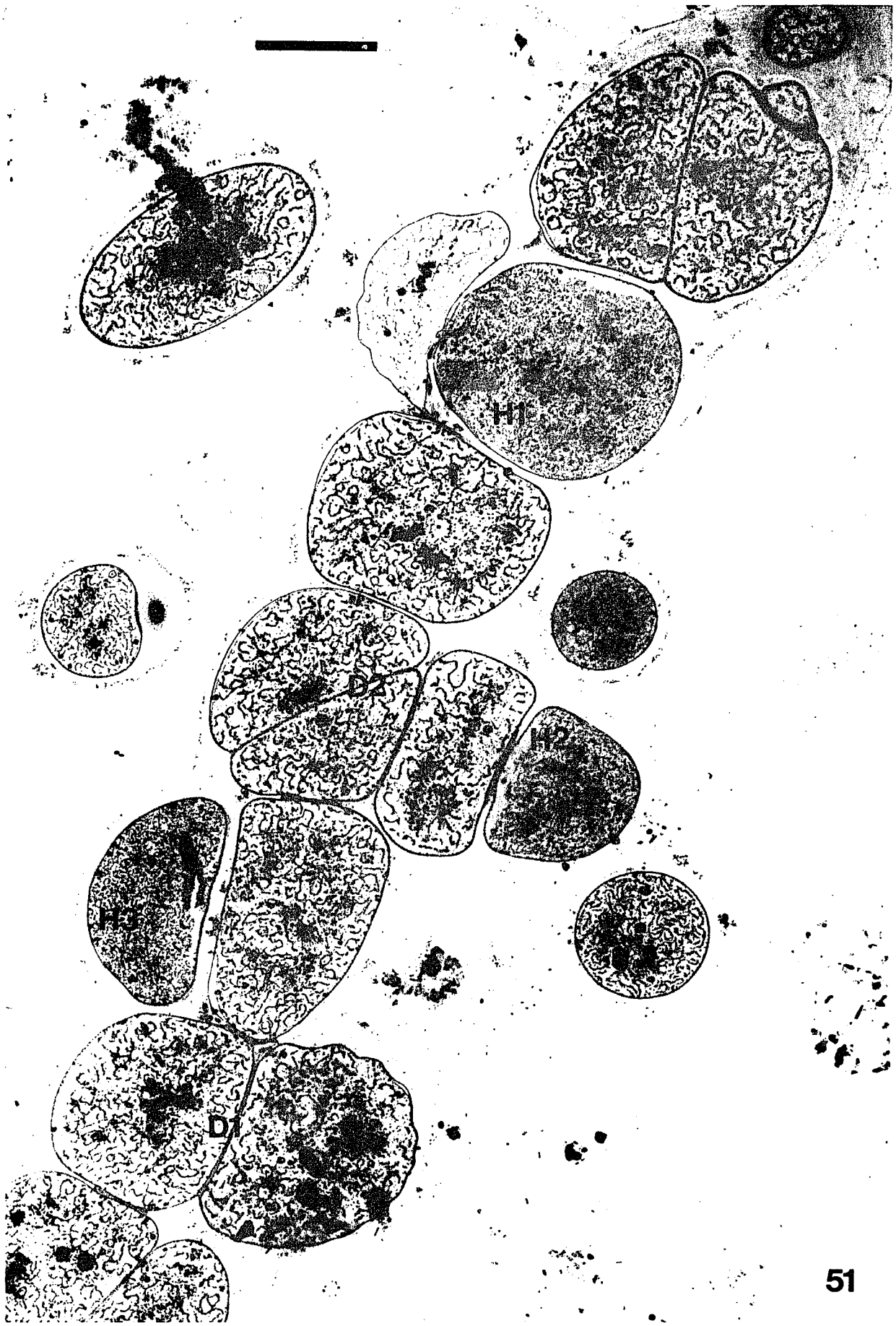


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TABLE 3. Changes in the Proportions of Cell Types Related to Heterocyst Differentiation During Nitrogen Starvation in M. laminosus

Time (h)	*Cell type (% of Total) VC	DC	PH	MH	Sample size
0	73.1	13.2	5.4	8.4	167
6	61.4	19.7	7.9	11.0	127
19	43.4	28.5	9.6	18.5	491
25	33.5	38.1	8.5	19.9	457
44	22.2	37.9	13.1	26.8	396

*Abbreviations: VC = vegetative cells; DC = differentiating cells; PH = proheterocysts; MH = mature heterocysts.

The proportions of vegetative cells, "differentiating cells", proheterocysts, and mature heterocysts (see above) observed during progressive N-starvation are given in Table 3. The proportion of differentiating cells dropped during the first 6 h after transfer from CO_2/N_2 to CO_2/argon, while the proportion of both proheterocysts and mature heterocysts increased. The proportion of mature heterocysts continued to increase sharply between 6 and 44 h, while the proportion of vegetative cells decreased sharply. At the end of the nitrogen starvation period, only 22.2% of the cells remained in the fully vegetative form, while 26.8% had become mature heterocysts. Assuming that the differentiating cells were in the process of becoming proheterocysts (see above), no less than 77.8% of the cells had at least started heterocyst differentiation by the end of the starvation period.

Formation of Multiple Adjacent Heterocysts. M. laminosus displayed a marked tendency to form sets of consecutive, adjacent heterocysts and proheterocysts (i.e., heterocysts and proheterocysts that were not separated from each other by intervening vegetative cells). In young filaments, this tendency was limited to the formation of double heterocysts or proheterocysts (Fig. 52). Both of the cells in paired proheterocysts always appeared to be at the same stage of differentiation. Triple or other multiple heterocysts did not occur in young filaments. Double heterocysts themselves were relatively rare, probably representing less than 5% of all heterocysts in young filaments.

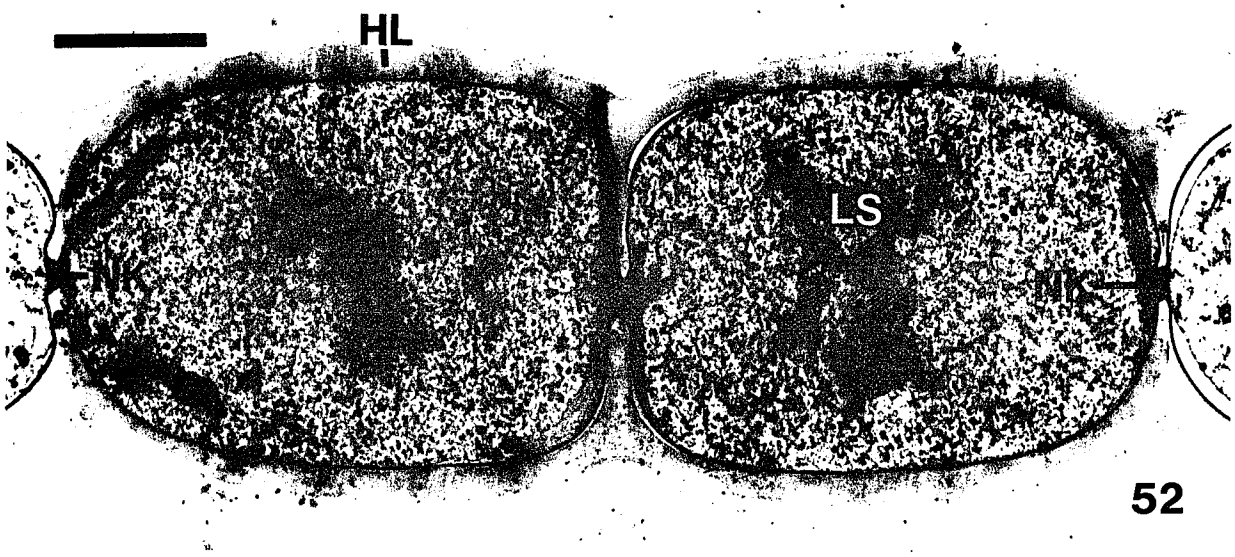
Old filaments produced multiple heterocysts considerably more frequently than did young filaments. At least 14-20% of the proheterocysts and mature heterocysts in old filaments were double or multiple. Moreover, the old filaments were not limited to the formation of double heterocysts. Triple heterocysts were seen frequently, while some filaments contained sets of 4, 5, or 6 adjacent heterocysts. Most of these multiple heterocysts were of the intercalary type, having differentiated necks on both ends of the cell (Fig. 53). They differed from those seen in young filaments (above) in that the individual heterocysts or proheterocysts within a multiple set were usually not all at the same stage of heterocyst differentiation (Fig. 53). It was possible that all of the cells in old filaments were at least capable of becoming heterocysts, since old filaments were found in which all of the cells appeared to be in the early stages of heterocyst differentiation.

Patterns of Morphogenesis in M. laminosus

Ultrastructure of the Cell Division Process. The ultrastructure of the cell division process in M. laminosus (Fig. 54) was quite similar to that reported for Fischerella ambigua by Thurston and Ingram (1971). Cell division was apparently initiated by the formation of mesosome-like structures on opposite sides of the cell (Fig. 54a). This was followed by simultaneous invagination of the cytoplasmic membrane and peptidoglycan layer to form a division septum (Fig. 54b). Septum formation continued until the cytoplasm was transected by a cross-septum of peptidoglycan material (Fig. 54c), which later began to thicken on both ends (Fig. 54d). The thickened

Figure 52. Longitudinal thin section through developing double heterocyst in relatively young N_2-grown M. laminosus filament. Both cells are at the same stage in proheterocyst development. HL = homogeneous extra wall layer; LS = bundle of lamellar membrane stacks; NK = developing neck. Bar = 2.0 μm.

Figure 53. Longitudinal thin section through multiple group of heterocysts and proheterocysts in old N-starved M. laminosus filament. Group includes three mature heterocysts (H1-H3) and one early proheterocyst (PH). Note double neck (DN) between two mature heterocysts. Bar = 3.0 μm.


HL
LS
52

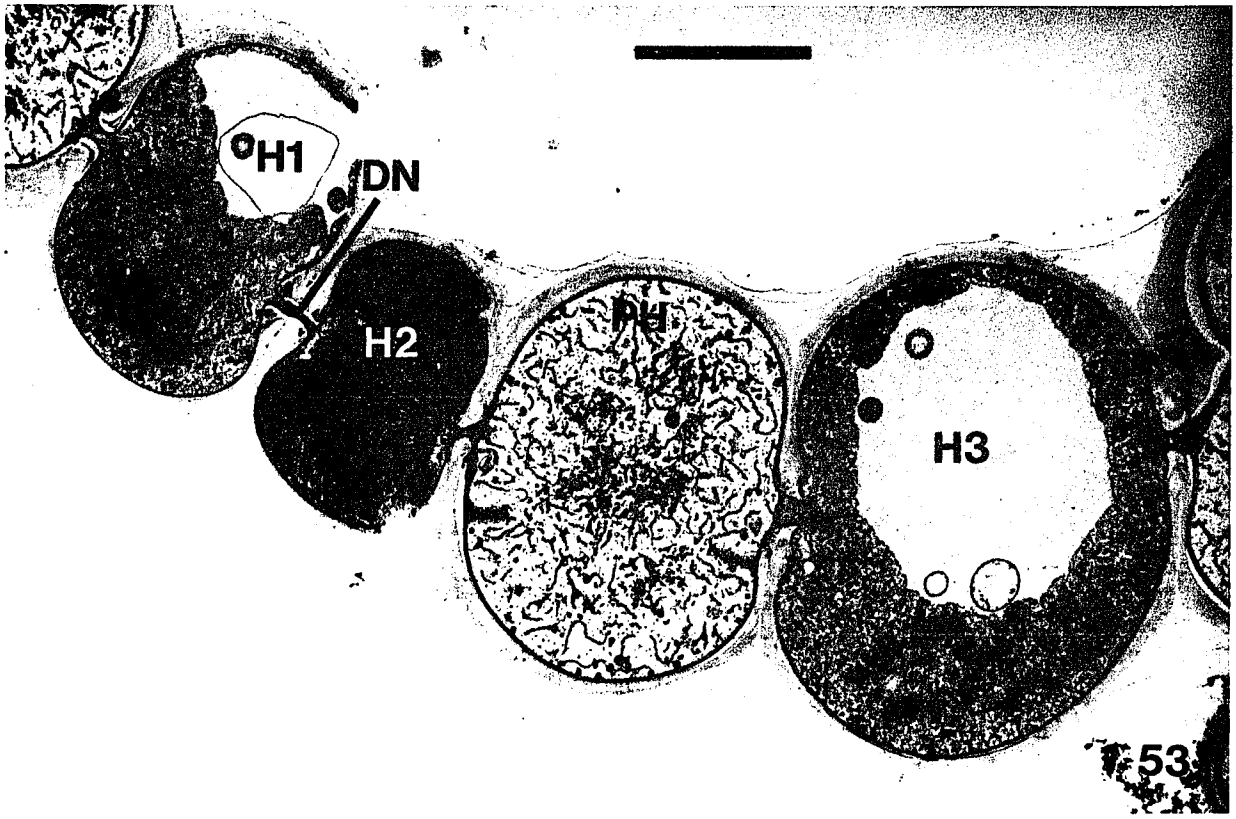

H1
DN
H2
H3
53

Figure 54. Thin sections through NO_3-grown M. laminosus filaments, depicting details of the cell division mechanism. CM = cytoplasmic membrane; M = mesosome-like structure; OM = outer membrane; PG = peptidoglycan layer; S = sheath; V = membranous vesicles on inner surface of sheath. Bars = 0.5 μm.

a. Mesosome-like structures on opposite sides of cell.

b. Ingrowth of septum (arrows) consisting of CM and PG.

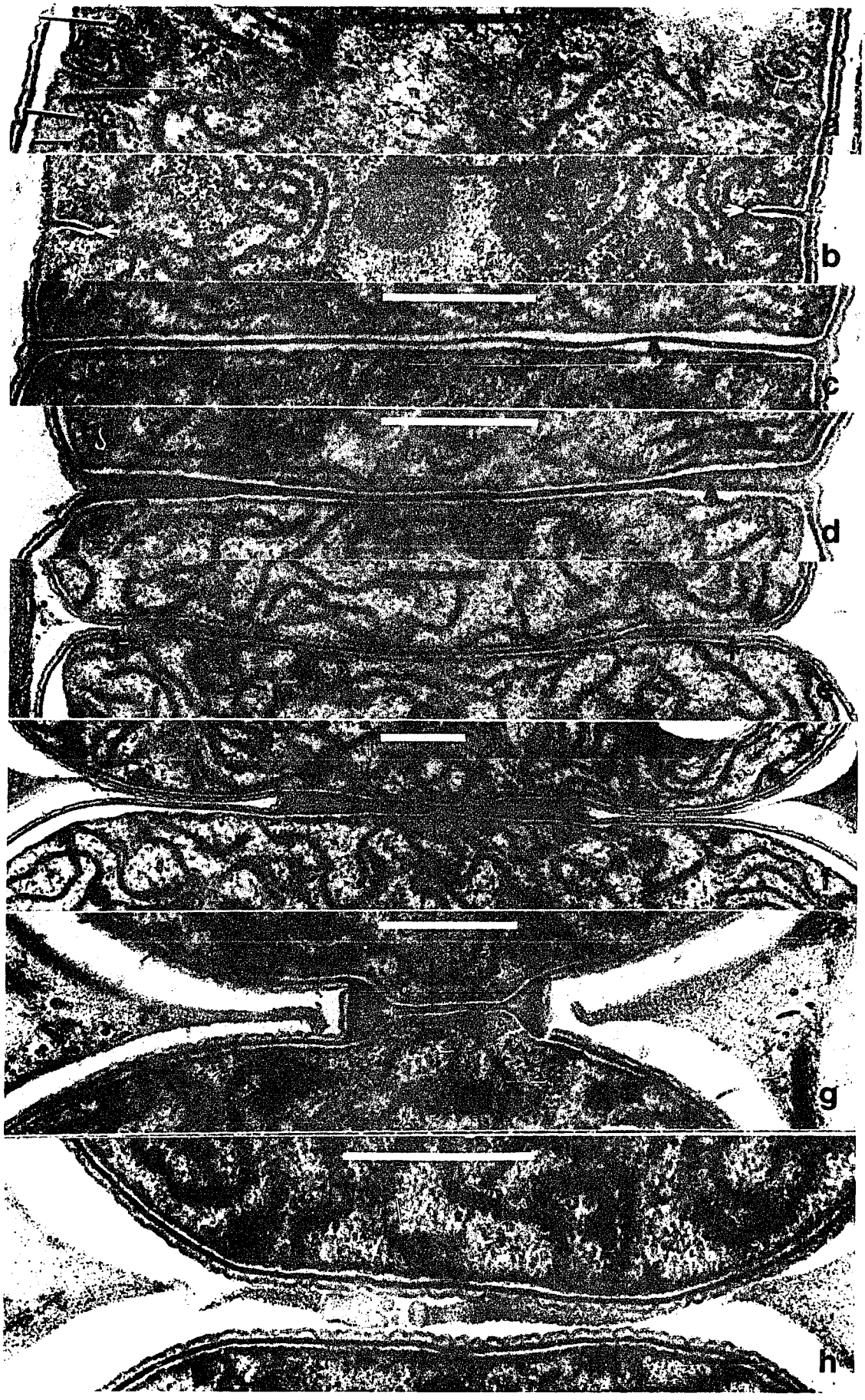
c. Complete cross-septum (arrow) consisting of CM and PG.

d. Thickening of PG layer at ends of cross-septum (arrow).

e. Splitting PG and ingrowth of OM (arrows). Note mesosome-like structure.

f. Ingrowth of sheath between layers of OM as PG thickening and splitting continues.

g. and
h. Final stages in separation of daughter cells.


b
c
d
g
h

portions of the cross-septum eventually split down the middle, leaving gaps into which the outer membrane and the innermost layer of the common sheath began to invaginate (Fig. 54e). Thickening of the peptidoglycan, splitting of the thickened peptidoglycan, ingrowth of the outer membrane, and ingrowth of the sheath all continued in sequence until the daughter cells were separated by a complete layer of sheath material (Figs. 54f-54h). Most of the cells in older, wider filaments were eventually separated by sheath material in this way.

Modes of Cell Division in Different Types of Filaments. Young cells divided repeatedly by first elongating to approximately twice their original length and then forming a division septum at the midpoint of the elongated cell (see above). Asymmetric septum formation did not occur, and septa formed only perpendicular to the long axis of the filament. This mode of cell division resulted in elongation of the filaments in the manner usually ascribed to trichome-forming cyanobacteria (Rippka et al., 1979; Stanier and Cohen-Bazire, 1977). Daughter cells could continue to elongate and divide as long as they retained the morphological characteristics of young cells (cylindrical shape, uniform diameter, and squared ends).

Old cells frequently underwent cell division, but only in a specific and somewhat unusual manner. These cells never formed conventional septa that ran perpendicular to the long axis of the filament. Instead, they divided either parallel to the long axis of the filament (most frequently) or at a 45^{o} diagonal to it (much less frequently; see Fig. 51). If an old cell underwent diagonal division, subsequent division of the daughter cells did not occur. Either

or both of these daughter cells sometimes differentiated into heterocysts, but they never divided. On the other hand, division of an old cell parallel to the long axis of the filament did not necessarily preclude subsequent division of the two daughter cells. The events which followed the division of an old cell parallel to the long axis of the filament included the following: (i) neither daughter cell underwent subsequent cell division (Fig. 51), (ii) one of the daughter cells subsequently divided parallel to the long axis of the filament, a process that eventually led to the formation of a lateral branch off the main filament as described below (Fig. 51), (iii) both daughter cells subsequently divided parallel to the long axis of the filament, but both of the outermost daughter cells resulting from the second round of division developed into heterocysts and stopped dividing (relatively rare: Fig. 49), or (iv) one daughter cell subsequently divided parallel to the long axis of the filament (leading to formation of a lateral branch), while the other cell underwent a diagonal division and then stopped dividing (Fig. 50). At no time did any of the old cells divide more than twice in any direction.

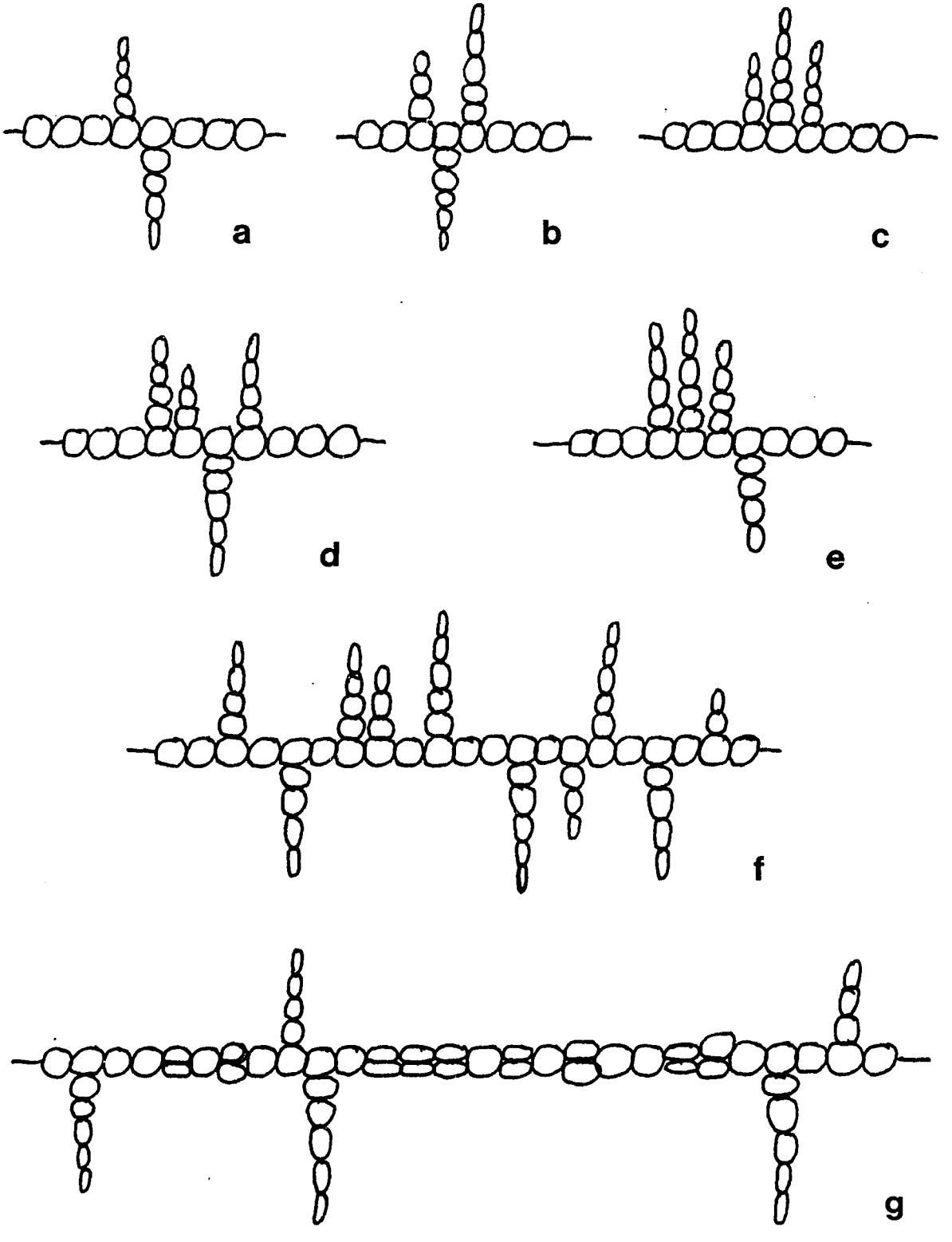
Cells in lateral branch filaments could only form division septa that were perpendicular to the long axis of the branch filament, at least while the branch filaments were still attached to their main filaments. Consequently, branch filaments did not produce secondary branches at any time. In contrast to the division patterns seen in the main filaments (above), division was perpendicular to the long axis of the (branch) filament regardless of cell morphology (young or old).

Morphology of Lateral Branching. Lateral branches off the main filament ranged in length from 2-16 cells, but the majority of them were between 4 and 7 cells long. Branch lengths did not appear to be affected by the position of a branch in relation to the other lateral branches of the same filament (see below). The frequency at which lateral branches formed in M. laminosus varied in a more-or-less random fashion; reproducible branching patterns were not observed. The most common variations are summarized diagrammatically in Fig. 55.

Ultrastructure of Lateral Branch Differentiation. The internal ultrastructural characteristics of cells within the main filament that had given rise to lateral branches were essentially identical to those of cells in the same filament that had not formed branches. Thus, there were no detectable ultrastructural changes preceding branch formation, aside from cell division parallel to the long axis of the main filament (Fig. 51). Branch formation was initiated when one of the two daughter cells formed by division parallel to the long axis of the main filament underwent a second round of cell division in the same direction. The two new cells formed by this process both had ultrastructural characteristics typical of old cells and, therefore, did not differ noticeably from other cells in the main filament (Fig. 56).

Elongation of newly formed lateral branches occurred when the terminal cell underwent division perpendicular to the long axis of the branch filament (Fig. 56). As long as a branch filament remained attached to its parent main filament, only the terminal cell could undergo division; the other cells in the branch filaments never

Figure 55. Diagrams depicting morphology of lateral branch formation in old filaments of M. laminosus. Branches usually formed on adjacent cells in the main filament (a-e). As many as four consecutive main filaments produced branches (d and e). Branches on consecutive cells could be on one side of the main filament (c) or both sides, (a, b, and d). No more than three consecutive branches formed on one side of the main filament (e). When branches were not on consecutive main filament cells (f and g), they were usually not separated by more than one or two nonbranching cells (f). In cases where more than one or two cells did separate the branches (g), many of the intervening cells had undergone cell division parallel to the long axis of the main filament (the first step in formation of a branch).



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underwent a second round of cell division while the branches were elongating.

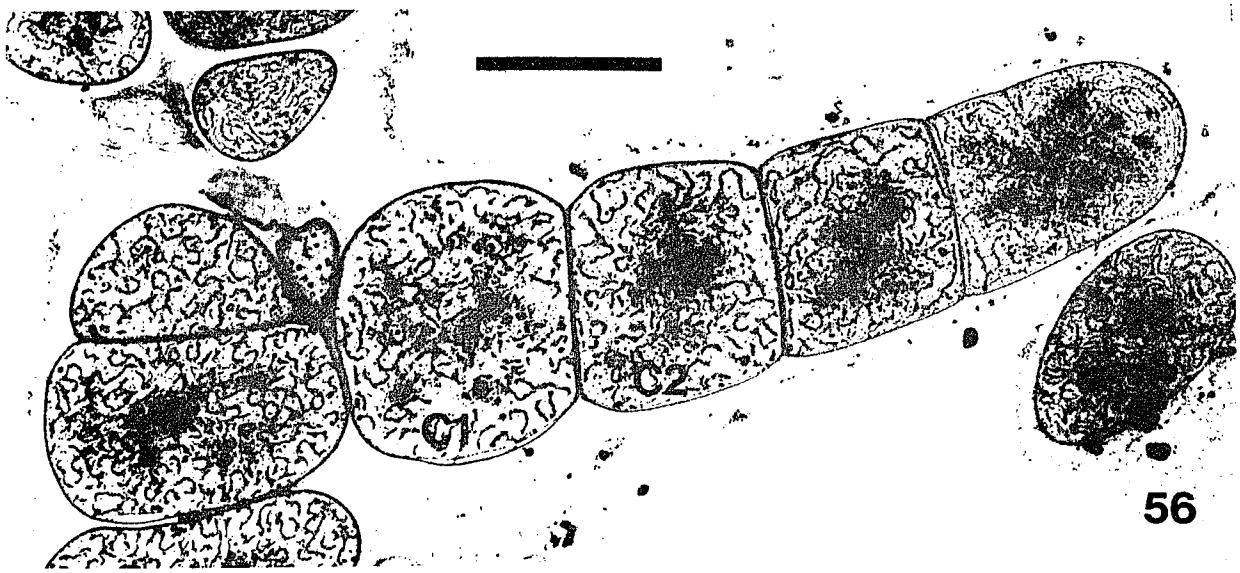
The morphological and ultrastructural characteristics of successive new cells changed as the lateral branches elongated (Fig. 56). After the formation of at least two cells with typical "old cell" characteristics (rounded, wide), a number of less rounded and increasingly cylindrical cells were produced. Specialized tapering cells that were seen only in branch filaments were sometimes formed between the older- and younger-looking cell types (Fig. 57). If the branch filaments elongated for a sufficient period of time, they ultimately differentiated into thin, cylindrical cells with typical "young cells" dimensions. Changes in the internal ultrastructural characteristics of the branch filament cells paralleled the morphological differentiation of these cells. Young-looking cells at the tips of the branches had more ribosomal and nuclear material, less polysaccharide, less fragmented thylakoids, and more carboxysomes than did the older-looking cells situated closer to the parent main filament (Fig. 56).

Release of Lateral Branches from the Main Filament. After branch filaments elongated, they were sometimes released from the main filament by a process similar to that seen in non-branching oscillatorian cyanobacteria (Lamont, 1969). The cell within the main filament that originally branched appeared to die and disintegrate in order to release the branch. The cell wall and cytoplasmic membrane of the "releasing cell" deteriorated first, leaving the cytoplasmic content of the cell (or a portion thereof) enclosed only

Figure 56. Longitudinal thin section through lateral branch off of main N_2-grown M. laminosus filament. Cells closest to filament (C1 and C2) have morphological and ultrastructural characteristics similar to those of old cells in main filament. Note differences in morphological and ultrasturctural characteristics of cells farther away from main filament. Terminal cell of branch is dividing, illustrating mechanism of branch elongation (see text). Bar = 4.0 μm.

Figure 57. Longitudinal thin section through M. laminosus branch filament with specialized tapering cell (TC) between older-looking (OL) and younger-looking (YL) cells in branch. Bar = 2.0 μm.

Figure 58. Longitudinal thin section through M. laminosus branch filament in the process of being released from the parent, main filament. "Releasing cell" (RC) is disintegrating in order to release branch. Note that, in this case, main filament will be split by the release process. Bar = 4.0 μm.



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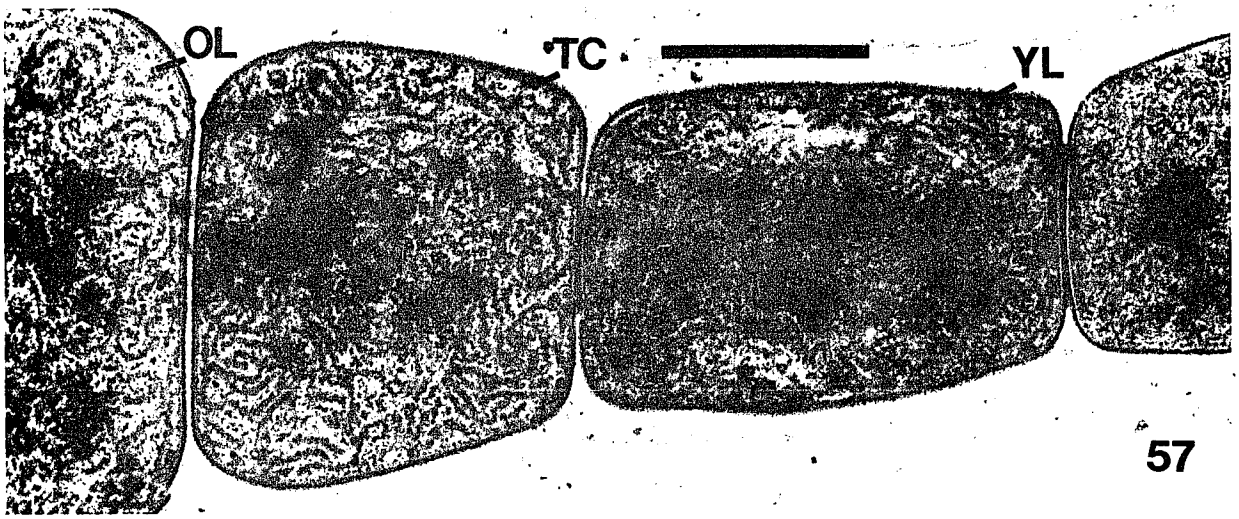

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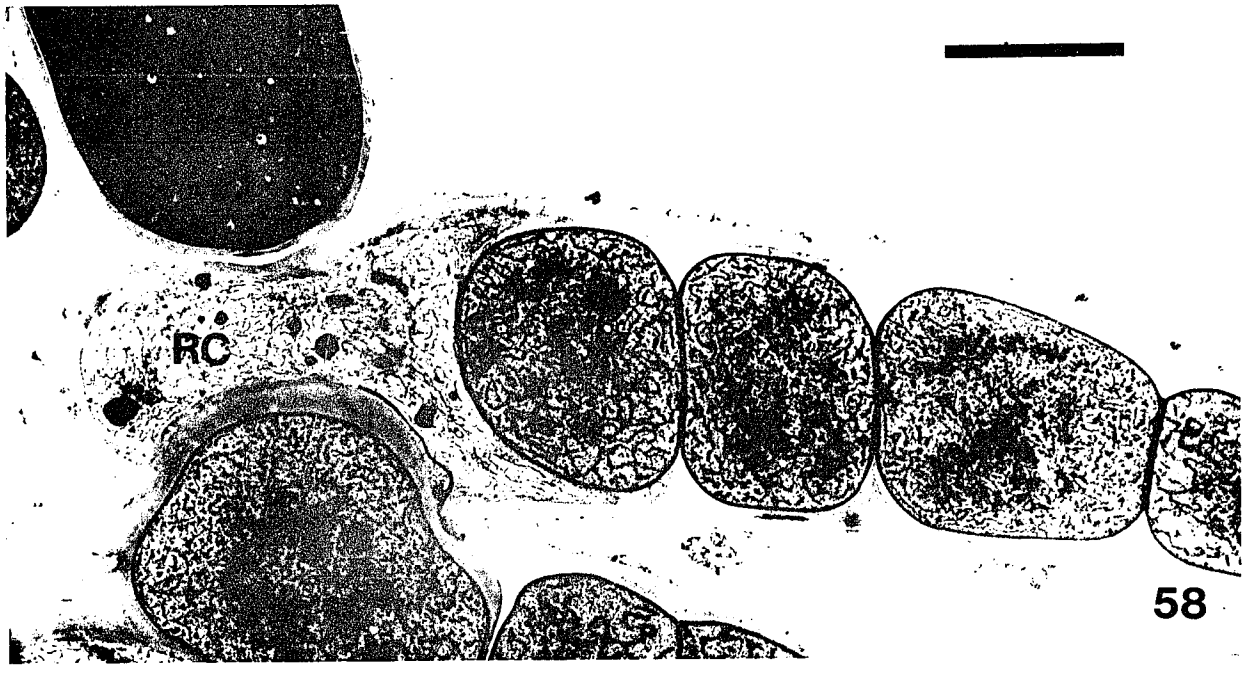

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by the common sheath layer. The branch was released when this sheath material eventually ruptured.

IV. DISCUSSION

Three-Dimensional Reconstruction of Agmenellum quadruplicatum

Assessment of Specialized Electron Microscopical Techniques

The three-dimensional reconstruction of A. quadruplicatum described in this study made use of two rather novel and unconventional electron microscopical techniques: high-voltage electron microscopy (HVEM) of thick sections and computer-aided reconstruction of serial thin sections (CARTOS). Since this was the first attempt to use either of these techniques to study the ultrastructure of a prokaryotic organism, it seems worthwhile to assess the effectiveness and utility of these methods.

High-Voltage Electron Microscopy of Thick Sections. The use of HVEM in this study significantly facilitated the three-dimensional reconstruction of A. quadruplicatum and served to provide important data regarding the basic ultrastructural characteristics of this organism. The most immediately obvious advantage of HVEM was its ability to provide high resolution images of relatively thick specimens. This permitted the use of serial thick sections (0.25-μm) to determine the three-dimensional distribution of inclusion bodies and other features throughout the A. quadruplicatum cell. Since relatively few thick sections were needed to cover an entire cell in comparison to the number of conventional thin sections that would

have been needed, it was possible to examine large numbers of complete cells in a reasonably short period of time. This was important in the present study because a large and representative sampling was needed to assess accurately and thoroughly the consistency of three-dimensional arrangements from one cell to another. It would have been impractical to obtain equally reliable and complete data by conventional thin sectioning, and therefore, would have been impossible to make equally definitive statements regarding the consistency of cell arrangements.

Several previous investigations have demonstrated that thick sectioning can provide three-dimensional information more rapidly than conventional thin sectioning (for a review, see King et al., 1981), but in the present study, thick sectioning also proved to have an unexpected advantage over thin sectioning. The thylakoid membranes were considerably easier to see in thick sections than in thin sections because they were better contrasted against their surroundings. Since the thylakoids were virtually transparent, their density did not increase with increasing section thickness. The density of the darkly stained materials around the thylakoids increased considerably, however, and this enhanced the contrast between the two structures. As a result of the increased visibility of the individual membranes, the entire thylakoid system was more completely or realistically depicted in the thicker sections. This made it easier to determine the overall three-dimensional arrangement of the entire system throughout the cell. It is reasonable to assume that HVEM and thick sectioning could be equally helpful in studies on thylakoid arrangements of other cyanobacteria.

An especially important advantage of HVEM and thick sectioning was the ability of this approach to reveal structures of ultrastructural details that could not be seen clearly in thin sections. Thick sections were vastly superior to thin sections in revealing the manner in which individual thylakoid membranes intersected with each other and in demonstrating the existence of interconnections between the thylakoid and cytoplasmic membranes. The latter structures were especially difficult to find in thin sections, being detected only quite rarely. This most likely occurred because the thylakoid-cytoplasmic membrane interconnections were relatively small structures that were located intermittently throughout the cell. Therefore, thick sections were more likely than thin sections to pass through one of these connections. For the same reason, Zapf (1974) was unable to locate surface membrane particles of post-synaptic receptor membrane in thin sections but readily observed them in thick sections. In the present study, the interconnections that were found in thin sections were not depicted very clearly in comparison to those seen in the thicker sections. This was probably the result of several factors, including: (i) the increased contrast in thicker sections (see above), (ii) the fact that larger portions of the cell were included in thick sections, thereby making it easier to understand the three-dimensional nature of the interconnections, and (iii) the fact that thick sections could be tilted about two perpendicular axes in order to obtain the clearest possible views of each interconnection. It was not possible to determine the effects of double tilting on thin sections because instruments capable of doing this were not available for the present

study. However, it is unlikely that tilting would have been very effective; other researchers have reported that it does comparatively little to clarify structures in thin sections (King et al., 1980).

HVEM and thick sectioning were not without disadvantages in regard to determining the complete three-dimensional architecture of A. quadruplicatum. The most serious of these was the inability of this approach to illustrate the overall arrangement of a complete cell. Ironically, the problem was caused by the electron-dense ribosomes and photopigments that served to increase the contrast of the thylakoids in 0.25-um sections. Thicker sections, which would have illustrated greater portions of cells or even complete cells, were rendered almost opaque by these dense inclusions and were unsuitable for examination of ultrastructural details (Materials and Methods). Similarly, HVEM was unable to provide clear images of critical-point-dried, whole cells (Balkwill and Nierzwicki-Bauer, unpublished results). The problem could have been avoided to some extent by degrading the dense ribosomes with ribonucleases prior to preparing the cells for electron microscopy. This was not done because the thylakoids of A. quadruplicatum have been shown to be sensitive to environmental or chemical stresses (Balkwill and Stevens, 1980b; Hardie et al., 1983a, b; Stevens et al., 1981a, b). Therefore, removal of the ribosomes from the cell most likely would cause an undesirable alteration of the thylakoids and give a false picture of thylakoid arrangements. Similarly, there are no selective fixatives currently available that could accurately preserve most of the features in a cyanobacterial cell while leaving the ribosomes either unpreserved or unstained. Therefore, HVEM remains unsuitable for resolving and displaying the three-dimensional architecture of properly

preserved or unaltered whole cells.

Computer-Aided Reconstruction of Serial Thin Sections. The CARTOS technique proved to be quite effective for illustrating the general three-dimensional arrangement of important substructures in whole cells, thereby counteracting the primary disadvantage of HVEM and thick sectioning (above). With CARTOS, electron-dense cell features (such as ribosomes and photopigments) that normally would have interfered with viewing an entire cell could be left out of the reconstruction images simply by programming the computer not to include them. Since the resulting reconstructions were still based on data obtained from sections through fully preserved cells, there was no possibility that "normal" cellular arrangements had been altered by this approach. The ability of CARTOS to display three-dimensional arrangements of entire cells in this way represented its primary advantage in the reconstruction project.

Unfortunately, the CARTOS approach alone was not sufficiently powerful to carry out a thorough and detailed three-dimensional reconstruction of A. quadruplicatum. The CARTOS images were based on thin sections, in which the details of thylakoid intersections were unclear and in which the connections between the thylakoid and cytoplasmic membranes were usually not resolved (see above). It would have been impractical to produce CARTOS reconstructions from serial thick sections because only a few sections were required to pass through an entire cell. The resulting CARTOS images would have lacked resolution and continuity. The ultrastructural details of complex and minute structures like the thylakoid-cytoplasmic membrane interconnections, then, could not be illustrated adequately by the

CARTOS technique.

CARTOS was further limited in its usefulness by its inability to display the thylakoid membranes as continuous, planar sheets. The contour lines used to display the thylakoids were often confusing, thereby necessitating manipulation of the reconstruction images to produce the clearest possible illustrations of three-dimensional arrangements within the cell. This limitation of CARTOS may be temporary, however, since researchers in Dr. Cyrus Levinthal's laboratory (in which the CARTOS system was invented) are currently developing a computer program capable of displaying planar objects as continuous sheets (C. Levinthal, personal communication).

General Conclusions Regarding Techniques. From the above, it should be apparent that a combination of HVEM and CARTOS was needed to complete successfully the three-dimensional reconstruction of A. quadruplicatum; neither approach was sufficient on its own. This is significant because the present study was the first to demonstrate the effectiveness of such a combination for studying three-dimensional ultrastructure. Many of the unexpected and significant biological findings discussed below were apparent only after complete three-dimensional data were obtained. Therefore, it is reasonable to suggest that three-dimensional analyses of other prokaryotic microorganisms will be worthwhile because they may lead to equally novel and significant conclusions. The combination of techniques developed in this study (HVEM and CARTOS) can serve to facilitate such investigations in the future.

Conclusions Regarding the Ultrastructure of A. quadruplicatum

This study has served to elucidate for the first time the complete three-dimensional arrangement of ultrastructural features in a cyanobacterium. To the best of my knowledge, it was also the first complete three-dimensional reconstruction of any prokaryotic organism. The overall significance and importance the reconstruction are tied to the fact that the three-dimensional data led to a number of unexpected and novel discoveries regarding the functional ultrastructure of A. quadruplicatum (discussed in detail below). These discoveries were novel, even though the ultrastructural characteristics of A. quadruplicatum and many other cyanobacteria have been studied for over two decades with more traditional methods. It was the three-dimensional reconstruction that led to the novel results. Similarly, three-dimensional reconstruction permitted certain definitive statements regarding the ultrastructure of A. quadruplicatum to be made in the present study (see below). In previous studies, many of these statements were merely speculations because the three-dimensional data needed to confirm them were not obtained.

Limitations Regarding the Results. It should be realized that A. quadruplicatum was grown under carefully controlled conditions for this investigation and that only exponentially growing cultures were examined. The ultrastructural characteristics of this organism are known to vary under different growth conditions (Hardie et al., 1983b; Stevens et al., 1981a, b). Therefore, further investigation would be required to understand fully the effects of cultural or other external factors on the three-dimensional arrangements described

in this report. It should also be realized that the three-dimensional ultrastructural characteristics of other cyanobacteria, especially the filamentous genera (Lang, 1968; Stanier and Cohen-Bazire, 1977; Wolk, 1973), probably differ from those of A. quadruplicatum. The other unicellular species may also differ from A. quadruplicatum, since they often appear to have distinct features in randomly cut thin sections (Allen, 1968; Baulina et al., 1981; Brown and Van Baalen, 1970; Cox et al., 1981; Echlin, 1964; Edwards et al., 1968; Holt and Edwards, 1972; Kats et al., 1979; Ris and Singh, 1961). Complete three-dimensional analyses of other unicellular cyanobacteria would be needed to determine how universally the specific features found in A. quadruplicatum occur in this group of organisms. Investigations of this nature would represent a logical and useful extension of the present study.

Intracellular Location of the Specialized Inclusion Bodies. One of the more interesting findings of this study was the fact that the specialized inclusion bodies of A. quadruplicatum consistently occupied specific locations within the cell. For example, polyphosphate bodies and carboxysomes were always confined to the central cytoplasmic region. Micrographs of randomly cut, individual sections have implied that this arrangement occurs in other unicellular cyanobacteria (Allen, 1968; Edwards et al., 1968; Gantt and Conti, 1969; Lewin et al., 1981), but but only the complete three-dimensional reconstructions carried out in the present study could demonstrate its existence conclusively. Direct contact between polyphosphate bodies and carboxysomes was seen occasionally and has been reported previously (Jensen, 1968). The unordered clustering of these two inclusion bodies that

was seen in three-dimensional reconstructions indicates that their occasional contact is more likely to occur by chance than as a result of any functional need. Similarly, the carboxysomes in A. quadruplicatum were never in direct contact with the thylakoid membranes. This is in contrast to the results of Peat and Whitton (1968) who found that such connections always occurred in Anabaenopsis sp. Since carboxysomes may be involved directly in CO_2 fixation (Beudeker and Kuenen, 1981; Stewart and Codd, 1975) and since thylakoids are known to produce the reducing power required for CO_2 fixation, contact between these two structures to increase efficiency is not an unreasonable possibility. Nevertheless, the present results have demonstrated clearly that contact is not required to maintain normal metabolic functions in rapidly growing cells.

Compartmentalization of the Cell. The central cytoplasmic region of A. quadruplicatum was a virtually independent compartment bounded by the innermost pair of thylakoid membranes. It may have had contact with the periphery of the cell at sites where thylakoid membranes merged and came in contact with the cytoplasmic membrane. However, the central cytoplasm and the surrounding thylakoid zone was separated from each other both physically and functionally. This separation was indicated by the absolute and consistent segregation of inclusion bodies into one region or the other. A physical separation was also indicated by the results of previous studies on nutrient limitation (Hardie et al., 1983b; Stevens et al., 1981a, b). In those studies, glycogen granules accumulated extensively throughout the thylakoid system but were not detected in the central cyto-

plasm until the thylakoid membranes began to deteriorate. The separation of the central cytoplasm from the rest of the cell in A. quadruplicatum is an example of true compartmentalization in a prokaryotic organism, possibly the first such example to be reported. It would be of interest to determine the functional advantage of compartmentalizing the cytoplasm in this way. More importantly, it will be necessary to reexamine the presently accepted definitions of prokaryotic and eukaryotic cells. Membrane-bound compartments are generally thought not to exist in prokaryotic organisms. The results of the present study contradict this belief and serve to blur the distinction between prokaryotic and eukaryotic forms of life.

Overall Nature and Arrangement of the Thylakoid System. The overall three-dimensional arrangement of the thylakoid membrane system in A. quadruplicatum was of major interest in this study because there has been considerable confusion over this topic in the literature. Most previous investigations have attempted to describe the three-dimensional arrangement of the thylakoid membranes in unicellular cyanobacteria by extrapolating from the appearance of these membranes in randomly cut, single sections. Several authors have described the thylakoids as a group of independent disks or flattened sacs (Echlin, 1964; Edwards et al., 1968; Holt and Edwards, 1972; Lang and Whitton, 1973), while others have considered them to be truly concentric structures (Allen, 1968; Baulina et al., 1981; Chang, 1980; Dohler et al., 1981; Rippka et al., 1974; Stanier and Cohen-Bazire, 1977). More recently, the thylakoid system has been described as "an anastomosing network of concentric shells" (Golecki

and Drews, 1982). The results of this study have proven that, at least for A. quadruplicatum, the first two concepts cited above are incorrect. The present data are consistent with the concept of an anastomosing network, but further show for the first time the exact appearance of that network. The thylakoids of A. quadruplicatum clearly joined together, but only at or very near the cytoplasmic membrane. The individual thylakoids emanating from these peripheral intersections did not branch at other points in the cell. In contrast, branching of thylakoids has been reported to occur in two other unicellular cyanobacteria, Anacystis and Synechococcus (Edwards et al., 1968; Holt and Edwards, 1972; Kats et al., 1979; Venkataraman et al., 1969). The significance of such branches, if any, is unknown because the present study has demonstrated that they are not required for normal thylakoid function.

Association of the Thylakoid System with the Cytoplasmic Membrane. The thylakoid network of A. quadruplicatum was connected to or associated with the cytoplasmic membrane at a number of locations in the cell. Connections between thylakoid and cytoplasmic membranes have been reported in a wide variety of cyanobacteria (Allen, 1968; Cox et al., 1981; Daft and Stewart, 1973; Golecki, 1979; Gromov and Mamkaeva, 1976; Kats et al., 1979; Lang and Rae, 1967; Neumann et al., 1970; Schnepf, 1964; Smith and Peat, 1967) although reviewers generally consider them to occur only rarely (Golecki and Drews, 1982; Stanier and Cohen-Bazire, 1977). Stanier and Cohen-Bazire (1977) stated that the thylakoids and the cytoplasmic membrane are topologically distinct, even in cyanobacteria where one would expect to detect interconnections easily if they were present. The inter-

connections were quite difficult to find in the present study, especially when randomly cut, individual thin sections were examined. Serial sections through complete cells were essential for detecting connections routinely and for determining their locations in the cell. The use of thick sections and HVEM also facilitated the discovery and location of connections for the reasons discussed above. The techniques used in the present study, then, might well detect previously unnoticed interconnections between the thylakoids and the cytoplasmic membrane in other cyanobacteria. In any event, this study has demonstrated conclusively that thylakoid-cytoplasmic interconnections can and do occur in cyanobacteria, despite the unwarranted claims of Stanier and Cohen-Bazire (1977).

The functional significance of connections between thylakoids and the cytoplasmic membrane cannot be determined from the present results. It has been suggested that thylakoids arise by invagination of the cytoplasmic membrane (Allen, 1968; Cox et al., 1981; Kats et al., 1979; Pankratz and Bowen, 1963; Smith and Peat, 1967; Jost, 1965); thus, the connections would most likely serve as sites for thylakoid synthesis. This suggestion might also explain why the lipid bodies, which could serve to store excess membrane components, were located only near the connections. As Golecki and Drews (1982) have pointed out, however, no one has demonstrated conclusively that thylakoids do originate from the cytoplasmic membrane. The techniques used in the present study might serve to resolve this issue if they were used to study thylakoid regeneration in nutrient-starved cells (Stevens et al., 1981a). If the connections seen in A. quadruplicatum were not invaginations or centers for thylakoid synthesis,

then their function may have been simply to maintain the three-dimensional arrangement of the thylakoid system. The merging of the thylakoids and their attachment to the cytoplasmic membrane allows the central cytoplasm to approach the periphery of the cell. This arrangement might facilitate segregation of nuclear material during cell division by providing sites for attachment of the genome to the cytoplasmic membrane, as is thought to occur in other bacteria. The thylakoid arrangement in A. quadruplicatum might also facilitate transport of substances in and out of the cytoplasm. If the thylakoids were arranged as independent and concentric cylinders without merging near the cytoplasmic membrane, then substances would have to cross several layers of semi-permeable membrane to enter or leave the central cytoplasm. Thus, the connections in A. quadruplicatum may allow the central cytoplasm to have limited contact with the external medium while still being compartmentalized from the rest of the cell.

Variations in Thylakoid Arrangements. Although the arrangement of thylakoids in A. quadruplicatum was quite consistent from cell to cell, two variations that require explanation were observed. The first of these was the existence of an alternate cross sectional pattern in about 30% of the cells. Since no intermediate or transitional forms were observed and since the two patterns were not related to cell division, it is assumed that they represent an inherent variation in the culture. There may have been two distinct genotypes present, or the alternate thylakoid arrangement may have been coded by a plasmid. Further study will be required to determine the cause of this variation. The second variation was seen in cell poles and

was clearly related to cell division. Since transitional forms between the "new" and "old" arrangements (Fig. 19) were detected, it is reasonable to assume that cell division left the thylakoid system in a slightly altered conformation that was later "corrected" by rearrangement of the membranes. A three-dimensional analysis of cell division would elucidate the details of this process, but was beyond the scope of the present study.

Consistency of Three-Dimensional Intracellular Architecture. Perhaps the most surprising general aspect of A. quadruplicatum's three-dimensional architecture is the fact that it was so consistent from one cell to another, even in the case of relatively complex or minute details. Such a high degree of intracellular organization within a prokaryotic organism must be the result of a very accurate control mechanism or it would not be reproduced so consistently in each cell. The mechanism by which the precise three-dimensional arrangement of A. quadruplicatum is maintained might be the most interesting and rewarding topic for future continuation of this work.

Morphology and Ultrastructure of Mastigocladus laminosus

This study has involved the first comprehensive ultrastructural analysis of a branching filamentous (stigonematacean) cyanobacterium. The morphology of these organisms at the light microscopical level has been described extensively (Lee, 1927; Marcenko, 1961; Martin and Wyatt, 1974; Rippka et al., 1979; Schwabe, 1960; Spearing, 1937), but relatively little is known about their ultrastructure (Butler and Allsopp, 1972; Marcenko, 1961; Ris and Singh, 1961). The only de-

tailed ultrastructural study to date has been that of Thurston and Ingram (1971) on Fischerella ambigua, and even that study was limited to cultures growing in the presence of nitrate. Because the branching filamentous cyanobacteria have received so little attention, virtually all of the present thinking regarding cyanobacterial ultrastructure has been based on studies involving only unicellular or nonbranching filamentous genera. This is important because the present investigation has revealed that M. laminosus possesses features that have not been accounted for by the conventional thinking on cyanobacterial ultrastructure. These unusual and novel aspects of ultrastructure in M. laminosus, along with other significant findings, are given detailed consideration in the following discussion.

Taxonomical Considerations

Rippka et al. (1979) recently proposed a new classification scheme for cyanobacteria in which it was suggested that the genera Fischerella and Mastigocladus be combined into a single genus: Fischerella Gomont 1895. The new combined genus was to be placed in "Group V" of their overall classification scheme, a group that included only Fischerella (multicellular, branching filaments) and Chlorogloeopsis (multicellular, irregular aggregates). The proposal to combine Mastigocladus and Fischerella was based on physiological similarities to a limited extent, but it rested primarily on morphological or ultrastructural characteristics such as formation of true lateral branches and the mechanism of cell division within the filaments. Since the present study has provided significant new information on the morphology and ultrastructure of Mastigocladus, it is

reasonable to re-evaluate the proposal of Rippka et al. (1979) in light of these new data.

The general morphological characteristics of M. laminosus, as observed by light and scanning electron microscopy, were basically similar to those reported for both Mastigocladus and Fischerella strains in previous light microscopical studies (Lee, 1927; Marcenko, 1961; Martin and Wyatt, 1974; Rippka et al., 1979; Schwabe, 1960; Spearing, 1937). Furthermore, the morphological features of NO_3-grown cultures were virtually identical to those of NO_3-grown Fischerella ambigua cultures (Thurston and Ingram, 1971). These results indicate that the strain of M. laminosus studied here clearly fits the definition of Fischerella Gomont given by Rippka et al. (1979), at least in terms of vegetative morphology. The morphological similarities between NO_3-grown M. laminosus and F. ambigua, then, support the proposal of Rippka et al. (1979) to combine Mastigocladus and Fischerella into a single genus. However, it should be realized that the N_2-grown M. laminosus cultures in the present study did not fit the definition of Fischerella Gomont given by Rippka et al. (1979) in one important morphological respect: they lacked the specialized resting cells known as akinetes. Rippka et al. (1979) indicated that all strains of Fischerella and Mastigocladus examined possessed akinetes, but these specialized cells were very difficult to recognize and identify because their only distinguishing characteristic in light microscopical preparations (electron microscopy was not done) was a "weaker pigmentation." This somewhat vague claim was not substantiated convincingly by the light micrographs that accompanied it. The present study failed to detect any evidence of akinete

formation in M. laminosus despite extensive examination of the organism with both light and electron microscopy. Similarly, none of the earlier studies on Mastigocladus or Fischerella cited above have presented conclusive evidence that stigonematacean cyanobacteria possess anything like the true, classic akinetes that are known to occur in nonbranching filamentous genera (Adams and Carr, 1981; Nichols and Adams, 1982). It appears from all of this that Rippka et al. (1979) were unjustified in defining Fischerella Gomont as an akinete-producing genus. Unless conclusive evidence for the existence of akinetes in Fischerella and Mastigocladus can be produced, this characteristic should be dropped from the official description of the proposed combination genus.

Prior to the completion of the present study, it was not possible to determine whether Mastigocladus and Fischerella resemble each other as closely on the ultrastructural level as they do on the morphological level because Mastigocladus had never been investigated in detail. Even now, the only valid comparison that can be made is between NO_3-grown M. laminosus (this study) and NO_3-grown F. ambigua (Thurston and Ingram, 1971). These two strains were similar with respect to the general arrangement of their ultrastructural features within the cell, but some of their specific ultrastructural details differed noticeably. The thylakoids in young cells of F. ambigua were vesiculated, producing electron-transparent "intrathylakoidal spaces". In contrast, the thylakoid pairs of M. laminosus were always close together. The thylakoids were peripheral in young cells of both organisms but, in the micrographs of F. ambigua, they seemed to be asymmetrically distributed toward one side of the cell. In M. laminosus,

they were clearly distributed around the entire periphery of the cell. The numbers of carboxysomes, lipid bodies, and polyphosphate granules all increased as young M. laminosus cells differentiated into old cells. Similar changes were not seen in F. ambigua, although cyanophycin granules increased in size and number as the cells aged. In contrast, cyanophycin granules were not observed in old cells of M. laminosus. These ultrastructural differences probably reflect physiological or cultural differences between the two strains rather than generic differences, considering the extent to which the detailed ultrastructure of M. laminosus was affected by cultural conditions in the present study (see below). In any case, the differences certainly were not great enough to warrant keeping Fischerella and Mastigocladus in separate genera. Furthermore, the present study demonstrated conclusively (for the first time) that (i) the detailed mechanism of cell division in M. laminosus was virtually identical to that of F. ambigua (Thurston and Ingram, 1971) and (ii) that M. laminosus formed true branches. These ultrastructural data provide very strong support for the proposal of Rippka et al. (1979) to combine the two genera because the traditional distinction between them was based on presumed differences in the mode of cell division and the ability to form true branches.

The present study has provided both morphological and ultrastructural evidence to support the proposal of Rippka et al. (1979) to combine Mastigocladus and Fischerella into a single genus (above), but at the same time, it has raised some questions regarding their description of the proposed combination genus. One of these, the issue of akinete formation, was discussed previously (see above).

A more serious problem is related to the morphology of heterocyst formation in these organisms. Rippka et al. (1979) stated that heterocysts in the main filaments were predominantly terminal or lateral, but this was not the case in the strain of M. laminosus studied here. In addition, the present study demonstrated that heterocyst formation in M. laminosus was considerably more complicated and extensive than in any of the strains described by Rippka et al. (1979). Whether or not this justifies keeping M. laminosus in a separate genus is debatable. The important point is that Rippka et al. (1979) should either leave these organisms in separate genera or write their description of the proposed combined genus with sufficient accuracy to cover all of the strains they intend to include within it.

Vegetative Ultrastructure and the Effects of Nitrogen

The vegetative cells of M. laminosus did not contain any unusual or remarkable features that have not been described previously. Individual M. laminosus vegetative cells, then, were ultrastructurally similar to those of most other cyanobacteria, especially those of other filamentous genera (Lang, 1968; Stanier and Cohen-Bazire, 1977; Wolk, 1973).

The ultrastructural characteristics of vegetative cells in M. laminosus were affected noticeably by both the availability of nitrogen and by the identity of the nitrogen source when it was available. The latter case was illustrated effectively by comparing the ultrastructural characteristics of NO_3^- and N_2-grown cultures. The most obvious differences were seen in the younger cells. The N_2-grown

cells contained fewer ribosomes, fewer lipid bodies, considerably more polysaccharide granules, and comparatively disorganized thylakoids. All of these ultrastructural characteristics are well-documented symptoms of phosphorous, iron, and nitrogen limitation in cyanobacteria. It is unlikely that the ultrastructural characteristics of N_2-grown cells were related to phosphorous limitation, however, because phosphorous limitation has been shown to result in an accumulation of cyanophycin granules (Jensen and Sicko, 1974; Stevens et al., 1981b). Cyanophycin granules were not observed in N_2-grown M. laminosus cells. Similarly, iron limitation is unlikely to have occurred in M. laminosus because it resulted in distinctive and unusual rearrangements of the thylakoid system in both Agmenellum quadruplicatum (Hardie et al., 1983b) and M. laminosus (Hardie and Balkwill, unpublished). Distinctive thylakoid rearrangements were not seen in the present study. Therefore, it seems most likely that the N_2-grown M. laminosus cells studied here were undergoing a relatively mild form of nitrogen limitation (de Vasconcelos and Fay, 1974; Giesy, 1964; Peat and Whitton, 1967; Stevens et al., 1981a). It could not have been a severe form of nitrogen starvation because the ultrastructural characteristics of nitrogen-starved cells were different from those of N_2-grown cells (see below). The vegetative cells of N_2-grown cultures, then, were not receiving as much nitrogen as were their NO_3-grown counterparts, perhaps because nitrogen fixation in M. laminosus was not as efficient as nitrate assimilation. As a consequence, N_2-grown cells were probably less metabolically active than NO_3-grown cells. Consistent with this idea was the fact that young N_2-grown cells were more similar ultrastructurally to their

older, less metabolically active counterparts than were the young cells in NO_3-grown cultures.

Nitrate starvation in M. laminosus led to several changes in vegetative cell ultrastructure beyond those apparently brought about by mild nitrogen limitation in N_2-grown cells (above). These changes were consistent with the results of previous studies on nitrogen starvation in cyanobacteria (de Vasconcelos and Fay, 1974; Giesy, 1964; Peat and Whitton, 1967; Stevens et al., 1981a), except for the marked disappearance of carboxysomes. This is significant because cyanobacterial carboxysomes have been shown to persist during prolonged starvation for a variety of nutrients, including nitrogen (as cited above), phosphorous (Jensen and Sicko, 1974; Stevens et al., 1981b), iron (Hardie et al., 1983b), and carbon (Miller and Holt, 1977). Therefore, it is unlikely that the disappearance of carboxysomes in the present study was caused by the nitrogen starvation. The only instance reported to date in which carboxysomes have consistently and completely disappeared from cyanobacterial cells has been during heterocyst differentiation. The complete disappearance of these structures in the present study, then, was indirect evidence that all of the cells in M. laminosus were at least capable of initiating heterocyst differentiation under certain stressful conditions such as severe nitrogen starvation.

Heterocyst Differentiation in M. laminosus

This study included the first detailed analysis of the heterocyst differentiation process in a branching filamentous (stigonematacean) cyanobacterium. This is important because virtually all of the detailed information now available on heterocysts and heterocyst

differentiation has come from studies on a limited number of Anabaena sp. (Adams and Carr, 1981; Carr, 1979; Carr and Bradley, 1973; Haselkorn, 1978; Haselkorn et al., 1980; Wolk, 1982). Adams and Carr (1981) speculated that significant deviations from the currently accepted picture would be discovered when heterocysts in a wider variety of cyanobacterial types were examined with the electron microscope. The present study has had the effect of advancing this speculation into the realm of established fact.

The Ultrastructure of Heterocyst Differentiation. The sequence of ultrastructural changes that took place during heterocyst differentiation in M. laminosus was distinctly different from that which has been described for Anabaena spp. (Kulasooriya et al., 1972; Lang, 1965; Wilcox et al., 1973b). The first stages of Anabaena heterocyst differentiation all involved external changes, namely (i) the appearance of an extra wall layer known as the "fibrous layer", (ii) the appearance of the homogeneous extra wall layer (equivalent to that seen in M. laminosus), and (iii) a "squaring" of the cell poles in order to form the "necks" leading to adjacent cells in the filament. In contrast, the first stages of M. laminosus heterocyst differentiation involved internal ultrastructural changes such as (i) a decrease in polysaccharide granules, (ii) an increase in ribosomal material, and (iii) an alteration of the thylakoid membranes. M. laminosus never produced a "fibrous" layer during heterocyst differentiation, while production of the homogeneous layer and formation of necks clearly occurred only after the internal changes listed above were in progress. It is possible that internal changes preceding the formation of extra wall layers went unnoticed in the Anabaena

studies, but this is unlikely in view of the fact that all three studies were in agreement on this point. M. laminosus and Anabaena heterocyst differentiation were similar in that all specialized inclusion bodies normally found in vegetative cells eventually disappeared, but in M. laminosus, the carboxysomes disappeared much earlier in the differentiation sequence. The two organisms also differed considerably with respect to development of the internal membrane system in heterocysts. In Anabaena, the thylakoids vesiculated (split apart) prior to the appearance of lamellar stacks of membranes. It was suggested (Kulasooriya et al., 1972) that the lamellar stacks of membranes then arose by de novo formation of membrane vesicles, followed by coalescence into organized lamellae. In contrast, the thylakoids of M. laminosus did not split apart during heterocyst differentiation, and the lamellar stacks of extra membranes originated through elongation of preexisting segments of thylakoid membrane. These results indicate that the currently accepted sequence of events for heterocyst differentiation in cyanobacteria may apply only to Anabaena species. Detailed examinations of a wide variety of heterocyst-forming genera may have to be carried out before a full appreciation and understanding of this process can be obtained.

One might expect from the above that the ultrastructural characteristics of mature M. laminosus and Anabaena (Kulasooriya et al., 1972; Lang, 1965; Lang and Fay, 1971) heterocysts would differ to some extent. M. laminosus heterocysts clearly lacked the "fibrous layer" and may also have lacked the "laminated layer" that is present in Anabaena heterocysts. It is not possible to make a definite statement about the laminated layer because this layer has been re-

ported to be preserved well only during potassium permanganate fixation, and permanganate fixations were not used in the M. laminosus portion of this study. Most M. laminosus heterocysts did not contain the cyanophycin "plugs" that are normally present in the necks leading to adjacent vegetative cells. This is somewhat disturbing because the plugs generally have been considered to be a standard component of the mature heterocyst cell (Adams and Carr, 1981; Stanier and Cohen-Bazire, 1977) and have been used as the sole means for identifying heterocysts in light microscopical studies (Rippka et al., 1979). The lack of cyanophycin plugs in heterocysts of N_2-starved M. laminosus cultures may have been the result of the nitrogen starvation, since cyanophycin granules are generally thought to be a form of reserve nitrogen. However, this would not explain why the plugs were also rare in heterocysts of N_2-grown cultures. The internal membranes of M. laminosus were considerably more extensive than those of Anabaena, eventually filling almost the entire heterocyst cell. In addition, M. laminosus heterocysts contained an unusual type of inclusion body that has not been reported to occur in the heterocysts of any other cyanobacterium. All of this leaves little doubt that Mastigocladus and Anabaena heterocysts differed significantly at the ultrastructural level. It is not yet known if these ultrastructural differences relate to important functional differences between the two types of heterocysts; perhaps they also differed in terms of their mechanisms of or capacities for nitrogen fixation. Further research on this question could prove to be a productive extension of the work described here.

Morphology of Heterocyst Differentiation. One of the more unusual aspects of heterocyst morphology noticed in this study was the fact that mature M. laminosus cultures contained heterocysts of widely varying shapes and sizes. In fact, it often appeared that several distinct types of heterocysts were present in this organism. This is in sharp contrast to what has been reported for Anabaena and Nostoc species (Adams and Carr, 1981; Carr, 1979; Haselkorn, 1978; Haselkorn et al., 1980; Wolk, 1982), in which heterocysts had reasonably uniform morphological characteristics. The situation in M. laminosus was probably just the result of heterocyst differentiation being initiated in different types of vegetative cells (i.e., young and narrow, old and wide, etc.); the heterocysts simply assumed the general morphological characteristics of the cells from which they developed. Since these different types of heterocysts did not differ in their ultrastructural characteristics or in the mechanisms by which they differentiated, it is unlikely that they would have differed functionally.

The most significant and surprising aspect of the morphology of heterocyst differentiation in M. laminosus was that it bore almost no resemblance to the morphology of heterocyst differentiation in Anabaena. It is admittedly somewhat difficult to compare these two organisms, since their basic vegetative morphologies are also somewhat different (the former consisting of branching filaments and the latter containing only unbranched filaments). Nevertheless, the morphology of heterocyst development in the two organisms differed greatly even when a comparison included only the young filaments of M. laminosus that were most analagous morphologically to Anabaena

filaments. It is now well established that heterocysts form only at specific locations within Anabaena filaments, thereby producing a repeatable "spacing pattern" (Adams and Carr, 1981; Mitchison and Wilcox, 1972; Wilcox et al., 1973a, 1975). The heterocysts are always separated by a minimum number of intercalary vegetative cells, the precise number being to some extent a species-specific characteristic. It is thought that the intercalary cells are prevented from differentiating into heterocysts by an unidentified inhibitory compound that is excreted by the heterocysts already present in the filament (Literature Review). In any event, heterocyst differentiation is controlled by a precise and reproducible mechanism in Anabaena and similar genera. In contrast, the young filaments of M. laminosus did not exhibit a reproducible spacing pattern at all. The number of vegetative cells between heterocysts varied by a factor of ten and, in some cases, two heterocysts formed immediately adjacent to one another. "Double" heterocysts of this sort have been seen in Anabaena filaments only after exposure to mutagens or other environmental stresses (Adams and Carr, 1981). The results of the present study, then, indicate that heterocyst differentiation in M. laminosus is controlled by an entirely different (and, apparently, less precise) mechanism than that which has been studied so extensively in Anabaena. This in no way detracts from the excellent work done on Anabaena; it simply indicates that the conclusions stemming from this work do not apply to M. laminosus and may not apply to the other heterocyst-forming genera that have yet to be examined closely. A considerable amount of new research will be required to elucidate the mechanism by which M. laminosus regulates the differentiation of heterocysts in its young filaments.

If control of heterocyst differentiation in young filaments of M. laminosus was imprecise, it must have been almost nonexistent in the older filaments. Double and multiple heterocysts were observed frequently and, in some filaments, all of the old cells were capable of differentiating into heterocysts. Because of this, old M. laminosus filaments contained far higher percentages of heterocysts than have been reported to occur in Anabaena or related genera under any growth conditions (see references cited above). The reason for this is not entirely clear, but it is probable that the large numbers of heterocysts in old M. laminosus filaments served to supply combined nitrogen to the lateral branch filaments. If this were the case, then the older filaments in N_2-fixing cultures had a specialized function distinct from those of younger filaments. This interesting possibility merits further investigation.

Aging and Morphogenesis in M. laminosus

As stated earlier, individual M. laminosus cells did not contain any unusual or novel ultrastructural features that would distinguish these cells from those in nonbranching filamentous cyanobacteria. However, a number of interesting and novel features were noticed when (i) the ultrastructural characteristics of all the cells in a complex, branching filament were examined comparatively or (ii) the way in which these complex, branching filaments developed morphologically was examined in detail.

Nonbranching filamentous cyanobacteria reproduce by "trichome elongation" (Stanier and Cohen-Bazire, 1977); that is, the vegetative cells within a filament repeatedly elongate and then divide to produce

an increasingly longer filament. Except for length, the morphological characteristics of these vegetative cells never change during this process. All of the cells within the filament remain active and continue to divide as long as the culture itself is growing exponentially. Therefore, the individual cells of actively growing, nonbranching, filamentous cyanobacteria are physiologically and morphologically equivalent to those of a unicellular cyanobacterium; the only difference is that the former happen to remain attached to one another in a filament that is sometimes surrounded by a common sheath layer. Surprisingly, the present study has demonstrated that the situation in M. laminosus is entirely different. Vegetative cells in a new, young filament continued to divide actively for only a limited period of time, even if the culture in its entirety was still growing exponentially. After this limited period of active cell division, some mechanism apparently prevented further cell division in the usual sense (i.e., cell division parallel to the long axis of the filament) without preventing further growth of the cell body. Consequently, the cells underwent a series of morphological changes during which the originally thin, cylindrical (young) cells became wide, spherical (old) cells. This was essentially a form of cellular aging or senescence in a prokaryotic organism, since the wide, spherical cells were unable to revert back to thin, cylindrical, actively dividing form. The ultrastructural changes that took place within the cells as they rounded and became wider also indicated that these cells were aging. The rounded cells had far fewer ribosomes and, possibly, less nuclear material. At the same time, they tended to have larger numbers of storage-related inclusion bodies. These ultrastructural changes would be expected if the rounded cells were no longer involved in

active cell division or extensive synthesis of new cell materials. The aged cells in M. laminosus may have had specific metabolic functions or roles, distinct from those of the younger, actively dividing cells. It was suggested earlier that, in N_2-fixing cultures, the older cells may all differentiate into heterocysts in order to provide nitrogen to the branch filaments. Further work would be required to elucidate fully the possible specific roles of these old cells. In any event, no equivalent to these aging cells has been observed in any of the unicellular or nonbranching filamentous cyanobacteria that have been examined extensively to date.

The aged or senescent cells in M. laminosus were not moribund in the sense that they could no longer reproduce. They were not able to revert back to an actively growing form, but they could undergo one last cell division after reaching the fully developed, rounded morphological form. This division could take place only in a direction perpendicular (usually) or diagonal to the long axis of the filament. In other words, the aged cells could divide only in such a way as to start the formation of a lateral branch. The development of lateral branches was quite interesting in itself because it appeared to have the effect of reversing the aging process that took place in the main filament. The first cells formed in the branch filaments were morphologically and ultrastructurally identical to the aged cells in the main filament. The new branch cells could only divide perpendicular to the long axis of the main filament (like the aged cells from which they originated), but since the branch filament itself was perpendicular to the main filament, this had the effect of elongating the branch filament. As branch development continued, the

newer cells became increasingly "younger" in terms of their morphological and ultrastructural characteristics. It is not at all clear why this type of morphogenesis took place, unless young (actively growing) cells were needed for survival of a branch filament after it was separated from the main filament by the release mechanism described in Results. This is a reasonable possibility because release of branch filaments appeared to be the only mechanism that M. laminosus had for propagating new filaments. Unlike other filamentous cyanobacteria, the organism did not produce akinetes (which could germinate to release young filaments) or hormogonia (specialized cells related to propagation of new filaments).

It is clear from the above considerations that the morphological development of M. laminosus (and, probably, other stigonematacean cyanobacteria) is considerably more complicated than that of the non-branching filamentous or the unicellular cyanobacteria. Because of this, it will be necessary to broaden presently accepted concepts regarding the diversity of structure and function in photosynthetic prokaryotes. In addition, the aspects of M. laminosus morphogenesis described in this study should lead to several very interesting areas of research in the future.

General Statement

This study involved two independent investigations, each of which dealt with certain aspects of ultrastructure and function in cyanobacteria. These two investigations were not directly related to one another, and it was not intended that they should be. They were linked only by the fact that they both involved aspects of cyanobacterial ultrastructure that have received little or no attent-

ion in the past. That is why the detailed results of these two investigations were discussed separately above. It should be noted, however, that both studies did produce a similar general result: the organisms examined proved to be considerably more complex and sophisticated in regard to their structural characteristics than might have been expected from all previous investigations on structure and function in the cyanobacteria. Considering how much is already known about cyanobacterial ultrastructure, it is surprising that the results of both investigations in the present study were novel. It is important to realize that these results do not necessarily contradict those of previous investigations; they merely indicate that (i) the cyanobacteria are more structurally diverse and complicated than was thought to be the case and (ii) it is necessary either to use novel techniques or to concentrate on genera that have received little attention in order to appreciate fully this diversity and complexity. It is hoped that, in the future, a few investigations directed toward obtaining such an appreciation will accompany the inevitably large number of studies directly strictly toward further examination of species or topics that have already been investigated in great detail.

V. SUMMARY

This study was undertaken to examine certain aspects of cyanobacterial morphology and ultrastructure that have not received very much attention during the past thirty years. Two independent investigations were carried out in order to do this: (i) a complete three-dimensional, ultrastructural reconstruction of the unicellular cyanobacterium Agmenellum quadruplicatum and (ii) a comprehensive morphological and ultrastructural analysis of the branching, filamentous, heterocystous cyanobacterium Mastigocladus laminosus.

The three-dimensional reconstruction of A. quadruplicatum was accomplished with a combination of two rather unconventional electron microscopical approaches, high-voltage electron microscopy of thick sections and computer-aided reconstruction of serial thin sections. This was the first successful three-dimensional reconstruction of a prokaryotic organism at the ultrastructural level, and it led to a number of novel and significant findings:

1. The combination of high-voltage electron microscopy and computer-aided reconstructions used in this study proved to be an especially effective approach for elucidating the three-dimensional architecture of Agmenellum quadruplicatum. It is suggested that this approach will prove equally effective when applied to other cyanobacteria or to other structurally complex prokaryotic organisms.

2. The specialized inclusion bodies of A. quadruplicatum occupied specific locations within the cell body. Carboxysomes and polyphosphate bodies were always centrally located, while lipid bodies were

always peripherally located.

3. The central cytoplasmic portion of the A. quadruplicatum cell was surrounded entirely by the innermost photosynthetic thylakoid membrane. It was separated from the rest of the cell in what appeared to be a form of true compartmentalization in a prokaryotic organism.

4. The individual membranes of the complete photosynthetic thylakoid membrane system were arranged as an anastomosing network of concentric shells. These shells tended to interconnect and approach the cytoplasmic membrane at three locations along the entire longitudinal axis of the cell.

5. The thylakoid membrane system was connected clearly to the cytoplasmic membrane at several locations throughout the A. quadruplicatum cell. It was not possible to determine whether the thylakoids invaginated from the cytoplasmic membrane or simply were attached to it at these locations.

6. The detailed three-dimensional architecture of A. quadruplicatum was exceedingly consistent from one cell to another. This indicated that control of intracellular organization within this organism must involve a very precise mechanism.

The morphological and ultrastructural characterization of M. laminosus involved only conventional microscopical approaches, but also led to important and unexpected results. These results were obtained because this was the first comprehensive effort to characterize the morphology and ultrastructure of a stigonematalean cyanobacterium. Stigonematalean species have been avoided by electron microscopists in the past, apparently because of their complex morphological characteristics. The most important findings of the present

study can be summarized as follows:

1. The basic ultrastructural characteristics of individual M. laminosus vegetative cells were quite similar to those of other cyanobacteria. They did not contain any unusual or previously undescribed features.

2. The ultrastructural characteristics of vegetative cells were affected noticeably by the availability of nitrogen and by the identity of the source when nitrogen was available. The effects of nitrogen on ultrastructure were generally similar to those observed previously in other types of cyanobacteria.

3. The ultrastructural characteristics of heterocysts in M. laminosus differed distinctly from those of heterocysts formed by other genera (primarily Anabaena) in several ways. These ultrastructural differences implied that functional differences may also exist and would be worthy of further investigation.

4. The sequence of ultrastructural changes that took place during heterocyst differentiation in M. laminosus was also quite different from that known to occur in Anabaena. This demonstrated that the vast amount of data produced on heterocyst differentiation in Anabaena is not necessarily relevant to other heterocystous genera.

5. The morphological aspects of heterocyst differentiation in M. laminosus were exceedingly different from those which have been described for other cyanobacteria. No regular heterocyst spacing patterns were formed, and it appeared that all of the cells in old filaments could differentiate into heterocysts. This indicated that control of heterocyst differentiation must not be exerted by the precise mechanism thought to function in Anabaena.

6. The morphogenesis of M. laminosus filaments was complicated, involving the formation of lateral branches and shifts in the plane of cell division. The process appeared to involve a prokaryotic equivalent to aging or senescence.

In general, this study has shown that cyanobacteria are more structurally diverse and complicated than has been thought to be the case previously. It has also demonstrated that it is necessary to either use novel techniques or to examine genera that have received relatively little attention in order to appreciate fully this diversity and complexity.

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