A study of the fine structure of the blue-green alga, Nostoc muscorum

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A STUDY OF THE FINE STRUCTURE OF THE
BLUE-GREEN ALGA, NOSTOC MUSCORUM

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INTRODUCTION

The cellular differentiation of the Cyanophyceae is similar in many respects to that of the bacteria and these two groups are distinct from all higher plants and animals. The cells lack the membrane-bound organelles such as nuclei, Golgi, mitochondria, plastids and endoplasmic reticulum found in the cells of higher organisms. Because of these differences it has been proposed that the blue-green algae and bacteria be considered together as the Procaryota as opposed to all cells with true nuclei, the Eucaryota (Stanier and Van Niel, 1962).

As more procaryotes are studied at the fine structure level, this separation seems to become more clearly affirmed and more widely accepted. The cell envelope of the blue-greens closely resembles the cell wall characteristic of gram-negative bacteria. Both of these wall types were found to contain a wide range of amino acids and diaminopimelic acid, but the latter compound has not yet been found in higher organisms (Work and Dewey, 1953; Frank et al., 1962b). In general, most compounds are shared by the procaryotes and eucaryotes. However, sterols have been reported to be absent from both blue-greens and bacteria although they are of general occurrence in the cells of higher organisms (Levin and Block, 1964). Sterols are commonly found in the intracellular membranes of eucaryotes, and it has been suggested that the lack of sterol content may be significant in explaining the lack of membrane-bound organelles in the procaryotes (Stanier, 1961).

The blue-greens share the basic photosynthetic pigments,
chlorophyll $a$ and $\beta$-carotene with higher plants and in both cases free oxygen is a product of photosynthesis. Both groups have $\omega$-linolenic acid-containing galactolipids in the photosynthetic apparatus (Levin et al., 1964). These particular compounds have been suggested to play a role in the process of oxygen evolution and are not found in bacteria. In bacteria other substances, such as sulfur, are reduced. The photosynthesis of blue-greens resembles that of bacteria in its relationship to respiration in that light appears to inhibit oxygen uptake by both groups (Stanier, 1961).

Ris and Chandler (1963) compared two major types of genetic systems which differ significantly in molecular organization. In procaryotes and viruses the genetic system consists of DNA not bound with histone-type proteins. In the eucaryotes the genome consists of chromosomes in which the DNA molecules are combined with basic proteins. De (1965) has suggested that this finding is understandable in view of the work on the role of histones in the differentiation of DNA and that the absence of histones in procaryotes may explain the diffuse state of their nuclear material.

Although the subcellular structure and organization observed in the blue-greens is quite different from that of higher organisms, the metabolic pathways so far investigated in blue-greens are in many respects similar to those recognized in higher organisms (Black et al., 1963; Holm-Hansen and Brown, 1963; Fujita et al., 1964; Susor and Krogman, 1964; Berns et al., 1966).

Exactly how the procaryote cells perform similar functions to
those of the eucaryote cells in which the membrane-bound organelles are recognized to spatially segregate certain pathways is interesting but little understood. In fact many of the structures recognized in blue-green cells remain at present chemically unidentified and their metabolic involvements are even less understood. The present study was undertaken primarily to study cytoplasmic inclusions which appear to be characteristic of blue-green cells. A better insight into the morphology of these structures, their structural relationships and possibly their chemical composition might aid in eventually delineating their functional relationships and giving a better understanding of the cellular organization and metabolism of these organisms.
LITERATURE REVIEW

Light Microscopy

The structure of blue-green algal cells has been studied for many years, and much of the light microscopy work has been reviewed by Fritsch (1945). According to these early studies, the cell envelope was divided into two layers, the inner investment which completely surrounded the protoplast, and the cell sheath which enveloped single cells in the coccoid forms or formed a cylinder around an entire trichome in the filamentous forms. The protoplast was generally divided into two areas, the centroplasm and the chromatoplasm. The centroplasm was reported to contain volutin granules, metachromatic granules, and polyphosphate bodies. Fuhs (1958a) suggested that the polyphosphate bodies were identical to the metachromatic and volutin granules of the older studies. These granules have been suggested variously to contain polyphosphates, organic bases or nucleic acid (Fritsch, 1945). Ebel and Muller (1958) determined that the presence of polyphosphates was characterized by a metachromatic coloration with some basic dyes including toluidine blue. They also stained with methyl green and pyronine which was used to stain nucleic acids. The most specific reagent for inorganic phosphate was found to be lead nitrate at pH 3.5. A positive reaction with this reagent in association with a positive metachromatic reaction permitted positive diagnosis of polyphosphate.

Talpasayi (1963) described polyphosphate bodies as determined by the above procedure. They were freed from the cell as distinct
granules and showed a red fluorescence of RNA when viewed with ultraviolet light after treatment with buffered acridine orange. No metachromatic staining was observed after extraction with 10% trichloroacetic acid (TCA). Fuhs (1958a) reported them to be intensely red when treated with the Feulgen reagent without hydrolysis but fully removed with HCl at 60°C. The high degree of polymerization gave a chemical reaction similar to nucleic acids, and Fuhs (1958a) considered that methyl green and pyronine could not be used to distinguish nucleic acid content because the polyphosphates gave an even darker stain. The polyphosphate bodies were not disturbed by either RNase or DNase, and Fuhs concluded that the granules contained polyphosphates but not nucleic acid. Cassel and Hutchinson (1954) also reported negative tests for metachromatin or polyphosphates after digestion in 1M HCl at 60°C. This treatment also removed the cyanophycin granules. The centroplasm also contained what was believed to be true chromatin in a loose three dimensional network whose appearance varied depending upon fixation and staining conditions. Mitotic figures have been reported, but it is now generally agreed that there is no true chromosome or membrane-bound nucleus. The exact mechanism of cellular division in these cells remains unsolved.

The boundary between centroplasm and chromatoplasm was ill-defined, but in general the chromatoplasm was peripheral and, as the name suggested, contained the pigments. In addition to chlorophyll, carotenoids, and phycocyanin, cells contained varying amounts of phycoerythrin and xanthophylls. The pigments were considered as
diffuse throughout the chromatoplasm or as contained in small vesicles or granules. The photosynthetic product, a glycogen-like substance, was located in the peripheral region, but perhaps the most conspicuous components of this area were the cyanophycin granules, generally suggested to be a food reserve substance (Fritsch, 1945).

**General Electron Microscopy**

The very small size of the vegetative cells, approximately 10 microns in diameter, made their study at the light microscope level difficult, particularly with respect to subcellular organization. Recent work at the electron microscope level has done much to clarify the structure of these cells. The many studies include those of Niklowitz and Drews (1956, 1957), Drews and Niklowitz (1956, 1957), Fuhs (1958, 1963), Hopwood and Glauert (1960), Lefort (1960a, 1960b), Ris and Singh (1961), Pankratz and Bowen (1963), Giesy (1964), and Jost (1965). A general pattern of organization has developed from these studies and each succeeding work seems to confirm and extend the general description. The same proliferation of terminologies which characterized the descriptions given by the light microscopists is evident in the electron microscope studies.

**Cell Wall**

Ris and Singh (1961) described the cell envelope of *Anacystis nidulans* as consisting of 1) an exterior sheath, 2) a 7 μm outer membrane which had the appearance of a typical unit membrane with varying degrees of undulation, 3) an intermediate, dense and rather homogeneous layer or inner investment which was 12-20 μm thick, and 4) the 7 μm
plasma membrane itself which was a unit membrane. The inner investment extended into the cross walls separating neighboring cells. The three inner layers were equivalent to the inner investment described by the light microscopists. Pankratz and Bowen (1963) reported that the outer layer was diffuse and, although it was moderately dense after OsO₄ staining, it did not stain well with KMnO₄ and was not believed to be a unit membrane. Jensen (1965) described papillae 30-45 μm wide and 30-150 μm long projecting from the outer membrane. They were common in several of the species examined, and he speculated on a possible role of these papillae in trichome movement within the sheath. Echlin (1963) also observed a series of 40-80 μm blebs or protrusions around the cell periphery. Their outer boundary was formed by the outer membrane, and the inner investment projected into the protrusions. The electron-dense inner membrane of 10-15 μm did not take part in the formation of the protrusions.

The structure of the cell envelope of the blue-greens was strikingly similar to the model of the cell wall of gram-negative bacteria as proposed by Claus and Roth (1964). In this model the 8 μm plasma membrane and the 9 μm cell wall membrane, both of unit nature, were separated by a 5-10 μm less dense inner layer suggested to contain a mucocomplex and to impart rigidity to the cell wall. Chemical comparisons in wall compositions have been made by Frank et al. (1962b) and Drews and Meyer (1964). The latter authors compared the walls of the gram-negative bacterium Escherichia coli with Phormidium uncinatum and Anacystis nidulans and found muramic acid, diaminopimelic acid,
glucosamine and eight amino acids in common. The fact that diamino-
pimelic acid and muramic acid have been reported only in the walls of
blue-greens and bacteria suggests a taxonomic relationship between
these groups (Fogg, 1956; Stanier and Van Niel, 1962). The blue-green
cell wall was observed by electron microscopic studies to have several
layers. Isolated cell walls of *Anacystis nidulans* were shown to contain
polysaccharides, proteins, and lipids in addition to mucopolymers. No
morphological positions were suggested for any of the components
(Drews and Gollwitzer, 1965).

Protoplasts were successfully produced from several blue-greens
by the action of lysozyme (Crespi et al., 1962), and it was suggested
that the lysozyme-sensitive mucopolymers shared by the blue-green algae
and bacteria were located between the plasma membrane and the inner
investment of the blue-green cells (Frank et al., 1962a). This muco-
polymer was suggested to be localized in the inner layer of gram-negative
bacterial walls (Claus and Roth, 1964).

**Photosynthetic Apparatus**

The elaboration of lamellae throughout the cell or its peripheral
regions has been noted by many workers. The concept of centroplasm
versus chromatoplasm became untenable when the extent of these lamellae
and the lack of a clear demarkation between the lamellae and the
anastomosing nucleoplasm was observed. Menke (1961a) termed these
lamellar units thylakoids. Each thylakoid consisted of a flattened
membrane-bound sac. In blue-greens the opposing unit membranes may be
quite closely appressed or be separated by an intrathylakoidal space
(Drawert and Metzner 1958; Hopwood and Glauert 1960; Lefort 1960b; Ris and Singh 1961; Pankratz and Bowen, 1963). Pankratz and Bowen observed flattened thylakoids averaging 18 μm wide after osmium fixation, the 6-7 μm unit membranes separated by a 4-5 μm less dense space. After KMnO₄ fixation the appressed thylakoid consisted of a 3 μm electron-dense median region separated from lateral 2 μm dense zones by 3 μm less dense zones. Jost (1965) studied the fine structure of Oscillatoria by several techniques including freeze-etching and reported that the unit membranes of the thylakoids exhibited a certain polarity. He observed globular particles on both sides of the membranes. Those on the outside were 50-70 Å in diameter and remained visible after negative staining. The 100-200 Å particles observed on the inside of the membranes were suggested to be similar to the quantasomes of Park and Biggins (1964) and were lost during the process of negative staining. Jost concluded that the thylakoids consisted of a support layer with 50-70 Å globular particles, a homogeneous lamellar portion, and the quantasomes. The question as to whether the quantasomes were in or on the membrane was left open. Menke (1965) suggested that the thylakoid membrane was asymmetric with an inhomogeneous layer of 35 Å globules on the outer side and a homogeneous layer of a lipid nature on the inner side. The center-to-center separation of the globular particles was 34-40 Å, and they were suggested to be protein.

The origin of the thylakoids is not yet clear. Pankratz and Bowen (1963) favored the idea that the thylakoids were subdivided during cytokinesis by the annular growth of the cross-wall, and Chapman and
Salton (1962) suggested they arose de novo from the central portion of the cell and migrated to the periphery. Several workers have suggested that they may arise from the plasma membrane (Pankaratz and Bowen, 1963; Echlin and Morris, 1965; Jost, 1965). Echlin considered that the thylakoids were a complex branching reticulum formed by extensive invagination of the plasma membrane. Jost proposed a model for the formation of the thylakoid membranes by such an invagination. Small vesicles were formed by the invagination, and the vesicles then expanded into the thylakoidal system of the cell.

Pigments including chlorophyll a, xanthophylls, carotenes, and phycobilins are found within the blue-green cell. The chlorophyll and carotene were observed to be associated with particulate elements pelleted from the cell homogenates (Calvin and Lynch, 1952; Petrack, 1959; Shatkin, 1960). Calvin and Lynch negatively stained the particles and observed rounded 2 u vesicles which they considered to be grana equivalent to those of higher plant chloroplasts, but Shatkin considered that the vesicles were a result of membrane disruption. Petrack observed that, in addition to the pigments, the photophosphororation activity was associated with the particles. Although the chlorophyll and carotenoids appeared to be associated with the internal membranes, the intracellular localization of the phycobilins was less obvious, particularly since they remained in the supernate after centrifugation. Bergeron (1963) suggested that the phycocyanins were located between the lamellae. Phycocyanin, a water soluble proteinaceous pigment was usually released upon cellular disruption. Recently Susor and Krogman
(1964) studied photosynthesis in pigment-containing particles from *Anabaena variabilis*. The phycocyanin remained with the chlorophyll and carotenoids associated with the membrane fragments if Ficoll were added to the homogenizing medium. O'hEocha (1965) reported that phycocyanins were more readily dissociated and denatured than the phycoerythrins. Fuhs (1964) reported that the lamellae consisted of layers of closely packed 80 A spherical units. A preparation of phycocyanin given to him by O'hEocha showed a 80 A molecule after negative staining. He concluded that the spherical units which formed the photosynthetic lamellae were partly phycocyanin particles.

Investigations by Gantt and Conti (1966) have revealed granules attached to the chloroplast lamellae of the red alga *Porphyridium cruentum* after aldehyde fixation. These 35 mu granules were arranged in repeating rows with a center-to-center distance of 40-50 mu. They were believed to be the site of phycoerythrin concentration within the cells. Such granules were observed in a marine oscillatoria-like organism but were not found in organisms which lacked phycobilins.

Lefort (1965) studied the endosymbiont *Glaucocystis nostochinearum* and reported dense 30 mu grains disposed regularly in the interlamellar spaces after OsO₄ fixation. She suggested that these grains represented a cross section of a series of long filamentous structures located on the external surface of the lamellae. They were peculiar to symbiotic blue-greens, and no suggestion was offered for their chemical composition or possible function.

The proposed functions of the thylakoids include not only photo-
synthesis but also respiration, nitrogen fixation, nitrate reduction, and protein synthesis. Cox et al. (1964) studied Anabaena cylindrica and found nitrogen fixation activity in the pellet sedimenting between 15,000 and 35,000 g for 20 minutes, and suggested that this fixation ability was located in the photosynthetic lamellae which contained chlorophyll and carotenoids. Cox (1965) reported that work with photosynthetic inhibitors suggested that some part of the nitrogen fixation process was allied to the respiratory process rather than the photosynthetic pathway. He further suggested that nitrate reduction may be as versatile as nitrate fixation, using either light or dark generated reducing power and ATP according to prevailing circumstances, unless spatial organization of the necessary enzymes prevented this.

Jost (1965) observed small chains of 110 A particles on the thylakoids in his freeze-etched material and suggested that they were polyribosomes. Jost and Matile (1966) reported RNA in a thylakoid fraction isolated by density gradient centrifugation.

Nucleoplasm

The presence of DNA in the central region of the cells as indicated by a positive Feulgen reaction has been observed by many workers including Cassel and Hutchinson (1954), Leak and Wilson (1960), Fuhs (1963), and Pankratz and Bowen (1963).

In a series of studies on the nature of the DNA, Biswas (1957a, 1957b) and Biswas and Meyers (1960a, 1960b) determined that the nucleic acids of the blue-greens contained the four common nucleotides and methyl cytidine. They also found a polysaccharide closely
associated with the nucleic acid. They suggested that the central body contained histones bound to the nucleic acids because neither pepsin nor trypsin digested the central body. Treatment with 5% TCA for 15 minutes at 90°C left the central body intact and suggested to Biswas that the nucleic acids were bound by structural proteins. De (1965) tested species of Oscillatoria, Aphanocapsa, Anabaena, and Polycystis with 3 histochemical tests specific for histone. None of the species tested showed a positive reaction, and he concluded that the central body lacked histone.

The lability of bacterial nucleoplasm toward fixation was first demonstrated by Kellenberger et al. (1958). Versene treatment after fixation led to a coarse-structured nucleoplasm but uranyl acetate treatment led instead to a fine fibrillar organization consisting of 25 Å fibrils. Similar observations held for the fixation of nucleoplasm of blue-green algae (Ris and Singh, 1961). They described the central region of the blue-green cell as composed of 25 Å fibrils and 10-15 μm ribosomes. The ribosomes are preserved with either aldehyde or osmium but not with KMnO₄ fixation. Ris and Chandler (1963) suggested that DNA fibrils which were not combined with basic protein tended to agglutinate during dehydration unless complexed with heavy metal ions such as the uranyl ion. De (1965) suggested that the absence of histones in bacteria and blue-green algae could explain the diffuse state of the nuclear material.

Inclusions

In addition to the thylakoids and nucleoplasm, the blue-green...
cell contains a number of inclusions, some of which appear to be common
to most blue-greens, and others of which appear to be species or
collection-specific.

Several workers have noted the appearance of small granules
positioned between the thylakoids (Niklowitz and Drews, 1957; Ris and
Ris and Singh reported that they were heavily stained with lead and had
an average diameter of 25 μm. He termed these granules 'granular
inclusions' and postulated that they were photosynthetic products.
Pankratz and Bowen (1963) described 30 μm medium-dense granules which
were frequently elongate, extended between the lamellae, and surrounded
the structured granules. They termed them α-granules and suggested that
they were a food reserve or had a specific metabolic function related
to photosynthesis or respiration. Giesy (1964) studied the inter-
lamellar granules in Oscillatoria and concluded that since their
appearance ranged from the α-granules (Pankratz and Bowen, 1963) through
granular inclusions (Ris and Singh, 1961) and crystalline structures
(Menke, 1961b) to the rod-shaped inclusions observed in his own study,
that these variously described inclusions were the same. Exposure
to 2% diastase for 20 minutes removed both the interlamellar granules
and a PAS-positive substance observed in the chromatoplasm by light
microscopy. Giesy concluded that the granules were polyglucoside in
nature, and on the basis of observations correlated with growth studies
suggested that they were food reserves. Jost (1965) described 'botuli'
which he considered to be the same as the interlamellar granules
described by Giesy (1964). The 'botuli' were 35 μ in diameter and approximately 300 μ long. They were often observed to be hexagonally packed and were suggested to be a reserve substance related to the developmental cycle of the thylakoids.

Several workers have described osmiophilic granules ranging in diameter from 30-90 μ (Drews and Niklowitz, 1956; Fuhs, 1958b; Shatkin, 1960; Pankratz and Bowen, 1963). Fuhs suggested that they were polyphosphate, and Shatkin suggested that they were storage products. Pankratz and Bowen (1963) reported them to be found most frequently near the cross walls and along the lamellae. On the other hand, Leak and Wilson (1965) reported that they were randomly distributed throughout the cell. Their electron density following osmium fixation and poor preservation after KMnO₄ fixation suggested that they were lipoidal in nature. Pankratz and Bowen (1963) termed these osmiophilic bodies P-granules and suggested that they were comparable to the osmiophilic globules observed in chloroplasts.

The osmiophilic globules of higher plant chloroplasts have been studied by many workers including Murakami and Takamiya (1962) and Greenwood et al. (1963). Murakami and Takamiya reported that the diameter of globules in spinach chloroplasts varied between 70 and 120 μ, and on the basis of solvent extraction procedures suggested that these globules contained carotenoids and were lipid in nature. Greenwood et al. reported that the globules of the *Vicia faba* chloroplast were 10-500 μ in diameter and were located throughout the chloroplast matrix. Isolated globules contained no measurable carotene and these
workers suggested that they functioned in the chloroplasts as a general
deposit of insoluble lipid much as starch served as the carbohydrate
storage.

Jensen and Bowen (1961) studied the centroplasm of *Nostoc*
pruniforme. One inclusion type regularly located in this area was
roughly isodiametric and exhibited a polygonal profile from 50-360 mu
in diameter when observed in cross section. Because of their
characteristic shape these bodies were termed polyhedral bodies. In
cells fixed with KMnO₄, they possessed a fairly uniform medium electron
density, showed no regular internal structure, and sometimes appeared
to be closely surrounded by an unstained sheath 20-30 mu wide. Similar
bodies have been termed formed bodies (Hall and Claus, 1962), crystal­
line granules (Wildon and Mercer, 1963), electron-dense bodies
(Kawamatu, 1965), and granular bodies (Leak and Wilson, 1965). Ris
and Singh (1961) considered that such bodies were the third component
(in addition to DNA and ribosomes) of the nucleoplasm with which they
usually appeared to be closely associated. Pankratz and Bowen (1963)
observed that polyhedral bodies in *Symploca muscorum* were occasionally
surrounded by a 5 mu clear space and a 3 mu dark line after osmium
fixation. They were preserved by a variety of fixations including
osmium, potassium permanganate, and formalin-osmium. Costerton (1960)
observed 250-500 mu bodies which were associated with the nucleoplasm
and, because they were removed by acid digestion, suggested that they
were protein bodies.

The function of the polyhedral bodies is little understood but
Echlin (1966) labels them protein reserves on the basis of Costerton's
work. Lang (1965), in a study of heterocyst development in *Anabaena*, reported a decrease in polyhedral body size and their gradual disappearance as the heterocysts matured.

Large dense granules have been reported often near the cross walls (Fuhs, 1958b) and periphery of osmium-fixed cells, and they correspond closely in size and position to the cyanophycin granules described by light microscopists. Drews and Niklowitz (1956, 1957) termed these granules 'structured granules', and on the basis of tetrazolium and Janus green reduction suggested that they were mitochondrial equivalents. Fuhs (1958b) suggested that more work should be done before they be considered mitochondrial equivalents. Fogg (1956) considered that the granules were proteinaceous and reported them to have a high arginine content. They have been suggested to be phospholipid in nature (Fuhs, 1958b; Marcenko, 1961/1962). Marcenko reported that cyanophycin granules were not stained with Sudan III or with Janus green. Ris and Singh (1961) considered the structured granules of Drews and Niklowitz to be a type of membrane differentiation distinct from that of the lamellae. Pankaratz and Bowen (1963) observed similar granules in *Symploca muscorum*, but failed to find evidence of a membranous internal organization or of a limiting membrane. The granules did show an internal pattern of irregular dense and less-dense regions, and they were poorly preserved by KMnO₄ fixation.

In addition to the inclusions previously described which were common to most blue-greens (β-granules, polyhedral bodies, α-granules, and structured granules), there have been many interesting inclusions
reported which were not as wide spread in occurrence and, in some cases, appeared to be specific to a given collection (Ueda, 1965; Jensen, 1965). Several blue-green algae contain highly refractile areas called gas or pseudovacuoles. Bowen and Jensen (1965) described hexagonally close-packed arrays of gas vesicles. Single vesicles had conical ends and ranged from 0.2 to 1.0 μ in length with an average diameter of 75 μ. These vesicles were limited by a single electron-dense membrane 2 μ wide and, unlike typical unit membranes, this membrane was not preserved by KMnO4 fixation. Jost (1965) described 'hohlspindeln' which he observed in freeze-etched preparations and which appeared to be similar to the gas vesicles observed by Bowen and Jensen (1965). The hohlspindeln were about 65 μ in diameter, of variable length, and had a complex outer surface structure consisting of 40 Å ribs.
MATERIALS AND METHODS

Culture Techniques

Early studies employed *Nostoc muscorum* strain 1037 obtained as a unialgal culture from the Indiana University culture collection. This culture was bacterially contaminated and a *Nostoc* sp. strain M-12.4.1 obtained as a unialgal culture from the Kaiser Research Foundation and purified by Dr. James Lauritius until it was bacteria free was adopted for routine use. This organism has recently been identified as *N. muscorum* by Dr. Francis Drouet. The strain exhibited very flocculent growth in both media employed and, since it formed no clumps or sheets, it was considered desirable for the current studies.

Two culture media were used routinely. Most of the cultures were grown in Chu (Chu, 1942) with soil extract added at the rate of 40 ml per liter. Other cultures were grown in Gerloff's medium (Gerloff et al., 1950). One ml of cell suspension was used as inoculum for 100 ml of medium in 250 ml Erlenmeyer culture flasks. Cultures were generally maintained on a 12-hour alternating dark and light regime. They were grown in high-light (100 foot candles) for 10-20 days and then transferred to low-light conditions (20 foot candles). The culture chamber contained fluorescent light banks and a single 25-watt incandescent bulb. The temperature was maintained at approximately 20°C. An effort was made to use cultures between 2 and 5 weeks old and to standardize the time of day at which cultures were harvested.
Fixation Procedures

Since the culture was finely suspended, whole cells were concentrated from the culture medium by centrifugation in a clinical centrifuge at approximately 1,000 rpm for 2 minutes. Cool 4% agar was usually used to solidify the concentrated cells into a block which could be cut into small pieces and handled more easily during the subsequent fixing, dehydrating, and embedding procedures. Occasionally the cells were fixed in the suspended state and then put into agar just prior to dehydration. All of the pellets of subcellular components prepared by the various centrifugation processes required agar embedding prior to fixation.

Several fixation procedures were employed, and the details of each are outlined in Appendix A. Most of the cells were fixed in 1% osmium tetroxide in veronal acetate buffer at pH 6.1 as described by Kellenberger et al. (1958) and modified for blue-greens by Pankratz and Bowen (1963). Tryptophan was omitted from the fixative in the current studies. Other fixatives employed included 4% unbuffered potassium permanganate, (Luft, 1956; Mollenhauer, 1959) 4% formaldehyde (Pease, 1964) or 3% phosphate-buffered glutaraldehyde (Sabatini et al., 1963) with or without post-fixation in osmium. Dehydration, infiltration, and embedding in Epon 812 were performed as described in Appendix A. Several enzyme digestion procedures were employed as outlined in text Figure 1.
Preparation of Cell Homogenates

The routine homogenizing procedure employed the Mickle disintegrator. Cells were concentrated from the culture medium by crude centrifugation, and the concentrated sample was then added to approximately a ten-fold volume of 0.2 mm glass Balbini homogenizing beads. The beads were presoaked in the chosen homogenizing medium, and the total volume of beads and sample filled the Mickle sample cup. Two similar samples were prepared and run in the cold for 5-25 minutes with the Mickle adjusted for maximum displacement. The resulting brei was used immediately for negative staining studies, diluted and subjected to differential centrifugation, or layered directly on a prepared gradient for density gradient studies. If sucrose or Ficoll (Pharmacia Fine Chemicals) were used during homogenization, however, the brei was not suitable for negative staining.

Centrifugation Procedures

In addition to routine harvesting of cells by low-speed centrifugation in a clinical centrifuge, several other centrifugation procedures were employed. A Spinco Model L preparative centrifuge with a #40 head was used for the differential centrifugation. A SW-50 swinging-bucket rotor was used on the same centrifuge for the density gradient preparations. Density gradients were prepared in 5 ml celluloid tubes by carefully pipetting layers of the desired sucrose or sucrose-Ficoll solutions. Continuous gradients were prepared by establishing a discontinuous gradient and permitting it to
diffuse overnight at 4°C. Discontinuous gradients were used immediately after preparation. A Sorvall SS-3 with a SM-24 head and 3 ml tubes was occasionally used for differential centrifugation.

Spectrophotometric Measurements

Absorption spectra were obtained using a Beckman DU spectrophotometer equipped with quartz cuvetts. The samples were diluted to fill the 2 ml cuvette. No quantitative determinations were made. The absorption spectra for the solvent extraction experiments were obtained using a Bausch and Lomb Spectronic 20 colorimeter.

Microscopy

Sections were cut with a DuPont diamond knife on an LKB Ultrotome. The 40-80 μm sections were routinely picked up on clean, etched, 400-mesh copper grids, but 150-mesh carbon-coated formvar-supported grids were used for the negative staining procedures. The sections were routinely stained 10-15 minutes in methanol-uranyl acetate (Stempak and Ward, 1964). Occasionally lead citrate stain was employed (Venable and Coggeshall, 1965).

Specimens were observed using an RCA EMU-3F electron microscope at 50 kV with either a 25 μ or a 30-40 μ objective aperture. Some of the negatively stained specimens were observed at 100 kV. Micrographs were taken at 14,000 to 17,000 machine magnification on Kodak high contrast plates which were developed for 2-4 minutes in Kodak D-19 developer, or Cronar film which was developed for 1-2 minutes. The negatives were routinely enlarged 4.2 times on Kodak F2-F5
Kodabromide paper.

Negative Staining Procedure

The crude brei prepared by homogenizing the cells in the Mickle disintegrator was decanted from the homogenizing beads and negatively stained by the loop-film technique described by Murray (1963). The suspension to be examined was mixed in a small staining dish as follows: three drops of suspension, three drops of 4% phosphotungstic acid (PTA) at pH 7.2, three drops of double-distilled water, and one drop of bovine serum albumin solution. The mixture was stirred and permitted to stand 1-3 minutes so that the larger particles settled. A 5 mm platinum transfer-loop was used to transfer a sample drop to a 150-mesh carboned formvar-coated grid. The specimen was examined immediately at either 50 or 100 kV.
OBSERVATIONS

Sectioned Cells

Osmium fixation

The general preservation of cellular components after osmium fixation and uranyl acetate staining appeared excellent (Figure 2).

Unlike other strains of Nostoc muscorum, the strain employed for the major part of this study seldom had an observable sheath. The wall complex consisted of two parts, the outer member and the inner investment. The outer membrane was often slightly undulatory but measured 6-8 μ in width and exhibited the tripartate profile of a unit membrane. The inner investment was usually observed as a medium dense homogeneous 6-9 μ zone approximately equidistant between the outer membrane and the plasma membrane. The 7-8 μ plasma membrane appeared in profile as a unit membrane and had a slightly undulatory appearance similar to that of the outer membrane (Figures 2 and 5). When sectioned in oblique planes, the inner investment was not always evident, and the region between the plasma membrane and the outer membrane appeared to contain a uniform less dense material (Figure 2).

The photosynthetic apparatus consisted of an irregular array of thylakoids extending throughout the cytoplasm but most often observed in the peripheral region of the cell. After osmium fixation and uranyl acetate staining, the thylakoids characteristically appeared as a 5-parted myelin-like complex 15-18 μ wide consisting of two exterior 2-3 μ dense zones separated from a central 5-6 μ dense zone by two
2-3 μm less dense areas (Figure 2). Although this appressed condition was most characteristic, occasionally the two unit membranes were separated by an intrathylakoidal space.

Spherical 30-120 μm electron-dense β-granules appeared scattered throughout the thylakoidal region (Figure 2). The 25-30 μm α-granules were of low electron-density and were usually located between the thylakoids (Figure 9), but they were seldom well preserved by osmium fixation. Many preparations in which the α-granules were not preserved showed electron-transparent areas in the interthylakoidal regions.

Thylakoids, when viewed in oblique section, were observed to support a regular array of rod or disk-like particles (Figure 5). The array usually appeared to consist of rods approximately 6-7 μm wide and 25-40 μm in length spaced 6 μm apart.

Relatively dense 9-12 μm particles assumed to be ribosomes appeared to be concentrated in the nucleoplasm. The anastomosing nucleoplasm had areas of less electron-density in which the only observable structure was that of a fine network consisting of DNA fibers (Figures 2 and 5). A third component observed within the nucleoplasm was the polygonal polyhedral body. Single moderately electron-dense polyhedral bodies were usually 200-300 μm in diameter and were often observed in clusters (Figures 2 and 3). A 3-5 μm dense zone appeared to limit these bodies but was seldom seen in section to completely surround them. Where this zone appeared to be interrupted, the polyhedral bodies appeared continuous with the rest of the
nucleoplasm (Figures 2 and 5).

One of the least electron-dense components of the cell was the structured granule (Figures 2 and 3). These granules were roughly spherical, ranged in size up to 500 μm, and were usually observed in the peripheral portions of the cells. The granules sometimes contained irregular, more electron-dense areas but no limiting membrane was ever observed.

Figure 6 shows the cellular components after OsO₄ fixation but without a subsequent stain. In addition to the general lack of contrast, several other differences were observed. The outer membrane and the inner investment were not readily visible, but the 7-8 μm plasma membrane was stained. Quite unlike material fixed the same way but stained with uranyl acetate (Figure 2), the thylakoids (Figure 6) appeared as a 15-17 μm complex consisting of two 6-8 μm dense lines separated by a 3-4 μm less dense space. The polyhedral bodies were of uniform low electron-density. A 2-3 μm slightly more dense zone was observed surrounding some of the bodies, but it appeared quite different from, and less dense than, the unit membranes of the thylakoids. The structured granules (Figure 6) appeared more electron-dense than after staining with uranyl acetate (Figure 3) and contained irregular areas of even greater electron-density. The β-granules were osmiophilic but the nucleoplasm appeared of low electron-density throughout.

Figure 7 shows the appearance of cells stained with lead citrate after OsO₄ fixation. The greatly increased staining of the 9-12 μm ribosomes was the most striking difference observed when compared with
the unstained cell (Figure 6). The \( \alpha \)-granules and the DNA fibrils were also stained, but there was little change in the appearance of the other cellular components.

Glutaraldehyde fixation

Glutaraldehyde fixation alone provided little contrast and the cells were barely distinguishable from the embedding medium. Such cells after staining with uranyl acetate (Figure 8) had a relatively high electron-density throughout but exhibited little contrast. Each thylakoid (Figure 8) appeared as a 4-5 \( \mu \) central dense line flanked on either side by less electron-dense zones 6 \( \mu \) wide. The nucleoplasm had a finely textured appearance, and the well preserved polyhedral bodies were surrounded by a 3 \( \mu \) electron-dense zone. The \( \alpha \)-granules and \( \beta \)-granules were not stained. Cells fixed in glutaraldehyde, post fixed in osmium, and stained with uranyl acetate (Figures 9 and 10), appeared similar to those fixed in osmium (Figure 2) but \( \alpha \)-granules were more frequently observed (Figure 9).

In the early work employing *Nostoc muscorum* 1037, small tubule-like structures 12-15 \( \mu \) in width and of undetermined length were occasionally observed after glutaraldehyde-osmium fixation (Figure 4). The tubules had no apparent association with other cellular structures and were not observed in *Nostoc muscorum* M-12.4.1 which was used for the major portion of this study.

Potassium permanganate fixation

Cells fixed with KMnO\(_4\) and subsequently stained with uranyl acetate
(Figure 11) had a contrast comparable to the osmium-uranyl acetate preparations (Figure 2). The nucleoplasm assumed a greater electron-density in comparison to the peripheral cytoplasm. The polyhedral bodies showed a greatly increased electron-density but no surrounding dense zone was observed after KMnO₄ fixation. The DNA strands were visible although they often exhibited a clumped appearance. The ρ-granules were represented by small round electron-transparent areas (Figure 11). The appearance of cells fixed in KMnO₄ but not subsequently stained is shown in Figure 12. Each thylakoid appeared as a 14-17 μm complex consisting of two 6-7 μm unit membranes separated by a 3-4 μm space. The polyhedral bodies appeared slightly electron-dense, and the nucleoplasm appeared finely granular with regions of low electron density, especially when compared to the post-stained cell (Figure 11). The ρ-granules (Figure 12) were represented by small electron-dense rings, but the structured granules were not preserved.

Solvent Extraction

Unfixed cells and cells previously fixed in glutaraldehyde were treated with several solvents (100% methanol, 100% acetone, or 20% acetone-80% methanol) in an attempt to extract pigments and to observe any correlated morphological change.

Unfixed cells were treated with 20% acetone-80% methanol as described by Gantt and Conti (1966) and fixed in osmium. The control consisted of similarly fixed cells from the same culture which had not been solvent treated. The control appeared similar to the osmium-fixed cells previously described (Figure 2), but the treated cells were
quite altered (Figure 13). They appeared quite crenated, and the outer membrane and inner investment were separated from the plasma membrane and the protoplast. The outer membrane appeared highly convoluted, and many small papillae were observed. The cell contents appeared clumped and there were myelin-like membranous whorls in the cytoplasm. The polyhedral bodies remained recognizable. The thylakoids consisted of two 3 μm electron-transparent layers separated by an electron-dense layer 4-6 μm wide. The 3 μm dense zones which flank this configuration in the control were hard to distinguish or were absent. The β-granules were also less apparent than those in the control.

Because of the apparent extensive damage to the solvent-treated unfixed cells, cells were prefixed with glutaraldehyde, then treated for 1 hour with either 100% methanol, 100% acetone, or 20% acetone-80% methanol, and post-fixed in osmium. In general, the cellular preservation was improved. The controls appeared similar to glutaraldehyde-osmium fixed cells previously described (Figure 10).

The cells treated with 100% acetone had a slightly granular appearance. Few electron-dense β-granules were observed after the solvent extraction and several less-dense areas were observed from which the β-granules may have been extracted (Figure 14). The appearance of the thylakoids and other structures was similar to that of the control. The absorption spectrum of the solvent extract indicated carotenoid extraction but little extraction of chlorophyll.

Cells treated with 100% methanol (Figure 15) showed a slight derangement of the thylakoids and the electron-dense outer zones of
the thylakoids were difficult to distinguish. Some of the peripheral $\beta$-granules were removed leaving less electron-dense areas. Many of the more interior $\beta$-granules remained intact but this could be accounted for if the solvent gradually penetrated the cell. A thin electron-transparent zone was observed interior to the inner investment, and the plasma membrane was not visible. This zone was not observed in either the control or the acetone-extracted cells. Other cell components were not visibly changed. The absorption spectrum of the extract indicated both carotenoid and chlorophyll extraction. Cells extracted with 20% acetone-80% methanol appeared similar to the methanol-extracted cells just described (Figure 15), and the absorption spectra of the extracts were also similar.

The absorption spectra of the cells after solvent extraction indicated that some of the chlorophyll and carotenoids remained unextracted, but the cells exposed to 100% methanol and to 20% acetone-80% methanol retained relatively little chlorophyll. The phycocyanins were not removed from the cells by the solvents employed.

**Enzyme Treatments**

Several enzymes including pepsin, trypsin, chymotrypsin, lipase, deoxyribonuclease (DNase) and ribonuclease (RNase) were used in an effort to extract known compounds from intact cells (Figure 1).

An initial experiment was done in which unfixed cells were exposed to RNase but the control (which had been exposed to buffer only) showed such extensive damage that subsequent digestions were performed using pre-fixed cells. The cells were fixed with either glutaraldehyde
or formaldehyde, rinsed, incubated in the enzyme or corresponding control conditions, post-fixed in OsO₄, dehydrated, and embedded.

A study of Figure 1 shows that, with one exception, no specific extraction attributable to the enzyme employed was attained. Of particular interest, however, were the cells prefixed in formaldehyde and exposed to RNase according to the method of Jacobson et al. (1963). Although the cellular preservation was less than desirable, the results were consistent. The nucleoplasm in the digested material (Figure 16) consisted of a single, moderately electron-dense mass and the polyhedral bodies could not be seen as discrete entities. The control (Figure 17) also exhibited a clumped appearance, but the polyhedral bodies remained structurally intact. In both instances the outer membrane of the cell was quite undulatory and sometimes formed papillae (Figures 16 and 17). The formation of papillae was also common in cells treated at 60°C (Figure 21) or with Tris buffer (Figure 20).

The remainder of the enzyme treatments failed to visibly alter the cellular components if the treated cells were carefully compared with their corresponding controls. However, several of the controls were of interest. Since formaldehyde-osmium fixation had not been employed in the current work prior to this study, it was interesting to note that it compared favorably with the glutaraldehyde-osmium fixation often employed (Figures 18 and 19).

In glutaraldehyde-fixed cells incubated at 60°C (Figure 21), peripheral electron-dense myelin-like whorls were observed. The structured granules usually observed in this region were not found.
<table>
<thead>
<tr>
<th>FIXATION*</th>
<th>ENZYME TREATMENT</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hr F</td>
<td>RNase (0.07%) in dist. H2O pH 6.6 4 hr. 60°C, TCA (5%, 4°C, 30 min).</td>
<td>Specific extraction. Figures 16 and 17. Poor fixation, crenation, papillae.</td>
</tr>
<tr>
<td>12 hr G</td>
<td>RNase (0.07%) in dist. H2O pH 6.6 4 hr, 60°C, TCA (5%, 4°C, 30 min).</td>
<td>No specific extraction. Figure 21. Fixation poor. Slight crenation and formation of papillae. Myelin-like whorls.</td>
</tr>
<tr>
<td>2 hr G</td>
<td>RNase (0.08%) in dist. H2O, pH 6.8 10 hr, 37°C.</td>
<td>No specific extraction. Fixation poor.</td>
</tr>
<tr>
<td>2 hr G</td>
<td>RNase (0.05%) in phosphate buffer pH 7.4, 4 hr, 32°C.</td>
<td>No specific extraction. Fixation poor.</td>
</tr>
<tr>
<td>4 hr F</td>
<td>RNase (0.07%) in Tris-HCl buffer pH 7.6, 18 hr, 20°C, TCA (5%, 4°C, 30 min).</td>
<td>No specific extraction. Fixation poor. Walls separated from cell. Cells crenated.</td>
</tr>
<tr>
<td>12 hr G</td>
<td>RNase (0.07%) in Tris-HCl buffer pH 7.6, 18 hr, 20°C, TCA (5%, 4°C, 30 min).</td>
<td>No specific extraction. Figure 20. Fixation poor. Slight crenation and formation of papillae.</td>
</tr>
<tr>
<td>2 hr G</td>
<td>Lipase (0.2%) in phosphate buffer at pH 7.4, 4 hr, 32°C.</td>
<td>No specific extraction. Fixation fair.</td>
</tr>
</tbody>
</table>

*F=Formaldehyde fixation  
G=Glutaraldehyde fixation  
Cells were post-fixed in OsO4 after treatment.

Figure 1. Enzyme Treatments Employed
<table>
<thead>
<tr>
<th>FIXATION</th>
<th>ENZYME TREATMENT</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hr G</td>
<td>Trypsin (0.1%) in phosphate buffer pH 8.0, 4 hr, 32°C.</td>
<td>No specific extraction. Fixation fair.</td>
</tr>
<tr>
<td>12 hr G</td>
<td>Chymotrypsin (0.05%) in Tris-HCl buffer pH 7.6, 18 hr, 20°C</td>
<td>No specific extraction. Figure 19. Fixation fair.</td>
</tr>
<tr>
<td>4 hr F</td>
<td>Chymotrypsin (0.05%) in Tris-HCl buffer pH 7.6, 18 hr, 20°C.</td>
<td>No specific extraction. Figure 18. Fixation fair.</td>
</tr>
<tr>
<td>2 hr G</td>
<td>Pepsin (0.15%) in 0.01 N HCl, 4 hr, 32°C.</td>
<td>No specific extraction. Figures 23, 24, and 25. Polyhedral bodies removed. Slight formation of papillae.</td>
</tr>
<tr>
<td>12 hr G</td>
<td>DNase (1%) in 0.003 M MgSO₄ pH 6.6, 4 hr, 20°C, TCA (5%, 4°C, 30 min.)</td>
<td>No specific extraction. Cells granular. Fixation poor.</td>
</tr>
<tr>
<td>4 hr F</td>
<td>DNase (1%) in 0.003 M MgSO₄ pH 6.6, 4 hr, 20°C, TCA (5%, 4°C, 30 min.)</td>
<td>No specific extraction. Fixation poor.</td>
</tr>
</tbody>
</table>

Figure 1. continued
Figures 23, 24, and 25 show the range of variation observed in the cells exposed to 0.01 N HCl which was the control for the pepsin treatment. There was a slight formation of papillae from the outer membrane (Figure 24). The nucleoplasm was very electron-dense and had a clumped rather than fibrillar appearance. The appearance of the polyhedral bodies varied greatly and was paralleled by similar changes in the inter-thylakoidal regions of the cell. In some cells they were totally removed (Figure 23), but in others they were observed as electron-transparent regions containing small clumped fibrils (Figure 25). The structured granules were also removed by this acid treatment (Figure 22).

Cell Homogenates

Sectioned pellets

Cells homogenized in various media, including distilled water and Tris buffer at pH 7.2, were subjected to differential centrifugation. In general, whole cells, wall fragments, and large thylakoid fragments were pelleted first. Increasing centrifugal forces brought down increasingly smaller thylakoidal fragments and finally small fibrils only. The blue phycocyanin pigment could not be pelleted from solution by forces as great as 105,000 g for 2 hours. The composition of the successive pellets overlapped considerably, giving no clear-cut separation of subcellular particles.

Several subcellular components including polyhedral bodies and structured granules were not observed in the early work, and since the
centrifugation procedures required considerable time to complete, it was thought that fixation of the cells prior to homogenization might stabilize these structures. Cells fixed in osmium or glutaraldehyde prior to homogenization were difficult to rupture. Whole cells and large fragments were common, but few components were released free into the medium and no satisfactory separation was achieved. However, fixation after cell homogenization but prior to centrifugation did not appreciably alter the appearance or behavior of the particles when compared with unfixed preparations.

Attempts to isolate polyhedral bodies following disruption of the cells in buffers or distilled water alone failed. The first successful isolation was achieved in a medium of 20% Ficoll-17% sucrose-0.01 M NaCl and subsequently in the same medium without Ficoll. The cell breakage was decreased in these viscous media. The polyhedral bodies were found distributed throughout the various pellets after differential centrifugation, but appeared most numerous in the pellet obtained between 5,000-8,000 g. The polyhedral bodies freed from the cells (Figures 26, 27, and 29) appeared similar to those observed in intact cells. They remained very electron-dense and their characteristic polygonal profile was observed. They were surrounded by 2-5 μm electron-dense 'membranes' but no unit nature was discerned. In several of the isolated polyhedral bodies (Figures 26 and 27), there appeared a clear zone between the body itself and the associated 'membrane'. Although most bodies appeared homogeneous (Figures 26 and 27), several appeared partially empty with only granular material
remaining (Figures 28 and 29). Occasionally profiles were observed of polyhedral body 'membranes' devoid of contents but retaining their angularity (Figure 28). In homogenized material, the bodies often appeared to retain the close association with one another (Figures 27 and 28) which was observed in sectioned cells (Figures 2, 3, and 10). The polyhedral bodies usually appeared free from other cellular components, but occasionally they appeared to be very closely associated with the thylakoidal fragments (Figure 26).

Structured granules have been observed in several preparations but relatively pure fractions were not obtained. Structured granules observed in glutaraldehyde-osmium fixed cell homogenates (Figure 30) appeared quite homogeneous with dense wrinkles near the periphery suggesting a rough surface. After osmium fixation (Figure 31), the same roughened surface was apparent, and there were small less dense areas within the granules. Structured granules fixed in osmium and exposed to ethanol (Figure 32) appeared to be partially dissolved and clumped together in aggregates.

In an effort to increase the degree of separation of various components, cell homogenates were subjected to density gradient centrifugation. The various layers obtained were harvested from the bottom of the tube by a puncture with a specially designed needle device. After centrifugation at 100,000 g on a 60-20% sucrose gradient there was a thick green pellet at the tube bottom and blue pigment in the sample layer at the tube top. The intermediate banding was quite variable, but there usually was a yellow band ranging from
distinct to rather diffuse near the tube bottom. When this band was harvested, it was found to have an absorption maximum near 440 μm. A pellet of this material prepared for electron microscopy (Figure 35) was observed to contain thylakoidal vesicles with an average diameter of approximately 250 μm and a high proportion of β-granules. Occasionally the β-granules appeared to be associated with the thylakoidal vesicles. In lower speed pellets from studies in which the thylakoidal system was less disrupted (Figure 33), the β-granules were associated with the thylakoids, and as many as three or four membranes appeared to intersect at a β-granule.

Fractions from the density gradient centrifugations were overlapping and the small volume of material obtained on harvest was easy to lose in the subsequent repelleting and preparation for electron microscopy.

Occasionally fragments of the cell envelope were observed (Figures 27, 35, and 37). Both the outer membrane and the plasma membrane retain the appearance of unit membranes (Figure 36). The inner investment is visible as a comparatively electron-dense zone. Often the plasma membrane does not appear with the wall fragments (Figure 27) but it is perhaps pulled away as the cell contents are lost (Figure 37).

In one of the early fractionation studies, Nostoc muscorum strain 1037 was employed and a high speed pellet was obtained (Figure 34) which contained 15 μm x 100 μm rod or tubule-like structures. These rods appeared to have 4-5 μm electron-dense zones on each side and an electron-transparent interior region giving the
appearance of a small tubule. Although such structures have not been regularly observed in homogenates of strain M-12.4.1, similar-appearing rods were occasionally observed (Figure 27). These rods were 12-15 μm wide and 100 μm in length but appeared to be of uniform electron-density.

Thylakoids separated from cells which were homogenized in water and subsequently prepared for electron microscopy, had a smooth surface (Figure 33) and most typically appeared as expanded vesicles. As the speed required to sediment the fragments was increased, the size of the vesicles obtained decreased. Most thylakoid fragments pelleted from a sucrose homogenizing medium also had a smooth surface and retained their 70 μm unit membrane profiles (Figure 41). When Ficoll was employed in the medium, isolated thylakoid fragments had a coarse or rough surface (Figure 38) and in certain cases were seen to be studded with 75-90 Å particles. The particles were seen on the outer surface of the expanded thylakoidal vesicle. Sometimes the thylakoids remained relatively unexpanded and appeared appressed (Figure 40) similar to those observed in sectioned cells. Elongate projections were observed on the extrathylakoidal surfaces but the intrathylakoidal spaces appeared structureless. These projections were 4-7 μm wide but varied in length. Cells which failed to be disrupted in the Ficoll medium appeared very well preserved (Figure 43). The thylakoids in these cells often appeared to be studded with 6-7 μm projections which were regularly spaced and about 6 μm apart (Figures 43 and 44). These projections closely resembled those observed in Figure 40 and
were further suggestive of the membrane particles observed in Figure 5.

An effort was made to stabilize cellular components by breaking the unfixed cells in a 1% solution of glutaraldehyde. The homogenization took 20 minutes and many of the cells remained intact or only slightly broken. The cell fragments which were released however were quite interesting. Few of the thylakoid fragments were fully expanded (Figures 39 and 42) and most retained a partially appressed profile with only narrow intrathylakoidal spaces. Some of the membranes (Figure 42) appeared to support 20-30 μm globular particles on their outer surface. In oblique section these particles appeared to be arranged in rows approximately 35 μm apart. In other instances the thylakoids appeared slightly more expanded (Figure 39), and small particles approximately 6-7 μm wide and 10 μm long were observed on the outer surface. These resembled the particles observed in Figure 40 but appeared better preserved. Granules roughly 15 x 45 μm also appeared to be attached to some of these membranes. It is not clear whether the presence of such granules was due to better preservation of the components or to a clumping effect not observed in other preparations.

Negatively stained preparations

Samples from unfixed homogenates which were prepared immediately for electron microscopic observation were far superior to those prepared from glutaraldehyde or osmium-fixed homogenates. However, the fresh
samples seemed to deteriorate rapidly, and by 30 minutes after cell rupture much of the potential detail was lost from the preparation. Cell homogenates prepared in the Mickle disintegrator and negatively stained with 1% uranyl formate or 4% PTA were placed on carboned formvar-coated grids and observed immediately.

The thylakoid fragments observed in these preparations varied from vesicular or tubular elements to flat sheets (Figures 45, 47, 48, and 49). Often the tubular regions showed an expanded bulbous end or holes at which bulbous or tubular evaginations could have broken off (Figure 48). The less disrupted fragments (Figure 45) were suggestive of a continuous anastomosing tubular network. The surface of the thylakoids varied from a smooth (Figures 47 and 48) to a stippled appearance (Figure 45 and 49). In the most pronounced case (Figure 49), the membrane particles had an average diameter of 7.5 μm. Small rod-shaped particles (Figures 47 and 48) averaging 14 x 93 μm were observed in association with some of the thylakoids. They appeared singly (Figure 47) or in packets of two or three (Figure 48). These rods appeared homogeneous and were similar in dimensions to those observed in sectioned homogenates of strain M-12.4.1 (Figure 27) and strain 1037 (Figure 34).

Electron-transparent globules ranging from 60-120 μm in diameter were observed in negatively stained preparations (Figures 45, 48, and 49) and were considered to be the β-granules. They sometimes appeared free from other cellular components but were often observed in association with the thylakoids (Figure 45).
Although α-granules were not observed in pellets of cell homogenates, they were observed after negative staining (Figure 46) and appeared similar to those observed in sectioned cells (Figure 9). They were composed of two approximately equal parts joined at a slight constriction (Figure 46). The isolated granules averaged 30 μm wide and 70 μm long and were of uniform density except at the more electron-dense midline. The substructure was much more evident after staining with uranyl formate (Figure 46) than with PTA (Figure 49).

No structures which correlated well with structured granules or polyhedral bodies were observed in negatively stained preparations.
DISCUSSION

The Cell Envelope

The absence of a sheath in the organism studied was surprising but may be related to the strain employed, the age of the culture, or its growth habit. *Nostoc muscorum* strain 1037 which was used in the early work was observed to have a sheath and tended to grow in thin sheets. *N. muscorum* strain M-12.4.1 exhibited a very flocculent growth and no sheath was observed. Cultures of M-12.4.1 were usually 2-3 weeks old when harvested for studies but older cultures of 1037 were employed.

The structure of the remainder of the cell envelope (Figures 2 and 5) is in general agreement with the observations by Ris and Singh (1961) on several related organisms. The dense inner investment in the *Nostoc* studied in the current work, however, averages 10 mu in width as compared to 20 mu for the *Nostoc* employed by Ris and Singh. This difference is not surprising since the inner investment was observed to vary in width from 10 mu in *Anacystis* to 200 mu in *Oscillatoria* (Ris and Singh, 1961). The inner investment was observed to be approximately equidistant (6 mu) from the outer membrane and the plasma membrane but the intervening regions appeared to be filled with a less electron-dense substance and did not appear to be an artifact of fixation as suggested by Echlin and Morris (1965).

In cell homogenates the cell envelope was sometimes observed intact (Figure 36) and the fact that the layers were associated and the outer membrane and plasma membrane retained their respective
distances from the dense inner investment further suggested that the intermediate regions were not artifact but indeed contained some less dense substance.

The general structure of the *Nostoc* cell envelope was similar to that proposed by Claus and Roth (1964) for the gram-negative bacteria in which the structurally similar wall and plasma membranes were separated by a 5-10 mu less dense 'inner layer'. In certain planes of section through blue-green walls, the region between the outer membrane and the plasma membrane appeared homogeneous but it was approximately 20 mu thick. The relationship of the dense inner investment usually observed within this region, to the 'inner layer' of the gram-negative bacteria is not clear. Claus and Roth (1964) suggested that the inner layer contained the mucocomplex which imparted rigidity to the wall. Although blue-greens are known to contain a similar mucopolymer and protoplasts have been formed in several species by the action of lysozyme (Crespi et al., 1962) it is not yet clear where this mucopolymer is located within the wall. Frank et al. (1962a) suggested that it was located in the region between the inner investment and the plasma membrane. The 7-8 mu plasma membrane was observed as described by Lefort (1960b) and behaved similarly to the unit membranes of the thylakoids with respect to fixation and staining procedures (Figures 5, 6, 7, 11, and 12). The outer membrane and the inner investment, however, were not visibly stained after OsO₄ (Figure 6) or KMnO₄ fixation (Figure 12) but did increase greatly in electron-density after the subsequent uranyl acetate staining (Figures 2 and 5).
This suggested a chemical difference between the outer membrane and the plasma membrane. This difference was also indicated by some of the solvent extraction studies. The plasma membrane was removed by methanol (Figure 15) or acetone-methanol treatment, but both the inner investment and the outer membrane were still clearly visible. Although the appearance of the outer membrane and the plasma membrane were similar in stained, fixed cells, it is suggested that their chemical compositions are quite different and that, in particular, the plasma membrane has a greater lipid content.

Papillae associated with the outer membrane and resembling those described by several workers (Echlin 1963a, 1963b; Jensen 1965) were observed. They were postulated by Jensen to play a role in filament movements. Most well preserved cells (Figures 2, 5, and 10) did not show these papillae but under several experimental conditions they were quite evident. The formation of papillae was observed in some of the preparations associated with enzyme studies, but the blebbing seemed to be most common in cells incubated at 60°C (Figures 20 and 21) or in acid conditions (Figure 24) and not correlated to the enzyme employed. Cells treated with 20% acetone-80% methanol prior to fixation showed the most prominent blebbing (Figure 13). The wall was separated from the underlying plasma membrane and protoplast, and although the undulatory outer membrane appeared to be in contact with the inner investment, the latter layer was not observed to extend into the projections as reported by Echlin and Morris (1965). The fact that well preserved cells failed to show the presence of papillae,
coupled with the observation that when present they were accompanied by rather poor fixation of the entire cell suggests that the appearance of such papillae in *Nostoc muscorum* is a fixation artifact rather than a normal condition of the cell.

**Photosynthetic Apparatus**

The photosynthetic units of *Nostoc muscorum*, the thylakoids, were distributed throughout the cytoplasm with the majority positioned near the cell periphery. Their distribution appeared to be more random than that observed by Pankratz and Bowen (1963) for *Symploca muscorum*. The thylakoid (Menke, 1961a) consists of a flattened membrane-bound vesicle, and the opposing membranes may appear closely appressed or separated by an intrathylakoidal space varying in width. The general appearance and dimensions of the thylakoids observed in sectioned material agreed with the observations of previous workers (Menke, 1961a; Pankratz and Bowen, 1963; Jensen, 1965).

Rod or disc-like particles 6 μm wide seen on the outer surfaces of obliquely sectioned membranes appear to be a previously undescribed thylakoid component. They were preserved by osmium, glutaraldehyde-osmium and formaldehyde-osmium fixations but have not been observed after KMnO₄ fixation. Similar particles were particularly evident in sectioned cells and thylakoid fragments isolated from cell homogenates prepared in a Ficoll-sucrose medium (Figure 40). Although this medium was employed by Susor and Krogman (1966) in order to retain the phycocyanin on the lamellae, it is not clear whether or not the rod-like elements preserved by this medium are the site of phycocyanin
association. The fact that these workers could prepare membrane fragments which retained the phycocyanin argues for at least a loose structural association between these pigments and the photosynthetic lamellae, although Costerton (1960) suggested the phycocyanins were confined to the chromatoplasm but not bound to membranes.

Fuhs (1964) reported that a protein layer of the thylakoid consisted of 80 A particles some of which were phycocyanin. In the present study isolated membrane fragments not possessing phycocyanin appeared as unit membranes and it seems unlikely that a major membrane component could have been removed.

Since purified phycocyanin particles were reported to be 80 A in diameter (Fuhs, 1964), it is possible the 75-90 A particles observed on negatively stained membrane surfaces (Figures 45 and 49) or the 60-70 A rod-like particles observed in sectioned cells (Figure 5) are the phycocyanins.

Phycoerythrin, an accessory pigment of red algae was reported to be localized in 30 mu particles on the surface of the chloroplast lamellae of Porphyridium cruentum (Gantt and Conti, 1966). Similar 30 mu particles have been observed on the outer surface of the thylakoids in the blue-green endosymbiont Glaucocystis nostochinearum (Lefort, 1965) but no function was suggested. Similar particles have not been observed in organisms which lack phycobilins as accessory pigments. The Nostoc muscorum employed in the present study does not exhibit an absorption at 565 mu indicative of phycoerythrins, and 30 mu granules have not been observed in sectioned cells. Gantt and Conti
(1966) indicated that glutaraldehyde fixation was necessary to preserve the granules. In the current study some of the thylakoids released into glutaraldehyde at the time of disruption exhibited an array of 30 μm spherical particles (Figure 42). Since the organism employed does not exhibit a phycoerythrin absorption and since such 30 μm particles have not been observed in sectioned cells, it is not suggested that the currently observed particles contain phycoerythrins. However, it is possible that phycocyanins and phycoerythrins are associated within the cell in similar aggregates, and that because the phycocyanins are more easily dissociated and denatured (O'hEocha, 1965) they have not been adequately preserved in the blue-green algae. This is particularly possible since the choice fixation for blue-greens is osmium, and osmium is particularly destructive to the phycobilins (Gantt and Conti, 1966).

Although Jost (1965) reported 10-20 μm closely packed spheroids which he considered to be analogous to quantaosomes on the inner side of the thylakoid after freeze-etching, no similar particles were observed in the current study. The intrathylakoidal spaces were observed to be empty after the treatments employed (Figures 38, 39, and 40).

Chains of 110 Å particles similar to those considered by Jost to be polyribosomes were not observed but this may have been due to the different preparative techniques. In negative staining preparations 75-90 Å particles were observed on thylakoidal surfaces (Figures 45 and 49) but these particles were small compared to the 9-12 μm ribosomes observed in sectioned cells (Figure 7). Since ribosomes
were preserved by the methods employed in the current study, it is curious that they were not observed to be associated with the thylakoids if indeed this were a regular occurrence. Jost and Matile (1966) supported Jost's earlier conjecture of ribosome association with the thylakoids by observing RNA in a thylakoid preparation. This observation may, however, be open to other interpretations. Although wall fragments, gas vacuoles and phycobilins were separated by centrifugation from the thylakoids, the authors did not suggest where other cell components including β-granules, α-granules, free ribosomes, structured granules, and polyhedral bodies were located. The difficulties encountered in the current study in separating components from the thylakoids suggest that the preparation by Jost and Matile may not be pure thylakoids.

One thylakoid fraction isolated by density gradient centrifugation was observed to have absorption maxima at 440 and 675 μm indicative of carotenoids and chlorophyll. The carotenoid absorption relative to that of chlorophyll was greatly increased compared to the spectrum obtained from whole cells, and the pellet obtained from this fraction contained a high proportion of β-granules (Figure 35) when examined by electron microscopy. Thylakoids have previously been observed to contain both carotenoids and chlorophyll (Calvin and Lynch, 1952; Shatkin, 1960; Susor and Krogman, 1964). The β-granules could be removed from the cell by either acetone or methanol (Figures 14 and 15), and carotenoid absorption appeared in the extracts. From the present study it is concluded that although the thylakoids contain
both carotenoids and chlorophyll, the $\beta$-granules also contain carotenoids.

The $\beta$-granules have not been isolated free from the thylakoid fragments. The apparently close association of these granules with the thylakoids, particularly in cases in which the thylakoids remained relatively intact (Figure 33), suggests that these two elements could be structurally associated. Although the $\beta$-granules never appeared to adsorb to each other the possibility that they adsorb to the membrane after homogenization remains.

The $\beta$-granules of blue-greens have been previously compared to the osmiophilic globules observed in the chloroplasts of higher plants (Pankratz and Bowen, 1963). Globules isolated from spinach by Murakami and Takamiya (1962) were not membrane associated but did exhibit a high carotenoid content. Other workers, however, have failed to detect carotene in isolated chloroplast globules (Greenwood et al., 1963). Both $\beta$-granules and the osmiophilic droplets appeared to be lipid in nature on the basis of fixation and staining procedures and are at least spatially associated with the photosynthetic apparatus. The cellular function of neither component is known.

Nucleoplasm

The nucleoplasm of *Nostoc muscorum* appeared much like that described by Ris and Singh (1961) for several blue-greens and consisted of an anastomosing network with 3-5 A DNA fibrils in the less dense regions and many ribosome-like particles in the more dense areas (Figures 2 and 5). Fuhs (1958a) identified RNA in the nucleoplasmic
region by staining with pyronine, and no staining was observed after
ribonuclease digestion. Identification of these particles at the
electron microscope level has not yet been performed but the 9-12
mu ribosomes (Figure 7) agree closely in size to other plant ribo-
somes (Szarkowski et al., 1960). Luft (1956) reported that K\text{MnO}_4
fixation failed to fix ribosomes. In the current work, ribosome
particles were not visible after such fixation (Figure 12), but if
the fixed cells were subsequently stained with uranyl acetate,
the nucleoplasm yielded a marked increase in density (Figure 11).
The DNA fibrils were stained, and a general increase in granularity
was observed. This granulation was quite fine and did not appear to
be intact 9-12 mu ribosomes. Ribosomes were most apparent after
osmium fixation and lead citrate staining (Figure 7), but even in this
preparation they were not observed to be associated with the thylakoid
membranes as suggested by Jost (1965).

Inclusions

The 30 mu interlamellar granules termed \(\alpha\)-granules (Pankratz and
Bowen, 1963) and shown to be polyglucoside in nature (Fuhs, 1963;
Giesy, 1964), are difficult to fix and stain in a consistent manner.
Glutaraldehyde fixation generally aids in their preservation (Figure 9),
but in osmium fixed preparations electron-transparent regions are
observed between the lamellae where the \(\alpha\)-granules are usually located
(Figure 2). The 'botuli' described by Jost (1965) and considered by
him to be equivalent to \(\alpha\)-granules were 35 mu wide and 300 mu long
with a globular surface appearance in negatively stained
preparations. Jost and Matile (1966) reported the presence of the 'botuli' after centrifugation procedures. In the present work no α-granules were recovered after the centrifugation treatments. They were probably dissolved before the pellets were prepared and fixed. They were, however, released intact from the cells, and negatively stained preparations revealed them to be 30 μm by 70 μm bipartate structures similar to those observed in sectioned cells (Figure 46). No structures similar to 'botuli' were observed. If indeed the 'botuli' are equivalent to the α-granules as observed by Pankratz and Bowen (1963), they may manifest a different physiological condition.

The structured granules observed in Nostoc muscorum appeared similar to those previously described by many workers including Pankratz and Bowen (1963) and Jensen (1965), but they were never observed to be membrane bound. Isolated structured granules were observed, some of which appeared to have holes as if they had been partially dissolved (Figure 31). This observation, coupled with their staining properties after OsO₄ fixation into more and less dense areas (Figure 6), suggested that they were not chemically homogeneous. Isolated structured granules fixed in osmium were much better preserved than those fixed in osmium and subsequently exposed to ethanol (Figure 32). The latter granules appeared to be much less dense. Structured granules observed in intact cells after OsO₄ fixation (Figure 6) were much more electron-dense than those subsequently exposed to methanol-uranyl acetate stain (Figures 2 and 3). The observation that the structured granules were moderately electron-dense after OsO₄
fixation but were not well preserved with KMnO4 fixation coupled with the, admittedly circumstantial, observation of decreased staining upon exposure to ethanol and methanol suggested that the structured granules were at least partially lipid in nature. Myelin-like areas were observed in some cells where one would expect to observe structured granules. Fuhs (1958b) suggested that the structured granules were phospholipid. Jensen (1965) observed the structured granules to be partially digested by pepsin and suggested that they were partially proteinaceous. In the present work, attempts to repeat this observation using cells which had not previously been embedded in Epon failed. The granules did indeed appear to be partially digested after exposure to the pepsin, but control cells exposed to 0.01 M HCl were equally affected (Figure 22). It is suggested that the low pH rather than the pepsin was responsible for the removal of the structured granules. The possibility of a proteinaceous component has neither been affirmed nor rejected on the basis of the current work.

The polyhedral bodies of Nostoc muscorum appeared as previously described for similar organisms by Jensen and Bowen (1961) and Pankratz and Bowen (1963). Individually they were usually between 100 and 400 μm in diameter but they often appeared in clusters up to 1 μm in size.

Halicki (1964) stated that the polygonal profile was 'definitely a result of compression', but from several of the present observations it is concluded that they have an angular shape independent of compression effects. Single angular polyhedral bodies (Figures 5 and
10) and angular bodies on the edges of clusters (Figures 6 and 12) are commonly observed. The characteristic polygonal shape was also retained upon isolation from the cell (Figures 26, 27 and 29).

The 3-5 μ limiting 'membrane' was seldom observed in sectioned cells (Figures 2, 5, and 10) to completely surround the body, but it was observed to be continuous in the isolated polyhedral bodies suggesting the plane of section was responsible for the former observation. This 'membrane' appeared not to be a mere interface between the body and the cytoplasm since it was observed surrounding isolated polyhedral bodies and also retained its polygonal profile when empty (Figure 28).

The relationship of the polyhedral bodies to each other and to the rest of the cellular components is not clear. Occasionally an isolated polyhedral body was seen in close association with a thylakoid membrane but it is possible that these were cases of adsorption rather than of structural relationship (Figure 26). A close spatial relationship was frequently observed in sectioned cells between a polyhedral body and a thylakoid (Figure 3), but no definite structural relationship was observed. In some sections there appeared to be a structural association via the polyhedral body 'membranes' between members of the same cluster, both in whole cells (Figures 2, 3, and 10), and in homogenates (Figures 27 and 28).

The nature of the 'membrane' surrounding these bodies is open to a certain degree of speculation but it does not exhibit dimensions or staining characteristics similar to a typical unit membrane. It does
not have a tripartate appearance nor is it preserved by KMnO$_4$ fixation (Figures 11 and 12). It is preserved by OsO$_4$ fixation (Figure 6) but becomes much more electron-dense after staining with uranyl acetate (Figure 2). There is a striking similarity between these 'membranes' and the 2-4 μ membrane surrounding the gas vesicles observed in some blue-greens and recently described by Bowen and Jensen (1965). These membranes exhibited fixation properties similar to the polyhedral body 'membranes' just described. They appeared to maintain the characteristic cylindrical shape of the vesicles which had conical ends. The 'membranes' of the polyhedral bodies may also be responsible for the characteristic shape of this cell component. The polyhedral bodies were not easily isolated from the cells unless the homogenization took place in a sucrose medium, suggesting that the 'membrane' may be quite osmotically labile. Jost and Matile (1966) reported the isolation of gas vesicles from Oscillatoria. The isolated vesicles apparently lost their rigidity and appeared after negative staining as flattened bladders of various sizes.

The characteristic association of the polyhedral bodies with the nucleoplasm and the notable lack of other structures in this area, suggested that they could be the polyphosphate bodies commonly observed in this region by the light microscopists. Although individual polyhedral bodies would be difficult to detect because of their small size, the culters which are often observed range up to 1 μ in size and would, therefore, be relatively easy to observe.

The polyphosphate bodies, metachromatic granules and volutin
granules have been suggested to be identical (Fuhs, 1958a). The polyphosphate bodies have been reported to be removed by exposure to 1 N HCl (Cassel and Hutchinson, 1954; Fuhs, 1958a). The fact that the polyhedral bodies were removed by HCl exposure in the current study further suggested that they were equivalent to polyphosphate bodies. Talpasayi (1963) and others observed that polyphosphate bodies were not present in mature akinetes or heterocysts. In an electron microscopic study of heterocyst development in Anabaena, Lang (1965) reported a gradual loss of polyhedral bodies as the heterocysts matured.

Although the above observations strongly suggest that the polyhedral bodies of the electron microscopists and the polyphosphate bodies of the light microscopists are the same cellular structure, the chemical composition of these structures is less clear. Although they are considered to be highly polymerized polyphosphate (Talpasayi, 1963), they also exhibit several reactions characteristic of nucleic acids: staining by methyl green and pyronine (Ebel and Muller, 1958), digestion with cold 10% TCA (Fuhs, 1958a), and red fluorescence when treated with acridine orange and viewed with ultraviolet light (Talpasayi, 1963). Polyphosphate bodies could not be removed from the cells with either RNase or DNase, however, and on this basis Fuhs (1958a) concluded that the bodies did not contain nucleic acids, but rather that the reactions shared by polyphosphates and nucleic acids were caused by the high degree of phosphate polymerization.

In the present study, exposure to RNase according to the method described by Jacobson et al. (1963) left the nucleoplasm uniformly
electron-dense but no polyhedral bodies were observed (Figure 16). Since the controls did retain polyhedral bodies (Figure 17), the experiment suggests that the bodies contain RNA. Several other digestion conditions were employed in which no alteration of the polyhedral bodies was observed (Figure 1).

Costerton (1960) reported structures, recognizable from his electron micrographs as polyhedral bodies, to be 'protein bodies' on the basis of light microscope studies. He observed granules in the centroplasm which were stained with mercuric bromphenol blue but not with alkaline fast green and subsequently concluded that the bodies consisted of a non-basic protein. The mercuric bromphenol blue reaction is considered specific for proteins provided proper controls such as pepsin extraction are carried out, but no such controls were reported.

The polyhedral bodies are characteristic of all the blue-green algae so far examined at the electron microscope level, and it is tempting to think that they play a prominent role in the cellular activities. They pose several interesting questions with regard to the nature, role, and origin of their unusual 'membrane', the origin and maintenance of their polygonal shape, and of course their chemical composition and metabolic involvements. On the basis of the current work, it is concluded that the polyhedral bodies are probably the polyphosphate bodies of the light microscopists and furthermore, that they may have an RNA component.

Tubule-like Elements

The 12-15 μ tubule-like elements observed in Nostoc muscorum after
glutaraldehyde-osmium fixation were similar to the 14 μm tubules observed in *Nostoc prunicorne* by Jensen (1965). They appear to be smaller than the 23-27 μm tubules reported in other plants (Ledbetter and Porter, 1963) but compare with the 15 μm spindle tubules or with bacterial flagella.

Although similar tubules are common in eucaryotes, they have only rarely been reported in procaryotic cells; their function in these cell is, as yet, unknown. The fact that they were observed in sectioned cells, cell homogenates, and negatively stained preparations, argues against the possibility that they could be fixation artifacts. Whether these tubules are a normal component of blue-green algal cells or the result of a viral infection, is not yet clear.
SUMMARY

1. The fine structure of the blue-green alga, *Nostoc muscorum*, was studied using several fixation methods, enzyme digestions, solvent extractions, and cell fractionation procedures.

2. The cell envelope was similar to that observed in gram-negative bacteria and consisted of an outer membrane, an inner investment, and a plasma membrane. Papillae were seen associated with the outer membrane as described by earlier workers, but varied with experimental treatment, and were therefore considered to be fixation artifacts. The plasma membranes and the outer membrane appeared structurally similar after fixation and staining; however only the plasma membrane was visible prior to uranyl acetate staining. On the basis of solvent extraction studies, the plasma membrane has a greater lipid content. Therefore it is concluded that the plasma membrane and the outer membrane are chemically different.

3. The inclusions commonly described for blue-green algae were observed: thylakoids, DNA fibrils, ribosomes, polyhedral bodies, structured granules, \(\alpha\)-granules, and \(\beta\)-granules.

4. Previously unreported rod or disc-shaped particles 6-7 \(\mu\) wide were observed on obliquely sectioned thylakoid membranes. As a working hypothesis, it is proposed that these particles are the phycocyanins.

5. Tubule-like elements 12-15 \(\mu\) wide were observed in sectioned cells from strain 1037, and in homogenates of strains 1037 and M-12.4.1. Whether the tubules are normal cellular components or a result of viral infection has not been determined. Although similar tubules are
common in eucaryotes, they have only rarely been reported in pro-
caryotic cells.

6. A fraction containing intact polyhedral bodies was isolated. The polyhedral bodies retained their characteristic polygonal pro-
files. The 2-5 μm surrounding 'membrane' was observed to be different from most biological membranes, both in dimensions and in fixing and staining properties. The polyhedral 'membrane' did, however, appear similar to the membranes of expanded gas vesicles observed in some blue-green algae. The polyhedral bodies are characteristically associated with the nucleoplasm and are dissolved by dilute HCl. On the basis of this work it is concluded that the polyhedral bodies are the polyphosphate bodies described by the light microscopists. Furthermore, on the basis of staining properties and enzyme digestion studies, it is concluded that they probably have an RNA component.

7. The structured granule appeared to be chemically heterogeneous and to have a lipid component.

8. Negative staining reveals that the polyglucosidic -granules are 30 by 70 μm ellipsoids with a median transverse constriction.

9. The β-granules were removed from the cells by lipid solvents. On the basis of absorption spectra obtained from the solvent extracts and from isolated β-granule fractions, it is concluded that the β-granules contain carotenoids.


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APPENDIX A: FIXATION, DEHYDRATION AND EMBEDDING

Fixation

1. Osmium tetroxide fixation

The procedure employed was modified for blue-greens by Pankratz and Bowen (1963) from that described for bacteria by Kellenberger et al. (1958). In the current work the tryptophan was omitted.

a. Preparation of Michaelis buffer:

sodium acetate \((\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O})\) 1.94 g
sodium veronal (Barbital) 2.94 g
sodium chloride 3.40 g
distilled water to 100 ml

b. Preparation of fixing buffer:

Michaelis buffer (above) 5 ml
distilled water 13 ml
0.1 N HCl 7 ml
1 M CaCl\(_2\) solution 0.25 ml

Final pH should be 6.1 to 6.2.

c. Preparation of Fixative:

Dilute fixing buffer (above) 1:1 with aqueous 2\% osmium tetroxide.

d. Fix specimens 1-3 hours at room temperature.

2. Potassium permanganate fixation

Unbuffered 4\% aqueous potassium permanganate (Luft, 1956; Mollenhauer, 1959) was employed. Specimens were fixed 3-5 minutes at room temperature.
3. Glutaraldehyde fixation

Phosphate buffered 3% glutaraldehyde (Sabatini et al., 1963) was usually employed with post-fixation in osmium.

a. Preparation of buffer:

0.1 M $\text{KH}_2\text{PO}_4$ 13 ml
0.1 M $\text{Na}_2\text{HPO}_4$ 37 ml

Final pH should be 7.2 to 7.4

b. Preparation of fixative:

Add 50% glutaraldehyde to above buffer in proper portion to obtain a fixing solution of 3% glutaraldehyde. (1 ml glutaraldehyde: 15 ml buffer).

c. Fix 1-12 hours at 4°C.

d. Rinse in several changes of phosphate buffer.

e. If post-fixation is desired, fix 1-3 hours at room temperature in osmium (previously described).

4. Formaldehyde fixation

Phosphate-buffered 4% formaldehyde (Pease, 1964) with osmium post-fixation was employed.

a. Preparation of stock solutions:

Stock-buffer salt solution: Monobasic sodium phosphate 2.26%
Stock alkali solution: sodium hydroxide 2.52%

b. Preparation of fixative (4% formaldehyde):

Stock buffer salt solution 83 ml
Stock alkali solution 17 ml
Paraformaldehyde (powder) 4 gm

Adjust pH to 7.2-7.4
c. Fix 1-12 hours at 4°C

d. Post-fix 1-3 hours in 1% osmium (previously described) at room temperature.

Dehydration and Embedding

Dehydration and embedding were performed as modified from Luft (1961) as follows:

1. Preparation of Epon mixtures:
   a. Mixture A
      62 cc Epon 812
      100 cc DDSA (Dodecenyl succinic anhydride)
   b. Mixture B
      89 cc NMA (Nadic methyl anhydride)
      100 cc Epon 812
   c. Add 3 parts mixture A to 2 parts mixture B and stir thoroughly.
      Add DMP-30 (catalyst) at the rate of 0.2 ml per 10 ml Epon mixture. Mix thoroughly.

2. Dehydration of specimens at room temperature immediately followed fixation.
   a. 5 minutes each in 50%, 70%, and 95% ethanol
   b. 3 changes of 2-5 minutes each in 100% ethanol
   c. 3 changes of 2-5 minutes each in propylene oxide

3. Infiltration was accomplished in 4 steps.
   a. 5-10 minutes in a mixture of 1 part Epon: 3 parts propylene oxide
   b. 10-20 minutes in 1 part Epon: 1 part propylene oxide
c. 20-40 minutes in 3 parts Epon: 1 part propylene oxide
d. 8-18 hours in 100% Epon. The specimen vials were slowly rotated during this period.

4. Specimens were embedded in shallow open aluminum foil boats.

5. The polymerization was performed in 3 steps.
   a. 8-12 hours at 35°C
   b. 12 hours at 45°C
   c. 3-6 days at 60°C
APPENDIX B: FIGURES

Key to all Figures

A - $\alpha$-granules
B - $\beta$-granules
D - DNA fibrils
G - Granules
IV - Inner investment
IT - Intrathylakoidal spaces
W - Myelin-like whorls
NP - Nucleoplasm
OM - Outer membrane
P - Papillae
Z - Peripheral clear zone
PM - Plasma membrane
PB - Polyhedral bodies
R - Ribosomes
SG - Structured granules
TH - Thylakoids
T - Tubule-like elements
Figure 2. Cell of *Nostoc muscorum* fixed in osmium and stained in uranyl acetate. The cell envelope consists of the outer membrane (OM), inner investment (IV), and plasma membrane (PM). The nucleoplasm (NP) contains ribosomes (R), DNA fibrils (D) and polyhedral bodies (PB) which are sometimes surrounded by a fine dense zone. Thylakoids (TH), $\beta$-granules (B), and a structured granule (SG) are also visible. 70,000 X.
Figure 3. Portion of a cell showing structured granules (SG), and a cluster of polyhedral bodies (PB). Arrow indicates a close spatial relationship between polyhedral body and a thylakoid. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 4. Portion of a cell of Nostoc muscorum strain 1037 showing tubule-like elements (arrows). Glutaraldehyde-osmium fixation and uranyl acetate staining. 70,000 X.

Figure 5. Portion of a cell showing rod-like structures on obliquely sectioned thylakoids (arrows). The cell envelope consisting of the outer membrane (OM), inner investment (IV) and plasma membrane (PM) is clearly visible. 140,000 X.
Figure 6. Portion of an osmium fixed cell otherwise unstained. The structured granules (SG) and A-granules (B) are osmiophilic. The polyhedral bodies are surrounded by a fine dense zone (arrow) and the cell envelope is unstained except for the plasma membrane (PM). 70,000 X.

Figure 7. Portion of an osmium fixed cell stained with lead citrate. The A-granules (A), DNA fibrils (D) and ribosomes (R) show an increased electron-density. The edges of several thylakoids (arrows) are visible. 70,000 X.
Figure 8. Cell fixed in glutaraldehyde and stained with uranyl acetate. The less dense areas (arrows) are suggested to have contained $\beta$-granules. 70,000 X.

Figure 9. Portion of a glutaraldehyde-osmium fixed cell showing good preservation of $\alpha$-granules (A). Uranyl acetate staining. 70,000 X.

Figure 10. Glutaraldehyde-osmium fixed cell showing preservation similar to that observed in osmium fixed cells (Figure 2). Uranyl acetate staining. 70,000 X.
Figure 11. Cell fixed in KMnO₄ and stained with uranyl acetate showing increased electron-density of nucleoplasm (NP) and polyhedral bodies (PB). The $\beta$-granules are not preserved (arrows). The intra-thylakoidal spaces appear slightly expanded (IT). 70,000 X.

Figure 12. Cell fixed in KMnO₄ but otherwise unstained showing plasma membrane (PM), poorly preserved $\beta$-granules (B), polyhedral bodies (PB), and less dense nucleoplasm (NP). 70,000 X.
Figure 13. Cell treated with 20% acetone-80% methanol and then fixed in osmium showing formation of papillae (P) from the outer membrane. Note the myelin-like membranous whorl (W), and polyhedral bodies (PB). 70,000 X.

Figure 14. Glutaraldehyde-fixed cell, treated with 100% acetone and post-fixed in osmium showing less dense areas from which \( \beta \)-granules have been removed (arrows). 70,000 X.

Figure 15. Glutaraldehyde-fixed cell treated with 100% methanol and post-fixed in osmium showing small less dense areas from which \( \beta \)-granules have been removed (arrows), and a peripheral clear zone (Z). No plasma membrane is visible. 70,000 X.
Figure 16. Formaldehyde-fixed cell incubated 4 hours at 60°C in RNase, extracted with TCA, and post-fixed in osmium. Note the central area of uniform electron-density. The outer membrane (OM) is slightly undulatory. 70,000 X.

Figure 17. Cell treated as indicated above except the RNase was absent from the incubation medium. Polyhedral bodies (PB) remain and a few papillae (P) are observed. 70,000 X.
Figure 18. Cell fixed in formaldehyde and post-fixed in osmium. 70,000 X.

Figure 19. Cell fixed in glutaraldehyde and post-fixed in osmium. 70,000 X.

Figure 20. Note papillae (P) on outer membrane of cell incubated at 60°C (see text Figure 1 for treatment details). 70,000 X.

Figure 21. Portion of glutaraldehyde-fixed cell incubated at 60°C showing myelin-like whorls (W), and a slightly papillate outer membrane. 70,000 X.

Figure 22. Portion of glutaraldehyde-fixed cell exposed to 0.01 N HCl showing removal of the structured granule (SG). 70,000 X.
Figures 23 to 25. Portions of cells fixed in glutaraldehyde and exposed to 0.01 N HCl. The polyhedral bodies (PB) have been entirely or partially removed. Post-fixed in osmium and stained with uranyl acetate. 70,000 X.
Figures 23 to 25. Portions of cells fixed in glutaraldehyde and exposed to 0.01 N HCl. The polyhedral bodies (PB) have been entirely or partially removed. Post-fixed in osmium and stained with uranyl acetate. 70,000 X.
Figure 26. Polyhedral body freed from cell in sucrose medium. The limiting 'membrane' is preserved but the contents appear pulled from edge leaving a clear space. Arrow indicates association of polyhedral body with thylakoid. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 27. Cluster of isolated polyhedral bodies. Note small tubules (T), and portion of wall containing outer membrane (OM) and inner investment (IV). Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 28. Polyhedral body 'membranes' devoid of contents and retaining angularity. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 29. Isolated polyhedral bodies may appear electron-dense throughout or with varying degrees of granularity. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 30. Isolated structured granules after glutaraldehyde-osmium fixation showing slight surface projections (arrow). Uranyl acetate staining. 70,000 X.

Figure 31. Isolated structured granules after osmium fixation showing occasional less dense areas (arrows). Uranyl acetate staining. 70,000 X.

Figure 32. Isolated structured granules after osmium fixation and exposure to ethanol showing decreased electron-density, decreased size and homogeneous appearance. Uranyl acetate staining. 70,000 X.
Figure 33. Thylakoid vesicles isolated in distilled water. Arrow indicates possible β-granule association with the thylakoids. Low speed pellet fixed in osmium and stained with uranyl acetate. 70,000 X.

Figure 34. Portion of high speed pellet from crude homogenate of Nostoc muscorum strain 1037 showing tubule-like elements (T). Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 35. Portion of pellet obtained after sucrose gradient centrifugation containing thylakoid vesicles and a high proportion of β-granules. Sample also showed high carotenoid absorption. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 36. Cell homogenate containing a portion of cell envelope showing the electron-dense inner investment (IV) and the unit nature of both the outer membrane (OM) and the plasma membrane (PM). Osmium fixation and uranyl acetate staining. 140,000 X.

Figure 37. Portion of cell envelope showing plasma membrane (PM) pulled away from the wall complex. Osmium fixation and uranyl acetate staining. 140,000 X.
Figure 38. Thylakoid vesicle showing 75 A particles (arrows) on the outer surface. Isolated in Ficoll-sucrose, fixed in osmium and stained in uranyl acetate. 70,000 X.

Figure 39. Thylakoids homogenized in 1% glutaraldehyde showing small particles associated with the outer surfaces (arrow). Occasionally, larger elongate granules (6) are observed. Individual thylakoids often remain closely appressed. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 40. Thylakoids isolated in Ficoll medium showing small projections from outer surface (arrow). Individual thylakoids remain closely appressed. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 41. Thylakoids isolated in sucrose medium are usually expanded but the unit nature of the membranes is evident. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 42. Thylakoids from cells homogenized in 1% glutaraldehyde showing 30 mu granules associated with the outer surface of the membrane. Osmium fixation and uranyl acetate staining. 70,000 X.

Figure 43. Portion of a whole cell from Ficoll homogenate fixed in osmium and stained with uranyl acetate. Projections 6-7 mu wide are observed on the outer surfaces of the thylakoids (arrows). 70,000 X.

Figure 44. Enlargement from Figure 43 of membrane projections (arrows). 140,000 X.
Figure 45. Thylakoid from crude homogenate showing $\beta$-granules (B) and knobby surface. Tubular system appears to anastomose (arrows). Negatively stained with PTA. 70,000 X.
Figure 46. Isolated $\alpha$-granules showing bipartate structure and electron-dense construction (arrows). Negatively stained in uranyl formate. 70,000 X.

Figure 47. Thylakoid fragment showing associated small rods or tubule-like elements (T). Negatively stained with PTA. 70,000 X.

Figure 48. Tubular thylakoid fragment showing a pair of small rods or tubule-like elements (T). Negatively stained with PTA. 70,000 X.

Figure 49. Preparation negatively stained with PTA showing $\beta$-granules (B), $\alpha$-granules (A) and thylakoid fragment with 75-90 A surface particles. 70,000 X.