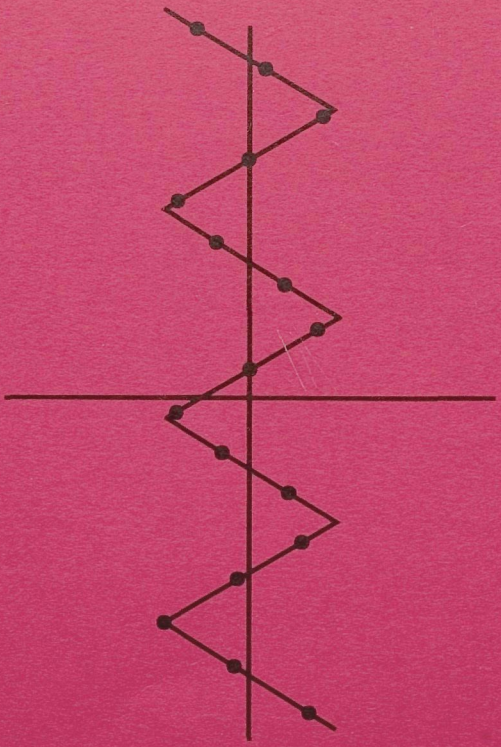
Spirulina platensis

MORPHOLOGY & ULTRASTRUCTURE



C. VAN EYKELENBURG

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE TECHNISCHE WETENSCHAPPEN AAN DE TECHNISCHE HOGESCHOOL DELFT, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. IR. B.P.TH. VELTMAN, VOOR EEN COMMISSIE AANGEWEZEN DOOR HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 20 NOVEMBER 1980 TE 16.00 UUR

DOOR

CAROLUS VAN EYKELENBURG
SCHEIKUNDIG INGENIEUR
GEBOREN TE ROTTERDAM

1980
Delftse Universitaire Pers

DIT PROEFSCHRIFT IS GOEDGEKEURD DOOR DE PROMOTOR PROFESSOR DR. A. FUCHS

DIT PROEFSCHRIFT WERD BEWERKT OP HET LABORATORIUM VOOR ALGEMENE EN TOEGEPASTE MICROBIOLOGIE EN HET LABORATORIUM VOOR ALGEMENE EN TECHNISCHE BIOLOGIE VAN DE TECHNISCHE HOGESCHOOL DELFT

VOORWOORD

Mijn dank gaat uit naar een ieder die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift of een onderdeel daarvan.

(Um zu erkennen, ob das Bild wahr oder falsch ist, müssen wir es mit der Wirklichkeit vergleichen. Nur so könnten wir a priori wissen, dass ein Gedanke wahr ist, wenn aus dem Gedanken selbst (ohne Vergleichsobjekt) seine Wahrheit zu erkennen wäre.

Sätze 2.223 und 3.05 Tractatus logico-philosophicus. Wittgenstein, L. J. J. 1921. Logisch Philosophische Abhandlung. Annalen der Naturphilosophie 14: 184-262.)

CONTENTS

I. GENERAL INTRODUCTION

HISTORY

Cyanobacteria* have been present on the surface of the Earth since the early Precambrian about 3.6 10^9 years ago (Stewart, 1977; Ford, 1980). Filamentous, cylindrical, unbranched, smooth and unlamellated forms with a diameter up to 10 µm and at least 25 times longer than wide are found in fossils from late Precambrian (1.1 10^9 years ago), hypersaline lagoons (Oehler, Oehler and Stewart, 1979).

In his second report to Spain (1520) Hernán Cortés (1485-1547) mentions tecuitlatl, a product which was eaten in considerable amounts by the pre-conquest inhabitants of Tenochtitlán (residence of the high priest Tenoch) (the present Mexico City), and which the Spaniards also found palatable. At the time of the conquest the local population in this area has been estimated to number 250,000, the feeding of which would exceed the capacity of the low levels of cattle breeding and agriculture practised. It is considered likely that tecuitlatl (Deevey, 1957) made from a cyanobacterium (Ancona, 1933) found in Lake Texcoco made up the staple part of the natives diet. In the sixteenth century this lake was twenty times larger than it is today (Ortega, 1972), and the cyanobacterium growing in it could well have provided the major source of protein.

The naturalist Francisco Hernandez (1513-1587), sent by the Council of the Indies to report on the flora, fauna and minerals of New Spain, was probably the first to give scientific information on the cyanobacteria which he, erroneously, thought to be a mineral. His report was compiled around 1550-1560, but the manuscripts were scattered and lost. In 1790, they were published incomplete

*Since 1971, the name blue-green alga is gradually being replaced in the literature by cyanobacterium (Stanier et al., 1971, see also Cohn, 1853); in 1978 a proposal was put forward (Gibbons and Murray, 1978) to validate the *Cyanobacteriales* as a new order of the kingdom of the *Procaryotae*, while Stanier et al. (1978) proposed the placing of the nomenclature of the cyanobacteria under the rules of the International Code of Nomenclature of bacteria. These proposals have been seriously questioned by Bourrelly (1979), Geitler (1979) and Golubic (1979).

(F. Hernandi, Opera, cum edita, tum inedita, Madrid, 1790). In his 'Essai Politique sur le Royaume de la Nouvelle Espagne' (1811), Von Humboldt refers to Bernardino de Sahagun who, about 1550, described the clear blue colour of tecuitlatl in his Universal History (Farrar, 1966, Ortega, 1972). We now know from the tecuitlatl still sold in the area that the cyanobacterium concerned is *Spirulina platensis*. It is an obligate photo-autotrophic organism, having an absolute requirement for light and there is no stimulation of growth or respiratory activity by reduced carbon compounds (Carr, 1979).

Reports on the presence of *Spirulina* in Africa date back to 1896 when West and West recorded the cyanobacterium in a collection taken from Lake Losuguta in Kenya. Jenkin (1929), who participated in the Percy Sladen Expedition to East Africa, collected and described the cyanobacterium from lakes Baringo, Naivasha, Nakuru and Elmenteita. Rich (1931, 1933) published an account on the phytoplankton collected from the lakes of Kenya and Uganda and confirmed the findings of Jenkin (1929). Ross (1953) described the water in the Ferguson Gulf of Lake Rudolph as having the appearance of green soup, due to a very thick population of *Spirulina* and *Anabaenopsis*.

Dangeard (1940) was the first to describe an edible cyanobacterium, collected and eaten by man in Central Africa. It proved to be a mass of helical filaments of a cyanobacterium now known to be *Spirulina platensis*.

During the winter of 1964-1965, members of the Belgian Sahara Expedition purchased, in the market of Fort Lamy near Lake Chad, flat cakes of a greenish edible substance called dihé. These appeared to consist solely of a cyanobacterium collected from the bottoms of seasonally dried up ponds in the North of Lake Chad and consumed by the local population. In the region of Ounianga Kébir, about 750 miles northeast of Fort Lamy, the members of the expedition were struck by the abundance of a microscopic alga in some lakes. Compère of the State Botanical Garden in Brussels examined the product and found it to consist solely of a cyanobacterium: *Spirulina platensis* (Léonard, 1966, Léonard and Compère, 1967). From chemical analysis carried out at the Laboratoire Intercommunal de Chimie et de Bacteriologie in Brussels it appeared to be very rich in protein and therefore very useful for consumption by the protein-deficient desert nomads. These findings drew considerable attention and led to investigations on the organism concerned.

Outside Africa and Mexico, *Spirulina platensis* appears to be less common. In Asia it has been harvested at Lahore (Pakistan) (Ghose, 1923; Rhandawa, 1936), Calcutta (India) (Biswas, 1927) and in Lake Beira (Sri-Lanka) (Holsinger, 1955) but never in the abundance seen in the African lakes. None of the authors who studied the organism in Asia mentioned human consumption.

The *Spirulina platensis* strain used in the present study originates from Lake Nakuru, a shallow soda lake in the Kenyan part of the African Rift Valley. The lake has no surface outlet and the level fluctuates in response to rainfall and evaporation. The surface area is about 35 km^2 when the lake is full. The electrical conductivity of the lake water is high (15-30 mS/cm at 20°C) and the pH varies from 9 to 11. There is a very high photosynthetic activity with a net primary production of 900-1800 tons fresh weight per day. *Spirulina platensis* is the dominating species and serves as the main source of food for the huge flocks of lesser flamingos (*Phoeniconaias minor*) for which the lake is famous (Källqvist and Meadows, 1978; Vareschi, 1978).

REMARKS ON TAXONOMY

The taxonomy of the cyanobacteria, or blue-green algae, has always caused frustration. Wallroth (1833) called the group *Myxophykae* but Stitzenberger (1860) changed this to *Myxophyceae*; Rabenhorst (1863) proposed *Phycochromophyces*, while Sachs (1874) called them *Cyanophyceae*, a name which Kirchner (1878) without success, tried to change to *Schizophyceae*.

The distinction within the cyanobacteria, between the genera *Spirulina* (Turpin, 1827) and *Arthrospira* (Stitzenberger, 1852) has long been subject of controversy. Traditionally, members of the *Oscillatoriaceae* lacking a sheath but with regularly helical (spiralized) septate trichomes have been placed in the genus *Arthrospira*, and those with non-septate regularly helical trichomes have been placed in the genus *Spirulina* (Smith, 1950; Prescott, 1951). Generic separation based on the presence or absence of cross-walls has been questioned by numerous workers dating back to early in the 20th century. Schmid (1920) and Figini (1925), working with species then described as *Spirulina*, showed that in many species septa are demonstrated after prolonged staining with neutral red. As a result, there was a tendency to place species of *Arthrospira* into *Spirulina*, since the name *Spirulina* has priority over that of *Arthrospira*. However, Crow (1927) maintained that certain species were non-septate and suggested that *Spirulina* should be reserved for such species, the septate forms being placed in the *Arthrospira* group. Such has been the practice of many authors, particularly in the USA. Using a special staining technique Gorbunova (1958) was able to show the presence of septa in *Spirulina major*, Kütz., a species commonly used to demonstrate the non-septate nature of typical *Spirulina* species, and on this basis again recommended combining *Arthrospira* and *Spirulina*. Holmgren, Hostetter and Scholes (1971) proposed an investigation of all species concerned in order to establish the presence or absence of septa, but this was not followed up.

Geitler (1932) did not recognize the generic distinction between *Arthrospira* and *Spirulina*. Iltis (1970) integrated the genus *Spirulina* (*Arthrospira*) into the genus *Oscillatoria* without any result.

In a paper on generic assignments, strain histories and properties covering 150 genera and well over 1000 species, Rippka et al. (1979) state that substantial differences in DNA base composition suggest a more solid genetic basis for recognizing two genera in the future. The generic subdivision of the filamentous, non-heterocystous cyanobacteria assigned to Section III (filamentous cyanobacteria; a trichome which grows by intercalary cell division; reproduction by random trichome breakage, by formation of hormogonia; a trichome always composed only of vegetative cells; division in only one plane) in the terminology of Rippka et al. (1979) provides three genera: *Oscillatoria*, *Pseudoanabaena* and *Spirulina* which can be distinguished on structural grounds. As stated by Herdman et al. (1979a), a clear-cut generic assignment for many of the strains cannot yet be made since these strains share, in various combinations, the properties which have been ascribed to the genera *Lyngbya*, *Plectonema* and *Phormidium*. These have been placed in a provisional category, termed the LPP group (see Rippka et al., 1979). *Oscillatoria* and *Pseudoanabaena* have relatively narrow and similar base compositional spans of 40 to 50 and 44 to 52 mol % GC, respectively. The two strains of *Spirulina* analysed by Herdman et al. (1979a) had DNA with 44 and 54 mol % GC. These two strains differ greatly in phenotypic respect: strain PCC (Pasteur Culture Collection) 7345 (*Arthrospira platensis*) contains gas vacuoles and forms very thick filaments up to 16 µm wide, whereas strain PCC 6313 (Kenyon, Rippka and Stanier, 1972) does not form gas vacuoles and has much thinner filaments (Herdman et al., 1979a and Rippka et al., 1979).

According to Herdman et al. (1979b), *Spirulina* differs from the majority of the Section III organisms (Rippka et al., 1979) in the possession of a small genome of $2.53\ 10^9$ dalton, whereas the average size for Section III is $3.79\ 10^9$ dalton with respect to genome size, while this Section is well separated from the LPP group.

SPIRULINA AS A SOURCE OF NUTRITIONAL PROTEIN

'Every attempt must be made to inform the scientific community of the increasing interest, as a potential source of food, in blue-green algae in general and the genus *Spirulina* in particular. Determination of the potential of algal protein in animal and human nutrition will require the examination of many strains, cultural conditions and processing techniques. In view of the meager knowledge of fine structure and physiological functions as taxonomical criteria for the blue-green algae, the fundamental knowledge of these organisms must be increased'. The statement above is the first paragraph of the 'Conclusions made at the conference "Preparing nutritional protein from *Spirulina*" in Stockholm, June 13-15, 1968'.

Since 1965, much work has been done on *Spirulina* species. Studies have been published on optimal medium composition (Zarrouk, 1966), on growth (Ogawa and Terui, 1970; Ogawa, Kozasa and Terui, 1972), on carbon requirement (Ivolgina, Meshcheryakova and Al'Bitskaya, 1972), on growth yield in continuous culture (Aiba and Ogawa, 1977), and on chemical factors influencing growth (Crance, Forin and Baron, 1977).

Besides these more technological studies, investigations into the possibilities of using *Spirulina* for human nutrition have been published. For instance, Clément, Durand-Chastel and Henny (1969) and Wachowicz and Zagrodzki (1976) have evaluated the proteins, amino-acid composition and nucleic acid content. Hedenskog et al. (1969) have investigated methods to increase digestibility. The variation in lipid composition was studied by Paoletti, Materassi and Pelosi (1971) while the mutational effects of ultraviolet rays and antibiotics on *Spirulina platensis* were examined by Pelosi, Pushparaj and Florenzano (1971).

Delpeuch, Joseph and Cavelier (1975) gave detailed information on where and how dihé is eaten in Chad, they also reported on the nutritional values of *Spirulina platensis* gathered in different seasons.

The acceptibility of various culinary products based on the alga *Spirulina* was tested by Sautier and Trémolières (1975). They conclude that *Spirulina is* little appreciated in France due to its offensive colour, smell and taste. However, no intestinal problems occurred, and the food did not modify the balances investigated, although faecal nitrogen increased to 2.08 g as compared with control period values between 1.33 g and 1.51 g when people were provided with 50% cyanobacterial protein. As the uric acid level in urine did not vary and the serum values increased only slightly (cf. Feldheim et al., 1973), they concluded that ingestion of *Spirulina* in small doses even over a long period of time should be tolerable for humans. Boudène, Collas and Jenkins (1975) could not find any evident toxicity related to the organism. Very low concentrations (2 to 3 ppb) of 3,4-benzpyrene in variously prepared dry *Spirulina* samples have been reported by Bories and Tulliez (1975). Jacquet (1975) showed that *Spirulina* preparations are accompanied by other micro-organisms, the species depending on the method of processing. However, faecal streptococci, Enterobacteriaceae, yeasts and mould spores are exceptional.

The protein content of dried *Spirulina* has been measured in numerous studies and varies between 60 and 72%. The dried, decolourized product can reach a pro-

tein content of 84.2% (Baron, 1975) without causing hygienic or nutritional inconvenience. Many studies, mainly by Russian and French authors, have been published on the nutritional value of *Spirulina* for animals (i.e. see Annales de la Nutrition et de l'Alimentation volume 29, 1975).

It should be emphasized that for single cell protein, *Spirulina platensis* is most economically grown in areas with high luminous flux, average temperatures above 25°C, and in natural waters such as Texcoco Lake near Mexico City, African lakes and Indian village ponds where 'low-cost technology' is used for cultivation (Seshadri and Thomas, 1978, 1979).

AIM OF THIS INVESTIGATION

As stated previously, *Spirulina platensis* might play a role as a source of nutritional protein. It is therefore appropriate to investigate nutritional, physiological and ultrastructural features and to gather as much technological data as possible on large-scale cultivation of the organism.

The aim of this thesis has been to establish the background of the helical structure of *Spirulina platensis* and the theoretical basis at the root of it. Furthermore, it was considered necessary to characterize the morphology and ultrastructure in relation to environmental factors. The environmental factors studied were chosen on the basis of the type of micro-organism and its natural habitat. Cyanobacteria are well known for their ability to withstand and to grow at extremes of temperatures. Together with green algae and diatoms, they are prominent in terrestrial habitats in the Arctic and Antarctic, and many are known to survive in hot springs at temperatures approaching boiling point. These properties make a study on the effect of temperature on the morphology and ultrastructure seem promising. The effect of light intensity was studied because cyanobacteria are phototrophic micro-organisms. The effect of available nitrate is of interest because this anion is not usually present in rocks of volcanic origin, the natural habitat of *Spirulina platensis* being volcanic lakes; nevertheless, nitrate is the best nitrogen source in cultivating this cyanobacterium (Zarrouk, 1966).

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INTRODUCTION TO II AND III

The present study was started with an investigation of the cell wall of the cyanobacterium *Spirulina platensis*. The helical morphology as observed under the light microscope is unusual as few species possess a helix at the cellular level. Molecular helices, on the other hand, are more common. The DNA double-helix is an outstanding example but many proteins and nucleoproteins are also arranged in this manner. These molecules owe their structure to hydrogen bonds and/or hydrophobic-hydrophilic interactions. Suborganismal helices were recognized by Roelofsen (1950) in the primary cell walls of *Phycomyces* sporangiospores, and their helical growth patterns were described by Gamov (1979). Helical arrangements of cellulose microfibrils are known to exist in many cells of higher plants, for example in cambial initials, conifer tracheids, vessels, phloem fibres and sisal fibres, as well as in a number of algae (see Middlebrook and Preston, 1952). In invertebrates, especially in the cylindrical cuticles of some pseudo- and eucoelomate worms, the strata fibres are often disposed in concentric helices of increasing diameter (Swanson, 1974). The latter examples are similar in that the helix is linked to the cell wall. As for the origin of these helices we may discriminate between cellulose or non-cellulose bound forms. Organismal helices are also cell wall-bound. The best known examples are the Spirillaceae, Spirochaetales and Spiroplasmas (Townsend et al., 1980), but certain mutants of *Bacillus* spp. (Mendelson, 1976; Tilby, 1977 and Fein, 1980) and *Seliberia*-like micro-organisms (Schmidt and Swafford, 1979) also occur as helices. The actual helical arrangement of wall components in these bacteria is not a prerequisite condition for helical growth and, if present, does not necessarily lead to a helical cell shape. The only factor required is a helical or rotational component in cell growth. Tilby (1977) favours the possibility of opposed helices of wall polymers with unequal stress in them to explain the tighter helical growth which appears to reverse in direction within the same chain of cells.

There are among the cyanobacteria a few examples of helically arranged trichomes, such as certain *Lyngbya* spp., *Anabaena helicoidea*, *Anabaena spiroides*, certain *Anabaenopsis* spp., *Oscillatoria ornata*, *Oscillatoria beggiatoiformis*, *Oscillatoria boryana*, *Phormidium antarcticum* and all *Spirulina* and *Arthrospira* spp. (Pascher, 1925) (see also Booker and Walsby, 1979). For a better understanding of the significance of the helix it seems appropriate to deal with its

geometry in some details.

A helix is the curve cutting the generators of a right circular cylinder under a constant angle β, ($x = r_o \cos\theta, y = r_o \sin\theta, z = r_o\theta \cot\beta$). Historically the helix is mentioned by Geminus (ca 70 BC), but a passage in Proclus (ca AD 460) suggests that it was already known to Apollonius (ca 225 BC). It was used by Pappus of Alexandria (ca AD 300) for producing the quadratrix of Hippias of Elis (a curve $r \sin\theta = (2a/\pi)\theta$, that may be used for dividing an angle into any number of equal parts). The orthogonal projection of the helix on a plane parallel to the axis of the cylinder is a curve as shown in Fig. a. In the literature often the word spiral is used, where helix is meant; however, a spiral (of Archimedes) (e.g. $r = a\theta + b$) is a two-dimensional curve which was discussed first by Archimedes about 225 BC.

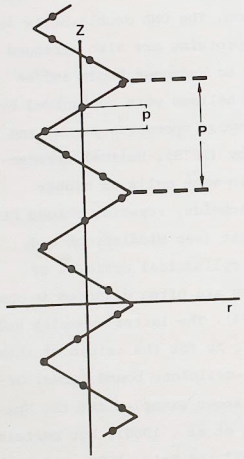

Fig. a. Side-on view (elevation) of a helix with an axial (z) repeat P and a separation p between structural subunits.

The main characteristic of a helix is its pitch, P ($2\pi r_o/\cot\beta$), which corresponds to the axial distance along the helix that gives a rotation of 2π (360°) on its surface. Another characteristic is the screw-angle of the helix Σ, the angular displacement of successive mathematical structural units. It is now evident that:

$$\Sigma = 2\pi p/P$$

where p is the separation between units. (As will be evident from Fig. a, Σ is the projection on the z,0-plane of the angle between the axis of two successive units.) In biomolecules these subunits may be nucleotides as in DNA. When viewed under the electron microscope, the image normally gives a side-on view (elevation) of a helix with an axial (z) repeat P and a radius r_o (see Klug, Crick and Wyckoff, 1958). A helix is usually defined in terms of the cylindrical polar coordinates r, φ and z (see Fig. b). The density variations in the helix satisfy the relation:

$$\rho(r,\phi,z) = \rho(r,\phi,z + P),$$

where P is the repeat distance of the helix in the z-direction.

Biological helices are not continuous distributions of density but are a

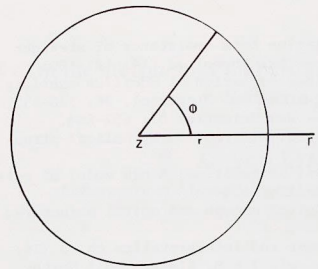

Fig. b. Z-projection of a helix in polar coordinates.

series of identical subunits (in this case, cells) separated in the z-direction by a distance, p, on a helix of pitch P as shown in Figure b. These subunits are successively rotated through the screw-angle Σ. Initially, it will be assumed that there is an exact number of subunits in a distance P, that is P/p is an integer, each subunit being rotated by $\Sigma = 2\pi p/P$ with respect to the one below or above it.

The discontinuous helix can be represented as the multiplication of a continuous helix by a one-dimensional lattice with a repeat distance p (Sherwood, 1976; see also Misell, 1978). Mendelson (1976) defines the helix angle of surface organization in *Bacillus* spp. as a tangent helix angle = pitch/circumference, where circumference equals D (D = diameter of the helix). Another characteristic is the helix length defined by $(\text{helix length})^2 = (\text{pitch})^2 (\text{circumference})^2$. Mendelson (1976) states that for helical growth morphology, it is necessary to assume that new cell surface is inserted along a helical path and thus the major wall components (for cyanobacteria peptidoglycan) must in some respect fit in this orientation. One simple possibility is that the glycan backbone of the peptidoglycan might be oriented in a helical path. This would provide a structurally sound organization for the cell wall.

Spirulina and *Arthrospira* spp. are the only cyanobacteria to have a well developed helical shape which is a constant property, and therefore the most suitable organisms for the study of the organismal biological helix. In this study *Spirulina platensis* was chosen for obvious reasons. Chapter II represents an integral study of the cell wall proper and the cross-wall. The latter did not fit previously described morphological models for cell wall shape. During the cell wall studies a fibrillar layer between the plasma membrane and the peptidoglycan layer was discovered. An additional study using pyrolysis mass-spectrometry was initiated to elucidate the chemical nature of these fibrils. This is described in chapter V. The in vitro shape of the cross-wall as found in II resulted in several hypotheses regarding the origin of its morphology. These are described and analyzed in chapter III.

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II. On the morphology and ultrastructure of the cell wall of *Spirulina platensis*

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van EYKELENBURG, C. 1977. On the morphology and ultrastructure of the cell wall of *Spirulina platensis*. Antonie van Leeuwenhoek **43:** 89–99.

The cell wall of the **cyanobacterium** *Spirulina platensis* was studied with the electron microscope using ultra-thin sectioning, shadowing, carbon-replication or freeze-etching techniques for specimen preparation. The cell wall could be resolved into four layers, L-I through L-IV. The L-I and L-III layers contain fibrillar material. The septum is a three-layered wall: an L-II layer sandwiched between L-I layers. The shape in vitro of isolated septa might be an artifact due to the preparation technique used. Certain structural properties of the septum seem to allow tangential stretching; they might be reflected in the flexible gliding mobility of *Spirulina* species. The outer, L-IV layer contains material longitudinally arranged along the trichome axis.

INTRODUCTION

Some cyanobacteria and green algae are considered to be potential sources of nutritional protein (Soeder, 1976). One of the most promising species is *Spirulina platensis* because of the technological advantages it offers (Soeder, 1976).

A review article by Wolk (1973) gives integrated information on the ultrastructure of the **cyanobacteria.** Since little is known about the morphology and ultrastructure of *Spirulina* species an electron microscopic study was initiated.

The cell wall of **cyanobacteria** consists of four layers, L-I through L-IV (Jost, 1965) though Jensen and Sicko (1972) reported an eight-layered wall for *Gloeocapsa alpicola.*

Allen (1968) suggested the L-I layer to be an artifact of preparation. The L-II layer contains peptidoglycan as has been repeatedly found in various species and which is generally accepted for **cyanobacteria** (Halfen, 1973). In this L-II layer rows of pores are frequently visible on both sides of the loci

of ingrowth of the septa in several filamentous cyanobacteria (Metzner, 1955; Halfen and Castenholz, 1971). Metzner (1955) suggested that these pores are involved in mucilage secretion. The L-III layer of *Oscillatoria princeps* was shown to be fibrillar and proteinaceous (Halfen, 1973). To our knowledge very little is known about the ultrastructure of the L-IV layer.

The present article deals with the morphology and ultrastructure of the cell wall and the septum of *Spirulina platensis*. The results were compared with literature data on other cyanobacteria. Special attention was paid to the shape of the septum, which was considered in relation to the helical shape of the trichome.

MATERIALS AND METHODS

Culture methods. An axenic strain of *Spirulina platensis* (from Lake Nakuru, Africa) and a xenic strain of *Spirulina laxissima* (from Lake Nakuru, Africa) were cultivated in a medium according to Ogawa and Terui (1970) using tap water instead of demineralized water. The organisms were grown in 100 ml Erlenmeyer flasks at 25 C. The light intensity of the fluorescent lamps by which the cultures were illuminated was 5 klux. The trichomes were harvested after 10 to 18 days.

Isolation of cell walls. Cells were disintegrated in a Mickle shaker (Hampton Middx, UK, 50 Cycles) with ballotini beads, 0.17–0.18 mm in diameter at 4 C for ten minutes in the presence of 0.001% (w/v) DNAse (from beef pancreas; NB cy). The suspension was made up in a 0.01 M EDTA solution. Cell walls were separated from plasma and cellular organelles by centrifugation at 2000 *g* at 4 C and washed twice with demineralized water. A fraction resistant to sodium dodecyl sulphate (SDS) was isolated by treatment of the isolated cell walls with a hot (100 C) 4% SDS solution for a few seconds.

Preparation for sectioning. Cell walls were fixed in 2.5% glutaraldehyde in a 0.2 M phosphate buffer (pH = 7.4) according to Hayat (1972) with 0.2% sucrose for 2 hours at 20 C. The material was washed twice in buffer followed by⊗ embedding in Spurr and ultra-thin sectioning followed. Sections were stained with lead citrate and uranyl acetate.

Preparation for shadowing. Cell walls and the SDS-resistant fraction were washed in demineralized water and platinum-shadowed at 10^{-5} Torr.

Preparation for replica technique. Replication was carried out with untreated material by the platinum/carbon replica technique. A 3.5% potassium dichromate solution in 25% sulphuric acid and a saturated sodium hypochlorite solution were used to clean the replicas. After washing the replicas with distilled water they were examined electron microscopically.

Preparation for freeze-etching. A 20% dimethyl sulfoxide (DMSO) solution in culture medium was used as a cryoprotectant. The cells were quickly frozen

⊗ post-fixation in 1% OsO_4 for 2 hours at 20 C after which

after a 20-minute treatment with DMSO. Etching was done at –105 C at 5.10^{-7} Torr and lasted 2 minutes. The freeze-etch replicas were cleaned as described before.

Experiments with pepsin (Merck, 10000 E/g, 7185), trypsin (Miles-Seravac, bovine), lysozyme (ELBG, 15351) and pronase (Calbiochem, 45000 PUK/g, 53702) were carried out according to Braun and Rehn (1969).

RESULTS

Electron microscopy of ultra-thin sectioned material of *Spirulina platensis* revealed the four-layered longitudinal cell wall (Fig. 1). Fig. 1 also shows the ingrowth of a septum in statu nascendi, which is three-layered: an L-II layer

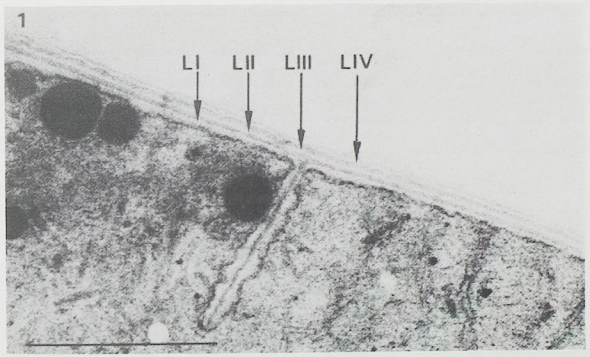
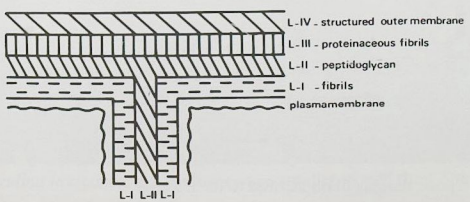


Fig. 1. Section through *S. platensis*. The cell wall is divided into four layers, the septum is divided into three layers.
The black bar indicates 500 nm unless otherwise stated.



Scheme 1. Model of the cell wall

sandwiched between L-I layers. L-I and L-III are electron transparent whereas the L-II and L-IV layers are electron-dense. All layers are 10 to 15 nm thick and therefore the whole wall ranges in thickness up to about 60 nm. The L-II layer in the septum and in the longitudinal cell wall equal each other in thickness but the L-I layers seem to have expanded.

Untreated septum material is almost always accompanied by fibrils as seen in Fig. 2. The diameter of these fibrils is 14 to 15 nm.

In untreated cell walls the L-III layer appears to be fibrillar (Fig. 3). The fibrils were continuous over the trichome surface in a right-handed helix. Their diameter was 8 to 10 nm.

Replica experiments revealed the outermost layer (L-IV) of the cell wall (Fig. 4) to be composed of linearly arranged material. The size of the elements of this arrangement is 12 to 15 nm and the direction of the array is parallel to the trichome axis. Distortion of the cell wall leads to a distortion in the normal

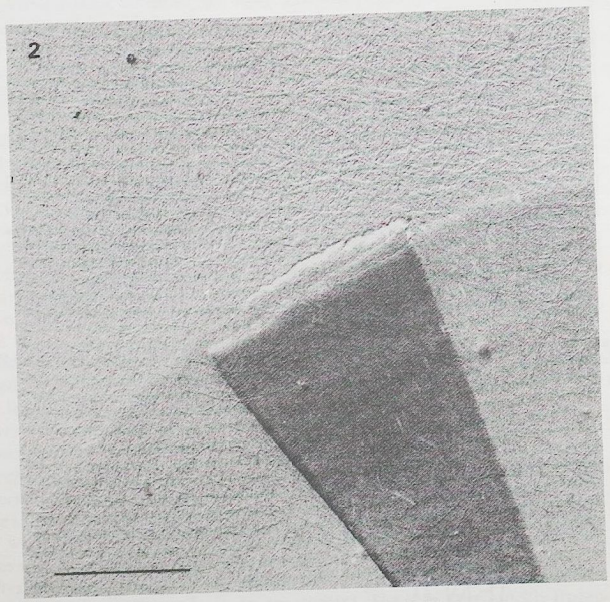


Fig. 2. Shadowed septum showing fibrils ascribed to the L-I layer.

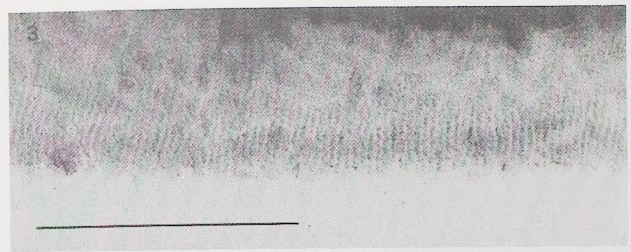


Fig. 3. Shadowed specimen of the L-III fibrils.

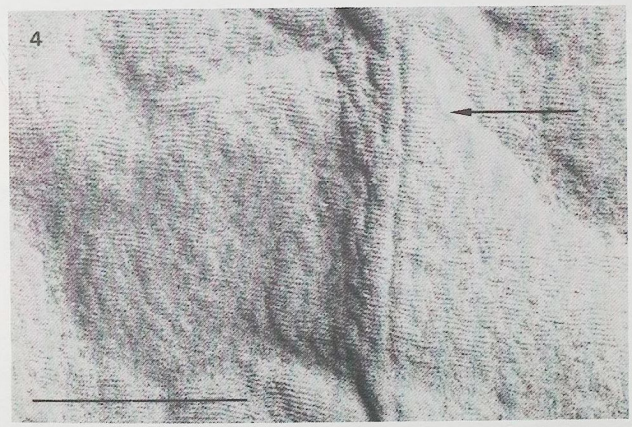


Fig. 4. Replicated trichome of *S. platensis* with linearly arranged material from the L-IV layer. The arrow indicates a septum.

arrangement (Fig. 5). Small areas are found in which the former arrangement is still present though these areas themselves form a distorted pattern.

Isolated septa visualized by shadowing are round and characteristically shaped (Fig. 6). They appear as thin discs which are folded in a sector covering about 5% of the total septum surface (Figs. 6 and 7). This implies that there is 10% more septum material than necessary for a flat septum.

Spirulina laxissima, a cyanobacterium smaller in size than *Spirulina platensis*

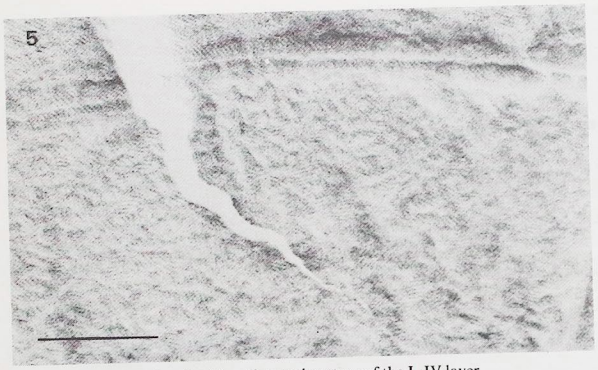


Fig. 5. Distorted cell wall with a distorted pattern of the L-IV layer.

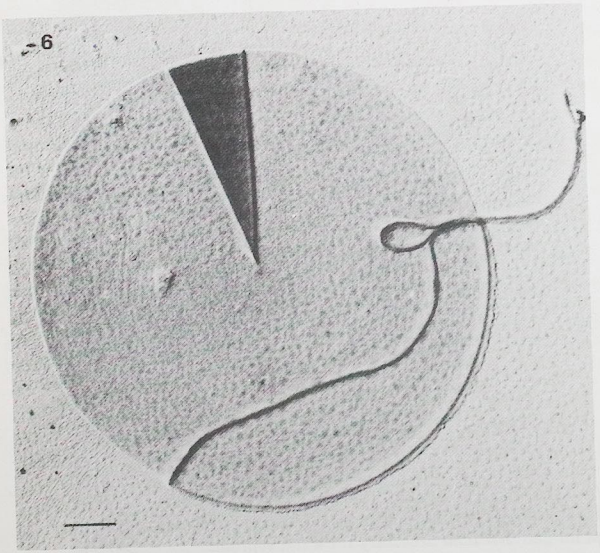


Fig. 6. Shadowed specimen of a septum with a fold and a zipper.

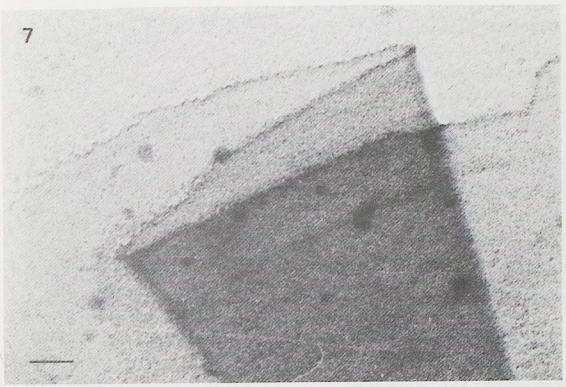


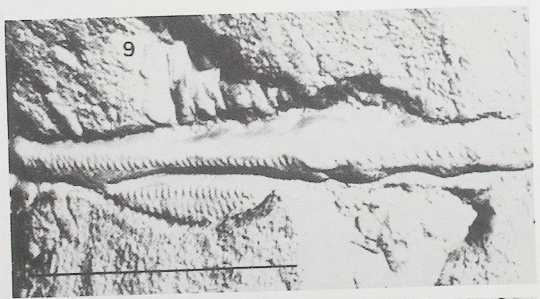
Fig. 7. Shadowed septum-part clearly showing the fold. The dark line represents 100 nm.

Fig. 8. Shadowed septum from *S. laxissima* with a relatively smaller fold.

but with a larger pitch was used to find a correlation between the shape of the septum and the trichome morphology. Fig. 8 shows a septum of *Spirulina laxissima* which is folded in a similar way while the sector covered is about 3% of the total surface.

The purpose of the freeze-etch experiments was to create a fracture face through all cell-wall layers to visualize the respective ultrastructural features. The fracture faces obtained were situated in the L-II layer, in the plasmalemma, or in the cytoplasma. The L-II layer could be recognized by the rows of pores near the septa.

Enzymes and chemicals were used in efforts to create another weak place in the cell wall leading to another fracture face. The experiments carried out with enzymes such as pepsine, trypsine, pronase and lysozyme, and with chemicals such as SDS and sodium hypochlorite in different concentrations failed to give the desired result. However, some results should be reported. Figs. 9 and 10 show a zipper-like structure in the L-II layer at a septum crossing. In Fig. 6 the zipper itself is wholly isolated from the longitudinal cell-wall but still half



Figs. 9 and 10. Freeze-etched zipper structures.

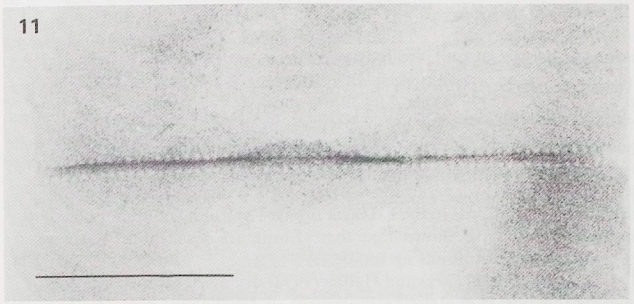


Fig. 11. Visualization of the rows of pores in ultra-thin sectioned material.

Fig. 12. Inner fracture face of the L-II layer showing the membrane-associated particles.

attached to a septum. In ultra-thin sectioned material rows of pores could be seen in the same place as shown in Fig. 11.

Another phenomenon to be reported is the characteristic pattern of membrane-associated particles in the L-II layer. Fig. 12 shows the distribution of these particles.

DISCUSSION

The four-layer structure of the longitudinal cell-wall and the three-layer structure of the septum are consistent with observations by Jost (1965) and by Halfen and Castenholz (1971) on *Oscillatoria* species.

The fibrils of Fig. 2 are assumed to originate from the L-I layer although they were never seen as a layer on the septum. This phenomenon could be due to the presence of a matrix in which these fibrils might be embedded, whilst the attachment of the L-I layer to the rest of the cell wall could be very loose. The assignment of the fibrils to the L-I layer is based on ruling out other possibilities. Experiments have shown that these fibrils are resistent to sodium dodecyl sulphate concentrations up to 4%.

Halfen and Castenholz (1971) and Halfen (1973) discussed an L-III fibrillar layer and its function in the gliding movement of *Oscillatoria princeps*. The diameter of the L-III fibrils of this organism was 5 nm. Halfen (1973) found these fibrils to be proteinaceous and wound helically around the trichome. The same conclusion applies to *Spirulina platensis*. The regular pattern in the L-IV layer was sofar unknown in cyanobacteria but resembles the ultrastructure of the outermost cell-wall layer of gram-negative bacteria described by Thornley, Glauert and Sleytr (1974). The distortion of the structure in the L-IV layer of *Spirulina platensis* is probably due to the construction of the layer and to the material of which it is composed.

The morphology of the septum in vitro might implicate a different morphology in vivo. However, it might merely be an artifact, due to the preparation technique used. Theoretical consideration of the exact shape of the septum in vitro suggests that it might result from tangential stretching of either one of the two layers in the septum or both of them during preparation. Certain structural properties of these layers common to both the cell wall proper and the septum might be responsible for the flexible gliding motion of *Spirulina* species on the one hand and the stretchability of the septum on the other. Work to provide evidence for this hypothesis is in progress (Van Eykelenburg, Fuchs and Schmidt, to be published). Remarkably, the size of the area covered by the fold is related to the pitch of the trichome. The larger the pitch the smaller the the folded area and vice versa. *Spirulina laxissima* was used to illustrate the hypothesis. Fig. 4 supports the correlation. The phenomenon described fits in the helical macro-morphology of the trichome or vice versa.

Metzner's (1955) idea that the rows of pores on both sides of the septa in the longitudinal cell-wall may play a role in mucilage secretion must be reconsidered. Figs. 6, 9, 10 and 11 suggest that the zipper-like structure is rather loosely built into the trichome and therefore could represent a trichome propagation device. The propagation mechanism might result in a tearing apart right through the L-II layer of the septum. Another mechanism could be that the septum as a whole together with the zipper is removed so that two cells would

be killed. The latter mechanism is supported by Fig. 6.

In general the cell wall of *Spirulina platensis* can be schematically represented as in Scheme 1.

Chemical characterization of the different types of cell-wall layers will be the next step in comparing *Spirulina* species with other cyanobacteria.

I wish to thank Ir B. H. A. van Kleeff, Dr Ir P. Kooiman and Drs P. J. Nieuwdorp for their kind support and Prof. Dr A. Fuchs and Prof. Dr T. O. Wikén for critically reading the manuscript.

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III. Some Theoretical Considerations on the *In Vitro* Shape of the Cross-walls in *Spirulina* spp.

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Electron micrographs obtained after shadow-preparation of the cross-walls of cyanobacteria belonging to the genus *Spirulina* showed that the walls have a perfect sectorial pleat, its size in different species being related to the magnitude of their trichome pitch. Further, shadow-preparation caused a decrease in diameter of the cells of *Spirulina platensis* of 40%, with a 10% extra shrinkage of the diameter of the cross-wall. Both phenomena no doubt reflect certain material properties of the peptidoglycan layer which is responsible for the inherent rigidity of the cell envelope, and also constitutes the cross-wall. Probably, these material properties determine both the *in vivo* helical shape and the *in vitro* occurrence of the overlap in *Spirulina* cross-walls, and also allow for flexibility and variability in shape of the organism as a whole.

Four hypotheses are put forward to explain the observed phenomena; on the basis of mechanical considerations it is most likely that the pleat in the cross-wall as observed *in vitro* must be ascribed to anisotropic shrinkage upon dehydration during shadow-preparation of the cross-walls. This anisotropy might be due to the polysaccharide chains of the peptidoglycan layer running in the circumferential direction and the peptide side-chains running in the radial direction. With this hypothesis the difference between the diameters *in vitro* of the cell envelope and the cross-wall can also be accounted for, within an error of 1·2%.

1. Introduction

In studying the morphology and ultrastructure of the cell wall of *Spirulina platensis* Van Eykelenburg (1977) observed a remarkable phenomenon.

Electron micrographs of the cross-walls of this spiralized—or rather helical—filamentous cyanobacterium showed them to have a perfect sectorial pleat with an angle α of 0·39 rad covering 6·2% of the plane. Thus, the cross-wall consists of 12·4% more material than needed to form a flat plane [Plate 1(a)]. The cross-walls of *Spirulina laxissima*, another cyanobacterium with a helical trichome, but with a larger trichome pitch, showed a smaller pleat, whereas those of non-spiralized species of the *Oscillatoriaceae*—i.e. with an infinite trichome pitch—had no pleat at all. From these, though limited, observations one might infer that there is a certain relationship between the magnitude of the pitch and the size of the overlap in the cross-wall, as seen after shadow-preparation. For *Spirulina platensis* this relationship proves to be linear, irrespective of growth temperature in the temperature range 13·5–40·0°C.

In vivo, the diameter of the cells of *Spirulina platensis* is 10 μm, whereas the diameter of the *in vitro* envelopes is 6·0 μm, as can be calculated from the size of empty envelopes as seen in the electron microscope. The diameter of the cross-wall *in vitro* is 5·4 μm. Hence, there is a 10% extra decrease of the radius of the cross-wall after shadow-preparation.

From micrographs of cross-walls of filamentous cyanobacteria as presented by Metzner (1955), Frank, Lefort & Martin (1962), Höcht, Martin & Kandler (1965), Jost (1965) and Van Eykelenburg (1977) it appears that the cross-walls are formed diaphragmatically and originate from the innermost layer† and the rigid peptidoglycan layer (for further details see also the review by Drews, 1973). The outer layers, on the other hand, are never part of the cross-walls in *Oscillatoriaceae* (Halfen & Castenholz, 1971). In other filamentous cyanobacteria cell-wall material of the outer layer does not protrude into the cross-walls until cell separation has begun (Ingram & Thurston, 1970).

The cross-walls of cyanobacteria consist of peptidoglycan which forms a layer coherent with the peptidoglycan layer in the cell wall and varying in thickness from less than 1 to over 10 nm in different species (Stanier & Cohen-Bazire, 1977) and 14 nm thick in *Spirulina platensis* (Van Eykelenburg, 1977). In the cell envelope of almost all bacteria peptidoglycans build up a comparable rigid and continuous structure. The polysaccharide part of these peptidoglycans consist of polymers of β-1,4-linked N-acetyl-glucosamine and N-acetyl-muramic acid residues. This structure forms a three-dimensional network through cross-linking with branched polypeptides. Via their carboxyl groups the lactic acid moieties of the N-acetyl-muramic acid building blocks are cross-linked with oligopeptides which vary in

† Considered to be an artefact of preparation (see the review by Stanier & Cohen-Bazire, 1977) or a very loosely attached fibrillar layer (Van Eykelenburg, 1977).

composition in different species of bacteria. In general, however, there is a remarkable consistency in structure and chemical composition of peptidoglycans throughout the bacterial world, as exemplified by the cyanobacteria and other gram-negative prokaryotes, whose structural and chemical conformity has been established (Frank *et al.*, 1962).

The occurrence of a pleat in the cross-wall of spiralized, filamentous cyanobacteria as observed after shadow-preparation no doubt reflects certain material properties of the peptidoglycan layer. Since this layer is part of both the cell wall proper and the cross-wall it might be possible that these material properties determine both the *in vivo* helical shape and the *in vitro* occurrence of the overlap in the cross-walls in *Spirulina* spp.

Fogg *et al.* (1973) distinguish seven possible ways of locomotion for filamentous cyanobacteria, viz. gliding, rotation, oscillation, bending, swaying, jerking and flicking. In addition, spiralized cyanobacteria should be able to stretch and tighten the helix. The latter movements will cause extra stresses in the cell wall, especially in the rigid peptidoglycan layer. Possibly, these various propelling movements can only be performed by virtue of elastic properties of the cell-wall material in spiralized cyanobacteria.

When placed on agar media *Spirulina platensis* forms aggregates with a true spiral shape [Plate 1(b)], as reported previously by Lazaroff & Vishniac (1961) for *Nostoc* spp.

In our opinion it is difficult to explain all these phenomena, without taking into account the physical properties of the peptidoglycan layer. These physical properties might not only be reflected in the *in vitro* shape of the cross-wall and in the overall *in vivo* shape of the organism as a whole, but also linked with the 'changeability' in shape of the latter. Apparently, two kinds of changeability should be clearly distinguished, viz. flexibility, enabling the different ways of locomotion, and variability in shape depending on environmental factors (f.i. a solid agar surface versus liquid medium).

In an attempt to more fully comprehend the morphology and ultrastructure of the spiralized, filamentous cyanobacteria and, in particular, the *in vitro* shape of the cross-walls several hypotheses are put forward.

2. Theory

For convenience, the change of the shape of the *Spirulina* cross-walls during the transition from the *in vivo* state to the *in vitro* state is considered to consist of two components. First, the diameter decreases from 10 μm to 5·4 μm. This shrinkage of 46% is due to dehydration; it reduces the size of the cross-wall, but it does not change its geometrical shape. We will call this deformation the main deformation. Second, since the pleat is very unlikely

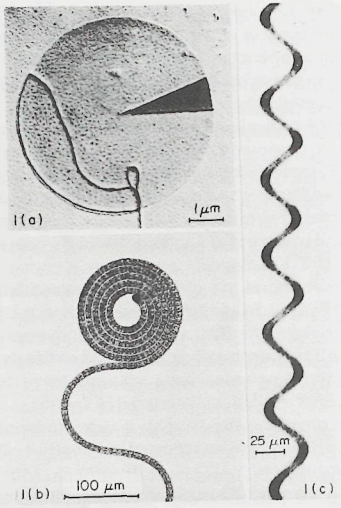

PLATE 1. (a) An isolated cross-wall from *Spirulina platensis* after shadow-preparation. Note the sectorial pleat and the concentric circles (electron micrograph). (b) *Spirulina platensis* on agar. Note the spiral trichome morphology and the end cell (light micrograph). (c) *Spirulina platensis* in liquid medium, showing its helical shape (light micrograph).

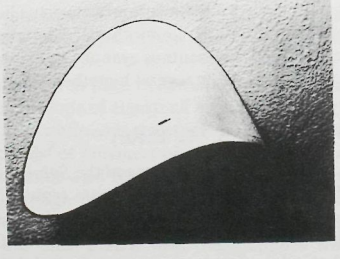

PLATE 2. A three-dimensional model of an *in vivo* cross-wall according to hypothesis I with $n = 2$ (see text); a so-called undulating saddle (see Stevens, 1974).

to be present already in the *in vivo* state, there must be an additional deformation. In this section, four hypotheses are put forward with respect to this deformation; some mechanical consequences of these hypotheses will be considered in the next section.

(I) One can assume that the additional deformation of the cross-wall takes place without straining it in its plane. Such a deformation is the only one the cross-wall allows for, if it is very resistant to straining in its plane. Clearly, the *in vitro* shape is locally developable†, except for the vertex of the pleat. Therefore, the *in vivo* shape must be developable too, also with the exception of its centre. Taking this condition into account, we construct a family of possible surfaces describing the *in vivo* shape. Let x, y and z be cartesian coordinates and let r and ϕ be polar coordinates for the x, y-plane. Then the elevation of the surface from the x, y-plane is:

$$z = c_n r \cos n\phi; \quad n = 2, 3, 4 \ldots .$$

Each value of the integer n corresponds to a member of the family of planes; n equals the number of the maxima of the elevation z on the circumference of the cross-wall. The surface with $n = 2$ is shown in Plate 2. The constant c_n is the maximal elevation divided by the radius of the cross-wall. The value of c_n depends on the angle α of the pleat. For *Spirulina platensis*, we have $\alpha = 0{\cdot}39$ rad and $c_2 = 0{\cdot}456$, $c_3 = 0{\cdot}267$, $c_4 = 0{\cdot}193$, *etc.*, and for large n we have $c_n \approx 0{\cdot}736/n$, as will be computed in the next section.

There is one major objection to this hypothesis: under the light microscope the cross-walls in the trichome appear as parallel straight lines perpendicular to the cell wall proper. Therefore, this hypothesis can be valid only if c_n is so small that the edge seems to be a straight line. From micrographs, not given in this paper, we have estimated that c_n may not exceed $0{\cdot}02$ and hence $n > 36$.

The following hypotheses have in common that the *in vivo* shape is assumed to be flat and that the additional deformation is a deformation with strain. The hypotheses differ with respect to the cause of the strain.

(II) One can assume a shrinkage due to dehydration to take place which is anisotropic: the strain in the radial direction exceeds the strain in the circumferential direction. Here, the main deformation and the additional one have the same origin. Therefore, they are indistinguishable and we can consider the deformation only as a whole. In the next section we will compute the difference between the strain in the radial direction and that in the circumferential direction from the value of the angle α.

† A surface is called developable, if it can be obtained from a flat plane by a deformation without straining it in its plane.

(III) One might assume the cross-wall to be under continuous stress in the *in vivo* state and to be elastically deformed upon disintegration of the cell. Thus, the *in vitro* morphology would be the result of the cross-wall 'snapping-back' to the stress-free state. This view is supported by the presence of slight constrictions in the trichome at the sites of the cross-walls as can be seen by light and electron microscopy. There is one known exception in an *Oscillatoria* sp. with annulated cross-walls, the margin of which protrudes slightly beyond the longitudinal cell wall of the filament (Mehrotra & Singh, 1977).

(IV) Finally, one might assume both anisotropic shrinkage due to dehydration during preparation and relaxation of *in vivo* stress together to be responsible for the *in vitro* shape of the cross-walls, thus combining hypotheses II and III.

3. Some Mechanical Aspects

In this section we derive some quantitative results to test the hypotheses stated in the previous section. The Roman numerals correspond to the hypotheses.

(I) Using cartesian coordinates x, y and z and polar coordinates r and ϕ for the x, y-plane (see Fig. 1), we can describe the surface of the *in vivo* cross-wall by the equations:

$$x = r\cos\phi; \quad y = r\sin\phi; \quad z = c_n r\cos n\phi; n = 2, 3, 4 \ldots . \tag{1}$$

Let R be the radius of the *in vitro* cross-wall, then the edge of the *in vivo* cross-wall after the main deformation is determined by the equations (1) and:

$$x^2 + y^2 + z^2 = R^2. \tag{2}$$

Substituting (1) into (2) we find for the edge:

$$r = r(\phi) = R(1 + c_n^2\cos^2 n\phi)^{-1/2}. \tag{3}$$

The length l of this edge follows from an integration and using (1) and (3):

$$l = \int_0^{2\pi}\left\{\left(\frac{dx}{d\phi}\right)^2 + \left(\frac{dy}{d\phi}\right)^2 + \left(\frac{dz}{d\phi}\right)^2\right\}^{1/2} d\phi = Rf(c_n, n) \tag{4}$$

where:

$$f(c_n, n) = \int_0^{2\pi}\{1 + c_n^2(\cos^2\phi + n^2\sin^2\phi)\}^{1/2}(1 + c_n^2\cos^2\phi)^{-1}\, d\phi. \tag{5}$$

Since we assume the additional deformation to be without strain, this length l must equal the length of the edge of the *in vitro* cross-wall, which is

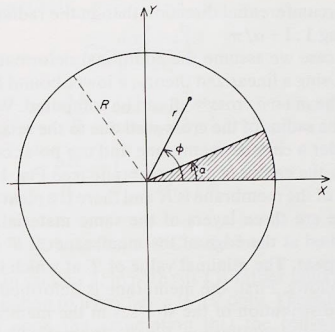

FIG. I. A circular membrane with pleat in the region $0 \leq \phi \leq \alpha$. R is the radius in the stress-free state.

$R(2\pi + 2\alpha)$, with the edge of the pleat reckoned three times. Therefore, the value of c_n is determined by the equation:

$$f(c_n, n) = 2\pi + 2\alpha \tag{6}$$

This equation has been solved numerically for $\alpha = 0{\cdot}39$, the results being given in the preceding section. The value of c_n tends to zero for $n \to \infty$, and we may approximate:

$$f(c_n, n) \approx \int_0^{2\pi}\{1 + (c_n n)^2\sin^2\phi\}^{1/2}\, d\phi, \; c_n \to 0. \tag{7}$$

Since the right side of (7) is a function of the product $c_n n$ only, c_n is inversely proportional to n for large n. For $\alpha = 0{\cdot}39$ we find $c_n \approx 0{\cdot}736/n$.

(II) Let the radius of the cross-wall be R *in vitro* and $\tilde{R}$ *in vivo*. Assuming the material of the cross-wall to be homogeneous (but anisotropic), we conclude that the length in the radial direction of an infinitesimal square of the material of the cross-wall is reduced by a factor $R/\tilde{R}$ during the transition to the *in vitro* state. The length of the edge of the cross-wall *in vivo* is $2\pi\tilde{R}$, and *in vitro* $R(2\pi + 2\alpha)$, with the edge of the pleat reckoned three times. Thus, the length in the circumferential direction of the infinitesimal square is reduced by a factor $(1 + \alpha/\pi)\, R/\tilde{R}$ during the transition to the *in vitro* state. Therefore after the transition an infinitesimal square of the material of the cross-wall in the *in vivo* state becomes a rectangle, which is

longer in the circumferential direction than in the radial direction, the ratio of its sides being $1:1+\alpha/\pi$.

(III) In this case we assume the additional deformation to be an elastic deformation. Using a linearized theory, a lower bound for the tensile stress at the edge of the *in vivo* cross-wall can be computed. We will also compute the change in the radius of the cross-wall due to the relaxation of this stress.

Let us consider a circular membrane and use polar co-ordinates r and ϕ with origin 0 in the centre of the membrane (see Fig. 1). In the stress-free state the radius of the membrane is R and there is a pleat so that in the region $0 \leq \phi \leq \alpha$ there are three layers of the same material. Suppose a tensile stress T is applied at the edge of the membrane. If T is large enough the pleat will disappear. The minimal value of T at which it disappears can be computed as follows. First, the membrane is deformed so that it becomes flat. Then, the distribution of the stresses in the membrane are computed using the equations of equilibrium. Finally, the stress T must be so large that all stresses in the membrane are tensile stresses.

Let e_r and e_ϕ be the strains of the material with respect to the stress-free state in the radial and tangential direction, respectively. The quantities σ_r and σ_ϕ denote the forces per length acting in a cross-section of the membrane in radial and tangential direction, respectively. We assume a linear relation between the stresses and the strains:

$$\sigma_r = c_r e_r + c_* e_\phi; \quad \sigma_\phi = c_* e_r + c_\phi e_\phi, \tag{8}$$

where c_r, c_ϕ and c_* are material constants. Using (8) in the equations of equilibrium, we find, after linearization in the strains, that the pleat disappears if

$$T > \frac{(c_r c_\phi - c_*^2)}{(c_\phi - c_r)} \frac{(\tilde{c} + c_*)(\mu - 1)}{(c_* \mu + c_\phi)} \frac{\alpha}{\pi}, \tag{9}$$

where $\tilde{c}$ is $(c_r c_\phi)^{1/2}$. Let the increase of the radius of the membrane, after removing the pleat, be ΔR. Then we find:

$$\frac{\Delta R}{R} = \frac{T}{\tilde{c} + c_*} + \frac{c_*(c_\phi - \tilde{c}) + c_\phi(\tilde{c} - c_r)}{(\tilde{c} + c_*)(c_\phi - c_r)} \frac{\alpha}{\pi} \tag{10}$$

and hence, by virtue of (9):

$$\frac{\Delta R}{R} > \left\{ \frac{\tilde{c}}{\tilde{c} + c_*} + \frac{c_*(c_\phi - c_*)(\tilde{c} - c_r)}{\tilde{c}(\tilde{c} + c_*)(c_\phi - c_r)} \right\} \frac{\alpha}{\pi}. \tag{11}$$

In the case of isotropic material, we have $c_r = c_\phi$ and the equations (9), (10)

and (11) become respectively

$$T > \frac{\alpha}{\pi} \frac{c_r^2 - c_*^2}{2c_r}, \tag{12}$$

$$\frac{\Delta R}{R} = \frac{T}{c_r + c_*} + \frac{1}{2} \frac{\alpha}{\pi}, \tag{13}$$

$$\frac{\Delta R}{R} > \left(\frac{1}{2} + \frac{c_r - c_*}{2c_r} \right) \frac{\alpha}{\pi}. \tag{14}$$

4. Discussion and Conclusions

As already stated in section 2, hypothesis I is rather unlikely to be true; here, we will pay attention to hypotheses II–IV only. These hypotheses are more or less based upon the assumption that the cell-wall material is anisotropic. We will first discuss the anisotropy from a molecular point of view.

Micrographs of isolated cross-walls show concentric circles [Plate 1(a)]; their presence seems to be plausible only if the polysaccharide backbone of the peptidoglycan is laid down in concentric circles with the peptide side chains being able to protrude in the perpendicular direction. As emphasized by Formanek, Formanek & Wawra (1974), there is the possibility of bulky interpeptide bridges consisting of several amino-acid residues between the carbohydrate chains of the peptidoglycan. These peptide bridges can be bent to the outside of the peptidoglycan layer perhaps causing the concentric circles. The ability of these peptide bridges to bend also implies that the rigidity constant c_r (see section 3) must be small. There is another possible explanation for the observed phenomenon, if the carbohydrate chains of the peptidoglycan are arranged radially; however, if this is so, then the concentric circles would be more pronounced at the centre of the cross-wall, which is in contradiction with the observations.

The former structure might provide us with an idea about the chemical background of the mechanical properties, such as c_r, c_ϕ and c_*, which are important in hypothesis III, and also of the anisotropic shrinkage upon dehydration, which is essential in hypothesis II. We will first discuss some consequences of hypothesis III; later we shall discuss hypothesis II.

As c_ϕ is the material constant determining the rigidity in the tangential direction, it applies to the polysaccharide backbone, whereas c_r, the material constant determining the rigidity in radial direction, applies to the peptide chains. Therefore, it is likely that c_ϕ is much larger than c_r, $c_\phi \gg c_r$, and also that the transverse contraction is small, $c_* \ll c_\phi$. Neglecting c_* and c_r with

respect to c_ϕ, equation (11) reduces to:

$$\frac{\Delta R}{R} > \frac{\alpha}{\pi}.$$

Thus, if hypothesis III holds true, the magnitude of the decrease of the radius of the cross-wall due to elastic properties of the material is at least $\alpha R/\pi$. For *Spirulina platensis* we have $\alpha/\pi = 0{\cdot}124$, so the size decrease must be at least 12·4%. In fact, we found an extra decrease upon shadow-preparation of 10% (see Introduction), which does not exceed the above-computed minimum 12·4%. However, if the cross-wall is elastic, the envelopes are probably elastic too, and they will then also show a size decrease due to elastic deformation. Therefore, the numbers given above do not contradict hypothesis III.

There is, however, another aspect which renders hypothesis III rather unlikely. We have computed the stress distributions for the *in vivo* cross-wall from the observed sectorial pleat in the *in vitro* state on the assumption that hypothesis III is valid. This stress distribution is non-homogeneous: both σ_r and σ_ϕ depend on the radial distance from the origin. However, the stress distribution in the *in vivo* cross-wall is inevitably strongly determined by the way the cross-wall is biosynthesized. From this point of view there is no reason to believe that the stresses in the cross-wall should be functions of the radial distance r, which follow from hypothesis III. If hypothesis III is valid, then any other shape of the pleat would be as likely to occur as a perfect sector of circle. Therefore, the elastic effects in the transition to the *in vitro* state are probably small, and hence hypothesis III and perhaps hypothesis IV are rather unlikely.

Finally, we come to hypothesis II. In this case the perfect sector of circle for the shape of the pleat follows from the assumption that the material of the cross-wall is homogeneous, but anisotropic for shrinkage due to dehydration. This anisotropy might be due to the polysaccharide chains running in the tangential direction, and the peptide side chains running in the radial direction.

With hypothesis II, we can also explain the difference between the diameter of the *in vivo* and the *in vitro* envelopes, if we make the assumption that the envelopes consist of polysaccharide chains running in the tangential direction and peptide side chains running in the direction of the axis of the cylinder. Then, the decrease of the diameter of the envelopes is determined by the shrinkage in the direction of the polysaccharide chains, whereas the decrease of the diameter of the cross-wall is determined by the shrinkage in the direction of the peptide side chains. The ratio of the shrinkages in the two directions is $1 : 1 + \alpha/\pi$, as was computed in section 3. For *Spirulina platensis* we have $1 : 1 + \alpha/\pi = 1 : 1{\cdot}124$, whereas the ratio of the diameters of the *in vitro* cross-wall and envelope is $5{\cdot}4 : 6{\cdot}0 = 1 : 1{\cdot}111$. Hence, the difference between the two diameters is explained within an error of 1·2% of the diameter of the *in vitro* cross-wall.

A consequence of hypothesis II is that an arbitrary wall consisting of polysaccharide chains in one direction and peptide side chains in between will shrink anisotropically upon dehydration. Its length in the direction of the polysaccharide backbone then decreases by a factor 0·61 and in the direction of the peptide side chains by a factor 0·54.

As the reader will have understood, we considered peptidoglycan as a rather simply constructed biopolymer. It should be realized that on a molecular basis peptidoglycan no doubt represents a more complicated structure.

The question why the pleat occurs in helical, but not in non-helical, cyanobacteria can probably be answered by assuming a large difference in chemical and physical properties of the peptidoglycan in both groups of organisms. Anisotropy might occur more frequently in the peptidoglycan of helical cyanobacteria than in non-helical ones. The same statement might be applicable to mutants of *Bacillus subtilis* which can grow as helices. Rogers & Thurman (1978) stated that in these mutants there must be a helical orientation of surface expansion. Though the bacterial species concerned is a gram-positive organism, and thus bears little relation to the gram-negative *Spirulina*, in our opinion the peptidoglycan layer of the former organism is constructed anisotropically in a way similar to the peptidoglycan layer of *Spirulina* spp.

The significance of this study lies in the general formulation of the consequences of anisotropy in peptidoglycan layers. Micro-organisms should not be looked upon as motile or non-motile, absolutely rigid "boxes" but as flexible living units which morphologically adapt to their environment by continuously "changing their shape". This adaptation can only take place when the cell-wall layer which is responsible for the cell's rigidity, i.e. the peptidoglycan layer, also endows the cell with an inherent changeability in shape.

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APPENDIX TO III (together with G.H. Schmidt)

In III, section 3, we have computed a lower bound for the tensile stress at the edge of the *in vivo* cross-wall (equations 9 and 12) and the change in the radius of the cross-wall due to relaxation of this stress (equations 11 and 14). For completeness and clarity, the derivation of equations 9 to 14 is given here.

Let us consider a circular membrane and use polar coordinates r and Φ with origin 0 at the centre of the membrane (see Figure 1). In the stress-free state, the radius of the membrane is R and there is a pleat so that in the region $0 \leq \Phi \leq \alpha$ there are three layers of the same material. Suppose a tensile stress, T, is applied at the edge of this membrane. A value for T at which the pleat disappears can be computed as follows; first, the membrane is deformed so that it becomes flat and then, the stress T must be so large that all stresses in the membrane are tensile stresses.

An expression for the strains on the material after the pleat is removed is given, assuming that these strains are independent of the Φ-coordinate. Let the material, which is at distance r from 0 in the stress-free state have a distance:

$$r = \tilde{r} + \rho(r) \qquad (8^*)$$

from 0 after removing the pleat. The function (r) is *a priori* unknown. Let e_r and e_Φ be the strains of the material with respect to the stress-free state in the radial and tangential direction, respectively. Then:

$$e_r(\tilde{r}) = \rho'(r) \quad \text{in which} \quad \rho' = \frac{d}{dr} \qquad (9^*)$$

The material in the stress-free state at a distance r from 0 was distributed in the stress-free state over a distance $(2\pi + 2\alpha)r$ in the Φ-direction. After removal of the pleat, this material is distributed over a distance $2\pi\tilde{r}$ in the Φ-direction. Therefore, the strain in the Φ-direction is:

$$e_\Phi(\tilde{r}) = \frac{2\pi\tilde{r} - (2\pi + 2\alpha)\, r}{(2\pi + 2\alpha)\, r} = \frac{\pi\rho(r) - \alpha r}{(\pi + \alpha)\, r} \qquad (10^*)$$

The quantities σ_r and σ_Φ denote the forces per unit length acting in a cross-section of the membrane in radial and tangential direction, respectively.

A linear relation between the stresses and strains is assumed.

$$\sigma_r = c_r e_r + c_* e_\phi \; ; \quad \sigma_\phi = c_* e_r + c_\phi e_\phi \qquad (11^*, \text{ equals } 8)$$

where c_r, c_ϕ and c_* are material constants. For isotropic material:

$$c_r = c_\phi = Eh/(1-\nu^2) \; ; \quad c_* = \nu Eh/(1-\nu^2) \qquad (12^*)$$

where E is Young's modulus, ν is Poisson's ratio and h is the thickness of the membrane. Substitution of (9^*-10^*) into (11^*) gives the stresses expressed in the unknown function $\rho(r)$:

$$\sigma_r(\tilde{r}) = c_r \rho'(r) + c_*(\pi\rho(r)/r - \alpha)/(\pi + \alpha) \qquad (13^*)$$

$$\sigma_\phi(\tilde{r}) = c_* \rho'(r) + c_\phi(\pi\rho(r)/r - \alpha)/(\pi + \alpha) \qquad (14^*)$$

The equation of equilibrium in the r-direction gives an equation for the stresses in the membrane:

$$\sigma_r(\tilde{r}) + \tilde{r} \frac{d}{d\tilde{r}} (\sigma_r(\tilde{r})) - \sigma_\phi(\tilde{r}) = 0 \qquad (15^*)$$

where, by (8^*):

$$\frac{d}{d\tilde{r}} = \frac{dr}{d\tilde{r}} \frac{d}{dr} = \frac{1}{1 + \rho'(r)} \frac{d}{dr} \qquad (16^*)$$

Substitution of (13^*-14^*), (8^*) and (16^*) into (15^*) gives a second order differential equation for $\rho(r)$:

$$rc_r\rho'' + (c_r + \frac{\alpha}{\pi + \alpha}(c_\phi - 2c_*))\rho' - \frac{\pi}{\pi + \alpha} c_\phi \frac{\rho}{r} +$$

$$(c_\phi - c_*) \frac{\alpha}{\pi + \alpha} + c_r\rho''\rho + (c_r - c_*)\rho'^2 + \frac{\pi}{\pi + \alpha}$$

$$(2c_* - c_\phi) \frac{\rho\rho'}{r} - \frac{\pi}{\pi + \alpha} c_* \frac{\rho^2}{r^2} = 0 \qquad (17^*)$$

At this point we linearize this equation with respect to α and ρ. In equation (17^*) we retain only first order terms, so that the equation becomes linear in :

$$rc_r\rho'' + c_r\rho' - c_\phi\rho/r + (c_\phi - c_*)\alpha/\pi = 0 \qquad (18^*)$$

The general solution which is bounded at r = 0 of this equation is:

$$\rho(r) = \lambda r + Ar^\mu \qquad (19^*)$$

where A is an arbitrary constant, and

$$\lambda = \frac{c_\phi - c_*}{c_\phi - c_r} \alpha/\pi \; ; \quad \mu = (c_\phi/c_r)^{\frac{1}{2}} \qquad (20^*)$$

The case of isotropic material where $c_r = c_\phi$ must be excluded here and will be discussed below. The value of A follows from the boundary condition for the radial stress at the edge of the membrane. Retaining only terms of first order in (13^*), this equation becomes:

$$\sigma_r(r) = c_r\rho'(r) + c_*(\rho(r)/r - \alpha/\pi) \qquad (21^*)$$

If we substitute (19^*), take r = R and give the left side of this equation the prescribed value T, we find:

$$A = R^{1-\mu} \{ \frac{T}{\tilde{c} + c_*} \frac{\alpha}{\pi} - \frac{c_r c_\phi - c_*^2}{(\tilde{c} + c_*)(c_\phi - c_r)} \} \qquad (22^*)$$

where:

$$\tilde{c} = (c_r c_\phi)^{\frac{1}{2}} \qquad (23^*)$$

Substituting this into (19^*), we determine the function $\rho(r)$; substitution of (19^*) into (21^*) then gives the radial stress as a function of r:

$$\sigma_r(r) = (\frac{r}{R})^{\mu-1} T + \frac{\alpha}{\pi} \frac{c_r c_\phi - c_*^2}{c_\phi - c_r} (1 - (\frac{r}{R})^{\mu-1}) \qquad (24^*)$$

The tangential stress as a function of r may be found in a similar way:

$$\sigma_\phi(r) = \frac{c_r c_\phi - c_*^2}{c_\phi - c_r} \frac{\alpha}{\pi} + \frac{c_*\mu + c_\phi}{\tilde{c} + c_*} \{ T - \frac{c_r c_\phi - c_*^2}{c_\phi - c_r} \frac{\alpha}{\pi} \} (\frac{r}{R})^{\mu-1} \qquad (25^*)$$

We will now investigate whether (24^*) and (25^*) represent tensile stresses, *i.e.* whether they are positive. The derivates of the right hand sides of (24^*) and (25^*) have fixed signs on $0 < r < R$. Therefore, it is sufficient to consider the signs of (24^*) and (25^*) at r = 0 and at r = R only. From energy considerations follows:

$$c_r c_\phi - c_*^2 > 0 \qquad (26^*)$$

which is used below. The cases $c_\phi > c_r$ and $c_r > c_\phi$ are discussed separately, starting with $c_\phi > c_r$.

If $c_\phi > c_r$, then $\mu > 1$ and the stresses are positive at r = 0 for all positive values of α. Hence, the stresses in the origin are tensile. The tangential stress at the edge is, by (25^*)

$$\sigma_\phi(R) = \frac{c_*\mu + c_\phi}{\tilde{c} + c_*} T + \frac{c_r c_\phi - c_*^2}{c_\phi - c_r} \left(1 - \frac{c_*\mu + c_\phi}{\tilde{c} + c_*}\right) \frac{\alpha}{\pi} \qquad (27^*)$$

The right-hand side of this expression is positive if:

$$T > \frac{c_r c_\phi - c_*^2}{c_\phi - c_r} \frac{(\tilde{c} + c_*)(\mu - 1)}{c_*\mu + c_\phi} \frac{\alpha}{\pi} \quad \text{or} \quad T > \frac{c_r c_\phi - c_*^2}{\tilde{c} + c_\phi} \frac{\alpha}{\pi} \qquad (28^*, \text{ equals } 9)$$

For the case $c_\phi > c_r$, the pleat will disappear when the stress T at the edge of the membrane exceeds the value given by the right-hand side of (28*).

If $c_r > c_\phi$, then $\mu < 1$ and the stresses are unbounded at $r = 0$. For all positive values of T and α, we find that $\sigma_r \to \infty$ and $\sigma_\phi \to \infty$ for $r \to 0$. Thus for all positive values of T and α, the stresses in the origin are tensile stresses. The value of $\sigma_\phi(R)$ is positive if and only if (28*) holds. Hence for $c_r > c_\phi$, the same condition (28*) for the disappearance of the pleat is valid.

The radius of the membrane, after removing the pleat is increased by $\Delta R = \rho(R)$, as follows from (8*). Substituting (20*) and (22*) into (19*), and taking $r = R$, gives:

$$\frac{\Delta R}{R} = \frac{T}{\tilde{c} + c_*} + \frac{c_*(c_\phi - \tilde{c}) + c_\phi(\tilde{c} - c_r)}{(\tilde{c} + c_*)(c_\phi - c_r)} \frac{\alpha}{\pi} \qquad (29^*, \text{ equals } 10)$$

and if the stress satisfies (28*):

$$\frac{\Delta R}{R} > \left\{ \frac{\tilde{c}}{\tilde{c} + c_*} + \frac{c_*(c_\phi - c_r)(\tilde{c} - c_r)}{\tilde{c}(\tilde{c} + c_*)(c_\phi - c_r)} \right\} \frac{\alpha}{\pi} \qquad (30^*, \text{ equals } 11)$$

Finally in the case of isotropic material, $c_r = c_\phi$, and the solution of (18*) is:

$$\rho(r) = \frac{\alpha}{\pi} \frac{c_* - c_r}{2c_r} r \log r + Ar, \qquad (31^*)$$

where A must be determined by the boundary condition for the radial stress. σ_r and σ_ϕ are found in a similar way:

$$\sigma_r(r) = T + \frac{\alpha}{\pi} \frac{c_*^2 - c_r^2}{2c_r} \log \frac{r}{R} \qquad (32^*)$$

$$\sigma_\phi(r) = T + \frac{\alpha}{\pi} \frac{c_*^2 - c_r^2}{2c_r} \left(\log \frac{r}{R} + 1\right) \qquad (33^*)$$

In the origin these stresses are unbounded but positive. The value of (R) is positive if:

$$T > \frac{\alpha}{\pi} \frac{c_r^2 - c_*^2}{2c_r} \qquad (34^*, \text{ equals } 12)$$

The increase of the radius of the membrane is now given by:

$$\frac{\Delta R}{R} = \frac{T}{c_r + c_*} + \tfrac{1}{2} \frac{\alpha}{\pi} \qquad (35^*, \text{ equals } 13)$$

and, if T satisfies (34*),

$$\frac{\Delta R}{R} > \left(\tfrac{1}{2} + \frac{c_r - c_*}{2c_r}\right) \frac{\alpha}{\pi} \qquad (36^*, \text{ equals } 14)$$

'Man sieht auch leicht ein, dass durch mechanische Hypothesen eine eigentliche Ersparnis an wissenschaftlichen Gedanken nicht erzielt werden kann. Selbst wenn eine Hypothese vollständig zur Darstellung eines Gebietes von Erscheinungen, z. B. der Wärmeerscheinungen ausreichen würde, hätten wir nur an die Stelle der tatsächlichen Beziehung zwischen mechanischen und Wärmevorgängen die Hypothese gesetzt. Die Zahl der Grundtatsachen wird durch eine ebenso grosse Zahl von Hypothesen ersetzt, was sicherlich kein Gewinn ist. Hat uns eine Hypothese die Erfassung neuer Tatsachen durch Substitution geläufiger Gedanken nach Möglichkeit erleichert, so ist hiermit ihre Leistungsfähigkeit erschöpft. Man gerät auf Abwege, wenn man von derselben mehr Aufklärung erwartet als von den Tatsachen selbst'

Mach, E. 1933 (1883). Die Mechanik historisch-kritisch dargestellt. Leipzig. pp 474-475.

INTRODUCTION TO IV

When dry proteins are exposed to air of high water content they rapidly bind water up to a maximum quantity, which differs for different proteins; usually it amounts to 10 to 20 percent of the weight of the protein. The hydrophilic groups of a protein are chiefly the positively charged groups in lysine and arginine and the negatively charged groups of aspartic and glutamic acid. Hydration may also occur at the hydroxyl groups of serine and threonine or at the amide groups of asparagine and glutamine. Hydrated water is essential to the structure of protein molecules. When proteins are completely dehydrated, the structure disintegrates with loss of biological function: denaturation. In aqueous solutions, proteins bind some of the water molecules very firmly; others are either very loosely bound or form islands between the loops of folded peptide chains. The water molecules may also form bridges between carbonyl and imino groups of adjacent peptide chains.

IV. RAPID REVERSIBLE MACROMORPHOLOGICAL CHANGES IN *SPIRULINA PLATENSIS*

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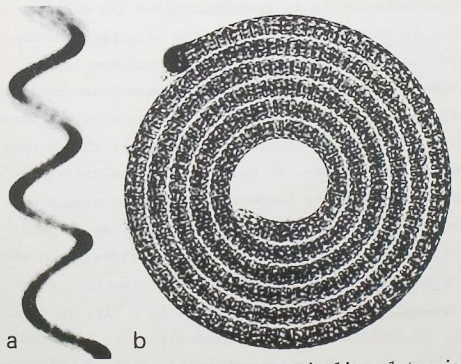
Cyanobacteria belonging to the genus *Spirulina* usually occur as helical filaments. For the species *S. platensis*, the helical parameters have been described (1, 2). When filaments are placed on agar the helical shape ($x=r_o\cos\theta$, $y=r_o\sin\theta$, $z=r_o\theta\cot\beta$) disappears and a true spiral ($r=a\alpha+b$) appears (2). A similar phenomenon of spiralization has been observed with *Nostoc muscorum* (3). For *S. platensis* the whole process takes 1 h or longer, depending on the prevailing humidity which is related to the agar concentration. The helix is always right-handed, and a spiral never occurs at both ends of the filaments at the same time, suggesting that there is a head-and-tail positioning involved. This is probably due to the fact that a helix does not have mirror-image symmetry.

If a spiral (see Fig. 1b) is rewetted, by slowly moving a drop of water placed on the agar surface to the spiral, it snaps back to a helix (see Fig. 1a) instantaneously. When a saturated NaCl solution is used, this reverse process takes seconds, while saturated sugar solutions do not influence the time of recoiling.



Fig, 1. (a) Helical filaments of *Spirulina platensis* (×3000), (b) a spiral occurring when *Spirulina* filaments are placed on agar (×5000)

As has been found in earlier experiments, *S. platensis* has fixed helical parameters (i.e., pitch length and helix diameter) that are dependent on growth temperature. When the organism was grown at 15°C the pitch length was 152 µm and the diameter of the helix 69 µm, while a growth temperature of 30°C induced a pitch length of 113 µm and a diameter of 36 µm (1). These parameters did not alter as a result of the reversible morphological changes described here.

The specific resistance and electrical charge of the surface on which the morphological alterations occur influence the spiralizing process. Agar, which has a negatively charged surface, induces separate filaments to spiralize; gelatin, with a neutral or positively charged surface, induces separate filaments to spiralize; gelatin, with a neutral or positively charged surface, causes filaments to adhere and to spiralize in groups.

From the observations described, it is evident that two different processes are involved in the morphological changes, i.e., a slow one from helix to spiral (a), and a very fast one from spiral to helix (b).

$$\text{Helix} \underset{b}{\overset{a}{\rightleftharpoons}} \text{Spiral}$$

Process (b) seems to depend on the ionic strength and thus on the water activity involved.

It is likely that changes in relative humidity cause these macromorphological changes. On lowering the humidity, the organism tends to minimize the surface area in contact with the liquid-air interface. Most probably, the resulting morphological changes are enabled by dehydration of the oligopeptides in the peptidoglycan. Peptidoglycan constitutes the major part of one of the cell-wall layers (L-II) (4) in cyanobacteria and is responsible for the rigidity and the morphology of the cell. It is assumed that when the oligopeptides are allowed to hydrate, they immediately return to their former conformation.

There seems to be a discrepancy between this phenomenon where conformational changes are brought about very quickly and the fact that filaments have a fixed morphology which, though dependent on growth temperature, does not change upon transferring the cells to another temperature. We believe that the difference in morphology of filaments grown at differing temperatures results from a different hydration state of the oligopeptides in the peptidoglycan. As is generally known, temperature significantly affects hydration of peptides. When an oligopeptide is biosynthesized at a given temperature its conformation, together with that of the peptidoglycan framework into which it is built, is determined by that temperature. The conformation of the peptidoglycan is fixed and temperature changes will not alter it to the extent that the macromorphology of the organism is affected. Dehydration of the oligopeptide will, however,

alter the conformation and thus the morphology of the organism. There is only one possible hydration state and this permits the oligopeptides to return to their original conformation, as their positions between the glycan chains in the peptidoglycan are fixed.

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Antonie van Leeuwenhoek **44** (1978) 321–327

V. A glucan from the cell wall of the cyanobacterium *Spirulina platensis*

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VAN EYKELENBURG, C. 1978. A glucan from the cell wall of the cyanobacterium *Spirulina platensis*. Antonie van Leeuwenhoek **44**: 321–327.

A polysaccharide was isolated from the cell wall of the cyanobacterium *Spirulina platensis*. Hydrolysis of the polysaccharide only yielded glucose. Curie-point pyrolysis mass spectrometry of the polysaccharide resulted in a spectrum very similar to that of β-1,2-glucan. Probably the glucan originates from the fibrillar inner layer of the cell wall.

INTRODUCTION

Spirulina platensis is one of the most promising species among the cyanobacteria to serve as a source of nutritional protein (Soeder, 1976).

In studying the ultrastructure of the cell wall of the cyanobacterium van Eykelenburg (1977) found the cell wall to be four-layered. The innermost (L-I) layer consists of fibrils, the L-II layer is a murein one, the L-III layer is fibrillar and the outermost (L-IV) layer is structured.

Polysaccharides have been detected in cyanobacterial cell walls. Drews and Gollwitzer (1965) described a phenol-water fractionated (lipo)-polysaccharide from the cell wall of *Anacystis nidulans*, which upon hydrolysis yielded mannose, glucose and, probably, galactose and fucose. Höcht, Martin, and Kandler (1965) examined three species of cyanobacteria for polysaccharide content and found only mannose in *Anacystis nidulans*, mannose and glucose in *Phormidium faveolorum* and mannose, glucose, and xylose in *Tolypothrix tenuis*. During heterocyst or spore formation in *Anabaena cylindrica* a glucose-rich polysaccharide-heteropolymer complex was produced which differed from the component of outer cell wall layers of vegetative cells (Dunn and Wolk, 1970). In the vegetative cell wall of this organism a polysaccharide was found consisting of 35% glucose, 50% mannose, 5% galactose, 8% xylose, and 2% fucose. Drews (1973) concluded that murein, lipopolysaccharides, and presumably protein are macromolecular

substances present in cyanobacterial cell walls. There is, however, no literature on homopolyglycans being present in cyanobacterial cell walls.

The present paper describes a polysaccharide isolated from the cell wall of *Spirulina platensis*. A technique appropriate to study biopolymers is Curie-point pyrolysis mass spectrometry and recent developments in this field by Meuzelaar, Kistemaker and Posthumus (1974) and Weyman (1976, 1977) make it possible to investigate polysaccharides and their structure in a fast and reproducible way.

MATERIALS AND METHODS

Cultures. The strain of *Spirulina platensis* originates from Lake Nakuru, Africa (van Eykelenburg, 1977). The cyanobacterium was grown in a modified medium of Ogawa and Terui (1970) at 30 C under continuous fluorescent light (1 mW/cm^2). The cells were harvested after 7 days of incubation. *Agrobacterium tumefaciens* strain LMD 53.15 was cultured in a modified medium according to Hisamatsu et al. (1977) for 5 days at 30 C.

Cell wall preparation. The cells of the filamentous cyanobacterium *Spirulina platensis* were homogenized repeatedly and cell walls were isolated as described by Golecki (1977). The purity of the cell walls was examined by electron microscopy.

Polysaccharide isolation. A polysaccharide was isolated from the cell wall of *Spirulina platensis* as described by Schaefer (1977). A β-1,2-glucan of *Agrobacterium tumefaciens* was isolated by concentrating the culture medium to one-twentieth of its original volume and precipitating the polysaccharide by adding an amount of ethanol (4 C). The latter polysaccharide was purified by repeatedly dissolving it in distilled water followed by precipitation with ethanol (4 C) and centrifugation. After five such treatments the polysaccharide was lyophilized.

Analytical procedures. To determine qualitative composition of the polysaccharide acid hydrolysis of the material was performed in 72% H_2SO_4 (w/w) for 1 h at room temperature; hydrolysis was completed in 2 N H_2SO_4 in a water bath at 100 C for 1 h. Sugars were identified by paper chromatography on Whatman no. 1 paper after neutralizing the sugar solution with $BaCO_3$ and after concentration of the hydrolysate. The solvent system used was butanol-1–pyridine–water (6:4:3). Spots were located using aniline- hydrogen phthalate (Partridge, 1949) or silver nitrate–alkali (Trevelyan, Proctor, and Harrison, 1950).

The polysaccharide isolated from *A. tumefaciens* was subjected to Nakanishi's test (1976) to demonstrate the absence of 1,3-glucan. The polysaccharides were analyzed directly using Curie-point pyrolysis mass spectrometry. This technique involves the use of a pyrolysis chamber, placed in the vacuum of a quadrupole mass spectrometer. Pyrolysis is achieved by Curie-point technique, involving rapid heating of a ferromagnetic wire in a high frequency field. The sample pyrolyzes when the equilibrium temperature is reached and mass spectrometry is directly performed. About 5 μg of sample was applied to the wires as small drops

from a suspension in methanol. The equilibrium temperature of the Fe-Ni wires used in this investigation was 510 C. To avoid excessive fragmentation during ionization the electron-impaction source of the mass spectrometer was operated at 13–15 eV. Therefore, most peaks in the resulting mass spectrum represent molecular ions rather than fragment ions (Weyman, 1976).

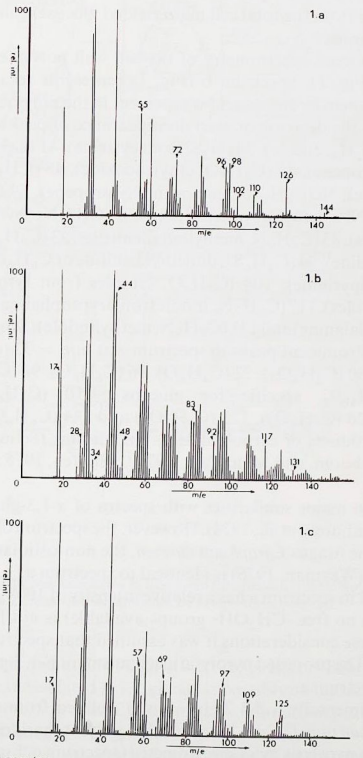

Fig. 1. Pyrolysis mass spectra
a. Polysaccharide isolated from the cell wall of *Spirulina platensis*.
b. Cell wall of *Spirulina platensis*.
c. β-1,2-glucan isolated from the culture medium of Agrobacterium tumefaciens LMD-53.15.

RESULTS

The amount of polysaccharide isolated from the cell wall of *Spirulina platensis* was less than 1% of the total cell mass calculated on a dry weight basis. Paper chromatography of the hydrolysate yielded only glucose. The hydrolysate of the polysaccharides isolated from the total cell mass yielded glucose, galactose, xylose, rhamnose and mannose.

Curie-point pyrolysis mass spectrometry of the cell wall polysaccharide resulted in spectrum a (Fig. 1). Spectrum b (Fig. 1) represents total cell wall material. Hardly any protein or amino acid was present in the polysaccharide as may be concluded from the decrease or even disappearance of peaks m/e = 17 (NH_3, ammonia), 28 (C_2H_4, ethene), 34 (H_2S, from cysteine), 41 (C_2H_3N, ethanenitrile), 42 (C_3H_6, propene), 44 (C_2H_4O, ethyleneoxide), 48 (CH_4S, methanethiol from methionine), 56 (C_4H_8, butene or methylpropene), 59 (C_2H_5NO, acetamide), 67 (C_4H_5N, methylpropenenitrile), 79 (C_5H_5N, pyridine), 81 (C_5H_7N, methylpyrroles), 83 (C_5H_9N, methylbutanenitrile), 92 (C_7H_8, toluene), 93 (C_6H_7N, methylpyridine), 94 ($C_2H_6S_2$, dimethyldisulfide or C_6H_6O, phenol), 107 (C_7H_9N, dimethylpyridine), 108 (C_7H_8O, cresoles from tyrosine), 109 ($C_7H_{11}N$, C_3-alkylpyrroles), 117 (C_8H_7N, indole from tryptophane and phenylacetonitrile from phenylalanine) and 131 (C_9H_9N, methylindole) (Schulten et al., 1973; Schulten, 1975). Prominent peaks in spectrum a at m/e = 32 (CH_4O), 43 (C_2H_3O), 55 (C_3H_3O), 60 ($C_2H_4O_2$), 72 (C_4H_8O), 96 (C_6H_8O), 96 (C_6H_8O), 98 ($C_5H_6O_2$), 102 ($C_5H_{10}O_2$, specific for glucans), 110 ($C_6H_6O_2$), 114 ($C_6H_{10}O_2$, $C_5H_6O_3$), 126 ($C_6H_6O_3$), 128 ($C_6H_8O_3$) and 144 ($C_6H_8O_4$) correspond with pyrolysis products of carbohydrates, viz. glucans (Schulten et al., 1973; Posthumus, Boerboom, and Meuzelaar, 1974; Schulten, 1975; Weyman, 1976, 1977; this paper).

Spectrum a shows no major similarities with spectra of α-1,3-glucan, β-1,4 glucan, or glycogen (Posthumus et al., 1974). However, the spectrum of a polysaccharide isolated from the fungus *Europhium aureum*, the non-ostiolate counterpart of *Ophiostoma* sp. (Weyman, 1976) is identical to spectrum a.

The peak at m/e = 32 in spectrum a has a relative intensity of 100% indicating that a 1,6-glucan (with no free -CH_2OH- groups available) is not likely to be involved. In view of these considerations it was assumed that spectrum a might represent a 1,2-glucan. The proposed pyrolytic mechanism for β-1,2-glucan (Fig. 2) is consistent with spectrum a.

To verify this experimentally a β-1,2-glucan was isolated from the culture medium of *Agrobacterium tumefaciens*. Like the other polysaccharides this β-1,2-glucan was subjected to pyrolysis mass spectrometry (spectrum c, Fig. 1). Spectrum c has the same prominent peaks as spectrum a namely m/e = 32, 43, 55, 60, 70, 72, 81, 82, 84, 85, 86, 96, 97, 98, 102, 110, 112, 113, 114, 126 and 144. However, spectrum c shows some contaminants, namely protein (P), lipid (L) and murein (M) at m/e = 17 (P), 28 (P, L), 42 (P, L) 44 (P), 57 (P, L), 69 (P, M, L), 97 (P), 109 (M) and 125 (M). Protein and amino sugar (109, 125) peaks probably originate from murein. Therefore the isolated β-1,2-glucan is possibly contaminated with lysate products of *A. tumefaciens* cells. Excluding the contaminant peaks, spectrum a is similar to the spectrum of β-1,2-glucan.

Cell walls of *Spirulina platensis* were subjected to NaOH treatment. Electron microscopically it was established that L-I fibrils were affected or even had disappeared depending on the concentration used.

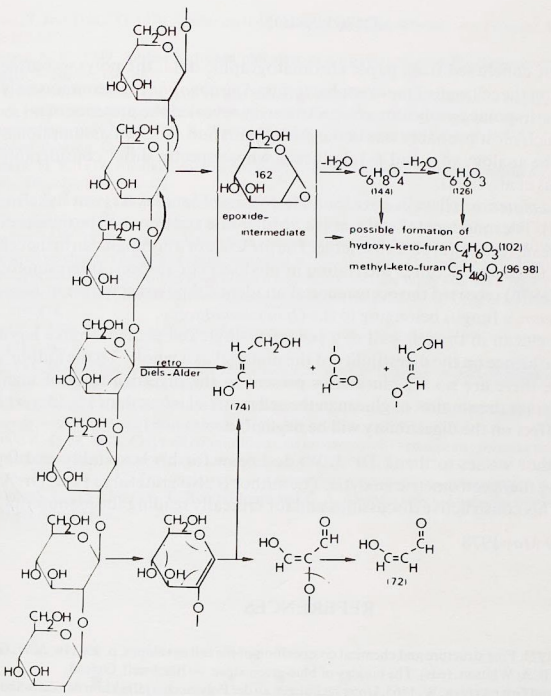

Fig. 2. Proposed pyrolytic mechanism for β-1,2-glucan (Shafizadeh, 1968). Numbers indicated refer to mass numbers in β-1,2-glucan; all substituents are in equatorial position.

DISCUSSION

As can be concluded from paper chromatographic data, the polysaccharide isolated from the cell wall of the cyanobacterium *Spirulina platensis* contains only glucose. Curie-point pyrolysis mass spectrometry revealed the presence of a 1,2-glucan, which most probably was in the β-conformation. The last assumption is made on the analogy of α and β-1,3-glucans whose spectra differ considerably (Posthumus et al., 1974).

Spirulina platensis cell walls were found to consist of four layers (van Eykelenburg, 1977). The innermost (L-1) layer is a fibrillar one and may well be the site of the 1,2-glucan, for both are alkali soluble. The presence of a homoglucan in the cell wall of an Oscillatoriacean is interesting in phylogenetic respect. Interestingly, Weyman (1976) reported the occurrence of an identical polysaccharide in *Europhium aureum*, a fungus belonging to the *Ophiostomataceae*.

A β-1,2-glucan in the cell wall of a potential single cell protein source has a negative influence on the digestibility of the material as a whole. To the author's knowledge there are no 1,2-glucanases present in the digestive tract of man. However, since the amount of glucan in the cell material is less than 1% (dry wt.) the total effect on the digestibility will be negligible.

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INTRODUCTION TO VI

In our research of the effects of temperature and light intensity on growth rate, biomass production, morphology and ultrastructure, we liked to compare as many parameters as possible at one time. Conventional algal and bacterial culture techniques were therefore not appropriate.

In 1956, Halldal and French introduced the principle of growing an alga on a uniformly seeded, large agar block under continuous gradients of light intensity and temperature (see Halldal and French, 1956, 1958). In 1964, Jitts et al. applied the same principle to test tube cultures. Edwards and Van Baalen (1970) embodied features of both systems into a new simplified design (see also Van Baalen and Edwards, 1973). Norland, Heldal and Dagestad (1977) introduced a water bath separated into compartments by acrylic walls. In this water bath, a temperature gradient was set up by maintaining the two extreme compartments at different temperatures. Thermal conduction was facilitated by copper rods running the length of the compartments.

For the present studies a simpler and more reliable apparatus was required. This is described in the next chapter.

Eriksen (1979) and Yarish, Lee and Edwards (1979) have since described improved models of the apparatus designed by Van Baalen and Edwards (1973).

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VI. AN 'ECOBOX' WITH A DISCONTINUOUS TEMPERATURE GRADIENT AND A CONTINUOUS LIGHT INTENSITY GRADIENT

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Summary. An apparatus is described for culturing micro-algae in a discontinuous temperature gradient and a continuous light intensity gradient. The apparatus provides 100 different combinations of these abiotic factors at 1 time. The cross-gradient culture apparatus is called 'ecobox'.

In studies on the cyanobacterium *Spirulina platensis*[1], cultures grown at different temperatures and light intensities were compared. To save time and space and to avoid variations in experimental results due to variable conditions over long time intervals, it was considered appropriate to culture the cyanobacterium at various combinations of temperature and light intensity at one time.

Edwards and Van Baalen[2] described an apparatus for the culture of benthic marine algae under varying regimes of temperature and light intensity. The apparatus described here involves an improvement, especially concerning heat transport and temperature regulation.

Materials and methods. Description of the apparatus (figure 1). A colourless anodized aluminium plate (15 mm thick) with 2 longitudinal borings, through which water of different temperatures is pumped, is used to achieve a tempera-

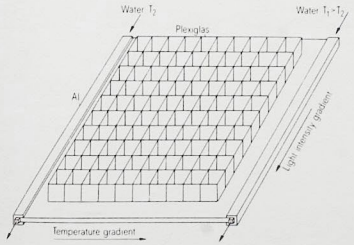

Fig. 1. The ecobox; an aluminium plate with sealed Plexiglas framework.

ture gradient. Aluminium is used because it is light, strong, non-toxic, easily machined, non-magnetic and highly corrosion-resistant. It is anodized in order to obtain a coating highly reflective for visible light and radiant heat; further, the coating soon forms a thin layer of the protective oxide and does not

deteriorate. The thermal conductivity of aluminium ranges from 2.36 W/cm °C at 0°C to 2.40 W/cm °C at 77°C. A Plexiglas framework consisting of 10x10 squares is sealed to the aluminium plate with silicon kit. Length and width of the framework are about 20% smaller than those of the plate to prevent temperature side effects. The actual dimensions of the framework are 40x40x4.0 cm forming 100 compartments with inner dimensions of 3.6x3.6x4.0 cm each. A glass plate is used to cover the framework. The glass plate can be treated with Dri-Film™ SC-87 (Pierce, Rockford, Illinois, USA) to prevent condensation at temperatures higher than the ambient temperature. 2 waterbaths (temperature accuracy, in the ranges used, 0.2°C) with circulation pumps are used to obtain a linear temperature gradient. The light intensity gradient is achieved by fluorescent lamps (Philips TL33, 40 W) suspended above the plate, with arrangements of lowering, raising and moving the lamps backwards and forwards. The 'ecobox' is insulated with polystyrene strips. Temperatures were measured with thermocouples; light intensities were estimated in lux using a Yokogawa luxmeter type 3281.

Results and discussion. Experiments with water only did not reveal any rise in temperature in the compartments due to the fluorescent lamps. Therefore, it was not necessary to cover the framework with an IR. reflecting glass plate. The temperature in any compartment was constant within 0.5°C; that means that there was no temperature range in a single compartment. The temperature constancy is certainly due to the high thermal conductivity of aluminium and the very low thermal conductivity of Plexiglas. Convection of the medium in the compartment resulted in the temperatures obtained. For the apparatus as a whole, it means that a discontinuous linear gradient of discrete temperatures was achieved (figure 2).

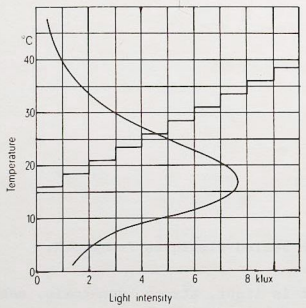

Fig. 2. Topview of the ecobox with the temperature gradient indicated by the scalariform curve and the light intensity gradient given by the smooth curve.

With 2 fluorescent lamps, it was possible to obtain a continuous light intensity gradient from 0.4 to 10.5 klx at the level of the growing cultures.

Figures 2 and 3 show the results of an experiment with *Spirulina platensis* at a continuous light intensity gradient given by the 'smooth' solid curve and at a discontinuous temperature gradient indicated by the scalariform solid curve; from figure 2 it is obvious that the maximum light intensity (7.6 klx) was provided at the 7th row from above and that the temperature increased stepwise from 16 to 38°C from left to right. The 7th row was chosen for maximum light intensity for 2 reasons: in order to obtain a clearly pronounced maximum and distinct minimum in the 1st row. The optimum temperature for the strain tested was 31.0±0.5°C. The maximum light intensity of 7.6 klx did not yield the maximum dry weight/compartment (figure 3). The apparatus can be used in

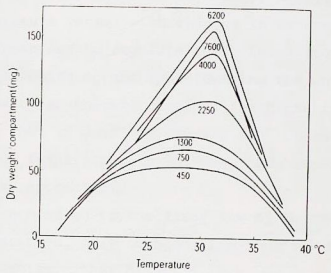

Fig. 3. Dry weight per compartment in relation to temperature; 7 curves with increasing light intensity. Curve 450 lx: 1st row, 750 lx: 2nd row, 1300 lx: 3rd row, 2250 lx: 4th row, 4000 lx: 5th row, 7600 lx: 7th row, 6200 lx: 6th row. Note: the maximum light intensity is not the optimum light intensity as shown by dry weight measurements; the optimum temperature for growth shifts to higher temperatures with increasing light intensity until the optimum is reached.

ecological experiments with photosynthetic microorganisms to study combined effects of temperature and light intensity on growth and yield, as well as on morphology and ultrastructure, and is, therefore, indicated in short as 'ecobox'.

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INTRODUCTION TO VII

For a better understanding of the effect of temperature and light intensity on biological processes, an introduction is presented.

Temperature is the expression of the amount of heat energy in a system and it is an intensive property of this system. Because of their low heat production in relation to mass and their extensive, poorly insulated surface area micro-organisms are essentially poikolothermic or ectothermic, i.e. their temperature varies with changes in external temperature. The inability of micro-organisms to regulate their temperature is shown dramatically by the loss of heat which occurs upon lowering the environmental temperature. The heat generated by exothermic chemical reactions in micro-organisms is dissipated so quickly that it does not cause a significant rise in their temperature. However, when respiration is intense, and heat loss prevented, their temperature may rise appreciably.

According to the first law of thermodynamics, energy cannot be created nor destroyed, only changed from one form to another. Heat energy is acquired by an organism either directly, e.g. by convection and conduction, or indirectly, i.e. by transformation of chemical energy in exothermic metabolic processes such as respiration, and conversion of radiant energy to heat. Heat may also be lost by convection and conduction and by emission of long-wave radiation.

When studying temperature effects, it is necessary to grow the organism in a controlled environment. If the temperature is kept constant the effects of other environmental factors may be studied, and, by precise regulation of the temperature, the interaction between temperature and such factors as light intensity can be investigated.

Temperatures at which *Spirulina platensis* was grown in the present study ranged from 11°C - 40°C. As is known, at 10°C - 12°C, the point where discontinuities are seen in Arrhenius plots, chilling injury takes place disrupting the entire physiolcgy of sensitive organisms. It seems likely that at the critical temperature, the cell membrane undergoes a transition from a normal flexible liquid crystal to a solid gel structure. This alteration may be expected to bring about a contraction of the membrane components causing the formation of holes and increased permeability. The phase transition may also increase the activation energy of membrane-bound enzymes leading to interference with meta-

bolic processes. Reduction in the supply of ATP coupled with increased rigidity of membranes may cause severe injury in life-supporting processes (imbalance in metabolism, accumulation of toxic metabolites, etc.) (see Lyons, 1973).

Spirulina platensis is not thermophilic being unable to survive long periods at temperatures in excess of about 40°C. A high temperature may damage the organism because of its differential effect on various metabolic processes. For example respiration usually has a higher temperature optimum than photosynthesis, and thus a temperature rise may disturb the balance between the two processes (see also Fogg et al., 1973). The temperature at which the two proceed at the same rate is called the temperature compensation point (TCP) (Larcher, 1975, Sutcliffe, 1977). Above this temperature, respiration is more rapid than photosynthesis and the food reserves eventually become exhausted, leading to starvation. This is especially pronounced at low light intensities.

There is evidence that the inability of organisms to grow properly above a critical temperature is sometimes due to failure in the synthesis of an essential metabolite. Such a biochemical lesion is known, for instance, in a mutant of *Neurospora crassa* (Munkres, 1979). Amelunxen and Lins (1968) showed that certain enzymes are more heat-stable when extracted from thermophiles than from mesophiles (see also Alexandrov, 1977). According to Levitt (1972), heat injury is due to protein denaturation at high temperature, leading to aggregation of protein molecules. As hydrophobic bond strength increases with temperature, denaturation must be brought about mainly by breakage of hydrophilic bonds, the strength of which is not so much affected by temperature.

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VII. The ultrastructure of *Spirulina platensis* in relation to temperature and light intensity

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VAN EYKELENBURG, C. 1979. The ultrastructure of *Spirulina platensis* in relation to temperature and light intensity. Antonie van Leeuwenhoek **45**: 369–390

The ultrastructure of *Spirulina platensis*, a cyanobacterium with a helical morphology, has been studied in relation to temperature and light intensity. An increase in temperature gives rise to a more tightly coiled trichome, an increase in sheath material formation and a decrease in cyanophycin (above 17 C) and polyglucan (above 20 C) granule concentration. An increase in light intensity leads to an increase in gas vesicle concentration while the phycobilisome content decreases. Furthermore, cylindrical bodies have been observed with a somewhat different ultrastructure from those found in other species of cyanobacteria. The occurrence, size and ultrastructure of polyhedral bodies, photosynthetic lamellae, mesosomes, lipid deposits and an unknown kidney-shaped inclusion in relation to temperature and light intensity are described.

INTRODUCTION

The cytology of the Cyanobacteriales (blue-green algae, blue-green bacteria, cyanobacteria, (Gibbons and Murray, 1978)), has been reviewed extensively (Carr and Whitton, 1973; Fogg et al., 1973; Wolk, 1973; Shively, 1974 and Stanier and Cohen-Bazire, 1977). Few papers have appeared on the ultrastructure and cytology of *Spirulina* spp. Busson (1971) made some observations on the orientation of the thylakoid membranes. Holmgren, Hostetter and Scholes (1971) described the cross walls in relation to taxonomy. Allen (1972) mentioned the mesosome. Chang and Allen (1974) isolated the rhapidosomes and Wildman and Bowen (1974) reported the phycobilisomes. Further, Titu, Paraschiv and Salageanu (1977) discussed the presence of the glycogen granules while Van Eykelenburg (1977, 1978) investigated the cell wall and its characteristics. Finally, Van Eykelenburg, Fuchs and Schmidt (1980) considered the implications of the in vitro shape of the cross walls of *Spirulina* spp. The present paper describes the

different organelles with reference to occurrence, size and ultrastructure in relation to temperature and light intensity.

To obtain reproducible material, and to save time, a so-called "ecobox" consisting of 10 × 10 identical compartments and provided with a discontinuous temperature gradient and a continuous light intensity gradient as described by Van Eykelenburg (1979) was used.

Materials and Methods

Culture methods

Spirulina platensis (Lake Nakuru, Africa) was cultivated in a basal medium containing: 16.8 g $NaHCO_3$, 0.5 g K_2HPO_4, 2.5 g $NaNO_3$, 1.0 g K_2SO_4, 1.0 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2.2H_2O$, 0.01 g $FeSO_4.7H_2O$ and 0.08 g EDTA per liter tap water. The basal medium was enriched with 1 ml/l of each of the following oligo-element solutions: A: 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.222 g $ZnSO_4.7H_2O$, 0.079 g $CuSO_4.5H_2O$ and 0.015 g MoO_3 per liter distilled water and B: $2.3 \cdot 10^{-2}$ g NH_4VO_3, $9.6 \cdot 10^{-2}$ g $K_2Cr_2(SO_4)_4.24H_2O$, $4.8 \cdot 10^{-2}$ g $NiSO_4.7H_2O$, $1.8 \cdot 10^{-2}$ g $Na_2WO_4.2H_2O$, $4.0 \cdot 10^{-2}$ g $Ti_2(SO_4)_3$ and $4.4 \cdot 10^{-2}$ g $Co(NO_3)_2.6H_2O$ per liter distilled water (Zarrouk, 1966 with modifications). The cells were uniformly inoculated in the 100 compartments of the "ecobox". The incident light intensity from cool-white fluorescent lamps (Philips TL-33, 40 Watt) ranged from 0.4 to 12 klux and the culture temperature from 13.5 to 40.0 °C. All cultures were harvested after seven days unless indicated otherwise.

Preparation for ultra-thin sectioning

The cells were pre-fixed in a mixture of 2.5 % formaldehyde and 2.0 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4 which contained 0.05 M NaCl for two hours at 20 °C. After several washings in buffer the cells were fixed in 1.0 % OsO_4 in the same buffer for two hours at 20 °C, washed again, embedded in Epon resin and sectioned on an LKB-Ultrotome. Sections were stained with uranyl acetate and lead citrate.

Preparation for freeze-etching

A 20 % dimethyl sulfoxide (DMSO) solution in culture medium was used as a cryoprotectant. After a twenty minute adjustment period to DMSO, the cells were quenched in liquid nitrogen. Etching was done at −105 °C at $5 \cdot 10^{-7}$ Torr and lasted two minutes. The platinum–carbon freeze-etch replicas were subsequently cleaned in a 3.5 % potassium dichromate solution in 25 % sulphuric acid and a saturated sodium hypochlorite solution.

Enzyme experiments

The experiments with pronase and trypsine were carried out according to Braun and Rehn (1969).

Results and Discussion

General morphology

The general morphology of *Spirulina* spp. is a helix (Fig. 1) the pitch of which is proportional to the size of the cross-wall overlap (see Van Eykelenburg et al.,

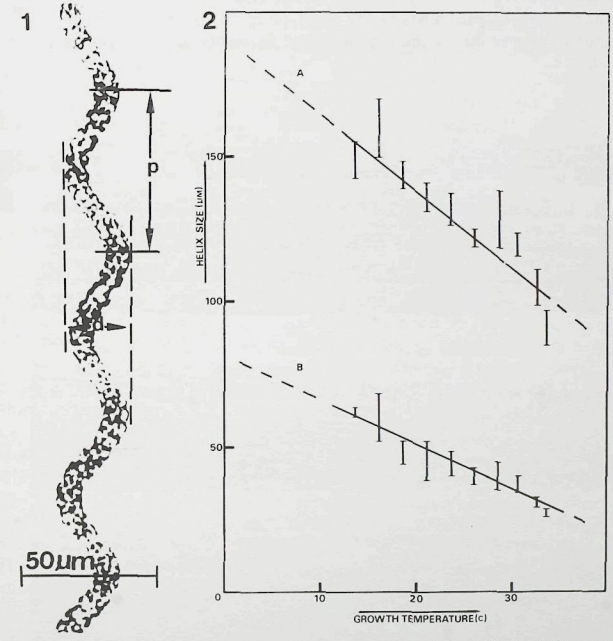


Fig. 1. Part of the helical trichome of *Spirulina platensis*; where p is the pitch and d the outer diameter of the helix.

Fig. 2. Effect of temperature on the pitch (A; correlation coefficient: −0.92) and the outer diameter (B; correlation coefficient: −0.97) of the helix of *Spirulina platensis*.

1980). Light intensity affects neither size of the pitch of the helix nor its diameter, while temperature influences both features (Fig. 2). From figure 2 it is evident that the pitch and diameter of the helix decrease with increasing temperature, i.e. the helix becomes more tightly coiled with increasing temperature.

Apparently something is changing in the rigid, shape-dependent, peptidoglycan layer of the cell wall. This change could result from a rearrangement of the polysaccharide chains or widening of this particular layer. However, there is no significant change in thickness of the peptidoglycan layer in *Spirulina platensis* grown at temperatures ranging from 13.5 to 38.5 C. The theoretical significance of the change in size of the helix will be discussed in a separate paper. With increasing temperature there is a tendency for the trichome to become shorter. At low temperatures, filaments have been observed up to 20 mm whereas at high temperatures (above 30 C) the length of the filaments never exceeds a few millimeters.

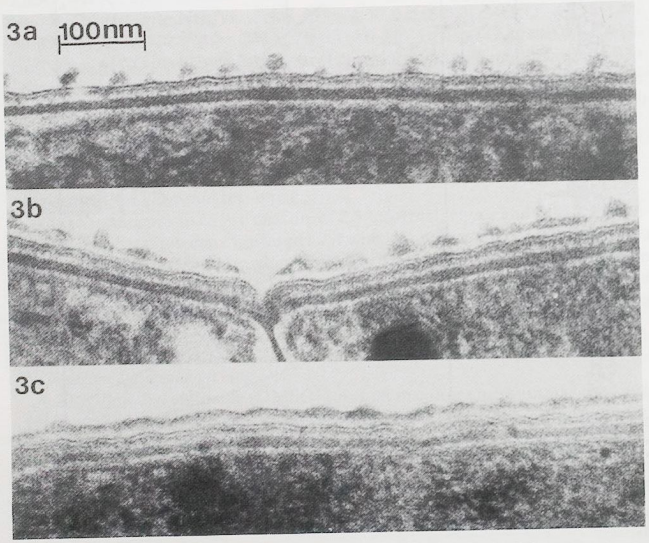


Fig. 3a. Cell wall and sheath of *Spirulina platensis* grown at 16.5 C; note the blebs.
Fig. 3b. The same organism grown at 25.0 C.
Fig. 3c. The organism grown at 37.0 C.

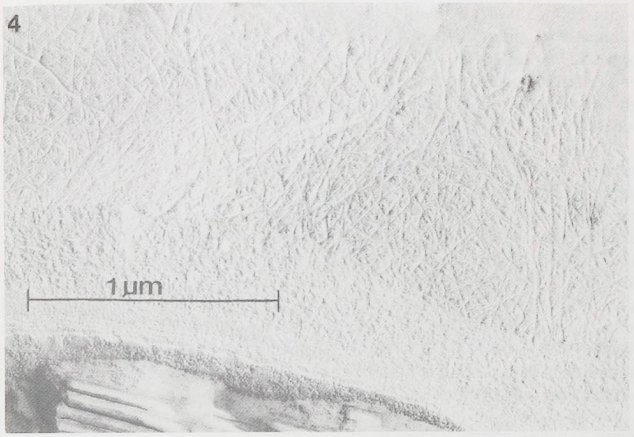


Fig. 4. Freeze-etch replica of *Spirulina platensis* grown at 37.0 C at 5 klux for 14 days, showing the fibrillar layer emanating from a continuous layer outside the cell.

The cell envelope

The cell wall of *Spirulina platensis* has been described by Van Eykelenburg (1977). No ultrastructural differences could be observed in ultra-thin sections of the cell wall of this cyanobacterium grown at different temperatures and different light intensities. It is known that temperature has an effect on the lipid composition of membranes of micro-organisms (Amelunxen and Murdock, 1978).

Cells grown at 16.5 C (Fig. 3a) show periodic "blebs" at the cell surface, most probably emanating from the pit-like pores in the cell surroundings (Brown and Bisalputra, 1969; Lamont, 1969*a*; Butler and Allsopp, 1972; Lang, 1977). These "blebs" (about 18 nm in diameter) are regularly distributed with about 35 nm interspaces. The pit-like pores could provide an avenue for diffusion of sheath precursors, in this case the "blebs" (Lamont, 1969*a*; Halfen and Castenholz, 1971; Lang, 1977). The sheath of cells grown at 25.0 C (Fig. 3*b*) shows a more continuous appearance while cells grown at 37.0 C have a continuous sheath of about 18 nm thick (Fig. 3c). Apparently at higher growth temperatures more sheath material is formed as a result of higher cellular activity and as a form of protection.

In freeze-etched material from cells grown at 37.0 C, 5 klux for 14 days, a fibrilllar (slime) layer can be seen (Fig. 4). This layer originates from a more continuous layer of about 340 nm thick and has itself a diameter of about 800 nm.

A similar layer could not be detected with cells grown at lower temperatures. In ultra-thin sections it was not possible to identify this particular layer. The smallest fibril diameter which could be measured was about 3 nm. Jost (1965), Leak (1967), Lamont (1969*b*), Tuffery (1969) and Wolk (1973), among others, described such a layer in different species of cyanobacteria. Halfen and Castenholz (1971) discussed views on the role of the sheath material in the gliding motion of these cyanobacteria.

Cyanophycin granules

Cyanophycin granules (structured or reserve granules) are normally located in the periphery of the cell, and are arranged along the cross walls, sometimes in constant numbers. They disappear upon cultivation in the dark (Fuhs, 1973). Fuhs (1973) also quotes Schussnig (1953) who states that in the genus *Spirulina* cyanophycin is generally not found.

Shively (1974) described granules without a limiting membrane and of variable size and shape with diameters greater than 500 nm in older cells (*cf.* Lang, Simon and Wolk, 1972). Simon (1971) isolated the granules from *Anabaena cylindrica* and found them to consist of 25000–100000 dalton molecular weight polypeptides containing only arginine and aspartic acid, in a 1:1 molar ratio.

In contrast to the observations of Schussnig (1953) we found *Spirulina platensis* to contain cyanophycin granules. They were observed throughout the temperature range and the light intensity gradient. They were abundant at temperatures up to 17.0 C (Fig. 5a) with low (0.5 klux) light intensities, but somewhat less profuse at the same temperatures and high light intensities (up to 12 klux). At temperatures higher than 17.0 C they appeared regularly in the cells irrespectively of the light intensity applied but then they were less readily stained and not abundant.

At temperatures below 17.0 C the granules can be as large as 2400 nm in diameter (Fig. 5b) while at higher temperatures the largest granule found was 630 nm in diameter. Figure 5c shows freeze-etched cyanophycin granules.

The high concentration of cyanophycin found at low temperatures is consistent with the view that it is a cellular nitrogen reserve material (Simon, 1973). During optimal growth, amino acids are incorporated into normal cellular proteins, but at low growth rates (caused by low temperature) nitrogen is stored in the cyanophycin polypeptide.

Polyglucan granules

Polyglucan granules (polyglucoside granules, glycogen granules or α-granules) are polymers of glucose (Orpin, 1973; Elbein and Mitchell, 1973; Shively, 1974). Orpin (1973) among others, found the polymer to be highly branched, of high molecular weight, and resembling either glycogen or amylopectin. In cyanobacteria, the granules generally appear as rods of about 30 nm in diameter and of different length. Jost (1965) described those of *Oscillatoria rubescens* as composed of 7.0 nm discs with a central pore. Priess (1969) and Stanier (1975) described their

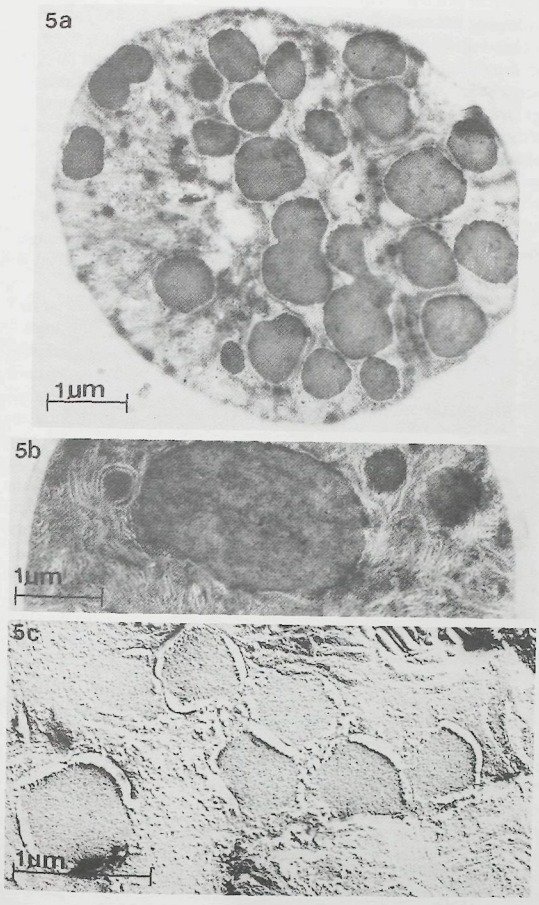


Fig. 5a. Cyanophycin granules, comprising up to 18 % of the cell volume in a cell grown at 15.5 C.
Fig. 5b. A cyanophycin granule with a longest diameter of 2400 nm.
Fig. 5c. A freeze-etch replica of cyanophycin granules.

synthesis by the following reactions:

ATP + α-glucose-1-P ⇌ A(U)DP-glucose + PP_i

ADP-glucose + α-1,4-glucan ⇌ ADP + α-1,4-glucosylglucan,

while Pritchard, Beauclerk and Smith (1975) claimed that cyanobacteria metabolize carbohydrates via the oxidative pentose phosphate cycle. Lehman and Wöber (1978) ascribed the breakdown of the glycogen in the dark to the action of at least three enzymes.

The polyglucan granules are located in the space between the thylakoids (Ris and Singh, 1961). Titu et al. (1977) described the granules in *Spirulina platensis*

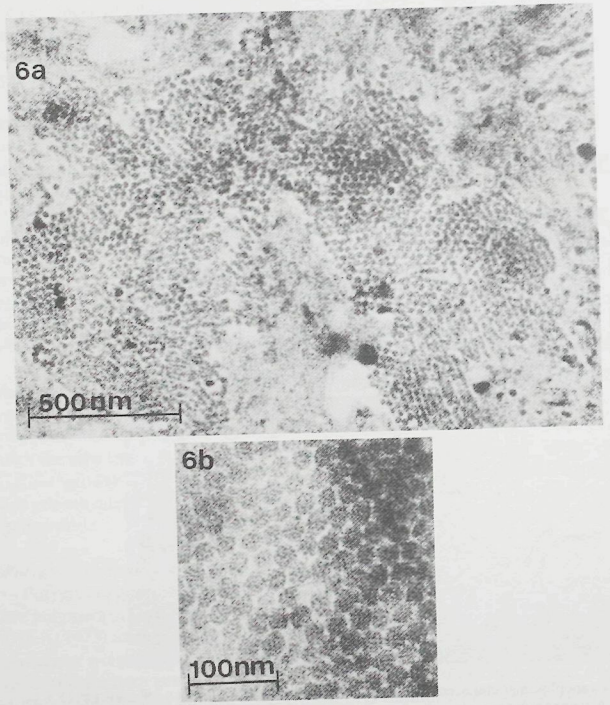


Fig. 6a. Polyglucan granules in cells grown at temperatures between 17.0 °C and 20.0 °C.
Fig. 6b. Polyglucan granules in hexagonal configuration.

(Nordst.) Geitl. as 25 nm wide rods, and up to 400 nm in length. In *Spirulina platensis* (Lake Nakuru) they appeared throughout the temperature and light intensity gradients used, but abundantly at temperatures between 17.0 and 20.0 °C (Fig. 6a near the cross wall), the diameter being about 25 nm and the length ranging to 450 nm. The rods are laid down in a hexagonal configuration (Fig. 6b).

As stated before, at temperatures up to 17.0 °C the cyanophycin granules were abundant in the cells; above this temperature they were abruptly replaced by polyglucan granules, the concentration of which decreased rapidly at temperatures higher than 20.0 °C. The metabolism apparently changes quite suddenly from nitrogen storage to carbohydrate storage.

The concentration of the polyglucan granules decreased with increasing light intensity. This has previously been reported by Giesy (1964) for *Oscillatoria chalybia*.

Cylindrical bodies

Pankratz and Bowen (1963) and Van Baalen and Brown (1969) reported cylindrical bodies in *Symploca muscorum* and *Trichodesmium erythraeum*, respectively. Wildman and Bowen (1974) also described a cylindrical body in *Symploca muscorum* when studying phycobilisomes. The bodies are composed of two concentric cylindrical zones of greater affinity for osmic acid than the nearby cytoplasm. They are seen singly or in clusters near the cell wall, and range from 100 to 140 nm in diameter and up to 1000 nm in length. Their function is not known and the possibility exists that they are intracellular parasites or symbionts (Pankratz and Bowen, 1963). Van Baalen and Brown (1969) gave a schematic representation of the cylindrical body in *Trichodesmium erythraeum*.

In *Spirulina platensis*, cylindrical bodies appeared throughout the temperature and light intensity gradients but were slightly more abundant at about 25 °C (Fig. 7a). They were found in the nucleoplasmic region, the diameter being constant (300 nm) but the length more variable (up to 1700 nm, Fig. 7b). In contrast to the observations of Van Baalen and Brown (1969), cylindrical bodies in *Spirulina platensis* were not found in close relation to polyhedral bodies. According to the latter authors, the bodies may be the site of phycobilisome biosynthesis because of the resemblance of the double lamellar structure of the two concentric membranes. Cylindrical bodies in *Spirulina platensis*, in contrast, have only one double lamellar membrane and an apparently "massive" core. Moreover, the bodies are surrounded by a rather osmiophobic region. (Fig. 7b). If this difference should be due to a different fixation technique (Van Baalen and Brown used also acrolein as a pre-fixative), then the two concentric double lamellar membranes might be of different chemical composition which is unlikely, because the structures are otherwise similar and therefore it is unlikely that different organelles are in question.

Some previously unreported features were observed c.q. a boomerang-shaped body (Fig. 7c, note that this particular cell is actively dividing by cross wall formation) and a spiral-shaped body (Fig. 7d).

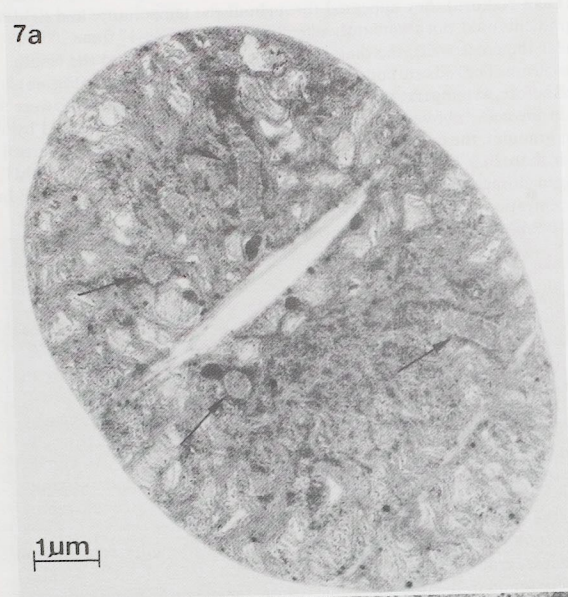


Fig. 7a. Cylindrical bodies in *Spirulina platensis* grown at 25.0 C.
Fig. 7b. A cylindrical body with one double lamellar membrane and a "massive" core. Note the osmiophobic outer region.

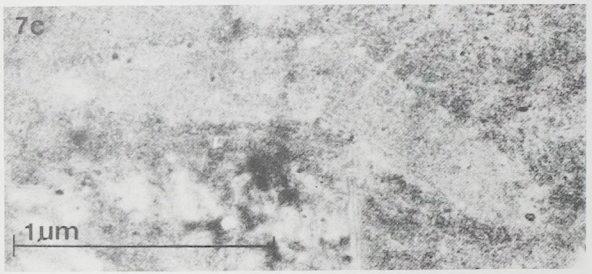
Fig. 7c. A cylindrical body in an actively dividing cell, as appears from the cross wall invagination. This organelle has a boomerang-like structure.
Fig. 7d. A top view of a cylindrical body with a spiral-like appearance.

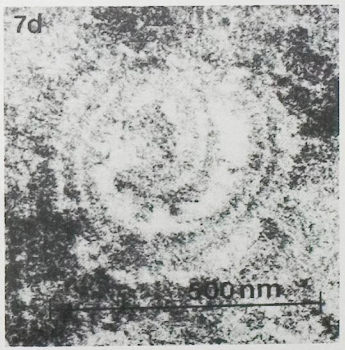
Polyhedral bodies

Polyhedral bodies (carboxysomes) are cytoplasmic inclusions up to 500 nm in diameter which have a distinct polygonal profile. They are commonly found in various cyanobacteria (Pankratz and Bowen, 1963; Fogg et al., 1973; Shively, 1974). The bodies have a granular substructure and are located in the nucleoplasmic region of the cell; they appear to be bounded by a non-unit membrane 3 to 4 nm thick (Shively, 1974).

Codd and Steward (1976) established in them the presence of ribulose-1,5-diphosphate carboxylase (RUBPCase); they emphasize the possible storage function of the bodies.

In *Spirulina platensis* these bodies were found irrespective of temperature or light intensity (Fig. 8a). Figure 8b shows the granular substructure.





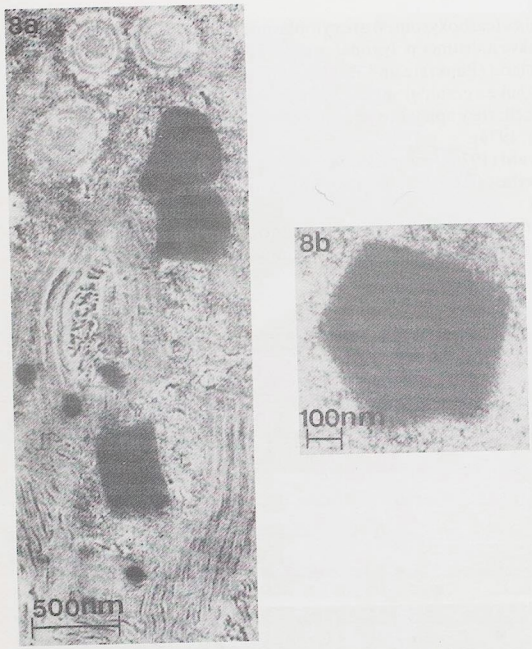


Fig. 8a. Polyhedral bodies (carboxysomes) with a polygonal profile. The bodies are densely stained. Note the three cylindrical bodies at the top.
Fig. 8b. A polyhedral body showing a granular substructure.

Gas vesicles

The ultrastructure, chemical composition and function of gas vesicles have been extensively reviewed, most recently by Walsby (1978). A gas vacuole consists of an array of substructures referred to as gas vesicles (Shively, 1974). These vesicles are hollow cylinders with conical ends. their diameter and length vary from 65 to 115 nm and 200 to 1,200 nm, respectively. The vesicle is surrounded by a membrane consisting solely of a coil of protein molecules resulting in an image of striated ribs with a periodicity of 4.0 to 5.0 nm.

In *Spirulina platensis*, the vesicles were generally found to have a diameter of 65 nm and a length up to 1 000 nm, the periodicity of the ribs being 4.0 nm (Fig. 9a).

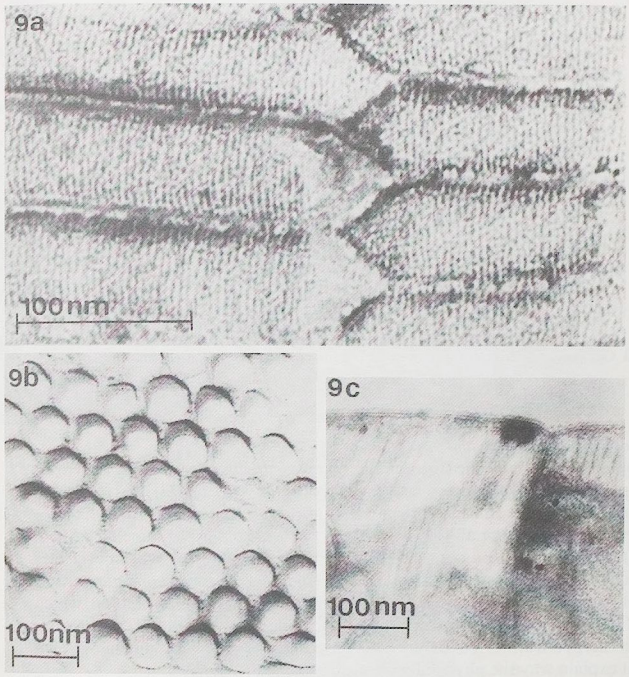


Fig. 9a. Gas vesicles in freeze-etch replica. The striated ribs have a periodicity of 4.0 nm. The vesicles are densely packed, note the conical ends in this respect.
Fig. 9b. Upper view of the gas vesicles in freeze-etch preparation. The vesicles are hexagonally packed.
Fig. 9c. Gas vesicles in ultra-thin section.

Figure 9b shows a top view of the gas vesicles from which it is clear that they are hexagonally packed. In ultra-thin sections, gas vesicles were not easily found (Fig. 9c) because upon centrifugation of the cell material most of the vesicles collapsed and flattened as illustrated in figure 9d, showing a freeze-etched replica of collapsed vesicles. Walsby (1978) points out that the absence of gas vesicle from prokaryotes occupying terrestrial habitats which are subject to high insolation, argues against their being efficient light shields. The only function which is generally accepted is their possibility of buoyancy.

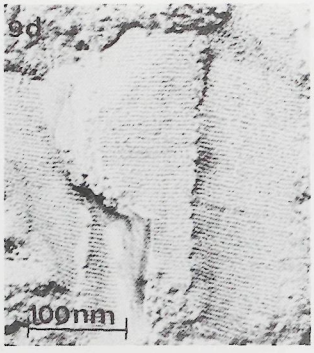


Fig. 9d. Gas vesicles after centrifugation of the cell material. The vesicles are flattened.

Photosynthetic lamellae and phycobilisomes

Photosynthetic lamellae (forming thylakoids) are closed discs and appear as two parallel lines following osmic acid fixation (Wolk, 1973) (for *Spirulina platensis*, see figures 10a and 10b). All, or almost all, cellular chlorophyll (chlorophyll *a*) and many or all the carotenoids are localized in the lamellae (Allen, 1968). In contrast to Busson (1971), no structural uniformity whatsoever was found in the orientation of the photosynthetic lamellae in *Spirulina platensis*.

Attached to the lamellae are the phycobilisomes (Gantt and Conti, 1966) which function as light-harvesting antennae (Gantt and Lipschultz, 1977). The excitation energy is transferred to chlorophyll *a* in the photosynthetic lamellae; which might explain why the phycobilisomes are attached to them.

The phycobilisome are high molecular aggregates of phycocyanin, *allo*-phycocyanin and phycoerythrin (with absorbance peaks at 615 nm, 650 nm and 565 nm, respectively). The overall size of the inclusions is 35 to 50 nm (Shively, 1974). Edwards and Gantt (1971) made a schematic three-dimensional representation of the phycobilisome ultrastructure in relation to the photosynthetic lamellae. They described them as rods, being 35 nm in diameter, consisting of closely packed heptamers of smaller rods, each of which is a stack of dimeric discs. Each disc of a dimer is about 3.0 to 3.5 nm thick with a diameter of about 10.5 to 12.5 nm (Wolk, 1973).

In *Spirulina platensis*, the phycobilisomes, each about 22 nm in diameter, appeared in close relation to the photosynthetic lamellae and frequently in pairs (Fig. 11). The distance between two pairs was about 41 nm while the photo-

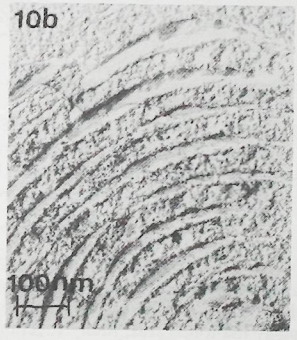


Fig. 10a. The photosynthetic lamellae; note that they are closed discs. On the lamellae no phycobilosomes are present probably because these cells have been treated with trypsin.
Fig. 10b. Part of a thylakoid in freeze-etch replica.

synthetic lamellae were spaced about 56 nm apart. From these observations, which were made on cells grown at 38.5 °C at 1.0 klux, it is clear that the phycobilisomes occupy about 4.5 % of the interlamellar space. At higher light intensities they are less abundant.

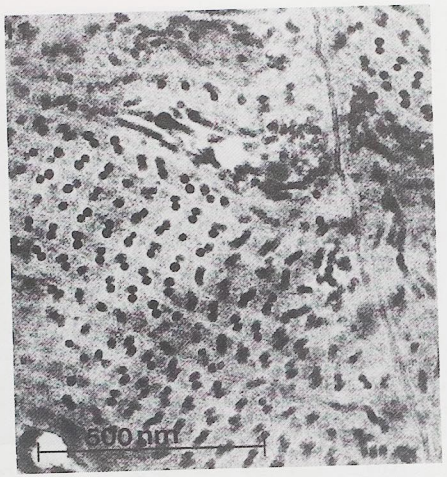


Fig. 11. Phycobilisomes in pairs at the surface of the photosynthetic lamellae.

Cells treated with pronase or trypsin were found to lack phycobilisomes on the photosynthetic lamellae (Fig. 10a, a trypsin-treated cell).

At the cell periphery, short unit membrane-like lamellae (8 nm thick) were found in close connection to phycobilisomes, with arranged structures (Fig. 12a and b). Perhaps the photosynthetic lamellae, although twice as thick as these unit membrane-like structures, are assembled together with the phycobilisomes.

Mesosomes

Mesosomes (Fitz-James, 1960) are unit membrane-like structures which have been observed in gram-positive as well as in gram-negative bacteria. Their structural and biochemical significance has been, and still is, a problem in bacterial ultrastructure and physiology (Salton, 1978). Many functions have been designated to these structures, but none of them has been generally accepted.

Allen (1972) observed mesosomes emanating from the cytoplasmic membrane in *Spirulina* (Berkeley strain 6313) when grown at 35 C about 11 klux.

Enzymes seem to be completely absent from the mesosomes (Salton and Owen, 1976). Salton (1978) detected the unique inhibitory action on the cardiolipid synthetase of the plasma membrane.

In *Spirulina platensis* (Lake Nakuru), mesosomes were frequently detected at lower light intensities (below 3 klux) throughout the temperature range (see Fig.

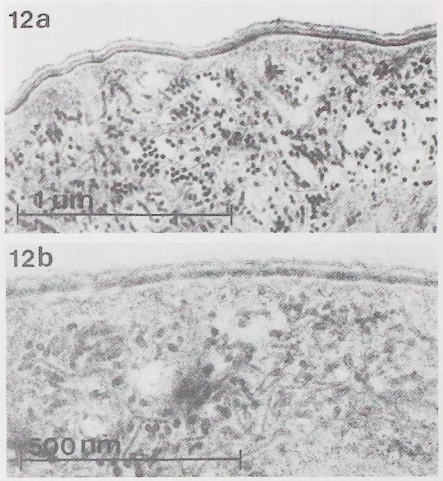


Fig. 12a. Phycobilisomes and unit membrane-like structures at the cell periphery.
Fig. 12b. Unit membrane-like appearance of the structures seen at the cell periphery in close relation to the phycobilisomes.

13a, b and c). The fact that mesosomes can be detected in freeze-etch replicas contradicts the view of Parks, Daneo-Moore and Higgins (1978) that mesosomes are artefacts caused by fixation techniques.

Other inclusions

Several other inclusions have been observed in the cyanobacterial cell such as polyphosphate granules, lipid deposits (which might consist of poly-β-hydroxy butyrate), etc. (Shively, 1974).

In *Spirulina platensis*, these inclusions were also present; figure 14 shows a lipid deposit. An unusual inclusion is seen in figure 15, this kidney-shaped body consists of fourteen lamella-like discs, 7 nm thick each enclosing another. The outer dimensions were 430 nm by 160 nm. This unusual, reniform object might be a mesosome, but the congruent lipophylic body is not consistent with the morphological descriptions. These inclusions were observed several times.

Rhapidosomes as reported by Chang and Allen (1974) in *Spirulina* (Berkeley strain 6313) have not been found in *Spirulina platensis* (Lake Nakuru).

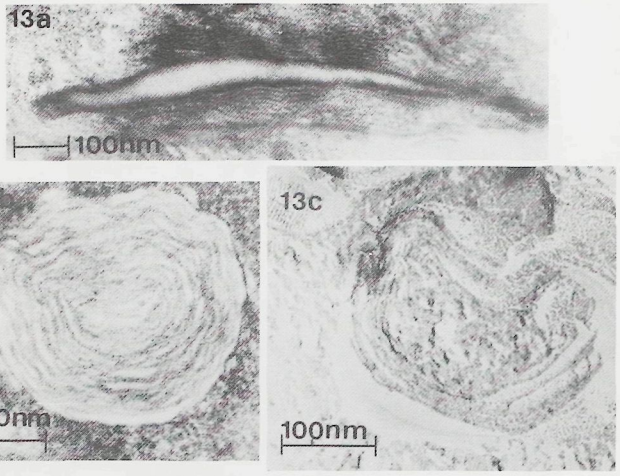


Fig. 13a. A mesosome in *Spirulina platensis* near the cross wall. Note the stretched morphology.
Fig. 13b. A mesosome with an oval morphology.
Fig. 13c. A mesosome in freeze-etch replica.

Concluding Remarks

The ultrastructure of the cyanobacterium *Spirulina platensis* resembles that of other Oscillatoriaceae.

Temperature influences occurrence and size of different organelles. With increasing temperature, the surrounding sheath becomes more pronounced while the pitch and diameter of the trichome helix decrease, indicating a change in the physical properties of the peptidoglycan layer. Up to 17.0 C the most abundant organelle is the cyanophycin granule which at that temperature is abruptly replaced by polyglucan granules. The underlying rapid change in metabolism is probably due to a change in activation energy of one or more of the enzymes involved. Growth temperature has no or little effect on the occurrence or size of cylindrical bodies, polyhedral bodies, gas vesicles, photosynthetic lamellae, phycobilisomes, mesosomes, polyphosphate bodies, lipid deposits or reniform objects. Light intensity has not as pronounced an influence as the growth temperature. An increase in light intensity causes an increase in gas vesicles but a decrease in phycobilisomes. The fact that more phycobilisomes are present at low

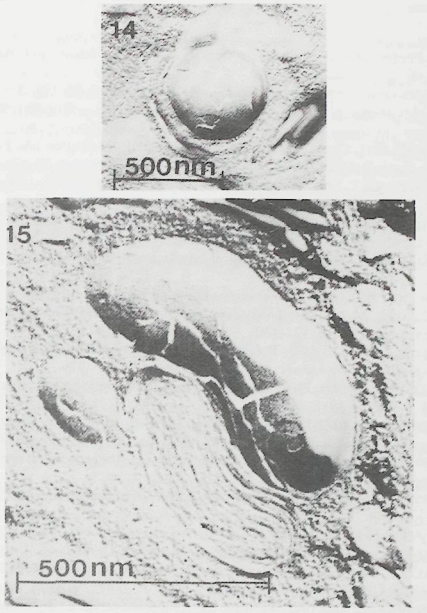


Fig. 14. A lipid deposit in freeze-etch replica. The layered structure indicates the lipophylic nature of the inclusion; furthermore, the global structure is an indication for the presence of lipids.
Fig. 15. An unknown inclusion in *Spirulina platensis* (perhaps a mesosome).

light intensities must be due to their function in the photosynthetic process. There is no effect on the helical morphology of the occurrence and size of the other organelles.

In conclusion, data on the occurrence and, especially, on the size of different organelles in cyanobacteria literature should be considered carefully in relation to growth temperature.

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INTRODUCTION TO VIII

Nitrate is the main source of nitrogen in the plant kingdom and its metabolism represents one of the most fundamental biological processes. The pathway from nitrate to ammonia via nitrite and hydroxylamine has two major metallo-enzymes: nitrate reductase and nitrite reductase, which catalyze in series the reduction of nitrate to nitrite and (hydroxylamine-)ammonia (see Beevers and Hageman, 1969; Hewitt, 1975 and Hewitt and Notton, 1980).

$$NO_3^- \xrightarrow{2e} NO_2^- \xrightarrow{6e} NH_3$$

These key enzymes of the corresponding reductive pathway can undergo rapid and drastic changes in activity as a consequence of reversible chemical modifications by oxidation-reduction reactions in response to the oxidation-reduction potential prevailing in the cell. In other words, the existence of efficient cellular regulatory mechanism allows the crucial enzymes to occur in two interconvertible forms, active or inactive, depending on their redox state (Lorimer et al., 1974 and Losada, 1974).

In cyanobacteria, the two-electron reduction of nitrate to nitrite is catalyzed by the flavo-molybdoprotein nitrate reductase, an enzyme complex of high molecular weight, with two moieties: reduced ferredoxin (a non-heme iron-sulphur protein with strong electron-negative redox potential) which acts as the natural electron donor for the second moiety: a molybdoprotein nitrate reductase (Losada, 1974). In addition, the nitrate reductase complex contains cytochrome-b557 as a functional component.

$$\text{red Fd} \longrightarrow \text{cyt b557} \longrightarrow \text{Mo(terminal } NO_3^-\text{Rase)} \longrightarrow NO_3^-$$

The terminal nitrate reductase moiety, which is remarkably resistant to heating and SH-binding reagents, can be totally and specifically inhibited by metal-chelating compounds, such as azide, carbamylphosphate, cyanate and cyanide. In all these cases, inhibition is of the competitive type with respect to nitrate (Vega et al., 1972).

Molybdenum is essential for the nitrate reducing activity of nitrate reductase (Hewitt, 1975). When molybdate is absent, the cells synthesize the whole enzyme complex, but only the first moiety is operative. Tungstate is a competi-

tive inhibitor of molybdenum in nitrate assimilation, but does not interfere when nitrite or ammonia are the nitrogen sources (Losada, 1974). It is believed that molybdenum binds to a polypeptide thus forming a low molecular co-factor which is common to molybdoproteins such as xanthine dehydrogenase and nitrogenase (Ketchum and Swarin, 1973, Lee et al., 1974).

In bacteria (Vega et al., 1973) and fungi (Lafferty and Garrett, 1974), the reduction step of nitrite to ammonia is catalyzed by the flavo-iron-protein NAD(P)H-nitrite reductase, an enzyme complex which utilizes NADH and NADPH, as the physiological reductant and requires FAD for maximal activity. In the transfer of electrons from NAD(P)H to nitrite catalyzed by the complex, two functional components can be distinguished: an initial NAD(P)H-oxidizing component with a marked specificity for reduced ferredoxins as the natural electron donor in cyanobacteria (Hewitt, 1975) and a terminal nitrite reducing component which contains iron. The first moiety is very sensitive to reagents which react with SH-groups, whereas the second is competitively inhibited with respect to nitrite by cyanide and carbon monoxide. In addition, the enzyme can exist in two interconvertible forms, active or inactive, depending on its redox state (Vega et al., 1973, Lafferty and Garrett, 1974). The functional difference with the nitrate reduction step is the prosthetic group molybdenum in the nitrate step and a heme component in the nitrite step.

The ferredoxin-nitrite reductase as it functions in cyanobacteria and green algae is probably a molecule which consists of two subunits (Ida and Morita, 1973). Murphy et al. (1974) have identified the heme prosthetic group of the enzyme as siroheme, an iron tetrahydroporphyrin with eight carboxylic acid containing side chains.

Ammonia, the end-product of the assimilatory pathway of nitrate reduction, is not only a nutritional repressor of the enzymes of the nitrate reduction system, but is the metabolite that promotes the reversible *in vivo* conversion of the oxidized active form of nitrate reductase into its reduced inactive form. By itself, ammonia does not have any effect on the enzyme *in vitro* (Lorimer et al., 1974, Hewitt, 1975). The latter authors also point out that the nitrate reductase inactivated *in vivo* by ammonia treatment is reduced but can be immediately re-activated to its initial activity by oxidation *in vitro* with ferricyanide. It is obvious that not only ammonia is metabolically in some way connected with the level of nitrate reductase activity, but that its mechanism of action must be related to its indirect reducing effect on the enzyme.

The formation of ammonia from nitrate is accompanied by the stoichiometric formation of ATP. In addition, however, ammonia is an uncoupler of photophosphorylation (Crofts, 1966).

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VIII. ECOPHYSIOLOGICAL STUDIES ON *SPIRULINA PLATENSIS*.

EFFECT OF TEMPERATURE, LIGHT INTENSITY AND NITRATE CONCENTRATION ON GROWTH AND ULTRASTRUCTURE

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Ecophysiological studies on *Spirulina platensis*. Effect of temperature, light intensity and nitrate concentration on growth and ultrastructure. C. van Eykelenburg. 1980. Antonie van Leeuwenhoek 46: 113-127.

The ultrastructure of the cyanobacterium *Spirulina platensis* was studied in relation to temperature, light intensity and nitrate concentration. The organism was able to grow in media supplied with nitrate in concentrations up to 250 mmol/l. High nitrate concentrations increased the yield and growth rate at temperatures above 35°C. Occurrence, distribution and abundance of cyanophycin granules, polyglucan granules, cylindrical bodies, carboxysomes and mesosomes varied widely in relation to the factors studied. At low temperatures (up to 17°C) cyanophycin was the abundant organelle, especially at high nitrate concentrations, whereas in the temperature range 17 - 20°C polyglucan was found in large quantities particularly at low nitrate concentrations. Special attention was paid to the cylindrical bodies, the ultrastructure of which was dependent on temperature. Three types of ultrastructure were distinguished each with several possible shapes.

INTRODUCTION

An abrupt metabolic change at 17 - 20°C was observed during studies on the morphology and ultrastructure of the cyanobacterium *Spirulina platensis* in relation to temperature and light intensity. At temperatures above 17°C, cyanophycin was replaced by polyglucan as the abundant storage material (Van Eykelenburg,1979b).

The utilization of nitrate is based upon an active nitrate-reducing system which catalyzes the reduction of nitrate via nitrite and hydroxylamine to ammonium ions. The activation of this system is mediated by light (Huffaker et al.,

1976; Tischner and Hüttermann, 1978). The enzymes involved, nitrate and nitrite reductases, are molybdoproteins. Reduced ferredoxin is probably the electron donor in the cyanophytes (Hewitt, Notton and Garner, 1979 and the review by Hewitt and Notton, 1980). The amount of nitrogen storage products such as cyanophycin (Shively, 1974) and biliproteins (Stewart and Lex, 1970; Boussiba and Richmond, 1979) depends largely on the activity of the nitrate-reducing system. This activity, in its turn, is dependent on temperature, light intensity and nitrate concentration, the effects of which are reported in the present paper.

MATERIALS AND METHODS

Culture methods. Spirulina platensis (see Van Eykelenburg, 1977) was cultivated in a mineral medium (see Zarrouk, 1966; Van Eykelenburg, 1979b) using an 'ecobox' as described by Van Eykelenburg (1979a). The ecobox used in the present study consisted of 10 x 10 cylindrical compartments. The incident light intensity from cool white fluorescent lamps (Philips TL-33, 40 Watt) ranged from 0.3 to 21 klux, and the culture temperature ranged from 15 to 39°C. All cultures were harvested after seven days unless otherwise indicated. Sodium nitrate (0 - 300 mmol/l) was used as a source of nitrogen.

Preparation for ultra-thin sectioning. The cells were prefixed in a mixture of 2.0% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for two hours at 20°C in 1% OsO_4 dissolved in the same buffer, washed again, dried in increasing concentrations of ethanol and 1,2-propylenoxide, embedded in Epon resin and sectioned on an LKB-Ultrotome. Sections were stained with uranylacetate in 50% ethanol (20 minutes) and lead citrate (5 minutes). The sections were examined using a Philips EM 201 electron microscope.

Nitrate measurements. Nitrate concentrations were measured by a nitrate ion electrode (model 92-07: ORION res Inc.) using a double junction reference electrode with a filling solution of 0.01 M KCl in the outer chamber.

Growth measurements. Growth was measured by determining dry weight. Each day the contents of ten compartments, differing only in temperature, were harvested, washed and air-dried at 70°C for 24 hours.

RESULTS AND DISCUSSION

Growth experiments

In order to establish the range of nitrate concentrations at which *Spirulina platensis* is able to grow, 'ecobox' tests were carried out. Growth occurred at concentrations from 0 - 250 mmol/l. Growth at 0 mmol/l can be explained by the

fact that the inoculum and oligoelement solution B (see Van Eykelenburg, 1979b) contained traces of nitrate (0.1 - 0.2 mmol/l). At concentration below 3 mmol/l, chlorosis occurred within a few days without, however, affecting the morphology of the organism. Nitrate was increasingly toxic at concentrations above 90 mmol/l, an observation which is in agreement with that of Zarrouk (1966). The effect of temperature on growth was measured for three nitrate concentrations (3, 30, 120 mmol/l) at a light intensity of 9 klux (see figure 1a, b and c). From these curves one may conclude that, of the three concentrations tested,

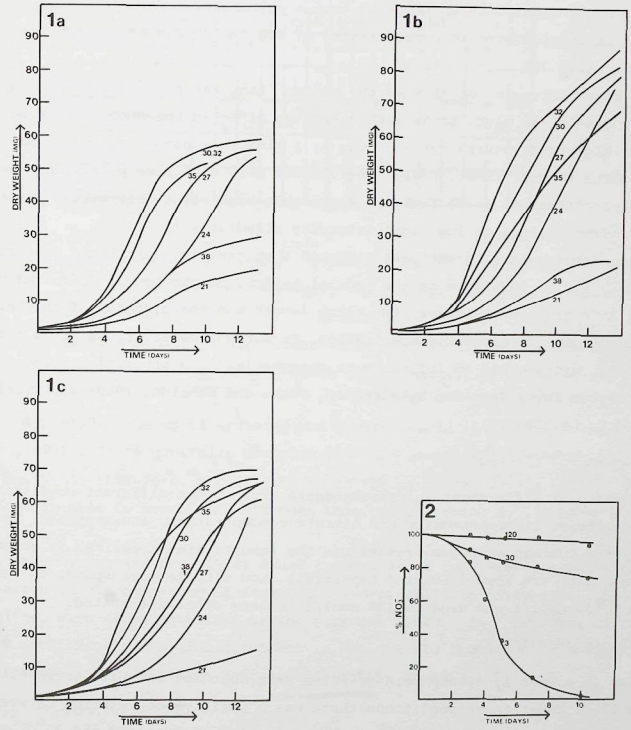

Fig. 1. Growth of *Spirulina platensis* measured at temperatures as indicated and at a light intensity of 9 klux. Nitrate concentration in the culture: a) 3 mmol/l, b) 30 mmol/l, c) 120 mmol/l.

Fig. 2. Nitrate consumption of *S. platensis* grown at 30°C and 9 klux in relation to initial nitrate concentrations.

30 mmol nitrate/l gave higher yields at all temperatures tested, except 35°C and 38°C. At these temperatures both 3 and 120 mmol nitrate/l gave higher yields.

Figure 2 shows nitrate consumption, expressed as percentage of the initial concentrations, for cultures grown at 30°C and 9 klux. Again, three initial nitrate concentrations were tested. At an initial concentration of 3 mmol/l, nitrate decreased within 7 days to 10% and within 10 days to 1% of its original value, whereas at an initial concentration of 30 mmol nitrate/l decreased to about 70% of this value after 10 days. At an initial nitrate concentration of 120 mmol/l the concentration remained between 90 and 95% of this value after 10 days. Chung et al. (1978) reported that a culture of *Arthrospira platensis* grown at an initial nitrate concentration of 4.8 mmol/l ceased to grow when there was still 71% nitrate left.

At a light intensity of ≲1 klux the minimal temperature of growth proved to be lower than at 10 klux. Light intensity also affected the number of lysed cells at high temperatures (38 - 39°C): at 1 klux this number exceeded that at 5 klux three to four times. These observations were made irrespective of the nitrate concentration used. Thus, it seems that high light intensities prevent the cell from lysing, or low light intensity stimulates it.

Light microscopical investigation showed that the various nitrate concentrations used had no influence on the general helical morphology of *Spirulina platensis*. At a given temperature, the pitch length and the diameter of the helix were independent of nitrate concentration. From these observations it is concluded that nitrate had no influence on the chemical and physical nature of the peptidoglycan layer (see Van Eykelenburg, Fuchs and Schmidt, 1980; Van Eykelenburg and Fuchs, 1980).

Ultrastructural observations

Ultrastructural differences in cyanobacteria grown under different conditions of temperature, light intensity and nitrate concentration, are manyfold. These prokaryotes contain numerous organelles. The results are summarized in scheme 1; all organelles are also discussed separately. Low nitrate corresponds to 3 mmol/l, medium to 30 mmol/l and high to 120 mmol/l, unless otherwise stated.

Cyanophycin granules

As shown in scheme 1, cyanophycin granules were abundant in cells grown between 15 and 17°C. Under these conditions there was a positive correlation between nitrate concentration and cyanophycin concentration. Between 17 and 25°C, many cyanophycin granules could be detected electron microscopically but low light intensity was a prerequisite in most cases (see figure 3a and b). In the temperature range 25 - 37°C, near or above the optimum temperature for growth, cyanophycin could not be detected in large quantities. This phenomenon could be due to the high growth rates found at these temperatures. Stanier (1977) stated that cyanophycin only accumulates in the cells when the culture approaches the stationary phase of growth. In the case of *Spirulina platensis*, this conclusion seems valid as the cultures were in or near the stationary growth phase at the relevant temperatures (see fig. 1 and scheme 1). Cyanophycin granules consist

T(°C) →	15_17			17_20			20_25			25_30			30_37		
$\|NO_3^-\|$ →	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
CYANOPHYCIN GRANULES	·	+	+	· I_L	·	·		· I_L	· I_L						
POLYGLUCAN GRANULES				+	+	+	·	·	·	· I_H	· I_H	· I_H			· I_H
CYLINDRICAL BODIES	·	· I_L	· I_L		· I_L	· I_L						·	·	· I_H	
CARBOXYSOMES			· I_H			· I_H			· I_H			· I_H			· I_H
MESOSOMES	·									· I_H		· I_H			

Scheme 1. Relative amount of organelles present in cells of *Spirulina platensis*. + = exuberant, + = abundant, · = present, no symbol = not detectable or present only in minor quantities. I_L = low light intensity (≲2 klux) and I_H = high light intensity (≳6 klux); nitrate concentrations low (L; 3 mmol/l), medium (M; 30 mmol/l) or high (H; 120 mmol/l).

of a polypeptide made up of arginine and aspartic acid in a 1 : 1 molar ratio (Simon, 1971). It is generally accepted that the granules serve as a reserve nitrogen pool; therefore, a cell growing in the presence of high nitrate concentrations is able to store more nitrogen than cells growing in low nitrate concentrations.

De Vasconcelos and Fay (1974) found a decrease in cyanophycin granule concentration in nitrogen-starved *Anabaena cylindrica*. It is interesting, that *Spirulina*, when grown with urea as the nitrogen source, is characterized by a higher methionine and arginine content in the overall protein than when grown with nitrate as the nitrogen source (Al'bitskaya et al., 1974).

Polyglucan granules

Polyglucan granules were abundant at temperatures ranging from 17 - 20°C (fig. 4), especially when the nitrate concentration was low (see scheme 1). Between 20 - 30°C polyglucan granules were present at all nitrate concentrations tested.

At temperatures above 25°C, large quantities were found only in cells grown at light intensities above 6 klux. In the range 30 - 37°C, polyglucan was absent from cells grown in low or medium nitrate concentrations.

Excess carbon dioxide fixation results in the production of polyglucan (Pelroy and Bassham, 1972), which apparently serves as a storage product. Batt and Brown (1974) investigated the effect of inorganic nitrogen on carbohydrate metabolism in *Anabaena cylindrica*. They found that, in the presence of nitrite and nitrate, the level of enzymes participating in the fixation of carbon dioxide increased. Hydroxylamine, an intermediate in the nitrate-reducing chain (Huffaker et al., 1976), has a positive influence on CO_2 fixation (Okabe, Codd and Stewart, 1979). It is evident that nitrate promotes CO_2 fixation and the production of cyanophycin at low temperatures. Nitrogen and carbon storage products were found in *Spirulina* grown under suboptimal temperature conditions (see scheme 1).

Cylindrical bodies

Cylindrical bodies were reported by Pankratz and Bowen (1963) and Wildman and Bowen (1974) in *Symploca muscorum*, by Van Baalen and Brown (1969) in *Trichodesmium erythraeum* and by Van Eykelenburg (1979b) in *Spirulina platensis*.

As indicated in table 1 and figures 5 and 6, the cylindrical body found in *Spirulina platensis* consists of two concentric cylinders individually arranged as unit membranes 21 nm thick, having an electron transparent layer of 5 nm sandwiched between two electron-dense layers each 8 nm thick. The cylinders are separated by an electron-transparent zone 30 nm wide. The body has an electron-dense central core with a diameter of 120 nm. The distance between central core and inner cylinder is 22 nm (see figure 6c). The minimum length of a cylindrical body was 140 nm, while the maximum was 3,400 nm. Three types of bodies were found: a type with a segmented structure which was found in cells grown below 19°C and above 37°C (figure 5c); one with a continuous structure, in cells grown between 19°C and 37°C (figures 5a and b) and a transition type, in cells grown at about 19°C or 37°C (figure 6a). Various differing shapes were found: 70% of the bodies being straight, 20% bent or partly bent (figure 5b) (the maximum curvature radius was 640 nm) and 10% either resembling a boomerang (figure 5c) or being irregular in shape (figure 6b). The maximum number of cylindrical bodies detected in one cell (per section) was six.

Scheme 1 summarizes the conditions at which cylindrical bodies were present in *Spirulina platensis*. At low temperatures (< 20°C) the bodies were abundant at low light intensities (≤2 klux). In the range 20 - 30°C, they were only found at nitrate concentrations above 30 mmol/l. At temperatures near the maximum growth temperature (> 37°C), and at high light intensities (≥ 20 klux), large quantities were found provided the nitrate concentration was not too high (≥ 90 mmol/l). Low nitrate concentrations favoured the occurrence of longer cylinders.

Table 1: Numerical data on cylindrical bodies (in nm)

ORGANISM	*Symploca muscorum*	*Symploca muscorum*	*Trichodesmium erythraeum*	*Spirulina platensis*
NUMBER CB/CELL	10	1	1-3	6
TOTAL DIAMETER	100-140	160	200-300	300±30
LENGTH	1000	no date	800	140-3400
CYLINDER WALL	15	40	80	21
TRANSPARENT ZONE	25	50	80	30
CORE DIAMETER	25	40	20	120
REFERENCES	Pankratz & Bowen (1963)	Wildman & Bowen (1974)+	Van Baalen & Brown (1969)	This paper

+ calculated from figure 24

Cylindrical bodies were more frequently found in cultures at the end of the exponential growth phase. The organelles were located in the nucleoplasm and/or in the region of active cell division (near the diaphragm of a closing cross-wall). These findings suggest that these structures are involved in the process of cellular division. Perhaps the cylinder walls provide a site of membrane-bound enzymes active in this process.

The morphology and number of cylindrical bodies per cell differs in response to different environmental factors. The cyanobacteria in which cylindrical bodies have been reported (table 1) all belong to the *Oscillatoriaceae* and have a benthic habitat. It would be interesting to investigate the presence of these organelles in a variety of cyanobacteria as a possible aid to the classification of these organisms (see Drouet, 1968).

Carboxysomes (polyhedral bodies)

Polyhedral bodies (carboxysomes) were found throughout the temperature range used, but only in considerable quantities at high nitrate concentrations (>90 mmol/l) and at a high light intensity (>9 klux) (fig. 6 and 7a) (scheme 1).

Lowering the nitrate concentration from 120 to 30 mmol/l increased the growth

rate (see fig. 1). It appears that polyhedral body material (ribulose-1,5-diphosphate carboxylase) (RuBPCase) is also present as a storage product (see Shively, 1974). It is interesting to note that Westphal et al. (1979) found DNA in the carboxysomes of *Nitrobacter* spp. In accordance with observations by De Vasconcelos and Fay (1974), polyhedral bodies were not abundant in *Spirulina platensis* cultures when nitrate levels were low. Under nitrogen starvation, these organelles disappeared and polyglucan was produced. RuBPCase is a prerequisite for polyglucan production. If polyglucan production is at a high level and carboxysomes are absent, RuBPCase must be present and active in the cytoplasm. This strongly supports the hypothesis that carboxysomes are storage products.

Okabe et al. (1979) found that hydroxylamine had an effect on ribulose-1,5-diphosphate carboxylase/oxygenase activities. The activity of RuBPCase, which catalyzes the photofixation of CO_2 in phototrophs, was markedly stimulated while the activity of RuBPCase, which catalyzes the light-dependent oxygen uptake (photorespiration) (Jensen and Bahr, 1977) was inhibited. In a study on the cyanobacterium *Agmenellum quadriplicatum* by Stevens and Van Baalen (1973), it was found that the rates of growth and nitrate reduction showed the same response to pH, temperature and light intensity. They also found that CO_2 was required for the reduction of nitrate, but only in trace amounts compared with the requirements for growth.

Mesosomes

Echlin (1964) was the first to report on mesosomes in cyanobacteria, and Allen (1972) detected these inclusions in *Spirulina* (Berkeley strain 6313). Echlin (1964) described the organelles as double membrane structures similar to the photosynthetic lamellae, while Allen (1972) described them as being quite distinct from the thylakoïd unit-membranes. In *Spirulina platensis* these inclusions were found regularly, at low temperatures (< 17°C) with low nitrate concentrations (see scheme 1) and between 25 - 30°C irrespective of nitrate concentration, but only at a high light intensity (10 klux) (see fig. 8a and b). Avakyan et al. (1978) described myelin-like structures in cyanobacteria, which appeared under extreme conditions. These myelin-like structures resemble the inclusion shown in figure 8b. Mesosomes have been considered artefacts caused by fixation techniques (Silva et al., 1976), but this view is doubtful since culture conditions affect their presence and appearance (see also Avakyan et al., 1978 and Van Eykelenburg, 1979b).

CONCLUDING REMARK

A general conclusion which can be drawn from scheme 1 is that, at low temperatures, low light intensity favoured the abundance of organelles while, at high temperatures, high light intensity was favourable. One exception is the abundance of carboxysomes for which a high light intensity is a prerequisite irrespective of growth temperature.

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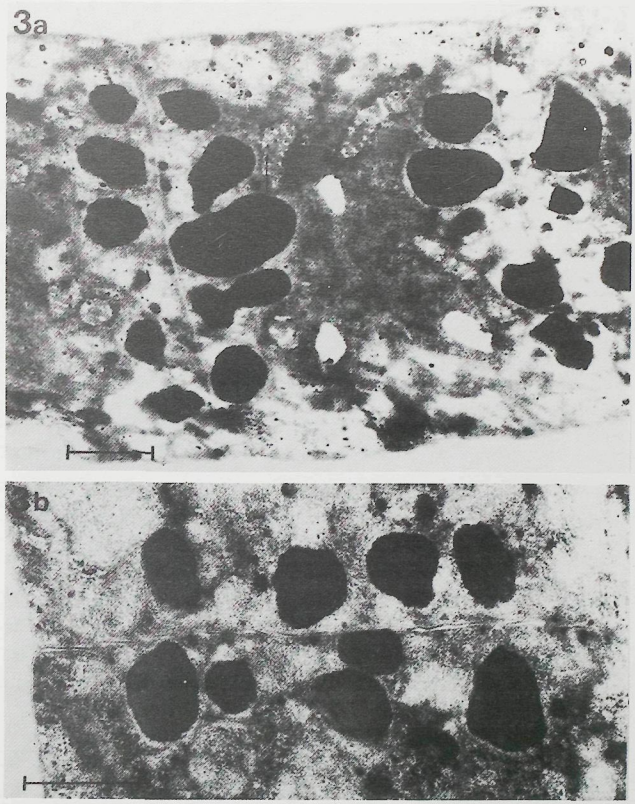


Figure 3. a) Ultra-thin section of a cell grown at 18°C, 0.35 klux and 0.5 mmol/l initial nitrate concentration, showing cyanophycin granules near the cross-wall. Note the irregular morphology of the cyanophycin granules and the segmented cylindrical bodies. b) A cell grown under the same conditions but with 3 mmol nitrate/l. Here, as in all other photographs, the marker represents 1 µm unless otherwise indicated.

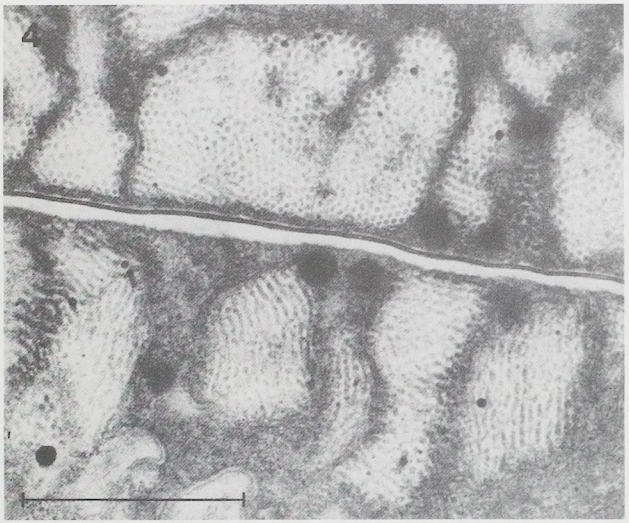
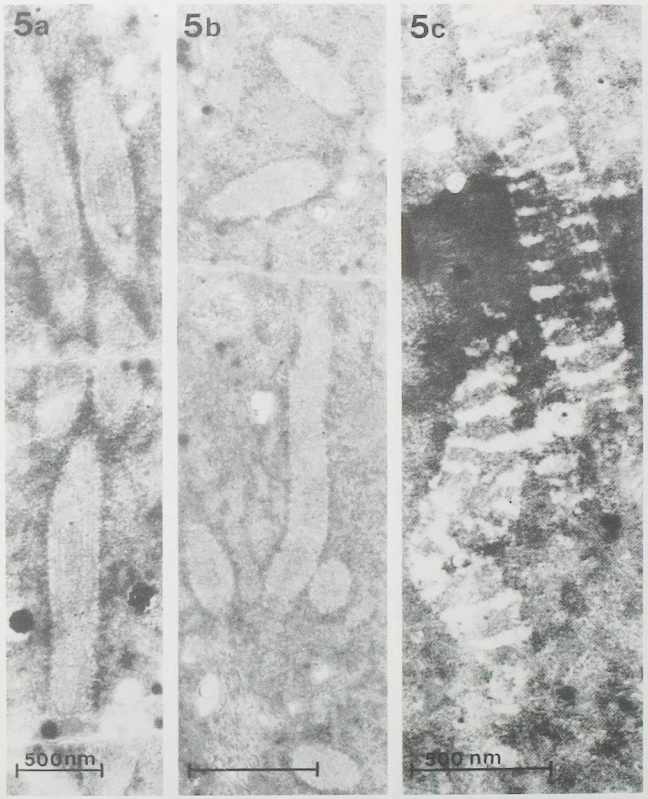
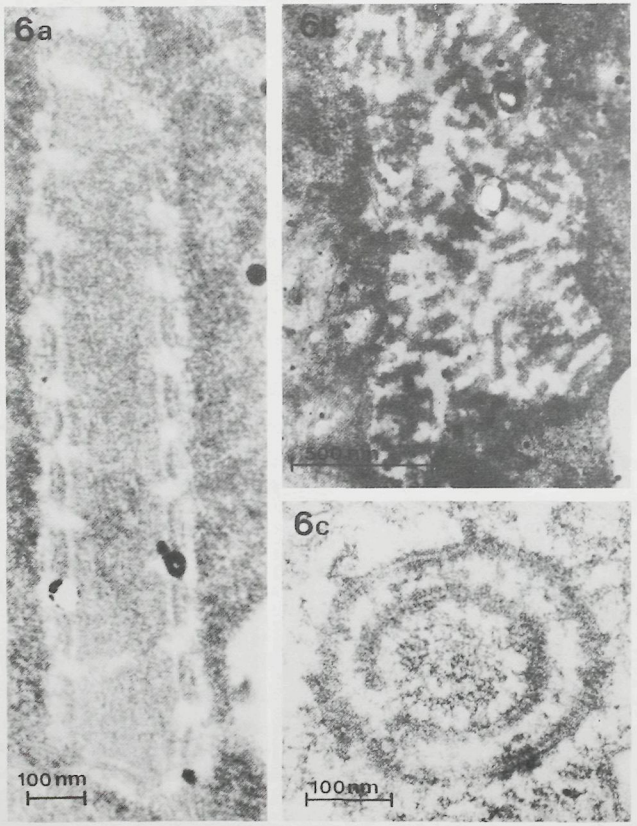


Figure 4. Conglomerates of polyglucan near the cross-wall of a cell grown at 18°C, 0.35 klux and 3 mmol nitrate/l. Note that the conglomerates are enclosed by the double membrane of the photosynthetic lamellae.

Figure 5. Cylindrical bodies. Cells grown at a) 30°C, 21 klux and 30 mmol nitrate/l; 'continuous type', b) 30°C, 21 klux and 3 mmol nitrate/l; 'continuous type', one partly bent, c) 37°C, 9 klux and 30 mmol nitrate/l; 'segmented type', one broken form.





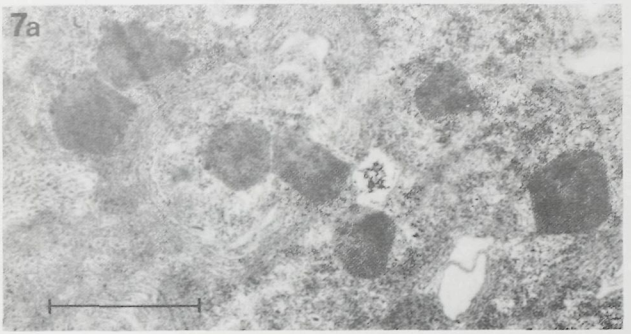


Figure 7. a) Polyhedral bodies with different polygonal profiles in a cell grown at 25°C, 10 klux and 120 mmol nitrate/l. The bodies are normally located near or enclosed within the photosynthetic membranes.

Figure 6. Cylindrical bodies. Cells grown at a) 19°C, 9 klux and 30 mmol nitrate/l; 'transition type', b) 17°C, 2 klux and 30 mmol nitrate/l; 'coagulated segmented type', c) cross-section through a cylindrical body, showing the double cylinder structure.

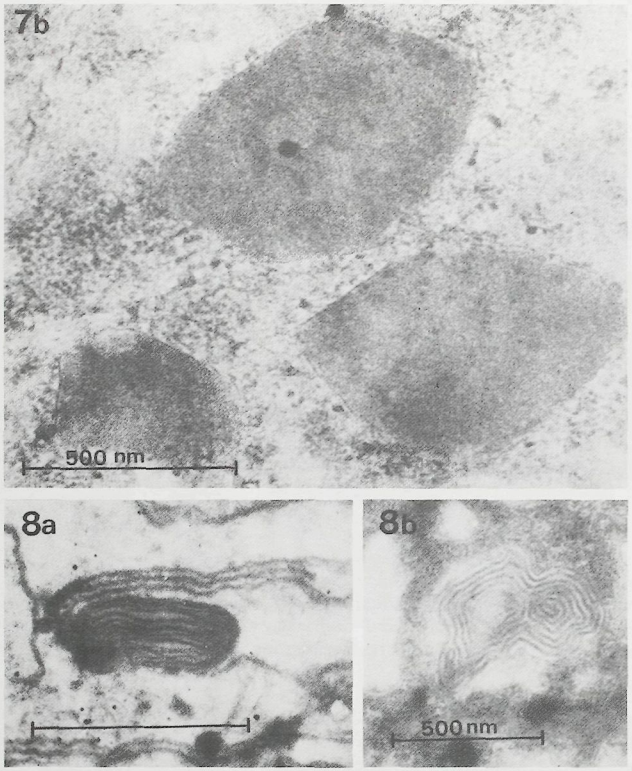


Figure 7. b) Higher magnification of the bodies. Note the single lamella round the bodies.

Figure 8. Two different types of mesosomes. a) A rod-shaped body with enclosed single lamellae. Note the photosynthetic membrane just above. b) An irregular body with single lamellae enclosing others in a cell grown at 28°C, 21 klux and 180 mmol nitrate/l.

SUMMARY AND DISCUSSION

This thesis deals with the morphology and ultrastructure of the cyanobacterium *Spirulina platensis* and the effect of several environmental factors on these features is described.

Spirulina platensis plays a role as a source of nutritional protein and in the future this role may become more important in countries with high luminous flux, temperatures above 25°C and natural water resources. It is most important that the people who consume the cyanobacterium increase the protein and vitamin content of their food. Potential countries are the savannah countries of Africa and South America, India, the Far-east and Mexico.

This thesis can be divided into four parts: the introduction (I), the morphological part (II, III and IV), the chemical part (V) and the ecological part (VI, VII,and VIII).

In the introduction the history and taxonomy of *Spirulina platensis* is discussed. Furthermore, its role as a source of nutritional protein is pointed out. It seems that this species is a perfect example of what are called single-cell protein organisms. It has all the advantages and only a few disadvantages.

The morphology of a micro-organism is dependent on its cell envelope and for cyanobacteria in particular on the peptidoglycan layer in the cell wall. Part 2 is devoted to the morphology and ultrastructure of the cell wall and in particular of the peptidoglycan layer. In order to familiarize the concept of the mathematical helix and its biological counterpart (i.e. *Spirulina platensis*) an introduction is given. In this introduction it becomes clear that a helix ($x=r_o\cos\theta$, $y=r_o\sin\theta$ and $z=r_o\theta\cot\beta$) is often confused with a spiral ($r=a\theta + b$). In II the morphology and ultrastructure of the cell wall of *Spirulina platensis* is described. The cell wall could be resolved into four layers, a structured outer membrane, a layer with proteinaceous fibrils, a peptidoglycan layer and an inner fibrillar layer. Each layer is about 15 nm thick, making the overall cell wall 60 nm thick. The most remarkable phenomenon is the shape of the *in vitro* cross-walls: a so-called undulating saddle. III describes in more detail the *in vitro* cross-wall morphology, which is characterized by a perfect sectorial pleat, its size being related to the magnitude of the trichome pitch. It is evident that shadow preparation causes the artificial *in vitro* shape. It is most probable that the material properties which determine the *in vitro* shape

of the cross-wall are also responsible for the *in vivo* helical shape of the organism as a whole. Four hypotheses are put forward to explain the observed phenomena. It is concluded that the *in vitro* shape must be ascribed to anisotropic shrinkage upon dehydration during shadow preparation of the cross-walls. It is suggested that this might be due to a fundamental difference in the glycan chains of the peptidoglycan running in circumferential direction and the peptide side-chains running in radial direction. For the longitudinal cell wall this means that the glycan chains also run in circumferential direction and the peptide chains run parallel to the trichome axis. Furthermore, *Spirulina* can rapidly and reversibly change its morphology from helix to spiral and back (IV). This phenomenon is ascribed to the *relative* water content (*relative* humidity) of the oligopeptides connecting the glycan chains in peptidoglycan. From III it can be concluded that the glycan chains and the peptide chains are oriented in a preferential direction (qualitative property) and from IV it is clear that the peptides must be able to bind and release water to a certain extent (qualitative *and* quantitative property). These properties must be responsible for the morphological behaviour of this organism and the species related to it.

In part 3 (V) the chemical structure of the fibrillar inner layer of the cell wall is elucidated and it turns out to consist at least in part of 1,2--glucan. For the analysis of this biopolymer pyrolysis mass spectrometry was used.

The ecophysiological studies on *Spirulina platensis* are described in VII and VIII. In order to perform these studies an apparatus ('ecobox') was designed and built in which micro-algae can be cultivated in such a way that the cells can grow in a discontinuous temperature gradient and a continuous light intensity gradient (VI).

In VII the ultrastructure of *Spirulina platensis* is described and its relation to temperature and light intensity. It is evident that the initial ambient temperature is responsible for the magnitude of the helical parameters. The higher the temperature, the more tightly coiled the trichome is. It was found that upon rise of the temperature more sheath material is formed. Furthermore, there is a change in nitrogen/carbon metabolism at 17 - 20°C. Upto 17°C cyanophycin is the abundant cell component whereas from 17 - 20°C polyglucan predominates. Special attention is paid to the cylindrical bodies which have been found in just a few other species of cyanobacteria (VII and VIII). In VIII special attention is paid to the effect of nitrate on growth and ultrastructure. The presence, abundance and ultrastructure of organelles is discussed in relation to the factors studied.

This thesis contributes to the knowledge on the biological helical structure

in general and on that of *Spirulina platensis* in particular. Furthermore, morphological and ultrastructural details are revealed which contribute to the general knowledge on cyanobacteria.

An important question remaining is *why* this cyanobacterium is helically coiled. The answer is at least partly given by the following reasoning. A helical form may provide some selective advantage in natural conditions, for example, in resisting predation or affecting the rate of vertical movement (Booker and Walsby, 1979). Due to this last property, helical forms have a relatively larger ability of survival over straight forms. A helical form will have a greater advantage in waters susceptible to periodic epilimnetic mixing. The advantage is to be found in the effect of low form resistance. Helical forms are hydrodynamically better adapted to waters which are liable to mixing:during calm periods they can move more rapidly to their preferred depth (Booker and Walsby, 1979; Reynolds and Walsby, 1975 and Walsby and Klemer, 1975). Velocities of vertical movements are directly proportional to changes in form resistance (following Stokes' law). Buoyant gas vacuolate filaments which form long coils will move upward more rapidly than filaments which are straight. As we know the extent of coiling in *Spirulina* spp. is dependent on growth temperature. The relation temperature-coiling-vertical movement provides the organism with a device which gives it selective advantage over straight forms.

For large-scale production of *Spirulina* single-cell protein the author recommends to use the strain studied. The morphological behaviour and the growth kinetics of this strain are more suitable than the same parameters published for other *Spirulina* strains now used for large-scale cultivation.

Booker, M.J. and Walsby, A.E. 1979. The relative form resistance of straight and helical blue-green algal filaments. — Br. Phycol. J. 14: 141-150.
Reynolds, C.S. and Walsby, A.E. 1975. Water-blooms. — Biol. Rev. 50: 437-481.
Walsby, A.E. and Klemer, A.R. 1974. The role of gas vacuoles in the microstratification of a population of *Oscillatoria agardhii* var. *isothrix* in Deming Lake, Minnesota. — Arch. Hydrobiol. 74: 375-392.

SAMENVATTING EN DISCUSSIE

De morfologie en de ultrastruktuur van de cyanobakterie *Spirulina platensis* vormen het onderwerp van dit proefschrift. Het hoge eiwitgehalte (tot 72%), het aminozuurpatroon dat vrijwel overeenkomt met de F.A.O.-normen voor menselijke voeding, en het hoge vitaminegehalte maken van dit organisme een potentiële bron van 'single-cell protein' voeding. In de toekomst kan het een belangrijke rol spelen in landen waar een gebrek bestaat aan hoogwaardige eiwitten. Voor een ekonomisch verantwoorde kweekwijze dient men wel gebruik te maken van natuurlijke wateren in landen met veel zon en een hoge gemiddelde temperatuur. Aan deze voorwaarden voldoen de savannelanden van Afrika en Zuid-Amerika, India, het Verre Oosten en Mexico.

Dit proefschrift bevat vier onderdelen: een introduktie (I), een morfologisch gedeelte (II, III en IV), een chemisch gedeelte (V) en een ekologisch gedeelte (VI, VII en VIII).

De geschiedenis van *Spirulina platensis* en de taxonomie ervan worden beschreven in de introduktie. Tevens wordt het belang verklaard van de rol als SCP--organisme. In dit opzicht heeft het organisme vele voordelen en slechts weinig nadelen.

Het tweede deel is gewijd aan de beschrijving van de morfologie en de ultrastruktuur van de celwand en de theoretische implikaties daarvan. De morfologie van een mikro-organisme is afhankelijk van de cel envelop en bij cyanobakteriën meer in het bizonder van de peptidoglycanlaag daarin. Daar *Spirulina* spp. een helische vorm bezitten is voorafgaand aan dit tweede deel een introduktie toegevoegd, waarin het begrip helix mathematisch verklaard wordt en enkele biologische voorbeelden worden gegeven. Tevens wordt gewezen op de verwarring rond de begrippen helix ($x=r_o\cos\theta$, $y=r_o\sin\theta$ en $z=r_o\theta\cot\beta$) en spiraal ($r=a\theta+b$). Uit II blijkt dat de cel envelop van *Spirulina platensis* opgebouwd is uit een buitenmembraan, een fibrillaire eiwitachtige laag, een peptidoglycan laag en een fibrillaire binnenlaag. Elke laag is ongeveer 15 nm dik, waardoor de cel envelop in totaal 60 nm dik is. Een opmerkelijke waarneming is de vorm van de tussenwanden (septa) *in vitro*. Deze vorm is enigszins te vergelijken met een zadel.

In III wordt de morfologie van het septum en het ontstaan ervan meer gedetailleerd behandeld. Het blijkt, dat een septum *in vitro* een overlap bezit in

de vorm van een sektor. De grootte van deze sektor is evenredig met de grootte van de spoed van de helix van het trichoom. Het is duidelijk, dat het proces van schaduwen deze kunstmatige *in vitro* vorm teweegbrengt. Het is zeer aannemelijk, dat de materiaaleigenschappen van het peptidoglycan de *in vitro* vorm van het septum bepalen en mede verantwoordelijk zijn voor de helische vorm van het organisme. Er worden vier hypothesen gesteld die de waarnemingen trachten te verklaren. Volgens de hypothese, die als juiste wordt aangemerkt, moet de *in vitro* vorm worden toegeschreven aan anisotrope krimp als gevolg van dehydratatie gedurende het schaduwproces. Dit zou een gevolg kunnen zijn van de oriëntatie van de glycanketens en de oligopeptide bruggen. De glycanketens moeten dan preferentieel in tangentiale richting georiënteerd zijn en de oligopeptideketens in radiale richting. Dit betekent, voor de longitudinale celwandlaag een tangentiale glycanoriëntatie en een peptideoriëntatie evenwijdig aan de helixas. *Spirulina platensis* kan zeer snel en reversibel overgaan van helix in spiraal (IV). Dit fenomeen wordt toegeschreven aan de *relatieve* hydratatie van de oligopeptiden in het peptidoglycan. Uit III kan gekonkludeerd worden dat de glycan- en peptideketens een voorkeursrichting hebben (kwalitatieve eigenschap) en uit IV is het duidelijk dat de peptiden in staat moeten zijn water meer of minder sterk te binden afhankelijk van de uitwendige omstandigheden (kwalitatieve en kwantitatieve eigenschap). Deze eigenschappen moeten verantwoordelijk zijn voor het morfologisch gedrag van dit organisme en aanverwante soorten.

In deel 3 (V) wordt de chemische struktuur van de fibrillaire binnenlaag van de celwand opgehelderd. Het blijkt, dat deze laag deels uit een 1,2-glucan bestaat. Voor de analyse is gebruik gemaakt van pyrolyse massa spektrometrie.

Het laatste deel van dit proefschrift is gewijd aan een ekofysiologische studie van *Spirulina platensis*. Om dit deel van het onderzoek snel en reproduceerbaar te kunnen uitvoeren is een apparaat ontwikkeld (een 'ecobox'), waarin mikro-algen gekweekt kunnen worden in een diskontinue temperatuurgradiënt en een kontinue lichtintensiteitsgradiënt (VI).

In VII wordt de ultrastruktuur van *Spirulina platensis* beschreven in relatie tot de temperatuur en de lichtintensiteit. Het blijkt, dat de initiële omgevingstemperatuur medeverantwoordelijk is voor de grootte van de helische parameters. Hoe hoger de temperatuur, hoe strakker het trichoom gewonden is. Als de temperatuur toeneemt, wordt er meer slijm geproduceerd, dat zich afzet tegen de buitenwand. Het metabolisme vertoont bij lage temperatuur een duidelijke omslag bij 17 - 20°C. Beneden 17°C is cyanophycine - een co-polymeer van arginine en asparaginezuur - het hoofdbestanddeel van de cel. Tussen 17 - 20°C is polyglucan het voornaamste reserveprodukt. Er wordt speciale aandacht besteed aan de cylindrische lichamen, die slechts in een gering aantal cyanobakteriesoorten

zijn aangetroffen (VII en VIII). In VIII komt de invloed van de nitraatkoncentratie op de ultrastruktuur aan de orde. De aanwezigheid en ultrastruktuur van de diverse organellen in relatie tot de bestudeerde faktoren wordt bediscussieerd.

Deze dissertatie draagt bij tot de kennis van de biologische helix in het algemeen en die van *Spirulina platensis* in het bijzonder. De morfologische en ultrastrukturele gegevens kunnen het begrip omtrent de bouw van cyanobakteriën verhelderen.

Een belangrijke vraag die overblijft is, *waarom* dit organisme een helix vorm heeft. Het antwoord kan gedeeltelijk gegeven worden met de volgende redenering. Een helische vorm kan een selektief voordeel bieden in natuurlijke omstandigheden, bijvoorbeeld als predatieweerstand of omdat het de snelheid van vertikale beweging beïnvloedt (Booker en Walsby, 1979). Een organisme met een helische vorm heeft een relatief grotere overlevingskans boven lineaire vormen. Er is een groter voordeel in wateren, die bloot staan aan epilimnische menging. Het voordeel bestaat uit het effekt van een *lage* vormresistentie. Helische vormen zijn hydrodynamisch beter aangepast in wateren waar laagmenging kan optreden. Gedurende rustiger perioden kan een helisch organisme zich sneller (dan een lineair organisme) naar de gewenste diepte bewegen (Booker en Walsby, 1979; Reynolds en Walsby, 1975 en Walsby en Klemer, 1974). De vertikale bewegingssnelheid is rechtevenredig met veranderingen in vormweerstand. Helische filamenten met gasvakuolen zullen sneller de gewenste diepte bereiken dan lineaire filamenten. Zoals reeds bekend, is de grootte van de spoed afhankelijk van de groeitemperatuur. De relatie temperatuur-heliciteit-vertikale beweging verschaft het organisme een mechanisme, dat selektief voordeel biedt boven lineaire vormen.

Het lijkt aanbeveling te verdienen voor industriële kweek gebruik te maken van de stam die onderwerp is geweest van deze studie. Het morfologische gedrag en de groeikinetiek van deze stam kunnen betere resultaten opleveren bij de kweek voor SCP dan de stammen, die tot nu toe daarvoor gebruikt zijn.

Booker, M.J. en Walsby, A.E. 1979. The relative form resistance of straight and helical blue-green algal filaments. — Br. Phycol. J. 14: 141-150.
Reynolds, C.S. en Walsby, A.E. 1975. Water-blooms. — Biol. Rev. 50: 437-481.
Walsby, A.E. en Klemer, A.R. 1974. The role of gas vacuoles in the microstratification of a population of *Oscillatoria agardhii* var. *isothrix* in Deming Lake, Minnesota. — Arch. Hydrobiol. 74: 375-392.