Nitrogen and Sulfur Starvation of the Cyanobacterium
Synechococcus 6301
An Ultrastructural, Morphometrical, and Biochemical Comparison

G. Wanner, G. Henkelmann, A. Schmidt, and H.-P. Köst
Botanisches Institut, Universität München, Menzinger Straße 67, D-8000 München 19
Z. Naturforsch. 41c, 741–750 (1986); received February 2/May 15, 1986

Cyanobacteria, Synechococcus Phycobiliproteins, Nitrogen Starvation, Sulfur Starvation

The effects of nitrogen and sulfur limitation on various cellular parameters of the cyanobacterium Synechococcus 6301 were studied by electron microscopy, morphometry and biochemical methods. Nitrate and sulfate starvation for 70 h results in a massive glycogen accumulation in parallel to a loss of soluble protein and chlorophyll. Phycobilisomes disappear prior to the degradation of photosynthetic membranes. For sulfate-starved cells, a formation of “storage granules” (poly-ß-hydroxy-butyric acid) is typical which amount up to 10% of the cell volume. The composition of polar lipids is simple: equal parts of C 16:0 and C 16:1 are present under all nutritional conditions; their amount is directly correlated with the total cellular membrane area as determined by morphometry.

Nitrate starved cells regenerate almost completely in structure and composition within 9 h after nitrate supplementation. Regeneration of sulfate starved cells is retarded; in spite of significant synthesis of phycocyanin within 9 h the cells still exhibit marked signs of starvation.

Introduction

The investigation of environmental influences on organisms is an area of increasing importance. In recent years, detailed information has been obtained from unicellular organisms by combining their structural and biochemical data.

Effects of nitrogen deprivation have been analyzed by both ultrastructural and biochemical methods in green algae [1], red algae [2–4] and cyanobacteria [5–7]. Carbon deficiency was investigated using the cyanobacterium Anacystis nidulans (synonymous to Synechococcus) [8], the effect of iron deficiency on Anacystis nidulans is described [9]. In spite of these detailed ultrastructural investigations, quantitative morphometric data are not available for cyanobacteria. This information would allow for a detailed correlation to changing biochemical data as shown for the red algae Rhodella violacea [2] and Porphyridium cruentum [3, 4].

Due to limited knowledge in sulfur metabolism, data on the effects of sulfur deficiency on cyanobacteria [10–12] and red algae [3, 4] are rare.

The effect of nitrogen starvation on cyanobacteria can be summarized as a loss of phycocyanin [5, 6, 13–15], reduction of cyanophycin polypeptide (= “multi-t-arginyl poly-t-aspartic acid”) in Aphanocapsa 608 [10] and reduction of thylakoid membranes and accumulation of polysaccharides in Agmenellum quadruplicatum [6]. Nitrate starvation induces heterocyst formation in Anabaena cylindrica [5] and Mastigocladus laminosus [7]. As reported earlier, sulfate starvation leads to a loss of phycocyanin in Synechococcus 6301 [11].

The following biochemical and morphometrical study was initiated to compare qualitative and quantitative aspects of nitrate and sulfate starvation and regeneration of Synechococcus 6301 (Anacystis nidulans).

Materials and Methods

Synechococcus 6301 cultures (obtained from the algal collection of the Institute Pasteur, Paris) were grown under axenic conditions in BG-11 medium (16). Cells were cultivated in 750 ml Pirson flasks at 30 °C and 6000 lux under aeration. For exponential growth, the medium contained 18 mmol/l NaN03 and 0.3 mmol/l MgSO4. For nitrogen starvation experiments, NaNO3 was omitted, for sulfur starvation, MgSO4 was replaced by MgCl2. For regeneration experiments, the limiting nutrient was injected as a sterile-filibrated solution. Starvation cultures were inoculated each with 2 ml of stock culture; thus a minor carry-over of sulfate and nitrate allowed for initial growth.

Reprint requests to G. Wanner and H.-P. Köst.
Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen 0341-0382/86/0700-0741 $ 01.30/0

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht:
Creative Commons Attribution-NoDerivs 3.0 Germany Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.
**Biochemical determinations**

**Fatty Acids**

*Synechococcus 6301* cultures were centrifuged (40 min, 14,000×g) and broken with a French pressure cell at 70,000–84,000 kPa (2 passages). After centrifugation of the homogenate, the pellet was extracted with chloroform/methanol (2:1) under nitrogen. The solvent was removed in the vacuum and the dry residue dissolved in a minimum amount of chloroform. To obtain fatty acid methyl esters, the solution was refluxed with 3% BF3/CH3OH for 1 h; as internal standard heptadecanoic acid was added.

After extraction with hexane/water the esterified fatty acids were determined by gas/liquid chromatography on a packed column: 10% EGSS-X on Gaschrom P, 100/120 mesh, 180 cm, ID = 6.4 mm, flow rate 30 ml N2/min; detector: FID with a flow rate of H2 and synthetic air 1200 ml/min; with C-19 fatty acid methyl ester as internal standard. Peaks were integrated by an Auto Lab System IV integrator and standardized by computer.

**Chlorophyll and biliproteins**

Chlorophyll was determined spectrophotometrically from the cell homogenate using εM, 621 nm = 3.33×10^5 M^−1 cm^−1 for hexameric C-PC (mol.-Weight of αβ-subunit = 36700) [18]. In *Synechococcus 6301* phycobilisomes the allo-phycocyanin content is 13.8% of total phycobiliprotein content (calculated from [18]). However the contribution of allo-phycocyanin to phycobilisome absorption at 621 nm amounts to only 5% [18].

**Cell titer and protein**

The cell titer was determined using a Neubauer hemocytometer. The content of total soluble protein was determined from the supernatant, according to the Coomassie brilliant blue method [19].

**Carbohydrate storage products**

Carbohydrate storage products were determined after enzymatic hydrolysis to glucose (Boehringer, Mannheim, GOD-Perid-Method, Testset. No. 124036).

Isolation and identification of poly-β-hydroxybutyric acid

An aliquot of the chloroform/methanol extract was used for the qualitative analysis of storage products. The extract was brought to dryness on a rotary evaporator and dried for 16 h in the desiccator (CaCl2). The residue was dissolved in chloroform and mixed with an equal part of ether (dried over sodium wire) and kept for 30 min at −20 °C. The precipitate (storage product) was recovered by centrifugation. The dissolution and precipitation steps were repeated for purification. The purified storage product was colourless.

**Infrared (IR) spectroscopy**

IR spectra were recorded with a Beckman IR-22 instrument after application of the storage product on NaCl plates as a thin film.

**Hydrolysis and 1H NMR spectroscopy**

For further characterization, 5 mg storage product were suspended in 5 ml ethanol. After addition of 5 ml 2 M NaOH, the mixture was refluxed for 1 h under nitrogen in the dark. It was acidified with 1 M H2SO4 and repeatedly extracted with ether. After solvent evaporation in a stream of nitrogen, the hydrolysate was esterified with 2 ml ethereal diazomethane solution. After 30 min, the solvent was removed and a 1H NMR spectrum recorded.

**Gas chromatography**

For routine experiments, polymeric storage product was transesterified with BF3 (20 per cent) in methanol. The formed ester was extracted with n-hexane and analyzed by gas chromatography. For apparatus set-up, see “fatty acids” (time programmed GC, starting temperature 50 °C, rate 4 °C/min). The retention time for the ester was 750 s.

**Soluble phosphate**

The total content of soluble phosphate was determined from the supernatant as molybdenum blue complex [20].

**Electron microscopy and morphometry**

Cells were harvested by centrifugation (14,000×g) and fixed for 90 min at room temperature with 3% glutardialdehyde in fixation buffer (100 mmol/l
sodium cacodylate, 2 mmol/l MgCl₂, pH 6.8). After the cells had been rinsed for several times with the same buffer, they were postfixed for 1 h in 2% osmium tetroxide in fixation buffer. The fixed cells were stained with 1% uranyl magnesium acetate in 50 mmol/l maleate buffer at pH 5.2 for 1 h. They were dehydrated in a graded series of either ethanol or acetone and embedded in Spurr’s low viscosity resin [21].

Thin layers of resin infiltrated cells were polymerized on glass slides for light microscopy and photography. Silvergrey and grey sections were cut with a diamond knife on an LKB Ultrotome II and mounted either on uncoated or Formvar coated copper grids. The sections were poststained with lead citrate. All pictures were taken with a Siemens Elmiskop 101 electron microscope. The magnification was calibrated with a cross-line grating replica. All measurements of micrographs were made with a semi-automatic measuring device (Kontron MOP AM 02). For the morphometrical evaluation of changes in cellular parameters, the sample estimate was considered to be adequate when for several consecutive calculations the standard deviation was approximately constant. Usually, 40 cross sections per cell were sufficient. For the determination of the cell volume, light microscopic micrographs of whole cells were evaluated (100 cells per sample). The amount of fatty acids per cell was calculated by relating the total membrane area per cell to the mean cross section area of C₁₆:₀ and C₁₆:₁ fatty acids (corresponding to 22 × 10⁻¹⁶ cm² resp. 48 × 10⁻¹⁶ cm²) [22].

### Results

#### Light microscopic observations

Cells grown in normal growth medium exhibit the blue-green colour which is typical for many cyanobacteria. Nitrate and sulfate starvation cause a bleaching of the cells which is most pronounced after 70 h of nitrate starvation. Size and shape of the cells do not change significantly as documented by the constant cell volume (see Table I). Regeneration for 9 h has a twofold effect on nitrate starved cells: the typical blue-green colour is regained and the cell volume is significantly increased (Table I). Granular inclusions with high refraction index are typical for sulfate starved cells. Sulfate starved and sulfate regenerated cells are difficult to distinguish by light microscopy.

#### Electron microscopic observations

In the early logarithmic phase, cells are cylindrical with a smooth cell wall attached to the cytoplasmic membrane. Two parallel sheets of thylakoids are peripherically arranged. Generally, phycobilisomes are difficult to distinguish, possibly due to dense package and lack of order. The cytoplasm is packed with ribosomes and polyhedral bodies; “polyphosphate” granules are observed in most cell sections. Cells of stagnating and nitrate starved cultures (9 h) are similar in appearance: the thylakoid-thylakoid interspace is more electron translucent, due to carbohydrate accumulation; “polyphosphate” granules are absent (see Fig. 2). Nitrate starvation for 70 h

<table>
<thead>
<tr>
<th>Cultivation time</th>
<th>Normal growth 30 h</th>
<th>Nitrate starvation 9 h</th>
<th>Regeneration after 70 h of nitrate starvation</th>
<th>Sulfate starvation 70 h</th>
<th>Regeneration after 70 h of sulfate starvation 9 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell titer [Cells per ml × 10⁻⁷]</td>
<td>0.6</td>
<td>2.0</td>
<td>5.4</td>
<td>5.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Cell volume [µm³]</td>
<td>1.7</td>
<td>1.4</td>
<td>5.4</td>
<td>5.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Fresh weight per cell [g × 10⁻¹³]</td>
<td>2.4</td>
<td>3.4</td>
<td>n.d.</td>
<td>5.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Dry weight per cell [g × 10⁻¹³]</td>
<td>1.1</td>
<td>1.1</td>
<td>n.d.</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Specific density (ρ) of dry cells [g × cm⁻³]</td>
<td>0.7</td>
<td>0.8</td>
<td>n.d.</td>
<td>1.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Cell volume = \( \left( \frac{\text{diameter}}{2} \right)^2 \times \pi \times \text{length} \).
severely influences the ultrastructure of Synechococcus cells: the thylakoid system is reduced and less parallel, glycogen granules occupy a large portion of the cell volume.

The cell wall is wavy and separated from the cytoplasmic membrane (Fig. 3). Again, “polyphosphate” granules are present. In contrast to nitrate starved cells, sulfate starved cells (70 h) additionally show spherical cytoplasmic inclusions resembling lipid bodies in higher plants [23] (Fig. 5). Their electron translucent matrix is completely structureless. At the surface of these “lipid-body” like inclusions, electron dense material is frequently accumulated. These (cup-shaped) “precipitates” (as proven by serial sectioning) are of extreme hardness, thus causing holes in the embedding resin during sectioning (Fig. 5).
Fig. 3. Electron micrograph of *Synechococcus 6301* deprived of nitrogen for 70 h. The thylakoid membranes are reduced in area and are no longer parallel to the cellular membrane (CM). In addition to the reduced pigment content, an enormous accumulation of glycogen (G) is responsible for the typical yellow colour of the bleached cells. During dehydration (EM procedure) the glycogen shrinks for 10% (in volume) which leads to an artificial separation of the plasma membrane from the cell wall and therefore causes a wavy appearance.

Fig. 4. Electron micrograph of *Synechococcus 6301* regenerated for 9 h after 70 h of nitrogen starvation. Most of the glycogen granules are degraded and therefore the cytoplasmic membrane is attached to cell wall (compare Fig. 3). The cell matrix is of similar appearance as the matrix of exponentially growing cells (compare Fig. 1); note the cellular elongation which is due to a prestate of cell division (arrows Fig. 4).

After addition of nitrate to the medium, nitrate starved cells regenerate almost completely in ultrastructure within 9 h and then resemble exponentially growing cells (compare Figs. 1, 4). Sulfate starved cells, however, regenerate slowly. After 9 h of regeneration, they still exhibit the appearance of starved cells: however, phycobilisome-sized electron dense granules can clearly be distinguished at the cell periphery (arrows, Fig. 6).
Nitrogen and Sulfur Starvation of the Cyanobacterium Synechococcus 6301

Fig. 5. Synechococcus 6301 deprived of sulfate for 70 h. The thylakoids are reduced (only one pair as compared with two pairs as typical for exponentially growing cells (Fig. 1). As observed in nitrogen starved cells, the cytoplasmic membrane (CM) is separated from the cell wall (CW), due to glycogen accumulation (and its shrinkage during dehydration; for explanation see Fig. 3). Very typical for sulfur starved cells: an accumulation of “storage granules” (S) which are composed of electron dense and electron translucent material (poly-β-hydroxy butyric acid).

Fig. 6. Electron micrograph of Synechococcus 6301 regenerated for 9 h after 70 h of sulfur deprivation. Sulfur starved cells regenerate very slowly in comparison to nitrogen starved cells (Fig. 4). Although phycocyanin is being resynthesized, the thylakoid system is still reduced. Electron dense granules are clearly visible in the cell periphery (arrows) in contrast to exponentially growing cells (Fig. 1). They possibly are phycobilisomes in “statu nascendi”.

Quantitative analysis of cellular parameters

The basic cellular parameters of Synechococcus 6301 in relation to changing cultural conditions are summarized in Table I. Concluding from the cell titer, 30 h and 70 h cultures represent exponentially growing and stagnating cells. The maximum cell density during nitrate and sulfate starvation is $3 - 5 \times 10^7$ cells/ml indicating that at the most, one additional cell division occurs in comparison to 70 h cultures. Within 9 h of regeneration, no cell divisions are observed (constant cell titer; see Table I).

Therefore, changes of the culture can directly be correlated with individual changes. The cell volume is relatively constant ($1.5 \mu m^3$) except for regenerating nitrate starved cells. Their increased cell volume...
(3 μm³) may indicate a prestate of division. During starvation and regeneration, fresh and dry weight of *Synechococcus* cells are significantly increased. Their increased specific weight during nitrate and sulfate starvation (Table I) indicates an accumulation of storage products.

The morphometric data confirm the striking qualitative changes and, in addition, reveal small but statistically significant differences (see Table II). After 70 h of nitrate (sulfate) starvation, the area of thylakoid membranes per cell volume is reduced to 60% (42%). After 9 h of regeneration (after nitrate or sulfate starvation), 72% of the maximally observed “thylakoid membrane density” (area per cell volume) is regained. The net thylakoid synthesis is more striking: the thylakoid membrane area per cell is doubled within 9 h of regeneration (Table II).

Although polyhedral bodies are constituents of all investigated cells, their relative volume is decreased during regeneration from sulfate or nitrate starvation respectively (Table II). “Lipid-body” like structures (see Fig. 3, 5) are minor during nitrate starvation; but are dominant during sulfate starvation (up to 10% of cell volume, see Table II).

**Biochemical analysis**

During nitrate and sulfate starvation, the chlorophyll content of *Synechococcus 6301* (g/cell) is drastically reduced to 10–15% (see Table III). Upon regeneration after nitrate starvation, the chlorophyll is resynthesized to 40–50% of its maximum within 9 h. Although sulfur starved cells also significantly increase their total thylakoid membrane area during regeneration (from 10.5 to 18.8 μm² per cell) almost no chlorophyll is synthesized within the same time. The fast response of *Synechococcus 6301* to changing cultural conditions is best reflected by its phycocyanin content (see Table III): reaching their stationary phase, the cells show marked signs of “senescence” as expressed by the 50% reduced phycocyanin level (g/cell; Table III). During nitrate starvation, the phycocyanin content is further decreased — already after 9 h — to a constant level of 10% and completely restored in the same period of time upon regeneration. Sulfate starvation even leads to a phycocyanin degradation of 97%; the cellular vitality is proven by the increase of the phycocyanin content within 9 h upon sulfate addition (Table III).

The protein content of *Synechococcus 6301* cells does not exclusively depend on their supply of nitrogen; their soluble cellular protein is reduced to 67% during stagnation (see Fig. 3).

This level is not significantly altered by 9 h or 70 h of nitrate starvation. Surprisingly, during sulfate starvation the soluble cellular protein is drastically reduced to 15%. Nitrate starved cells recover com-

### Table II. Structural parameters and their changes in correlation with nitrate and sulfate starvation conditions and regeneration after starvation. These parameters are directly determined from electron micrographs. For explanation see text.

<table>
<thead>
<tr>
<th>Cultivation time</th>
<th>Normal growth</th>
<th>Nitrate starvation</th>
<th>Regeneration after 70 h of nitrate starvation</th>
<th>Sulfate starvation</th>
<th>Regeneration after 70 h of sulfate starvation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 h</td>
<td>6.9</td>
<td>3.1</td>
<td>10.2</td>
<td>5.7</td>
<td>7.2</td>
</tr>
<tr>
<td>70 h</td>
<td>6.2</td>
<td>9.1</td>
<td>9.2 ± 2</td>
<td>24.5</td>
<td>10.5</td>
</tr>
<tr>
<td>9 h</td>
<td>3.1</td>
<td>11.8 ± 2</td>
<td>12.2 ± 2</td>
<td>24.5</td>
<td>18.8</td>
</tr>
<tr>
<td>70 h</td>
<td>6.3</td>
<td>9.2 ± 2</td>
<td>10.4 ± 1</td>
<td>14.7</td>
<td>10.5 ± 1</td>
</tr>
<tr>
<td>Regeneration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after 70 h of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitrate starvation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 h</td>
<td>10.0</td>
<td>9.2 ± 2</td>
<td>7.5 ± 1</td>
<td>14.7</td>
<td>10.5 ± 1</td>
</tr>
<tr>
<td>70 h</td>
<td>10.2</td>
<td>12.2 ± 2</td>
<td>10.5 ± 1</td>
<td>24.5</td>
<td>18.8</td>
</tr>
<tr>
<td>Density of thylakoid membranes per cell volume [μm²×μm⁻³]</td>
<td>14.4 ± 1</td>
<td>11.8 ± 2</td>
<td>9.1 ± 1</td>
<td>9.2 ± 2</td>
<td>10.4 ± 1</td>
</tr>
<tr>
<td>Volume of polyhedral bodies per cell [%]</td>
<td>1.6 ± 0.4</td>
<td>1.3 ± 0.6</td>
<td>1.6 ± 0.6</td>
<td>1.6 ± 0.5</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Volume of “polyphosphate”* per cell [%]</td>
<td>0.8 ± 0.3</td>
<td>0</td>
<td>1.6 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Volume of “storage product”* per cell [%]</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.7</td>
<td>0.6 ± 1</td>
<td>0</td>
</tr>
</tbody>
</table>

* = Calculated from volume density of electron dense material.

* = Calculated from volume density of electron translucent material (biochemically characterized as poly-β-hydroxy butyric acid).
Table III: Changes in the content of chlorophyll, phycocyanin soluble protein and phosphate of *Synechococcus 6301* cells in correlation to nitrate and sulfate starvation.

<table>
<thead>
<tr>
<th>Cultivation time</th>
<th>Normal growth 30 h</th>
<th>Nitrate starvation 70 h</th>
<th>Regeneration after 70 h of nitrate starvation 9 h</th>
<th>Sulfate starvation 70 h</th>
<th>Regeneration after 70 h of sulfate starvation 9 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) [g/cell (\times 10^{14})]</td>
<td>2.5 3.0 1.2 0.4 1.2</td>
<td>0.3 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) g per (\mu\text{m}^2) thylakoid membrane area (\times 10^{16})</td>
<td>10.2 18.2 12.0 2.7 3.8</td>
<td>2.9 2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phycocyanin content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[g/cell (\times 10^{14})]</td>
<td>25.3 12.2 2.2 2.7 16.7</td>
<td>0.9 2.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Content of soluble protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[g/cell (\times 10^{14})]</td>
<td>27 18 11 17 36</td>
<td>4 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Content of inorganic phosphate per cell volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[mol (\times 10^{15}) (\times \mu\text{m}^{-3})]</td>
<td>3.2 0.1 n.d. 1.9 1.1</td>
<td>0.3 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Poly-\(\beta\)-hydroxybutyric Acid as Storage Product

Morphometric determinations have shown that predominantly under sulfur starvation conditions a lipid-like storage product is formed (Table II). From the main infrared absorption band at 1725 cm\(^{-1}\) which is typical for ester bands a first characterization of the polymer as a polyester compound is given. The spectrum is identical to the one of poly-\(\beta\)-hydroxybutyric acid published by Lundgren *et al.* [24]. A proof of the identity of the storage product with poly-\(\beta\)-hydroxybutyric acid is given by the \(^1\)H NMR spectra which are in agreement with those recorded by Capon *et al.* [25]. As control served the preparation of the esterified hydrolysis product and its chromatographic (GC) and spectroscopic comparison (\(^1\)H NMR) with a synthetic reference prepared from the sodium salt.

**Lipid analysis**

The cells of *Synechococcus 6301* exhibit a constancy of fatty acid composition independent from cultural conditions. The total amount of fatty acids per cell is about \(7 \times 10^{-14}\) g when determined by GLC (Table IV). The same amount is calculated from

Table IV: Total amounts of fatty acids per cell. The composition of fatty acids of *Synechococcus 6301* is given in relation to nitrate and sulfate starvation.

<table>
<thead>
<tr>
<th>Cultivation time</th>
<th>Normal growth 30 h</th>
<th>Nitrate starvation 70 h</th>
<th>Regeneration after 70 h of nitrate starvation 9 h</th>
<th>Sulfate starvation 70 h</th>
<th>Regeneration after 70 h of sulfate starvation 9 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount of fatty acids per cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[g (\times 10^{14})]</td>
<td>6.8 7.0 7.3 8.9</td>
<td>6.2 6.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Determination by GLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) Calculated from total cellular membrane area</td>
<td>8.8 6.4 5.9 11.7</td>
<td>4.5 7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[% of total]</td>
<td>C(_{16:0}) 49.2 42.4 56.0 37.0</td>
<td>50.8 45.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{16:1})</td>
<td>41.5 44.7 43.5 34.7</td>
<td>43.9 43.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others*</td>
<td>9.3 12.9 0.5 29.3</td>
<td>5.3 11.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Identified as: C\(_{12:0}\); C\(_{14:0}\); C\(_{14:1}\); C\(_{18:0}\); C\(_{18:1}\); each less than 5% of total.
morphometrical data (Table IV) using the total cellular membrane area and molecular dimensions of fatty acids (see Material and Methods). This agreement demonstrates the precision of both independent methods. An increased amount of fatty acids per cell is observed in regenerating (nitrate starved) cells only. However, these are bigger and contain more cellular membranes (compare Tables I, II and IV). The fatty acid pattern of Synechococcus 6301 is relatively simple with C:16 dominating and equal parts of C 16:0 and C 16:1. Other fatty acids (C 12:0, C 14:0, C 14:1, C 18:0, and C 18:1) are present in traces (each less than 5% of total) except for cells recovering from nitrate starvation (Table IV).

Discussion

In general, starvation of cyanobacteria and unicellular algae results in formation of different storage products (see Introduction). A massive storage of carbohydrates is clearly manifested in an increased dry weight per cell (Table I) resulting in a specific density of \( \rho = 1.7 \) (g/ml) what in a natural environment leads to sedimentation and eventual formation of algal deposits. This may be a general ecological effect, since in lakes “starvation” conditions in terms of algal deposits. This may be a general ecological effect, since in lakes “starvation” conditions in terms of phosphorus, nitrogen and sulfur should be considered as “normal events”.

Whereas under nitrate starvation starch (glycogen) is the dominant storage product, sulfur-starved cells accumulate both starch and poly-ß-hydroxybutyric acid (PHB) as storage product (Table II). The lipid-body homologue storage product for bacteria is poly-ß-hydroxybutyric acid. However, it is documented only for a few cyanobacterial species that poly-ß-hydroxybutyric acid is a storage product, but it may be widespread (for review see [26]).

A comparison of the total amounts of fatty acids per cell demonstrates a surprising constancy of about \( 7 \times 10^{-14} \) g/cell. These fatty acids are exclusively located within the cellular membranes as calculated from total membrane areas (Table IV).

Synechococcus 6301, is very conservative concerning its fatty acid composition: under all cultural conditions, the dominant fatty acids of the membranes are C 16:0 and C 16:1 in equal parts (Table IV); this is in good agreement with data for Synechococcus lividus during normal growth and carbon dioxide deprivation [8]. This ratio is valid for thylakoid membranes as well as for the cytoplasmic membrane, as can be concluded from its constancy in spite of differing ratios: cytoplasmic membrane area per cell to total thylakoid membrane area per cell (compare Tables II and IV). Only under regeneration conditions after nitrate starvation, a portion of 30% of fatty acids with different chain lengths is found. This is seen as an expression of a de-novo synthesis of fatty acids during regeneration as additionally supported by the increase in total amounts of fatty acids per cell (Table IV). Comparable starvation conditions (sulfate- and nitrate-starvation and -regeneration) for Porphyridium cruentum (a red alga) is characterized by a massive accumulation of starch and lipids; the fatty acid composition of the membrane lipids (mainly thylakoids) varies widely [3].

The function of polyhedral bodies (carboxysomes) during starvation and regeneration of Synechococcus 6301 is totally unclear. Under all conditions tested, they maintain a more or less constant fraction of about 1.5% of the cell volume (Table II). It has been shown that polyhedral bodies are unaffected in the cyanobacteria Agmenellum quadruplicatum and Chlorogloea fritschii under nitrogen limitation [6, 27] and in Synechococcus lividus under carbon dioxide deprivation [8]. This indicates an essential role of polyhedral bodies under many nutritional conditions and excludes their function as an easily accessible nitrogen store [6]. This is also supported by the observation that Anacystis nidulans (Synechococcus spec.) reduces its polyhedral bodies under iron deficiency although nitrogen is not limiting at all [9]. From these studies, it is assumed that polyhedral bodies may be required for optimal carbon dioxide fixation (for detailed discussion see [9]).

The discussion on the function of polyphosphate granules during N- and S-starvation of Synechococcus 6301 is problematic: although it is widely accepted that all electron-dense granules within cyanobacteria are polyphosphate their analytical proof is rare. The chemical quantification of inorganic phosphate (g per cell) (see Table III) made in parallel to ultrastructural investigations represents only soluble phosphate. A comparison of the data of Tables II and III suggests that polyphosphate granules are rare during exponential growth; most of the phosphate is present in soluble form. During nitrogen and sulfur starvation, most of the phosphate is deposited in granules. During regeneration, two thirds of the polyphosphate are mobilized and obviously ana-bolized as indicated by the reduced phosphate con-
tent per cell (Table III). There is little known on the significance and the eventual fate of polyphosphate. It has been shown for the cyanobacterium *Plectonema boryanum* [28] that polyphosphate granules are formed under phosphate supplementation and degraded during phosphate starvation. Nitrogen limitation did not effect polyphosphate bodies of the cyanobacterium *Agmenellum quadruplicatum* [6].

In general, nutritional starvation of cyanobacteria and various algal eucaryonts results in two main effects: formation of storage products and degradation of cellular structures. Degradation of pigments (biliproteins and chlorophylls) is the first visible sign of nitrogen starvation of various species: e.g. *Rhodella violacea* [2], *Porphyridium cruentum* [3], *Anacystis nidulans* [14], *Aphanocapsa 6308* [10], *Synechococcus 6301* [15]. Membrane structures, especially thylakoids are degraded next: *Rhodella violacea* [2], *Porphyridium cruentum* [4], *Anabaena cylindrica* [5], *Agmenellum quadruplicatum* [6].

The formation of storage products under starvation conditions obviously is of evolutionary advantage. In contrast, a drastic reduction of essential photosynthetic pigments and structures, however, appears to be less sensible as an intact photosynthetic apparatus would enable a more rapid regeneration. However, the high in order thylakoids are thermodynamically instable and the maintenance of integrity is coupled to active metabolisms. Reduced utilization of light energy for photosynthesis will increase membrane damage caused by excessive photooxidation; this may be compensated for by a reduction of thylakoid membranes. As a consequence of this hypothesis, starvation conditions should generally lead to comparable changes in cellular parameters, such as accumulation of storage products and degradation of cellular structures. A differentiation for individual effects of various starvation conditions and environmental factors is, therefore, difficult and will require a combination of biochemical and morphometric methods.

**Acknowledgements**

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG). We are indebted to Dr. H. Formanek for valuable support and helpful discussions.