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C-Phycocyanin as a Storage Protein in the Blue-Green Alga *Spirulina platensis*

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Abstract. The possibility that c-phycocyanin serves as a nitrogen source in *Spirulina platensis* during nitrogen starvation was studied. The following evidence was obtained in support of this idea. 1. Under favourable conditions for growth, c-phycocyanin existed in large excess in the algal cells. 2. When the supply of nitrogen was low, about 30–50% of the c-phycocyanin disappeared without any effect on the maximal growth rate. 3. A culture which was deprived of nitrogen continued to grow unaffectedly for a period, the duration of which depended on the c-phycocyanin content in the cell before nitrogen starvation was initiated. 4. c-phycocyanin was the only nitrogenous compound that was depleted during the course of nitrogen starvation when growth was yet unaffected. 5. When protein synthesis was inhibited either by nitrogen starvation or by methionine sulfoximine (MSO), phycocyanin content began to decline immediately and growth continued at normal rates as long as c-phycocyanin did not decline below 50%. 6. The decrease in c-phycocyanin content during nitrogen starvation was accompanied by an increase in proteolytic activity.

Key words: Nitrogen starvation – c-phycocyanin – Blue-green alga – *Spirulina platensis*.

The pigment c-phycocyanin is present in all blue-green algae (Chapman, 1973; Glazer, 1976; Stanier and Cohen Bazire, 1977). Its concentration in the cell depends on environmental conditions (Myers and Kratz, 1955). c-phycocyanin is generally believed to be an accessory pigment with which light energy is captured and is transfered it to chlorophyll *a* (Bogoarad, 1975). However, the existence of mutants lacking the pigment, or part of it, but still having the same growth rate and photosynthetic efficiency (Stevens and Myers, 1976; Rogers et al., 1977) suggest that this biliprotein pigment may have other functions in addition to its recognized role in photosynthesis. Abeliovich and Shilo (1972) suggested that phycocyanin may function as an antioxidant and Miller and Holt (1977) proposed it to serve as a carbon-storage material.

Allen and Smith (1969) were the first to point out the possibility that phycocyanin may be a nitrogen storage compound in the cell. Other works brought somewhat inconclusive findings for this possibility (Van Gorkom and Donze, 1971; Stewart and Lex, 1970; Van Liere et al., 1977).

Ownby et al. (1979) recently reported on experiments with *Anabaena* in which pulse labeling and treatment with methionine sulfoximime (MSO) were used to trace the course of protein degradation during nitrogen starvation. They concluded that cyanobacteria rapidly degrade proteins in response to nitrogen starvation as well as in response to treatment with MSO utilizing the amino acids thus released for the synthesis of new proteins. They hypothesized that the major form of protein hydrolyzed in nitrogen starved cells – or MSO treated cells was phycocyanin. Lau et al. (1977) were the first to present evidence that spectrophotometric determinations of phycocyanin reflect (at last in the case of nitrogen starvation) events at the level of appoprotein degradation.

In the present study, we sought to bring new physiological evidence for the thesis that c-phycocyanin acts as a nitrogen-storage compound. Our experiments were directed at establishing a correlation between nitrogen supply and the concentration of c-phycocyanin, as reflected by growth parameters. Consequently, in most of our experiments we used a continuous culture of *Spirulina platensis*, which allowed us to control all growth factors.

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Materials and Methods

Organism and Growth Conditions. Spirulina platensis (Lb 1475/4a) was obtained from the Cambridge Culture Collection. Zarouk's medium (1966) was used as the growth medium with the following modifications: the concentration of $NaHCO_3$ was 4.2 g/l, and that of K_2HPO_4 was 0.185 g/l, $NaNO_3$ was used at various concentrations. (See Results and Discussion.)

The algae were grown at 35°C either in continuous culture in a turbidostat or in batch cultures. The pH was maintained at 8.5–9.0, and the cultures were stirred by means of air stream (4 l/min) containing 1.5% CO_2. Illumination at various intensities was provided by cool white lamps, as described in the text. Cultures were allowed to grow for at least 10 doubling times at which stage, regarded to be steady state, the experiments began.

Nitrogen Starvation Experiments and Methionine Sulfoximime (MSO) Treatment of Cells. Cells were harvested by centrifugation, then re-suspended in nitrogen-free medium, centrifuged again and washed thoroughly with distilled water. For treatment with MSO, cells were resuspended in full medium and MSO was added to a final concentration of 5 μM.

Concentration of Pigments. For the determination of c-phycocyanin, the cells were first disrupted by one of two methods: 1. Sonication in 0.1 M Na-phosphate buffer, pH 7.0, or 2. treatment with a solution of lysozyme, 100 μg/ml of 0.1 M Na-phosphate buffer, containing 100 mM NaEDTA. The absorbance of the resulting suspension was read spectrophotometrically at 620 nm (Myers and Kratz, 1955), and the concentration of c-phycocyanin was then calculated from the specific absorption coefficient $E\,1\% = 73$ (Boussiba and Richmond, 1979).

Chlorophyll *a* was extracted by sonication in 80% acetone and its concentration was determined from its absorbance at 663 nm and an extinction coefficient of 0.82 (McKinney, 1941).

Nucleic Acid Determination. RNA and DNA were determined according to the methods of Burton (1956) and Schneider (1957) respectively.

Measurements of Oxygen Evolution. Cells were suspended in fresh medium and the suspension was transferred to a 3.0-ml Perspex cell. The rate of oxygen evolution was measured at 30°C by the use of a Clark-type Oxygen Electrode (YSI 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio) connected to a recorder (Servogor 310). The light intensity at the surface of the cell was 10^5 ergs $m^{-2} \cdot s^{-1}$.

Specific growth rate was calculated as $\ln 2 \cdot dt^{-1}$ (Pirt, 1975). The output rate was obtained by multiplying the specific growth rate by the cell density (mg/ml).

Cell density was maintained at 0.5 mg d.wt/ml in all the continuous culture experiments.

Protein and Free Amino Acid Measurements. Protein was determined according to the procedure of Lowry et al. (1955). Free amino acids and other nitrogen containing compounds, such as amides and ammonia, were estimated by the ninhydrin test (Rosen, 1957). Cells were disrupted by sonication, which was followed by precipitation in 10% trichloroacetic acid (TCA). Leucine was used as the standard.

Dry weight (d.wt) was measured by filtering culture samples through 8-mμ membrane filters. The filters were weighed, then dried at 80°C for 1 h and reweighed.

Determination of Protease Activity During Nitrogen Starvation. Six-day-old cultures growing in Zarouk's medium containing $NaNO_3$, 2.5 g/l, were harvested and re-suspended in nitrogen-free medium after thorough washing with distilled water. Two-liter samples were taken at intervals. Each sample was centrifuged, and the sediment was re-suspended in a small volume of 0.1 M Na-phosphate buffer, pH 7.0. The cells were then disrupted in a mechanical cell homogenizer (Brawn Model MSK) for 3 min, and the suspension was centrifuged at 10,000 × *g* for 15 min. The precipitate, which included cell walls and debris, was discarded. Protease activity was determined in the resulting crude extract.

Protease Assay. According to Foulds and Carr (1977) pure (620/280 > 4) [^{14}C]c-phycocyanin (7×10^5 cpm/ml), 324 μg/ml, was used as the standard solution for the assay. Each assay solution usually contained 20 μl of the standard c-phycocyanin solution, 0.9 ml of the crude extract and 80 μl of 0.1 M Na phosphate buffer, pH 7.0. The test tubes containing the assay solutions were incubated in a shaking bath at 30°C for 1.5 h. The reaction was terminated by addition of an equal volume of 10% TCA and the precipitate was removed by centrifugation. Aliquots of the supernatant, containing degradation products of c-phycocyanin, were placed in 5-ml vials containing scintillation fluid [0.4 g of 2.5-diphenyloxazole (PPO) and 50 mg of 1,4-bis-(5-phenyloxazoly) benzene (POPOP) per liter in toluene]. Radioactivity was counted in a Packard liquid scintillation spectrometer (model 3320). The counting efficiency for [^{14}C]c-phycocyanin was 85%. Protease activity was expressed as counts released per mg protein per h.

Results and Discussion

Effect of NO_3^- Concentration at Different Light Intensities

Continuous cultures of *S. platensis* were grown at two different concentrations of $NaNO_3$, 29.4 mM and 3.7 mM each at high and low light intensities, 175 and 80 micro Einstein $\cdot$ $m^{-2} \cdot s^{-1}$, respectively (Table 1).

The responses of the growth parameters at the two light regimes were similar – growth rate and output rate as well as total protein content and chlorophyll concentration were maintained at the same level at both nitrogen concentrations – but the c-phycocyanin content was markedly reduced at the lower concentration of NO_3, i.e. 50 and 30% of the c-phycocyanin disappeared at the high and low intensities, respectively. Nevertheless, the absolute amount of c-phycocyanin which declined was the same, i.e. 14–15 μg per 500 μg dry weight, under the two light regimes. It was, therefore, concluded that c-phycocyanin is present in excess and can be depleted without impairing the maximal growth rate under the conditions of the experiment.

Effect of Nitrogen Starvation at Different Light Intensities

When continuous cultures grown at the high nitrogen (29.4 mM), under either high or low light intensities, were deprived of nitrogen, they continued to grow for one doubling cycle at the growth rate they had displayed prior to the deprivation (Fig. 1a, b). The photosynthetic potential of the cells, as indicated by the rate of net O_2 production, decreased only when the level of c-phycocyanin fell below 14.5 or 35.7 μg $\cdot$ ml^{-1}, in

Table 1. Effect of NO_3^- concentration on growth parameters and content of protein, chlorophyll and c-phycocyanin in continuous cultures of *Spirulina platensis*

Light intensity μEinsteins $\cdot m^{-2} \cdot s^{-1}$	NO_3^- conc. in medium (mM)	Specific growth rate (h^{-1})	Output rate (mg d.wt. $\cdot ml^{-1} \cdot h^{-1}$)	Total protein ($\mu g \cdot ml^{-1}$)	Chlorophyll ($\mu g \cdot ml^{-1}$)	c-phycocyanin	
						($\mu g \cdot ml^{-1}$)	%
High							
175	29.4	0.073	3.5	250	2.85	28.4	100
	3.7	0.073	3.5	245	2.80	14.5	51
Low							
80	29.3	0.049	2.5	250	5.70	50	100
	3.7	0.050	2.6	250	5.90	35	70

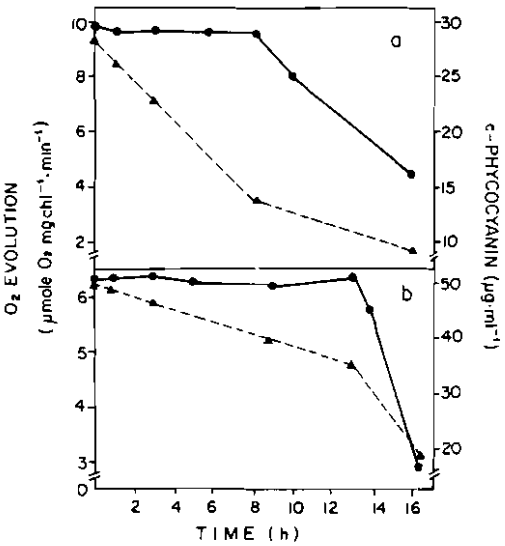

Fig. 1. Effect of nitrogen starvation on oxygen evolution and c-phycocyanin content in cultures growing under high NO_3^- (29.4 mM) at high (**a**) or low light intensity (**b**). ●——● Oxygen evolution, ▲——▲ c-phycocyanin content

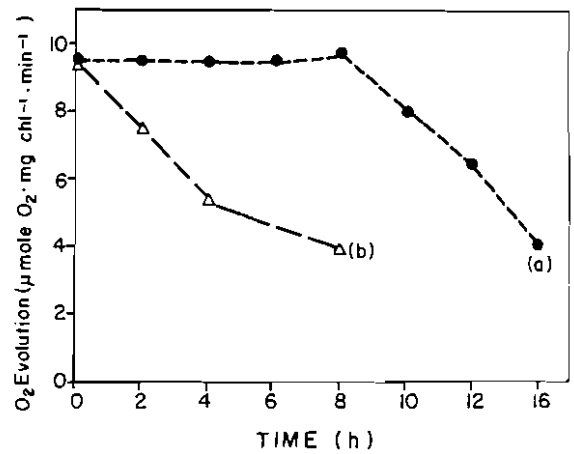

Fig. 2. Effect of nitrogen starvation on oxygen evolution in cultures growing at high light intensity under high NO_3^- – 29.4 mM (**a**) ●——● or low NO_3^- – 3.7 mM (**b**) △——△

cultures grown under high or low light intensity, respectively.

We tested the hypothesis that the length of the period of normal growth and development before the effect of nitrogen deprivation is manifested, is related to the cellular content in c-phycocyanin. For this purpose, cultures were grown in continuous culture with either high or low nitrogen and were exposed to high light intensity. These cells, which contained either high or low c-phycocyanin concentrations, were transferred to a nitrogen free medium. As expected, the growth rate of cells with the relatively low concentration of c-phycocyanin was reduced immediately on exposure to nitrogen deficiency (Fig. 2).

The role of c-phycocyanin as a storage substance for nitrogen is further supported by analysis of the kinetics of dry weight, protein and c-phycocyanin content in batch culture when transferred in the logarithmic phase of growth to a nitrogen-free medium (Fig. 3). After the transfer, the cells continued to grow at the initial growth rate for one generation (Fig. 3a). The amount of total protein did not increase (Fig. 3b) while c-phycocyanin decreased continuously during the course of the experiment (Fig. 3c). The amount of c-phycocyanin which disappeared during starvation was quantitatively sufficient to maintain the concentration of non-c-phycocyanin protein at a constant level: at the beginning of starvation, protein content was 50 $\mu g \cdot ml^{-1}$, of which 11 $\mu g \cdot ml^{-1}$ were c-phycocyanin, while after 72 h of growth in a nitrogen-free medium, the concentration of protein was the same (50 $\mu g \cdot ml^{-1}$), but that of c-phycocyanin had fallen to as little as 2 $\mu g \cdot ml^{-1}$. In parallel with the decline of c-phycocyanin, a marked increase of proteolytic activity was observed (Fig. 4).

Still further support for this role of c-phycocyanin as a storage compound was obtained in experiments that tested the effect of MSO on cell growth and

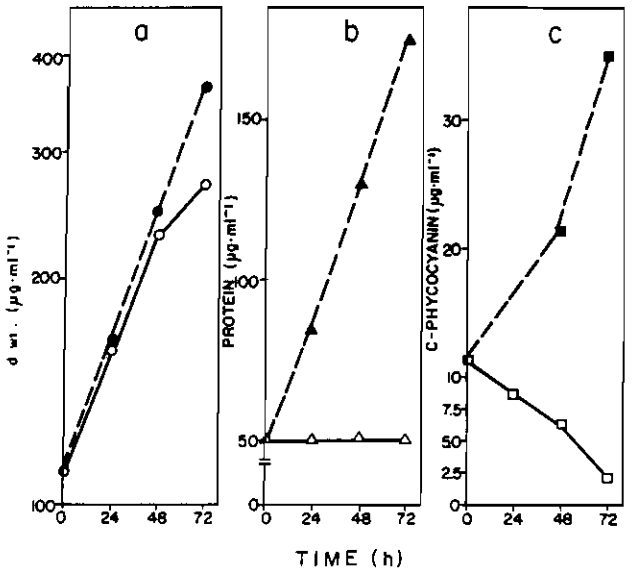

Fig. 3. Effect of nitrogen starvation on (a) dry weight (b) protein content and (c) c-phycocyanin content. Full symbols – control treatment, empty symbols – nitrogen starvation treatment

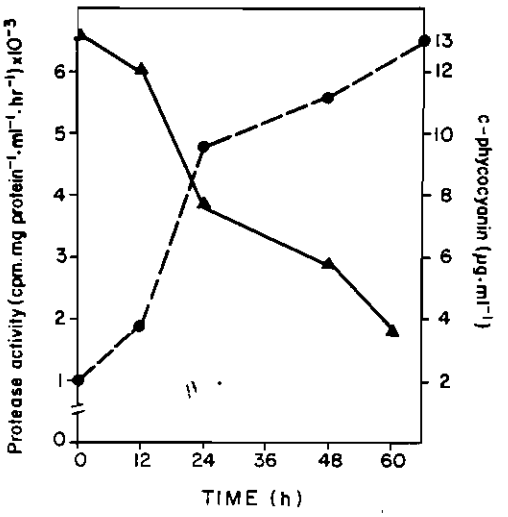

Fig. 4. Effect of nitrogen starvation on protease activity and c-phycocyanin content. ●——● Protease activity, ▲——▲ c-phycocyanin content.

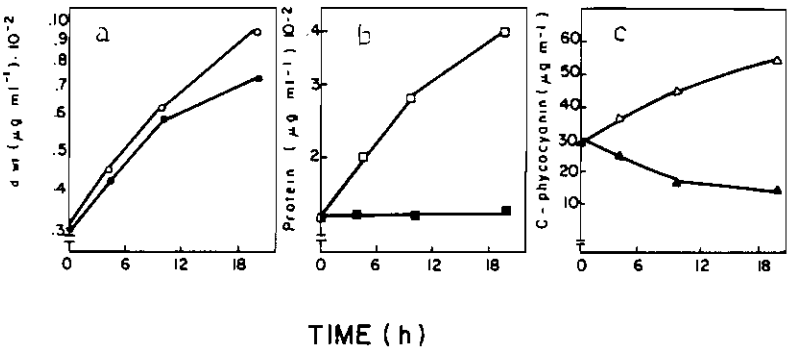

Fig. 5
The effect of MSO on (a) d.wt. (b) protein content (c) c-phycocyanin content. Empty symbols – control treatment, full symbols – cell treated with 5 µM MSO

Table 2. Effect of nitrogen starvation on the content of ninhydrin-reactant substances under high and low light intensities

Starvation (h)	Content of ninhydrin-reactant substances[a] (µmole · ml⁻¹)	
	High intensity	Low intensity
0	0.188	0.180
1		0.214
2		
3	0.200	0.196
4		
5		0.188
6	0.175	
8		
10	0.180	0.185
12		
16		
24	0.185	0.185

[a] Leucine equivalents

phycocyanin depletion. In close similarity to the effect of nitrogen starvation on phycocyanin content, MSO caused an immediate decline in phycocyanin content but growth was not altered unless phycocyanin fell below 50% (Fig. 5).

The content of ninhydrin reactant substances, i.e. free amino acids, amides and ammonia, was very small and did not change significantly throughout the course of nitrogen deprivation (Table 2). The amount of nucleic acids (7–8% of dry weight) also remained the same. Clearly, the only nitrogen compound which decreased significantly during the course of the deprivation and which could, therefore, have served as a nitrogen-storage molecule, was c-phycocyanin.

It may be worth noting that in the blue-green algae, c-phycocyanin differs from other known storage materials in that it has a dual role in the cell and that its synthesis occurs largely during the logarithmic phase of

growth and practically ceases during the stationary phase (Simon, 1973). This is in contrast to other storage products which are usually accumulated at the end of the growth phase or in the final stage of the life cycle (Lehmann and Wober, 1976).

The advantage of c-phycocyanin as a storage form of reduced nitrogen skeletons may be speculated: When cells which grow logarithmically with an abundant nitrogen source are exposed to a temporary shortage of nitrogen, they would be able to grow unhindered for up to another generation before their growth ceases. Also, when cells of these algae so multiply as to cause dense blooms, a portion of c-phycocyanin becomes potentially a storage material, which facilitates immediate resumption of accentuated growth when cells from such blooms are dispersed and light ceases to be limiting to growth.

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