

The Phycobiliproteids in *Cyanophora paradoxa* as Accessoric Pigments and Nitrogen Storage Proteins

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The phycobiliproteids of cyanobacteria have two functions. They are accessoric pigments for the light-dependent photosynthetic electron transport, and, secondly, they are storage proteins. *Cyanocyta korschikoffiana*, the endocyanelle of *Cyanophora paradoxa*, a hardly adapted endocytobiotic cyanobacterium, is responsible for the photoautotrophy of the host flagellate. The biosynthesis of the phycobiliproteids takes place in the endocyanelles and is reversible. Under nitrogen starvation the phycobiliproteids were disintegrated again, in contrast to the carotenoids (and in a lower degree to chlorophyll), whose contents remain more constant in the cells, as shown by *in vivo* measurements. Therefore, it is concluded that similar to the function in free living cyanobacteria the phycobiliproteids of *C. paradoxa* also serve as storage substances ("stress proteins"). This opinion is supported by experiments with chloramphenicol.

Introduction

The apoplastidic flagellate *Cyanophora paradoxa* hosts from two to six (to eight) endocyanelles depending on the age and the physiological condition of the cells. The endocyanelles, which are undoubtedly of prokaryotic nature (endocytobiotic cyanobacteria) [1–8], have received the systematic name *Cyanocyta korschikoffiana* Hall and Claus [1]. With the help of these assimilators, the cyanome is able to live completely autotrophically. At present it is not possible to decide whether the observed phototrophy of the flagellate [9] is to be regarded as evidence of the far-reaching mutual adaptation of the symbiotic partners or as an indication of the loss of original existing plastids in the eukaryotic host. All eukaryotic photosynthetic cells observed so far are able to synthesize reserve carbohydrates, whose chemical structure is often typical for the systematic position of the organism regarded. In *C. paradoxa* reserve carbohydrates were found in the cytoplasm of the host, not in the "chloroplast-like" [10, 11] endocyanelle. Additionally, reference is made [1] to polyphosphate granula and oil troplets as reserve substances in the endocyanelles. It remains to be seen whether the phycobiliproteids are also reserve

substances for the endocytobiotic consortium, as is the case with free living cyanobacteria [12–14].

Materials and Methods

1. Analytically pure substances (Merck, Darmstadt) were used as chemicals. Origin and Culture of the algae: *C. paradoxa* (K. G. Grell, Institute of Biology II, D-7400 Tübingen) was grown at 24 °C in 300 ml cultures [15] and gassed with an air/carbon-dioxide mixture (98:2, v:v), light intensity 1200 lx (0,55 mW/qcm). Culture medium see [16]. For variation of the nitrogen concentration in the medium the nitrate concentration in the original medium was simply varied. For transfer to the different media the flagellate was centrifuged (900 × g, 5 min), and then the new medium was added to its pellet.

2. *In vivo* estimation of pigment concentration (phycocyanin, allophycocyanin, chlorophyll and carotenoids):

a) Method: After dilution with new nutrition medium the cultures of the flagellate (ca. 1×10^6 cells/ml) were investigated, taking every 3 ml probes every 3 h. These probes were measured directly by *in vivo* VIS-spectroscopy in a Beckmann spectrophotometer, Model Acta V. The glass cuvettes, with a roughed surface on the front side for diffusion of the incident light, were positioned immediately in front of the photomultiplier. Their content was stirred.

Abbreviations: APC, allophycocyanin; Chl, chlorophyll a, mr, molar ratio; PC, C-phycocyanin; PCP, phycocyanin-proteids (= phycobiliproteids); RCN, relative cell number.

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For quantitative estimation of the pigment concentrations the data for optical density were taken out of the *in vivo* spectra at 750 nm (dispersion), 678 nm (chlorophyll *a*), 653 nm (allophycocyanin), 626 nm (C-phycocyanin) and 490 nm (carotenoids).

b) Calculation: For the equations (1–3) we have taken the basic data given by Schneider and Bogorad [17]. Whereas aqueous solutions of phycocyanin (*C. paradoxa*) have an absorption maximum at 618 nm [18], respectively at 620 nm [19] and our results) and allophycocyanin at 653 nm [18], respectively 650 nm [19], *in vivo* VIS-spectra of *C. paradoxa* show a maximum at 630 nm [20], also if chlorophyll is in the minimum (Fig. 1, I). All absorption values for the different pigments were corrected (!) by subtraction of the dispersion value at 750 nm:

$$\text{Chl } (\mu\text{g/ml}) = 13.7 \text{ OD}_{678} - 3.2 \text{ OD}_{653} + 0.7 \text{ OD}_{626}, \quad (1)$$

$$\text{APC } (\mu\text{g/ml}) = -20.8 \text{ OD}_{678} + 199.0 \text{ OD}_{653} - 40.5 \text{ OD}_{626}, \quad (2)$$

$$\text{PC } (\mu\text{g/ml}) = -19.7 \text{ OD}_{678} - 84.3 \text{ OD}_{653} + 186.6 \text{ OD}_{626}, \quad (3)$$

$$\text{Car } (\mu\text{g/ml}) = 6.27 (\text{OD}_{490} - \text{OD}_{490_{\text{pig}}}). \quad (4)$$

The estimation of chlorophyll was compared with the method of Arnon [21] (chlorophyll extraction in 80% acetone). The results available from these two methods are in good agreement (standard deviation: $\pm 4.7\%$). This is also true of the estimation of the total amount of the carotenoids in *C. paradoxa* using the absorption value at 490 nm (Eq. (4)). For the tetrapyrrolpigments, also absorbing at 490 nm, a term for pigment correction ($\text{OD}_{490_{\text{pig}}}$) was introduced in Eq. (4):

$$\text{OD}_{490_{\text{pig}}} = \text{OD}_{490_{\text{chl}}} + \text{OD}_{490_{\text{apc}}} + \text{OD}_{490_{\text{pc}}}, \quad (5)$$

$$\text{OD}_{490_{\text{chl}}} = 0.00277 \times [\text{Chl}], \quad (5a)$$

$$\text{OD}_{490_{\text{apc}}} = 0.00012 \times [\text{APC}], \quad (5b)$$

$$\text{OD}_{490_{\text{pc}}} = 0.00026 \times [\text{PC}]. \quad (5c)$$

The only important carotenoids in *C. paradoxa* are β -carotene and zeaxanthin [18, 22]. These two pigments are found in *C. paradoxa* in similar quantities and, additionally, their extinction coefficients ($\text{ml/mg} \times \text{cm}$) at 480 nm [23] (according to our observations the shoulder at 480 nm in the

carotenoid spectra is shifted in the *in vivo* VIS-spectra to 490 nm) are in relatively good agreement: β -carotene (209.6), zeaxanthin (197.2). At first we used their median size (203.4) leading to a multiplication factor 4.92 in the equation (4). (Using the data of Röbbelen [24] the factor should be 5.17.) But comparison with the acetone extraction method [25] shows that the “*in vivo*” values are ca. 27% lower than the values in acetone extracts. Therefore it seems necessary to replace the “theoretical” factor 4.92 in Eq. (4) by the “practical” factor 6.27. Now the results of Eq. (4) correspond to those in equation (25):

$$\begin{aligned} C_{\text{car}} (\mu\text{g/ml}) &= 4.20 \times E_{452.5} - 0.0264 \times [\text{Chl } a] \\ &\quad - 0.496 \times [\text{Chl } b] \end{aligned}$$

with a standard deviation of $\pm 4.5\%$ in the limits for OD between 0 and 0.5. The more reproducible data were produced by the *in vivo* method (in our laboratory). Such data can help to overview the variation of the relation between the amounts of the different pigments during an investigation. Notwithstanding, these calculations are only to be regarded as approximations of the real amounts of pigments because we have not yet investigated the different *in vivo* extinction coefficients of the pigments in *C. paradoxa* in more detail.

c) Nephelometric approximation of the relative cell number of *C. paradoxa* in aqueous cultures: For estimation of the relative cell number (RCN) during long series of measurements a simple relation between the dispersion at 750 nm and the relative cell number of cultured *C. paradoxa* was used. For comparison, the relative cell number was estimated in a counting chamber “THOMA”. A linearity between the dispersion and the relative cell number was found using optical density at 750 nm only in the limits 0.01–0.4:

$$\text{RCN (cells/ml)} = 3.1 \times 10^7 \times \text{OD}_{750}. \quad (6)$$

Results and Discussion

The growth behaviour of the flagellate, cultivated in air/carbondioxide gassed mass cultures, illuminated with continuous white light (1200 lx), is, in relation to the relative cell number (RCN), similar to that described by Marten *et al.* [26] for 3500 lx light conditions. But as a result of the lower nitrogen

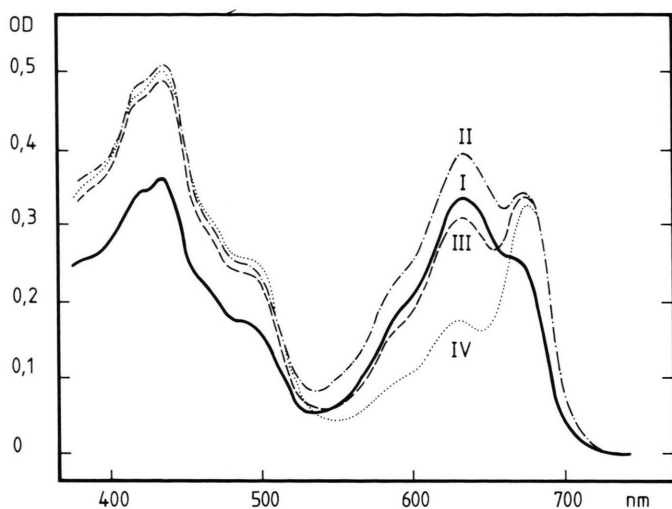


Fig. 1. *In vivo* VIS-spectra of *C. paradoxa* (normalized at OD₇₅₀ = 0). I. Spectrum of exponential phase of growth, II. S. of early stationary phase, III. S. of late stationary phase, IV. S. under nitrogen starvation.

content of our inorganic medium, cultures finally show, after about 5–7 days or longer, a green (Fig. 1, III) or yellow-green colour (Fig. 1, IV). If *C. paradoxa* is diluted at the end of the stationary phase, a variation of the culture colour from green before dilution to a fresh blue-green colour ca. 1.5 days later is observed (Fig. 1, I). This phenomenon can be investigated quantitatively by *in vivo* VIS-spectroscopy. The variation of the colour is dependent on the relation of the concentrations of phycobiliproteids to the other pigments. The blue-

green colour of cultures is observed if the absorption of phycobiliproteids at 630 nm is higher than that of chlorophyll at 678 nm. The culture's colour changes to yellowish green if the absorption at 630 nm becomes lower than that at 678 nm. In the course of these variations, the overall concentration of carotenoids only increases slightly. This shows that the concentration of phycobiliproteids in comparison to the other pigments must be regulated by faster velocities of synthesis and decomposition (Figs. 2 and 3). The calculation of pigment concen-

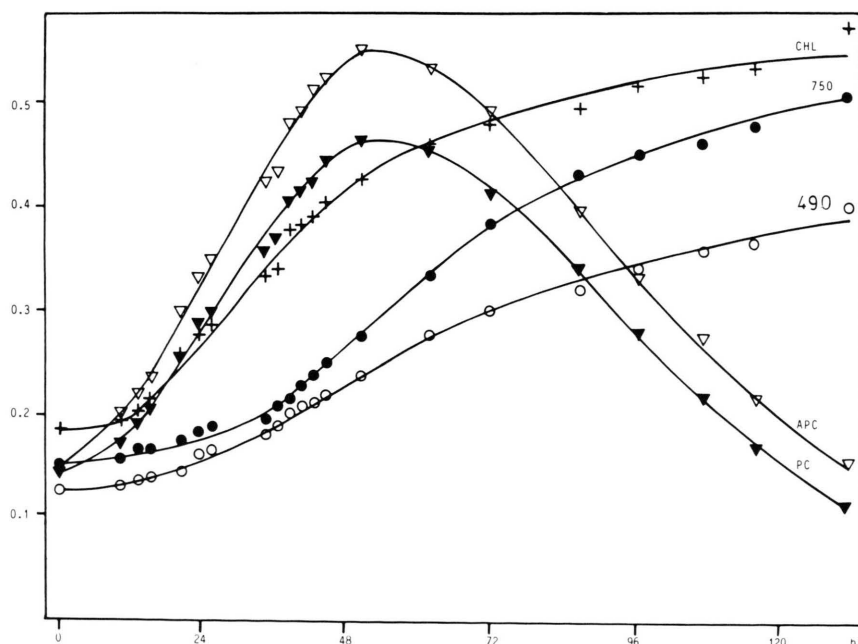


Fig. 2. Variations of pigment concentrations in a culture of *C. paradoxa* dependent on cultivation time. Scale values of ordinate: a) optical density at 490 nm (carotenoid shoulder) and at 750 nm (dispersion, RCN at zero time 4.4×10^6 cells/ml, after 5.5 days: 1.6×10^7 cells/ml), b) Chl concentration ($\mu\text{g/ml}$), c) PC and APC concentration ($\times 100 \mu\text{g/ml}$, e.g. 0.5 = 50 $\mu\text{g/ml}$).

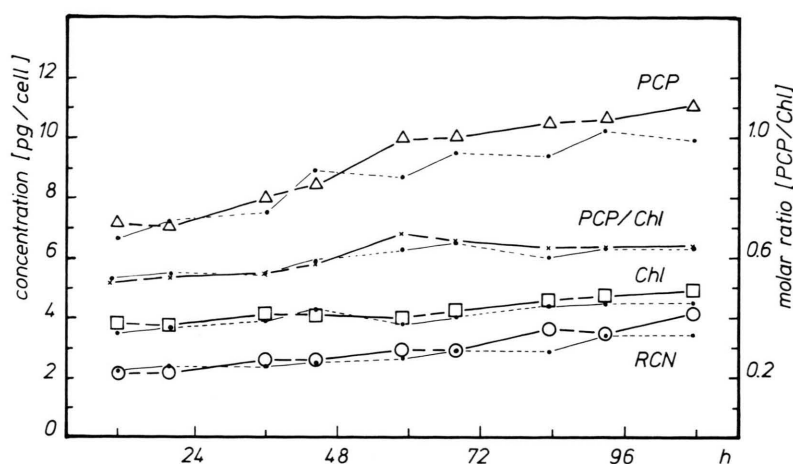


Fig. 3. *C. paradoxa*: Variations of tetrapyrrolpigment content in two cultures with inverse light-dark conditions (nitrogen content, see normal medium: 4 mM KNO_3). Broken line: dark conditions. PCP/Chl = molar ratios in the sum of phycochromoproteids to chlorophyll.

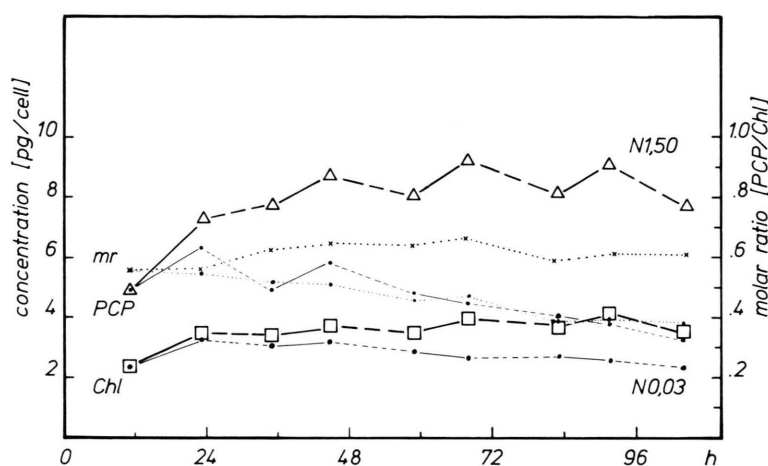


Fig. 4. *C. paradoxa*: Variation of tetrapyrrolpigment content per cell in two cultures with different nitrogen concentrations: N1,5 = 6 mM KNO_3 , N0,03 = 0.12 mM KNO_3 .

trations on the basis of the *in vivo* VIS-spectra leads to results similar to those given for *Anacystis nidulans* [14]:

1) Following the dilution of the cultures with new medium, the biosynthesis of pigments begins after ca. 10 to 12 h.

2) The velocities of biosynthesis for the various pigments differ.

3) The division rate of cells accelerates ca. 10 h later than the biosynthesis of pigments. Similar observations were made by Marten *et al.* [26, 27] for the synthesis of chlorophyll.

4) A rapid decomposition of phycochromoproteids can be observed after 2 to 5 days.

5) The molar ratio (mr) of phycochromoproteids to chlorophyll is very constant, normally approx. 0.63 (it increases in the exponential growth phase and

decreases in the late stationary phase), the molar ratio of APC to PC is also constant and higher as referred [19], approx. 1–1.3:1.

It is now a question of the extent to which the concentrations of phycochromoproteids vary with the age of the culture, with light conditions (light-dark alterations) or with the concentration of nitrate in the medium (NH_4 ions are a very toxic [20] substance for the cells of *C. paradoxa* ($\text{LC}_{100} < 1.3$ mM) and, therefore, they play no part as a source of nitrogen in *C. paradoxa*). The cultivation of *C. paradoxa* in media with different concentrations of nitrate (Fig. 4) demonstrates a significant dependency of the absolute concentration of phycochromoproteids in the culture and in the cells respectively, not only on nitrate content in the medium, but also on the age of the culture and the

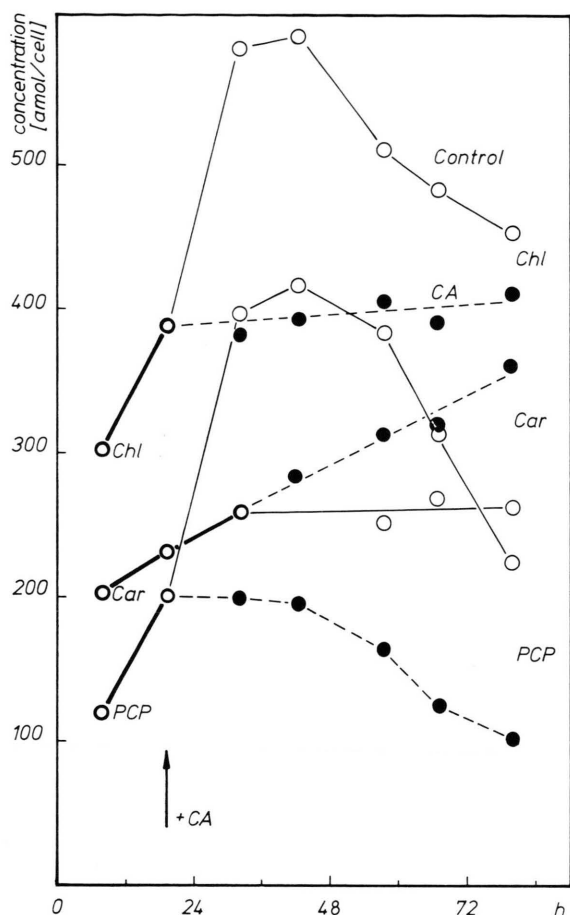


Fig. 5. *C. paradoxa*: Different influence of Chloramphenicol on the contents of various pigments in the cyanelles.

cultivation time. The variation of concentrations dependent on nitrogen and light conditions is very similar for both PC and APC, therefore the concentration values of phycochromoproteids are combined in the Figs. 3 and 4. These two figures show that in *C. paradoxa* the biosynthesis of phycochromoproteids, and to a lesser extent that of chlorophyll, is dependent on a high energy level in the cells of the symbiotic consortium (and therefore

indirectly on light). This is in contrast to the biosynthesis of the carotenoids which remains more or less constant at ca. 140 fg/cell. It seems that the cyanelles synthesize their tetrapyrrolpigments dependent on the stored energy, preferably in the light. During the first 9 h after darkening of the cultures the concentration of phycochromoproteids remains constant, but then the decomposition of phycochromoproteids begins and after more than 24 h the decrease in the content of phycochromoproteids in the cells can be clearly observed. This effect can be demonstrated equally so impressively by inhibition of the prokaryotic translation with chloramphenicol (Fig. 5). In this case the concentration of Chl remains constant, that of the carotenoids increases, whereas the PCP content per cell decreases after 1-day incubation time. The eukaryotic cells survive 0.2 mg chloramphenicol/ml culture for more than a week. After that time the flagellate shows a high mobility under microscopic identification, whereas the endocyanelles are bleached to yellow. If the flagellate is now transferred to a fresh medium without chloramphenicol the cells will green again. It is notable that during treatment of cultures with cycloheximide the content of phycochromoproteids is not influenced to the same high degree as is the case with chloramphenicol [28–29].

We conclude that the phycobiliproteids of *C. paradoxa*, in addition to their function as accessory pigments, also serve as nitrogen storage proteins, but usually only in the strong stress situation of nitrogen starvation ("stress proteins"). The manner in which the cytoplasm of the eukaryotic host cell is able to regulate the concentration of phycobiliproteids in the cyanobacterial endocytobiotic guest is still unclear. Perhaps there is a complicated equilibrium, based on diffusion exchanges between the endocyanelles and the cytoplasm, between distinct amino acids and the phycobiliproteids, catalyzed by enzymes, which under deficiency of these amino acids in the cytoplasm leads to preferred decomposition of the phycobiliproteids in the endocyanelles.

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