

Physiological Characterization of *Cyanophora paradoxa*, a Flagellate Containing Cyanelles in Endosymbiosis

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Culture conditions are described under which *Cyanophora paradoxa* grows with a generation time of less than two days. The organism is an obligate photoautotrophic flagellate unable to degrade exogenous carbohydrates via respiration. It does not synthesize nitrogenase but can form a hydrogenase with similar properties as in blue-green algae. The photosynthetic O₂-evolution proceeds with essentially the same rate as in green or blue-green algae. Besides CO₂, *p*-benzochinon, nitrate and nitrite also stimulate the photosynthetic O₂-evolution. Nitrite reduction is strictly light-dependent where the stoichiometry between nitrite-disappearance and photosynthetic O₂-evolution is 1:1.5. It is concluded that the cyanelles supply the eucaryotic host both with carbon and nitrogen compounds.

Cyanophora paradoxa is a small, unicellular flagellate difficult to categorize systematically. It was classified either with cryptomonads [1] or with primitive dinoflagellates [2], and there were also proposals to consider an own class of organisms comprising *Glaucocystis* and *Gloeochaete* besides *Cyanophora* [3, 4]. More interesting to note, *Cyanophora* contains 1–6 endosymbiotic, blue-greenish structures which are termed cyanelles, which reproduce in an autonomous way [5] and which obviously take over functions of the chloroplasts of higher plants [6]. The nature of these cyanelles is also unclear at present. Cytological investigations [7] and the pigment analysis [8] indicated that the cyanelles were of cyanophycean origin. However, Schnepf and Brown [9] considered the cyanelles as chloroplasts and the intact organisms as primitive algae of the rhodophycean type. A recent study [10] of the DNA content and of the genome size also indicated that the cyanelles are chloroplast type organelles rather than endosymbiotic blue-green algae.

The unresolved taxonomic affinity is not the only feature which deserves attention with *Cyanophora*. Such an organism where blue-green algae or chloroplast type organelles are housed in an eucaryotic cell may constitute an ancient relict in the evolution of chloroplasts and mitochondria. Moreover, there

are now attempts to bring nitrogen fixing blue-green algae into protoplasts of higher plants [11]. These have as their final goal the establishing of stable symbiotic organisms which would be self-supplying in nitrogen compounds. With *Cyanophora*, nature may already have provided such a stable symbiosis which was, however, not yet investigated with respect to its nitrogen metabolism. This was probably due to the fact that *Cyanophora* and all other organisms containing cyanelles grew very slowly which impeded physiological experiments.

The present investigation was undertaken to improve the culture conditions for *Glaucocystis geitleri* and *Cyanophora paradoxa*. Whereas all attempts to obtain short growth rates failed with the former organism, conditions were found where *Cyanophora* consistently grew with a generation time of less than two days. Thus sufficient quantities of cells were available for a physiological characterization of *Cyanophora*. This communication reports experiments on photosynthesis, respiration, hydrogen metabolism and nitrogen assimilation with emphasis on the latter process.

Materials and Methods

Cyanophora paradoxa was generously given to us in pure culture by Dr. Kies, Hamburg. *Glaucocystis geitleri* was No. 229–5 of the Sammlung von Algenkulturen of the Pflanzenphysiologisches Institut, University of Göttingen, Germany. *Cyanophora* was grown in sterile media with three different nitrogen sources. The nitrate-medium contained the following salts in M: KNO₃, 10^{–3}; MgSO₄ × 7 H₂O,

Abbreviations: Chl, chlorophyll; DCMU, N'-(3,4-dichlorophenyl) N,N-dimethylurea; FCCP, carbonylcyanid-*p*-trifluoromethoxyphenyl-hydrazone (= *p*-CF₃-CCP).

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4×10^{-5} ; $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 5×10^{-5} ; CoCl_2 , 10^{-6} ; H_3BO_3 , 10^{-5} ; $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$, 10^{-5} ; $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$, 10^{-6} ; $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$, 10^{-7} ; $\text{NaMoO}_4 \times 2 \text{H}_2\text{O}$, 10^{-7} ; $\text{K}_2\text{HPO}_4 \times 3 \text{H}_2\text{O}$, 7.5×10^{-5} ; FeSO_4 , 2.5×10^{-5} as the EDTA complex, vitamin B_1 , 4×10^{-6} ; vitamin B_{12} , 1.2×10^{-8} ; a mixture of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ adjusted to pH 8.0, 8×10^{-4} . In the ammonia-medium, nitrate was replaced by $(\text{NH}_4)_2\text{HPO}_4$, 3.75×10^{-4} , and the nitrate-ammonia medium contained KNO_3 , 1×10^{-3} plus $(\text{NH}_4)_2\text{HPO}_4$, 7.5×10^{-5} . The phosphate salts and the iron-EDTA complex were autoclaved separately, and the vitamins were added after sterile filtration. With all media, the pH-value was adjusted to 8.0. The same media were also tried for *Glaucocystis* with different pH-values varying from 6.5 to 8.0.

Cyanophora was grown at 24° or 18°C and 1100 lux in 100 ml Erlenmeyer or 2 l Fernbach flasks closed with cotton wool, and diluted with fresh culture medium at the rate of approximately 1:5 every three to four days. The motile cells distributed almost homogeneously throughout the culture medium, although some accumulation was often observed on the surface of the solution. For the experiments, the cultures were concentrated 10–20 fold of the original volume by centrifuging at $5000 \times g$ and 4°C for 10 min and suspending the cells in the medium desired in the assays.

The photosynthetic and respiratory activities were assayed by conventional Warburg manometry. The photosynthetic O_2 -evolution rate was determined under argon. Before the start of the experiments, the cells were routinely illuminated in the vessels for 30–60 min. This completely terminated the O_2 -evolution activity probably caused by the endogenous CO_2 which could not be removed by gassing. After tipping the Hill-reagents from the side arms of the vessels, the assays were conducted at 25°C for 1–2 h and 35 000 lux. Such high light intensities did not affect the photosynthetic activities of the organism in these short-termed experiments. In order to determine the respiratory O_2 -uptake capabilities, the center well of the Warburg vessels contained 0.2 ml of 10% KOH to bind the CO_2 evolved.

The measurement of the C_2H_2 -reduction (nitrogen fixation) and H_2 -uptake activities were described in detail in the preceding publications [12, 13].

In order to determine the nitrate and nitrite assimilation rates, the organisms were washed twice in a medium free of nitrate before the assays were

conducted for 1 h and 25°C . They were terminated by boiling the organisms for 15 min to completely release the nitrate and nitrite not assimilated. After centrifugation, the amount of nitrite could directly be measured with Gries-Ilovay's reagent (α -naphthylamine and sulfanilic acid) in the supernatant. Nitrate was also determined as nitrite. For this purpose, it had to be converted enzymatically with nitrate reductase from *Escherichia coli* using a slightly modified version of the procedure described by Ohmori and Hattori [14]. The reaction mixture to convert nitrate to nitrite contained in a final volume of 3.8 ml in μmol : Tris-HCl-buffer pH 7.6, 200; $\text{Na}_2\text{S}_2\text{O}_4$, 30; methyl viologen, 3; NaHCO_3 , 30; NaNO_3 to be converted 0–5; and 2 ml of the nitrate reductase preparation (see below). The reaction mixture was incubated for 30 min at 30°C in test tubes, and during this period care was taken that the solutions remained deep blue due to reduced methyl viologen. To end the reaction, the dye was oxidized by air, and the test tubes were boiled. After centrifugation, the Gries-Ilovay's reagent was added to the supernatant, a resulting precipitate was removed by centrifugation and the extinction of the diazo couple was determined at 546 nm 15 min after the addition of the reagent. As a reference, a standard of nitrate, treated in exactly the same way as the samples, was required, since nitrate was only partially recovered as nitrite in these assays with *Escherichia coli* nitrate reductase.

Nitrate reductase was either purchased from Sigma, St. Louis, No. N 3255 or (preferentially) prepared by suspending 2 g of *Escherichia coli* cells (obtained from Merck, Darmstadt, No. M 1132) in 10 ml of 0.2 M Tris-HCl buffer pH 7.6 containing 2 mg of dithiothreitol. The cells were broken by sonication and centrifuged for 10 min at $10\,000 \times g$. The supernatant was used as the nitrate reductase preparation.

Chlorophyll was determined in cells broken by sonication using the extinction coefficient of $82 \text{ mm}^{-1} \times \text{cm}^{-1}$ at 663 [15]. Protein was measured according to Lowry *et al.* [16]. Cell numbers were counted in a Thoma chamber after having immobilised the organisms by adding a drop of formaldehyde to the suspension. Chlorophyll and carotinoids were separated by thin layer chromatography and phycobiliproteins by gel-filtration on Sephadex G-200. The spectra were recorded with a Perkin-Elmer 200 photometer and compared to standards.

Results

In the literature [6], *Cyanophora* was reported to grow very slowly. Using the three media described under Materials and Methods, the organism consistently showed fast growth (Fig. 1). The exponential phase lasted from the second to the sixth day after the inoculum, and the generation time was 1.8–2.4 days in the nitrate-ammonia medium and 1.6 to 2.0 days when nitrate was the only source for the cultures. In the ammonia-medium, the generation time was somewhat longer with 2.0–3.0 days. *Cyanophora* grew strictly photoautotrophically. The addition of organic carbon compounds (glucose, fructose, lactose, galactose, maltose, sucrose, succinate, pyruvate, acetate) to the media did not enhance the growth rate; on the contrary, concentrations above 0.1 osmolar of all these compounds caused the organisms to bleach out and finally to die.

Vitamin B₁ and B₁₂ were routinely added to the media but the absolute dependence of the growth on these substances was not seriously checked. The temperature could vary between 18 ° and 24 °C and low light intensities (under 1500 lux) were required. *Cyanophora* was easy to handle and thus is an organism suitable for physiological experiments. By comparison, the generation time is 20 h for the nitrogen fixing species *Anabaena cylindrica* and *Nostoc muscorum*.

Contrary to the situation with *Cyanophora*, the generation time of *Glaucocystis geitleri* was 15–20 days under best obtainable conditions with all nutrient solutions tried. Moreover, this organism tended to adhere tightly to the glass of the vessels which could only partially be prevented by covering the bottom of the flasks with a sheath of agar. In addition, the cells did not homogeneously suspend in solutions. Therefore the following experiments were exclusively done with *Cyanophora*.

The pigment analysis confirmed the results of Chapman [8]. *Cyanophora* contained chlorophyll a, c- and allo-phycoerythrin, β -carotene and zeaxanthin. Chlorophyll b, lutein, echinenone as well as myxoxanthophyll were not detected. In NO₃⁻-grown cultures harvested from the exponential growth phase, 1 mg chlorophyll corresponded to 3.25×10^9 cells and to 95 mg soluble protein, whereas the ratio between chlorophyll, cell number and protein content was $1:2.43 \times 10^9:62$ in NH₄⁺-grown cells.

The photosynthetic O₂-production at the expense of NaHCO₃ was approximately 120 μ mol/h \times mg chlorophyll and hence is in the same range of the rates observed with blue-green or green algae. Of the other electron acceptors tested (Table I), *p*-benzochinon, nitrite and to some extent also nitrate could stimulate the photosynthetic O₂-production,

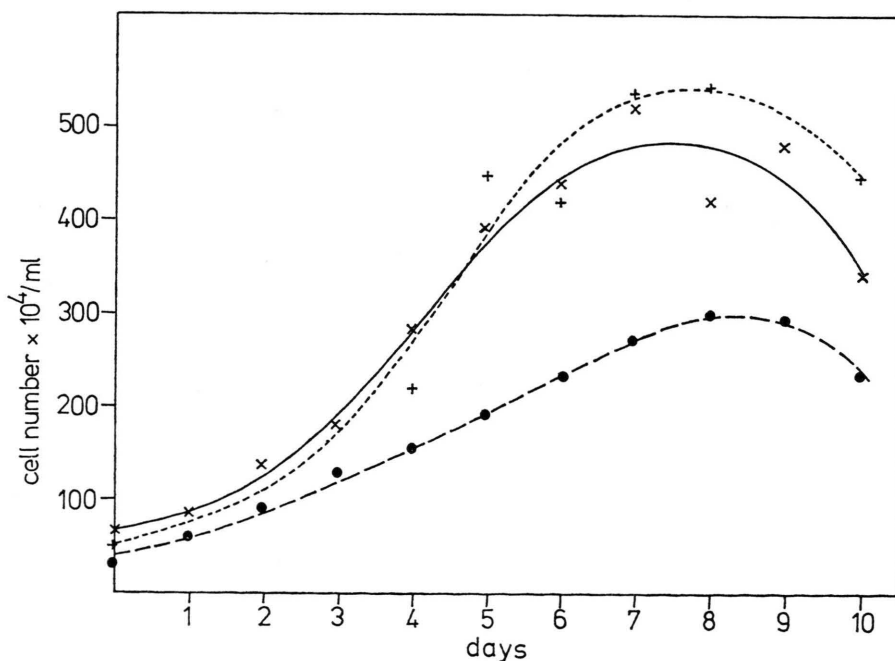


Fig. 1. Growth of *Cyanophora paradoxa* in three different media.
 ×—× NO₃⁻-medium;
 ●—● NH₄⁺-medium;
 +---+ NO₃⁻+NH₄⁺-medium.

Table I. The photosynthetic O_2 -evolution by intact *Cyanophora paradoxa* in the presence of various electron acceptors. The Warburg experiments were conducted for 30–60 min at 25 °C and 35 000 lux under argon. The vessels contained in a final volume of 3 ml: chlorophyll, 0.03–0.09 mg; electron acceptors, 10 μ mol, unless indicated otherwise. DCMU was 1×10^{-5} M in No. 9 and 10.

Electron acceptor	μ mol O_2 evolved/ h \times mg Chl
1. —	— 3
2. $NaHCO_3$	+122
3. $K_3Fe(CN)_6$	+ 3
4. <i>p</i> -benzochinon	+ 33
5. $K_3Fe(CN)_6$ + <i>p</i> -benzochinon (0.5 μ mol)	+ 28
6. <i>p</i> -benzochinon (0.5 μ mol)	+ 29
7. $NaNO_3$	+ 13
8. $NaNO_2$ (5 μ mol)	+ 55
9. <i>p</i> -benzochinon + DCMU	— 7
10. $NaNO_2$ + DCMU	\pm 0

whereas $K_3Fe(CN)_6$ did not do so. The latter compound was also ineffective in the presence of catalytic amounts of *p*-benzochinon, contrary to the situation in *Anacystis nidulans* [17]. The O_2 -production in *Cyanophora* was affected by the herbicide DCMU similar as in other photosynthetic organisms having two light reactions.

The endogenous respiratory rate accounted to 15 μ mol O_2 consumed/h \times mg chlorophyll in the dark. This activity was not stimulated by glucose, fructose, maltose, galactose, sucrose and succinate, which might reflect the obligate photoautotrophic

nature of this organism. Some enhancement of the rates was observed by pyruvate (18 μ mol O_2 consumed/h \times mg chlorophyll) and acetate (22 μ mol) which was, however, marginal. The endogenous respiration was not stimulated by the uncouplers dinitrophenol and FCCP.

Table II indicates that *Cyanophora* did not reduce acetylene when the cultures were kept either aerobically or anaerobically in the absence of any combined nitrogen. Since the organism did not grow under these conditions, it is obviously unable to synthesize nitrogenase. It can, however, form hydrogenase. This enzyme was not present in air-grown cells but was induced by anaerobic conditions and became fully active when the cultures were continuously flushed with molecular hydrogen (Table II).

Table II. H_2 -consumption and N_2 -fixation (C_2H_2 -reduction) by *Cyanophora paradoxa*. The organism was grown for 3–6 days in the nitrate medium a) anaerobically under flushing with a mixture of 20% H_2 / 75% N_2 / 5% CO_2 , b) anaerobically under flushing with 95% N_2 / 5% CO_2 , c) aerobically without flushing, in 1 liter flasks at 22 °C and 1100 lux. Rates are given per h \times mg Chl.

Culture condition	μ mol C_2H_4 formed	μ mol H_2 consumed
anaerobically $H_2/N_2/CO_2$	0	52
anaerobically N_2/CO_2	0	6
aerobically —	0	0

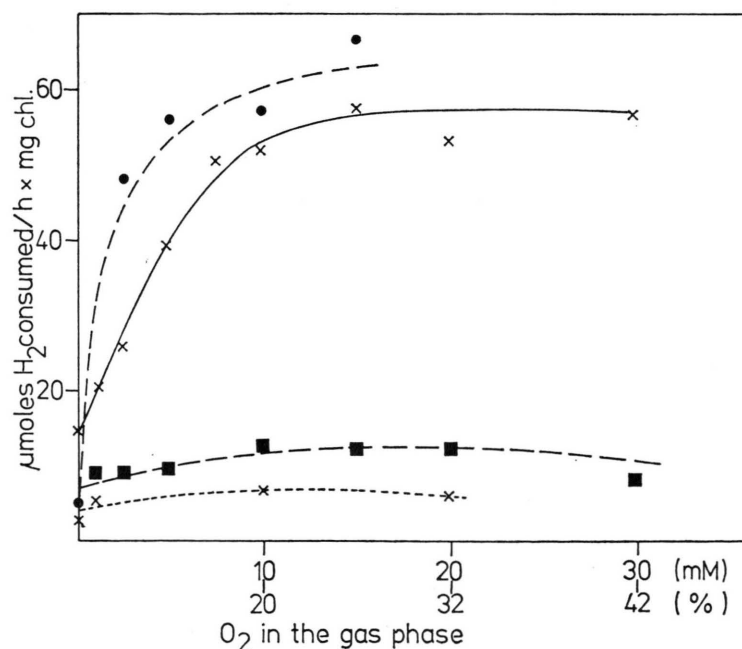


Fig. 2. Dependence of the H_2 -consumption on the presence of varying concentrations of oxygen. a) *Cyanophora* grown under $H_2/N_2/CO_2$ (see Table II) ●—● assayed in the dark; ×—× assayed in the light. b) *Cyanophora* grown under N_2/CO_2 . ■—■ assayed in the dark; ×—× assayed in the light.

Similar as recently observed for several blue-green algae [18], hydrogen uptake in *Cyanophora* was dependent on the presence of oxygen in the vessels. Fig. 2 shows that the optimal concentration of this gas was approximately 10 mmolar which corresponds to 20% in the gas phase. High levels of oxygen in the assays did not significantly decrease the H_2 -consumption activity, indicating that hydrogenase in intact *Cyanophora* is virtually insensitive to exposure to oxygen.

Since *Cyanophora* grew on nitrate as the sole source of nitrogen, it is able to form the assimilatory nitrate and nitrite reductases. Table III shows that nitrate disappeared in the assays conducted in the dark. The rate of nitrate reduction was enhanced more than twofold when the samples were illuminated, and this light-dependent activity was accompanied by a production of oxygen. Contrary to nitrate, no nitrite was consumed in the dark, whereas the assimilation rate was high in illuminated samples (Table III). This already indicated that nitrite reduction was linked to the photosynthetic electron transport system of the cyanelles. The situation appeared to be similar as in chloroplasts of higher plants where nitrite is reduced at the acceptor side of photosystem I in a ferredoxin dependent reaction [19]. This would mean that nitrite reduction in *Cyanophora* should be accompanied by a stoichiometric release of oxygen according to the following equations:

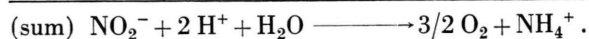
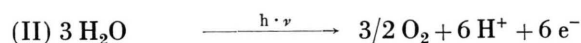
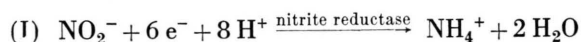


Table III. The reductions of nitrate and nitrite by *Cyanophora paradoxa*. The assays were conducted at 25 °C, 1 h and 35 000 lux or in the dark. The vessels contained in a final volume of 3 ml: nitrate or nitrite, respectively, 5 μmol , *Cyanophora* with 0.05 mg Chl. Negative data in the table are due to endogenous respiration.

	Light	Dark
<i>nitrate reduction</i>		
a) nitrate consumption	24	10
b) O_2 -evolution	6	-5
<i>nitrite reduction</i>		
a) nitrite consumption	40	0
b) O_2 -evolution	56	-5

Table IV. Stoichiometry between nitrite disappearance and photosynthetic O_2 -evolution. Data are given in μmol . Experimental conditions as in Table III.

1. NO_2^- added at the start of the experiment	2	5	10
2. NO_2^- recovered at the end of the experiment	0.8	2.9	8.3
3. NO_2^- consumed	1.2	2.1	1.7
4. O_2 evolved photosynthetically	1.8	2.9	2.7
5. ratio O_2 formation: NO_2^- consumption	1.5:1	1.4:1	1.6:1

Table IV shows that the theoretical ratio of 1.5:1 between photosynthetic O_2 -production and nitrite disappearance closely matched the results of the experiments with three different nitrite concentrations. Thus the conclusion is valid that nitrite reduction is exclusively confined to the cyanelles of *Cyanophora*.

Dinitrophenol is known to cause green algae to excrete nitrite to the medium [20]. This effect cannot yet unequivocally be interpreted, but it appears to be restricted to eucaryotic cells. Table V shows that such an excretion of nitrite was observed with the green alga *Ankistrodesmus braunii* as a control, but neither in the blue-green algae *Anabaena cylindrica* and *Anacystis nidulans* nor in *Cyanophora paradoxa*, regardless of whether high or low cell numbers were tried in the assays. From this we tentatively conclude that nitrate assimilation in *Cyanophora* follows the same mechanism as in blue-green algae, although more organisms should be investigated before such a generalization is safe.

Discussion

The present investigation shows that *Cyanophora* is an organism easy to grow and to handle. It is an obligate photoautotrophic flagellate which is unable to consume sugars by respiration. Concentrations

Table V. Nitrite excretion caused by 2–4 dinitrophenol at the nitrate reduction of some algae. Data are given in nmol NO_2^- formed/vessel. The assays contained the following cell numbers of algae: *Ankistrodesmus*, 2×10^7 ; *Anacystis*, 600×10^7 ; *Anabaena cylindrica* B 629, 30×10^7 ; *Cyanophora*, 20×10^7 .

Alga	Concentration of dinitrophenol		
	—	$1 \times 10^{-4} \text{ M}$	$2 \times 10^{-4} \text{ M}$
1. <i>Ankistrodesmus braunii</i>	0.2	11.7	24.0
2. <i>Anacystis nidulans</i>	0.5	0.2	0.2
3. <i>Anabaena cylindrica</i>	0.5	0.0	0.0
4. <i>Cyanophora paradoxa</i>	0.5	0.5	0.4

of these substances above 0.1 osmolar cause irreversible damage to the organism, probably due to osmotic effects. It is possible that the permeases for the transport of sugars into the cells are lacking in *Cyanophora* or in the cyanelles similar to the situation in most blue-green algae [21].

The obligate photoautotrophic nature is not the only feature which *Cyanophora* shares with these organisms. There are close similarities in the pigment garniture, although echinenone and myxoxanthophyll, which are typical for blue-green algae [22], are lacking in *Cyanophora*. Molecular hydrogen is consumed in an oxygen-dependent, Knallgas-type reaction, and hydrogenase is virtually insensitive to exposure to oxygen, as in blue-green algae [18], but contrary to the situation in eucaryotic algae [23]. Also nitrate assimilation shares common characteristics. The experiments reported in this communication showed that nitrite release to the medium caused by dinitrophenol is demonstrable in the green algae *Ankistrodesmus*, but not in *Anacystis* and *Anabaena* and also not in *Cyanophora*. In the blue-green alga *Nostoc* [24], nitrate as well as nitrite reductions are particle-bound and ferredoxin-dependent, whereas the formation of nitrite from nitrate catalyzed by nitrate reductase of higher plants requires NADH and is probably located in the cytoplasm [25]. In *Cyanophora* assayed in the light, both nitrate and nitrite reductions were accompanied by a release of oxygen produced in photosynthesis which should be expected if both reaction are ferredoxin dependent as in *Nostoc*. It should be stressed here, however, that these observations about the physiology are still too isolated to allow conclusions on the taxonomy of *Cyanophora*. More physiological

as well as genetic and cytological investigations are required to establish the relationship between *Cyanophora* and blue-green algae or rhodophyta.

Schenk and Hofer [26] showed that *Cyanophora* has a regular Calvin cycle to fix carbon dioxide. It is obvious from the obligate photoautotrophy of this organism that the cyanelles must provide the eucaryotic cell with an organic carbon compound. The host must also be dependent on a supply of nitrogen from the organelles. This latter statement is based on the observation presented here that nitrite reduction is strictly light-dependent and coupled to the photosynthetic O₂-formation and therefore must occur in the cyanelles. It is unlikely that the disappearance of nitrite in the light is an artifact or a side-reaction to get rid of poisonous nitrite from *Cyanophora*, since the compound was shown to be an intermediate of the assimilatory nitrate reduction with all organisms so far investigated.

Further experiments have to show which carbon and nitrogen compounds are transferred from the cyanelles to the eucaryotic cell. Such a study requires the isolation of metabolic active cyanelles which is attempted. It is possible that *Cyanophora* will turn out to be one of the most useful systems for the study of the interrelationship between an organism of procaryotic nature and an eucaryotic host.

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