Is cyanophycin involved in the integration of nitrogen and carbon metabolism in the cyanobacteria *Anabaena cylindrica* and *Gloeotheca* grown on light/dark cycles?

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(Received 19 December 1989; revised 30 April 1990; accepted 6 July 1990)

In *Anabaena cylindrica*, protein synthesis continued during dark periods at 80% of the rate observed in the light. Since nitrogen fixation ceases in the dark, this implies that fixed nitrogen accumulates during the light periods to supply amino acids for protein synthesis in the dark. Measurements of cyanophycin and the distribution of nitrogen in subcellular fractions indicated that cyanophycin does not represent a significant proportion of total cell nitrogen, nor does its concentration vary across the light/dark cycle. Similar results were obtained with *Gloeotheca*; there was no evidence that cyanophycin accumulated during the dark to support protein synthesis in the light. Cyanophycin does not, therefore, appear to serve as a dynamic temporary storage form of newly fixed nitrogen in the integration of light and dark metabolism. We also investigated an immunological approach to the measurement of cyanophycin and its turnover. Cyanophycin is immunogenic, and in the pure state could be assayed by radioimmunoassay, which had greater sensitivity than traditional assay procedures. The insolubility of cyanophycin at neutral pH, however, prevented the successful development of methods for quantifying cyanophycin turnover in cell extracts.

Introduction

Cyanobacteria are unique in that they are capable of both nitrogen fixation and oxygenic photosynthesis. The oxygen sensitivity of nitrogenase requires that these processes be separated in nitrogen-fixing cyanobacteria. Heterocystous cyanobacteria achieve this by confining nitrogen fixation to heterocysts while CO₂ fixation occurs in vegetative cells. Thus a spatial separation exists between the two processes. When heterocystous organisms such as *Anabaena cylindrica* are grown on light/dark (L/D) cycles, both CO₂ and nitrogen fixation occur in the light. In contrast, non-heterocystous cyanobacteria, such as *Gloeotheca* and *Synechococcus*, separate CO₂ and nitrogen fixation temporally (Mullineaux *et al.*, 1981*a*; Mitsui *et al.*, 1986). When these organisms are grown on L/D cycles, nitrogen fixation occurs in the dark, and CO₂ fixation occurs in the light.

Balanced growth has been defined as the state where the concentrations of all cellular components increase at

the same rate (Shuter, 1979), and if an organism is to achieve this, nutrients must be provided in the correct proportions at the appropriate time and place (Carr, 1988; Romero & Lara, 1987). In this paper, balanced growth during L/D cycles is defined as the synthesis of protein, nucleic acids, chlorophyll and structural carbohydrate. It does not include the glycogen synthesized during light periods, which is used to provide energy to support cellular processes in the dark (cf. Mur, 1983). Because the strategies adopted by nitrogen-fixing cyanobacteria to protect nitrogenase from oxygen involve the separation of nitrogen and carbon fixation, if growth is to occur throughout the L/D cycle the supplies of fixed carbon and nitrogen must be appropriately regulated. Fixed carbon can be stored temporarily in the form of sugars and glucan, and Foy & Smith (1980) showed that two *Oscillatoria* species incubated under L/D cycles continued to grow in the dark at the expense of glycogen accumulated during the preceding light period. The supply of nitrogen required for protein synthesis in the dark also depended partly on nitrate taken up during the light, which implies that the nitrogen was stored in some form. The nature of this temporary storage form of fixed nitrogen is not yet known.

Abbreviations: butyl-PBD, 2(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole; TBS, Tris-buffered saline; TTBS, TBS containing 0.1% Triton X-100; L/D, light/dark.

If nitrogen-fixing *A. cylindrica* were to continue growing during the dark phase of a L/D cycle it would also need a supply of fixed nitrogen in addition to the supply of glycogen, since nitrogen fixation ceases in the dark in this strain (Mullineaux *et al.*, 1981*a*). In contrast, *Gloeotheca* fixes nitrogen in the dark at the expense of glycogen accumulated in the light. The supply of glycogen is limited, however, and protein synthesis measurements in *Gloeotheca* have indicated that most protein synthesis occurs in the light (Mullineaux *et al.*, 1981*b*). Therefore it would appear that both *A. cylindrica* and *Gloeotheca* need to be able to accumulate a temporary store of fixed nitrogen to buffer their metabolism against the interruptions in nutrient supply which growth on L/D cycles imposes.

The experiments described in this paper investigate the possibility that cyanophycin might act as a temporary store of fixed nitrogen in the integration of nitrogen fixation and photosynthesis. Cyanophycin is the copolymer of aspartate and arginine, first isolated by Simon (1971). Most cyanobacteria accumulate cyanophycin at some point in their growth cycle (Lawry & Simon, 1982; Allen, 1984). Simon (1973) showed that cyanophycin accumulated in stationary-phase *A. cylindrica* and that it was degraded when such cells were used to reinitiate growth. He proposed that cyanophycin served as a nitrogen reserve under these conditions. It has also been suggested that cyanophycin may serve as a dynamic reservoir of newly fixed nitrogen to buffer nitrogen-fixing cells against fluctuations in environmental supply (Carr, 1983, 1988; Carr & Wyman, 1986). This hypothesis is based on the observation that both the synthetic and degrading enzymes for cyanophycin are more active in heterocysts of nitrogen-fixing *Anabaena* species than in the vegetative cells (Gupta & Carr, 1981). Carr (1983) proposed that 'cyanophycin serves, in both heterocysts and vegetative cells, as a reservoir of newly assimilated nitrogen' which is then used for other cell processes. This hypothesis would make cyanophycin an integral part of the interplay between nitrogen and carbon metabolism.

The aim of the experiments reported in this paper was to establish whether cyanophycin levels altered during the L/D cycle in a manner consistent with cyanophycin acting as a temporary store of fixed nitrogen. The paper reports measurements of growth, protein synthesis, nitrogen incorporation and cyanophycin levels made in *A. cylindrica* grown on L/D cycles under nitrogen-fixing conditions and measurements of nitrogen incorporation and cyanophycin levels in *Gloeotheca* grown similarly.

The investigation of cyanophycin metabolism has been hampered by the lack of specificity and relative insensitivity of the assay procedure (Simon, 1987). This has been a continuing problem in our work with cyanophycin (for example see Daday *et al.*, 1988). Simon

(1987) has reported that cyanophycin is antigenic and this paper also reports our attempts to develop suitable immunoassays for cyanophycin.

Methods

Cyanobacterial growth. *A. cylindrica* (ATCC 27899) was obtained from the American Type Culture Collection and grown as previously described (Daday *et al.*, 1977, 1985) in the medium of Allen & Arnon (1955) diluted eightfold for all components except phosphate (full strength, 2 mM) and NiSO_4 ($4 \times$ full-strength, $0.68 \mu\text{M}$) at a light density of $150 \mu\text{E m}^{-2} \text{s}^{-1}$. Cultures were sparged with 0.3% CO_2 in air or in air alone as specified for individual experiments. *Gloeotheca* (ATCC 27152) was obtained from the American Type Culture Collection. Sheathless *Gloeotheca* (PCC 6909-1, Kallas *et al.*, 1983) was a gift from Dr J. Gallon, Swansea, UK. Both strains were grown in full-strength Allen & Arnon (1955) medium supplemented with 0.5 mM- NaHCO_3 and $4 \times$ full-strength NiSO_4 ($0.68 \mu\text{M}$). Cells were sparged with air and grown at a light intensity of $20 \mu\text{E m}^{-2} \text{s}^{-1}$ (photosynthetically active radiation). Both organisms were grown on 12 h light/12 h dark cycles. For each experiment two identical cultures were inoculated and grown with the light periods 12 h out of phase.

Growth measurements. Growth was measured either by light scattering with a Klett-Summerson colorimeter (no. 66 filter, Mallette, 1969), by cell dry weight (Daday *et al.*, 1988) or by protein concentration. Protein concentrations were measured using the method of Bradford (1976), standardized with bovine plasma gamma-globulin (Bio-Rad reagents). Protein values are reported for the supernatant obtained after cells were broken with the French pressure cell (138 MPa) and centrifuged at 27000 g for 15 min.

Cyanophycin extraction and assay. Cyanophycin was extracted by the method of Simon (1973). Cell samples were concentrated approximately 10-fold by centrifugation and resuspension in 10 mM-HEPES/NaOH, 1 mM- Na_2EDTA , pH 7.5. Concentrated cell suspensions were stored frozen until cyanophycin extractions were done. Thawed cells were broken by a French pressure cell at 138 MPa. The cyanophycin content in the pooled acid extracts was determined with the Sakaguchi assay for arginine (Simon, 1973; Messineo, 1966). Arginine.HCl was used as the standard. The conversion factor between measured arginine and cyanophycin was determined by amino acid analysis and arginine assay of representative samples. One microgram of arginine in the colorimetric assay corresponds to $4.2 \mu\text{g}$ of cyanophycin measured by amino acid analysis.

Nitrogen content. Nitrogen was determined by a modification of the micro-Kjeldahl procedure (Bergersen, 1980; Daday *et al.*, 1985). When ammonia was recovered by diffusion, plastic centre wells (Kontes Scientific Glassware/Instruments, cat. no. 882320-0000) were used to hold the acid-soaked filter paper strips. For some samples ammonia was determined without the diffusion procedure, as described by Bergersen (1980), except that the concentration of NaOH in colorimetric reagent B was 95 g l^{-1} rather than 40 g l^{-1} .

Cells (50–100 Klett units) were concentrated approximately 10-fold by centrifugation, and resuspended in 10 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5. Samples (5 ml) of the concentrated cells were used to determine whole cell N-content. The remaining cells were frozen overnight and broken with the French pressure cell (138 MPa). The cell-free extract was centrifuged (27000 g, 15 min) and the pellet and supernatant were separated. Cyanophycin was extracted from the pellet fraction (Simon, 1973) and the nitrogen content of the resulting acid extract was determined. A portion of the supernatant was made 5% (w/v) in trichloroacetic acid (TCA). The pellet from this step was resuspended

in warm 0.5 M-NaOH (Ownby *et al.*, 1979) and the nitrogen content was determined in appropriate samples. Aliquots of the TCA-supernatant were also assayed. All samples were dried at 85 °C before acid digestion.

Protein synthesis in *A. cylindrica*. Cells were grown to 50–80 Klett units. At suitable times, duplicate 15 ml aliquots were transferred to 25 ml flasks, and incubated in growth cabinets with sparging. [^{14}C]Leucine or [^{14}C]protein hydrolysate [2.2 μCi (82 kBq) in 100 μl , Amersham] was added to each flask, and samples (100 μl cells) were taken at 0, 30, 60 and 90 min. Each sample was immediately frozen and thawed twice in liquid nitrogen, and then transferred to a glass-fibre disk. Protein was precipitated with cold 5% (w/v) TCA. Disks were washed twice with cold 5% (w/v) TCA, and three times with ethanol, then dried and transferred to scintillation vials containing 10 ml scintillant (toluene/2-methoxyethanol, 60:40, v/v, containing 6 g butyl-PBD l^{-1}). Radioactivity incorporated into TCA-precipitable material was counted with a Beckman LS2800 scintillation counter. Counting efficiency was 80%.

Antibody production. Outbred Swiss white mice were injected intraperitoneally with either 0.1 ml cyanophycin (1 mg ml^{-1} emulsified in sterile 0.15 M-NaCl using a sonic cleaning bath) or 0.1 ml cyanophycin emulsified with 0.1 ml Freund's incomplete adjuvant (Difco). The injections were repeated after 2, 15, 22 and 29 d. ELISA tests on serum obtained from blood via the optic plexus showed that antibodies were produced in mice which had received the cyanophycin/adjuvant mixture. On day 26 these mice were injected with Sarcoma 180 cells (approx. 0.1 ml of a 10% suspension) (Tikasingh *et al.*, 1966). Antibody-rich ascitic fluid was collected after approximately 7–14 d accumulation. The ascitic fluid was defatted, pooled, and stored in 100 μl aliquots at -20°C .

Enzyme-linked immunoassay (ELISA). ELISA assays were done in 96-well microtitration trays using standard methods. Microtitration plates were prepared by adsorbing 50 μl cyanophycin (40 $\mu\text{g ml}^{-1}$) in Tris-buffered saline (TBS, 10 mM-Tris/HCl, 50 mM-NaCl, pH 7) to each well for 1 h at room temperature. Unadsorbed antigen was removed by washing the wells three times with TBS. The wells were treated with 300 μl 0.5% defatted milk powder in TBS (room temperature, 30 min) to block non-specific binding, and then washed three times with 0.1% Tween 20 in TBS (TTBS). Serum or ascitic fluid was diluted into antibody buffer (5% milk powder in TTBS), added as appropriate to the wells and incubated for 1 h at room temperature. Alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin solution (diluted 1 in 3000) was used with *p*-nitrophenol phosphate for detection of the primary antibody according to the instructions of Bio-Rad Laboratories. The trays were screened using a Dynatech Model 2250 EIA reader.

Radioimmunoassay for cyanophycin. This assay was an adaptation of the methods of Davis *et al.* (1984) and La Duca *et al.* (1986). A square (10 $\times$ 10 cm) of nitrocellulose membrane filter was cut and washed in distilled water for 15 min. The filter was then marked with a soft pencil to produce a grid of squares (1 $\times$ 1 cm). After a further wash (15 min) in distilled water, the filter was cut into strips (10 $\times$ 1 cm) which were thoroughly dried before use. Samples (1–5 μl) containing cyanophycin were spotted onto the strips; if necessary larger volumes could be applied by drying the strips between applications. Once dry, the strips could be stored at 4 °C for at least 2 months.

Prior to use, the strips were washed for 15 min in TBS and for 30 min in blocking solution (0.5% milk powder in TBS). Ascitic fluid was diluted 1 in 300 into antibody buffer and the strips were incubated in this solution for 90 min at 37 °C. The strips were washed with TTBS and then incubated with ^{125}I -labelled sheep anti-mouse IgG solution [approx. 1 μCi (37 kBq) in 20 ml antibody buffer] for 2 h at room

temperature with gentle agitation. The strips were washed in five changes of TTBS over a period of 1 h and then allowed to dry before being cut into squares. Bound radioactivity was counted in an LKB Model 1270 Rackgamma II counter; all results are expressed as c.p.m.

Results

Patterns of growth and protein synthesis during light/dark cycles

It was first necessary to establish whether growth (as defined in the Introduction) of nitrogen-fixing *A. cylindrica* and *Gloeotheca* occurred predominantly during light periods or across both light and dark periods. Growth measurements using the methods of light scattering, dry weight and increase in total protein were not sufficiently sensitive to give a clear answer to this question (results not shown).

Consequently, protein synthesis in the light and dark was assessed in *A. cylindrica* by measuring the rate of incorporation of [^{14}C]leucine or other ^{14}C -labelled amino acids into TCA-precipitable material as described in Methods. The results of these measurements are shown in Fig. 1. Rates of protein synthesis in the dark were comparable to the rates in the light for both labels used.

Measurement of protein synthesis in the sheathed strain of *Gloeotheca* (ATCC 27152) was attempted using the same procedure as used for *A. cylindrica*. However, the thick carbohydrate sheath protected the cells against lysis by freezing and thawing, and thus the washing procedures following TCA-precipitation were not effective. Since Mullineaux *et al.* (1981b) have reported results for protein synthesis in *Gloeotheca* using a different method (Mullineaux *et al.*, 1980), these experiments were not pursued.

Cyanophycin levels during light/dark growth

Growth and protein synthesis measurements with *A. cylindrica* indicated that cells need to accumulate fixed nitrogen in some form during the light period to support the protein synthesis which occurs in the dark while nitrogen fixation is not active. Results of experiments to determine if fixed nitrogen accumulated as cyanophycin are also shown in Fig. 1. Cyanophycin did not accumulate significantly in nitrogen-fixing, exponentially-growing *A. cylindrica* at any time during light or dark periods irrespective of the growth conditions used. The following growth conditions all gave similar results: 12 h L/12 h D sparged with air or 0.3% CO_2 in air, 12 h L/12 h D with low light intensity (80 $\mu\text{E s}^{-1} \text{m}^{-2}$) and 6 h L/18 h D sparged with 0.3% CO_2 in air. The maximum value for cyanophycin shown in Fig. 1 corresponds to approximately 0.1% of dry weight. The detection limit for cyanophycin measurements typically corresponded

Fig. 1. Protein synthesis in nitrogen-fixing *A. cylindrica*. Protein synthesis was measured by incorporation of [^{14}C]leucine (●) and ^{14}C -labelled amino acids (○) into TCA-precipitable material. Plotted points represent rates of incorporation of label relative to cell dry weight. Initial rates of incorporation were linear for at least 60 min. The error bars represent the standard error of these slopes. Cells were grown in air under nitrogen-fixing conditions to initial cell densities of 66 Klett units (a) and 71 Klett units (b). Dry weights were measured at each time point. Cyanophycin contents of the cells are also shown (■). The maximum concentration of cyanophycin shown in (b) corresponds to approximately 0.1% of dry weight. Dark bars on the x-axes of this and Figs. 2 and 3 indicate dark periods.

Fig. 2. Distribution of nitrogen in cell fractions of nitrogen-fixing *A. cylindrica* grown on 12 h L/12 h D cycles under 0.3% CO_2 in air. Measurements were made during the light (a, b) and dark (c, d) periods. Initial Klett values were 82 (a, b) and 62 (c, d). (a, c) Changes in cell dry weight (▲). (b, d) Distribution of nitrogen in whole cells (●), in TCA-precipitable material (○), in TCA-supernatant (□) and in cyanophycin (■). Results are means $\pm$ SD.

to cyanophycin contents of 0.05% of dry weight. Similarly, cyanophycin did not accumulate in nitrogen-fixing *Gloeotheca* (not shown). In fact cyanophycin was frequently not detectable at all in either organism under these conditions of exponential growth in L/D cycles (cf. Simon, 1987).

Measurement of total nitrogen

Figs. 2 and 3 show the distribution of nitrogen in different cellular fractions of *A. cylindrica* and *Gloeotheca*.

thece, respectively, as a function of time. Cyanophycin accounted for less than 1% of total cell nitrogen and it did not vary across the L/D cycle in either organism. In *A. cylindrica* the total nitrogen content increased predominantly in the light period (Fig. 2b), which is consistent with the known pattern of nitrogen fixation in this organism. Newly-fixed nitrogen was found in the protein fraction (TCA-precipitable material), which would be consistent with the interpretation that this nitrogen was incorporated into amino acids, and used immediately for protein synthesis. Protein nitrogen also

Fig. 3. Distribution of nitrogen in cell fractions of nitrogen-fixing *Gloeotheca* grown on 12 h L/12 h D cycles under air. Measurements were made during the light (a, b) and dark (c, d) periods. Initial Klett values were 47 (a, b) and 52 (c, d). (a, c) Changes in cell dry weight ($\blacktriangle$). (b, d) Distribution of nitrogen in whole cells ($\bullet$), in TCA-precipitable material ($\circ$), in TCA-supernatant ($\square$) and in cyanophycin ($\blacksquare$). Results are means $\pm$ SD. Twenty-four hours after the first samples were taken, the cell densities had increased to 54 and 60 Klett units, respectively.

increased during the dark period. The increase ($2.5 \mu\text{g ml}^{-1}$) was about 80% of that observed in the light ($3.2 \mu\text{g ml}^{-1}$), which is consistent with the measured rates of protein synthesis (Fig. 1). The increase in protein nitrogen in the dark was also significantly greater than the observed increase in total nitrogen ($1.5 \mu\text{g ml}^{-1}$), which means that non-protein nitrogen must have been used to support the observed protein synthesis. If cyanophycin were the source of this nitrogen its pool size at the end of the light period would need to have been of the order of $1 \mu\text{g ml}^{-1}$ rather than the measured level ($0.1 \mu\text{g ml}^{-1}$). There is no evidence that the TCA-soluble fraction could have supplied this nitrogen, so the source of extra nitrogen remains unknown (cf. Foy & Smith, 1980).

In *Gloeotheca*, total nitrogen increased in the dark by $1.4 \mu\text{g ml}^{-1}$ (Fig. 3d), which agrees with the expected dark fixation of nitrogen under these conditions. The newly-fixed nitrogen apparently went straight into the protein pool, which increased by $1.0 \mu\text{g ml}^{-1}$ (Fig. 3d); there was no indication that any newly-fixed nitrogen entered the cyanophycin pool. As would be expected, total nitrogen remained constant in the light. Little protein synthesis occurred in the light period (Fig. 3b), which is perhaps explicable if all the fixed nitrogen had already been used for protein synthesis in the dark period. Both Klett units and dry weight increased, however (Fig. 3a). These data imply that glycogen accumulates during the light to support nitrogen fixation and protein synthesis in the dark.

Antigenicity of cyanophycin

Fig. 4 shows the results of a typical ELISA measurement of cyanophycin, using the ascitic fluid produced in mice. The antigen concentration was $2 \mu\text{g}$ per well, and the ascitic fluid was serially diluted from 1:15 to 1:30000 in antibody buffer. On the basis of these results it was concluded that antibodies directed against cyanophycin had been produced, and that cyanophycin was antigenic.

Development of a radioimmunobinding assay for cyanophycin

The radioimmunobinding assay procedure described in Methods was first tested on purified cyanophycin, which was spotted on to the nitrocellulose in amounts ranging from 0 to $4 \mu\text{g}$. These concentrations are below the limit of detection by the usual chemical assay for cyanophycin. In this concentration range, bound radioactivity increased linearly with cyanophycin (the correlation coefficient in a linear least squares analysis was 0.9939; result not shown). The lower limit of detection was $0.1 \mu\text{g}$ of cyanophycin.

Experiments were performed to assess the suitability of the assay for detection of cyanophycin in crude cell extracts. These experiments involved comparing results of a radioimmunoassay of crude extracts with conventional assays of cyanophycin purified from the same extracts. Unfortunately, considerable non-specific binding of the primary antibody to proteins in the crude extracts occurred. Such non-specific binding was re-

Fig. 4. ELISA assay for anti-cyanophycin antibody. Antibodies were raised in mice and assayed as described in the Methods. The x-axis shows successive 1 in 3 dilutions of ascitic fluid starting from 1 in 15.

duced somewhat by washing the nitrocellulose strips with Triton X-100 (0.1%) or 3 M-urea after addition of the primary antibody, but detection of cyanophycin was still strongly masked by non-specific binding. It was concluded that the radioimmunoassay has limited applicability for assaying cyanophycin in crude extracts. Its main virtue at this stage is that it extends the detection limit of extracted cyanophycin from approximately 2 µg (arginine assay) to approximately 0.1 µg.

An immunochemical approach to cyanophycin turnover

Experiments were done to find a suitable immunoprecipitation assay for cyanophycin which might allow quantitative measurement of cyanophycin turnover by pulse-chase methodology using labelled arginine. Immunoprecipitation using *Staphylococcus aureus* protein A (Langone, 1982) was based on the specific procedure of Rice & Strauss (1982).

To ensure that arginine transport into cells was not rate-limiting in such experiments, the rate of uptake of [³H]arginine was determined. When 0.25 µCi (9.2 kBq) [³H]arginine (9.6 pmol) was added to 2.5 ml *A. cylindrica* cells (140 Klett units), arginine uptake was rapid and essentially complete within 10–15 min (result not shown).

Despite many experiments, a useful and reliable immunoprecipitation procedure was not developed. The insolubility of cyanophycin itself masked any precipitation of cyanophycin-antibody-protein A conjugates.

Discussion

The results presented here indicate that when nitrogen-fixing *A. cylindrica* is grown on L/D cycles significant

amounts of protein synthesis occur in the dark. This implies the presence of a temporary store of fixed nitrogen to supply nitrogen for that protein synthesis in the dark. On the basis of measurements of cyanophycin by two methods, the arginine assay (Fig. 1) and total nitrogen measurements (Fig. 2), it is quite clear that cyanophycin does not supply this nitrogen in exponentially-growing cells. The results also agree with the report by Simon (1987) that cyanophycin levels are always very low during the exponential growth phase in nitrogen-fixing *A. cylindrica*, although he was probably referring to cultures grown in continuous light.

The source of nitrogen for protein synthesis during the dark is not clear. The total nitrogen recovered in the cell fractions (protein, soluble fraction and cyanophycin) accounted for approximately 75% of the nitrogen detected in the whole-cell samples. The pellet fraction contained some green material, indicating the presence of small numbers of unbroken cells, and implying the loss of some nitrogen during the washing steps which preceded cyanophycin extraction. In addition, the cell walls remained insoluble during the washing steps, and therefore the peptidoglycan fraction was not included in totals of subcellular nitrogen. However, although these factors help to explain the discrepancy between whole-cell nitrogen and the subcellular fractions, they do not clarify the source of the nitrogen which supports protein synthesis in the dark.

In *Gloeotheca*, nitrogen would be fixed during the dark period, and since most of it appeared to enter the protein pool directly, *Gloeotheca* would not appear to need to accumulate a store of fixed nitrogen in the dark to support protein synthesis in the light. This result disagrees with the data of Mullineaux *et al.* (1981b), who reported that dark protein synthesis in *Gloeotheca* was only 15% of the rate in the light. Their result appears to have been obtained with *Gloeotheca* that had been grown in continuous light and then darkened for the first time during the course of the experiment. Since cells require several days to adapt to alterations in their lighting regime (e.g. Post, 1986, 1987), such cells may not represent suitable material for the experiment.

The ability to accumulate cyanophycin as a temporary store of fixed nitrogen has been suggested to buffer nitrogen-fixing cells against variations in the supply of newly assimilated nitrogen (Carr, 1983; Carr & Wyman, 1986). The growth of *A. cylindrica* on L/D cycles represents such conditions; the supply of NH₃ from nitrogenase is certainly not constant, and protein synthesis makes continued demands for amino acids. There is no indication, however, of net accumulation of cyanophycin during the light to provide fixed nitrogen in the dark. Although nitrogen may cycle into and out of cyanophycin as proposed by Carr (1988), it must turn

over very rapidly, and consequently could not be used as a temporary nitrogen store under the conditions prevailing in the experiments described here.

To date there is still only circumstantial evidence to support the hypothesis (Carr, 1988) that dynamic cyanophycin metabolism is important in heterocystous cyanobacteria under N_2 -fixing conditions (Meeks *et al.*, 1977, 1978; Stewart *et al.*, 1975). The ability to synthesize and degrade cyanophycin constitutively would ensure that cyanobacteria are always poised to synthesize cyanophycin if an imbalance in nutrient supply occurs. However, if such a mechanism operates, an imbalance in growth conditions should lead to cyanophycin accumulation. The accompanying paper describes conditions where variations in the supply of fixed nitrogen lead to dynamic cyanophycin metabolism (Mackerras *et al.*, 1990).

The ability to measure cyanophycin turnover would allow Carr's hypothesis to be tested directly under conditions where nitrogen might cycle rapidly into and out of a cyanophycin pool. Our attempts to develop immunological methods for isolating and quantifying cyanophycin were pursued with this aim. However, the insolubility of cyanophycin prevented the use of this approach.

The definition and measurement of growth in photosynthetic cells subjected to L/D cycles is complicated. Light-scattering measurements are biased towards increases in total and individual cell mass, rather than increase in cell number (Brock *et al.*, 1984; Mallette, 1969). Any measured increase in biomass during the light period in cells grown on a L/D cycle cannot be considered purely as growth, because it also includes carbohydrate which accumulates to cope with the coming dark period (Mur, 1983; Smith, 1983). The protein synthesis observed in the dark must occur at the expense of glycogen stored during the preceding light period. Net dry weight would not increase under these conditions and, in fact, should decrease because CO_2 is released as respiratory processes supply the necessary energy. Consequently light-scattering measurements alone do not show the whole picture. Similar problems with definitions of growth have occurred during studies of nitrogen starvation. It is well known that carbohydrate accumulates under these conditions (e.g. Allen & Smith, 1969; Lehmann & Wober, 1976) but this cannot accurately be called growth since protein accumulation ceases (cf. Boussiba & Richmond, 1980).

The experiments reported in this paper were designed to test the possibility that cyanophycin synthesis and degradation were important in nitrogen-fixing cells grown on L/D cycles where the ability to temporarily fill and deplete a store of fixed nitrogen would indeed buffer metabolic requirements against variations in nitrogen

supply. Although the results indicate that *A. cylindrica* needs a store of fixed nitrogen to support protein synthesis in the dark, there was no evidence that cyanophycin fulfilled that role. Thus, the high activities of cyanophycin synthetase and cyanophycinase found in heterocysts and the role of the polar plugs thought to be rich in cyanophycin remain to be explained.

We thank Mrs Nola M. de Chazal for technical assistance. We also acknowledge the financial assistance of the Australian Research Grants Scheme and the Faculties Research Fund of the Australian National University.

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