

## Cyanophycin Granule Polypeptide Formation and Degradation in the Cyanobacterium *Aphanocapsa* 6308

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The effect of a number of conditions on the amount of cyanophycin granule polypeptide [multi-L-arginyl poly(L-aspartic acid)] formed in the unicellular cyanobacterium *Aphanocapsa* 6308 was determined. Light, CO<sub>2</sub>, sulfur, and phosphorus starvation as well as the addition of arginine to culture media increased the amount of cyanophycin granule polypeptide in cells when compared with that in cells grown under conditions optimal for growth. Nitrogen limitation and reduction of growth temperature to 30°C decreased the amount of cyanophycin granule polypeptide on a dry-weight basis. Shift-up and shift-down experiments suggest cyanophycin granule polypeptide may be a reserve nitrogen polymer in *Aphanocapsa* 6308.

Cyanophycin granules have long been observed in cyanobacteria by microscopy (3), but were not chemically identified until 1971 as high-molecular-weight copolymers of L-aspartic acid and L-arginine (6) in *Anabaena cylindrica*. More recent studies have shown the material to be multi-L-arginyl poly(L-aspartic acid) (10). Granules of similar structure and composition have also been identified in a non-nitrogen-fixing species, *Aphanocapsa* 6308 (2). Quantitative data for *A. cylindrica* show that the maximal rate of cyanophycin granule polypeptide (CGP) synthesis occurs at the end of exponential growth of a culture, and the maximum amount of CGP is found in stationary-phase cells. CGP is synthesized by a chloramphenicol-insensitive, ribosome- and tRNA-independent enzyme system (7); the enzyme responsible for CGP synthesis has been purified 100-fold and partially characterized (9). Simon (8) also showed, in *A. cylindrica*, that soluble protein decreased during stationary phase, correlating with an increase in CGP and that CGP largely disappeared when an old culture was diluted and growth resumed; this suggested that CGP was a cellular nitrogen reserve.

The present work describes conditions for the formation and breakdown of CGP in the non-nitrogen-fixing cyanobacterium *Aphanocapsa* 6308.

### MATERIALS AND METHODS

**Organism and growth conditions.** *Aphanocapsa* 6308 (ATCC 27150) was routinely grown in liquid medium no. 11 (1) supplemented with 2.4 g of sodium carbonate per liter at 35°C in 10,800 lx of cool

white fluorescent light with bubbling of 5% CO<sub>2</sub> in air; these are considered standard conditions. Cultures grown with 0.03% CO<sub>2</sub> had air bubbled through them or were grown in side-arm flasks with constant shaking. CO<sub>2</sub> was eliminated from the side-arm flasks by suspending small test tubes containing KOH within the flask and sealing the flasks with rubber stoppers and petrolatum. CO<sub>2</sub>-free conditions were maintained in Roux bottles by bubbling the cultures with air that had previously been bubbled through two consecutive containers of 20% KOH or NaOH. Light intensity was varied by changing the distance between the lights and the cultures. In some experiments the medium was supplemented with 5 to 50 mM arginine or with 5 to 50 mM aspartic acid or with both. Low-phosphate medium was prepared, using medium no. 11 modified by the use of 40 mg of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.5, 0.039 g of KCl, and 1 mg of K<sub>2</sub>HPO<sub>4</sub> per liter. Low-nitrogen medium was prepared by using medium no. 11 with 1/40 the amount of NaNO<sub>3</sub> in the complete growth medium. Low-sulfur medium consisted of medium no. 11 with MgCl<sub>2</sub>·6H<sub>2</sub>O replacing MgSO<sub>4</sub>·7H<sub>2</sub>O. In some experiments chloramphenicol, in concentrations varying from 1 to 10 µg/ml, was added to cells in exponential phase.

Growth was routinely assessed by measurement of cell densities by absorbance at 750 nm with a Gilford 240 spectrophotometer or with a Klett colorimeter, using a Kodak 88A Wrattan filter. Absorbance was linear with dry-weight measurements to an optical density (OD) of 0.5; at this absorbance, the dry weight was 125 µg/ml. Cultures with higher densities than 0.5 were diluted before absorbance measurements were made. At least two experiments of each type were carried out. Radioactive incorporation studies were carried out with uniformly labeled L-[<sup>14</sup>C]arginine (specific activity, 277 mCi/mmol) or with L-[<sup>14</sup>C]aspartate (specific activity, 204 mCi/mmol; New England Nuclear Corp.) added to medium no. 11. Continuous cultures were grown in a Biotec continuous culture apparatus (30°C) with light as the sole growth-

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limiting factor. Shift-up and shift-down experiments (35°C) were carried out using inocula grown at a low enough light intensity to cause CGP formation during logarithmic growth. Portions of logarithmically growing cells containing 2 to 3% CGP were transferred to 10,800-lx light, washed in distilled water before transfer to nitrogen-free medium, transferred to microaerophilic conditions (N<sub>2</sub> flushing), or transferred to anaerobic conditions (10<sup>-5</sup> M DCMU plus N<sub>2</sub> flushing); dry weight and CGP content were measured with time after shift.

**Chemical analyses.** CGP was measured by the method of Simon (8). Variations in percent CGP in triplicate analyses on any portion were in the first decimal place. Greater variation was seen from one batch culture to another because of differences in the percent CGP in the inocula. Dry-weight determinations were made by filtering cells through washed, dried, and tared Whatman GF/C glass fiber filters which were then dried 48 h at 80°C. At least two replicate samples were analyzed at each point in each experiment. Protein determinations (4) were carried out on supernatant fractions and recorded as soluble protein after sonication. Cells were fractionated by the method of Roberts et al. (5). At various times after shift to minus nitrogen medium, cells were removed by centrifugation, and the medium was concentrated 10 to 50 times by vacuum evaporation and tested for arginine and citrulline by thin-layer chromatography or chemical analysis as already described (12).

## RESULTS

**Effect of variation in light intensity on growth and CGP content.** Figure 1 shows growth curves for batch cultures at two light intensities, 10,800 and 3,240 lx; growth becomes light limited in each case. High-light cells grow faster and to higher growth yields than do low-light cells. The amount of CGP at various cell densities during such growth curves was measured, and Table 1, in which growth is correlated with CGP content, shows that as cells were more light limited, they reached stationary phase later in time and at a lower growth yield. Cells grown at lower light intensity formed a greater percentage of CGP at lower cell densities, suggesting that light limitation causes an increase in CGP content. A 10-fold dilution of a light-limited stationary culture caused a threefold decrease in percent CGP (dry weight) within 7 h, a 10-fold decrease in 24 h, and a decrease in the amount of granulation observed microscopically, whereas suspension of washed cells into the same volume of fresh medium did not. No cell lysis was observed. Cells grown in continuous culture, with light as the sole growth-limiting factor, at a specific growth rate of 0.04 h<sup>-1</sup>, formed no microscopically visible granules, and the CGP content was at the high-light exponential growth level of 1.5%.

**Effect of temperature variation on CGP**

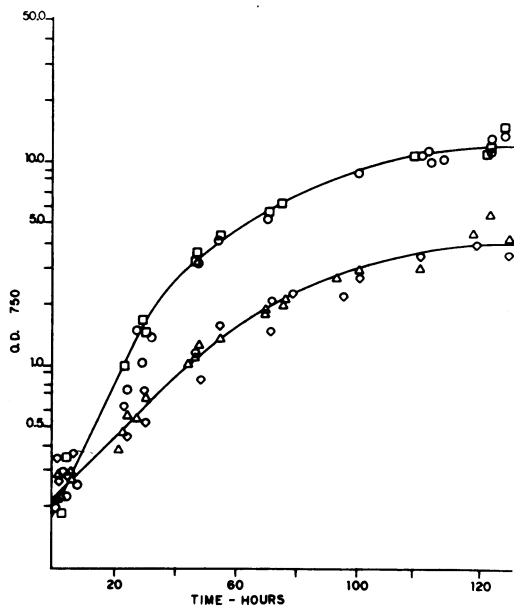


FIG. 1. Growth curves of *Aphanocapsa* 6308 at 35 and 30°C at 10,800 and 3,240 lx. Growth was analyzed by OD<sub>750</sub>. Data for each growth condition are from four separate experiments. Symbols: □, 35°C, 10,800 lx; ○, 30°C, 10,800 lx; △, 35°C, 3,240 lx; ▽, 30°C, 3,240 lx.

**content.** Reduction of the growth temperature from 35 to 30°C caused a slightly lower growth rate for 5% CO<sub>2</sub>-grown cells at 10,800 lx, but approximately the same growth yield (Fig. 1 and Table 2). The CGP content in cells grown under high light intensity at 30°C was lower than that of cells grown at 35°C.

The light intensity effect on CGP content observed at 35°C (Table 1) was not seen at 30°C; in other words, light-limited cells grown at low light intensity at 30°C did not show an increase in CGP content at lower cell densities (Table 2). At 30°C, low levels of CGP (1.1%) were found in cells which were at approximately the same low cell density (OD<sub>750</sub> = 4.66) as those grown at 35°C where a higher level of CGP was found (4.8% CGP at OD<sub>750</sub> = 3.98).

**Effect of inorganic carbon on CGP content.** At 35°C, CO<sub>2</sub> was growth limiting at 0.03% in either high light or low light (Table 3). Cells formed a greater percentage of CGP at lower cell densities with 0.03% CO<sub>2</sub> than with 5% CO<sub>2</sub>. The presence of excess carbonate had little effect on growth when culture densities were compared for each set of growth conditions; however, it did effect an increase in CGP content when CO<sub>2</sub> was limiting at 3,240 lx. With 5% CO<sub>2</sub> supplied, light became the growth-limiting factor both at 3,240 lx and at 10,300 lx. Very little growth was ob-

TABLE 1. Correlation of growth with CGP content in batch cultures of *Aphanocapsa* 6308<sup>a</sup>

Light intensity (lx)	Day stationary phase reached	Growth yield (mg/ml)	% CGP at the following OD <sub>750</sub> during growth period:					
			1.5	2.75	3.4	5.6	7.8	10
10,800	7	1.61	1.5	1.5	1.5	2.6	11.5	16.6
8,100	8	1.01	1.5	2.0	3.0	7.4	—	—
5,400	9	0.86	5.2	7.2	8.5	—	—	—
2,700	10	0.63	7.1	9.3	10.3	—	—	—

<sup>a</sup> 5% CO<sub>2</sub> in air, 35°C. Batch cultures were sampled at various times in their growth period at each light intensity. Portions were analyzed in triplicate for OD<sub>750</sub>, for dry weight and for CGP content.

TABLE 2. Effect of temperature on CGP formation<sup>a</sup>

Temperature (°C)	Light intensity (lx)	OD <sub>750</sub>	% CGP (dry weight)
30	10,800	11.4	6.9
35	10,800	11.22	11.1
30	3,240	4.66	1.1
35	3,240	3.98	4.8

<sup>a</sup> 5% CO<sub>2</sub>, 2.4 g of carbonate per liter in all media. Sampling was at 140 h after inoculation. Cultures grown at high light were in stationary phase, whereas cultures in low light were in late logarithmic phase of growth. Data for each growth condition are from four different experiments.

served in the absence of CO<sub>2</sub>.

At 30°C, under conditions of low light, as the CO<sub>2</sub> concentration was increased, there was more cell growth, but levels of CGP remained low (Table 3). Even in late stationary phase under conditions of 0.03% CO<sub>2</sub> without excess carbonate, where cells grew to an OD<sub>750</sub> of 8.76 in 337 h, only 0.02% CGP was formed. The presence of excess carbonate had some effect on increasing CGP levels when CO<sub>2</sub> was limiting (Table 3) but had little effect on growth except in the case of cells grown with 0.03% CO<sub>2</sub> in low light, where the presence of excess carbonate decreased the amount of growth.

**Effect of other limiting nutrients on CGP content.** Both sulfur and phosphorus limitation caused an increase in CGP content (Table 4). Data are given for cells sampled at 47 h, when the control and low phosphorus cultures were in late logarithmic phase, and also for cells sampled at 122 h when all cells were in stationary growth. The amount of CGP in sulfur- and phosphate-limited cells was greater than in the more dense control cultures, and it increased with time. In nitrogen-limited cells the amount of CGP reached a low level. The concentration of soluble protein decreased in control cells with growth, but decreased to lowest levels in starved cultures.

**Effect of aspartate and arginine on CGP content.** Cultures grown photo-autotrophically in medium no. 11 with 5% CO<sub>2</sub> at 10,800 lx and

35°C showed the same growth rate (0.07 h<sup>-1</sup>) and growth yield (1.6 mg/ml) with or without the addition of aspartate or arginine to the medium. Higher levels of CGP were consistently formed in media containing arginine; a typical experiment showed 16% CGP in medium no. 11 plus arginine and 12% in medium no. 11 after 14 days growth. Table 5 shows the distribution of label in cell fractions after Roberts extraction of logarithmically grown cells (1 day) and of stationary, granulating cells (14 days) grown with [<sup>14</sup>C]aspartate or [<sup>14</sup>C]arginine in the medium. Roberts extraction of purified granules from <sup>14</sup>C-labeled cells showed 91 to 94% of the label in the ethanol-soluble, ether-insoluble fraction (2). Cells in early logarithmic growth incorporate both aspartate and arginine into the major CGP-containing fraction (ethanol soluble, ether insoluble) at low levels and into the residual protein fraction (hot trichloroacetic acid insoluble) at high levels, whereas cells from stationary phase have more label in the ethanol-soluble, ether-insoluble fraction and less label in the hot trichloroacetic acid-insoluble fraction.

**Shift-up and shift-down experiments.** The data in Table 1 suggest that cells grown in low light accumulate CGP during logarithmic growth. When these cells were shifted up to high light or shifted down to nitrogen-free media, the amount of CGP decreased from between 2 and 3% to between 0.6 and 0.7% in 10 h; this decrease was more rapid than the increase in cellular dry weight (Fig. 2). The amount of soluble protein decreased from 55 to 35% of cellular dry weight in 10 h in the nitrogen-free cultures; phycocyanin concentration did not decrease over this time period. Analyses on portions sampled every 10 min during the first 2 h after shift showed the CGP content decreased linearly by 29% during this time period, whereas protein and phycocyanin concentrations did not fall. Cells shifted down to microaerophilic conditions (continuous nitrogen flushing) or anaerobic conditions [3'-(3,4-dichlorophenyl)1',1'-dimethyl urea (DCMU) plus continuous nitrogen flushing] showed the same growth rate and CGP and protein concentration as the control for at least

TABLE 3. Effect of inorganic carbon on CGP formation<sup>a</sup>

% CO <sub>2</sub>	Excess carbonate (2.4 g/liter)	Light intensity (lx)	OD <sub>750</sub> at 35°C	% CGP (dry wt) at 35°C	OD <sub>750</sub> at 30°C	% CGP (dry wt) at 30°C
0.03 <sup>b</sup>	+	10,800	1.37	7.5	2.39	2.3
0.03	- <sup>c</sup>	10,800	1.49	8.0	2.34	1.2
5 <sup>d</sup>	+	10,800	11.22	11.1	11.4	6.9
5	- <sup>c</sup>	10,800	10.38	11.9	11.13	6.6
0.03	+	3,240	1.1	7.6	0.708	3.2
0.03	- <sup>c</sup>	3,240	2.25	0.3	3.08	0.005
5	+	3,240	4.08	5.8	6.84	1.5
5	- <sup>c</sup>	3,240	4.54	6.4	6.03	1.4

<sup>a</sup> Sampling was at 138 to 145 h after inoculation.<sup>b</sup> Air bubbled through cultures.<sup>c</sup> pH adjusted to 11.5 with NaOH.<sup>d</sup> 5% CO<sub>2</sub> in air bubbled through cultures.TABLE 4. Effect of limiting sulfur, phosphorus, or nitrogen on *Aphanocapsa* 6308<sup>a</sup>

Treatment <sup>b</sup>	47 h			122 h		
	Cell concn <sup>c</sup>	Protein (% dry wt)	CGP (% dry wt)	Cell concn <sup>c</sup>	Protein (% dry wt)	CGP (% dry wt)
Control	0.78	61.7	1.8	1.06	37.5	5.2
Low sulfate <sup>b</sup>	0.2	12.7	7.6	0.12	16.3	9.3
Low phosphate <sup>b</sup>	0.46	18.0	5.7	0.72	14.4	9.9
Low nitrate <sup>b</sup>	0.03	22.2	0.2	ND <sup>d</sup>	ND	ND

<sup>a</sup> 35°C, 10,800 lx, 5% CO<sub>2</sub> in air. Times refer to hours after inoculation.<sup>b</sup> Concentrations are given in the text.<sup>c</sup> Milligrams (dry weight) per milliliter.<sup>d</sup> ND, Not determined.TABLE 5. [<sup>14</sup>C]arginine and [<sup>14</sup>C]aspartate incorporation in *Aphanocapsa* 6308<sup>a</sup>

Treatment	% Incorporation of:			
	[ <sup>14</sup> C]arginine		[ <sup>14</sup> C]aspartate	
	Day 1	Day 14	Day 1	Day 14
Cold TCA soluble	31	36	20	17
Ethanol soluble, ether insoluble	0	29	4	26
Ethanol and ether soluble	11	4.5	8	8
Hot TCA soluble	9	15	34	32
Hot TCA insoluble	49	16	33	16

<sup>a</sup> Cells grown in radioactive medium from day zero. TCA, Trichloroacetic acid.

50 h after the shift. No arginine or citrulline could be detected in media after the shift to minus nitrogen conditions.

**Effect of chloramphenicol on growth and CGP content.** Chloramphenicol inhibited growth of *Aphanocapsa* 6308 at all concentrations tested (Fig. 3). Whereas concentrations of 2 and 4 µg/ml allowed slow growth, 5 µg of chloramphenicol per ml caused complete inhibition of growth. Figure 4 shows the effect of chloramphenicol on CGP content during the growth experiment described in Fig. 3. All concentrations showed an increase in CGP content for at least 20 h; cultures grown with 10, 8, and

6 µg of chloramphenicol per ml then showed a decrease in granule content. The CGP content in cells treated with 2, 4, or 5 µg of chloramphenicol per ml increased as long as experiments were carried out, increasing with increasing chloramphenicol concentrations. Figure 5 shows the soluble protein and CGP contents on a per cell basis in cells sampled 48 h after treatment with 0 to 5 µg of chloramphenicol per ml. Increasing chloramphenicol concentration effected decreases in soluble protein and increases in CGP contents.

## DISCUSSION

Growth conditions have great effects on the level of CGP produced by cells of *Aphanocapsa* 6308. At 35°C, the optimal temperature for growth, limitation of light, sulfur, phosphorus, or CO<sub>2</sub> caused an increase in the amount of CGP when expressed as percent dry weight of cells and compared with unstarved controls. Continuous culture data suggest that CGP formation is not caused simply by a slow division rate of cells. At 30°C, little CGP was formed in any tested condition except in cells grown at high light intensity with 5% CO<sub>2</sub>; here the amount of CGP was still less than that observed in cells grown under the same conditions at 35°C (Table 3), although each culture reached the same growth

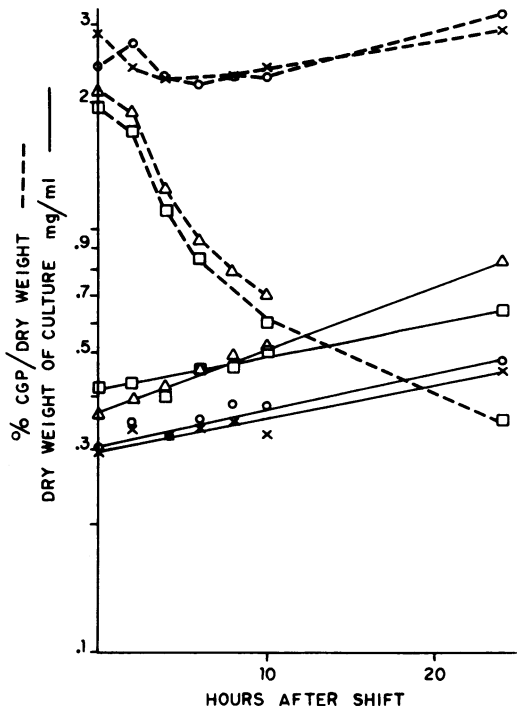


FIG. 2. Shift-up and shift-down experiments. Cells grown logarithmically at low light intensity, containing 2 to 3% CGP (described in the text), were shifted up to higher light intensity or shifted down by nitrogen flushing or to nitrogen-free conditions. Dry-weight increase, indicative of cell growth, and CGP content were measured with time after the shift. Symbols: x, logarithmically growing control from which portions were shifted up or down, Δ, shift-up to high light; ○, shift-down to flushing with nitrogen, □, shift-down to minus nitrogen medium.

yield. In limiting light, increased CO<sub>2</sub> concentrations at 30°C caused increased growth but low CGP contents. A higher growth temperature allowed more CGP accumulation. At both temperatures, addition of excess carbonate to growth media did not significantly affect the growth of cells (except in the case of 0.03% CO<sub>2</sub>, low-light cells), but a higher percentage of CGP was formed in media with excess carbonate when cells were CO<sub>2</sub> limited. This suggests that added carbonate is not readily used as a carbon source for growth, even when CO<sub>2</sub> is growth limiting. Carbonate has been shown to enter cells and to be metabolized at pH 11.0 (11). Addition of excess carbonate (pH 11 after autoclaving) or addition of base (pH 11 to 11.5) to media was necessary to allow growth of *Aphanocapsa* 6308 in 5% CO<sub>2</sub>. After 6 h with 5% CO<sub>2</sub>, the pH dropped to 7.8, where it remained for at least 173 h.

Growth is limited by light only when high

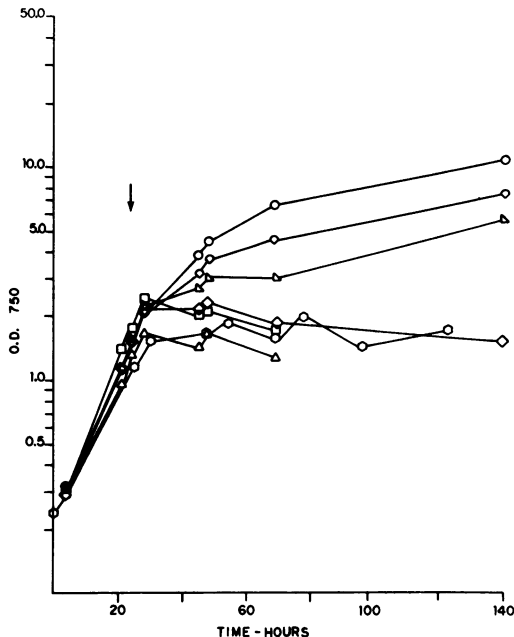


FIG. 3. Growth curves of cultures to which chloramphenicol was added 24 h after inoculation. Symbols (chloramphenicol concentration in micrograms per milliliter): ○, control; ▽, 2; △, 4; ◇, 5; □, 6; Δ, 8; Δ, 10.

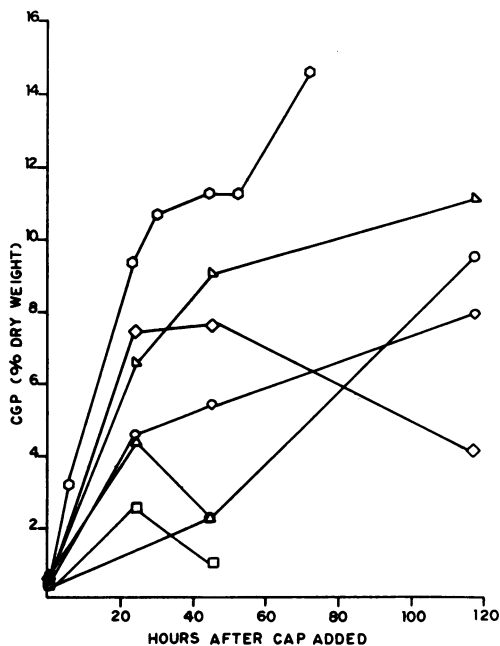


FIG. 4. CGP content of cultures in Fig. 3. CGP content (Simon analysis) was measured at each indicated hour after chloramphenicol (CAP) was added. Symbols are as described in the legend to Fig. 3.

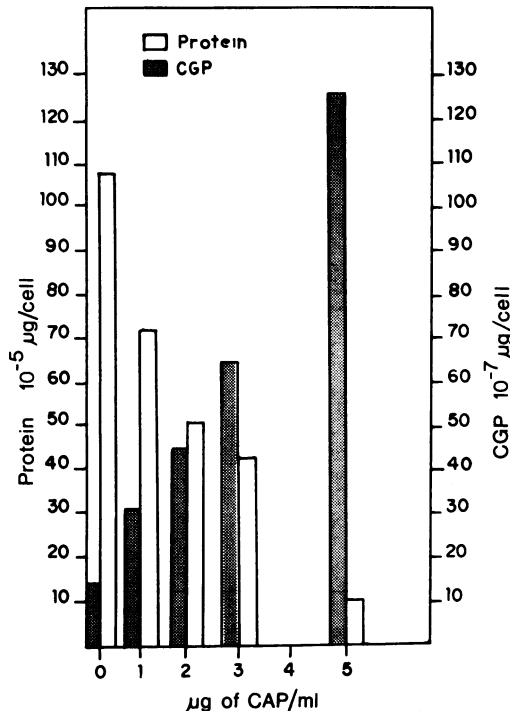


FIG. 5. Effect of chloramphenicol on protein and CGP content per cell. Various chloramphenicol (CAP) concentrations were added to cell populations. Protein (Lowry analysis) and CGP (Simon analysis) were measured after 48 h and compared with cell numbers (hemacytometer analysis).

levels of CO<sub>2</sub> are available. At 35°C in 5% CO<sub>2</sub>, the generation time varies with light intensity (Fig. 1), but in low CO<sub>2</sub>, light does not limit growth since the growth yields are approximately equal at 10,800 or 3,240 lx (Table 3). At both light intensities with 0.03% CO<sub>2</sub>, growth yields are much lower than with 5% CO<sub>2</sub>. In 5% CO<sub>2</sub>, the growth yield at high light intensity is the same at either 35°C or 30°C, but at low light intensity the growth yield is higher at 30 than at 35°C.

The presence of arginine in media allows an increase in growth yield only when CO<sub>2</sub> is limiting (12); however, CGP content increases in the presence of arginine only when CO<sub>2</sub> or carbonate concentrations are high. This suggests that arginine is used for growth, but not CGP synthesis, when there is not sufficient CO<sub>2</sub> for maximum photosynthesis. Arginine has been shown to be metabolized by *Aphanocapsa* 6308 by the arginine dihydrolase pathway forming ATP, CO<sub>2</sub>, ammonia, and ornithine, or by the arginine-urease pathway forming urea, ornithine, CO<sub>2</sub>, and ammonia (12); results suggested that the CO<sub>2</sub> from arginine was then used for photosynthesis.

The synthesis of CGP is not dependent on new ribosomal protein synthesis in *Aphanocapsa* 6308 (Fig. 4 and 5), as was also shown in *A. cylindrica* (7). As in *A. cylindrica*, Fig. 5 suggests that CGP is a storage substance in *Aphanocapsa* 6308 formed from the breakdown of cellular protein, since decrease in protein content correlates with increase in CGP content. Further support for this hypothesis are the <sup>14</sup>C incorporation data in Table 5 which show that there is a shift in labeling pattern from incorporation into the hot trichloroacetic acid-insoluble protein to the ethanol-soluble, ether-insoluble, CGP-containing fraction when cells form high levels of CGP in stationary growth. Furthermore, 1-min <sup>14</sup>CO<sub>2</sub> fixation experiments (11; P. J. Weathers, unpublished data) during early light-limited stationary phase showed that little <sup>14</sup>C (1.4%) of the total extractable by the Roberts treatment is incorporated into CGP, even though <sup>14</sup>CO<sub>2</sub> is incorporated into the cells and large amounts of CGP are being synthesized (Tables 1 and 2). This suggests that CGP could be synthesized from protein breakdown, not from newly synthesized photosynthetic products of which aspartate is a major product (11) in early stationary phase.

The low concentration of CGP in nitrogen-starved cells (Table 4) suggests that CGP may be a nitrogen storage reserve. Shift-up and shift-down experiments (Fig. 2) show that CGP can be broken down by cells; breakdown was observed within 10 min after transfer to nitrogen-free medium or to high light intensity. During the first 2 h after shift, neither phycocyanin nor protein concentrations decreased. CGP does not appear to be broken down to excreted arginine or citrulline since none was found in concentrated media after the shift. The possibility that CGP is synthesized microaerophilically and anaerobically is suggested by data showing that cells grow under nitrogen and DCMU plus nitrogen for at least 50 h and that they increase twofold in their CGP content.

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