Studies on the Formation of Phycocyanin, Porphyrins, and a Blue Phycobilin by Wild-Type and Mutant Strains of Cyanidium Caldarium

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Received August 26, 1965.

Summary. The synthesis of chlorophyll a and the bile-pigment and protein moieties of phycocyanin were arrested in illuminated cells of Cyanidium caldarium, strain III-D-2, incubated with chloramphenicol, ethionine, p-fluorophenylalanine, and p-chloromercuribenzoate. Pigment synthesis was similarly retarded in illuminated cells provided with nutrient medium lacking nitrogen.

Porphobilinogen, porphyrins, and a blue phycobilin were excreted into the nutrient medium by illuminated and unilluminated cells of wild-type and mutant C. caldarium strains incubated with δ-aminolevulinic acid in darkness. Pigment production from δ-aminolevulinic acid was sensitive to treatment with chloramphenicol and ethionine.

Cells of C. caldarium excreted 7 red-fluorescing porphyrins into the suspending medium during incubation with δ-aminolevulinic acid. Three of these porphyrins were identified as uroporphyrin III, coproporphyrin III, and protoporphyrin on the basis of their spectral properties and by paper chromatography with standards.

The blue phycobilin was characterized spectrally and compared with biliverdin. The algal phycobilin displayed properties of a pigment with a violin-type structure. The phycobilin may be an immediate precursor of phycocyanobilin, the phycocyanin chromophore, or identical to it.

Phycocyanins, allophycocyanins, and phycoerythrins are water soluble, bile-pigment-protein complexes found in blue-green and red algae, and in cryptomonads (25). The chromophoric moieties of biliproteins belong to a class of bile-pigmens termed phycobilins (15). Phycocyanobilin, the phycocyanin chromophore, is thought to be related to the bilidene bile-pigments (15). The sequence of substituents on the pyrrole rings of phycocyanobilin suggests that the molecule could be formed by oxidative removal of the α-bridge carbon between pyrrole rings A and B of type IX proto or mesoporphyrin (9).

There are no reports in the literature on the biosynthesis of biliproteins in vitro. Our present understanding of their formation has come largely from studies on the involvement of light in biliprotein biosynthesis (2).

The present report describes A) the effect of inhibitors and nitrogen starvation on the formation of chlorophyll a and phycocyanin by C. caldarium, B) the formation of phycobilin, porphobilinogen (PBG), and porphyrins which are excreted into the suspending medium during incubation of the alga with ALA, and C) the characterization of ALA utilization products.

Materials and Methods

The Organism. The wild type of C. caldarium contains chlorophyll a, phycocyanin, allophycocyanin, and carotenoids. Nichols and Bogorad (22) have partially characterized a number of Cyanidium mutants produced by ultraviolet irradiation of the wild-type. Strain III-D-2, which like the wild-type accumulates chlorophyll a and phycocyanin in the light, was used in most of the experiments. This strain grows more rapidly and accumulates more pigment per cell than does the wild-type. The other strains used in some experiments with ALA were: the wild-type; GGB, a chlorophyll-less strain; III-C, a phycocyanin-less strain; and GGB-Y, a strain which has lost the ability to make phycocyanin and chlorophyll a. This latter strain was produced by ultraviolet irradiation of strain GGB.

Cultural Procedures. The nutrient medium used for the culture of all the Cyanidium strains was simil-
ilar to that of Allen (1) except that iron was supplied as the ethylenediamine tetraacetate acid chelate (Fe-EDTA) and NH₄Cl was used in place of (NH₄)₂SO₄. The cells for all experiments were grown in darkness at 40 to 45° in nutrient medium containing 1% glucose. One liter of medium was contained in 3-liter Fernbach flasks which were agitated by a rotary shaker for 9 to 12 days. The dark-grown cells were harvested by centrifugation and were resuspended in fresh nutrient medium lacking glucose. The cell density was then adjusted to 5 x 10⁸ cells per ml. Aliquots of this suspension were used in subsequent experiments.

Chlorophyll a and Phycoerythrin Determinations. Attempts to make quantitative measurements of chlorophyll a and phycoerythrin from broken-cell preparations of C. caldarium met with limited success. Incomplete disruption of cells was observed with conventional methods of cell breakage such as prolonged sonication, passage through the French pressure cell, treatment with detergents, or grinding with alumina, dry ice, or glass beads. Chlorophyll a and phycoerythrin were therefore estimated from spectra of intact cells in a Cary (model 11) recording spectrophotometer using the opal glass technique of Shibata (28). The method which proved useful for pigment determination in aqueous extracts of Anacystis nidulans (19) was adapted for calculation of pigment content from spectra of whole cell suspensions of Cyanidium; the specific extinction coefficients used for chlorophyll a were those of Mackinney (17) and the values used for calculating phycoerythrin concentrations were those reported by Hattori and Fujita (8).

To determine the differences, if any, in the extinction of a given quantity of chlorophyll a and phycoerythrin in vivo, in broken cells, and after partial purification the following experiments were performed. The absorption spectrum (350-800 nm) of fresh or frozen and thawed cells (in 0.1 M phosphate buffer, pH 6.5) which had been grown autotrophically, was determined. The cell suspensions were sonicated in a Raytheon 10 K.C. sonic oscillator for as long as 90 minutes. The optical density (OD) at 675 and 620 nm of a suspension sonicated for 60 minutes differed from the initial sample by 6 and 3%, respectively. Sonication for longer than 90 minutes resulted in slightly greater discrepancies. Following centrifugation (25,000 x g, 30 min) the sum of the ODs at 675 and 620 nm of the supernatant and resuspended pellet were generally equal to the ODs at 675 and 620 nm of the original broken cell suspension. Furthermore, the spectrum of a clear phycoerythrin solution (OD = 0.450) using an opal glass diffusing plate between the sample and the photomultiplier compared favorably to the spectrum of the same solution (OD = 0.420) without opal glass.

Estimation of Products of ALA Utilization. The ALA remaining in the suspending medium after 24-hour incubations with Cyanidium cells was determined by the method of Mauzerall and Granick (18). Porphobilinogen (PBG) was estimated by the Ehrlich reaction (18). The total porphyrin present was calculated from the OD at 406 mλ of an acidified aliquot of the suspending medium (27). Porphobilin yields were estimated by reading the OD of a neutral chloroform extract of the suspending medium at 615 mλ. It was assumed, for lack of better information, that the specific absorption coefficient of the algal pigment in neutral chloroform was the same as that of biliverdin in the same solvent (11).

Results

Pigment Formation in Cell Suspensions. The accumulation of chlorophyll a and phycoerythrin was followed in cell suspensions of dark-grown C. caldarium, strain III-D-2, exposed to light to determine the rate of pigment synthesis under the experimental conditions. As noted before (23), trace quantities of chlorophyll a are present in dark-grown cells of this strain (fig 1). The lag in chlorophyll a and phycoerythrin synthesis extended from 12 to 18 hours after the onset of illumination and the duration of the lag was at least in part dependent on the density of the cell suspension. Synthesis of both pigments appeared to begin concomitantly. After illumination for 48 hours, the chlorophyll a concentrations increased about 50-fold. Phycoerythrin was spectrally undetectable in dark-grown cells but attained a concentration of about 1.4 x 10⁻⁵ μmoles/ml after illumination for 48 hours. Additional pigments failed to form after 48 hours. Cell counts made throughout the greening process indicated that the cells were not dividing during this period.

In an attempt to determine whether protein synthesis is necessary for pigment accumulation to occur, suspensions of dark-grown cells were exposed to chloramphenicol, ethionine, or p-fluorophenylalanine, or deprived of inorganic nitrogen during illumination. Inhibitors were preincubated with 10 ml aliquots of
diluted suspensions of dark-grown cells for 6 hours prior to illumination for 48 hours under standard conditions (43°, ca. 160 ft-c General Electric 90 w fluorescent tubes). For experiments on nitrogen starvation, dark-grown cells were washed 3 times with distilled water and suspended in nutrient medium without nitrogen and illuminated for 48 hours.

Chlorophyll a and phycocyanin failed to accumulate when nitrogen was omitted from the suspending medium during illumination (table 1). p-Chloromercuribenzoate also inhibited pigment production. Pigment synthesis in illuminated cultures could be stopped even after accumulation had begun by adding chloramphenicol (fig 1) or resuspending the cells in fresh nutrient medium without nitrogen.

After deprivation of nitrogen or incubation with inhibitors for 48 hours, the cells were collected by centrifugation and resuspended in fresh complete nutrient medium in order to determine whether the cells were living. Pigment synthesis resumed during illumination for another 48 hours although the total chlorophyll a and phycocyanin produced was less than in cell suspensions illuminated for the same time but never exposed to inhibitors or deprived of nitrogen. The formation of pigment by the treated and washed cells showed that most of them had survived 48-hour exposures to nitrogen starvation or to inhibitors.

**Incubations with ALA.** In view of the probable origin of the phycocyanin chromophore from a porphyrin, a metalloporphyrin (e.g. heme), or a metalloporphyrin-protein complex (e.g. hemoprotein), experiments were conducted to determine the fate of ALA administered to *C. caldarium* cell suspensions (strain III-D-2) which had been maintained in darkness or in light (ca. 160 ft.-c) for 24 hours.

In these experiments, 1.0 ml of a 7.0 × 10^{-2} m ALA solution was added to 10 ml of dark-grown or previously illuminated cell suspensions; an equal volume of water was added to controls. All treatments were then incubated in darkness for another 24 hours.

During the 24-hour incubations with ALA, the alga excreted PBG, porphyrins, and a blue phycobilin (bile-pigment) into the suspending medium; neither these nor similar compounds were produced in detectable amounts by cells not provided with ALA. The ALA utilization products were formed by both dark-grown and preilluminated cells but the yields of porphyrin and of bile-pigment were generally 2 times greater in the latter (table II). The recovery of ALA in control flasks from which cells were omitted was 91 to 100%. The PBG, porphyrins and bile-pigment present in the suspending medium of preilluminated and unilluminated cells incubated with ALA accounted for 24 and 19% of the substrate supplied, respectively.

After removal from the suspending medium, preilluminated ALA treated cells absorb more light at 675 and 615 mµ than do untreated cells (fig 2; curve 2). A soret band at 400 mµ was observed both in dark-grown and preilluminated ALA-treated cells. This shows that some of the products of ALA utilization were not excreted into the suspending medium but remained within the cells. Figure 2 (curve 3) shows the spectrum of a suspending medium following the removal of ALA-treated cells. The broad absorption

<table>
<thead>
<tr>
<th>Table I. Effect of Inhibitors and Nitrogen Starvation on Phycocyanin and Chlorophyll a Formation in C. caldarium, Strain III-D-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment</td>
</tr>
<tr>
<td>Conditions*</td>
</tr>
<tr>
<td>1. Unilluminated cells</td>
</tr>
<tr>
<td>2. Illuminated cells</td>
</tr>
<tr>
<td>(48 hrs)</td>
</tr>
<tr>
<td>A) + Ethionine 10^{-3} m</td>
</tr>
<tr>
<td>B) + p-Fluorophenylalanine, 10^{-3} m</td>
</tr>
<tr>
<td>C) + Chloramphenicol, 10^{-3} m</td>
</tr>
<tr>
<td>D) - Nitrogen**</td>
</tr>
<tr>
<td>* Each treatment consisted of 10 ml of cell suspension. The cell density was 5 × 10^{8} cells per ml. Inhibitors were preincubated with the cells for 6 hours prior to illumination.</td>
</tr>
<tr>
<td>** NaCl was substituted for NH_{4}Cl in the nutrient medium.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table II. PBG, Porphyrins, and Bile-Pigment in the Suspending Medium of C. caldarium, Strain III-D-2, after 24-Hour Incubations with ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells were grown heterotrophically in darkness for 10 days. Some cells were illuminated for 24 hours before incubation with ALA in darkness for an additional 24 hours.</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Dark-grown cells μmoles/ml × 10^{8}</td>
</tr>
<tr>
<td>1. PBG</td>
</tr>
<tr>
<td>2. Porphyrin**</td>
</tr>
<tr>
<td>3. Bile-pigment***</td>
</tr>
<tr>
<td>* The bile-pigment yield was determined by the OD of a chloroform extract of the suspending medium. It was assumed that the extinction of the bile-pigment in this solvent is the same as reported for biliverdin (11)</td>
</tr>
<tr>
<td>** The ALA equivalent is the μmoles of ALA necessary to account for porphyrin and bile-pigment in the suspending medium at the conclusion of the experiment.</td>
</tr>
<tr>
<td>*** Porphyrins estimated by the method of Shenin (27).</td>
</tr>
</tbody>
</table>
Table III. ALA Utilization by Variously Treated III-D-2 Cell Suspensions

<table>
<thead>
<tr>
<th>Conditions*</th>
<th>Porphyrins** $\mu$mole/ml $\times 10^3$</th>
<th>Bile-pigment*** $\mu$mole/ml $\times 10^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dark-grown cells</td>
<td>5.60</td>
<td>16.30</td>
</tr>
<tr>
<td>2. Preilluminated cells</td>
<td>14.32</td>
<td>38.30</td>
</tr>
<tr>
<td>A) + Ethionine, $10^{-2}$ M</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>B) + Chloramphenicol, $10^{-3}$ M</td>
<td>0.42</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Conditions described in table II.
** Porphyrins estimated by the method of Shenin (27).
*** The bile-pigment yields were determined by the OD of the pigment in chloroform (see footnote 1, table II).

The ability of wild-type and mutant Cyanidium strains to produce porphyrins and the bile-pigment was tested. All strains were grown heterotrophically in darkness for 10 days in a glucose-containing medium. The cells were harvested by centrifugation, resuspended in fresh nutrient medium and incubated in the dark or preilluminated for 24 hours. One ml of an ALA solution ($7.0 \times 10^{-2}$ M) was added and all treatments were incubated for another 24 hours (table IV).

Regardless of their normal pigmentation when grown in the light, all of the various Cyanidium strains produced PBG, porphyrins, and a bile-pigment when incubated in darkness with ALA. With the exception of strain III-D-2, which utilized ALA less efficiently in the dark, and more efficiently when the cells were preilluminated, the ALA utilization of similarly treated cells of the various strains did not differ significantly.

Characterization of ALA Utilization Products. In an attempt to show that the Ehrlich color in the suspending medium of strain III-D-2, grown under standard conditions (43°, 160 ft.-c fluorescent lights), resulted from PBG and not from dipyrromethanes, or other polymeric products, PBG was isolated by chromatography on Dowex 2 $\times$ 8 columns (18). About 90% of the Ehrlich reacting material in the

Table IV. Porphyrins and Phycobilin in the Suspending Medium of Wild-Type and Mutant C. caldarium Cells after Incubation of 24 Hours with ALA ($7.0 \times 10^{-2}$ M)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal pigmentation</th>
<th>Chl.**</th>
<th>P.C.**</th>
<th>Total porphyrins*</th>
<th>(umoles/ml $\times 10^3$)</th>
<th>Phycobilin**</th>
<th>(umoles/ml $\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. III-D-2</td>
<td>+</td>
<td>+</td>
<td></td>
<td>3.62</td>
<td>18.88</td>
<td>8.54</td>
<td>37.20</td>
</tr>
<tr>
<td>2. Wild-type</td>
<td>+</td>
<td>+</td>
<td></td>
<td>6.28</td>
<td>14.72</td>
<td>10.85</td>
<td>17.80</td>
</tr>
<tr>
<td>3. III-C</td>
<td>+</td>
<td>-</td>
<td></td>
<td>5.96</td>
<td>15.00</td>
<td>9.39</td>
<td>19.70</td>
</tr>
<tr>
<td>4. GGB</td>
<td>-</td>
<td>+</td>
<td></td>
<td>5.12</td>
<td>14.48</td>
<td>9.10</td>
<td>26.40</td>
</tr>
<tr>
<td>5. GGB-Y</td>
<td>-</td>
<td>-</td>
<td></td>
<td>4.00</td>
<td>14.72</td>
<td>8.15</td>
<td>23.20</td>
</tr>
</tbody>
</table>

* Pigments yields were determined in the manner described in footnotes 1 and 3 of table II.
** Chl. = chlorophyll a; P.C. = phycocyanin.
suspended medium was recovered in acidic eluates from the columns. The column eluates were concentrated by lyophilization and the pH adjusted to 9.0 with 1 N NH₄OH. The eluates were compared to PBG by zone electrophoresis. The electrophoretic runs were performed at a potential of about 20 volts per cm on Whatman No. 3 paper using 0.04 M sodium barbital buffer, pH 9.0, at 2 °C. After each run the paper was dried and sprayed with Ehrlich's reagent. Only 1 spot with Ehrlich color was observed in the column eluates. This substance was indistinguishable from PBG on the basis of its behavior electrophoretically and with the Ehrlich reagent.

Seven porphyrins were detected in the suspending medium of AlA-treated cells by descending paper chromatography. The chromatograms were developed for 24 to 30 hours in 2.6-lutidin, 1.08 N ammonia, water 1 N EDTA (25: 10.5: 7: 0.05, v/v). Three of the algal porphyrins moved with similar Rₜ values to the uroporphyrin III (uro III), coproporphyrin III (copro III), and protoporphyrin IX (proto IX) markers (fig 3). Three other porphyrins produced by the algae were located between the uro III and copro III markers. The Rₜ values of these suggested they may have been porphyrins with 7, 6, and 5 carboxyl groups per molecule. Another red-fluorescing porphyrin was located between the copro III and proto IX markers. This substance may have been a 3 carboxyl porphyrin.

To characterize the algal porphyrins further, the suspending medium was extracted 3 times with chloroform to remove the blue phycoobilin. The aqueous phase was then adjusted to pH 4.0 and shaken 3 times with diethyl ether. The ether was washed with water and the red-fluorescing porphyrins were extracted with various concentrations of HCl (0.01-2.5 N). Much of the red fluorescence was removed from the ether by 0.04 to 0.08 N HCl. The major constituent in this HCl fraction was spectrally identical to copro III (table V) and it chromatographed with the same Rₜ value as copro III in the lutidine solvent system (fig 3). The remaining fluorescence was removed from the ether by 2.0-2.5 N HCl. This HCl fraction contained a porphyrin which absorbed at 408, 554, and 598 μm, as does proto IX (table V). The identity of this porphyrin with proto was substantiated by paper chromatography (fig 3).

After shaking with chloroform and ether, the aqueous phase of the suspending medium was passed through a 2.0 × 0.5 cm talc column. The water soluble porphyrin in the aqueous phase remained at the top of the column. It was eluted with 1 N NH₄OH. In 0.5 N HCl, the water soluble porphyrin absorbed maximally at 405, 552, and 594 μm, as did a reference sample of uro III in the same solvent (table V).

The methyl ester of the algal uro obtained from the talc column was prepared (26). The uro methyl ester (0.48 μmole) from the alga was compared to uro I and III octamethyl esters by the method of Cornford and Benson (4). Figure 4 shows that the uro ester produced by the ALA-treated algae moved with the same Rₜ value as the octamethyl ester of isomer III of uro.

The algal uro methyl ester was hydrolyzed in 7.0 N HCl and decarboxylated to copro (5). The algal copro (0.40 μmole) recovered from the decarboxylation was spectrally indistinguishable from copro III in ethylacetate and in 0.5 N HCl (table V). It also chromatographed with the same Rₜ value as copro III (fig 3). This shows that the uro produced by the ALA-treated algae was isomer number III.

The blue phycoobilin produced by C. caldarium could be precipitated from the suspending medium of the ALA-treated cells by making the aqueous solution 80% saturated with ammonium sulfate. The blue precipitate faded and disappeared during dialysis against distilled water for 6 hours at 2 °C.

The blue phycoobilin was completely removed from the algal culture medium (pH 2.0) by 3 chloroform extractions. A white precipitate formed at the

| Table V. Data on the Spectral Properties of Porphyrin Standards and Algal Porphyrins |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| Porphyrin std. | Solvent λ max (μm) | Soret* | 1. | 2. | 3. | 4. |
| Uro III | 0.5 N HCl | 405 | 552 | 594 | | 2.70 |
| Copro III | 0.1 N HCl | 399 | 548 | 590 | | 2.53 |
| | Ethylacetate | 394 | 495 | 525 | 565 | 620 |
| Proto IX | 2.5 N HCl | 408 | 554 | 598 | | 2.34 |
| | 1.0 N HCl | 405 | 552 | 594 | | 2.72 |
| | Ether soluble | 0.1 N HCl | 399 | 548 | 590 | | 2.55 |
| | HCl Fraction | 2.0-2.5 N HCl | 2.5 | 5 N HCl | 408 | 555 | 600 |
| | Fraction | 0.1 N HCl | 399 | 548 | 590 | | 2.52 |
| | Decarboxylated | Ethylacetate | 394 | 495 | 525 | 565 | 620 |

* The respective values for λ max of the standards are those of Rimington (1960).
Fig. 3. Tracing of a paper chromatogram of porphyrin standards and porphyrins produced by ALA-treated algae. 1. Porphyrins from the suspending medium of ALA-treated algae. 2. Copro I and III standards. 3. Copro III and proto IX standards. 4. Uro obtained from the suspending medium by chromatography on talc column. 5. Algal porphyrin fraction transferred from ether to 0.04 to 0.08 N HCl. 6. Algal porphyrin fraction transferred from ether to 2.0 to 2.5 N HCl. 7. Algal copro produced by decarboxylation of uro.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Solvent</th>
<th>Max (mµ)</th>
<th>1</th>
<th>2</th>
<th>E₃/£</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biliverdin*</td>
<td>Chloroform</td>
<td>645–655</td>
<td>378–380</td>
<td>3.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>645–655</td>
<td>378–380</td>
<td>3.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol-HCl 5 %</td>
<td>665–675</td>
<td>375</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>Phycocyanobilin**</td>
<td>Chloroform</td>
<td>612</td>
<td>360</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform-protonated</td>
<td>630</td>
<td>365</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanidium</td>
<td>Chloroform</td>
<td>610–625</td>
<td>367–372</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td>Phycobilin</td>
<td>Methanol</td>
<td>610–625</td>
<td>367–375</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol-HCl 5 %</td>
<td>680–690</td>
<td>370</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol zinc complex</td>
<td>675–690</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table VI. Data on the Spectral Properties of Biliverdin, Phycocyanobilin, and the Blue Phycobilin Produced by C. caldarium, Strain III-D-2, after Incubation with ALA (7.0 × 10⁻⁵ M) in Darkness for 24 Hours

* Biliverdin prepared by the method of Gray et al. (11).
** Data from O'Horocha (24).

Fig. 4. Tracing of a paper chromatogram uro I and III octamethyl esters and the uro ester obtained from ALA-treated algae. Sample 1 and 2 are methyl esters of algal uro: 3 and 4 are uro I methyl ester; 5 and 6 are uro III methyl ester. The concentration of porphyrin in samples 2, 4, and 6 is doubled.

chloroform water interphase during transfer of the bile-pigment to chloroform. Once in chloroform, the blue pigment was no longer soluble in water, suggesting the possibility that it had been loosely bound to a hydrophilic substance (protein) in the supernatant of the ALA-treated cells.

The spectral properties of the Cyanidium phycobilin in chloroform, in methanol, and in acidic methanol are shown in table VI and figure 5. In chloroform and in methanol, the maximum of the broad absorption band of the phycobilin in the visible portion of the spectrum (610–625 mµ) was at shorter wavelengths than that of biliverdin in chloroform (640–655 mµ) but at about the same wavelength (612 mµ) reported for phycocyanobilin (24).

The absorption maximum of the algal pigment in 5% HCl in methanol was shifted to 680–690 mµ, whereas the peak for biliverdin in the same solvent occurred at about 670 mµ (table VI). The absorption peaks of the protonated phycobilin in methanol at 370 and 680 to 690 mµ and at 367 and 610 to 625
m\textsubscript{\mu} in neutral methanol, are close to the values reported for mesobiliverdin (16).

The greater extinction of the algal pigment at 367–375 m\textsubscript{\mu} than at 610–625 m\textsubscript{\mu} in chloroform and methanol further substantiates its identity as a bile-pigment which is similar if not identical to phycocyanobilin, the phycocyanin chromophore.

The *Cyanidium* bile-pigment did not fluoresce under ultraviolet light (3600 A) in neutral chloroform or in methanol. It developed an intense red fluorescence, however, when the zinc complex of the pigment was prepared (10). The absorption maximum of the zinc complex in methanol was shifted from 610 to 625 m\textsubscript{\mu} to 675 to 690 m\textsubscript{\mu} (table VI). In agreement with the findings of Gray et al. (11) the development of red fluorescence did not accompany a similar spectral shift in the visible absorption maximum of the biliverdin zinc complex. The formation of a red-fluorescing zinc complex suggested a violin-type structure for the algal tetrapyrrole (10).

**Discussion**

Our present understanding of biliprotein biosynthesis is based largely on studies of the action spectrum for phycocyanin formation in a chlorophyll-less *C. caldarium* mutant (23) and on studies of the effect of pre-illumination with red (600–700 m\textsubscript{\mu}) or green (500–550 m\textsubscript{\mu}) light on the formation of phycocyanin and phycoerythrin precursors, respectively, in *Tolyphothrix tenus* (6). Myers and Kratz (19) have reported that the phycocyanin content of *Anacystis nidulans* was higher when the alga was grown under low light intensity. Bogorad (2) has speculated on biliprotein biogenesis from a hemoprotein precursor on the basis of studies on bile-pigment production by mammalian systems in vivo and in vitro.

The conversion of hematin and heme-proteins but not protoporphyrin IX into biliverdin in vitro (12), and the enzymatic formation of a possible precursor of biliverdin from pyridine-hemichromogen and hemoglobin-haptoglobin (20), or myoglobin (21) suggests that the phycocyanin chromophore may also be produced from a complex of a protein with type IX protoporphyrin or heme, rather than either free acid. The protein moiety of the presumptive hemoprotein precursor in the *C. caldarium* system (2) may function as an enzyme for the production of the phycobilin (e.g. open-chain tetrapyrrole), thus representing an enzyme-substrate complex. Alternatively, the hemoprotein may be the actual precursor of the entire native bile-pigment-protein complex (phycocyanin); after the enzymatic oxidation of the heme moiety of the precursor into a phycobilin, the protein-bile-pigment complex could undergo further alterations to become phycocyanin.

**Pigment Biosynthesis and Inhibitors.** The inhibition of phycocyanin and chlorophyll a accumulation by chloramphenicol in illuminated *C. caldarium* cells suggests that protein synthesis is required for pigment formation to occur. This presumes that the effect of the antibiotic in the algal system is similar to its effect on bacterial ribosomes (13) and reticulocyte ribosomes (29). Inhibition of pigment accumulation in *C. caldarium* was observed with a wide range of chloramphenicol concentrations \((10^{-2}–10^{-3} \text{m})\). A similar inhibition of biliprotein synthesis by chloramphenicol treatment was reported to occur in *T. tenus* (7).

The inhibitory effects of nitrogen starvation, chloramphenicol, \(p\)-fluorophenylalanine, and ethionine, on phycocyanin formation in *Cyanidium* lends further support to the idea that protein synthesis is needed for greening of this alga. The failure of cells incubated with inhibitors to produce pigment in the light and the inability of soluble protein extracts from dark-grown cells to react immunochemically with antiserum to the phycocyanin protein (Bogorad and Troxler, unpublished data) suggests that the phycocyanin protein is engendered de novo during greening of dark-grown cells.

There have been numerous reports on the formation of PBG and porphyrins from ALA by bacteria (14) and by algae (3). As described in this report, cells of *Cyanidium* produce a blue bile-pigment as well as PBG and porphyrins during incubation with ALA. The algal phycobilin was produced by dark-grown and by preilluminated cells of the wild-type and mutant *C. caldarium* strains: preillumination appeared to stimulate ALA utilization. This suggests that the concentration of enzymes for phycobilin synthesis and ALA utilization is lower in dark-grown than in illuminated cells.

Porphyrin and phycobilin synthesis from ALA was obstructed by treating cells of *C. caldarium* with
chloramphenicol or ethionine. These data suggest that a synthesis of proteins (e.g., enzymes) is necessary for the production of bile-pigment from ALA to occur.

Synthesis of the phycocyanin protein and chromophore (e.g., phycocyanobilin) normally requires light. Phycobilin formation can occur in darkness, however, if ALA is supplied. On the basis of these observations, it is concluded that: A) bile-pigment synthesis (i.e., phycocyanobilin chromophore production) in ALA-treated cells does not occur via photochemical reactions similar to the photooxidation of the etioporphyrin sodium complex into etiobiliverdin (16); B) light is probably required for the synthesis of ALA or its precursors; and C) in view of the inability of cells treated with inhibitors to utilize ALA, it is likely that phycobilin synthesis from ALA by Cyanidium occurs enzymatically rather than by chemical reactions described in model systems for bile-pigment formation (16).

**AL-4 Utilization by Cyanidium Mutants.** As observed previously (tables II, III, IV) more porphyrins and bile-pigments were formed by suspensions of dark-grown cells of Strain III-D-2 which had been illuminated prior to incubation with ALA than by unilluminated cells: wild-type *C. caldarium* and variously pigmented mutant strains behaved similarly (table IV).

It is of particular interest that phycobilin was produced from ALA by strain III-C, which normally produces no phycobiliproteins, and by strain GGB-Y, which lacks both phycobiliproteins and chlorophyll a. Apparently the required enzymes are present. The behavior of III-C suggests that the control of bile-pigment and of chlorophyll production may be separate at a very early point, i.e., ALA formation.

Bile pigment production from ALA by strain GGB, which forms phycobiliproteins but lacks chlorophyll a, and by wild-type cells is not surprising.

**Products of ALA Utilization.** Although 4 isomers of uroporphyrin and coproporphyrin have been prepared synthetically, only the I and III isomers have been isolated from biological sources (2). The ALA-treated Cyanidium cells excreted primarily uroporphyrin and coproporphyrin into the suspending medium. It was shown by paper chromatography with standards that both algal porphyrins were the III isomers of uro and coproporphyrin.

If the algal porphyrins originated by the nonenzymatic condensation of PBG or dipyrromethanones, other porphyrins in addition to the III isomers would have been detected.

The spectral properties of the *C. caldarium* phycobilin in neutral chloroform resemble those of phycocyanobilin, the phycocyanin chromophore (24). The visible absorption maximum of the protonated *Cyanidium* phycobilin in methanol (680-690 μM) was observed at longer wavelengths than protonated phycocyanobilin (max = 630 μM) in chloroform described by O’HöEocha (24). The pigment described as phycocyanobilin (phycobilin 630) by O’HöEocha (25), however, was obtained by hydrolysis of phycocyanin in concentrated HCl for 30 minutes. Since phycobilin 630 was shown to undergo isomerization in concentrated HCl for 7 hours (24), the identity of this phycobilin with the native chromophore could be questioned. The *Cyanidium* phycobilin which was obtained under milder conditions may be an immediate precursor of the phycocyanin chromophore, or perhaps identical to it.

On the basis of its red-fluorescing zinc complex, the *C. caldarium* phycobilin was tentatively judged to be a violin-type pigment (10). The phycobilin produced by the ALA-treated *Cyanidium* cells reacted similarly to biliverdin in the Omelin reaction, however, suggesting it is a bilirethene pigment, in contradiction to the evidence for its presumptive violin-type structure. Further characterization of the *C. caldarium* bile-pigment is in progress.

**Literature Cited**


