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Biosynthesis of Phycocyanobilin*

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ABSTRACT: Phycocyanin synthesis was studied in *Cyanidium caldarium* cells incubated with the porphyrin–bile–pigment precursor, Δ-aminolevulinic acid-4-C^{14} (ALA). ^{14}C-labeled phycocyanin from sonicated, ALA-treated cells was purified by ammonium sulfate fractionation and column chromatography on brushite and diethylaminoethylcellulose. Isotopically labeled phycocyanobilin, a bile–pigment-like chromophore of phycocyanin was cleaved from protein, methylated, and purified by thin layer chromatography on silica gel. The *C. caldarium* phycocyanobilin dimethyl ester was spectrally and chromatographically indistinguishable from the corresponding pigment prepared from *Phormidium luridum* phycocyanin. Furthermore, melting point depressions were not observed when *C. caldarium* phycocyanobilin dimethyl ester-^{14}C was cocrystallized with methylated *P. luridum* pigment. These results establish that ALA is a *direct* precursor of phycocyanobilin and support the idea that phycocyanins of differing algal species have one common chromophore. *Cyanidium caldarium* cells exposed to sufficiently high ALA concentrations excrete porphobilinogen, porphyrins, and a blue, protein-free bile pigment into the suspending medium. The blue pigment was methylated and purified by thin layer chromatography on silica gel. The methylated blue pigment absorbed maximally at 685 and 375 mμ in methanol–5% HCl, as did phycocyanobilin dimethyl ester from *P. luridum* and *C. caldarium* phycocyanin. Methylated blue pigment migrated on silica gel with the same R_F value in three solvent systems as did methylated phycocyanobilin from both algal species. These data suggest that the excreted blue pigment is phycocyanobilin, a phycocyanin chromophore. The specific activities of excreted coproporphyrin III and phycocyanobilin were nearly identical, and were four times that of porphobilinogen. Porphobilinogen contained twice as much radioactivity per micromole as did Δ-aminolevulinic acid-4-^{14}C. This established that ALA is incorporated directly into phycocyanobilin, and that porphobilinogen and coproporphyrinogen III are intermediates in the biosynthesis of this bile pigment.

Blue-green and red algae synthesize an accessory photosynthetic pigment called phycocyanin (Haxo, 1960). Phycocyanin consists of a protein (estimated molecular weight 300,000) to which are bound approximately 20–30 residues of the chromophore phycocyanobilin (O'hEocha, 1965). Phycocyanobilin is a blue tetrapyrrole in which the pyrrole rings are joined by three carbon bridges conferring an "open-chain" configuration resembling that of mammalian bile pigment (Lemberg and Bader, 1933).

In mammals, bile pigment is formed by degradation of the iron porphyrin (heme) which is the prosthetic group of hemoglobin and other heme-containing proteins (Lester and Schmid, 1964). Because of its similarity to mammalian bile pigment, it has been suggested that phycocyanobilin may also be formed by the degradation of a protein-bound metalloporphyrin precursor (Troxler and Bogorad, 1966). The alga *Cyanidium caldarium* is an attractive subject for studies on phycocyanin and phycocyanobilin formation. Wild-type cells of this alga grown in the dark lack chlorophyll a and phycocyanin, but pigment formation occurs when the cells are placed in the light (Nichols and Bogorad, 1962). On incubation with Δ-aminolevulinic acid (ALA),[1] both wild-type and mutant *C. caldarium* cells excrete a protein-free pigment which closely resembles phycocyanobilin (Troxler and Bogorad, 1967), and which can be extracted readily from the suspending medium. The excretion of this pigment is dependent on the ALA concentration and on the number of cells in the suspension (Troxler and Bogorad, 1966). As a result of these several factors pigment synthesis in *C. caldarium* can be fully controlled by appropriate manipulation of the culture conditions.

In the present paper, phycocyanin and phycocyano-

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[1] Abbreviations used: ALA, Δ-aminolevulinic acid; PGB, porphobilinogen.

bilin synthesis were studied in *C. caldarium* cells incubated with ALA-4-^{14}C. Phycocyanin was isolated and characterized chromatographically. ^{14}C-labeled phycocyanobilin, cleaved from protein, was methylated, chromatographed, and crystallized. The purified material was characterized and compared to phycocyanobilin of another species and to the pigment excreted into the suspending medium by ALA-treated cells of *C. caldarium*.

Materials and Methods

The Organism. Cells of *C. caldarium* (mutant III-D-2 and GGB) were used in the present investigation. When cultured in the light, mutant III-D-2 contains more chlorophyll a and phycocyanin per cell than does the wild type (Nichols and Bogorad, 1960). Illuminated cells of mutant GGB make phycocyanin but are unable to make chlorophyll a. When grown in darkness, chlorophyll a and phycocyanin are not produced in significant amounts by either strain although carotenoids occur in both mutants regardless of the culture conditions.

Incubations with Δ-Aminolevulinic Acid-4-^{14}C. In all experiments the algal cells were grown in a sterile glucose-containing medium (Allen, 1959) at 40–45° in darkness for 7–11 days (standard conditions). The suspensions were agitated in erlenmeyer flasks by means of a rotary shaker. Dark-grown cells were collected by centrifugation, washed once with distilled water, and resuspended in the same nutrient medium lacking glucose. For studies on synthesis of ^{14}C-labeled phycocyanin and phycocyanin chromophore (*i.e.*, phycocyanobilin), both mutants GGB and III-D-2 were used. ALA-4-^{14}C (9.35×10^{-3} mM, sp act. 10.7 mc/mmole) was added to 60-ml suspensions of dark-grown cells which were shaken and illuminated for 48 hr under standard conditions. Flasks were placed 15 cm from General Electric 15-w, cool white fluorescent tubes.

Experiments on pigment excretion by cells treated with an excess of ALA were performed with mutant III-D-2. Green cells were incubated in darkness with ALA-4-^{14}C to which unlabeled carrier was added (final value: 8.38×10^{-1} mM, sp act. 0.024 mc/mmole).

Preparation of Phycocyanin. Following exposure to ALA-4-^{14}C and light for 48 hr the pigmented cells were washed twice with 0.1 M phosphate buffer (pH 6.5), resuspended in three volumes of the same buffer, and disrupted for 10 min with a Branson 20-kcycle sonic oscillator. The large particles in the broken cell preparations were removed by centrifugation at 38,000 *g* for 30 min. The phycocyanin in the blue supernatant was precipitated with ammonium sulfate (50% saturation). The precipitated phycocyanin was dissolved in a small volume of distilled water and dialyzed for 24 hr at 4° against distilled water.

The crude phycocyanin was applied to 1.0 × 10 cm brushite columns (Siegelman and Firer, 1964) and eluted stepwise with phosphate buffers of increasing ionic strength. Samples (1.8 ml) of column effluent were obtained with the aid of a fraction collector with a drop-counting device.

In several experiments the phycocyanin obtained by chromatography on brushite columns was pooled, dialyzed, and chromatographed on 1.0 × 10 cm DEAE-cellulose columns. The DEAE columns were equilibrated with 0.01 M Tris-HCl buffer (pH 7.2). Following application of the phycocyanin sample, the DEAE columns were developed by linear gradient from ionic strength of the starting buffer (240 ml) to 0.5 M NaCl in the same buffer (240 ml). Fractions of 8.0 ml were collected.

The optical density at 620, 410, and 280 mμ of fractions from brushite or DEAE columns was read on a Beckman DB recording spectrophotometer. The radioactivity in each fraction obtained from the columns was determined in 0.05-ml samples dissolved in 1 ml of Hyamine (Rapkin, 1961), 2 ml of methanol, and 15 ml of dilute scintillation fluid.[2] These were counted on a (Packard Tri-Carb) liquid scintillation spectrometer. The quenching in each sample was calculated by the addition of a known quantity of internal standard.

Preparation of the Phycocyanobilin. ^{14}C-labeled phycocyanobilin was obtained from phycocyanin-^{14}C to which unlabeled carrier phycocyanin was added. Phycocyanin chromophore was cleaved from pigment protein by refluxing for 16 hr in 1 l. of absolute methanol according to the procedure of Siegelman *et al.* (1966). The chromophore so obtained was separated from protein residue and methylated in 7% BF_3. Impurities were separated from the phycocyanobilin dimethyl ester by preparative thin layer chromatography on silica gel (Siegelman *et al.*, 1966).

In experiments in which high concentrations of ALA (ALA-4-^{14}C plus carrier) were administered to III-D-2 cells, the ^{14}C-labeled pigment excreted into the suspending medium was extracted with chloroform, methylated, and purified by thin layer chromatography. Melting point determinations (uncorrected) on crystalline pigments were determined with Zeiss WL polarizing microscope on a Kosler microheating stage.

Results

Preparation of Phycocyanin-^{14}C. The "profile" of a brushite column chromatogram of protein from "greened," ALA-treated, GGB cells showed three major isotopically labeled components designated as fractions I–III (Figure 1). Fraction II contained phycocyanin. In the tubes (Figure 1, 34–37) containing the greatest amount of phycocyanin more isotope was found than in the others (Figure 1). Fraction I (Figure 1) was faintly blue and contained a small amount of phycocyanin as well as a hemoprotein with a soret band at 408 mμ. The hemoprotein was tentatively

[2] Liquiflor, diluted 25 times with toluene, Pilot Chemicals, Inc., Watertown, Mass.

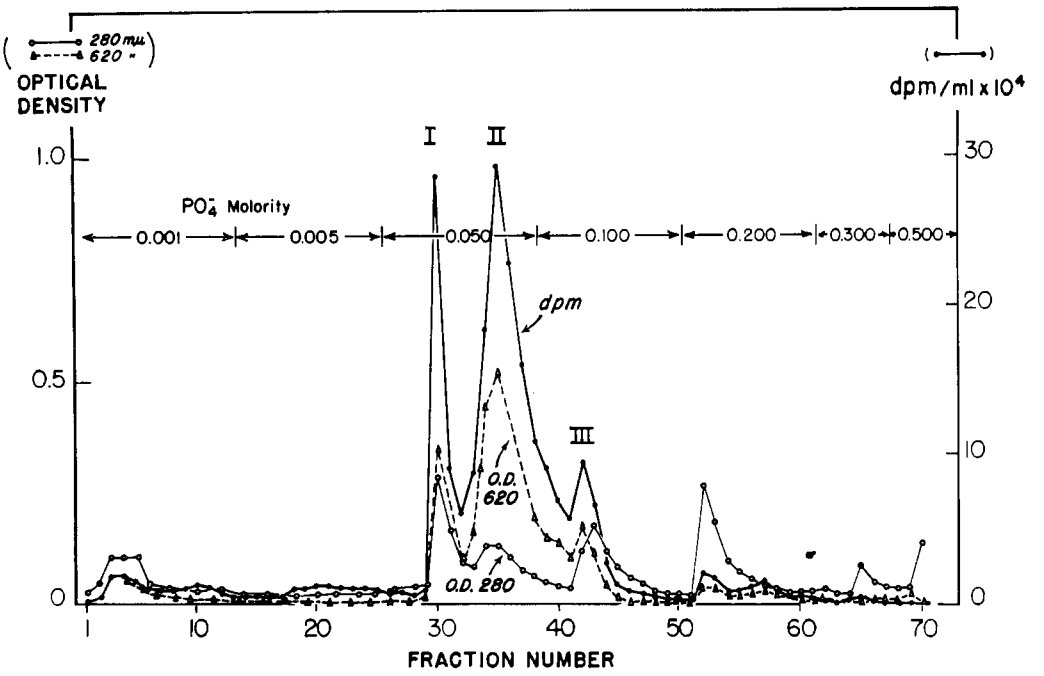

FIGURE 1: Brushite column chromatogram of protein from *C. caldarium*, mutant GGB. The optical density at 280 mμ (○), 620 mμ (△), and dpm/ml × 10^4 (●) is shown. The column was developed stepwise with phosphate buffers (PO_4^-) of increasing ionic strength. Each fraction contained 1.8 ml.

identified as a peroxidase on the basis of its ability to produce guaiacol color and to degrade H_2O_2 (Crean, 1966). Fraction III consisted of a mixture of equal amounts of phycocyanin and the closely related bile pigment–protein complex, allophycocyanin.

The ratio of the optical density at 620 mμ to that at 280 mμ has been used as a criterion for purity of phycocyanin. The $OD_{620\,m\mu}$:$OD_{280\,m\mu}$ ratio of crystalline phycocyanin from *Plectonema calothricoides* is reported to be approximately 4.0 (Berns and Scott, 1966). The $OD_{620\,m\mu}$:$OD_{280\,m\mu}$ ratio of phycocyanin in tubes 34–37 in Figure 1 was greater than 3.0 indicating that at least by this criterion, the phycocyanin was not appreciably contaminated with other proteins.

To determine whether pigment obtained from brushite columns contained radioactive material other than phycocyanin, the phycocyanin-^{14}C from several runs was pooled, dialyzed, and chromatographed on DEAE-cellulose. The phycocyanin began to move on the DEAE column as a diffuse band when the chloride concentration had reached about 0.18 M (Figure 2). Elution from the column was complete when the chloride ion concentration had reached about 0.25 M. It is evident from Figure 2 that the isotope and the blue pigment moved together on the DEAE column.

The $OD_{620\,m\mu}$:$OD_{280\,m\mu}$ ratio of the phycocyanin prior to and after chromatography on DEAE columns was about 3.0. Because this ratio did not change significantly and the migration of the blue pigment coincided with that of the radioactivity, it seemed likely that the phycocyanin samples were essentially free from contaminant.

To determine the differences, if any, in the ability of *C. caldarium* strains containing and lacking chlorophyll a to utilize exogenously supplied ALA for phycocyanin chromophore biosynthesis, dark-grown cells of the chlorophyll-containing mutant III-D-2 were illuminated and exposed to ALA-4-^{14}C for 48 hr. Crude phycocyanin was prepared as described above and purified by column chromatography. Two major isotope-containing fractions were obtained (Figure 3). Fraction IIA (Figure 3) contained phycocyanin and fraction IIIA contained a mixture of phycocyanin and allophycocyanin. The qualitative difference in the chromatograms of mutant III-D-2 and GGB protein is the lack of a hemoprotein–phycocyanin fraction in the former strain. Also apparent is the quantitative difference in the allophycocyanin content observed in the preparations from the two mutants.

Preparation of Phycocyanobilin from Phycocyanin. The free chromophore liberated by refluxing about 1 g of *C. caldarium* phycocyanin in absolute methanol for 16 hr was methylated in 7% BF_3. The methylated chromophore was separated from altered pyrrolic derivatives by thin layer chromatography on silica gel. Phycocyanin contains only about 5% phycocyanobilin by weight (Brody and Brody, 1961). The recovery of pigment from the methanol reflux was estimated to be about 10% but significant additional losses occurred during methylation. Because milligram quantities of *C. caldarium* phycocyanobilin dimethyl ester were not available the pigment was cocrystallized with the corresponding pigment from *P. luridum*.

The chromatographic behavior of noncrystalline,

TABLE I: Chromatographic Behavior of Methylated Phycocyanin Chromophores.

Phycocyanobilin Dimethyl Ester	R_F Values[a]		
	CCl_4–Methyl Acetate (2:1) I	Benzene–Ethanol (9:1) II	Methylene Dichloride–Ethyl Acetate (8:2) III
C. caldarium[b]	0.28	0.60	0.35
P. luridum[c]	0.28	0.60	0.35
C. caldarium and *P. luridum*[c]	0.28	0.60	0.35

[a] Silica gel 0.75 mm in thickness. [b] Noncrystalline pigment. [c] Crystallized twice from chloroform–methanol.

isotopically labeled methylated *C. caldarium* phycocyanobilin was identical with the pigment prepared by the same procedure from *Phormidium luridum* phycocyanin. The distance of migration (R_F) of the colored spots was identical in three solvent systems (Table I), and 80–94% of the measurable radioactivity on the chromatographic surface coincided with the colored areas (Table II). Small amounts of radioactivity, presumably in part representing degradation products, were located at the origin and trailed behind the blue pigment.

The spectral properties of *C. caldarium* and *P. luridum* phycocyanin chromophores were identical. Both pigments absorbed maximally at 685 and 375 mμ in methanol–5% HCl, as did the corresponding pigment from *Plectonema boryanum* phycocyanin (Siegelman *et al.*, 1966). Neither the phycocyanin chromophores of *P. luridum* nor *C. caldarium* fluoresced when their methanol solutions were irradiated with ultraviolet light (3600 A). Both pigments formed nonfluorescing zinc complexes on addition of Schlesinger reagent to their methanol solutions. The melting point of *P. luridum* phycocyanobilin dimethyl ester was 219–223° and that of mixed crystals of phycocyanobilin dimethyl ester from *C. caldarium* and *P. luridum* was 218–226°. Therefore, on the basis of their chromatographic behavior, spectral properties, and melting point data the chromophores of *P. luridum* and *C. caldarium* phycocyanins appear to be identical.

Preparation of Blue Pigment Excreted into the Suspending Medium. The blue pigment excreted by cells of mutant III-D-2 during 48-hr incubations with ALA-4-^{14}C and carrier ALA was isolated, methylated, and

TABLE II: Thin Layer Chromatography of *P. luridum* and *C. caldarium* Phycocyanobilin Dimethyl Ester on Silica Gel.[a]

R_F Value	Dpm/R_F Increment as a % of the Total					
	Solvent I[a]		Solvent II[a]		Solvent III[a]	
	1[b]	2[c]	1[b]	2[c]	1[b]	2[c]
0.0–0.1	7	14	2	0	2	0
0.1–0.2	6	2	1	0	5	13
0.2–0.3	87	83	2	0	10	3
0.3–0.4	1	1	6	6	78	81
0.4–0.5	0	0	7	2	4	3
0.5–0.6	0	0	48	92	1	0
0.6–0.7	0	0	32	0	0	0
0.7–0.8	0	0	1	0	0	0
0.8–0.9	0	0	1	0	0	0
0.9–1.0	0	0	0	0	0	0

[a] Silica gel 0.75 mm in thickness. Solvents I–III correspond to those in Table I. [b] Noncrystalline *C. caldarium* pigment. [c] Cocrystalline *P. luridum* and *C. caldarium* pigment.

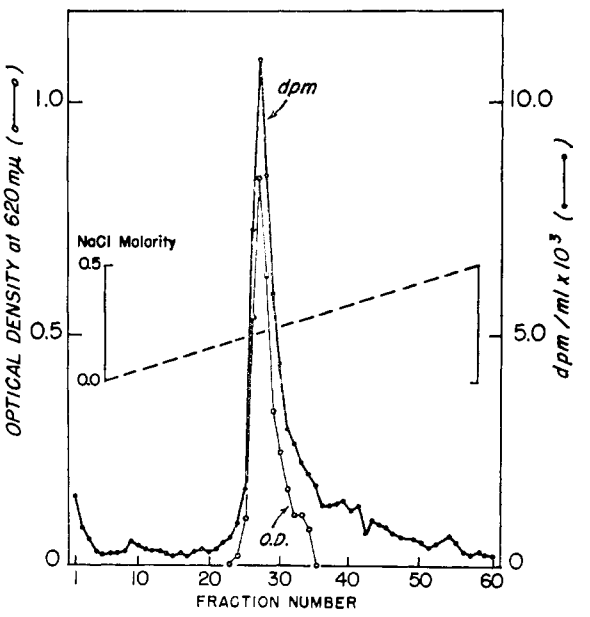

FIGURE 2: Chromatogram of phycocyanin-^{14}C from *C. caldarium*, mutant GGB on DEAE-cellulose showing the optical density at 620 mμ (○) and the dpm/ml × 10^3 (●) in each 8.0-ml fraction. Elution of pigment was accomplished by means of a linear NaCl gradient (see text).

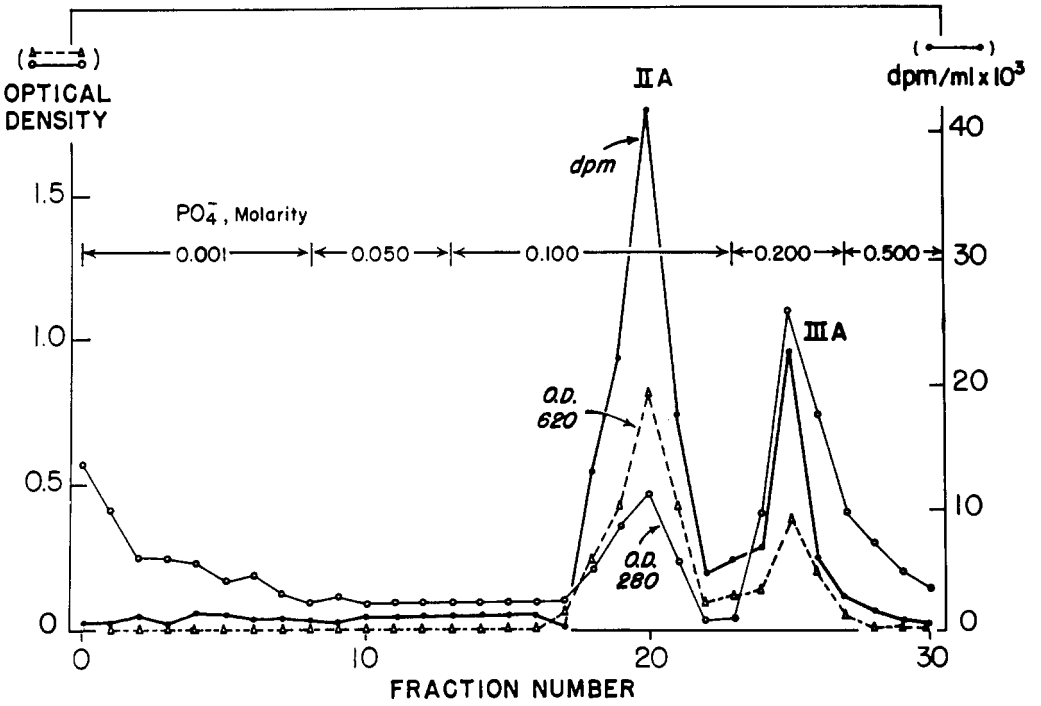

FIGURE 3: The brushite column elution pattern of protein from *C. caldarium*, mutant III-D-2, showing the optical density at 280 mμ (○), 620 mμ (△), dpm/ml × 10^3 (●) in each 5.0-ml fraction.

chromatographed. In three solvent systems the isotope was located in the region corresponding to that of the blue pigment. The R_F values of this pigment were the same as those of *C. caldarium* and *P. luridum* phycocyanobilin dimethyl esters (Table III). The pigment absorbed maximally at 685 and 375 mμ in methanol–5% HCl and did not fluoresce in absolute methanol but formed a nonfluorescing zinc complex when treated with Schlesinger reagent. These data indicate that the excreted pigment is identical with the chromophore of phycocyanin.

TABLE III: Thin Layer Chromatography of *P. luridum* Phycocyanobilin Dimethyl Ester and Methylated Pigment Excreted by Cells of *C. caldarium*, Mutant III-D-2.

Compound	R_F Values[a]		
	Solvent I[a]	Solvent II[a]	Solvent III[a]
P. luridum phycocyanobilin dimethyl ester	0.28	0.60	0.35
Methylated blue pigment[b]	0.28	0.60	0.35

[a] Silica gel bed 0.75 mm. The solvents correspond to those numbered I, II, and III in Table I. [b] Noncrystalline pigment.

Wild-type and mutant *C. caldarium* cells excrete porphobilinogen (PBG) and porphyrins into the suspending medium during incubations with ALA (8.38×10^{-1} mM) in addition to the blue bile pigment (Troxler and Bogorad, 1966). To determine whether these pyrrolic pigments are related biosynthetically to the bile pigment their specific activities were estimated. The methods for extraction and quantitation have been described (Troxler and Bogorad, 1966). Isotope measurements were performed in 15 ml of Bray's (1960) solution in a Tri-Carb liquid scintillation spectrometer.

As shown in Table IV, the specific activity of excreted coproporphyrin III and blue bile pigment were almost identical. These values are about four times that of PBG and eight times that of ALA as would be predicted if they were intermediates in the same pathway and were produced from ALA directly.

Discussion

Mammalian bile pigment is probably derived not only from the heme of hemoglobin (Ostrow *et al.*, 1962) but also from the prosthetic groups of cytochrome B_5 and P-450 (Omura and Sato, 1964), catalase, peroxidase (Kench, 1954), myoglobin (R. Lester, J. Daly, and R. F. Troxler, in preparation), and other heme-containing proteins (Bogorad, 1963). Although it is accepted that bile pigment biogenesis involves porphyrin-ring formation and ring cleavage, the mechanisms

TABLE IV: Specific Activity of Pyrrolic Pigments Excreted by Cells of *C. caldarium*, Mutant III-D-2, during 48-hr Incubation with Δ-Aminolevulinic acid-4-^{14}C.

	dpm/μmole	Theoretical Sp Act. Based on Δ-Amino-levulinate
Δ-Aminolevulinic acid[a]	9.5×10^4	
Porphobilinogen[a]	1.8×10^5	1.9×10^5
Coproporphyrin III[a]	7.7×10^5	7.6×10^5
Blue bile pigment[b]	7.5×10^5	7.6×10^5

[a] Prepared as described by Troxler and Bogorad (1966). [b] ϵ 38,000 at 680 mμ in 5% HCl–methanol.

permitting the formation of "open-chain" tetrapyrroles from metalloporphyrins have not been elucidated. It has been proposed that heme degradation proceeds through a series of steps leading to the oxidative removal of the α-methene bridge carbon of protoheme and to subsequent loss of iron. The evidence for this hypothesis is largely based on spectrophotometric identification of intermediates and end products formed during the coupled oxidation of pyridine hemochromogen with ascorbic acid or hydrazine (Lemberg, 1956). Isolation of these proposed intermediates and rigorous proof of the hypothetical biochemical pathway have not been performed. Although an enzyme (so-called heme α-methenyl oxygenase) which converts pyridine hemochromogen, hemoglobin–haptoglobin (Nakajima *et al.*, 1963), and myoglobin (Nakajima, 1963) to bile pigment has been described, recent investigations cast doubt on the validity of these data (Levin, 1967; Murphy *et al.*, 1967).

Several lines of evidence suggest that algal phycocyanobilins are produced by a mechanism similar to those involved in the conversion of heme to bile pigment. (1) As with *i*-urobilin, phycoerythrobilin, the chromophore of phycoerythrin, is oxidized by chromic acid to ethylmethylmaleimide, hematinimide, and succinic acid (O'hEocha, 1967). This indicates that both end rings in phycoerythrobilin contain β-methyl and β-ethyl substituents, that one or more central rings have β-methyl and β-carboxyethyl groups. Assuming that phycocyanobilin and phycoerythrobilin are produced by similar mechanisms, one could argue in view of the above that algal bile pigments derive from porphyrin rings cleaved only at the IXα position rather than by polymerization of single pyrrole units or by random oxidation at any bridge carbon in the porphyrin ring. (2) Algal bile pigments treated chemically form derivatives similar or identical with known derivatives of mammalian bile pigment, *e.g.*, mesobiliverdin is released from phycocyanin boiled in 10% methanolic alkali (Lemberg and Legge, 1949), and urobilin is released from phycoerythrin, a red algal chromoprotein, during prolonged treatment with hot, concentrated hydrochloric acid (Lemberg, 1930).

While the foregoing suggest strongly that algal phycocyanobilin and mammalian bile pigment biogenesis proceeds through steps involving porphyrin ring formation and α-methene bridge cleavage, it is unclear whether heme is an intermediate common to both pathways. The spectral response curve for phycocyanin formation in a chlorophyll-less *C. caldarium* mutant resembles the absorption spectrum of a hemoprotein (Nichols and Bogorad, 1962). This might suggest that the heme moiety of an algal hemoprotein is the direct precursor of the phycocyanin chromophore. Alternatively, this postulated hemoprotein could serve as a photoreceptor for a light-requiring step necessary for the synthesis of phycocyanin protein, or chromophore, or both.

There is no reason *a priori* to assume that phycocyanobilin formation, like mammalian bile pigment formation, proceeds through a heme (*i.e.*, iron protoporphyrin IX) intermediate. Although the end-product phycocyanobilin is an "open-chain," IXα tetrapyrrole with a structure resembling mammalian bile pigment (Cole *et al.*, 1967a,b), in the algae this sequence could conceivably derive from a metal-free meso- or protoporphyrin intermediate, or more likely, it could derive from a porphyrin-containing magnesium or another metal. Despite these potential differences, the similarities between open-chain tetrapyrrole formation in algae and mammals suggest the suitability of phycocyanobilin synthesis as a model of bile pigment formation. The data presented in this paper provide additional information on which to base further study of this subject.

The column chromatographic data, by showing that counts coincide with phycocyanin migration, and that the 620:280 mμ ratios remain constant after passage through a second column, indicate that the preparative procedures yield a relatively pure end-product phycocyanin. Labeled allophycocyanin is also synthesized by both algal mutants (GGB and III-D-2), but because of its lower concentration and since less data are available on its chromophore, it is a less suitable compound to study. In the third labeled fraction synthesized by mutant GGB, a hemoprotein was observed which degraded H_2O_2 and produced guaiacol color, and is probably a peroxidase (Crean, 1966).

The phycocyanin chromophores of at least two algae (*C. caldarium* and *P. luridum*) are indistinguishable by spectral and chromatographic means. That these chromophores are identical is further supported by the failure of mixed crystals of phycocyanobilin dimethyl ester from the two species to show melting point depressions. This in turn is consistent with the finding that phycocyanobilins obtained from a variety of blue-green and red algae are the same open-chain tetrapyrrole (H. W. Siegelman, personal communication).

The isotopic studies described above show that ALA, a specific porphyrin–bile pigment precursor, is incorporated into the chromophore of phycocyanin. Al-

though the biosynthetic intermediates between ALA and phycocyanobilin have not been described, one can guess with some confidence what they might be from the recently proposed structure of this bile pigment based on nuclear magnetic resonance spectra and on mass spectral analysis (Cole *et al.*, 1967a,b). The probable intermediates (*e.g.*, uroporphyrinogen III, coproporphyrinogen III, protoporphyrin IX, and possibly heme) present in *C. caldarium* cells actively synthesizing phycocyanin (and therefore phycocyanobilin) have not been detected despite considerable efforts to find them by extracting 100-g quantities of cells, or attempting to block synthesis at an intermediate step with inhibitors or by exposing cells to mineral deficient conditions.

However, the specific activities of the excreted blue bile pigment, which is indistinguishable from *C. caldarium* phycocyanobilin by several criteria and is probably identical with it, and of excreted coproporphyrin III and PBG, suggest that all these pyrrolic pigments are made in the same biochemical pathway directly from ALA. Assuming that excreted blue bile pigment is phycocyanobilin, then PBG and coproporphyrinogen III can be assigned as intermediates in phycocyanobilin biosynthesis.

Finally, the data showing that ALA-treated *C. caldarium* cells produce and excrete a pigment which is identical with the chromophore of phycocyanin prepared by the "methanol reflux" procedure is of particular interest. The possibility of a minor molecular alteration being induced in the chromophore during the reflux procedure is appreciable and would be difficult to detect. The pigment produced by ALA-treated *C. caldarium* cells is identical with the purest preparations of phycocyanin chromophore, and is excreted by the algal cells free from protein. This pigment is obtained in pure form simply by extraction of the cell-suspending medium with chloroform. The identity of this material with the material obtained from phycocyanin directly, by the more rigorous procedure, provides additional evidence that the extractive procedure does not alter the structure and that the end product is indeed the native phycocyanin chromophore.

Acknowledgment

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