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Synthesis of Bile Pigments in Plants. Formation of Carbon Monoxide and Phycocyanobilin in Wild-Type and Mutant Strains of the Alga, *Cyanidium caldarium*†

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ABSTRACT: Wild-type cells of the alga, *Cyanidium caldarium*, lack photosynthetic pigments when grown in darkness but produce phycocyanin and chlorophyll a when placed in the light. During synthesis of phycocyanobilin, the bile pigment prosthetic group of phycocyanin, wild-type cells evolved equimolar quantities of carbon monixide (CO) at identical rates. Cells of the wild-type, mutant III-D-2 (which makes more pigment per cell than the wild type), and mutant GGB strains (which makes phycocyanin but not chlorophyll a), administered δ-aminolevulinic acid-5-^{14}C (ALA), produced equimolar quantities of labeled CO phycocyanobilin in which the specific activity of CO was one-seventh that of phycocyanobilin. This suggests that CO and algal bile pigment are derived from the carbon skeleton of protoporphyrin IX. Mutants which are unable to make phycocyanin did not produce CO in the light. Wild-type cells incubated with unphysiologic concentrations of ALA-5-^{14}C in the dark excreted labeled phycocyanobilin and evolved labeled CO at comparable rates. The specific activity of evolved CO was one-seventh that of excreted phycocyanobilin. This shows convalent linkage of phycocyanobilin to phycocyanin apoprotein is not an essential step in production of algal bile pigment, and that the mechanism of porphyrin ring opening and CO formation appears to be similar whether phycocyanobilin is apoprotein bound or apoprotein free. The probable involvement of a metal complex of protoporphyrin IX as the direct precursor of phycocyanobilin is discussed, as is the relation of algal CO production to CO exhalation in rats, dogs, and man as a consequence of administered heme and hemoglobin conversion to mammalian bile pigment.

Phycocyanin and phycoerythrin are bile pigment–protein-complexes found in the photosynthetic apparatus of red, blue-green, and cryptomonad algae (O'hEocha, 1966). These algal biliproteins are thought to serve as accessory pigments by virtue of their promotion of photosynthetic oxygen evolution and a chlorophyll a fluorescence (Myers, 1971). Biliproteins exist *in vivo* as large aggregates called phycobilisomes, which appear in stained sections in the electron microscope as granules, 350–450 Å in diameter, located on the surfaces of thylakoid membranes in chloroplasts (Gantt and Conti, 1967). Biliprotein molecules are comprised of subunits which associate and dissociate in aqueous solution into larger or smaller molecular species depending on the pH, ionic strength of buffer, and pigment concentration (O'hEocha, 1965; Hattori *et al.*, 1965). The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis of phycocyanin suggest that the molecule consists of two distinct subunits with molecular weights of 16,000 and 17,000, respectively (Bennett and Bogorad, 1971). Each subunit is believed to contain one residue of the bile pigment chromophore, phycocyanobilin. Phycocyanobilin is structurally related to bilirubin (Cole *et al.*, 1967; Crespi *et al.*, 1967), the principal mammalian bile pigment derived from hemoglobin in senescent erythrocytes (Lester and Troxler, 1969). Phycocyanobilin is covalently linked to phycocyanin apoprotein (Siegelman *et al.*, 1967), and comprises 3.6–4.0% (by weight) of the phycocyanin molecule (Troxler and Lester, 1967; Crespi *et al.*, 1968). The "biosynthetic unit" of phycocyanin, therefore, consists of a polypeptide chain in the 16,000 molecular weight range (*ca.* 130 amino acid residues) to which 1 residue of phycocyanobilin (molecular weight of the free acid = 586; Cole *et al.*, 1967; Crespi *et al.*, 1967) is covalently linked. It is not known whether phycocyanobilin and phycocyanin apoprotein are synthesized separately and then joined, or if a "biosynthetic unit" of phycocyanin is derived from conversion of a metalloporphyrin–protein complex to a phycocyanobilin–protein complex *via* porphyrin ring opening *in situ*, although either alternative is possible (Bogorad and Troxler, 1967). Studies on phycocyanin biosynthesis are complicated by the fact that (a) it is a conjugated protein whose moieties are end products of different metabolic pathways, (b) the nature of the covalent linkage between bile pig-

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TABLE I: Pigmentation of Wild-Type and Mutant *C. caldarium* Cells in the Light.[a]

Genotype	Chloro-phyll a	Phyco-cyanin	Carot-enoids
Wild type	+	+	+
III-D-2	+	+	+
GGB	−	+	+
III-C	+	−	+
GGB-Y	−	−	+

[a] The + and − signs indicate the presence and absence, respectively, of the pigment indicated. None of the strains produce significant quantities of either chlorophyll a or phycocyanin when grown in the dark. Carotenoids are present regardless of the culture conditions.

ment and protein has not been established unequivocally (Crespi *et al.*, 1968; Rudiger and O'Carra, 1969), and has precluded quantitative separation and recovery of both constituents, (c) phycocyanobilin and phycocyanin apoprotein are produced in a 1:1 molar ratio due to coordinate regulation of the pathways for porphyrin and protein synthesis (Troxler and Brown, 1970), and (d) most algae which make phycocyanin are obligate photoautotrophs which produce pigments concomitantly with cell division in the light. Thus, most algal species are "constitutive" with regard to phycocyanin formation and in many respects are unsuitable for metabolic studies on this pigment. Moreover, phycocyanin is found in both prokaryotic blue-green algae and in eukaryotic red and cryptomonad algae, groups in which the degree of biological organization is completely different, as may be the subcellular compartmentalization of bile pigment and protein synthesis.

The unicellular alga, *Cyanidium caldarium*, is an anomalously pigmented green alga (Allen, 1959) whose characteristic mode of growth overcomes complication (d) above. Cells of this alga kept in darkness divide rapidly with the production of progeny lacking chlorophyll a and phycocyanin (Troxler and Bogorad, 1966). Dark-grown cells placed in light stop dividing and begin synthesizing photosynthetic pigments. Phycocyanobilin (linked to phycocyanin apoprotein) synthesis can be easily quantitated spectrophotometrically and is sensitive to treatment with inhibitors of protein synthesis (Troxler and Bogorad, 1966). *C. caldarium* cells incubated with unphysiologic concentrations of the porphyrin–bile pigment precursor, δ-aminolevulinic acid (ALA),[1] excrete porphobilinogen (PBG), porphyrins, and protein-free phycocyanobilin into the suspending medium (Troxler and Bogorad, 1966, 1967). Thus, under abnormal culture conditions, synthesis of phycocyanobilin and phycocyanin apoprotein can be separated, and complications (b) and (c) above avoided in part.

A novel feature of mammalian heme catabolism is cleavage and oxidation of the α-methyne bridge carbon to carbon monoxide (CO) (Sjostrand, 1949). It has now been established that the rates and amounts of CO excreted *via* the lungs of rats and dogs correlate nicely with the corresponding values for administered heme-^{14}C destroyed (Landaw *et al.*, 1970; Coburn *et al.*, 1967). Production of CO concomitantly with phycocyanobilin in algae during phycocyanin biosynthesis would suggest a similar or identical mechanism of porphyrin ring opening in mammals and in plants. The present paper describes some quantitative and kinetic parameters of CO and phycocyanobilin formation in wild-type and mutant strains of the alga, *Cyanidium caldarium*.

[1] Abbreviations used are: ALA, δ-aminolevulinic acid; PBG, porphobilinogen.

Materials and Methods

Organism. C. caldarium is an anomalously pigmented, unicellular chlorophyte which is found in acid hot springs and in hot soils (Doemel and Brock, 1971). Wild-type cells of this organism grow rapidly in darkness with the production of cells lacking chlorophyll a and phycocyanin but when placed in light, the cells stop dividing and begin synthesizing photosynthetic pigments (Troxler and Bogorad, 1966). Pigment synthesis in light occurs as a consequence of chloroplast development, and chlorophyll a and phycocyanin accumulation appears to cease when differentiation of these organelles is complete (Mercer *et al.*, 1962; R. F. Troxler, unpublished data). The pigmentation of light-grown, wild-type, and mutant *C. caldarium* strains used in this work is shown in Table I. Mutant III-D-2 grows faster and contains more chlorophyll a and phycocyanin per cell than does the wild type. Mutant GGB is unable to make chlorophyll but can make phycocyanin in the light. Mutant III-C makes chlorophyll a in the light but is unable to make phycocyanin. Mutant GGB-Y is unable to make either chlorophyll a or phycocyanin in the light.

Experimental Conditions. Algal cells were grown in darkness for 8 days at 43° in 14 l. of nutrient medium supplemented with sucrose in an MF-114 New Brunswick fermentator (Troxler, 1971). Dark-grown cells were collected by centrifugation in a Szent-Györgi and Blum continuous-flow system, resuspended in 14 l. of sucrose-free medium, and illuminated for 40–96 hr with fluorescent tubes mounted on a manifold located behind the glass fermentator tank. During the illumination period, 2 l. of 95% O_2–5% CO_2 (Medical-Technical Gases, Medford, Mass.) were cycled continuously between a gas reservior and the cell suspension with a Masterflex pump (Cole-Parmer Instrument Co, Chicago, Ill.).

For studies on synthesis of labeled phycocyanobilin and CO, 10 μCi of δ-aminolevulinic acid-5-^{14}C; (ALA-5-^{14}C; 25 Ci/mole; New England Nuclear) was added to the cell suspension at the beginning of the illumination period. For studies on phycocyanobilin and CO production in darkness, 1 μCi of carrier diluted ALA-5-^{14}C (5×10^{-2} M, 0.4 μCi/mmole; New England Nuclear) was added to 5 ml of packed cells in 45 ml of sucrose supplemented medium. The resulting suspension was placed in a tightly stoppered 2-l. flask and incubated in darkness at 43° for 96 hr. At 24-hr intervals, evolved ^{14}CO and excreted phycocyanobilin-^{14}C were quantitated and assayed radiochemically as described below.

Spectrophotometric Measurements of Pigments in Vivo and in Vitro. Chlorophyll a and phycocyanobilin were quantitated *in vivo* in suspension aliquots by the opal glass method of Shibata (1959) on a Beckman DB-GT recording spectrophotometer. The absorption maxima of chlorophyll a and phycocyanobilin *in vivo* in *C. caldarium* cells are at 675 and 620 nm, respectively. The quantity of chlorophyll a present per 14-l. suspension was determined by dividing the optical density at 675 nm by the specific absorption coefficient (84 l./(g cm), Myers and Kratz, 1955) which gives the grams of chlorophyll per liter. The μg of chlorophyll per l. divided by 893.5 g/mole (Merck Index, 1960) and multiplied by 14 (l.) gives the μmoles present per 14-l. cell suspension.

The specific absorption coefficient for *C. caldarium* phycocyanin ($E^{0.1\%}_{1\ cm}$) is 7.74 in 0.1 M potassium phosphate buffer (pH 6.5) (Troxler and Lester, 1968). The extinction of phycocyanobilin (bound to apoprotein) *in vivo* and *in vitro* is approximately the same (Troxler and Bogorad, 1966), phycocyanobilin constitutes 3.6% of phycocyanin by weight (Troxler and Lester, 1968), and the molecular weight of phycocyanobilin is 586 (Cole *et al.*, 1967; Crespi *et al.*, 1967). Thus

$$\text{phycocyanin (mg/14 l.)} = \frac{OD_{620\ nm}}{7.74\ ml/(mg\ cm)} \times 14{,}000$$

$$\text{phycocyanobilin } (\mu\text{moles/14 l.}) = \frac{(\text{total phycocyanin})(0.036)}{586\ mg/mmole} \times 1000\ \mu moles/mmole$$

From these expressions, the total chlorophyll a (micromoles) and phycocyanobilin (micromoles) present in cell suspensions throughout the illumination period could be computed and compared to the corresponding values for CO produced.

Preparation of Phycocyanin and Phycocyanobilin. At the end of illumination periods, pigmented algal cells which have been given ALA-5-^{14}C were collected by centrifugation, suspended in 0.1 M phosphate buffer (pH 6.5), and disrupted by sonic vibration with a Branson sonic oscillator. The broken cell preparation was centrifuged at 100,000*g* for 1 hr, phycocyanin in the supernatant was precipitated by the addition of solid ammonium sulfate to 50% saturation, and the precipitated pigment was dialyzed against distilled water for 24 hr. Phycocyanin was purified by chromatography on brushite columns (2 × 20 cm) developed with phosphate buffers of increasing ionic strength as described previously (Cole *et al.*, 1968; Troxler and Lester, 1967). Phycocyanin in tubes of column eluate in which the $E_{620}/_{280}$ ratio was greater than 3.5 was crystallized from 30% ammonium sulfate at 4°.

Phycocyanobilin was prepared by refluxing phycocyanin denaturated with 1% trichloroacetic acid in absolute methanol (1 g of pigment/500 ml of solvent) for 18 hr (Cole *et al.*, 1968). Under these conditions, 10–40% of the phycocyanobilin residues was split from phycocyanin apoprotein. Liberated phycocyanobilin was converted to the dimethyl ester derivative in 7% BF_3, and purified by thin-layer chromatography (tlc) on Absorbosil-5 plates (Applied Science Laboratories, State College, Pa.) developed with chloroform–methyl acetate (2:1) (Cole *et al.*, 1968). The phycocyanobilin dimethyl ester was eluted from Absorbosil-5 in absolute ethanol, quantitated spectrophotometrically in methanol 5% HCl (ϵ 38,000 at 685 nm), and counted in 15 ml of Bray's solution (1960) in a Tri-Carb liquid scintillation spectrometer (Troxler and Lester, 1967).

Measurement of CO. CO production in algal cells was followed in one of two ways. Algal CO which had accumulated in the 2-l. reservior containing 95% O_2–5% CO_2 was estimated spectrophotometrically in a Beckman 315A infrared analyzer which had been precalibrated with a certified CO standard (Matheson Gas Products, E. Rutherford, N. J.).

Labeled CO, evolved by algal cells administered ALA-5-^{14}C in light, was quantitated on a train apparatus consisting of the following components connected in series (Troxler, 1971): (a) baralyme granules (National Cylinder Gas, Chicago, Ill.), (b) ascarite (Arthur Thomas, Philadelphia, Pa.), (c) an alkali test trap to check for incomplete CO_2 removal, (d) hopcalite catalyst (Mine Safety Appliances, Pittsburgh, Pa.) to convert ^{14}CO to $^{14}CO_2$, and (e) a series of alkali traps to collect $^{14}CO_2$ derived from labeled CO. Derived $^{14}CO_2$ was quantitated by manual titration of residual alkali to pH 8.5 with 1 N HCl on a Radiometer pH meter (PHM 28b) with a GK 2303C combined glass electrode. After titration, derived bicarbonate-^{14}C was driven from solution by acidification with 2 N H_2SO_4, collected in 5 ml of ethanolamine–methoxyethanol (2:1), and counted in 15 ml of toluene–methoxyethanol (2:1) containing 4 g/l. of 2,5-bis(2-*tert*-butylbenzoxazolyl)-β-thiophene (Packard Instrument Co., Downers Grove, Ill.).

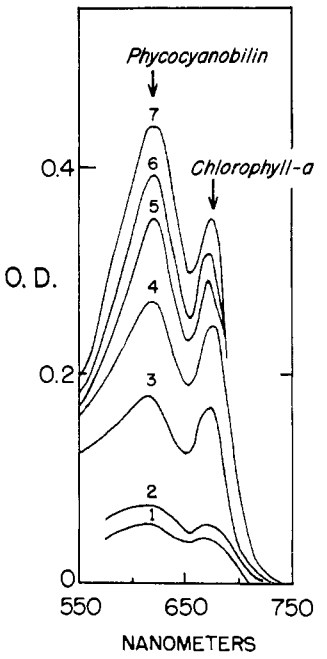


FIGURE 1: Opal glass scans 1–7 show absorption spectra of *C. caldarium* cells after 10, 14, 19, 28, 42, 51, and 68 hr, respectively, in the light. Spectral measurements of chlorophyll a and phycocyanobilin in this and subsequent Figures 2, 3, and 5–9 were made as described in Materials and Methods. The cell density of suspensions ranged from 0.8 × 10^8 to 3.0 × 10^8 cells per ml.

Results

Studies on Illuminated Cells of C. caldarium. Chlorophyll a and phycocyanobilin absorb maximally at 675 and 620 nm, respectively, in wild-type *C. caldarium* cells. Opal glass scans from 750 to 550 nm of cells after increasing exposure to light displayed a continuous rise in the optical density at the absorption maxima of these pigments (Figure 1). While reference is made here and below to phycocyanobilin synthesis, it should be understood that, unless specified, changes in the optical density of cells at 620 nm *in vivo* represent formation of the entire bile pigment–protein complex, *i.e.*, phycocyanin. The molar ratio of chlorophyll a to phycocyanobilin was 1.2 to 1.8 throughout illumination. The difference in extinction of the two pigments accounted for the lower optical density values observed for chlorophyll a.

Wild-type *C. caldarium* cells evolved CO and synthesized phycocyanobilin at identical rates in the light (Figure 2). A 1:1 stoichiometry between the micromoles of CO and the micromoles of bile pigment was observed, as described in a preliminary communication (Troxler *et al.*, 1970). CO was not evolved by cells during the 8-day growth period in darkness or after phycocyanobilin synthesis in the light had ceased.

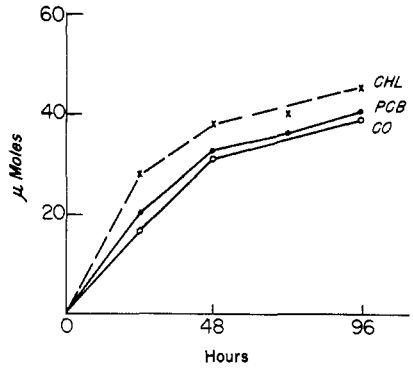


FIGURE 2: Synthesis of chlorophyll a (Chl) and phycocyanobilin (PCB) and evolution of CO (CO) in wild-type *C. caldarium* cells in the light. CO measurements were made on the train apparatus. Values in Figures 2, 3, and 5-9 express the μmoles of pigment and CO per 14-l. suspension.

At the conclusion of the 96-hr illumination period the cell suspension was made millimolar with respect to potassium ferricyanide and incubated in darkness for 4 hr. Neither CO nor phycocyanobilin was produced under these conditions.

The recovery of known quantities of added CO from the fermentator–reservoir–pump assembly ranged from 90 to 98% in ten determinations. Values greater than 90% were obtained when the assembly contained sterile medium lacking in the light or medium containing growing cells in the dark. These results show that in the dark, the cells did not destroy added CO in detectable quantities. The recovery of added CO was essentially the same in calibration experiments lasting from 1 to 24 hr. Quantitation of added CO in recovery experiments and of algal CO described in Figure 2 was performed on the train apparatus (Troxler, 1971).

To establish the identity of algal CO by a different method, a comparable experiment was performed in which CO determinations were performed in a Beckman 315A infrared analyzer. Mutant III-D-2 was used. III-D-2 cells evolved CO and phycocyanobilin at identical rates and in equimolar amounts in the light (Figure 3). The kinetics and stoichiometry observed with the wild type and III-D-2 establish the identity of CO as an algal metabolite and demonstrate the quantitative relationship between CO and phycocyanobilin in two *C. caldarium* genotypes.

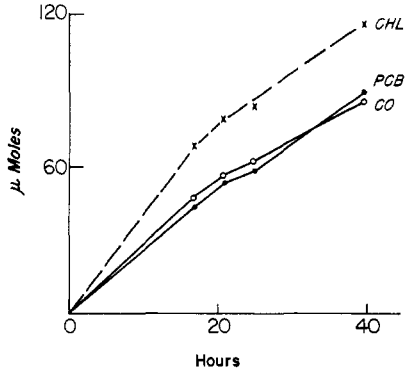


FIGURE 3: Synthesis of chlorophyll a (Chl) and phycocyanobilin (PCB), and evolution of CO (CO) in cells of *C. caldarium*, mutant III-D-2, in the light. CO measurements were made on a Beckman 315A infrared analyzer. The error due to the presence of 5% CO_2 in the reservoir gas was 2%, and machine error with the certified CO standards was 1%.

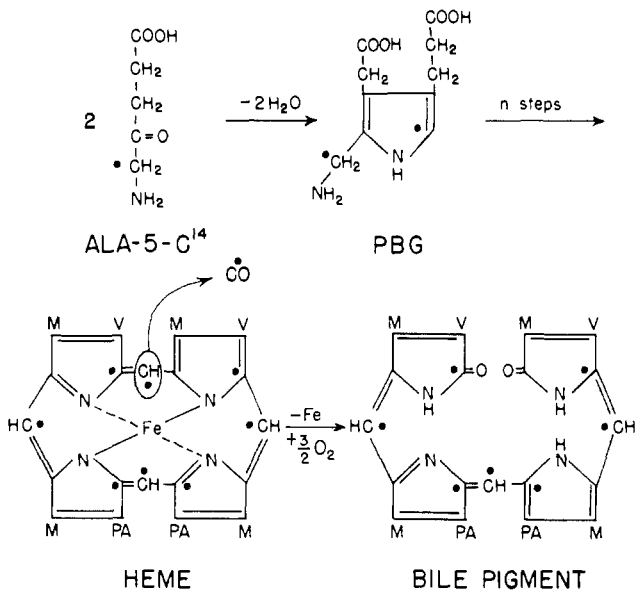


FIGURE 4: Theoretical pattern of incorporation of ALA-5-^{14}C into PBG, heme, CO, and bile pigment. M = methyl; V = vinyl; and PA = propionic acid.

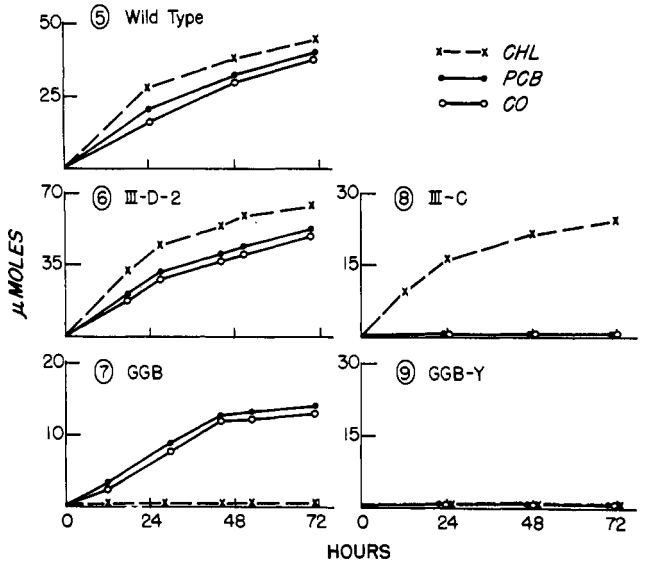
CO evolution in additional *C. caldarium* mutants unable to make phycocyanobilin was investigated. Cells of each genotype which had been grown in darkness were placed in light in the fermentator–reservior–pump assembly in nutrient medium to which trace qualities of ALA-5-^{14}C (10 μCi/14 l.) were added. Theoretically, 8 moles of ALA-5-^{14}C is required to make 1 mole of protoporphyrin IX (or heme), with four labeled carbon atoms in the pyrrole rings and four labeled carbon atoms in the methyne bridges (Figure 4). Phycocyanobilin and CO derived from heme so labeled would contain seven and one labeled carbon atoms, respectively. Furthermore, the specific activity (given in disintegrations per minute per micromole) of phycocyanobilin would be seven times greater than the specific activity of CO.

The radiochemical and kinetic parameters of CO and phycocyanobilin biosynthesis in wild-type and mutant *C. caldarium* cells administered ALA-5-^{14}C in the light are shown in Figures 5–9 and in Table II. Cells of the wild type, mutant

TABLE II: Total Micromoles and Specific Activity[a] of CO and Phycocyanobilin Produced by Wild-Type and Mutant *C. caldarium* Cells Given ALA-5-^{14}C in the Light.[b]

Genotype	Chlorophyll a (μmoles)	Phycocyanobilin		CO	
		μmoles	dpm/μmoles	μmoles	dpm/μmole
Wild type	45	40	7600	39	7359
III-D-2	65	56	8000	54	8100
GGB	0	33	8800	35	9000
III-C	25	0		0	
GGB-Y	0	0		0	

[a] The specific activity of phycocyanobilin has been divided by 7. [b] The data for pigment and gas in genotypes 1–5 come from the experiments illustrated kinetically in Figures 5–9.



FIGURES 5–9: Formation of chlorophyll a (Chl) and phycocyanobilin (PCB), and evolution of CO (CO) or lack of it in the wild type, mutant III-D-2, mutant GGB, mutant III-C, and mutant GGB-Y, respectively. The cells of each strain which have been grown previously in darkness were placed in light in 14-l. suspensions and administered 10 μCi of ALA-5-^{14}C.

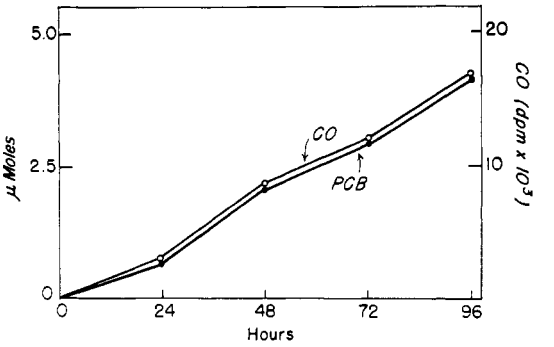


FIGURE 10: The rates of phycocyanobilin-^{14}C excretion and ^{14}CO evolution from wild-type *C. caldarium* cells incubated with 5×10^{-2} M ALA-5-^{14}C in the dark. The values express the μmoles of phycocyanobilin and CO produced per 50 ml of suspension containing 5 ml of packed cells. Prior to the experiment, the cells had been illuminated for 4 days and contained the full compliment of chlorophyll a and phycocyanobilin. ^{14}CO was assayed radiochemically as "derived" CO_2 on the train apparatus.

III-D-2, and mutant GGB evolved ^{14}CO and synthesized labeled phycocyanobilin in equimolar quantities at identical rates (Figures 5–7). Cells of mutant III-C, which are unable to make phycocyanin, and of mutant GGB-Y, which have lost the ability to make either phycocyanin or chlorophyll a, failed to evolved CO in detectable quantities (Figures 8 and 9).

The specific activity of CO and of phycocyanobilin produced in the wild-type, mutant III-D-2, and mutant GGB is shown in Table II. The specific activity of CO was obtained by dividing the total radioactivity recovered in CO by the total micromoles of gas evolved. The comparable values for phycocyanobilin represent the disintegrations per minute per micromole in the dimethyl ester derivative prepared from phycocyanin isolated and purified from cells at the end of the illumination period. The specific activity of phycocyanobilin has been divided by 7 so that it can be compared directly to that of CO. These data show that total incorporation of ALA-5-^{14}C into phycocyanobilin as well as the specific activity of this bile pigment was seven times greater than the total incorporation into (and the specific activity of) CO evolved by cells of the three genotypes.

In these experiments, 10 μCi of ALA-5-^{14}C (25 Ci/mole) was added to the 14-l. suspensions of wild-type and mutant cells. This represents 0.4 μmole of exogenously added ALA. The wild-type, mutant III-D-2, and mutant GGB produced 40, 56, and 33 μmoles of phycocyanobilin, respectively. Mutant III-D-2 and the wild-type produced at least as many μmoles of chlorophyll a and cells of all three genotypes produced at least two cytochromes and two other hemoproteins (Crean, 1966; Kupelian, 1964), whose prosthetic groups are also derivatives of ALA and protoporphyrin IX. Since 1 mole of heme corresponds to 8 equiv of ALA, and since mutant GGB minimally produced 33 μmoles of porphyrin derivative, it is evident that most of the phycocyanobilin, CO, and chlorophyll a (in wild-type and in mutant III-D-2) were synthesized from endogenous ALA, *i.e.*, the labeled ALA administered was present in trace quantities relative to the total ALA equivalents of tetrapyrrole produced. The wild-type, mutant III-D-2, and mutant GGB incorporated approximately 2.43×10^6, 3.56×10^6, and 2.23×10^6 dpm into phycocyanobilin and CO, which represents 11, 16, and 10% of the 22×10^6 dpm in ALA added. The labeling in chlorophyll a was not measured.

Studies on C. caldarium Cells Given ALA-5-^{14}C in the Dark. Light-grown, wild-type *C. caldarium* cells (5 ml of packed cells) were placed in 45 ml of nutrient medium containing sucrose (1×10^{-1} M) and ALA-5-^{14}C (5×10^{-2} M, 1 μCi, 0.4 μCi/mmole) and incubated for 96 hr in darkness in a 2-l. stoppered flask containing air. Under these conditions, the efficiency of ALA utilization was greater when the flasks contained air rather than 95% O_2–5% CO_2. At 24-hr intervals, CO in the air above the cells and phycocyanobilin excreted into the suspending medium were measured. CO determinations were performed on the train apparatus. Excreted, protein-free phycocyanobilin in the suspending medium after removal of cells by centrifugation was determined spectrophotometrically (ε 38,000 at 685 nm). At the end of the experiment, phycocyanobilin-^{14}C was transferred to chloroform, methylated, and purified by tlc, and the specific activity was determined as with phycocyanobilin split from phycocyanin apoprotein.

Wild-type *C. caldarium* cells given 5×10^{-2} M ALA-5-^{14}C in darkness excreted protein-free phycocyanobilin-^{14}C and evolved ^{14}CO at comparable rates (Figure 10). The values for CO produced represent the disintegrations per minute present in the air above the cells at each sampling time. Attempts were not made to measure CO as "derived" CO_2 by titration directly because experimental error by this method is ±2 μmoles and total CO production anticipated was only 4 μmoles (Figure 10). However, the micromoles of excreted phycocyanobilin were well within the limits required for spectrophotometric measurement, and it was assumed that the micromoles of CO evolved was equivalent to the micromoles of phycocyanobilin excreted.

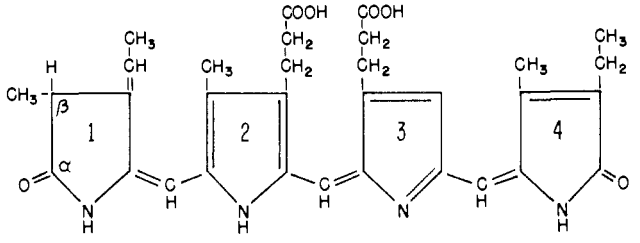
The specific activity of excreted phycocyanobilin and CO in this experiment and in another (expt II) is shown in Table III. Total dpm and the specific activity of phycocyanobilin have been divided by 7. These results show that seven times as much label from ALA-5-^{14}C was incorporated into excreted phycocyanobilin as was incorporated into evolved CO. If

TABLE III: Phycocyanobilin Excretion and CO Evolution by ALA-Treated, Wild-Type *C. caldarium* Cells.[a]

Compound	Experiment I			Experiment II		
	Total dpm	μmoles	dpm/μmole	Total dpm	μmoles	dpm/μmole
Phycocyanobilin	15,000[b]	4[c]	3750[b]	4700[b]	0.65	6890[b]
CO	14,000	4[c]	3500	4854	0.65[c]	7480

[a] The data in expt I are illustrated kinetically in Figure 10. [b] The total dpm and dpm/μmole of phycocyanobilin have been divided by 7. [c] The μmoles of CO evolved were too small to measure accurately by titration and were assumed to be equal to the μmoles of phycocyanobilin excreted.

the micromoles of CO were equivalent to the micromoles of phycocyanobilin, as would be expected from the behavior of *C. caldarium* cells in the light, then the disintegrations per minute per micromole of CO was one-seventh as great as the disintegrations per minute per micromole of phycocyanobilin excreted by the ALA-treated cells. Thus, the kinetics and labeling pattern of phycocyanobilin and CO produced by wild-type *C. caldarium* cells given unphysiologic quantities of ALA in the dark was the same as the labeling pattern observed in these compounds synthesized by cells given trace quantities of labeled precursor in the light. These data show that covalent linkage to phycocyanin apoprotein is not an obligatory step in the biosynthesis of phycocyanobilin in *C. caldarium*.



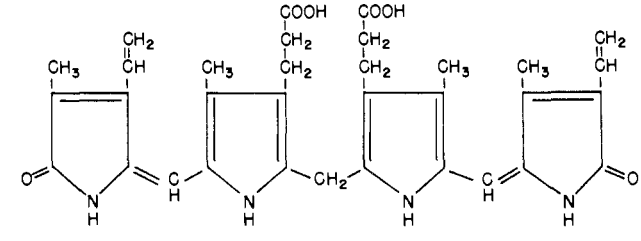


FIGURE 11: The authors' interpretation of the structures proposed for phycocyanobilin (Cole *et al.*, 1967, 1968; Crespi *et al.*, 1967, 1968) and bilirubin (Jansen and Stoll, 1971; Rudiger, 1969). Note that in bilirubin the double bond at the central bridge has been reduced and that, in phycocyanobilin, ethylidine and ethyl groups on rings 1 and 4 replace vinyl groups at these positions in bilirubin.

Discussion

The structural similarity between protoporphyrin IX, heme and bile pigment has been recognized for decades. It has been established that hemoglobin in sequestered erythrocytes and intravenously administered hemoglobin, myoglobin, and methemalbumin can undergo nearly quantitative conversion to bilirubin in the intact animal (Ostrow *et al.*, 1962; Daly *et al.*, 1967; Snyder and Schmid, 1965). The kinetics of hematin and hemoglobin conversion to bile pigment are similar, suggesting that binding to globin is not essential for splitting the ferriprotoporphyrin ring (Snyder and Schmid, 1965). However, protoporphyrin IX is converted slowly and inefficiently to bile pigment in dogs, suggesting that metal chelation is an essential step in cleavage of the porphyrin ring *in vivo* (Ibrahim *et al.*, 1966).

Sjostrand (1949) observed that the quantitative aspects of CO exhalation in the breath of man correlated nicely with heme calculated to have been destroyed as a result of red cell turnover. It has been shown recently that rats and dogs administered hematin-^{14}C produced equimolar quantities of labeled CO and bilirubin (Landaw *et al.*, 1970; Coburn *et al.*, 1967). These results provided direct evidence for the biologic origin of CO and bile pigment from the carbon skeleton of protoporphyrin IX in heme.

Tenhunen *et al.* (1968, 1969) have described an enzyme, microsomal heme oxygenase, which converts hematin, methemoglobin, and the α and β chains of hemoglobin to bile pigment and CO. This enzyme requires NADPH, is inhibited by CO, and contains cytochrome P-450 which serves as the terminal oxidase mediating the insertion of molecular oxygen into heme during cleavage of the porphyrin ring at the α-bridge carbon atom. The level of microsomal heme oxygenase in spleen is present in excess of that needed for the kinetic requirements of hemoglobin turnover in rats. This suggests the relation of microsomal heme oxygenase to physiologic heme catabolism *in vivo*.

Microsomal heme oxygenase, or for that matter, cytochrome P-450, has not been described in plants. Nevertheless, bile pigments comprise the prosthetic groups of algal biliproteins (O'hEocha, 1965) and of phytochrome, a bile pigment–protein complex in higher plants involved in photocontrol of metabolic and developmental processes (Butler *et al.*, 1965). The concentration of algal biliproteins is frequently very high, and can account for as much as 40% of the total cell protein (Myers and Kratz, 1955). The quantity of phytochrome in higher plants is low, and the phytochrome molecule is extremely labile (Hillman, 1967), thereby limiting its value in studies on the biosynthesis and metabolism of linear tetrapyrroles in plants.

The structure of phycocyanobilin (Cole *et al.*, 1967; Crespi *et al.*, 1967) and of bilirubin (Jansen and Stoll, 1971; Rudiger, 1969) is shown in Figure 11. These bile pigments are termed IXα isomers because the linear sequence of substituents at the β position on the pyrrole rings could arise only by removal of the α-methyne bridge from the carbon skeleton of the ferriprotoporphyrin IX ring. Bridge cleavage at other positions would result in a different sequence of substituents at the β position on the pyrrole rings. Thus, solely on structural grounds, protoporphyrin IX or heme can be implicated as precursors of plant and mammalian bile pigment.

The results of the present investigation have shown that CO and phycocyanobilin are produced concomitantly in wild-type cells of the alga, *Cyanidium caldarium* (Figures 2 and 5). The identical quantitative and kinetic parameters observed

for CO and phycocyanobilin formation suggest that these compounds were derived from a common intermediate. The fact that phycocyanobilin is a IXα bile pigment suggests that algal CO was derived from the α-methyne bridge carbon atom of a metallo derivative of protoporphyrin IX. In this work, CO was established as an algal metabolite directly by infrared spectroscopy and indirectly by titration of "derived CO_2" (Figures 2 and 3). Failure to detect additional CO after treatment of cells with ferricyanide showed that CO-binding hemoproteins, if present, did not interfere with CO determinations. The nearly quantitative recovery of added CO (Matheson) from cell suspensions in the fermentator–reservior–pump assembly showed that the cells did not destroy CO in detectable quantities. This suggests that the 1:1 stoichiometry observed between CO evolution and phycocyanobilin synthesis was not fortuitous (Figures 2 and 3).

The relationship between algal CO and phycocyanobilin observed in the wild-type was extended in work with four *C. caldarium* mutants (Figures 3 and 5–9). Mutant III-D-2 and mutant GGB produced equimolar quantities of CO and phycocyanobilin at identical rates, as did the wild-type (Figures 3 and 5–7). Mutants GGB-Y and III-C which are unable to make phycocyanin failed to evolve CO in the light (Figures 8 and 9). The results with mutants III-D-2 and GGB confirm those obtained with the wild-type strain (Figures 5–7). The behavior of mutant III-C suggests that CO is not a by-product of chlorophyll a synthesis (Figure 8). The inability of mutant GGB-Y to produce CO indicates that CO production cannot be ascribed to some other metabolic process occurring concomitantly with the synthesis of algal bile pigment (Figure 9).

Cells of the wild-type, mutant III-D-2, and mutant GGB strains administered trace quantities of ALA-5-^{14}C in the light, and cells of the wild type incubated with unphysiologic quantities of this precursor in the dark, produced labeled CO in which the specific activity was one-seventh as great as the specific activity of apoprotein-bound, or apoprotein-free, phycocyanobilin (Tables II and III). These results are what would be expected if CO and phycocyanobilin were derived directly from the ferriprotoporphyrin IX ring of a metalloporphyrin precursor. The labeling pattern observed, therefore, shows that both CO and phycocyanobilin are produced *via* the porphyrin pathway.

Reference has been made above to the probable origin of algal bile pigment from heme, or from another metalloporphyrin, rather than from protoporphyrin IX directly. Support for this speculation comes from the observation that metal-free porphyrin conversion to bilirubin is minimal in dogs (Ibrahim *et al.*, 1966). While these latter results do not necessarily mean that the iron complex of protoporphyrin IX is *the* cyclic precursor of phycocyanobilin, they do suggest this possibility. Further support for this idea comes from the observation that the action spectrum for phycocyanin formation in *C. caldarium*, mutant GGB, resembles the absorption spectrum of a hemoprotein (Nichols and Bogorad, 1962). On the other hand, the potential involvement of other metal chelates of protoporphyrin IX in phycocyanobilin biosynthesis is suggested by at least two model systems. Fischer and Bock (1938) described the photochemical oxidation of the etioporphyrin sodium complex to a bile pigment and dipyrrylmethenes. More recently, Barrett (1967) showed that magnesium protoporphyrin dimethyl ester was converted to protobiliviolin in another reaction driven by light. The relation of these photoreactions to synthesis of prosthetic groups in biliproteins is suggested by the fact that most algal species synthesize phycocyanobilin exclusively in the light, and concomitantly with cell division, as they are obligately photoautotrophic. The difficulty with this interpretation rests with the finding that ALA-treated *C. caldarium* cells (Table III and Figure 9) and chromatically adapted cells of the blue-green alga, *Tolypothrix tenuis* (Fujita and Hattori, 1963), synthesize phycocyanobilin in the dark. These results show that light is not an essential requirement for bile pigment formation at least in these algal species.

Bogorad and Troxler (1967) suggested two possible ways one might view the formation of a "biosynthetic unit" of phycocyanin:

$$\text{hemoprotein} \xrightarrow[+{}^3/_2O_2]{-CO,\ -Fe} \text{phycocyanobilin–apoprotein complex} \quad \text{(a)}$$

$$\text{heme} \xrightarrow[+{}^3/_2O_2]{-CO,\ -Fe} \text{phycocyanobilin} + \text{apoprotein} \longleftarrow$$

$$\downarrow$$

$$\text{phycocyanobilin–apoprotein complex} \quad \text{(b)}$$

Scheme a proposes that heme is catabolized to phycocyanobilin *in situ* on phycocyanin apoprotein. Ring cleavage in this case would be mediated by another enzyme or by phycocyanin apoprotein directly. Scheme b indicates that heme is first converted to bile pigment which is subsequently linked covalently to phycocyanin apoprotein. The incorporation of ALA-5-^{14}C into excreted phycocyanobilin and CO (Figure 10 and Table III) and the involvement of microsomal heme oxygenase in physiologic heme catabolism in mammals (Tenhunen *et al.*, 1968, 1969) favor the biologic reality of scheme b. However, scheme a cannot be completely dismissed until an enzyme comparable to microsomal heme oxygenase is described in plants. It is conceivable that the hypothetical hemoprotein precursor suggested by the action spectrum for phycocyanin formation in mutant GGB (Nichols and Bogorad, 1962) is analogous to the cytochrome P-450 prosthetic group of an algal "heme oxygenase" (scheme b) or to another hemoprotein (scheme a). The peaks of effectiveness in the action spectrum at 420 and 550 nm could also represent absorption maxima of one or more photoreceptors for processes unrelated to phycocyanin formation directly, or possibly of a magnesium or cobalt protoporphyrin chelate whose function would be consistent with either scheme a or b. The overall significance of an enzyme in plants comparable to microsomal heme oxygenase in mammals need not be universal, however, because the prokaryotic blue-green algae which make phycocyanin do not have an endoplasmic reticulum.

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