The Biosynthesis of Chlorobium Chlorophylls-660. The Production of Magnesium Protoporphyrin Monomethyl Ester, Bacteriochlorophyll, and Chlorobium Pheoporphyrins by *Chlorobium thiosulfatophilum*-660*

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ABSTRACT: Concentrated suspensions of *Chlorobium thiosulfatophilum*-660, when incubated in a medium containing glycine, succinate, ethionine, Tween 80, and ferrous ion, have been found to excrete high concentrations of protoporphyrin monomethyl ester, its magnesium derivative, and bacteriochlorophyll, in addition to porphyrins and metalloporphyrins with from 3 to 8 carboxyl groups. Under certain conditions, a photochemical conversion of chlorobium pheophytins to the pheoporphyrins (farnesol esters) also occurred and was greatly enhanced by whole cells or active cell particles.

On the basis of these observations, we suggest that the biosynthesis of chlorobium chlorophylls-650 and -660 may proceed not only through magnesium protoporphyrin monomethyl ester, but through bacteriochlorophyll or one of its immediate precursors as well.

Uro- and coproporphyrins have been isolated from the media of the nonsulfur purple bacteria *Rhodopseudomonas spheroides* (Lascelles, 1955, 1956; Gibson *et al.*, 1962b) and *Rhodopseudomonas capsulata* (Cooper, 1963) and the green sulfur bacterium *Chlorobium thiosulfatophilum*-660 (Richards and Rapoport, 1966; Uspenskaya and Kondrat'eva, 1964; Uspenskaya, 1965a,b) when organic tetrapyrrole precursors such as glycine and succinate or 2-ketoglutarate were included. In addition traces of metallocopro- and uroporphyrins and porphyrins with 2, 3, 5, 6, and 7 carboxyl groups were detected with *C. thiosulfatophilum*-660 (Richards and Rapoport, 1966). Protoporphyrin has been found in the medium of *R. spheroides* when incubated with δ-aminolevulinic acid, succinate, and iron (Lascelles, 1955, 1956) and evidence for the presence of protoporphyrin along with its magnesium derivative in the medium of the green bacteria *Chloropseudomonas ethylicum* and *C. thiosulfatophilum* has been presented by Uspenskaya (1965b) and Godnev *et al.* (1966), respectively. Magnesium protoporphyrin monomethyl ester was produced by purple bacteria when Tween 80, methionine, and iron (Cooper, 1963) or 8-hydroxyquinoline (Jones, 1963a) were included in the medium. The latter procedure has also resulted in the detection of two additional compounds, a probable precursor of bacteriochlorophyll, magnesium 2,4-divinylpheoporphyrin a_5 monomethyl ester (Jones, 1963b,c), and 2-devinyl-2-hydroxyethylpheophorbide *a* (Jones, 1964) which may have been derived from the corresponding magnesium-containing intermediate.

Although the major photosynthetically active chlorophylls (Vernon and Seely, 1966) of green bacteria, chlorobium chlorophylls-650 and -660, are quite distinct from the chlorophyll of the purple bacteria, a minor chlorophyll recently found in green bacteria (Olson and Romano, 1962) appears to be bacteriochlorophyll (Holt *et al.*, 1963). Using the Tween 80 medium of Cooper (1963), with ethionine in place of methionine, we have observed that the green bacterium, *C. thiosulfatophilum*-660, excreted a high concentration of protoporphyrin monomethyl ester, its magnesium derivative, and bacteriochlorophyll, in addition to porphyrins and metalloporphyrins with from 3 to 8 carboxyl groups. Under certain conditions, a photochemical conversion of chlorobium pheophytins to the pheoporphyrins (farnesol esters) also occurred and was greatly enhanced by whole cells or active cell particles. On the basis of these observations we suggest that the biosynthesis of the chlorobium chlorophylls may proceed through the intermediates protoporphyrin, magnesium protoporphyrin, magnesium protoporphyrin monomethyl ester, and bacteriochlorophyll or one of its immediate precursors, before the addition of the "extra" carbon atom side chains to form chlorobium chlorophylls-650, and *meso*-alkylation to form chlorobium chlorophylls-660.[1]

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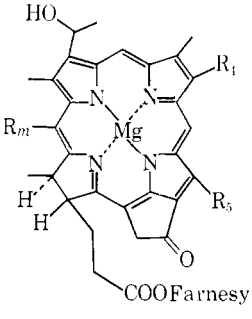
[1] There is still some question as to the position of the *meso*-alkyl substituents; see footnote 1 of Richards and Rapoport (1966).

TABLE I: Products of Incubation of *C. thiosulfatophilum*-660 with Media[a] Containing Tween 80.

Expt	Special Conditions[b]	Time (hr)	Abs Ratio Soret/668 mμ	Excreted Products[c] (μM) Pheophyt	Pheoporph	Bact ch	PROTO	COPRO
1[d]		90	17.4	2	10			19
2[d]	No Tween 80	90						8
3		82	15.5	3	18			
4	Dark	82	2.4	50	4			
5	Sonicate	82	12.1	3	16			
6	Sonicate, dark	82	2.5	29	3			
7	Sonicate, TCA	82	2.4	24	2			
8	Sonicate, TCA, dark	82	2.2	50	0			
9	Chlorophyll only	82	3.3	7	4			
10	Chlorophyll only dark	82	2.4	39	3			
11	10 × Fe	81	4.5	6	5			6
12	10 × Fe, methionine	81	3.4	10	5			
13	10 × Fe, methionine, no ethionine	81	2.8	13	4			sl+
14[e]	Plus Na_2S and NaH_2PO_4	135	3.70	1	11	sl+		
15[f]		281				+	+	++
16[f]		216				++	++	++

[a] All incubations were in medium E in the light and in 60-ml bottles at bacteria concentration of 1 mg (dry wt)/ml and 25° except where noted. [b] Special conditions described in Experimental Procedures; TCA is trichloroacetic acid, 0.01 M; 10 × Fe is ferrous citrate, 0.2 mM; methionine is DL-methionine, 0.5 mM; ethionine is DL-ethionine; Na_2S (2.8 mM) and NaH_2PO_4 (0.14 M) added to expt 14. [c] Products estimated as described in Experimental Procedures; pheophyt and pheoporph are chlorobium pheophytins-660 and pheoporphyrins-660, respectively; Bact ch is bacteriochlorophyll; PROTO is protoporphyrin monomethyl ester and the magnesium derivative; COPRO is a mixture of etio-type porphyrins with from 3 to 8 carboxyl groups. [d] Incubation bottle (1 l.) at bacteria concentration 0.8 mg/ml. [e] Flask (22 l.) at 30° and bacteria concentration 0.4 mg/ml. [f] Flask (22 l.) at 30° and bacteria concentration 0.25 mg/ml.



chlorobium chlorophylls-650

$R_{m_{650}}$ = H

chlorobium chlorophylls-660

$R_{m_{660}}$ = CH_3, C_2H_5

R_4 = C_2H_5, *n*-C_3H_7, *i*-C_4H_9; R_5 = CH_3, C_2H_5

Experimental Procedures

Growth and Incubation of the Organism. C. thiosulfatophilum-660 was grown and harvested as previously described (Richards and Rapoport, 1966). Incubations were carried out in the light in Tween 80 containing media (medium E below) in 60-ml, 1-l., and 22-l. flasks at the bacterial concentrations specified in Table I, and the product estimation procedure was the same as previously described (Richards and Rapoport, 1966) except that three 250-w reflector flood lamps instead of two were used for the 22-l. incubations. The following extinction coefficients were employed for the product estimation reported in Table I: coproporphyrin, ϵ_{396} 1.8 × 10^5; chlorobium pheophytin, ϵ_{416} 1.0 × 10^5; and chlorobium pheoporphyrin, ϵ_{428} 1.0 × 10^5. The ratio of the Soret (416 mμ) to the 668-mμ peak for chlorobium pheophytin is 2.2; hence in experiments where both pheophytin and pheoporphyrin were found, the absorption at 668 mμ was multiplied by 2.2 and the product was used for calculation of pheophytin concentration; it was also subtracted from the absorbance of the Soret (between 416 and 428 mμ), the remainder of which was assumed to be due to the pheoporphyrin absorption. If coproporphyrin was also present, the following formulas were used: $OD_{coproporphyrin} = OD_{396} - 0.3(OD_{430})$, and $OD_{pheoporphyrin} = OD_{430} - 0.04(OD_{396})$.

Incubation Medium. MEDIUM E. Mixture B of Cooper (1963) contained inorganic salts and glycine (0.01 M),

disodium succinate (0.04 M), ferrous citrate (0.02 mM), Tween 80 (polyoxyethylene sorbitan monooleate) (0.2%, v/v), but DL-ethionine (0.5 mM) in place of DL-methionine, and no nicotinic acid (pH 6.6).

ADDITIONS. When indicated in Table I, DL-methionine was added at 0.5 mM; NaOH (1.0 M) was added to all media for pH adjustment.

SPECIAL CONDITIONS. As indicated in Table I, in expt 2, Tween 80 was omitted. In expt 5–8 the bacteria were sonicated in 30–40-ml suspensions for 5 min at 50 w with a Bronwill "Biosonik" sonicator at ice temperature. In expt 7 and 8, trichloroacetic acid (0.01 M) was added to precipitate the proteins. In expt 9 and 10, chlorobium chlorophyll extract (Mathewson *et al.*, 1963a) was added at a concentration of 42 μM instead of a cell suspension. In expt 11–13, ferrous citrate was added at 0.2 mM, ten times the normal concentration. In expt 14, sodium sulfide was added at a concentration of 2.8 mM and NaH_2PO_4 at 0.14 M to retard deterioration of the cells. Tests for porphyrinogens were made by adding 1 ml of a 2 mM solution of iodine in ethanol to 9 ml of the medium to be tested, for a final concentration of 0.2 mM.

Extraction Procedure. The extraction procedure was similar to that employed by Cooper (1963). The centrifuged medium was brought to pH 5.5 with 85% phosphoric acid and shaken with an equal volume of 50% (v/v) ethyl ether in 95% ethanol. For the 60 ml and 1-l. media, the extraction was repeated with an equal volume of 75% (v/v) ethyl ether in 95% ethanol. The combined organic phases were then washed three times with an equal volume of 30% aqueous ethanol. The combined water phases from the 30% ethanol washes were back-extracted once with *ca.* one-fourth the volume of ethyl acetate, and the organic phase was combined with the organic phase from the 30% ethanol washes. This combined fraction was termed the "organic-soluble" fraction. The solvent was removed *in vacuo* to a water–Tween 80 residue, brought to pH 4 with 85% phosphoric acid, and employed for polyethylene chromatography.

The water phase from the ether–ethanol extracts was brought to pH 3.5 with 85% phosphoric acid and extracted with ethyl acetate and then extracted with cyclohexanone at pH 1.8 as previously described (Richards and Rapoport, 1966). The ethyl acetate and cyclohexanone extracts were both extracted with 1 M ammonia; these ammonia extracts were combined and termed the "water-soluble" fraction.

Purification of Extracts. Polyethylene chromatography with aqueous lutidine solvents and countercurrent distribution with a 200 tube Craig apparatus were employed as previously described (Richards and Rapoport, 1966) except aqueous HCl–ether systems were employed for the countercurrent separations and the distribution diagram in Figure 3 was constructed by measuring the absorbance at the Soret peak of either phase (appropriately diluted) on a Cary Model 14 spectrophotometer. Since the partition coefficient is known, the total concentration in each tube can be calculated from the concentration in either phase. Primarily, the ether phase was used. Purification by silicic acid chromatography (Jeffery, 1963) was carried out after esterification of porphyrin material in 5% (v/v) concentrated sulfuric acid in anhydrous methanol (Falk, 1963). The esterified sample was chromatographed on a column (4 × 50 cm) prepared from a slurry of silica gel powder and Celite Super-Cel (5:2, w/w) in chloroform (1 l./200 g of adsorbent) which had been shaken together well and allowed to settle in the column under nitrogen pressure. The chloroform was drained to the top of the column, the column was washed with petroleum ether (bp 40–50°), and the sample was added to the column from a petroleum ether solution. (Solubility in this solvent was obtained by the presence of Tween 80.) The adsorbed sample was washed with petroleum ether on the column and then developed with 5% chloroform in petroleum ether (for pheoporphyrins) or 70% chloroform in petroleum ether (for copro- and protoporphyrins). Unesterified impurities could be recovered from the column with methanol. The solvent was removed from the fraction *in vacuo* and final traces of Tween 80 and methyl pheophorbide were removed from the methyl pheoporphyrin fraction by precipitation of the methyl pheoporphyrin from the oily residue with *n*-pentane, centrifugation, and washing the precipitate with *n*-pentane. The precipitation was repeated from concentrated solutions of the least amount of chloroform, and the precipitate was centrifuged again, washed, recentrifuged, collected, and dried.

Paper and Thin Layer Chromatography. The paper chromatographic systems for free acid (Eriksen, 1953) and methyl esters (Chu and Chu, 1957) of porphyrins were employed as previously described (Richards and Rapoport, 1966). Silica gel thin layer plates were prepared from 40 g of silica gel G and 70 ml of water, shaken for 30 sec, and applied to 10 × 20 cm plates as a 0.25-mm film. Alumina thin layer plates were prepared similarly from 50 g of aluminum oxide G and 100 ml of water. Both were heated for 0.5 hr at 110°. Alumina plates were activated for a few minutes at 110° prior to use. Solvent systems employed were 25% (v/v) acetone in chloroform with silica gel plates and 40% (v/v) benzene in chloroform with alumina plates; development was for *ca.* 0.5 hr.

Instrumental Measurements. Electronic spectra were recorded on a Cary Model 14 spectrophotometer; nuclear magnetic resonance spectra were recorded on a Varian A-60 spectrometer; pH measurements were taken with a Beckman Zeromatic pH meter.

Materials. PORPHYRINS AND CHLOROPHYLL SAMPLES. Chlorobium pheoporphyrin-660 was prepared from crude unseparated chlorobium pheophorbides-660 (Mathewson *et al.*, 1963a) by the iron–formic acid reduction and air reoxidation procedure for vinylpheoporphyrin a_5 of Fischer and Stern (1940). The ether extract from this preparation was purified by polyethylene chromatography. The adsorbed sample was washed on the column with 25% aqueous methanol, eluted with 90% aqueous methanol, and esterified

with 5% concentrated sulfuric acid in methanol. Chlorophylls *a* and *b* were obtained from spinach and bacteriochlorophyll from *Rhodospirillum rubrum* (Mathewson *et al.*, 1963b). Bacteriopheophorbide was prepared by the method of Fischer and Stern (1940) and purified by polyethylene chromatography by elution with 80–99% aqueous methanol. Mesoporphyrin was purchased from Mann Research Laboratory, Inc., New York, N. Y.

SOLVENTS AND CHEMICALS. All solvents were distilled before use. Ether was distilled from solid ferrous sulfate and washed with 10% (w/v) aqueous ferrous sulfate just before use to remove peroxides. The kerosene used for paper chromatography was a fraction with bp 40–80° at 1 mm. Tween 80 was purchased from Mann Research Laboratory, Inc., New York, N. Y. Chromatographic adsorbents were: polyethylene, Dow Chemical Co., Experimental Resin QX 2187; silica gel powder, Baker, or silicic acid, 100 mesh, Mallinckrodt (column); silica gel G, Research Specialities Co., Richmond, Calif. (thin layer); Celite Super-Cel, Johns Manville; and aluminium oxide G, Merck.

Metal Analyses. Metalloporphyrins were treated with 1 ml of concentrated sulfuric acid, the acid was neutralized with NaOH, and the liberated porphyrin was extracted into ethyl acetate at pH 3.5. The sulfate salt was obtained from the residue and dried *in vacuo*. Metal analyses on the salt were performed by spectrochemical analyses.

Results

Incubation with Media Containing Tween 80. Typical results of incubations with media containing Tween 80, which does not lyse the cells, are presented in Table I. It was found that if cells of *C. thiosulfatophilum*-660 were left in the dark in the Tween 80 medium, the *in vivo* chlorobium chlorophyll-660 absorption maximum at *ca.* 745 mμ gradually disappeared to a level of 0–10% of that present at the start of the incubation and that over a period of 70–90 hr, chlorobium pheophytin at a concentration of about 50 μM appeared in the medium. Between 10 and 20 μmoles of additional pheophytin could be extracted from the centrifuged cells with 80% aqueous acetone. If no Tween 80 were present, the *in vivo* chlorophyll spectrum remained intact. In the Tween 80 medium in the light (expt 1 and 3) there was a gradual decrease in the pheophytin spectrum (main absorption at 668 and 416 mμ) and a gradual appearance of a material, later shown to be a mixture of the chlorobium pheoporphyrins, with Soret absorption at *ca.* 428 mμ. However, there was essentially no conversion in the dark (expt 4). The active region for this conversion seemed to be in the infrared region of the spectrum. However the total appearance of the pheoporphyrin never equalled the amount of pheophytin which disappeared. Furthermore, it appeared that the pheophytin was being converted in the presence of light to an intermediate compound with much smaller extinction maxima at *ca.* 500 and 460 mμ. This intermediate could be oxidized by iodine (final concentration 0.2 mM) to the pheoporphyrin.

In order to test whether intact cells were necessary for this photochemical conversion, experiments with sonicated cells were carried out. The sonicated cell suspensions in the Tween 80 medium (in which it was assumed that enzyme activity had not been completely destroyed) still produced the pheoporphyrin in the light (expt 5) but not in the dark (expt 6) and not when the sonicate had been treated with trichloroacetic acid (expt 7 and 8), which precipitated the proteins but not the pheophytin from the medium. Extracted chlorobium chlorophyll-660 added to the medium without bacterial cells (expt 9 and 10) quickly lost its magnesium, but showed essentially no conversion to the pheoporphyrin in the light or dark.

It was found that if methionine was used in place of ethionine in the Tween 80 medium (expt 13) or when methionine and ethionine were both present (expt 12), the methionine did not appear to have any inhibitory effect on the production of the pheoporphyrin.

In addition to the pheoporphyrin, a mixture of other porphyrins was usually observed although there was a considerable variation in the types of porphyrins produced. In some of the experiments (3–10) no additional porphyrins were observed; in the others, porphyrins and metalloporphyrins with from 3 to 8 carboxyl groups were present. In all cases, there was increased fluorescence and adsorption at 396–400 mμ upon treatment with dilute iodine or oxygen indicating the presence of porphyrinogen or dipyrrylmethene-like intermediates in the medium (Cooper, 1963; Richards and Rapoport, 1966). The variation in the types of porphyrins produced seemed to be due to the activity of the bacteria when the cells were harvested for the incubation. Most of the experiments were carried out with 3-day cultures, at which time the bacteria had already entered the stationary phase of growth. However, two experiments (15 and 16) were carried out with cultures harvested after only 2 days of growth and the bacteria were in the log phase of growth. In addition, these incubations were made with more dilute suspensions and for longer periods of time, 281 and 216 hr, respectively. In both cases, in addition to porphyrins with 3–8 carboxyl groups, a large band corresponding to a porphyrin with one carboxyl group was observed during polyethylene chromatography, and was shown to be a mixture of protoporphyrin IX monomethyl ester and its magnesium derivative. However, after these longer term incubations, no pheoporphyrin was detected, but a compound spectroscopically resembling bacteriochlorophyll (with traces of bacteriopheophytin) was observed instead.

In expt 11–13 ferrous ion was present at ten times the concentration present in expt 1, and coproporphyrin production (expt 11) was inhibited by about two-thirds. Omission of Tween 80 (expt 2) also inhibited coproporphyrin production. As had been found previously with methionine at 2 mM (Richards and Rapoport, 1966), methionine at 0.5 mM also inhibited the produc-

TABLE II: Visible Spectra of Metallo- and Pheoporphyrin Fractions from Polyethylene Chromatography.[a]

Component[b]	Type[c]	$\lambda_{max}^{aq\ lutidine}$ (mμ)			
		Red Bands[d]	I	II	Soret
I	Chlorin	668 (1.00), 607 (0.63)	575 (0.81)	532 (0.87)	429 (12.0)
II	Metallo	637 (0.23), 610 (0.21)	572 (0.84)	536 (1.00)	405 (13.8)
Magnesium etioporphyrin[e]			580 (0.62)	550 (1.00)	407 and 421
III	Metallo	640 (0.10), 622 (0.10)	560 (1.00)	525 (0.56)	398 (12.2)
Copper etioporphyrin[f]			563 (1.00)	527 (0.57)	398 (9.4)

[a] Relative absorption in parentheses. [b] Components I and II were bands from 10 to 0.25% lutidine fractions from polyethylene chromatography of organic-soluble porphyrins of expt 1 of Table I. Component III was a 5% lutidine fraction from polyethylene chromatography of water-soluble porphyrins of expt 1. [c] For explanation of spectra types, see Falk (1963). [d] Red bands due to interaction of metalloporphyrins with nitrogenous bases; *cf.* Allison and Becker (1963). [e] Spectrum in pyridine of Allison and Becker (1963). [f] Spectrum in dioxane of Erdman and Corwin (1946).

tion of coproporphyrin both in the presence (expt 12) and absence (expt 13) of ethionine.

Characterization of the Products of the Incubations. The media from expt 1 and 14 were centrifuged and extracted as described in the experimental procedure. The organic-soluble fraction of expt 1 was chromatographed on polyethylene; however, considerable difficulty was encountered in getting the material adsorbed on the column due to interference from Tween 80. After about three washes through a polyethylene column, a separation was obtained into a 1% and a green 10% aqueous lutidine fraction (I, Table II). The 1% lutidine and the water-soluble fraction of expt 1 were both chromatographed separately on polyethylene and good separations were obtained. The 1% lutidine fraction yielded a metallocoproporphyrin III band (II, Table II) which ran in front of the coproporphyrin III band with 0.25% lutidine, a tricarboxylic porphyrin, and green material eluted with 5–10% lutidine. The latter material had a spectrum consisting of a mixture of pheoporphyrin and pheophytin absorption and was identical with the 10% lutidine fraction of the previous chromatography (I, Table II). The water-soluble fraction yielded a metallouroporphyrin band which ran in front of the uroporphyrin with lutidine–0.1 M KH_2PO_4 buffer (pH 6.75), bands with porphyrins containing 7, 6, and 5 carboxyl groups, coproporphyrin III, and a metallocoproporphyrin III (III, Table III) which ran behind the coproporphyrin band with 0.25% lutidine. It was completely removed with 5% lutidine. Both fractions II and III (Table II) contained coproporphyrin III, as determined by paper chromatography of the methyl esters of the metal-free porphyrins (Richards and Rapoport, 1966). A metal analysis of the sulfate salt detected copper with only minor amounts of magnesium in both samples as the only detectable metals. The isomer of the uroporphyrin was not determined. A summary of pertinent spectra of the chromatographic fractions is presented in Table II.

The combined green material of the 10% lutidine fractions contained a minor amount of material with one and two carboxyl groups but consisted mostly of material which was fully esterified (probably with farnesol). The latter was, however, converted to a dicarboxylic material upon treatment with 1 M NaOH or under the conditions of the phase test (Holt, 1958).

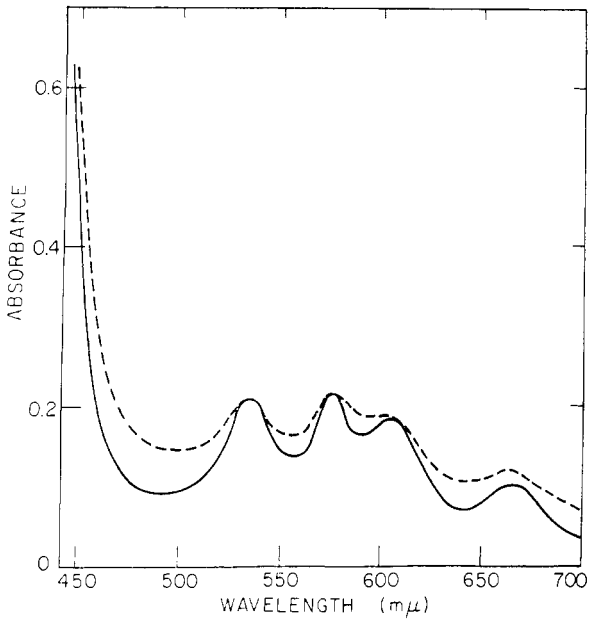


FIGURE 1: Comparison of electronic spectra of chlorobium pheoporphyrins in chloroform from medium E (Tween 80 medium), purified by silica gel–Celite chromatography; and from iron–formic acid reduction and air oxidation of the pheophorbide. (——) From Tween 80 medium and (- - - -) prepared from pheophorbide.

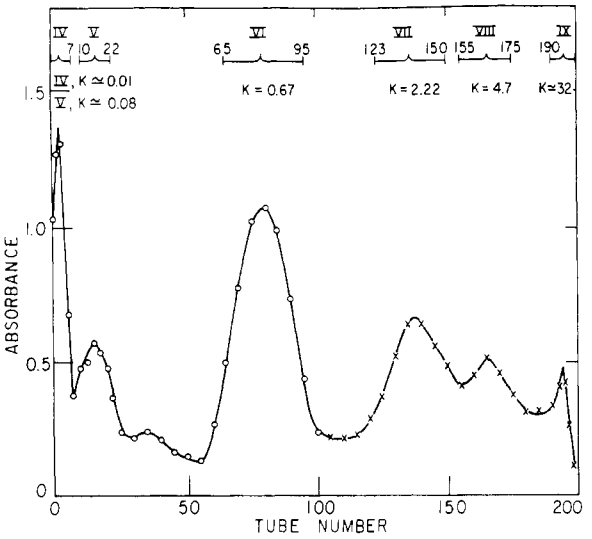


FIGURE 2: Countercurrent distribution diagram for separation of chlorobium pheoporphyrins-660 from incubation of *C. thiosulfatophilum*-660 with medium E (Tween 80 medium). The phases were ether–aqueous HCl (0.85 M); transmittance of aqueous (O) or ether (X) phases was measured by a Coleman photometer with a 430-mμ filter.

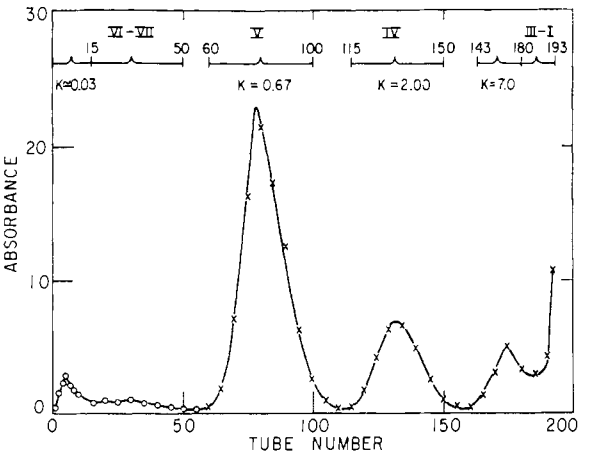


FIGURE 3: Countercurrent distribution diagram for separation of chlorobium pheophorbides-660. The phases were ether–aqueous HCl (2.2 M); absorbance of aqueous (O) or ether (X) phases was measured by a Cary Model 14 spectrophotometer. Tubes 0–15 contained chlorobium pheoporphyrins. Tubes 163–180 contained a chlorobium pheophorbide-650 component. The roman numerals refer to the fractions obtained by Holt *et al.* (1963).

These observations were paralleled by synthetic pheoporphyrin and by chlorobium pheophytin and methyl pheophorbide, which, while not undergoing a visible phase test, were converted to a mixture of mono- and dicarboxylic material. Chlorophylls *a* and *b* and bacteriochlorophyll were converted to a mixture of di- and tricarboxylic material. These results confirmed that the chlorobium compounds contained no 10-carbomethoxy group (Holt and Morley, 1960; Holt and Hughes, 1961; Mathewson *et al.*, 1963a) but indicated that the phase test conditions were adequate to cause a cleavage of the isocyclic ring E. The treatment of the pheoporphyrins with base was accompanied by an irreversible shift of the Soret band to 408 mμ and a loss in extinction. However, acid treatment caused no such irreversible shift.

The methyl esters of the 10% lutidine fraction (not treated with alkali) were prepared by transesterification in 5% concentrated sulfuric acid in methanol, and chromatographed on silica gel–Celite. There was still some interference by Tween 80 in the main band but it and traces of methyl pheophorbide were effectively removed by repeated precipitations with *n*-pentane. A comparison of the *n*-pentane-insoluble precipitate with synthetic chlorobium pheoporphyrin is presented in Figure 1. The methyl esters of both the synthetic and natural pheoporphyrins were identical in two thin layer chromatography systems, having R_F values of 0.62 on alumina and 0.74 on silica gel plates.

The pheoporphyrin isolated from the Tween 80 medium (methyl ester) was hydrolyzed in 6 M hydrochloric acid and was subjected to two countercurrent distribution purifications. The first partition (2.0 M aqueous HCl–ether) removed a small amount of a substance with a bacteriopheophorbide spectrum (in addition to at least one fraction of a more ether-soluble pheoporphyrin) from the major portion of the pheoporphyrins which remained in the first 25 tubes. The second partition (0.85 M aqueous HCl–ether) separated the pheoporphyrins into six fractions. The distribution diagram is shown in Figure 2. For comparison, a separation of chlorobium pheophorbides-660 by countercurrent distribution (2.2 M aqueous HCl–ether) is given in Figure 3 to show the similarity of the fractions obtained. Electronic spectra of the separated pheoporphyrins (components IV–IX) are given in Table III.

The medium from expt 15 was centrifuged and extracted as described in the experimental procedure. The organic-soluble fraction was evaporated of water *in vacuo* and the residue was esterified in 5% concentrated sulfuric acid in methanol. A silica gel–Celite chromatography yielded a fraction consisting of mostly coproporphyrin III tetramethyl ester but with traces of a dicarboxylic porphyrin ester, and a fraction (eluted with pure chloroform) which contained a material with absorption characteristic of bacteriopheophorbide at 757 mμ. However, no pheoporphyrin was found. The incubation was repeated (expt 16) but this time the bacteria were not centrifuged and the medium was filtered directly through a 7.5 × 30 cm column of polyethylene. Material absorbed on the polyethylene was removed with acetone. The acetone-soluble fraction was then purified by polyethylene chromatography. Bands of porphyrins with from 4 to 8 carboxyl groups

TABLE III: Visible Spectra of Chlorobium Pheoporphyrin Fractions Separated by Countercurrent Distribution.[a]

Component[b]	Type[c]	$\lambda_{max}^{CHCl_3}$ (mμ) I	II	III	IV	Soret
IV	Rhodo	650 (0.38)	596 (0.85)	570 (0.99)	530 (1.00)	427 (19.4)
V	Rhodo	652 (0.46)	595 (0.78)	572 (1.00)	529 (0.96)	427 (21.7)
VI	Rhodo	653 (0.40)	597 (0.81)	571 (1.00)	529 (1.00)	428 (20.2)
VII	Rhodo	653 (0.38)	598 (0.72)	572 (1.00)	530 (0.99)	428 (22.8)
VIII	Etio	670 (0.40)	608 (0.52)	577 (0.76)	535 (1.00)	434 (20.9)
IX	Etio	660 (0.42)	601 (0.75)	572 (0.84)	533 (1.00)	432 (13.6)
Pheoporphyrin a_5[d]	Oxorhodo	634 (0.12)	583 (0.79)	562 (1.00)	521 (0.59)	417 (12.0)
Vinylpheoporphyrin a_5[d]	Oxorhodo	638 (0.12)	588 (0.79)	567 (1.00)	525 (0.47)	419 (10.9)
Divinylpheoporphyrin a_5[e]	Oxorhodo	642 (0.10)	590 (0.67)	568 (1.00)	527 (0.59)	422 (11.0)
2-Acetylpheoporphyrin a_5[f]	Oxorhodo	646	597	571	527	

[a] Relative absorption in parentheses. [b] Components IV–IX are fractions 1–6, respectively, of countercurrent distribution purification of chlorobium pheoporphyrins (Figure 2). [c] For explanation of spectra types (see Falk, 1963). [d] Spectra of monomethyl esters in dioxane of Granick (1950). [e] Spectrum of monomethyl ester in dioxane of Jones (1963c). [f] Spectrum of dimethyl ester in pyridine–ether of Fischer and Stern (1940).

TABLE IV: Visible Spectra of Protoporphyrin and Bacteriochlorophyll Fractions from Polyethylene Chromatography.[a]

Component[b]	$\lambda_{max}^{solvent}$ (mμ) I	II	III	IV	Soret
X ($CHCl_3$)	630 (0.36)	575 (0.50)	541 (0.80)	505 (1.00)	408 (11.7)
Protoporphyrin[c] (dioxane)	630 (0.38)	575 (0.46)	537 (0.79)	503 (1.00)	
XI (10% lutidine)	591 (0.90)	551 (1.00)			416
Magnesium protoporphyrin[d] (basic 50% ethanol)	590 (0.93)	551 (1.00)			418 (14.3)
XII (acetone)	770 (1.00)	679 (0.47)	573 (0.40)	520 (0.28)	387 (1.38), 357 (1.63)
Bacteriochlorophyll[e] (ether)	770 (1.00)	705 (0.10)	573 (0.12)		393 (0.54), 357 (0.82)
Bacteriopheophorbide[e] (ether)	750 (1.00)	675 (0.19)	615 (0.10)	522 (0.49)	385 (1.27), 357 (3.56)

[a] Relative absorption in parentheses. [b] Components X–XII were 10 and 10–15% aqueous lutidine and acetone fractions, respectively, from polyethylene chromatography of products from expt 16 of Table I; solvent in parentheses. [c] Spectrum of dimethyl ester of Stern and Wenderlein (1934). [d] Spectrum of Cooper (1963). [e] From *R. rubrum* (*cf.* Experimental Procedures).

were observed but not studied further. The dark red main band was eluted with 10% lutidine and a trailing band (bright red with an orange fluorescence) was eluted with 10–15% lutidine. A blue-gray material still left on the column was removed by acetone. The 10% lutidine fraction showed a spectrum of protoporphyrin (X, Table IV) but the porphyrin contained only one carboxyl group as determined by paper chromatography (Eriksen, 1953). Hence it was assumed to be the monomethyl ester derivative. The trailing bright red compound showed a metalloporphyrin spectrum of the magnesium type (XI, Table IV) and also contained only one free carboxyl group; hence it was assumed to be magnesium protoporphyrin monomethyl ester. The acetone fraction showed a bacteriochlorophyll spectrum with traces of bacteriopheophytin (XII, Table IV). This material contained no free carboxyl groups. Table IV shows the spectra of these three fractions.

The protoporphyrin monomethyl ester was esterified

in 5% concentrated sulfuric acid in methanol and the dimethyl ester showed R_F values of 0.93 (first solvent) and 0.58 (second solvent) by paper chromatography (Richards and Rapoport, 1966; solvent system A, Table IV). With this system mesoporphyrin dimethyl ester showed R_F values of 0.93 (first solvent) and 0.69 (second solvent).

The nuclear magnetic resonance spectrum (δ values, referred to internal tetramethylsilane = 0) of the protoporphyrin dimethyl ester (*ca.* 0.17 M in $CDCl_3$) showed singlet peaks for ring methyl (3.45) and ester methyl (3.71) protons, triplet peaks for propionic β-methylene (3.11) and propionic ring methylene (4.29) protons, unresolved multiplets for vinyl (6.10 and 6.40) protons, and three singlets in a 1:2:1 ratio for *meso* (9.65, 9.74, and 9.84) protons similar to the reported spectrum (Becker *et al.*, 1961) of protoporphyrin IX dimethyl ester.

Discussion

It has been previously reported (Richards and Rapoport, 1966) that *C. thiosulfatophilum*-660 excreted porphyrins into the medium when concentrations of the bacterium were suspended in glycine, succinate, and 2-ketoglutarate (coproporphyrin III with traces of uroporphyrins I and III); δ-aminolevulinic acid (uroporphyrin I, with lesser amounts of the III isomer, coproporphyrins I and III, and porphyrins with 5, 6, and 7 carboxyl groups); and glycine, succinate, and ethionine (coproporphyrin III and uroporphyrin). In addition, metalloporphyrin derivatives of copro- and uroporphyrins were detected during countercurrent distribution purification, but only traces of di- and tricarboxylic porphyrins were found.

In the present work, medium E employed essentially mixture B of Cooper (1963) except that ethionine was substituted for methionine because we had found that methionine did not stimulate coproporphyrin production in *C. thiosulfatophilum*-660. Cooper (1963) could not relate the effect of methionine in *R. capsulata* to the known effect of methionine (Tait and Gibson, 1961; Gibson *et al.*, 1962a,b) in providing the methyl ester group of magnesium protoporphyrin monomethyl ester in *R. spheroides*. He suggested, however, that its effect on porphyrin metabolism might come from sulfur compounds other than methionine, as methionine could be completely replaced by glutathione (1 mM) and partially by L-cysteine (1 mM) in its effects on stimulating coproporphyrinogen, magnesium protoporphyrin monomethyl ester, and bacteriochlorophyll synthesis in *R. capsulata;* however, the effect of ethionine in *R. capsulata* was not studied.

Gibson *et al.* (1962b) found that coproporphyrin excretion in *R. spheroides* was increased up to 20 times by the addition of between 0.5 and 5.0 mM ethionine to Lascelles' (1956) mixture I. However, although no magnesium protoporphyrin monomethyl ester was excreted by *R. spheroides* during incubations with ethionine, bacteriochlorophyll synthesis (while reduced 75% by 0.5 mM ethionine) was not inhibited completely until greater than 1 mM ethionine was added. Although we have found no magnesium protoporphyrin monomethyl ester excretion by *C. thiosulfatophilum*-660 when incubated with 2 mM ethionine (Richards and Rapoport, 1966) the stimulatory effect for coproporphyrin excretion (*ca.* three to six times) was not as great in this organism as in *R. spheroides*. However, in the present work, ethionine at only one-fourth this concentration (0.5 mM) in the presence of Tween 80 not only increased coproporphyrin excretion, but led to the excretion of magnesium protoporphyrin monomethyl ester and bacteriochlorophyll.

Although the bacteriochlorophyll was identified only by a spectroscopic comparison with an authentic sample (Table IV), it had already been shown that the tetrahydroporphyrin (chlorobium chlorophyll-770; Olson and Romano, 1962) occurring naturally in *C. thiosulfatophilum* is bacteriochlorophyll (Holt *et al.*, 1963) and not a mixture of tetrahydroporphyrins of the chlorobium chlorophyll-660 type. Therefore, it is probable that the bacteriochlorophyll is being synthesized by the same pathway which operates in the purple bacteria. However, it is unlikely that an intermediate between magnesium protoporphyrin monomethyl ester and bacteriochlorophyll is inhibiting the δ-aminolevulinic acid synthetase system in *C. thiosulfatophilum*-660 as suggested by Gibson *et al.* (1962b) for *R. spheroides*.

If *C. thiosulfatophilum* synthesizes chlorobium chlorophylls-650 and -660 through the intermediacy of bacteriochlorophyll or one of its immediate precursors, then subsequent alkylation steps would be required, whereby the "extra" carbon atom side chains at the 4, 5, and *meso* positions are attached to the tetrapyrrole nucleus. Further, if ethionine were functioning as it does in *R. spheroides* (Gibson *et al.*, 1962b, 1963) in inhibiting the methylation of magnesium protoporphyrin by *S*-adenosylmethionine, then it might also be inhibiting *C* methylation of the bacteriochlorophyll or one of its immediate precursors in *C. thiosulfatophilum*-660 by some similar but unknown mechanism, and hence causing an accumulation of bacteriochlorophyll in the medium.

Cooper (1963) has attributed the solubilization by Tween 80 of metalloporphyrins or porphyrin esters in the medium as a possible explanation for the fact that this agent increased magnesium protoporphyrin monomethyl ester production in *R. capsulata* 10–20 times. We have confirmed that there is an increase in the proportion of metalloporphyrin excretion in Tween 80 medium with the bacterium *C. thiosulfatophilum*-660. The metallocoproporphyrins (II and III, Table II) were probably not separated well by the extraction and polyethylene procedures. However, the more organic-soluble metallocoproporphyrin III (II, Table II) had a magnesium-type spectrum (for a definition of spectral types, see Falk, 1963), while the more water-soluble metallocoproporphyrin III (III, Table II) was copper coproporphyrin III as indicated by its spectrum and metal analysis. The complex of the magnesium porphyrin may have been more unstable and may have

lost its magnesium during extraction prior to the metal analysis. This instability of the magnesium complex was also found with the magnesium protoporphyrin monomethyl ester sample, which lost its magnesium either during extraction from 10 to 15% lutidine solution at pH 4 with ethyl acetate or during attempts to remove traces of lutidine from the extract. Tait and Gibson (1961) and Gibson *et al.* (1962b, 1963) have shown by enzymatic work that the true precursor of magnesium protoporphyrin monomethyl ester in *R. spheroides* is magnesium protoporphyrin and not protoporphyrin monomethyl ester. Therefore, the presence of the magnesium-free protoporphyrin monomethyl ester in the Tween 80 medium of *C. thiosulfatophilum*-660 was most probably due to the instability of the magnesium complex and consequent loss of magnesium during the isolation procedure.

We have found no trace of unesterified protoporphyrin or its magnesium derivative. In contrast, Uspenskaya (1965b) reported finding an unesterified dicarboxylic porphyrin and its magnesium derivative in the culture medium of *C. ethylicum*. The spectrum of this porphyrin and of the magnesium porphyrin after the metal had been removed indicated that the porphyrin contained no vinyl groups. However, the isolation procedure involved a prior 0.15 M HCl extraction of coproporphyrin from the ether extract which may have hydrolyzed the methyl ester and/or hydrated any protoporphyrin monomethyl ester in that extract to hematoporphyrin (*cf.* Falk, 1963). Also, Godnev *et al.* (1966) report evidence (but no experimental details) for the formation of protoporphyrin and its magnesium derivative but were unable to establish the presence of any methyl esters in cultures of green bacteria to which threonine had been added. On this basis, they suggested that magnesium protoporphyrin was the last common intermediate in the biosynthesis of chlorobium chlorophylls and other chlorophylls.

We found that in addition to the solubilization of metalloporphyrins in the medium, Tween 80 caused chlorobium chlorophyll to be leached into the medium in the form of the magnesium-free pheophytin. Photobleaching of the pheophytin occurred with or without whole cells, and with sonicated cells with or without the presence of trichloroacetic acid. In all cases some pheoporphyrin was also produced and more could be generated from an intermediate with absorption at 460 and 500 mμ by the addition of iodine upon opening the incubation bottles. However, in the case of the whole or sonicated cells there was an enzymatic enhancement of the pheoporphyrin formation which was inhibited by trichloroacetic acid. We are unable to fully explain the significance of this chlorobium pheoporphyrin production. Further evidence, however, that the pheoporphyrins were being derived from the pheophytins and not being synthesized from the organic precursors, glycine and succinate, lay in the fact that the pheoporphyrins were produced even in those experiments (3–10) in which no other porphyrins (*e.g.*, uro-, copro-, or protoporphyrin) were excreted into the medium. In addition, the pheoporphyrins were, for the most part, fully esterified (with farnesol?) and apparently consisted of a mixture of homologous derivatives (Figure 2) quite similar to that observed for the chlorobium pheophorbides-660 (Figure 3). Hence the reaction may be considered similar to the reverse of the photochemical production of chlorophyll a from protochlorophyll, but with the magnesium-free derivatives instead.

As shown in Table III, the chlorobium pheoporphyrins separated by countercurrent distribution (Figure 2) had spectra with maxima of absorption at longer wavelengths than the pheoporphyrins obtained from the previously isolated biosynthetic intermediates, magnesium vinylpheoporphyrin a_5 monomethyl ester (Granick, 1950) and magnesium 2,4-divinylpheoporphyrin a_5 monomethyl ester (Jones, 1963b,c). Although a closer coincidence of the maxima was observed with 2-acetylpheoporphyrin a_5 (Fischer and Stern, 1940), all of the pheoporphyrins of the a_5 series mentioned above exhibited an oxorhodo-type spectrum. The first four fractions of the chlorobium pheoporphyrins (components IV–VII), on the other hand, exhibited a rhodo-type spectrum (Table III) which must have been due to the effect of the *meso*-alkyl substituent. The fifth fraction (component VIII, Table III), however, was shifted 5–20 mμ further to the red from the firs four fractions, but exhibited an etio-type spectrum. It therefore may have contained further conjugation such as an acetyl group in place of the α-hydroxyethyl group. We are unable to predict, however, what effect this should be on the spectral type. Component IX appeared to be a mixture of both types of spectra.

We have not been able to obtain any further information on the tetracarboxylic porphyrins with phyllotype spectra which were observed in trace amounts from incubations of *C. thiosulfatophilum*-660 with glycine and 2-ketoglutarate (Richards and Rapoport, 1966). Hence, with the present results we must consider these porphyrins to be artifacts, as it is apparent that *meso* alkylation has not occurred prior to the magnesium protoporphyrin monomethyl ester stage, and possibly not until after bacteriochlorophyll has been synthesized. It is likely that *C. thiosulfatophilum*-660 synthesizes regular bacteriochlorophyll by the same biosynthetic pathway as do the purple bacteria. *meso* alkylation and the addition of the "extra" carbon atom side chains may occur at subsequent stages, although it seems more likely that a branch point occurs before the bacteriochlorophyll stage. For example, 2-devinyl-2-hydroxyethylpheophorbide *a* has been detected in *R. spheroides* by Jones (1964) and the magnesium complex is probably an intermediate very close to bacteriochlorophyll. This intermediate or its corresponding magnesium pheoporphyrin or even magnesium protoporphyrin monomethyl ester itself could possibly be a branch point for the chlorobium-type magnesium pheoprobides or pheoporphyrins, at which stage *meso* alkylation and/or the addition of an "extra" carbon atom side chain occurred, perhaps one methyl group at a time. Therefore if this *C* alkylation were being blocked by ethionine, an excess of the branch intermediate would be available for the necessary additional

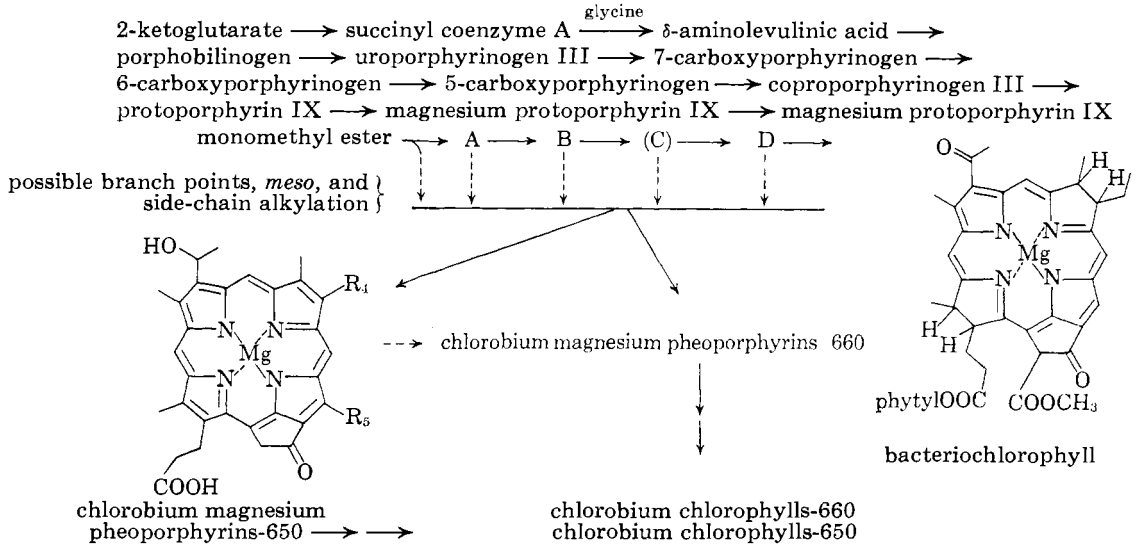


FIGURE 4: Suggested scheme for the biosynthesis of chlorobium chlorophylls-650 and -660. A = magnesium 2,4-divinylpheoporphyrin a_5 monomethyl ester? B = magnesium vinylpheoporphyrin a_5 monomethyl ester? C = hypothetical intermediate: magnesium 2-devinyl-2-α-hydroxyethylpheoporphyrin *a* monomethyl ester or chlorophyllide *a*? D = 2-devinyl-2-α-hydroxyethylchlorophyllide *a*?

reactions including esterification with phytol and isomerization of the hydroxyethylchlorin to the acetyltetrahydroporphyrin for conversion to bacteriochlorophyll. Once the chlorobium-type intermediates had been synthesized, the mixture of homologs could then be esterified with farnesol and lose the 10-carbomethoxy group to form the chlorobium chlorophylls-650 and -660. It is not known, however, whether the chlorobium (magnesium) pheoporphyrins are true biosynthetic intermediates which are subsequently converted by a photochemical reaction to the chlorobium chlorophyll-(ide)s, or whether the chlorobium-type intermediates are already at the pheophorbide level of reduction. A summary of the suggested scheme is presented in Figure 4.

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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.