Pyrroles and Related Compounds. Part 39.[1] Structural and Biosynthetic Studies of the *Chlorobium* Chlorophylls-660 (Bacteriochlorophylls *c*). Incorporations of Methionine and Porphobilinogen [2]

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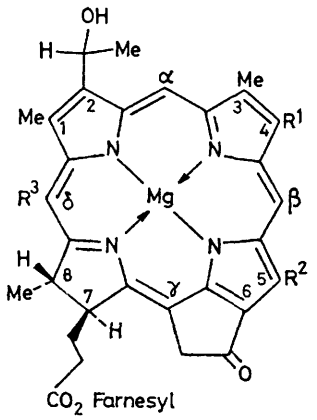
Treatment of the phaeofarnesins (4) isolated from the *Chlorobium* chlorophylls-660 (ex. *Chloropseudomonas ethylicum*) with sulphuric acid in methanol gives the methyl phaeophorbides (1) which can be dehydrated by refluxing in benzene in the presence of acid to afford the 2-vinyl derivatives (2): prolonged refluxing gives the novel cyclised compounds (6). By-products from handling the pigments are the 8'-acetylbilitrienes (5), obtained by photo-oxygenation. [$^{14}CH_3$]-, [$^{13}CH_3$]-, and [$^{13}C^2H_3$]-L-methionine are efficiently incorporated into the *Chlorobium* chlorophylls-660, showing that the extra methylation present in the 8-methyl, 4-isobutyl, 4-n-propyl, and 5-ethyl groups of these pigments is methionine derived. Using 6-^{14}C labelled material, porphobilinogen is also confirmed as an important building block in these bacterial chlorophylls.

THE *Chlorobium* chlorophylls-660 † (Figure 1) (recently renamed [3] as bacteriochlorophylls *c*) are a series of polymethylated chlorophylls derived [4] from certain sulphur bacteria. A dispute has arisen over the precise structures of these chlorophylls, particularly with regard to the positioning and nature of the *meso*-alkyl substituent. The 650 † chlorophylls (bacteriochlorophylls *d*[3]), produced [5] by *Chlorobium thiosulphatophilum* strain L, lack the *meso*-alkyl substituent, but other strains of *C. thiosulphatophilum*, as well as *C. limicola* and *Chloropseudomonas ethylicum* (said [6] to consist of *C. limicola* together with an unknown colourless non-photosynthetic bacterium), all produce the *Chlorobium* chlorophylls-660. Degradative [7] and synthetic [8,9] work had clearly shown the *meso*-alkyl groups in the 660 series to be present in the δ-position, but an alternative positioning (either α or β) was suggested by Mathewson *et al.*,[10] who showed by n.m.r. spectroscopy that one of the two *meso*-protons was readily exchangeable with deuterium in acid; thus on the basis of previous work in the literature [11] it seemed reasonable to propose [10] that the δ-proton (normally exchanged readily [11]) was still present, and therefore that the alkyl substituent must be in the α- or β-position. On the basis of n.m.r. spectra, Holt *et al.*[7] had already suggested δ-substitution because the highest field *meso*-proton [12] was missing in the *Chlorobium* phaeophorbides; more recently, δ-substitution has been conclusively confirmed [2,13,14] by n.m.r. methods, and a rationalisation of the reasons for exchange of the α-proton in δ-methylchlorins has been presented.[2]



Band	R^1	R^2	R^3	% of total
1	Bu^i	Et	Et(?)	0.5
2	Bu^i	Et	Me	0.5
3	Pr^n	Et	Et(?)	2
4	Pr^n	Et	Me	16
5	Et	Et	Me	71
6	Et	Me	Me	10

FIGURE 1 Proposed [15] structures for *Chlorobium* chlorophylls-660

In Part 19 [9] we questioned the allocation of a δ-ethyl

† The numbers 660 and 650 refer to the wavelength (in nm) of the maximum in the red region of the electronic absorption spectra of the pigments in ether solution.

[1] Part 38, G. W. Kenner, J. Rimmer, K. M. Smith, and J. F. Unsworth, *J.C.S. Perkin I*, 1977, 332.

[2] Part of this work was reported in the Royal Society Discussion Meeting on the Biosynthesis of Porphyrins, Chlorophyll, and Vitamin B_{12}, London, 1975; G. W. Kenner, J. Rimmer, K. M. Smith, and J. F. Unsworth, *Phil. Trans. Roy. Soc.*, 1976, **273B**, 255.

[3] A. Jensen, O. Aasmundrud, and K. E. Eimhjellen, *Biochim. Biophys. Acta*, 1964, **88**, 466.

[4] M. B. Allen, in ' The Chlorophylls,' eds. L. P. Vernon and G. R. Seely, Academic Press, New York, 1966, p. 516.

[5] A. S. Holt, in ' The Chemistry and Biochemistry of Plant Pigments,' ed. T. W. Goodwin, Academic Press, New York, 1965, p. 3, and references therein.

[6] V. N. Shaposhnikov, E. N. Kondrateva, and V. D. Fedorov, *Nature*, 1960, **187**, 167.

[7] A. S. Holt and D. W. Hughes, *J. Amer. Chem. Soc.*, 1961, **83**, 499; A. S. Holt and H. V. Morley, *ibid.*, 1960, **82**, 500; A. S. Holt, D. W. Hughes, H. J. Kende, and J. W. Purdie, *ibid.*, 1962, **84**, 2835; *Plant Cell Physiol.*, 1963, **4**, 49; H. V. Morley and A. S. Holt, *Canad. J. Chem.*, 1961, **39**, 755.

[8] J. L. Archibald, D. M. Walker, K. B. Shaw, A. Markovac, and S. F. MacDonald, *Canad. J. Chem.*, 1966, **44**, 345.

[9] M. T. Cox, A. H. Jackson, and G. W. Kenner, *J. Chem. Soc.* (*C*), 1971, 1974.

[10] J. W. Mathewson, W. R. Richards, and H. Rapoport, *J. Amer. Chem. Soc.*, 1963, **85**, 364; *Biochem. Biophys. Res. Comm.*, 1963, **13**, 1.

[11] R. B. Woodward and V. Škarić, *J. Amer. Chem. Soc.*, 1961, **83**, 4676.

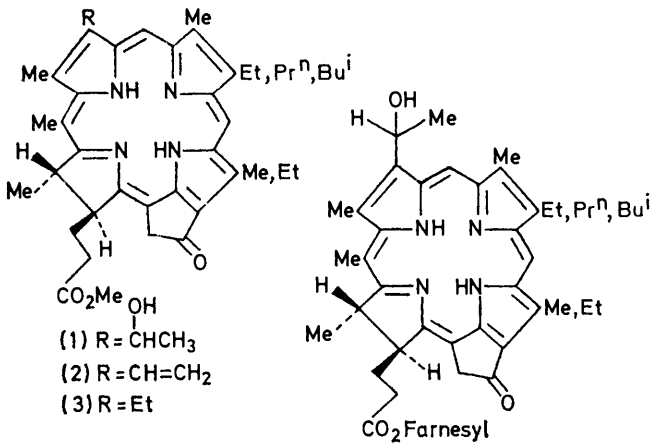
[12] For a review of porphyrin and chlorin n.m.r. spectroscopy, see H. Scheer and J. J. Katz, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 399.

[13] K. M. Smith and J. F. Unsworth, *Tetrahedron*, 1975, **31**, 367.

[14] H. Brockmann, jun., *Phil. Trans. Roy. Soc.*, 1976, **273B**, 277.

group to bands 1 and 3 of the 660 chlorophylls, and there is no reason to expand on the stated arguments, or to alter our conclusions that structural assignments [15] featuring *meso*-ethyl groups are incorrect. In this paper we describe our work on the isolation and chemistry of the *Chlorobium* chlorophylls-660, and report on biosynthetic experiments which, using radiochemical labels, establish methionine and porphobilinogen as primary building blocks.

Structural Studies.—Initially, the source of *Chlorobium* chlorophylls-660 used in the present work was *Chlorobium thiosulphatophilum* (NCIB cat. no. 8346), but after a short time this was replaced by *Chloropseudomonas ethylicum*, a hardier anaerobe which produces the pigments much more prolifically. Attempts to isolate the chlorophylls themselves were thwarted by ready demetallation, so in order to facilitate the examination of products from feeding experiments, as well as to prepare the pigments for separation of the individual homologues,* it was decided to develop efficient degradative procedures to give the methyl phaeophorbides (1), methyl 2-vinylphaeophorbides (2), and methyl mesophaeophorbides (3).



(4)

Extraction of the growing cultures with acetone after filtration on Celite was found to be preferable to the methanol used by previous workers because smaller volumes were required. Partition between water and methylene chloride, followed by evaporation of the methylene chloride gave the phaeofarnesins (4). Mass spectrometry indicated the presence of a series of homologues with mass ions corresponding to the proposed [15] structures (Figure 1) with the exception of band 1. Characteristic losses in the spectra were 18 and 204 mass units due to dehydration and removal of the farnesyl ester respectively. The phaeofarnesins were transesterified in 5% concentrated sulphuric acid in methanol to give the methyl phaeophorbides (1). In this procedure *ca.* 5% of the 1-hydroxyethyl material was

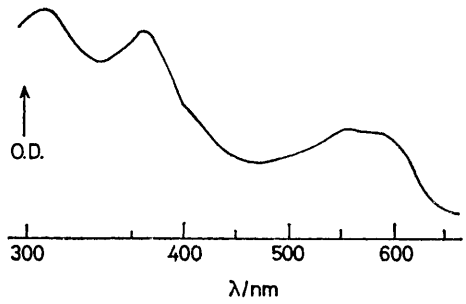
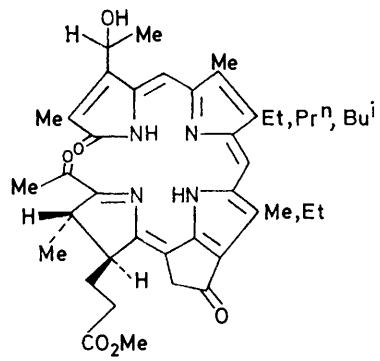


FIGURE 2 Electronic absorption spectrum (in $CHCl_3$) of the total mixture of 8′-acetylbilitrienes (5) from *C. ethylicum*

transformed into vinyl, but the compounds (1) and (2) were readily separable by column chromatography on alumina; ^1H and ^{13}C n.m.r. spectroscopy confirmed the proposed structure for band 5 (Figure 1, major component). A characteristic feature of the t.l.c. of either the methyl (1) or farnesyl phaeophorbides (4) when elution was carried out with 30% acetone in light petroleum (b.p. 60—80°) was resolution of a purple band which was more polar than the green-black phaeophorbide band. Preparative t.l.c. (elution with 5% tetrahydrofuran in methylene chloride) gave a clear separation and the purple material possessed an electronic absorption spectrum (Figure 2) characteristic of an open-chain bile pigment or a macrocycle with interrupted conjugation. ^1H N.m.r. showed two high field methine protons, and the methyl group resonances were at higher field than those of the methyl phaeophorbides (1) attesting to the absence of a ring current.[12] Mass spectrometry confirmed that the purple pigment was a homologous mixture of 8′-acetylbilitrienes (5), derived presumably by photo-oxygenation at the δ-methyl-bearing methine



(5)

position. Similar photo-oxygenations of magnesium porphyrins [17] and zinc chlorins [18] have been reported,

* Holt and his co-workers [7] have reported band separations using distribution between hydrochloric acid and ether on Celite columns; however, this separation is not completely efficient.[16] All work described in the present paper was carried out on the intact mixture, the composition of which is shown in Figure 1.

[15] A. S. Holt, J. W. Purdie, and J. W. F. Wasley, *Canad. J. Chem.*, 1966, **44**, 88.

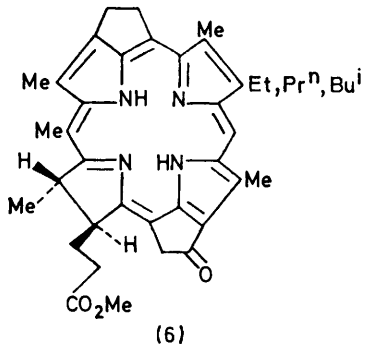
[16] R. A. Chapman, M. W. Roomi, T. C. Morton, D. T. Krajcarski, and S. F. MacDonald, *Canad. J. Chem.*, 1971, **49**, 3544.

[17] J.-H. Fuhrhop and D. Mauzerall, *Photochem. Photobiol.*, 1971, **13**, 453.

[18] J.-F. Fuhrhop, P. K. W. Wasser, J. Subramanian, and U. Schrader, *Annalen*, 1974, 1450.

and in the latter case both possible formylbilitrienes have been isolated. Moreover, we have synthesised [19] δ-methyl-*trans*-octaethylchlorin and have obtained both possible acetylbilitrienes.[20] However, in the case of the *Chlorobium*-660 methyl phaeophorbides only one isomer is obtained, namely that with the acetyl group attached to the reduced ring, and similar conclusions have been reached by Brockmann.[14] Oxygenation of methyl phaeophorbides in daylight during one week accomplishes transformation into the acetylbilitrienes (5) in 71% yield.

Hydroxyethylporphyrins can be transformed into vinylporphyrins by heating with toluene-*p*-sulphonic acid in benzene [21] or *o*-dichlorobenzene.[22] Treatment of the methyl phaeophorbides (1) for 1 h in refluxing benzene containing toluene-*p*-sulphonic acid gave a 77% yield of the 2-vinyl product (2). However, more prolonged heating in benzene and acid, followed by treatment with diazomethane to rectify some partial hydrolysis of the methyl ester, gave a different product in 79% yield. The electronic absorption spectrum showed a hypsochromic (11 nm) shift of the long wavelength peak from that of the 2-vinyl compounds (2), and the mass spectrum featured the same mass ions as in (2). ^{1}H N.m.r. spectroscopy, however, showed only one *meso*-proton and no vinylic hydrogens, but some extra badly defined signals in the τ 6—7 region were present. From this data it was apparent that cyclisation of the 2-vinyl group in (2) to the α-*meso*-position had occurred, and that the new products were the α-ethylenephaeophorbides (6). ^{13}C N.m.r. spectra also confirmed this structural assignment. It is notable that when methyl pyrophaeophorbide *a* (7) (obtained [23] by degradation of



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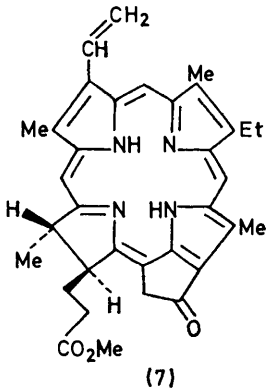


(7)

phaeophytins) was subjected to the same reaction conditions no similar vinyl cyclisation was apparent; this therefore establishes that the δ-methyl substituent is essential for the reaction. Several explanations for this novel cyclisation can be advanced, and these range from purely electronic to mainly steric effects.

For example, one could argue that the well established electron-donating capacity of the methyl group was raising the overall electron density in the macrocycle and that cyclisation with a primary carbonium ion obtained by protonation of the 2-vinyl (followed by loss of the proton originally on the α-carbon) would give (6); cyclisation of the more likely secondary carbonium ion would give a sterically impossible four-membered ring. In support of this type of electronic argument it has been noted [13] that change from $CDCl_3$ solvent to trifluoroacetic acid in the carbon n.m.r. spectra of chlorins causes a downfield shift of the vinyl CH_2 of *ca.* 8 p.p.m. compared with no shift for the vinyl CH peak. On the other hand, steric effects of the δ-methyl group would certainly be expected to distort the macrocycle so as to render the α-position susceptible to electrophilic attack. A final possibility is that protonation could occur at the δ-carbon, and this might be expected to relieve steric compression in that area of the molecule; a simple electrocyclic reaction, followed by deprotonation, would give the required product.

Both the photo-oxidative ring cleavage to give the acetylbilitrienes (5), and the acid catalysed vinyl cyclisation to give (6), are important reactions from the structural standpoint. The presence of a 8′-acetyl group in (5) further confirms that the *meso*-methyl group is in the δ-position in the chlorophylls. The fact that the vinyl group in the 2-position can be cyclised to give (6) adds yet more evidence that the *meso*-methyl group is not present at the α-position. Possibly more important, compounds (6) no longer possess the photochemically labile [24] vinyl substituent, and their ready accessibility makes them useful as substrates upon which to attempt band separations without fear of decomposition during handling. The mesophaeophorbides (3), which are equally useful in this context, are also readily available by hydrogenation of the methyl 2-vinylphaeophorbides (2) (96% yield).

Biosynthetic Studies.—Methionine. Though methionine has been shown [25] to enhance the growth of *Chlorobium* bacteria, and ethionine has been shown [26] to be an inhibitor, no conclusive proof of the involvement of methionine (as *S*-adenosylmethionine) in the biosynthesis of the *Chlorobium* chlorophylls using modern labelling techniques has been gathered. [$^{14}CH_3$]- and [$^{13}CH_3$]-L-methionine were prepared [27] from L-homocystine using sodium in liquid ammonia, and the appropriately labelled methyl iodide. [$^{14}CH_3$]-L-Methionine was administered to *C. ethylicum* using a variety of

[19] M. J. Bushell, B. Evans, G. W. Kenner, and K. M. Smith, *Heterocycles*, 1977, **7**, 67.
[20] M. J. Bushell, unpublished results.
[21] P. S. Clezy and J. Barrett, *Biochem. J.*, 1961, **78**, 798.
[22] G. W. Kenner, S. W. McCombie, and K. M. Smith, *Annalen*, 1973, 1329.
[23] G. W. Kenner, S. W. McCombie, and K. M. Smith, *J.C.S. Perkin I*, 1973, 2517.
[24] *E.g.*, J. Barrett, *Nature*, 1959, **183**, 1185; H. H. Inhoffen, H. Brockmann, jun., and K.-M. Bliesener, *Annalen*, 1969, **730**, 173.
[25] V. E. Uspenskaya and E. N. Kondrateva, *Doklady Akad. Nauk S.S.S.R.*, 1964, **157**, 678; T. N. Godnev, E. N. Kondrateva, and V. E. Uspenskaya, *Izv. Akad. Nauk S.S.S.R. (Ser. Biol)*, 1966, **31**, 525. See also ref. 26.
[26] W. R. Richards and H. Rapoport, *Biochemistry*, 1966, **5**, 1079; 1967, **6**, 3830.
[27] V. du Vigneaud, H. M. Dyer, and J. Harmon, *J. Biol. Chem.*, 1933, **101**, 719.

methods. Feeding to growing cultures, whether at the time of subculturing or batchwise during growth (and particularly during the exponential region of a regularly measured time–growth curve), gave reproducible incorporations into the farnesyl phaeophorbides (4) of *ca.* 9%. Incubations with concentrated cell suspensions to which lysozyme had been added (to disrupt the cell wall) also gave 9% incorporations, but disruption of the cell wall by freeze–thawing before feeding gave a decreased incorporation of only 3%. A blank experiment was performed involving administration of [$^{14}CH_3$]-L-methionine to a solution of phaeofarnesins, quinones, and carotenoids from *C. ethylicum*; an ' incorporation ' of 0.005% gave an indication of the level of cross contamination and helped to confirm the incorporations laid out above.

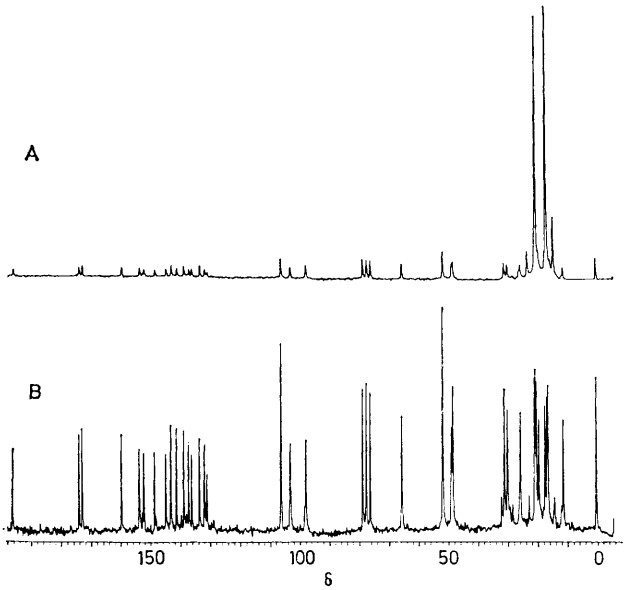


FIGURE 3 ^{13}C N.m.r. spectrum (Varian XL-100, proton decoupled) of the total mixture of methyl phaeophorbides (1) from *C. ethylicum*: A, obtained from feeding [$^{13}CH_3$]-L-methionine; B, at natural abundance

Encouraged by the high radiochemical incorporations, we decided to feed [$^{13}CH_3$]-L-methionine to growing cultures, this being the most straightforward method as well as the one giving the best incorporations. [$^{13}CH_3$]-L-Methionine (91% mol) was added to a growing culture (13 l), and the pigments were extracted. The ^{13}C n.m.r. spectrum of the derived methyl phaeophorbides (1) is shown in Figure 3A, and Figure 3B shows the spectrum of the phaeophorbides at natural abundance. Two greatly enhanced peaks are immediately apparent in Figure 3A; the resonance at 20.1 p.p.m. is attributed to the δ-methyl group, and that at 16.6 p.p.m. to the methyl of the 5-ethyl function in 660 bands 1—5 (Figure 1). The peaks are 17—19 times more intense than the natural abundance resonances (measured with respect to the line at 51.5 p.p.m.). A closer examination of the spectrum shows two other signals to be significantly enhanced, namely those at 14.4 and 23.1 p.p.m. These peaks could not be assigned to band 5 (Figure 1), but comparison (Figure 4) of a greatly intensified spectrum (Figure 4B) with that of fairly pure band 4 methyl phaeophorbide * (Figure 4A) enables the peak at 14.4 p.p.m. to be assigned to the terminal methyl of the 4-n-propyl group in 660 band 4 (Figure 1); good comparison with the terminal methyl in the ^{13}C n.m.r. spectrum (14.0 p.p.m.) of n-propylbenzene is also obvious. The signals at 23.1 p.p.m. could also be assigned to the terminal methyl groups of an isobutyl function (*cf.* isobutylbenzene, 22.4 p.p.m., though a better model is required [28]). Therefore, the enhancements at 14.4 and 23.1 p.p.m. are due to incorporation of methyl units from methionine into the terminal positions of n-propyl and isobutyl groups present in bands 1—4 (Figure 1) of the total phaeophorbide mixture. No evidence for the existence of *meso*-ethyl groups (which, if produced from two labelled methyl groups would show large ^{13}C–^{13}C couplings) in the complete mixture was provided from these results. Thus, this experiment clearly establishes the origin of the δ-methyl group and the terminal methyl groups in the 4-n-propyl, 4-isobutyl, and 5-ethyl functions of the *Chlorobium* chlorophylls-660 (ex. *C. ethylicum*) as L-methionine.

We have proposed [2] a route (Scheme 1) by which methyls can be incorporated into the 660 chlorophylls to give n-propyl or isobutyl functions. It is possible, by analogy with the known [29] formation of the 2- and 4-vinyls in protoporphyrin-IX from the 2- and 4-propionates in coproporphyrinogen-III, that unsaturated groups (Scheme 1) are precursors of the propyl and butyl functions. The same is true for the 5-ethyl group, and a pathway (assuming [30] hydroxylation followed by decarb-

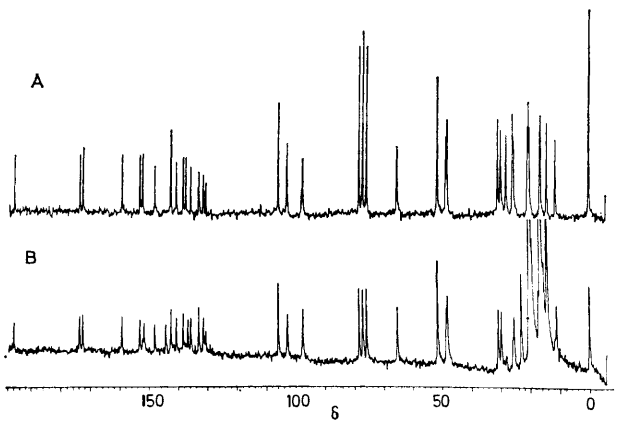


FIGURE 4 ^{13}C N.m.r. spectrum (Varian XL-100, proton decoupled) of the *Chlorobium* chlorophyll methyl phaeophorbides: A, 660 series, band 4 (see Figure 1) from *Chlorobium thiosulphatophilum*; B, the total mixture from *C. ethylicum* after feeding of [$^{13}CH_3$]-L-methionine (note that this spectrum is the same as shown in Figure 3A, but plotted at much higher intensity)

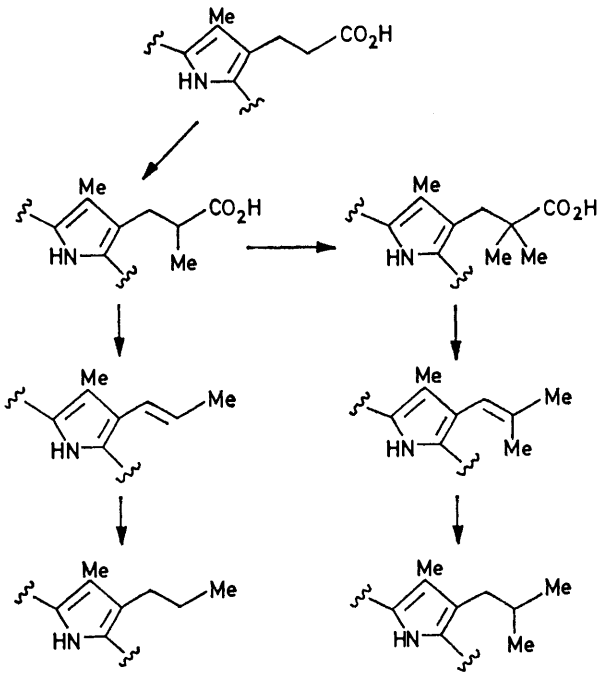
* Material kindly supplied by Drs. A. S. Holt and J. W. F. Wasley.

[28] J. Rimmer, work in progress.

[29] *E.g.* J. A. S. Cavaleiro, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1974, 1188 and references therein.

[30] S. Sano, *J. Biol. Chem.*, 1966, **241**, 5276.

oxylation and dehydration) is outlined in Scheme 2. In order to investigate the possibility presented in Scheme 2, we prepared [$^{13}C^2H_3$]-L-methionine from



SCHEME 1

$^{13}C^2H_3I$ as described earlier, and fed this to a growing medium. The pigments produced were transformed into the methyl phaeophorbides and their ^{13}C n.m.r. spectrum was measured, using deuterium decoupling.* Figure 5

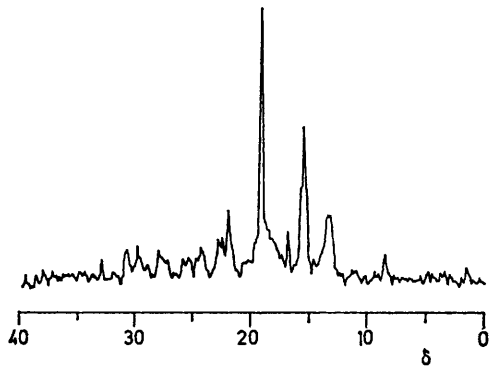
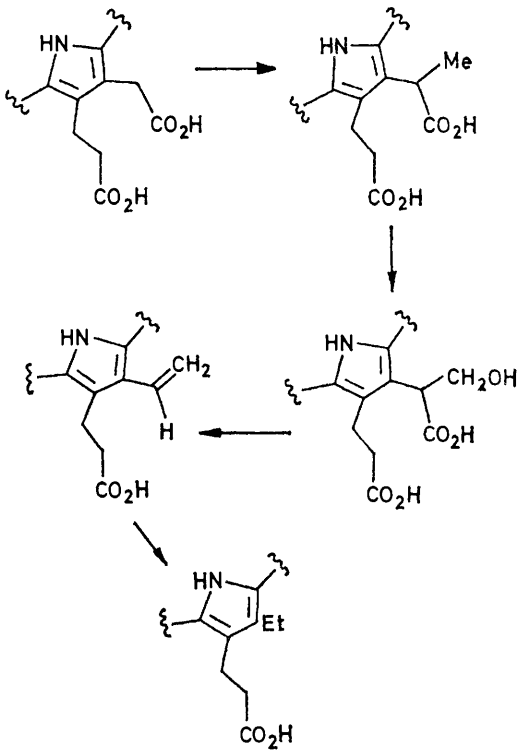


FIGURE 5 ^{13}C N.m.r. spectrum (Varian XL-100, deuterium decoupled) of the total mixture of methyl phaeophaeophorbides (1) from *C. ethylicum* after feeding with [$^{13}C^2H_3$]-L-methionine. Only the 0—40 p.p.m. range is shown, and the spectrum was measured with an external ^1H lock; the spectrum was referenced to ' imaginary ' tetramethylsilane by assigning the $CDCl_3$ line a value of δ 76.9

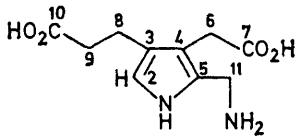
shows the spectrum (0—40 p.p.m. region only) obtained; the peak at 19.29 p.p.m. is assigned to the δ-methyl group, incorporated as expected, intact. The triplet at 15.63 p.p.m. is also immediately assigned to a $^{13}C^2H_3$ function attached to a CH_2; the coupling (J 4.8 Hz) is as expected [31] for a carbon-13 nucleus coupling with protons attached to an adjacent carbon atom (*i.e.* $^{13}C^2H_3$–$^{12}CH_2$). Thus, both methyl groups are incorporated intact and the proposition advanced in Scheme 2 is excluded in favour of the general type of mechanism



SCHEME 2

involved in the transformation of uroporphyrinogen-III into coproporphyrinogen-III.[32] The additional peaks in Figure 5 at 22.07 and 13.54 p.p.m. can also be assigned to the terminal methyl groups in the isobutyl and n-propyl side-chains in bands 1—4 (Figure 1).

Porphobilinogen (PBG) (7). It was recognised that feeding to growing cultures would be unsatisfactory for substrates such as porphyrinogens. They would require



(7)

a more active cell preparation. [6-^{14}C]PBG was synthesised [1] and used [2] to develop such a versatile system.

* We thank Professor E. A. V. Ebsworth and Dr. A. S. Boyd for this spectrum.

[31] G. C. Levy and G. L. Nelson, ' ^{13}C Nuclear Magnetic Resonance for Organic Chemists,' Wiley, New York, 1972; J. B. Stothers, ' ^{13}C Nuclear Magnetic Resonance Spectroscopy,' Academic Press, New York, 1972; H. Scheer and J. J. Katz, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 490.

[32] A. R. Battersby and E. McDonald, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 61.

Administration of [6-^{14}C]PBG to growing cultures and cell suspensions in much the same way as described above for methionine gave only very small incorporations into the *Chlorobium* chlorophylls-660. Feeding to sonicated cell suspensions, however, proved more successful, the derived methyl phaeophorbides (1) showing incorporations of between 3 and 7%. Experiments using [6-^{13}C]PBG, designed to confirm these results, are in hand.

EXPERIMENTAL

Electronic absorption spectra (solutions in methylene chloride or ether) were measured using a Unicam SP 800 spectrophotometer. Since the materials used were a mixture of at least six homologous fractions, combustion analyses on the products have not been obtained. For the same reason, accurate extinction coefficients are not quoted; however, relative intensities are given. ^{1}H N.m.r. spectra were determined (usually in $CDCl_3$ solution with tetramethylsilane as internal standard) with a Varian HA-100 instrument, and ^{13}C n.m.r. spectra (conditions as previously described [13]) with a Varian XL-100 spectrometer. Mass spectra were measured using an A.E.I. MS 12 instrument (direct insertion probe; 70 eV, 50 μA, and source temperatures *ca.* 200°). The activities of radioactive samples were determined with a Packard (model 3003) Tri-Carb liquid scintillation counter. To maximise counting efficiency, coloured samples were bleached before counting. This was done by dissolving in tetrahydrofuran (*ca.* 3 ml), adding benzoyl peroxide (*ca.* 1 mg), and allowing the solution to stand in sunlight until colourless. Then, an organic scintillator solution (*ca.* 7 ml of 0.6% butyl-PBD in toluene) was added and the sample counted. The efficiency of counting was determined using [^{14}C]-n-hexadecane as internal standard. Background counts were obtained over long periods prior to counting radioactive samples. Incorporations are calculated as (counts recovered × 100)/(counts fed).

Growth of Chloropseudomonas ethylicum.—The strain was obtained from the Botany Department, King's College, London. The medium used consisted of four solutions A—D in distilled water which were autoclaved at 15 lb in^{-2} for 15 min before use. Solution A contained potassium dihydrogen phosphate (1.5 g), ammonium chloride (1.5 g), magnesium chloride hexahydrate (7.5 g), sodium chloride (30 g), calcium chloride (68 mg), iron(III) chloride hexahydrate (3.6 mg), boric acid (0.84 mg), zinc sulphate hexahydrate (0.66 mg), cobalt(II) nitrate hexahydrate (0.38 mg), copper(II) sulphate pentahydrate (0.03 mg), and manganese(II) sulphate (0.03 mg) in water (1 500 ml). Solution B contained iron(II) sulphate heptahydrate (3.8 mg) in water (7.5 ml). Solution C contained sodium sulphide nonahydrate (0.15 g) in water (7.5 ml). Solution D contained sodium hydrogencarbonate (2 g) in water (75 ml). Solutions B—D were added under sterile conditions to solution A. The pH was adjusted to 7.3 if necessary using sterile dilute phosphoric acid. The resulting medium was inoculated with well grown *C. ethylicum* (10—20 ml) and grown under 30 W fluorescent lamps at 30°. The cells were harvested after 7 days or else subcultured into fresh medium after 10 days to maintain the culture.

Isolation of Chlorobium *Chlorophylls*-660.—Seven day old culture (4.5 l) was filtered through a Celite bed (4 cm deep, 40 cm diameter; 3 parts Celite 545 to 1 part 505). After drying the Celite bed under vacuum for 1 h it was stirred with acetone (2 l). The resulting suspension was filtered and the Celite re-extracted with acetone (3 × 2 l). The green filtrates were combined, methylene chloride was added, and the mixture was washed with saturated aqueous sodium chloride. The organic layer was dried (Na_2SO_4) and evaporated to dryness to give chlorophylls contaminated with carotenoids, quinones, *etc.*, $\lambda_{max.}$ (ether I, 660; II, 625; III, 431; IV, 411 nm (III > I > IV > II), together with absorptions at 462 and 493 nm due to carotenoids, *etc.* A very concentrated solution of crude chlorophyll showed an additional $\lambda_{max.}$ (CH_2Cl_2) at 765 nm. Mass spectra measured on this material were uninterpretable.

Farnesyl 5-Ethyl-2-(1-hydroxyethyl)-5-demethyl-δ-methyl-2-devinylpyrophaeophorbide a *and Homologues* (*Farnesyl Phaeophorbides or Phaeofarnesins from* C. ethylicum) (4).—A solution of crude *Chlorobium* chlorophyll-660 from *C. ethylicum* (20 l of medium) in methylene chloride (200 ml) was shaken vigorously with 1% v/v concentrated HCl in water (200 ml). An immediate colour change from green to brown was observed. The organic layer was washed with water (2 × 100 ml), dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on neutral alumina (grade V); elution was first with petroleum (b.p. 60—80°) to remove carotenoids, quinones, *etc.*, and then with methylene chloride–petroleum (1 : 1) to remove the black phaeofarnesin band. The eluates were then evaporated to dryness, τ (0.1M in $CDCl_3$) 0.20 and 0.58 (2 *meso*-H), 3.70 (C*H*OH), 4.86 (2 H, s, CH_2CO), and 6.25, 6.64, and 6.82 (3 × Me); other resonances were unresolved; $\lambda_{max.}$ (CH_2Cl_2) I, 669; II, 614; III, 557; IV, 525; V, 492; and VI, 424 nm, (VI > I > III > IV > II > V); *m/e*, 812 (M^+, 6%), 798 (M^+, 28), 796 (7), 794 (5), 784 (M^+, 100), 782 (26), 780 (23), 768 (74), 766 (74), 754 (6), 752 (5), and a corresponding set of peaks from *m/e* 608 to 548.

Methyl 5-Ethyl-2-(1-hydroxyethyl)-5-demethyl-δ-methyl-2-devinylpyrophaeophorbide a *and Homologues* (*Methyl Phaeophorbides from* C. ethylicum) (1).—The foregoing phaeofarnesins were left overnight at 25° in 5% v/v concentrated sulphuric acid in methanol (100 ml). Methylene chloride (100 ml) and water (100 ml) were added and the organic layer was washed with saturated aqueous sodium hydrogencarbonate, then with water, dried (Na_2SO_4), and finally evaporated to dryness to give a black oil. Chromatography on alumina (Fluka, neutral, Brockmann Grade II) (elution with methylene chloride) gave two fractions. The first was a minor product, the dehydrated phaeophorbides (2). The second band was collected and rechromatographed on six 20 cm × 20 cm × 1.5 mm preparative t.l.c. plates (silica G), developing with 5% tetrahydrofuran in methylene chloride. Two bands were obtained; the black methyl phaeophorbides (1) (R_F 0.4) were removed from the silica gel by stirring in 5% methanol in methylene chloride, and after evaporation of the solvent, they were crystallised by scratching under hexane to give a black powder (180 mg), τ (0.1M) 0.19 (β-*meso*-H), 0.56 (α-*meso*-H), 3.69 (q, C*H*OH), 4.88 (s, CH_2CO), 5.54 and 6.03 (m, 7- and 8-H), 6.03 (q, 5-CH_2), 6.25 (δ-Me), 6.46 (OMe), 6.54 and 6.81 (1- and 3-Me), 6.37 (q, 4-CH_2), 7.5—8.2 (m, CH_2CH_2), 8.00 and 8.33 (t, 4- and 5-Me), and 8.64 (d, 8-Me); $\lambda_{max.}$ (CH_2Cl_2) I, 669; II, 614; III, 557; IV, 525; V, 492; and VI, 424 nm (VI > I > III > IV > II > V); *m/e* 622 (M^+, 1%), 608 (M^+, 6), 606 (7), 604 (6), 594 (M^+, 15), 592 (31), 590 (30), 580 (38), 578 (100), 576 (88), 564 (19), 562, (21), and a corresponding set of peaks from *m/e* 535 to 475.

The second product (R_F 0.1) was removed from the silica

gel as above. Filtration and evaporation gave a purple solid (20 mg) identified as 1,3,8-*trimethyl*-4,5-*diethyl*-2-(1-*hydroxyethyl*)-6-c-(1-*oxoethylene*)-7,8-*dihydro*-7-(2-*methoxycarbonylethyl*)-8′-*acetylbilitriene* and homologues (5), τ (0.05M) 3.16 and 3.58 (methine Hs), 4.9—5.0 (m, C*H*OH), 6.34 (OMe), 6.46 (10-CH_2), 7.2—8.1 (8 H, m, CH_2CH_2 and 4- and 5-CH_2), 7.82, 7.93, and 8.14 (1- and 3-Me and CH_3-CO), 8.41 (d, 8-Me), and 8.75 (6 H, t, 4- and 5-Me); $\lambda_{max.}$ (ether) I, 590; II, 561; III, 364; IV, 314 nm (IV > III > II > I); *m/e* 654 (M^+, 4%), 640 (M^+, 19), 626 (M^+, 100), 612 (M^+, 15), 611 (8), 597 (23), 583 (69), 569 (11), 539 (9), 497 (4), 419 (4), 368 (7), 356 (4), 325 (9), 313 (8), and 260 (17), *m** 556.9, 543.0, and 529.0.

The 8′-acetylbilitrienes were prepared more efficiently as follows. A solution of methyl phaeophorbides (1) (80 mg) in methylene chloride (200 ml) was purged with oxygen for 1 week. The solution was evaporated to dryness and the residue was chromatographed on four 20 × 20 cm × 1.5 mm preparative t.l.c. plates of Kieselgel G (elution with 5% tetrahydrofuran in methylene chloride). A small amount of starting material soon separated from the required product, and after removal from the silica and standing in hexane, the purple band afforded a purple solid (60 mg, 71%), identical in all respects with the material (5) described above.

Methyl 5-*Ethyl*-5-*demethyl*-δ-*methylpyrophaeophorbide* a *and Homologues* (*Methyl* 2-*Vinylphaeophorbides from* C. ethylicum) (2).—Methyl phaeophorbides (1) (180 mg) in benzene (150 ml) containing toluene-*p*-sulphonic acid hydrate (1 g) were refluxed under nitrogen for 1 h. Chloroform (300 ml) was added and the solution was washed with saturated aqueous sodium hydrogencarbonate and water. The organic layer was dried (Na_2SO_4) and evaporated to dryness. Analytical t.l.c. showed that partial hydrolysis of the methyl esters had occurred, so the product was treated with ethereal diazomethane. The resulting solution was evaporated and the residue chromatographed on alumina (Grade II) (elution with methylene chloride). Evaporation of the eluates and scratching of the residue under hexane gave a black powder (140 mg, 77%), τ (0.1M) 0.55 (β-*meso*-H), 0.63 (α-*meso*-H), 2.1—2.4 and 2.8—3.1 (m, $CH{=}CH_2$), 4.80 (10-CH_2), 5.50 and 5.95 (m, 7- and 8-H), 5.95 (q, 5-CH_2), 6.21 (δ-Me), 6.41 (q, 4-CH_2), 6.46 (OMe), 6.46 and 6.86 (1- and 3-Me), 7.4—8.2 (m, CH_2CH_2), 8.06 and 8.35 (t, 4- and 5-Me), and 8.55 (d, 8-Me), $\lambda_{max.}$ I, 671; II, 615; III, 553; IV, 522; V, 491; and VI, 420 nm. (VI > I > III > IV > II > V); *m/e* 604 (M^+, 6%), 590 (M^+, 22), 576 (M^+, 100), and 562 (M^+, 10).

Methyl 2-α-*Ethylene*-5-*ethyl*-5-*demethyl*-δ-*methyl*-2-*devinylpyrophaeophorbide* a *and Homologues* (*Methyl* 2-α-*Ethylenephaeophorbides from* C. ethylicum) (6).—A solution of methyl phaeophorbides (1) (100 mg) in benzene (100 ml) containing toluene-*p*-sulphonic acid (1 g) was refluxed overnight under nitrogen. The product (80 mg, 79%) was isolated as for the methyl 2-vinylphaeophorbides (2), τ (0.1M) 0.76 (β-*meso*-H), 4.82 (10-CH_2), 5.5—5.7 and 5.8—6.0 (m, 7- and 8-H), 6.0—7.3 (8 H, m, 2-, 4-, 5-, and α-CH_2), 7.5—8.2 (CH_2CH_2), 6.36 (δ-Me), 6.46 (OMe), 7.07 and 7.57 (1- and 3-Me), 8.08 and 8.49 (t, 4- and 5-Me) and 8.49 (d, 8-Me); $\lambda_{max.}$ (CH_2Cl_2) I, 660; II, 608; III, 563; IV, 529; V, 420 nm (V > I > III > IV > II); *m/e* 604 (M^+, 3%), 590 (M^+, 20), 576 (M^+, 100), and 562 (M^+, 18).

Methyl 5-*Ethyl*-5-*demethyl*-δ-*methylmesopyrophaeophorbide* a *and Homologues* (*Methyl Mesophaeophorbides from* C. ethylicum) (3).—Methyl 2-vinylphaeophorbides (2) (140 mg) in acetone (25 ml) were hydrogenated during 1 h at 25° and 1 atm over 10% palladised charcoal (50 mg). Filtration through Celite and evaporation gave a black residue which soon solidified in hexane to afford a black powder (135 mg, 96%), τ (0.1M) 0.55 (β-*meso*-H), 0.75 (α-*meso*-H), 4.82 (10-CH_2), 5.5—5.7 and 5.9—6.1 (m, 7- and 8-H), 5.95 (q, 5-CH_2), 6.39 (4 H, q, 2- and 4-CH_2), 6.24 (δ-Me), 6.49 (OMe), 6.71 and 6.81 (9 H, t, 2-, 4-, and 5-Me), and 8.58 (d, 8-Me); $\lambda_{max.}$ (CH_2Cl_2) I, 667; II, 613; III, 557; IV, 525; V, 492; and VI, 419 nm (VI > I > III > IV > II > V); *m/e*, 606 (M^+, 4%), 592 (M^+, 20), 578 (M^+, 100), and 564 (M^+, 6).

[$^{13}CH_3$]-L-*Methionine*.—Liquid ammonia (30 ml) was distilled into L-homocystine (750 mg, 1 equiv.) while stirring and cooling in a dry-ice–acetone bath. Nitrogen was passed through the solution and pieces of sodium were added until there was a permanent blue colouration. [^{13}C]H_3]Methyl iodide (91% g atom; 1.0 g, 1.2 equiv.) was added. After 20 min the ammonia was allowed to evaporate and water (20 ml) was added. The solution was filtered and brought to pH 6.0 with hydriodic acid. Boiling ethanol was added and the solution was cooled to 0°. Platelets (550 mg, 64%) were collected and the material was shown to be identical with authentic unlabelled L-methionine by t.l.c. An amino-acid analysis of the labelled material showed L-methionine to be the only amino-acid present, τ (D_2O; unlabelled methionine) 5.69 (t, CH), 6.8—7.4 (m, CH_2CH_2), and 7.40 (s, Me); τ (D_2O; ^{13}C labelled methionine) 5.7 (m, CH), 6.9—7.5 (m, CH_2CH_2), and 7.50 (d, $^{13}CH_3$, *J* 140 Hz) (shifts appeared to be concentration dependent).

A sample of [$^{14}CH_3$]-L-methionine was prepared in a similar fashion using L-homocystine and $^{14}CH_3I$.

Feeding Experiments.—[$^{14}CH_3$]-L-*Methionine*. (i) Incubation in the presence of lysozyme. *C. ethylicum* (1.5 l; grown for 70 h after subculturing) was centrifuged at 10 000 r.p.m. for 40 min at 0°. The centrifugate was resuspended in 0.1M-potassium phosphate buffer (20 ml; pH 7.4) and to this was added magnesium chloride hexahydrate (24 mg), adenosine triphosphoric acid disodium salt (ATP) (64 mg), lysozyme (70 mg), and a known quantity (*ca.* 200 mg) of aqueous [$^{14}CH_3$]-L-methionine solution (specific activity 4 mCi mol^{-1}). The resulting suspension was incubated during 12 h under normal growth conditions, then stirred for 15 min with methanol (200 ml). Methylene chloride (250 ml) was added and the organic layer was washed with saturated aqueous sodium chloride and 1% v/v concentrated HCl in water (500 ml), then dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on alumina (Grade V) (elution with petroleum first and then with 1 : 1 petroleum–methylene chloride). The dark eluates were evaporated and rechromatographed on two 20 × 20 cm × 1.5 mm preparative silica t.l.c. plates [elution with 30% acetone in petroleum (b.p. 60—80°)]. Two green-black bands were observed, the methyl and farnesyl phaeophorbides. A radiochemical scan of the plates showed activity in both bands. The actual amounts were estimated from spectrophotometry of the Soret bands and red absorption maxima. This indicated that the two compounds had approximately the same specific activity, and therefore that methionine had been incorporated into the chlorin nucleus, and not just into the farnesyl group. The methyl and farnesyl phaeophorbides were removed from the silica gel by stirring in 5% methanol in methylene chloride. Both samples were then dissolved in tetrahydrofuran (2 ml),

bleached in the presence of benzoyl peroxide, and counted: total activity fed in the form of [$^{14}CH_3$]-L-methionine 3.5 μCi, total activity recovered in the form of methyl and farnesyl phaeophorbides 0.32 μCi, incorporation 9.2%.

(ii) Incubation with growing cultures. Two batches [(a) and (b)] of *C. ethylicum* (1.5 l) were subcultured. To (a) was added, just prior to subculturing, a solution (*ca.* 200 mg) of [$^{14}CH_3$]-L-methionine in water (specific activity 4 mCi mol^{-1}) diluted with inactive L-methionine (5 mg). To (b) was added a similar solution in four equal batches, at the time of subculturing, and 24, 48, and 72 h later. (The total activity administered in each feeding was approximately equal.) The cultures were harvested after 7 days growth by filtration through Celite and extraction with methanol. The methyl and farnesyl phaeophorbides were bleached and counted as before; (a) total activity fed 4.9 μCi, total activity recovered in pigments 0.46 μCi, incorporation 9.5%; (b) total activity fed 4.6 μCi, total activity recovered 0.43 μCi, incorporation 9.2%.

(iii) Incubation with a freeze–thawed cell extract. The procedure was as described for the lysozyme incubation except that prior to addition of cofactors, the buffered cell suspension was frozen at −30° and then allowed to thaw at 25°, and the lysozyme was omitted: total activity fed 1.1 μCi, total activity recovered in pigments 0.032 μCi, incorporation 3.0%.

(iv) Blank experiment. To a solution of farnesyl phaeophorbides, quinones, carotenoids, *etc.*, obtained from *C. ethylicum* culture (1.5 l) was added [$^{14}CH_3$]-L-methionine in aqueous solution (*ca.* 200 mg; specific activity 4 mCi mol^{-1}). Methanol (200 ml) was added and the pigments were isolated as for the lysozyme incubation: total activity 'fed' 3 μCi, total activity recovered in pigments 1.6×10^{-4} μCi, 'incorporation' 0.005%.

[$^{13}CH_3$]-L-*Methionine*. Four batches of *C. ethylicum* (3.25 l each) were subcultured. Just prior to subculturing, [$^{13}CH_3$]-L-methionine (50 mg) was dissolved in distilled water and added to each batch. The cultures were harvested after 9 days growth and the *Chlorobium* chlorophyll-660 was extracted and degraded to the methyl phaeophorbides (120 mg) and 8′-acetylbilitrienes (10 mg), as described earlier. The ^{13}C n.m.r. spectrum of the methyl phaeophorbides is shown in Figures 2 and 3.

[6-^{14}C]*Porphobilinogen*. (i) Incubation with growing culture. To *C. ethylicum* culture (400 ml) (48 h after subculturing) was added [6-^{14}C]PBG (10 mg, specific activity 9.8 mCi mol^{-1}). The culture was harvested after 5 days growth and the pigment was isolated as described earlier to obtain the methyl and farnesyl phaeophorbides which were bleached and counted: total activity fed 0.46 μCi, total activity recovered in pigments 1.1×10^{-4} μCi, incorporation 0.02%.

(ii) Incubation in the presence of lysozyme. The procedure was the same as for the incubation of [$^{14}CH_3$]-L-methionine except that (a) 400 ml of culture was centrifuged, and (b) to the buffered cell suspension (5 ml) was added magnesium chloride hexahydrate (6 mg), ATP (16.5 mg), lysozyme (30 mg), [6-^{14}C]PBG (500 mg, specific activity 0.16 mCi mol^{-1}) in ammonium acetate solution, and the reduced form of glutathione (5 mg): total activity fed 0.32 μCi, total activity recovered in pigments 2.24×10^{-4} μCi, incorporation 0.07%.

(iii) Incubation with a sonicated cell extract. *C. ethylicum* culture (250 ml) grown for 48 h after subculturing was centrifuged at 10 000 r.p.m. for 40 min at 0°. The centrifugate was resuspended in 1M-Tris buffer (8 ml; pH 7.3) in a sonication tube. ATP (16 mg), magnesium chloride hexahydrate (6 mg), and L-methionine (25 mg) were added and the suspension was sonicated (8 microns peak to peak) for 30 s while cooling in an ice–salt bath (in 5 s bursts of sonication with 15 s intervals). A known amount of [6-^{14}C]PBG (*ca.* 2 mg; specific activity 7.5 mCi mol^{-1}) dissolved in ammonium acetate solution (0.5 ml) was added immediately after sonication and the cell suspension was incubated for 18 h under normal growth conditions. This was then shaken with acetone (100 ml), diluted with methylene chloride (100 ml), and washed with water (3×100 ml) and saturated aqueous sodium chloride (2×250 ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness to give a residue which was transesterified to the methyl phaeophorbides (1) as previously described. Chromatography (one 20×20 cm $\times$ 1.5 mm silica plate; elution with 5% tetrahydrofuran in methylene chloride) gave a clean phaeophorbide band which was extracted from the silica gel, evaporated to dryness, and the residue was dissolved in tetrahydrofuran (3 ml), bleached, and then counted. The results from four separate experiments are as follows:

	1	2	3	4
Total activity fed (μCi)	0.07	0.059	0.076	0.07
Total activity in recovered methyl phaeophorbides (μCi)	2.66×10^{-4}	2.4×10^{-3}	2.4×10^{-3}	0.49×10^{-2}
Incorporation (%)	0.4	4.0	3.1	7.1

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