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Studies on the biosynthesis of the *Chlorobium* chlorophylls

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The *Chlorobium* chlorophylls (660) from *Chloropseudomonas ethylicum* are shown by ^{13}C n.m.r. spectroscopy and certain chemical transformations to be *meso*-methylated at the δ-position. Earlier work, which proposed that the *meso*-alkyl group was present at the α or β positions, is shown to be experimentally correct, but incorrectly interpreted. On the basis of ^{14}C and ^{13}C feeding experiments, the novel methyl groups in the (660) chlorophylls are shown to be derived from methionine in all cases. For most of the homologous mixture of (660) chlorophylls, the branch point from the biosynthetic pathway to chlorophyll *a* appears to lie between uroporphyrinogen III and coproporphyrinogen III; earlier workers had suggested that *Chlorobium* chlorophyll biosynthesis proceeded through magnesium protoporphyrin IX mono-methyl ester and possibly also via bacteriochlorophyll *a* or one of its immediate precursors. Evidence against this proposal, and a working hypothesis explaining feeding results, is presented. On the basis of this hypothesis, proposals for the structures of certain fractions of the (660) chlorophylls which are currently in dispute, are presented.

Introduction

Of the ten or so species of the family of green photosynthetic bacteria, Chlorobacteriaceae, the photosynthetic pigments of only the three best known, *Chlorobium thiosulphatophilum*, *Chlorobium limicola* and *Chloropseudomonas ethylicum*, have been investigated (Jensen, Aasmundrud & Eimhjellen 1964). The chlorophylls isolated in great profusion from these sulphur bacteria are called *Chlorobium* chlorophylls, and are subdesignated according to the wavelength (in nm) of their red electronic absorption band measured in ether solution; the two series are therefore termed (650) and (660). Several strains of *C. thiosulphatophilum* and *C. limicola* have been isolated, and with the exception of only one strain of *thiosulphatophilum*, all produce *Chlorobium* chlorophyll (660) (Stanier & Smith 1960). The only known bacterium which produces *Chlorobium* chlorophyll (650) is *C. thiosulphatophilum*, strain L. Doubt has recently been cast upon the identity of *Chloropseudomonas ethylicum*, cultures of which have been shown (Shaposhnikov, Kondrat'eva & Federov 1960) to consist of *C. limicola* together with an unknown colourless non-photosynthetic bacterium. In addition to *Chlorobium* chlorophylls these green photosynthetic bacteria produce small quantitites of bacteriochlorophyll *a* (Jensen *et al.* 1964); *Chlorobium* chlorophylls (650) and (660) are now termed bacteriochlorophylls *d* and *c* respectively.

Structural studies

After extensive degradative work (Holt & Morley 1960; Morley & Holt 1961; Holt & Hughes 1961; Holt, Hughes, Kende & Purdie 1962, 1963) the phaeophorbides (obtained by demetallation and hydrolysis) of *Chlorobium* chlorophylls (650) and (660) were shown to be homologues of 2-devinyl-2-(1-hydroxyethyl)pyrophaeophorbide *a*, the (660) series differing from the (650)

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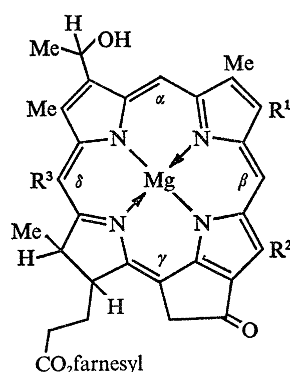
compounds in having a *meso* alkyl substituent. The esterifying alcohol of both series was shown (Holt *et al.* 1962) to be all-*trans* farnesol, and this has been confirmed (Katz *et al.* 1972) for the chlorophylls from *Chloropseudomonas ethylicum* by a detailed mass spectrometric analysis.

Distribution between hydrochloric acid and ether on Celite columns was used by Holt *et al.* to separate both the (650) and (660) chlorophylls from strains of *C. thiosulphatophilum*, each into six components or bands (Holt *et al.* 1962; Hughes & Holt 1962). After classical oxidative degradation to maleimides and *trans*-dihydrohaematinic acid, and to pyrro- and phyllo-porphyrins, as well as ^1H n.m.r. studies of the phaeophorbides, the chlorophylls were assigned the structures shown in table 1*a*, *b* (Purdie & Holt 1965; Holt 1965).

Table 1. Structural assignments for *Chlorobium* chlorophylls

((*a*) (650) Series (Purdie & Holt 1965; Holt 1965); (*b*) (660) Series (Purdie & Holt 1965; Holt 1965); (*c*) Revised (660) assignments (Holt, Purdie & Wasley 1966).)



band	(650) series R¹	(650) series R²	(660) series R¹	R²	R³	R¹	R²	R³
1	i-Bu	Et	i-Bu	Et	Me	i-Bu	Et	Et
2	n-Pr	Et	i-Bu	Et	Et	i-Bu	Et	Me
3	i-Bu	Me	n-Pr	Et	Me	n-Pr	Et	Et
4	Et	Et	n-Pr	Et	Et	n-Pr	Et	Me
5	n-Pr	Me	Et	Et	Me	Et	Et	Me
6	Et	Me	Et	Me	Me	Et	Me	Me
	(*a*)		(*b*)			(*c*)		

Abbreviations: Me = CH_3; Et = CH_2CH_3; n-Pr = $CH_2CH_2CH_3$; i-Bu = $CH_2CH(CH_3)_2$

Our group in Liverpool first encountered the *Chlorobium* chlorophylls in 1965 when Dr Holt kindly provided samples of degradation products from all the *Chlorobium* chlorophyll (650) fractions and from (660) fractions 2, 4, 5 and 6, in order that we might extend our investigations of the mass spectra of porphyrins and chlorins (Jackson *et al.* 1965) while at the same time confirming the proposed structures for these novel pigments.

Merely by consideration of molecular and prominent fragmentation ions we were able to add confirmation (Smith 1967) to the structural proposals for all of the (650) chlorophylls and for (660) bands 5 and 6. However, (660) bands 2 and 4 possessed molecular ions which were 14 mass units too low for them to be identifiable with the proposed structures (table 1*b*). This information caused Holt, Purdie & Wasley (1966) to interchange their proposals for fractions 1 and 2,

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and for fractions 3 and 4, giving the structures shown in table 1*c*. This outcome was somewhat unsatisfactory because it assigned *meso*-ethyl groups, of which we had always been suspicious for a variety of reasons, to fractions for which samples were either scarce or non-existent.

As a result of painstaking synthetic work (Archibald *et al*. 1966) the structural assignments of all of the (650) chlorophylls, and of (660) fractions 5 and 6 are beyond question; yet more confirmation has recently been provided (Chapman *et al*. 1971) by 'reductive C-alkylation'. However, the structures for (660) bands 1 and 3 (table 1*c*) had been assigned by default and there was no firm evidence for the *meso*-ethyl substituent in these bands. We therefore synthesized (Cox, Jackson & Kenner 1971) the phylloporphyrins derived from the structures proposed for (660) bands 3 and 4 (table 1*c*). The two synthetic porphyrins were fully characterized but attempted identification with (660) degradation products was not entirely satisfactory. Some grounds were obtained for feeling that the synthetic *meso*-methyl porphyrin was identical with one of the (660) phylloporphyrins, but the *meso*-ethyl porphyrin could not be identified with any degradation product from the (660) series; the conclusion was drawn (Cox *et al*. 1971) that the structural proposals including *meso*-ethyl substituents in the *Chlorobium* chlorophylls (660) must be discarded. Alternative suggestions for the identities of (660) fractions 1 and 3 are made later.

Our initial source of *Chlorobium* chlorophyll (660) was *C. thiosulphatophilum* (NCIB cat. no. 8346). It is an obligate anaerobe and growth under a layer of liquid paraffin was required. Since it has been shown (S. F. MacDonald 1972, personal communication) that fractions separated using Holt's hydrochloric acid–ether partition on Celite are contaminated with neighbouring homologous bands, we have investigated other means for separation of the mixture of (660) pigments. Thick layer chromatography on silicagel offered no separation, and neither did preliminary investigations with high-pressure liquid chromatography, though we have successfully separated mixtures of porphyrin type-isomers using this method (Cavaleiro, Kenner & Smith 1974*b*). Thus, all of our work to date has employed the intact (660) mixture, which contains *ca*. 80% of band 5 (table 1*c*) with much smaller amounts of the other homologues. We can operate quite satisfactorily in our preliminary investigations with the mixture, but in the long term we shall have to tackle the major problem of separation, probably by high pressure liquid chromatography. To this end we are at present building up gram quantities of the (660) phaeophorbide mixture.

Chloropseudomonas ethylicum produces the (660) chlorophylls much more prolifically than *C. thiosulphatophilum* and is also a hardier anaerobe. We soon changed to *ethylicum*, which grows healthily without the need to have a layer of liquid paraffin, which contaminates the pigments and causes difficulties in crystallization.

The positioning of the *meso*-alkyl group in the (660) chlorophylls has been a topic of debate, and possibly more so than the actual nature of the alkyl group itself. Synthetic (Archibald *et al*. 1966; Cox *et al*. 1971) and degradative evidence (Chapman *et al*. 1971) now clearly place the *meso*-methyl group in the δ-position, but an alternative structure was proposed by Mathewson, Richards & Rapoport (1963). Loss of the high-field *meso*-proton n.m.r. signal led Holt *et al*. to presume δ-substitution, but Mathewson *et al*. (1963) soon discovered that one of the two remaining *meso*-protons in the phaeophorbides from *Chlorobium* chlorophyll (660) was readily exchangeable with deuterium. Since it is known (Woodward & Škarić 1961) that the *meso*-protons adjacent to the reduced ring (D) in chlorins are readily exchangeable, Mathewson *et al*. (1963) concluded that the *meso*-methyl group in the (660) chlorophylls must be in the

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α or β position. Any argument which refutes this assignment must be able to explain the undoubtedly correct experimental observation that one of the two remaining *meso*-protons is unexpectedly labile towards exchange.

During the course of an extensive investigation into the ^{13}C n.m.r. spectra of chlorins and other chlorophyll degradation products (Smith & Unsworth 1975) we identified a characteristic and reproducible downfield shift of the resonances from the α and β *meso*-carbons of chlorins in trifluoroacetic acid (TFA) relative to deuteriochloroform ($CDCl_3$); the γ and δ carbons are only slightly shifted in acid. Table 2 shows the ^{13}C shifts of *meso*-carbon atoms in some typical chlorins. A pronounced downfield shift of the lines from the α and β carbon atoms is apparent when one changes from $CDCl_3$ to TFA, and this is also the case in the methyl mesophaeophorbides (**3**) from *Chloropseudomonas ethylicum*. This allows the α, β and γ, δ pairs of resonance to be unambiguously identified. In the undecoupled spectrum, *both* of the γ and δ resonances remain as singlets (i.e. they are quaternary carbons), and this definitely identifies the δ-carbon as that bearing the methyl group because the γ-carbon is already substituted with the methylene of the isocyclic ring. Furthermore, by carrying out the analysis on the *meso* monodeuteriated sample it is possible to identify the α-hydrogen as that which exchanges with deuterium; the β-proton is presumably reluctant to exchange owing to the local electron-withdrawing effect of the isocyclic ring carbonyl function.

Table 2. *Meso*-carbon resonances in ^{13}C n.m.r. spectra of chlorins in deuteriochloroform ($CDCl_3$) and trifluoroacetic acid (TFA)

compound	solvent	*meso*-carbons† α	β	γ	δ
octaethylchlorin (reduced ring D)	$CDCl_3$	98.3 (α, β)		92.5 (γ, δ)	
	TFA	106.1 (α, β)		93.5 (γ, δ)	
methyl phaeophorbide *a*	$CDCl_3$	96.4	103.6	104.8	92.6
	TFA	105.7	113.0	104.9	94.1
methyl pyrophaeophorbide *a*	$CDCl_3$	96.4	103.2	105.4	92.4
	TFA	105.3	112.8	106.5	93.9
methyl mesophaeophorbides from *C. ethylicum*	$CDCl_3$	95.1[d]‡	102.3[d]	105.3[s]	105.0[s]
	TFA	105.8[d]	111.4[d]	105.4[s]	105.4

[d] Signal appears as a doublet in undecoupled spectrum.
[s] Signal appears as a singlet in undecoupled spectrum.
† In parts/10^6 downfield from tetramethylsilane.
‡ Signal absent in sample pretreated with deuterioacetic acid.

Yet more evidence for the presence of enhanced nucleophilic character at the α-position is available. Scheme 1 shows the straightforward chemistry that we routinely apply to chlorophyll samples. The isolated chlorophylls are kept overnight in 5 % sulphuric acid in methanol, and this affords the methyl phaeophorbides (**1**) slightly contaminated (*ca.* 5 %) with the corresponding vinyl derivative (**2**). These two series of pigments can be separated from each other by preparative thick layer chromatography on silicagel, but it is often convenient to convert all of the material into the vinyl compound (**2**) and then, by catalytic hydrogenation, into the methyl mesophaeophorbides (**3**). The vinylation is carried out by heating **1** for 1 h in benzene containing toluene *p*-sulphonic acid, and affords a 77 % yield of the methyl vinylphaeophorbides (**2**) after treatment with diazomethane. However, prolonged refluxing in benzene–toluene *p*-sulphonic acid leads to a completely different compound, isolated in 79 % yield. The

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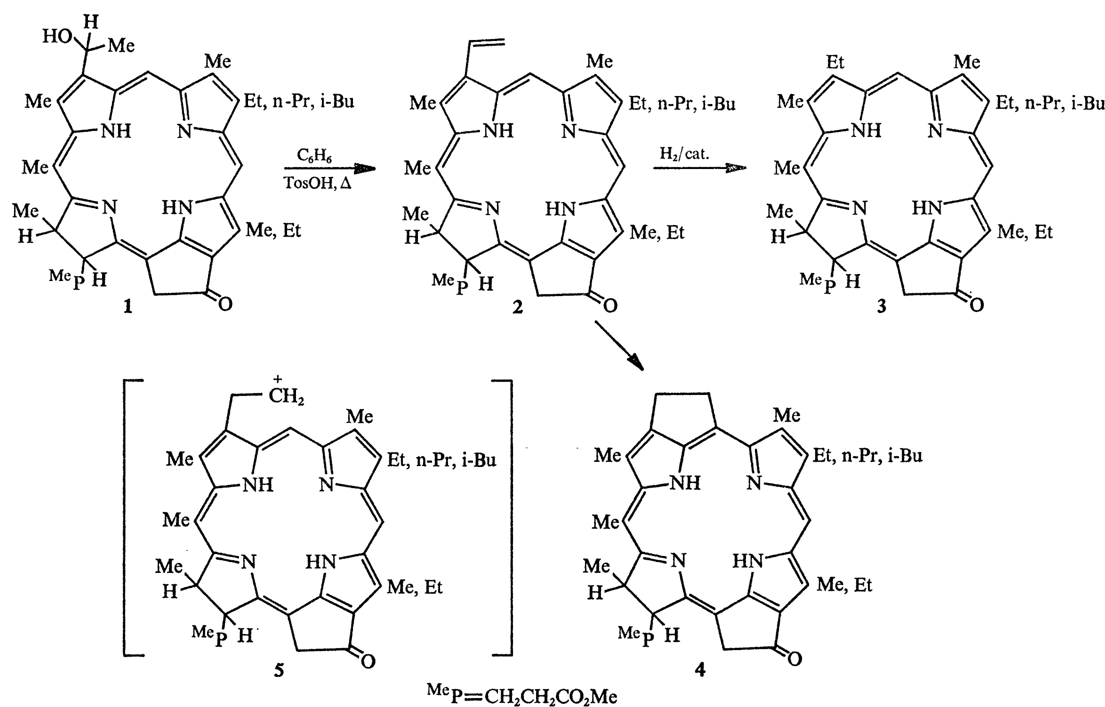
visible absorption spectrum of this new material showed a hypsochromic shift of 11 nm from the vinyl derivative (**2**) (suggesting absence of the vinyl), but the mass spectrum indicated the same molecular masses as in the vinyl phaeophorbides. The ^{1}H n.m.r. spectrum showed only one *meso*-proton resonance, no vinyl resonances, and a new, badly defined set of signals in the aromatic-methylene region. All of this evidence suggests cyclization of the vinyl group to give the 2,α-ethylenephaeophorbides (**4**). This proposal was fully confirmed in the ^{13}C n.m.r. spectrum.



SCHEME 1

Mechanistically, it seems likely that the reaction proceeds by nucleophilic attack of the α-*meso* carbon on the terminal carbon of the 2-vinyl group. In acidic solution, one would expect the carbonium ion with the positive charge on the carbon adjacent to the aromatic nucleus to be most favoured, but the cyclization to give **4** requires the carbonium ion (**5**). It may be that owing to the presence of equilibrium proportions of *N*,*N'*-diprotonated species in the acidic solution the more insulated carbonium ion (**5**) is favoured, and it is an experimental fact that in the ^{13}C n.m.r. spectra of vinyl-substituted chlorins (Smith & Unsworth 1975) the terminal carbon of the vinyl group undergoes a characteristic downfield shift of 8 ± 1 parts/10^6 in TFA relative to $CDCl_3$, whereas the vinyl methine carbon is virtually unaltered. Methyl phaeophorbide-*a* does not undergo this novel vinyl cyclization reaction.

The enchanced nucleophilicity at the α-carbon, discovered but not recognized by Rapoport, is a new aspect of chlorin chemistry. It is probably due to the electron-donating influence of the δ-methyl substituent; chlorins lacking the electron-withdrawing carbonyl groups at position 5 should exhibit increased nucleophilicity at both the α and β positions. Experiments to test this with γδ-dimethyl-chlorins are in hand.

The change of species from *C. thiosulphatophilum* to *Chloropseudomonas ethylicum* as our source of

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(660) chlorophylls presented us with an unexpected bonus. The *ethylicum* cultures, grown under bright light without rigorous exclusion of air, produce approximately 10 mg of pigments per litre of culture solution. When the phaeophorbides (**1**) were chromatographed (thick plates, silicagel, elution with 30 % acetone–70 % petroleum ether) a new, previously unobserved purple band, slightly more polar than the greenish-black phaeophorbide fraction was observed. An enhanced separation between these bands could be obtained by chromatography with 5 % tetrahydrofuran–95 % methylene chloride for elution, and the purple material could be isolated to the extent of *ca.* 1 mg per litre of culture. Visible absorption spectroscopy (figure 1) indicated a macrocycle with interrupted conjugation or a bile pigment. The ^{1}H and ^{13}C n.m.r.

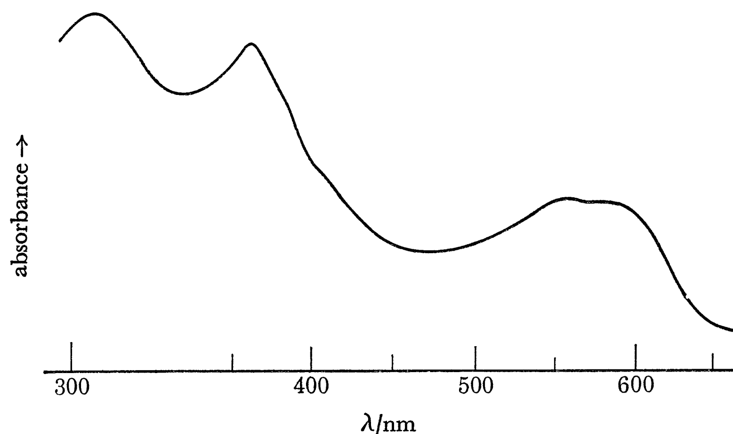
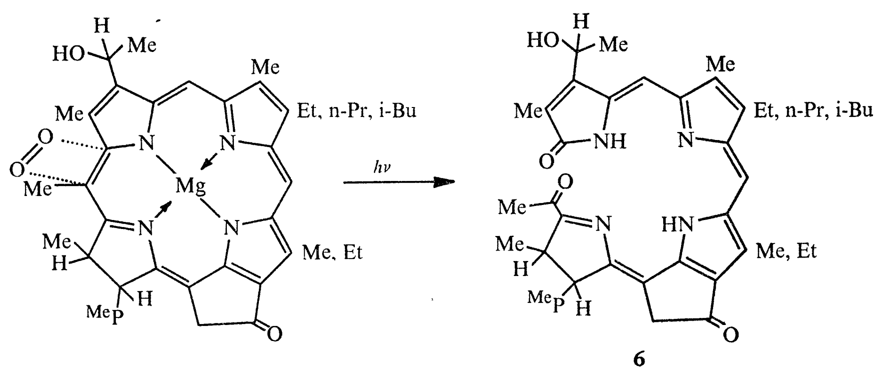


FIGURE 1. Electronic absorption spectrum (measured in ether) of the acetylbilitriene (**6**).

spectra suggested the 8′-acetylbilitriene (**6**), and this was strikingly confirmed by the mass spectrum which showed all of the expected fragmentation pathways. Wasser & Fuhrop (1973) have shown that photo-oxygenation of zinc chlorins leads to the formation of zinc formyl-bilitrienes, but demetallation of these compounds cannot be accomplished without random decomposition. Addition of oxygen (scheme 2) to the macrocycle, in exactly the same way as described for zinc chlorins (Wasser & Fuhrhop 1973) would, after demetallation, give the acetylbilitriene (**6**). It should be noted that magnesium can be removed from macrocycles much more readily than zinc, and that magnesium chlorins have lower oxidation potentials than their zinc analogues.

The isolation of **6** in the *Chlorobium* chlorophyll (660) series from *Chloropseudomonas ethylicum* is presumably a consequence of the growing culture not being completely anaerobic during



SCHEME 2

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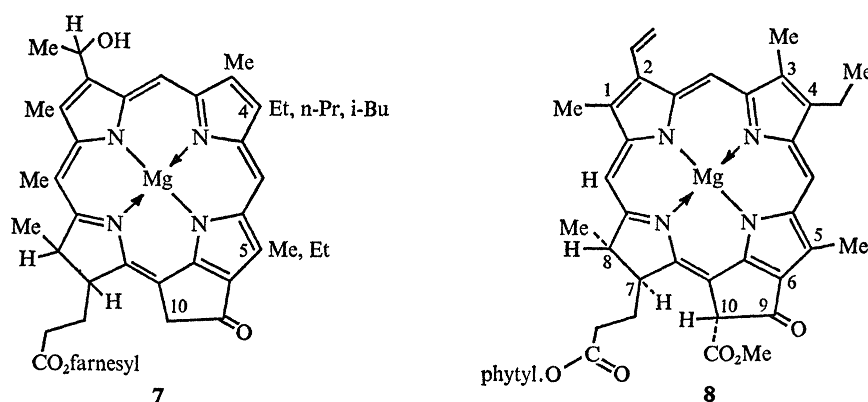
irradiation required for active growth. There is an important structural point which can be deduced from the isolation of the *acetyl*bilitriene (**6**). In the zinc chlorin case, the photo-oxygenation involves rupture of the chlorin macrocycle at a methine bridge adjacent to the reduced ring D, the methine carbon becoming a carbonyl (aldehyde) group in the product. The appearance of an acetyl group in the open-chain product from the (660) chlorophylls shows that the *meso*-carbon adjacent to the reduced ring is substituted with a methyl group, once more confirming that the (660) chlorophylls are methylated at the δ-position. It is interesting that no acetylbilitriene with the acetyl group attached to the original ring A of the chlorophyll is isolated (cf. the two isomeric formyl-bilitrienes obtained by Wasser & Fuhrhop from the zinc chlorin photo-oxygenation). We consider that this first demonstration of ring-opening of natural chlorophylls under normal conditions is significant and may offer a clue to the pathway for catabolism of the plant chlorophylls in senescent leaves.

Biosynthetic studies

Introduction

Our attention has been focused on the two most novel features of the structure of the *Chlorobium* chlorophylls (660), namely the δ-methyl substituent and the extra methyl units attached to the 4 and 5 side chains. The (660) chlorophylls (**7**) also show several other, but less striking,



Scheme 3

differences from chlorophyll *a* (**8**). For example, the 2-vinyl group in (**8**) is hydrated, the methoxycarbonyl group at C10 is absent, and the esterifying alcohol in the *Chlorobium* chlorophylls is all-*trans* farnesol, and not phytol as is found in chlorophyll *a*. However, all of these relatively minor differences almost certainly arise (as with bacteriochlorophyll *a*) in the terminal stages of the biosynthesis. That is not to say that these final stages lack importance; the reactivity of the γ and δ-*meso* positions in chlorins and metallochlorins towards electrophiles is well established (Woodward & Škarić 1961), and it would therefore seem probable that the δ-methyl group in the (660) chlorophylls is introduced at a very late stage, possibly the *last* stage in the biosynthetic pathway. We have therefore concentrated our initial efforts in the direction of the origin of the homologous methylations of the 4 and 5 side chains, but, once we have established a firm foundation in this area, progression to these other aspects should be easier.

To date, most of the experiments concerned with *Chlorobium* chlorophyll biogenesis have

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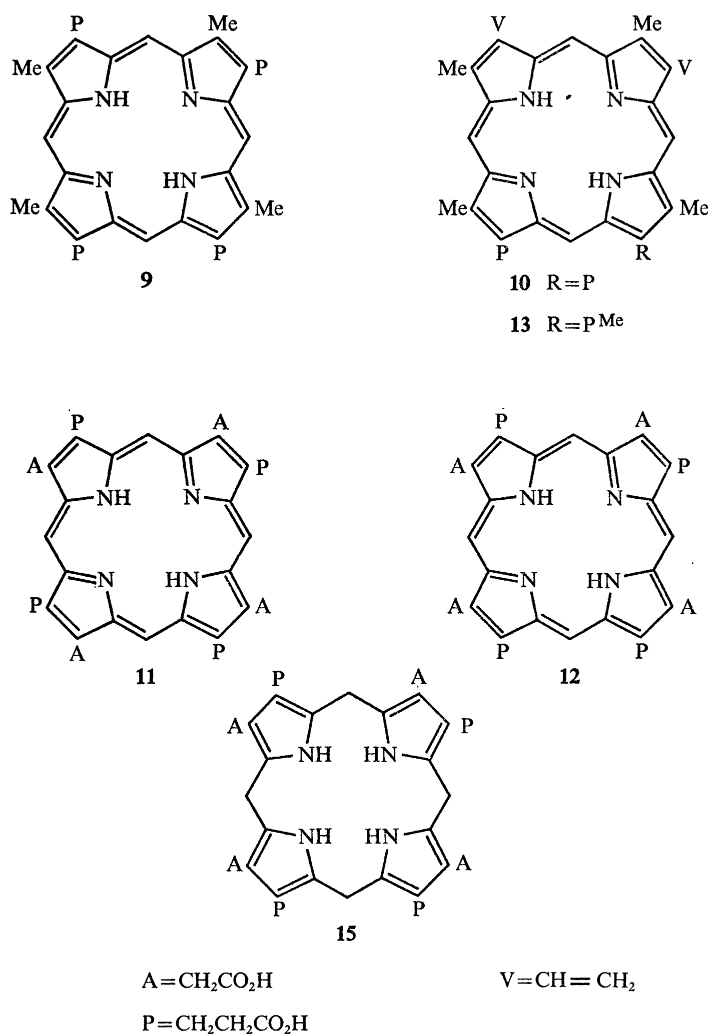
involved isolation and identification of porphyrins excreted by green photosynthetic bacteria under a variety of conditions. Uspenskaya & Kondrat'eva (1964) found that growing cultures of *C. ethylicum* and *thiosulphatophilum* secreted free porphyrins into the medium, notably coproporphyrins I and III (**9**), and protoporphyrin IX (**10**). Furthermore, it was found (Godnev, Kondrat'eva & Uspenskaya 1966) that addition of protoporphyrin IX to the cultures stimulated



SCHEME 4

chlorophyll formation in light and in darkness, and that addition of threonine inhibited culture growth, with the result that magnesium protoporphyrin IX was accumulated. These workers therefore concluded that the *Chlorobium* chlorophylls and chlorophyll *a* share a common biosynthetic pathway as far as magnesium protoporphyrin IX. Richards & Rapoport (1966, 1967) made a thorough examination of porphyrin excretion. Concentrated suspensions of *C. thiosulphatophilum* (660) when incubated with glycine and succinate excreted coproporphyrin III (**9**); addition of methionine reduced the level of this, and some uroporphyrin was also excreted (Richards & Rapoport 1966). Similar incubations with δ-aminolaevulinic acid (ALA) and

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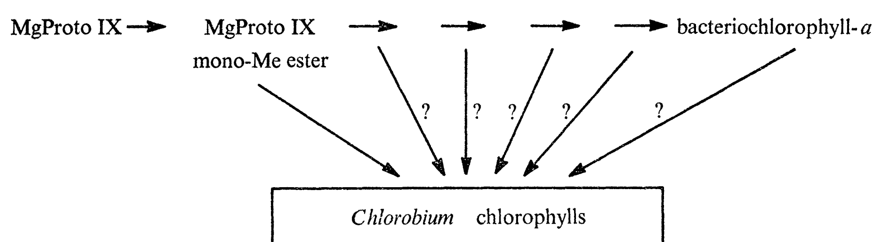
succinate led to higher porphyrin excretion, mainly uroporphyrin I (**11**) and uroporphyrin III (**12**). Further experiments with glycine, succinate, ethionine, and Tween 80 (polyoxyethylene sorbitan mono-oleate) led (Richards & Rapoport 1967) to the isolation of protoporphyrin IX monomethyl ester (**13**), its magnesium complex, and bacteriochlorophyll, as well as porphyrins and metalloporphyrins with from three to eight carboxylic acid groups. Under these conditions, *Chlorobium* phaeo 'farnesins'† and phaeoporphyrins were also excreted into the medium. It was therefore concluded (Richards & Rapoport 1967) (scheme 5) that the biosynthesis of the *Chlorobium* chlorophylls proceeded not only through magnesium protoporphyrin IX monomethyl ester but also through bacteriochlorophyll *a* or one of its immediate precursors. Some evidence in favour of this was also provided by Jones (1968), who detected the magnesium protoporphyrin IX methylating enzyme in *C. thiosulphatophilum.*



SCHEME 5. Tentative proposal (Richards & Rapoport 1967) for the biosynthesis of the *Chlorobium* chlorophylls.

Bacteriochlorophyll *a* is formed along with the *Chlorobium* chlorophylls in green photosynthetic bacteria (Jensen *et al.* 1964). Furthermore, the bacteriochlorophyll *a*–protein complex from *Chloropseudomonas ethylicum* and *C. thiosulphatophilum* (650) which may contain the *in vivo* photochemical reaction centre, has been isolated (Olson 1966). Thus it appears that bacteriochlorophyll *a* is jointly involved in photosynthesis along with the *Chlorobium* chlorophylls, and that the former is not merely an accumulated intermediate on the biosynthetic pathway to the *Chlorobium* chlorophylls.

Uro- and coproporphyrins have been isolated (Lascelles 1955, 1956; Gibson, Neuberger & Tait 1963; Cooper 1963) from the media of the bacteriochlorophyll *a* producing organisms *R. spheroides* and *R. capsulata*. Protoporphyrin IX has been found (Lascelles 1955, 1956) in the medium of *R. spheroides* when incubated with ALA and succinate, and magnesium protoporphyrin IX monomethyl ester was produced by purple bacteria when Tween 80 and methionine (Cooper 1963) or 8-hydroxyquinoline (Jones 1963) were included in the medium. Thus, with the exception of the *Chlorobium* phaeofarnesins and phaeoporphyrins, all porphyrins isolated by Uspenskaya & Kondrat'eva (1964) and Godnev *et al.* (1966) and by Richards & Rapoport (1966, 1967) could have been produced largely from the bacteriochlorophyll *a* biosynthetic pathway. Moreover, addition of ethionine to the medium might be expected to block the methylation steps in *Chlorobium* chlorophyll biosynthesis in much the same way that it inhibits methylation of magnesium protoporphyrin IX in *R. spheroides* (causing (Gibson, Neuberger & Tait 1962) excretion of coproporphyrin) and thereby divert materials towards bacteriochlorophyll *a* biosynthesis.

† In this instance, the normal term 'phaeophytin' (magnesium-free chlorophyll derivative) is inappropriate because the esterifying alcohol in the *Chlorobium* chlorophylls is farnesol and not phytol.

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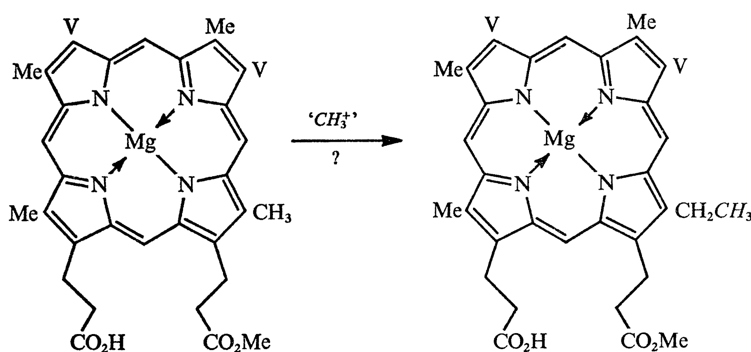
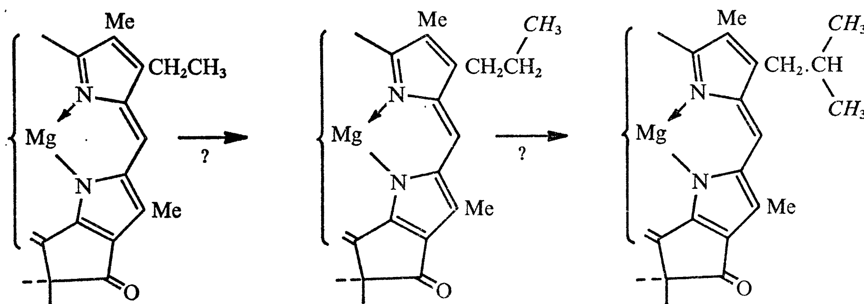
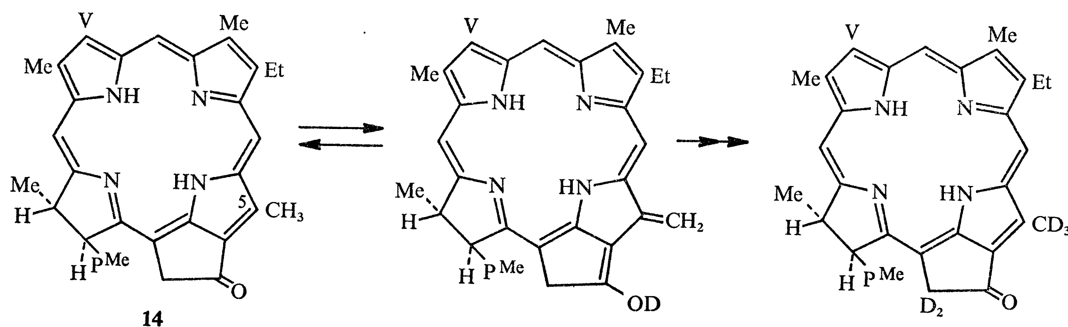
We were also suspicious of the conclusions outlined in scheme 5 on purely chemical grounds. Scheme 6 considers both extremes of this proposal. Methylation of the 5-methyl group in protoporphyrin IX, or one of its derivatives to give a 5-ethyl group would be an uncommon biosynthetic transformation. Admittedly there is chemical evidence that such a 5-methyl group is activated when adjacent to a chlorophyll-like carbocyclic ring; methyl pyrophaeophorbide *a* (**14**) undergoes deuterium exchange at the 5-methyl by prolonged reflux in pyridine containing deuterium oxide (Mengler 1967). The mechanism (scheme 7) presumably involves keto-enol tautomerism, and shows that such groups can be activated by the presence of carbonyl functions. At the other extreme of the proposal in scheme 5, methylation of an ethyl group (scheme 6) firstly to give an *n*-propyl, and subsequently an iso-butyl group seems unlikely. On the other hand, two such methylations of a vinyl group, for example in protoporphyrin IX could be contemplated. However, taking everything into account, on chemical grounds, mono-





SCHEME 6



SCHEME 7

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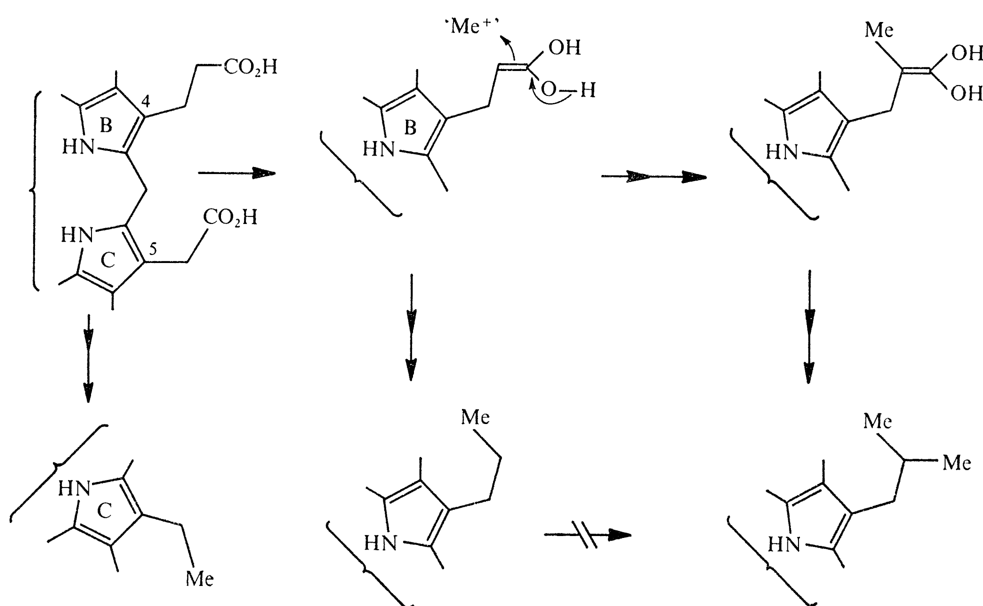
and dimethylation of vinylporphyrin in conjunction with monomethylation of a 5-methyl group seems an unlikely proposition.

In our view the side chains to be methylated (presumably by *S*-adenosylmethionine) must be activated towards electrophilic attack, and though this activation is not present in protoporphyrin IX or bacteriochlorophyll *a*, or their respective derivatives, the activation is indeed available in the side chains of known biosynthetic precursors of these compounds, for example, uroporphyrinogen III (**15**). Scheme 8 shows how acetic and propionic acid side chains may be activated via the corresponding enol forms. The *n*-propyl group in ring B can be obtained by enolization of the propionic acid side chain followed by methylation and decarboxylation. It must be recognized that decarboxylation may yield initially an olefinic side chain which is subsequently reduced. If the monomethylated side-chain is re-enolized (before decarboxylation) and then methylated a second time, then after decarboxylation, an iso-butyl substituent results.



SCHEME 8. Schematic proposal for methylation of activated side chains in *Chlorobium* chlorophyll biosynthesis.

Similarly, methylation of the enolized acetic acid side chain in ring C, followed by decarboxylation, would produce the 5-ethyl group present in all but one (table 1*c*) of the *Chlorobium* chlorophylls (660). It is of course inherent in this hypothesis that the *n*-propyl substituent itself cannot be methylated to afford the isobutyl group.

On the basis of this reasoning, it is possible to arrive at the tentative hypothesis shown in scheme 9, namely that uroporphyrinogen III should be a precursor of all (650) and (660) chlorophylls, and that in the (660) series which is our major concern, protoporphyrin IX and coproporphyrinogen III should be precursors only of band 6. This hypothesis is based on the structural assignments indicated in table 1*c*, which, as has been mentioned earlier, are not unequivocal. (The ways in which alternative structural proposals for (660) bands 1 and 3 affect this hypothesis are discussed later.)

It was therefore clear which labelled compounds it would be necessary for us to synthesize in order that the hypothesis in scheme 9 might be tested.

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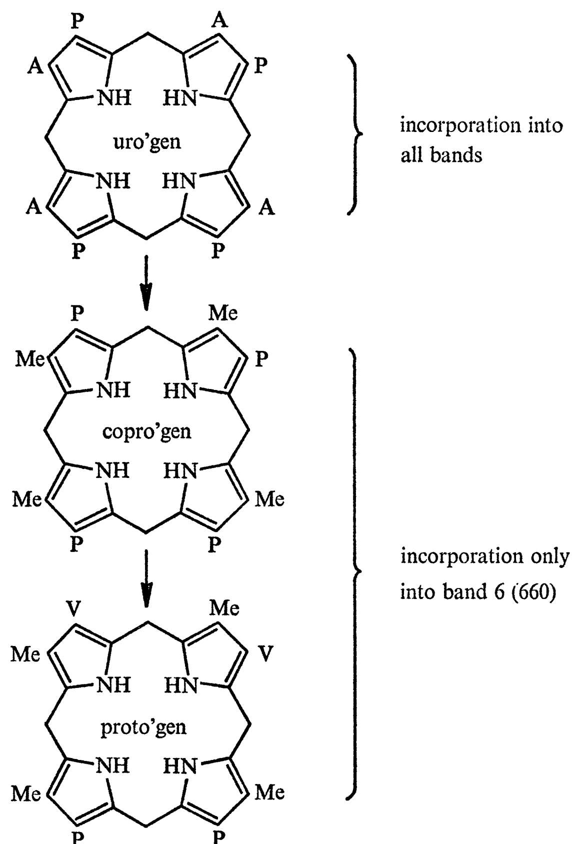
Synthetic aspects

(i) [^{14}C]- *and* [^{13}C]*methionine*

None of the earlier work had shown conclusively (i.e. by incorporation of labelled material) that methionine was indeed the origin of the novel methyl groups, though a great deal of circumstantial evidence for this view had been accumulated. Both [^{14}C]- and [^{13}C]L-methionine were prepared in good yield by treatment of L-homocystine with sodium in liquid ammonia, followed by addition of the appropriate methyl iodide (du Vigneaud, Dyer & Harmon 1933; Melville, Rachele & Keller 1947).



SCHEME 9. Working hypothesis for *Chlorobium* chlorophyll biosynthesis.

(ii) [6-^{14}C]*porphobilinogen*

It was anticipated that incorporations of methionine could be obtained merely by feeding the appropriate substrate to a growing culture of *Chloropseudomonas ethylicum*. However, this technique could not be expected to be satisfactory for substrates such as porphyrinogens. To enable development of a suitable preparation or cell suspension, we therefore embarked upon a synthesis of ^{14}C-labelled porphobilinogen, a substrate which must be satisfactorily incorporated before we could consider feeding more elaborate precursors.

We have already shown (Kenner, Smith & Unsworth 1973) that pyrroles bearing readily accessible acetyl side chains can be transformed into the corresponding methoxycarbonylmethylpyrroles by treatment with thallium(III) nitrate in methanol, and furthermore that this

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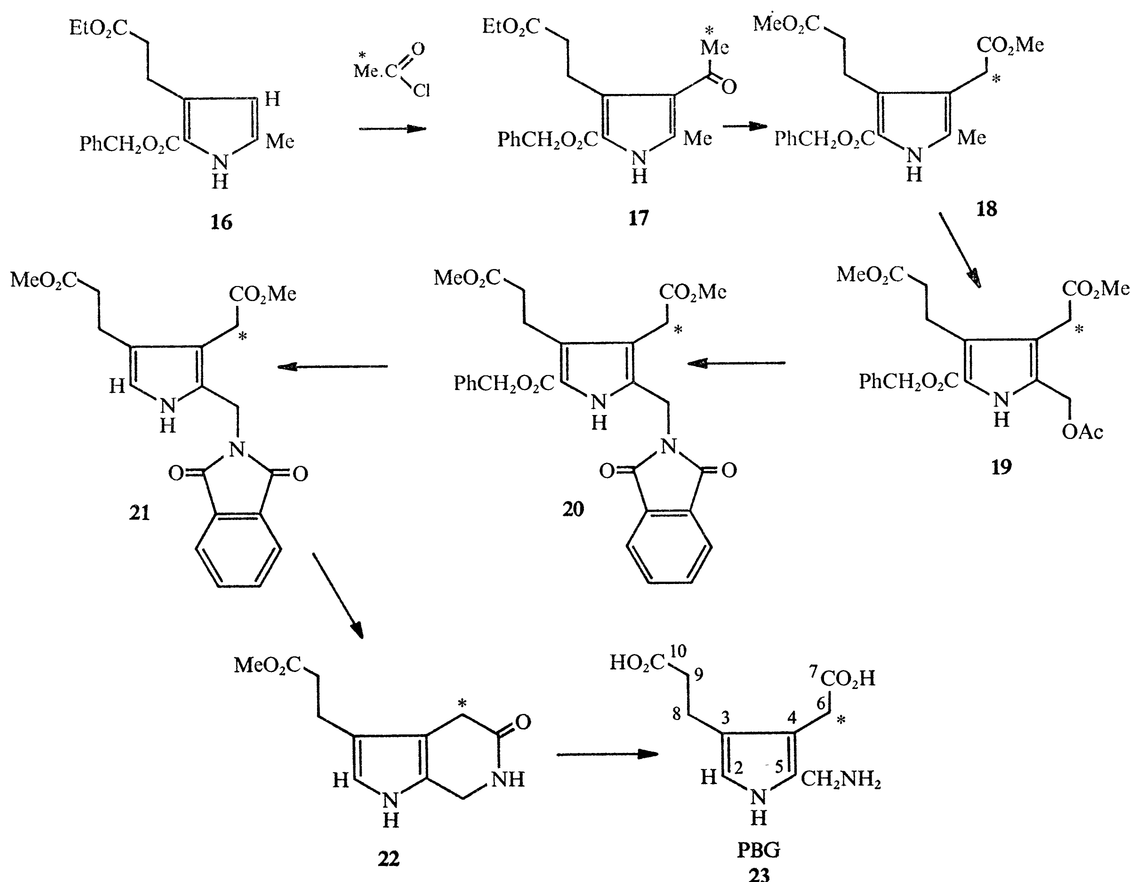
involves an aryl migration whereby the methyl group of the acetyl is converted into the methylene group of the product. Using this approach, we have developed a new synthesis of porphobilinogen, and this is outlined for the C6 labelled derivative in scheme 10.

Thus, treatment of the 3-unsubstituted pyrrole (**16**) with [2-^{14}C]acetyl chloride (obtained from the corresponding sodium acetate by treatment with oxalyl chloride) and aluminium trichloride gave the labelled acetylpyrrole (**17**) which was treated with 1.1 mol of thallium(III) nitrate in methanol containing nitric acid (Kenner *et al.* 1973) to give an 83 % yield of the pyrrole (**18**). With lead tetra-acetate, a high yield of the acetoxymethylpyrrole (**19**) was obtained, and this reacted smoothly with 1 mol of potassium phthalimide in dimethylsulphoxide to afford an 89 % yield of the phthalimidomethylpyrrole (**20**). At this stage it had been intended to remove the benzyl ester function by catalytic hydrogenation; surprisingly, attempts to accomplish this with either palladized charcoal or Raney Nickel were unsuccessful. We were, however, able to fall back on a method of deprotection which we have used (Sutton 1973) successfully in the porphyrin series. By using a 1:1 mixture of 10 % sulphuric acid in trifluoroacetic acid and anisole a good yield of the 5-unsubstituted pyrrole (**21**) was obtained. The phthalimido protecting group was next removed by treatment with hydroxylamine hydrochloride and this furnished a good yield of PBG lactam methyl ester (**22**). Alkaline hydrolysis (cf. Frydman, Reil, Despuy & Rapoport 1969) furnished [6-^{14}C]porphobilinogen (**23**).



SCHEME 10. Synthesis of [6-^{14}C]porphobilinogen.

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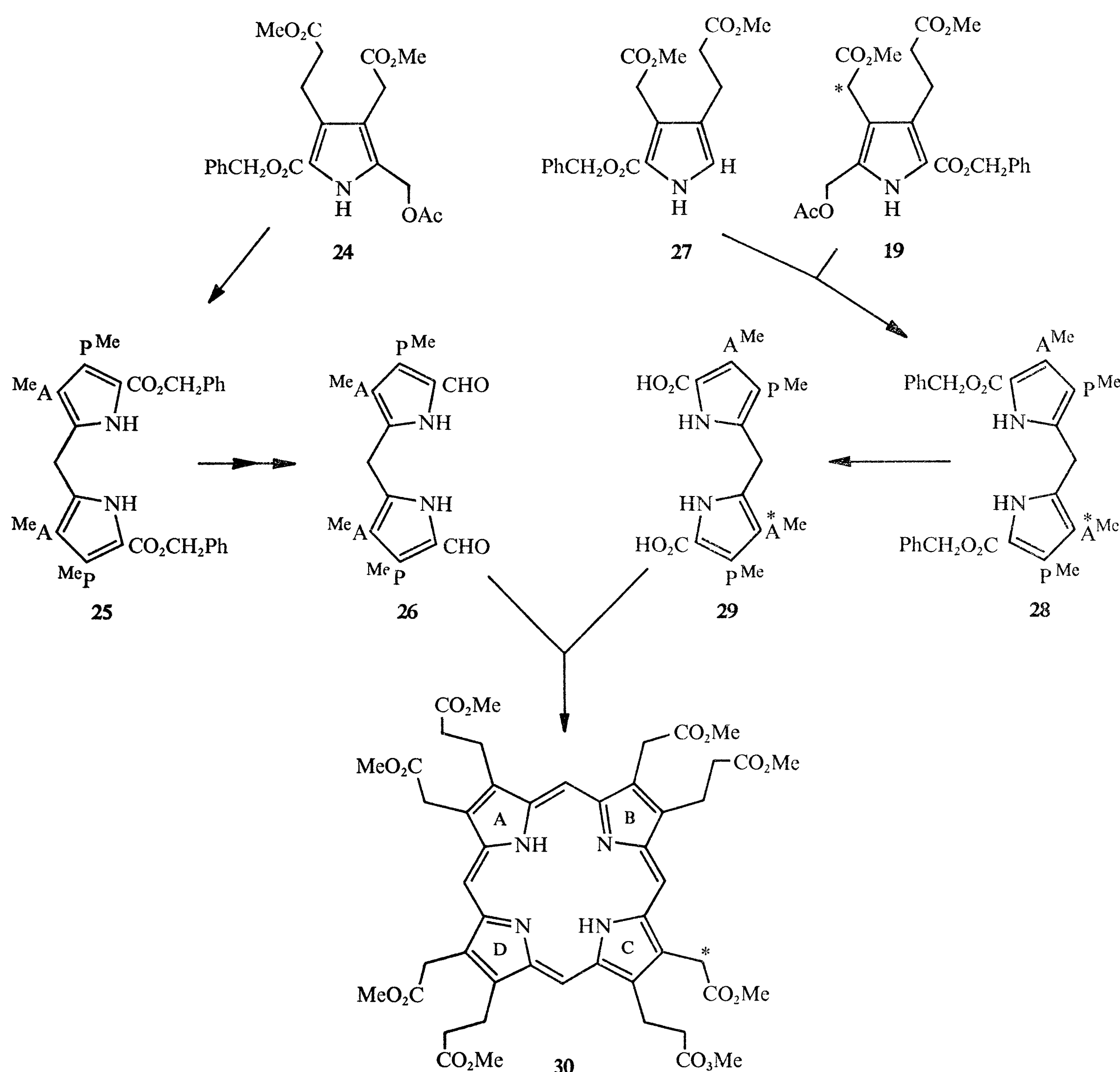
Direct application of this new synthetic route to the preparation of [6-^{13}C]PBG is in hand; indeed, in the ethyl ester series, the Friedel-Crafts acylation with [^{13}C]acetyl chloride, and the thallium promoted rearrangement have already been successfully carried out (Kenner *et al.* 1973).



SCHEME 11. Synthesis of ^{14}C-labelled uroporphyrin III octamethyl ester.

(iii) [^{14}C]*uroporphyrin* III

The most successful synthetic approach to uroporphyrin III which has so far been reported is that of Tarlton, MacDonald & Baltazzi (1960) employing the condensation of a 5,5′-di-unsubstituted pyrromethane with a 5,5-diformylpyrromethane. We employed a slightly modified version of this, as outlined in scheme 11. In principle, a carbon label can be incorporated at any of several different points; our strategy made use of the ready availability of the labelled [^{14}C]acetoxymethylpyrrole (**19**). Thus, treatment of the acetoxymethylpyrrole (unlabelled, **24**) with hydrochloric acid in methanol gave a high yield of the symmetrically substituted pyrro-

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methane (**25**), which was debenzylated by hydrogenation over palladized charcoal and then diformylated by treatment with dimethylformamide and benzoyl chloride, followed by hydrolysis of the intermediate bis(imine salt), giving a good yield of the 5,5′-diformylpyrromethane (**26**). The unsymmetrically substituted half (**28**) of the uroporphyrin III molecule was obtained by condensation of the 2-unsubstituted pyrrole (**27**) with the labelled acetoxymethylpyrrole (**19**) in acetic acid containing a catalytic quantity of toluene-*p*-sulphonic acid (Cavaleiro, Gonsalves, Kenner & Smith 1973); hydrogenation over palladized charcoal gave a quantitative yield of the pyrromethane 5,5′-dicarboxylic acid (**29**), which was condensed with the diformylpyrromethane (**26**) in methylene chloride and methanol containing toluene-*p*-sulphonic acid to furnish a 58 % yield of uroporphyrin III octamethyl ester (**30**) after methanolysis in 5 % sulphuric acid in methanol.

Yields of porphyrin in the pyrromethane condensations in presence of toluene-*p*-sulphonic acid are usually around 30–40 % (Cavaleiro *et al.* 1974*b*). It is notable, however, that even with hydriodic acid as the catalyst, yields of uroporphyrin are usually much higher than this value, and we consider this to be a peculiarity of uroporphyrins in which the bulky nature of the acetate and propionate side chains force the initially formed *b*-bilene into a coiled conformation which facilitates intramolecular cyclization to porphodimethene (and eventually, by oxidation, to porphyrin) rather than unwanted intermolecular condensation to polymeric material.

(iv) *Meso-tritiated uroporphyrin III, coproporphyrin III, and protoporphyrin IX*

We have already described total syntheses of coproporphyrin III tetramethyl ester (Abraham, Barnett, Bretschneider & Smith 1973; Jackson, Kenner & Wass 1972) and protoporphyrin IX dimethyl ester (Cavaleiro, Gonsalves, Kenner & Smith 1974*a*); these compounds can also be obtained from haematoporphyrin IX (Kenner, McCombie & Smith 1973). *Meso*-tritiated samples of uroporphyrin III octamethyl ester, coproporphyrin III tetramethyl ester, and protoporphyrin IX dimethyl ester were prepared by treatment of these porphyrins with hexapyridyl magnesium di-iodide in pyridine containing tritiated water (Cavaleiro *et al.* 1974*b*; Kenner, Smith & Sutton 1973).

Feeding experiments

(i) *Methionine*

A concentrated cell suspension of *Chloropseudomonas ethylicum* (centrifuged during the exponential part of its growth) was incubated with [^{14}C]L-methionine and lysozyme in the presence of co-factors ATP and magnesium chloride. Extraction of the pigments with methanol followed by demetallation led to a mixture of methyl and farnesyl phaeophorbides which were separated by preparative thick layer chromatography. The farnesyl and methyl phaeophorbides exhibited roughly the same specific activities, indicating that incorporation from methionine had taken place into the chlorin substituents and not merely into the farnesyl side chain; a total incorporation of 9.2 % was obtained. Substantially poorer results were obtained when the cell suspension was disrupted using freeze-thawing in place of lysozyme. The validity of the incorporations were tested by performing a 'blank' experiment involving admixture of labelled methionine, quinones, and carotenoids in a solution of phaeofarnesins, followed by the normal work-up. An 'incorporation' of 0.005 % gave an indication of the level of cross-contamination occurring and confirmed the incorporations mentioned above.

Since larger quantities of materials need to be isolated in order to monitor ^{13}C incorporations, preliminary experiments were carried out using addition of [^{14}C]L-methionine to growing

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cultures and reproducible incorporations between 9.2 and 9.5 % were obtained. Thus, [^{13}C]L-methionine (91 % mol; 200 mg) was added to 13 l of growing culture medium. Work-up to obtain methyl phaeophorbides was carried out; figure 2 shows the natural abundance ^{13}C n.m.r. spectrum of the mixture of methyl phaeophorbides from *Chloropseudomonas ethylicum*, and above it, the spectrum of the (660) phaeophorbides obtained from feeding [^{13}C]L-methionine. The resonances attributed to the δ-methyl group (20.1 parts/10^6) and methyl group of the 5-ethyl function (16.6 parts/10^6) of the methyl phaeophorbides are enhanced *ca.* 17 and 19 times natural abundance with respect to the resonance at 51.5 parts/10^6.

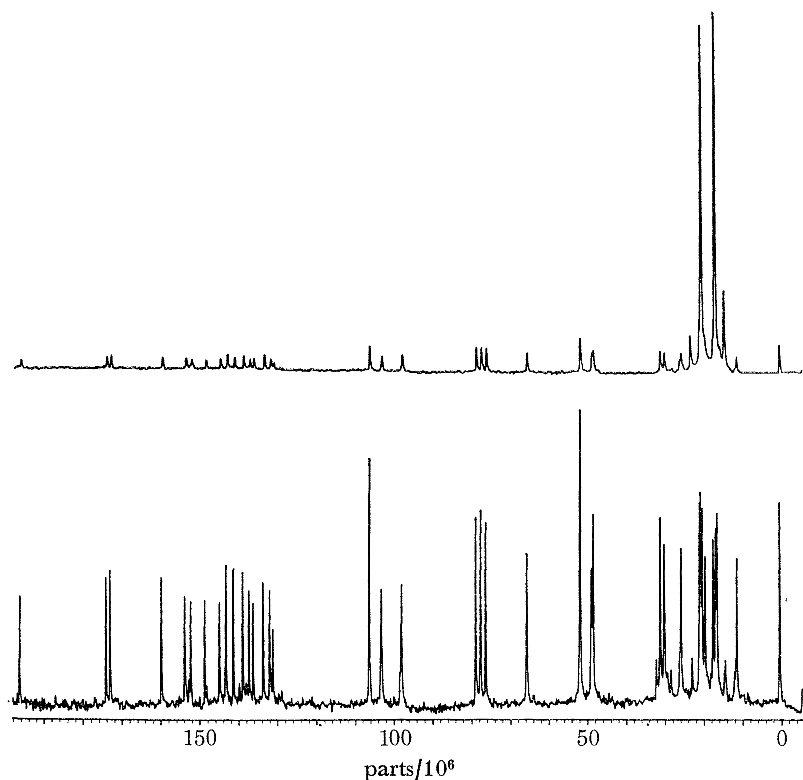

FIGURE 2. ^{13}C n.m.r. spectra (in $CDCl_3$) of (upper) *Chlorobium* chlorophyll (660) phaeophorbide methyl esters from [^{13}C]L-methionine feeding and (lower) (660) phaeophorbide methyl esters at natural abundance.

Further examination of the ^{13}C enriched and natural abundance spectra (figure 2) shows two other signals to be significantly enhanced, namely those at 14.4 and 23.1 parts/10^6. Neither of these signals can be assigned to (660) band 5 (the major homologue present in the mixture), nor do they have significant intensity in the natural abundance spectrum. Figure 3 (lower spectrum) shows the enriched spectrum once again, recorded with increased intensity, and above it the natural abundance spectrum of the methyl phaeophorbide from *C. thiosulphatophilum* (660) band 4. It can be seen that the signal at 14.4 parts/10^6 corresponds with the resonance assigned to the terminal methyl in the 4-*n*-propyl group in (660) band 4. Furthermore, this chemical shift is not far removed from the terminal carbon in the ^{13}C n.m.r. spectrum of *n*-propyl-benzene (14.0 parts/10^6). The enhanced signal at 23.1 parts/10^6 (figure 2) is also at a chemical shift which is comparable with that (22.4 parts/10^6) corresponding to the terminal methyl groups in the spectrum of isobutylbenzene, and we therefore conclude that the two enhanced signals in the enriched spectrum in figure 2 arise from the presence of small amounts

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of bands 1 and 2 (isobutyl) and 3 and 4 (*n*-propyl) in the phaeophorbide mixture. It is notable that, barring any coincident resonances, there is no evidence of methyl groups from *meso*-ethyl phaeophorbides.

The [^{13}C]L-methionine feeding also provided a small sample of the acetylbilitriene (**6**); the ^{13}C n.m.r. spectrum of this showed two greatly enhanced peaks at 15.6 parts/10^6 (from the 5-ethyl) and 26.1 parts/10^6 (acetyl), together with a less enhanced line at 14.1 parts/10^6, presumably corresponding to ring-opened material bearing an *n*-propyl substituent.

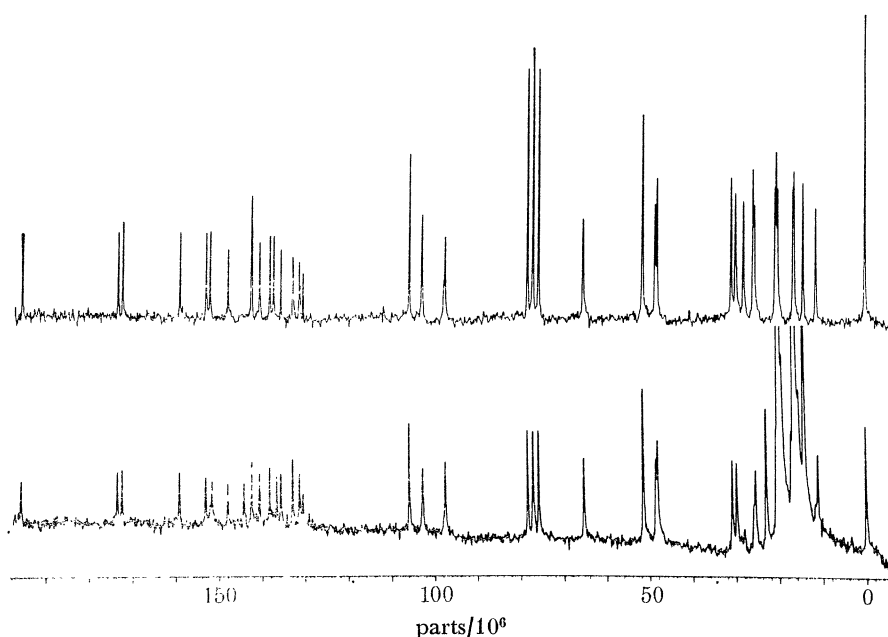

FIGURE 3. ^{13}C n.m.r. spectra (in $CDCl_3$) of (upper) the methyl phaeophorbide from *Chlorobium* chlorophyll (660) band 4 (*C. thiosulphatophilum*) and (lower) the methyl phaeophorbide mixture from feeding [^{13}C]L-methionine to *Chloropseudomonas ethylicum.*

Thus, the biosynthetic origin of the terminal methyl groups in the 4-*n*-propyl, 4-isobutyl, and 5-ethyl functions and the δ-*meso*-methyl is established as L-methionine for the (660) chlorophylls from *Chloropseudomonas ethylicum.*

(ii) *Porphobilinogen*

Only slight incorporations of [6-^{14}C]PBG were observed when it was administered to the growing culture or a cell suspension treated with lysozyme. After further experiments, we decided to use sonication as the method for disrupting the cell walls in concentrated cell suspensions, and furthermore to add, along with the co-factors ATP and magnesium chloride, a quantity of unlabelled methionine because we had established that this was required for *Chlorobium* chlorophyll biosynthesis. Thus, incubation of [6-^{14}C]PBG with a concentrated cell suspension in 1 M tris buffer (pH 7.5) (co-factors ATP, magnesium chloride, L-methionine) after sonication (total time 30 s), followed by work-up for methyl phaeophorbides as described above, gave a 5.8 % incorporation into the pigments.

We considered that this level of incorporation was sufficiently high to allow us to confirm the feeding using [6-^{13}C]PBG, and the synthesis of this material along the lines shown in scheme 10

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is in hand. Perhaps more significantly, this incorporation indicated that our cell suspension might be sufficiently active to tolerate more ambitious feedings of precursors such as porphyrinogens.

(iii) *Uroporphyrinogen III, coproporphyrinogen III, and protoporphyrinogen IX*

Porphyrin methyl esters were hydrolysed and then reduced to the porphyrinogen carboxylic acids using sodium amalgam as described earlier (Cavaleiro *et al.* 1974*b*). All labelled samples were incubated with concentrated cell suspensions in presence of ATP, magnesium chloride, and L-methionine after disruption of the cell walls by sonication as described in the PBG feedings. After methanolysis, the methyl phaeophorbides were purified by preparative thick layer chromatography and then counted after bleaching (cf. Cavaleiro *et al.* 1974*b*). Uroporphyrinogen III (^{14}C-labelled) gave reproducible incorporations around 2 %, and occasionally as high as 5.4 %. Though, on the basis of the structures in table 1*c* and the arguments presented in schemes 8 and 9, incorporations of coproporphyrinogen III and protoporphyrinogen IX would be expected to be equal, our preliminary results with tritium-labelled porphyrinogens (and assuming 50 % loss of tritium on oxidation of porphyrinogen up to porphyrin, i.e. an enzymic reoxidation), coproporphyrinogen III gave 0.4 % incorporation and protoporphyrinogen IX 0.3 %.

Conclusions from preliminary feeding experiments

It is clear that the incorporations of uroporphyrinogen III are sufficient to encourage us to repeat the feeding experiments with ^{13}C-labelled uroporphyrinogen III; the synthesis of this material is in hand. We can also extrapolate, and deduce that any precursor which is likely to be incorporated into the major component of the (660) mixture (i.e. band 5) will be amenable to investigation by ^{13}C studies.

While it is not wise to read deeply into preliminary experiments which were carried out to test our enzymic preparation, we may comment upon certain trends. The results appear to confirm the hypothesis in scheme 9 that the biosynthesis of most of the *Chlorobium* chlorophyll fractions branches from normal chlorophyll biosynthesis somewhere between uroporphyrinogen III and coproporphyrinogen III. Since the loss of four molecules of carbon dioxide from uroporphyrinogen III is known to occur in a stepwise manner, our results do not necessarily indicate that the branch of *Chlorobium* chlorophyll biosynthesis from chlorophyll *a* biosynthesis occurs *at* the uroporphyrinogen III stage. According to scheme 8, the novel methylations require intact carboxylic acid functions on the 4 and 5 positions; thus it is possible to construct the working hypothesis shown in scheme 12, the key intermediate being the pentacarboxylic porphyrinogen (**31**) which should be capable of incorporation into all of the (660) chlorophylls. This hypothesis suggests that uroporphyrinogen III loses three molecules of carbon dioxide to give (**31**) which is then attacked by a methylating system which accomplishes methyl insertion and decarboxylation (cf. scheme 8) to give the 5-ethylporphyrinogen (**32**). Since it is known (Cavaleiro *et al.* 1974*b*) that the 2-vinyl is formed before the 4-vinyl in the biosynthesis of protoporphyrin IX from coproporphyrinogen III, it seems sound to postulate that the 5-ethylporphyrinogen (**32**) is vinylated at the 2-position, to give (**33**), before (i) vinylation at position 4 with subsequent conversion into (660) band 5, or (ii) monomethylation of the propionic side chain, followed by decarboxylation and conversion into (660) band 4, or (iii) di-methylation of the 4-side chain, and then decarboxylation and transformation into (660) band 2. Any pentacarboxylic acid porphyrinogen (**31**) which escapes methylation at position 5

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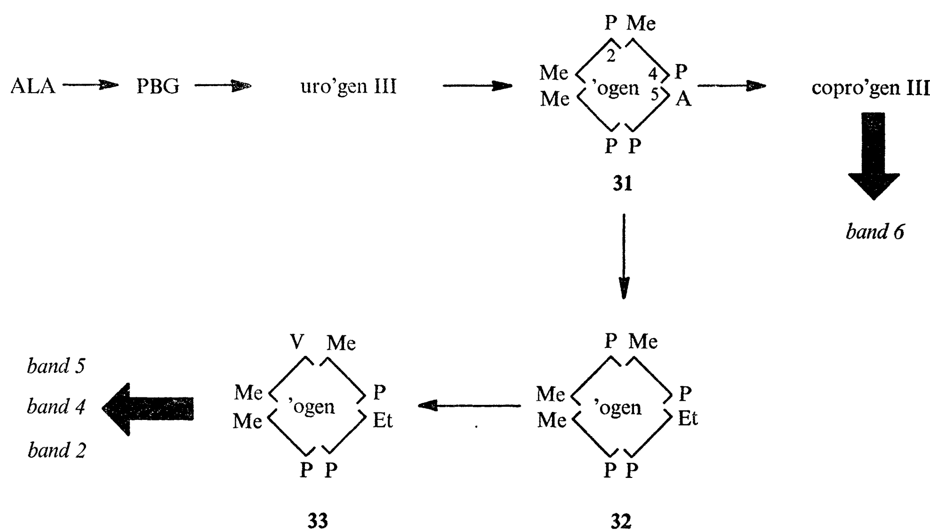
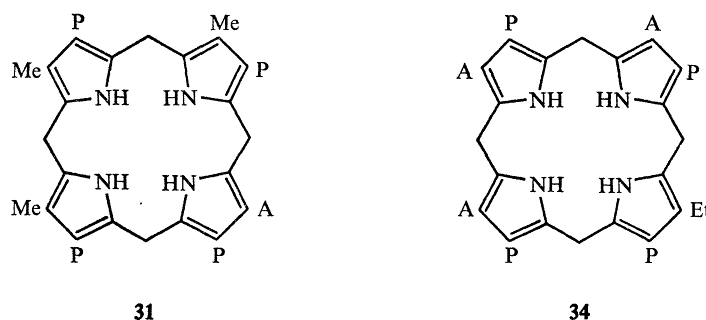
will afford coproporphyrinogen III which can still, probably via protoporphyrin IX, be converted into (660) band 6.

Thus, the hypothesis put forward in scheme 12 can be tested by synthesis and feeding of the pentacarboxylic porphyrinogen (**31**). However, should our proposal prove incorrect because the branch from normal chlorophyll metabolism is indeed actually *at* the uroporphyrinogen III stage, the porphyrinogen (**34**) bearing a 5-ethyl group might be expected to be on the biosynthetic pathway to most of the *Chlorobium* chlorophylls.



SCHEME 12. Extended working hypothesis for the biosynthesis of *Chlorobium* chlorophyll (660) bands 2, 4, 5 and 6.



SCHEME 13

Finally, the working hypothesis presented in scheme 12 allows us to make certain proposals regarding the identity of the (660) fractions for which *meso*-ethyl functions have been suggested. It is clear that these *meso*-ethyl groups were postulated to make fractions 1 and 3, which were obviously chromatographically different from bands 2 and 4, structurally different in a manner that would be least incompatible with the degradative evidence; the *meso*-carbon atoms and any substituents on them were lost in the degradation to maleimides. There are two main possibilities for the identities of the unknown bands, and these are presented in scheme 14. The first possibility centres once again on the key pentacarboxylic acid porphyrinogen (**31**); if the 5-acetic acid side chain were to be dimethylated (rather than monomethylated) prior to

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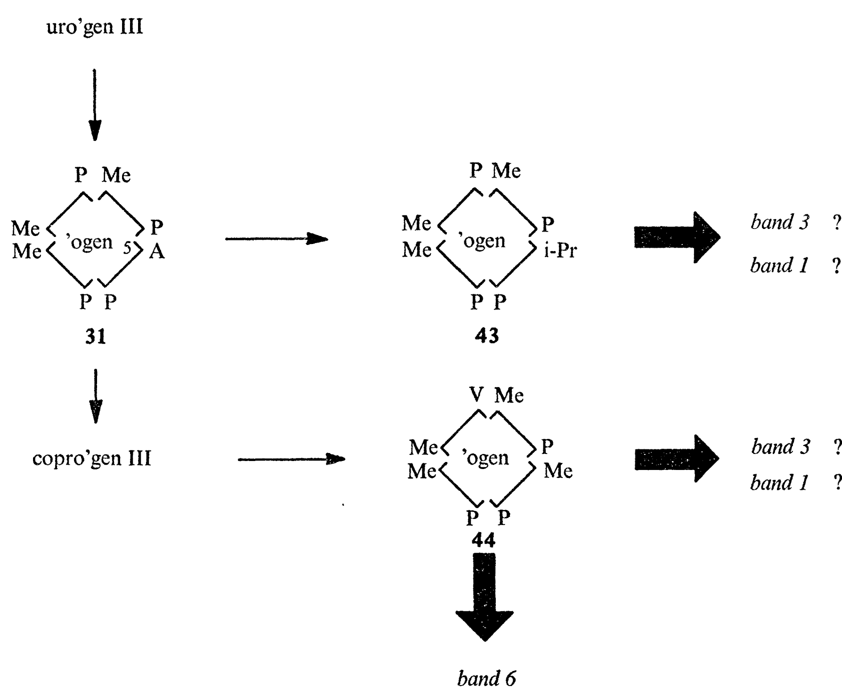
decarboxylation, then that would lead to a porphyrinogen (**43**) bearing a 5-isopropyl function, and this in theory should be capable of eventual transformation into *Chlorobium* chlorophyll (660) bands bearing 4-ethyl-5-isopropyl, 4-*n*-propyl-5-isopropyl, and 4-isobutyl-5-isopropyl substituents. This gives us three possibilities where we only require two, but it should be mentioned that the (660) chlorophylls have often (e.g. Aronoff 1966) been said to consist of at least *seven* fractions. Alternatively, coproporphyrinogen III might be a precursor of the unknown fractions; the only fraction said (table 1*c*) to have a 5-methyl group is band 6. However, if coproporphyrinogen III or harderoporphyrinogen (**44**), which we have synthesized (Cavaleiro *et al.* 1974*b*), were to be monomethylated or dimethylated (cf. scheme 8) at the 4 position, and then decarboxylated, then the two porphyrinogens which result should eventually yield (660) bands bearing 4-*n*-propyl-5-methyl, and 4-isobutyl-5-methyl substituent patterns, and these might indeed be the identities of the unknown fractions. On balance, we favour this second proposal. The (650) chlorophylls (which we feel differ mainly from the (660) series in that the *meso*-methylation is omitted at a *very late* stage in their biosynthesis) contain fractions which have 4-*n*-propyl-5-methyl (band 5) and 4-isobutyl-5-methyl (band 3) substituent arrays; *meso*-methylation of these would lead directly to the structures put forward in our second proposal.



SCHEME 14. Structural proposals for (660) bands 1 and 3 based on extrapolations from scheme 12.

There are several other possibilities for the structures of the unknown bands; some of these involve methylations at entirely different points of the molecule. However, we consider that these methylations must conform with the basic proposals of scheme 8. In this context, we therefore await with interest the identification of the hexacarboxylic acid porphyrin occurring between uroporphyrinogen III and coproporphyrinogen III in normal porphyrin metabolism. (The full structural assignments for the porphyrinogens (bearing seven, six, and five carboxylic acid groups) which occur in normal porphyrin biosynthesis between uroporphyrinogen III and

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coproporphyrinogen III have been made by Professor Jackson at this meeting. It is particularly significant that the pentacarboxylic porphyrinogen is identical with **31**, the compound which we have postulated to be the key intermediate in the biosynthesis of the *Chlorobium* chlorophylls.)

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Discussion

A. H. Jackson (*University College, P.O. Box* 78, *Cardiff, CF*1 1*XL*). I was very interested to hear of your elegant work and interesting conclusions concerning the origin of the *Chlorobium* chlorophylls particularly in relation to our own results, and the accumulation of a pentacarboxylic porphyrin in some forms of porphyria. I wonder if the unexpected ready exchange of the α-*meso* proton in the *Chlorobium* chlorophylls of the 660 series might be due in part to steric factors as well as electronic? The δ-methyl group might well distort the ring sufficiently to render the α-position more susceptible to electrophilic attack, e.g. as shown by the deuteriation experiments, and cyclization of the 2-vinyl group which you described.

K. M. Smith. That is a good possibility, though one might expect protonation to occur at the δ-position rather than at α. However, as mentioned in the manuscript of this lecture, we intend to investigate this novel reactivity with model γδ-dimethylchlorins.

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