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The mechanism of the attachment of esterifying alcohol in bacteriochlorophyll *a* biosynthesis

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The mechanism through which the C-17[3] carboxy group of bacteriochlorophyllide *a* is esterified to produce bacteriochlorophyll a_{phytyl} of *Rhodopseudomonas spheroides* and bacteriochlorophyll $a_{geranylgeranyl}$ of *Rhodospirillum rubrum* was studied by using 5-aminolaevulinate labelled with ^{18}O at its C-1 carboxy oxygen atoms. The latter species was prepared by an exchange reaction in which 5-aminolaevulinate hydrochloride was heated in $H_2{}^{18}O$ in an autoclave. A method for the determination of the ^{18}O content of the C-1 oxygen atoms of 5-aminolaevulinate was developed. As a prelude to the mechanistic work, a systematic study was undertaken to establish the optimal conditions under which a significant proportion of the bacteriochlorophyll *a* of the two photosynthetic organisms originated from the exogenously added 5-aminolaevulinate. It was found that, when *Rps. spheroides* and *Rsp. rubrum* were grown in the presence of about 0.15mM- and 1.2mM-5-aminolaevulinate respectively, 30–40% of their chlorophyll was derived from the added precursor. In these conditions, 5-amino[1,4-$^{18}O_3$]laevulinate was incorporated into bacteriochlorophyll a_{phytyl} and bacteriochlorophyll $a_{geranylgeranyl}$ by the relevant organisms. The samples of chlorophylls were then hydrolysed with alkali to obtain phytol and geranylgeraniol, which were converted into the corresponding trimethylsilyl derivatives and analysed by gas chromatography–mass spectrometry. The data were used to deduce that the alcohols contained 90–95% of the ^{18}O originally present at each of the C-1 oxygen atoms of the precursor 5-aminolaevulinate. In the light of these results it is suggested that the ester bond at C-17[3] is formed, not by a chlorophyllase type of enzymic reaction, but by a process involving the nucleophilic attack by the C-17[3] carboxylate group of the chlorophyllide on the activated form of an isoprenyl alcohol.

During the course of their monumental work on the elucidation of the structure of chlorophylls, Willstätter & Stoll (1910) exploited an earlier discovery by Borodin (1882) of the formation of ethylchlorophyllide from ethanol extracts of green leaves, and established that the ethyl chlorophyllide was produced by the action of an enzyme, chlorophyllase, on chlorophyll *a* present in leaves.

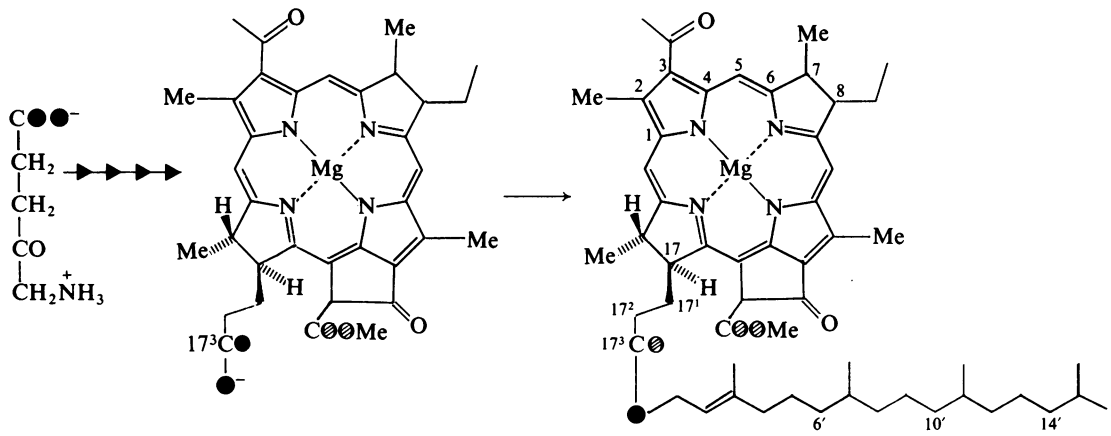
The enzyme, which was solubilized in aq. 40% acetone and was functional in this mixture, catalysed the hydrolysis of chlorophyll *a* to chlorophyllide *a* and phytol in a reversible process and also participated in a variety of transesterification reactions (e.g. reactions 1 and 2):

$$\text{Chlorophyll } a \rightleftharpoons \text{chlorophyllide } a + \text{phytol} \quad (1)$$

$$\text{Methylchlorophyllide } a + \text{phytol} \rightleftharpoons \text{chlorophyll } a + \text{methanol} \quad (2)$$

With regard to the biological function of this enzyme, it was assumed that chlorophyllase was intimately involved in the degradation of chlorophylls. A quarter of a century later, studies by Granick and colleagues, performed by combining enzymological approaches with the elegant use of *Chlorella* mutants, revealed that, in plants, chlorophyllide *a* was the penultimate biosynthetic inter-

Abbreviations used: ALA, 5-aminolaevulinate; g.c.–m.s., gas chromatography–mass spectrometry.



(I) ALA (II) Bacteriochlorophyllide *a*

(Ia) ● = ^{16}O

(Ib) ● = ^{18}O

(IIIa) Bacteriochlorophyll *a* from *Rps. spheroides*

(IIIb) Bacteriochlorophyll *a* from *Rsp. rubrum*; esterifying group = geranylgeranyl with double bonds at $\Delta^{6',10',14'}$

(IIIc) Plant chlorophyll *a*, vinyl group at C-3 and double bond at Δ^{7}

Scheme 1. *Path of C-1 oxygen atoms of ALA during bacteriochlorophyll a biosynthesis*
The origin of the hatched oxygen atoms (⊘) in (II) and (III) has not yet been experimentally determined, though it could be argued that these are also derived from C-1 of ALA.

mediate tetrapyrrole, which merely required esterification for conversion into chlorophyll *a* (Granick, 1950; for a review see Granick & Beale, 1978). Chlorophyllase now acquired a new significance, and it appeared that the enzyme, through the reversal of reaction (1), may function in a biosynthetic role for the attachment of phytol to the C-17^3 carboxy group of chlorophyllide *a* (structure of type II; Scheme 1). Support for this view came from the experiments performed by Holden (1961), who showed that the activity of chlorophyllase was negligible in etiolated leaves, but that when the leaves were transferred to light the activity of the enzyme increased and paralleled the accumulation of chlorophyll. A careful scrutiny of the literature, however, reveals that in highly purified states not all preparations of chlorophyllase catalyse the reversal of reaction (1) (Ichinose & Sasa, 1973, and references cited therein). This observation, although conflicting with a biosynthetic role for the enzyme, could be explained by assuming that *in vivo* the enzyme functions in a lipid environment from which water can be excluded, thus favouring the reversal process, and that such a situation is unattainable in aqueous solutions.

We envisaged that the question of how the esterification process occurs in living cells might be resolved by using an approach in which a species of chlorophyllide containing ^{18}O in the strategic C-17^3 carboxy group (see structure II) is generated *in vivo* and then the fate of the ^{18}O during the conversion of the chlorophyllide into chlorophyll is investigated through the mass-spectrometric analysis of phytol obtained from the chlorophyll. The present paper is an account of such a study, and in addition describes methods for the preparation of [1-$^{18}O_2$]ALA (Ib) and incorporation of the latter into two types of bacteriochlorophyll *a* (IIIa and IIIb) under conditions in which a significant proportion of the intracellular bacteriochlorophyll originates from the exogenously added precursor. This work is taken in part from a Ph.D. Thesis (Ajaz, 1983), and a preliminary account of part of this work is already published [Abid (now Ajaz) *et al.* (1980)].

Results

Preparation and analysis of [1,4-$^{18}O_3$]- and [1-$^{18}O_2$]-ALA (Scheme 2)

A key requirement for the investigation was the availability of a sample of ALA in which its two C-1 carboxy oxygen atoms were labelled with ^{18}O. To obtain such a species, ALA was heated in $H_2{}^{18}O$ in the presence of an acid catalyst, and the equilibrated material was recovered by freeze-drying. Studies with authentic ALA had shown that, owing to problems of dimerization during the formation of derivatives, the structure of ALA

precluded the direct determination of its isotopic content by mass-spectrometric analysis. An indirect method was therefore developed in which ALA was treated under carefully controlled conditions with $NaIO_4$, and the resulting succinic acid, as its bistrimethylsilyl derivative (V; Scheme 2), was subjected to g.c.–m.s. Bistrimethylsilyl succinate gave a major ion at *m/z* 247 due to the $[M-15]^+$ fragment (VI) (Corina & Harper, 1977), and the species obtained from the ALA equilibrated in $H_2{}^{18}O$ gave additional peaks at *m/z* 249, 251 and 253, indicating that the C-4 as well as the two C-1 oxygen atoms of the ALA contained ^{18}O.

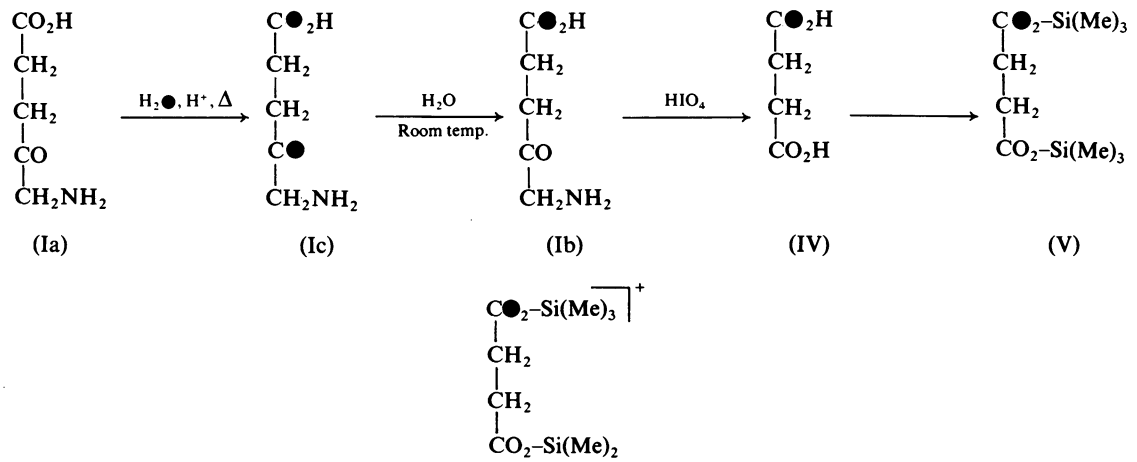
Because of the relative lability of the C-4 carbonyl oxygen of ALA in aqueous media and the symmetry of its degradation product, succinic acid, it was not possible to obtain an accurate measurement, via succinate, of the ^{18}O content of the carboxy oxygen atoms at C-1 of triply labelled ALA (IC). Because only the carboxy oxygens of ALA are those incorporated into the positions of interest in the chlorophylls, it was decided to try and selectively exchange out the C-4 oxygen atom of ALA in order to measure accurately and exclusively the carboxy oxygen atoms at C-1 of ALA (it was, though, estimated that in the species Ic all three oxygen atoms were labelled with an average of 70–75 atom% excess ^{18}O).

When the sample of [1,4-$^{18}O_3$]ALA (Ic) was kept at 30°C in water for 24h and the material was then oxidized with $NaIO_4$ to succinate, the latter, after formation of the bistrimethylsilyl derivative, was found to contain fragments having *m/z* 247, 249 and 251. This confirmed, as expected, a ready exchange of the C-4 oxygen atom of the ALA with the oxygen atom of water, allowing the resulting material to be formulated as [1-$^{18}O_2$]ALA (Ib) with an average of 64–67 atom% excess of ^{18}O in each of the two carboxy oxygen atoms. In some experiments such samples were further equilibrated with $H_2{}^{18}O$ to give ALA with up to 93% enrichment of ^{18}O.

Studies on the incorporation of [4-^{14}C]ALA into the chlorophylls of Rps. spheroides and Rsp. rubrum

Preliminary screening with a number of photosynthetic organisms indicated that *Rps. spheroides* and *Rsp. rubrum* were the most promising systems for the incorporation of exogenously added ALA into their respective chlorophylls. These two bacteria therefore were chosen for a detailed study, the purpose of which was to optimize the incorporation of [4-^{14}C]ALA into the pigments. It should be noted that *Rps. spheroides* and *Rsp. rubrum* synthesize bacteriochlorophyll a_{phytyl} and bacteriochlorophyll $a_{geranylgeranyl}$ respectively (Rapoport & Hamlow, 1961; Brockmann & Knobloch, 1973).

A series of incubations were inoculated with a fully grown culture of the relevant organism, supplemented with various amounts of [4-^{14}C]-ALA and kept in light at 32°C. When the stationary phase had been reached (3 days), the bacteria were harvested, and the bacteriochlorophyll was purified and its total amount determined spectrophotometrically. The proportion of the pigment arising from the exogenously added ALA was estimated by comparing the specific radioactivity of the precursor with that of the product. The data in Table 1 for *Rps. spheroides* highlight the fact that, although in the presence of increasing amounts of ALA the net biosynthesis of bacteriochlorophyll *a* was greatly impaired, the proportion



(VI, *m/z* = 247 and 251 for ● = ^{16}O and ^{18}O respectively)

Scheme 2. *Preparation and analysis of [1-$^{18}O_2$]ALA*
● = ^{18}O.

of the pigment arising from the exogenously added precursor progressively increased. For instance, in the presence of 0.3mM-ALA (Table 1, Expt. 6), when the biosynthesis of bacteriochlorophyll *a* had decreased by 91.7%, nearly 55.0% of the pigment produced by the organism had originated from the exogenously added ALA. The inhibition of bacteriochlorophyll *a* synthesis by ALA has been observed previously (Lascelles & Hatch, 1969), and from more-recent work can be attributed to the inhibition of cell growth by exogenous ALA (Ajaz, 1983).

A similar trend was noted for *Rsp. rubrum* except that in this case there was less inhibition of bacteriochlorophyll $a_{geranylgeranyl}$ synthesis; in the presence of 2.7mM-ALA, the pigment content had decreased by only 60% (Table 2, Expt. 6).

Labelling experiments with [4-^{14}C,1,4-$^{18}O_3$]ALA

In the light of the above results and the knowledge that in 1–0.5μmol of bacteriochlorophyll *a* there should not be more than a 4-fold dilution of the ^{18}O label in order to obtain a reliable isotopic analysis, the following experiments were undertaken.

Rps. spheroides was grown with samples of multiply labelled ALA, containing ^{14}C in addition to ^{18}O, as described in Table 3, and it was found that, for different experiments, between 0.97 and 2.4μmol of bacteriochlorophyll *a* could be obtained in a purified form. By comparing the ^{14}C specific radioactivity of the precursor with that of the product in these experiments 35–58% of the pigment was shown to have originated from the added precursor (Table 3). The bacteriochlorophyll *a* was then hydrolysed with NaOH to transfer the bridge oxygen of the ester bond to phytol. The use of an alkaline-hydrolysis procedure ensured that the transfer of the bridge oxygen will occur without exchange with oxygen of the medium. The phytol was subjected to preliminary purification by t.l.c. and then after conversion into the trimethylsilyl ether was analysed by g.c.–m.s. Authentic phytol or that obtained from a control experiment

Table 1. *Effect of the concentration of ALA on the biosynthesis of bacteriochlorophyll* a_{phytyl} *(IIIa) in Rps. spheroides*
Each incubation consisted of medium MS (360ml) (Lascelles, 1959) containing a fully grown culture of *Rps. spheroides* (40ml) supplemented with [4-^{14}C]ALA as detailed below. The growth was allowed to proceed at 32°C under light for 3 days. After this period, the micro-organisms were harvested, bacteriochlorophyll *a* was isolated, purified and quantified spectrophotometrically, and a sample was taken for the determination of radioactivity. In the examination of the data below it should be borne in mind that 8 mol of ALA produce 1 mol of bacteriochlorophyll *a*.

Sample no.	ALA added (μmol)	ALA added [d.p.m. (×10^{-6})]	ALA incorporated into the chlorophyll [d.p.m. (×10^{-6})]	ALA incorporated into the chlorophyll (μmol)	ALA incorporated into the chlorophyll (%)	Total chlorophyll formed (μmol)	Chlorophyll formed from the exogenous ALA (μmol)	Chlorophyll formed from the exogenous ALA (%)
1	0.119	2.98	1.162	0.064	39.0	3.6	–	–
2	20	3.5	0.42	2.4	12.0	3.0	0.3	10.0
3	40	3.81	0.503	5.28	13.2	2.4	0.66	27.5
4	60	4.9	0.453	5.55	9.25	1.9	0.7	36.5
5	90	4.7	0.213	4.08	4.53	1.0	0.51	51
6	120	4.88	0.054	1.31	1.1	0.3	0.164	54.5

Table 2. *Effect of the concentration of ALA on the biosynthesis of bacteriochlorophyll* $a_{geranylgeranyl}$ *(IIIb) in Rsp. rubrum*
The incubations consisted of medium (75ml; see the Experimental section) containing a fully grown culture of *Rsp. rubrum* (15ml) supplemented with various quantities of [4-^{14}C]ALA and left under light for 3 days. Other details were as described in the Experimental section.

Sample no.	ALA added (μmol)	ALA added [d.p.m. (×10^{-6})]	ALA incorporated into the chlorophyll [d.p.m. (×10^{-6})]	ALA incorporated into the chlorophyll (μmol)	ALA incorporated into the chlorophyll (%)	Total chlorophyll formed (μmol)	Chlorophyll formed from the exogenous ALA (μmol)	Chlorophyll formed from the exogenous ALA (%)
1	Trace	2.8	0.21	–	7.39	2.8	–	–
2	20	2.9	0.162	1.12	5.6	2.08	0.14	6.7
3	50	3.3	0.143	2.16	4.32	1.95	0.27	13.8
4	80	3.7	0.193	4.16	5.2	1.78	0.52	29.1
5	120	3.6	0.121	4.04	3.37	1.39	0.505	36.3
6	180	4.1	0.10	4.33	2.4	1.13	0.541	47.9
7	250	4.3	0.0413	2.395	0.96	0.51	0.299	58.7

Table 3. *Incorporation of ^{18}O into the side-chain alcohols of bacteriochlorophylls a elaborated by Rps. spheroides and Rsp. rubrum from [1,4-$^{18}O_3$]ALA*

(*a*) The incorporation of [4-^{14}C,1,4-$^{18}O_3$]ALA into bacteriochlorophyll a_{phytol} of *Rps. spheroides* was performed essentially as indicated in Table 1. Nine independent incorporation experiments were carried out during a 3-year period (Ajaz, 1983), but the details of only four are given below for economy of space. In all cases, quantitative data on the chlorophyll were obtained after purification by rechromatography. For the determination of the ^{18}O content, phytol was isolated, then converted into its trimethylsilyl derivative, and peak heights due to $[M-15]^+$ fragments at *m/z* 353 and 355 were used for quantification as described in the Experimental section. It should be noted that, whereas [1,4-$^{18}O_3$]ALA was used in the incubation, the ^{18}O content at its C-1 carboxy oxygen atoms was determined, in each experiment, after the exchange of the C-4 carbonyl oxygen with the medium. The reasons for this are described in the text. (*b*) The incubations with *Rsp. rubrum* were carried out as indicated in Table 2 in conjunction with the details as described above, except that in the analysis of geranylgeraniol trimethylsilyl ether molecular-ion peaks at *m/z* 362 and 364 were used for quantification. With respect to the transfer of ^{18}O to the side-chain alcohol, results similar to those detailed below were obtained in at least six other independent experiments.

		ALA			Chlorophyll		% ^{18}O content	
Expt. no.	Sample volume (ml)	Added (μmol)	(% ^{18}O at each C-1 oxygen)	Incorporated into the chlorophyll (μmol)	Total amount isolated (μmol)	(% from exogenous ALA)	Theoretical*	Experimental
(*a*)							Phytol	
1	400	60	None	0.423	0.97	43.61	None	None
2	400	60	65	0.418	1.17	35.73	23.22	22.50
3	200	94	54	0.242	0.70	34.57	18.66	18.67
4	200	60	66	0.81	1.40	57.90	38.20	36.10
5	200	51	92	0.80	2.31	34.64	31.87	30.64
(*b*)							Geranylgeraniol	
1	100	91	None	0.25	1.21	20.66	None	None
2	100	122	65	0.337	0.88	38.29	24.90	23.40
3	75	118	66	0.495	1.42	34.90	23.00	21.60
4	75	113	67	0.498	1.47	33.90	22.70	21.00

* Assuming that the side chain at C-17^3 is elaborated via mechanism 3 (Scheme 3).

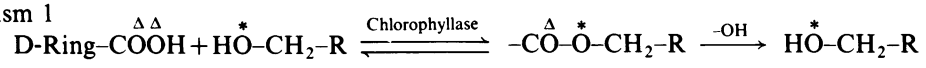
[Table 3(*a*), Expt. 1] gave an intense $[M-15]^+$ peak at *m/z* 353 due to the dimethylsilyl species $[(CH_3)_2$–Si–O–$C_{20}H_{39}]^+$. Natural abundance gave a peak at 2 mass units higher (*m/z* 355) at 7% of the intensity of *m/z* 353. The phytol obtained from the ^{18}O-labelling experiment gave a strong peak at *m/z* 355, showing the presence of [1-^{18}O]phytol. When allowance was made for the dilution of the bacteriochlorophyll *a* produced from ^{18}O-labelled ALA with that arising from endogenous biosynthesis, then the ^{18}O content of phytol, and hence that of the bridge oxygen of the chlorophyll, corresponded to the incorporation of one atom of oxygen from the C-1 carboxy group of ALA into this position.

The results on the incorporation of [1-^{14}C,1,4-$^{18}O_3$]ALA into bacteriochlorophyll $a_{geranylgeranyl}$ of *Rsp. rubrum* in Table 3(*b*) are strikingly similar to those described above for *Rps. spheroides* and prove that the geranylgeranyl and phytyl side chains of the two species of bacteriochlorophylls are introduced by analogous mechanisms.

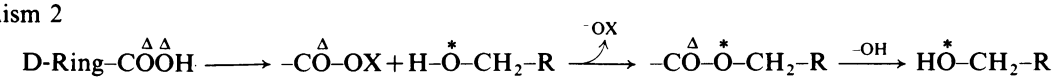
Discussion

There is now a general agreement that the addition of the final building block in the biosynthesis of all classes of chlorophylls involves the esterification of the ring-D carboxy group. The alcohol component varies considerably and has been shown to be phytol for plant chlorophylls and bacteriochlorophyll *a* of *Rps. spheroides* (Rapoport & Hamlow, 1961), geranylgeraniol for bacteriochlorophyll *a* of *Rsp. rubrum* (Brockmann & Knobloch, 1973) and farnesol for *Chlorobium* chlorophylls (Caple *et al.*, 1978). In the present paper, the mechanism of the esterification process was investigated in *Rps. spheroides* and *Rsp. rubrum*. The study was based on the well-founded assumption that C-1 of ALA will become the C-17^3 carboxy group of bacteriochlorophyllide *a* (II). It was not known whether the two oxygen atoms at C-1 of ALA would remain intact during the transformations leading to bacteriochlorophyllide *a*; however, this stability has now been demonstrated by the ^{18}O results obtained. Thus it has been shown that the bridge oxygen at the ester bonds of bacteriochlorophyll a_{phytyl} and bacteriochlorophyll $a_{geranylgeranyl}$ is derived intact from one of the C-1 oxygen atoms of ALA. In the light of this finding and precedents from biochemistry for related esterification reactions, we examined the various possible mechanisms (Scheme 3) through which bacteriochlorophyllide *a*→bacteriochlorophyll *a*

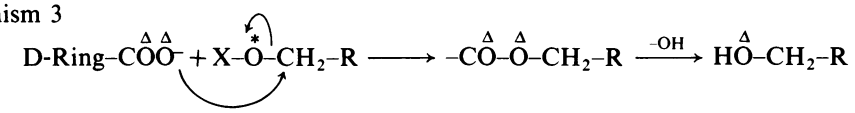
Mechanism 1



Mechanism 2



Mechanism 3



Scheme 3. *Three possible mechanisms for the esterification step in bacteriochlorophyll biosynthesis*
R–CH_2– represents a C_{20} isoprenyl unit; OX is a biological leaving group. The origin of the bridge oxygen in the ester bond is revealed by the subsequent analysis of the C_{20} alcohol released by alkaline hydrolysis as shown in the last step.

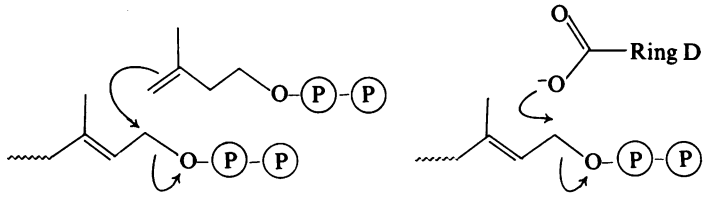
conversion may occur. Mechanism 1 assumes that the esterification process is merely the reversal of the hydrolytic reaction catalysed by chlorophyllase (for details see the introduction). According to such a mechanism the bridge oxygen of the ester bond would be expected to originate from the alcohol moiety. The present study, showing this not to be the case, excludes this mechanism as well as a related pathway (mechanism 2) in which the acyl transfer to phytol (or geranylgeraniol) occurs after the conversion of the chlorophyllide carboxy group into an active ester intermediate (see Granick & Beale, 1978). It may be noted that in excluding mechanism 1 we have tacitly assumed that the chlorophyllase-catalysed reaction occurs via an acyl-oxy fission process. Although there is no direct experimental proof for such an assertion, the fact that chlorophyllase catalyses a variety of transesterification reactions may be cited as evidence in support of the operation of an acyl-oxy fission process for this enzyme.

The final mechanism in which the ester-bond formation occurs by a carboxy-alkyl transfer reaction involving the participation of an active form of the isoprenyl unit requires that both the parent oxygen atoms of the carboxy group are retained in the product. We have not yet experimentally shown that the carbonyl as well as the bridge oxygen atom at C-17[3] of bacteriochlorophyll *a* is derived from C-1 of ALA, and hence C-17[3] of bacteriochlorophyll *a*. However, the convincing demonstration that the bridge oxygen has originated from ALA gives support to the view that a mechanism of type 3 operates for the isoprenylation step in the formation of the chlorophylls of *Rps. spheroides* and *Rsp. rubrum*. The close agreement between the experimentally determined and theoretically predicted values for the ^{18}O contents of the bridge oxygen in Table 3 also gives quantitative information on the biosynthetic flux through the pathway operating *in vivo* via mechanism 3. This is estimated to be 90–95% for these two organisms. As far as we are aware, such a dogmatic conclusion could not have been drawn by using any other existing technique.

The work of Rüdiger *et al.* (1980) on the synthesis of chlorophyll *a in vitro* by crude homogenates from maize shoots and oat-seedling etioplast membranes lends further support for the operation of mechanism 3, and also shows that the isoprenylation reaction occurs through the intermediacy of a pyrophosphorylated terpenoid alcohol. These workers have demonstrated the presence in the etioplast preparations of an enzyme that catalyses the transfer of C_{20} units from phytyl pyrophosphate or geranylgeranyl pyrophosphate to chlorophyllide *a*. The enzyme participating in this process has been named chlorophyll synthetase.

The stage at which the three double bonds of the geranylgeranyl unit are reduced to produce the phytyl moiety is not yet known. Recent studies have revealed that such a transformation could occur before or after the ester-bond formation, since two types of enzyme activities have been demonstrated in spinach, one converting geranylgeranyl pyrophosphate into phytyl pyrophosphate and the other chlorophyll $a_{\text{geranylgeranyl}}$ into chlorophyll a_{phytyl} (Soll *et al.*, 1983; see also Schoch & Schafer, 1978).

Thus the results from our laboratory and those reported by the German workers complement each other and highlight the fact that polyisoprenyl pyrophosphates produced in the biosynthesis of terpenoids are used for the transfer of a C_{20} polyisoprenyl group to a carboxylate nucleophile. In broad mechanistic terms this chlorophyll synthetase-catalysed reaction is equivalent to the transfer of isoprenyl units in the formation of geranyl pyrophosphate, farnesyl pyrophosphate and geran-



Scheme 4. *Transfer of isoprenyl units to olefinic and carboxylate nucleophiles*

ylgeranyl pyrophosphate except that in these steps the displacement reaction involves the participation of an olefinic nucleophile (Scheme 4).

The preceding discussion further points to the fact that *in vivo* the tetrapyrrole-biosynthetic pathway leading to the formation of chlorophyllide must be strictly co-ordinated with the polyisoprenoid biosynthesis so that the C_{20} pyrophosphates are only produced on demand and do not accumulate intracellularly.

Experimental

Growth and harvesting of bacteria

Rps. spheroides (strain N.C.I.B. 8253) and *Rsp. rubrum* (unknown strain) were grown and maintained as described by Lascelles (1959) and Ormerod *et al.* (1961) respectively. The bacteria were harvested by centrifugation at 12000*g* for 25min.

Extraction, purification and determination of bacteriochlorophyll a

The pellet (2–5g) was suspended in acetone/methanol (7:2, v/v) (80–100ml) in a separating funnel and was shaken vigorously. Diethyl ether (200ml) was added and the contents were gently shaken. The pigments were transferred into the ether layer by addition of water (100ml). The diethyl ether was separated, washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. Bacteriochlorophyll *a* was purified by cellulose-20 (microgranular; Sigma Chemical Co.) column chromatography. The pigments were dissolved in light petroleum (b.p. 60–80°C) and applied gently on to the column (1.3cm × 12cm). When the pigments had adsorbed, the column was washed with light petroleum (b.p. 60–80°C) (30ml), then with light petroleum (b.p. 60–80°C)/acetone (33:1, v/v) (50ml) to remove most of the carotenoids. The bright-grey band of bacteriochlorophyll *a* was eluted with light petroleum (b.p. 60–80°C)/acetone (20:1, v/v) (100ml). The eluate, containing bacteriochlorophyll *a*, was evaporated to dryness, and the residue was kept in the dark (at 0°C) after flushing with N_2 gas. In those experiments in which the incorporation of ^{18}O-labelled ALA was performed, the samples of bacteriochlorophylls were usually rechromatographed.

The quantity of purified bacteriochlorophyll *a* was determined spectrophotometrically (Sauer *et al.*, 1966) at 770–775nm in a Pye–Unicam SP.800 spectrometer. This was achieved by dissolving the bacteriochlorophyll *a* in acetone/methanol (7:2, v/v) (5ml) and measuring the absorbance at 770–775nm of a suitably diluted sample in a quartz cell, against a solvent blank. A molar absorption coefficient of $69.2 mM^{-1} \cdot cm^{-1}$ at the above wavelength was used in quantifying bacteriochlorophyll *a*.

Isolation of the side-chain alcohol from bacteriochlorophyll a

The bacteriochlorophyll *a* (0.5–10μmol) was dissolved in methanol (10ml) and was hydrolysed with 30% (w/v) KOH in methanol (20–50ml). The solution was incubated at room temperature for 30min. After the addition of water, the reaction mixture was extracted with diethyl ether, and the organic layer was washed with water (2 × 100ml), dried over anhydrous Na_2SO_4 and evaporated to dryness.

The residue was dissolved in diethyl ether (50–250μl) and the solution was applied to silica-gel plates (PF-254 Brockman grade; 0.5mm; 10cm × 20cm), which were developed in diethyl ether/light petroleum (b.p. 40–60°C) (1:1, v/v). In this solvent the R_F values for phytol and geranylgeraniol were 0.65 and 0.52 respectively. The bands were detected either by keeping the plate in I_2 vapour or spraying a portion of the plate with 2.5% (v/v) H_2SO_4 in ethanol. The remaining band was then eluted with diethyl ether/acetone (5:1, v/v), and the alcohol was retained for g.c.–m.s. analysis (see below).

Measurement of radioactivity

Bacteriochlorophyll *a* was dissolved in diethyl ether (5ml), and a sample (50–100μl) was taken in a counting vial containing 10ml of butyl-PBD [5-(4-bisphenylyl)-2-(4-t-butyphenyl)-1-oxa-3,4-dia-

zole]/toluene (15g of butyl-PBD in 2.5 litres of toluene) and its ^{14}C radioactivity counted in an Intertechnique SL-40 liquid-scintillation counter.

Preparation of [1,4-$^{18}O_3$]ALA

ALA hydrochloride (75mg, 450μmol) was placed in a 5ml constricted test tube containing $H_2{}^{18}O$ (100μl; 98–99 atom% excess ^{18}O) and HCl (0.2M; 2μl). The tube was sealed carefully under vacuum and heated in a pressure cooker for 2–3h. The tube was cooled, then opened, and the contents were immediately freeze-dried.

When ALA with very high ^{18}O content (>90% at each C-1 carboxy oxygen) was required, the ^{18}O-labelled ALA prepared above was once again heated with $H_2{}^{18}O$ (100μl; 99 atom% excess ^{18}O) and the contents were freeze-dried.

Oxidative degradation of [$^{18}O_3$]ALA to [$^{18}O_3$]-succinate

[$^{18}O_3$]ALA (2mg, 12μmol) was added to a test tube containing a solution of $NaHCO_3$ (20%, w/v; 1ml) and $NaIO_4$ (5.4mg, 25μmol). The contents were shaken for 3–5min and then adjusted to pH2 with conc. HCl. NaCl (250mg) was added to the mixture, and [$^{18}O_3$]succinate was extracted with ethyl acetate (3×8ml), which was then water-washed, dried over anhydrous Na_2SO_4 and evaporated to dryness.

Equilibration of [1,4-$^{18}O_3$]ALA

[$^{18}O_3$]ALA (2mg) was dissolved in water (5ml), and the solution was left at 30°C for 24h. At the end of this period the equilibrated material was converted, by the method described above, into succinate and the material was analysed by g.c.–m.s.

Analysis of ^{18}O-labelled succinate and ^{18}O-labelled isoprenyl alcohols by g.c.–m.s.

Succinic acid was silylated and analysed by g.c.–m.s. as described previously (Corina & Harper, 1977). Isoprenyl alcohols were silylated with pyridine/hexamethyldisilazane/chlorotrimethylsilane (5:1:1, by vol.) and analysed by g.c.–m.s. on a Pye 104–Kratos MS 30 instrument, with a 2.7m×4mm (internal diam.) glass g.c. column packed with 3% OV-1 on Diatomite CQ (100–120 mesh), He carrier flow rate 40ml/min and injector temperature 300°C. Retention times were approx. 3.5min at g.c. temperatures of 245°C (phytoltrimethylsilyl ether) and 275°C (geranylgeranioltrimethylsilyl ether).

Determination of ^{18}O content

The isotopic abundance of the ^{18}O-labelled species was calculated from the $[M-15]^+$ ions for bistrimethylsilyl succinate and for phytol trimethylsilyl ether and from the M^+ ion for geranylgeraniol trimethylsilyl ether by using established methods (Caprioli, 1972) by comparison with unenriched standards.

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