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Mechanistic Studies on the Phytylation and Methylation Steps in Bacteriochlorophyll *a* Biosynthesis: An Application of the ^{18}O-Induced Isotope Effect in ^{13}C NMR†

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ABSTRACT: The high-resolution ^{13}C NMR spectrum of bacteriochlorophyll *a* biosynthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid by growing cells of *Rhodopseudomonas sphaeroides* has shown both the C-17[3] and C-13[3] resonances consist of three additional components upfield shifted from the —^{16}O—^{13}C=^{16}O resonance. By comparison with the ^{13}C NMR spectrum obtained for phytyl acetate containing ^{13}C and ^{18}O selectively in the ester linkage, these components have been identified as the bridge (—^{18}O—^{13}C=^{16}O), nonbridge (—^{16}O—^{13}C=^{18}O), and dual-labeled (—^{18}O—^{13}C=^{18}O) isotopomers. These results have been interpreted to suggest that both the ester bonds of bacteriochlorophyll *a* are produced by a carboxy–alkyl transfer process.

A common characteristic of all known chlorophylls is the esterification of the D-ring propionate carboxy group at C-17[3] with a long-chain alcohol moiety (for example, see structure III, Figure 1) (Akhtar & Jordan, 1979). The precise nature of this alcohol varies considerably, and it has been shown to be predominantly *all-trans*-farnesol (in association with smaller quantities of other long-chain alcohols) for bacteriochlorophylls *c* and *d* (Rapoport & Hamlow, 1961; Caple et al., 1978), geranylgeraniol for bacteriochlorophyll *a* from *Rhodospirillum rubrum* (Katz et al., 1976; Walter et al., 1979), tetrahydrogeranylgeraniol for bacteriochlorophyll *b* from *Ectothiorhodospira halochloris* (Steiner et al., 1981), and phytol for bacteriochlorophyll *a* from *Rhodopseudomonas sphaeroides* (Brockman, 1971) and for the plant chlorophylls (Brockman, 1971; Fischer & Wenderoth, 1939).

Historically, investigations on the esterification process at C-17[3] can be traced back to Willstätter and Stoll (1910), who exploited an earlier discovery of Borodin (1882), noting the formation of ethyl chlorophyllide from ethanol extracts of green leaves, and established that the former was produced by the action of chlorophyllase, an enzyme present in the leaves, on chlorophyll *a*. The enzyme, which was solubilized in 40% aqueous acetone and was functional in this mixture, catalyzed the hydrolysis of chlorophyll *a* to chlorophyllide *a* and phytol in a reversible process and participated in a variety of transesterification reactions. Since the equilibrium constant for the former reaction in aqueous solution favored chlorophyll hydrolysis, the major biological function of chlorophyllase was assumed to be in chlorophyll degradation. However, following the identification by Granick and colleagues that, in plants, chlorophyllide *a* was the penultimate tetrapyrrole intermediate which merely required esterification for conversion to chlorophyll *a*, chlorophyllase acquired a new significance—that of a role in chlorophyll synthesis via a reversal of the hydrolytic reaction [Granick, 1950; for a review see Granick and Beale (1978)].

Support for this view came from the experiments of Holden (1961), which showed that the activity of chlorophyllase

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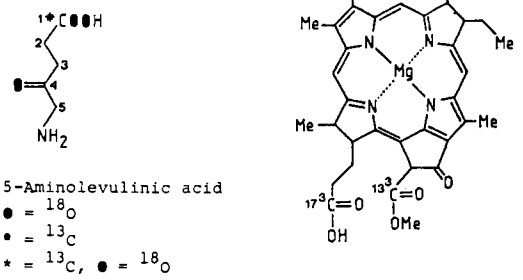
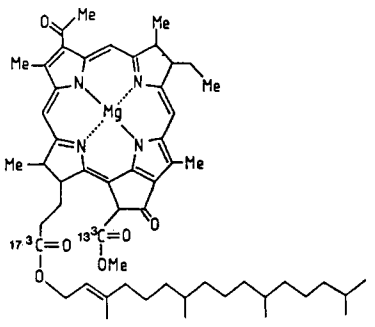


Ia, 5-Aminolevulinic acid
Ib, ● = ^{18}O
Ic, • = ^{13}C
Id, * = ^{13}C, ● = ^{18}O

II, Bacteriochlorophyllide *a*

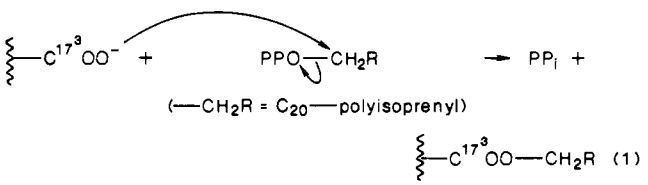


III, Bacteriochlorophyll *a*

FIGURE 1: Structures of bacteriochlorophyll *a* and its precursors.

paralleled chlorophyll accumulation in greening leaves. Following the work of Ellsworth (1971, 1972a), which culminated in the partial resolution of the hydrolytic and esterification activities within a chlorophyllase preparation from wheat seedlings (Ellsworth, 1972b), it became apparent that not all preparations of chlorophyllase catalyze a reversal of the hydrolytic reaction (Ichinose & Sasa, 1973), thus questioning a biosynthetic role for the enzyme. The existence of a novel enzyme (a "chlorophyll synthetase") distinct from chlorophyllase that catalyzed the final step in the biosynthesis of the chlorophylls was first shown by Rüdiger et al. (1980) via the incorporation of the pyrophosphorylated form of [1,1-$^3H_2$]geranylgeraniol into chlorophyll *a* in a preparation from maize seedlings and was shown independently by Akhtar et al. (1984) by incorporation of [1,1,4-$^{18}O_3$]-5-aminolevulinic acid (Ib, Figure 1) into bacteriochlorophyllide *a* (II) followed by demonstration of ^{18}O in the phytyl moiety of the formed bacteriochlorophyll *a* (III). The latter work highlighted that the chlorophyll synthetase catalyzed process must represent the predominant course for the elaboration of the C-17^3 side chain.

A mechanism based on these results has been proposed in which the carboxy anion at C-17^3 of bacteriochlorophyllide *a* (II) attacks the polyisoprenyl pyrophosphate to yield bacteriochlorophyll *a* (III):



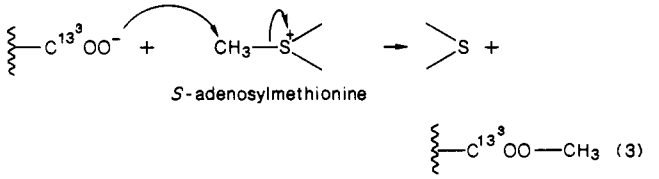
(—CH_2R = C_{20}—polyisoprenyl)

(1)

Studies on the enzymology of the methylation process occurring at C-13^3 have shown that the reaction

Mg–protoporphyrin IX →
Mg–protoporphyrin IX monomethyl ester (2)

involves the participation of *S*-adenosylmethionine (Gibson et al., 1963; Radmer & Bogorad, 1967). Chemical considerations predict that this process would also occur via a carboxy–alkyl transfer:



S-adenosylmethionine

(3)

However, Rebeiz and Castelfranco (1971) found that in a cell-free extract from etiolated cucumber cotyledons, the formation of magnesium protoporphyrin IX monomethyl ester was dependent on the availability of methanol, thus engaging a number of alternative mechanisms for the esterification process. Investigations on the mechanism of this reaction (Akhtar et al., 1985) have shown that during the transformation the bridge oxygen of the ester bond originates from the parent tetrapyrrole, which is consistent with the tenets of reaction 3.

A mandatory requirement of the proposed phytylation and methylation mechanisms is that *both* oxygen atoms at C-17^3 and C-13^3 of bacteriochlorophyll *a* (III) derived from [1,1,4-$^{18}O_3$]-5-aminolevulinic acid (Ib) are labeled with ^{18}O. The traditional method utilized for such an analysis has been mass spectrometry; however, despite recent technical advances, the mass spectral analysis of multiply isotopically enriched chlorins remains difficult.

Isotope shift effects have long been observed in NMR in the gas phase, in liquids, and in solution [for a review see Batiz-Hernandez and Bernheim (1967)]. Significantly, the ^{18}O isotope shift on the ^{31}P NMR signal of phosphate esters has provided a means to assess the stereochemical course at phosphorus for a number of biologically important transformations (Lowe, 1983). Although the prediction of a similar ^{18}O isotope shift effect on ^{13}C NMR spectra by Jameson (1977) was not demonstrated experimentally until 1979 (Risley & Van Etten, 1979), the technique has been increasingly utilized particularly in the delineation of secondary metabolite biosynthesis (Hutchinson et al., 1981; Cane et al., 1981; Lane et al., 1982; Ajaz & Robinson, 1983; McIntyre et al., 1984; Simpson et al., 1984).

Thus the stage was set for the application of ^{18}O-induced isotope shift in ^{13}C NMR to analyze the isotopic status of both bridge (—*O—C=O) and nonbridge (—O—C=*O) oxygen atoms at C-17^3 and C-13^3 in bacteriochlorophyll *a* biosynthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid (Id) and hence allow a critical scrutiny of the proposed mechanisms in reactions 1 and 3 (Akhtar et al., 1984, 1985). Fundamental to the success of our experimental approach was the resolution in the ^{13}C NMR spectrum of species containing ^{18}O in the bridge oxygen (—^{18}O—C=O), nonbridge oxygen (—O—C=^{18}O), and both oxygen atoms (—^{18}O—C=^{18}O) of the ester linkages at C-13^3 and C-17^3 of bacteriochlorophyll *a*. In the light of this requirement, samples of phytyl acetate containing ^{18}O selectively in bridge oxygen, nonbridge oxygen, and both oxygen atoms of the ester bond were prepared and utilized to optimize the ^{13}C NMR conditions prior to analysis of the biosynthetic bacteriochlorophyll *a* derived from [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid.

A preliminary report of part of this work is available (Emery & Akhtar, 1985).

Experimental Procedures

Chemicals. All deuteriated solvents for NMR spectroscopy and barium manganate were purchased from Aldrich Chem-

ical Co., Gillingham, Dorset. $H_2{}^{18}O$, [1,3-$^{13}C_2$]diethyl malonate, and [4-^{14}C]-5-aminolevulinic acid were from Amersham International, Amersham, Bucks. *R. sphaeroides* (NCIB 8253) was from Torry Research Station, Aberdeen. All other chemicals were from either Sigma Chemical Co., Poole, Dorset, or BDH Chemicals Ltd., Eastleigh, Hants.

[1-^{13}C,1,1-$^{18}O_2$]Acetic Acid. [1-^{13}C]Sodium acetate (10 mg; 112 μmol; 90% ^{13}C) was placed in a 5-mL constricted test tube, and $H_2{}^{18}O$ (98 atom % excess, 0.1 mL) followed by glacial acetic acid (30 μL; 600 μmol) was added. The solution was sealed under vacuum and then left for 24 h at 160 °C to effect the exchange reaction. Following cooling, the vial was opened and the contents were neutralized with an equimolar quantity of sodium hydroxide (1 M) and then lyophilized to yield [1-^{13}C,1,1-$^{18}O_2$]sodium acetate. Analysis of the product as its benzyl ester by gas chromatography–mass spectrometry (GC–MS) (*m/z* for unlabeled species 150) gave the following excess of the two isotopes: ^{13}C, 23 atom %; ^{18}O, 73 atom % (specifically, 16.4% $^{16}O_2$, 31.6% $^{18}O^{16}O$, and 53% $^{18}O_2$).

[1-^{13}C,1,1-$^{18}O_2$]Sodium acetate (18.6 mg; 0.22 mmol) was placed in the left arm (LA) of an apparatus shaped to enable the transfer of liquid under vacuum, and benzoic acid (300 mg; 2.45 mmol) was layered upon it. A glass wool plug compressed the solids to reduce the possibility of contamination by sublimation of the benzoic acid. The apparatus was then evacuated and the right arm (RA) placed in liquid nitrogen. Heat was applied to the LA by using a micro-Bunsen burner, and as a result the benzoic acid melted, converting sodium acetate into acetic acid, which condensed in the cooled RA. When all the benzoic acid had melted, the heat was removed and the apparatus maintained under vacuum for a further 5 min. The [1-^{13}C,1,1-$^{18}O_2$]acetic acid (85% conversion) was carefully dissolved in dry dichloromethane (2 mL), five molecular sieves (type 3A) were added to adsorb any moisture present, and then the solution was used for the synthesis of phytyl acetate (vide infra).

Phytal. Phytol was oxidized by the method described by Firouzabadi and Ghaderi (1978). Phytol (200 mg; 0.67 mmol) was dissolved in dry dichloromethane (10 mL) and stirred for 6 h in the dark at room temperature with barium manganate (2 g; 6.7 mmol). At the end of the reaction the barium manganate was removed by filtration and washed extensively with dry dichloromethane. Evaporation of the solvent in vacuo afforded phytal as a yellow oil (194 mg; 98%) that was chromatographically homogeneous [silica gel PF_{254} plates developed in light petroleum (bp 60–80 °C)/acetone (20:1 v/v) gave phytal, R_f 0.4, when visualized under UV light].

[1-^{18}O]Phytol. Phytal (125 mg; 0.43 mmol) was placed in a 5-mL constricted test tube and $H_2{}^{18}O$ (0.1 mL, 98 atom % excess) added, followed by freshly distilled tetrahydrofuran (1.4 mL) to achieve a homogeneous solution. After the contents were sealed under vacuum, the solution was heated for 1 h in a domestic pressure cooker at a setting corresonding to 15 psi. When cool, the tube was opened, redistilled methanol (1 mL) was added, followed by sodium borohydride (20 mg; 0.5 mmol) in methanol (1 mL), and the solution was left at room temperature for 20 min. Methanol was removed in vacuo and the residue extracted into diethyl ether (3 × 15 mL). The latter was washed with water (20 mL) and then dried over anhydrous sodium sulfate. Evaporation of the diethyl ether gave a yellow oil (78 mg; 62%) that was chromatographically and spectrally identical with authentic phytol. Analysis of the product as its trimethylsilyl ester by GC–MS gave peaks at *m/z* 353 (M − 15) and 355, yielding an ^{18}O enrichment of 45%.

Phytyl Acetate. Phytyl acetate was synthesized by the esterification procedure described by Neisis and Steglich (1978). Phytol (52 mg; 0.175 mmol) was dissolved in dry dichloromethane (5 mL), and then *N,N'*-dicyclohexylcarbodiimide (36.1 mg; 0.175 mmol) and 4-pyrrolidinopyridine (2.6 mg; 0.175 mmol) were added, followed by acetic acid (10.5 mg; 0.175 mmol). The mixture was stirred at room temperature for 5 h. At the end of the reaction dicyclohexylurea was removed by filtration and the resulting filtrate evporated in vacuo to afford phytyl acetate as an oil (53 mg; 90%) that was chromatographically homogeneous [silica gel PF_{254} plates developed in light petroleum (bp 60–80 °C)/acetone (20:1 v/v) gave phytyl acetate, R_f 0.3, when visualized with sulfuric acid/methanol (5% v/v)]. The ^{1}H NMR spectrum of the phytyl acetate in deuteriated chloroform exhibited a resonance at 2.0 ppm corresponding to the acetyl methyl hydrogen atoms, in addition to the ^{1}H NMR resonances expected for phytol (Boxer et al., 1974).

Phytyl acetate samples selectively enriched with ^{18}O and containing ^{13}C in the acetyl carbonyl carbon were prepared as above by combining the appropriately enriched reactants.

[1-^{13}C]-5-Aminolevulinic Acid Hydrochloride (Hydrochloride of Ic). The synthesis of [1-^{13}C]-5-aminolevulinic acid was achieved by adapting the method of Pichat et al. (1966). Production of 1-bromo-3-phthalimidopropanone (XII) followed the aforementioned method; however, introduction of the crucial ^{13}C label is described in detail below.

Ethyl 2-(Ethoxycarbonyl)-4-oxo-5-phthalimido[1,3-$^{13}C_2$]Pentanoate (XIII). Sodium hydride (80%; 42 mg; 1.4 mmol) was washed with dry light petroleum (bp 60–80 °C) under argon to remove traces of oil and then dried under argon. Dry dimethylformamide (4 mL) was added followed by a solution of [1,3-$^{13}C_2$]diethyl malonate (0.25 g; 1.56 mmol) in dimethylformamide (5.5 mL). The resulting cloudy suspension was stirred for 1 h under argon, during which time a clear solution was obtained.

To the above diethyl malonate anion was added, dropwise and with stirring, a solution of XII (401 mg; 1.42 mmol) in dimethylformamide (14.5 mL) via a 20-mL syringe. Stirring was continued for 24 h under argon, during which time the reaction mixture became slightly orange in color. The solvent was removed by rotary evaporation in vacuo and the resulting orange-brown liquid dissolved in dichloromethane/*n*-hexane (1:1 v/v; 2 mL), and after filtration the solution was subjected to chromatography using a silica gel 60H column (50 × 25 mm) previously equilibrated in dichloromethane/*n*-hexane (1:1 v/v). The product was eluted with a 0–60% linear gradient of diethyl ether in the application solvent (total volume of gradient, 150 mL). Analysis of the column eluant by TLC on silica gel PF_{254} plates developed in dichloromethane/methyl acetate (9:1 v/v) and visualized under UV light showed that fractions corresponding to an elution volume of 50–70 mL contained the condensation product (R_f 0.45). The relevant fractions were evaporated in vacuo to yield XIII as white crystals (307 mg; 60%), mp 132–133 °C.

[1-^{13}C]-5-Aminolevulinic Acid Hydrochloride (Hydrochloride of Ic). Compound XIII was deprotected and decarboxylated in a mixture of concentrated hydrochloric acid (8 mL) and glacial acetic acid (8 mL) for 14 h under reflux. Acetic acid was removed by evaporation and azeotroped with water (10 mL). The remaining solution was continuously ether extracted for 24 h to remove phthalic acid and then the aqueous phase was evaporated in vacuo to yield a light brown oil, which was dissolved in water (5 mL) and lyophilized. This procedure was repeated twice more to remove traces of hy-

drochloric acid. After lyophilization the off-white solid was recrystallized from methanol/ethyl acetate to yield white crystals of [1-^{13}C]-5-aminolevulinic acid hydrochloride (136 mg; 98%), mp 144 °C [for the unlabeled compound, Neuberger and Scott (1954) quote mp 145 °C].

[1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid Hydrochloride (Hydrochloride of Id). The preceding hydrochloride (53 mg; 0.316 mmol) was placed in a 5-mL constricted test tube and dissolved in $H_2{}^{18}O$ (98 atom % excess; 0.1 mL). After the addition of hydrochloric acid (0.2 M; 7 μL) the vial was sealed under vacuum and then heated for 1.5 h in a domestic pressure cooker at a setting corresponding to 15 psi. The vial was cooled, and its contents were lyophilized to give a cream-colored residue that was recrystallized from methanol/ethyl acetate to produce white crystals of [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid hydrochloride (49 mg; 93%) with the following excess of each isotope: ^{13}C, 90 atom %; average ^{18}O, 73.5 atom % (specifically, 11% $^{16}O_2$, 36% $^{18}O^{16}O$, and 53% $^{18}O_2$).

Assessment of ^{18}O in the product was either by ^{13}C NMR directly or by oxidative degradation to succinic acid followed by gas chromatography–mass spectrometry, as previously described (Akhtar et al., 1984).

Incorporation of [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid (Id) into Bacteriochlorophyll a. A McCartney bottle containing 8 mL of Lascelles medium S (Lascelles, 1960) was inoculated by *R. sphaeroides* and incubated at 29 °C beneath a 60-W tungsten lamp. When the culture had reached mid-logarithmic phase of growth, it was used to inoculate 40 mL of culture medium contained within a 100-mL conical flask. The 40-mL culture, when grown, was then utilized to inoculate 360 mL of culture medium in a 500-mL conical flask to yield an absorption at 700 nm between 0.4 and 0.7. At this juncture a mixture of [4-^{14}C]- and [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid hydrochloride (30 μmol; isotopic status as described above, 7.3×10^4 dpm ^{14}C/μmol) was added, and the cells were allowed to grow for a further 24 h (final A_{700} = ca. 2.8). After the 24-h incubation, cells were harvested by centrifugation at 10000*g*, and bacteriochlorophyll *a* was extracted and purified as previously described (Akhtar et al., 1984).

^{13}C NMR Parameters. All spectra were obtained by using a Bruker WH400 spectrometer operating at 100.53 MHz in the ^{13}C mode with quadrature detection and broad-band decoupling. All NMR shifts are expressed relative to tetramethylsilane with the resonant positions of either deuteriated acetone or deuteriated methanol used as the internal standard.

Phytyl acetate samples (25 mg) were dissolved in deuteriated trichloromethane (0.6 mL) and the spectra accumulated at 303 K in a 5-mm-bore tube by using a sweep width of 200 Hz with an 8K data block, 296 scans, a pulse angle of 70°, and an acquisition time of 2.56 s. For resolution enhancement a Gaussian multiplier of 0.25 was applied; 0.049 Hz/point was used, and the free induction decay (FID) was zero filled to 16K prior to Fourier transformation.

[1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic acid hydrochloride (1 mg) was dissolved in deuteriated methanol (0.5 mL), and spectra were accumulated at 297 K by using a sweep width of 254 Hz, 178 scans, a pulse angle of 48°, and an acquisition time of 4.033 s.

Bacteriochlorophyll *a* (25 mg, ^{13}C enrichment 23%) was dissolved in deuteriated acetone/deuteriated methanol (4:1 v/v; 0.6 mL) and the spectrum accumulated at 297 K by using a sweep width of 1000 Hz with 32 K data block, 1464 scans, a pulse angle of 48°, and an acquisition time of 4.096 s.

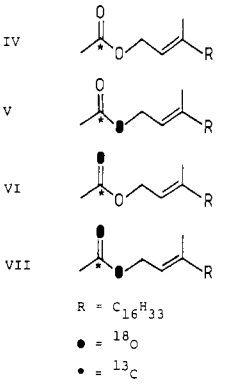


FIGURE 2: Structures of phytyl acetate used for ^{13}C NMR analyses.

The spectra of the same bacteriochlorophyll *a* sample dissolved in acetone/deuteriated pyridine (3:1 v/v; 0.6 mL) were accumulated at 297 K by using a sweep width of 452 Hz with 16K data block, 969 scans, a pulse angle of 60°, and an acquisition time of 4.526 s.

In both cases, resolution enhancement was achieved by applying a line broadening factor of −0.5 together with a Gaussian multiplier of 0.17; 0.055 Hz/data point was used, and the FID was zero filled to 4K prior to Fourier transformation.

Results and Discussion

Synthesis and ^{13}C NMR Analysis of Phytyl Acetate Samples Enriched with ^{13}C and ^{18}O. The semimicrosynthesis of phytyl acetate was conveniently achieved by coupling phytol and acetic acid via dicyclohexylcarbodiimide in the presence of 4-pyrrolidinopyridine (Neisis & Steglich, 1978). Suitably ^{18}O-enriched reactants were prepared by exchange with $H_2{}^{18}O$ (for details see Experimental Procedures) and then combined appropriately to produce the desired phytyl acetate samples (IV–VII, Figure 2). The high-resolution partial ^{13}C NMR spectrum of each species is shown in Figure 3. The enriched carbon resonance (170.796 ppm) of V, containing ^{18}O only in the bridge oxygen atom (—^{18}O—^{13}C=^{16}O), exhibited an upfield shift of 1.233 Hz relative to its ^{16}O counterpart (Figure 3A) whereas VI containing ^{18}O in the nonbridge oxygen atom (—^{16}O—^{13}C=^{18}O) exhibited an upfield shift of 3.714 Hz relative to —^{16}O—^{13}C=^{16}O (Figure 3B). In light of these data the spectrum obtained for VII containing ^{18}O in both oxygen atoms could be rationalized (Figure 3C) since the shift (5.079 Hz) in this case is equal to the sum of the shifts for two single-labeled species.

The abundance of each isotopomer was deduced from peak intensities (or peak integration) in the ^{13}C NMR spectrum and then compared with the mass spectral data obtained for the same species (phytyl acetate, m/z 335; data not shown). In all cases both mass spectral and NMR estimations of isotopic abundance correlated within 5%. These experiments suggested that, under appropriate conditions, NMR resonances due to all the three ^{18}O-containing phytyl acetate isotopomers (V–VII) can be resolved from each other. Also, since the NMR data and mass spectral analyses were in accord, the conditions used to accumulate the spectra clearly did not produce too much saturation, nor did the individual isotopomers exhibit differential relaxation.

Synthesis of [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid (Id) and Its Incorporation into Bacteriochlorophyll a. The synthesis of [1-^{13}C]-5-aminolevulinic acid (Ic) as its hydrochloride was achieved by using the method described by Pichat et al. (1966), except that the ^{13}C label was introduced as [1,3-

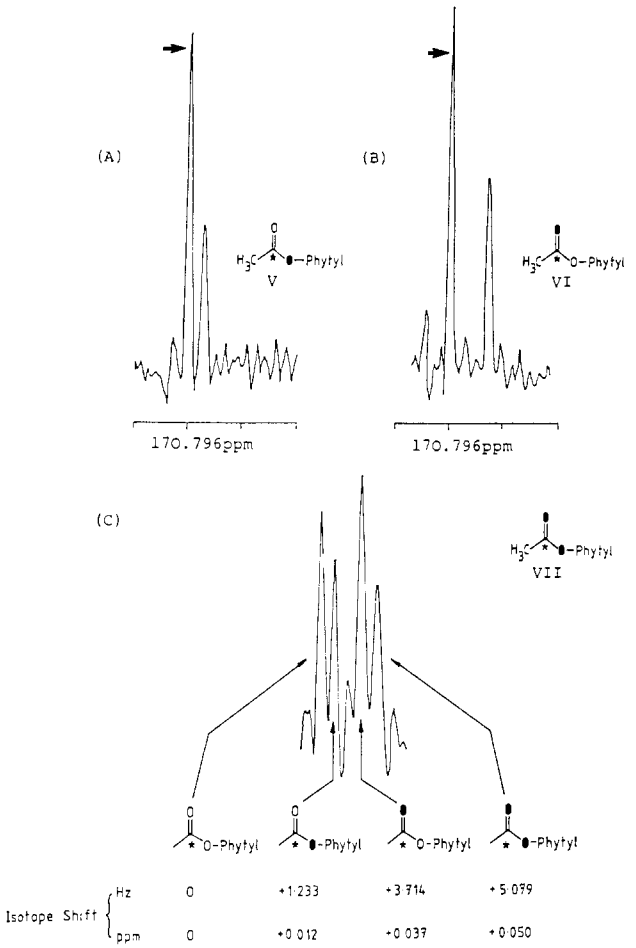


FIGURE 3: Partial ^{13}C NMR spectrum of phytyl acetate samples containing ^{18}O in the bridge oxygen (A), nonbridge oxygen (B), and both oxygen atoms (C). The $—^{16}O—^{13}C=^{16}O$ resonance at 170.796 ppm is indicated by an arrow for the spectra shown in (A) and (B); each division of the scale in (A) and (B) is 0.05 ppm. ^{13}C NMR parameters are given under Experimental Procedures.

$^{13}C_2$]diethyl malonate at the penultimate stage of the synthesis (Figure 4). All reactions proceeded smoothly to give pure [1-^{13}C]-5-aminolevulinic acid in an overall yield of 60%, based on [1,3-$^{13}C_2$]diethyl malonate. The [1-^{13}C]-5-aminolevulinic acid was enriched with ^{18}O by autoclaving a solution of the former in $H_2{}^{18}O$ for 1.5 h in the presence of a trace of HCl. The resulting material, which contained ^{18}O at C-4 as well as C-1, was either analyzed directly by ^{13}C NMR to yield the distribution of ^{18}O at C-1 or incubated at pH 6.9 for 24 h to exchange the relatively labile ^{18}O at C-4 and then oxidized with $NaIO_4$ to give succinic acid (Akhtar et al., 1984). The latter, after conversion into its bis(trimethylsilyl) ester derivative, was analyzed by gas chromatography–mass spectrometry. Both analyses produced similar values for ^{18}O enrichment (±5%), yielding an average ^{18}O content at C-1 of 73.5% (specifically, 11% $^{16}O_2$, 36% $^{18}O^{16}O$, and 53% $^{18}O_2$).

Conditions for the manipulation of *R. sphaeroides* to preferentially incorporate exogenously added 5-aminolevulinic acid have previously been established in our laboratory (Akhtar et al., 1984, 1985; Emery & Akhtar, 1985). Using this experience, we supplemented a culture medium (360 mL) containing a freshly grown inoculum of *R. sphaeroides* (40 mL) with a mixture of [4-^{14}C]- and [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid, and growth was allowed to proceed for 24 h at 29 °C under illumination from a tungsten lamp. The cells were harvested and processed to give after purification bacteriochlorophyll *a* (3.4 μmol) whose specific activity revealed that 22% of the biosynthetic pigment had originated from

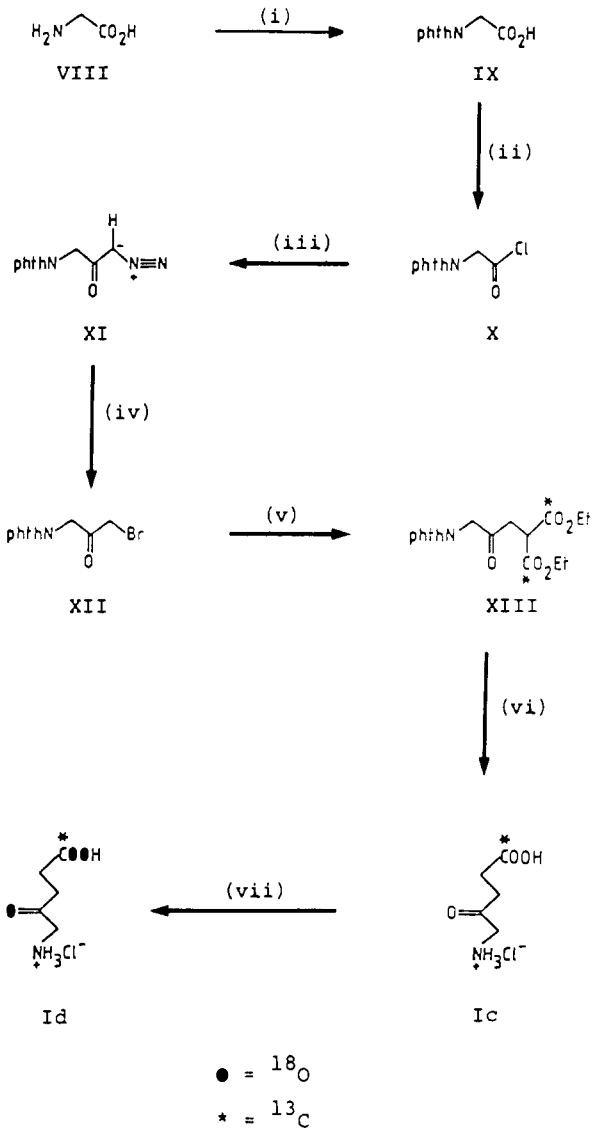


FIGURE 4: Synthetic route to [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid. Reagents: (i) phthalic anhydride; (ii) $SOCl_2$; (iii) CH_2N_2; (iv) HBr; (v) [1,3-$^{13}C_2$]diethyl malonate; (vi) AcOH, 7 M HCl; (vii) $H_2{}^{18}O$, HCl.

exogenously added [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid. In order to aid facile NMR analysis, eight parallel 400-mL incubations were performed, each containing 30 μmol of [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid, and yielding ca. 27 μmol of bacteriochlorophyll *a* in total.

^{13}C NMR Analysis of Bacteriochlorophyll a Derived from [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid. The high-resolution 100.53-MHz ^{13}C NMR spectrum of the biosynthetic sample of bacteriochlorophyll *a* showed the C-17[3] resonance at 173.86 ppm to consist of four components corresponding to the $—^{16}O—^{13}C=^{16}O$, $—^{18}O—^{13}C=^{16}O$, $—^{16}O—^{13}C=^{18}O$, and $—^{18}O—^{13}C=^{18}O$ species, respectively (Figure 5B). The upfield isotope shifts for these species (1.509, 3.802, and 5.275 Hz) are consistent with the values obtained for the model phytyl acetate samples (see Figure 3) and other compounds (Risley & Van Etten, 1980; Vederas, 1980). A comparison of the isotopic ratios determined from the intensities of the ^{13}C resonances for C-17[3] (13.5% $—^{16}O—^{13}C=^{16}O$, 19.5% $—^{18}O—^{13}C=^{16}O$, 18.5% $—^{16}O—^{13}C=^{18}O$, and 48.5% $—^{18}O—^{13}C=^{18}O$) with those for C-1 of the starting 5-aminolevulinic acid demonstrated that retention of ^{18}O at *both* oxygen atoms was in excess of 90% (means of six independent experiments, Table I). As expected, the intensities of C-17[3]

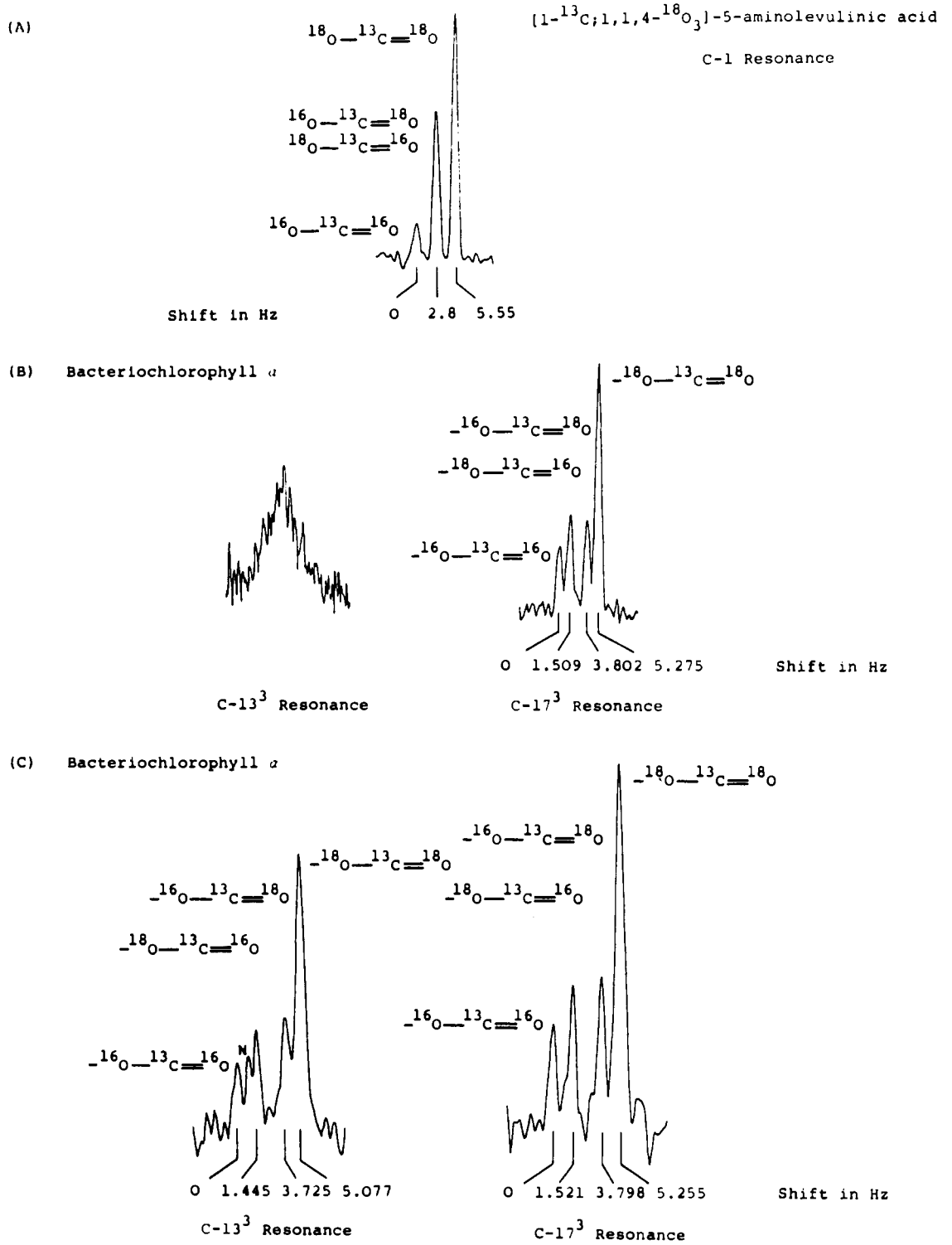


FIGURE 5: Partial ^{13}C NMR spectrum of the following: (A) [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid, illustrating the isotopic distribution at C-1 (176.273 ppm). (B) Bacteriochlorophyll *a* biosynthesized from the [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid above, dissolved in deuteriated acetone/deuteriated methanol (4:1 v/v) and illustrating the isotopic distribution at C-17^3 (173.860 ppm). It should be noted that under these conditions the C-13^3 resonance (172.260 ppm) remains unresolved. (C) As in (B) except dissolved in deuteriated acetone/deuteriated pyridine (3:1 v/v) to reveal the isotopic distribution at C-17^3 (173.078 ppm) and C-13^3 (171.41 ppm). ^{13}C NMR parameters are detailed under Experimental Procedures.

signals due to —^{18}O—^{13}C=^{16}O and —^{16}O—^{13}C=^{18}O were equal, and their sum (38%) is equivalent to the intensity of single-labeled oxygen species in the starting 5-aminolevulinic acid (36%).

Unfortunately, under the conditions used to obtain the spectrum of C-17^3 (bacteriochlorophyll *a* dissolved in deuteriated acetone/deuteriated methanol, 7:2 v/v) the C-13^3 resonance at 172.26 ppm, despite possessing the same peak area (as judged by integration) as the C-17^3 resonance, was substantially broadened such that an accurate assessment of ^{18}O was impossible. In order to comprehend this aspect of the spectrum and, hence, circumvent it, an inspection of the coordination chemistry of the chlorophylls is required. Inorganich chemistry dictates the magnesium prefers to be five- or six-coordinate (Katz, 1968, 1978; Fong & Koester, 1975); however, only four sites are occupied by the pyrrolic nitrogens

Table I: Summary of Intensities of Individual Isotopomers within the C-13[3] and C-17[3] ^{13}C NMR Resonances of Bacteriochlorophyll *a* Biosynthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid[a]

isotopomer and position	intensity (%)			
	—^{16}O—^{13}C=^{16}O	—^{18}O—^{13}C=^{16}O	—^{16}O—^{13}C=^{18}O	—^{18}O—^{13}C=^{18}O
5-aminolevulinic acid				
C-1	11	36[b]		53
bacteriochlorophyll *a*				
C-13[3]	12.5	18.2	20	49.3
C-17[3]	14	18.5	19.5	48

[a] For comparison, the intensity of isotopomers within C-1 of the starting 5-aminolevulinic acid is shown. [b] Combined intensity due to the two single-labeled species —C(=O)—^{18}OH and —C(=^{18}O)—OH.

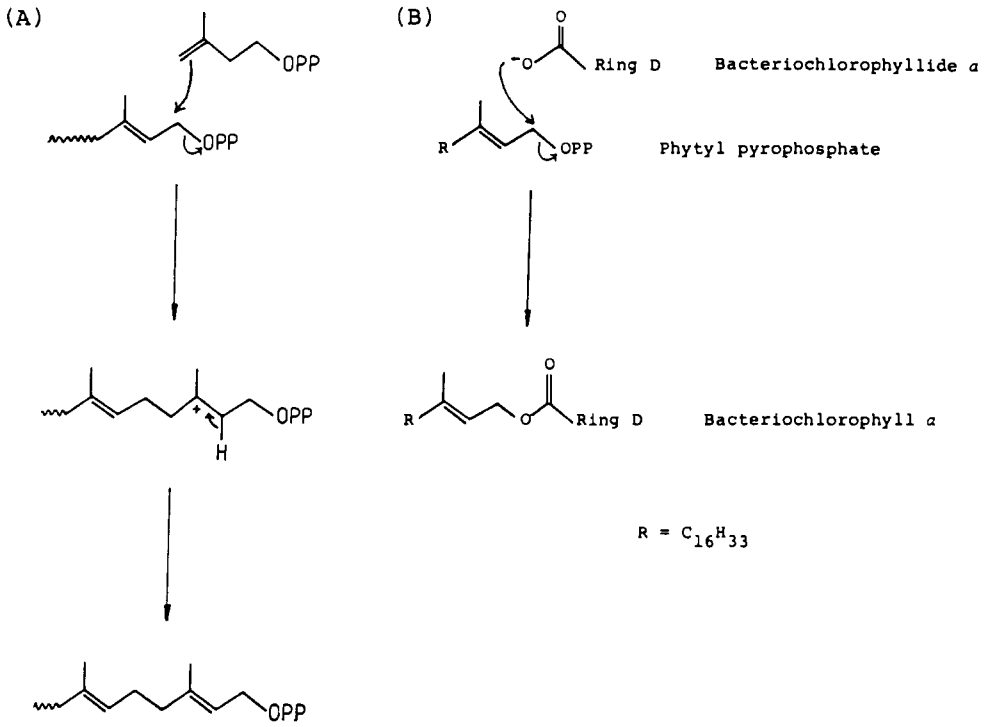


FIGURE 6: Comparison of the transfer of isoprenyl units to olefinic nucleophiles during the biosynthesis of the polyisoprenoids (A) with the esterification of the D-ring carboxylate nucleophile of bacteriochlorophyllide *a* by phytyl pyrophosphate to yield bacteriochlorophyll *a* (B).

in bacteriochlorophyll *a*. In the absence of external basic ligands, the vacant coordination sites are occupied by further bacteriochlorophyll *a* molecules coordinating directly via the E-ring carbonyl group or indirectly via a water molecule that hydrogen bonds to the E-ring carbonyl. Hence all chlorophylls tend to self-aggregate, leading to line broadening in the ^{13}C NMR spectra. As expected in the ^{13}C NMR spectrum of the biosynthetic bacteriochlorophyll *a* in [$^2H_2$]dichloromethane, neither the C-17[3] nor the C-13[3] resonances were resolved into their individual components (data not shown). Solutions containing external ligands such as methanol reduce aggregation and clearly resolve the C-17[3] but not the C-13[3] resonances (Figure 5B). It can, therefore, be expected that both C-17[3] and C-13[3] resonances will be resolved in the presence of stronger basic ligands, e.g., pyridine (Katz, 1968, 1978; Katz et al., 19766; Fong & Koester, 1975; Brereton & Saunders, 1983a,b).

With the above information assimilated, the ^{13}C NMR spectrum of the biosynthetic bacteriochlorophyll *a* dissolved in deuteriated acetone/deuteriated pyridine (3:1 v/v) was obtained and is shown in Figure 5C. Two differences emerge from a comparison of this spectrum with that shown in Figure 5B: first, the absolute resonant positions of C-17[3] and C-13[3] are shifted downfield by ca. 0.8 ppm and now resonate at 173.078 and 171.411 ppm, respectively, and second, both the C-17[3] and C-13[3] resonances are resolved into their constituent

Table II: Summary of Upfield Isotope Shifts Observed for the C-1 Resonance of [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid Dissolved in [$^2H_4$]Methanol and for the C-13[3] and C-17[3] Resonances of Bactriochlorophyll *a* Biosynthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-5-Aminolevulinic Acid Dissolved in [$^2H_6$]Acetone/[$^2H_5$]Pyridine (3:1 v/v)

isotopomer	5-aminolevulinic acid, C-1	bacteriochlorophyll *a*	
		C-13[3]	C-17[3]
—^{16}O—^{13}C=^{16}O	0	0	0
—^{18}O—^{13}C=^{16}O	2.8	1.445	1.521
—^{16}O—^{13}C=^{18}O		3.725	3.798
—^{18}O—^{13}C=^{18}O	5.55	5.077	5.255

isotopomers. The C-13[3] resonance appears as four components corresponding to the four isotopomers expected. The upfield isotope shifts for the ^{18}O-bearing species (1.445, 3.725, and 5.077 Hz) are almost identical with those obtained for the isotopomers at C-17[3] (1.521, 3.798, and 5.255 Hz) except for a spike, interpreted as noise, at 0.827 Hz (Figure 5C and Table II). A comparison of the isotopic ratios determined from the intensities of the ^{13}C resonances for C-13[3] above (12.5% —^{16}O—^{13}C=^{16}O, 18.2% —^{18}O—^{13}C=^{16}O, 20% —^{16}O—^{13}C=^{18}O, and 49.3% —^{18}O—^{13}C=^{18}O) with those for the C-1 of the starting aminolevulinic acid demonstrates that retention of ^{18}O at both oxygen atoms was in excess of 90% and that the sum of the intensities of the single-labeled species (38.2%)

is equal to the intensity of single-labeled oxygen species in the starting aminolevulinic acid (36%) (Table I).

Conclusions

Continuing our work on the elucidation of the mechanisms of enzymic reactions that participate in tetrapyrrole biosynthesis (Akhtar et al., 1976; Akhtar & Jordan, 1979; Barnard & Akhtar, 1979; Jones et al., 1984; Seehra et al., 1984), we have now studied the chemistry through which the two ester bonds at positions C-13^3 and C-17^3 of chlorophylls are produced. For this purpose, a sample of 5-aminolevulinic acid labeled at its C-1 with ^{13}C and ^{18}O was synthesized and incorporated into bacteriochlorophyll *a* of *R. sphaeroides*. Since the C-13^3 and C-17^3 atoms of chlorophylls originate from C-1 of 5-aminolevulinic acid (see Figure 1), the mechanism of the esterification reaction may be inferred from knowledge of the fate suffered by the carboxyl oxygen atom(s) of the appropriately labeled 5-aminolevulinic acid during the incorporation of the latter into bacteriochlorophyll *a*. The ^{18}O contents of the precursor and the product were determined through the application of the ^{18}O-induced shift in ^{13}C NMR in conjuction with mass spectrometry. When the three ^{18}O-containing isotopomers ($-^{18}O-^{13}C=^{16}O$, $-^{16}O-^{13}C=^{18}O$, and $-^{18}O-C=^{18}O$) of the two ester bonds at C-13^3 and C-17^3 in a sample of biosynthetic bacteriochlorophyll *a* were viewed by ^{13}C NMR, their relative proportions were identical with those of the corresponding species present in the precursor 5-aminolevulinic acid (Table I). The results clearly show the retentions of the two carboxyl oxygen atoms in the formation of the ester bonds at C-13^3 and C-17^3, suggesting that these bonds are elaborated through carboxy–alkyl transfer processes exemplified in reactions 1 and 3 of the introduction. Since the retention of both oxygen atoms at C-13^3 and C-17^3 was in excess of 90%, under in vivo conditions the biosynthetic flux through the pathway operating via these mechanisms must be at least 90% in *R. sphaeroides*.

Our ^{18}O results for C-13^3 are compatible with earlier findings of Gibson et al. (1963) who showed that in vitro the methylation of Mg–protoporphyrin IX requires *S*-adenosylmethionine. This is because methyl transfer in such a reaction would be expected to occur via a carboxy–alkyl transfer process leading to the retention of both the original oxygen atoms of the acid in the ester. It should however be emphasized that the results could also be used to rationalize the participation of methanol (Rebeiz & Castelfranco, 1971) in the methylation process by making the assumption that methanol is activated for methyl transfer by conversion either to a phosphorylated derivative or to *S*-adenosylmethionine.

The results presented for C-17^3 showing the retention of both the oxygen atoms extend our previous work in which the retention of only the bridge oxygen of bacteriochlorophyll *a* was demonstrated (Akhtar et al., 1984) and combined with the results of Rüdiger et al. (1980) allow the mechanism shown in reaction 1 to be vindicated. At present, there is conflicting evidence as to whether the chlorophyll synthetase catalyzed esterification of chlorophyllide *a* at C-17^3 (reaction 1) occurs with geranylgeranyl pyrophosphate, which is subsequently reduced to yield the phytyl side chain, or whether reduction occurs prior to esterification since both reaction pathways have been demonstrated in spinach (Soll et al., 1983; Shioi & Sasa, 1983). In broad mechanistic terms, the chlorophyll synthetase catalyzed esterification process (reaction 1) is equivalent to the transfer of isoprenyl units in the formation of geranyl, farnesyl, and geranylgeranyl pyrophosphates except that in these latter examples the displacement reaction involves the participation of an olefinic nucleophile (Figure 6).

Acknowledgments

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Registry No. Ic, 98599-93-0; Ic·HCl, 106213-17-6; Id, 98599-94-1; Id·HCl, 106213-18-7; III, 17499-98-8; XII, 6284-26-0; XIII, 106213-16-5; $H_2{}^{18}O$, 14314-42-2; [1-^{13}C,1,1-$^{18}O_2$]acetic acid, 77934-46-4; [1-^{13}C]sodium acetate, 23424-28-4; [1-^{13}C,1,1-$^{18}O_2$]sodium acetate, 83587-72-8; phytal, 13754-69-3; phytol, 150-86-7; [1-^{18}O]phytol, 106213-15-4; phytyl acetate, 10236-16-5; acetic acid, 64-19-7; [1,3-$^{13}C_2$]diethyl malonate, 77386-82-4.

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