Mechanism of the Phytylation Step in Bacteriochlorophyll a Biosynthesis

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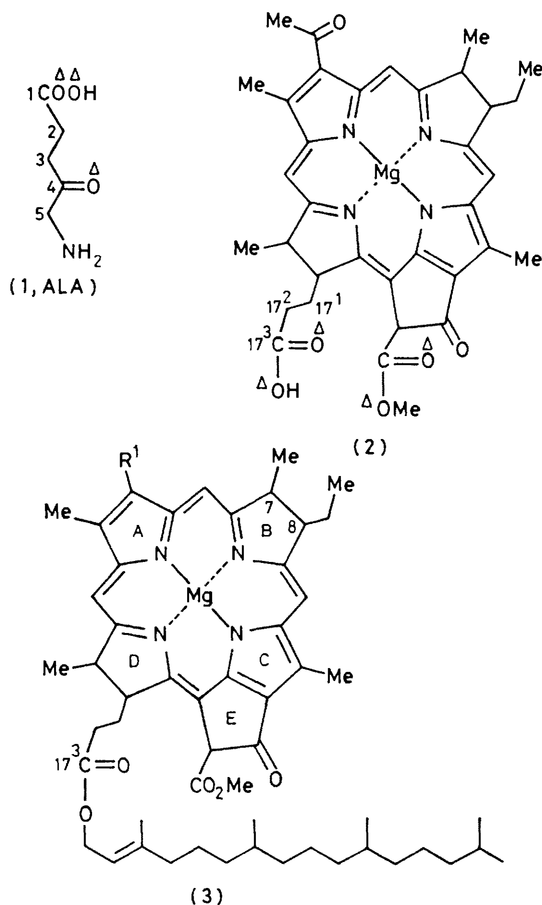
Summary It is shown that the bridge oxygen (*) of the ester bond of bacteriochlorophyll a, –CO–$\overset{*}{O}$–phytyl, originates from one of the C-17^3 carboxy-oxygens of bacteriochlorophyllide a (**2**).

THE ring D propionate-carboxy-groups of all naturally occurring chlorophylls (**3**) are esterified[1] with long chain alcohols The nature of the esterifying alcohol varies considerably and has been shown to be phytol for plant



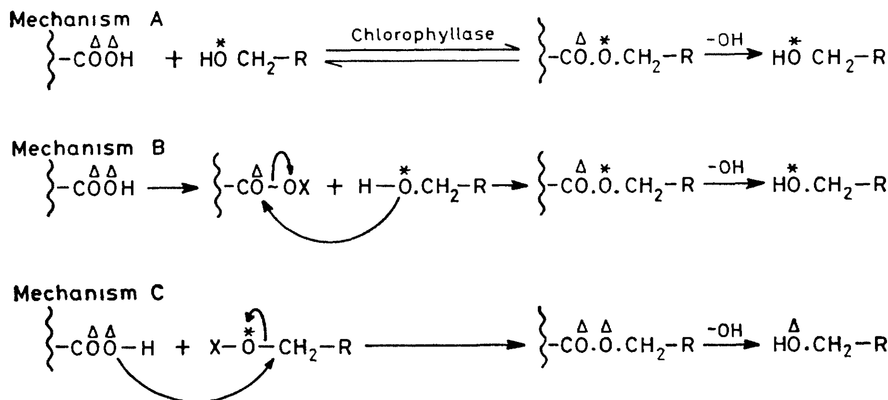
(**3a**) Plant chlorophyll a, R^1 = vinyl, Δ^7
(**3b**) Bacteriochlorophyll a from *R spheroides*, R^1 = Ac, no Δ^7

chlorophylls[2] and bacteriochlorophyll a from *R spheroides*,[3] geranylgeraniol for bacteriochlorophyll a from *R rubrum*,[4] and farnesol for *Chlorobium* chlorophylls[5] It is generally agreed that the esterification process occurs during the final stages of the biosynthesis of chlorophylls On the basis of chemical analogies, three types of mechanism (Scheme) may be considered Mechanism A assumes that the esterification step is merely the reversal of the hydrolytic reaction catalysed by chlorophyllase This was the view that prevailed[6] for nearly half a century, but has been questioned in recent years because of the unfavourable equilibrium of the chlorophyllase-catalysed reaction in the direction of esterification and the consideration that biosynthesis and degradation reactions are usually catalysed by separate enzymes[1,7] Consequently, two other mechanisms may be considered which involve the participation of either an activated-ester (mechanism B) or an activated-alcohol (mechanism C), producing the ester bond by acyl-oxy or carboxyl-alkyl transfers, respectively

We envisaged that these mechanistic alternatives may be differentiated by studying the fate of the C-17^3 oxygen atoms of a chlorophyllide [structure of type (**2**)] during its conversion into the corresponding chlorophyll (**3**) The key feature of our approach involved the production, under *in vivo* biosynthetic conditions, of a species of chlorophyllide containing $^{18}O_2$ at C-17^3 (**2**, $O^\Delta = {}^{18}O$) This was achieved by manipulating *R spheroides* preferentially to incorporate exogenously added [1-$^{18}O_2$]-5-aminolaevulinate (ALA, **1**, $O^\Delta = {}^{18}O$) into bacteriochlorophyllide a (**2**) and thence into bacteriochlorophyll a (**3b**)

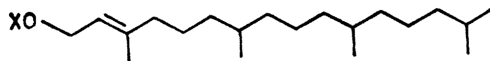
[1—$^{18}O_2$]ALA was synthesized by autoclaving a solution of ALA (50 mg) in ^{18}O-water (0 1 ml, containing 84 atom% excess of ^{18}O) for $2\frac{1}{2}$ h in the presence of a trace of HCl The resulting material, which contained ^{18}O at C-4 as well as C-1, was incubated at pH 6·9 for 24 h to exchange the relatively labile ^{18}O at C-4 and then oxidised with $NaIO_4$ to give succinic acid The latter, after conversion into its bis-(trimethylsilyl) ester derivative, was analysed by g c -m s which showed that the parent ALA (**1**) contained 66 atom% excess of ^{18}O at each of the two C-1 oxygen atoms

A culture medium[8] (360 ml) containing a freshly grown inoculum of *R spheroides* (40 ml) was supplemented with [4—^{14}C, 1,4-$^{18}O_3$]ALA (60 μmol, ^{14}C specific activity, 2 5 × 10^4 d p m /μmol, 66 atom% excess of ^{18}O at each of the two C-1 oxygen atoms) and the growth allowed to proceed for 24 h at 27 °C under illumination from a 150 W tungsten lamp The cells were harvested and processed to give, after purification, bacteriochlorophyll a (2 4 μmol) containing 0 16 × 10^6 d p m of ^{14}C, thus showing that 33·3%



SCHEME Three possible mechanisms for the esterification step in chlorophyll biosynthesis The partial structure represents the D ring propionate carboxyl group of chlorophyllide R–CH_2– represents a C_{20} isoprenyl unit, OX is a biological leaving group

of the biosynthetic pigment had originated from exogenously added ALA. The bacteriochlorophyll a was hydrolysed with NaOH and the resulting phytol converted into its trimethylsilyl ether (**4**). The latter, when analysed by g.c.–m.s., gave a fragment at *m/e* 353 (intensity normalized to 100) due to the dimethylsilyl phytol radical (**5**) and an



(**4**) X = Me_3Si—
(**5**) X = $Me_2\dot{S}i$—

isotope peak at *m/e* 355 (relative intensity 23·3 corrected for natural abundance, see below) due to the corresponding derivative produced from [1-^{18}O]phytol. From the cumulative data the phytol was calculated to have 19 atom% excess of ^{18}O which compares favourably with the predicted value of 21·9 atom% for the case where the bridge oxygen of the ester bond of bacteriochlorophyll a originated from [1—$^{18}O_2$]ALA *via* [17^3–$^{18}O_2$]bacteriochlorophyllide a (**2**)† ($O^{\Delta} = {}^{18}O$). In control experiments, when commercial phytol or phytol isolated from bacteriochlorophyll a biosynthesised as above but from [1–$^{16}O_2$]ALA, was analysed by mass spectrometry, the intensity of the peak at *m/e* 355 was that expected for natural abundance (5·2% of that of the 353 peak). These findings were confirmed in six biosynthetic experiments using three independently prepared batches of [1–$^{18}O_2$]ALA.

The cumulative results are in accord with mechanism C in which the ester bond formation occurs by the nucleophilic attack of the 17^3-carboxylate anion on the activated form of a isoprenyl alcohol. That the activated species may be an isoprenylpyrophosphate derivative is suggested by the recent findings of Rüdiger and co-workers[9] who, using a cell-free system from maize seeds, showed the incorporation of geranylgeranylpyrophosphate into a chlorophyll fraction, albeit in low yield (about 1%). Our experiments, which show an exceptionally high transfer of ^{18}O from the C-1 of ALA to the bridge position of the ester bond of bacteriochlorophyll a, not only shed light on the mechanism of the isoprenylation step but also give quantitative information on the biosynthetic flux through the pathway operating *via* mechanism C under *in vivo* conditions. This is estimated to be at least 90%‡ for *R. spheroides*.

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† Mechanistic considerations require that both the oxygen atoms of the CO_2Me group of (**2**) are also labelled with ^{18}O, but this has not yet been demonstrated experimentally.

‡ Based on an average value from six experiments.

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