Stereochemical Course of the Formation of the *C(7)*-Formyl Group from a Chiral Methyl Group during the Transformation of Chlorophyllide *a* into Chlorophyllide *b*

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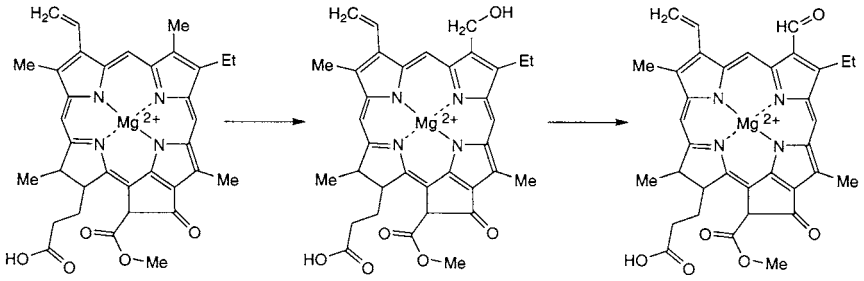
In appreciation of the contribution by *Duilio Arigoni* to the understanding of the stereochemistry of enzyme-catalysed reactions.

The biosynthesis of chlorophyll *a* and chlorophyll *b* from (2*R*,3*R*)- and (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid in greening barley has established that chlorophyllide *a* oxidase catalyses the transformation of the methyl group at C(7) of chlorophyllide *a* into the CHO group of chlorophyllide *b* with the loss of H^{Si} from the 7-(hydroxymethyl)chlorophyllide intermediate.

Introduction. – Chlorophyll *a* differs from chlorophyll *b* only in that the Me side chain at C(7) has been oxidised to a formyl (CHO) group. Chlorophyll *b* is essential for photosynthesis in all higher plants. Intuitively, the transformation of the Me group of chlorophyll *a* into a CHO group would occur in two distinct enzyme-catalysed oxidative steps, the first oxidation involving the insertion of an O-atom to form a hydroxymethyl (CH_2OH) intermediate, and the second, yielding the CHO moiety (*Scheme 1*).

Scheme 1. *Transformation of Chlorophyllide* a *to Chlorophyllide* b via *the 7-(Hydroxymethyl) Intermediate Catalysed by Chlorophyllide* a *Oxidase* (CAO)



Early investigations suggested that O_2 is important for the *in vitro* conversion of [^{14}C]chlorophyll *a* to chlorophyll *b* [1], and more-recent studies [2][3] established conclusively that O_2 is incorporated as the formyl O-atom of chlorophyll *b*. Little information was available, however, about the nature of the enzyme responsible for the

conversion, and whether the true substrate involved was chlorophyllide *a* or chlorophyll *a*. Suggestions that chlorophyllide *a* may be the substrate arose from the observation that labeled phytol was more favorably incorporated into chlorophyll *b* than into chlorphyll *a* [4].

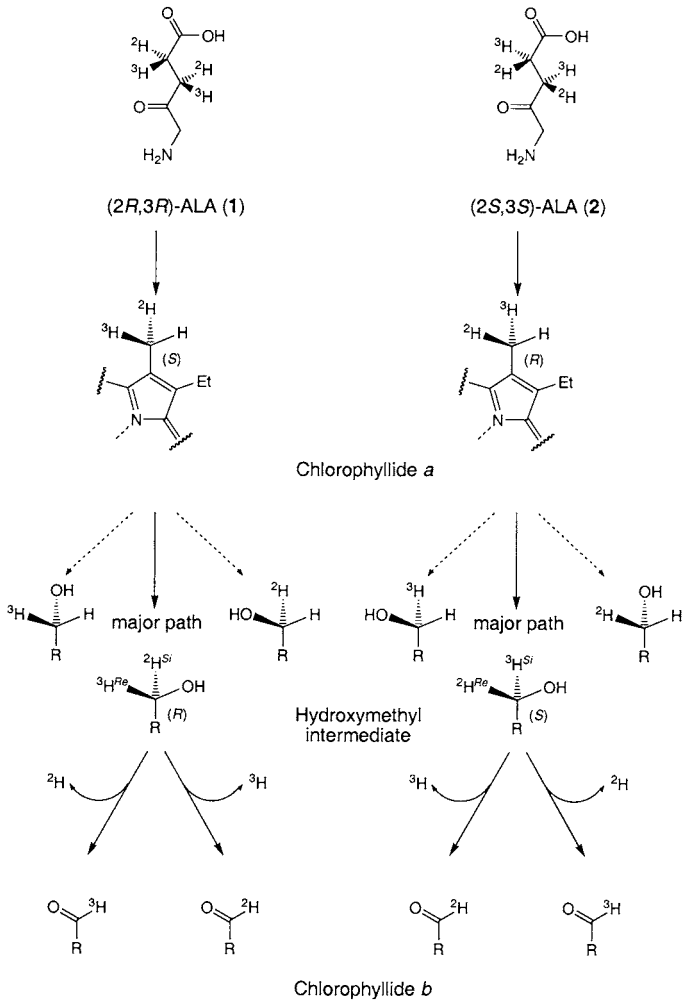
The isolation of chlorophyll *b*-less mutants of *Chlamydomonas reinhardtii* by insertional mutagenesis [5] has led to the identification of a gene specifying a protein with an amino acid sequence similar to that of other methyl monooxygenases. The sequence contains a putative *Reiske* motif for a (2Fe/2S) centre and a mononuclear Fe binding site. Similar, highly conserved genes have also been found in other photosynthetic organisms extending from algae to higher plants [6]. Expression of the equivalent gene from *Arabidopsis thaliana* in the form of a recombinant protein in *Escherichia coli* [7] has allowed the enzyme, termed chlorophyllide *a* oxidase (CAO), to be isolated and its reaction specificity investigated. When the recombinant enzyme was incubated with chlorophyllide *a*, the main product was chlorophyllide *b*, with small amounts of 7-(hydroxymethyl)chlorophyllide. Neither chlorophyll *a* nor protochlorophyllide *a* acted as substrates, confirming CAO as a chlorophyllide *a* oxidase.

Initial indications that both the oxidation of the Me group at C(7) of chlorophyllide *a* to the CH_2OH intermediate as well as the oxidation (dehydrogenation) of the CH_2OH group to the CHO moiety are catalysed by the same enzyme have been proposed on the basis of genetic evidence [5]. Experiments with recombinant CAO have substantiated this premise in that both steps require the presence of an NADPH-generating system, *i.e.*, ferredoxin and ferredoxin reductase [7].

To obtain mechanistic and stereochemical information about the conversion of the Me group of chlorophyllide *a* to chlorophyllide *b*, we adopted a chiral Me approach, originally conceived by *Arigoni* [8] and adopted by others [9]. A chiral Me group at C(7) of chlorophyllide *a* can be generated *in vivo* by incorporating the tetrapyrrole precursor, 5-aminolevulinic acid (= 5-amino-4-oxopentanoic acid; ALA), appropriately labelled with ^2H and ^3H at C(2) (*Scheme 2*). Methods for the preparation of such labelled samples of ALA have been developed by *Akhtar* and co-workers [10]. Since the steric course for the decarboxylation of the CH_2COOH group of uroporphyrinogen III into coproporphyrinogen III has been deduced [11][12], the sense of the chirality of the Me group at C(7) of chlorophyllide *a*, synthesised from such a chiral ALA, would also be known. Thus, the biosynthesis of chlophyllide *a* from (2*R*,3*R*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid (**1**) would generate a chiral Me group at C(7) of chlorophyllide *a* with the (*S*)-configuration. Conversely, (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid (**2**) would furnish a Me group with the (*R*)-configuration.

Because the enzyme-catalysed insertion of O_2 at C–H bonds occurs by a mechanism involving retention [13], and, assuming that there is isotopic discrimination between the three H isotopes within the chiral Me group (there is no reason to assume otherwise), then unique families of labelled forms of CH_2OH intermediates will be generated from each chiral sample of labelled chlorophyllide *a* during the chlorophyll oxidase reaction. Thus, three labelled CH_2OH intermediates would arise from **1** (*Scheme 2*, left) and three labelled CH_2OH intermediates would arise from **2** (*Scheme 2*, right). In each case, due to the isotope effect, the major species would arise from O insertion at the C–^1H bond of the Me group at C(7). Taking these considerations into account, analysis of the labelling of chlorophyllide *b* compared with

Scheme 2. *Theoretical Labelling Patterns of 7-(Hydroxymethyl)chlorophyllide Intermediates Generated from the Hydroxylation of Chlorophyllide* a *Biosynthesised from* a) *(2*R,*3*R*)-5-Amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic Acid* (left); b) *(2*S,*3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic Acid* (right), *and the Subsequent Labelling of Chlorophyllide* b. The labelling pattern reflects an isotopic discrimination favoring the preferential insertion of O at the C–^{1}H bond in the first step.



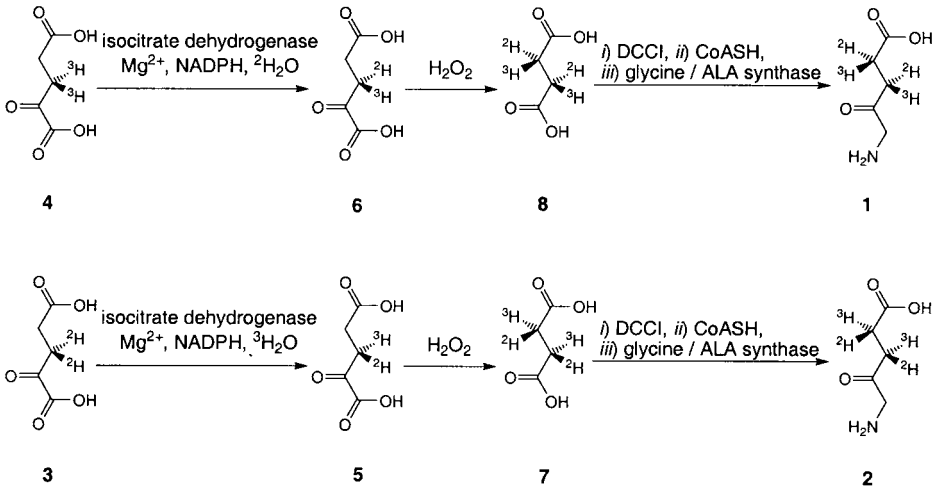
chlorophyllide *a* would permit the steric course of the oxidation (dehydrogenation) of the chiral (hydroxymethyl)-chlorophyllide to chlorophyllide *b* to be deduced. This paper describes the synthesis of **1** and **2** and their incorporation into chlorophylls *a* and *b* in greening barley.

Results and Discussion. – *Synthesis of (2*R,*3*R*)-5-Amino[2,3-$^{14}C_2$,2,3-$^{2}H_3$,2,3-$^{3}H_2$]levulinic Acid* (**1**) *and (2*S,*3*S*)-5-amino[2,3-^{14}C,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic Acid* (**2**). The generation of a chiral Me group at C(7) of chlorophyll *a*, necessary to explore the

steric course of CHO group formation, requires the stereospecific labelling of the CH_2 group of the CH_2COOH group of porphobilinogen, and, thence, uroporphyrinogen III, with ^2H and ^3H. This may be achieved by using **1** or **2** as biosynthetic precursor [10]. Since the decarboxylation of the CH_2COOH group of uroporphyrinogen III is known to occur with retention of configuration [11][12], by carrying out the reaction in ^1H_2O (as would occur *in vivo*), a chiral Me group of known absolute configuration would be generated from each labelled precursor.

The initial introduction of label into 5-aminolevulinic acid (ALA) was achieved by a protocol involving both enzymic and nonenzymic steps (*Scheme 3*). Initially, incorporation of ^2H or ^3H at C(3) of 2-oxoglutarate was carried out by nonenzymic exchange in either ^2H_2O, or ^3H_2O, respectively, as described in the *Exper. Part.* In the case of the 2-oxo[3,3-$^2H_2$]glutarate (**3**), the extent of the ^2H labelling was assessed by oxidative decarboxylation to succinate in H_2O_2 and by mass-spectrometric analysis of the bis-TMS-succinate derivative (data not shown). The mole fractions (%) of mono- and dideuterated species were 7.1% of mono-deuterated and 91.4% of dideuterated species. With respect to the tritiated counterpart, 2-oxo[3,3-$^3H_2$]glutarate (**4**), the specific activity was found to be 110×10^6 dpm ^3H/μmol.

Scheme 3. *Synthetic Route for the Preparation of (2*R*,3*R*)- and (2*S*,3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic Acids*



The stereogenic centre in 2-oxoglutaric acid was generated by exploiting the enzyme isocitrate dehydrogenase, which transforms (2*R*,3*S*)-isocitrate into 2-oxoglutarate. In this reaction, the H^{Si} atom at C(3) of 2-oxoglutarate is derived from the medium and, thus, the overall reaction from (2*R*,3*S*)-isocitrate proceeds with retention of configuration at C(3) [14][15]. The pig-heart enzyme catalyses a partial reaction in which the H^{Si}-atom at C(3) of 2-oxoglutarate is exchanged with the protons of the medium in the presence of Mg^{2+} and NADPH [16]. Thus, enzyme-catalysed exchange of 2-oxo[3,3-$^3H_2$]glutarate (**4**) in ^2H_2O furnished (3*R*)-2-oxo[3-^{2}H,3-^{3}H]glutarate (**6**),

whereas exchange of 2-oxo[3,3-$^2H_2$]glutarate (**3**) in ^3H_2O resulted in (3*S*)-2-oxo[3-^{2}H,3-^{3}H]glutarate (**5**). Having introduced the stereogenic centres, **6** and **5** were decarboxylated in H_2O_2, **6** giving (2*R*,3*R*)-[2,3-$^2H_2$,2,3-$^3H_2$]succinate (**8**) and **5** giving (2*S*,3*S*)-[2,3-$^2H_2$,2,3-$^3H_2$]succinate (**7**; *Scheme 3*)[1]).

The doubly labelled succinates **8** and **7** were mixed with [2,3-$^{14}C_2$]succinate to give a ^{3}H/^{14}C ratio of *ca.* 10, and the resulting (2*R*,3*R*)-[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]succinate and (2*S*,3*S*)-[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]succinate were converted to the respective succinic anhydrides by treatment with Ac_2O. After removal of excess Ac_2O, the succinic anhydrides were reacted with coenzyme A to form (2*R*,3*R*)- and (2*S*,3*S*)-[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]succinyl-CoA. The triply labelled succinyl-CoA samples were then transformed into (2*R*,3*R*)- and (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acids (*Scheme 3*) with highly purified 5-aminolevulinic acid synthase isolated from *Rhodobacter spheroides* [17]. Compounds **1** and **2** were purified from residual succinic acid by ion-exchange chromatography on *Dowex 1X2* chloride, and non-labelled carrier was added. The overall radiochemical yield of labelled 5-aminolevulinic acids in the enzymic reaction from succinyl-CoA was typically 25–40%. Details of the above procedures are covered in the *Exper. Part*.

Biosynthesis of Chlorophyll a *and Chlorophyll* b *from (2*R*,3*R*)- and (2*S*,3*S*)-5-Amino[2,3-^{14}C,2,3-$^2H_2$,2,3-$^3H_2$]levulinic Acids in Greening Barley.* Freshly cut, etiolated barley leaves were mixed with each **1** and **2**, and the leaves were illuminated for 4 h in a current of air to aid the rapid uptake of the substrate (see *Exper. Part*). After another 9 h of illumination for a complete incorporation into chlorophylls *a* and *b*, the leaves were extracted with organic solvents, and the chlorophylls were purified by TLC as pheophytin *a* and pheophytin *b*. The ^{3}H/^{14}C ratios of all starting materials and products were determined by scintillation counting with appropriate quench corrections. The results are shown in the *Table*.

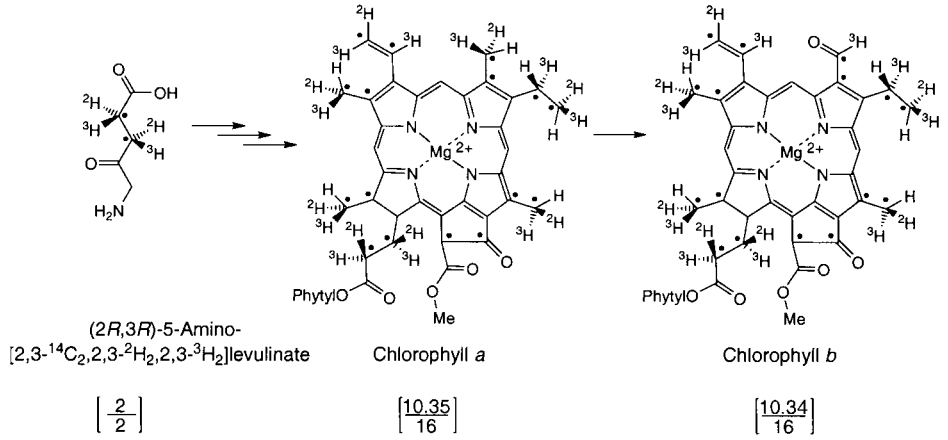
Table. *The Incorporation of (2*R*,3*R*)- and (2*S*,3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic Acid into Pheophytin* a *and Pheophytin* b

Compound used/isolated	Amount [μmol]	^{3}H/^{14}C	Atomic ratio	
			Observed	Predicted
(2*R*,3*R*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid	0.73	9.10	2 : 2	
Pheophytin *a*	1.31	5.89	10.35 : 16	10 : 16
Pheophytin *b*	0.25	5.88	10.34 : 16	10 : 16 or 9 : 16
(2*S*,3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid	0.48	6.22	2 : 2	
Pheophytin *a*	1.10	3.10	7.98 : 16	8 : 16
Pheophytin *b*	0.30	2.84	7.31 : 16	7 : 16 or 8 : 16

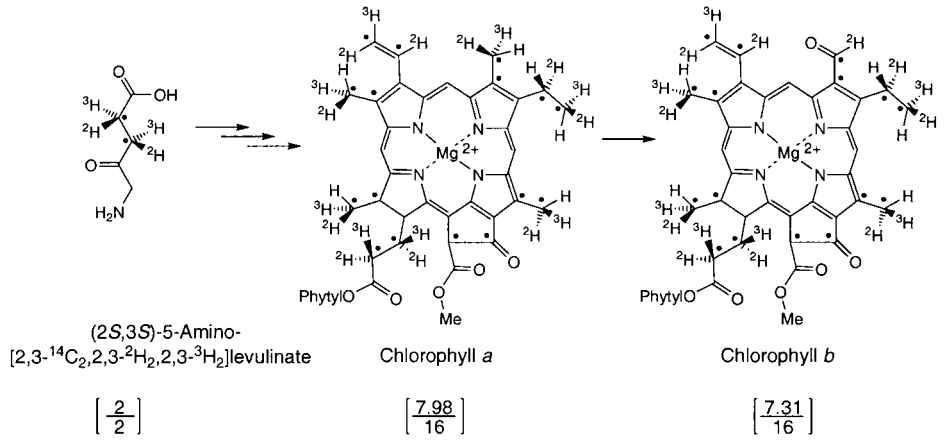
The Steric Course of Formation of the CHO Group at C(7) of Chlorophyllide b *from Chlorophyllide* a. The results shown in the Table can be rationalised by considering *Schemes 4* and *5*. Radiochemical analysis of the chlorophyll *a* that had been biosynthesised from (2*R*,3*R*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid (corrected

[1]) Note that the symmetrical nature of succinate results in the apparent labelling of both C(2) and C(3) although, in reality, only one position is labelled in any given molecule.

Scheme 4. *Incorporation of Label from (*2R,*3*R*)-5-Amino[2,3-*$^{14}C_2$*,2,3-*$^{2}H_2$*,2,3-*$^{3}H_2$*]levulinic Acid into Chlorophyll* a *and Chlorophyll* b (determined as pheophytins) *Showing the Radiochemical Atomic Ratios for Both Pigments* (it should be noted that any ^{3}H (or ^{2}H) initially incorporated at the acidic C(13^2) H-atoms between the ketone and ester carbonyl groups would be rapidly lost during isolation, if not before)



Scheme 5. *Incorporation of (*2S,*3*S*)-5-Amino[2,3-*$^{14}C_2$*,2,3-*$^{2}H_2$*,2,3-*$^{3}H_2$*]levulinic Acid into Chlorophyll* a *and Chlorophyll* b *Showing the Radiochemical Atomic Ratios for Both Pigments* (see *Scheme 4*)



atomic ratio 2:2) showed an observed atomic ratio of 10.35:16 (*Scheme 4*), a figure that is close to the predicted value of 10:16 expected from the known stereochemical courses of uroporphyrinogen III decarboxylase [11][12] and coproporphyrinogen III oxidase enzymes [18][19]. The species of chlorophyll *a* in this experiment would contain a chiral Me group at C(7) (and C(2)) with the (*S*)-configuration. Since the transformation of chlorophyllide *a* to chlorophyllide *b* involves only the oxidation of the Me group at C(7) to a CHO residue, no other losses of labeled H-atoms are

expected[2]). The experimental observation that chlorophyll *a* and chlorophyll *b* derived from (2*R*,3*R*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid both have the same $^{3}H/^{14}C$ ratio indicates that no loss of ^{3}H had occurred during the conversion of the Me group of chlorophyllide *a* to the CHO group of chlorophyllide *b*. Thus, the H^{Si}-atom of the CH_2OH intermediate must have been retained in chlorophyllide *b*.

Radiochemical analysis of the chlorophyll *a* that had been biosynthesised from (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid (*Scheme 5*) showed an atomic ratio of 7.98 : 16, close to the theoretical value of 8.00 : 16. This reflects the anticipated loss of two ^{3}H-atoms, H^{Si} from C(3^1) and C(8^1), during the coproporphyrinogen III oxidase reaction to form vinyl groups in these positions [18][19]. The radiochemical analysis of the pigments showed that the conversion of chlorophyllide *a* into chlorophyllide *b* is accompanied by the further loss of 0.67 of a ^{3}H-atom from the C(7) of chlorophyllide *a* derived from (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid. This result indicates that the chlorophyllide *a* oxidase catalyses the formation of a chiral CH_2OH intermediate from which the H^{Si}-atom is stereoselectively abstracted to form chlorophyllide *b* in the subsequent oxidation (dehydrogenation) reaction. Despite the complex nature of the incorporpation of labelled H-atoms into chlorophyll *a*, the results are remarkably predictable, and their validity is made even more secure because the chlorophyll *a* and chlorophyll *b* are generated in the *same* experiments and, therefore, act as internal checks for one another.

The experiments with (2*R*,3*R*)- and (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acids mutually reinforce one another and conclusively demonstrate that the H^{Si}-atom is removed from the CH_2OH intermediate during the overall transformation of chlorophyllide *a* into chlorophyllide *b* (*Scheme 6*).

Insufficient information is currently available about the nature of the chlorophyll *a* oxidase enzyme to understand the detailed mechanism of two-step reaction. The first step involving the attack on the Me at C(7) group by an O-atom bound to a non-heme Fe-centre is by far the more energetically difficult of the two steps and is expected to proceed with retention of configuration, as discussed above. The same non-heme Fe-centre has also been implicated in the further oxidation of the CH_2OH intermediate [7], presumably, *via* a gem-diol intermediate (*Scheme 7*) that then dehydrates to give the CHO group. Although a straightforward dehydrogenation of the CH_2OH intermediate would also generate the CHO group (*Scheme 7*), the presence of the CH_2OH intermediate at the active site of the CAO may make a second O addition (hydroxylation) inevitable. These possibilities may be resolved by determining whether any ^{18}O is incorporated into the CHO group when the CH_2OH intermediate is incubated with CAO in the presence of $^{18}O_2$. Whether the mechanism of the second step involves oxygen insertion (hydroxylation) or dehydrogenation, the results presented above clearly indicate that the CH_2OH intermediate is positioned within the active site of the CAO enzyme so that H^{Si}-atom is stereospecifically removed.

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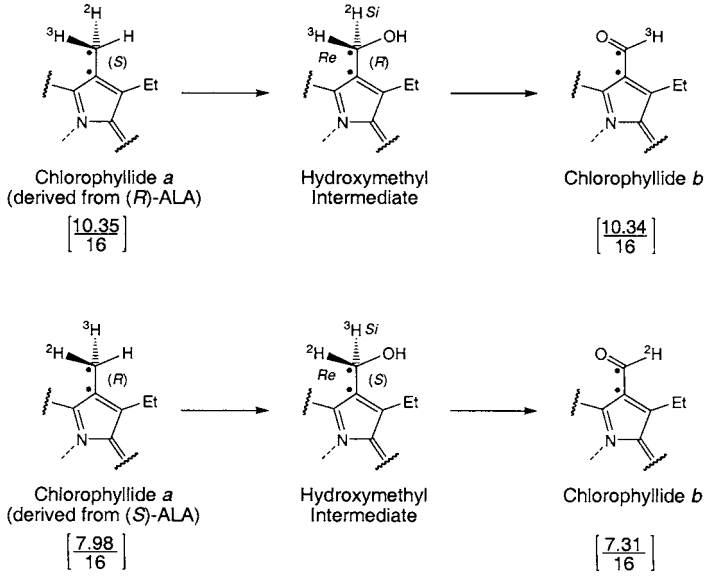
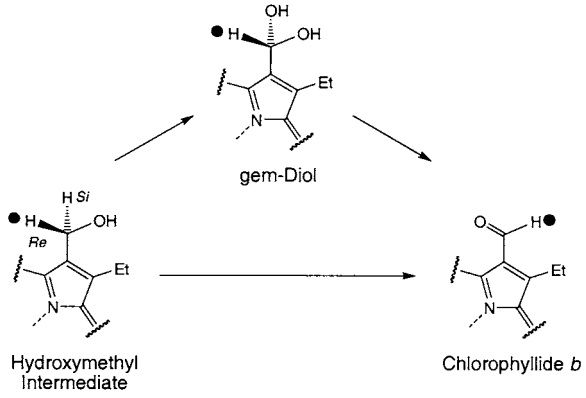
[2]) It should be noted that both ^{3}H labels are lost from the carboxyethyl side chain at C(13), one as a result of the formation of the ketone group at C(13^1) and the other due to rapid nonenzymic exchange of the acidic H-atom at C(13^2). Thus, out of the 16 possible ^{3}H-atoms, six are lost in the biosynthesis of chlorophyll *a* from (2*R*,3*R*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid and eight are lost during the biosynthesis from (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^{2}H_2$,2,3-$^{3}H_2$]levulinic acid (see also *Schemes 4* and *5*).

Scheme 6. *Summary of the Steric Course of the Transformation of Chiral Me Groups at C(7) of Chlorophyllide* a *into CH_2OH Intermediates and Loss of H^{Si}-Atom to Generate the CHO Group in Chlorophyllide* b



Scheme 7. *Alternative Mechanisms for the Transformation of Chlorophyllide* a *into Chlorophyllide* b. A double O-insertion mechanism involving the formation of a gem-diol intermediate as suggested in [7] (*upper path*) or a single O-insertion/dehydrogenation mechanism (*lower path*).



Experimental Part

Preparation of Labelled Substrates. Disodium 2-oxo[3,3-$^3H_2$]glutarate (**4**) was prepared by exchange of the H-atoms at C(3) of 2-oxoglutarate in ^3H_2O (specific radioactivity of the product 110×10^6 dpm $^3H/\mu mol$).

*Disodium (2*R*,3*R*)-[2,3-$^2H_2$,2,3-$^3H_2$]succinate* (**8**) was prepared by the stereospecific exchange of the H^{Si}-atom at C(3) **4** in ^2H_2O catalysed by isocitrate dehydrogenase according to established protocols [10][12][14][20]. The release of ^3H was monitored by constantly removing aliquots from the mixture and chromatography on *Dowex-1* chloride that retains 2-oxoglutaric acid and allows the radioactivity in the eluted

water to be determined. After 50% of the ^3H label had been equilibrated, the resulting doubly labelled *(3*R*)-2-oxo[3-^2H,3-^3H]glutarate* (**6**) was decarboxylated by treatment with 5% H_2O_2 and **8**[1]) was isolated by anion-exchange chromatography (specific radioactivity 55×10^6 dpm ^3H/μmol).

Disodium 2-oxo[3,3-$^2H_2$]glutarate (**3**) was prepared by exchange of the H-atoms at C(3) of 2-oxoglutarate in ^2H_2O (99.8 atom excess).

*Disodium (2*S,*3*S*)-[2,3-$^2H_2$,2,3-$^3H_2$]succinate* (**7**) was prepared by the stereospecific exchange of the H^{Si}-atom at C(3) of **3** in ^3H_2O catalysed by isocitrate dehydrogenase as described above. After 50% of the ^2H label had been equilibrated, the resulting doubly labelled (3*S*)-2-oxo[3-^{2}H,3-^{3}H]glutarate (**5**) was decarboxylated by treatment with H_2O_2 and purification by anion-exchange chromatography, as described before, to afford **7**[1]) (specific radioactivity 5.3×10^6 dpm ^3H/μmol).

*Disodium (2*RS,*3*RS*)-[2,3-$^2H_2$,2,3-2 H_2]succinate* was prepared by the nonenzymic decarboxylation of **3** in 5% H_2O_2 as described above. After purification by anion-exchange chromatography the ^2H content was determined by GLC/MS as the bis(trimethylsilyl) ether (*m*/*z* 247 [M – 15]; with ^2H- and $^2H_2$-labelled species giving *m*/*z* values of 248 and 249, resp.[1]).

*Conversion of Chiral Succinates into (2*R,*3*R*)- and (2*S,*3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic Acid* (**1** and **2**, resp.) *and Their Incorporation into Chlorophylls* a *and* b. The chiral succinates prepared as described above were mixed with [2,3-$^{14}C_2$]succinic acid and converted to the corresponding anydrides. These were treated with coenzyme A, and the resulting succinyl-CoA samples were transformed into chiral 5-aminolevulinic acids with purified 5-aminolevulinic acid synthase [17]. The resulting (2*R*,3*R*)- and (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid samples were mixed with non-labelled 5-aminolevulinic acid, as a carrier, and purified by *Dowex 1X8* chloride ion-exchange chromatography [20].

*Transformation of (2*R,*3*R*)- and (2*S,*3*S*)-5-Amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic Acids into Chlorophylls* a *and* b *in Greening Barley.* Barley seeds (*Bonus* variety) were grown for 7 d in moist compost in the dark until *ca.* 10-cm high. The leaves were then cut and placed in 10-ml glass tubes containing the labelled substrate in five batches of 40 leaves, each containing 300 μl of either (2*R*,3*R*)- or (2*S*,3*S*)-5-amino[2,3-$^{14}C_2$,2,3-$^2H_2$,2,3-$^3H_2$]levulinic acid (1 μmol in 10 mM K_3PO_4 buffer, pH 6.8). The leaves were illuminated with white light from a 40-W fluorescent tube placed 20 cm above the leaves. Transpiration was accelerated by placing the leaves in a current of air for 3–4 h until all the substrate soln. had been taken up, after which 1 ml of 10 mM potassium phosphate buffer, pH 6.8, was added. The leaves were allowed to remain in the light for a further 9 h to maximise the incorporation of the labelled substrates into the chlorophylls.

Extraction and Purification of Labelled Chlorophylls a *and* b *as Their Pheophytin Derivatives.* All operations were carried out in the dark. Leaf material (40 g) was homogenised in 3×20 ml acetone/H_2O 70:20 (*v*/*v*) and, after centrifugation, the combined supernatants were diluted with 200 ml of NaCl (5% *w*/*v*) and the chlorophylls were extracted quantitatively into 100 ml of Et_2O/petroleum ether 50:50 (*v*/*v*). Chlorophylls were converted to their respective pheophytins by washing the org. soln. with 30 ml of HCl (30% *w*/*v*), followed by removal of acid with 3×100 ml extractions with H_2O. The org. layer was dried (Na_2SO_4) and evaporated to dryness. Pheophytin *a* and *b* were purified by TLC (*Merck Kieselgel 60G*; 0.5 mm). An initial development to remove carotenoids was performed with benzene/AcOEt/EtOH 80:18:12 (*v*/*v*), followed by a second separation with petroleum ether (60–80°)/acetone/PrOH 90:10:1 (*v*/*v*) as the developing system. Pheophytin *a* (R_f 0.30) and pheophytin *b* (R_f 0.20) were scraped from the silica and rechromatographed to constant specific activity. A typical experiment afforded pheophytin *a* (2.0 mg, 2.3 μmol) and pheophytin *b* (0.2 mg, 0.24 μmol).

Determination of Radioactivity. Radiochemical analysis was performed on an *Intertechnique SL33* liquid scintillation counter programmed for very accurate quench correction to disintegrations per minute (dpm) Samples were counted in toluene containing 2-([1,1′-biphenyl]-4-yl)-5-(4-butylphenyl)-1,3,4-oxadiazole (6 g/l) as scintillant. Aq. samples were made miscible with the scintillation fluid by addition of MeOH (final concentration 20% *v*/*v*). All vials were counted after storage in the dark for 24 h and at the same quench value to ensure accurate comparisons between $^3H/^{14}C$ ratios.

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