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TETRAPYRROLE BIOSYNTHESIS IN *ANACYSTIS NIDULANS*; INCORPORATION OF [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]- AND [2-^{13}C, 2-$^{2}H_3$] ACETATE*

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Key Word Index—*Anacystis nidulans*; Cyanophyta; blue–green algae; biosynthesis; stable isotopes; ^{13}C NMR; tetrapyrroles; chlorophyll *a*; phycocyanobilin; tricarboxylic acid cycle.

Abstract—Labelling experiments with [2-^{13}C]- and [1,2-^{13}C]acetate showed that both photopigments of *Anacystis nidulans*, chlorophyll *a* and phycocyanobilin, share a common biosynthetic pathway from glutamate. The fate of deuterium during these biosynthetic events was studied using [2-^{13}C, 2-$^{2}H_3$]acetate as a precursor and determining the labelling pattern by ^{13}C NMR spectroscopy with simultaneous [^{1}H, ^{2}H]-broadband decoupling. The loss of ^{2}H (*ca* 20%) from the precursor occurred at an early stage during the tricarboxylic acid cycle. After formation of glutamate there was no further loss of ^{2}H in the assembly of the cyclic tetrapyrrole intermediates or during decarboxylation and modification of the side-chains. Thus the labelling data support a divergence in the pathway to cyclic and linear tetrapyrroles after protoporphyrin IX.

INTRODUCTION

The efficacy of NMR spectroscopy in conjunction with precursors labelled with stable isotopes such as ^{13}C and ^{15}N for the study of complex biosynthetic pathways is well established [1]. A recent advance has been the detection of isotopic incorporation from precursors labelled with deuterium, directly by ^{2}H NMR spectroscopy [2], indirectly *via* simultaneously ^{1}H, ^{2}H broadband decoupled ^{13}C NMR spectroscopy [3–5], or less satisfactorily by observation of ^{1}H decoupled ^{13}C NMR spectra [6]. By using this labelling approach it is possible to trace the fate of hydrogen from an early precursor through a series of biochemical transformations to the final metabolic product. In this report we wish to extend our earlier observations [7] of tetrapyrrole biosynthesis in *Anacystis nidulans* as well as trace the fate of hydrogen from acetate through glutamate and aminolevulinic acid (ALA) into the cyclic tetrapyrrole chlorophyll *a*, and into the linear tetrapyrrole phycocyanobilin.

RESULTS

It was shown that when *A. nidulans* was grown as already described [7] there was a substantial incorporation of exogenous [1-^{14}C] acetate into chlorophyll *a* and phycocyanobilin. The experiment was repeated several more times using [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]- and [2-^{13}C, 2-$^{2}H_3$]acetate and following the same feeding procedures. In the ^{13}C NMR spectrum of chlorophyll *a* labelled from [1-^{13}C]acetate, only two low field resonances at δ 171.4 and 173.4 appeared more intense (*ca* 13 times, see Table 1) due to incorporation of exogenous label from the precursor. These resonances were previously assigned to C-13^{3} and C-17^{3} respectively [8 and references therein].

In the ^{13}C NMR spectrum of chlorophyll *a* labelled from [2-^{13}C]acetate the intensity of seven side-chain resonances C-2^{1}, C-3^{2}, C-7^{1}, C-8^{2}, C-12^{1}, C-13^{2}, C-17^{2} and C-18^{1} were considerably enhanced over natural abundance (*ca* 24 times) through incorporation of exogenous label from the precursor. (The signals for C-2^{1} and C-12^{1} were superimposed.) Neither of the two carbonyl atoms, labelled from [1-^{13}C]acetate, were labelled from [2-^{13}C]acetate, and there was no indication of labelling at any other position in the pyrrole nucleus or phytol side-chain.

In the spectrum of chlorophyll *a* labelled from [1,2-^{13}C]acetate, the resonances for the propionic side-chain carbons C-13^{2} and C-13^{3}, and C-17^{2} and C-17^{3} were each accompanied by satellites arising from ^{13}C–^{13}C coupling through incorporation of an intact acetate unit into the side chain. The resonances of the remaining labelled positions C-2^{1}, C-3^{2}, C-7^{1}, C-8^{2}, C-12^{1} and C-18^{1} contained no satellites but appeared as intense singlets (*ca* 27 times natural abundance). Since these singlet resonances were also labelled from [2-^{13}C]acetate, the combined results of the labelling experiments with [2-^{13}C]- and [1,2-^{13}C]acetate reveal that eight units of exogenous acetate are incorporated into each molecule of chlorophyll *a* but that six of these units are cleaved during the biosynthetic process. Furthermore the data in Table 1 clearly indicate that there was no scrambling of the precursor before or after incorporation into the tetrapyrrole.

Analagous results were obtained with phycocyanobilin (**2**). In the ^{13}C NMR spectrum of **2** labelled from [1,2-^{13}C]acetate, the resonances for the pairs of carbons C-8^{2}

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Table 1. ^{13}C chemical shift (δ_c) and ^{13}C enrichment (% ^{13}C) in chlorophyll *a* labelled with ^{13}C from various labelled acetate precursors

		% ^{13}C from labelled precursor			
C	δ_c*	[1-^{13}C]Acetate	[2-^{13}C]Acetate	[1,2-^{13}C]Acetate†	Id/(Is + Id)‡
7^1	11.1	—	21	27	
12^1	12.6	—	20	25	
2^1	12.6	—	20	25	
8^2	18.1	—	26	31	
18^1	23.8	—	25	31	
17^2	30.9	—	26	28	0.92
13^2	65.8	—	27	25	0.93
3^2	120.1	—	26	32	
13^3	171.3	12	—	22	0.91
17^3	173.4	14	—	25	0.92
Av. error		±2	±4	±5	±0.03

*Relative to TMS; in Me_2CO-d_6: FT-80.

†^1J(C-17^2, C-17^3) = 57.5 Hz, ^1J (C-13^2, C-13^3) = 59.0 Hz (error ± 0.3 Hz).

‡Is, Intensity of singlet resonance; Id, intensity of doublet due to incorporation of intact doubly-labelled unit. Values shown indicate no scrambling of precursor (for which Id/(Is + Id) = 0.90) before incorporation.

Table 2. ^{13}C chemical shifts (δ_c), ^{13}C enrichments (% ^{13}C), ^2H distribution and ^2H retention (F_D %) in chlorophyll *a* labelled with ^{13}C and ^2H from acetate

			Species from labelled precursor			
C	δ_c*	% ^{13}C from precursor	$^{13}C^1H_2$	$^{13}C^1H^2H$	$^{13}C^2H_2$	F_D(%)
7^1	11.4	6.8	0.07	0.27	0.66 ± 0.06	80
12^1	12.9	7.1	0.06	0.26	0.68	81
2^1	13.0	6.5	0.05	0.27	0.68	82
8^2	18.3	6.8	0.08	0.31	0.61 ± 0.06	77
18^1	23.3	7.8	0.08	0.27	0.65 ± 0.05	79
17^2	30.2	7.0	0.07	0.26	0.67	80
13^2	66.5	9.8	0.79	0.21	—	21
3^2	119.9	7.5	0.08	0.28	0.63 ± 0.05	77
Av. error		±0.8	±0.03	±0.03	±0.03	±4

*In Me_2CO-d_6 + C_6F_6 + $Cr(acac)_3$; relative to TMS.

and C-8^3, and C-12^2 and C-12^3, were accompanied by satellites arising from ^{13}C–^{13}C coupling. Six other resonances assigned to the methyl groups at C-1^1, C-3^2, C-7^1, C-13^1, C-17^1 and C-18^2 were enhanced (*ca* 3.6 times) above natural abundance (Table 3) but contained no satellites. Just as with chlorophyll *a* the labelling results indicate that eight acetate units are incorporated into phycocyanobilin, and that six are cleaved during the assembly process.

Cultivation of *A. nidulans* exactly as before but with [2-^{13}C, 2-$^2H_3$]acetate added to the medium yielded labelled chlorophyll *a* and phycocyanobilin. The isotopic distribution within each pigment was obtained by analysis of the respective ^1H, ^2H broadband decoupled ^{13}C NMR spectra [4, 5] and the results are listed in Tables 2 and 4. In chlorophyll *a* (**1**), only carbons derived from the methyl carbon of acetate displayed isotopically shifted resonances due to deuterium labelling. In addition to the regular protiated carbon signal the methyl resonances C-

Table 3. ^{13}C chemical shift (δ_c) and ^{13}C enrichment (% ^{13}C) in phycocyanobilin labelled from [1,2-^{13}C]acetate

C	δ_c	% ^{13}C from precursor	
2^1	15.6	3.2	
3^2	14.9	3.3	
7^1	9.3	3.6	
8^2 / 12^2	35.3	3.8	$^1J_{cc}$ = 57.4 ± 0.3 Hz; Id/(Is + Id) = 0.88 ± 0.3
8^3 / 12^3	173.2	3.4	$^1J_{cc}$ = 57.4 ± 0.3 Hz; Id/(Is + Id) = 0.93 ± 0.3
13^1	9.8*	3.7	
17^1	9.7*	3.7	
18^2	13.0	3.4	
Av. error		±0.4	

*Assignments may be reversed.

Table 4. ^{13}C chemical shifts (δ_c), ^{13}C enrichments (% ^{13}C), ^{2}H distribution and ^{2}H retention (F_D %) in phycocyanobilin labelled with ^{13}C and ^{2}H from acetate

C	δ_c	% ^{13}C from precursor	Species from labelled precursor			F_D(%)
			$^{13}C^1H_2$	$^{13}C^1H^2H$	$^{13}C^2H_2$	
2^1	15.6	15.0	0.06	0.22	0.72	83
3^2	15.0	13.1	0.24	0.34	0.42	59
7^1	9.3	13.6	0.00	0.32	0.68	84
13^1	9.7	13.7	0.07	0.24	0.69	81
17^1	9.8					
18^2	13.0	15.3	0.11	0.26	0.63	76
8^2	35.3	13.8	0.09	0.25	0.66	79
12^2						
Av. error		±3	±0.04	±0.04	±0.04	±5

2^1, C-7^1, C-8^2, C-12^2 and C-18^1 each contained two upfield isotopically-shifted components corresponding to the species $^{13}C^1H_2{}^2H$ and $^{13}C^1H^2H_2$. There was no evidence of a $^{13}C^2H_3$ component. In addition, the α-carbon of the propionic side-chain (C-8^1) and the terminal carbon of the vinyl group (C-3^2) both displayed isotopically shifted resonances corresponding to the species $^{13}C^2H_2$ and $^{13}C^1H^2H_1$, whereas the resonance for C-13^2 showed only one isotopically-shifted component corresponding to a $^{13}C^2H_1$ species.

The labelling results with [1,2-^{13}C]acetate showed that C-17^2 and C-17^3 are derived from an intact unit of acetate, and the fraction of ^{2}H retained at C-17^2 was 0.80 ± 0.04. Although C-13^2 and C-13^3 are also derived from an intact unit of acetate the ^{2}H retention at C-13^2 was only 0.21 ± 0.04 in this case, indicating substantial loss of ^{2}H from this labile centre. On the other hand, the retention of ^{2}H at the methyl carbons C-2^1, C-7^1, C-8^2, C-12^1 and C-18^1 is the same as C-17^2 (Table 2), although each methyl is derived from an acetate unit which has undergone cleavage.

From the same labelling experiment with [2-^{13}C, 2-$^{2}H_3$]acetate, free glutamate was isolated following aqueous ethanol extraction of cells previously extracted for chlorophyll *a*. The ^{1}H, ^{2}H broadband decoupled ^{13}C NMR spectrum of the purified glutamate showed that only C-4 was labelled and contained two isotopically shifted resonances corresponding to a $^{13}C^2H_2$ and a $^{13}C^2H_1{}^1H_1$ species. No other position in the amino acid was isotopically enriched and the ^{2}H retention at C-4 was 0.80 ± 0.04.

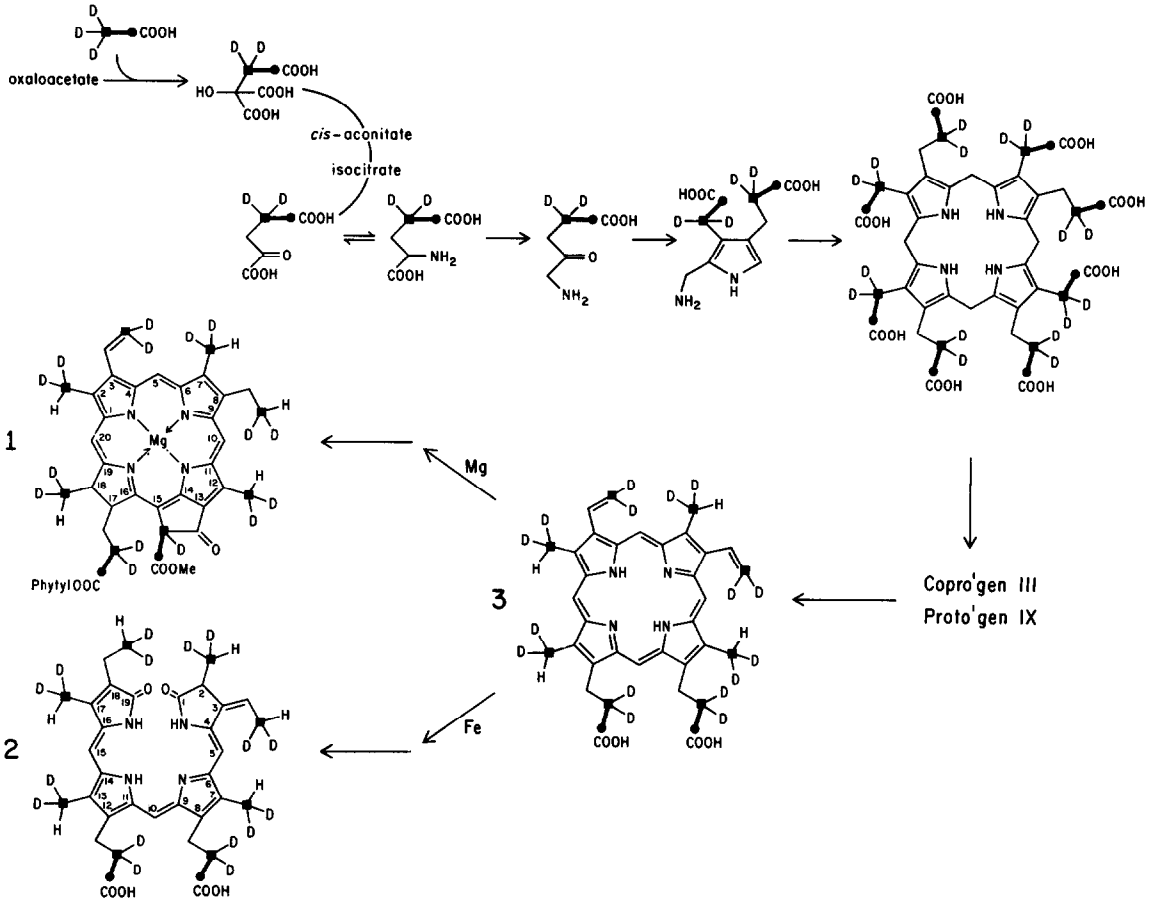
In a separate experiment, phycocyanobilin was labelled from [2-^{13}C, 2-$^{2}H_3$]acetate. The ^{1}H,^{2}H broadband decoupled ^{13}C NMR spectra of the labelled linear tetrapyrrole showed that, as in chlorophyll *a*, all the positions labelled from the methyl group of acetate contained a maximum of two deuteriums (Table 4). The fraction of ^{2}H retained at all the labelled centres was about 0.8, except at C-3^2 where ^{2}H retention was only 0.59 ± 0.05.

DISCUSSION

Our previous studies [7] on the biosynthesis of phycocyanobilin in *A. nidulans* supported the classical assembly of the tetrapyrrole from four porphobilinogen (PBG) units which in turn were derived from aminolevulinic acid (ALA). Moreover these studies indicated that ALA itself was assembled from the five carbon skeleton of α-ketoglutarate or glutamate [9–11] rather than by the more established glycine–succinate route [12]. This latter observation was based principally upon the fact that label from [1-^{13}C]acetate was confined to the carboxyl carbons of the two propionic side-chains in the bile-pigment. Thus, as a result of the incomplete tricarboxylic acid cycle in *A. nidulans* [13–15], label from C-1 of acetate is restricted to C-5 of ketoglutarate hence C-1 of ALA and the carboxyl carbons of phycocyanobilin (Scheme 1). If succinate was a precursor of ALA then, through its generation from isocitrate or malate, it is possible to predict that label from [1-^{13}C]acetate would be distributed between C-1, C-3, C-6, C-8, C-8^3, C-12, C-12^3, C-14, C-16 and C-18.

In the current series of experiments, exogenous [2-^{13}C]acetate labelled eight positions in the side-chains of chlorophyll *a* (Table 1). There was no labelling of any aromatic carbons. This labelling pattern, in which only certain side-chain carbons are enriched, is another illustration of the incomplete tricarboxylic acid cycle in *A. nidulans* and assembly of ALA *via* α-ketoglutarate. Thus label from the methyl group of acetate appears only in C-4 of ketoglutarate and then in C-2 of ALA (Scheme 1). If the glycine–succinate route were in operation then *all* the side-chain carbons (except the two derived from [1-^{13}C]acetate) and four aromatic carbons would be labelled.

The sensitivity and accuracy with which isotope enrichments can be measured is significantly improved when doubly-labelled precursors are used [1]. Bonded ^{13}C–^{13}C units present in a precursor can be traced throughout an entire pathway, and the presence of appropriate satellite signals in a metabolite is convincing evidence that this component of the precursor has been incorporated intact. Their loss is a clear indication that bond scission has occurred. Following incorporation of [1,2-^{13}C]acetate only two intact acetate units were found in chlorophyll *a* (C-13^2, C-13^3 and C-17^2, C-17^3) and phycocyanobilin (C-8^2, C-8^3 and C-12^2, C-12^3). Six other resonances in each tetrapyrrole appeared as intense singlets due to bond scission of the initially incorporated units. This C–C bond cleavage occurs during decarboxylation of the acetic and propionic side-chains of coproporphrinogen III to yield protoporphrinogen IX, the penultimate precursor of the fully aromatised intermediate protoporphyrin IX (3). At this stage it is proposed that a divergence in the pathway to chlorophyll *a* and the bilins occurs [16]. Insertion of



Scheme 1.

Fe^{2+} to form haem, followed by a series of oxidations, produces the linear tetrapyrroles, whereas insertion of Mg^{2+} yields magnesium protoporphyrin IX, a precursor of chlorophyll (Scheme 1).

By using [2-^{13}C, 2-$^{2}H_3$]acetate as a precursor it is possible to trace the fate of hydrogen during the biosynthetic route. If both tetrapyrroles initially share a common biosynthetic pathway and are formed from the same biosynthetic intermediate, protoporphyrin IX (3), the distribution and retention of deuterium in both pigments should be similar. With two exceptions, every carbon in both pigments labelled from the methyl group of acetate contained a maximum of two deuteriums and all retained approximately 80% of the isotope. The similar distribution of isotopic label in each pigment strongly supports the proposal [16] that cyclic and linear tetrapyrroles share a common biosynthetic pathway, and that a divergence late in this pathway leads to either chlorophyll or bile pigments.

There was considerable loss of ^{2}H at the labile centre C-13^2 in chlorophyll *a* in which 20% of the isotope was retained. In phycocyanobilin, the methyl group at C-3^2 contains significantly less isotope compared with the other labelled positions. However, it has been shown [17] that the methyl hydrogens of this ethylidene group are exchanged in refluxing methanol, the same condtions used in this work for separating the chromophore from the protein.

The partial loss of ^{2}H from the other labelled positions could occur during the decarboxylation steps to generate methyl, ethyl and vinyl side chains or at an earlier stage before formation of the first cyclic intermediate. In *Rhodopseudomonas spheroides*, the stereochemistry of the decarboxylation step is known to occur with retention of configuration [18, 19]. Although no mechanism has been firmly established, generation of the methyl groups is most likely an acid-catalysed process. The ethyl groups are formed by a stereochemically controlled decarboxylation process yielding a vinyl side-chain which is subsequently reduced. Our results with chlorophyll *a* show that the loss of ^{2}H does not occur during either of these decarboxylation steps since the retention of isotope in the methyl, ethyl and vinyl side chains is the same as that in the remaining unaltered propionic side chain (Table 2). A similar comparison of the ^{2}H retention in the side chains of phycocyanobilin leads to the same conclusion (Table 4). Thus in *A. nidulans*, as in *R. spheroides* [20], there is little or no loss of isotope after formation of ALA.

We are left with the conclusion that loss of ^{2}H from the precursor must occur before or during formation of ALA. To clarify this point the free glutamate present in the cells was isolated. The ^{1}H, ^{2}H decoupled ^{13}C NMR

spectrum of the amino acid showed two isotopically shifted resonances for C-4 corresponding to C^1H^2H and C^2H_2 species, in which the retention of ^2H was 80%. Hence, there is no loss of isotope during the conversion of ketoglutarate to ALA.

It follows that the isotope loss must occur at an early stage during the assembly of ketoglutarate from acetate *via* the tricarboxylic acid cycle. We have already observed that during the biosynthesis of fatty acids in *A. nidulans* from [2-^{13}C, 2-$^2H_3$]acetate, approximately 10% of the ^2H is lost from the acetyl CoA used for fatty acid synthesis [5]. If the acetyl CoA used in the condensation step with oxalacetate to form citrate is drawn from a common pool this would account for about half the loss of isotope we observe in the tetrapyrroles. In the condensation step it is known that the rate determining step is formation of acetyl CoA carbanion, which can occur only in the presence of enzyme and oxaloacetate [21, 22]. Once the anion is formed the reaction proceeds with inversion of configuration at the methyl group of acetyl CoA [23]. Our results clearly confirm that two methyl protons from acetyl CoA are retained in citrate. This condensation step, during which carbanion is generated, is a plausible stage during which further isotope loss could occur. In fact it has been shown using water and substrates labelled with tritium that exchange of methyl hydrogen with protons of the medium does occur [21, 22].

Recently it has been reported [24] that both pathways (glutamate and glycine–succinate) to tetrapyrroles operate in *Euglena gracilis*. The enzymes for both pathways appear to be compartmentalised and under light-grown conditions chlorophyll *a* is generated from the glutamate pathway, whereas heme *a*, a mitochondrial product, is generated by the glycine–succinate route. In our study, in which every carbon position in the tetrapyrroles can be monitored, there is no evidence for participation of the glycine–succinate route, and under the growth conditions employed, acetate is utilised via the glutamate pathway only, to generate ALA and the tetrapyrroles. Although the sensitivity of the labelling experiments with phycocyanobilin is less than for chlorophyll *a* there is no evidence in the labelling patterns to indicate that there has been any metabolic turnover of the cyclic or linear tetrapyrrole.

EXPERIMENTAL

Culture, maintenance and growth. The culture of *A. nidulans*, its maintenance and growth were exactly as previously described [7].

Isolation of chlorophyll a. After harvesting, all experiments and manipulations were performed in subdued light. Freeze-dried cells (14.7 g) were placed in a Waring blendor and extracted with petrol–MeOH (4:1; 300 ml). After filtration the residue was further extracted with the same organic mixture (7 × 300 ml) until all the blue–green colour was removed. The filtered extracts (2.4 l.) were combined and partitioned twice against 20% aq. MeOH (800 ml). The aq. MeOH fractions were discarded and Et_2O (100 ml) was added to the petrol fraction prior to washing with distilled H_2O (3 × 500 ml). The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under vacuum to yield a dark coloured extract (434 mg). Pure chlorophyll *a* (135 mg) was isolated following open CC on icing sugar and elution with petrol (30–60°) containing increasing amounts of *n*-PrOH (up to 0.4%). Purity was checked by HPLC on C_{18} μ-Bondapak and elution with 5% aq. MeOH.

Isolation of phycocyanobilin. After removal of chlorophyll *a* as described above, pure phycocyanobilin dimethyl ester (*ca* 10 mg) was isolated from the remaining blue-coloured cell material as already described [7].

Isolation of glutamic acid. Following removal of chlorophyll *a* (see above) the cell material (*ca* 14 g) was extracted with 80% aq. EtOH (2 × 100 ml). The combined EtOH solubles were taken to dryness under vacuum, suspended in citrate buffer (0.2 M; 10 ml; pH 3.5) then applied to a Dowex 50 column (2.5 × 80 cm) and eluted with the same buffer. Fractions containing glutamate were separated by high voltage electrophoresis and detected by ninhydrin staining. The combined glutamate fractions were acidified to pH 1 with 6 N HCl, diluted with H_2O (600 ml) and Dowex 50 (10 g; H^+ form) was added with stirring. After the resin was washed thoroughly with cold deionised H_2O, glutamate was eluted with M NH_4OH and the solvent removed under vacuum. The residue was dissolved in H_2O, clarified with charcoal, filtered through celite, and adjusted to pH 3 with 6 N HCl before evaporation to dryness. The colourless residue crystallized from aq. EtOH to yield colourless crystals (11 mg).

Isotope labelling. Sodium [2-^{13}C]acetate (90 atom % enriched) and sodium [1,2-^{13}C]acetate (90 atom %) were obtained from Merck, Sharp and Dohme, Pointe Claire, P.Q. Sodium [2-^{13}C, 2-$^2H_3$]acetate (90 atom % ^{13}C; > 98 atom % ^2H) was obtained from sodium [2-^{13}C]acetate by repeated exchange with KOD–D_2O as already described [5]. The labelled compounds were autoclaved separately and added 30 hr after inoculation of the medium. Cells were usually allowed to grow for 96 hr, but in the labelling experiment with [1,2-^{13}C]acetate they were harvested after 69 hr.

Isotopic measurement by NMR. Carbon-13 NMR spectra were obtained: (A) with a Varian XL-100/15 spectrometer at 25.16 MHz, spectral width (SW) 5120 Hz, acquisition time (AT) 1.6 sec, flip angle (FA) *ca* 40°, (90° pulse 44 μsec), ^1H broadband decoupling, 5 mm tubes, internal lock to ^2H in solvent; or (B) with a Varian FT80A spectrometer at 20.0 MHz, SW 4132 Hz, AT 0.99 sec, FA *ca* 60° (90° pulse 11 μsec), ^1H broadband decoupling, 5 mm tubes. Special conditions for each sample follow.

(i) Chlorophyll *a* at natural isotopic abundance: conditions (A) above, 42 mg, solvents Me_2CO-d_6, Me_2CO-d_6–C_6F_6 (4:1) and Me_2CO-d_6–C_6F_6 (4:1) plus 5 mg/ml chromium (III) acetylacetonate [$Cr(acac)_3$], *ca* 0.3 ml in 5 mm tube, 29° and −4°, delay between acquisitions (PD) 0, 1.6 or 3.2 sec, the latter two with suppression of NOE and addition of $Cr(acac)_3$ for comparison of ^{13}C resonance intensities with those of labelled material, internal lock to ^{19}F when C_6F_6 in solvent; also conditions (B) above, 0°.

(ii) Chlorophyll *a* labelled from [1-^{13}C]-, [2-^{13}C]- or [1,2-^{13}C]acetate: conditions (B) above, 38–40 mg, Me_2CO-d_6, 0.8 ml soln, 9 mg/ml Cr(acac), 0°.

(iii) Chlorophyll *a* labelled from [2-^{13}C, 2-$^2H_3$]acetate: conditions (A) above, 38 mg, 4/1 Me_2CO-d_6–C_6F_6, without and with $Cr(acac)_3$ added (*ca* 4 mg/ml), −4°, internal lock to ^{19}F, simultaneous broadband decoupling of ^1H and ^2H; also ^1H-decoupling only, PD 0, 1.6 and 3.2 sec with NOE suppression in the latter two cases.

(iv) Phycocyanobilin at natural isotopic abundance: conditions (A) above, 12 mg, C^2HCl_3, 29°.

(v) Phycocyanobilin labelled from [1,2-^{13}C]acetate: conditions (A) above, 20 mg, C^2HCl_3, without and with 10 mg/ml $Cr(acac)_3$ added, PD 1.6 and 3.2 sec, NOE-suppression.

(vi) Phycocyanobilin labelled from [2-^{13}C, 2-$^2H_3$]acetate: conditions (A) above, 3 mg in 0.2 ml 4/1 C^2HCl_3–C_6F_6 with 20 mg/ml $Cr(acac)_3$ added, 29°, simultaneous broadband decoupling of ^1H and ^2H, ^{19}F lock.

The methods by which isotopic enrichments and proportions of labelled species are calculated from integrals of expanded ranges of these spectra have been described previously [4, 5, 25].

The ^{13}C resonances of chlorophyl *a* (**1**) were assigned from previously reported data [8]. There was little or no perturbation of the chemical shifts resulting from C_6F_6 present in the solvent to provide the ^{19}F field frequency lock. The results of Wray *et al.* [26] were used to assign the resonances of phycocyanobilin (**2**).

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