Phytochemistry, Vol. 20, No. 7, pp. 1547–1549, 1981.
Printed in Great Britain.

0031-9422/81/071547-03 $02.00/0

δ-AMINOLAEVULINATE BIOSYNTHESIS IN THE CYANOBACTERIUM *SYNECHOCOCCUS* 6301

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(*Received* 1 *November* 1980)

Key Word Index—*Synechococcus* 6301; cyanobacterium; blue-green alga; biosynthesis; δ-aminolaevulinate.

Abstract—The cyanobacterium (blue-green alga) *Synechococcus* 6301 incorporated a large amount of isotope from [1-^{14}C] and [2-^{14}C]acetate into phaeophorbide *a* obtained from chlorophyll *a* and into glutamate in cell protein; very little radioactivity was present in aspartate in cell protein. This distribution of isotope indicates that aspartate and the tetrapyrrole of chlorophyll *a* are not derived from a common C_4 precursor. The ratios of the specific radioactivities of phaeophorbide *a* to glutamate for organisms grown in the presence of [1-^{14}C] and [2-^{4}C] acetate were 2.5:1 and 10:1 respectively. These are close to the theoretical values for the C_5 route to δ-aminolaevulinate which indicates that this is the only pathway to the tetrapyrrole precursor in *Synechococcus* 6301.

INTRODUCTION

There are two distinct routes from common metabolites to δ-aminolaevulinic acid (δ-ALA) which is the first intermediate unique to the biosynthesis of tetrapyrroles [1]. In bacteria and animals it is produced from glycine and succinate while in oxygenic photosynthetic organisms δ-ALA is formed from a C_5 precursor closely related to glutamic acid. As prokaryotes cyanobacteria would be expected to use the succinate–glycine route but as oxygenic photosynthetic organisms they are more likely to exploit the C_5 pathway. The route to δ-ALA in cyanobacteria has been investigated recently [2–4] using the labelling procedure employed by Beale *et al.*, [5] and by Meller *et al.*, [6]; this involves the determination of the distribution of isotope from specifically labelled potential precursors among the carbon atoms of δ-ALA accumulated in the presence of laevulinic acid. The interpretation of data obtained by applying this technique to cyanobacteria has been complicated not only by the relatively inefficient incorporation of organic compounds by these organisms, but also by the incomplete recovery of isotope from the partial degradation of the labelled δ-ALA. While the results obtained by this technique are consistent with the operation of the C_5 route in cyanobacteria, they do not eliminate the possibility of a contribution from the alternative pathway to the synthesis of the tetrapyrrole precursor.

Because the conversion of α-oxoglutarate to succinate is blocked in cyanobacteria [7] the route for δ-ALA biosynthesis in these organisms can be established by comparing the labelling of tetrapyrroles by radioactive acetate with that of glutamate and aspartate. If the C_5 route is operating, the amount of isotope in these compounds will be in the order tetrapyrrole > glutamate ≫ aspartate whereas the labelling will be glutamate ≫ tetrapyrrole > aspartate if these organisms produce δ-ALA from glycine and from succinate derived from oxaloacetate. This paper describes the results of an investigation of the labelling of glutamate, aspartate and phaeophorbide-*a* isolated from the cyanobacterium *Synechococcus* 6301 grown in the presence of [1-^{14}C] and [2-^{14}C]acetate.

RESULTS AND DISCUSSION

During growth in the light on CO_2 in the presence of [1-^{14}C] and [2-^{14}C]acetate *Synechococcus* 6301 incorporated *ca* 90% of the labelled substrate into cell material. Half of the isotope was recovered in the photosynthetic pigment fraction obtained by extracting organisms with acetone (Table 1). Most of the remaining isotope was present in the hydrolysate of cell protein (Table 1). A similar distribution of label among the cell constituents of cyanobacteria grown in the presence of [2-^{14}C] acetate has been described by Hoare and Moore [9]. While aspartate isolated from cell protein contained relatively little isotope, glutamate was very heavily labelled by both [1-^{14}C] and [2-^{14}C] acetate (Table 2). In

Table 1. Fractionation of *Synechococcus* 6301 grown in the presence of [1-^{14}C]- and [2-^{14}C]-acetate

	Organisms grown in the presence of	
	[1-^{14}C]-acetate	[2-^{14}C]-acetate
	(% total radioactivity)	
Acetone	50.8	52.6
Ethanol	4.6	6.1
Ethanol–diethyl ether	5.5	5.5
Hot trichloroacetic acid	2.1	1.8
Acid ethanol–diethyl ether	4.0	3.9
Hydrolysate of the cell residue in 6 M HCl	33.0	30.1

The amount of radioactivity in the washed whole cells recovered from cultures containing [1-^{14}C]- and [2-^{14}C]-acetate was 2.52×10^8 and 2.16×10^8 dpm respectively. Organisms were extracted with H_2O–Me_2CO (1:4) followed by the reagents used by Roberts *et al.* [8].

Table 2. Labelling of aspartate, glutamate and phaeophorbide *a* isolated from *Synechococcus* 6301 grown in the presence of [1-^{14}C]- and [2-^{14}C]-acetate

Labelled acetate	Specific radioactivity of aspartate (10^{-2} × dpm/μmol)	glutamate (10^{-4} × dpm/μmol)	phaeophorbide *a* (10^{-5} × dpm/μmol)	Ratio of specific radioactivities: phaeophorbide *a* / aspartate	phaeophorbide *a* / glutamate
[1-^{14}C]	3.74	7.61	1.89	5.1×10^2	2.5
[2-^{14}C]	0.76	6.41	6.51	8.6×10^3	10.2

Chlorophyll *a* isolated from organisms grown in the presence of specifically labelled acetates was converted to phaeophorbide *a*, and aspartate and glutamate were isolated from hydrolysates of cell protein as described in the Experimental.

both cases the specific radioactivity of the glutamate was *ca* half that of the labelled acetates indicating that dilution by endogenous substrates was significant but not extensive. The differential labelling of glutamate and aspartate by radioactive acetate is a direct result of the incomplete tricarboxylic acid cycle in these organisms; it is blocked at the conversion of α-oxoglutarate to succinate [7]. A further consequence of this is that carbon atoms 4 and 5 of glutamate are derived from carbon atoms 2 and 1 of acetate [10]. Phaeophorbide-*a* was heavily labelled by [1-^{14}C] and [2-^{14}C] acetate (Table 2) and its specific radioactivity was much greater than that of aspartate isolated from the same samples of cell material. This is inconsistent with the synthesis of δ-ALA from glycine and from succinate derived from a C_4 precursor related to oxaloacetate. The large amount of isotope in phaeophorbide-*a* can be attributed to the operation of the C_5 route to δ-ALA in *Synechococcus* 6301. A similar differential distribution of isotope from labelled acetate in phaeophorbide-*a* and aspartate was also found in two other cyanobacteria, *Chlorogloeopsis* 6912 and *Nostoc* strain Mac.

During the synthesis of chlorophyll-*a*, certain of the carbon atoms of δ-ALA are lost with the result that the ratio of the specific radioactivities of phaeophorbide-*a* to glutamate will not be the same for organisms grown in the presence of [1-^{14}C] and [2-^{14}C] acetate; the theoretical values are 2:1 and 8:1 respectively. For *Synechococcus* 6301 the values obtained were 2.5:1 and 10:1 which are close to those predicted. These data are wholly consistent with the synthesis of δ-ALA via the C_5 route in which carbon atoms 1 and 2 of δ-ALA are derived from carbon atoms 5 and 4 of glutamate. From this it follows that carbon atoms 1 and 2 of δ-ALA are derived from carbon atoms 1 and 2 of acetate respectively; this is consistent with the observation that only 8% of the isotope of [2-^{14}C]acetate incorporated into δ-ALA by *Fremyella diplosiphon* was present in the C_5 position [2]. In addition, the close agreement between the theoretical and the experimental values for the specific radioactivity ratios indicates that any contribution to the synthesis of δ-ALA from the succinate–glycine route is insignificant. These data therefore provide independent confirmation of the conclusion drawn from the labelling of δ-ALA by [^{14}C]glutamate [2–4]. Although cyanobacteria are prokaryotic organisms they resemble eukaryotic algae and higher plants not only in terms of the mechanism of photosynthesis but also with respect to the pathway for δ-ALA biosynthesis.

EXPERIMENTAL

Growth of organism. Synechococcus 6301 (ATCC 27144) was obtained originally from Dr. Mary Allen, Department of Biological Sciences, Wellesley College, Wellesley, Massachusetts U.S.A. It was grown in the light in a total vol. of 1.6 l. in the presence of [1-^{14}C] and [2-^{14}C] acetate (*ca* 0.07 μCi/μmol) at a final concn of 1 mM as described previously [7].

Isolation of phaeophorbide-a. After washing with H_2O, the organisms (0.35 g dry wt) were extracted with H_2O–Me_2CO (1:4) and the chlorophyll-*a* in the pigment extract (100 ml) was transferred to petrol by mixing with one vol. of the latter and one vol. of H_2O. The epiphase was washed several times with H_2O, and when the chlorophyll-*a* started to ppt. the epiphase was mixed with three vol. of MeOH. Chlorophyll-*a* was isolated from this prepn [11] and converted to phaeophytin-*a* [12] and then to phaeophorbide-*a* [13]. At each stage the product was purified by chromatography on columns (22 cm × 1.5 cm diam.) of confectioners sugar. For phaeophytin-*a* the columns were developed with *iso*-PrOH–petrol (1:200) and for the isolation of phaeophorbide-*a*, with *iso*-PrOH–$CHCl_3$–petrol (1:60:139). When the band of pigment had moved halfway down the column, development was stopped, the column contents were extruded and the pigment zone extracted with Et_2O (phaeophytin-*a*) or Me_2CO (phaeophorbide-*a*).

Isolation of glutamate and aspartate. The material remaining after the extraction of the photosynthetic pigments with aq. Me_2CO was further fractionated [8] and the final residue of protein hydrolysed under reflux with 6 M HCl for 18 hr. The hydrolysate was taken to dryness by rotary evapn, dissolved in H_2O and the procedure repeated × 3 to remove residual acid. The hydrolysate was taken up in 0.5 M HOAc, decolourised with Norit K charcoal prewashed with HOAc–H_2O (1:1), and taken to dryness. Ion exchange chromatography with a column (22 cm × 1.5 cm diam.) of Amberlite CG400 resin (type II, 200 mesh) in the acetate form was used to separate glutamic acid and aspartic acid from other amino acids in the hydrolysate and from each other [14].

Estimation of amino acids, phaeophorbide-a *and* [^{14}C]. The identity and purity of the compounds isolated from cell material were confirmed by chromatography and electrophoresis in the case of glutamate and aspartate [9] and by spectrophotometry and TLC in the case of phaeophorbide-*a* [15]. The amino acids were estimated by the ninhydrin method [16] using a glutamate standard. Phaeophorbide-*a* was determined from the absorbances of solns in Me_2CO at 667 nm using a molar extinction coefficient of 43.4 × 10^3 [17]. The amount of isotope in various samples including purified glutamate, aspartate and phaeophorbide-*a* was determined by spotting replicate samples on to metal planchets. When dry these were counted, with an

efficiency of 20% in a planchet counter. Na [1-^{14}C] acetate and Na [2-^{14}C]acetate obtained from the Radiochemical Centre, Amersham, England were purified before use [18]. All of the organic solvents were dried and redistilled before use.

Acknowledgements—C. Lucas was the recipient of a University of Wales postgraduate studentship. The authors thank Miss Myrtle Williams for expert technical assistance.

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