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THE BIOSYNTHESIS OF PHYCOCYANOBILIN IN *ANACYSTIS NIDULANS**

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Key Word Index—*Anacystis nidulans*; Cyanophyta; blue-green algae; biosynthesis; tetrapyrroles; aminolevulinic acid; phycocyanobilin.

Abstract—Aminolevulinic acid, the common precursor of linear and cyclic tetrapyrroles, can arise either by condensation of succinate and glycine or from the entire carbon skeleton of glutamate. Both pathways appear to operate in algae. In cultures of *Anacystis nidulans*, [1-^{14}C]α-ketoglutarate labelled the linear tetrapyrrole pigment, phycocyanobilin, more efficiently than [1,4-^{14}C]succinate or [2-^{14}C]glycine; [1-^{14}C]acetate was even more efficiently utilized. When the culture was supplemented with [1-^{13}C]acetate, ^{13}C NMR analysis of the resulting labelled phycocyanobilin showed that only the carbonyl carbons of the pigment were labelled, indicating that the glutamate pathway operates in this organism.

INTRODUCTION

It has been shown in a wide variety of organisms, including bacteria, yeast, plants and mammals [1] as well as algae [2], that δ-aminolevulinic acid (ALA) is an intermediate in porphyrin biosynthesis. In bacteria, yeast and mammals, ALA can be formed by a condensation of glycine and succinyl CoA catalysed by ALA synthetase. However, in greening plants, this enzyme has not been detected and ALA can arise from the entire 5-carbon skeleton of glutamic acid (or α-ketoglutarate) [3–7]. The biogenesis of ALA in algae is ambivalent: *Cyanidium caldarium* [8] and *Euglena gracilis* [9] use the glutamic acid pathway while glycine and succinate were incorporated preferentially into regreening nitrogen-starved *Chlorella fusca* [10]. Both pathways operate to generate ALA for chlorophyll synthesis in the green alga *Chlorella vulgaris* [11] and in a pigment mutant of *Scenedesmus obliquus* [12].

A recent report [13] suggests that the glutamate pathway is the principal route to ALA in the blue-green alga *Agmenellum quadruplicatum*, whereas there is evidence [11] that both pathways operate in another blue-green alga, *Fremyella diplosiphon*. The manner of ALA biosynthesis poses a particularly intriguing question with blue-green algae because of their phylogenic relationship to bacteria and because many of them do not possess a complete tricarboxylic acid cycle [14–16].

RESULTS AND DISCUSSION

Anacystis nidulans was grown axenically for 100 hr in 30 l. batches using the apparatus shown in Fig. 1. The yield of freeze-dried cells was 12–14 g, from which 5–15 mg of purified phycocyanobilin dimethyl ester was obtained by the refluxing methanol treatment [17] of partially purified phycocyanin.

In bacteria and mammals, ALA and hence porphyrins, are biosynthesized from glycine and succinate [1] whereas in plants ALA is synthesized from glutamate [2]. Both pathways have been shown to operate in green algae [10–12], and although ALA is produced by the blue-greens [18], the current evidence [11, 13] indicates that both routes may operate in this group. To obtain information about ALA biosynthesis, both sets of precursors that could plausibly generate phycocyanobilin via ALA were fed to illuminated cells of *A. nidulans*. Table 1 summarizes the incorporation of label from these precursors into phycocyanobilin. Both [1-^{14}C]keto-glutarate and [1,4-^{14}C]succinate labelled the pigment, though radioactivity in ketoglutarate was *ca* three times more efficiently incorporated. [2-^{14}C]Glycine, on the other hand, failed to label the bilin. [1-^{14}C]Acetate, which is known to be efficiently incorporated by cells of blue-green algae [19], labelled the chromophore heavily. When the experiment with labelled acetate was repeated using [1-^{13}C]acetate as a supplement, ^{13}C NMR analysis of the resulting phycocyanobilin dimethyl ester showed that only one resonance (δ_c 173.18 ppm) was enhanced (Fig. 2). From several ^{13}C NMR studies of porphyrins [20] and bilirubins [21–23], this signal can be assigned to the carbonyl resonances of the two equivalent carbomethoxy groups.

The tricarboxylic cycle is incomplete in *A. nidulans* due to absence of the two enzymes α-ketoglutarate dehydrogenase and succinyl CoA synthetase [14–16]. Nevertheless, succinate can be produced by a series of reactions from oxaloacetate and by cleavage of isocitrate (Scheme 1). Condensation of acetyl CoA with the glyoxalate formed in the latter reaction yields malate which can be converted to more succinate. Both isocitrate lyase and malate synthetase have been detected in small amounts in *A. nidulans* [15]. Succinyl CoA is generated from succinate by CoA transfer from acetyl CoA in

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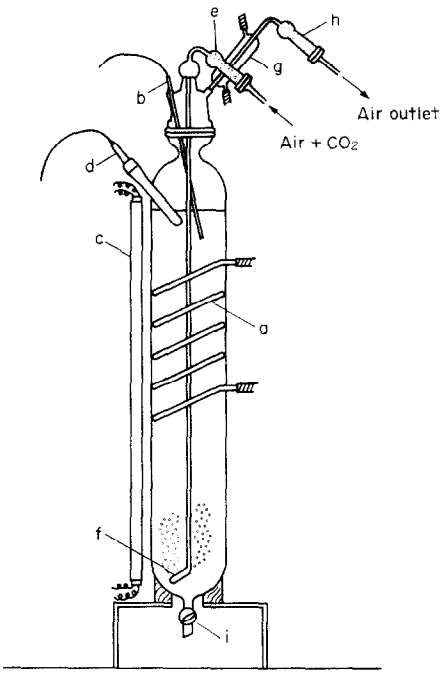


Fig. 1. The 30 l. culture apparatus consisted of a vertically mounted glass tube (18 × 130 cm), fitted with a hemispherical cap with a ground glass joint. A glass cooling coil (a) controlled the temperature of the culture via a solenoid valve which regulated the flow of water to the coil. A temperature sensing probe (b) was connected to a control unit which operated the solenoid valve. Sixteen (40 W) fluorescent tubes (c) mounted vertically 2 cm from the vessel provided sufficient heat to maintain the culture at 41°. pH control was maintained using a 30 cm combination electrode (d) and a Radiometer pH stat. The titrator valve regulated the flow of CO_2 to a diaphragm air pump. The natural increase of the culture pH during growth was controlled by the addition of CO_2 to a constant value of pH 7.6 throughout the experiment. Air was pumped into the culture through a sterile cotton filter (e) and a glass dispersion tube (f) and exited through a small water cooled condenser (g) and a second sterile cotton filter (h). At the end of the growth period the cells were collected through the tap (i) at the bottom of the vessel.

another blue-green alga, *Anabaena variabilis* [16], which also possesses an incomplete tricarboxylic cycle.

Labelling of ALA from [1,4-^{14}C]succinate in an organism which utilizes the α-ketoglutarate pathway has already been observed with maize [6], *Chlorella vulgaris* and *Fremyella diplosiphon* [11]. It probably occurs by conversion of succinate to α-ketoglutarate since the label was located at C-5 in ALA rather than being equally distributed between C-1 and C-4. Thus the incorporation of some label in phycocyanobilin when *A. nidulans* was grown on [1,4-^{14}C]succinate would be expected, even if the glycine–succinate route to ALA were not used.

Although we found that incorporation of glycine into the cells of *A. nidulans* was quite low, the possibility that this glycine is a precursor of ALA is contradicted by the observation that, of the radioactive glycine that did penetrate the cells, none was incorporated into phycocyanobilin (Table 1).

The efficient photometabolism of acetate by *A. nidulans* is well documented [19], and at 1 mM concentration, incorporation of [1-^{14}C]acetate into phycocyanobilin was substantial. We did not observe isotope scrambling when labelled acetate was incorporated into the fatty acids of *A. nidulans* [24], but to verify that the labelling of phycocyanobilin was not the result of a secondary mode of incorporation, such as re-incorporation of respired $^{14}CO_2$, [1-^{13}C]acetate was administered under the same conditions as used for the radioactive precursor. No scrambling of the isotope was observed and only the carboxyl carbons were labelled (Fig. 2). This is interpreted to mean that because of the interrupted cycle, label from C-1 of acetate is restricted to C-5 of α-ketoglutarate and glutamate [14–16] and hence to C-1 of ALA and the carboxyl carbons of phycocyanobilin (Scheme 1). Although the interruption also prevents the generation of succinate from α-ketoglutarate, any succinate in the cell can be converted by the remainder of the cycle to malate and eventually α-ketoglutarate and glutamate. However, if succinate were a direct precursor, then label acquired in C-4 from [1-^{13}C]acetate would be expected to equilibrate into C-1 before the CoA ester was formed. Thus C-1 and C-4 of ALA would be labelled, resulting in an equal distribution of isotope between C-8, C-12 and C-8^{III}, C-12^{III} in phycocyanobilin. (The numbering system for bile pigments follows the corrin system nomenclature, see [23].)

Table 1. Specific incorporation of radioactivity from labelled substrates into phycocyanobilin dimethyl ester*

Supplement	Activity (μCi/mmol)	Phyco-cyanobilin activity (μCi/mmol)	Specific incorporation† (× 100)
[1,4-^{14}C]Succinic acid	1.41	0.0013	0.09
[1-^{14}C]Sodium ketoglutarate	0.35	0.001	0.29
[2-^{14}C]Glycine	1.16	0	0
[1-^{14}C]Sodium acetate	0.87	0.32	36.7

* For details of feeding conditions see Experimental.

† Specific incorporation is calculated as the specific activity of phycocyanobilin dimethyl ester divided by the specific activity of the precursor.

In a separate experiment, production medium containing [2-^{14}C]glycine (2.33 μCi/mmol; 1880 cpm/ml medium) yielded radioactively labelled cells of *A. nidulans* (5500 cpm/g freeze-dried cells).

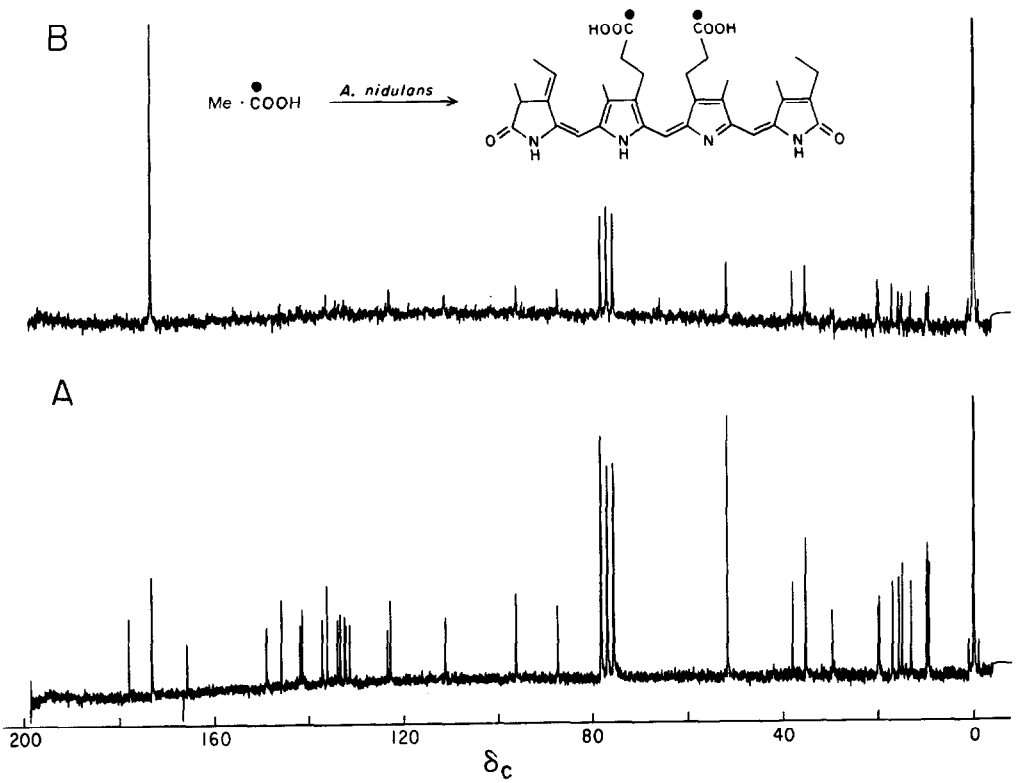
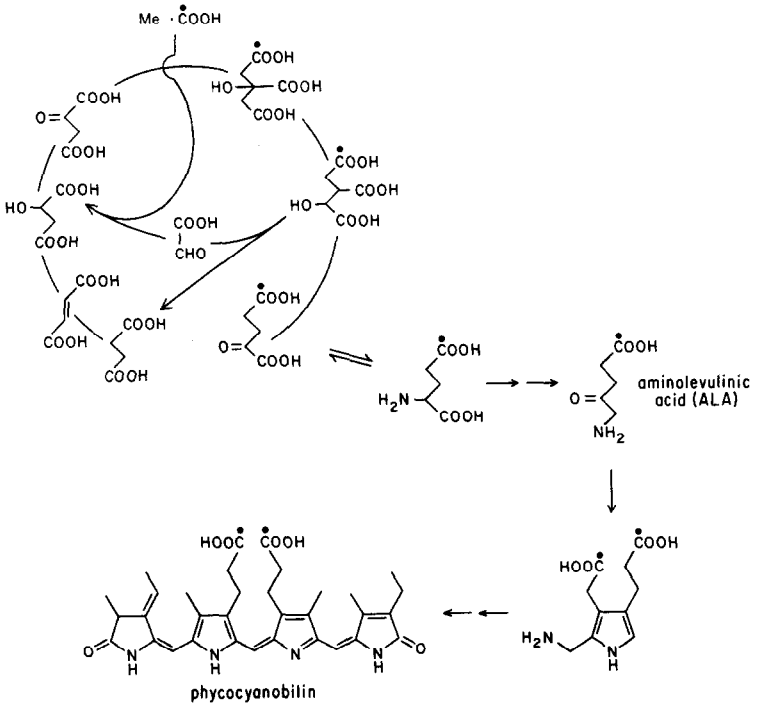


Fig. 2. Proton noise-decoupled ^{13}C NMR spectra of phycocyanobilin in $CDCl_3$. (A) at natural abundance, (B) biosynthesized from [1-^{13}C]acetate.



Scheme 1.

These results support the earlier observations [14–16] that *A. nidulans* does not possess a complete tricarboxylic cycle. They also show, for the first time in this blue-green alga, that ALA and, therefore, phycocyanobilin, are biosynthesized by the glutamate pathway.

The biosynthesis of ALA is the same in *C. caldarium* [8] and *E. gracilis* [9] and follows the alternative glutamate pathway of plants [3–7] as opposed to the glycine–succinate route of bacteria [1].

EXPERIMENTAL

Culture. Anacystis nidulans was obtained from Dr. Ford Doolittle, Department of Biochemistry, Dalhousie University, Halifax, and maintained on agar slants as described below.

Maintenance and growth conditions. A. nidulans was maintained on agar (5 g/250 ml) slants prepared with Allen's media [25]. A production inoculum was prepared by transferring these cells to the liquid medium of ref. [26]. All cultures were maintained at 36° for 10–14 days with constant illumination before transferring to fresh media.

For production runs or labelling expts, the glass culture vessel (Fig. 1) was autoclaved or steamed for 48 hr and the modified Kratz and Meyers' medium (30 l.) was introduced aseptically through a millipore filter (0.3 μm). The inoculum (100 ml) was added after the medium was adjusted to pH 7.6 and the tank had reached 41°. After addition of the inoculum the pH was initially adjusted to 7.2 by the addition of CO_2, and then maintained at 7.6 during production with the flow of CO_2 controlled by an automatic titrator. Cells were grown for *ca* 100 hr before harvesting. The culture yielded, on average, 14 g of freeze-dried material.

Extraction and purification of phycocyanobilin. Freeze-dried cells (*ca* 14 g) were suspended in H_2O (200 ml) and disrupted with a Branson Sonifier (100 W) for 5 × 1 min intervals at 0–5°. Brushite ($CaHPO_4 \cdot H_2O$) slurry [14] was added to the suspension, then filtered through a bed of Brushite (1–2 cm) on a Buchner funnel (15 cm). With sufficient Brushite, all the phycocyanin was retained. However, if the filtrate was blue the filter bed was resuspended and refiltered through a bed made with fresh Brushite. After washing with H_2O (200 ml), phycocyanin was eluted with 0.2 M PO_4^{2-} buffer, pH 7 (500 ml). An equal vol. of satd $(NH_4)_2SO_4$ soln was added to the filtrate and the suspension centrifuged. The supernatant was discarded and the blue pellet of phycocyanin, dissolved in a minimum vol. of H_2O, was dialysed overnight against H_2O. The contents of the dialysis bag were carefully concd *in vacuo* (< 35°) to yield a blue-coloured solid which was stored overnight in a desiccator. The solid was scraped from the flask walls and washed with dry MeOH (× 2). The residue was refluxed overnight with dried MeOH (25 ml/g). The blue supernatant was removed by filtration and then treated with BF_3–MeOH soln (5 ml) for 5 min. The soln was concd *in vacuo*, dissolved in CH_2Cl_2 and washed thoroughly with H_2O (> × 4) to remove any traces of reagent. Phycocyanobilin diMe ester was purified by TLC using $Me_2CO-C_6H_6$ (1:4). Before NMR analysis, the pigment was dissolved in a small vol. of $CHCl_3$ and washed with EDTA (10^{-3} M, × 2).

Isotopic labelling and measurement. The following radio-labelled compounds were purchased from New England Nuclear Corporation, Boston, MA: [1-^{14}C]Na acetate, [1-^{14}C]α-keto-glutarate, [1,4-^{14}C]succinic acid, and [2-^{14}C]glycine. [1-^{13}C]Na acetate (90 atom % enriched) was obtained from Merck, Sharp & Dohme, Ponte Claire, P.Q., Canada.

The labelled compounds were autoclaved separately and added just after inoculation to give a final concn in the production medium of 1 mM. The blue pigment was isolated and purified as described above. The results of the radio-tracer expts are shown in Table 1. To examine the efficiency of incorporation of [2-^{14}C]glycine into *A. nidulans*, harvested cells were washed × 6 with cold media, then with H_2O and freeze-dried. The cells (1 g) were decolourized by treating them for 2–3 min in hot 6 M HCl (10 ml) and H_2O_2 (1 ml; 100 vol.) The suspension was evapd to dryness *in vacuo* and resuspended in EtOH–toluene scintillant containing CAB-O-SIL.

^{13}C NMR pulse-Fourier transform spectra of phycocyanobilin (10–15 mg) in $CDCl_3$ were recorded at *ca* 30° under the following conditions: frequency 25.16 MHz, acquisition time 1.6 sec (data accuracy ±0.3 Hz), spectral width 5120 Hz, internal ^{2}H lock to solvent, int. ref. to TMS.

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REFERENCES

1. Granick, S. and Beale, S. I. (1978) *Adv. Enzymol.* **46**, 33.
2. Troxler, R. F. and Lester, R. (1967) *Biochemistry* **6**, 3840.
3. Beale, S. I. and Castelfranco, P. A. (1974) *Plant Physiol.* **53**, 297.
4. Beale, S. I., Gough, S. P. and Granick, S. (1975) *Proc. Nat. Acad. Sci. U.S.A.* **72**, 2719.
5. Wellburn, A. R. (1975) *Phytochemistry* **14**, 699.
6. Meller, E., Belkin, S. and Harel, E. (1975) *Phytochemistry* **14**, 2399.
7. Lohr, J. B. and Friedmann, H. C. (1976) *Biochem. Biophys. Res. Commun.* **69**, 908.
8. Jurgenson, J. E., Beale, S. I. and Troxler, R. F. (1976) *Biochem. Biophys. Res. Commun.* **69**, 149.
9. Salvador, G. F. (1978) *C. R. Acad. Sci. Ser. D* **286**, 49.
10. Porra, R. J. and Grimme, L. H. (1974) *Arch. Biochem. Biophys.* **164**, 312.
11. Meller, E. and Harel, E. (1978) in *Developments in Plant Biology. 2 Chloroplast Development* (Akoyungolou, C. and Argyroudi-Akoyungolou, eds.) p. 51. Elsevier, Amsterdam.
12. Klein, O. and Senger, H. (1978) *Plant Physiol.* **62**, 10.
13. Kipe-Nolt, J. A. and Stevens, S. E. (1980) *Plant Physiol.* **65**, 126.
14. Hoare, D. S., Hoare, S. L. and Moore, R. B. (1967) *J. Gen. Microbiol.* **49**, 351.
15. Pearce, J. and Carr, N. G. (1967) *J. Gen. Microbiol.* **49**, 301.
16. Pearce, J., Leach, C. K. and Carr, N. G. (1969) *J. Gen. Microbiol.* **55**, 371.
17. Beuhler, R. J., Pierce, R. C., Freidman, L. and Siegelman, W. (1976) *J. Biol. Chem.* **251**, 2405.
18. Kipe-Nolt, J. A., Stevens, S. E. and Stevens, C. L. R. (1978) *J. Bacteriol.* **135**, 286.
19. Smith, A. J. (1973) in the *Biology of Blue-Green Algae* (Carr, N. G. and Whitton, B. A., eds.) p. 1. Blackwell Scientific, Oxford.
20. Abraham, R. J., Hawkes, G. E. and Smith, K. J. (1974) *J. Chem. Soc. Perkins Trans.* 2, 627.
21. Severini-Ricca, G., Manitto, P., Monti, D. and Randall, E. W. (1975) *Gazz. Chim. Ital.* **105**, 1273.
22. Hansen, E. P., Thiessen, H. and Broderson, R. (1979) *Acta Chem. Scand. Ser. B* **33**, 281.
23. Wray, V., Gossauer, A., Grüning, B., Reifenstahl, G. and Zilch, H. (1979) *J. Chem. Soc. Perkins Trans.* 2, 1558.
24. McInnes, A. G., Walter, J. A. and Wright, J. L. C. (1979) *Tetrahedron Letters* 3245.
25. Allen, M. M. (1968) *J. Phycol.* **4**, 1.
26. Kratz, W. A. and Myers, J. (1955) *Am. J. Botany* **42**, 282.