Phytochemistry, 1977, Vol. 16, pp 351–354. Pergamon Press Printed in England

BIOSYNTHESIS OF HETEROCYST GLYCOLIPIDS OF *ANABAENA CYLINDRICA*

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(*Received* 21 *July* 1976)

Key Word Index— *Anabaena cylindrica*; Cyanophyceae; blue-green alga; biosynthesis; heterocyst glycolipids; long chain alcohols.

Abstract—The incorporation of sodium acetate-[1-^{14}C] into the heterocyst glycolipids of *Anabaena cylindrica* cultures from 60–234 hr old is reported. Incorporation of radioactivity was maximal in 88 hr old cultures. In 60 hr and 88 hr cultures about 90% of the radioactivity of the heterocyst glycolipids was found in the non-saponifiable glycolipid fraction, whereas in older cultures this fraction contained only 75% of the radioactivity. Acid hydrolysis of non-saponifiable heterocyst glycolipid fractions showed that in 60 hr cultures, 81% of the radioactivity occurs in the lipid moiety, whereas in older cultures a greater proportion (40–53%) of the radioactivity was found in the sugar residue. The lipid fraction obtained by acid hydrolysis contained a mixture of labelled long chain mono-, di- and trihydric alcohols. In young (60 hr) cultures the primary alcohol fraction was most heavily labelled (57.3% of the radioactivity in the non-saponifiable glycosides) with much smaller amounts in the diol and triol (8.4 and 15.1% respectively), whereas in older cultures (234 hr) the primary alcohol (23.6%) diol (22.5%) and triol (18.9%) fractions contained *ca* equal amounts of radioactivity.

INTRODUCTION

Blue–green algae grown in the presence of fixed nitrogen have a relatively simple polar acyl lipid composition, similar to that of the chloroplasts of higher plants [1]. When grown under nitrogen fixing conditions, however, both filamentous heterocystous [2] and unicellular blue–green algae [3, 4] produce a unique group of glycolipids, which, in heterocystous Cyanophyceae, are found exclusively in the heterocyst [5, 6] where they form the 'laminated layer' of the heterocyst envelope [6] and can be termed 'heterocyst glycolipids'. The major component of this group of heterocyst glycolipids in *Anabaena cylindrica* has been identified as the glycoside 1-(*O*-α-D-glucopyranosyl)-3,25-hexacosanedio [7, 8]. Minor amounts of the glycoside 1-(*O*-α-D-glucopyranosyl)-3,25,27-octacosanetriol were also detected. In addition to these non-saponifiable glycolipids, smaller amounts of a second class of glycolipids which were alkali labile were also detected in *A. cylindrica*. The major component of this class was identified as 25-hydroxyhexacosanoic acid (1-α-D-glucopyranose) ester [8]. In addition, an as yet unidentified polar, alkali-labile acyl lipid, which does not appear to contain a sugar residue [2], is also specifically produced when *A. cylindrica* is grown under nitrogen fixing conditions. Neither the heterocyst glycolipids nor the unidentified acyl lipid can be detected in *A. cylindrica* when the organism is grown in the presence of fixed nitrogen [3, 5].

It has been shown that very long chain acyl lipid and hydrocarbons are formed via an elongation pathway in higher plants [9, 10], whereas a condensation mechanism seems to operate in hydrocarbon biosynthesis in *Sarcina lutea* [11] and in *br*-hydroxyacid formation in *Corynebacterium diphtheriae* [12]. However, systematic studies of the biosynthesis of the very long chain lipid components of the heterocyst glycolipids of Cyanophyceae have not yet been reported and in view of their possible physiological role in the heterocyst [6, 8, 13], a study of the biosynthesis of this group of compounds has been undertaken.

RESULTS AND DISCUSSION

The amount of sodium acetate-[1-^{14}C] substrate incorporated into the total lipid of *A. cylindrica* was fairly constant in the older cultures (88 hr, 28%; 136 hr, 34%; 234 hr, 31.2%) but much lower (11.5%) in the 60 hr culture. Analysis of the radioactivity in the total lipid (Table 1) showed that the heterocyst glycolipid fraction was optimally labelled in the 88 hr culture, while incorporation into this fraction decreased sharply in the 234 hr culture. Optimal incorporation of label into the heterocyst glycolipids in the young cultures is consistent with the observation that in 60–88 hr cultures 'proheterocysts' are readily visible in the filaments at a frequency of 1 to ~10 vegetative cells, whereas in the 10-day-old cultures only mature heterocysts are visible at a frequency of 1:25 vegetative cells. At all time periods examined, the unidentified acyl lipid (U_1) also contained significant amounts of radioactivity and was most heavily labelled in the 60 hr culture. Alkaline hydrolysis of labelled U_1 isolated by preparative-TLC gave a radioactive fraction with R_f consistent with a long chain diol, the identity of which is under further investigation. At all ages of culture examined monogalactosyl diglyceride and sulphoquinovosyl lipid were the major labelled components. This distribution is in contrast to that reported earlier using sodium acetate-[2-^{14}C] in *A. cylindrica* [14], in which phosphatidylglycerol and

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Table 1. Distribution of radioactivity in the total lipid of *Anabaena cylindrica* cells of different ages after incubation with sodium acetate-[1-^{14}C]

Component	% of Total lipid radioactivity Age of culture (hr) 60	88	136	234
Origin	9.9	10.1	5.1	7.8
Sulpholipid	11.8	25.4	26.5	16.3
Phosphatidylglycerol	7.8	6.1	7.6	7.4
Digalactosyldiglyceride	3.8	2.7	2.2	1.4
Heterocyst glycolipid	8.2	13.6	8.8	4.0
Unidentified acyl lipid	3.8	1.3	0.9	2.8
Monogalactosyldiglyceride	35.5	32.9	40.0	49.1
'Neutral lipid'	18.9	8.0	9.1	9.4

monogalactosyl diglyceride each contained *ca* 25% of the total radioactivity. The difference in the incorporation may be due to the different stage of development of the cultures used in that study.

The heterocyst glycolipid fraction from each incubation was isolated by preparative-TLC and subjected to mild alkaline hydrolysis. On TLC in the polar solvent system the glycosidic, non-saponifiable glycolipids retained their R_f values, while the acyl moieties cleaved from the saponifiable, glycose ester glycolipids ran to the solvent front. This distribution of radioactivity showed that in 60-hr and 88-hr-old cultures about 90% of the radioactivity was present in the glycosidic glycolipid fraction, whereas in the older cultures (136 and 234 hr) the glycosidic glycolipids contained a smaller proportion of the total label (*ca* 70%). This result suggests that the formation and accumulation of glycose ester glycolipids may increase with the age of the maturing heterocyst.

Sodium acetate-[1-^{14}C] labels both the lipid and the sugar moieties of the heterocyst glycolipids. In order to determine the incorporation into the lipid moiety of the glycoside fraction the labelled non-saponifiable glycolipid fraction from each incubation was purified by preparative-TLC and subjected to acid hydrolysis. TLC of the radioactive products in the polar system gave two fractions, one corresponding to the methyl glycoside of the sugar residue which remained on the origin, and the other representing the lipid moieties which travelled to the solvent front. Hydrolysis was essentially quantitative as judged by the absence of starting material. The resulting distribution of radioactivity showed that the lipid fraction was most heavily labelled in the 60 hr culture (86%) and that there was a large decrease in incorporation into the lipid moieties in the 88 hr culture (46.7%) followed by a slow rise in the older cultures (61–65%). It thus appeared that sodium acetate-[1-^{14}C] was incorporated into the lipid moieties of the glycosidic glycolipid most effectively in the 60-hr-old culture.

The qualitative composition of the lipid fraction obtained by acid hydrolysis of non-saponifiable heterocyst glycolipid fractions was investigated; the glycoside fraction was isolated from the heterocyst glycolipids of a 60 l. 14-day-old *A. cylindrica* culture, as described by Bryce *et al.* [7]. TLC analysis (non-polar system) revealed a single major component, with an R_f (0.18) corresponding with a long chain triol. GC–MS of the TMSi ethers of this triol fraction gave a single major component with a fragmentation pattern characteristic of hexacosane-1,3,25-triol [7, 8] (*m/e* 615, 540, 525, 513, 450, 219, 117). Thus the major lipid moiety of the non-saponifiable heterocyst glycolipids of 14-day-old *A. cylindrica* produced under our growth conditions was the C_{26}-triol reported by other workers [7, 8]. However, radio-TLC analysis of the products obtained by acid hydrolysis of the non-saponifiable glycolipid isolated from 234-hr-old *A. cylindrica* cultures incubated with sodium acetate-[1-^{14}C] revealed the presence of three, rather than one, mobile labelled fractions, each containing *ca* equal amounts of radioactivity (Fig. 1), which together comprised 65% of the total radioactivity in this fraction. The most polar fraction (Fig. 1, 18.9% of total radioactivity) which co-chromatographed with authentic hexacosane-1,3,25-triol and, after acetylation, gave a labelled product which co-chromatographed with the triacetate of hexacosane-1,3,25-triol, was identified as a long chain triol fraction. The least polar fraction (Fig. 1) which contained 23.6% of the total radioactivity, co-chromatographed with authentic hexacosanol and on acetylation and TLC in the same solvent gave a labelled product which co-chromatographed with 1-acetoxyhexacosane, and was identified as a long chain primary alcohol fraction. A sample was purified by preparative-TLC and subjected to GC–RC as its TMSi ether derivative (Fig. 2), which revealed that hexacosanol was the major labelled primary alcohol (49%) with lesser amounts in the C_{22} (27%), C_{24} (13%) and C_{28} (1%) homologues.

The mobile fraction of intermediate polarity, (22.5% radioactivity, Fig. 1), was slightly more polar than tetracosane-1,15-diol but did not separate from hexacosane-1,2-diol. Treatment with $NaBH_4$ did not change its chromatographic properties, indicating the absence of a BH_4^- sensitive carbonyl function, but acetylation yielded a derivative less polar than the starting material and slightly more polar than authentic docosane-1,22-diol diacetate. These chromatographic properties suggested

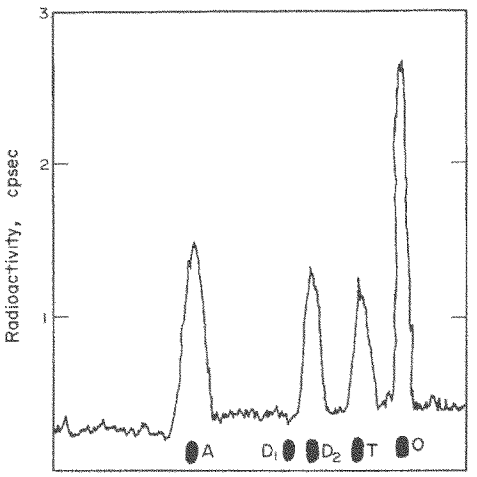

Fig. 1. Radio thin-layer chromatogram of the products obtained on acid hydrolysis of the labelled, non-saponifiable heterocyst glycolipid fraction from incubation of sodium acetate-[1-^{14}C] with a 234 hr old *A. cylindrica* culture. Reference compounds A = hexacosanol, D_1 = tetracosane-1,15-diol, D_2 = hexacosane-1,2-diol, T = hexacosane-1,3,25-triol, O = 1-0-methyl glucose.

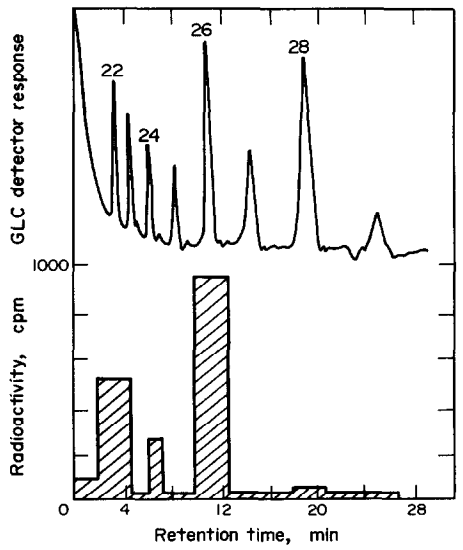

Fig. 2. Radio gas chromatography of the TMSi ether derivative of the labelled primary alcohol fraction obtained from the non-saponifiable heterocyst glycolipid.

that the fraction contained long chain diols in which the OH groups were either close together, e.g. C_{26}-1,3-diol, or far apart, e.g. C_{26}-1,25-diol. If such diols are formed by an elongation mechanism, as is indicated by preliminary inhibitor studies [15], then from C_{26}-1,3-diol Cr_2O_3 oxidation should yield a mixture of long chain fatty acids, whereas C_{26}-1,25-diol should produce long chain dioic acids. TLC of the Cr_2O_3 oxidation products revealed two labelled products, one of which co-chromatographed with 1,22-docosanedioic acid (84% radioactivity), while the second (16% of radioactivity) co-chromatographed with hexacosanoic acid. This result is consistent with the presence of both types of long chain diol in the original fraction, with C_{26}-1,25-diol predominating. Further work is in hand to fully characterise this fraction.

While variations in the distribution of radioactivity in the long chain primary alcohol, diol and triol fractions were observed in separate incubations with *A. cylindrica* cultures of the same age, these classes of compound were always the major labelled lipid components. These results suggest that a range of primary alcohols from C_{22}–C_{26} is produced and can be glycosylated, suggesting that the enzymes involved have relatively low specificity for their lipid substrate. Whether these primary alcohol glycosides were in fact present in the heterocyst envelope or were precursors of tri-droxy alcohol glycosides located elsewhere, cannot be deduced from the above study, although they have not been detected in chemical analyses on mature *A. cylindrica* heterocysts [8]. However, it has been established that the qualitative and quantitative composition of the hydroxy fatty acid phytopolymer cutin in very young tissue is markedly different from that in older tissue [10], so it may be that primary alcohol glycosides are major components of the envelopes of 'pro-heterocysts'. In support of this hypothesis is the observation that although the lipid components of the labelled non-saponifiable glycolipid isolated from incubation of 60-hr old *A. cylindrica* with sodium acetate-[1-^{14}C] contained a larger amount of the total radioactivity in the glycolipid fraction (80.8%) than was the case with the 234-hr-old culture, the distribution was strikingly different with the primary alcohol fraction predominating (57.3%) and much smaller amounts in the diol (8.4%) and triol (15.1%) fractions. Work is in progress to analyse the chemical composition of glycolipids of 'pro-heterocysts'.

The formation of glycosides of long chain primary alcohols suggests that further hydroxylation at C-3 and C-25 may occur after glycosylation of the primary alcohol rather than before. One of the suggested functions of the glycolipids in the heterocyst envelope is the maintenance of an anaerobic environment in the heterocyst by excluding diffusion of oxygen [8]. It may be that introduction of further hydroxyl groups into the methylene chain via a mixed function oxygenase catalysed reaction represents a mechanism of removing molecular oxygen and preventing its access to the heterocyst.

EXPERIMENTAL

A. cylindrica (strain 1403/2A) was obtained from the Cambridge collection of Algae and Protozoa. Cells were grown in ASM-1 medium [16] as modified in ref. [17]. Cultures for incubation were grown in 250 ml conical flasks containing 100 ml of medium in an orbital incubator (100 rpm) under a constant light source of 2500 lx at 25°. Cells for extraction were grown in 20 l. vessels containing 17 l. of medium which was aerated and circulated by a stream of compressed air [17]. Growth was initiated by a 1% inoculum of 14-day-old *A. cylindrica* cells.

Incubation conditions. Cells from 100 ml culture medium were isolated by centrifugation (5000 *g* 10 min) at 5°, resuspended in culture medium (2 ml) and transferred to 25 ml conical flasks. Normally 10 μCi of sodium acetate-[1-^{14}C] (sp. act. 57 Ci/mol) was added and the total vol. made up to 3 ml with medium. Flasks were loosely stoppered with cottonwool and incubated for 5 hr on a rotary incubator at 25° and 2500 lx.

Extraction of lipids and isolation of authentic heterocyst glycolipids. Incubations were terminated by addition of excess (15 ml) *iso*-PrOH and lipid extracted by standard procedures [18]. Total lipid was isolated from the large scale cultures as described in ref. [7].

TLC. Examination of the total lipid extracts and the alkaline and acid hydrolysis products of the purified heterocyst glycolipids was carried out on Si gel G using $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:4) (polar TLC system). The acid hydrolysis products of the purified non-saponifiable glycolipid and their acetate derivatives were analysed using Et_2O–hexane (7:4) (non-polar system). Samples were visualised either by spraying with α-naphthal–H_2SO_4, H_2SO_4 charring or dichlorofluorescein with UV inspection.

Determination of radioactivity. TLCs were scanned directly for radioactivity with a scanner. Radioactive zones were scraped from chromatograms and transferred directly to counting vials containing 10 ml of a sol of PPO in EtOH–toluene (3:7) (4 g/l.). Serial 1 cm bands were scraped from the chromatogram of the products of Cr_2O_3 oxidation of the labelled diol and assayed. Radioactive solns were assayed by counting a small aliquot in the same scintillant system. ^{14}C counting efficiency was normally 85% and samples were assayed to a 2 σ confidence level of 1% in a liquid scintillation spectrometer. Corrections were made for quenching by use of an internal standard of hexadecane-[1-^{14}C] where necessary.

Preparation of standards and derivatives of metabolic products. Authentic hexacosane-1,3,25-triol was isolated by the method described in ref. [7]. Me tetracos-15-enoate was converted to tetracos-15-en-1-ol, a 1:1 mixture of tetracosane-1,15-diol and tetracosane-1,16-diol, and to tetracosane-1,15,16-triol by standard procedures [19]. The C_{24}-diols are positional isomers of

very similar chromatographic properties and do not separate on the TLC systems employed. For simplicity the mixture is referred to as tetracosane-1,15-diol. Me hexacosanoate, Me 2-hydroxyhexacosanoate and a mixture of C_{22}–C_{34} fatty acid Me esters were converted to their corresponding alcohols by $LiAlH_4$ reduction. Acetates and TMSi ethers were prepared by standard procedures. Cr_2O_3 oxidn was as in ref. [19].

Hydrolyis of metabolic products. Compounds were treated with 2M methanolic KOH (3 ml) at room temp. for 3 hr. The reaction mixture was diluted with H_2O, acidified with 5 M HCl, and extracted into $CHCl_3$ (3 × 50 ml). Acid hydrolysis was carried out by refluxing with 30 ml 2 M H_2SO_4 in MeOH–H_2O (9:1) for 28 hr. The reaction mixture was diluted with H_2O (50 ml) and extracted with $CHCl_3$ (3 × 100 ml).

GLC—trapping. A 1.5 m 3 mm id glass column packed with 3% OV-1 was used with a N_2 flow rate of 70 ml/min at 250°. The injector and detector splitter oven were maintained 70° higher than the column. The instrument was equipped with an effluent splitter which gave an effective ratio of 10:1 (trap:detector). Components were trapped at an efficiency of *ca* 50% by passing the effluent stream through a Pasteur pipette lightly packed with glass wool moistened with scintillant soln. The trapped sample was transferred to a counting vial by passing 10 ml of scintillant soln through the Pasteur pipette.

GC–MS. This was carried out on an instrument equipped with a silicone rubber membrane separator system and linked to a data system. Spectra were determined at 70 eV. The column system used was a 3 m glass column packed with 1% SE-30 operating at 257° with a He flow rate of 40 ml/min.

Acknowledgement—The authors thank Dr. J. A. Ballantine of the Chemistry Department of this University for determination of GC–MS data.

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