Environmental Factors Affecting the Formation of Mesityloxiide, Dimethylallylic Alcohol and Other Volatile Compounds Excreted by *Anabaena cylindrica*

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Anabaena cylindrica excretes a large number of volatile products. The major components are dimethylallylic alcohol and mesityloxiide, while the minor components detected include various nor-carotenoids and lipid degradation products. The formation of mesityloxiide is strictly light-dependent, and ceases in the dark. A direct association with photosynthesis can be ruled out since 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) does not affect the formation of mesityloxiide during the first hour after application, but immediately inhibits the evolution of oxygen.

INTRODUCTION

Cyanobacteria contain a broad spectrum of different carotenoids, some resembling those of the green algae, others being unique to this group of organisms (Stransky & Hager, 1970; Hertsberg *et al.*, 1971). Apart from these tetraterpenoids, very few smaller terpenoids have been detected in cyanobacteria. Initially, steroids were stated to be absent from cyanobacteria (Levin & Bloch, 1964), but later work has characterized a small number of these compounds (Reitz & Hamilton, 1968; Nadal, 1971). Reports on regular di-, sesqui- and monoterpenes are still lacking. However, irregular mono- and sesquiterpenes have been detected. β -Cyclocitral is the major excretion product of *Microcystis* (Jüttner, 1981*a*), and its carbon skeleton indicates biosynthesis of this monoterpene from carotenes by oxidative cleavage. Other components described are 2-methylisoborneol, a bicyclic C₁₁ compound, and geosmin, a C₁₂ terpene alcohol (Gerber, 1968). As far as is known, the latter are liberated exclusively by filamentous cyanobacteria (Safferman *et al.*, 1967; Kikuchi *et al.*, 1973; Tabachek & Yurkowski, 1976). Though biosynthetic studies have not been conducted, a subsequent introduction of a methyl group and loss of an alkyl group, respectively, is evident for these two substances. Their strong muddy odour together with their extremely low odour thresholds (Persson, 1980) gives rise to serious problems in fisheries and in the production of drinking water. A significant correlation between the number of *Oscillatoria agardhii* present in the phytoplankton and off-flavours in bream has been demonstrated (Persson, 1979). The results of a survey on North American water supplies (Sigworth, 1957) indicated that odour problems are most frequently caused by diatoms of the genera *Asterionella* and *Synedra*, followed by *Anabaena* species and other cyanobacteria.

During the course of studies on volatile excretion products of cyanobacteria and algae we have found two terpenoid substances which were identified as mesityloxiide and dimethylallylic alcohol and which were excreted in exceptionally high amounts by the well-studied species *Anabaena cylindrica*.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

METHODS

Organism. *Anabaena cylindrica*, identical to strain 1403/2a, Culture Centre of Algae and Protozoa, Cambridge, U.K., was obtained from Dr N. G. Carr, Department of Biochemistry, Liverpool, U.K.

Culture medium and conditions. The cyanobacteria were cultivated in 300 ml culturing tubes at 27 °C, gassed with a 0.27% (v/v) CO₂/air mixture from below and illuminated with one fluorescent tube (1000 lx). After each transfer, the axenic nature of the cultures was tested by streaking samples on nutrient agar plates (DEV-nutrient agar, Merck no. 11471). The medium used contained: 0.6 mM-CaCl₂, 8 mM-NaNO₃, 0.4 mM-K₂HPO₄, 0.4 mM-MgSO₄, 10 µM-NaFeEDTA (from Fluka, Neu-Ulm, Germany), 10 µM-H₃BO₃, 10 µM-MnCl₂, 2 µM-Na₂MoO₄, 0.2 µM-ZnSO₄, 0.2 µM-CuSO₄ and 0.2 µM-CoSO₄, in distilled water.

Determination of chlorophyll a and protein. The cyanobacteria were extracted with ethanol and chlorophyll *a* was determined by using the absorption coefficient given by Seely & Jensen (1965). The protein content of the extracted residue was determined with a biuret reaction using bovine serum albumin as a standard.

Identification of the volatile substances. The volatile components of 100 ml of *A. cylindrica* suspension in the declining exponential growth stage were stripped after addition of 20 g NaCl in a closed loop system, adsorbed on an odour trap, then thermally transferred into a gas chromatograph and separated on a glass capillary column as described previously (Jüttner & Wurster, 1979; Jüttner & Hahne, 1981).

Reference compounds and chemicals. Chemicals and reference compounds were either purchased or synthesized as stated previously (Jüttner, 1981b). 6-Methylhept-5-en-2-ol was synthesized from 6-methylhept-5-en-2-one which was a kind gift of the BASF/Ludwigshafen/Germany. The alcohol was obtained in high yield after reduction with NaBH₄ in acetonitrile/water.

Quantitative determination of the volatile components in fermenter cultures. The quantitative determination of the volatile components in fermenter cultures was performed as described by Jüttner & Hahne (1981). The medium used here was supplemented with 20 mM-NaHCO₃. At the beginning of the experiment, aeration was stopped and 20 ml samples were withdrawn for the determination of the volatile components.

Quantitative determination of the growth-dependent formation of the volatile components. Samples (200 ml) of suspension of *A. cylindrica* in the exponential growth stage were diluted with 1000 ml of fresh medium, divided into twelve 300 ml Erlenmeyer flasks closed with aluminum foil and incubated in an incubation shaker [27 °C, 110 strokes min⁻¹, 1400 lx, gassing at 500 ml min⁻¹ with 0.27% (v/v) CO₂ in air]. At one-day intervals flasks were withdrawn and the volatile components determined in 20 ml samples as described above.

RESULTS

Though cultures of *Anabaena cylindrica* have only a weak odour, the gas chromatogram (Fig. 1) demonstrates the presence of an appreciable number of volatile components which are excreted. The mass fragmentation patterns along with the retention times were used to identify the substances. A compound was regarded as being positively identified when it could not be separated on a glass capillary column spiked with the authentic substance. The nature of peak 18 remains unknown. The mass spectrum exhibited features of a branched ketone but the data available were not sufficient to determine its structure.

Difficulties arose initially in differentiating between pollution products which are always present in distilled water, nutrient salts, stoppers and aeration gas, and the excretion products of the cyanobacteria. The best method for determining the origin of single components was to follow their generation during the growth of a culture. Pollution products were present in either stable or erratic concentrations, while cyanobacterial products showed growth-dependent formation. The concentrations of the volatile excretion products present in *A. cylindrica* cultures during exponential growth, declining exponential growth and the beginning of the stationary phase are presented in Figs 2 and 3. Extremely high concentrations of mesityloxide (4-methylpent-3-en-2-one) and dimethylallylic alcohol (3-methylbut-2-en-1-ol) were observed, together representing the bulk of volatile excretion products of *A. cylindrica*. Most of the other components were present in concentrations of at least an order of magnitude lower.

The formation of mesityloxide was found to be light-dependent. In an experiment in which cultures were exposed to alternating light and dark conditions, no significant synthesis of this compound could be observed in the dark (Fig. 4). In order to investigate whether formation of mesityloxide is directly coupled to photosynthesis, DCMU, a specific and effective inhibitor of photosynthesis, was added and the formation of mesityloxide followed. The concentration of DCMU (10⁻⁶ M) applied was sufficient to suppress the oxygen evolution completely; however, for at least one hour after application, mesityloxide production was little affected and declined only later.

Fig. 1. Volatile compounds isolated from a shaking culture of *A. cylindrica* on the eighth day after inoculation ($15 \mu\text{g}$ chlorophyll *a* ml^{-1} ; $130 \mu\text{g}$ protein ml^{-1}) separated on a 25 m glass capillary column (UCON 50 HB 5100). Pollution products are printed in *italics*. 1, Penten-3-one; 2, *toluene*; 3, dimethyldisulphide; 4, *cis*-pent-2-enal; 5, hexanal; 6, *trans*-pent-2-enal; 7, mesityloxide; 8, *butan*-1-ol; 9, pent-1-en-3-ol; 10, pentan-1-ol; 11, *cyclohexanone*; 12, dimethylallylic alcohol; 13, 6-methylhept-5-en-2-one; 14, hexan-1-ol; 15, 6-methylhept-5-en-2-ol; 16, *benzaldehyde*; 17, *2-ethylhexan*-1-ol; 18, ketone (unidentified); 19, β -cyclocitral; 20, *benzyl alcohol*; 21, β -ionone.

Fig. 2. Time course of growth ($\circ$, protein; $\blacksquare$, chlorophyll *a*) and the formation of volatile norcarotenoids (β -ionone, 6-methylhept-5-en-2-one, mesityloxide, 6-methylhept-5-en-2-ol) and dimethylallylic alcohol in illuminated shaking cultures of *A. cylindrica*. The concentrations of protein and chlorophyll *a* are in mg l^{-1} ; those of the other components are in $\mu\text{g l}^{-1}$.

Fig. 3. Time course of the formation of lipid degradation products (pentan-1-ol, pent-1-en-3-ol, hexanal, *trans*-pent-2-enal, hexan-1-ol and *cis*-pent-2-enal) of *A. cylindrica* in the same experiment as Fig. 2.

Fig. 4. Concentration of mesityloxiide (plotted as the integrated area of the flame ionization detector signal) in a 2 litre fermenter culture of *A. cylindrica* during alternating light and dark conditions.

DISCUSSION

The volatile substances determined in the medium of *Anabaena cylindrica* can be divided into two major groups, terpenoid compounds and fatty acid degradation products, according to their presumptive biosynthetic origin. Mesityloxiide and 6-methylhept-5-en-2-one belong to the first group and might result from an oxidative cleavage of open-chain carotenes as has been demonstrated in tomatoes (Stevens, 1970). A reduction reaction mediated by an alcohol dehydrogenase could result in the formation of 6-methylhept-5-en-2-ol, which was observed in much higher concentrations than the corresponding ketone. On the other hand, the alcohol corresponding to mesityloxiide could not be detected. Nor-carotenoids derived from β -carotene were represented by β -cyclocitral and β -ionone. β -Cyclocitral has already been detected as the major excretion product of the cyanobacterium *Microcystis* (Jüttner, 1981a) and β -ionone and

6-methylhept-5-en-2-one have been found in the primitive rhodophyte *Cyanidium caldarium* (Jüttner, 1979). A different biosynthetic pathway involving the action of phosphatase on dimethylallylic pyrophosphate may be responsible for the generation of the extremely high amounts of dimethylallylic alcohol observed. Both major components, mesityloxide and dimethylallylic alcohol, have not been found to constitute major excretion products in any other micro-organism.

The kinetics of formation of the isoprenoid components during different stages of growth were rather similar to one another. High rates were observed during exponential and late-exponential growth and a sharp decline in the stationary growth phase. This agrees with the observation that mesityloxide is synthesized by *Anabaena cylindrica* exclusively in the light. However, photosynthesis is not directly involved in its formation, as indicated by the small effect of DCMU. Indeed, cessation of its production may be due to depletion of a precursor terpenoid which cannot be synthesized in the dark.

The group of compounds derived from fatty acids (straight-chain alcohols, alkenols, alkanals and alkenals) exhibit kinetics of formation different from those of the aforementioned terpenoids; however, individual compounds show similar kinetics. Markedly increased rates were noticed with the onset of the stationary growth phase. Polyunsaturated fatty acids present in all strains of *Anabaena* (Kenyon *et al.*, 1972) including *A. cylindrica* (Nichols & Wood, 1968) can be regarded as precursors of these components. The action of lipoxygenase (Galliard & Metthew, 1977) together with a hydroperoxide lyase (Heimann *et al.*, 1975; Vick & Zimmermann, 1976; Hatanaka *et al.*, 1979) on linoleic acid results in the generation of hexanal, as has been demonstrated in higher plants. The enzymic reactions which would generate the C₅ compounds are less well investigated. However, α -linoleic acid, present in high amounts in *Anabaena* (Kenyon *et al.*, 1972), can be assumed to be the precursor.

The only sulphur-containing component present was dimethyldisulphide, which has previously been detected in cyanobacteria (Bechard & Rayburn, 1979). The quantities produced, however, were too low to follow its formation during growth.

The ecological significance of the excretion of substantial amounts of volatile compounds into the environment can only be guessed at. Inhibiting effects of nor-carotenoids on the uptake and respiration of glucose were observed with aquatic heterotrophic bacteria (Reichardt, 1981). β -Ionone was demonstrated to inhibit the growth of the chlorophyte *Nannochloris* though the site of inhibition is not known (Jüttner, 1979). The physiological effects of mesityloxide and dimethylallylic alcohol, each reaching concentrations as high as 0.25 p.p.m. in the medium, have not yet been studied intensively.

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