Arch. Mikrobiol. 90, 323—332 (1973)

Nitrogen Fixation by *Anabaena cylindrica*

I. Localization of Nitrogen Fixation in the Heterocysts

N. M. Weare and John R. Benemann

Department of Chemistry, University of California, San Diego, La Jolla, California 92037

Received December 27, 1972

Summary. Blending *Anabaena cylindrica* cultures results in a loss of nitrogenase activity which is correlated with the breakage of the filaments at the junctions between heterocysts and vegetative cells. Oxygen inhibition of nitrogen fixation was significant only above atmospheric concentrations. Nitrogen-fixation activities in the dark were up to 50% of those observed in the light and were dependent on oxygen (10 to 20% was optimal). Nitrogenase activity was lost in about 3 h when cells were incubated aerobically in the dark. Re-exposure to light resulted in recovery of nitrogenase activity within 2 h. Blending, oxygen, or dark pre-incubation had similar effects upon cultures grown under air or nitrogen and did not inhibit light-dependent CO_2 fixation. We conclude that heterocysts are the sites of nitrogenase activity and propose a model for nitrogen fixation by *Anabaena cylindrica*.

While fixing atmospheric nitrogen most filamentous blue-green algae contain heterocysts, characteristic cells evenly spaced among the vegetative cells in the filaments. Fay *et al.* (1968) suggested that heterocysts are the sites of nitrogen fixation in filamentous blue-green algae. This theory is supported by the correlation between heterocyst formation and nitrogen fixation (Fogg, 1949; Stewart *et al.*, 1968; Neilson *et al.*, 1971; Kulasooriya *et al.*, 1972), by the reducing environment observed inside heterocysts (Talpasayi, 1967; Stewart *et al.*, 1969), and by the absence of an oxygen-evolving photosystem II (Thomas, 1970; Donze *et al.*, 1972). Suitable anaerobic conditions for the oxygen-labile nitrogenase enzyme and reaction are therefore attributed to heterocysts. However, several workers believe vegetative cells to be capable of nitrogen fixation (Smith and Evans, 1970; Wolk, 1970; Kurz and LaRue, 1971; Millbank, 1972; Ohmori and Hattori, 1971), even if only under anaerobic conditions (Stewart, 1971; Van Gorkom and Donze, 1971; Thomas and David, 1972). Evidence supporting these views are the low nitrogenase activities in purified heterocyst preparations (Stewart *et al.*, 1969; Wolk and Wojciuch, 1971), the presence of nitrogenase in vegetative cell fractions (Smith and Evans, 1970; Bothe, 1970; Haystead *et al.*, 1970), the reducing environment in vegetative cells under anaerobic conditions (Thomas and David, 1972),

the oxygen sensitivity of nitrogen fixation by anaerobic cultures (van Gorkom and Donze, 1971), and the ability of *Plectonema boryanum*, a nonheterocystous, filamentous, blue-green alga, to fix nitrogen anaerobically (Stewart and Lex, 1970).

The physiological experiments described below indicate that heterocysts are the main, probably exclusive, sites of nitrogen fixation in both air- and N_2-grown cultures of *Anabaena cylindrica*. Our data also suggest that vegetative cells supply substrates needed for nitrogen fixation. We propose a model for nitrogen fixation by *Anabaena cylindrica*.

Methods

Culture of Algae

Axenic cultures of *Anabaena cylindrica* (B629, obtained from Dr. D. C. Yoch) were grown at 28 to 30° C in 2.5-l glass bottles in 2 l of a modified Allen and Arnon (1955) medium. (Modifications: 2×10^{-3} M K_2HPO_4 and 5×10^{-3} M $NaHCO_3$.) The cultures were magnetically stirred and bubbled (100 to 300 ml/min) with 0.3% CO_2 in either air (aerobic cultures) or nitrogen (anaerobic cultures). The cultures were illuminated with fluorescent lamps; the light intensity at the sides of the vessels was 2×10^4 ergs cm^{-2} sec^{-1}. The pH was 7.8—8.2. (The characteristic pH rise of the anaerobic cultures was corrected by addition of HCl.) Cell densities were measured with a Klett-Summerson colorimeter with a red (660 nm) filter. Chlorophyll determinations were carried out in 90% acetone using a Turner Fluorometer. Doubling times ranged between 14 to 18 h. Above 100 Klett units exponential growth ceased and cultures were considered old. However, stationary phase was not achieved until cell densities of over 200 Klett units.

Nitrogen and CO_2 Fixation Assays

Nitrogen fixation was measured with the acetylene-reduction assay using 2 ml of culture in 7 ml serum stoppered flasks containing 12% acetylene (balance air, argon, or argon-oxygen mixtures as indicated). The flasks were shaken in holders in a Warburg apparatus. The assay temperature was 30° C and the light intensity 6×10^4 ergs cm^{-2} sec^{-1}. Dark activities were assayed in tinfoil-wrapped flasks. Reactions were terminated with 0.2 ml of 20% trichloroacetic acid. Ethylene determinations were carried out as previously described (Benemann *et al.*, 1972). CO_2 fixation was measured concurrently with acetylene reduction by injecting 50 µl of 10 µc/ml $NaH^{14}CO_3$ into the assay flasks. At the end of the assay 1 ml aliquots were removed and filtered on Whatman GF/C filters for the CO_2 fixation determinations by scintillation counting.

Results

Effect of Blending

Blending *Anabaena cylindrica* cultures drastically reduced filament lengths and also resulted in a drop of nitrogen fixation, but not CO_2 fixation, activities (Fig. 1). Average filament length decreased from about 100 to about 10 cells within 10 sec. As filament length decreased higher speeds and longer times were required between blending points to

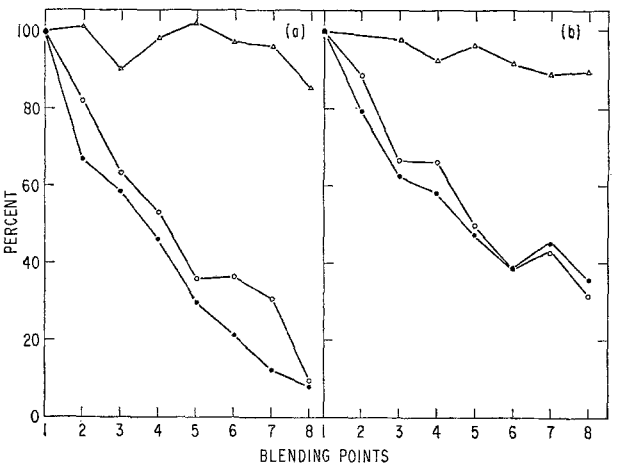

Fig. 1 a and b. Effect of blending on *Anabaena cylindrica*. 70 ml of culture was blended in a Sorvall Omni-mixer under air (aerobic cultures) or argon (anaerobic cultures) with increasing times and speeds. At each point a small aliquot was removed for heterocyst counting and for N_2- and CO_2-fixation assays. The blending was then resumed. The times between blending points were: 5, 5, 5, 10, 10, 15 and 30 sec, and the speeds were: 90, 100, 110, 120, 120, 140 and 140 (Powerstat settings). C_2H_2-reduction activities (•——•), CO_2-fixation activities (△——△) and the percentage of middle heterocysts (vegetative cells attached to both ends of the heterocyst)(o——o) are given as the percentage of values before blending (point 1). a Aerobic (air/CO_2) culture blended and assayed under air (15 min assay after 15 min pre-incubation). Klett = 15; chlorophyll a = 375 µg/l. Before blending (point 1), the culture had activities of 0.723 nmoles acetylene reduced/ml/min, 10335 ^{14}C-counts per min/ml/15 min assay time and 90.7% of heterocysts counted were middle heterocysts. b Anaerobic (N_2/CO_2) culture blended and assayed under argon (15 min assay after 15 min pre-incubation). Klett = 30; chlorophyll a = 675 µg/l. Before blending the culture had activities of 1.488 nmoles of acetylene reduced/ml/min, 19000 ^{14}C-counts per min/ml/15 min assay time, and 78.7% of heterocysts counted were middle heterocysts

continue lowering nitrogenase activity. The loss of nitrogen fixation activity was correlated with an increase in free and terminal heterocysts and a decrease in middle heterocysts. (Terminal heterocysts have one attached vegetative cell, middle heterocysts have vegetative cells attached to both poles.) When normalizing nitrogen fixation and heterocysts frequencies, the correlation between nitrogenase activity and number of middle heterocysts is very close (Fig. 1). Similar results are obtained whether the blending and assays are carried out aerobically or anaerobically (under argon).

N_2-grown cultures had more terminal and isolated heterocysts before blending and lost less nitrogen-fixation activity during blending than did

air-grown cultures. Such differences in heterocysts distribution and relative loss of nitrogenase activity during blending were observed also between young and old cultures. However, in all cases, loss of nitrogen fixation always correlated with filament breaks at the heterocysts. The undiminished CO_2-fixation activity suggests that vegetative cells were unaffected by the blending.

Effects of Oxygen

Oxygen inhibits nitrogen fixation, but not CO_2 fixation, in the light (Fig. 2). In the dark, when CO_2 fixation was over 95% inhibited, nitrogen-fixation activities showed a requirement for oxygen. The optimal oxygen concentration was between 10—20%; higher oxygen concentrations inhibited nitrogenase activities in the dark even more strongly than in the light. The magnitudes of these dark activities were surprising, in young cultures about 50% of those measured in the light. Dark activities decreased during growth and aging of the cultures, in older cultures no dark activities could be detected.

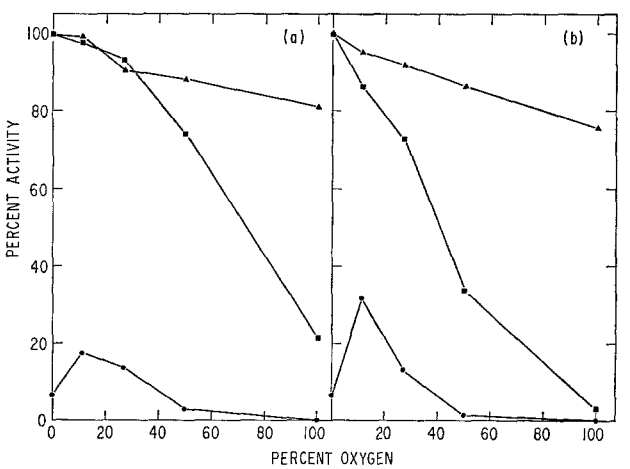

Fig. 2a and b. Effect of oxygen on *Anabaena cylindrica*. Cultures were assayed under an argon atmosphere containing varying amounts of oxygen for 15 min after a 10 min pre-incubation. CO_2-fixation (▲——▲), N_2-fixation in the light (■——■), and N_2-fixation in the dark (●——●) are given as a function of percent oxygen added to the assay flasks. The CO_2 and N_2 fixation (both light and dark) data are given as the percentage of values under argon in the light. a Aerobic (air/CO_2) cultures. Klett = 96; chlorophyll a = 1875 µg/l. 100% activities: 3.1 nmoles of acetylene reduced/ml/min and 11720 ^{14}C counts per min/ml/15 min assay time. b Anaerobic (N_2/CO_2) cultures. Klett = 73; chlorophyll a = 1380 µg/l. 100% activities: 3.06 nmoles of acetylene reduced/ml/min and 17180 ^{14}C count per min/ml/15 min assay time

Although nitrogenase activities by N_2-grown cultures were more strongly inhibited by oxygen than air-grown cultures, the differences are only significant above 20% O_2. The age of the cultures is the determining factor in the sensitivity of the nitrogenase activities to oxygen. After exponential growth ceased, nitrogen fixation continues at substantial rates for days, even weeks. Oxygen inhibition becomes pronounced in these cultures even at atmospheric concentrations. However, in *Anabaena cylindrica* cultures actively growing under air or nitrogen, oxygen at atmospheric pressures does not inhibit nitrogenase activity to a significant extent.

Effect of Dark Incubation

The loss of nitrogenase activity after transferring cultures to the dark, and the recovery of this activity after return to the light, are shown in Fig. 3. Aerobic, but not anaerobic, incubation in the dark decreased nitrogenase activities measured both in the light and in the dark. CO_2

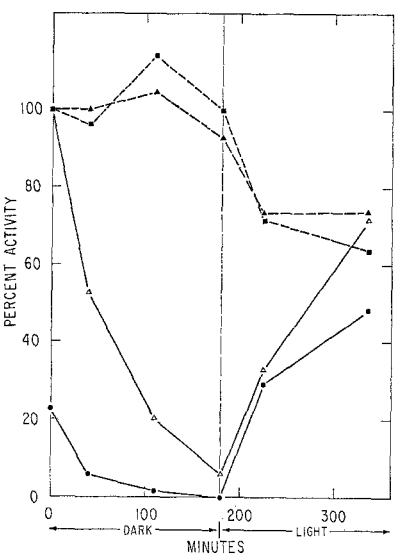

Fig. 3. Effect of dark incubation on *Anabaena cylindrica*. An air/CO_2-grown culture (Klett = 96; chlorophyll a = 1875 µg/l; N_2 fixation-activity in light under air = 3.01 nmoles of acetylene reduced/ml/min; CO_2 fixation activity 6950 ^{14}C counts per min/ml in 10 min) was placed in the dark under air and aliquots were assayed after varying times for 10 min under air for CO_2 fixation in the light (■——■), N_2 fixation in the light (△——△) and N_2 fixation in the dark (●——●). Another portion of the culture was placed in the dark under argon and assayed after varying times for 10 min for N_2 fixation in the light under air (▲——▲). After 3 h cultures were returned to the light. All activities are reported as percentage of values at zero time

fixation assayed in the light was not affected by dark pre-incubations. When cultures with most of their nitrogen-fixing activity lost by aerobic dark incubation for 3 h were returned to the light, the recovery of both light and dark nitrogenase activities took place at approximately the same initial rate (Fig. 3). Similar results were obtained using cultures grown under air/CO_2 or N_2/CO_2. Recovery of nitrogenase activity took place, although to a lesser extent, in the presence of chloramphenicol, indicating that biosynthesis alone cannot account for the recovery. In older cultures loss of nitrogenase activities under dark aerobic conditions was fast and irreversible.

Discussion

The blending experiments indicate that filament breakage occurs preferentially at the junction between vegetative cells and heterocysts, and that breaking this connection results in a loss of nitrogenase activity by the culture. The sonication experiments of Stewart *et al.* (1969) showed that nitrogenase activity was lost faster than the number of attached heterocysts decreased. Our data give similar results when attached (middle and terminal) heterocysts are correlated with nitrogenase activity. Therefore, only heterocysts attached at both ends to vegetative cells appear to be functional. The correlation of filament breakage at the heterocysts with loss of nitrogen fixation activity was found with both air- and N_2-grown cultures and under aerobic and anaerobic blending and assay conditions. These experiments support the suggestion of Fay and Lang (1971) that isolating heterocysts by differential disruption of the vegetative cells results in damage to the heterocyst pores and leakage of their cellular material into vegetative cell preparations.

Oxygen tensions up to 20% have only minor effects on nitrogen fixation by air- or N_2-grown cultures. Since there is no evidence that vegetative cells have an oxygen protection mechanism, like that supposed to operate in heterocysts, loss of nitrogenase activity in the presence of oxygen should be rapid and pronounced if vegetative cells were capable of fixing nitrogen in N_2-grown cultures. Our results therefore support the suggestion of Kulasooriya *et al.* (1972) that even under anaerobic conditions heterocysts are the main site of nitrogen fixation. The term „anaerobic" can be misleading since strict anaerobiosis is impossible to achieve in the light with these oxygen-evolving organisms. Therefore „anaerobic", although widely used in the literature, must be qualified to indicate that when gassing with nitrogen or argon low oxygen tensions exist in the *Anabaena* cultures. Whether under strict anaerobic conditions, like those reported in the presence of hydrogen (Van Gorkom and Donze,

1971) or of sulfide (Stewart and Pearson, 1970), nitrogen fixation is restricted to heterocysts remains to be established.

The oxygen requirement for nitrogen-fixation activities in the dark suggests that oxidative phosphorylation can supply the ATP requirement of nitrogenase. Leach and Carr (1969, 1970) and Biggins (1969) have demonstrated NADPH-mediated oxidative phosphorylation in *Anabaena*. However, these findings have not yet been extended to heterocysts. The absence of any sustained dark nitrogenase activities under anaerobic conditions excludes fermentation as a significant source of ATP for nitrogen fixation.

All three types of experiments reported in this paper represent short term assays and in all CO_2 and N_2 fixation behaved differently. This supports previous work which indicated an indirect relationship between photosynthesis and nitrogen fixation (Cox and Fay, 1969).

The kinetics of the loss of nitrogen-fixation activity during aerobic dark incubation and its recovery after transfer back to the light (Fig. 3) can be explained in terms of movement of reductant- and ATP-generating supplies from the vegetative cells to heterocysts. The data supports the view that in the dark aerobic assays (Fig. 2) ATP was the limiting substrate of nitrogenase whereas after 3 h of dark aerobic incubation (Fig. 3) the reductant supply became limiting. In autoradiography experiments, Wolk (1968) showed movement of labeled carbon from vegetative cells to heterocysts. Although the nature of these metabolites is not known, they should be capable of reducing NADP, which then can generate reduced ferredoxin (Benemann and Valentine, 1972) and, under dark aerobic conditions, ATP (Leach and Carr, 1970; Biggins, 1969). This argument rests on the finding that NADPH is the electron donor in *Azotobacter vinelandii* (Benemann *et al.*, 1970, 1971), and on the work in the same laboratory extending this finding to *Anabaena cylindrica* (Bothe, 1970). The potential role of NADPH in nitrogen fixation by *A. cylindrica* has been confirmed by Smith *et al.* (1971) and Haystead and Stewart (1972), who also show that light-driven ferredoxin reduction could support nitrogen fixation. It has been argued (Smith *et al.*, 1971) that light-driven electron transport supplies most (95%) of the reductant for nitrogenase. Our data showing high dark activities together with the reported absence of photosystem II in heterocysts (Thomas, 1970; Donze *et al.*, 1972) do not support this contention. The stimulatory effect of light on nitrogen fixation might better be ascribed to ATP synthesis by photosystem I photophosphorylation (Fay and Walsby, 1966; Fay, 1970).

We have summarized our current views about nitrogen fixation by *Anabaena cylindrica*, and other heterocystous blue-green algae, in the model shown in Fig. 4. In this hypothetical model vegetative cells provide

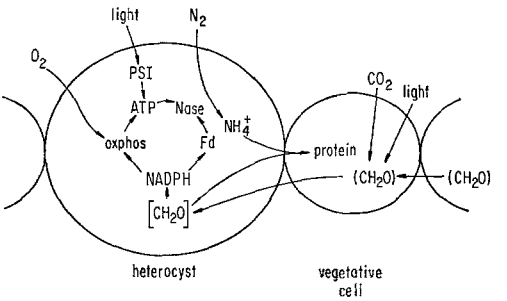

Fig. 4. Proposed model for nitrogen fixation by *Anabaena cylindrica*

reduced carbon compounds to the heterocysts which utilize them to generate NADPH which is then able to reduce nitrogenase through ferredoxin. Under dark aerobic conditions NADPH also serves as the source for ATP; in the light photophosphorylation generates the ATP required for nitrogen fixation. Pyruvate has been reported to stimulate nitrogenase activity in whole and broken *Anabaena* filaments (Cox and Fay, 1967, 1969). An *Anabaena* pyruvate: ferredoxin oxidoreductase has been recently described (Leach and Carr, 1971). Which metabolite, pyruvate or NADPH, is the predominant and immediate electron donor in an electron carrier mediated nitrogenase reaction still remains to be settled for both *Anabaena* and *Azotobacter*. The ammonia produced by nitrogenase is transported to the vegetative cells, probably in a combined form. This model applies to heterocystous blue-green algae actively growing under aerobic or semi-aerobic conditions. Whether it also applies to old or strict anaerobic cultures is not yet known.

Acknowledgements. We thank Dr. M. D. Kamen for his support and encouragement and S. DeNardis for his technical assistance. This work was supported by grants to Dr. M. D. Kamen (NSF GB-25050 and NIH GM-18528) and by an Atomic Energy Commission grant (Contract No. AT (11-1) GEN 10, P.A. 20) to the Food Chain Research Group of the Scripps Institute of Oceanography. N. W. gratefully acknowledges O. Holm-Hansen for this support.

References

Allen, M. B., Arnon, D. I.: Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. **30**, 366—372 (1955).

Benemann, J. R., McKenna, C. E., Lie, R. F., Traylor, T. G., Kamen, M. D.: The vanadium effect in nitrogen fixation by *Azotobacter*. Biochim. biophys. Acta (Amst.) **264**, 25—38 (1972).

Benemann, J. R., Valentine, R. C.: The pathways of nitrogen fixation. Advanc. Microbiol. Physiol. **8**, 59—102 (1972).

Benemann, J. R., Yoch, D. C., Valentine, R. C., Arnon, D. I.: Azotoflavin as an electron carrier in nitrogen fixation by *Azotobacter*. Fed. Proc. **29**, 404 (1970).

Benemann, J. R., Yoch, D. C., Valentine, R. C., Arnon, D. I.: The electron transport system in nitrogen fixation by *Azotobacter*. III. Requirements for NADPH-supported nitrogenase activity. Biochim. biophys. Acta (Amst.) **226**, 205—212 (1971).

Biggins, J.: Respiration in blue-green algae. J. Bact. **99**, 570—575 (1969).

Bothe, H.: Photosynthetische Stickstoffixierung mit einem zellfreien Extrakt aus der Blaualge *Anabaena cylindrica*. Ber. dtsch. bot. Ges. **83**, 421—432 (1970).

Cox, R. M., Fay, P.: Nitrogen fixation and pyruvate metabolism in cell-free preparations of *Anabaena cylindrica*. Arch. Mikrobiol. **58**, 357—365 (1967).

Cox, R. M., Fay, P.: Special aspects of nitrogen fixation by blue-green algae. Proc. roy. Soc. B **172**, 357—366 (1969).

Donze, M., Haveman, J., Schiereck, P.: Absence of photosystem 2 in heterocysts of the blue-green algae *Anabaena*. Biochim. biophys. Acta (Amst.) **256**, 157—161 (1972).

Fay, P.: Photostimulation of nitrogen fixation in *Anabaena cylindrica*. Biochim. biophys. Acta (Amst.) **216**, 353—356 (1970).

Fay, P., Lang, N. J.: The heterocysts of blue-green algae. I. Ultrastructural integrity after isolation. Proc. roy. Soc. B **178**, 185—192 (1971).

Fay, P., Stewart, W. D. P., Walsby, A. E., Fogg, G. E.: Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature (Lond.) **220**, 810—812 (1968).

Fay, P., Walsby, A. E.: Metabolic activities of isolated heterocysts of the blue-green alga *Anabaena cylindrica*. Nature (Lond.) **209**, 94—95 (1966).

Fogg, G. E.: Growth and heterocyst production in *Anabaena cylindrica* Lemm. II. In relation to carbon and nitrogen metabolism. Ann. Bot. **13**, 241—259 (1949).

Gorkom, H. J. van, Donze, M.: Localization of nitrogen fixation in *Anabaena*. Nature (Lond.) **234**, 231—232 (1971).

Haystead, A., Robinson, R., Stewart, W. D. P.: Nitrogenase activity in extracts of heterocystous and non-heterocystous blue-green algae. Arch. Mikrobiol. **74**, 235—243 (1970).

Haystead, A., Stewart, W. D. P.: Characteristics of the nitrogenase system of the blue-green alga *Anabaena cylindrica*. Arch. Mikrobiol. **82**, 325—336 (1972).

Kulasooriya, S. A., Lang, Norma J., Fay, P.: The heterocysts of blue-green algae. III. Differentiation and nitrogenase activity. Proc. roy. Soc. B **181**, 199—209 (1972).

Kurz, W. G. W., LaRue, T. A.: Nitrogenase in *Anabaena flos-aquae* filaments lacking heterocysts. Naturwissenschaften **58**, 417 (1971).

Leach, C. K., Carr, N. G.: Oxidative phosphorylation in extracts of blue-green algae. Biochem. J. **112**, 125—126 (1969).

Leach, C. K., Carr, N. G.: Electron transport and oxidative phosphorylation in the blue-green alga *Anabaena variabilis*. J. gen. Microbiol. **64**, 55—70 (1970).

Leach, C. K., Carr, N. G.: Pyruvate: ferredoxin oxidoreductase and its activation by ATP in the blue-green alga *Anabaena cylindrica*. Biochim. biophys. Acta (Amst.) **245**, 165—174 (1971).

Millbank, J. W.: Nitrogen metabolism in lichens. IV. The nitrogenase activity of the *Nostoc* phycobiont in *Peltigera canina*. New Phytol. **71**, 1—10 (1972).

Neilson, A., Rippka, R., Kunisawa, R.: Heterocyst formation and nitrogenase synthesis in *Anabaena* sp. Arch. Mikrobiol. **76**, 139—150 (1971).

Ohmori, H., Hattori, A.: Nitrogen fixation and heterocysts in the blue-green alga *Anabaena cylindrica*. Plant Cell Physiol. **12**, 961—967 (1971).

Smith, R. V., Evans, M. C. W.: Soluble nitrogenase from vegetative cells of the blue-green alga *Anabaena cylindrica*. Nature (Lond.) **225**, 1253—1254 (1970).

Smith, R. V., Noy, A. J., Evans, M. C. W.: Physiological electron donor systems to the nitrogenase of the blue-green alga *Anabaena cylindrica*. Biochim. biophys. Acta (Amst.) **253**, 104—109 (1971).

Stewart, W. D. P.: Physiological studies on nitrogen-fixing blue-green algae. Plant Soil, Spec. Vol., pp. 377—391 (1971).

Stewart, W. D. P., Fitzgerald, G. P., Burris, R. H.: Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. **62**, 336—348 (1968).

Stewart, W. D. P., Haystead, A., Pearson, H. W.: Nitrogenase activity in heterocysts of blue-green algae. Nature (Lond.) **224**, 226—228 (1969).

Stewart, W. D. P., Lex, M.: Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch. Mikrobiol. **73**, 250—260 (1970).

Stewart, W. D. P., Pearson, H. W.: Effects of aerobic and anaerobic conditions on growth and metabolism of blue-green algae. Proc. roy. Soc. B **175**, 293—311 (1970).

Talpasayi, E. R. S.: Localization of ascorbic acid in heterocysts of blue-green alga. Current Sci. **7**, 190 (1967).

Thomas, J.: Absence of the pigments of photosystem II of photosynthesis in heterocysts of a bluegreen alga. Nature (Lond.) **228**, 181—183 (1970).

Thomas, J., David, K. A. V.: Site of nitrogenase activity in the blue-green alga *Anabaena* sp. L-31. Nature (Lond.) **238**, 219—221 (1972).

Wolk, C. P.: Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. J. Bact. **96**, 2138—2143 (1968).

Wolk, C. P.: Aspects of the development of a blue-green alga. Ann. N. Y. Acad. Sci. **175**, 641—647 (1970).

Wolk, C. P., Wojciuch, E.: Photoreduction of acetylene by heterocysts. Planta (Berl.) **97**, 126—134 (1971).

Dr. John R. Benemann
Department of Chemistry
Revelle College
University of California
La Jolla, California 92037
U.S.A.