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H_2, N_2, and O_2 Metabolism by Isolated Heterocysts from *Anabaena* sp. Strain CA†

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Metabolically active heterocysts isolated from wild-type *Anabaena* sp. strain CA showed high rates of light-dependent acetylene reduction and hydrogen evolution. These rates were similar to those previously reported in heterocysts isolated from the mutant *Anabaena* sp. strain CA-V possessing fragile vegetative cell walls. Hydrogen production was observed with isolated heterocysts. The ratio of C_2H_4 to H_2 produced ranged from 0.9 to 1.2, and H_2 production exhibited unique biphasic kinetics consisting of a 1 to 2-min burst of hydrogen evolution followed by a lower, steady-state rate of hydrogen production. This burst was found to be dependent upon the length of the dark period immediately preceding illumination and may be related to dark-to-light ATP transients. The presence of 100 nM $NiCl_2$ in the growth medium exerted an effect on both acetylene reduction and hydrogen evolution in the isolated heterocysts from strain CA. H_2-stimulated acetylene reduction was increased from 2.0 to 3.2 μmol of C_2H_4 per mg (dry weight) per h, and net hydrogen production was abolished. A phenotypic Hup^- mutant (N9AR) of *Anabaena* sp. strain CA was isolated which did not respond to nickel. In isolated heterocysts from N9AR, ethylene production rates were the same under both 10% C_2H_2–90% Ar and 10% C_2H_2–90% H_2 with or without added nickel, and net hydrogen evolution was not affected by the presence of 100 nM Ni^{2+}. Isolated heterocysts from strain CA were shown to have a persistent oxygen uptake of 0.7 μmol of O_2 per mg (dry weight) per h, 35% of the rate of whole filaments, at air saturating O_2 levels, indicating that O_2 impermeability is not a requirement for active heterocysts.

Heterocysts are differentiated cell forms produced by several species of filamentous cyanobacteria (blue-green algae) when grown under diazotrophic conditions. It is the heterocyst, by virtue of its unique physiological and biochemical constitution, that allows the reduction of dinitrogen to the level of NH_4^+ for subsequent utilization by an organism (23). Specifically, the heterocyst creates an environment that optimizes conditions for the enzymes of the nitrogenase complex.

The heterocyst possesses an outer envelope composed of a highly branched polysaccharide (3), an unknown "fibrous" composition, and a "laminated" glycolipid layer (14). Collectively, these layers may serve as a physical barrier to oxygen diffusion (14). Heterocysts are deficient in photosystem-II components (9, 23) and so are believed no longer capable of photosynthetic oxygen evolution. They may possess oxygen "scavenging" mechanisms in the form of respiratory or Knallgas-type reactions (2, 21). The large requirement of ATP for nitrogenase is generally thought to originate with cyclic photophosphorylation, and the ultimate source of reductant is still quite controversial.

From a physiological standpoint, the nitrogenase complex has additional features of interest. During nitrogen fixation, there is an obligatory hydrogen formation (4, 22), and the theoretical stoichiometric relationship between nitrogen fixation and hydrogen production is related by the equation $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$. This yields a 1:1 ratio for H_2 produced to N_2 reduced (22, 29). A comparison of the rates of acetylene reduction (11) and hydrogen production (30) in whole filaments of *Anabaena* sp. strain CA shows a 1:1 ratio.

It was the purpose of this work to confirm the earlier observations of Kumar et al. (19, 20) on isolated heterocysts and to extend them from the fragile-vegetative cell wall mutant, CA-V, to the wild-type strain, CA. Second, we examined the kinetics of hydrogen production and nitrogen fixation (acetylene reduction) in the isolated heterocysts and the possibility that the isolated heterocysts possess an active O_2 uptake capacity.

MATERIALS AND METHODS

Organism. The organism used for these studies was *Anabaena* sp. strain CA (ATCC 33047), a filamentous, heterocyst-forming, marine cyanobacterium.

Growth conditions. Cultures were routinely grown in Pyrex culture tubes (22 by 175 mm) in 20 ml of ASP-2 medium (25) with no combined nitrogen and a NaCl content of 5 g/liter. Filter-sterilized $NiCl_2 \cdot 6H_2O$ or NH_4NO_3 was added to a final concentration of 100 nM or 10 mM, respectively, when required. The cultures were grown in 1% CO_2-in-air at 39 ± 0.1°C. The growth bath was illuminated with four F36T12/D/HO fluorescent lamps (Westinghouse, Bloomfield, N.J.) on either side of the bath 12 cm from the center of the growth tube at an average intensity of 300 $\mu E/m^2$ per s. The growth rate was determined turbidimetrically with a model 402 Lumetron colorimeter equipped with a broad-band pass filter set with a peak transmission centered at 600 nm. Dry weights were determined by harvesting whole filaments or isolated heterocysts on a 0.4-μm polycarbonate filter and drying to a constant weight in a vacuum oven at 45°C over P_2O_5.

Mutant isolation. For the strain designated N9AR, muta-

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genesis was performed as described before (12). 1-Methyl-3-nitro-1-nitrosoguanidine (40 μg/ml final concentration)-treated cell suspensions were plated on medium ASP-2 containing 100 nM $NiCl_2 \cdot 6H_2O$, 1% water-washed agar (Bacto-Agar, 0140, Difco Laboratories, Detroit, Mich.) and no added nitrogen source. The plastic petri dishes were then incubated in 0.5 to 1.0% CO_2-in-air in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) at 39°C in the light. Colonies were picked and restreaked several times to ensure segregation and then were screened for H_2 production. Then 2 ml of a culture grown in medium ASP-2 containing 100 nM $NiCl_2 \cdot 6H_2O$ was transferred to a 7-ml serum-stoppered tube and incubated in the light under 1% CO_2-in-air; the H_2 formed was measured by gas chromatography (see below). Stock cultures of the appropriate mutants were established by replating the liquid cultures.

Heterocyst isolation. The procedure for heterocyst isolation was essentially that of Kumar et al. (19). A suspension of 20 to 30 ml of cells at a density of 0.10 to 0.12 mg (dry weight) per ml was washed two times in ASP-2 medium with the total concentration of KCl raised to 0.03 M and NaCl to 0.37 M (assay medium). Exogenous thiols were not required and therefore were not added during heterocyst isolation or in any of the experiments. The cells were resuspended in 5 ml of the assay medium containing 1 mg of lysozyme per ml and sparged for 5 min with either 1% CO_2–99% N_2 or 1% CO_2–99% Ar, depending upon the assay to follow. Cells were then transferred to a 16-ml serum-stoppered tube containing the same gas mixture, using a gastight syringe, and the tube was placed in a 39°C light bath equipped with a linear rotating bar having a 1-inch throw at 48 rpm. The bath was illuminated from the bottom by six F48T12/CW/HO fluorescent lamps (Sylvania, Danvers, Mass.) at an average intensity of 300 $\mu E/m^2$ per s. After 30 min, the suspension was sonicated for 15 s on a model W-10 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) set at full power. The sonicated suspension was transferred to a 5-ml serum-stoppered centrifuge tube and centrifuged at 2,000 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in 4 ml pre-gassed assay medium and centrifuged at 1,500 rpm for 5 min. The final pellet was resuspended in either a 2-ml volume for acetylene reduction assays or a 3-ml volume for use on the hydrogen electrode or oxygen electrode. Heterocysts to be assayed amperometrically for hydrogen were preconditioned under 1% CO_2–99% N_2 by shaking in the light for 20 min in a sealed, 7-ml, serum-stoppered tube before transfer to the electrode chamber. This was done since acetylene reduction assays with isolated heterocysts indicated that the maximal activity was reached 10 to 20 min immediately after the isolation procedure.

Acetylene reduction. Suspensions of 2 ml of either whole filaments or isolated heterocysts were placed in 7-ml serum-stoppered tubes containing either 10% C_2H_2–90% H_2 or 10% C_2H_2–90% Ar and placed in the same shaker bath as that used for heterocyst isolation. Ethylene was measured by injection of 0.2 ml of the gas phase into an Antek model 464-IPC gas chromatograph equipped with a 182-cm Chromosorb 104 column (Johns-Manville, Celite Division, Denver, Colo.) at 50°C with helium as the carrier gas (11).

Hydrogen and oxygen measurements. Amperometric measurements of hydrogen and oxygen were performed as described previously (18, 27, 30), using a no. 5331 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted into a 1.6-ml water-jacketed chamber held at 39°C. The suspensions were continuously mixed with a magnetic stirring bar. The electrode signal was monitored and amplified by a Keithly model 150B microvolt-ammeter (Keithley Instruments, Inc., Cleveland, Ohio) and recorded. Actinic light was provided by a projector with a DAY-DAK 500-W lamp (Sylvania, Winchester, Ky.) screened by a no. 34-01-2 hot mirror (Baird Atomic Inc., Bedford, Mass.). Light intensity incident upon the chamber was 1,000 $\mu E/m^2$ per s and was varied with screens. Gassing of the sample was performed directly in the electrode chamber for 2 min before an assay.

Hydrogen was also measured by gas chromatography with a model 8515 GC (Carle Instruments Inc., Anaheim, Calif.), using a Spherocarb (Analabs, Inc., North Haven, Conn.) column (213 by 0.33 cm) at 37°C with N_2 as the carrier gas.

Chemicals. Biochemicals were purchased from the Sigma Chemical Co., St. Louis, Mo. and were of reagent grade quality or better. Gases were obtained from Big-3 Industries, Houston, Tex.

RESULTS

Isolation of heterocysts from wild-type *Anabaena* sp. strain CA. The use of strain CA-V, a fragile vegetative cell wall mutant derived from the wild-type strain CA, facilitated earlier work on the isolation of highly active heterocysts. With the understanding developed in strain CA-V of the requirements for the isolation of active heterocysts, it became possible to isolate heterocysts from the wild-type strain CA that were as active in acetylene reduction as those from strain CA-V. A linear rate of 2 μmol of C_2H_4 formed per mg (dry weight) of heterocysts per h was reproducible from day to day and stable for more than 3 h. The isolation of active heterocysts from strain CA eliminates any argument that prior results depended upon some unknown genotypic characteristic of mutant CA-V. It also allows direct comparison of results obtained with heterocysts isolated from any chosen mutant of strain CA with wild-type heterocysts.

Isolation of a mutant deficient in hydrogen uptake capacities. The presence of nickel in the growth medium of *Anabaena* sp. strain CA has recently been shown to repress, presumably by controlling uptake hydrogenase activity, net aerobic hydrogen production (1, 31). It was of interest to isolate a mutant strain of CA which, even in the presence of nickel, continued to evolve hydrogen aerobically (see data below). One such mutant, designated strain N9AR, was, in contrast to the wild type (31), found to be capable of aerobic hydrogen evolution in the presence of 100 nM $NiCl_2$. In growth rate, chlorophyll *a* content, acetylene reduction rate, and photosynthetic capacity (O_2 evolution), the mutant did not differ greatly from the wild-type strain (Table 1), but H_2 uptake was undetectable. Isolate N9AR displayed the phenotypic characteristics expected of a Hup^- mutant.

Acetylene reduction by isolated heterocysts from strains CA and N9AR. Figure 1 shows the typical time course of acetylene reduction in isolated heterocysts from either strain CA or N9AR with or without nickel and under an atmosphere of either 10% C_2H_2–90% H_2 or 10% C_2H_2–90% Ar. The rates of acetylene reduction for CA with or without nickel under 10% C_2H_2–90% Ar and N9AR with or without nickel and under either 10% C_2H_2–90% Ar or 10% C_2H_2–90% H_2 are identical. However, the rate of acetylene reduction for CA without nickel under 10% C_2H_2–90% H_2 is higher than that of heterocysts under Ar. The highest rate of ethylene production was seen in heterocysts isolated from cultures of strain CA purposely grown with 100 nM Ni^{2+} and under an atmosphere of 10% C_2H_2–90% H_2. These observations on

TABLE 1. Characteristics of parent strain *Anabaena* sp. strain CA and its mutant N9AR grown under nitrogen-fixing conditions

Strain	Generation time (h)	Chlorophyll α (%[dry wt])	Photosynthesis (μmol of O_2 released per mg [dry wt] per h)	C_2H_4 formed (μmol/mg [dry wt] per h)	H_2 production[a] With 100 nM Ni^{2+}	H_2 production[a] Without Ni^{2+}	H_2 uptake[a] With 100 nM Ni^{2+}	H_2 uptake[a] Without Ni^{2+}
CA	4.6	0.85	9.8	1.7	0	1.3	0.45	0.045
N9AR	5.2	0.82	9.3	1.3	1.3	1.3	0	0

[a] Hydrogen production was measured in the light and uptake was measured in the dark; both parameters are expressed as micromoles per milligram (dry weight) per hour.

hydrogen metabolism in strains N9AR and CA imply that strain CA contains one Ni^{2+}-requiring or Ni^{2+}-activated hydrogenase (31). It should be noted that acetylene reduction in isolated heterocysts was always strictly light dependent (data not shown).

Hydrogen production by isolated heterocysts. Figure 2 shows typical hydrogen electrode tracings of suspensions of heterocysts isolated from strains CA and N9AR, both grown without added nickel. In all preparations, the curve was biphasic with an initially higher rate of hydrogen production. This burst typically lasted only 1 min but in the most active preparations could last up to 2 min. The lower, steady-state rate was constant for at least 10 min on the hydrogen electrode. Bubbling of the samples for 2 min with 1% CO_2–99% Ar immediately increased the hydrogen production at least fourfold. The kinetics of hydrogen production were not affected by the Ar bubbling.

The transient burst was found to be dependent on the length of the dark period immediately preceding the light period (Table 2). A finite time of at least 60 s was necessary for maximum burst rate.

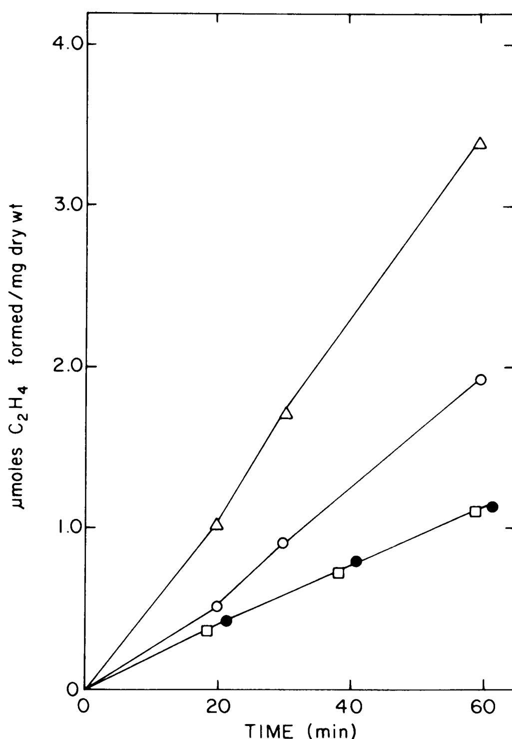

FIG. 1. Time course of acetylene reduction by isolated heterocysts from strain CA and mutant N9AR with or without added nickel in the growth medium and under an atmosphere of either 10% C_2H_2–90% Ar or 10%C_2H_2–90% H_2. $NiCl_2$ was added to 100 nM upon culture transfer, and the cells were allowed to grow for at least five generations in the presence of nickel. Symbols: □, strain CA with or without nickel under 10%C_2H_2–90% Ar; ○, strain CA without nickel under 10% C_2H_2–90% H_2; △, strain CA with nickel under 10% C_2H_2–90% H_2; ●, strain N9AR with or without nickel and under 10% C_2H_2–90% Ar or 10% C_2H_2–90% H_2.

Effect of Ni^{2+} on hydrogen production in heterocysts iso-

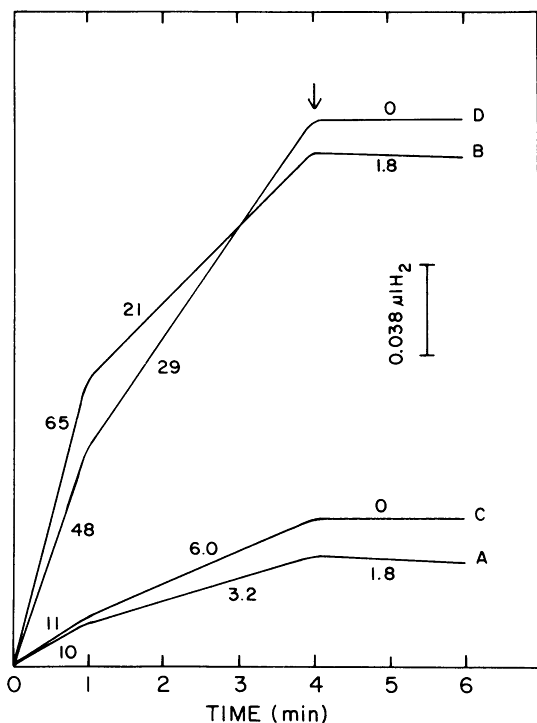

FIG. 2. Tracing of original electrode records (reduced in scale) of hydrogen production by isolated heterocysts from strains CA and N9AR. Cells were grown in medium ASP-2 without added Ni^{2+}. After isolation, heterocysts were incubated in the light at 39°C under 1% CO_2–99% N_2 for 20 min before assay. Heterocysts were then transferred directly to the electrode chamber, where they were gassed with 1% CO_2–99% N_2 for 2 min. In all cases, gassing and measurement of the sample under 1%CO_2–99% Ar followed immediately the measurement of hydrogen evolution under 1% CO_2–99% N_2. The electrode chamber was held at 39°C; the light intensity incident upon the chamber was 1,000 μE/m² per s. Lines: A, heterocysts from strain CA under 1% CO_2–99% N_2; B, heterocysts from strain CA under 1% CO_2–99% Ar; C, heterocysts from strain N9AR under 1% CO_2–99% N_2; D, heterocysts from strain N9AR under 1% CO_2–99% Ar. The light was on at time zero, and the down arrow indicates lights off. Numbers beside the line represent the calculated rates of hydrogen evolution or uptake in microliters per milligram (dry weight) per hour.

TABLE 2. Effect of the length of the dark period on the hydrogen burst in isolated heterocysts upon illumination[a]

Dark period (s)	Burst rate	Steady-state rate
5	21.4	21.4
10	27.5	21.5
20	34.5	22.9
40	40.2	24.8
60	48.5	26.0
120	46.0	24.6
180	47.0	25.4

[a] Rates were measured under 1% CO_2–99% Ar and are expressed as microliters of H_2 per milligram (dry weight) per hour.

lated from CA and N9AR. Similar to whole filaments (Table 1), isolated heterocysts from a culture of strain CA grown in the presence of 100 nM $NiCl_2$ exhibited no net hydrogen production under 1% CO_2–99% N_2 (Fig. 3). Upon bubbling of the sample with 1% CO_2–99% Ar for 2 min, a small initial burst of hydrogen was apparent before evolution ceased and uptake began. However, the heterocysts isolated from the mutant strain N9AR grown in the presence of Ni^{2+} were able to evolve hydrogen at the same rate as those heterocysts without nickel. No evidence for uptake hydrogenase activity could be found in heterocysts from strain N9AR when H_2-saturated ASP-2 medium was injected directly into the electrode chamber in the dark at levels from 12 to 25 μM H_2.

Oxygen uptake by isolated heterocysts from CA. There were several observable characteristics of oxygen uptake in isolated heterocysts. The heterocysts were capable of decreasing the level of oxygen in the assay medium in the light or in the dark. Upon repeated injections of small amounts of O_2-saturated assay medium into the electrode chamber, giving 5 to 8% O_2 saturation, the heterocysts consistently removed oxygen to less than 2% of air saturation levels. When heterocyst suspensions were purposely air-saturated, the O_2 uptake rate increased to 0.7 μmol/mg (dry weight) per h. The dark O_2 uptake rate of whole filaments from N_2-grown cultures was 2 μmol/mg (dry weight) per h. At an average value of 11% heterocysts, and assuming the same rate of O_2 uptake for heterocysts and vegetative cells, the heterocyst O_2 uptake rate should be about 0.2 μmol/mg (dry weight) per h, not 0.7 μmol. Evidently the heterocyst is not restricted in O_2 permeability and has a much more active O_2 uptake capacity than has been commonly supposed.

DISCUSSION

Nickel exerted a dramatic effect on both acetylene reduction and hydrogen evolution in heterocysts from strain CA. Nickel has most often been associated with functional hydrogenases in nitrogen-fixing organisms (5, 10, 16, 24). In the cyanobacteria, though, the presence of a nickel-dependent hydrogenase has not been sufficiently addressed, and indeed the actual number of hydrogenases is ambiguous (2). The most obvious effect was the twofold increase in ethylene production in heterocysts under 10% C_2H_2–90% H_2 isolated from a culture of strain CA grown with 100 nM Ni^{2+} over those grown without added Ni^{2+}. Clearly, the rate of acetylene reduction in heterocysts of strain CA grown without nickel was limited by H_2 uptake capacity and not because of damage during isolation. The acetylene reduction rate of Ni^{2+}-sufficient heterocysts of strain CA under hydrogen (3.2 μmol/mg [dry weight] per h) represents a 40% recovery of whole filament activity. However, heterocysts from cultures grown without nickel formed 2 μmol of C_2H_4 per mg (dry weight) per h, suggesting the possibility of two hydrogenases or increased activation of an existing hydrogenase by the addition of nickel, or the restriction of the full activity of a single hydrogenase by contaminating Ni^{2+} level in unsupplemented medium ASP-2 (26). The latter argument seems quite plausible given the observations on heterocysts isolated from strain N9AR: (i) the acetylene reduction was the same with or without nickel under either 10% C_2H_2–90% H_2 or 10% C_2H_2–90% Ar, and these rates were the same as those from heterocysts of strain CA under 10% C_2H_2–90% Ar; (ii) hydrogen production was not influenced by the presence of nickel; (iii) no dark- or light-stimulated hydrogen uptake could be demonstrated in isolated heterocysts from Ni^{2+}-sufficient cultures. We have no evidence for a reversible hydrogenase in strain CA at this time.

Heterocysts were also found to produce hydrogen in a light-dependent reaction. The rate of hydrogen evolution increased dramatically when heterocysts under 1% CO_2–99% N_2 were bubbled with 1% CO_2–99% Ar for 2 min. Theoretically, the ratio of hydrogen produced to nitrogen reduced is 1:1 (22, 29). This ratio can be demonstrated by the ratio of ethylene produced to hydrogen evolved in isolated heterocysts since both processes are two electron transfers under conditions of maximal electron flow through the nitrogenase complex. In our preparations, the C_2H_4 to H_2 ratio was 0.9 to 1.2, a further indication that our heterocysts are metabolically competent. It is also interesting to note that the

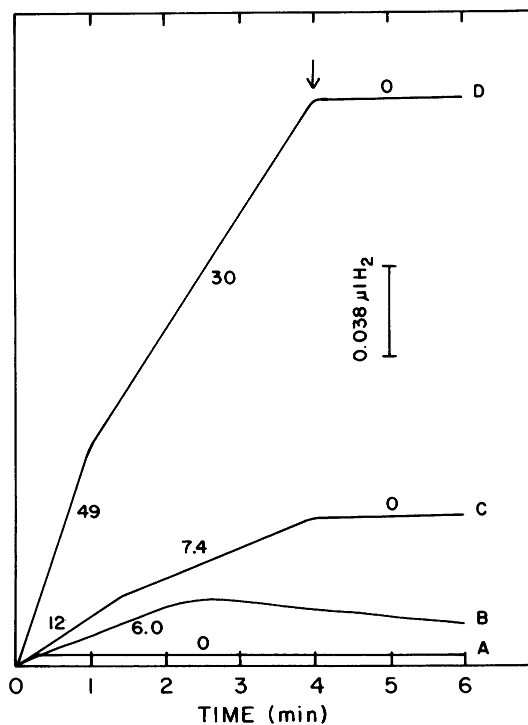

FIG. 3. Tracing of original electrode records (reduced in scale) of hydrogen production by isolated heterocysts from strains CA and N9AR grown in the presence of 100 nM $NiCl_2$. Assay conditions were as described in the legend to Fig. 2. Lines: A, heterocysts from strain CA under 1% CO_2–99% N_2; B, heterocysts from strain CA under 1% CO_2–99% Ar; C, heterocysts from strain N9AR under 1%CO_2–99% N_2; D, heterocysts from strain N9AR under 1% CO_2–99% Ar.

observed Ni^{2+} effects on H_2 uptake activity in whole filaments (31) reside in the heterocyst (cf. Fig. 2 and 3).

The biphasic kinetics of hydrogen production in the isolated heterocysts is a new observation. The burst pattern was not seen with a whole filament suspension on the H_2 electrode. An immediate burst of hydrogen has been seen in many seemingly disparate systems. Greenbaum et al. (13) saw a "gush" of hydrogen in cultures of *Chlamydomonas* 0-5 anaerobically adapted in the light. Their explanation was the "dehydrogenation of an organic substrate in a photosystem-I type reaction." Additionally, a burst of hydrogen can be seen when the two subunits of the nitrogenase complex are brought together under conditions of low electron flux or when the Fe protein is limiting in vitro in *Klebsiella* (4) or when crude *Azotobacter* preparations of nitrogenase are ATP limited (29). In isolated heterocysts, low electron flow does not seem to be the case since we were able to increase the hydrogen production fourfold by simply shifting the gas phase from 1% CO_2–99% N_2 to 1% CO_2–99% Ar. Rather, it seems better to invoke the influence of a limited ATP supply. Ernst et al. (7) reported a rapid depletion and subsequent accumulation of ATP pools in heterocysts upon transition from dark to light conditions. Our work shows the requirement of a finite time period in the dark for maximum burst, i.e., too short a time in the dark may not allow ATP pools to go below a level that would produce a burst of hydrogen upon illumination. This also agrees with the strict light dependence of acetylene reduction in isolated heterocysts since it is reasonable to assume that a primary role of light is to supply the large ATP demand. Indeed, the often-quoted photosystem-I-like action spectrum of nitrogen fixation (8) may be a reflection of the large demand for cyclic photophosphorylation.

Oxygen metabolism was also investigated in the isolated heterocysts. It has been implied that the heterocyst may possess an active respiratory pathway coupled to O_2 (6, 15, 17, 21). In contrast to these observations is the perennial suggestion that the heterocyst is impermeable to O_2 (14). We observed a persistent and significant oxygen uptake in heterocysts that is inconsistent with the heterocyst having a restricted O_2 uptake. The simultaneous function of an oxygen-resistant hydrogenase (5, 28) and the efficient O_2 uptake system seen here could impart significant advantages to the nitrogenase complex in the form of increased ATP production, reductant supply, and oxygen protection.

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