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MODES OF REDUCTION OF NITROGENASE IN HETEROCYSTS ISOLATED FROM *ANABAENA* SPECIES

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Summary

N_2 fixation (acetylene reduction) has been studied with heterocysts isolated from *Anabaena cylindrica* and *Anabaena* 7120. In the presence of ATP and at very low concentrations of sodium dithionite, reducing equivalents for activity of nitrogenase in these cells can be derived from several compounds. In the dark, D-glucose 6-phosphate, 6-phosphogluconate and DL-isocitrate support acetylene reduction via NADPH. In the light, reductant can be generated by Photosystem I.

Introduction

With few exceptions, the capacity for N_2 fixation by blue-green algae under aerobic conditions is restricted to heterocyst-forming species (see ref. 1). In recent years it has become clear that heterocysts are principal loci of N_2 fixation in these organisms [2—8], although it remains unclear whether they are the only sites of N_2 fixation within the algal filaments under those conditions.

N_2 fixed by heterocysts is incorporated into glutamine, which is subsequently transferred to vegetative cells [8,9]. Isolated heterocysts lack the

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Abbreviations: Chl, chlorophyll *a*; CoASH, reduced form of coenzyme A; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine dihydrochloride; DCIP ($DCIPH_2$), oxidized (reduced) 2.6-dichlorophenol-indophenol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N'*-bis-2-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

capacity for oxygenic photosynthesis [7,10] and for carboxylation of ribulose bisphosphate [11—13]. Movement of photosynthate from vegetative cells into heterocysts has been shown in vivo by autoradiography [14]. It has therefore been suggested that under aerobic, N_2-fixing conditions vegetative cells and heterocysts are interdependent: heterocysts provide vegetative cells with fixed nitrogen and depend on vegetative cells for reducing equivalents and reduced carbon compounds.

The oxidative pentose phosphate pathway is the main glucose-oxidizing pathway in blue-green algae [15—17], and enzymes of this pathway are present in heterocysts at high levels [13]. In contrast, the activity of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the Embden-Meyerhof pathway, is low in heterocysts compared with vegetative cells [13]. It seems likely that carbon compounds entering heterocysts as carbohydrate [12] would be metabolized by the oxidative pentose phosphate pathway and might thus provide reducing equivalents for N_2 fixation.

Although blue-green algae can reduce N_2 in the dark, the rate of reduction is stimulated by light [18]. Photosystem I has been shown to be present in heterocysts and to be capable of both photoreduction and photophosphorylation [3,7,10,19,20]. The precise relationship between Photosystem I of heterocysts and N_2 fixation remains unclear, however.

Bradley and Carr [21] reported that acetylene reduction by heterocysts isolated in the absence of dithionite could be stimulated by phosphoenolpyruvate and $DCIPH_2$/ascorbate (an electron donor system to Photosystem I). The reported specific rates are low (1—3%, on a dry weight basis) as compared to the specific rates of intact filaments. Recently methods have been published for the isolation of metabolically active heterocysts with specific activities for nitrogenase exceeding those of the filaments from which they were derived [6—8]. In these experiments with isolated heterocysts, the reducing equivalents for nitrogenase activity were provided by the non-physiological reductant sodium dithionite [22], which at the same time protects nitrogenase from inactivation by O_2. In this communication we show that heterocysts isolated from two different strains of *Anabaena* by modifications of two of these isolation procedures can use reducing equivalents provided by intermediates of the oxidative pentose phosphate pathway and by isocitrate for N_2 fixation (acetylene reduction). The rates of acetylene reduction in these systems are typically one-third of the dithionite-supported rates and are enhanced by light. We show also that the Photosystem I of heterocysts photoreduces nitrogenase when provided with artificial electron donor systems.

Materials and Methods

Algal strains and culture conditions. Anabaena cylindrica Lemm. and *Anabaena* 7120 were grown autotrophically with N_2 as the sole nitrogen source in illuminated fermentors sparged with air or air enriched with 0.5% CO_2. *Anabaena* 7120 was cultured in the medium of Allen and Arnon [23] at 34°C (minimum doubling time 12 h); *A. cylindrica* was cultured in an 8-fold dilution of this medium [23] at 30°C (minimum doubling time 16 h). Cultures were harvested by centrifugation and used immediately (*A. cylindrica*) or stored in

liquid N_2 until used (*Anabaena* 7120). The methods used were those which had evolved for the two different organisms in two different laboratories.

Isolation of heterocysts. Heterocyst preparations were carried out under strictly anaerobic conditions. Heterocysts of *A. cylindrica* were isolated under Ar by a modification of the procedure of Thomas et al. [8]. Algal material was washed in 10 mM TES/NaOH, pH 7.6. The algae were resuspended at a concentration of 80—160 μg Chl per ml in a medium consisting of 10 mM TES/NaOH, 26 mM Na_2EDTA, 0.5 mM $Na_2S_2O_4$, 650 mM D-mannitol, pH 7.6, and were incubated at 30°C for 45 min in a water bath shaker at 75 rev./min. 20—25 ml of this suspension were cavitated for 8—15 s per ml of suspension (occasionally up to 20 s/ml) in a Model S 125 Sonifier (3.5 A output) or a Model W 185 Sonifier (setting 4) (Heat Systems-Ultrasonics Inc., Plainview, L.I., N.Y.), cooled with tap water, until virtually all of the vegetative cells were destroyed. Heterocysts were sedimented twice at 150 × *g* for 10 min in 15 ml of 10 mM TES/NaOH, pH 7.6, containing 10 mM sodium ascorbate, and were finally resuspended in 4—8 ml of this buffer.

Heterocysts of *Anabaena* 7120 were isolated under H_2 by a modification of the procedure described by Tel-Or and Stewart [7]. Algal filaments were washed and resuspended in a solution composed of 30 mM HEPES, 30 mM PIPES/KOH, 1 mM $MgCl_2$, 1 mM $Na_2S_2O_4$, 350 mM D-mannitol, pH 7.2, at a concentration of 200—400 μg Chl/ml. Lysozyme was added to a final concentration of 1 mg/ml, and the pH adjusted to 7.5 with KOH to retard decomposition of the $Na_2S_2O_4$. The suspension was then incubated for 30 min at 35°C, after which it was cavitated with a Branson Sonifer at setting 6 or 7 for 4.5 s/ml to break vegetative cells. The heterocysts were sedimented 3 or 4 times at 500 × *g* for 5 min, and then resuspended in 20 ml of buffer. Contamination, by vegetative cells, of heterocysts isolated by either of these procedures never exceeded 5%.

In order to observe the effects of potential physiological electron donor systems in support of acetylene reduction by heterocysts, the $Na_2S_2O_4$ concentration in the medium had to be drastically reduced. For *A. cylindrica*, this was done by resuspension in the above buffer lacking $Na_2S_2O_4$. This procedure resulted in occasional loss of nitrogenase activity, presumably due to traces of O_2. Isolated heterocysts from *Anabaena* 7120 were resuspended in the aforementioned buffer which had been diluted 11-fold with deaerated buffer lacking $Na_2S_2O_4$. This procedure yielded consistently active preparations. Maximal acetylene reduction activities, as percent of in vivo activities, were comparable to those reported earlier [6,8].

Assays of acetylene reduction. Heterocysts from *A. cylindrica* were incubated under 90% Ar, 10% acetylene in 5-ml serum vials containing 2 ml of a reaction mixture consisting of 51.5 mM TES/NaOH, 11.5 mM sodium ascorbate, 5 mM ATP, 5 mM $MgCl_2$, 25 mM creatine phosphate, and 15 units of creatine phosphokinase, pH 7.6. Reactions were run for 20 or 30 min at 30°C in a water bath shaker. Incandescent illumination of 4000 foot candles was supplied where indicated; for dark assays, reaction vials were wrapped in aluminum foil. Reactions were terminated by injection of 0.5 ml of 15% (w/v) trichloroacetic acid. Ethylene in the gas phase was assayed with a Varian Aerograph Model 1200 gas chromatograph equipped with a flame ionization detector.

Heterocysts of *Anabaena* 7120 were assayed under 83% H_2, 17% acetylene at 30°C in 21-ml vaccine bottles containing 2 ml of a reaction mixture consisting of 30 mM HEPES, 30 mM PIPES/KOH, 350 mM D-mannitol, and except in the experiment shown in Table IV, 5 mM ATP, 8 mM $MgCl_2$, 20 mM creatine phosphate, and 6 units of creatine phosphokinase, pH 7.2. Other details are as described by Peterson and Burris [6].

Chlorophyll *a* was determined by extraction with methanol or 80% acetone employing the extinction coefficients given by Mackinney [24] and Vernon [25], respectively.

Glycolic acid was obtained from Calbiochem, Los Angeles, Calif., and creatine phosphate used with *Anabaena* 7120, from Pierce Chemical Co., Rockford, Ill. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Other chemicals were of the highest purity commercially available. DAD was recrystallized from 6 M HCl as the dihydrochloride.

Results

Acetylene reduction in the dark

In the absence of any added reductant to nitrogenase, the rate of acetylene reduction by anaerobically isolated heterocysts in the dark is low (compare the first two lines in Table I). This low rate can be enhanced by NADPH, but not by NADH. Addition of glucose 6-phosphate, in the presence of NADPH, further enhances acetylene reduction to 39% of the rate obtained with the artificial reductant, sodium dithionite (Table I). When $NADP^+$ is added instead of NADPH, acetylene reduction depends largely on the addition of D-glucose 6-phosphate, with saturation achieved at a concentration of about 5 mM glucose 6-phosphate. Acetylene reduction by isolated heterocysts with D-glucose 6-phosphate as the electron source requires $NADP^+$. As shown in Fig. 1, maximal rates are obtained at $NADP^+$ concentrations in the range of 50—300 μM, higher concentrations being inhibitory.

Using $^{15}N_2$, it was confirmed that the stimulation of acetylene reduction by the D-glucose 6-phosphate/$NADP^+$ system is the result of an enhanced nitrogenase activity. $^{15}N_2$ fixation by heterocysts from *Anabaena* 7120 in the presence of ATP requires the addition of both $NADP^+$ and D-glucose 6-phosphate for

TABLE I

EFFECTS OF NADH AND NADPH ON ACETYLENE REDUCTION BY HETEROCYSTS ISOLATED FROM *A. CYLINDRICA*

Reactions were run in the dark. Each reaction mixture contained heterocysts with 5.7 μg chlorophyll *a*.

Additions	μmol ethylene formed/mg chlorophyll *a* per h
—	0.36
10 mM $Na_2S_2O_4$	11.50
0.125 mM NADH	0.42
1.0 mM NADH	0.32
0.125 mM NADPH	1.53
1.0 mM NADPH	2.31
0.125 mM NADPH, 5 mM Glc-6-*P*	4.47

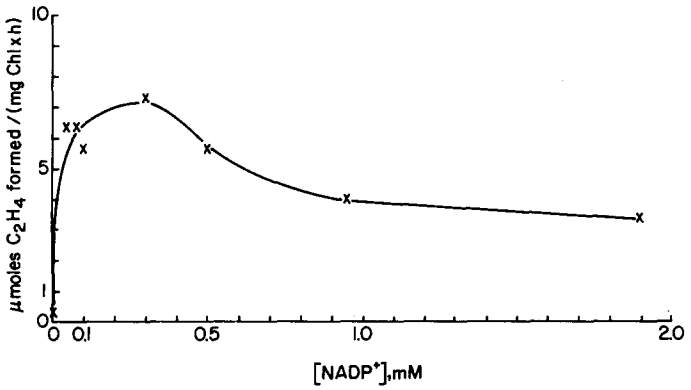

Fig. 1. Dependence on $NADP^+$ concentration of acetylene reduction by heterocysts isolated from *Anabaena* 7120, with glucose 6-phosphate as the substrate. Reactions were run in the dark. Each reaction mixture contained 5 mM glucose 6-phosphate and heterocysts with 9.9 μg chlorophyll *a*.

maximal activity (Table II), showing the same characteristics as does acetylene reduction by heterocysts from both organisms used in this study.

Besides D-glucose 6-phosphate, 6-phosphogluconate and DL-isocitrate were found to provide electrons to nitrogenase (Table III). With each of these electron donors, acetylene reduction was increased by addition of $NADP^+$. Pyruvate, tested with heterocysts from *Anabaena* 7120 at concentrations of 10 and 30 mM, in the absence and presence of 300 μM $NADP^+$, 20 μM CoASH, 1 mM DL-dithiothreitol, and a crude ferredoxin preparation from *Anabaena* 7120, and tested with heterocysts from *A. cylindrica* at a concentration of 5 mM,

TABLE II

FIXATION OF $^{15}N_2$ BY HETEROCYSTS ISOLATED FROM *ANABAENA* 7210, SUPPLIED WITH AN ELECTRON DONOR SYSTEM CONSISTING OF GLUCOSE 6-PHOSPHATE AND $NADP^+$

Assays were conducted in the dark at 30°C for 30 min under an atmosphere of 50% $^{15}N_2$, 50% Ar in 8-ml Warburg flasks sealed with rubber stoppers. 0.2 ml of 0.18 M pyrogallol in 36% KOH was present in the center well to absorb O_2. The final volume of the reaction mixture was 1.0 ml. The samples were subjected to one cycle of freeze-thawing prior to assay. ATP and its generating system was present in all cases (see Materials and Methods). Incubations were terminated by injection of 1.0 ml of 3.75 M H_2SO_4. Control replicates were inactivated at time zero. ^{15}N analyses were performed with a Consolidated-Nier mass spectrometer according to ref. 39. Total heterocyst N was calculated after subtraction of the total Kjehldahl nitrogen content (determined according to 39) of a blank containing the complete reaction mixture but no heterocysts.

Additions	Total heterocyst N (μg)	Gas phase atom % ^{15}N	Sample atom % ^{15}N	Sample atom % ^{15}N excess	nmol N_2 fixed/ mg N per 30 min
—	805	68.0	0.383	0.000	0
	1865	77.3	0.382	—0.001	0
0.6 mM $NADP^+$	805	77.3	0.383	0.000	0
	805	69.5	0.384	0.001	1.4
10 mM Glc-6-*P*	725	80.0	0.387	0.004	5.4
	745	80.5	0.391	0.008	10.5
0.6 mM $NADP^+$, 10 mM Glc-6-*P*	805	79.5	0.426	0.043	54.0
	415	75.0	0.394	0.011	23.6

TABLE III

ACETYLENE REDUCTION BY HETEROCYSTS ISOLATED FROM *A. CYLINDRICA* AND *ANABAENA* 7120 SUPPORTED BY INTERMEDIATES OF THE OXIDATIVE PENTOSE PHOSPHATE PATHWAY AND THE TRICARBOXYLIC ACID CYCLE

Reactions were run in the dark. Reaction mixtures contained heterocysts with 2.8 μg chlorophyll *a* (*A. cylindrica*) or 27.8 μg chlorophyll *a* (*Anabaena* 7120).

(a) *A. cylindrica*			(b) *Anabaena* 7120		
Addition	μmol ethylene mg Chl per h		Addition	μmol ethylene mg Chl per h	
	$-NADP^+$	+50 μM $NADP^+$		$-NADP^+$	+300 μM $NADP^+$
—	0.22	0.70	—	0.00	0.00
5 mM Glc-6-*P*	0.18	3.09	10 mM Glc-6-*P*	0.00	6.21
5 mM 6-*P*-gluconate	0.40	3.19			
10 mM DL-isocitrate	0.10	2.04	10 mM DL-isocitrate	0.00	6.72
10 mM $Na_2S_2O_4$	8.74	—	1 mM $Na_2S_2O_4$	18.42	—

with and without 7.5 μM CoASH, did not enhance acetylene reduction (data not shown). Acetylene reduction by heterocysts from *A. cylindrica* was also not increased by 5 mM DL-malate, with or without 50 μM $NADP^+$ (data not shown).

Effect of light

Acetylene reduction by heterocysts isolated from *Anabaena* 7120 and (to a

TABLE IV

EFFECT OF LIGHT ON ACETYLENE REDUCTION BY HETEROCYSTS ISOLATED FROM *A. CYLINDRICA* AND *ANABAENA* 7120

Heterocysts were isolated and assays performed as described under Materials and Methods. Note that in the case of heterocysts from *A. cylindrica*, but not from *Anabaena* 7120, the basic reaction mixture already contained ATP, its generating system and 50 μM $NADP^+$. Where indicated, the incubation mixtures were illuminated with white light of an intensity of 4000 foot candles in the case of heterocysts from *A. cylindrica* or of 600 foot candles in the case of heterocysts from *Anabaena* 7120. Reaction mixtures contained heterocysts with 2.2 μg chlorophyll (*A. cylindrica*) or 20.8 μg chlorophyll (*Anabaena* 7120).

(a) *A. cylindrica*			(b) *Anabaena* 7120		
Addition	μmol ethyleme mg Chl per h		Addition	μmol ethylene mg Chl per h	
	Dark	Light		Dark	Light
—	0.00	0.78	—	0.09	1.26
5 mM Glc-6-*P*	4.72	5.75	ATP and its generating system	0.15	3.99
5 mM Glc-6-*P*, 1.6 units Glc-6-*P*-dehydrogenase	4.27	5.29	5 mM Glc-6-P, 300 μM $NADP^+$	0.09	0.90
10 mM $Na_2S_2O_4$ ($NADP^+$ omitted)	7.82	9.63	ATP and its generating system, 5 mM Glc-6-P, 300 μM $NADP^+$	7.11	9.09

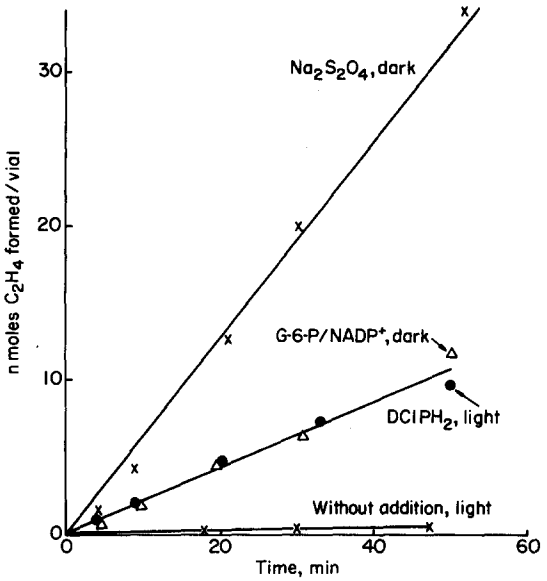

Fig. 2. Time courses of acetylene reduction by heterocysts isolated from *A. cylindrica* supplied with various electron donor systems. The concentration of electron donors was: $Na_2S_2O_4$, 10 mM; glucose 6-phosphate, 5 mM, plus $NADP^+$, 50 μM; DCIP, 0.5 mM (as noted in Materials and Methods, the reaction mixture in each case contained 11.5 mM sodium ascorbate). Each reaction mixture contained heterocysts with 5.7 μg chlorophyll *a*. Where indicated, vials were illuminated with white light (4000 foot candles).

lesser extent) *A. cylindrica*, supplemented or not with physiological electron donors, is enhanced by light (Table IV). In *Anabaena* 7120, the effect of light is further enhanced by the addition of ATP and an ATP-generating system. In

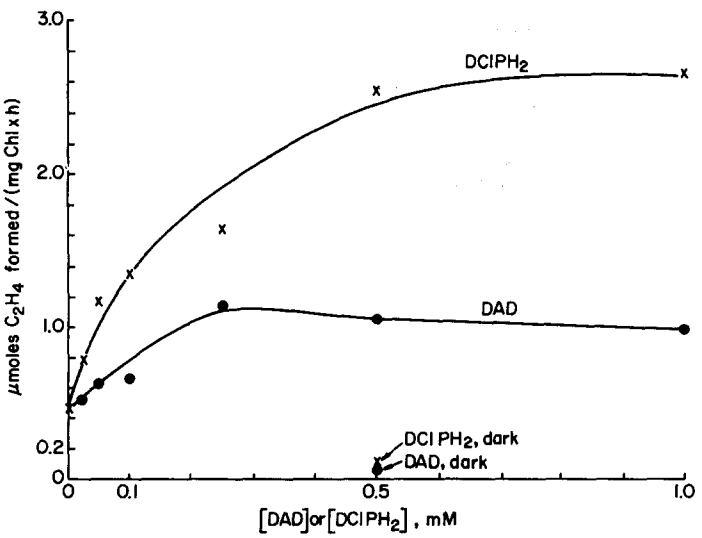

Fig. 3. Photoreduction of acetylene by heterocysts isolated from *A. cylindrica*: dependence on concentration of DCIP and DAD. Each reaction mixture contained 11.5 mM sodium ascorbate (see Materials and Methods) and heterocysts with 2.7 μg chlorophyll *a*. Except where indicated, vials were illuminated with white light (4000 foot candles).

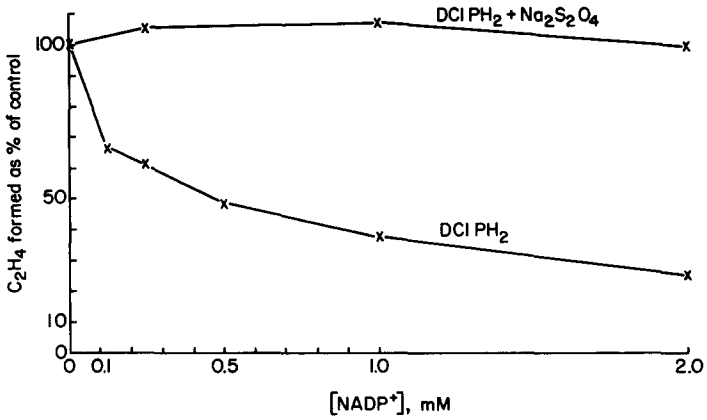

Fig. 4. Inhibition, by $NADP^+$, of the DCIP-stimulated photoreduction of acetylene by heterocysts isolated from *A. cylindrica*. The electron donor system was 0.5 mM DCIP plus 11.5 mM sodium ascorbate. Where indicated, the reaction mixtures contained, in addition, 10 mM $Na_2S_2O_4$. The vials contained heterocysts with 2.8 μg chlorophyll *a* and were illuminated with white light (4000 foot candles). Control rates (= 100%); 1.9 μmol ethylene formed/mg chlorophyll *a* per h in the absence, and 10.3 μmol ethylene formed/mg chlorophyll *a* per h in the presence, of $Na_2S_2O_4$.

the dark, and in the presence of ATP, an ATP-generating system and of either D-glucose 6-phosphate plus $NADP^+$, or dithionite, acetylene is reduced more rapidly than in the light in the absence of added reductants (Table IV).

As is illustrated in Figs. 2 and 3, artificial electron donor systems to Photosystem I increase several-fold the light-dependent (Table IV, Fig. 3) reduction of acetylene by heterocysts supplied with ATP and its generating system. The rates of acetylene reduction with dithionite or with D-glucose 6-phosphate plus $NADP^+$ in the dark or with ascorbate plus DCIP in the light are linear with time for up to 50 min (Fig. 2). For different batches of heterocysts, the rate of the $DCIPH_2$-dependent photoreduction of acetylene varied from 40 to 100% of the glucose 6-phosphate/$NADP^+$-dependent rate. DAD/ascorbate, an alternate electron donor couple to Photosystem I, was, in most experiments, less effective than $DCIPH_2$/ascorbate (Fig. 3). Reduction of acetylene in the light by heterocysts isolated from *A. cylindrica* was not stimulated by 5 mM DL-malate, succinate, glycolate or pyruvate.

TABLE V

LACK OF ADDITIVITY OF DCIP/ASCORBATE- AND GLUCOSE 6-PHOSPHATE/$NADP^+$-DEPENDENT ACETYLENE REDUCTION BY HETEROCYSTS ISOLATED FROM *A. CYLINDRICA*

All assays were performed in the light (4000 foot candles). Each reaction mixture contained heterocysts with 6.5 μg chlorophyll *a*.

Additions	μmol ethylene/mg chlorophyll *a* per h
—	0.10
0.5 mM DCIP	1.24
5 mM Glc-6-*P*, 50 μM $NADP^+$	2.96
5 mM Glc-6-*P*, 50 μM $NADP^+$, 0.5 mM DCIP	2.54
5 mM $Na_2S_2O_4$	12.01

Reduction of acetylene with $DCIPH_2$/ascorbate as the electron donor system in the light is inhibited by $NADP^+$ at concentrations of 100 μM and above (Fig. 4). The rates of reduction of acetylene supported by D-glucose 6-phosphate/$NADP^+$ and by $DCIPH_2$/ascorbate are not additive (Table V), although they are several-fold lower than the $Na_2S_2O_4$-supported rates.

Discussion

The immediate electron donor to nitrogenase from blue-green algae is thought to be ferredoxin [26—28]. Several attempts have been made to determine the source(s) of electrons for N_2 fixation in *Anabaena*. The action spectrum for photostimulation of acetylene reduction by intact filaments corresponds mainly to Photosystem I [29]. Photostimulation by Photosystem I light is not enhanced by Photosystem II light [30], and is much less sensitive to *p*-chlorophenyl-1,1-dimethyl urea (CMU) or 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), inhibitors of linear photosynthetic electron transport, than is fixation of CO_2 [31,32]. These data can be interpreted as indicating that Photosystem I contributes to N_2 fixation by cyclic photophosphorylation. However, it has been argued [3,18,33] that under certain physiological conditions Photosystem I may supply nitrogenase with reductant. Exogenously supplied pyruvate was also found to stimulate nitrogenase activity of whole filaments [31,34,35]. In vitro experiments using cell-free extracts from whole filaments showed that reducing equivalents for N_2 fixation could be supplied by pyruvate [28,36,37], isocitrate [28], glucose 6-phosphate [26] and Photosystem I [26, 28]. However, because the biochemical activities present in filaments may be unequally distributed between the heterocysts and vegetative cells of N_2-fixing filaments, these in vitro results are of uncertain relevance to understanding of the physiology of intact filaments. Because no conclusive data support the localization of active nitrogenase in vegetative cells of aerobically grown filaments [1], an investigation of nitrogenase activity using preparations of heterocysts seems justified.

Firm criteria for evaluating the intactness of heterocysts are not available at present. Heterocysts isolated by methods involving cavitation retain enzymatic activities, some at higher specific activities than recovered from whole filaments [6,8,13]. However, because dithionite and ATP support the nitrogenase activity of isolated heterocysts [2,6,8], the latter appear to be permeable to substances of low molecular weight, and therefore presumably lose such substances during isolation. Heterocyst preparations such as used in this study are therefore not directly equivalent to heterocysts in vivo. It is also possible that much of the reductant generated in heterocysts in vivo, although not in our experiments, is utilized to keep the heterocyst anaerobic.

Our results show that in heterocysts isolated from two different strains of *Anabaena*, electrons for nitrogenase activity in the dark may be derived from NADPH. The pathway of electron transfer presumably involves ferredoxin-$NADP^+$ oxidoreductase and ferredoxin, known to be present in heterocysts at sufficient activities [7]. Because glucose 6-phosphate, 6-phosphogluconate and isocitrate stimulate reduction of acetylene in the presence of $NADP^+$, we conclude that the corresponding dehydrogenases can keep $NADP^+$ in heterocysts

essentially fully reduced (see also Table I). To what extent the supply of substrates is rate limiting in vivo is unknown. Lack of support of acetylene reduction by pyruvate under a variety of conditions may be attributable to a deficiency of pyruvate-ferredoxin oxidoreductase or pyruvate oxidase in the heterocysts of our strains of *Anabaena*. Alternatively, these enzymatic activities may be lost during the isolation of the heterocysts.

Light increases acetylene reduction by heterocysts isolated from both strains of *Anabaena* to a variable and limited extent. In the presence of ATP and its generating system, rates of acetylene reduction are higher in the dark in the presence of added electron donors (e.g. glucose 6-phosphate plus $NADP^+$, dithionite) than in the light in the absence of added reductant. When reductant is omitted, the supply of ATP is therefore not rate limiting, so that the stimulation by light may be attributable to photogeneration of reductant rather than to photophosphorylation. However, an alternative interpretation of these data is possible. Our preparations may contain heterocysts of different degrees of intactness. That is, one subpopulation of heterocysts, freely permeable to dithionite, ATP, and other reagents, may give the dark responses observed, as well as the light-mediated response to $DCIPH_2$. A second subpopulation of heterocysts, much less permeable to those substances, and retaining intact systems for generating reductant and for photophosphorylating, may be responsible for a stimulation by light, independent of added electron donors. Because the responses to light vary with the additions to the medium (Table IV), the second interpretation, in the form presented, would have to be an oversimplification.

Two observations indicate that in heterocysts, the Photosystem I-dependent reduction with an artificial electron donor has one or more intermediates in common with the pathway of the NADPH-dependent reduction of nitrogenase. First, NADPH-dependent reduction in the light is not further stimulated by electron donor systems to Photosystem I, and second, $NADP^+$ inhibits the photoreduction. The most likely common intermediate is ferredoxin.

If the principal function of Photosystem I in N_2 fixation by heterocysts is to catalyze cyclic photophosphorylation, the activity of the photosystem might be regulated by the availability of reducing equivalents to nitrogenase. Insufficient reduction of the common pool of ferredoxin would deplete Photosystem I of electrons, thus decreasing the rate of cyclic photophosphorylation. However, the suggestion that Photosystem I may provide nitrogenase with reductant from organic substances in the light [33] remains an interesting possibility. According to Murai and Katoh [38], several organic acids act as electron donors to Photosystem I in lamellar fragments isolated from *Anabaena variabilis*. We have as yet not found such a physiological substance capable of donating electrons to the Photosystem I of heterocysts. It therefore remains unclear to what extent photoreduction occurs in vivo.

In conclusion, working with isolated heterocysts rather than with extracts derived from whole filaments, we have demonstrated that dehydrogenases of the oxidative pentose phosphate pathway and the tricarboxylic acid cycle can reduce nitrogenase via NADPH. Substrate(s) of these pathways are likely to supply heterocysts with reducing equivalents for N_2 fixation. Photosystem I may contribute by photoreduction and/or by photophosphorylation.

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