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Effect of ammonia on nitrogen fixation by the blue-green alga *Anabaena cylindrica* [1]

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Ammonia at a concentration of 1×10^{-3} M completely inhibited nitrogenase activity, as measured by acetylene reduction, in the blue-green alga *Anabaena cylindrica*. Free ammonia was undetectable in cells grown either on N_2 or ammonia within the limits of precision of the method used. Glutamic acid formed a major amino acid pool in N_2-grown cells, and basic amino acids, i.e. lysine, histidine and arginine were abundant in ammonia-grown cells. A 10-fold increase in the amounts of labile amino compound(s) was observed when N_2-grown cells were exposed to ammonia.

When cells were incubated under anaerobic conditions, the acetylene-reducing activity increased 2-fold or more; ammonia had no effect. Oxygen was required for ammonia to inhibit acetylene reduction.

Modes of inhibition by ammonia on acetylene reduction were compared with those by chloramphenicol, puromycin, cycloheximide, DCMU and CCCP. On the basis of these comparisons we concluded that ammonia not only acts as a suppressor of nitrogenase synthesis but also inhibits acetylene-reducing activity by lowering the supply of reductant and/or of energy for the nitrogenase system.

It has been reported that ammonium ion prevents the de novo synthesis of nitrogenase in *Klebsiella pneumoniae* without affecting the activity of the enzyme already formed (*1*). Dalton and Mortenson (*2*) hypothesized that nitrogenase synthesis is controlled by the level of intracellular ammonia. No nitrogenase components were detected in cells of *Clostridium pasteurianum* grown on ammonia (*3*). However, Hardy et al. (*4*) found that ammonium ion had a direct inhibitory effect on nitrogenase activity in *Azotobacter vinelandii*. This is not the case with a cell-free enzyme preparation. Daesch and Mortenson (*5*) later demonstrated that *C. pasteurianum* grown in a chemostat under ammonia-limited conditions contains three times more nitrogenase than do cells grown under nitrogen-fixing conditions. A high nitrogenase activity has also been reported with *Azotobacter chroococcum* (*6*) and *Rhodospirillum rubrum* (*7*) grown under ammonia-limited conditions.

In the present study, the effect of ammonia on nitrogen fixation in the blue-green alga *Anabaena cylindrica* was investigated, with special reference to the correlation between intracellular ammonia or the amino acid pool and nitrogen-fixing activity. The mode of inhibition by ammonia was compared to modes of inhibitors of protein synthesis as well as those of inhibitors of photosynthetic electron transport and phosphorylation.

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Materials and methods

Algal culture

Anabaena cylindrica Lemm. (Fogg strain IAM M-1) was used as the test organism. Culturing was conducted at 28±1°C in the light (5000 lux) using the N-free medium of Allen and Arnon (*8*) as described previously (*9*). Cultures were continuously bubbled with air containing 0.5% CO_2. If necessary, NH_4Cl was added to the medium at a concentration of 1×10^{-3} M. Growth was followed by measuring the optical densities at 750 nm with a Shimadzu-Bausch & Lomb Spectronic 20 spectrophotometer.

Acetylene reduction

Unless otherwise indicated, 4-day old cells were used. Nitrogen-fixing activity was measured by an acetylene reduction technique (*10*). Suspensions of harvested cells, 5 or 10 ml each, washed (2–3 mg protein/ml) or unwashed (0.2–0.3 mg protein/ml), were placed in Warburg type flasks (30 ml capacity) fitted with rubber stoppers. Flasks were evacuated several times, refilled with argon, and finally filled with a 1 : 9 mixture of acetylene (The Matheson Co. New Jersey) and argon. Flasks were incubated with shaking at 30°C in the light at an intensity of 22,000 lux. After a 30 min incubation, the reaction was stopped by introducing 0.2 ml of 4 N NaOH with a hypodermic syringe through the rubber stopper. Production of ethylene from acetylene was determined with a Shimadzu GC-3AL gas chromatograph, as described in a previous paper (*11*).

CO_2 uptake

Cells were incubated in the same manner as in the acetylene reduction measurements, except that flasks were filled with pure argon. The reaction was stopped by introducing 0.2 ml of 6 N H_2SO_4. CO_2 released into the gas phase was measured by the gas chromatograph, using the same column as that used for the measurement of acetylene reduction. CO_2 uptake was estimated from the difference between the amounts of CO_2 before and after incubation.

Chemical analyses

Ammonia was determined by the indophenol method of Lubochinsky and Zalta (*12*). When necessary, the microdiffusion technique of Conway was used to separate the ammonia. Protein was determined by the Folin method as described by Lowry et al. (*13*). Free amino acids in cells were extracted with boiling 80% ethanol for 15 min. Extraction was repeated three times. Extracts were combined, then the amino acids were separated using an Amberlite IR-120-B column (*14*). Amino acid composition was determined with a JLC-6AH automatic amino acid analyzer of Japan Electronic Optics Laboratory Co., Ltd.

Results

Effect of ammonia on acetylene reduction

When ammonia was added to the 4-day old culture, the acetylene-reducing activity was reduced by 50% after 3 hr and was almost completely abolished after

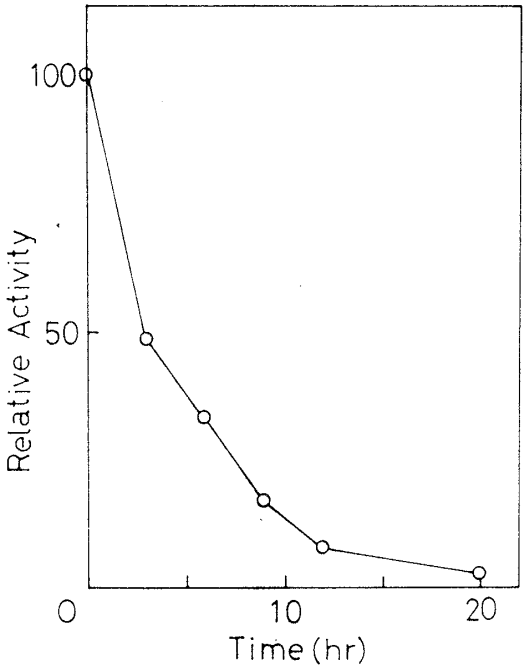

Fig. 1. *Change in acetylene-reducing activity by* Anabaena cylindrica *during growth on ammonia (5000 lux, 28°C).* 1×10^{-3} M ammonia was added at time 0 to 4-day old cells grown on N_2.

20 hr (Fig. 1). The protein content increased from 236 to 296 μg/ml during the 20 hr culture period. Thus, the decrease in the specific activity for acetylene reduction cannot be explained by the dilution of nitrogenase by newly formed protein. When 8×10^{-4} M of ammonia was added to the harvested algal suspension (3 mg protein/ml), it was completely used up within 2 hr at 30°C in the light (22,000 lux) (Fig. 2). The decline in activity for acetylene reduction continued even after exhaustion of external ammonia. The activity for acetylene reduction rose again 2 hr after the exhaustion of ammonia. In another experiment, we observed that when cells grown in the presence of ammonia (1×10^{-3} M) were incubated, after washing, in an N-free medium, acetylene reduction recommenced after a time lag of 1 to 2 hr.

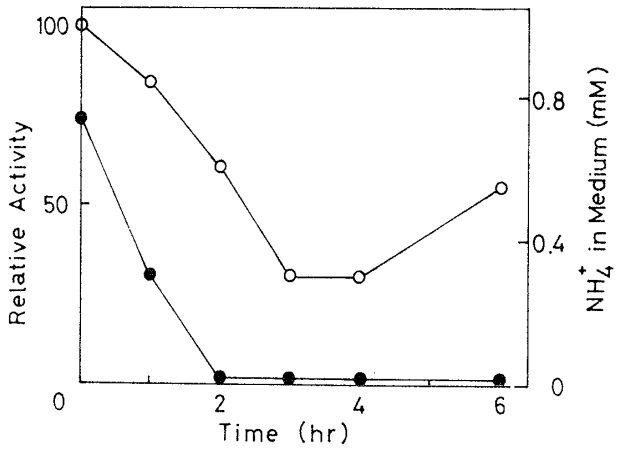

Fig. 2. *Change in acetylene-reducing activity by* Anabaena cylindrica *during incubation with ammonia at 30°C in the light (22,000 lux).* Ammonia (8×10^{-4} M) was added at time 0 to 4-day old cells grown on N_2. Open circles: relative activity for acetylene reduction, closed circles: ammonia concentration in the medium.

Changes in levels of cellular ammonia and free amino acids

If intracellular ammonia is responsible for the regulation of nitrogenase synthesis, the ammonia level should be correlated with the nitrogen-fixing activity. Therefore, variations in the level of free ammonia was followed, using cells incubated in the presence or absence of ammonia. Free ammonia in the trichloroacetic acid (TCA)-soluble fraction of the cell was undetectable both in the presence and absence of ammonia in the culture medium. The TCA-extract contained a certain labile amino compound(s) which was degraded to produce ammonia when the extract was exposed to the high pH of 12 in Conway's microdiffusion units. This labile amino compound(s) was present at a level of 1 to 4 nmoles ammonia/mg dry cells grown in the absence of a bound form of nitrogen. Adding ammonia to the medium raised the level of the compound(s) to 20 nmoles ammonia/mg dry cells or more after 1 hr of incubation (Fig. 3.).

The size of the amino acid pool was not substantially altered by the provision of ammonia to N_2-grown cells. Some differences were observed, however, with respect to amino acid composition (Table 1). Glutamic acid was a main component in cells grown on N_2. When these cells were incubated with ammonia for 3 hr, the relative amounts of glutamic acid and alanine decreased and those of basic amino acids increased. A possible alteration in the metabolic pathway for amino acid synthesis in the presence of a large amount of ammonia is suggested.

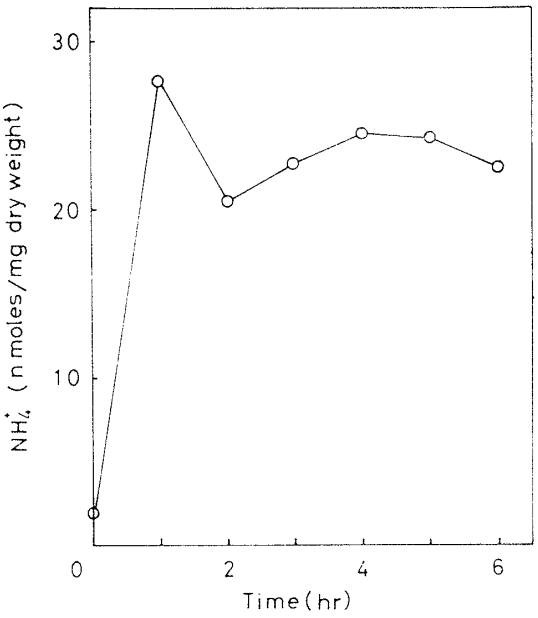

Fig. 3.

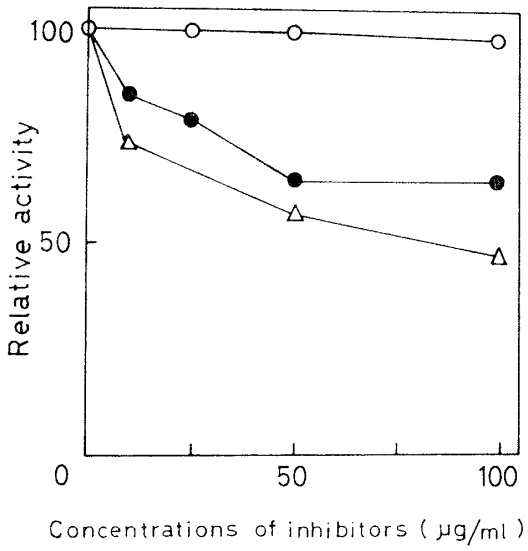

Fig. 4.

Fig. 3. *Change in concentration of a labile amino compound(s) in* Anabaena cylindrica *cells during growth with ammonia at 28°C in the light (5000 lux).* Ammonia (1×10^{-3} M) was supplied at time 0 to 4 day-old cells grown on N_2.

Fig. 4. *Effects of chloramphenicol, puromycin and cycloheximide on acetylene reduction by* Anabaena cylindrica. Cells were incubated for 3 hr at 30°C in the light (22,000 lux) in the presence of inhibitors, then acetylene-reducing activity was assayed. -○-: Cycloheximide, -●-: puromycin, -△-: chloramphenicol.

Table 1 *Composition of free amino acids in* Anabaena cylindrica *incubated in the presence and absence of ammonia* [a]

	Ammonia present		Ammonia absent	
	nmoles [b]	%	nmoles [b]	%
Lys	790	18.8	377	10.6
His	759	18.0	422	11.9
Arg	207	4.9	48	1.4
Unknown-1 [c]	591	14.0	484	13.7
Asp	210	5.0	134	3.8
Thr (Gln, Asn)	175	4.1	64	1.8
Ser	102	2.4	98	2.8
Glu	590	14.0	1237	34.9
Pro	—	—	—	—
Gly	143	3.4	154	4.3
Ala	56	1.3	110	3.1
Cys	—	—	—	—
Val	58	1.4	59	1.7
Met	46	1.1	54	1.5
Ileu	44	1.0	24	0.7
Leu	44	1.0	27	0.8
Tyr	26	0.6	48	1.4
Phe	15	0.4	19	0.5
Other unknown-2 [c,d]	357	8.4	184	5.2
Total	4312	100	3543	100

[a] 4-day old cells were incubated for 3 hr in the presence (5×10^{-3} M) and absence of ammonia. Light intensity: 22,000 lux, temperature: 30°C.
[b] On the basis of 100 mg dry weight of cells.
[c] Expressed in terms of leucine equivalents.
[d] Consisted of several amino compounds.

The increase in amounts of labile amino compound(s) observed when cells were exposed to ammonia may be due to an increased production of a labile amine such as glutamine. In fact, the amino content in the fraction containing glutamine was significantly higher in ammonia-grown cells than in N_2-grown cells (Table 1). Unfortunately, however, threonine and asparagine were also present in this fraction, so that we could not decide which component actually increased in the presence of ammonia.

Effects of amino acids on acetylene reduction

At a concentration of 1×10^{-2} M, arginine, citrulline and serine had no significant effect on the activity for acetylene reduction (Table 2). The activity was not affected by the addition of free amino acids extracted from any of the ammonia-grown cells examined. However, activity increased about 50% when cells were grown in the presence of 10^{-2} M of glutamic acid.

Table 2 *Effects of amino acids on acetylene reduction by* Anabaena cylindrica [a]

Exp. No.	Compounds added (M)		Relative activity
1 [b]	None		100
	NH_4Cl	5×10^{-3}	28
	Glutamine	1×10^{-2}	90
	Arginine	1×10^{-2}	87
	Citrulline	1×10^{-2}	97
	Serine	1×10^{-2}	89
2 [c]	None		100
	NH_4Cl	1×10^{-3}	21
	Glutamic acid	1×10^{-3}	113
		1×10^{-2}	156
3 [c]	None		100
	Amino acids extracted from N_2-grown cells	ca. 1×10^{-3}	107
	Amino acids extracted from NH_4-grown cells	ca. 1×10^{-3}	105

[a] 10 ml lots of cell suspension were incubated for 3 hr at 30°C in the light (22,000 lux) in the presence of the indicated compounds then the acetylene-reducing activity was measured.
[b] Cell concentration: 0.2–0.3 mg protein/ml.
[c] Cell concentration: 2–3 mg protein/ml.

Effects of oxygen and bicarbonate on ammonia inhibition

When cells were incubated in argon or N_2 for 3 hr, their acetylene-reducing activities increased markedly, irrespective of the presence or absence of ammonia (Table 3). Under anaerobic conditions, the ammonia concentration in the medium remained unchanged, while excretion of ammonia from the cells occurred if they were incubated in an ammonia-free medium (Table 3). Under aerobic conditions, ammonia was rapidly assimilated, accompanied by suppression of the acetylene-reducing activity (Tables 3 and 4).

Table 3 *Changes in ammonia content in the medium and in the acetylene-reducing activity in* Anabaena cylindrica *cells incubated under different atmospheres*

Exp. No.	Incubation conditions [a]		Ammonia in medium (mM)		Relative activity of acetylene reduction after incubation
	Ammonia in medium	Gas phase	Before incubation	After incubation	
1	−	Air	0.00	0.00	100
	−	Ar	0.00	0.14	235
	+	Air	1.94	0.30	24
	+	Ar	1.94	1.89	221
2	−	Air	0.00	0.00	100
	−	N_2	0.00	0.39	145
	+	Air	1.70	0.66	14
	+	N_2	1.70	1.68	140

[a] Cells were incubated for 3 hr at 30°C in the light (22,000 lux).

Table 4 *Effects of O_2 and HCO_3^- on acetylene reduction by* Anabaena cylindrica [a]

Compounds added	Acetylene-reducing activity [b]	
	nmoles/mg protein·30 min	Relative activity
None [c]	83	100
NH_4Cl 3×10^{-3} M [c]	13	15
$NaHCO_3$ 2×10^{-3} M [c]	121	143
NH_4Cl 3×10^{-3} M, $NaHCO_3$ 2×10^{-3} M [c]	13	15
None [d]	280	327
NH_4Cl 3×10^{-3} M [d]	248	278

[a] Sodium-potassium phosphate buffer (final 0.01 M, pH 7.5) was added to the incubation medium.
[b] After 3 hr of incubation.
[c] Gas phase: Ar-O_2 (9 : 1).
[d] Gas phase: Ar.

The acetylene-reducing activity increased by about 40% when cells were incubated for 3 hr in the presence of bicarbonate (2×10^{-3} M). However, bicarbonate did not protect the inhibitory action of ammonia (Table 4).

Effects of inhibitors on acetylene reduction

Cells were incubated for 3 hr in the presence of inhibitors of protein synthesis, i.e. chloramphenicol, cycloheximide and puromycin (Fig. 4). Acetylene-reducing activity decreased when chloramphenicol and puromycin were supplied at concentrations higher than 10 μg/ml. Cycloheximide had no effect even at a concentration as high as 100 μg/ml. No inhibition of acetylene-reducing activity was observed at the beginning of incubation with these antibiotics, which suggests that they do not directly inhibit the enzyme system. This is also the case when the inhibitors were replaced by ammonia.

Cells were incubated for 1 hr in the presence of ammonia (5×10^{-3} M), chloramphenicol (10 μg/ml) or cycloheximide (10 μg/ml) at different light intensities (Table 5). Ammonia and chloramphenicol suppressed the acetylene-reducing

Table 5 *Effects of ammonia, chloramphenicol and cycloheximide on acetylene reduction at different light intensities* [a]

Compounds added	400 lux		22,000 lux	
	C_2H_4 [b] formed	Relative activity	C_2H_4 [b] formed	Relative activity
None	127	100	543	100
NH_4Cl [c]	20	15	456	84
Chloramphenicol [c]	132	104	440	81
Cycloheximide [c]	125	99	576	106

[a] *Anabaena cylindrica* cells were incubated in the presence of the indicated compound for 1 hr at 30°C, then the acetylene-reducing activity was measured.
[b] Expressed in terms of nmoles/mg protein·30 min.
[c] NH_4Cl, chloramphenicol and cycloheximide were added at concentrations of 5×10^{-3} M, 10 μg/ml and 20 μg/ml, respectively.

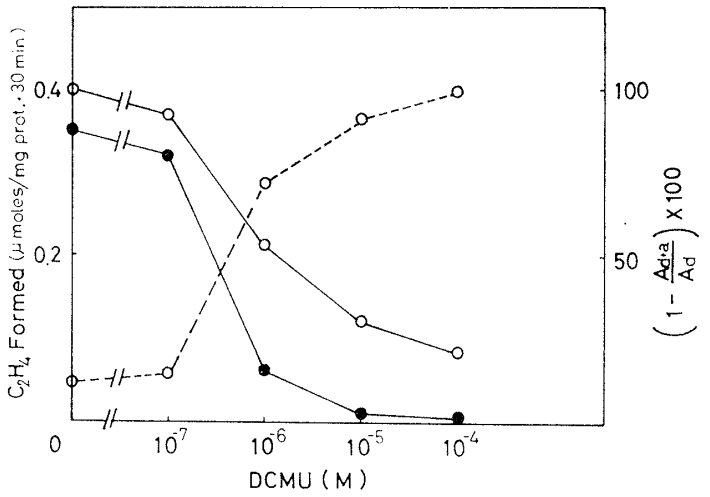

Fig. 5. *Effects of DCMU on acetylene reduciton by* Anabaena cylindrica. Cells were incubated for 30 min in the presence or absence of DCMU and ammonia before the assay of acetylene reduction. -○-: DCMU alone (A_d), -●-: ammonia (5×10^{-3} M) was added with DCMU (A_{d+a}), --○--: $(1-A_{d+a}/A_d)\times100$ (%).

activity to a similar extent at 22,000 lux. At 400 lux, ammonia reduced the activity by 85%, while chloramphenicol had no effect.

Fig. 5 and 6 show effects of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), an inhibitor of photosynthetic electron transport, and carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP), an uncoupler of phosphorylation, on the acetylene-reducing activity. These compounds were added 30 min before the start of activity measurements. 50% inhibition was observed at ca. 1×10^{-6} M of DCMU and at ca. 5×10^{-6} M of CCCP. This supports the view that the reductant and/or energy

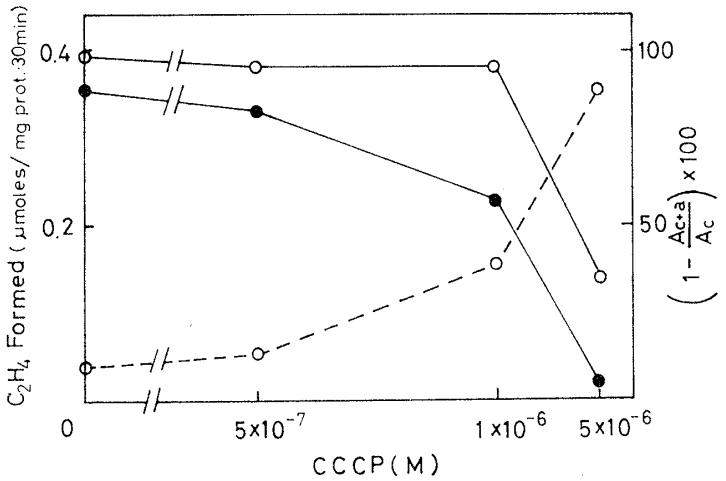

Fig. 6. *Effects of CCCP on acetylene reduction by* Anabaena cylindrica. Cells were incubated for 30 min in the presence or absence of CCCP and ammonia before the assay of acetylene reduction. -○-: CCCP alone (A_c), -●-: ammonia (5×10^{-3} M) was added with CCCP (A_{c+a}), --○--: $(1-A_{c+a}/A_c)\times100$ (%).

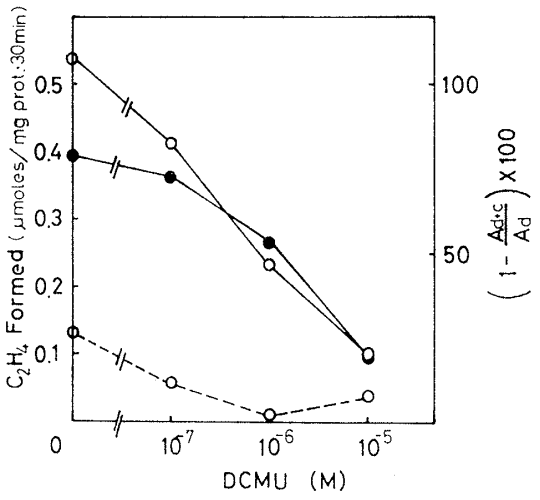

Fig. 7. *Effects of DCMU and chloramphenicol on acetylene reduction by* Anabaena cylindrica. Cells were incubated for 30 min in the presence or absence of DCMU and chloramphenicol before the assay of acetylene reduction. -○-: DCMU alone (A_d), -●-: chloramphenicol (50 μg/ml) was added with DCMU (A_{d+c}), --○--: $(1-A_{d+c}/A_d)\times 100$ (%).

necessary for the reduction of N_2 or acetylene are produced through the photosynthetic process.

When ammonia was supplied together with DCMU or CCCP, the acetylene-reducing activity was significantly reduced during an incubation period as short as 30 min. The higher the concentration of DCMU or CCCP, the more intense was the effect of ammonia (Fig. 5 and 6). These results are consistent with the enhanced ammonia-inhibition observed under weak light conditions. Both the presence of DCMU or CCCP and the reduction of light intensity bring about a lowering of the level of reductant and ATP. No such synergistic effect was observed when chloramphenicol was added together with DCMU (Fig. 7). Ammonia stimulated CO_2 uptake whereas DCMU and CCCP suppressed it (Table 6).

Discussion

The hypothesis that the size of the intracellular ammonia pool regulates nitrogenase synthesis cannot be directly applied to nitrogen fixation in *A. cylindrica*, since free ammonia was hardly detectable, within the limits of precision of our ammonia determination (1×10^{-11} mole per mg dry cells), in cells of *A. cylindrica* even when culturing was conducted in the presence of 10^{-3} M of ammonia.

Table 6 *Effects of ammonia, CCCP and DCMU on CO_2 assimilation by* Anabaena cylindrica

Compounds added (M)		μl CO_2 used/5 ml cell suspension [a]
None		32
NH_4Cl	5×10^{-3}	62
CCCP	1×10^{-6}	39
CCCP	1×10^{-5}	16
DCMU	1×10^{-5}	4

[a] 5 ml cell suspension (0.2 mg protein/ml) was incubated in the presence of the indicated compound for 45 min in argon. Light intensity: 22,000 lux, temperature: 30°C.

However, the level of labile amino compound(s) increased ten times when ammonia was supplied to N_2-grown cells of *A. cylindrica*. This suggests that, in *A. cylindrica*, assimilated ammonia is rapidly metabolized to produce a labile amino compound(s). If this is the case, the pool of labile amino compound(s) instead of the ammonia pool, as postulated by Dalton and Mortenson (*2*), would form the key regulator of nitrogenase synthesis.

Glutamic acid forms a major free amino acid in *A. cylindrica* grown under nitrogen-fixing conditions (Table 1), as was also observed in both Gram-negative and -positive bacteria (*15*). But, basic amino acids such as lysine, histidine and arginine are more abundant in cells grown on ammonia (Table 1). Under N_2-fixing conditions, glutamic acid is probably synthesized by the action of glutamate dehydrogenase and/or glutamate synthetase (*16*). The decrease in glutamic acid content observed when ammonia was added to N_2-grown cells can be explained if ammonia is assimilated through the pathway for glutamine synthesis from glutamic acid and ammonia, as has been found in root systems of green plants (*17*). Accumulation of glutamine may in turn accelerate the formation of arginine and histidine.

When incubated for 3 hr in the presence of ammonia (1×10^{-3} M), the acetylene-reducing activity of *A. cylindrica* decreased to about 50% (Fig. 1). Under these conditions, the doubling time of this organism was over 24 hr. Therefore, the observed drop in activity cannot be due to dilution of the preformed nitrogenase as discussed above. A similar rapid decline in nitrogenase activity, as compared to growth, in the presence of ammonia was also reported with *Azotobacter chroococcum* (*18*), *Rhodospirillum rubrum* (*19*) and *Azotobacter vinelandii* (W. J. Brill, cited in ref. *2*). Some other mechanism must be found to account for the function of ammonia in the regulation of nitrogen fixation.

Chloramphenicol and puromycin have been suggested to inhibit protein synthesis by blocking the translation on 70S ribosomes, and cycloheximide by blocking the translation on 80S ribosomes (*20*). As in the bacteria and some blue-green algae (*21*, *22*), our data also suggest that nitrogenase formation in *A. cylindrica* proceeds on the 70S ribosomes.

Ammonia and chloramphenicol suppress the activity for acetylene reduction to a similar extent under strong light conditions (Table 5). Under weak light conditions, chloramphenicol shows no inhibitory effect on acetylene reduction, whereas inhibition by ammonia is enhanced (Table 5). When ammonia was added simultaneously with DCMU, marked enhancement of inhibition took place (Fig. 5). In this case, ammonia almost completely suppressed the acetylene-reducing activity for a short incubation period of 30 min (Fig. 5). Chloramphenicol did not show this synergistic effect when it was added together with DCMU (Fig. 7). These results provide evidence which supports the view that ammonia acts not merely as an inhibitor of nitrogenase synthesis but also as a metabolic inhibitor.

The fact that CCCP is effective in reducing activity for acetylene reduction at a concentration of 5×10^{-6} M (Fig. 6) is evidence that photochemically produced ATP is used for nitrogen fixation (*23*, *24*). The addition of ammonia together with CCCP also enhances the inhibition of acetylene reduction (Fig. 6). CCCP suppresses the CO_2 uptake, but ammonia accelerates it (Table 6). Stimulation of CO_2 and malate uptake in *R. rubrum* by ammonia was reported by Shick (*19*).

Oxygen has a strong inhibitory effect on nitrogen fixation in intact cells (Table 4, ref. *25*) and in a cell-free system (*26*). Stewart and Pearson (*27*) reported that, in *Anabaena* and *Nostoc*, acetylene reduction occurred most actively at pO_2 below 0.2 atm. Oxygen is also indispensable for the inhibition of nitrogen fixation by ammonia. Under anaerobic conditions in the light, the addition of ammonia had no inhibitory effect on acetylene reduction (Table 3). Oxygen may, directly or indirectly, contribute to the assimilation of ammonia or the formation of the labile amino compound(s) in the cell.

Dalton and Mortenson (*2*) stated that control over nitrogenase is exerted in two ways. *The first is a coarse control in which synthesis of the enzyme is repressed by an excess of one of the products of its activity (ammonia); and the other is a fine control in which the activity of the existing nitrogenase is controlled by one of the products, ADP; or more specifically by the ratio of ATP to ADP* (*28*, *29*). No experimental evidence is available with respect to the second mechanism for blue-green algae. If ammonia is assimilated by *A. cylindrica* through the pathway for glutamic acid synthesis or through similar processes, then a reductant such as reduced pyridine nucleotide as well as ATP would be consumed. If this is so, then a supply of ammonia would result in a reduction in the size of the reductant and ATP pools and, in turn, in a lowering of the level of acetylene reduction. This is another possible control mechanism for nitrogen fixation.

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