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Formation of Glutamine from [^{13}N]ammonia, [^{13}N]dinitrogen, and [^{14}C]glutamate by Heterocysts Isolated from *Anabaena cylindrica*

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A method is described for the isolation of metabolically active heterocysts from *Anabaena cylindrica*. These isolated heterocysts accounted for up to 34% of the acetylene-reducing activity of whole filaments and had a specific activity of up to 1,560 nmol of C_2H_4 formed per mg of heterocyst chlorophyll per min. Activity of glutamine synthetase was coupled to activity of nitrogenase in isolated heterocysts as shown by acetylene-inhibitable formation of [^{13}N]NH_3 and of amide-labeled [^{13}N]glutamine from [^{13}N]N_2. A method is also described for the production of 6-mCi amounts of [^{13}N]NH_3. Isolated heterocysts formed [^{13}N]glutamine from [^{13}N]NH_3 and glutamate, and [^{14}C]glutamine from NH_3 and [^{14}C]glutamate, in the presence of magnesium adenosine 5'-triphosphate. Methionine sulfoximine strongly inhibited these syntheses. Glutamate synthase is, after nitrogenase and glutamine synthetase, the third sequential enzyme involved in the assimilation of N_2 by intact filaments. However, the kinetics of solubilization of the activity of glutamate synthase during cavitation of suspensions of *A. cylindrica* indicated that very little, if any, of the activity of that enzyme was located in heterocysts. Concordantly, isolated heterocysts failed to form substantial amounts of radioactive glutamate from either [^{13}N]glutamine or α-[^{14}C]ketoglutarate in the presence of other substrates and cofactors of the glutamate synthase reaction. However, they formed [^{14}C]glutamate rapidly from α-[^{14}C]ketoglutarate by aminotransferase reactions, with various amino acids as the nitrogen donor. The implications of these findings with regard to the identities of the substances moving between heterocysts and vegetative cells are discussed.

Filamentous cyanobacteria that have been shown to be capable of fixing nitrogen gas (N_2) aerobically are characterized by the presence of differentiated cells called heterocysts. A large amount of indirect evidence (6, 8, 22, 25, 27, 36) favors the hypothesis that heterocysts are major loci of nitrogen fixation in the filaments. This hypothesis was substantiated by autoradiographic experiments with [^{13}N]N_2 (32). Early attempts (7, 10, 20, 21) to demonstrate substantial nitrogenase activity in isolated heterocysts free of vegetative cells were unsuccessful. Recently, however, Peterson and Burris (16), working with a strain of *Anabaena*, described a procedure for isolating heterocysts that can reduce C_2H_2, an alternate substrate for nitrogenase, at up to 1,840 nmol per mg of chlorophyll (Chl) per min. When calculated on the basis of the heterocyst frequency in filaments, this rate accounted for 13% of the rate of C_2H_2 reduction by intact filaments (1,060 nmol per mg of Chl per min). Tel-Or and Stewart (23) found up to 45% of the activity of whole filaments in heterocysts isolated from *Anabaena cylindrica*, but the specific activity of nitrogenase in whole filaments reported by the latter workers was only 0.194 nmol of C_2H_4 per mg of protein per min. This rate is approximately 6 nmol of C_2H_4 per mg of Chl per min if one assumes a ratio of protein to chlorophyll of approximately 30 (4).

The enzymatic pathway consisting of glutamine synthetase and glutamate synthase (glutamine amide:α-ketoglutarate amidotransferase) (24) was shown to be the major route of metabolism of ammonia in heterotrophic nitrogen-fixing bacteria (15). Using [^{13}N]N_2, we recently established that this pathway also me-

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diates the metabolism of N_2-derived ammonia in *A. cylindrica* (26, 34). The presence of the two constituent enzymes in cyanobacteria had previously been demonstrated (3, 11).

In our experiments (34), ammonia derived from [^{13}N]N_2 was incorporated into glutamine so rapidly that the latter substance had as much ^{13}N as had ammonia after about 2 s of fixation. In contrast, approximately 90 s elapsed before glutamate became as highly labeled as glutamine. These results suggested that glutamine synthetase is located close to the site of N_2 fixation. The activity of glutamine synthetase in heterocysts had been found to be 1.7 times as great as that in vegetative cells, per milligram of protein (3). However, the localization of glutamate synthase in filamentous nitrogen-fixing cyanobacteria has not heretofore been investigated.

In this paper we report that isolated heterocysts reduce N_2 gas at a substantial fraction of intact-filament rates. Moreover, the nitrogenase activity in these heterocysts is metabolically coupled to activity of glutamine synthetase, so that the heterocysts produce [^{13}N]glutamine. However, the heterocysts produce very little or no glutamate, and concordantly have very low or no glutamate synthase activity. It was possible to obtain these results because we had developed a new method for isolating heterocysts that had high metabolic activity and contained no more than 2% as many vegetative cells as heterocysts. Also important was development of a method for production of highly radioactive [^{13}N]ammonia.

MATERIALS AND METHODS

Cultures of *A. cylindrica* Lemm. Cultures were grown aerobically in fermentors (for details, see 32) with N_2 as nitrogen source, with an exponential doubling time of about 18 h, to a density of 0.5 to 0.76 μg of Chl per ml, except where noted otherwise. For experiments in which whole filaments were exposed to [^{13}N]N_2, the cyanobacterium was concentrated to 27 μg of Chl per ml by centrifugation at 200 $\times$ *g* for 5 min and was incubated aerobically, as described earlier (32), until used.

Isolation of heterocysts. Heterocysts were isolated as follows. The cyanobacterial suspension was sedimented at 200 $\times$ *g* for 25 min. The pellet was resuspended to 27 to 100 μg of Chl per ml in a portion of the supernatant fluid or in 5 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer, pH 7.6. Ten milliliters of the concentrated suspension was thrice evacuated to 50 μm of mercury and regassed to 1 atm with argon. Further processing of the suspension was done under these strictly anaerobic conditions, with solutions and reagents degassed as described above. Biochemicals, unless otherwise specified, were obtained from Sigma Chemical Co., St. Louis, Mo. One milliliter of solution containing 2 or 5 mM TES, pH 7.6, and 200 mM sodium dithionite ($Na_2S_2O_4$) was added to the 10-ml algal suspension, followed by 20 ml of a solution containing TES, D-mannitol (Fisher Scientific Co., Fair Lawn, N.J.), and Na_2EDTA [(ethylenedinitrilo)tetraacetic acid, disodium salt]. The resulting suspension, containing 1.4 to 5 mM TES, 6.5 mM sodium dithionite, 26 mM Na_2EDTA, and 650 mM D-mannitol, was incubated on a water bath shaker (75 rpm) at 30°C. After 45 min, most of the vegetative cells were detached or broken, but a large number of short filaments still remained intact. Twenty to 25 ml of the suspension was then subjected to sonic disruption under argon for periods of 6 s/ml (on occasion up to 14 s/ml) at setting 4 of a Sonifier Cell Disruptor (model W185, Heat Systems-Ultrasonic Inc., Plainview, N.Y.). The suspension was then centrifuged for 10 min at 200 $\times$ *g* in degassed, 15-ml conical centrifuge tubes. The pellets were washed twice with, and finally resuspended in 2 to 5 ml of, a solution containing 10 mM sodium dithionite and 2 or 5 mM TES. For certain experiments, dithionite either was omitted from all solutions or was replaced by sodium ascorbate (final concentration, 2.5 or 5 mM) throughout.

The number of heterocysts per milliliter of suspensions of whole filaments and of isolated heterocysts was determined with an eosinophil counter. Suspensions of isolated heterocysts (Fig. 1) contained apparently intact heterocysts as well as heterocysts that were apparently damaged (empty-looking, or with broken walls or displaced polar bodies).The sum of such "intact" and "damaged" heterocysts is referred to as "total" heterocysts. No more than 2% as many vegetative cells as heterocysts were present together with the heterocysts isolated by the procedure described above.

Acetylene reduction assay. Acetylene reduction by whole filaments of *Anabaena* was determined as described (36), under a gas phase of argon/C_2H_2 (9:1, vol/vol), after storage of the concentrated suspension under argon for 3 to 4.5 h, during which time the process of isolating heterocysts was completed. Comparisons are thus made between suspensions of filaments and heterocysts with the same history of exposure to aerobic and anaerobic conditions. Such suspensions of filaments reduced acetylene at least as rapidly, per milligram of Chl, as when assayed immediately after withdrawal from a fermentor.

Reduction of acetylene by isolated heterocysts was normally measured anaerobically in the presence of dithionite, adenosine 5'-triphosphate (ATP), and an ATP-generating system (see Table 1, footnote *a*). On occasion, certain of the components were omitted or replaced by others.

Labeling with [^{13}N]N_2 and [^{13}N]NH_3. ^{13}N was generated by irradiation of 18.6 mg of ^{13}C with protons (1, 32). To form [^{13}N]N_2, the ^{13}C target was subjected to Dumas combustion (1, 32). Alternatively, [^{13}N]NH_3 was generated by a modification of the method of Carangal and Varner (2; J. E. Varner, personal communication). The target was transferred to a 100-ml Kjeldahl flask preheated to 100°C on a heating mantle. Potassium dichromate, 0.4 g, and 0.2 g of potassium sulfate were added to the

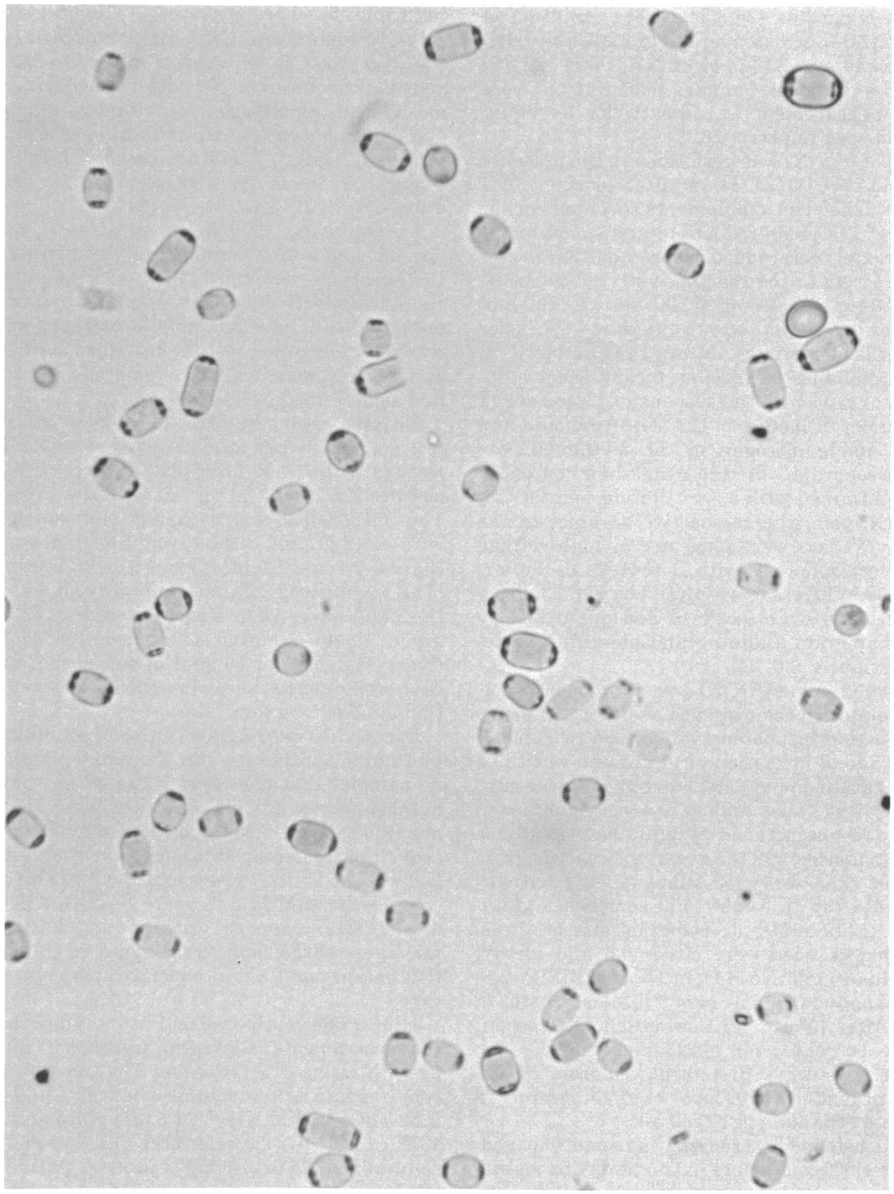

FIG. 1. *Photomicrograph of heterocysts isolated from A. cylindrica and active in reduction of [13N]N_2 and acetylene. The heterocysts are suspended in a solution containing 2.5 mM sodium ascorbate and 5 mM TES, pH 7.2. A low frequency of broken heterocysts and less than 2% vegetative cells are present as contaminants.* ×920.

flask and mixed well with the target, and 2 ml of concentrated sulfuric acid (specific gravity, 1.84) saturated with potassium dichromate was then added. The temperature in the flask rose steadily, reaching nearly 250°C at the end of 10 min. The flask was then removed from the heating mantle, and 5 ml of saturated sodium borate (pH 10), 8 ml of 40% (wt/vol) NaOH, and 2 ml of saturated silver sulfate solution were added in rapid succession. [^{13}N]NH_3 was distilled under vacuum, at 50°C, from the resulting blackish-green alkaline reaction mixture. A side-arm test tube immersed in liquid nitrogen served as receiving tube. Distillation of [^{13}H]NH_3, monitored with a model ABG-10KG-SB ionization gauge (Jordan Electronics, Division of Victoreen Instruments Co., Alhambra, Calif.), is complete within 10 to 15 s after application of vacuum from a tap-water aspirator. Bumping and "boiling over" of the reaction mixture is prevented by permitting a feeble stream of air to enter the

distillation apparatus (28). The frozen solution of [^{13}N]NH_3, containing an average of 6 mCi of $^{13}NH_3$ and approximately 0.3 μmol of NH_3, was thawed and diluted to 1 to 2 ml. Samples of 25 to 100 μl were taken for measurement of radioactivity by liquid scintillation spectrometry (26, 34).

Fixation of [^{13}N]N_2 was performed in the presence of argon and 1% CO_2 (26, 34), or after addition of 0.1 atm of acetylene. The supplements specified earlier for assays of acetylene reduction, and sometimes 1 mM glutamate, were added before addition of isolated heterocysts to the reaction vial. Suspensions, 0.25 ml, of filaments (27 μg of Chl/ml, without supplements) or heterocysts were exposed to [^{13}N]N_2 for 0.25 min or 2 (sometimes 15) min, respectively, in the presence of 4,000 lx from an incandescent bulb. At the end of exposure, the suspensions were mixed with 4 volumes of methanol (26, 34). Ammonia and glutamine amide nitrogen in the methanolic extracts were separately distilled, and their content of ^{13}N was determined with a scintillation counter (26, 34). Alternatively, the radioactive organics in the methanolic extracts were separated by high-voltage (3,000 V) electrophoresis with 70 mM borate buffer, pH 9.2, on thin layers of cellulose (26, 34). Radioactive constituents were localized and quantified by radiochromatogram scanning and identified as described previously (26, 34).

Experiments using [^{13}N]NH_3 were done with heterocysts isolated in the presence of either dithionite or ascorbate and incubated as described in Table 6. When the rate of formation of glutamine was to be quantified as nanomoles per 10^6 heterocysts per min, 2 mM NH_4Cl was also added. When evidence was sought for the occurrence of glutamate synthase activity in nonbroken heterocysts, the reaction mixture in the vials was also supplemented with α-ketoglutarate (up to 5 mM) and sometimes glutamine (up to 10 mM), or combinations of these. Electron donors tried were either 0.5 mM methyl viologen plus 10 mM $Na_2S_2O_4$ or 50 μM DCPIP (2,6-dichlorophenolindophenol) plus 5 mM ascorbate, in the light. After 10 min of illumination, the suspensions were processed for electrophoresis (26, 34), with heterocyst lipids first displaced from ^{13}N-labeled constituents by thin-layer chromatography in chloroform-methanol, 1.3:1 (vol/vol).

The suspension of heterocysts was sometimes sedimented at 1,000 $\times$ g before extraction with methanol, and the supernatant and pellet fractions were processed separately for electrophoretic examination of labeled products. Where indicated, the radioactive compound co-electrophoresing with stable glutamine was eluted from the thin-layer plate. The ^{13}N radioactivity of its amide nitrogen was then determined by steam distillation in the presence of alkali and scintillation counting of the distillate (26, 34).

Labeling with [^{14}C]glutamate and α-[^{14}C]ketoglutarate. Formation of [^{14}C]glutamine from [^{14}C]-glutamate (New England Nuclear Corp.; 230 μCi/μmol) by isolated heterocysts was determined in the presence of ATP, an ATP-generating system, and N_2, NH_3, or no exogenous nitrogen source (details in Table 5). Formation of [^{14}C]glutamate from α-[^{14}C]ketoglutarate (New England Nuclear Corp.; 255 μCi/μmol) by heterocysts in the absence of any added electron carrier was determined with various amino acids as nitrogen donor and with or without 5 mM aminooxyacetate (an inhibitor of aminotransferases; 12, 14) or 1 mM azaserine (an inhibitor of glutamine amide transferases; 17, 34; details in Table 7).

Assays using ^{14}C-labeled substrates were performed at 30°C for 30 min in 1-ml Reactivials (Pierce Chemical Co., Rockford, Ill.), with magnetic stirring. The reactions were terminated by addition of methanol, and the methanolic extracts were processed for electrophoresis as described above. Radioactive spots were scanned, visualized, and quantified as described earlier (26, 34).

Enzyme assays in vitro. Fermentor cultures, 0.6 to 1.4 μg of Chl per ml, harvested at 40,000 $\times$ g and washed with 5 mM TES, pH 7.2, were resuspended at 90 to 125 μg of Chl per ml with the same buffer. The suspension was degassed and supplemented with $Na_2S_2O_4$ and dithiothreitol to final concentrations of 2.5 and 1.0 mM, respectively. For breakage of all vegetative cells and essentially all heterocysts, the algal suspension was cavitated at 12°C under argon at 3.0-A output of a model S-125 Sonifier (Heat Systems) for 60 s/ml of suspension (29). The cavitated preparations were clarified by centrifugation at 5,000 $\times$ g for 5 min.

For studies on the time course of solubilization of glutamine synthetase and glutamate synthase, 12-ml samples that had been cavitated for the desired number of seconds per milliliter were centrifuged anaerobically at 5,000 $\times$ g for 5 min. The supernatant fluid was assayed for enzymatic activities (see below). The pellets, resuspended with 12 ml of buffer containing 5 mM TES, 2.5 mM $Na_2S_2O_4$, and 1.0 mM dithiothreitol, were cavitated for an additional 60 s/ml. These suspensions were again clarified by centrifugation, and their supernatant fluid was assayed.

Glutamine synthetase activity was determined by the formation of [^{14}C]glutamine from [^{14}C]glutamate as with isolated heterocysts. The reaction mixture contained, in a final volume of 0.2 ml: 5 mM ATP, 15 mM $MgCl_2$, 5 mM NH_4Cl, 2.5 mM glutamate (0.92 to 1.66 μCi of ^{14}C), 20 mM TES, pH 7.2, and extract containing 0.06 to 0.33 mg of protein. Activity in the crude extract was proportional to time for 30 min and proportional to protein concentration to 1.75 mg of protein/ml.

Glutamate synthase activity was determined by the formation of [^{14}C]glutamate from α-[^{14}C]ketoglutarate and stable glutamine in the presence of aminooxyacetate (11). The reaction mixture contained, in a final volume of 0.2 ml: 5 mM glutamine, 1 mM methyl viologen, 12.5 mM $Na_2S_2O_4$, 2.5 mM α-ketoglutarate (0.92 to 1.73 μCi of ^{14}C), 5 mM aminooxyacetate, 28 mM TES, pH 7.2, and extract containing 0.20 to 0.62 mg of protein. Activity was linear with time to 30 min and was a linear function of protein concentration in the range of 0.15 (essentially nil activity) to 1.0 mg of protein per assay.

RESULTS

Acetylene reduction by isolated heterocysts. Heterocysts isolated in the presence of TES, sodium dithionite, EDTA, and mannitol accounted normally for about 13%, but up to 34%, of the acetylene-reducing activity of whole filaments (Table 1, experiment 1). The maximum percent activity measured was 41 if only intact heterocysts were considered. The activity of 11.9 nmol of C_2H_4 per 10^6 total heterocysts per 20 min (Table 1, experiment 1) corresponds, on the basis of 0.30 pg of Chl per heterocyst (33), to an activity of 1,560 nmol of C_2H_4 per mg of Chl per min. Incubation in the presence of TES-dithionite-EDTA-mannitol for 45 min yielded active heterocysts. After only 5 min of incubation in this mixture, sonic treatment resulted in preparations of heterocysts with very low nitrogenase activity (Table 1, experiment 2), perhaps due to lesser permeation of cofactors.

Optimum acetylene-reducing activity of isolated heterocysts was obtained in the presence of ATP, an ATP-generating system, and dithionite (Table 2; cf. reference 21). Although ATP itself was required for reduction of acetylene (Table 2), acetylene was reduced at a constant but lower rate in the absence of an ATP-generating system (Fig. 2, Table 2).

Replacement of sodium dithionite (13 experiments) with sodium ascorbate (9 experiments) during the isolation procedure resulted in heterocysts with, on the average, lower acetylene-reducing activity (Table 2). However, we have not found a physiological substance that can replace dithionite as an electron donor during the acetylene reduction assay. Heterocysts isolated in the absence of a reductant, and assayed in the presence of dithionite, had less than 4% of the activity of heterocysts isolated in the presence of dithionite. Ascorbate plus DCPIP could not replace dithionite as an electron donor in the light (Table 2).

Fixation of $[^{13}N]N_2$ and coupling to glutamine formation by isolated heterocysts. Heterocysts isolated in the presence of dithionite or ascorbate reduced $[^{13}N]N_2$ in the light. Scans of electrophoretograms of methanolic extracts showed only a single organic product of fixation of $[^{13}N]N_2$, which co-electrophoresed with [^{14}C]glutamine (Fig. 3). Vacuum distillation at pH 10 of methanolic extracts of heterocysts labeled for 120 s with $[^{13}N]N_2$ was followed by steam distillation in the presence of alkali. These distillation procedures showed the presence of $[^{13}N]NH_3$ and ^{13}N-labeled amide nitrogen (Table 3; see 26, 34). Addition of 0.1 atm of acetylene to the reaction vial containing heterocysts inhibited $[^{13}N]N_2$ fixation by about 99%.

Radioactivity of the amide nitrogen steam-distilled as described above accounted for up to 67% of the combined distillates for ammonia and amide nitrogen after fixation of $[^{13}N]N_2$ for 120 s by heterocysts in the presence of 1 mM glutamate (Table 3). Radioactivity in the combined distillates of extracts of heterocysts accounted for up to 23% of the label found in the combined distillates (NH_3 and amide nitrogen) of extracts of whole filaments exposed to $[^{13}N]N_2$, per heterocyst and per minute of fixation (Table 4), or for about 20% of the ^{13}N radioactivity present in whole filaments, if the α-amino-^{13}N label of glutamate and glutamine is considered (see reference 34). We used only 15-s

TABLE 1. *Acetylene reduction by isolated heterocysts and whole filaments of Anabaena cylindrica*

Expt no.	Conditions for isolation of heterocysts	nmol of C_2H_4 formed/20 min[a]		
		Per 10^6 total[b] isolated heterocysts	Per 10^6 intact[b] isolated heterocysts	Per 10^6 heterocysts of whole filaments[c]
1	Normal procedure: 45-min incubation in 1.4 mM TES, 6.5 mM $Na_2S_2O_4$, 26 mM Na_2EDTA, 650 mM D-mannitol	11.9 (34)	14.3 (41)	35.0 (100)
2	As in 1, but only 5-min incubation	0.7 (1.5)	—[d]	47.7 (100)

[a] Acetylene reduced at 30°C under 4,000 lx of incandescent light in 5-ml serum vials containing 1.8×10^6 to 1.9×10^6 heterocysts isolated in the presence of dithionite, or containing suspensions of whole filaments having 0.94×10^6 to 2.3×10^6 heterocysts. Vials containing isolated heterocysts were supplemented with 0.5 mg of creatine phosphokinase, 50 mM creatine phosphate, 5 mM ATP, 5 mM $MgCl_2$, 10 mM $Na_2S_2O_4$, and 50 mM TES in 2 ml, final volume. Gas phase: 90% Ar, 10% C_2H_2. All values are means of duplicate determinations. Values in parentheses are percentages relative to intact filaments.

[b] "Intact" and "total" heterocysts are defined in Materials and Methods.

[c] Filaments concentrated to 27 to 100 μg of Chl/ml were stored under Ar for 3 to 4.5 h until the isolation of heterocysts was completed and were then assayed for reduction of C_2H_2.

[d] Not determined.

TABLE 2. *Requirements for acetylene reduction in the light by heterocysts isolated in the presence or absence of sodium dithionite or sodium ascorbate*

Assay conditions	nmol of C_2H_4 formed/10^6 total isolated heterocysts in 20 min		
	10 mM dithionite[a]	5 mM ascorbate[a]	None[a]
Complete supplements[b]	13.3	3.74	0.49
$-Na_2S_2O_4$ + 5 mM sodium ascorbate + 50 μM DCPIP		0.40	0.06
-CrP-CrK[c]	9.4	2.35	0.23
-CrP-CrK-ATP	0.0	-[d]	-

[a] Reductant present during isolation.
[b] As in Table 1.
[c] CrP, creatine phosphate; CrK, creatine phosphokinase.
[d] -, Not determined.

exposures of filaments to [^{13}N]N_2 so that the great majority of fixed ^{13}N would be distillable and longer exposure of heterocysts so as to increase fixation and thereby reduce the statistical error in determining the radioactivity fixed. Examination under a microscope indicated that approximately 50% of the population of isolated heterocysts used in this experiment could be considered damaged. If N_2-fixing activity were restricted to intact heterocysts, the nitrogenase activity in such heterocysts would have accounted for about 40% of the N_2-fixing activity of whole filaments.

When isolated heterocysts were incubated in the light for 30 min in the presence of [^{14}C]glutamate and other supplements, and with either N_2 or no other added source of nitrogen, about 22% as much [^{14}C]glutamine was formed as in the presence of 5 mM ammonia (Table 5). Addition of 1 mM methionine sulfoximine inhibited formation of [^{14}C]glutamine by nearly 90%.

Assimilation of [^{13}N]ammonia by isolated heterocysts. Isolated heterocysts formed, from [^{13}N]NH_3, a ^{13}N-labeled substance that co-electrophoresed with stable and with ^{14}C-labeled glutamine. Addition of stable glutamate to the reaction mixture resulted in greatly increased radioactivity of [^{13}N]glutamine (Table 6). When the region of the thin-layer plate bearing ^{13}N radioactivity was eluted and the eluate was steam-distilled in the presence of alkali (26, 34), all of the original radioactivity, within experimental error, could be accounted for as amide ^{13}N. The average rate of formation of glutamine observed in the presence of 1 mM glutamate and 2 mM NH_4Cl was 0.1 nmol per 10^6 heterocysts per min, whereas N_2-fixing filaments supplemented with 1 mM glutamate and 2 mM NH_4Cl formed an average of 10 nmol of glutamine per 10^6 heterocysts per min. When the suspension of heterocysts in the reaction vial was sedimented, after incubation with [^{13}N]NH_3, glutamate, and other cofactors, and the pellet and supernatant fluid were processed separately, 97% of the radioactive glutamine was found in the supernatant fluid (Table 6). Thoroughly extracted pellets also contained slight radioactivity. In comparable experiments in which isolated heterocysts were incubated with [^{14}C]glutamate and NH_3, the [^{14}C]glutamine that was formed and the [^{14}C]glutamate that remained were both distributed 0.96:0.04 between the supernatant and pellet fractions. In the presence of 1 mM methionine sulfoximine, very little (Table 6) ^{13}N-labeled substance detectable by scanning of electrophoretograms was formed.

Localization of glutamate formation. Heterocysts were incubated for 10 min with [^{13}N]NH_3 and other cofactors (see above) for synthesis of [^{13}N]glutamine. Adding α-ketoglutarate (up to 5 mM), glutamine (up to 10 mM), and methyl viologen plus dithionite or DCPIP plus ascorbate in the light as electron donor, and decreasing the concentration of glutamate from 1 mM to 0.1 or 0 mM, resulted in a ratio of [^{13}N]glutamate to [^{13}N]glutamine of at most 0.02. These results provided no evidence for the idea that heterocysts have substantial glutamate synthase activity.

We therefore attempted to localize the site of glutamate synthase activity by the technique of

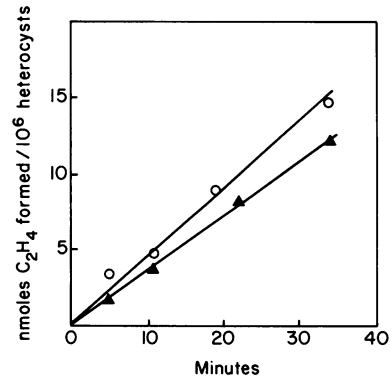

FIG. 2. *Time course of reduction of acetylene by heterocysts isolated from A. cylindrica. Reaction vials contained 1.75 × 10^6 heterocysts in a total volume of 2 ml. Details of assay and supplements present were as specified for Table 1* (○), *or creatine phosphokinase and creatine phosphate were omitted* (△).

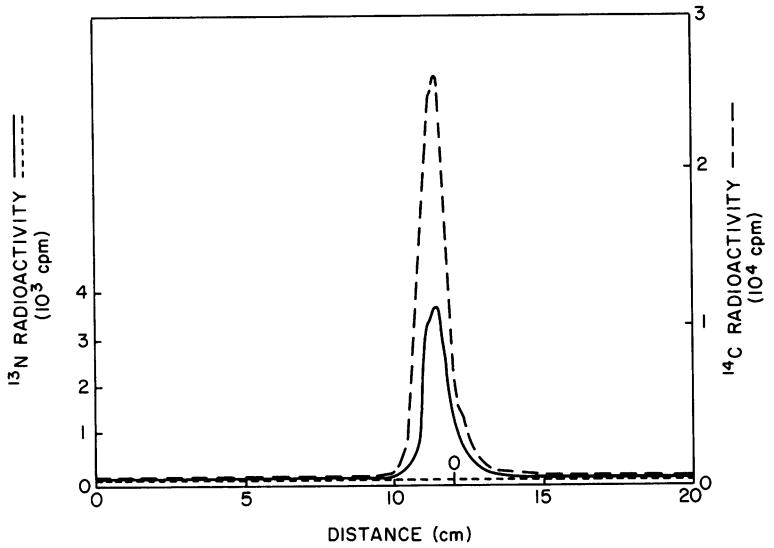

FIG. 3. *Scan of radioactivity from ^{13}N (——) in an electrophoretogram of compounds extracted from a suspension of heterocysts isolated from A. cylindrica in the presence of dithionite and allowed to fix [^{13}N]N_2 for 15 min at 4,000 lx of incandescent light. The 1-ml reaction vial contained 7.1 × 10^6 heterocysts and supplements and gas phase as stated in footnote b of Table 3. An 80% methanolic extract of the contents of the reaction vial, supplemented with ten unlabeled amino acids and [^{14}C]glutamine as markers, was applied in a thin strip (at 0) 12 cm from the positive (left-hand) end of a cellulose thin-layer plate (20 by 5 cm). Lipids were displaced from the region of the origin by chromatography in chloroform-methanol (1.3:1, vol/vol). The extract was then subjected to electrophoresis at 3,000 V for 12 min in 70 mM sodium borate buffer, pH 9.2. The plate was scanned at 5 cm/min, τ = 1 s. After decay of the ^{13}N, the plate was rescanned as before (- - -). A 127-μm layer of aluminum covering the detector was then removed, and the electrophoretogram was scanned for radioactivity from ^{14}C (— — —). The plate was then dried at 90°C and sprayed with a solution of ninhydrin to localize the stable marker amino acids. The peak of ^{13}N radioactivity in the figure co-electrophoresed with glutamine. Glutamate was present at the 6.7-cm position after electrophoresis.*

differential disruption of vegetative cells and heterocysts (29, 35). Enzymatic activities associated with supernatant and pellet fractions after cavitation for periods of 1 to 25 s/ml of a suspension of filaments were assayed in cell-free extracts as described in Materials and Methods. As previously shown (29), the number of apparently intact heterocysts decreased exponentially during cavitation, with 50% of the heterocysts remaining apparently intact after cavitation of a suspension for about 15 s/ml, whereas the amounts of chlorophyll (Fig. 4) and protein (not shown) sedimentable at 5,000 × *g* showed a much more rapid decrease. Glutamate synthase activity is solubilized very rapidly from the filaments, in that 50% of the glutamate synthase activity is found in the supernatant fluid after cavitation of a suspension for close to 1 s/ml and essentially 100% is found after cavitation for 5 s/ml. Vegetative cells are destroyed comparably rapidly (35). After 5 s of cavitation per ml and centrifugation for 5 min at 5,000 × *g*, 75 to 80% of the heterocysts remained apparently intact and were sedimented, whereas approximately 20% of the glutamine synthetase activity and approximately 24% of the total protein and chlorophyll were sedimented. Cavitation for up to 25 s/ml of suspension continued to solubilize glutamine synthetase activity, approximately in parallel with the "solubilization" of chlorophyll (Fig. 4) and protein (not shown).

Aminotransferase activity of isolated heterocysts. Isolated heterocysts, incubated for 30 min in the presence of stable glutamate and α-[^{14}C]ketoglutarate, formed [^{14}C]glutamate at a substantial rate (Table 7). This activity was extensively inhibited by 5 mM aminooxyacetate, but was not affected by 1 mM azaserine. The formation of [^{14}C]glutamate, also unlike the glutamate synthase reaction in vitro, was not dependent on the presence of an exogenous reductant such as dithionite-reduced methyl viologen. The aminotransferase activity could also be supported by glutamine, at a lower rate than by glutamate, and by asparate, but not measurably by alanine. Glutamine-supported transferase activity was also unaffected by azaserine and inhibited by aminooxyacetate. Some formation of [^{14}C]glutamate from α-[^{14}C]keto-

TABLE 3. *Distribution of ^{13}N radioactivity in products distilling as ammonia and amide nitrogen after fixation of [^{13}N]N_2 by heterocysts isolated from A. cylindrica*

Vial contents	Cpm/μCi[a]	
	[^{13}N]NH_3	[^{13}N]amide
Standard[b] + heterocysts	15.93 ± 0.29[c]	31.74 ± 0.23[c]
Standard + heterocysts + 0.1 atm of C_2H_2	0.18 ± 0.02	0.28 ± 0.03
Standard − heterocysts	−0.04 ± 0.04	0.04 ± 0.13

[a] After fixation of [^{13}N]N_2 for 120 s, the suspension was extracted with 80% methanol and the extract was subjected first to vacuum distillation at pH 10, to determine [^{13}N]NH_3, and then to steam distillation in the presence of NaOH, to determine [^{13}N]amide. Cpm per microcurie represents cpm of [^{13}N]NH_3 or cpm of [^{13}N]amide in the fixation vial (measured with approximately 74% counting efficiency), corrected to the time of the start of fixation and normalized to equal amounts of ^{13}N (in microcuries) in the vial.

[b] One-milliliter reaction vials contained, in a volume of 0.25 ml, 1 mM glutamate, 4 mM ATP, 4 mM $MgCl_2$, 40 mM phosphocreatine, 0.05 mg of creatine phosphokinase, 10 mM $Na_2S_2O_4$, 24 mM TES, pH 7.2, and, as indicated, 2.55 × 10^6 total or no heterocysts, under an atmosphere of 2% N_2, 1% CO_2, and 97% argon.

[c] Mean ± standard deviation of the mean after correction for background (approximately 18 cpm) and time of decay, calculated from replicate readings of three separate samples of [^{13}N]NH_4^+ distillate (uncorrected values, 2,352 to 3,564 cpm) and [^{13}N]amide distillate (uncorrected values, 2,480 to 3,578 cpm).

TABLE 4. *Comparison of rates of fixation of [^{13}N]N_2 by filaments and by heterocysts isolated from the same culture*

Culture fraction	Determination[a]			
	Cpm/μCi[b]	Fixation time (min)	Total heterocysts/vial	Cpm/μCi per min per 10^6 heterocysts
Filaments	2.20	0.25	1.09 × 10^6	8.07 (100)[c]
Heterocysts[d]	8.56	2.00	2.27 × 10^6	1.88 (23)

[a] Distillate [^{13}N]NH_3 plus [^{13}N]amide.

[b] See Table 3, footnote *a*.

[c] Numbers in parentheses are percentages.

[d] The reaction mixture was the same as described in footnote *b* of Table 3, but with glutamate omitted.

glutarate occurred in the absence of exogenous amino donors.

DISCUSSION

Nitrogenase activity of heterocysts. Our results with isolated heterocysts provide additional direct evidence that these cells are major sites of nitrogen fixation during aerobic photosynthetic growth of filamentous cyanobacteria. Fixation of N_2 by isolated heterocysts had not heretofore been demonstrated. The isolated heterocysts in our experiments accounted for a higher fraction of intact-filament nitrogenase activity than did the heterocysts isolated from a different organism by Peterson and Burris (16), but were of slightly (ca. 15%) lower specific activity and had a far higher specific activity than that reported (23) for heterocysts isolated

TABLE 5. *Formation of [^{14}C]glutamine by isolated heterocysts in the light*

Assay conditions[a]	Percent of NH_3-supported activity[b]
Standard	23.2 (4)[c] ± 6.9
−ATP	7.5 (3) ± 2.7
$-N_2$ + Ar	20.9 (4) ± 6.9
$+NH_3$	100.0
$+NH_3$ + MSX	10.0 (3) ± 5.6

[a] Reaction mixtures contained, in a volume of 0.2 ml, 5 mM ATP, 15 mM $MgCl_2$, 50 mM creatine phosphate, 0.05 mg of creatine phosphokinase, 15 mM $Na_2S_2O_4$, 60 mM TES, pH 7.2, 20 to 36 μM [^{14}C]glutamate, and 0.7 × 10^6 to 2.2 × 10^6 heterocysts under N_2 ("standard"). Where indicated, ATP was not included, N_2 was replaced with Ar, or 1 mM methionine sulfoximine (MSX) and/or 5 mM NH_4Cl was added.

[b] Rate of NH_3-supported formation of [^{14}C]glutamine by isolated heterocysts: 3.1 ± 0.3 nmol per 10^6 heterocysts per 30 min (four observations).

[c] Numbers in parentheses are number of observations.

TABLE 6. *Effect of added glutamate and methionine sulfoximine on the incorporation of [^{13}N]ammonia into glutamine by heterocysts isolated from A. cylindrica*

Additions to reaction vial[a]	Dpm of [^{13}N]glutamine recovered[b] dpm of [^{13}N]NH_3 added[b]
1. 5 mM ATP, 8 mM $MgCl_2$, 13 mM TES, 50 μM DCPIP, 5 mM sodium ascorbate, 5 mM α-ketoglutarate.	0.032
2. As in 1, plus 1 mM glutamate.	1.21
3. As in 2; heterocysts and medium separated.	0.031[c] 1.16[d]
4. As in 2, plus 1 mM methionine sulfoximine.	<0.023

[a] Reaction vials each contained 2.27 × 10^6 heterocysts, isolated in the presence of sodium ascorbate, and supplements, in a total volume of 0.4 ml. Reactions were initiated by the addition of [^{13}N]ammonia, 6.32 × 10 dpm,[b] and were incubated for 10 min in the presence of 4,000 lx of incandescent light.

[b] Corrected to the time of distillation of [^{13}N]NH_3.

[c, d] In experiment 3, the suspension of heterocysts was sedimented by centrifugation at 1,000 × *g*, and the pellet (*c*), and supernatant fluid (*d*) were separately extracted and processed for electrophoresis.

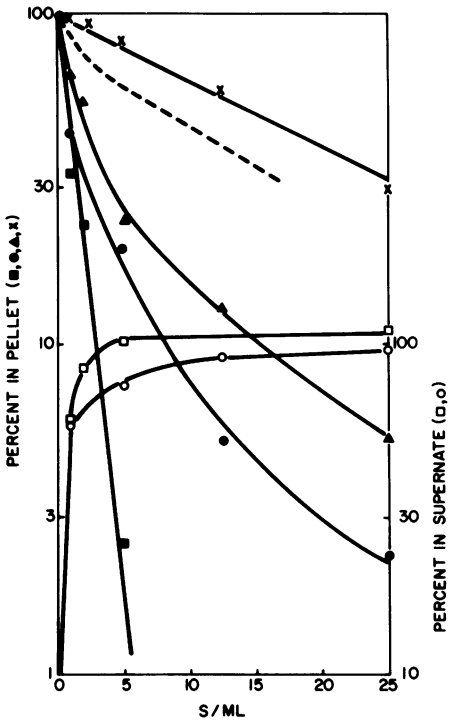

FIG. 4. *Time course of solubilization of glutamine synthetase and glutamate synthase activities during cavitation. Heterocysts apparently intact as percentage of initial number of heterocysts (×); chlorophyll in 5-min, 5,000 × g pellets as percentage of total chlorophyll (▲); activities of glutamine synthetase (●) and glutamate synthase (■) associated with such pellets (left-hand ordinate; see below) as percentage of total activity; and activities of glutamine synthetase (○) and glutamate synthase (□) in the supernatant fluid (right-hand ordinate), assayed as described in Materials and Methods, as percentage of total activity, after cavitation for varying periods of time. Total activities of glutamine synthetase and glutamate synthase were taken to be the activities of the supernatant fluid after cavitation of a cyanobacterial suspension for 60 s/ml and centrifugation for 5 min at 5,000 × g. These activities were 25.4 and 5.2 nmol per mg of protein per min, respectively. Enzymatic activities associated with the pellets were measured by resuspending the pellets, cavitating for an additional period of 60 s/ml, centrifuging as before, and assaying the supernatant fluid. The time course of solubilization of glucose-6-phosphate dehydrogenase (- - -), an activity associated largely with heterocysts, is repeated from an earlier paper (29) for comparison.*

from a different strain of *A. cylindrica*. Hydrogen, included in the gas phase over the suspensions and in the assay vials used by Peterson and Burris (16), may have stimulated acetylene reduction activity (cf. 36). Our procedure, designed to obtain heterocyst preparations that would reduce $[^{13}N]N_2$ as well as acetylene, omitted hydrogen because of its inhibitory effect on N_2 fixation.

Intact heterocysts in our suspensions accounted for up to 40% of the nitrogenase activity of intact filaments. The initial localization of the remaining activity is unknown. The results of autoradiographic experiments with $[^{13}N]N_2$ (32) and of experiments on the time course of solubilization of nitrogenase during cavitation (35) were consistent with, but did not prove, the interpretation that the remaining activity is located in the vegetative cells. However, sonic disruption is known to damage heterocysts (5). By preincubating in TES-dithionite-EDTA-mannitol, we have been able to reduce the time of sonic disruption required to isolate heterocysts to 6 s/ml. This much cavitation may, however, still damage the functioning of the heterocysts. In the absence of a reliable procedure for evaluating the proportion of truly intact, isolated heterocysts, it is difficult to arrive at a firm conclusion about the percentage of nitrogenase located in heterocysts in vivo.

Enzymes involved in assimilation of NH_3 by heterocysts. Dharmawardene et al. (3) showed that the specific activity of glutamine synthetase in isolated heterocysts is 1.7 times greater than in vegetative cells. We have found that after 12.5 or 25 s of sonic disruption per ml, the ratio of sedimentable glutamine synthetase activity per intact heterocyst (no vegetative cells remain intact), corrected to the initial number of heterocysts, accounts for about 8% of the initial activity of glutamine synthetase (Fig. 4). This figure is slightly greater than the initial ratio of heterocysts to total cells. We have thus

TABLE 7. *Aminotransferase activity of heterocysts isolated in the presence of ascorbate*[a]

Supplements[b]	nmol of glutamate formed/10^6 heterocysts per 30 min
5 mM Glu	1.26
5 mM Glu + 5 mM aminooxyacetate	0.01
5 mM Glu + 1 mM azaserine	1.22
5 mM Gln	0.84
5 mM Gln + 5 mM aminooxyacetate	0.00
5 mM Gln + 1 mM azaserine	0.94
5 mM asparate	1.24
5 mM alanine	0.18
None (no exogenous N donor)	0.20

[a] Reaction mixtures contained, in 0.2 ml: 25 mM TES, pH 7.2, 18 to 34 μM α-[^{14}C]ketoglutaric acid, plus supplements as indicated. Incubation was for 30 min at 30°C.

[b] Abbreviations: Glu, glutamate; Gln, glutamine.

confirmed that in contrast to enzymes of the oxidative pentose phosphate cycle (13, 19, 29), there is not a great difference in the specific activity of glutamine synthetase in heterocysts compared with vegetative cells. A number of observations, taken together, show that glutamine synthetase is involved in the assimilation of ammonia by heterocysts. First, radioactive glutamine is formed from [^{13}N]NH_3 and from [^{14}C]glutamate, the synthesis being in both cases largely inhibited by the addition of methionine sulfoximine. We have not attempted to account for the limited formation of [^{14}C]glutamine in the presence of methionine sulfoximine. Second, synthesis of [^{13}N]glutamine is enhanced by addition of glutamate, and synthesis of [^{14}C]glutamine is enhanced by addition of ammonia. Finally, the ^{13}N in the glutamine synthesized was all, or nearly all, in the amide group.

Moreover, using [^{13}N]N_2, we have demonstrated that nitrogenase is indeed coupled metabolically to glutamine synthetase in heterocysts. Addition of 0.1 atm of acetylene, an inhibitor of nitrogen fixation (18), decreased the ^{13}N radioactivity in ammonia plus glutamine by about 99%, indicating that glutamine is formed from N_2-derived ammonia. Because formation of [^{14}C]glutamine from [^{14}C]glutamate by suspensions of heterocysts is little affected by substitution of argon for N_2 in the gas phase (Table 5), an endogenous source of NH_3 appears also to be present in the suspensions. Although isolated heterocysts were observed to metabolize two-thirds of their newly fixed nitrogen to glutamine, this figure may underestimate the in vivo fraction. That is, if the isolation procedure increases the "leakiness" of heterocysts to substances of low molecular weight, N_2-derived [^{13}N]NH_3 may leak out of isolated heterocysts before it can react with glutamine synthetase.

When heterocysts were incubated with ^{13}N-labeled or stable NH_3 and with stable or ^{14}C-labeled glutamate, the suspension was sedimented, and the supernatant fluid and pellet were examined separately for (extractable) radioactivity, it was found that 96 to 97% of the radioactive glutamine formed was in the supernatant fluid. Thus, the glutamine synthesized in the isolated heterocysts is rapidly lost from those cells.

In extracts of N_2-grown filaments (11), but not in suspensions of heterocysts, α-ketoglutarate is aminated to form glutamate in the presence of glutamine, dithionite, and methyl viologen. Massive accumulation of ^{13}N radioactivity in glutamine was observed in suspensions of heterocysts (Table 6) with very little or no appearance of radioactivity in glutamate. α-[^{14}C]ketoglutarate easily enters isolated heterocysts, as shown by its extensive transamination to form glutamate. This reaction is indeed a transamination, because it is greatly inhibited by aminooxyacetate (12, 14). Dithionite and ATP are presumed to enter heterocysts because of the dependence of nitrogenase activity on their presence. However, whether methyl viologen, ascorbate, and DCPIP can penetrate the heterocysts is unknown.

We therefore tested by means of in vitro reactions whether glutamate synthase activity is concentrated in heterocysts. Our results (Fig. 4) appear to show that cavitation renders glutamate synthase activity nonsedimentable much more rapidly than it does glutamine synthetase activity, which is in turn much more rapidly solubilized than are enzymes of the oxidative pentose phosphate pathway (29). Accordingly, we suggest that the specific activity of glutamate synthase, the third enzyme in the initial pathway of assimilation of N_2 in *A. cylindrica* (26, 34), is much greater in vegetative cells than in heterocysts. It therefore appears that glutamine newly synthesized in heterocysts is transported to vegetative cells, where glutamate is synthesized. It further appears that part of the glutamate formed then returns to the heterocysts to be amidated or, alternatively, that α-ketoglutarate and aminated substrates of the aminotransferase of heterocysts react within heterocysts to form glutamate, which is then amidated.

Possible implications for pattern formation. It is known that heterocysts inhibit nearby cells in the same filament from differentiating into heterocysts (30, 31), that the inhibition is mediated by a diffusible inhibitor produced by heterocysts (31), and that methionine sulfoximine, which inhibits synthesis of glutamine from ammonia (22, 34), prevents exogenously supplied ammonia (9) from inhibiting heterocyst formation (22). It is therefore possible that the inhibitor that moves from cell to cell may be glutamine or a derivative of glutamine. If this suggestion were correct, it might be that either glutamine itself or some derivative of it is directly involved, in vegetative cells, in inhibiting differentiation.

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