MSUCL-248
August 1977

The pathways of assimilation of $^{13}NH_4^+$ by the cyanobacterium, *Anabaena cylindrica**

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Running title: Assimilation of NH_4^+ by *Anabaena*

The principal initial product of metabolism of ^{13}N-labeled ammonium by *Anabaena cylindrica* grown with either NH_4^+ or N_2 as nitrogen source is amide-labeled glutamine. The specific activity of glutamine synthetase is approximately half as great in NH_4^+-grown as in N_2-grown filaments. After 1.5 min of exposure to $^{13}NH_4^+$, the ratio of ^{13}N in glutamate to ^{13}N in glutamine reaches a value of approximately 0.1 for N_2- and 0.15 for NH_4^+-grown filaments, whereas after the same period of exposure to $[^{13}N]N_2$, that ratio has reached a value close to unity and is rising rapidly. During pulse-chase experiments, ^{13}N is transferred from the amide group of glutamine into glutamate, and then apparently into the α-amino group of glutamine. Methionine sulfoximine, an inhibitor of glutamine synthetase, inhibits the formation of glutamine. In the presence of the inhibitor, direct formation of glutamate takes place, but accounts for only a few percent of the normal rate of formation of that amino acid; and alanine is formed about as rapidly as glutamate. Azaserine reduces formation of $[^{13}N]$-glutamate approximately 100-fold, with relatively little effect on the formation of $[^{13}N]$glutamine. Aminooxy acetate, an inhibitor of trans-aminase reactions, blocks transfer of ^{13}N to aspartate, citrulline and arginine. We conclude, on the basis of these results and others in the literature, that the glutamine synthetase/glutamate synthase pathway mediates most of the initial metabolism of ammonium in *A. cylindrica*, and that glutamic acid dehydrogenase and alanine dehydrogenase have only a very minor role.

In *Anabaena cylindrica*, the major enzymatic pathway for the assimilation of NH_4^+ derived from N_2 consists of glutamine synthetase and glutamate synthase (1,2). This pathway was previously shown to be the major route of assimilation of N_2-derived ammonium in heterotrophic nitrogen-fixing bacteria (3,4) and of exogenous ammonium by nitrogen-limited chemostat cultures of marine pseudomonads (5) and *Rhizobium* (6). The levels of extractable activities of glutamine synthetase and glutamate synthase decline during growth with high levels of exogenous ammonium, while the level of extractable glutamic acid dehydrogenase activity increases 5,6; cf. 4). The alteration in the activities of extractable enzymes has been taken to indicate that the major route of ammonium assimilation changes from the glutamine synthetase/glutamate synthase pathway to catalysis by glutamic acid dehydrogenase (7). In contrast to the results just cited, enzymological studies of *A. cylindrica* have indicated that the extractable activity of glutamine synthetase remains high in ammonium-grown (8,9) and N_2-grown, ammonium-treated (10) cultures, compared to untreated, N_2-grown cells. These data imply that glutamine continues to be synthesized by glutamine synthetase in NH_4^+-grown as well as N_2-grown cells of *A. cylindrica*.

The specific activities of glutamic acid dehydrogenase (NADPH) which have been reported in *A. cylindrica* (11) and *A. variabilis* (12) are rather low; the level of this enzyme is greater in nitrate-grown than in N_2-grown *Gloeocapsa* strain 6909 (13). Alanine dehydrogenase is relatively active in *A. cylindrica*, although with a low affinity for ammonium, and the specific activity increases in N_2-fixing and nitrogen-starved cultures compared to NH_4^+-grown cells (14). It is not

known whether in the cyanobacteria these dehydrogenases function in an assimilatory or dissimilatory manner. It is also unknown whether glutamate synthase activity (15) is present in ammonium-grown cyanobacteria.

Much, perhaps all, of dinitrogen assimilation by *A. cylindrica* takes place in differentiated cells called heterocysts (16-18), where it is coupled to synthesis of glutamine (18). Glutamine synthetase of N_2-grown filaments is present largely in the vegetative cells, although it is also present in heterocysts and in fact at higher specific activity (10,18); glutamate synthase appears to be present essentially only in vegetative cells (18). Ammonium is maintained at a very low intracellular concentration during N_2 fixation (1). However, the intracellular concentration of NH_4^+ may be much higher when NH_4^+ is present in the medium. Moreover, filaments in our ammonium-grown fermentor cultures lack heterocysts (see (19)) so that assimilation of NH_4^+ by such filaments takes place exclusively in vegetative cells. The pathway(s) of assimilation of exogenous NH_4^+ may therefore differ from the pathway operative during N_2 fixation.

Using cultures of a different strain of *Anabaena cylindrica*, which had been grown on N_2 and then starved for nitrogen and pre-labeled with $^{14}CO_2$, Lawrie et al. (20) studied the kinetics of incorporation of ^{14}C into various amino acids after exposure of the cyanobacterium to 1 mM NH_4Cl. Addition of ammonium led to a rapid, protracted and extensive increase of ^{14}C in glutamine and (to a lesser extent) aspartate, but had much less of an effect on, and in the dark even led to a decrease in, the radioactivity in glutamate. The results were interpreted as indicating that glutamine synthetase catalyzes the most important

initial reaction of ammonium assimilation in this organism. The origin of the nitrogen in aspartate, and the role of glutamate in the assimilation of ammonium, remained obscure.

Thus, enzymological studies and experimentation with isotopes have not established unequivocally which pathways for the assimilation of exogenously supplied NH_4^+ are operative in either NH_4^+-grown or N_2-fixing filaments of cyanobacteria. We have therefore investigated the initial pathway(s) of assimilation of $^{13}NH_4^+$. So as to eliminate the complications resulting from the presence of two kinds of cells, the filaments were normally grown with ammonium, but for comparison, a small number of experiments were also performed using N_2-grown filaments.

Our results show that glutamine synthetase, the specific activity of which is about half as great in NH_4^+-grown as in N_2-grown *A. cylindrica*, catalyzes the first step of assimilation of $^{13}NH_4^+$ by that cyanobacterium grown with either nitrogen source. By demonstrating the formation of limited amounts of glutamate and alanine in the presence of an inhibitor which virtually prevents the formation of glutamine, we show also that both glutamate dehydrogenase and alanine dehydrogenase function in an assimilatory manner, albeit at a low rate. Glutamate synthase in vegetative cells (18), which functions in assimilation of NH_4^+ derived from N_2 (1,2), also plays a major role in the assimilation of exogenously supplied ammonium.

MATERIALS AND METHODS

Preparation of cyanobacteria for labeling

Anabaena cylindrica Lemm. was grown photosynthetically in fermentors as semi-continuous cultures with N_2 (air) as the nitrogen source

as described earlier (17), or as continuous cultures with NH_4^+ as the nitrogen source. For growth on N_2, the basal medium was that of Allen and Arnon (21), diluted 8-fold. The doubling time was approximately 18 h, and the density varied between 0.24 and 0.60 μg chlorophyll $(Chl)\cdot ml^{-1}$. Continuous ammonium-grown cultures, in which the basal medium was supplemented with 2 mM NH_4Cl plus, as buffer, 4 mM sodium N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonate (TES), pH 7.2, were incapable of fixing N_2. The flow rate of the 5.1-liter, continuous culture was 4.75 $ml\cdot min^{-1}$, corresponding to a 12-h exponential doubling time with the culture density maintained at 0.70 μg $Chl\cdot ml^{-1}$. N_2-grown cultures were concentrated by centrifugation at 240 x g for 5 min and resuspended in supernatant fluid (growth-conditioned basal medium) to 27 μg $Chl\cdot ml^{-1}$, 10 to 15 min prior to use, under a gas phase of $Ar/O_2/CO_2$ (80:19:1, v/v/v). Ammonia-grown cultures were similarly concentrated, washed once, and resuspended to 27 μg $Chl\cdot ml^{-1}$ in growth-conditioned or fresh basal medium under the same gas phase. In some experiments, 2 mM O-diazoacetyl-L-serine (azaserine), aminooxy acetate, or L-methionine-DL-sulfoximine was added to the cyanobacterial suspension at the time of resuspension. These substances are inhibitors of, respectively, glutamine amide transferases (22), amino transferases (15,23), and glutamine synthetase (22). Sometimes, at least 5 min after addition of methionine sulfoximine, aminooxy acetate was also added to the same suspension. Azaserine was purchased from Calbiochem (La Jolla, Calif.), and all other biochemicals from Sigma Chemical Co. (St. Louis, Mo.).

Labeling with $[^{13}N]NH_4^+$

^{13}N was generated by irradiation of 18.6 mg ^{13}C with protons (24). $^{13}NH_4^+$ was generated by acid digestion of the target, and vacuum

distillation, as described earlier (18). The resulting solutions contained about 0.3 μmoles NH_4^+ and an average of 6 (and up to 18) mCi ^{13}N, in a volume of 0.5 to 1.5 ml. The amount of ^{13}N was measured with a model ABG-10KG-SB ionization gauge (Jordan Electronics Division, Victoreen Instruments Co., Alhambra, Calif.). Approximately 50 (Fig. 1A) to 200 dpm of a long-lived radioactivity were present together with the ^{13}N. This amount of contaminating radioactivity was too small to affect our observations significantly: no long-lived component was observed in amide nitrogen (Fig. 1B). Virtually all of the ^{13}N in the distillate migrated as NH_4^+ when subjected to electrophoresis at pH 2 (1). The largest labeled impurity, approximately 5×10^{-4} of the ^{13}N, had the electrophoretic mobility of nitrate at pH 9.2 (1).

Assimilation of $^{13}NH_4^+$ by the suspended cyanobacteria took place in light (1) in 1.0-ml reaction vials fitted with stopcocks (1) under $Ar:CO_2$ (99:1, v/v) or air, or in 15-ml conical centrifuge tubes under air. Up to five reactions were run with a preparation of $^{13}NH_4^+$. Reactions were initiated by addition of cyanobacterial suspension (50 to 250 μl) or of $^{13}NH_4^+$ (50 to 250 μl), and were terminated by mixing the suspension with four volumes of methanol. In some experiments, the $^{13}NH_4^+$ was supplemented with stable NH_4Cl prior to addition of cyanobacterial suspension, to give a final concentration of 2 mM NH_4^+. In certain other experiments, the suspensions were supplemented with 5 mM NH_4Cl or 10 mM methionine sulfoximine after 3 s of assimilation of $^{13}NH_4^+$. When 10 mM methionine sulfoximine was present, the suspensions of cyanobacteria were first filtered on glass fiber filters to remove excess inhibitor that would have interfered with thin layer electrophoresis; the cyanobacteria were then washed, and extracted with 2 ml of 80% methanol. The radioactive products in methanolic extracts were separated by electrophoresis, and sometimes also by chromatography in an orthogonal direction, on thin layers of cellulose (1,2). Those products were

identified by coelectrophoresis and cochromatography with stable and ^{14}C-labeled substances, and their content of ^{13}N quantified by radiochromatogram scanning and scintillation spectrometry, as has been described earlier (1,2). In certain experiments, ^{13}N-containing material which coelectrophoresed with aspartate, glutamate and/or glutamine was eluted from the thin layer plate. The radioactivity of the original eluates and of the amide and α-amino nitrogen recovered from the glutamine-containing eluate were determined by scintillation spectrometry (1,2). The amide nitrogen in a measured portion of the eluate from the glutamine region was recovered by steam distillation in the presence of alkali (1,2). In certain experiments, the α-amino nitrogen from a second measured portion of the eluate from that region was recovered by reaction with ninhydrin, release of NH_3 from the ninhydrin by H_2O_2, and vacuum-distillation at pH 10 (1).

Two dimensional scans

In some experiments the thin layer plates were scanned in two dimensions (x and y) by a newly developed scanner (R. G. Markham, S. M. Austin, and M. Stya, to be published) using the following principles. A 200-mm by 2.4-mm slit is placed with its long dimension (x-axis) parallel to the long dimension of the thin layer plate, and is backed by a proportional counter. The slit is repeatedly moved back and forth along the y axis so as to sample decays from the entire plate. When a decay is detected, its y coordinate is determined with a resolution of 2.4 mm by the position of the slit at the time of decay. The x coordinate is determined by position-sensitive proportional-counter techniques. Cascade electrons induced in the counter gas by the decay positrons are collected by two counter wires, one 5 mm, the other 10 mm from the thin layer plate. The position of the event on each wire is

determined by charge division (25) and the x coordinate of the decay position is then fixed with a resolution of 2-3 mm by ray tracing. Because the scanner can accept a large solid angle (0.5-1.0 sr) without substantial degradation of resolution, the overall efficiency is about 0.3%, comparable to that of the usual one-dimensional scanners.

<u>In vitro enzyme assays</u>

Fermentor cultures were harvested at 40,000 x g, washed with 5 mM TES, pH 7.2, and resuspended to 90-125 μg Chl·ml^{-1} in the same buffer. The suspensions were degassed (evacuated and regassed) with argon as described previously (18). All solutions and reagents used were degassed, and the cyanobacterial suspension further processed under anaerobic conditions to prevent oxidation of the sodium dithionite used as reductant in the glutamate synthase assay. The degassed cyanobacterial suspension was supplemented with sodium ascorbate and dithiothreitol to final concentrations of 2.5 and 1.0 mM, respectively. In order to break all vegetative cells and essentially all heterocysts, the cyanobacterial suspensions were cavitated at 12° C under argon with a Model S-125 Sonifier (Heat Systems-Ultrasonic Inc., Plainview, N.Y.) at a setting of 3.0 amp for 60 s·ml^{-1} of suspension (18). The cavitated preparations were clarified by centrifugation at 5,000 x g for 5 min.

Glutamine synthetase activity was determined by the formation of [^{14}C]glutamine from [^{14}C]glutamate (230 mCi·mmol^{-1}, New England Nuclear Corp., Boston, Mass.) as previously described (18). 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 (0.92-1.66 μCi ^{14}C) and 20 mM TES, pH 7.2.

Glutamate synthase activity was determined by the formation of [^{14}C]glutamate from [^{14}C]α-ketoglutarate (255 mCi·mmol^{-1}, New England Nuclear Corp.) and stable glutamine in the presence of aminooxy acetate (15,18). 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 (0.92-1.73 μCi ^{14}C), 5 mM aminooxy acetate, and 28 mM TES, pH 7.2.

RESULTS

<u>Kinetics of labeling</u>

The time-course of appearance of $^{13}NH_4^+$-derived label in metabolic pools was the same, within experimental error, whether the filaments were suspended in fresh or growth-conditioned basal medium, and whether O_2 was present as 20% of the gas phase or in only small quantity, dissolved in the algal suspension and in the $^{13}NH_4^+$ solution used. Representative results are presented in Fig. 2. When NH_4^+-grown filaments were incubated with $^{13}NH_4^+$ for up to 900 s (Fig. 2-4), ^{13}N was incorporated extensively into only five constituents which could be extracted with 80% methanol and resolved by our experimental techniques. These constituents coelectrophoresed and cochromatographed with stable and ^{14}C-labeled glutamine, glutamate, aspartate, citrulline and arginine, and will be so designated in the following discussion. An additional peak, similarly identified as alanine, was observed in the presence of methionine sulfoximine (see below). Under certain experimental conditions

additional, minor peaks appeared which electrophoresed between glutamate and asparagine, and between alanine and arginine, at pH 9.2.

After 15 s, or less, of assimilation of $^{13}NH_4^+$, appreciable ^{13}N was present only in glutamine and, to a much lesser extent, glutamate (Fig. 2). Of the radioactivity in glutamine, 88 ± 4% could be accounted for as amide-^{13}N after 3 s of fixation, and approximately 50% after 120 s of fixation or longer. Only negligible ^{13}N was present in aspartate at 15 s, but aspartate was approximately as radioactive as was glutamate after 120 s of labeling, and was usually more radioactive than glutamate after 900 s. Peaks of ^{13}N corresponding to citrulline and arginine became evident between 120 s and 360 s of fixation. Normally seen as a shoulder on the glutamine peak, the peak of citrulline could be resolved by lengthening the duration of electrophoresis at pH 9.2, by combining electrophoresis in one dimension with chromatography in a second dimension (Fig. 3), or in certain chase experiments (see below).

For filaments grown with either N_2 or NH_4^+, the ratio of ^{13}N in glutamate to ^{13}N in glutamine was approximately 0.04 after 1 s of assimilation of $^{13}NH_4^+$ (Fig. 4). After 15 s of assimilation of $^{13}NH_4^+$, that ratio reached a value of approximately 0.08 in N_2-grown filaments and one of approximately 0.12 in NH_4^+-grown filaments. With NH_4^+-grown cultures, increasing the initial exogenous concentration of NH_4^+ to 2 mM led to no significant increase (1.31 ± 0.51 times) in incorporation of ^{13}N into glutamate relative to glutamine during 15 s of assimilation, although total formation of ^{13}N-labeled organic compounds was reduced about 12-fold. However, a separate peak, tentatively identified as alanine and containing approximately 0.1 as much ^{13}N as did the peak of glutamine, was

observed after 15 s of assimilation of $^{13}NH_4^+$ by NH_4^+-grown filaments in the presence of 2 mM NH_4^+.

Pulse-chase experiments

Pulse-chase experiments were performed as follows. After 3 s of assimilation of $^{13}NH_4^+$, without added carrier, by NH_4^+-grown *A. cylindrica*, the extracellular concentration of NH_4^+ was increased to 5 mM by addition of stable NH_4Cl, and incubation then continued. There was an initial rapid decrease in glutamine amide-^{13}N together with an immediate increase in [^{13}N]glutamate (Fig. 5). The ^{13}N content of the α-amino group of glutamine increased more slowly than did [^{13}N]glutamate during the first 15 s of chasing. After 15 s of chase, there was a decrease in [^{13}N]glutamate together with an increase in the ^{13}N content of the α-amino group of glutamine. The α-amino group of glutamine sometimes appeared to be at least as radioactive as the amide group and as glutamate after 120 s of chasing. Citrulline and arginine became conspicuously labeled after 120 s or (in experiments which are not shown) 360 s of chasing. The total amount of organic ^{13}N extractable with 80% methanol increased monotonically approximately 2.4-fold during the 120-s chase period.

Studies with inhibitors

Azaserine reduced incorporation of $^{13}NH_4^+$ into glutamine about 40%; and reduced incorporation into glutamate 99% after 120 s of assimilation, and less extensively after longer periods of assimilation (Table 1, experiments 1 and 2). Transfer of ^{13}N into aspartate, citrulline, and arginine was greatly reduced, or prevented, by 1 mM aminooxy acetate (Table 1, experiment 3). Incorporation of ^{13}N into glutamine

was decreased at least 99.6% (average: 99.85%), and into glutamate about 98% in the presence of methionine sulfoximine (Table 1, experiment 4). Alanine became clearly visible as an independent peak in the presence of this inhibitor, and the ratio of alanine to glutamate varied from 0.2 to 1.0 after 900 s of assimilation in the presence of methionine sulfoximine. Aminooxy acetate, added in conjunction with methionine sulfoximine, had no major further effect on the synthesis of glutamate or alanine (Table 1, experiment 5).

In certain experiments, methionine sulfoximine (final concentration, 10 mM) was added after filaments had assimilated $^{13}NH_4^+$ for 3 s. Incubation was then continued (Table 2). Assimilation of ^{13}N into glutamine continued, although at a decreasing rate, for more than 30 s, and even after 120 s of "chase" with methionine sulfoximine, glutamine was still more radioactive than glutamate and aspartate combined. By 360 s of "chase", glutamate had much more ^{13}N than had glutamine (averaged over 3 experiments, nearly 5-fold more), whereas in 360-s, unchased controls, glutamate had 0.5 times as much ^{13}N as glutamine.

Enzymatic assays

The mean specific activity of glutamine synthetase from NH_4^+-grown cultures of A. cylindrica, 18.6 ± 1.9 nmol·mg protein^{-1}·min^{-1} (mean ± standard deviation of the mean), is approximately half of that of N_2-grown cultures, 37.5 ± 3.6 nmol·mg protein^{-1}·min^{-1}. However, the mean specific activity of glutamate synthase does not differ significantly between the two growth conditions: 8.6 ± 1.5 and 8.0 ± 1.7 nmol·mg protein^{-1}·min^{-1}, respectively.

DISCUSSION

The principal assimilatory pathway

The first major product of assimilation of $^{13}NH_4^+$ by both N_2- and NH_4^+-grown filaments of Anabaena cylindrica is glutamine (Fig. 2). After 1 to 2 s of assimilation, glutamine accounts for about 96% of the organic ^{13}N extractable with 80% methanol, and the ^{13}N is present predominately in the amide nitrogen (Figs. 2 and 4). Thus, the quantitatively most important first step of assimilation of exogenous ammonium is catalyzed by glutamine synthetase. Glutamine is also the principal initial product when $^{13}NH_4^+$ is assimilated in the presence of 2 mM NH_4Cl, in confirmation of the conclusion drawn by Lawrie et al. (20) for N_2-grown A. cylindrica on the basis of ^{14}C-labeling experiments. A primary role of glutamine synthetase was earlier deduced for N_2-derived ammonium (1,2).

Glutamate, the second major organic product of assimilation of N_2, is also the second major product of assimilation of ammonium. However, the kinetics of appearance of ^{13}N in glutamine and glutamate differ extensively for the two nitrogen sources, $[^{13}N]N_2$ and $^{13}NH_4^+$. During fixation of $[^{13}N]N_2$, the ratio of ^{13}N in glutamate to ^{13}N in glutamine increases beyond a value of unity after 90 s (top curve in Fig. 4, recalculated from reference (1)). In contrast, during assimilation of $^{13}NH_4^+$ by both N_2- and NH_4^+-grown cultures, that ratio remains at a much lower value (Fig. 4).

The simplest interpretation of the difference in kinetics of

labeling is that N_2 and exogenously supplied NH_4^+ are assimilated principally at different sites in the filaments. For example, the pool of glutamine that derives its radioactivity rapidly from $[^{13}N]N_2$ would be in heterocysts plus, possibly, certain vegetative cells, and could be smaller than the pool of glutamate (principally in vegetative cells: 18) that receives its α-amino group by amide transfer from the glutamine, whereas the pool of glutamine (e.g., in all vegetative cells) that becomes labeled from exogenous $^{13}NH_4^+$ could be larger than that pool of glutamate.

According to this interpretation, the delay (ca. 90 s) before glutamate becomes as radioactive as glutamine, starting from $[^{13}N]N_2$ (1), corresponds to the time required for fixation of N_2, amidation of glutamate, and movement of $[^{13}N]$glutamine to those vegetative cells where amide transfer takes place (18). (If all fixation of N_2 takes place in heterocysts, it cannot be principally $^{13}NH_4^+$ that moves from heterocysts to vegetative cells, because in that case, as in the case where cells are exposed to $^{13}NH_4^+$, the concentration of $[^{13}N]$glutamate should not approach that of $[^{13}N]$glutamine, in contrast to what we observe with $[^{13}N]N_2$.) Thus, the characteristic time for movement of N_2-derived ^{13}N into vegetative cells is below 90 s. In earlier, autoradiographic experiments (17), a total of about 300 s elapsed between the initiation of labeling and the completion of drying in preparation for autoradiography. There was therefore sufficient time for 75 to 80 percent of the ^{13}N, the percent detected in vegetative cells, to have moved there from heterocysts. However, as noted in that paper, very rapid movement of ^{13}N between vegetative cells would then have been required, in order to produce the observed distribution of ^{13}N along the filaments.

That glutamate is labeled principally by a reaction in series with, rather than in parallel with, glutamine synthetase, is supported by four types of observations. First, increasing the concentration of exogenous, stable NH_4^+ to 2 mM, a value approaching the K_m ($[NH_4^+] \approx$ 12.5 mM: (11)) of glutamic acid dehydrogenase, and greater than the K_m ($[NH_4^+] \approx .1$ mM: (10)) of glutamine synthetase, has little effect on the relative rates of appearance of ^{13}N in glutamate and glutamine. Second, methionine sulfoximine and azaserine, inhibitors, respectively, of formation of and amide transfer from glutamine, greatly reduce the rate of formation of glutamate (Table 1). Third, if formation of glutamine takes place before the inhibition by methionine sulfoximine becomes fully effective a transfer of ^{13}N from glutamine to glutamate can be observed (Table 2). Fourth, when 3-s pulses of $^{13}NH_4^+$ are chased with 5 mM NH_4Cl, ^{13}N is transferred from the amide group of glutamine into glutamate and then, after a lag period, into the α-amino group of glutamine (Fig. 5).

The specific activity of glutamate synthase extractable from certain heterotrophic bacteria (5,6), but not the activity from _A. cylindrica_, declines immediately when those bacteria are transferred from nitrogen-limited or N_2-based growth to growth on ammonium. In some but not all heterotrophic bacteria, the immediate decline in the activity of glutamine synthetase upon the addition of high levels of ammonium is effected by adenylylation of the enzyme (22). However, there is presently no support for the idea that the activity of glutamine synthetase from _A. cylindrica_ is regulated by adenylylation (10). How growth in the presence of 2 mM exogenous NH_4^+ approximately halves the specific activity of glutamine synthetase in both our (Table 3) and Fogg's (8,9) strains of _A. cylindrica_ is unknown. Because heterocysts contain only between one and two times

as much glutamine synthetase activity per cell as do vegetative cells (10,18), and account for at most eight percent of total cells under N_2-fixing conditions, differentiation of heterocysts cannot alone account for the doubling of the specific activity of extractable glutamine synthetase observed under these conditions. The limited difference in specific activity of glutamine synthetase, and essential constancy of specific activity of glutamate synthase, when A. cylindrica is grown with N_2 or NH_4^+ as nitrogen source (Table 3), supports our conclusion that these enzymes play an important role in assimilation of nitrogen under both conditions of growth.

Other anabolic reactions

Nonetheless, that some flux of ^{13}N into glutamate and alanine is catalyzed by glutamic acid dehydrogenase and alanine dehydrogenase is indicated by the following observations. Incorporation of ^{13}N into these amino acids was much less inhibited by methionine sulfoximine than was incorporation into glutamine. The residual labeling of glutamate and alanine was not eliminated by aminooxy acetate, an inhibitor of transamination reactions. Moreover, a peak tentatively identified as alanine was clearly resolved by electrophoresis at pH 9.2 after assimilation of $^{13}NH_4^+$ for 15 s in the presence, but not the absence, of 2 mM exogenous NH_4^+.

The ^{13}N in aspartate might have been derived directly from ammonium, by transamidation from glutamine, or by transamination from glutamine or glutamate. The observation that formation of [^{13}N]aspartate was inhibited more than 90% by the presence of aminooxy acetate, whereas formation of glutamine and glutamate was relatively little affected (Table 1, experiment 3), implied that formation of aspartate is dependent upon a transamination reaction. The inhibition of labeling of aspartate by methionine sulfoximine

and azaserine is presumably based on the fact that these inhibitors greatly reduce the rate of formation of [^{13}N]glutamate and α-[^{13}N]amino glutamine. We have shown that heterocysts of A. cylindrica have an amino-transferase activity capable of donating nitrogen from the α-amino group of glutamine to α-ketoglutarate, thereby generating glutamate and, presumably, α-ketoglutaramate (18). The ^{13}N in aspartate could conceivably be derived from the α-amino group of glutamine in a transamination reaction with oxaloacetate, generating [^{13}N]α-ketoglutaramate as well as aspartate. [^{13}N]α-ketoglutaramate might migrate near serine during electrophoresis at pH 9.2; we have often observed low radioactivity in this region.

As shown by the results of "chasing" 3-s pulses of $^{13}NH_4^+$ for periods of 2 min and less with methionine sulfoximine, even 10 mM inhibitor failed to arrest amide formation sufficiently rapidly to prevent substantial appearance of ^{13}N in the α-amino group of glutamine. The results of Table 2 therefore do not permit us to identify whether ^{13}N in aspartate is derived from [^{13}N]glutamate or α-[^{13}N]amino glutamine.

The observations that ^{13}N appears more rapidly in citrulline than in arginine (Table 1, experiment 1), and that the syntheses of [^{13}N]citrulline and [^{13}N]arginine are extensively inhibited by methionine sulfoximine, azaserine and aminooxy acetate (Table 1), are consistent with the ideas that arginine is derived from citrulline, and that the formation of citrulline is dependent upon amide- and amino-transferase reactions. In cyanobacteria, citrulline is formed by condensation of carbamyl phosphate with ornithine (26); the inhibition of the formation of citrulline by

aminooxy acetate may therefore be due to inhibition of the formation of ornithine. Although [^{13}N]carbamyl phosphate might be expected thereupon to accumulate, and should survive processing (1), we were unable to detect this product under these conditions.

The pathways of initial metabolism of ammonium by A. cylindrica, as determined by the experiments with ^{13}N described in this paper, are presented in Fig. 6. As noted above, the amide group of glutamine is the major site of entry of ammonium nitrogen into cellular metabolism, with much of the nitrogen then being channeled via glutamate. We have not attempted to identify other metabolites which may have been derived from glutamine by transamidation. Much of the newly synthesized glutamate is apparently amidated to form additional glutamine, with other portions being utilized in various anabolic reactions. Aspartate and arginine, which are labeled early and extensively, can be copolymerized to form multi-L-arginyl-poly(L-aspartic acid), the principal nitrogenous reserve product of cyanobacteria (27).

REFERENCES

1. Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M., and Galonsky, A. (1976) J. Biol. Chem. 251, 5027-5034
2. Thomas, J., Wolk, C. P., Shaffer, P. W., Austin, S. M., and Galonsky, A. (1975) Biochem. Biophys. Res. Commun. 67, 501-507
3. Nagatani, H., Shimizu, M., and Valentine, R. C. (1971) Arch. Mikrobiol. 79, 164-175.
4. Dilworth, M. J. (1974) Ann. Rev. Plant Physiol. 25, 81-114
5. Brown, C. M., Macdonald-Brown, D. S., and Stanley, S. O. (1972) J. mar. biol. Ass. U.K. 52, 793-804
6. Dilworth, M. J., and Brown, C. M. (1976) in Proc. 1st Int. Symp. Nitrogen Fixation, (W. E. Newton and C. J. Nyman, eds.), p. 476, Wash. State Univ. Press, Pullman, Wash.
7. Brown, C. M., Macdonald-Brown, D. S., and Meers, J. L. (1974) in Advances in Microbial Physiology (A. H. Rose and D. W. Tempest, eds.) 11, p. 1, Academic Press, New York
8. Batt, T., and Brown, D. H. (1974) Planta (Berlin) 116, 27-37
9. Dharmawardene, M. W. N., Stewart, W. D. P., and Stanley, S. O. (1972) Planta (Berlin) 108, 133-145
10. Dharmawardene, M. W. N., Haystead, A., and Stewart, W. D. P. (1973) Arch. Mikrobiol. 90, 281-295
11. Haystead, A., Dharmawardene, M. W. N., and Stewart, W. D. P. (1973) Plant Sci. Lett. 1, 439-445
12. Pearce, J., Leach, C. K., and Carr, N. G. (1969) J. gen. Microbiol. 55, 371-378
13. Neilson, A. H., and Doudoroff, M. (1973) Arch. Mikrobiol. 89, 15-22

14. Rowell, P., and Stewart, W. D. P. (1976) Arch. Microbiol. 107, 115-124

15. Lea, P. J., and Miflin, B. J. (1975) Bch. Soc. Trans. 3, 381-384

16. Fay, P., Stewart, W. D. P., Walsby, A. E., and Fogg, G. E. (1968) Nature 220, 810-812

17. Wolk, C. P., Austin, S. M., Bortins, J., and Galonsky, A. (1974) J. Cell Biol. 61, 440-453

18. Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M., and Chien, W.-S. (1977) J. Bacteriol. 129, 1545-1555

19. Fogg, G. E. (1949) Ann. Bot. n.s. 13, 241-259

20. Lawrie, A. C., Codd, G. A., and Stewart, W. D. P. (1976) Arch. Microbiol. 107, 15-24

21. Allen, M. B., and Arnon, D. I. (1955) Plant Physiol. 30, 366-372

22. Prusiner, J., and Stadtman, E. R. (1973) The Enzymes of Glutamine Metabolism, Academic Press, N.Y.

23. Bognstad, R., and Clark, D. G. (1974) Arch. Biochem. Biophys. 161, 638-646

24. Austin, S. M., Galonsky, A., Bortins, J., and Wolk, C. P. (1975) Nucl. Inst. and Meth. 126, 373-379

25. Miller, G. L., Williams, N., Senator, A., and Stensgaard, R. (1971) Nucl. Inst. and Meth. 91, 389-396

26. Wolk, C. P. (1973) Bact. Rev. 37, 32-101

27. Simon, R. D., and Weathers, P. (1976) Biochim. Biophys. Acta 420, 165-176

FOOTNOTES

*We thank Prof. Roger G. Markham of the Cyclotron Laboratory and Dept. of Physics, M.S.U., for his dominant role in development of the two-dimensional scanner, and for making it available for these experiments, and Ms. Marga Stya for her contribution to construction and use of the scanner. This research was supported by the U.S. Energy Research and Development Administration under Contract EY-76-C-02-1338, and by the U.S. National Science Foundation.

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Figure 1. Decay curves. (A) Radioactivity associated with a solution of $^{13}NH_4^+$ formed by the reaction $^{13}C(p, n)^{13}N$, digestion of the product by a modified Kjeldahl procedure (18), and vacuum distillation of the ammonia formed. The radioactivity in 60% of the volume of $^{13}NH_4^+$ distilled was measured with a scintillation counter (average background: 81 cpm). Each count is a 5-min average, from which the asymptotic value of 111 cpm (30 cpm above background) has been subtracted. Zero time on the abscissa corresponds to the time at which digestion of the target was begun, and was approximately 3 min after the target was removed from the proton beam. (B) After assimilation of $^{13}NH_4^+$ for 900 s, filaments were washed by centrifugation, extracted with 80% methanol, and the content of $^{13}NH_4^+$ and ^{13}N-labeled amide nitrogen in the extract determined by distillation and scintillation spectrometry $^{13}NH_4^+/[^{13}N]amide \cong 0.19$). The decay of 21 percent of the amide-derived $^{13}NH_4^+$ was then followed. Each count is a 10-min average, from which the average background, 69 cpm, has been subtracted. Error bars indicate one standard deviation from the mean values shown. The sloping lines correspond to a 10-min half-life.

Figure 2. Time course of the appearance of $^{13}NH_4^+$-derived ^{13}N in metabolites of NH_4^+-grown _A. cylindrica_ suspended in basal medium. The radioactivity in the amino acids was determined by integration of peaks in scans of electrophoretograms of 80% methanolic extracts. After the scans were completed, the radioactivity in the peak corresponding to glutamine was eluted, ~~and the fraction of ^{13}N corresponding to $[^{13}N]$amide~~ in the eluate determined by distillation in the presence of alkali, and scintillation spectrometry. The ordinate expresses the amount of ^{13}N present in a constituent of the extract, as a percentage of the ^{13}N added to the cyanobacterial suspension. All radioactivities were decay-corrected to the same standard time. A logarithmic scale is used on the abscissa to permit clear representation of a large range of times. ^{13}N in: (x) both nitrogens of glutamine; (■) glutamine $[^{13}N]$amide; (•) glutamate plus aspartate, the latter contributing substantially only at 900 s; (□) arginine; (▲) citrulline; and (o) unidentified compounds.

Figure 3. Two-dimensional scan following two-dimensional separation of ^{13}N-containing substances extracted with 80% methanol after 900 s of assimilation of $^{13}NH_4^+$ by filaments of NH_4^+-grown Anabaena cylindrica. After the extract was spotted (at the short white line under serine), together with stable amino acid standards, lipids were removed from the origin by chromatography in methanol-chloroform (1:3, v/v). Water-soluble substances were then separated by electrophoresis at pH 9.2 and at 3000 v for 9 min (positive pole at the right). The 20 x 5 cm thin layer plate was dried, and subjected to chromatography in the orthogonal (short) direction, in phenol-water (3:1, v/v) equilibrated with 3% aqueous NH_4OH, for 17 min. The plate was scanned in two dimensions using the scanner described in Materials and Methods. The rays arising from a given area element were summed. After the scan was completed, the sums were printed in a corresponding two-dimensional array. The standard amino acids were visualized with ninhydrin and their positions in the two-dimensional scans determined by reference to points (•) labeled with radioactivity on a second thin-layer plate. Peaks of radioactivity were localized by drawing isorads (contours connecting points of equal radioactivity) on the printout at 3 (.....), 9 (-.-.-), 24 (——), 63 (——), and 149 (▬▬) disintegrations per area element. The mean background during the 20-min scan was approximately 0.08 counts per area element.

Figure 4. Time course of the ratio of the sum of [^{13}N]glutamate plus [^{13}N]aspartate to [^{13}N]glutamine during the assimilation of $^{13}NH_4^+$ by NH_4^+-grown (▲) and N_2-grown (o) filaments of A. cylindrica, and during the assimilation of [^{13}N]N_2 by N_2-grown filaments (•; recalculated from the data of reference (1)). Error bars indicate ± 1 S.D. of the mean values. [^{13}N]-Aspartate constitutes a large fraction of the aspartate-plus-glutamate peak only in NH_4^+-grown filaments after relatively long periods of assimilation. The radioactivity in the amino acids was determined as in Fig. 2, and ratios then calculated. Typically, 50 to 250 µl of cyanobacterial suspension were incubated with 50 to 250 µl of $^{13}NH_4^+$ in water, for the times indicated.

Figure 5. Time-course of the fractions of extractable ^{13}N in amide (Δ) and α-amino (□) groups of glutamine, and in glutamate plus aspartate (●) and other constituents (○), during a "chase" with 5 mM NH_4Cl after 3 s of assimilation of $^{13}NH_4^+$ by NH_4^+-grown Anabaena cylindrica in the absence of supplemental NH_4Cl. Glutamate plus aspartate, and total glutamine, were determined from scans of radioactivity from ^{13}N in electrophoretograms. The regions corresponding to glutamine were then eluted, and the fraction of ^{13}N in amide nitrogen determined by distillation in the presence of alkali. Non-distillable ^{13}N in the glutamine region was taken to be α-[^{13}N]amino nitrogen.

Figure 6. Diagram of the major and minor routes of initial metabolism of ammonium by A. cylindrica, based on experiments with ^{13}N. The heavy lines represent the major pathway of the initial metabolism of ammonium. Minor and secondary pathways of metabolism of ammonium are represented by light lines, uncertain pathways by dashed lines, and presumed amide transferase reactions with a dotted line. Certain of the enzymes involved are numbered: (1) glutamine synthetase, (2) glutamate synthase, (3) glutamic acid dehydrogenase, and (4) alanine dehydrogenase. The sites of inhibition by methionine sulfoximine (MSX), azaserine (AS), and aminooxy acetate (AOA) are indicated.

Table 1. Principal radioactive constituents observed after assimilation of $^{13}NH_4^+$ by NH_4^+-grown A. cylindrica in the absence of inhibitors, or presence of 1 mM azaserine (AS), aminooxy acetate (AOA), or methionine sulfoximine (MSX), added individually or in combination. Values were determined by integration of peaks of radioactivity from ^{13}N after electrophoresis at pH 9.2, in comparison with radioactivity in $^{13}NH_4^+$ determined by scintillation counting.

Expt.	Inhibitor	Time, s	^{13}N found in compound, as percentage of the ^{13}N added						
			Aspartate	Glutamate	Glutamine	Citrulline	Alanine	Unknowns	Arginine
1	None	120	a	1.67[a]	4.62	0.46[b]	-[c]	0.05	-
	AS	120	-	0.025	4.09	-	-	-	-
2	None	360	a	1.56[a]	3.80	0.94	-	0.23	0.78
	AS	360	-	0.06	1.56	-	-	-	-
3	None	900	2,85	2.33	8.95	1.31	-	0.03	0.06
	AOA	900	0.19	2,22	16.70	-	-	-	0.05
4.	None	900	4.11	1.76	5.29	0.86	-	0.27	1.54
	MSX	900	-	.044[d]	-	-	.009[d]	.009	-
5	MSX	900	-	.042	.008	-	.014	.021	-
	MSX+AOA[e]	900	-	.024	-	-	.040	.015	-

a. Peak principally glutamate, but with aspartate shoulder.

b. Shoulder of glutamine peak, value inaccurate.

c. No (separate) peak observed.

d. Significantly higher radioactivity was found in both glutamate and alanine in two out of eleven experiments.

e. AOA added at least 5 min after addition of MSX.

Table 2. Principal products observed after assimilation of $^{13}NH_4^+$ for 3 s by NH_4^+-grown A. cylindrica, followed by additional incubation in the presence or absence of 10 mM methionine sulfoximine (MSX). The averaged results from three experiments are presented.

Incubation time, s		^{13}N found in compound, as percentage of the ^{13}N added					
-MSX	+MSX	Asp[a]	Glu	Gln	Cit	Arg	Other
3	0		.02	0.19			
3	15		.15	.60[b]			
3	120	.50	.61	1.97[c]			
3	360	.36	1.41	.29	.26	.07	.03
360	0	.92	2.10	4.10	.38	.15	.02

a. Determined, following thin layer electrophoresis at pH 9.2, by visualization of the aspartate and glutamate regions with ninhydrin, elution of the two regions, and scintillation counting of the eluates.

b. 12% α-[^{13}N]amino.

c. 33% α-[^{13}N]amino.

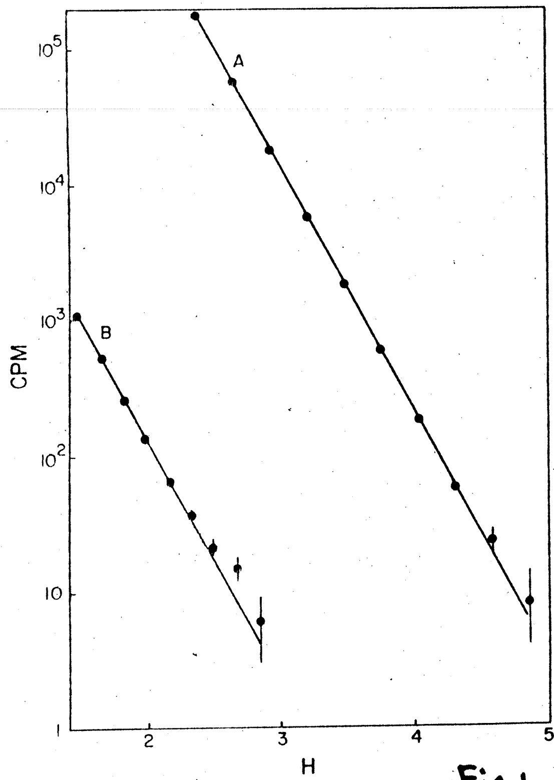

Fig. 1

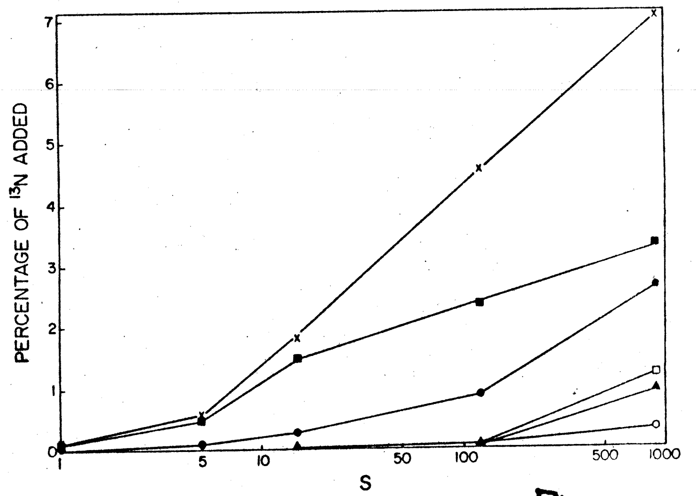

Fig. 2

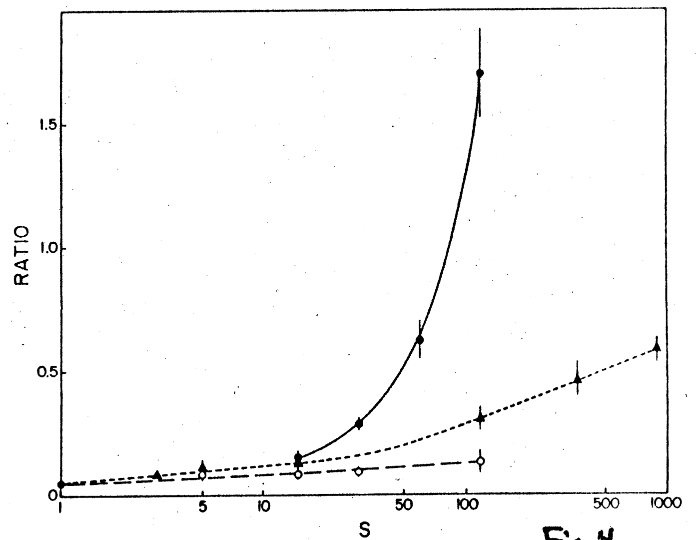

Fig. 4

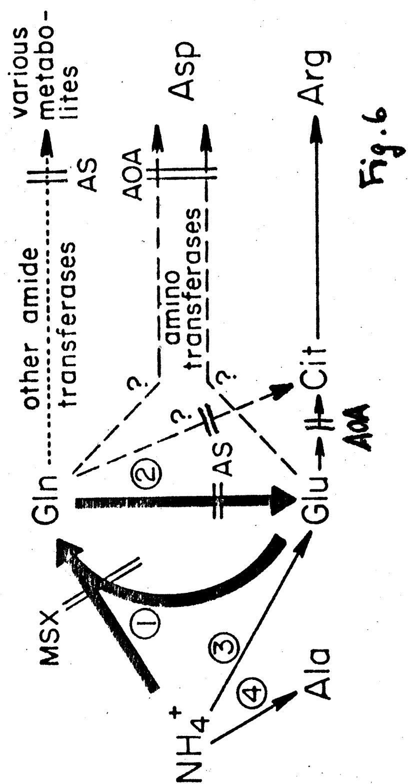

Fig. 6

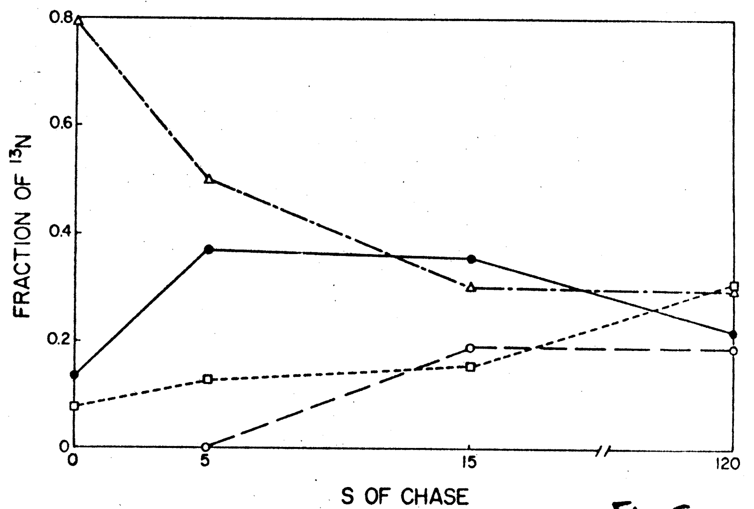

Fig. 5

LYS + ARG

CIT

GLN

ASN