The Pathways of Assimilation of $^{13}$NH$_4^+$ by the Cyanobacterium, Anabaena cylindrica*

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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 $^{15}$NH$_4^+$, the ratio of $^{15}$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 $^{15}$N, that ratio has reached a value close to unity and is rising rapidly. During pulse-chase experiments, $^1$N is transferred from the amide group of glutamine into glutamate, and then apparently into the $\alpha$-amino group of glutamate. 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 per cent of the normal rate of formation of that amino acid: and alanine is formed about as rapidly as glutamate. Aza-serine reduces formation of $^{15}$N-glutamate approximately 100-fold, with relatively little effect on the formation of $^{15}$N-glutamine. Aminooxyacetate, an inhibitor of transaminase reactions, blocks transfer of $^1$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.

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the intracellular concentration of NH\textsubscript{3} may be much higher when NH\textsubscript{4}\textsuperscript{+} is present in the medium. Moreover, filaments in our ammonium-grown fermentor cultures lack heterocysts (see Ref. 19) so that assimilation of NH\textsubscript{4}\textsuperscript{+} by such filaments takes place exclusively in vegetative cells. The pathway(s) of assimilation of exogenous NH\textsubscript{3} may therefore differ from the pathway operative during N\textsubscript{2} fixation.

Using cultures of a different strain of A. cylindrica, which had been grown on N\textsubscript{2} and then starved for nitrogen and prelabeled with \textsuperscript{14}CO\textsubscript{2}, Lawrie et al. (20) studied the kinetics of incorporation of \textsuperscript{14}C into various amino acids after exposure of the cyanobacterium to 1 mm NH\textsubscript{4}Cl. Addition of ammonium led to a rapid, protracted and extensive increase of \textsuperscript{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\textsubscript{4}\textsuperscript{+} are operative in either NH\textsubscript{4}\textsuperscript{+}-grown or N\textsubscript{2}-fixing filaments of cyanobacteria. We have therefore investigated the initial pathway(s) of assimilation of \textsuperscript{15}NH\textsubscript{3} 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\textsubscript{4}\textsuperscript{+} derived from N\textsubscript{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 somocontinu-uous cultures with N\textsubscript{2} (air) as the nitrogen source as described earlier (17), or as continuous cultures with NH\textsubscript{4}\textsuperscript{+} as the nitrogen source. For growth on N\textsubscript{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 0.26 and 0.60 mg of chlorophyll ml\textsuperscript{-1}. Continuous ammonium-grown cultures, in which the basal medium was supplemented with 2 mM NH\textsubscript{4}Cl plus, as buffer, 4 mM sodium N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate, pH 7.2, were incapable of fixing N\textsubscript{2}. The flow rate of the 5.1-liter, 10 to 15 min prior to use, under a gas phase of Ar/O\textsubscript{2}/CO\textsubscript{2} (99:1, v/v/v) or air, or in 15-ml conical centrifuge tubes under 0.19). The decay of 21% of the amide-derived \textsuperscript{15}NH\textsubscript{3} was then followed. 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 approxi-mately 3 min after the light was turned off. B, after assimilation of \textsuperscript{15}NH\textsubscript{3} for 900 s, filaments were washed by centrifugation, extracted with 80% methanol, and the content of \textsuperscript{15}NH\textsubscript{3} and \textsuperscript{15}N-labeled amide nitrogen in the extract determined by distillation and scintillation spectrometry (\textsuperscript{15}NH\textsubscript{3}/\textsuperscript{15}Namide = 0.19). The decay of 21% of the amide-derived \textsuperscript{15}NH\textsubscript{3} was then followed. Each count is a 5-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.

**FIG. 1. Decay curves.** A, radioactivity associated with a solution of \textsuperscript{15}NH\textsubscript{3} formed by the reaction \textsuperscript{14}C(p, n)\textsuperscript{15}N, digestion of the product by a modified Kjeldahl procedure (18), and vacuum distillation of the ammonia formed. The radioactivity in 80% of the volume of \textsuperscript{15}NH\textsubscript{3} distilled was measured, with a liquid scintillation counter (aver-age 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 approxi-mately 3 min after the light was turned off. B, after assimilation of \textsuperscript{15}NH\textsubscript{3} for 900 s, filaments were washed by centrifugation, extracted with 80% methanol, and the content of \textsuperscript{15}NH\textsubscript{3} and \textsuperscript{15}N-labeled amide nitrogen in the extract determined by distillation and scintillation spectrometry (\textsuperscript{15}NH\textsubscript{3}/\textsuperscript{15}Namide = 0.19). The decay of 21% of the amide-derived \textsuperscript{15}NH\textsubscript{3} was then followed. Each count is a 5-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.
Assimilation of \( \text{NH}_4^+ \) by \textit{Anabaena}

Reactions were initiated by addition of cyanobacterial suspension (20 to 250 \( \mu \)l) of \( \text{NH}_4^+ \) (50 to 250 \( \mu \)l) and were terminated by mixing the suspension with 4 volumes of methanol. In some experiments, the \( \text{NH}_4^+ \) was supplemented with stable \( \text{NH}_4\text{Cl} \) prior to addition of cyanobacterial suspension, to give a final concentration of 5 mM \( \text{NH}_4^+ \). In certain other experiments, the suspensions were supplemented with 10 mM \( \text{NH}_4\text{Cl} \) or 10 mM methionine sulfoximine after 3 s of assimilation of \( \text{NH}_4^+ \). 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 co-electrophoresis and co-chromatography with stable and \(^{14}\text{C}\)-labeled substances, and their content of \(^{15}\text{N} \) quantified by radiochromatogram scanning and scintillation spectrometry, as has been described earlier (1, 2). In certain experiments, \(^{15}\text{N}\)-containing material which co-electrophoresed with aspartate, glutamate, or glutamine, or all of these, was eluted from the thin layer plate. The radioactivity of the original eluates and of the amide and \( \alpha \)-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 \( \alpha \)-amino nitrogen derived from a second measured portion of the eluate from that region was recovered by reaction with ninhydrin, release of \( \text{NH}_3 \) from the ninhydrin by \( \text{H}_2\text{O} \), and vacuum distillation at pH 10 (1).

**RESULTS**

**Kinetics of Labeling** — The time course of appearance of \(^{15}\text{N}\)-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 \( \text{O}_2 \) was present as 20% of the gas phase or in only small quantity, dissolved in the algal suspension and in the \( \text{NH}_4^+ \) solution used. Representative results are presented in Fig. 2. When \( \text{NH}_4^+ \)-grown filaments were incubated with \( \text{NH}_4^+ \) for up to 900 s (Figs. 2 to 4), \(^{15}\text{N} \) was incorporated extensively into only five constituents which could be extracted with 80% methanol and resolved by our experimental techniques. These constituents co-electrophoresed and co-chromatographed with stable and \(^{14}\text{C}\)-labeled glutamine, glutamate, aspartate, citrulline, and arginine, and will be so designated in the following discussion. An additional peak, similarly identified as alamine, was observed in the presence of methionine sulfoximine (see below). Under certain experimental conditions additional, minor peaks appeared which coelectrophoresed between glutamate and aspartate, and between alanine and arginine, at pH 9.2.

After 15 s, or less, of assimilation of \(^{15}\text{NH}_4^+ \), appreciable \(^{15}\text{N} \) 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 \(^{15}\text{N} \)amide after 3 s of fixation, and approximately 50% after 120 s of fixation or longer. Only negligible \(^{15}\text{N} \) was present in aspartate at 15 s, glutamate from \(^{15}\text{C} \)glutamate (250 mCi mmol\(^-1, \) New England Nuclear Corp.) and stable glutamine in the presence of aminoxyacetate (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\(_2\)SO\(_4\), 2.5 mM \( \alpha \)-ketoglutarate (0.92 to 1.73 \( \mu \text{Ci} \) of "\( ^{14} \text{C} \)), 5 mM aminoxyacetate, and 28 mM TES, pH 7.2.

**In Vitro Enzyme Assays** — Filament cultures were harvested at 40,000 \( \times \) g, washed with 5 mM TES (pH 7.2), and resuspended to 90 to 120 \( \mu \text{g} \) of chlorophyll \( \text{ml}^{-1} \) 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 diaminoxyacetate in final concentrations of 2.5 and 10 mM, respectively. In order to break all vegetative cells and essentially all heterocysts, the cyanobacterial suspensions were cavitated at 12°C under argon (see below). Under certain experimental conditions additional, minor peaks appeared which coelectrophoresed between glutamate and aspartate, and between alanine and arginine, at pH 9.2.

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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 $^{15}$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 $NH_3^+$, the ratio of $^{15}$N in glutamate to $^{15}$N in glutamine was approximately 0.04 after 1 s of assimilation of $^{15}NH_3^+$ (Fig. 4). After 15 s of assimilation of $^{15}NH_3^+$, that ratio reached a value of approximately 0.08 in $N_2$-grown filaments and one of approximately 0.12 in $NH_3^+$-grown filaments. With $NH_3^+$-grown cultures, increasing the initial exogenous concentration of $NH_3^+$ to 2 mM led to no significant increase ($1.31 \pm 0.51$ times) in incorporation of $^{15}$N into glutamate relative to glutamine during 15 s of assimilation, although total formation of $^{15}$N-labeled organic compounds was reduced about 12-fold. However, a separate peak, tentatively identified as alanine and containing approximately 0.1 as much $^{15}$N as did the peak of glutamate, was observed after 15 s of assimilation of $^{15}NH_3^+$ by $NH_3^+$-grown filaments in the presence of 2 mM $NH_3^+$.

**Pulse-Chase Experiments**—Pulse-chase experiments were performed as follows. After 3 s of assimilation of $^{15}NH_3^+$, without added carrier, by $NH_3^+$-grown *Anabaena cylindrica*, the extracellular concentration of $NH_3^+$ was increased to 5 mM by addition of stable $NH_4Cl$, and incubation then continued. There was an initial rapid decrease in glutamine [$^{15}$N]amide together with an immediate increase in [$^{15}$N]glutamate (Fig. 5). The $^{15}$N content of the a-amino group of glutamine increased more slowly than did [$^{15}$N]glutamate during the first 15 s of chasing. After 15 s of chase, there was a decrease in [$^{15}$N]glutamate together with an increase in the $^{15}$N content of the a-amino group of glutamine. The a-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 $^{15}$N extractable with 80% methanol increased monotonically approximately 2.4-fold during the 120-s chase period.

**Studies with Inhibitors**—Azaserine reduced incorporation of $^{15}NH_4^+$ into glutamine about 40%; and reduced incorporation into glutamate 99% after 120 s of assimilation, and less extensively after longer periods of assimilation (Table I, Experiments 1 and 2). Transfer of $^{15}$N into aspartate, citrulline, and arginine was greatly reduced, or prevented, by 1 mM aminooxyacetate (Table I, Experiment 3). Incorporation of $^{15}$N into glutamate was decreased at least 99.8% (average: 99.85%), and into glutamine about 98% in the presence of methionine sulfoximine (Table I, 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. Aminooxyacetate, added in conjunction with methionine sulfoximine, had no major further effect on the synthesis of glutamate or alanine (Table I, Experiment 5).

In certain experiments, methionine sulfoximine (final concentration, 10 mM) was added after filaments had assimilated \(^{15}\)NH\(_3\) for 3 s. Incubation was then continued (Table II). Assimilation of \(^{15}\)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 \(^{15}\)N than had glutamine (averaged over three experiments, nearly 5-fold more), whereas in 360-s, unchased controls, glutamate had 0.5 times as much \(^{15}\)N as glutamine.

Enzymatic Assays—The mean specific activity of glutamine synthetase from \(^{15}\)N-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 \(^{15}\)N-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.

FIG. 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 \(^{15}\)NH\(_3\) by \(^{15}\)N-grown (A) and \(^{15}\)N-grown (O) filaments of \(A.\) cylindrica, and during the assimilation of \(^{15}\)N\(_2\) by \(^{15}\)N-grown filaments (O, recalculated from the data of Ref. 1). Error bars indicate ±1 S.D. of the mean values. \([^{15}\)N]Aspartate constitutes a large fraction of the aspartate-plus-glutamate peak only in \(^{15}\)N-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 \(\mu L\) of cyanobacterial suspension were incubated with 50 to 250 \(\mu L\) of \(^{15}\)NH\(_3\) in water, for the times indicated.

TABLE I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inhibitor</th>
<th>Time</th>
<th>Aspartate</th>
<th>Glutamate</th>
<th>Glutamine</th>
<th>Citrulline</th>
<th>Alanine</th>
<th>Unknowns</th>
<th>Arginine</th>
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<tr>
<td>1</td>
<td>None</td>
<td>120</td>
<td>-</td>
<td>1.67(^{d})</td>
<td>4.62</td>
<td>0.46(^{c})</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
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<tr>
<td>2</td>
<td>Azaserine</td>
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<td>-</td>
<td>0.25</td>
<td>4.09</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>360</td>
<td>-</td>
<td>1.56(^{d})</td>
<td>5.68</td>
<td>0.94</td>
<td>-</td>
<td>0.23</td>
<td>0.78</td>
</tr>
<tr>
<td>4</td>
<td>Azaserine</td>
<td>360</td>
<td>-</td>
<td>0.06</td>
<td>1.56</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5</td>
<td>None</td>
<td>900</td>
<td>2.85</td>
<td>3.33</td>
<td>8.95</td>
<td>1.31</td>
<td>-</td>
<td>0.03</td>
<td>0.60</td>
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<td>6</td>
<td>Aminooxyacetate</td>
<td>900</td>
<td>0.19</td>
<td>2.22</td>
<td>16.70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>Methionine sulfoximine</td>
<td>900</td>
<td>4.11</td>
<td>1.76</td>
<td>5.29</td>
<td>0.86</td>
<td>-</td>
<td>0.27</td>
<td>1.54</td>
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<tr>
<td>8</td>
<td>Methionine sulfoximine</td>
<td>900</td>
<td>-</td>
<td>0.044(^{d})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>9</td>
<td>Methionine sulfoximine + aminooxyacetate</td>
<td>900</td>
<td>-</td>
<td>0.024</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.040</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\(^a\) 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 2 out of 11 experiments.

DISCUSSION

Principal Assimilatory Pathway—The first major product

FIG. 5. Time course of the fractions of extractable \(^{15}\)N in amide (\(\Delta\)) and \(\alpha\)-amino (O) groups of glutamine, and in glutamate plus aspartate (O) and other constituents (C), during a "chase" with 5 mM NH\(_4\)Cl after 3 s of assimilation of \(^{15}\)NH\(_3\) by \(^{15}\)N-grown \(A.\) cylindrica in the absence of supplemental NH\(_4\)Cl. Glutamate plus aspartate, and total glutamine, were determined from scans of radioactivity from \(^{15}\)N in electrophoretograms. The regions corresponding to glutamine were then eluted, and the fraction of \(^{15}\)N in amide nitrogen determined by distillation in the presence of alkali. Nondistillable \(^{15}\)N in the glutamine region was taken to be \(\alpha\)-\(^{13}\)N amino nitrogen.
in contrast to what we observe with $^{15}$N.] Thus, the characteristic time for movement of $N_2$-derived $^{15}$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% of the $^{15}$N, the per cent detected in vegetative cells, to have moved there from heterocysts. However, as noted in that paper, very rapid movement of $^{15}$N between vegetative cells would then have been required, in order to produce the observed distribution of $^{15}$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$_4^+$] = 12.5 mM (11)) of glutamic acid dehydrogenase, and greater than the $K_m$ ([NH$_4^+$] = 1 mM (10)) of glutamine synthetase, has little effect on the relative rates of appearance of $^{15}$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 I). Third, if formation of glutamine takes place before the inhibition by methionine sulfoximine becomes fully effective, a transfer of $^{15}$N from glutamine to glutamate can be observed (Table II). Fourth, when 3-s pulses of $^{12}$NH$_4^+$ are chased with 5 mM NH$_4$Cl, $^{15}$N is transferred from the amide group of glutamine into glutamate and then, after a lag period, into the $\alpha$-amino group of glutamate (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 adenyllylation 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 adenyllylation (10). How growth in the presence of 2 mM exogenous NH$_4^+$ approximately halves the specific activity of glutamine synthetase in both our and Fogg's (8, 9) strains of A. cylindrica is unknown. Because heterocysts contain only between 1 and 2 times as much glutamine synthetase activity per cell as do vegetative cells (10, 18), and account for at most 8% 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, 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 $^{15}$N into glutamate and alanine is catalyzed by glutamic acid dehydrogenase and alanine dehydrogenase is indicated by the following observations. Incorporation of $^{15}$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 aminooxycetate, an inhibitor of transamination reactions. Moreover, a peak tentatively identified as alanine was clearly resolved by electrophoresis at pH 9.2 after assimilation of $^{15}$NH$_4^+$ for 15 s
in the presence, but not the absence, of 2 mm exogenous NH₄⁺.

The ¹³N in aspartate might have been derived directly from ammonium, by transamination from glutamine, or by transamination from glutamine or glutamate. The observation that formation of [¹³N]aspartate was inhibited more than 90% by the presence of aminooxyacetate, 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 [¹³Nlglutamate and α-[¹³Nlaminoglutamine. We have shown that heterocysts of A. cylindrica have an aminotransferase activity capable of donating nitrogen from the α-amino group of glutamine to α-ketoglutarate, thereby generating glutamate and, presumably, α-ketoglutarate (18). The [¹³N] in aspartate could conceivably be derived from the α-amino group of glutamate in a transamination reaction with oxaloacetate, generating α-[¹³Nlketoglutarate as well as aspartate. α-[¹³NlKetoglutarate 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 [¹⁵NlNH₄⁺ 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 [¹⁵N] in the α-amino group of glutamine. The results of Table II therefore do not permit us to identify whether ¹³N in aspartate is derived from [¹⁵Nlglutamate or α-[¹³Nlaminoglutamine.

The observations that [¹³N] appears more rapidly in citrulline than in arginine (Table I, Experiment 1), and that the syntheses of [¹⁵Nl]citrulline and [¹⁵Nl]arginine are extensively inhibited by methionine sulfoximine, azaserine, and aminooxyacetate (Table I), are consistent with the ideas that arginine is derived from citrulline, and that the formation of citrulline is dependent upon amide- and aminotransferase reactions. In cyanobacteria, citrulline is formed by condensation of carbamyl phosphate with ornithine (26); the inhibition of the formation of citrulline by aminooxyacetate may therefore be due to inhibition of the formation of ornithine. Although [¹³Nlcarbamyl 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 [¹³N] as 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 glutamate by transamination. 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 co-polymerized to form multi-α-arginyl-poly-(α-aspartic acid), the principal nitrogenous reserve product of cyanobacteria (97).

**Fig. 6.** Diagram of the major and minor routes of initial metabolism of ammonium by Anabaena cylindrica, based on experiments with ¹³N. 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, glutamate synthetase; 2, glutamate synthase; 3, glutamic acid dehydrogenase; and 4, alanine dehydrogenase. The sites of inhibition by methionine sulfoximine (MSX), azaserine (AS), and aminooxyacetate (AOA) are indicated.

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The pathways of assimilation of $^{13}$NH$_4^+$ by the cyanobacterium, Anabaena cylindrica.

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