AUTORADIOGRAPHIC LOCALIZATION OF $^{13}$N AFTER FIXATION OF $^{13}$N-LABELED NITROGEN GAS BY A HETEROCYST-FORMING BLUE-GREEN ALGA

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ABSTRACT

$^{13}$N, generated by proton bombardment of $^{13}$C powder, is rapidly and easily converted to $^{13}$N-N$_2$, 0.01 atm pressure, ca. 10 mCi/ml, by automated Dumas combustion. $^{13}$N fixed (as $^{13}$N-N$_2$) by algal filaments was localized by an autoradiographic technique which permits track autoradiography with isotopes having short half-lives. Our findings show directly that a minimum of about 25% of the N$_2$ fixation by intact, aerobically grown filaments of Anabaena cylindrica is carried out by the heterocysts. If all of the N$_2$ fixation takes place in the heterocysts, then the movement of nitrogen along the filaments can be characterized by a constant $\tau < \text{ca. } 5 \text{ s (cell}^{-2}).$

INTRODUCTION

The site of fixation of molecular nitrogen by aerobically-grown heterocyst-forming blue-green algae has been a matter of controversy. The results of a variety of indirect experiments (Fay et al., 1968; Gorkom and Donze, 1971; Pringsheim, 1968; Stewart et al., 1969; Stewart and Lex, 1970; Weare and Benemann, 1973) have been interpreted as indicating that all of the nitrogen fixation takes place in the heterocysts. However, other observations have suggested that heterocysts are not important for nitrogen fixation: Anabaena flos-aquae was observed to fix nitrogen actively despite a paucity of heterocysts (Kucz and LaRue, 1971); suspensions of Anabaena cylindrica with vegetative cells detached from heterocysts developed nitrogenase activity in less time than was required—in a separate experiment—for new heterocysts to form (Ohmori and Hattori, 1971); and at least one genus in which heterocysts have not been found fixes nitrogen under aerobic conditions (Rippka et al., 1971; Wyatt and Silvey, 1969), although most such genera do not (Kenyon et al., 1972; Stanier et al., 1971). Heterocysts have been isolated from filaments which had assimilated $^{15}$N-labeled N$_2$ for 5 min. The concentration of $^{15}$N in the heterocysts was no higher than in intact filaments (Ohmori and Hattori, 1971), but $^{15}$N might have been solubilized from the heterocysts during their isolation. Localization without disruption would be preferable.

Wolk and Wojciuch (1971a) have shown that heterocysts from sonically disrupted filaments of A. cylindrica have the capacity to account for 25% of the acetylene-reducing (and therefore, presumably, nitrogen-fixing; see Dalton and Mortenson, 1972) activity of intact filaments. We have let algal
filaments assimilate nitrogen gas labeled with radioactive nitrogen (\(^{13}\text{N}\): 1.20 MeV maximum \(\beta^+\) energy, \(t_{1/2} = 10\) min), and by track autoradiography have localized the nitrogen fixed. Attainment of track frequencies much greater than background was achieved by batch-processing of \(^{13}\text{N}\) from bombarded targets of \(^{12}\text{C}\), and by the development of appropriate autoradiographic techniques. The results obtained demonstrate directly that relative to vegetative cells, heterocysts in intact filaments have a high activity for reducing \(\text{N}_2\), the physiologically important substrate of nitrogenase.

MATERIALS AND METHODS

*Anabaena cylindrica* Lemm. was grown aerobically in fermentors as described previously (Wolk and Wojciuch, 1971 b). Where noted, the medium was supplemented with 2 mM NH\(_4\)Cl and 4 mM buffer [sodium \(n\)-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonate, pH 7.2 (Sigma Chemical Co., St. Louis, Mo.), or with 5 mM KNO\(_3\), 5 mM NaNO\(_3\) and 2.5 mM Ca(NO\(_3\))\(_2\)]. These supplements had little effect on the growth rate. The nitrogenase activity of cultures grown with and without fixed \(\text{N}_2\) was tested, one of two vials was filled with 1% CO\(_2\)-19.9% Ar, and a second vial with 0.1% CO\(_2\)-19.9% Ar-80% \(\text{N}_2\). When inhibition by H\(_2\) was tested, one of two vials was filled with 1% CO\(_2\)-99% H\(_2\). The competitive inhibition of fixation of \(^{13}\text{N}-\text{N}_2\) by nonradioactive nitrogen was measured by filling one vial with 0.1% CO\(_2\)-99.9% Ar and, a second vial with 0.1% CO\(_2\)-19.9% Ar-80% \(\text{N}_2\). Vials then received 0.25 ml of algal suspension, and were incubated in the light (400 foot candles incandescent illumination) for 2 or 15 min, during which time they were stirred with magnets. The radioactive gas was then evacuated and replaced with 1% CO\(_2\)-99% Ar. In some experiments, the algae were then autoradiographed. Sometimes, the gas phase was replaced with 1% CO\(_2\)-99% \(\text{N}_2\), and incubation was continued in the light for 15 min more before autoradiography. During the illumination, the temperature of the vials stayed close to 23°C.

Because of the low ratio (see below) of disintegrations to cells, disintegrations were recorded as individual tracks, so as to be rendered visible in the presence of a background of randomly distributed silver grains. To record tracks, a relatively thick layer of emulsion is required. Because \(^{13}\text{N}\) has a half-life of only 10 min, the layers of emulsion had to be prepared in advance. Microscope slides (1 inch \(\times\) 3 inches) were coated with 1.5 cc of Ilford G-5 emulsion (Ilford Ltd., Essex, England) which had been melted at 50°C, and the emulsion was then gelled at 6°C. After drying for 8-20 h at 23°C in the presence of anhydrous calcium chloride, the emulsions were hypersensitized by treatment with triethanolamine for 20 min (Barkas, 1963), so as to decrease the grain spacing in positron tracks. Hypersensitization and all subsequent steps were performed in complete darkness. The pellicle of hypersensitized emulsion was dried for 24 h in the presence of anhydrous calcium chloride at 6°C before use. The layer of emulsion was then about 50 \(\mu\)m thick.

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For autoradiography, the algal suspension was diluted 10-fold, and a portion of 10 or 25 µl was injected through a serum stopper onto an emulsion-coated microscope slide. The opaque slide-holder was shaken to spread the algal filaments over the emulsion surface and was then evacuated. Within 20 s, the added liquid had evaporated. Three consequences of evacuation to dryness were: diffusion of nitrogen was stopped; the algal filaments became affixed to the emulsion surface strongly enough that most were not dislodged during development of the emulsion; and sensitivity of the emulsion, which is greatly decreased when the emulsion is moist, was maintained at a high level. The slides of emulsion were developed after an exposure of 2-12 h.

For development, slides were soaked in Bristol developer (Barkas, 1963), ca. 50 ml per slide, for 10 min at 6°C, and then an equal volume of 23°C developer was added. The solution was permitted to warm toward 23°C for 50 min; development was stopped with 0.2% acetic acid (10 min); and fixation was performed with Barkas' (1963) fixer for 2 h at 6°C. Pellicles were then washed for 1 day with distilled water, which was changed once. Developed slides were stored at 4°C, 100% relative humidity, in the dark. Tracks were localized with a microscope, usually with the senior author and an additional observer concurring on each accepted track. Only tracks directed from the alga, having a first silver grain within one cell radius of the edge of a cell, and consisting of no less than six grains, were accepted. With few exceptions, the accepted tracks consisted of many more than six silver grains.

The radioactivity of the cyclotron targets was measured with a Jordan Electronics ionization gauge model ABG-10KG-SB (Jordan Electronics, Div. Victoreen Instrument Co., Alhambra, Calif.). Readings were transformed to radioactivities by the relationship: 1 R/h at 1 m corresponds to 1.7 Ci. The radioactivity of purified 13N-N2 was measured with the same instrument at early time points, and after extensive decay was measured using a Beckman CPM-100 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) set to count 13P. For the latter measurements, the gas was compressed into serum vials which were then filled to 1 atm with Beckman Cocktail D and were counted in scintillation vials containing Cocktail D. The radioactivity of algal suspensions was assayed in Cocktail D with the CPM-100. To determine the radioactivity of algal filaments, portions of algal suspensions were filtered onto 0.45-µm pore size Millipore filters (Millipore Corp., Bedford, Mass.), washed with nutrient solution, and the radioactivity on the filters was assayed with the scintillation counter. In certain experiments, the fraction of 13N extractable from Millipore-filtered algae with 80% methanol was determined by scintillation counting of the extract, with a filter present; of the extracted filtered filaments; and of unextracted, filtered filaments, in each case with 2 ml of 80% methanol present. In yet other experiments, the radioactivity of the emulsion to which algae had been applied, and of filtered algae, was counted with a Nuclear-Chicago planchet counter model 1042/8703 (Nuclear-Chicago Corp., Des Plaines, Ill.)

The pressure, Km, for half-maximal rate of N2 fixation was calculated from the relationship p = \( \frac{p_{\text{max}}}{K_m + p} \). The partial pressure (p) of nitrogen in the fixation vials was set by the amount of carrier nitrogen added to the combustion tube. The maximal rate (p_{\text{max}}) of fixation of nitrogen was determined approximately from the aerobic growth rate of the alga (about one doubling per day) and the amount of algal nitrogen per vial. Finally, the rate (p) of fixation of all nitrogen was determined by measuring the rate of fixation of 13N by the alga with a scintillation counter and clock and by multiplying this rate by the ratio of N2 per vial to 13N-N2 per vial (determined with an ionization meter).

The products of combustion of K14NO3 and K15NO3 were measured with a GD 150 mass spectrometer (Varian/MAT, Bremen, Germany) equipped with an HTE/DE-150 inlet system. Peak heights corresponding to N2 and NO were corrected for differences in electron impact ionization cross sections (Kieffer and Dunn, 1966).

RESULTS

The nuclear reaction 13C(p,n)14N has a high yield of 13N/µA relative to alternative nuclear reactions which generate 15N (Austin, Bortins, Galonsky, and Wolk, unpublished calculations). Only six radioactive byproducts with half-lives greater than 1 s are possible. Formation of these isotopes (3H, 7Be, 10Be, 14C, 15C, and 15O) from 14C is, however, not energetically possible at a bombarding energy of ≤ 15 MeV. Absence of significant quantities of radioactive byproducts in our experiments was confirmed by the observations that the earliest measured half-life of the target was 10 min (Fig. 1, A), and that the 13N-N2 produced decayed to background with a half-life of 10 min (Fig. 1, C: mean half-life 10.03 ± 0.16 min). Radioactivity fixed into the algal suspension (Fig. 1, B) and into algae filtered from suspension also exhibited a 10-min half-life. Thus, observed tracks (other than background) from algal cells resulted from decay of atoms of 15N.

Algal fixation of 15N was sharply reduced by carbon monoxide, by hydrogen, by excess stable N2, and by prior growth of the alga in the presence of fixed nitrogen (Table I).

As little as 2-4% of the 15N generated remained
FIGURE 1  Decay curves after generation of \(^{13}\)N by the reaction \(^{13}\)C(p,n)\(^{13}\)N. (A) Decay curve of the target material after bombardment of 20 mg of 60 atom % \(^{13}\)C with a 0.82 \(\mu\)A beam of protons. (B) A similar target was bombarded with 2 \(\mu\)A of protons, the purified \(^{13}\)N-N\(_2\) compressed into a single vial, N\(_2\) assimilated by an algal suspension (0.25 ml, 26.8 \(\mu\)g chlorophyll/ml), and the radioactivity in 10% of the volume of the suspension measured after removal of \(^{13}\)N-N\(_2\). (C) A 16.6-mg target of 90 atom % \(^{13}\)C was irradiated with a 4 \(\mu\)A current of protons. Of the \(^{13}\)N generated, 6.3% (corrected for decay) was compressed into a vial, and the radioactivity in the vial measured (each count is a 10-min average).

in the combustion tube of the Nitrogen Analyzer, and ca. 13% was detected in the liquid nitrogen trap (percentages corrected for decay time). The \(^{13}\)N-N\(_2\) obtained was compressed into 1-ml vials at a concentration of up to 22 mCi per vial, and normally 3-10 mCi per vial.

Plentiful radioactivity was fixed into algal cells to permit autoradiographic determination of the sites of the \(^{13}\)N atoms incorporated (Fig. 2). The track densities observed were much higher than background. \(^{13}\)N was not released from emulsion by evacuation (Table II), but a maximum of 13% (see Discussion) of the tracks expected were found and traceable to a cell of origin. The resolution of the site of origin of tracks which could be traced to the filament was \(\pm 0.2\) - \(\pm 0.5\) \(\mu\mbox{m}\) (Figs. 3, 4).

The value of \(K_m\)\(_{\text{N}_2}\) obtained for nitrogen fixation was 0.076 \(\pm 0.016\) atm (mean of eight determinations). These values of the \(K_m\) are in agreement with the range of values published for the blue-green alga \textit{Nostoc muscorum} (Burris and Wilson, 1946) and for other organisms (Parejko and Wilson, 1971). The values of \(K_m\) were not corrected for two factors: incorporation of \(^{13}\)N not due to \(^{13}\)N-N\(_2\) (see below), and release of \(^{12}\)N from the filaments after fixation. These factors would have had opposite, small effects on the computation.

Table III shows the frequency distributions of tracks originating \(n\) cells from the nearest conjoined heterocyst and of cells located \(n\) cells from the nearest conjoined heterocyst both (A, B) 3 min after a 2-min exposure to \(^{13}\)N-N\(_2\), and (C) ca. 18 min after that exposure, with an intervening 15-

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TABLE I  Conditions Decreasing Algal Assimilation of \(^{13}\)N

| Condition | Observed\* | Expected for \(^{15}\)N |
|-----------|------------|-----------------------|
| Growth on NH\(_4^+\) | 1.1 | <0.5\§ |
| Growth on NO\(_3^-\) | 7 | 5 ||
| Fixation in the presence of 0.013 atm CO | 21 (19) | 39§ |
| Fixation in the presence of a high partial pressure of H\(_2\) | 13 | low||
| Fixation in the presence of a high partial pressure of N\(_2\) | 11 | 8¶ |

Fixation of \(^{13}\)N by algae grown aerobically with N\(_2\) as nitrogen source, and incubated for 2 min with \(^{13}\)N-N\(_2\), CO\(_2\) and Ar (see Materials and Methods), was compared with fixation by equivalent algae in the presence of CO, excess N\(_2\), or H\(_2\), or with fixation by algae grown with sources of fixed nitrogen. Counts per minute (corrected to a standard time) per milliliter of 14N per milliliter gas phase, and ratios thereof, were computed for Millipore-filtered, washed samples of algae or for portions of algal suspensions.

\* Corresponds to incorporation into alga.

\§ Additional incorporation into supernate. The nitrate-grown alga must excrete much of the \(^{13}\)N it fixes.

\¶ Ratio of acetylene reduction by the same concentrated algal suspensions used for \(^{13}\)N assays.

\|| See text.

\¶ Assumes \(K_m\)\(_{\text{N}_2}\) = 0.076 atm.
min chase with stable N$_2$. In Table III B and C, cells in terminal sequences of vegetative cells, such as at the right-hand end of the filament in Fig. 4, were excluded because these might have become detached from a heterocyst after incubation with $^{13}$N-N$_2$. At the earlier time point, heterocysts (ca. 7% of total cells) had 22.6 ± 1.4% of the total $^{13}$N in filaments. During the 15-min period of “chasing” with 1% CO$_2$-99% N$_2$ in the light, the fraction of the total $^{13}$N found in the heterocysts decreased to 13.3 ± 2.1%. With the same batch of algae 4.5 ± 0.5 min after a 2-min exposure to $^{13}$N, heterocysts had 22.1 ± 1.7% (3 slides, 722 tracks total) of the $^{13}$N. In the determinations of the ratio of tracks originating at heterocysts to total tracks, all cells were included, and track frequencies were corrected for background.

3 min after a 2-min pulse of $^{13}$N-N$_2$, 79 ± 4% of the $^{12}$N was extractable from Millipore-filtered algae with 80% methanol.

Portions from an algal suspension were incubated in 1-ml vials for 2 min under 1% CO$_2$-balance Ar in the presence and absence of $^{13}$N-N$_2$. The gas phases were then evacuated, replaced with 1% CO$_2$-balance Ar, and 10% C$_2$H$_2$ added. Ethylene production in the two vials during 20 min was equal, within the experimental error of the C$_2$H$_2$ reduction technique.

In certain experiments, unlabeled filaments were processed for autoradiography, in parallel with
TABLE II

|                          | Scintillation counter | Percent | Planchet counter | Percent | Relative counts per minute, percent |
|--------------------------|-----------------------|---------|------------------|---------|------------------------------------|
| Filtered alga            | 129,500               | 87.6    | 52,200           | 89.5    | 40.3*                              |
| Total suspension         | 147,800               | 100     |                  |         |                                    |
| Evacuated emulsion       |                       |         | 58,400           | 100     | 39.5                               |

Portions of an algal suspension were processed in four ways. One portion was filtered onto a 0.45-µm pore size Millipore filter, washed, the filter added directly to a scintillation vial containing Cocktail D, and counted. A second portion was filtered and washed like the first, the filter was glued to a planchet, and counted. A third portion was added directly to Cocktail D, a filter was added, and the vial was counted. A fourth portion was applied to emulsion in the dark, and evacuated until dry. The portion of emulsion with a surface layer of algae was excised from its microscope slide, glued to a planchet, and counted. Each sample was counted at least twice, and all counts were corrected to a standard time.

* An independent determination of the relative counting efficiency of planchet counter and scintillation counter using 32P-labeled algae gave a figure of 39.7%.

15N-labeled filaments. In one such experiment, 27,656 cells were examined and 37 countable tracks found. The background density of tracks was 0.58 (±0.04) × 10^-4 tracks/µm^2 at the emulsion surface, or 38.5 tracks expected on the basis of a per-cell projected area of 24 µm^2.

DISCUSSION

Generation and Purity of 15N-N2

An average cell of *A. cylindrica* (mass 1.6 × 10^-11 g [Dunn and Wolk, 1970], 11% N) contains 7.7 × 10^10 atoms of nitrogen. When growing at a rate of one doubling per day (0.8 atm N2), the average cell incorporates 3.7 × 10^7 atoms of nitrogen/min. If 15N-N2 is present at 3.2 × 10^-4 mol gas (with a radioactivity of 1 mCi), if N2 is present at 0.013 atm, and if Km(N2) = 0.076 atm, 15N will be fixed at a rate of 0.29 15N/cell/min. Exposure of algae to 2π sr of emulsion 3 min after a 2-min pulse can, therefore, result in a maximum of about 0.24 tracks/cell per mCi 15N/ml.

In practice, the production by β-rays (from 15N and 32P) of recordable tracks was maximally about 13% of the number expected for 2π geometry. The efficiency of track production was low for several reasons. The origin of some tracks could not be determined with certainty. For example, tracks for which the displacement of the first grain away from a filament exceeded one cell radius, as a consequence of very high β-ray energy or nearly horizontal trajectory, were excluded. Some tracks were obscured by insensitive regions in the emulsion, by locally high background grain density, or—if the tracks lay beneath the alga—by optical distortion. It is clear that tracks were not reduced in number due to volatilization of 15N, because evacuated emulsion retained 100% of the radioactivity (corrected for decay time) applied to it (see Table II). Because of the inefficiency of production of traceable tracks, a maximum of ca. 0.03 tracks could be recorded/cell per mCi 15N/ml. Background levels, maximally 2 × 10^-4 tracks/µm^2, or 5 × 10^-3 tracks/cell, accounted normally for on the order of 10% of the tracks observed. We know that the physical process of autoradiographing algal filaments did not generate tracks, because the number of tracks traceable to unlabeled algae was consistent with the number expected on the basis of the observed density of background tracks.

Before our work, 15N-N2 had been produced in a continuous stream from a gas-swept target. The nuclear reaction 12C(d, n)15N, with a 40-μA deuterium current, produced 15N-N2 continuously at 0.03-0.06 mCi/ml (Nicholas et al., 1961; see also Carangal and Varner, 1959, and Campbell et al., 1967), a concentration which would have resulted in a track level little above background. To increase the concentration of 15N, we generated 15N-N2 by a batch process, and used a more efficient nuclear reaction.

We chose the 14C(p, n)15N reaction because of its high yield, the ready availability of proton beams, and the disadvantages inherent in other
Figure 3  Sequence of photomicrographs depicting positron tracks derived from decay of atoms of $^{13}$N, and illustrating the resolution of their sites of origin. After a 2-min pulse of $^{13}$N-$\text{N}_2$, $^{13}$N in vegetative cells was not found to be concentrated near heterocysts. The line drawing is a tracing of the filament as seen in a, with heterocysts (H) and tracks localized with a microscope. Although all tracks indicated are clearly identified with a microscope, not all are shown equally clearly by the series of optical sections. X 370.
TABLE III
Frequency Distributions of the Origins of Tracks and of the Position of Cells

| Treatment | (A) 2-min pulse + 3-min delay | (B) The same as A | (C) The same 2-min pulse as A, B + 15-min chase with stable $N_2$ + 3.5-min delay† |
|-----------|-------------------------------|------------------|----------------------------------|
|           | Tracks observed | Cells examined | Tracks/Cell‡ | Tracks/Cell | Tracks observed | Cells examined | Tracks/Cell‡ |
| Position of cell* |                      |                  |                |            |                      |                  |                |
| H         | 205                 | 1,668            | 0.118          | 42         | 1,328               | 0.028            |
| n = 1     | 126                 | 2,821            | 0.040          | 62         | 2,265               | 0.024            |
| 2         | 131                 | 2,754            | 0.043          | 46         | 2,104               | 0.018            |
| 3         | 119                 | 2,675            | 0.040          | 33         | 1,924               | 0.013            |
| 4         | 76                  | 2,545            | 0.025          | 20         | 1,686               | 0.008            |
| 5         | 70                  | 2,234            | 0.027          | 28         | 1,354               | 0.017            |
| 6         | 74                  | 1,884            | 0.035          | 16         | 985                 | 0.013            |
| 7         | 45                  | 1,489            | 0.026          | 9          | 590                 | 0.012            |
| 8         | 31                  | 1,142            | 0.023          | 10         | 364                 |                  |
| 9         | 19                  | 842              | 0.018          | 1          | 183                 |                  |
| 10        | 16                  | 610              | 0.022          | 1          | 81                  |                  |
| 11        | 14                  | 475              | 0.025          | 1          | 33                  | 0.014            |
| 12        | 10                  | 360              | 0.023          | 0          | 20                  |                  |
| 13        | 10                  | 286              |               | 0          | 10                  |                  |
| 14        | 3                   | 205              | 0.021          | 0          | 4                   |                  |
| 15        | 3                   | 135              |               | 0          | 0                   |                  |
| 16        | 4                   | 96               |               |            |                      |                  |
| 17        | 0                   | 69               |               |            |                      |                  |
| 18        | 0                   | 55               |               |            |                      |                  |
| 19        | 1                   | 33               |               |            |                      |                  |
| 20        | 0                   | 18               | 0.019          |            |                      |                  |
| 21        | 1                   | 12               |               |            |                      |                  |
| 22        | 1                   | 8                |               |            |                      |                  |
| 23        | 0                   | 7                |               |            |                      |                  |
| 24        | 0                   | 4                |               |            |                      |                  |
| >24       | 0                   | 0                |               |            |                      |                  |
| x         | 19                  | 539              | 0.031          |            |                      |                  |

* H = heterocyst, n = 1 is a vegetative cell adjacent to a heterocyst, n = 2 is a vegetative cell one cell removed from a heterocyst, etc. x = cells in sequences lacking heterocysts.

† Combined data from two slides of same algal suspension.

‡ Corrected for background = 0.0046 ± 0.0003 tracks per cell.

§ Not here tallied: cells and tracks of indeterminate position relative to a heterocyst before autoradiography.

¶ Corrected for background = 0.0037 ± 0.0002 tracks per cell.

nuclear reactions. Thus (a) the $^{12}$C(p, $\gamma$)$^{13}$N reaction (Carangal and Varner, 1959) has a low yield (Austin, Bortins, Galonsky, and Wolk, unpublished calculation). (b) The biological usefulness of the $^{14}$N + p → $^{15}$N reactions (Campbell et al., 1967) is greatly reduced because the resulting $^{14}$N, if present at a partial pressure approaching or greater than the $K_a$ for fixation, competitively inhibits fixation of $^{15}$N-N$_2$. (c) $^{15}$NO would be expected to be a major contaminant present in $^{15}$N-N$_2$ generated by the reaction $^{16}$O(p, $\alpha$)$^{15}$N, at least with certain oxygen-containing target materials, for the following reason: $^{15}$N generated by bombardment of an oxygen-rich target material, Li$_2$CO$_3$, is present largely as oxides, whereas this is not the case for $^{15}$N generated from a carbon target (Süe, 1949). So to estimate the extent to which labeled nitrogen present in combination with oxygen in a target would remain in combination with oxygen as a contaminant in purified N$_2$ derived from the target, we combusted K$^{15}$NO$_3$ with the Nitrogen Analyzer. Ca. 0.015% of the gas produced had the mass of $^{15}$N$^{16}$O (bp, $-152^\circ$C). This percent was of the same order of magnitude.
as the percent of available N₂ fixed by the algae in our experiments. Because a liquid nitrogen trap (-196°C) was present in the nitrogen purification train, all other oxides of nitrogen (mp, > -103°C), and cyanides (mp, > -35°C), were absent or were present at much lower concentrations. No product with mass 31 was detected when K¹⁴NO₃ was combusted.

With any target, measurable amounts of ¹⁵N-labeled contaminants can form after purification of ¹⁵N-N₂. Thus, at 10 mCi/ml, 0.01 atm N₂, there are $3.2 \times 10^{14}$ ¹⁵N¹⁴N and $2.7 \times 10^{17}$ N₂ present/ml, so that at least $3.8 \times 10^5$ ¹⁵N₂/ml would be expected. Since random pairing of nitrogen atoms is not always obtained with the Coleman Nitrogen Analyzer (Desaty et al., 1969), $3.8 \times 10^5$...
represents a lower limit. During the 3 min between purification and the end of fixation, 34% of the $^{13}\text{N}_2$ molecules decompose as a consequence of radioactive decay. Many of the resulting free $^{13}\text{N}$ atoms would be expected to react with the gas and liquid in the reaction vials, thereby forming products (e.g., oxides) not removable by evacuation, and which might be assimilated by the algae. To reduce the percentage of the total "fixation" of $^{13}\text{N}$ attributable to molecular species other than $\text{N}_2$, the algae were concentrated 10- to 20-fold before being exposed to $^{13}\text{N}$, and $\text{O}_2$ was omitted from the experimental vials. Because of the short duration of our experiments (< 20 min), nitrogenase activity would not have been diminished by 0.2 atm $\text{O}_2$ (Wolk, 1970), as was confirmed by a preliminary experiment with $^{13}\text{N}_2$.

In order to ascertain whether fixation of $^{13}\text{N}$ was largely as $^{13}\text{N}_2$, boiled (Campbell et al., 1967; Nicholas et al., 1961; Ruben et al., 1940) and cyanide-treated organisms (Nicholas et al., 1961) and genera lacking nitrogenase (Campbell et al., 1967) were used as controls in previous reports. However, incorporation of a particular contaminant may require active growth or the presence of an enzyme system possibly missing from a different organism.

We have used several different means to test what fraction of the $^{13}\text{N}$ fixed is fixed as $^{13}\text{N}_2$: (a) Growth of the alga in the presence of ammonium ion and nitrate ion. Nitrogenase activity is thereby reduced, more extensively by $\text{NH}_4^+$ than by $\text{NO}_3^-$.

Nitrification activity is also reduced by growth of the alga on $\text{NH}_4^+$, but is enhanced by growth on $\text{NO}_3^-$ (Hattori, 1962). Comparable reduction of $^{13}\text{N}$ assimilation and of nitrogenase activity (Table I) is consistent with essentially all, but not quite all, $^{13}\text{N}$ being incorporated as $\text{N}_2$. In particular, no significant incorporation of $^{13}\text{N}$ as NO (Fewson and Nicholas, 1960)—e.g., after oxidation and reaction with water to form $\text{NO}_3^-$, or by reaction with nitrogenase—seems possible.

(b) Inhibition by carbon monoxide. In long-term experiments, a concentration of 0.003 atm carbon monoxide inhibited nitrogen fixation by $N. \text{muscorum}$ 95-98%, while 0.02 atm did not inhibit the uptake of combined nitrogen (Burris and Wilson, 1946). We found that reduction of $\text{C}_2\text{H}_2$ (0.05 atm, 20 min) was inhibited 95% by 0.013 atm CO. Our observation that fixation of $^{13}\text{N}$ was only slightly less inhibited than was $\text{C}_2\text{H}_2$ reduction by CO in short-term experiments (Table I) was again consistent with essentially all $^{13}\text{N}$ assimilated being fixed as $\text{N}_2$. Hydrogen, an inhibitor of reduction of $\text{N}_2$—but not of $\text{CaH}_2$—also sharply reduced assimilation of $^{13}\text{N}$. The range of inhibition predictable on the basis of published studies (Burris and Wilson, 1946) is broad, and brackets the observed values. (c) Inhibition of $^{13}\text{N}$ uptake by excess stable $\text{N}_2$. If two vials were to contain $\text{N}_2$ at partial pressures $p_1$ (low) and $p_2$ (high), and if a contaminant were fixed in both vials at a rate equal to a fraction $f$ of the rate of fixation of $^{13}\text{N}_2$ in the vial with a low pressure of $\text{N}_2$, the ratio of the rates of $^{13}\text{N}$ fixation in the two vials would be

$$\frac{1 + f}{K_m + p_1 + f}.$$  

It may be calculated from Table I that $f = 3-4\%$. However, because of the uncertainty in the value of $K_m(\text{N}_2)$ and because the apparent value of $f$ would be increased by any slight leakage of stable $\text{N}_2$ into the reaction vials, we consider that the results of this type of experiment were consistent with essentially all $^{13}\text{N}$ being incorporated as $\text{N}_2$. The concurrence of the results of the different methods of estimation indicates that a negligible percentage (1-3%) of the $^{13}\text{N}$ fixed was fixed in a form other than $\text{N}_2$.

Movement of Nitrogen out of Heterocysts and along a Filament

Exposure to high levels of radioactivity might conceivably have impaired the $\text{N}_2$ fixation system differentially in the two types of cells. This possibility was shown not to have affected our experiments, because $\text{CaH}_2$ reduction by an algal suspension was unaltered by prior incubation of the suspension with $^{13}\text{N}_2$. The fact that $^{13}\text{N}$ was present at a much higher concentration in heterocysts than in vegetative cells (Table III A, B) thus demonstrates directly that heterocysts are special sites of nitrogen fixation in intact filaments.

The heterocyst, because it is a nongrowing cell, cannot continually fix nitrogen at a greater rate than its neighbors and retain the nitrogen it fixes. Intercellular transfer of nitrogen via the medium seems highly unlikely because it would entail the risk, under natural conditions, of interception by other organisms. If the $^{13}\text{N}$ were secreted, the consequent extensive dilution would make it very difficult to reassimilate, e.g., 90% (Table II) of the labeled nitrogen in the few minutes before autoradiography. Furthermore, the lipid layer which
surrounds the cell wall and protoplast of the heterocyst, except at the junction(s) with vegetative cells, probably constrains nitrogenous substances released from heterocysts to move directly into neighboring cells, rather than into the medium (Winkenbach et al., 1972).

To analyze the movement of nitrogen along a filament between heterocysts, we make the following simplifying assumptions: (a) $^{15}$N present in small molecules, and at a concentration $C(x, t)$ ($t =$ time, $x =$ distance measured in units of cells), moves with a flux proportional to its concentration gradient. If this were the only assumption, $C$ would obey the diffusion equation $\frac{\partial C}{\partial t} = \frac{1}{2\pi r} \left( \frac{\partial^2 C}{\partial x^2} \right)$, but only approximately, because filaments are not homogeneous. Here, $r$ is the time ($= t/x^2$) during which, if $^{15}$N moves from a point, the mean square distance of movement increases by unity. (b) The small, $^{15}$N-labeled molecules undergo biochemical reactions at a rate $kC$, proportional to $C$. This assumption modifies the equation governing the concentration $C$ to the form $\frac{\partial C}{\partial t} = \frac{1}{2\pi r} \left( \frac{\partial^2 C}{\partial x^2} \right) - kC$. The result of these reactions is that $^{15}$N is immobilized in macromolecules, at a concentration $C'(x, t)$. That is, $\frac{\partial C'}{\partial t} = kC$.

The distribution of $^{15}$N after fixation of a pulse of label may therefore be calculated as the sum, $C + C'$, of the solutions of the last two equations. The ratio diffusible-$^{15}$N/total-$^{15}$N is equal, by definition, to the ratio $\int_{-\infty}^{\infty} C' dx / \int_{-\infty}^{\infty} (C + C') dx$.

\[ \frac{\partial}{\partial t} \int_{-\infty}^{\infty} C' dx = k \int_{-\infty}^{\infty} C dx, \]

\[ \frac{dN/dt}{dt} = kN, \]

where $k'$ is the algal growth constant—is $\approx 8 \times 10^{-4}$ s$^{-1}$. There is evidence that $k'$ is approximately independent of position (Mitchison and Wilcox, 1972). Almost all cellular nitrogen is present in the form of substances of high molecular weight. For that reason, and in analogy with the integrated equations, the half-time for movement of $^{15}$N out of heterocysts at that time was much greater than 1.5 min.

During exponential growth, the rate of increase, $dN/dt$, of total cell nitrogen (N) is equal to $k'N$, where $k'$—the algal growth constant—is $\approx 8 \times 10^{-4}$ s$^{-1}$. The ratio $N/N_p$, at least to a first approximation, where $N_p$ is precursor-pool nitrogen. Thus, $k'N \approx kN_p$. The ratio $N/N_p \approx 400$ (Dharmawardene et al., 1972, and W. D. P. Stewart, personal communication). Because $k$ is therefore $\approx 32 \times 10^{-4}$ s$^{-1}$ during exponential growth, the experimentally determined “immobilization of $^{15}$N” can be approximately accounted for in terms of growth of vegetative cells during the labeling period. Concordantly, much of the $^{15}$N in heterocysts 3 min after the labeling period could be “chased” out by incubation of filaments with stable N$_2$ (Table III B, C). The half-time for movement of $^{15}$N out of heterocysts at that time was much greater than 1.5 min.

Because $N_p$ was fixed at a low partial pressure in our $^{15}$N experiments, in the absence of a chase there may have been little tendency for $^{15}$N to move out of heterocysts. Thus, it is possible that the actual fraction of total nitrogen fixation accounted for by heterocysts was only slightly greater than the 23% of total tracks observed to originate from heterocysts (cf. also Wolk and Wojciuch, 1971 a). If there is more $^{15}$N per cell in vegetative cells close to heterocysts than in those farther away (Table III, A), the differential could be due to movement of nitrogen from heterocysts, or to stimulation by heterocysts of nitrogen fixation in nearby cells (cf. Wolk, 1970; Winkenbach and Wolk, 1973).

The occurrence of nitrogenase activity in vegetative cells would be consistent with the observation that only about half of the nitrogenase assayable in
vitro appears to be associated with heterocysts as determined by sonic rupture of vegetative cells (Wolk and Wojciuch, 1971b), whereas about 78% of the activity of glucose-6-phosphate dehydrogenase in filaments appears to be associated with heterocysts according to a similar type of experiment (Winkenbach and Wolk, 1973).

The sum $C + C'$ (corrected for background) was calculated for values of (i.e., for vegetative cells at) $x = 1, 2, 3, \ldots, 13$, for the 240-s period between the midpoint of the pulse and the initiation of autoradiography, and for various values of $\tau$, using either the measured value of $k$ or the value $k = 0$, and assuming that $^{13}$N appearing in a cell is derived from heterocysts to both sides of that cell and spaced 25 cells apart (Fig. 5). The approximate probability of generating observed track frequencies by background-corrected, expected frequencies was determined as the product of probabilities calculated with the $\chi^2$ distribution where both frequencies were $\geq 5$; and by the Poisson distribution for the infrequent tracks distant from heterocysts, in order to accord those tracks their full biological import, rather than group them so as to use the $\chi^2$ distribution. If $\tau > 5$ s, the relative probability that a calculated distribution could generate the observed track distribution (Table III B) was very low ($<5 \times 10^{-4} = 7.6 \times 10^{-7} / 1.7 \times 10^{-4}$) compared with the probability that the observed distribution of $^{13}$N in vegetative cells was generated by fixation by vegetative cells. The relative probability was approximately 0.25 if $\tau = 3$ s. Thus, if all fixation of $N_2$ is by heterocysts, the value of $\tau$ is less than about 5 s.

Unfortunately, the true value of $\tau$ is unknown. However, $\tau$ can be estimated on the basis of experiments of Gorkom and Donze (1971; and M. Donze, Winkenbach and Wolk, 1973).

Figure 5  Distribution of relative number of tracks per cell as a function of distance (in number, $n$, of cells) from a heterocyst. The mean number expected on the basis that all vegetative cells fix $N_2$ at the same rate, and account for 77.4% of the fixation by filaments, is taken as standard (relative number = 1) (•). Other curves: tracks observed (○; data from Table III, B); and mean number expected, calculated on the basis that all $N_2$ fixation takes place in the heterocysts, and that 77.4% of the amount of nitrogen fixed at $t = 0$ diffuses along filaments for 240 s, with ($k = 8 \times 10^{-4} s^{-1}$) or without ($k = 0$) simultaneous immobilization into macromolecules, according to equations presented in the text. $k = 0 s^{-1}$, $\tau = 6 s$ (△), $k = 8 \times 10^{-4} s^{-1}$ and $\tau = 3 s$ (●), 5 s (▲), 10 s (●), and 60 s (□).
personal communication). These authors found that nitrogen-starved filaments of *A. cylindrica* appeared to lose their biliprotein pigments. Filaments subsequently exposed to N₂ gas under aerobic conditions for 14 h showed gradients of reformed biliproteins, from a normal level adjacent to heterocysts, to none or a trace midway between heterocysts. Heterocysts accounted for 1 cell in 14, so that the gradients had a root mean square length less than 3.3 cells. It may be shown—again by integration of the equations governing C and C'—that

\[ \int_\infty^{-\infty} x^2(C + C')dx / \int_\infty^{-\infty} (C + C')dx = (1 - e^{-\kappa t}) / \kappa t. \]

According to this analysis, the root mean square distance of migration of N should not exceed \((\kappa r)^{-1/2}\) cells. Assuming that \(k < 32 \times 10^{-8}\) s⁻¹ (see above), \(r\) would have had a value >28 s. The discrepancy between this value of \(r\) and the value derived from our experiments can be accounted for in two ways. Heterocysts, and perhaps adjacent vegetative cells, may fix only a disproportionately large fraction of the total N₂ fixed. Alternatively, N₂ fixation may be restricted to the heterocysts, but only if the value of \(k\) or of \(r\) is greater in their experiments than in ours.

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