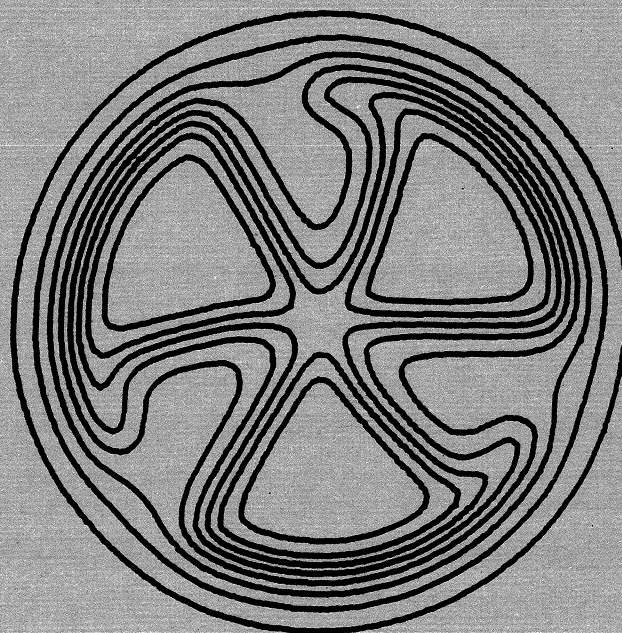


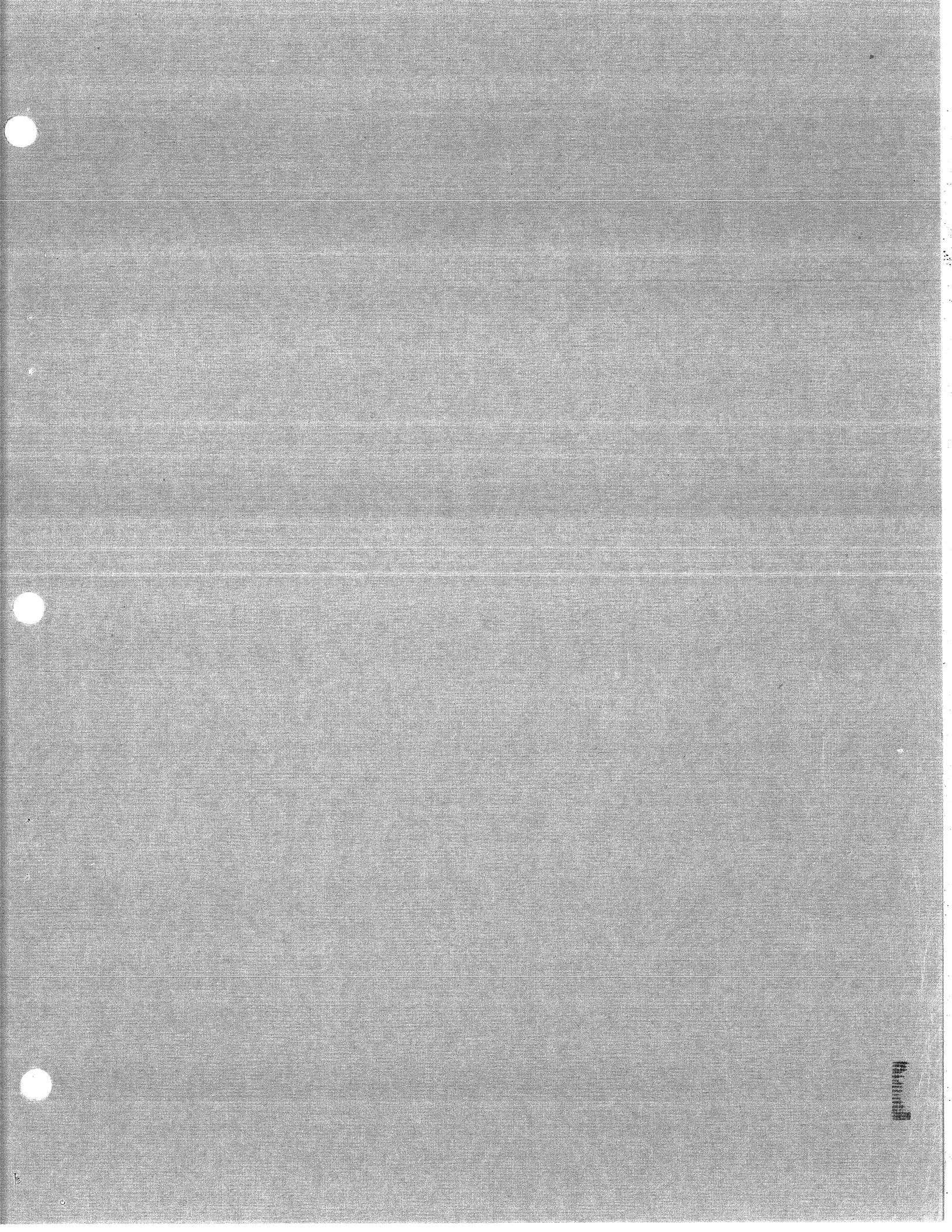
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AUTORADIOGRAPHIC LOCALIZATION OF ^{13}N
FOLLOWING FIXATION OF ^{13}N -LABELED NITROGEN GAS
BY A HETEROCYST-FORMING BLUE-GREEN ALGA

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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; Fringsheim, 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 (Kurz and LaRue, 1971); suspensions of A. 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

Autoradiographic Localization of ^{13}N Following Fixation of ^{13}N -labeled Nitrogen Gas by a Heterocyst-forming Blue-green Alga

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Let algal filaments assimilate nitrogen gas labeled with radioactive nitrogen (^{13}N : 1.20 MeV maximum β^+ 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}N from bombarded targets of ^{13}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 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, 1971b). Where noted, the medium was supplemented with 2 mM NH_4Cl and 4 mM buffer [sodium n-tris(hydroxymethyl)-methyl-2-aminoethane sulfonate (Sigma), pH 7.2], or with 5 mM KNO_3 , 5 mM NaN_3 and 2.5 mM $\text{Ca}(\text{NO}_3)_2$. These supplements had little effect on the growth rate. The nitrogenase activity of cultures grown with and without fixed nitrogen was measured by the acetylene reduction technique (Dalton and Mortenson, 1972; Wolk and Wojciuch, 1971a). The alga was concentrated from 1.3-2.6 to 26 μg chlorophyll per ml growth medium by centrifugation at 1000 x G for 1-2 min, and was kept in the light under an atmosphere of 19.9% O_2 -0.1% CO_2 -80% Ar until used, usually within less than 0.5 h. Despite the absence of nitrogen, the acetylene reducing activity of such suspensions did not increase prior to use.

Nitrogen-13 was generated by proton bombardment of ^{13}C , i.e., by the nuclear reaction $^{13}\text{C}(p,n)^{13}\text{N}$. Beams of 11-MeV protons from the M.S.U. Cyclotron ranged from 0.8 to 4.0 μamp , and bombardments lasted about 30 min. The targets were 16.6 mg (in early experiments, 20 mg) of amorphous ^{13}C (60 and 90 atom % from Prochem, 97 atom % from Monsanto, Mounds Laboratory), arranged so that the beam left the target after losing 6 MeV of energy. The target cell (Austin, Bortline, Galonsky, and Wolk, unpublished) was designed to permit rapid remote transfer of the ^{13}C target to the combustion tube used for further processing, and to permit the proton beam to penetrate through the target for focussing and for measurement of current. In addition, the cell could be positioned in, and removed from, the beam by means of a pneumatic tube. The ^{13}N was recovered from the targets by a slight modification of the technique used by Barsdate and Dugdale (1965) for ^{15}N . The target was thoroughly mixed with fine CuO powder and, together with 0.18 mg KNO_3 to provide carrier nitrogen, was subjected to automated Dumas combustion (800°C; rear oven 580°C) using a Coleman Nitrogen Analyzer, model 29, with all ovens adjusted to setting no. 5. The combustion products were passed through an extra 13 cm of CuO and 20 cm of Cu at 500°C, carrier CO_2 and minor products of the combustion were removed with a liquid nitrogen trap, and a Toeppler mercury pump was used to compress the N_2 into 1-ml evacuated vials. The process from the end of bombardment to exposure of algae took ca. 16 min.

The 1-ml vials--which had received ca. 0.01 atmospheric ml of ^{13}N -labeled N_2 ($^{13}\text{N}-\text{N}_2$)--were filled to 1 atm with a gas mixture (Matheson), usually 1% CO_2 -99% Ar. In tests of the inhibition of ^{13}N -fixation by carbon monoxide, one of a pair of vials received 10 μl of CO. 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 non-radioactive 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% N_2 .

Vials then received 0.25 ml of algal suspension, and were incubated in the light (400 fc 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. In others, the gas phase was replaced with 1% CO_2 -99% 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}N has a half-life of only 10 min, the layers of emulsion had to be prepared in advance. Microscope slides (1" x 3") were coated with 1.5 cc of Ilford G-5 emulsion which had been melted at 50°C, and the emulsion was then gelled at 6°C. After drying for 8-20 hr 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 hr in the presence of anhydrous calcium chloride at 6°C prior to use. The layer of emulsion was then about 50 μm thick.

For autoradiography, the algal suspension was diluted 10-fold, and an aliquot 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 sec, 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 sufficiently strongly 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 hr.

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 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 hr at 6°C. Pellicles were then washed for 1 da 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 AGB-10KG-SB. Readings were transformed to radioactivities by the relationship: $1 \text{ R/hr at } 1 \text{ m}$ corresponds to 1.7 Ci . The radioactivity of purified $^{13}\text{N-N}_2$ was measured with the same instrument at early time points, and after extensive decay was measured using a Beckman CPM-100 liquid scintillation counter set to count ^{32}P . 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, aliquots of algal suspensions were filtered onto $0.45\text{-}\mu\text{m}$ pore size Millipore filters, washed with nutrient solution, and the radioactivity on the filters assayed with the scintillation counter. In certain experiments, the fraction of ^{13}N 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.

The pressure, K_m , for half-maximal rate of N_2 fixation was calculated from the relationship $v = v_{\text{max}} \frac{D}{K + D}$. 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 (v_{max}) of fixation of nitrogen was determined approximately from the aerobic growth rate of the alga (about 1 doubling per day) and the amount of algal nitrogen per vial. Finally, the rate (v) of fixation of all nitrogen was determined by measuring the rate of fixation of ^{13}N by the alga with a scintillation counter and clock and by multiplying this rate by the ratio of N_2 per vial to $^{13}\text{N-N}_2$ per vial (determined with an ionization meter).

The products of combustion of K^{14}NO_3 and K^{15}NO_3 were measured with a GD 150 mass spectrometer (Varian/NMT, Bremen, Germany) equipped with an HTE/DE-150 inlet system. Peak heights corresponding to N_2 and NO were corrected for differences in electron impact ionization cross-sections (Kieffer and Dunn, 1966).

Results

The nuclear reaction $^{13}\text{C}(p,n)^{13}\text{N}$ has a high yield of ^{13}N per amp relative to alternative nuclear reactions which generate ^{13}N (Austin, Bortins, Galonsky, and Wolk, unpublished). Only six radioactive byproducts with half-lives greater than 1 s are possible. Formation of these isotopes (^3H , ^7Be , ^{10}Be , ^{10}C , ^{11}C , and ^{14}O) from ^{13}C or ^{12}C is, however, not energetically possible at a bombarding energy of $\leq 15 \text{ MeV}$. 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. 1A), and that the $^{13}\text{N-N}_2$ produced decayed to background with a half-life of 10 min (Fig. 1C; mean half-life $10.03 \pm 0.16 \text{ min}$). Radioactivity fixed into the algal suspension (Fig. 1B) 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 ^{13}N .

Algal fixation of ^{13}N was sharply reduced by carbon monoxide, by hydrogen, by excess stable N_2 , and by prior growth of the alga in the presence of fixed nitrogen (Table 1).

As little as 2 to 4% of the ^{13}N generated remained in the combustion tube of the Nitrogen Analyzer, and ca. 13% was detected in the liquid nitrogen trap (percentages corrected for decay time). The $^{13}\text{N-N}_2$ obtained was compressed into 1-ml vials at a concentration of up to 22 mc per vial, and normally 3 to 10 mc 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 2), 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 ± 0.2 to $\pm 0.5 \mu\text{m}$ (Figs. 3, 4).

The value of $K_m(\text{N}_2)$ obtained for nitrogen fixation was 0.076 ± 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 *Nostoc muscorum* (Burriss 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 $^{13}\text{N-N}_2$ (see below), and release of ^{13}N from the filaments following fixation. These factors would have had opposite, small effects on the computation.

Table 3 shows the frequency distributions of tracks originating in cells from the nearest conjoined heterocyst and of cells located in cells from the nearest conjoined heterocyst both (A, B) 3 min after a 2-min exposure to $^{13}\text{N-N}_2$, and (C) ca. 18 min after that exposure, with an intervening 15-min chase with stable N_2 . In Table 3, 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 $^{13}\text{N-N}_2$. At the earlier time point, heterocysts (ca. 7% of total cells) had $22.6 \pm 1.4\%$ 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 $13.3 \pm 2.1\%$. With the same batch of algae 4.5 ± 0.5 min after a 2-min exposure to ^{13}N , heterocysts had $22.1 \pm 1.7\%$ (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.

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

Aliquots 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 $^{13}\text{N-N}_2$. The gas phases were then evacuated, replaced with 1% CO_2 -balance Ar, and 10% C_2H_2 added. Ethylene production in the two vials during 20 min was equal, within the experimental error of the C_2H_2 reduction technique.

In certain experiments, unlabeled filaments were processed for autoradiography, in parallel with ^{13}N -labeled filaments. In one such experiment, 27656 cells were examined and 37 countable tracks found. The background density of tracks was $0.58 (\pm 0.04) \times 10^{-4}$ tracks/ μm^2 at the emulsion surface, or 38.5 tracks expected on the basis of a per-cell projected area of $24 \mu\text{m}^2$.

Discussion

Generation and Purity of $^{13}\text{N}-\text{N}_2$

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 N_2), the average cell incorporates 3.7×10^7 atoms of nitrogen/min. If $^{13}\text{N}-\text{N}_2$ is present at 3.2×10^{10} /ml gas (with a radioactivity of 1 mc), if N_2 is present at 0.013 atm, and if $K(\text{N}_2) = 0.076$ atm, ^{13}N will be fixed at a rate of $0.29 \text{ }^{13}\text{N}/\text{cell}/\text{min}$. Exposure of algae to 2 π steradians of emulsion 3 min after a 2-min pulse can therefore result in a maximum of about 0.24 tracks/cell per mc $^{13}\text{N}/\text{ml}$.

In practice, the production by β -rays (from ^{13}N and ^{32}P) of recordable tracks was maximally about 13% of the number expected for 2 π 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 ^{13}N , because evacuated emulsion retained 100% of the radioactivity (corrected for decay time) applied to it (see Table 2). Because of the inefficiency of production of traceable tracks, a maximum of ca. 0.03 tracks could be recorded/cell per mc $^{13}\text{N}/\text{ml}$. 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.

Prior to our work, $^{13}\text{N}-\text{N}_2$ had been produced in a continuous stream from a gas-swept target. The nuclear reaction $^{12}\text{C}(d,n)^{13}\text{N}$, with a 40-kamp deuteron current, produced $^{13}\text{N}-\text{N}_2$ continuously at 0.03-0.06 mc/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 ^{13}N , we generated $^{13}\text{N}-\text{N}_2$ by a batch process, and used a more efficient nuclear reaction.

We chose the $^{13}\text{C}(p,n)^{13}\text{N}$ reaction because of its high yield, the ready availability of proton beams, and the disadvantages inherent in other nuclear reactions. Thus (1) the $^{12}\text{C}(p,\gamma)^{13}\text{N}$ reaction (Carangal and Varner, 1959), has a low yield (Austin, Bortins, Galonsky and Wolk, unpublished). (11) The biological usefulness of the $^{14}\text{N} + p \rightarrow ^{13}\text{N}$ reactions (Campbell et al., 1967) is greatly reduced because the resulting $^{14}\text{N}_2$, if present at a partial pressure approaching

or greater than the K_m for fixation, competitively inhibits fixation of $^{13}\text{N-N}_2$. (iii) ^{13}NO would be expected to be a major contaminant present in $^{13}\text{N-N}_2$ generated by the reaction $^{16}\text{O(p,}\alpha)^{13}\text{N}$, at least with certain oxygen-containing target materials, for the following reason: ^{13}N generated by bombardment of an oxygen-rich target material, Li_2CO_3 , is present largely as oxides, whereas this is not the case for ^{13}N generated from a carbon target (Süe, 1949). So as 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 $^{15,16}\text{N}_2\text{O}$ (b.p. -152°C). This per cent was of the same order of magnitude as the per cent of available N_2 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 (m.p. $> -103^\circ\text{C}$), and cyanides (m.p. $> -35^\circ\text{C}$), were absent or were present at much lower concentrations. No product with mass 31 was detected when K^{14}NO_3 was combusted.

With any target, measureable amounts of ^{13}N -labeled contaminants can form after purification of $^{13}\text{N-N}_2$. Thus, at 10 mc/ml, 0.01 atm N_2 , there are 3.2×10^{11} $^{13,14}\text{N}$ and 2.7×10^{17} N_2 present/ml, so that at least 3.8×10^5 $^{13}\text{N}_2$ /ml would be expected. Since random pairing of nitrogen atoms is not always obtained with the Coleman Nitrogen Analyzer (Desaty et al., 1969), 3.8×10^5 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}N atoms would be expected to react with

the gas and liquid in the reaction vials, thereby forming products which could not be removed by evacuation, and which might be assimilated by the algae. To reduce the percentage of the total "fixation" of ^{13}N attributable to molecular species other than N_2 , the algae were concentrated 10- to 20-fold before being exposed to ^{13}N , and 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 O_2 (Volk, 1970), as was confirmed by a preliminary experiment with $^{13}\text{N-N}_2$.

In order to ascertain whether fixation of ^{13}N was largely as $^{13}\text{N-N}_2$, boiled (Campbell et al., 1967; Nicholas et al., 1961) and 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}N fixed is fixed as $^{13}\text{N-N}_2$: (1) Growth of the alga in the presence of ammonium ion and nitrate ion. Nitrogenase activity is thereby reduced, more extensively by NH_4^+ than by NO_3^- . Nitrate reductase activity is also reduced by growth of the alga on NH_4^+ , but is enhanced by growth on NO_3^- (Hattori, 1962). Comparable reduction of ^{13}N assimilation and of nitrogenase activity (Table 1) is consistent with essentially all, but not quite all, ^{13}N being incorporated as N_2 . In particular, no significant incorporation of ^{13}N as NO (Fevson and Nicholas, 1960)—e.g., following oxidation and reaction with water to form NO_3^- , or by reaction with

nitrogenase--seems possible. (ii) Inhibition by carbon monoxide. In long-term experiments, a concentration of 0.003 atm carbon monoxide inhibited nitrogen fixation by Nostoc muscorum 95-98%, while 0.02 atm did not inhibit the uptake of combined nitrogen (Burris and Wilson, 1946). We found that reduction of C_2H_2 (0.05 atm, 20 min) was inhibited 95% by 0.013 atm CO. Our observation that fixation of ^{15}N was only slightly less inhibited than was C_2H_2 reduction by CO in short-term experiments (Table 1) was again consistent with essentially all ^{15}N assimilated being fixed as N_2 . Hydrogen, an inhibitor of reduction of N_2 --but not of C_2H_2 --also sharply reduced assimilation of ^{15}N . The range of inhibition predictable on the basis of published studies (Burris and Wilson, 1946) is broad, and brackets the observed values. (iii) Inhibition of ^{15}N uptake by excess stable N_2 . If two vials were to contain 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 ^{15}N -- N_2 in the vial with a low pressure of N_2 , the ratio of the rates of ^{15}N fixation in the two vials would be $\frac{1+f}{\frac{K_m + P_1}{K_m + P_2} + f}$. It may be calculated from Table 1 that $f = 3-4\%$. However, because of the uncertainty in the value of $K_m(N_2)$ and because the apparent value of f would be increased by any slight leakage of stable N_2 into the reaction vials, we consider that the results of this type of experiment were consistent with essentially all ^{15}N being incorporated as N_2 . The concurrence of the results of the different methods of estimation indicates that a negligible percentage (1-3%) of the ^{15}N fixed was fixed in a form other than N_2 .

Movement of Nitrogen out of Heterocysts and along a Filament

Exposure to high levels of radioactivity might conceivably have impaired the N_2 -fixation system differentially in the two types of cells. This possibility was shown not to have affected our experiments, because C_2H_2 reduction by an algal suspension was unaltered by prior incubation of the suspension with ^{15}N -- N_2 . The fact that ^{15}N was present at a much higher concentration in heterocysts than in vegetative cells (Table 3A, B) thus demonstrates directly that heterocysts are special sites of nitrogen fixation in intact filaments.

The heterocyst, because it is a non-growing 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 ^{15}N were secreted, the consequent extensive dilution would make it very difficult to reassimilate ca. 90% (Table 2) of the labeled nitrogen in the few minutes prior to 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 (Hinkenbach et al., 1972).

To analyze the movement of nitrogen along a filament between heterocysts, we make the following simplifying assumptions:

(1) ^{13}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 $\frac{\partial C}{\partial t} = \frac{1}{2\tau} \frac{\partial^2 C}{\partial x^2}$, but only approximately, because filaments are not homogeneous. Here, τ is the time ($= t/x^2$) during which, if ^{13}N moves from a point, the mean square distance of movement increases by unity. (ii) The small, ^{13}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 $\frac{\partial C}{\partial t} = \frac{1}{2\tau} \frac{\partial^2 C}{\partial x^2} - kC$. The result of these reactions is that ^{13}N is immobilized in macromolecules, at a concentration $C'(x,t)$. That is, $\frac{\partial C'}{\partial t} = kC$.

The distribution of ^{13}N following fixation of a pulse of label may therefore be calculated as the sum, $C + C'$, of the solutions of the last two equations.¹ The ratio diffusible- ^{13}N /total- ^{13}N is equal, by

¹If at $t = 0$, all ^{13}N were concentrated at $x = 0$, these equations would

have the solutions $C(x,t) = \frac{x}{2\tau} e^{-\frac{x^2}{2\tau t}} e^{-kt}$ and

$$C'(x,t) = \sqrt{\frac{k\tau}{2}} \left(e^{-|x|\sqrt{2k\tau}} [0.5 + \phi(\sqrt{2k\tau} - |x|\sqrt{\tau/t})] - e^{-|x|\sqrt{2k\tau}} [0.5 - \phi(\sqrt{2k\tau} + |x|\sqrt{\tau/t})] \right)$$

where $\phi(u) = \frac{1}{\sqrt{\pi}} \int_0^u e^{-v^2/2} dv$. Modification of the equations governing

C and C' so as to take into account the radioactive decay of ^{13}N leads to no change in the relative spatial distribution of ^{13}N predicted by these equations.

definition, to the ratio $\int_{-\infty}^{\infty} C dx / \int_{-\infty}^{\infty} (C + C') dx$, and it is easily shown—by integration from $x = -\infty$ to $x = \infty$ of the equations governing C and C' —that the latter ratio is equal to e^{-kt} . These equalities permit experimental determination of the value of k . The fraction of ^{13}N present in easily diffusible compounds was estimated as the fraction of ^{13}N extractable from Millipore-filtered algae with 80% methanol. We thereby determined that, averaged over a 2-min pulse and a 3-min delay, $k = 8 \times 10^{-4} \text{ s}^{-1}$.

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 $8 \times 10^{-6} \text{ s}^{-1}$. 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 equation

$$\frac{\partial}{\partial t} \int_{-\infty}^{\infty} C' dx = k \int_{-\infty}^{\infty} C dx, \quad dN/dt \text{ is also } = kN_p, \text{ at least to a first approxi-}$$

mation, where N_p is precursor-pool nitrogen. Thus, $k'N \approx kN_p$. The ratio $N/N_p = 400$ (Dharmawardene *et al.*, 1972, and W. D. P. Stewart, personal communication). Because k is therefore $\approx 32 \times 10^{-4} \text{ s}^{-1}$ during exponential growth, the experimentally determined "immobilization of ^{13}N " can be approximately accounted for in terms of growth of vegetative cells during the labeling period. Concordantly, much of the ^{13}N in heterocysts 3 min after the labeling period could be "chased" out by incubation of filaments with stable N_2 (Table 3B, C). The half-time for movement of ^{13}N out of heterocysts at that time was much greater than 1.5 min.

Because N_2 was fixed at a low partial pressure in our ^{13}N experiments, in the absence of a chase there may have been little tendency for ^{13}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 Wojdlich, 1971a). If there is more ^{13}N per cell in vegetative cells close to heterocysts than in those farther away (Table 3A), 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; Winkembach 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 Wojdlich, 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 (Winkembach and Wolk, 1973).

The sum $C + C'$ (corrected for background) was calculated for values of k (i.e., for vegetative cells at) $x = 1, 2, 3, \dots, 13$, for the 240 s period between the midpoint of the pulse and the initiation of autoradiography, and for various values of r , 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 χ^2 distribution where both frequencies

≥ 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 χ^2 distribution. If $r \geq 5s$ the relative probability that a calculated distribution could generate the observed track distribution (Table 3B) was very low ($< 5 \times 10^{-4}$ to 7.6×10^{-7} to 1.7×10^{-3}) 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 $r = 3s$.

Unfortunately, the true value of r is unknown. However, r can be estimated on the basis of experiments of Gorkom and Donze (1971; and M. Donze, personal communication). These authors found that nitrogen-starved filaments of *A. cylindrica* appeared to lose their biliprotein pigments. Filaments subsequently exposed to N_2 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

$$\int_{-\infty}^{\infty} x^2 (C + C') dx / \int_{-\infty}^{\infty} (C + C') dx = (1 - e^{-kr})/kr.$$

According to this

analysis, the root mean square distance of migration of N should not exceed $(kr)^{-1/2}$ cells. Assuming that $k \leq 32 \times 10^{-4} \text{ s}^{-1}$ (see above), r would have had a value $> 28s$. 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 N_2 fixed. Alternatively, N_2 fixation may be restricted to the heterocysts, but only if the value of k or of τ is greater in their experiments than in ours.

Acknowledgments

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Summary

^{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 mC/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 *A. 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 filament can be characterized by a constant $\tau < \text{ca. } 5\delta \text{ (cell}^{-2}\text{)}$.

Table 1.--Conditions decreasing algal assimilation of ^{13}N . 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 materials in the presence of CO , excess N_2 , or H_2 , or with fixation by algae grown with sources of fixed nitrogen. Counts per min (corrected to a standard time) per mC ^{13}N per ml gas phase, and ratios thereof, were computed for Millipore-filtered, washed samples of algae or for aliquots of algal suspensions.

Condition	Per cent of control	
	Observed ^a	Expected for ^{13}N
Growth on NH_4^+	1.1	$< 0.5^c$
Growth on NO_3^-	21 (19) ^b	39 ^c
Fixation in the presence of 0.013 atm CO	7	5 ^d
Fixation in the presence of a high partial pressure of H_2	-13	low ^d
Fixation in the presence of a high partial pressure of N_2	11	8 ^e

^a Corresponds to incorporation into alga.

^b Additional incorporation into supernatant. The nitrate-grown alga must excrete much of the ^{13}N it fixes.

^c Ratio of acetylene reduction by the same concentrated algal suspensions used for ^{13}N assays.

^d See text.

^e Assumes $K_m(N_2) = 0.076 \text{ atm}$.

Table 2.--Effect of evacuation on ¹³N content of sample applied to emulsion. Aliquots of an algal suspension were processed in four ways. One aliquot was filtered onto a 0.45- μ m pore size Millipore filter, washed, the filter added directly to a scintillation vial containing Cocktail D, and counted. A second aliquot was filtered and washed like the first; the filter was glued to a planchet, and counted. A third aliquot was added directly to Cocktail D, a filter was added, and the vial was counted. A fourth aliquot 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.

	Scintillation counter	%	Planchet counter	%	Relative cpm, %
Filtered alga	129500	87.6	52200	89.5	40.3 ^a
Total suspension	147800	100			
Evacuated emulsion			58400	100	39.5

^aAn independent determination of the relative counting efficiency of planchet counter and scintillation counter using ⁵⁹Fe-labeled algae gave a figure of 39.7%.

Table 3.--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 ¹³ N + 2- and 3.5-min delay ^b			
	Position of cell	Tracks observed	Cells examined	Tracks/Cell	Tracks ^d observed	Cells ^d examined	Tracks ^c /Cell	Tracks ^d observed	Cells ^d examined	Tracks ^c /Cell		
H	n=1	205	1668	0.118	205	1668	0.118	42	1328	0.028		
1	126	2821	0.040	126	2821	0.040	62	2265	0.024			
2	131	2754	0.043	126	2681	0.042	46	2104	0.018			
3	119	2675	0.040	112	2529	0.040	33	1924	0.013			
4	76	2545	0.025	72	2276	0.027	20	1686	0.008			
5	70	2234	0.027	59	1804	0.028	28	1354	0.017			
6	74	1884	0.035	58	1312	0.039	16	985	0.013			
7	45	1489	0.026	37	861	0.038	9	590	0.012			
8	31	1142	0.023	18	471	0.034	10	364				
9	19	842	0.018	8	243	0.028	1	183				
10	16	610	0.022	3	92		1	81				
11	14	475	0.025	3	35	0.050	0	33				
12	10	360	0.023	2	15		0	20	0.014			
13	10	286		0	4		0	10				
14	3	205	0.021	0	0		0	4				
15	3	135		0	0		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									

^aHeterocyst, 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.
^bCombined data from two slides of same algal suspension.
^cCorrected for background = 0.0046 \pm 0.0003 tracks/cell
^dNot here tallied: cells and tracks of indeterminate position relative to a heterocyst prior to autoradiography.
^eCorrected for background = 0.0037 \pm 0.0002 tracks/cell

Figure Legends

Fig. 1. Decay curves following generation of ^{13}N by the reaction $^{13}\text{C}(p,n)^{13}\text{N}$. **A** Decay curve of the target material following bombardment of 20 mg of 60 atom per cent ^{13}C with a 0.82 μamp beam of protons. **B** A similar target was bombarded with 3 μamp of protons, the purified $^{13}\text{N-N}_2$ compressed into a single vial. N_2 assimilated by an algal suspension (0.25 ml, 26.8 μg chlorophyll/ml), and the radioactivity in 10% of the volume of the suspension measured following removal of $^{13}\text{N-N}_2$. **C** A 16.6-mg target of 90 atom per cent ^{13}C was irradiated with a 4 μamp 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).

Fig. 2. Positron tracks resulting from decay of ^{13}N atoms incorporated by algal filaments during 15 min of fixation of $^{13}\text{N-N}_2$. The tracks are concentrated in the vicinity of the algal filaments, the ends of which are indicated with white arrows. $\times 312$.

Fig. 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 $^{13}\text{N-N}_2$, ^{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 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. $\times 812$.

Fig. 4. Sequence of photomicrographs depicting positron tracks. Four min after a 2-min pulse of $^{13}\text{N-N}_2$, heterocysts still have nearly 25% of the ^{13}N in algal filaments. Vegetative cells more than half-way to the end of a filament from the nearest conjoined heterocyst, and tracks from such vegetative cells (e.g., at the right-hand side of the filament illustrated), were not included in Table 3, B and C, because a heterocyst might have become detached from the end of the filament following fixation and movement of ^{13}N . See also the commentary in the legend of Fig. 3. $\times 336$.

Fig. 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) (\bullet). Other curves: tracks observed (\circ ; data from Table 3B); 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 ($k = 8 \times 10^{-4} \text{s}^{-1}$) or without ($k = 0$) simultaneous immobilization into macromolecules, according to equations presented in the text. $k = 0 \text{s}^{-1}$, $\tau = 6 \text{s}$ (Δ). $k = 8 \times 10^{-4} \text{s}^{-1}$ and $\tau = 3 \text{s}$ (\times), 5s (Δ), 10s (\square), and 60s (\boxtimes).

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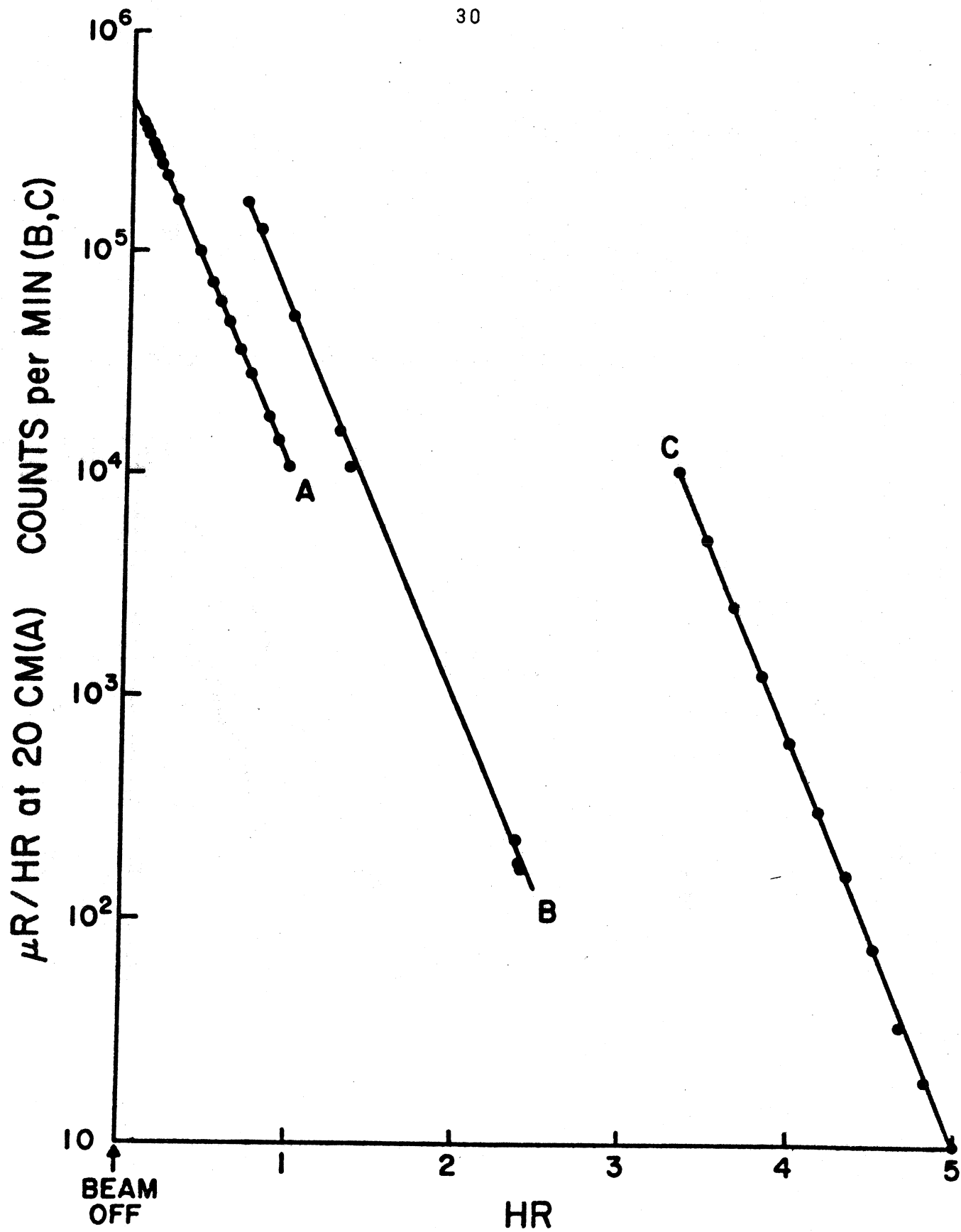


Fig. 1

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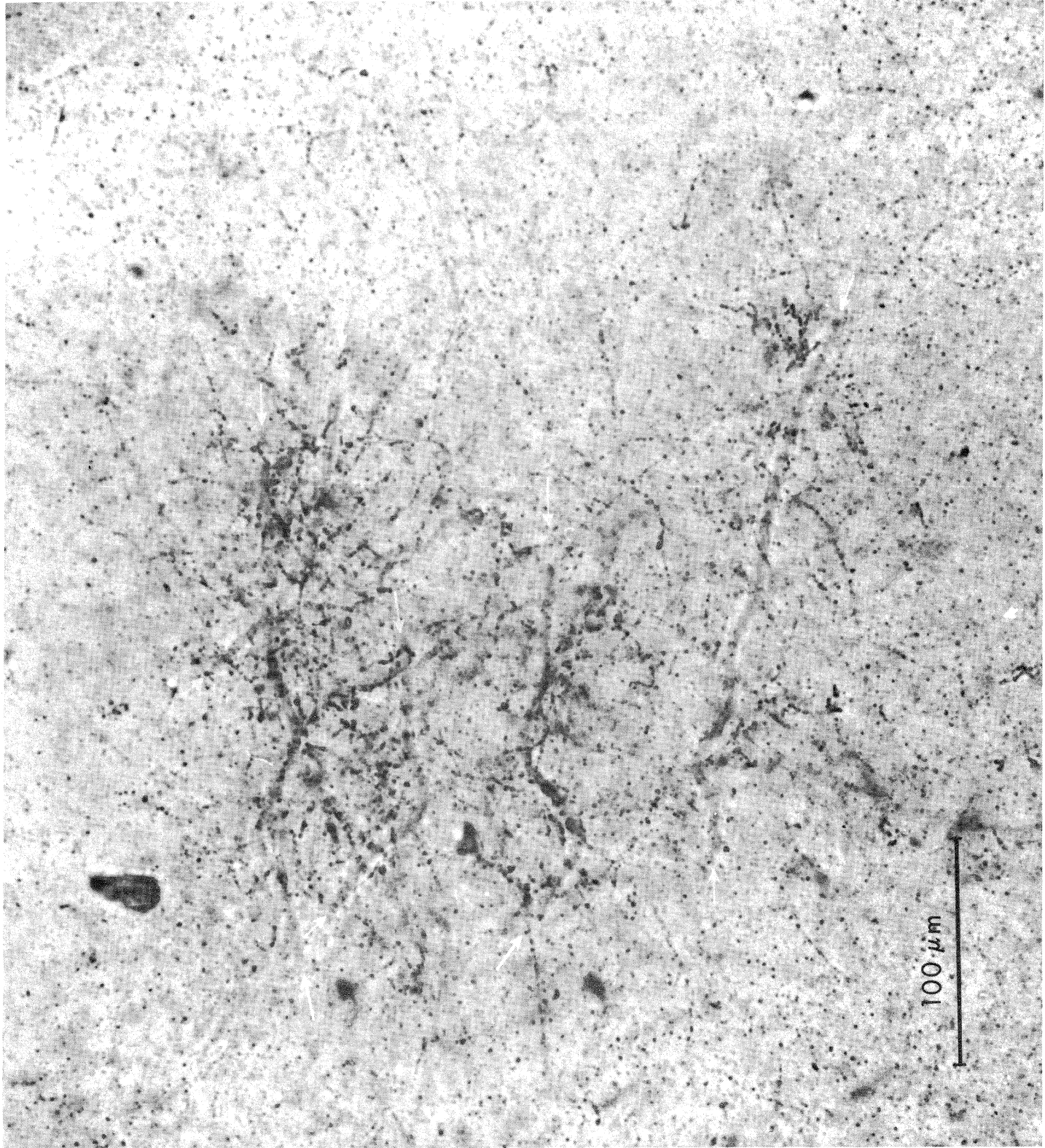
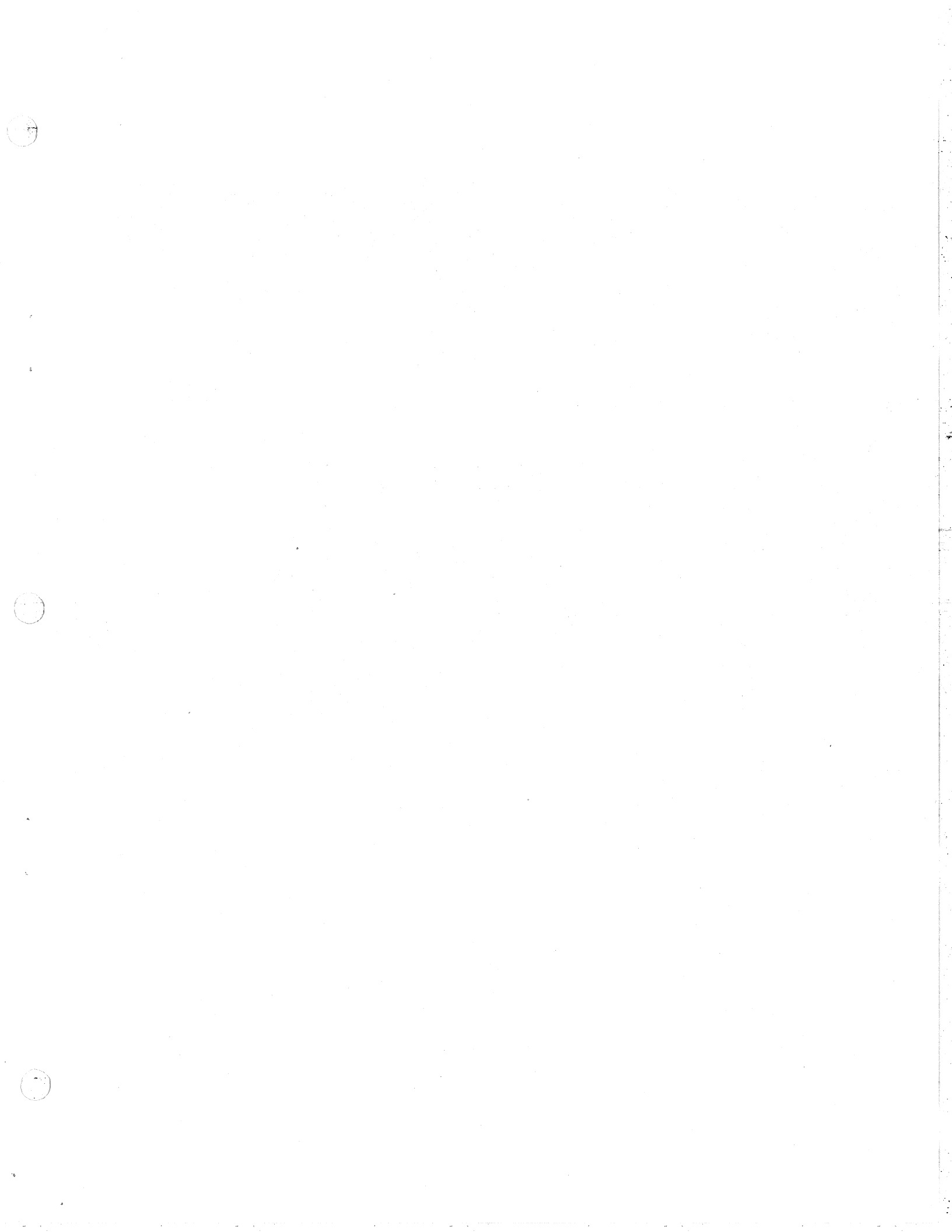


Fig. 2



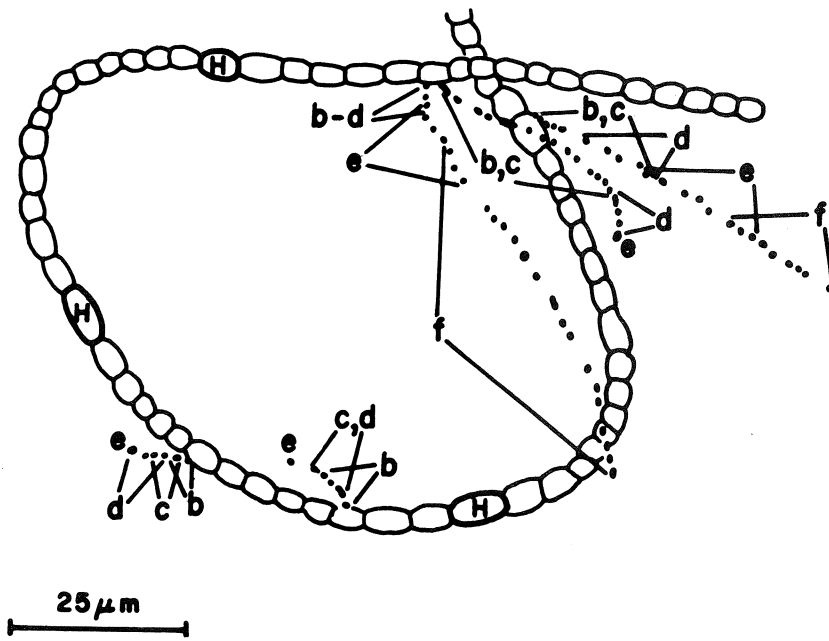
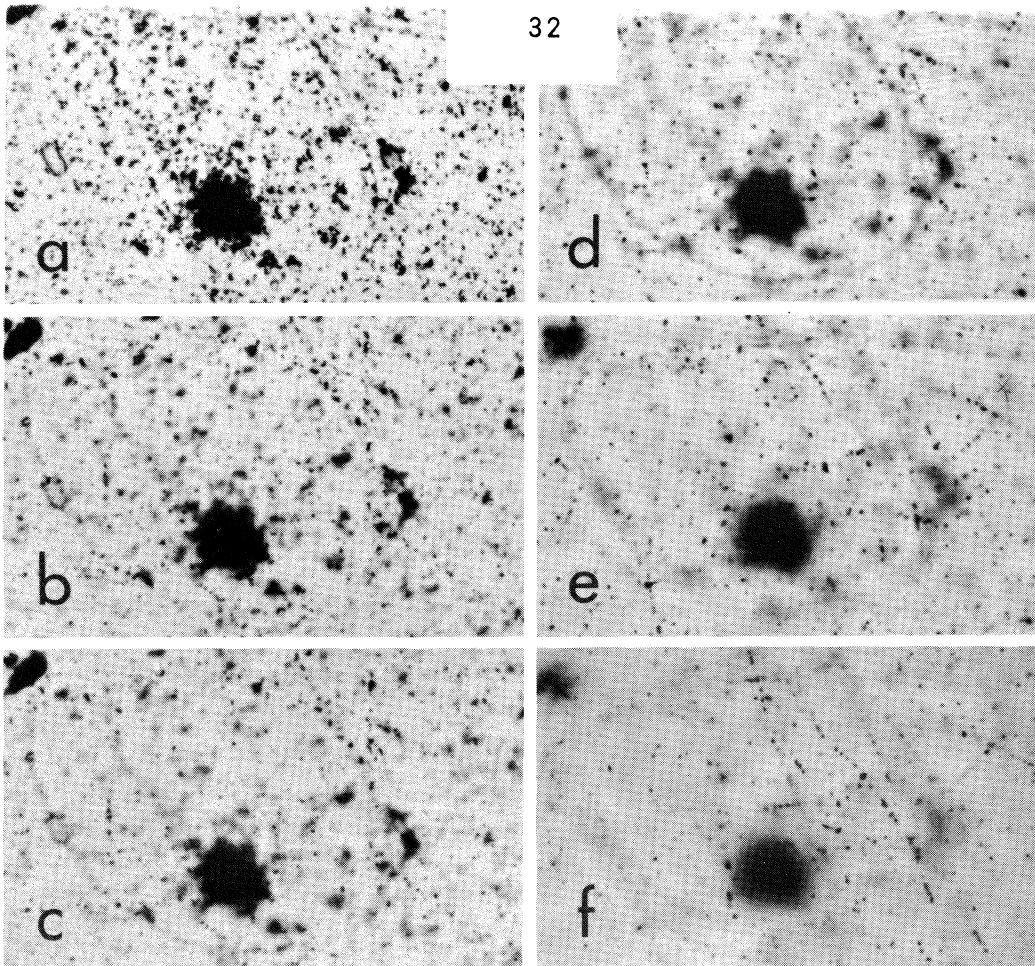
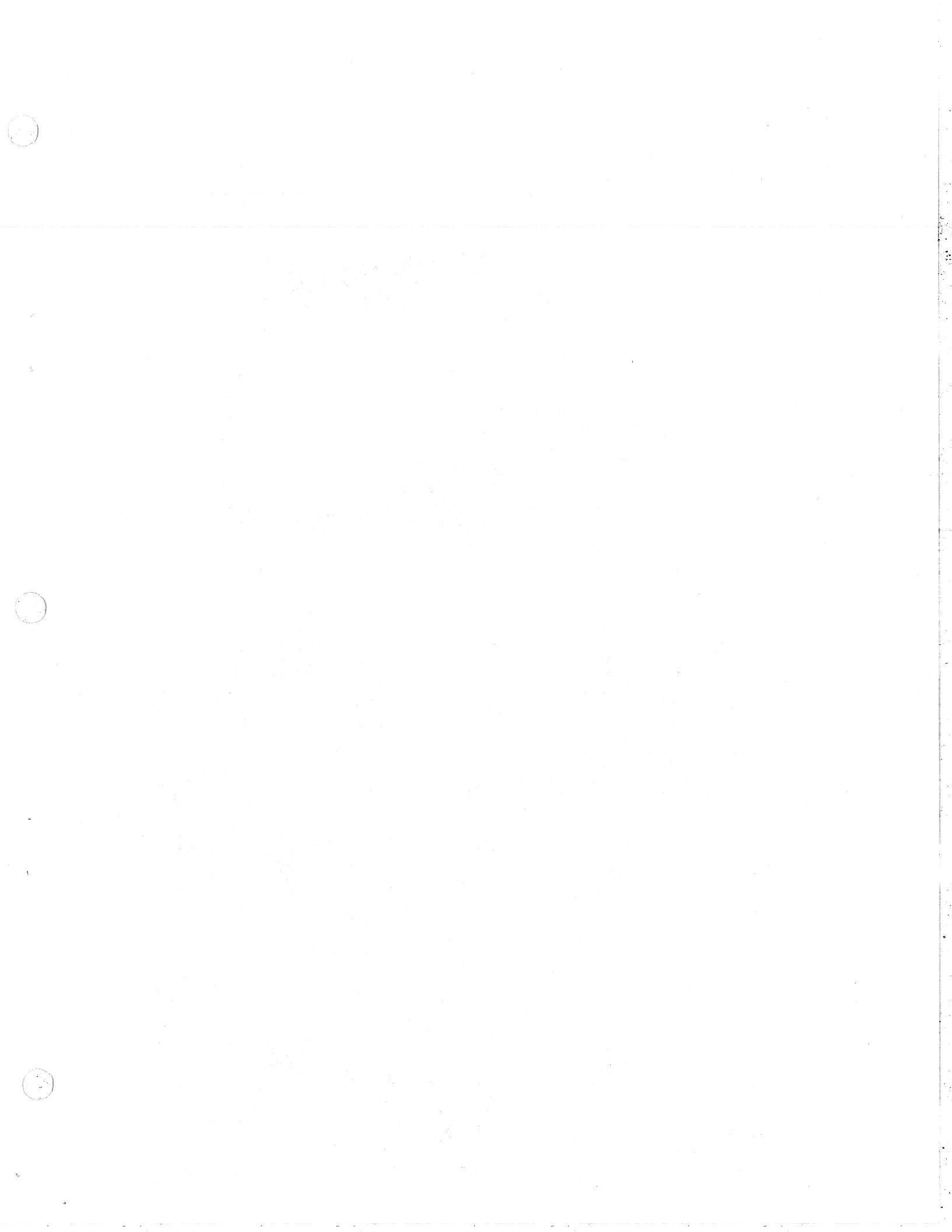


Fig. 3



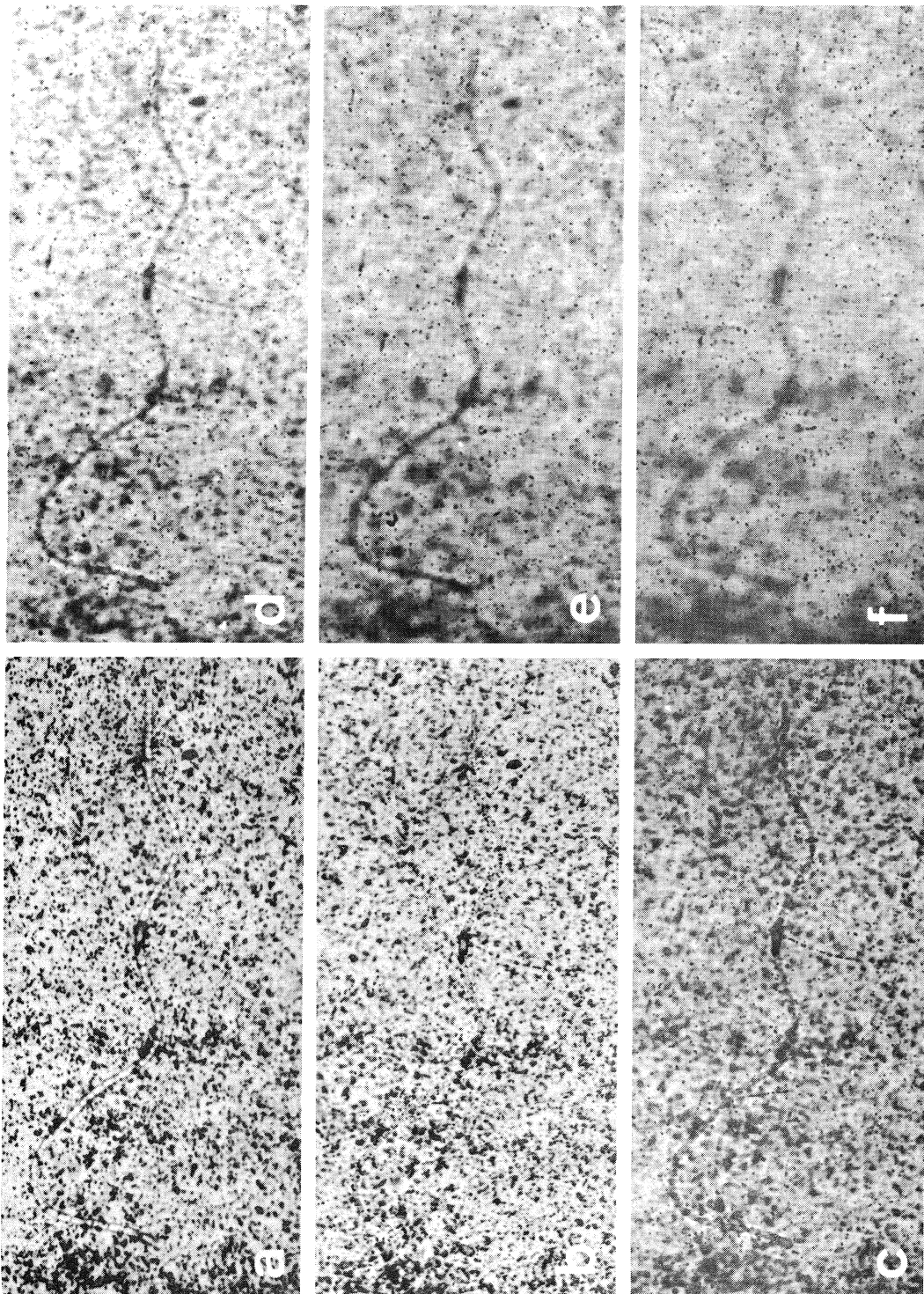


Fig. 4

