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The pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium, Anabaena cylindrica

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Methods have been developed for identifying the pathway of assimilation of N_2-derived nitrogen. The products of fixation of ^{13}N-labeled nitrogen gas (^{13}N-N_2), and the distribution of ^{13}N within glutamine, were determined after short periods of labeling (ca. 1 to 120 s) and also in pulse-chase experiments. Ammonia, the amide nitrogen of glutamine, and the α-amino nitrogen of glutamate, in that order, were the first observed products of fixation of N_2 by the cyanobacterium (blue-green alga), Anabaena cylindrica. This sequence of the formation of nitrogenous products was confirmed by the use of inhibitors. The presence of 1 mM methionine sulfoximine permitted continued formation of $^{13}NH_3$, while virtually preventing ^{13}N-labeling of amino acids. In the presence of 1 mM azaserine, glutamine was labeled, but not other amino acids. Our observations demonstrate unequivocally that N_2-derived nitrogen fixed by this organism is metabolized initially by the glutamine synthetase/glutamace synthase pathway.

Most studies of the pathways considered potentially capable of initial metabolism of nitrogen in cyanobacteria (blue-green algae) have been enzymological. Activities of glutamic acid dehydrogenase and alanine dehydrogenase have been measured in Anabaena cylindrica (1,2) and other cyanobacteria (3-5); glutamine synthetase activity was observed in A. cylindrica at levels higher than required to account for in vivo rates of nitrogen assimilation (6; cf. also 1); and glutamate synthase (glutamine amide: 2-oxoglutarate amido transferase, or GOGAT) activity was detected in the same cyanobacterium only when ferredoxin was used as reductant (7). Glutamine synthetase (8), like nitrogenase (9), is present at relatively high specific activity in heterocysts, differentiated cells which are major loci of nitrogen fixation in aerobically grown filaments (10-15). Little else is known about how the cellular localization of the enzymes assayed compares with the localization of the initial metabolism of fixed nitrogen. It is therefore not possible at this time to reach firm conclusions, on the basis of enzymological studies, about pathways operative in the metabolism of N_2-derived fixed nitrogen.

Addition of L-methionine-DL-sulfoximine, in other organisms an inhibitor of glutamine synthetase, to cultures of A. cylindrica (16) led to a great decrease in the size of the total intracellular pool of glutamine, and to about a halving of the glutamate pool, over a 12-h period. The concentrations of aspartate and of glycine plus alanine changed very little. Secretion of NH_3 was increased greatly. These changes, being slow, could have been indirect, but they did

support the idea that N_2-derived NH_3 is metabolized initially by the glutamine synthetase/glutamate synthase pathway.

Early studies of products of fixation of ^{15}N-labeled nitrogen gas, by Magee and Burris (17), showed that in hydrolysates of protein from the cyanobacterium Nostoc muscorum, the highest specific activity (atom percent excess) of ^{15}N was in glutamic acid. Because the products were analyzed only after 1.5 h of fixation, these studies could only suggest possible candidates for the earliest products of fixation. More recently, Stewart et al. (18) have referred to investigations involving 10-min exposures to ^{15}N-labeled N_2. Although the period of labeling was such that several organic products had become labeled, the results were consistent with operation of the glutamine synthetase/glutamate synthase pathway. In such studies, as increasingly shorter intervals of labeling are employed, increasingly smaller amounts of fixed ^{15}N must be detected and quantified. Efforts to compensate for decreased fixation by increasing the quantity of N_2-fixing cells are hampered by self-shading of the suspension of pigmented, photoautotrophic cells. In common practice, the content of ^{15}N in fixed nitrogen is measured, after reoxidation of the nitrogenous products to form N_2, either by emission spectrometry or by mass spectrometry. Even slight leakage of N_2 from the air into the analytical equipment can hinder accurate measurement of small, ^{15}N-derived signals.

We have circumvented these problems by making use of radioactive, ^{13}N-labeled nitrogen gas (^{13}N-N_2: 15, 19, 20). Products of the fixation of ^{13}N-N_2 are separated rapidly by high-voltage thin layer

electrophoresis. These products can then be quantified easily and accurately, with low background, by laboratory apparatus that is standard for work with radioisotopes. The brief report of our results which has appeared (20) shows that although the half-life of ^{13}N is only 10 min, this property of the isotope is not as limiting to experimentation as it might appear to be. Processing times of 1 h are routinely possible; under favorable circumstances, peaks of radioactivity much above background can be obtained 1.5 h after fixation. In this paper, we provide a complete account of our methodology and results. The results firmly establish the glutamine synthetase/glutamate synthase pathway as the initial pathway of metabolism of N_2-derived fixed nitrogen in Anabaena cylindrica.

MATERIALS AND METHODS

Labeling and extraction

Anabaena cylindrica Lemm. was grown aerobically with N_2 as nitrogen source, concentrated to 27 μg chlorophyll per ml, and stored for ca. 15 min under aerobic conditions prior to exposure to ^{13}N-N_2, as described earlier (15). In some experiments, 1 mM L-methionine-DL-sulfoximine (see above; Sigma Chemical Co., St. Louis, Mo.) or o-diazoacetyl-L-serine (azaserine; Calbiochem, La Jolla, Calif.) was added to the cyanobacterial suspension. Azaserine is an inhibitor of amide transfer from glutamine (21). Fixation of ^{13}N-N_2 was performed in a 1-ml Reactivial (Pierce Chemical Co., Rockford, Ill.), sealed with a serum stopper. The cylindrical space within the vial tapers as a cone to a point. The lower end of the vial was ground off to the bottom of the cone, and cemented with epoxy to the flat-ground top of a stopcock. From the stopcock, a short length of tubing led through a serum stopper to a centrifuge tube containing 1 ml of methanol. The reaction vial received ca. 0.02 atm ^{13}N-N_2 and was filled to 1 atm, usually with a gas mixture consisting of 0.95% CO_2, balance Ar (North American Cryogenics, Willoughby, Ohio). Where indicated, the vial was filled instead with a mixture of 1% CO_2, balance N_2. One-quarter ml of Anabaena suspension was then added to the vial. The suspension was stirred by means of a magnetic stirring bar mounted on a triangular piece of teflon (Pierce Chemical Co.) set in the cone of the vial. The suspension was illuminated with incandescent light at an intensity of 4000 lux. The amount of ^{13}N in the vial was monitored with a model ABG-10KG-SB ioniza-

tion gauge (Jordan Electronics, Div. Victoreen Instrument Co., Alhambra, California). Periods of exposure of the suspension to ^{13}N-N_2 varied from about 1 to 120 s inclusive of an interval of ca. 0.5 s required for transfer of the suspension into the methanol. In certain experiments, the gas phase was evacuated after 15 s, and replaced with a gas phase of 1% CO_2, balance N_2, after which incubation was continued for up to 5 min before the suspension was transferred to the methanol.

If the ^{13}N-N_2 had not been evacuated, the centrifuge tube containing the methanolic suspension was flushed with gas to remove most of the residual free ^{13}N-N_2. The suspension was then mixed for 1 min on a vortex micromixer, and centrifuged at 1000g for 2 min. The supernatant fluid was decanted. In some experiments, the resulting pellet was washed twice with 4 ml of 80% methanol, resuspended, transferred to a scintillation vial, 10 ml of "Cocktail D" (Beckman Instruments, Irvine, Calif.) added, and the radioactivity in the suspension measured with a CPM-100 scintillation counter (Beckman). Portions of 10 to 50 µl were removed from the original 80% methanolic centrifugal supernatant fluid for determination of the total ^{13}N radioactivity of the extracts. All measurements of radioactivity were corrected for background and for time of decay. Where indicated, solutions of $NaNO_2$, $NaNO_3$, stable amino acids (Sigma Chemical Co.), ^{14}C-labeled carbamyl phosphate (New England Nuclear Corp., Boston, Mass.), ^{14}C-labeled amino acids (ICN Pharmaceuticals, Inc., Cleveland, Ohio, and New England Nuclear Corp.), and ^{3}H-glutamate (New England Nuclear Corp.) were added to the extract at this point of the processing.

Separation of ^{13}N-labeled constituents of extracts

The remaining supernatant fluid, or a measured portion of it, was then treated in either of two ways: (i) The fluid, supplemented with appropriate reagents, was subjected to vacuum distillation to recover ammonia, and then to steam distillation to recover amide nitrogen (22). The results of different labeling experiments made within the same day were compared quantitatively by normalizing to equal amounts of radioactivity in the fixation vial. Two-ml portions of the distillates were added to scintillation vials containing 20 ml of Cocktail D, and their radioactivity measured with the scintillation counter. (ii) Alternatively, in preparation for electrophoresis, the fluid was dried under vacuum at 50°C, the residue dissolved in 200 µl of 80% methanol, the solution dried as before, and the residue dissolved in 50 µl of 80% methanol. The final methanolic solution was carefully spotted on a 2x10 mm area of a 5x20 cm glass plate bearing a 0.1 mm layer of cellulose (E. Merck, W. Germany). The 10-mm dimension of the spot normally paralleled the 5-cm dimension of the thin layer plate. However, when electrophoresis was to be followed by chromatography, the long dimension of the spot paralleled the long dimension of the plate.

Relative and absolute recoveries of ^{14}C-glutamine and ^{3}H-glutamate, added to cyanobacterial extract and adsorbed to the walls of the test tube during drying, were determined by scintillation

spectrometry, with measurements to 0.1% counting accuracy. No significant differential loss of glutamine or glutamate to the walls of the test tube was detected. Recovery was not as complete when only a one-step concentration procedure was employed.

The lipid-soluble substances extracted by the methanol were found to impede the separation of amino acids, and were therefore displaced from the region of spotting by chromatography for 2 to 3 min with chloroform-methanol (3:1, v/v) in the direction of the short dimension of the thin layer plate. The plate was then dried, sprayed with buffer, and subjected to electrophoresis at 3000 V for 1.5 to 12 min in a model Q11 SAE-3202 High Voltage Electrophoresis Apparatus (Shandon Scientific Co., London, England). Buffers used were 70 mM sodium borate ($Na_2B_4O_7 \cdot 10\ H_2O$), pH 9.2, and 0.75 M formic acid, pH 2.0 (23). The distribution of ^{13}N on the thin layer plates was determined, after electrophoresis, with a model 7201 Radiochromatogram Scanner (Packard Instrument Co., Downers Grove, Ill.) Plates were scanned at 2 cm/min, unless noted otherwise, with a 2.5-mm slit and a 3-sec time constant. In order to scan only ^{13}N, when ^{14}C was present, a 127-μm layer of aluminum was interposed between the thin layer plate and the collimator. Removal of the aluminum foil permitted detection and scanning of the distribution of ^{14}C, after the ^{13}N had decayed. Stable amino acids were localized by spraying with a 0.5% (w/v) solution of ninhydrin in a 1:1 mixture of acetone and 0.7 M formic acid.

On occasion, following initial chromatography and electrophoresis, scanning (for ^{13}N) and drying of a plate, chromatography was again

performed parallel to the short dimension of the thin layer plate. The duration of this chromatography was 16-30 min. The solvent system was phenol-water (3:1, v/v) equilibrated with 3% aqueous NH_4OH (24). This chromatographic system is suitable for resolving citrulline, glutamine, asparagine, glutamate and aspartate (respective R_f's ≈ 0.66, 0.55, 0.42, 0.22 and 0.14) in this short a time. After chromatography, sections of the plate were excised which, according to the earlier scan, contained zones of ^{13}N radioactivity. Such a section had as its long dimension the 5 cm width of the original plate. A residual portion of the original extract, labeled with ^{14}C-glutamate and ^{14}C-glutamine which had been added as standards, and with ^{13}N, was spotted close to the short dimension of the section to serve as a reference marker for position. The section was then scanned for ^{13}N, and later for ^{14}C, along its 5-cm dimension, and was subsequently sprayed in order to localize ninhydrin-positive material. In certain experiments, the thin layer plate was dried after chromatography. Regions corresponding to specific amino acids were localized with ninhydrin, and the cellulose in those regions scraped from the plate and suspended in one ml of water. The suspensions were agitated with a vortex mixer for one minute, and centrifuged to remove the cellulose. Measured portions of the supernatant fluid were then assayed for their content of ^{13}N using the scintillation counter.

Chemical reactions of separated constituents of extracts

In other experiments, ^{13}N-containing bands localized by scanning after electrophoresis were scraped from the thin layer plates and

suspended in 2 ml of water. Substances containing ^{13}N were separated from the cellulose, and their radioactivity measured, as described above. A measured portion of the solution eluted from the band containing glutamine was supplemented with 40% NaOH; the amide nitrogen of the glutamine was hydrolyzed, and was distilled as ammonia, with steam (22). Alternatively, the glutamine contained in the measured portion of solution was reacted with ninhydrin at pH 2.5, and the product digested with H_2O_2, whereby the α-amino nitrogen was released as ammonia (25). The ammonia was then vacuum-distilled at pH 10 (22). The ammonia released was trapped in a 4% solution of boric acid, and assayed for radioactivity with the scintillation counter. In some experiments, known amounts of stable glutamine were added to the eluted solution to serve as internal standard, and the recovery of amide or α-amino nitrogen as ammonia was determined colorimetrically (26).

The solution eluted from a rapidly migrating, ^{13}N-containing band was assayed for its content of $^{13}NO_2^-$ and $^{13}NO_3^-$ by means of steam distillation in the presence of the appropriate reducing agents (22). Twenty-five μg of $NaNO_2$, added to and electrophoresed with the extract, was localized by means of a spray consisting of a 1:1 (v/v) combination of 1% sulfanilamide in 3 N HCl and 0.02% N-(1-naphthyl)ethylene-diamine dihydrochloride (27). $NaNO_3$, added to and electrophoresed with the extract, was localized by elution of 1-cm bands of cellulose, reduction to nitrite with a cadmium-copper catalyst, and chromogenic reaction of the nitrite with sulfanilamide (28). The electrophoretic mobilities of NO_2^- and NO_3^- were nearly equal in borate buffer, pH 9.2.

RESULTS

The organic products observed after varying periods of fixation of ^{13}N-N_2, and separation at pH 9.2, are portrayed qualitatively in Fig. 1 and quantitatively in Fig. 2. Although many amino compounds fail to separate at pH 2, others separate better than at pH 9.2 (see Fig. 3).

After 2 min of fixation of ^{13}N-N_2, various radioactive organic products of fixation were detectable. During electrophoresis at pH 9.2, these products migrated with stable standards of arginine, citrulline and/or glycine, glutamine, glutamate and, indicated by a shoulder on the peak of glutamate radioactivity, aspartate (Fig. 1A). After electrophoresis at pH 2, glycine appeared to be unlabeled, and label could be detected in ammonium ion (Fig. 3). Label was sometimes detected in the regions of alanine (Figs. 1, 3) and lysine (Fig. 3).

After one minute of fixation, and electrophoresis at pH 9.2, essentially only two organic products of fixation were detected (Figs. 1B, 4). These two products co-electrophoresed with ^{14}C-labeled glutamine and glutamate (Fig. 4). ^{14}C-labeled glutamine, glutamic acid and carbamyl phosphate had been added to the extract in the proportion (μCi/ml) of 1.00:0.59:2.12. They were recovered in the approximate proportion 1.00:0.48:1.85 (mean from three scans, one of which is shown in Fig. 4). No ^{13}N-labeled product was detected at the position of migration of ^{14}C-labeled carbamyl phosphate. When electrophoresis at pH 9.2 was followed by chromatography in phenol-water-ammonia, the peak with lower electrophoretic mobility

co-chromatographed with ^{14}C-glutamine, and the peak with higher electrophoretic mobility co-chromatographed with ^{14}C-glutamate (Fig. 5). Glutamine and glutamic acid, both labeled with ^{14}C, both co-electrophorese at pH 2 with the predominant peak of activity of ^{13}N (Fig. 6).

The same two organic products were observed after progressively shorter periods of fixation, although the substance with lower electrophoretic mobility became increasingly dominant (Fig. 1B, C, and 2). Of the ^{13}N in the dominant peak after 1 min of fixation, up to 87% (statistical range: ±3%) was steam-distillable in the presence of alkali; higher percentages of the ^{13}N in that peak were steam-distillable after shorter periods of fixation. After reaction with ninhydrin and digestion of the product, approximately 14±2% (average of two experiments, corrected for 88% efficiency of recovering ammonia from the α-amino group of stable glutamine added as internal standard) of the ^{13}N from this peak was distillable at pH 10 in vacuo, also after 1 min of fixation.

The net rate of labeling of the ammonium-ion pool decreases rapidly during the first few seconds of fixation, whereas the pool of ^{13}N-labeled amide nitrogen increases approximately linearly during the first 15 s of fixation (Fig. 7). When results from many experiments were collated, it was found that the amount of ^{13}N in glutamine, normalized to equal amounts of ^{13}N in the fixation vial, increases nearly linearly with time during the first min of labeling, but much more slowly thereafter.

When 1 mM methionine sulfoximine was added to the suspension of cyanobacteria 10 to 15 min before the suspension was exposed to

^{13}N-N_2, the amount of ^{13}N in ammonium ion present in the suspension was 16- to 25-fold greater than after 1 min of fixation in the absence of methionine sulfoximine (Table 1). Glutamine and glutamate were not detectable by scanning of thin layer plates. In one experiment, in which cyanobacteria treated with methionine sulfoximine were exposed to an unusually high level of ^{13}N for 1 min, traces of organic products co-electrophoresing with alanine and aspartate were detected. Following the addition of 1 mM azaserine to the suspension of cyanobacteria, glutamine was the only organic metabolite, the formation of which was observed (Fig. 8).

The suspension of cyanobacteria was exposed to ^{13}N-N_2 for 15 s, the gas evacuated, and the suspension then exposed to a gas mixture containing 99% N_2, 1% CO_2. Glutamate-^{13}N thereupon increased and glutamine-^{13}N decreased as a fraction of total methanol-extractable, organic ^{13}N (Fig. 9). Small quantities of ^{13}N were incorporated by an unknown substance, and by substances co-electrophoresing with aspartate and arginine. In one such experiment, a small peak (6% of organic ^{13}N) was detected in the carbamyl phosphate region of the thin layer plate. After a chase of 105 s, ^{13}N not extractable with 80% methanol accounted for ca. 8% of organic ^{13}N.

When distilled water, rather than a suspension of algae, was added to a fixation vial containing ^{13}N-N_2, and the water processed for electrophoresis, a peak was observed with an electrophoretic mobility, toward the anode, of about 2 cm/min at pH 9.2. The same peak was detected during brief electrophoresis of ^{13}N-labeled algal extracts at pH 9.2. The peak accounted for approximately half of the

^{13}N detected by electrophoresis after 15 s of fixation. When $NaNO_2$ and $NaNO_3$ were added prior to, and localized after, electrophoresis, the ^{13}N-labeled material in this peak was found to migrate about as rapidly as did the nitrite and nitrate. Steam distillation showed that the unknown could be accounted for, within experimental error, as nitrite and nitrate. When 0.1 ml of used growth medium was exposed for 1 min to ^{13}N-N_2, the radioactive gas evacuated and flushed, and the cyanobacterium exposed for 1 min to the residual solution, the resulting formation of organic metabolites was only about 1% of that found when the organism was exposed directly to the ^{13}N-N_2.

As determined with ^{3}H-glutamate and ^{14}C-glutamine internal standards, approximately 20% of the radioactivity in extracts was lost during concentration. No peaks other than those already mentioned were observed when the duration of electrophoresis was decreased. The total ^{13}N-radioactivity in methanolic extracts could be accounted for, within experimental error, as the sum of the radioactivities of the observed constituents.

When ^{13}N-N_2 was fixed for 15 s in the presence of 1% CO_2, balance N_2, 86±6% of the organically bound ^{13}N extractable with 80% methanol migrated with glutamine during electrophoresis at pH 9.2, and 9±2% with glutamate; after fixation for 2 min, the corresponding figures were 27±5% with glutamine and 64±4% with glutamate.

DISCUSSION

Because the product of assimilation of N_2 by nitrogenase, *in vitro*, is NH_3 (29), it was anticipated that the first product of fixation of ^{13}N-N_2 would be ^{13}N-labeled NH_3. By reducing the period of fixation to about 1.25 s, we were able to demonstrate that ammonia is labeled more rapidly than is any other metabolic product (Fig. 7). These results were corroborated by our finding that methionine sulfoximine, while permitting continued production of ^{13}N-labeled ammonia, largely prevents the formation of other metabolic derivatives of ^{13}N during 1 min of fixation of ^{13}N-N_2. Our results confirm the observations made by Stewart and Rowell (16) upon treating cultures of *A. cylindrica* for a number of hours with methionine sulfoximine.

The first two organic products of fixation of ^{13}N-N_2 are glutamine and glutamate. The identification of these products is based upon co-chromatography, and co-electrophoresis at pH 9.2 and pH 2.0, with stable and ^{14}C-labeled glutamine and glutamate (Figs. 3-5). No other amino acid is known to migrate with glutamine or glutamate in all three of the separation systems used (23, 24). The identification of one of the products as glutamine is based also upon the fact that after short periods of fixation of ^{13}N-N_2, most of the ^{13}N in this product reacted as amide nitrogen. That is, it could be steam-distilled in the presence of alkali, and trapped in a 4% solution of boric acid. The residual ^{13}N in this product could be accounted for as α-amino nitrogen. Analysis after long periods of labeling (not technically feasible in these experiments), should show that close to half of

the ^{13}N in glutamine is present in the amide nitrogen group.

Glutamine becomes labeled, in its amide nitrogen, much more rapidly than does any other organic molecule, presumably by amidation of glutamate with $^{13}NH_4^+$. Next, glutamate becomes labeled, presumably by amination of α-ketoglutarate. The appearance of ^{13}N in the α-amino group of glutamine can be accounted for by subsequent amidation of α-amino-labeled glutamate.

Three observations indicate that the α-amino nitrogen of glutamate is derived from the amide nitrogen of glutamine. Methionine sulfoximine, which inhibits the formation of glutamine, also prevents the formation of glutamate. Azaserine, an inhibitor of amide transfer from glutamine (21), permits formation of glutamine, but prevents biosynthesis of glutamate. In addition, glutamate becomes labeled at the expense of glutamine during a chase experiment. The ratio of glutamine-^{13}N to total organic ^{13}N decreases rapidly at first during a chase, and later much more slowly. These observations suggest that amide-^{13}N is lost during the first period, and that during the second period, much of the ^{13}N in glutamine is in the α-amino group, and is derived from α-$^{13}NH_2$-labeled glutamate.

The enzymes corresponding to the above sequence of reactions, starting with N_2 as substrate, are (i) nitrogenase, (ii) glutamine synthetase, and (iii) glutamate synthase (GOGAT). The activities of all of these enzymes have been measured in A. cylindrica (2, 7-9, 30-32).

Fixation of ^{13}N-N_2 is reduced competitively approximately 9-fold by the presence of 0.8 atm of stable nitrogen (15). In consequence, certain of the experiments reported here would not have been possible, for technical reasons, at normal atmospheric partial pressures of N_2. However, the possibility existed that pressures of N_2 greater than those which we normally used would increase the intracellular concentration of NH_4^+ and would, as a result, lead to substantial direct formation of glutamate, alanine, or other products, by their respective dehydrogenases. We therefore examined the products of short periods of fixation of ^{13}N-N_2 in the presence of 0.99 atm N_2. The results obtained closely resembled those obtained with 0.02 atm N_2. Our results at both pressures of N_2 gas, supported by the enzymatic analyses and by a variety of less direct measurements (16, 18), demonstrate that the glutamine synthetase/glutamate synthase pathway is principally responsible for the metabolism of N_2-derived fixed nitrogen by A. cylindrica.

By the end of two min of labeling, additional compounds become labeled. ^{13}N-radioactivity was detected in the citrulline-glycine region after electrophoresis at pH 9.2, whereas after electrophoresis at pH 2.0, the glycine region was essentially devoid of ^{13}N. Thus, ^{13}N may be labeling citrulline within 2 min. The carbamyl group of citrulline is heavily labeled after 5 min of fixation of $^{14}CO_2$ by Nostoc muscorum (33, 34). Radioactivity was detected in a region co-electrophoresing with arginine after some but not all 2-min

fixations of ^{13}N-N_2. Enzymatic synthesis of carbamyl phosphate by cell-free extracts of A. cylindrica and N. muscorum has been demonstrated (2, 35), as has transcarbamylation of ornithine to form citrulline (1, 35-37), a precursor of arginine (37). There was therefore reason to suspect that carbamyl phosphate might be present in extracts following one minute of fixation but might have gone undetected due to its great lability (38). Carbamyl phosphate labeled with ^{14}C was added to the extract of ^{13}N-labeled cyanobacteria, and carried through the normal procedure for concentrating and electrophoresing the ^{13}N-labeled compounds. Compared with the recovery of ^{14}C-glutamine, about 87% of the carbamyl phosphate was recovered. The fact that ^{13}N-labeled carbamyl phosphate could not be detected on thin layer plates after electrophoresis could not, therefore, be explained as due to extensive degradation of that substance. Either carbamyl phosphate had not yet become labeled, or it was present at very low levels. In the experiment reported, there was also an apparent loss of ^{14}C-glutamate relative to ^{14}C-glutamine. We attribute this result to the inherent variability in the output of the scanner used.

That aspartate is labeled with ^{13}N after 2 min of fixation was given support, following two-dimensional separation, by localization with ninhydrin of the aspartate spot (well-separated from the glutamate spot), elution from the aspartate spot, and scintillation counting of the eluate. Traces of ^{13}N were in one instance detected in bands co-electrophoresing with aspartate and alanine after exposure of A. cylindrica to ^{13}N-N_2 in the presence of methionine sulfoximine, suggesting that these amino acids may be formed, at least in part, by aspartate dehydrogenase (1) and alanine dehydrogenase (1, 3, 5, 18). The formation of measurable quantities of these two amino acids after 1 min of fixation in the presence of methionine sulfoximine may have been a consequence of the augmentation in the concentration of NH_4^+ which results from the presence of the inhibitor.

Heterocysts, although they do not grow, fix much more N_2 per cell than do vegetative cells (10-15). In vivo, much of the nitrogen fixed by the heterocysts moves out from them (15). The lipid layer in the envelope of heterocysts probably prevents direct release of fixed nitrogen to the medium (39). We infer that nitrogen is transferred from heterocysts to vegetative cells. The size of the ammonia pool is very small (cf. also 40), and ammonia is very rapidly incorporated into glutamine. We therefore doubt that nitrogen moves from cell to cell principally in the form of ammonia. Carbon fixed by vegetative cells passes into heterocysts, where it is metabolized (41). Enzymological evidence (42, 43) supports the idea that part of the carbon moves in the form of a sugar or sugar derivative. Because there is very little glyceraldehyde phosphate dehydrogenase activity in heterocysts (42, 43), these cells may be unable to produce a large amount of carbon skeletons from sugars for the formation of glutamate and glutamine. We therefore suggest that the functioning of glutamine synthetase may be coupled to transfer of the carbon skeleton of glutamine (in the form of glutamate, α-ketoglutarate, or a precursor thereof) into heterocysts and to transfer

of glutamine and/or glutamate out of heterocysts as vehicles for net transfer of fixed nitrogen into vegetative cells.

The ^{13}N-labeled peak which we have detected in the absence of cyanobacteria presumably results from a reaction with water, because it forms when the labeled gas is in contact with distilled water, and because unlike the gas supplied, it remains following evacuation. We conclude that the peak contains ionic oxides of nitrogen, $^{13}NO_2^-$ and $^{13}NO_3^-$, on the basis of its distillation properties and its electrophoretic mobility. The nitrite and nitrate presumably account for the ca. 1% of total ^{13}N-fixation attributable to a nitrogen source other than N_2 (15). This interpretation is supported quantitatively by the present experiments in which growth medium was exposed to ^{13}N-N_2, the gas evacuated, cyanobacterial suspension then added to the medium, and the incorporation of ^{13}N into organic metabolites by the suspension measured. ^{13}N-labeled nitrite and nitrate may have been derived from trace quantities of ^{13}NO and/or $^{13}N_2$ (15).

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Figure 1. Scan of radioactivity from ^{13}N in electrophoretograms of organic compounds extracted from Anabaena cylindrica with 80% methanol after (A) 120 s, (B) 60 s, and (C) 20 s of fixation of ^{13}N-labeled nitrogen gas (ca. 0.02 atm) in immediately consecutive, and therefore directly comparable, experiments. The extracts, supplemented with ten unlabeled amino acids as markers, were applied to cellulose thin layer plates in a thin strip (origin = 0) 7 cm from the positive end (at the right) of the plate. Lipids (L) were displaced from the origin by chromatography in chloroform-methanol (3:1, v/v). The extracts were then subjected to electrophoresis at 3000 V for 12 min in 70 mM sodium borate buffer, pH 9.2. The plates were scanned at 2 cm/min (τ = 3 s) from - to +, and were then dried, and sprayed with a solution of ninhydrin to localize the marker amino acids. The ^{13}N-containing substances in (B) and (C) co-electrophoresed with glutamine and glutamate, as did the two major ^{13}N-containing substances in (A). The extract in (A) also contained ^{13}N-labeled substances which co-electrophoresed with stable arginine, citrulline and/or glycine, and (appearing as shoulders on major peaks) alanine and aspartate. A quantitative evaluation of electrophoretograms such as those of Fig. 1 is presented in Fig. 2.

Figure 2. Distribution of ^{13}N in organic substances soluble in 80% methanol after fixation of $^{13}N\text{-}N_2$ by A. cylindrica for varying periods of time. After 15, 30, 60, and 120 s of fixation, extraction, and electrophoresis at pH 9.2, radioactivity was quantified by integration of peak areas and/or by elution and liquid scintillation spectrometry, with corrections applied for decay. Values presented are means, ± standard deviation of the mean, of the fraction of organic-^{13}N co-electrophoresing with stable glutamine (o) and glutamate (•), and in all other organic compounds extracted (■).

Figure 3. Scans of radioactivity from ^{13}N following electrophoresis of compounds extracted from A. cylindrica with 80% methanol after 120 s of fixation of $^{13}N\text{-}N_2$. The experimental procedures were the same as those of Fig. 1, except that the origin was 3 cm from the positive end of the plate, and electrophoresis was for 6.5 min in 0.75 M formic acid, pH 2.0. The peak corresponding to NH_4^+ was scanned first (——). The peak corresponding in position to lysine, and the major peak were scanned 13.8 and 31.7 min, respectively, after the NH_4^+ peak, during subsequent scans (----, -·-·).

Figure 4. Scan of radioactivity from ^{13}N (——) in an electrophoretogram of organic compounds extracted from A. cylindrica after fixation of ^{13}N-N_2 for 60 s. Electrophoresis was performed as in Fig. 1, in the presence of ^{14}C-labeled standards, but for only 8 min. The supplemented extract was applied 8 cm from the negative (left-hand) end of the plate. Radiations from ^{14}C were blocked from the detector, during scanning of radioactivity from ^{13}N, with a 127-μm layer of aluminum. Once the ^{13}N had decayed to background, the aluminum was removed, and the thin layer plate rescanned for radioactivity (----) from ^{14}C-labeled glutamine (at left), glutamate (center) and carbamyl phosphate (right).

Figure 5. Scan of radioactivity from ^{13}N following two-dimensional separation of organic compounds extracted from A. cylindrica after fixation of ^{13}N-N_2 for 60 s. A majority of the extract, supplemented with stable and ^{14}C-labeled glutamine (GLN) and glutamate (GLU), was applied at an origin (O), and chromatographed with a lipid solvent to displace lipids and pigments (P). The substances applied were then subjected to electrophoresis for 7 min at pH 9.2, in the direction shown by the two-headed arrow (↔). A scan (similar to Figs. 1B and 3, but not shown) of the thin layer plate was then made at 5 cm/min (τ = 1 s), with 127 μm of aluminum between plate and slit, to localize the two bands of ^{13}N-radioactivity. The plate was then dried and subjected to chromatography for 16 min in phenol-water (3:1, v/v), equilibrated with a 3% solution of NH_4OH, in a direction (double-shafted arrows: =>) perpendicular to the direction of electrophoresis. The solvent front (F) is marked. The plate was then cut (at C_1, C_2, and C_3) to either side of the two original ^{13}N-containing bands. The remaining extract was applied as position-reference markers (R_1, R_2) close to the bottoms of the pieces of the thin layer plate. The pieces of thin layer plate were scanned for radioactivity from ^{13}N (——) parallel to the direction of chromatography. A second scan, five half-lives after the first (....) shows that radioactivity from ^{14}C is blocked from the detector. Subsequently, the aluminum foil was removed, the radioactivity from ^{14}C (----) was scanned, and the pieces of thin layer plate were sprayed with ninhydrin to visualize amino acids.

Figure 6. Scan of radioactivity from ^{13}N (——) in an electrophoretogram of compounds extracted from A. cylindrica with 80% methanol after 60 s of fixation of ^{13}N-N_2. The experimental procedures were the same as those of Fig 3 except that the extract was supplemented with ^{14}C-labeled glutamine and glutamate (which do not separate at pH 2.0), and electrophoresis was for only 5 min. After decay of the ^{13}N, the region of the peak was rescanned (····). The 127-μm layer of aluminum blocking the detector was then removed, and the electrophoretogram scanned for radioactivity from ^{14}C (----).

Figure 7. Time course of incorporation of ^{13}N into pools of NH_3 and amide nitrogen. After fixation of ^{13}N-N_2 for 15, 5, and (approximately) 1.25 s, the suspension of cyanobacteria was extracted with 80% methanol, and the extract subjected first to vacuum distillation at pH 10, to determine $^{13}NH_3$ (-o-), and then to steam distillation in the presence of 40% NaOH, to determine amide-^{13}N (-•-). The ordinate represents cpm $^{13}NH_4^+$ or cpm amide-^{13}N in the fixation vial (measured with approximately 74% counting efficiency), corrected to the time of the start of fixation, and normalized to equal amounts of ^{13}N (in μCi) in the vial. At the "1.25-s" time point, and after corrections for background (ca. 15 cpm) and time of decay, three separate samples of $^{13}NH_4^+$ distillate (uncorrected values: 1347, 1264, 1131 cpm) agreed to within 2% of their mean; three separate samples of amide-^{13}N (uncorrected values: 384, 339, 294 cpm) agreed to within 5% of their mean; and the mean value for $^{13}NH_4^+$ was 43% greater than the mean value for amide-^{13}N. The curves are extrapolated to the origin because negligibly little ^{13}N distilled as NH_3 or as amide nitrogen when used growth medium was exposed to ^{13}N-N_2 and then processed in place of a suspension of cyanobacteria.

Figure 8. Scans of radioactivity from ^{13}N in an electrophoretogram of organic compounds extracted from A. cylindrica with 80% methanol after 120 s of fixation of ^{13}N-N_2 in the presence of 1 mM azaserine. Electrophoresis and scanning were performed as in Fig. 1. First scan: ——; second scan, begun 14 min after the first: ----. Only a single ^{13}N-containing substance, co-electrophoresing with stable glutamine, was found. Stable glutamate was present at the 16.7-cm position, and azaserine at the 13.4-cm position, after electrophoresis.

Figure 9. Distribution of ^{13}N in organic substances extractable with 80% methanol when fixation of ^{13}N-N_2 for 15 s was followed by evacuation, and then by fixation of 99% N_2, 1% CO_2 for varying periods of time. Methanolic extracts were electrophoresed at pH 9.2, the thin layer plates scanned, and the peaks in the scans integrated and corrected for decay. After 45 s of chase, a very small shoulder, attributable to aspartate, was observed on, and is here added to, the glutamate peak (o); peaks appeared in the arginine (■) and citrulline (Δ) regions of the electrophoretograms; and an unknown substance (□), which migrated between arginine and alanine, was also labeled. The fraction of radioactivity in glutamine (●) decreased greatly during the chase.

Table 1. Effect of 1 mM methionine sulfoximine on the concentration of $^{13}NH_4^+$ in a suspension of Anabaena cylindrica exposed to $^{13}N\text{-}N_2$ for 1 min.

	cpm $^{13}NH_3$ [a]	mCi $^{13}N\text{-}N_2$ [b]	Ratio (cpm/mCi)	$\frac{(cpm/mCi),+MSX}{(cpm/mCi),-MSX}$
+MSX	254000 ± 2.6%	9.0	28200	18
-MSX	17200 ± 4.1%	10.7	1610	

a. Vacuum-distillable cpm in the extract (mean ± standard deviation of the mean of six measurements) corrected to the starting time of exposure to $^{13}N\text{-}N_2$

b. Radioactivity, in mCi, of the N_2 in the fixation vial at the start of fixation, calculated from the relationship: 1 R/h at 1 m corresponds to 1.7 Ci. The 1-ml vials contained the same amount of N_2, ca. 0.02 atmospheric ml

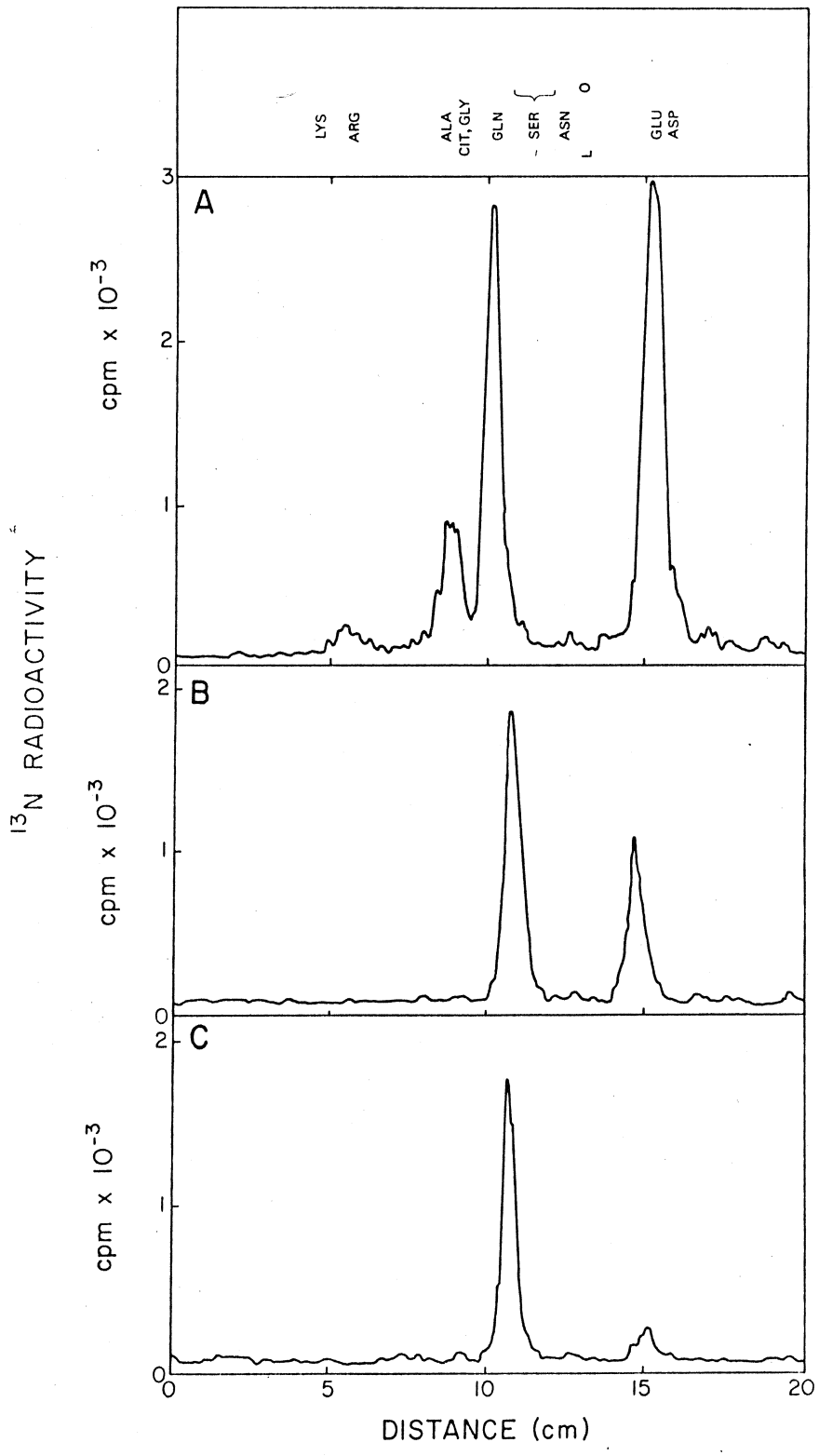
LYS
ARG
ALA
CIT, GLY
GLN
SER
ASN
O
L
GLU
ASP
A
B
C
cpm x 10-3
cpm x 10-3
cpm x 10-3
13N RADIOACTIVITY
0
1
2
3
0
1
2
0
1
2
0
5
10
15
20
DISTANCE (cm)

Fig. 1

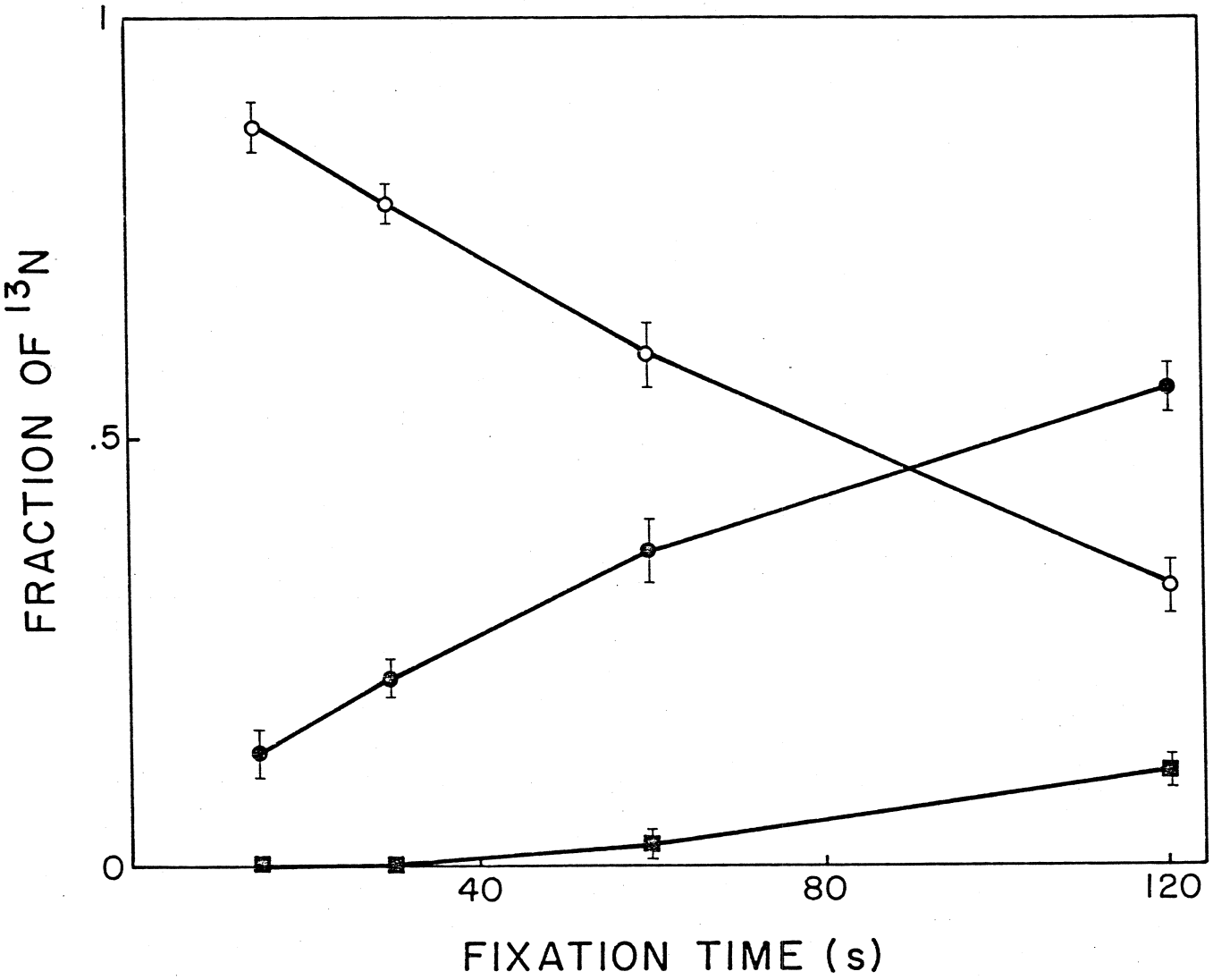

Fig. 2

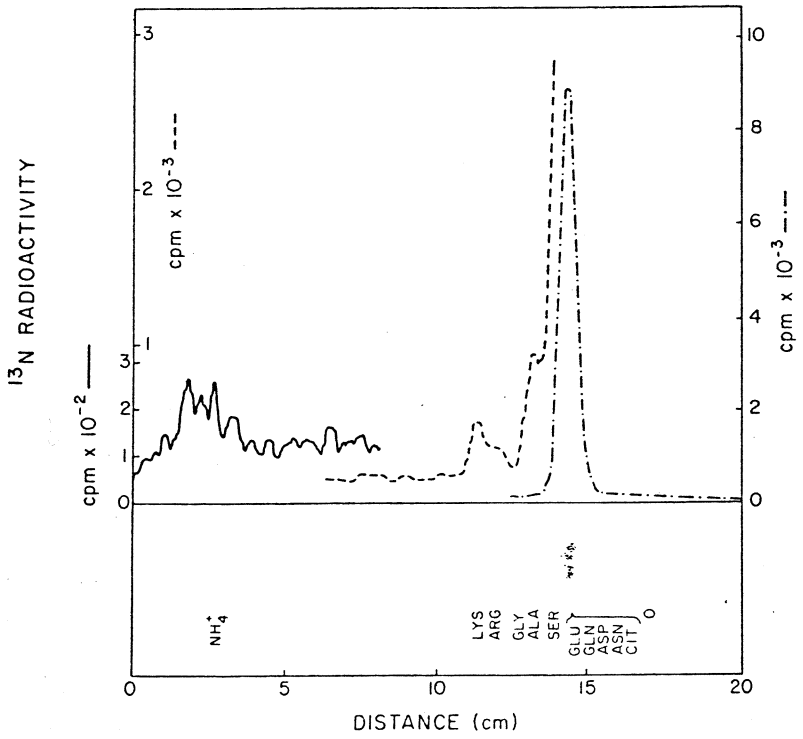
^{13}N RADIOACTIVITY
cpm x 10^{-2}
cpm x 10^{-3}
cpm x 10^{-3}
NH_4^+
LYS
ARG
GLY
ALA
SER
GLU
GLN
ASP
ASN
CIT
O
DISTANCE (cm)

Fig.3

^{14}C RADIOACTIVITY (cpm x 10^{-4})---
^{13}N RADIOACTIVITY (cpm x 10^{-3}) ——
DISTANCE (cm)
0
5
10
15
20
1
2
3

Fig. 4

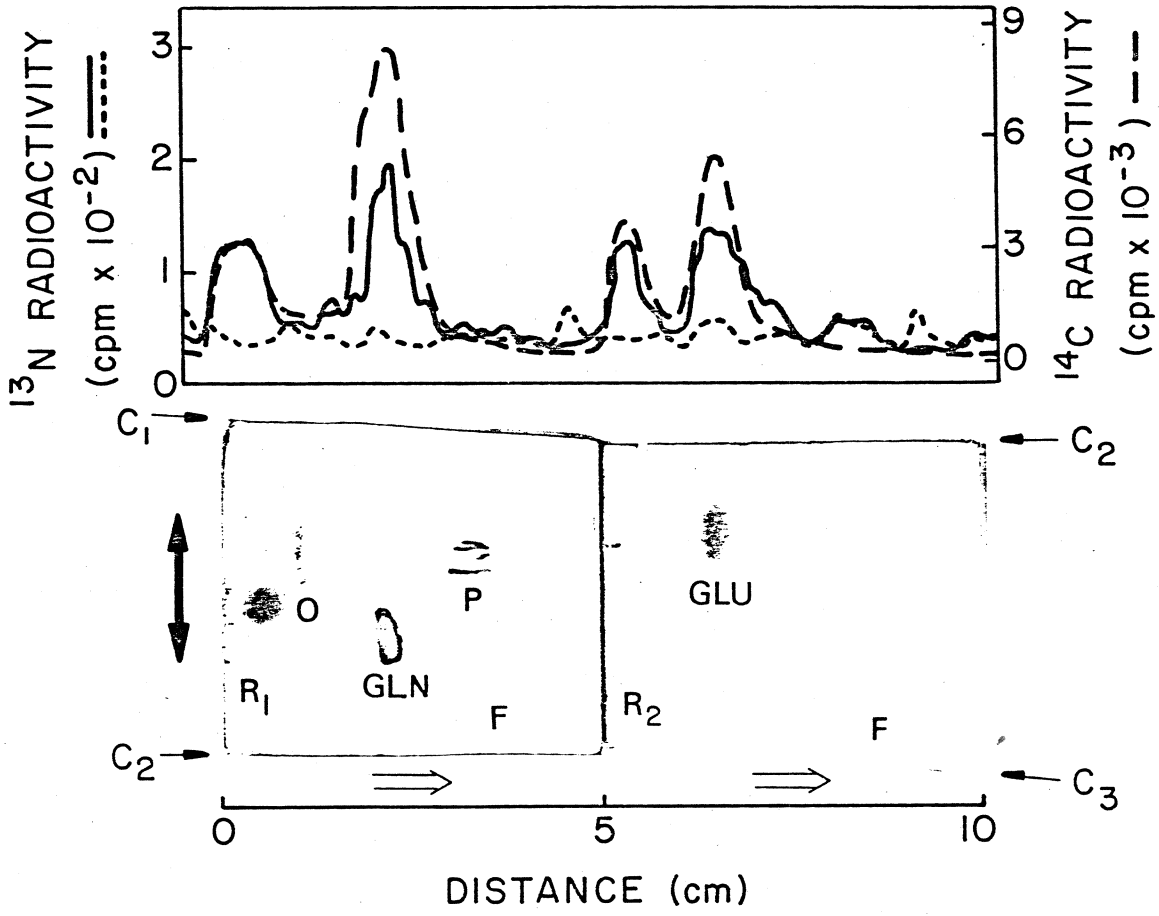
^{13}N RADIOACTIVITY (cpm x 10^{-2}) ------
^{14}C RADIOACTIVITY (cpm x 10^{-3}) — —
0
1
2
3
0
3
6
9
C_1
C_2
O
P
GLU
GLN
R_1
R_2
F
F
C_2
C_3
0
5
10
DISTANCE (cm)

Fig. 5

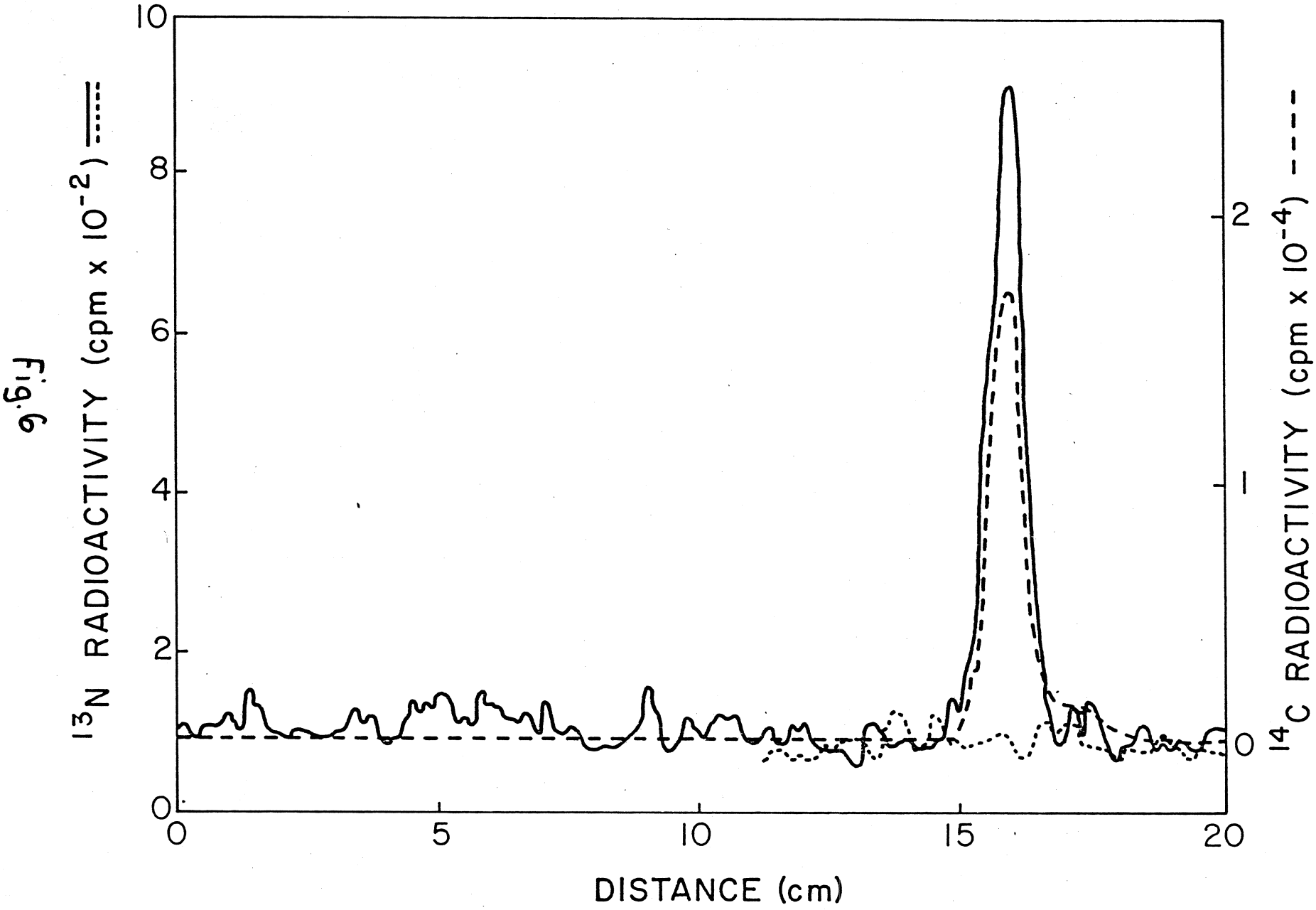
Fig. 6
^{13}N RADIOACTIVITY (cpm x 10^{-2}) ------
^{14}C RADIOACTIVITY (cpm x 10^{-4}) ----
DISTANCE (cm)
0
2
4
6
8
10
0
1
2
0
5
10
15
20

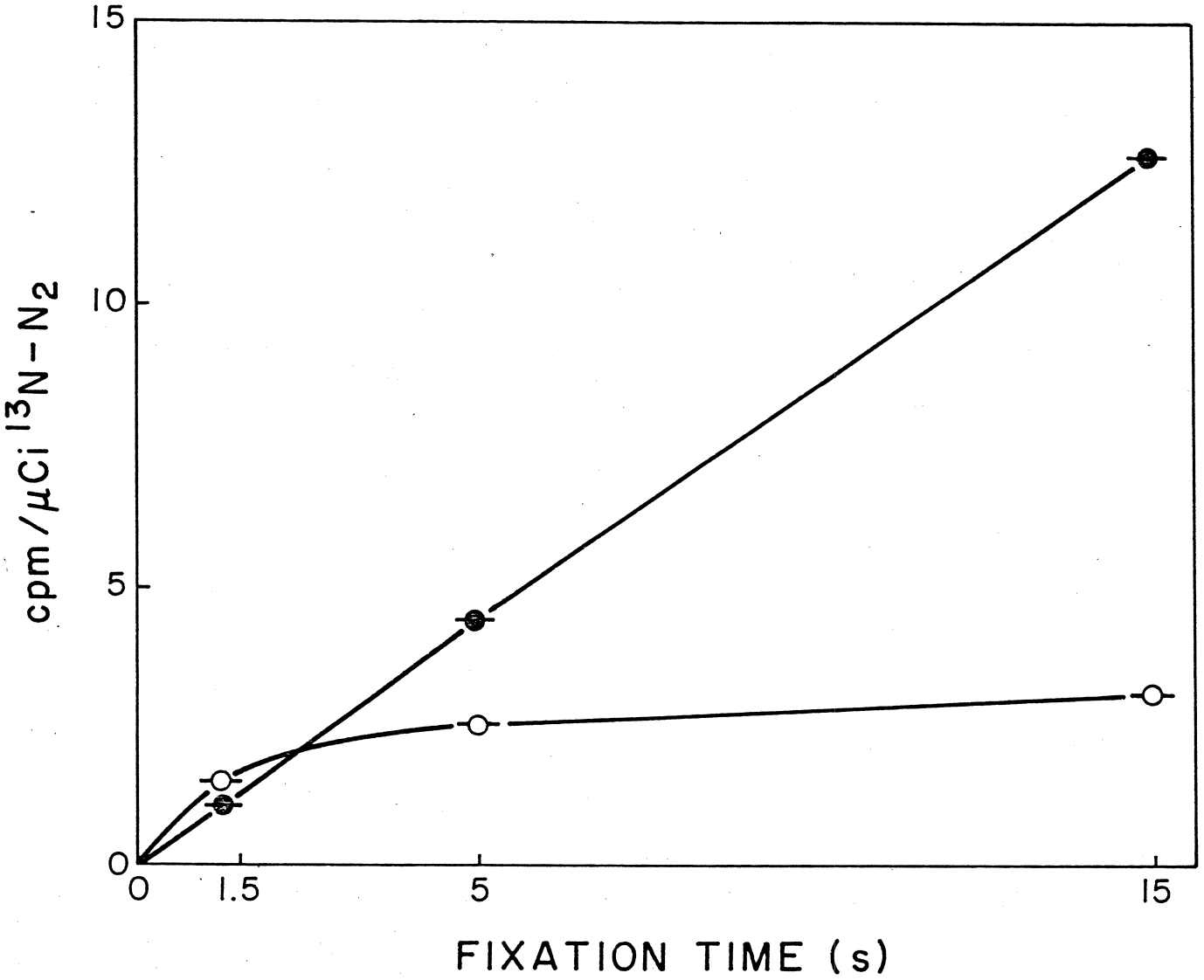

Fig. 7

^{13}N RADIOACTIVITY (cpm x 10^{-2}) ——
^{13}N RADIOACTIVITY (cpm x 10^{-3}) ----
0
3
6
9
0
5
10
15
20
DISTANCE (cm)

Fig. 8

Fig. 9