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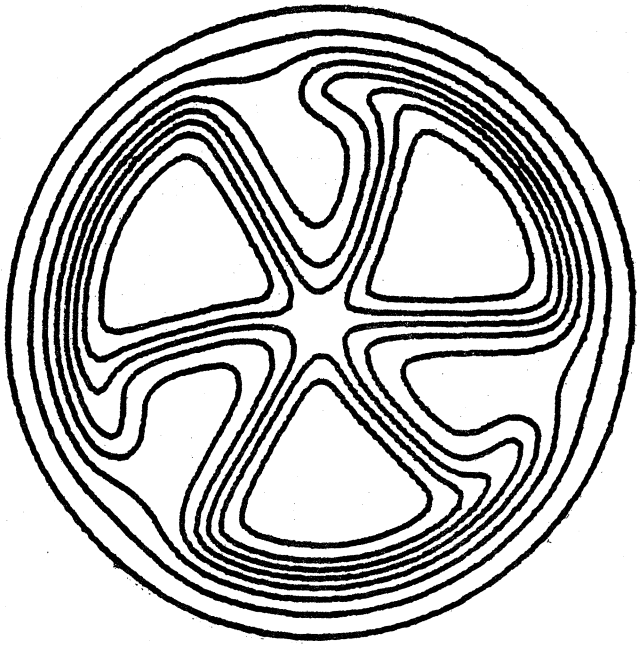

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THE INITIAL ORGANIC PRODUCTS OF FIXATION OF ^{13}N-LABELED NITROGEN GAS BY THE BLUE-GREEN ALGA ANABAENA CYLINDRICA

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SUMMARY

Methods have been developed for the rapid isolation and characterization of the first organic products of fixation of ^{13}N-labeled N_2. In experiments with the blue-green alga, Anabaena cylindrica, glutamine is the first ^{13}N-labeled organic product observed, and glutamate is the second. The results indicate that the glutamine synthetase/glutamate synthase pathway is operative in this blue-green alga.

The primary products of biological nitrogen fixation have been investigated earlier, using N_2 labeled with the stable isotope ^{15}N, in nitrogen-fixing aerobic (1) and anaerobic (2) bacteria and in legume nodules (3, 4). Although products of fixation of ^{15}N-labeled N_2 ($[^{15}N]N_2$) were examined only after a minimum of 45 s of fixation (3, 4), these studies suggested that ammonia was the first nitrogenous product of fixation. However, the conclusion that glutamate was the first organic product of nitrogen fixation is now open to doubt. Recent evidence favors the operation of

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the glutamine synthetase/glutamine amide: 2-oxoglutarate amido transferase (glutamate synthase or GOGAT) pathway (5) in nitrogen-fixing microorganisms (6).

Both of the enzymatic activities required for this pathway have been measured in extracts of Anabaena cylindrica (7, 8). In other efforts to determine enzymologically the pathway of assimilation of N_2-derived NH_3 in blue-green algae, activities of glutamic acid dehydrogenase (9, 10) and alanine dehydrogenase (9, 10, 11) were measured. Attempts (11) to employ $[^{15}N]N_2$ to determine the earliest products of nitrogen fixation by blue-green algae do not appear to have been entirely successful because several nitrogenous compounds had become labeled after 10 min, the shortest period of fixation reported. It has recently been shown that addition of methionine sulfoximine, an inhibitor of the glutamine synthetase from nitrogen-fixing bacteria (12), to cultures of A. cylindrica led after some hours to an increase in the secretion of NH_3, while the intracellular concentration of glutamine decreased greatly, and the concentration of glutamate was approximately halved. The concentrations of aspartate and of glycine plus alanine changed very little (13).

Wolk et al. have developed methodology for producing radioactive, ^{13}N-labeled N_2 ($[^{13}N]N_2$) of high specific activity and purity, and have employed this gas in studies of the localization of nitrogen fixation in filaments of A. cylindrica (14, 15). In this paper we identify the first organic products of nitrogen fixation by this blue-green alga, as determined by kinetic studies of $[^{13}N]N_2$ fixation in the time range of 15 s to 2 min.

MATERIALS AND METHODS

Anabaena cylindrica Lemm. was grown aerobically in the absence of combined nitrogen. Details of cultivation, sampling, concentration, and conditions of short-term (ca. 10 min) storage of algal material prior to use were as described earlier (15). One-ml vials containing $[^{13}N]N_2$ (ca. 0.02 atm) and filled to one atmosphere with a gas mixture containing 0.95%

CO_2, balance Ar (Matheson Scientific Div., Will Ross Inc., East Rutherford, N.J.) were prepared as reported (15). Algal suspension (0.25 ml, 27 µg chlorophyll/ml) was injected into such vials and was stirred in incandescent light (4000 lux), by magnetic bars, for periods of 15, 30, 60 or 120 s.

At the end of incubation the algal suspension was transferred in less than 1 s, through a stopcock in the base of the vial, into a centrifuge tube containing 1 ml of methanol. The centrifuge tube was flushed with the gas mixture to remove residual free $[^{13}N]N_2$, and the suspension then mixed for 1 min on a vortex micromixer and centrifuged at 1000 g for 2 min. The supernatant fluid was decanted and dried under vacuum at 50°C. The residue after drying was dissolved in 200 µl of 80% methanol, dried again as before, and redissolved in 50 µl of 80% methanol. Next, the extract was carefully spotted on an area of 2 x 10 mm on a 5 x 20 cm glass plate coated with a thin (0.1 mm) layer of cellulose (E. Merck, West Germany).

The lipid-soluble substances extracted by the methanol were found to interfere with further processing of the nitrogenous products. These substances 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 dried, sprayed with buffer, and subjected to electrophoresis at 150 V/cm for 5-12 min in a Shandon High Voltage Electrophoresis Apparatus, model Q11 SAE-3202 (Shandon Scientific Co., London, England). High-voltage thin-layer electrophoresis was the method of choice for initial separation because its rapidity permitted further analyses of the first organic products of fixation. Buffers used were 70 mM sodium borate ($Na_2B_4O_7.10H_2O$), pH 9.2, or 750 mM formic acid, pH 2.0 (17). In certain experiments, electrophoretic separation was followed by chromatography in phenol-water (3:1, v/v) saturated with ammonia vapor, at a right angle to the direction of electrophoresis. Stable (Sigma Chemical Co., St. Louis, Mo.) and ^{14}C-labeled (ICN Pharmaceuticals, Inc., Cleveland, Ohio, and New England Nuclear Corp., Boston, Mass.) amino acids were sometimes co-electrophoresed and co-chromatographed with the ^{13}N-labeled extracts. On completion of electrophoresis and/or chromatography, the plates were scanned for radioactivity with a Packard Radiochromatogram Scanner, model 7201 (Packard Instrument Co., Downers Grove, Ill.), using a 2.5-mm wide slit. In order to scan ^{13}N in the presence of ^{14}C, the detector slit was covered with a 127 µm layer of aluminum. After decay of the ^{13}N, the aluminum foil was removed, permitting the detection and scanning of ^{14}C. In experiments involving co-migration of stable amino acids, the plates were then sprayed with ninhydrin to locate the amino acids.

When desired, the glutamine and glutamate zones (see RESULTS) of the thin layer plate were scraped separately into 2-ml portions of water, mixed and centrifuged. Aliquots of the supernatant liquid were assayed for ^{13}N activity using a Beckman CPM 100 liquid scintillation counter (Beckman Instruments, Irvine, Calif.). The remaining solution of ^{13}N-labeled glutamine was supplemented with 40% NaOH, and the glutamine amide nitrogen hydrolyzed and distilled with steam (16). The $[^{13}N]NH_3$ released was trapped in a 4% solution of boric acid and assayed for radioactivity with the scintillation counter. Further details of experimental procedure will be published later.

RESULTS

The 10-min half-life of ^{13}N necessitated the development of rapid techniques for the isolation and separation of the likely primary products

of nitrogen fixation. Ten amino acids, aspartic acid, glutamic acid, asparagine, glutamine, citrulline, serine, glycine, alanine, arginine and lysine, plus carbamyl phosphate were examined for their mobility and separability in various one- and two-dimensional electrophoretic and chromatographic solvent systems. Electrophoresis with 70 mM sodium borate buffer, pH 9.2, at 150 V/cm, provided excellent separation of 8 of the 10 amino acids in 12 min on thin layers of cellulose (Fig. 1). Only glycine and citrulline overlapped. Carbamyl phosphate was separable at shorter times, but migrated off of the plate in 12 min.

After two min of fixation, at least six compounds had become labeled, but after 60 s of fixation, ^{13}N was detected in only two organic compounds, glutamine and glutamate. The identities of these amino acids were established by co-electrophoresis with ^{14}C-glutamine and ^{14}C-glutamic acid, in the presence of 10 stable amino acids, at pH 9.2 (Fig. 2) and at pH 2, and by co-chromatography (data not shown). The relative amounts of ^{13}N in these two amino acids were determined, following increasing periods of fixation, either by radioscanning of thin-layer electrophoretograms, or by liquid scintillation counting of ^{13}N eluted from the corresponding zones of electrophoretograms. As the period of fixation of $[^{13}N]N_2$ was reduced progressively to 15 s, an increasing percentage of the radioactivity appeared in glutamine, with much less in glutamate (Table 1).

When, after 1 min of fixation, glutamine was isolated by electrophoresis on and elution from the thin layer plates, and supplemented with 40% NaOH, up to 87% of its content of ^{13}N was steam-distillable as ammonia. After fixation of $[^{13}N]N_2$ for 1 min in the presence of 1 mM L-methionine-DL-sulphoximine, ^{13}N-labeled glutamine and glutamate were undetectable.

DISCUSSION

The development of suitable methodology has permitted the use of the radioisotope ^{13}N for detecting nitrogenous compounds formed after only 15 s of nitrogen fixation. Experiments with even shorter periods of fixation appear feasible.

The kinetics of ^{13}N-labeling of glutamine and glutamate (Table 1; cf. also Fig. 2) demonstrate that glutamine is the first detectable organic product, and glutamate the second. After short periods of fixation, the great majority of the ^{13}N in glutamine is located in the amide group. Methionine sulphoximine, which inhibits glutamine synthetase activity, greatly decreases assimilation of ^{13}N into organic metabolites. These results indicate that the glutamine synthetase/glutamate synthase pathway, for which the requisite enzymatic activities have been detected (7, 8) *in vitro*, is operative in *A. cylindrica in vivo*. That is, N_2-derived, fixed nitrogen is assimilated first as the amide nitrogen of glutamine, and is then transaminated to form the α-amino group of glutamate. Part of the glutamate is then recycled as a substrate for further synthesis of glutamine.

ACKNOWLEDGMENT

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Table 1. Ratio of glutamate to glutamine after short-term fixation of $[^{13}N]N_2$ by Anabaena cylindrica

Fixation time (seconds)	Glutamate/ glutamine*
15	0.19 ± 0.04 (3)
30	0.27 ± 0.07 (7)
60	0.52 ± 0.26 (5)
120	1.64 ± 0.37 (4)

* Mean values ± standard deviations; number of experiments is indicated in parentheses.

After fixation of $[^{13}N]N_2$, algal material was extracted with 80% methanol, and the extracts subjected to high voltage electrophoresis. For any particular experiment, the ^{13}N-labeled amino acids were located by scanning, and their radioactivity quantified from the scans and/or by elution and liquid scintillation counting.

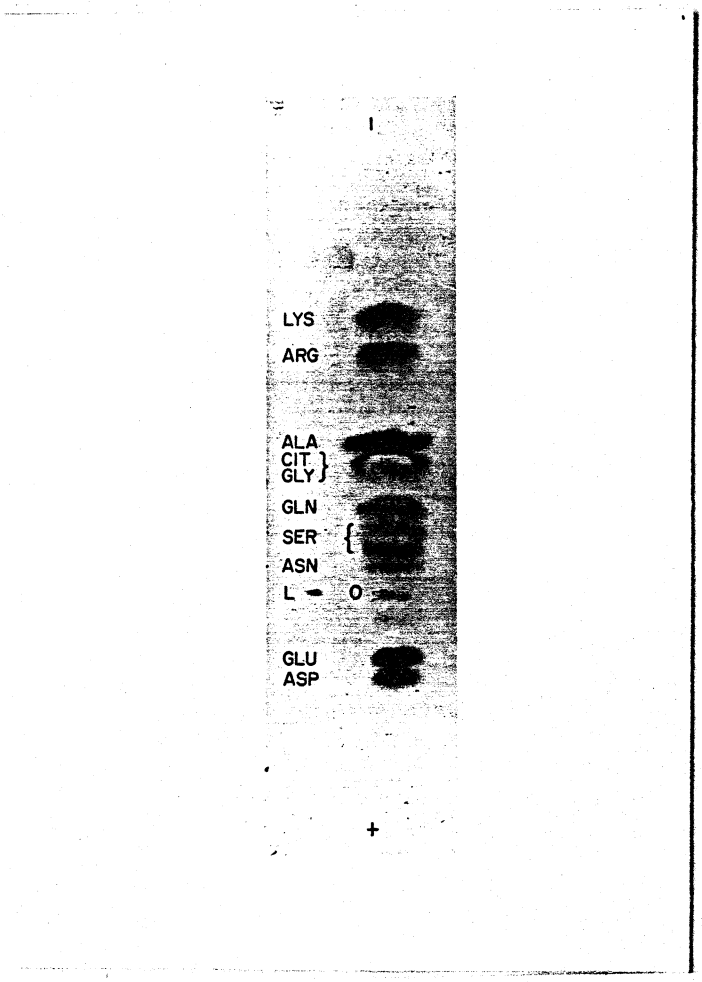

Fig. 1. A thin-layer electrophoretogram of an 80% methanolic extract of Anabaena cylindrica, with ten stable amino acids added to the extract. The supplemented extract, applied in a thin strip (at 0) 7 cm from the positive end (+) of a 5 x 20 cm thin layer (0.1 mm cellulose) plate was subjected to chromatography in chloroform-methanol (3:1, v/v) to displace lipids (L), and then to electrophoresis at 150 V/cm for 12 min in 70 mM sodium borate buffer, pH 9.2. The plate was dried, and sprayed with a 0.5% (w/v) solution of ninhydrin in a 1:1 (v/v) mixture of acetone and 0.7 M formic acid.

The direction of electrophoretic migration of glutamine, serine and asparagine varied from one production lot of thin-layer plates to another (compare Figs. 1 and 2), but the sequence of the ten amino acids remained constant.

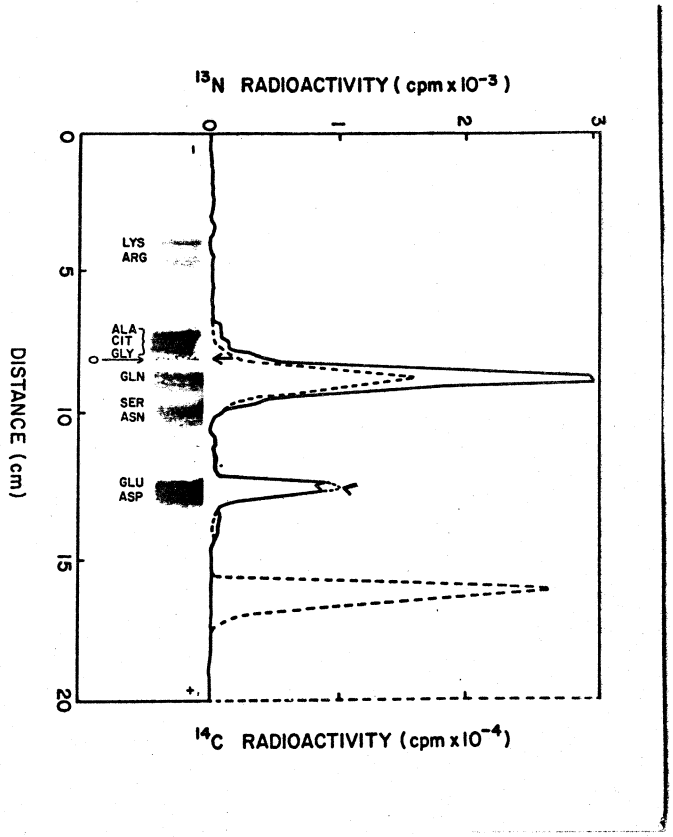

Fig. 2. Scan of radioactivity from ^{13}N (——) in an electrophoretogram of organic compounds extracted from Anabaena cylindrica with 80% methanol after 60 s of fixation of ^{13}N-labeled nitrogen gas. Electrophoresis was performed as in Fig. 1, but for only 8 min. The extract was applied 8 cm from the negative end (-) of the plate. (With regard to the direction of electrophoretic migration, see the legend of Fig. 1.) Arbitrarily chosen amounts of glutamine, glutamate and (at the 17-cm position in the figure) carbamyl phosphate, all labeled with ^{14}C, and ten stable amino acids were co-electrophoresed, as markers. The marking ∨ indicates the height of the peak of ^{13}N-labeled glutamate, corrected for decay to the time of appearance of the peak of glutamine. The thin layer plate was scanned for radioactivity from ^{13}N using a 127 μm cover of aluminum to block ^{14}C-radiations from the detector. After decay of the ^{13}N, the aluminum foil was removed, and the plate re-scanned for radioactivity from ^{14}C (- - -).