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Assimilation of $^{13}NH_4^+$ by *Azospirillum brasilense* Grown under Nitrogen Limitation and Excess

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The specific activities of glutamine synthetase (GS) and glutamate synthase (GOGAT) were 4.2- and 2.2-fold higher, respectively, in cells of *Azospirillum brasilense* grown with N_2 than with 43 mM NH_4^+ as the source of nitrogen. Conversely, the specific activity of glutamate dehydrogenase (GDH) was 2.7-fold higher in 43 mM NH_4^+-grown cells than in N_2-grown cells. These results indicate that NH_4^+ could be assimilated and that glutamate could be formed by either the GS-GOGAT or GDH pathway or both, depending on the cellular concentration of NH_4^+. The routes of in vivo synthesis of glutamate were identified by using ^{13}N as a metabolic tracer. The products of assimilation of $^{13}NH_4^+$ were, in order of decreasing radioactivity, glutamine, glutamate, and alanine. The formation of [^{13}N]glutamine and [^{13}N]glutamate by NH_4^+-grown cells was inhibited in the additional presence of methionine sulfoximine (an inhibitor of GS) and diazooxonorleucine (an inhibitor of GOGAT). Incorporation of ^{13}N into glutamine, glutamate, and alanine decreased in parallel in the presence of carrier NH_4^+. These results imply that the GS-GOGAT pathway is the primary route of NH_4^+ assimilation by *A. brasilense* grown with excess or limiting nitrogen and that GDH has, at best, a minor role in the synthesis of glutamate.

The heterotrophic, microaerobic diazotroph *Azospirillum brasilense* has been found in abundant numbers in the rhizosphere of specific grasses (1) as well as in the intercellular spaces in the roots of certain cereals (15). These relationships are viewed as associative symbioses (6), in which the bacteria receive nonspecific photosynthate carbon from the plant and, in turn, provide the plant with fixed nitrogen.

In addition to nitrogenase (3, 5, 13), the nitrogen-metabolizing enzymes glutamine synthetase (GS; EC 6.3.1.2) (4, 5, 14), glutamate synthase (GOGAT; EC 1.4.1.13) (2, 14, 17), and glutamate dehydrogenase (GDH; EC 1.4.1.2) (3, 14) have been detected in extracts of *A. brasilense*. The observations that the activity of GS decreases and that the activity of GDH increases in cells grown with NH_4^+ relative to N_2 supported the idea that the route of glutamate formation by *A. brasilense* varies with the amount of assimilable nitrogen in the environment (14); at low concentrations of available NH_4^+ (growth on N_2), the GOGAT cycle (sequential activities of GS and GOGAT) is thought to predominate, while the GDH route is the primary reaction at high NH_4^+ concentrations. Such NH_4^+ concentration-dependent changes in enzymatic pathways of glutamate synthesis have been well documented in bacteria (20) and originate from the studies of Meers et al. (11). The idea of two pathways of glutamate formation in *A. brasilense* was reinforced by the isolation of mutants that had low GOGAT activities and were capable of growth in NH_4^+ medium but that were pleiotrophic in their inability to grow with N_2 and NO_3^- as well as with organic forms of nitrogen, including glutamate (2). However, the mutants also failed to derepress the synthesis of GS and nitrogenase (2), thus implying possible regulatory alterations that complicate an analysis of metabolic pathways.

In this study we determined the specific activities of GS, GOGAT, and GDH in crude extracts of *A. brasilense* cells grown with different sources of nitrogen and correlated these activities with the products of $^{13}NH_4^+$ assimilation. We conclude that the GOGAT cycle is the predominant route of NH_4^+ assimilation by *A. brasilense* irrespective of the nitrogen source for growth and that GDH functions primarily in a dissimilatory manner.

MATERIALS AND METHODS

Cultures. *A. brasilense* Sp7 was obtained from the American Type Culture Collection, Rockville, Md., as ATCC 29145. Cultures were grown in basal salts medium DD (21) supplemented with 0.5% (wt/vol) L-glutamate (DDGlu) or L-glutamine (DDGln) or 43 mM NH_4^+ (DDN). When NH_4^+ was present as the nitrogen source, the carbon source was 0.5% (wt/vol) L-malate (DDNM), L-glutamate (DDNGlu), or L-glutamine (DDNGln) or combinations of 0.5% (wt/vol) L-malate and 0.1% (wt/vol) L-glutamate (DDNMGlu) or 0.5% (wt/vol) L-malate and 0.1% (wt/vol) L-glutamine (DDNMGln). When cells were grown with N_2 as the nitrogen source, 0.5% (wt/vol) L-malate was the carbon source (DDM); in most experiments the medium for N_2-dependent growth was also supplemented with low levels (0.005% [wt/vol]) of yeast extract (DDMYE) (Difco Laboratories, Detroit, Mich.). In experiments in which N_2 served as the nitrogen source, the cultures were sparged with nitrogen gas containing 1 to 2.5% (vol/vol) O_2. The concentrations of gases were obtained by mixing the gases in a model 7351 flow meter (Matheson Gas Products, Joliet, Ill.) and passing them through 0.45-μm-pore-sized in-line filters (Millipore Corp., Bedford, Mass.) before humidification and introduction into cultures. Growth of cultures incubated microaerobically with N_2 was started in 600 ml of DDMYE medium in 1-liter

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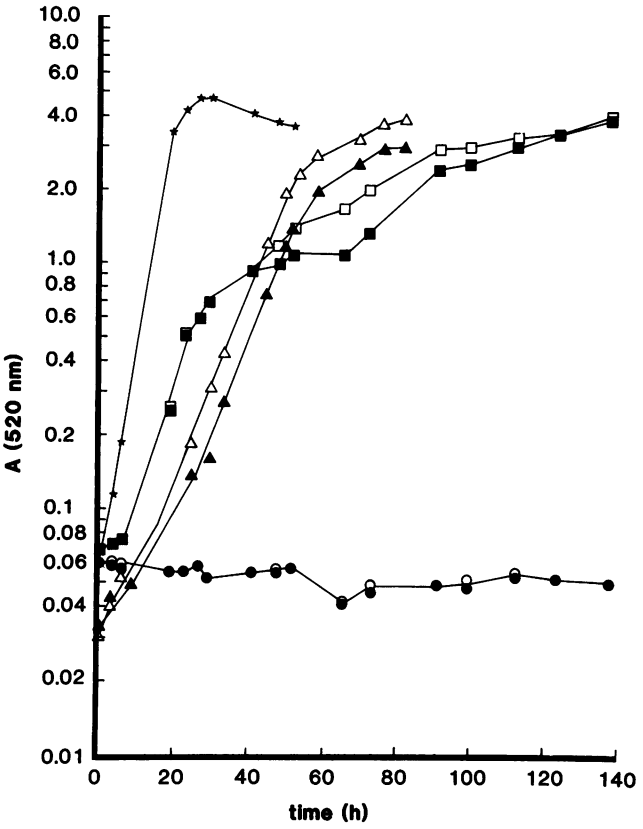

FIG. 1. Growth of *A. brasilense* Sp7 on glutamine and glutamate as sources of carbon or sole sources of carbon and nitrogen in comparison with malate as the source of carbon. The concentrations of glutamine, glutamate, and malate were 0.5% (wt/vol) and, when added, NH_4^+ was 43 mM; all were used in basal medium (DD). Symbols: ■, DDGlu; □, DDNGlu; △, DDGln; ▲, DDNGln; ★, DDNM; ●, DD; ○, DDN.

flasks; incubation was at room temperature (ca. 25°C). Aerobic cultures for enzyme assays and radiolabeling experiments were started in 250 ml of DD medium in 1-liter flasks and shaken at 150 rpm on a gyratory shaker; incubation was at room temperature. Growth experiments were done in batch cultures (100 ml of medium in 300-ml flasks) by monitoring changes in optical density at 520 nm with a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). These flasks were incubated at 30°C with shaking (150 rpm), and the readings were made directly at low cell densities or after dilution at high cell densities.

Enzyme assays. When culture densities reached 3×10^8 to 1×10^9 CFU ml^{-1} (21), the cells were harvested by centrifugation at 8,000 × *g* for 10 min at 5°C. The pellet fractions were washed twice in 50 mM Tris hydrochloride (pH 8.0) by centrifugation as described above and resuspended in the same buffer. Crude cell extracts were obtained by sonic cavitation by using either a model W-225R apparatus (Heat Systems Ultrasonics, Inc.) with a microprobe at an output of about 80 W or a model S-110 apparatus (Branson Sonic Power Co., Danbury, Conn.) at 110 W. The cavitated suspensions were clarified by centrifugation at 12,000 × *g* for 15 min at 5°C, and the supernatant fraction was immediately used for assays.

The assay of GS activity was the transferase reaction described by Stadtman et al. (19) and subsequently modified by Kustu and McKereghan (7). GOGAT and GDH activities were monitored by pyridine nucleotide oxidation in α-ketoglutarate-glutamine-, and NADPH-dependent (GOGAT) reactions or α-ketoglutarate-, NH_4^+-, and NADH-dependent (GDH) reactions as described by Meers et al. (11). All assays were done at room temperature (ca. 25°C).

Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as a standard. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Assimilation of $^{13}NH_4^+$. ^{13}N was generated at the Crocker Nuclear Laboratory, University of California, Davis, by the $^{16}O(p,\alpha)^{13}N$ reaction (9). The major product, $^{13}NO_3^-$, was concentrated and converted to $^{13}NH_3$ by using the Devarda alloy (10). $^{13}NH_3$ was vacuum distilled and trapped in 2 ml of 5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH adjusted to 3.4 by the inclusion of 7 mM formic acid; the pH of the distillate was adjusted to 7.0, and the volume was increased as necessary with distilled water (10). The radioactivity (15 to 50 mCi), radiochemical purity (>99.9%), and carrier NH_4^+ concentration (105 μM) were determined as described before (10).

Approximately 60 min prior to exposure to $^{13}NH_4^+$, 4 to 6 ml of exponentially growing cultures of *A. brasilense* were harvested by centrifugation at 7,000 × *g* for 5 min at 20°C; the pellets were washed once with DDM medium that had been diluted 10-fold with sterile distilled water and were suspended to about 5×10^8 CFU ml^{-1} (ca. twice the original cell density) in the 10-fold-diluted DDM medium, with or without supplements. In certain experiments, at about 30 to 60 min before exposure to $^{13}NH_4^+$, one of the following compounds was added to the cell suspensions at the stated final concentration: 0.5 to 1 mM L-methionine-DL-sulfoximine (MSX, an irreversible inhibitor of GS; 18) or 1 mM 6-diazo-5-oxo-L-norleucine (DON, an irreversible inhibitor of glutamine amide transfer reactions; 16). Assimilation took place in air in 15-ml conical centrifuge tubes placed on a gyratory shaker at room temperature. The reaction was initiated by adding 0.25 ml of $^{13}NH_4^+$ solution to 0.25 ml of cell suspension and was terminated after 2 to 5 min of incubation by the addition of 2 ml of methanol coupled with vigorous micromixing. The methanolic extracts were processed for separation of amino acids by thin-layer electrophoresis and quantitation by radioelectrophoretogram scanning as described previously (22), except that the chromatography prior to electrophoresis was done in chloroform-methanol at a 4:1 ratio.

TABLE 1. Specific activities of GS, GOGAT, and GDH in extracts of *A. brasilense* grown with limiting (N_2) or excess (NH_4^+) inorganic nitrogen and with glutamate and glutamine

Growth conditions[a]	Sp act[b] of:			GDH/GOGAT ratio
	GS	GOGAT	GDH	
N_2, malate	0.991	0.084	0.021	0.25
NH_4^+, malate	0.236	0.039	0.057	1.46
Glu	0.265	0.024	0.067	2.79
Glu, NH_4^+, malate	0.468	0.029	0.037	1.28
Gln	0.257	0.029	0.062	2.14
Gln, NH_4^+, malate	0.520	0.032	0.060	1.88

[a] Glutamate (Glu) and glutamine (Gln) concentrations were 0.5% (wt/vol) when used as sole sources of carbon and nitrogen; they were 0.1% (wt/vol) when media were supplemented with malate at 0.5% (wt/vol). NH_4^+ was 43 mM. DD basal salts were used in all instances.

[b] Reported as micromoles $minute^{-1}$ milligram of $protein^{-1}$, assayed as described in Materials and Methods. The values reported are means of two to six replicates taken from at least two separately grown cultures.

TABLE 2. Radioactive products of assimilation of $^{13}NH_4^+$ by *A. brasilense* grown with limiting (N_2) or excess (NH_4^+) nitrogen

Nitrogen for growth[a]	Supplements to incubation medium[b]	cpm in compound/10^3 cpm of $^{13}NH_4^+$ added mg of protein^{-1}[c]			
		Glu	Gln	Ala	Total
NH_4^+	None	88.8 ± 18.9	160.9 ± 36.9	35.0 ± 7.4	284.0 ± 57.0
NH_4^+	1 mM MSX	14.2 ± 2.4 (84)	6.5 ± 3.1 (96)	9.0 ± 5.0 (74)	30.0 ± 2.0 (90)
NH_4^+	1 mM DON	10.0 ± 4.0 (89)	41.9 ± 7.1 (74)	8.3 ± 3.2 (76)	60.3 ± 7.9 (79)
NH_4^+	1 mM NH_4^+	5.7 ± 2.0 (94)	6.6 ± 7.0 (96)	4.2 ± 1.7 (88)	16.5 ± 2.6 (94)
N_2	None	47.3 ± 3.7	169.3 ± 38.8	16.1 ± 3.2	224.9 ± 54.4
N_2	0.5 mM MSX	15.8 ± 2.8 (67)	16.8 ± 2.4 (90)	4.1 ± 0.5 (75)	36.7 ± 5.6 (84)

[a] The carbon source for growth in DD basal salts was malate at 0.5% (wt/vol); NH_4^+ was 43 mM, and N_2 was 0.78 to 0.98 atm (ca. 79.0 to 99.3 kPa) under microaerobic growth conditions.

[b] MSX and DON were added 30 to 60 min before exposure to $^{13}NH_4^+$; carrier NH_4Cl was added at the same time as $^{13}NH_4^+$.

[c] Incubation times were 2 and 5 min for NH_4^+ and N_2 cultures, respectively. The values were determined by integration of peaks in radioscans, with decay corrected to a standard time, and are expressed as the amount of ^{13}N present in a constituent normalized to the amount of $^{13}NH_4^+$ added and to cell protein; they represent the means ± standard errors of the means of two to four replicates. Glu, Glutamate; Gln, glutamine; Ala, alanine; Total, sum of the radioactivity recovered in methanolic extracts. Values in parentheses represent the percent inhibition of no addition (supplement).

RESULTS

Growth experiments. The results presented in Fig. 1 establish that both glutamate and glutamine can serve as carbon and nitrogen sources for the growth of *A. brasilense*. When glutamate or glutamine served as only a source of carbon, the growth rates were 1.9- to 2.3-fold lower than that supported by malate. The rates of growth and yield of cells at the stationary phase in batch cultures were similar regardless of whether glutamate or glutamine served as a source of both carbon and nitrogen or only carbon. However, it took longer for cultures grown with glutamate than with glutamine as the carbon source to reach the final cell density. There was no apparent growth in the absence of a source of organic carbon.

Enzyme activities. The specific activities of GS, GOGAT, and GDH in crude extracts of cells grown with various sources of carbon and nitrogen are shown in Table 1. The specific activity of the transferase reaction of GS was highest, by a factor of 3.7 to 4.2, in extracts of cells grown with N_2 as the nitrogen source and malate as the source of carbon, relative to cells grown with malate plus NH_4^+ or with glutamate or glutamine as the sole source of carbon and nitrogen. When the medium was supplemented with glutamate or glutamine in the additional presence of NH_4^+ and malate, the GS transferase specific activity was about 50% that of N_2-grown cells.

The specific activity of GOGAT was also higher, by a factor of 2.2, in cells grown with N_2 and malate than in cells grown with NH_4^+. The GOGAT activity did not vary markedly in extracts of cells from all other growth conditions and was similar to that in NH_4^+-grown cells.

The variations in the specific activity of GDH were nearly inverse to those of GOGAT. That is, GDH activity was low in N_2-grown cells and increased up to 2.9-fold in cells grown with NH_4^+, glutamate, or glutamine. The exception was a less-than-2-fold increase in cells grown with malate plus glutamate and NH_4^+.

$^{13}NH_4^+$ assimilation. Radioactivity was associated with glutamine, glutamate, and alanine after the assimilation of $^{13}NH_4^+$ for 2 and 5 min, respectively, by NH_4^+- and N_2-grown cells of *A. brasilense* (Table 2). These products were identified by their comigration with nonradioactive standards during electrophoresis at pH 9.2 (9, 10, 22). Glutamine accounted for 56 and 75% of the total radioactivity recovered in NH_4^+- and N_2-grown cells, respectively. In cells grown with either nitrogen source, glutamate was always the next highest radioactive compound, followed by alanine.

The assimilation of $^{13}NH_4^+$ into total organic metabolites by cells grown with both N_2 and NH_4^+ was lowered in the presence of 0.5 to 1.0 mM MSX (Table 2). The radiolabeling of glutamine was inhibited 90 to 96% by MSX, while there was a somewhat lower inhibition of ^{13}N incorporation into glutamate (67 to 84%) or alanine (75%). Exposure of NH_4^+-grown cells to 1 mM DON also lowered the total assimilation of $^{13}NH_4^+$ (Table 2). The formation of [^{13}N]glutamate and [^{13}N]alanine was inhibited to a similar extent by the presence of DON or MSX, but [^{13}N]glutamine formation was slightly less inhibited by DON. Inclusion of 1 mM NH_4^+ in the incubation medium resulted in a 94% decrease in the total assimilation of $^{13}NH_4^+$, and the incorporation of ^{13}N into the respective amino acids decreased in parallel (Table 2).

DISCUSSION

On the basis of the following lines of evidence from the two experimental approaches of in vitro enzyme assays and in vivo ^{13}N radiolabeling of metabolic intermediates, we conclude that *A. brasilense* assimilates NH_4^+ primarily by the GOGAT cycle, irrespective of the nitrogen source for growth.

The model of two routes for the steady-state synthesis of glutamate, depending on the cellular concentration of NH_4^+, was proposed from studies with *Klebsiella pneumoniae* (12), *Enterobacter aerogenes* (11), and *Pseudomonas fluorescens* (11). In these organisms, the differences in the specific activity of GDH and in the ratio of GDH and GOGAT activities were shown to be greater than 25-fold when growth was compared under limiting to excess NH_4^+, with GDH being predominant at high NH_4^+ concentrations. Although an increase of about 2.7-fold was observed in the GDH activity of NH_4^+-grown *A. brasilense* as compared with N_2-grown *A. brasilense* (Table 1), with an accompanying increase of 5.8-fold in the ratio of GDH/GOGAT activities, the differences were relatively small. These results are similar to those reported for *Clostridium pasteurianum* (12) and *A. brasilense* by others (3, 14, 17). Thus, with respect to NH_4^+ concentration-dependent changes in enzyme specific activities, there is only equivocal evidence for two major routes of glutamate formation in *A. brasilense*.

The results of in vivo ^{13}N labeling experiments showed that glutamine and glutamate were the most highly radioactive metabolites after short incubation times. The extensive accumulation of radioactivity in glutamine is indicative of active assimilation by GS in cells grown with both limiting and excess nitrogen (Table 2). However, [^{13}N]glutamate

could have been formed by either GDH or GOGAT. To distinguish these possibilities, we examined the products of $^{13}NH_4^+$ assimilation in the presence of the analogs MSX (an inhibitor of GS; 18) and DON (an inhibitor of GOGAT; 16). MSX inhibited the formation of [^{13}N]glutamine by 90% (N_2-grown cells) and 96% (NH_4^+-grown cells) and of [^{13}N]glutamate by 67% (N_2-grown cells) and 84% (NH_4^+-grown cells). The lower inhibition of [^{13}N]glutamate formation by MSX implies that a fraction of its synthesis could be dependent on GDH. However, the 89% inhibition of [^{13}N]glutamate formation by DON confirms that, even in NH_4^+-grown cells with high in vitro GDH activity (Table 1), only a minor fraction of glutamate is formed by GDH-catalyzed direct amination. The 74% inhibition of radiolabeling of glutamine in the presence of DON presumably resulted from depletion of the pool of glutamate as a consequence of inhibition of GOGAT activity (9). The results of these experiments support the model of the formation of glutamate primarily by the GOGAT cycle by *A. brasilense* grown with both limiting and excess nitrogen.

A further distinguishing characteristic of the GOGAT cycle as compared with the GDH route in the formation of glutamate is the affinity of the primary assimilating enzyme for NH_4^+; GS has considerably greater affinity for NH_4^+ than does GDH (20). The addition of 1 mM NH_4^+ to the $^{13}NH_4^+$, incubation solution resulted in a parallel decline in the radiolabeling of glutamine and glutamate in NH_4^+-grown cells of *A. brasilense* (Table 2). This result supports the model of assimilation primarily by the GOGAT cycle.

The observation that the formation of [^{13}N]alanine decreased in parallell to decreases in the radiolabeling of glutamine and glutamate (Table 2) implies that alanine was most probably synthesized principally from glutamate in an aminotransferase reaction rather than by direct amination in *A. brasilense*.

We conclude from the radiolabeling studies that, under our experimental conditions, GDH has only a minor role in the biosynthesis of glutamate in *A. brasilense*. Either glutamine or glutamate supports the growth of *A. brasilense* in the absence of other sources of carbon and nitrogen (Fig. 1). Thus, a physiological role for GDH in these cells could be catabolic.

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