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Effect of the Nitrogen Source on Glutamine and Alanine Biosynthesis in *Neurospora crassa*

AN *IN VIVO* ^{15}N NUCLEAR MAGNETIC RESONANCE STUDY*

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The influences of different nitrogen sources on the relative rates of biosynthesis of glutamine and alanine have been studied by ^{15}N nuclear magnetic resonance spectroscopy of intact *Neurospora crassa* mycelia suspensions. The rate of glutamine synthesis was fastest after growth in media deficient in free ammonium ion, whereas it was slowest following growth in media containing both glutamic acid and glutamine. The reverse trend was observed for the biosynthesis of alanine. A competition between the two biosynthetic pathways for the same substrate, glutamic acid, was found to limit the rate of alanine synthesis when glutamine synthesis was rapid. The observed *in vivo* rates of these reactions are compared to the reported specific activities of the enzymes catalyzing the reactions, and implications of these results for nitrogen regulation of these pathways under various physiological conditions are discussed.

For *Neurospora crassa*, as for most microorganisms, glutamic acid and glutamine play crucial roles in assimilating ammonia and acting as nitrogen donors for the synthesis of other metabolites (1). The biosynthetic pathways of glutamic acid and glutamine are shown in Fig. 1. Glutamic acid serves as the donor of the α-amino group for other amino acids. Among these, alanine is one of the most abundant amino acids in *N. crassa*, but little is known about the physiological function of the free alanine pool or regulation of its biosynthesis (2). Glutamine, on the other hand, is important not only as the nitrogen donor for a large number of other metabolites, but also for its possible role in the regulation of other nitrogen-related enzymes. It has been proposed that glutamine is responsible for "nitrogen metabolite repression" in *N. crassa*, a coordinated mechanism of control wherein a number of nitrogen-related enzymes are repressed by a common metabolite under conditions of nitrogen sufficiency (3). Thus, glutamine may serve as an intracellular indicator of the degree of availability of nitrogen and, in this way, assist the organism in regulation of other nitrogen-related enzymes for maximum cellular economy.

In view of their crucial roles in nitrogen metabolism, it is important to understand how biosynthesis of these amino acids is regulated as a function of the nature and concentrations of the various possible nitrogen sources. Beside the obvious influence of intracellular concentrations of substrates and cofactors, the rate of a particular metabolic pathway depends on (i) inhibition or activation of regulatory enzymes in the pathway, and (ii) changes in enzyme concentrations as the result of induction or repression. These levels of metabolic control are normally studied with purified enzymes *in vitro* or in cell-free extracts. For example, glutamine synthetase of *N. crassa* has been shown to be subject to cumulative feedback inhibition by end products such as ADP, L-histidine, and CTP (4, 5). The effect of growth on different nitrogen sources on the induction and repression of glutamate dehydrogenase (6, 7), glutamine synthetase (8–10), and glutamate synthase (11) in *N. crassa* has been studied by measurements of their specific activities in cell-free extracts. Valuable as such experiments are, specific activities in extracts are not a sure indication of the role of the enzyme in living microorganisms, nor can the relative flow of metabolites through alternate or competing pathways be judged from enzyme activities. A different approach is required to observe the rates of given pathways in living microorganisms and to evaluate which of the different levels of control (normally studied separately) is important in regulating the rates of given pathways under physiological conditions.

NMR spectroscopy is a useful technique for observing the flow of metabolites through pathways *in vivo*, as has been demonstrated by ^{13}C-NMR studies of glycolysis and gluconeogenesis in bacteria and mammalian liver (12–14). For example, the flux of glutamate through the glutamine synthetase pathway in mammalian liver can be studied from the time lag in the appearance of a specific ^{13}C labeling pattern in glutamine compared to glutamate (15). For observation of the course of ammonium assimilation in living systems, ^{15}N nuclear magnetic resonance spectroscopy is particularly important. The feasibility of observing small metabolites such as amino acids in intracellular fluids of intact mycelial suspensions of *N. crassa* by ^{15}N-NMR has recently been demonstrated (16, 17), and nitrogen metabolism in plants and lyophilized mycelia of *N. crassa* has been studied by solid state ^{15}N-NMR (18, 19). We report here the use of ^{15}N-NMR to study the influence of different nitrogen sources in the medium on the relative rates of biosynthesis of glutamine and alanine in intact *N. crassa* and the concomitant information the results provide as to the regulation of these pathways *in vivo*.

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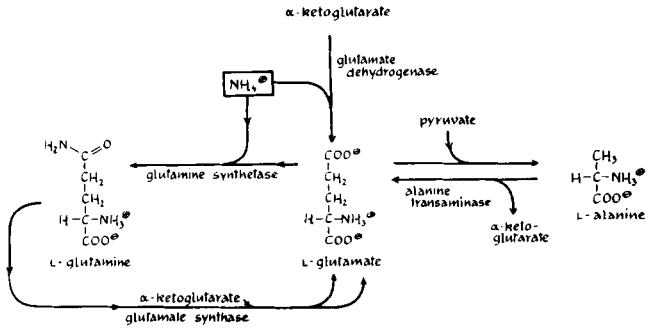

FIG. 1. **Biosynthetic pathways of L-glutamate, L-glutamine, and L-alanine in *N. crassa*.**

EXPERIMENTAL PROCEDURES

N. crassa, strain LA1 (wild type, 74A), was from the collection of one of the authors (R. L. W.). The medium was Vogel's minimal medium supplemented with 1.5% (w/v) sucrose (20). What we call a "nitrogen-free medium" is the same, except that it contains no ammonium nitrate. Where specified, the nitrogen source in Vogel's minimal medium (25 mM ammonium nitrate) was substituted or supplemented with glutamic acid, glutamine, or alanine. The ^{15}N-labeled ammonium chloride (99% enriched in ^{15}N) was purchased from Merck. L-Methionine-DL-sulfoximine was purchased from Sigma.

Cultures were inoculated with an aqueous suspension of washed conidia to a final concentration of approximately 1×10^7 conidia/ml. Conidia were germinated in 1-liter baffled flasks containing 500 ml of culture medium, with aeration provided by gyratory shaking. Germination and subsequent mycelial growth were performed at room temperature for 12–14 h or until growth of the culture was logarithmic as measured by culture turbidity. Where specified, growing mycelia were transferred to nitrogen-free medium for 3 h.

Cycloheximide[1] was added to the culture medium to a final concentration of 20 μg/ml a few minutes before transfer to a medium containing [^{15}N]ammonium ions. The mycelia were collected by filtration, and equal amounts of mycelia (770 mg, wet weight) from each culture were transferred to 20 ml of N-free medium supplemented with 0.2% [^{15}N]ammonium chloride and 20 μg/ml of cycloheximide. The cultures were shaken for specified periods with aeration in 125-ml baffled flasks at room temperature to facilitate assimilation of [^{15}N]ammonium ion. Where specified, L-methionine-DL-sulfoximine was added to the culture to a final concentration of 5 mM. For NMR measurements, the 20-ml mycelial suspensions were transferred to 25-mm outer diameter NMR sample tubes and the spectra taken over the specified time intervals. The mycelial concentration in each NMR sample was checked by measurement of optical density at 420 nm to ensure uniformity of concentration among cultures.

The ^{15}N-NMR spectra were obtained with a Bruker WH-180 spectrometer operating in the Fourier transform mode at 18.25 MHz (21). Chemical shifts are reported in parts per million upfield of a solution made up to have 1 M $H^{15}NO_3$ in ^2H_2O. The external standard was contained in a 5-mm tube mounted co-axially in the sample tube. The operating conditions were 30-μs pulse (40° flip angle) with a 2-s delay and with full proton decoupling. The sample temperature was maintained at 25 ± 2 °C. After each NMR experiment, the mycelial suspension was returned to the 125-ml baffled flask for further growth.

The intracellular concentrations of ADP and CTP in *N. crassa* were measured by the method described previously (22). Amino acid pools in boiling water extracts of *N. crassa* were determined as previously described (23).

RESULTS

Fig. 2 shows representative ^{15}N spectra of intact *N. crassa* mycelia which were grown in media containing different nitrogen sources (preincubation) and then transferred to media containing [^{15}N]ammonium ion to observe the rates of biosynthesis of glutamine, alanine, and glutamic acid. During the preincubation period, the enzymes involved in the biosynthesis of amino acids undergo induction or repression depending on the nitrogen source. This results in different concentrations of these enzymes among these cultures. To preserve these enzyme concentrations, cycloheximide, an inhibitor of protein synthesis, was added to each culture (16). Equal amounts of mycelia were collected from each culture and transferred to a fresh medium containing [^{15}N]ammonium chloride as the sole nitrogen source. The ^{15}N spectrum of each culture was taken at the indicated times to observe the relative rates of biosynthesis of ^{15}N-enriched glutamine, alanine, and glutamic acid. The course of the biosynthesis could be inferred from the increase in the resonance intensities of γ-^{15}N of glutamine (264 ppm), α-^{15}N of alanine (332 ppm), and α-^{15}N of glutamic acid (334.44 ppm), which is resolvable from α-^{15}N of glutamine (334.25 ppm) when plotted on an expanded scale (see below), even though these two resonances appear as a single peak in Fig. 2.

For each amino acid, the intensity of its ^{15}N resonance is proportional to its intracellular concentration because all of the spectra were taken under identical operating conditions. To estimate the relative intracellular concentrations of *different* ^{15}N-labeled amino acids from their peak intensities in the proton-decoupled ^{15}N spectra, it is necessary to take into account the respective spin-lattice relaxation times and contributions of nuclear Overhauser enhancement to the peak intensities. The γ-^{15}N of intracellular glutamine and α-^{15}N of alanine in suspensions of intact *N. crassa* mycelia have nuclear Overhauser enhancement values of −3.9 and −3.6, and T_1 values of 4.1 and 5.1 s, respectively, at 10 °C (24). These results suggest that the relationships between the ^{15}N peak intensities and intracellular concentrations are not substantially different for the γ-^{15}N of glutamine and α-^{15}N of alanine, but, because the spectra in Fig. 2 were obtained at 25 °C, accurate correlation of peak intensities with intracellular concentrations among different amino acids must await measurement of their T_1 and nuclear Overhauser enhancement values at this temperature. Here the ^{15}N peak intensities will only be used to estimate the relative intracellular concentrations of *each* ^{15}N-labeled amino acid under different preincubation conditions.

The steady state intracellular concentration of a metabolite depends on the rates of its biosynthesis and utilization. In the mycelia studied here, the amino acids are not utilized for protein synthesis because of the presence of cycloheximide. Degradation of amino acids by L-amino acid oxidases is expected to be minimal under these conditions because of the low levels of these enzymes during exponential growth in media containing a high level (5 μg/liter) of biotin (25, 26). The γ-nitrogen of glutamine is utilized for the biosynthesis of the ω-nitrogen of arginine, π-nitrogen of histidine, the indole nitrogen of tryptophan, as well as for some nucleotides. Among these substances, arginine is most abundant in *N. crassa*. However, its ω,ω′-^{15}N peak is undetectable in most spectra in Fig. 2, except for the culture (*II*) grown on ammonium nitrate at t = 385 min, even though this particular arginine nitrogen has a large nuclear Overhauser enhancement of −3.6 and a much shorter T_1 than γ-^{15}N of glutamine at 10 °C (24). The consequence is that the rate of utilization of γ-^{15}N of glutamine for the biosynthesis of other metabolites must be slow compared to the rate of the biosynthesis of glutamine itself under these experimental conditions. Thus, the observed differences in the resonance intensities of γ-^{15}N of glutamine among the cultures in Fig. 2 can be assumed to reflect differences in the rate of its biosynthesis. Alanine, biosynthesized by reversible transamination from glutamic acid, does not act as a precursor to any known metabolite in *N. crassa*, but can be reconverted to glutamic acid by alanine transaminase (Fig. 1).

For glutamine, it is clear from the growth of its γ-^{15}N

[1] The trivial name used is: cycloheximide, 4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione.

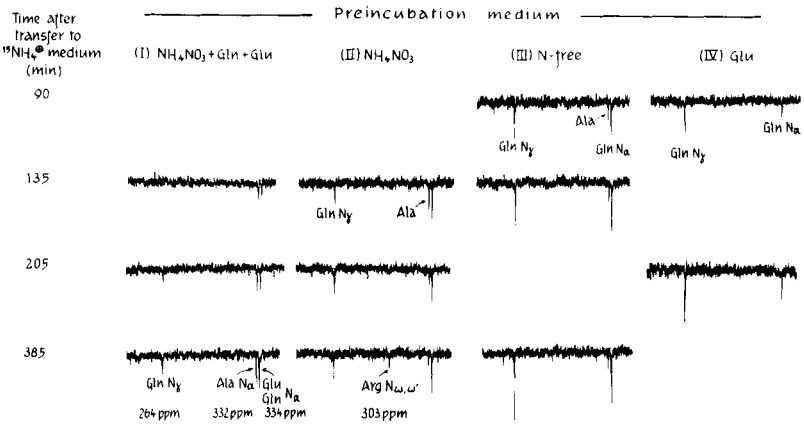

FIG. 2. **Proton-decoupled ^{15}N spectra of suspensions of intact *N. crassa* mycelia in [^{15}N]ammonium ion media after preincubation in media containing 25 mM ammonium nitrate + 6.8 mM glutamic acid + 6.8 mM glutamine (*I*), 25 mM ammonium nitrate (*II*), 25 mM ammonium nitrate followed by 3 h of nitrogen starvation (*III*), and 12 mM glutamic acid as nitrogen source (*IV*) (not enriched in ^{15}N).** After preincubation and addition of cycloheximide (20 μg/ml), equal amounts of mycelia (770 mg) collected from each culture were transferred to fresh N-free medium (20 ml) supplemented with 0.2% [^{15}N]ammonium chloride and 0.002% cycloheximide, and ^{15}N spectra of each culture were taken at the indicated time. Each spectrum represents an accumulation of 250 scans (8 min).

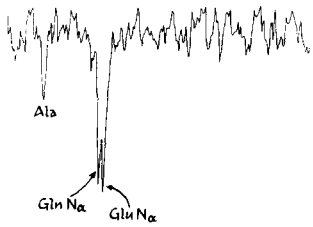

FIG. 3. **Proton-decoupled ^{15}N spectrum of α-^{15}N of alanine (332.04 ppm), α-^{15}N of glutamine (334.25 ppm), and α-^{15}N of glutamic acid (334.44 ppm) in suspensions of intact *N. crassa* mycelia plotted on an expanded scale to show resolved peaks for α-^{15}N of glutamine and glutamic acid.**

resonance that its biosynthesis is rapid in the culture grown on glutamic acid (see Fig. 2, *IV*) and in the nitrogen-starved culture (*III*), intermediate in the culture grown on ammonium nitrate (*II*), and slow in the culture grown on (ammonium nitrate + glutamic acid + glutamine) (*I*).

For alanine, by contrast, the reverse trend holds. The rate of its biosynthesis is fastest in the culture grown on (ammonium nitrate + glutamic acid + glutamine) (*I*), slow in the culture that had been nitrogen-starved (*III*), and undetectable in the culture grown on glutamic acid (*IV*). The culture grown on ammonium nitrate (*II*) is also interesting because its alanine, first synthesized in large amounts, decreases with time.

For intracellular glutamic acid, the α-^{15}N resonance has a chemical shift (334.44 ± 0.05 ppm) very close to that of the α-amino nitrogen of glutamine (334.25 ± 0.05 ppm). Nevertheless, the two peaks can be resolved when their intensities are comparable, as shown in the expanded scale spectrum of Fig. 3. In the spectra of Fig. 2, α-^{15}N of glutamic acid was observed in the culture grown on (ammonium nitrate + glutamic acid + glutamine), but in all of the other cultures, the α-amino peak at 334 ppm was predominantly that of glutamine. Therefore, while the steady state concentration of glutamic acid is substantial in those cultures where glutamine synthesis is slow, in others, glutamic acid appears to be rapidly converted to glutamine. Further evidence for conversion of glutamic acid to glutamine is found in the intracellular concentrations of these amino acids determined in boiling water extracts of *N. crassa* mycelia grown under the same conditions as *III* in Fig. 2. The intracellular concentration of glutamic acid (1.3 nmol/mg of mycelia, wet weight) was small compared to that of glutamine (24 nmol/mg, wet weight) 2 h after the transfer to ammonium chloride medium.

One of the remarkable features in Fig. 2 is the slow rate of glutamine synthesis combined with the enhancement of alanine synthesis in the (ammonium nitrate + glutamic acid + glutamine) culture (*I*). To determine whether this results from the presence of glutamic acid, glutamine, or both, in the preincubation medium, similar ^{15}N-NMR experiments were performed on cultures grown on various combinations of glutamic acid, glutamine, and ammonium nitrate, and the relative peak intensities of γ-^{15}N of glutamine and α-^{15}N of alanine at 135 min after the transfer to [^{15}N]ammonium ion medium were compared (Table I). The peak intensities of γ-^{15}N of glutamine were 59 and 63 in cultures preincubated in (ammonium nitrate + glutamine) and in (ammonium nitrate + glutamic acid), respectively, but negligible in those preincubated in (ammonium nitrate + glutamic acid + glutamine) or (glutamic acid + glutamine). Clearly, glutamine synthesis is only repressed significantly when both glutamic acid and glutamine are present in the preincubation medium.

Another feature of Fig. 2 is the significant enhancement of glutamine synthesis in the cultures that have been nitrogen-starved or grown on glutamic acid, both of which are deficient in free ammonium ion. The same effect was observed in a culture grown on alanine (Table I). In contrast, the culture grown on glutamine, which can provide two nitrogens/molecule, has an intermediate rate of glutamine synthesis, comparable to that observed in mycelia grown on ammonium nitrate (Table I). These results suggest that significant enhancement of glutamine synthesis occurs when the preincubation medium is deficient in free ammonium ion or in alternative preferred nitrogen sources, such as glutamine.

For alanine, the slow or undetectable rate of its synthesis in glutamic acid-grown or nitrogen-starved cultures could be caused by repression of alanine transaminase or by deficiency of the substrate glutamic acid as a result of its utilization for the competing glutamine synthesis pathway. To investigate which of these factors is important, rates of alanine synthesis were measured in nitrogen-starved and glutamic acid-grown mycelia with entry of glutamic acid into the competing pathway being prevented by the addition of L-methionine DL-sulfoximine, an inhibitor of glutamine synthetase (27). The results are shown in Table I. When glutamic acid was prevented from being used for glutamine synthesis, the synthesis

TABLE I

The relative ^{15}N resonance intensities of γ-^{15}N of glutamine, α-^{15}N of alanine, α-^{15}N of glutamine, and α-^{15}N of glutamic acid in suspensions of intact N. crassa mycelia grown on the indicated nitrogen sources and transferred to [^{15}N]ammonium ion medium

For each amino acid, the resonance intensity, measured 135 min after the transfer for each culture, is proportional to its intracellular concentration.

Nitrogen source in the preincubation medium	Relative ^{15}N resonance intensity[a]			
	[γ-^{15}N]Gln	[α-^{15}N]Ala	[α-^{15}N]Gln[b]	[α-^{15}N] Glu[b]
NH_4NO_3[c]	61	71	98	
NH_4NO_3 + N-free[d]	145	57	135	
Glu (12 mM)	153	ND[e]	106	
Ala (5 mM)	105	ND	78	
Gln (5 mM)	58	52	26	
NH_4NO_3 + Gln (6.8 mM)	59	55	42	
NH_4NO_3 + Glu (6.8 mM)	63	71	102	
Glu + Gln (6.8 mM each)	ND	73		61
NH_4NO_3 + Glu + Gln	ND	48		46
Glu (12 mM) + MS[f]	ND	53		44
NH_4NO_3 + MS[f]	ND	140		79
NH_4NO_3 + N-free + MS[f]	ND	145		73

[a] A relative intensity of 100 corresponds to a normalized peak height of 50 mm. The intensity is accurate to ±10%.

[b] The intensity of the predominant peak is shown.

[c] 25 mM in all cultures.

[d] Grown in NH_4NO_3 followed by 3 h of nitrogen starvation.

[e] ND, not detectable.

[f] L-Methionine DL-sulfoximine added a few minutes before transfer to [^{15}N]ammonium ion medium.

of alanine increased substantially both in the glutamic acid-grown and nitrogen-starved cultures. In the latter, as much alanine was synthesized as in the culture grown on ammonium nitrate (with L-methionine DL-sulfoximine). These results show clearly that neither nitrogen starvation nor growth on glutamic acid causes significant repression of alanine transaminase. The major factor limiting the rate of alanine synthesis in the nitrogen-starved and glutamic acid-grown cultures appears to be the existence of a highly efficient competing pathway for the consumption of glutamic acid. The ^{15}N spectra of the culture grown on ammonium nitrate (Fig. 2, *II*) also suggest such competition; at $t \approx 135$ min, glutamine and alanine were both present, but as glutamine synthesis increased, [α-^{15}N]alanine decreased ($t = 385$ min). The decrease was not due to depletion of the nitrogen source because the bulk of [^{15}N]ammonium chloride remained in the medium. The most reasonable explanation is that alanine was reconverted to glutamic acid as the latter was used for glutamine synthesis. More definitive evidence for (or against) such reconversion might be obtained by transferring the mycelia containing [α-^{15}N]alanine at $t = 135$ min to [^{14}N]ammonium chloride medium and tracing the fate of [α-^{15}N]alanine by the isotope-chase method. This study is now under way.

The rates of enzymatic reactions depend not only on the availability of substrates and the concentrations of enzymes, but also on the presence of metabolites which act as feedback effectors of regulatory enzymes. To determine whether the difference in the rate of glutamine synthesis between the nitrogen-starved culture (*III*) and the (ammonium nitrate + glutamic acid + glutamine) culture (*I*) could be due to differences in the intracellular concentrations of potential inhibitors such as ADP and CTP under the different growth conditions, their intracellular concentrations in nanomoles/mg of protein were measured for the two cultures. No significant differences were found; ADP, 7.3 (*I*) and 6.9 (*III*); CTP, 2.0 (*I*) and 1.6 (*III*).

DISCUSSION

The ^{15}N-NMR studies described here indicate a significant enhancement of the rate of glutamine synthesis in intact mycelia of *N. crassa* after growth in a medium deficient in free ammonium ion or glutamine. The observed *in vivo* rates are in qualitative agreement with the reported specific activity of glutamine synthetase in cell-free extracts. The specific activity increased 4-fold after 12 h of growth in limited (9 mM) ammonia (8) or in 5 mM glutamic acid (9) relative to that grown in 25 mM ammonium nitrate. Our results show that 3 h of nitrogen deprivation is sufficient to enhance the intracellular rate of glutamine synthesis significantly.

At first sight, it is surprising that the synthesis of glutamine should be so enhanced, at the expense of other metabolites, during nitrogen deficiency. Glutamine is but one of the two major nitrogen-donor metabolites, the other being glutamic acid. The predominance of glutamine synthesis becomes understandable, however, if, under nitrogen deficiency, glutamic acid is synthesized via glutamine by the glutamate synthase pathway (Fig. 1), which has been recently identified as an alternative pathway of glutamic acid synthesis in *N. crassa* (28). The reported 4-fold increase in the specific activity of glutamate synthase during growth in limited ammonia (11) supports this assumption. By contrast, under conditions of ammonia sufficiency, much glutamic acid may be formed directly from ammonium ion and α-ketoglutarate by the glutamate dehydrogenase pathway. A study of the relative contributions of these two pathways under ammonia sufficiency and deficiency as observed in intact *N. crassa* by ^{15}N-NMR is under way.

One of the interesting findings of the present ^{15}N-NMR work is that, when both glutamine and glutamic acid are directly available in the medium, the rate of glutamine synthesis decreases significantly, much more so than in the presence of glutamine alone. Although the specific activities of glutamine synthetase and glutamate synthase have been shown to decrease in the presence of glutamine alone (9), it is not known how the specific activities are affected when both glutamine and glutamic acid are present in the medium. The ^{15}N-NMR results suggest that these two metabolites may exert a coordinated mechanism of control over the synthesis of glutamine synthetase and, possibly, of glutamate synthase as well. Such a coordinated mechanism may constitute more efficient regulation because the biosynthetic pathways of these two amino acids are closely interlinked.

The ^{15}N-NMR results show that alanine is synthesized in significant quantities under conditions where the necessary substrate, glutamic acid, is not rapidly used in the competing pathway of glutamine synthesis. The observed predominance of the glutamine synthetase pathway over the alanine transaminase pathway is in accord with the relative importance of the two amino acids in intermediary metabolism. Unlike glutamine, alanine is not a precursor to any known metabolite in *N. crassa* and, beyond its role as a protein residue, little is known about the physiological function of the large free alanine pool. Because alanine can be reconverted to glutamic acid with release of pyruvic acid, a key intermediate in carbon metabolism, alanine may be a convenient reservoir of both the amino groups and pyruvic acid which is stored during nitrogen and carbon sufficiency and, when glutamic acid is depleted for glutamine synthesis, can be used to supply am-

monia for glutamic acid synthesis. The ^{15}N-NMR spectra of *N. crassa* in Fig. 2 (*II*), wherein alanine can be seen to decrease as glutamic acid is used for glutamine synthesis, are suggestive of such interconversions of metabolites at work in the intact microorganism.

The utility of ^{15}N-NMR is clear for observing relative rates of biosynthesis of amino acids in intact *N. crassa*. The *in vivo* rates, combined with knowledge from *in vitro* studies of the enzymes involved, allow evaluation of the relative importance of substrate availability, inhibition of enzymes, or repression and induction of enzymes in regulating the rate of a given pathway under physiological conditions.

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