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^{15}N NMR studies of nitrogen metabolism in intact mycelia of *Neurospora crassa*

(amino acid biosynthesis/metabolic turnover)

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ABSTRACT **Suspensions of intact mycelia of *Neurospora crassa* grown in medium containing $^{15}NH_4Cl$ have been found to give well-resolved ^{15}N nuclear magnetic resonance spectra for metabolites that play crucial roles in intermediary nitrogen metabolism. These include the amide nitrogen of glutamine, the α-amino nitrogens of glutamate and other amino acids, the guanidino nitrogens of arginine, the ureido nitrogen of citrulline, the side-chain nitrogens of ornithine or lysine, or both, and uridine diphosphates. The turnover time of glutamine *in vivo* was estimated to be less than 1 hr by using nuclear magnetic resonance spectroscopy in conjunction with tracer methodologies. Applications of these techniques to the study of nitrogen metabolism are discussed.**

High-resolution Fourier transform NMR has found increasing application in the study of *in vivo* metabolism of small molecules with improvements in the sensitivity and versatility of the available instrumentation. Spectra obtained with ^{31}P, ^{13}C, and ^{1}H nuclei have provided detailed information about the internal environment of living cells, such as the pH of various cellular compartments (1), the flux of metabolites in various metabolic pathways (2–4), and estimates of the intracellular viscosity (5). Applications to metabolic sequences have not been used very much because of the availability of radioisotopes that provide sensitivities not easily matched by nuclear magnetic resonance. Studies of nitrogen metabolism, while extensive, have been hindered by a lack of a radioisotope of nitrogen with sufficient half-life to permit the types of tracer studies that have proven to be useful for the radioisotopes of carbon, hydrogen, phosphorus, and sulfur. High-resolution Fourier transform NMR allows observation of cells *in vivo* with little or no perturbation, and NMR spectra of the stable nucleus ^{15}N have the potential of providing a unique opportunity to apply tracer methodologies to the metabolism of nitrogen *in vivo*.

In vivo, use of ^{15}N NMR so far has been limited to observations of macromolecular species labeled with ^{15}N, such as proteins (6, 7) and bacterial cell-wall components (8–10), although Schaefer *et al.* (6) reported a broad α-amino resonance of amino acids from soybeans in the intact seeds or lyophilized pods and leaves. Choice of these materials for study reflects the difficulty of observing ^{15}N resonances because of its low sensitivity and long relaxation times in aqueous environments. The work reported here is an application of ^{15}N NMR to the study of *in vivo* small-molecule metabolism in microorganisms.

Neurospora crassa was chosen for our studies because it is easily cultured in the laboratory and is capable of synthesizing all nitrogen-containing components (with the exception of the vitamin biotin) from ammonia. The organism is also amenable to genetic manipulation, and a large variety of mutants, defective at individual, well-defined steps in many metabolic pathways, are available. Finally, unlike prokaryotic organisms, *N. crassa* accumulates large pools of a variety of nitrogen-containing metabolites when cultured in medium containing only minimal essential nutrients (11). This latter aspect greatly facilitates the observation and identification of resonances observed in labeled cultures.

The aim of the present study has been to demonstrate the feasibility of using NMR in conjunction with tracer methodologies to elucidate details of nitrogen metabolism *in vivo* that are not readily accessible by other standard techniques. Such details include the turnover of various nitrogen-containing metabolites such as glutamine, the flow of nitrogen along various competing pathways as it occurs *in vivo*, and the extent to which alternative pathways of nitrogen metabolism can occur *in vivo* under a variety of culture conditions.

EXPERIMENTAL PROCEDURES

The ureaseless strain (LA44) of *Neurospora crassa* used in these studies was obtained from R. H. Davis. The medium was Vogel's minimal medium supplemented with 1.5% (wt/vol) sucrose (12). Nitrogen-free medium is the same without the addition of NH_4NO_3. The ^{15}N-substituted ammonium chloride (99% enriched in ^{15}N) was purchased from Kor Isotopes.

Cultures were inoculated with an aqueous suspension of washed conidia to a final concentration of approximately 1×10^7 conidia per ml. Conidia were germinated in 1-liter baffled flasks containing 500 ml of culture medium, with aeration provided by shaking at 150 rpm at 16°C for a period of 16–20 hr. The temperature was then raised to 30°C for 3 hr or until growth of the culture was logarithmic as measured by culture turbidity. In some experiments, cultures were exposed to $^{15}NH_4Cl$ for the entire germination period. Alternatively, growing mycelia were transferred to nitrogen-free medium for 3 hr prior to the addition of $^{15}NH_4Cl$. Where specified, cycloheximide {4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione} was added to the culture medium to a final concentration of 20 μg/ml a few minutes before the addition of $^{15}NH_4Cl$ to inhibit the incorporation of ^{15}N-labeled amino acids into proteins (13). The mycelial suspension for the NMR measurements was prepared by collecting the mycelia by filtration and resuspending them in enough medium to make 18 ml of mycelial suspension, which was placed in a 25-mm NMR sample tube. The volume ratio of wet mycelium to medium in the NMR sample tube was approximately 1:1.

Amino acid pools were determined by extraction of mycelia with boiling water (14). Pool extracts were evaporated to dryness, redissolved in 0.2 M sodium citrate (pH 2.2), and analyzed on a Beckman 120C amino acid analyzer. Protein determinations were made by the method of Lowry *et al.* (15) after overnight suspension of mycelia in 0.5 M NaOH at room temperature to solubilize the protein.

The ^{15}N NMR spectra were obtained with a Bruker WH 180

spectrometer operating at 18.25 MHz (16). Chemical shifts are reported in ppm upfield of 1 M $H^{15}NO_3$ in ^2H_2O mounted coaxially with the sample tube in a capillary. The operating conditions, unless otherwise specified, were 70-μsec pulses (90° flip angle) with a 2-sec delay and with full proton decoupling. The sample temperature was maintained at 10 ± 2°C, unless otherwise specified.

To check the viability of mycelia during the NMR experiments, the doubling time and ability of the mycelia to accumulate arginine from the culture medium were compared before and after exposure to conditions similar to those produced by the NMR experiment; no change was observed in either of these properties, which shows that the mycelia retain full viability.

RESULTS

To investigate the types of ^{15}N-labeled metabolites in *N. crassa* that can be observed *in vivo* by ^{15}N NMR, spectra were taken of intact mycelia grown in $^{15}NH_4Cl$-containing medium. *N. crassa* was grown in minimal medium containing 0.2% $^{15}NH_4Cl$ as the sole nitrogen source for 20 hr, and a mycelial suspension was prepared for NMR measurement as described above.

Fig. 1*a* shows the broadband proton-decoupled ^{15}N spectrum of these mycelia. A number of well-resolved resonances of ^{15}N-labeled intermediates and products were observed. Assignments of the ^{15}N resonances of the intracellular metabolites were made by comparison with those of the corresponding compounds in aqueous solution (17–21). Because of variations in spin-lattice relaxation times and nuclear Overhauser enhancements of the various chemical species in the presence of broadband proton-decoupling, accurate correlation of peak intensities with intracellular concentrations was not possible. Table 1 shows the pool sizes of several nitrogen-containing intracellular metabolites that were obtained from boiling water extracts of *Neurospora* cultures and analyzed as described above.

The intense peaks observed in the spectrum in Fig. 1*a* arise from the side-chain nitrogens of amino acids (both free amino acids and residues in proteins): glutamine N_γ (262.4 ppm); arginine N_δ (291.0 ppm) and $N_{\omega,\omega'}$ (303.5 ppm); lysine N_ε or ornithine N_δ, or both (342.6 ppm); and the α-amino nitrogens of free amino acids such as (*i*) proline (321.9 ppm); (*ii*) alanine (332.3 ppm); glutamine, glutamate, lysine, and arginine (≈334.3 ppm); and (*iii*) valine or serine, or both (338.9 ppm). The most shielded peak at 354.5 ppm represents ammonium ion. Significantly, one can also detect small ^{15}N resonances from some intermediates of arginine biosynthesis such as citrulline N_δ (287.7 ppm) and N_ω (300.6 ppm).

The resonance at 251.1 ppm can be assigned to *N*-acetyl-D-glucosamine either as a free metabolite or as a component of fungal cell walls (22). Among the nitrogens in cellular nucleic acids and free nucleotides, only the N3 resonance of uridine (216.7 ppm) was observed; this resonance probably arises from uridinediphosphoacetylglucosamine and uridinediphosphoglucose, both of which occur in significant amounts in *N. crassa* (see ref. 23, Table 1) and are intermediates in the biosynthesis of chitin, among other things.

The broadband proton-decoupled ^{15}N spectrum of Fig. 1*a* shows no peaks corresponding to the numerous backbone amide nitrogens of cellular proteins, although these nitrogens appeared as a broad peak around 253 ppm when the spectrum was obtained with gated proton decoupling as shown for comparison in the *Inset* of Fig. 1*a*.

To investigate the rates of turnover of the various pools, the mycelia used to obtain the spectrum shown in Fig. 1*a* were collected by filtration, washed, and incubated for 2 hr in a medium containing $^{14}NH_4Cl$ as a sole nitrogen source. Degradation of

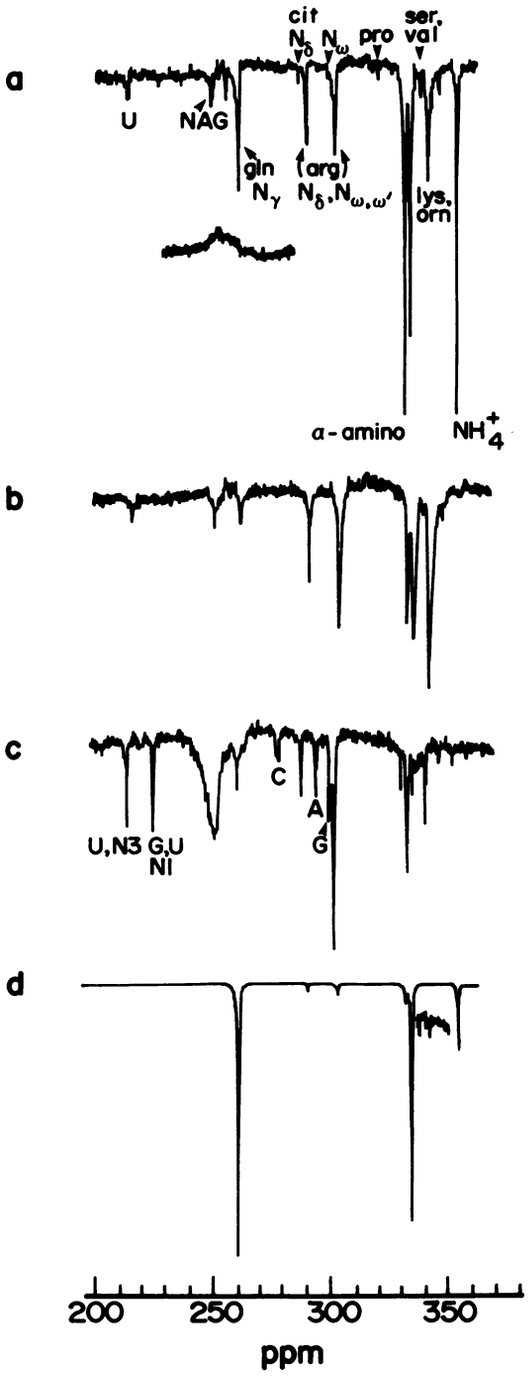

FIG. 1. Proton-decoupled ^{15}N NMR spectrum in ppm upfield of 1 M $H^{15}NO_3$. (*a*) Intact *N. crassa* mycelia germinated on minimal medium containing 0.2% $^{15}NH_4Cl$; the *Inset* shows a spectrum from the same sample around 253 ppm obtained with gated proton decoupling (5867 scans). Cit, Citrulline; NAG, *N*-acetyl-D-glucosamine. (*b*) The same sample of intact *N. crassa* mycelia after incubation for 2 hr in medium containing 0.2% $^{14}NH_4Cl$ (21,790 scans). (*c*) Boiling water extract of *N. crassa* mycelia used to obtain spectrum *b* (17,607 scans). (*d*) Intact *N. crassa* mycelia germinated on minimal medium, transferred to nitrogen-free medium for 3 hr and then incubated with cycloheximide (20 μg/ml) and $^{15}NH_4Cl$ (0.4%) for 3 hr; the *Inset* shows an expansion of the region of this spectrum around 343 ppm (4854 scans).

^{15}N-labeled protein was expected to be negligible during this period because most proteins turn over with half-lives on the order of days under the culture conditions used (24). By con-

Table 1. Pools of various nitrogen-containing compounds in *N. crassa*

Metabolite	Amino acid pool,* nmol/mg of protein		
	Medium		Cycloheximide/ NH_4Cl addition
	Minimal	Nitrogen-free	
Aspartate	10	26	38
Threonine	9	10	96
Serine	48	18	69
Asparagine	15	6	100
Glutamate	330	50	300
Glutamine	626	10	2500
Glycine	28	8	38
Alanine	455	51	438
Methionine	28	6	25
Isoleucine	10	5	10
Leucine	7	10	10
Ornithine	88	<10	174
Lysine	14	14	122
Arginine	150	4	449
Citrulline	9^{27}	ND	ND
UDP GNAc	8^{23}	ND	ND
UDP Glucose	6^{23}	ND	ND

* Amino acid pools were extracted from mycelia germinated in minimal medium, then transferred to nitrogen-free medium for 3 hr, and treated by addition of cycloheximide (20 μg/ml) and ammonium chloride (0.1%) for 3 hr. UDP GNAc, Uridinediphosphoacetylglucosamine; UDP Glucose, uridinediphosphoglucose.

trast, those free ^{15}N-labeled amino acids that are rapidly metabolized can be expected to show a decrease in ^{15}N peak intensities due to metabolic turnover. Fig. 1*b* shows the proton-decoupled ^{15}N spectrum of these mycelia. The most significant change relative to Fig. 1*a* is the marked loss in the intensities of the glutamine N_γ peak (262.4 ppm) and the N_α peaks (332.3, 334.3 ppm) of free amino acids.

To determine whether the amino acid side-chain resonances that did not turn over rapidly represented amino acid residues in protein or were slowly metabolized pools of free amino acids, the mycelia whose spectrum is shown in Fig. 1*b* were collected, and the soluble metabolites were extracted with boiling water as described above. The ^{15}N spectrum of this extract is shown in Fig. 1*c*. After correction for the differences in the number of transients, much of the pools responsible for the resonances of the N_δ and $N_{\omega,\omega'}$ of arginine and the resonance of the N_ε of lysine or the N_δ of ornithine, or both, remain soluble after the 2-hr "chase" of unlabeled ammonium ion.

Several resonances were observed (Fig. 1*c*) that were not detected in the spectra of intact mycelia. Among these are N1 of uridine and guanosine (228.8 ppm), cytidine NH_2 (281.8 ppm), adenosine NH_2 (297.3 ppm), and guanosine NH_2 (303.1 ppm). The last-named appeared as a distinct shoulder on the larger arginine $N_{\omega,\omega'}$ peak (304.5 ppm). The uridine N3 resonance (217.8 ppm) is also substantially increased in intensity. These peaks probably arise mainly from the bases in transfer RNAs that, on loss of base pairing by heat denaturation, assumed a more flexible conformation in which the individual nitrogen nuclei have shorter correlation times and, hence, larger (negative) nuclear Overhauser enhancement values. The broad peak around 253.2 ppm could be assigned to amide nitrogens of small oligopeptides that were not precipitated by the boiling-water extraction.

In intact *N. crassa*, it is possible to study the assimilation of $^{15}NH_4^+$ into free amino acids while restricting their incorporation into proteins. This can be accomplished by addition of cycloheximide to the culture medium before the addition of $^{15}NH_4Cl$. Under such conditions, the mycelia can biosynthesize metabolites such as amino acids from $^{15}NH_4Cl$ using preexisting enzymes, but *de novo* synthesis of proteins using ^{15}N-labeled amino acids will be inhibited (13). Thus, the biosynthesis of free ^{15}N-labeled amino acids *in vivo* can be studied unobscured by the contribution of ^{15}N-labeled amino acid residues in proteins.

N. crassa was grown in minimal medium for 17 hr, then transferred to nitrogen-free medium for 3 hr to deplete the intracellular pools of unlabeled amino acids. After addition of cycloheximide (20 μg/ml), the mycelia were exposed for an additional 3 hr to $^{15}NH_4Cl$ (0.4%) as the sole nitrogen source. Fig. 1*d* shows the proton-decoupled spectrum of these mycelia. The most conspicuous differences from the spectrum obtained from mycelia grown without cycloheximide (Fig. 1*a*) are (*i*) the glutamine N_γ peak (262.4 ppm) is much larger relative to those of arginine N_δ (291.0 ppm) and $N_{\omega,\omega'}$ (303.5 ppm) and (*ii*) the peak corresponding to lysine N_ε or ornithine N_δ, or both (342.6 ppm), is extremely small, being just distinguishable from the baseline (Fig. 1*d*, *Inset*).

DISCUSSION

Fig. 1*a* demonstrates the feasibility of observing ^{15}N NMR resonances from a variety of nitrogen metabolites *in vivo*. Resonances of the amide nitrogens of proteins, which should be present in relative abundance under the conditions employed in this experiment, are not observed in broadband proton-decoupled spectra but can be observed in spectra obtained with gated proton decoupling. This nulling effect for these resonances arises because the amide nitrogens of cellular proteins have, on the average, a nuclear Overhauser enhancement value of approximately zero. A similar effect has been observed in the ^{15}N spectra of bacterial cell walls (8).

Whereas numerous side-chain resonances of amino acids are seen in Fig. 1*a*, it must be emphasized that these resonances may represent contributions from both free amino acids and amino acid residues in proteins.

In anabolic pathways of nitrogen metabolism in *N. crassa*, glutamate and glutamine play crucial roles in assimilating NH_4^+ and act as precursors for many important nitrogenous cellular components (25). Large pools of both glutamate and glutamine (and other nitrogenous metabolites) are maintained in *N. crassa* (26, 27). The size of these pools is determined by a steady state between synthesis and utilization. Each pool is dynamic and turns over or renews itself in a manner determined by the relative rates of catabolism and anabolism associated with the particular pool. This rate of turnover can be measured by the method of isotope dilution. The labeled pool is subjected to a chase of unlabeled precursor material, and loss of label from the pool is monitored.

Fig. 1*b* shows the results of such an isotope dilution experiment in which the precursor $^{14}NH_4Cl$ is allowed to chase ^{15}N-labeled metabolites from the various nitrogen metabolite pools observed in Fig. 1*a*. The results clearly indicate that the glutamine N_γ peak observed in Fig. 1*a* arises predominantly from free intracellular glutamine molecules and not from glutamine residues in proteins. Moreover, the glutamine N_γ shows a very rapid turnover rate. It is clear from comparison of the glutamine N_γ peak intensities in Fig. 1 *a* and *b* (after correction for the difference in the number of transients) that the half-life of free glutamine in *N. crassa* growing in NH_4Cl must be less than 1 hr. Citrulline (an intermediate in arginine biosynthesis) shows rapid loss of intensity in the N_δ and N_ω peaks, indicating that this pool, too, turns over rapidly. The decrease in the intensities of the α-amino nitrogen resonances of amino acids is no doubt mainly caused by their incorporation into proteins and to trans-

aminations of the glutamate α-amino group to other nitrogenous metabolites.

The persistence of the N_δ and $N_{\omega,\omega'}$ peaks of arginine and the peak of lysine N_ε or ornithine N_δ, or both, (Fig. 1*b*) was originally thought to indicate the continuous presence of the arginine and lysine side-chain nitrogen resonances in protein after the chase period. However, the persistence of the resonances in protein-free extracts prepared from the chased cultures (Fig. 1*c*) shows that a significant amount of these peak intensities arise from oligopeptides and free amino acids. The bulk (>98%) of the arginine and ornithine pools in *N. crassa* have been shown to be sequestered within the vacuoles (11). This is true of lysine as well, although the exact distribution of this amino acid between the cytosolic and vacuolar compartments has not yet been determined. The persistence of label in these amino acids after the chase may reflect slow turnover of vacuolar pools relative to cytosolic pools such as glutamine and citrulline. ^{15}N NMR provides a method of examining the turnover of these sequestered pools and the manner in which this turnover is regulated.

To eliminate the problems of interpretation associated with the incorporation of labeled amino acids into protein, the incorporation of $^{15}NH_4Cl$ by cultures was examined in the presence of cycloheximide. The possible effect of the inhibition of protein synthesis that results from this treatment is the premature feedback inhibition of the biosynthetic pathways which generate these metabolites, because such metabolites as amino acids may accumulate more rapidly in the absence of protein synthesis and because of the failure of some inducible pathway enzymes to increase in response to the presence of an inducer. This latter effect is unlikely to be important in the experiments reported here because inducible enzymes are generally confined to catabolic pathways and because the activity of these enzymes would change significantly from pretreatment levels only if they turned over rapidly, relative to the time course of the labeling period involved.

The results of this experiment are shown in Fig. 1*d*. It is seen that, under these conditions, the glutamine N_γ resonance relative to the arginine N_δ and $N_{\omega,\omega'}$ peaks is larger than it is in the absence of cycloheximide (Fig. 1*a*); in addition, the resonance at 342.6 ppm corresponding to lysine N_ε or ornithine N_δ, or both, is much reduced. The large intensity of the glutamine N_δ peak may result because the enzyme catalyzing the synthesis of glutamine, glutamine synthetase, is not feedback-inhibited by glutamine itself (28). The peak also may be larger because of decreased utilization of glutamine by other biosynthetic pathways under these conditions.

The appearance of resonances corresponding to arginine N_δ and $N_{\omega,\omega'}$ in this culture (Fig. 1*d*) reflects the ability of *N. crassa* to sequester the majority of the amino acid in its vacuoles, thus circumventing the sensitive feedback inhibition of the regulatory enzyme acetylglutamate kinase for this pathway (29).

Therefore the decrease in intensity of the peak of lysine N_ε or ornithine N_δ, or both, observed in Fig. 1*d* is somewhat difficult to explain because, like arginine, these amino acids are compartmentalized within the vacuoles (11). Moreover, substantial pools of both amino acids are accumulated under these conditions (Table 1), and *de novo* synthesis of ornithine is demonstrated by the appearance of the arginine N_δ resonance, which is derived from ornithine N_δ. Apparently, in the presence of cycloheximide, accumulated pools of ornithine and lysine may largely arise from unlabeled sources such as protein degradation rather than from *de novo* synthesis, and newly biosynthesized ornithine is used preferentially for arginine biosynthesis rather than for compartmentation. Modifications of the techniques described, using ^{15}N-labeled ornithine, offer a means for further investigation of this possibility.

Whereas much is now known about nitrogen metabolism, some kinds of investigation have been hindered by the lack of a useful radioactive isotope of nitrogen. These include measurement of (*i*) the turnover of nitrogenous metabolites, such as glutamine and glutamate, that are central to the myriad pathways of nitrogen assimilation, (*ii*) the manner in which nitrogen from such sources as glutamine and glutamate moves among the various competing pathways, and (*iii*) where two or more pathways lead to the same products, the extent to which the alternative pathways operate simultaneously under various growth conditions. Many of these unresolved questions may be answered by use of ^{15}N NMR spectroscopy.

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