\textbf{15}N-NMR study of ammonium assimilation in \textit{Agaricus bisporus} \\
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\textbf{Abstract} \\
Ammonium assimilation was studied by feeding [\textsuperscript{15}N]ammonium to actively growing mycelium of \textit{Agaricus bisporus}. Products of ammonium assimilation were analysed using \textsuperscript{15}N-NMR. Participation of glutamine synthetase, glutamate synthase and NADP-dependent glutamate dehydrogenase was determined by inhibiting glutamine synthetase with phosphinothricin and glutamate synthase with azaserine. Our results clearly indicate that, under the conditions used, ammonium assimilation is mainly catalysed by the enzymes of the glutamine synthetase/glutamate synthase pathway. No indications were found for participation of NADP-dependent glutamate dehydrogenase. Furthermore, \textsuperscript{15}N-labelling shows that transamination of glutamate with pyruvate to yield alanine is a major route in nitrogen metabolism. Another major route is the formation of N-acetylglucosamine. Compared to the formation of N-acetylglucosamine there was only a limited formation of arginine. \\

\textbf{Keywords:} Nuclear magnetic resonance spectroscopy; Mushroom; \textit{Agaricus}; Ammonium assimilation \\

\textbf{1. Introduction} \\
A large number of studies \cite{1,2} have demonstrated the existence of two systems for nitrogen assimilation in microorganisms: the reductive amination of 2-oxoglutarate catalysed by NADP-dependent glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4.) with the synthesis of glutamate (GDH-pathway), and the amidation of glutamate catalysed by glutamine synthetase (GS; EC 6.3.1.2.) with the formation of glutamine. Consequently, the amino acids glutamine and glutamate are primary products of nitrogen assimilation and serve as donors of amide nitrogen and in transamination reactions. \\

An alternative to the NADP-GDH pathway for glutamate production is offered by the reaction catalysed by glutamate synthase (GOGAT; EC 1.4.7.1.). This reaction involves the reductive transfer of the amide nitrogen of glutamine to 2-oxoglutarate. By coupling the GOGAT reaction with GS an essentially irreversible pathway (GS/GOGAT-pathway) for the formation of glutamate is achieved. \\

Until 1980 ammonium assimilation in fungi was believed to be catalysed by NADP-GDH \cite{1}. However, more recent studies on \textit{Neurospora crassa} have shown that ammonium assimilation in fungi can also be mediated by the joint operation of GS and GOGAT \cite{3-5}. Furthermore, studies on \textit{Cenococcum geophilum} \cite{6}, \textit{Aspergillus nidulans} \cite{7} and \textit{Stropharia semiglobata} \cite{8} have shown that ammonium assimilation in fungi can be catalysed by the concurrent activity of the NADP-GDH pathway and the GS/GOGAT pathway. \\

Although the cultivation of the commercial mushroom \textit{A. bisporus} has developed into an industry over the past decades, only a limited amount of basic knowledge on its nitrogen metabolism is available. Recently Baars et al. \cite{9} made an inventory of the nitrogen-assimilating enzymes in \textit{A. bisporus}. Besides high transaminating activities, NADP-GDH, GS and GOGAT activities could be demonstrated in cell-free extracts. So \textit{A. bisporus} has the enzymic potential to produce glutamate by either the NADP-
GDH pathway or the GS/GOGAT pathway. In order to determine the relative contribution of both pathways for ammonium assimilation in more detail, the incorporation of $^{15}$N-ammonium by *A. bisporus* was studied by nuclear magnetic resonance spectroscopy ($^{15}$N-NMR).

2. Materials and methods

Organism and culture conditions. *Agaricus bisporus* strain Horst$^\text{®}$ U1 was used throughout this study. Stock cultures were stored at 4°C on slants of wheat agar. Mycelium was grown at 24°C in static cultures using Fernbach flasks containing 100 ml of liquid medium. Two slightly different media were used. Medium A contained 2 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 0.5 mM CaCl$_2$, 0.134 mM Na$_2$EDTA, 2.5 µM FeSO$_4$, 5 µM ZnSO$_4$, 5 µM MnSO$_4$, 4.8 µM H$_3$BO$_3$, 2.4 µM KI, 52 nM Na$_2$MoO$_4$, 4 mM CuSO$_4$, 4 mM CoCl$_2$, 0.5 µM thiamine-HCl and 0.1 µM D(-)-biotin. In most cases a modification of the medium described by Dijkstra et al. [10] was used (medium B). In this medium the amino acids were omitted. Both media contained 100 mM glucose. As a nitrogen source either 20 mM glutamate or 10–20 mM (NH$_4$)$_2$SO$_4$ was used.

Liquid cultures were inoculated with mycelium grown for 7 days on agar plates containing compost extract medium prepared according to Rainey [11] and solidified with 1.5% (w/v) of bacto-agar. The plates were overlayed with a cellophane disk and inoculated at 7 points per petri dish. After growth the mycelium was scraped off the plates with a sterile spatula and fragmented in a Waring blender for 30 s. Aliquots of the homogenate were used as an inoculum for the liquid media. Unless stated otherwise, cultures were harvested after 22 days by filtration over nylon gauze (100 µm pore size).

$^{15}$N-ammonium feeding procedure. Mycelium harvested from liquid cultures was dried by pressing gently between nylon gauze layered upon filter paper and parafilm. After weighing, equal portions (2–4 g fresh weight) were incubated in 50 or 100 ml medium B in which the nitrogen source was replaced by 5 or 10 mM $^{15}$NH$_4$Cl (99% enriched in $^{15}$N, ICN Biomedicals, Cleveland, OH, USA). In addition, penicillin G (50 µg·ml$^{-1}$) and streptomycin (50 µg·ml$^{-1}$) were added to prevent bacterial growth. The cultures were shaken at 50 rpm for specified periods in 1000 ml baffled flasks at room temperature. Where indicated, azaserine (AZS) or phosphinothricin (PPT) were added to the culture to final concentrations of 1 and 5 mM, respectively.

Harvest and extraction of nitrogen compounds. After $^{15}$N-ammonium feeding mycelia were harvested either by filtration over nylon gauze (100 µm pore size) or by centrifugation (12000 × g for 10 min). After washing the mycelia with 0.15 M NaCl and demineralised water, nitrogen compounds were extracted as described by Martin et al. [12]. Briefly, mycelia were ground and extracted in 25 ml of ice-cold methanol/chloroform/water (12:5:3, v/v/v). The homogenate was centrifuged (10000 × g for 15 min) and the pellet was extracted a second time. The supernatants were combined and dried at 40°C using a rotavapor. The samples were dissolved in 0.5 ml of 0.01 M HCl. The acidic solution was washed with 1 ml of ice-cold chloroform to remove compounds interfering with the $^{15}$N-NMR measurement and stored at −20°C.

When amino acid compositions of the mycelia were to be determined, 1 µmol norleucine was added to the mycelia as an internal standard before the extraction procedure. The extracted pellet was dried overnight at 80°C to estimate the amount of mycelium extracted.

$^{15}$N-nuclear magnetic resonance. The $^{15}$N-NMR spectra were obtained with a Bruker AMX-600 spectrometer operating at 60.816 MHz. Proton decoupling by the WALTZ-16 composite pulse sequence was used. The $^{15}$N-NMR spectra were obtained with the following settings: 90° pulse, 2 s recycle delay, and 16 K data points were recorded per free induction decay (FID). The number of scans accumulated per FID differs from one sample to the other and is given in the legend of the figures. The FIDs were zero-filled once and multiplied by exponential window function with 10 Hz line broadening prior to Fourier transformation leading to spectra with 16 K real data points. Spectra were recorded in 20% D$_2$O to provide a lock signal. Chemical shifts were reported relative to liquid ammonia at 25°C, where 0 ppm has been obtained by multiplying the 0 ppm $^1$H TSP frequency by 0.10132914 [13]. Temperature was maintained at 298 K. Assignments of resonances were made by comparison with published $^{15}$N-NMR data [14–17] and by analysing spectra of extracts from $^{15}$N-glutamate-fed mycelia.

Enzyme assays. Laccase (EC 1.14.18.1) activity of culture supernatants was used as a measure of mycelium growth and was determined spectrophotometrically according to a modification of the method of Wood [18]. Briefly, up to 0.5 ml of culture fluid was added to 2 ml substrate buffer (0.1 M Na-acetate, pH 5.6, containing 1 mg·ml$^{-1}$ N,N-dimethyl-p-phenylenediamine sulfate) in a total volume of 2.5 ml. Absorbance of the red quinone product was measured at 552 nm. A change in absorbance of 0.1 min$^{-1}$ was defined as one unit of enzyme activity (U). Activities of NADP-GDH and GS were determined according to the methods described by Baars et al. [9]. GS activity was measured by the transference reaction.

Analytical procedures. Ammonium was determined by the method described by Bergmeyer and Beutler [19]. Glutamate was determined by a modification of the method described by Beutler [20]. Instead of using diaphorase and 2-(4-iодophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), we used 0.75 mM N-methyldibenzopyrazine methyl sulfate (PMS) and 3.75 mM (3-[4,5-dimethylthiazol]-2-yl)-2,5-diphenylyltetrazolium bromide (MTT).

After recording the NMR spectra amino acid composi-
Fig. 1. $^{15}$N-NMR spectrum of nitrogen compounds extracted from mycelia of *A. bisporus* growing actively on $[^{15}\text{N}]$ammonium as a nitrogen source (27059 scans). Resonance frequencies: $\text{NH}_3^+$, 20.6 ppm; lysine, ornithine or $\gamma$-aminobutyrate ($\gamma\text{ABA}$), 32.7 ppm; $\alpha$-amino nitrogens ($\text{Na}^*$) of glutamine, glutamate, lysine and arginine, 38.9 ppm; alanine, 40.74 ppm; arginine $\omega,\omega'\text{-N}$, 71.04 ppm and $\delta\text{-N}$, 83.61 ppm; glutamine $\gamma\text{-N}$, 112.18 ppm and $N$-acetyl-$D$-glucosamine (NAG) 124.75 ppm.

Fig. 2. $^{15}$N-NMR spectra of extracts from *A. bisporus* obtained after feeding $[^{15}\text{N}]$ammonium chloride (99% $^{15}$N) at time zero (3072 scans). Resonance frequencies: $\alpha$-amino nitrogens ($\text{Na}^*$) of glutamine/glutamate, 40.6 ppm; alanine, 42.7 ppm; glutamine $\gamma\text{-N}$, 111.8 ppm.

After derivatization with 9-fluorenyl-methyl chloroformate (FMOC) the amino acids were separated on a TSK gel ODS 80 T$_M$ column (250 × 4 mm, TosohHaas, Mont-
3. Results

3.1. Growth with $[^{15}\text{N}]$ammonium as a nitrogen source

Mycelium was grown on medium A with 20 mM $[^{15}\text{NH}_4]$Cl as a nitrogen source to study the major pools of $[^{15}\text{N}]$-labelled compounds. Actively growing mycelium (3.4 g fresh weight) was harvested after 9 days of growth. Soluble compounds were extracted and subjected to NMR spectrometry (Fig. 1). Several peaks can be identified from the spectrum. Resonances were found at 32.7 ppm (lysine, ornithine or γ-aminobutyrate); 38.9 ppm ($α$-amino nitrogens of glutamine, glutamate, lysine and arginine); 40.7 ppm (alanine); 71 ppm (arginine $ω, ω'$-N); 83.6 ppm (arginine $δ$-N); 112.2 ppm (glutamine $γ$-N) and 124.7 ppm ($N$-acetyl-$D$-glucosamine).

3.2. Time-dependent incorporation of $[^{15}\text{N}]$ammonium

To study the uptake of $[^{15}\text{NH}_4]^+$ and its time-dependent incorporation, mycelium was grown in medium B, using 150 mM glucose and 10 mM ($NH_4$)H$_2$PO$_4$ as carbon and
nitrogen source, respectively. After 20 days of culture the medium was replaced by fresh medium and laccase activity was determined to measure mycelial growth [18]. From day 20 to 23, a linear increase in laccase activity from 10 to 25 U · ml⁻¹ was observed. Thereafter laccase activity hardly increased and the mycelium was harvested for the incorporation experiment at day 24. In the freshly harvested mycelium activities of NADP-GDH and GS were 0.038 U · mg⁻¹ and 0.032 U · mg⁻¹, respectively. Equal portions of mycelium (2.8 g fresh weight) were incubated in 100 ml nitrogen-free medium B supplemented with 5 mM [₁⁵N]NH₄Cl and antibiotics for a limited number of time intervals. Uptake of [₁⁵N] was followed by enzymatic determination of residual [₁⁵N]NH₄⁺ in the incubation medium. After 27 h about 1 mM was taken up by the mycelium, while after 48 h the [₁⁵N]NH₄⁺ concentration in the incubation medium was decreased to 0.3 mM. Mycelia were harvested at different time intervals from the incubation medium, their soluble compounds were extracted and [₁⁵N]NMR spectra were recorded (Fig. 2). Spectra of samples taken over a period of 27 h show the gradual appearance of a number of labelled compounds. Three peaks are observed: a resonance at 111.8 ppm due to glutamine γ-N and resonances at 42.7 and 40.6 ppm due to α-N from alanine and glutamate/glutamine, respectively. The resonance from glutamine γ-N is the most prominent over the complete period of labelling and increases with time. The signal-to-noise ratio of the glutamine γ-N peak increases from 19.8 after 2 h of incubation to 39.8, 47.6 and 57.4 after 4, 8 and 27 h, respectively. Labelling of glutamate/glutamine α-N lags behind but in time becomes almost as intense as the resonance of glutamine γ-N.

3.3. Inhibition of [₁⁵N]ammonium incorporation by phosphinothricin and azaserine

Ammonium assimilation can be influenced in vivo by adding specific inhibitors of GS and GOGAT [7,22]. The biosynthetic reaction catalysed by GS purified from A. bisporus is inhibited strongly by phosphinothricin (PPT; \( K_i = 17 \) \( \mu \)M, [23]). GOGAT activity in cell-free extracts was inhibited completely by 1 mM azaserine (AZS, [9]). In vitro NADP-GDH activity was not affected by addition of PPT (5 mM) to the NADP-GDH assay mixture, while addition of AZS (1 mM) caused about 25% inhibition of NADP-GDH activity. So it is unlikely that these inhibitors exert a significant effect on NADP-GDH activity in vivo. The effect of inhibitors on the ammonium assimilatory pathway was tested with mycelium pre-grown on medium B with 100 mM glucose and 20 mM (NH₄)H₂PO₄. After 22 days of cultivation, actively growing mycelium, as followed from laccase activity, was harvested. At this time the residual ammonium concentration of the medium was 10 mM. For incubation with [₁⁵N]NH₄⁺ the harvested mycelium was divided into 3 equal portions (4.1 g fresh weight per portion) and resuspended in 50 ml of medium

<table>
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<tr>
<th>Amino acid</th>
<th>No inhibitor</th>
<th>Azaserine (1 mM)</th>
<th>Phosphinothricin (5 mM)</th>
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<tr>
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<td>1.4</td>
</tr>
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<td>Asparagine</td>
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<td>1.9</td>
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</tr>
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<tr>
<td>γ-Aminobutyrate</td>
<td>3.7</td>
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</table>

Mycelium was pre-grown on medium B with 100 mM glucose and 20 mM (NH₄)H₂PO₄. After 22 days of cultivation actively growing mycelium was harvested and divided in 3 equal portions (4.1 g fresh weight) and resuspended in 50 ml of medium B with 10 mM [₁⁵N]NH₄Cl, antibiotics and additions. After 19.5 h soluble nitrogen pools were extracted from the mycelium and amino acids were analysed by HPLC.

B with 10 mM [₁⁵N]NH₄Cl and antibiotics. After 19.5 h soluble nitrogen pools were extracted from the mycelium.

The addition of inhibitors of ammonium assimilation had marked effects on the uptake of [₁⁵N]NH₄⁺. Addition of 1 mM AZS to ammonium-grown mycelium inhibited uptake by 60%. Addition of 5 mM PPT completely inhibited uptake of [₁⁵N]NH₄⁺ and even resulted in an increase of extracellular ammonium of 0.05 mM. When inhibitors were absent, peaks could be observed at 40.62 ppm and 111.84 ppm in the spectra (Fig. 3A). Also some other peaks were observed, which could hardly be distinguished from the noise. These peaks became more pronounced after prolonged incubation (Fig. 1). Inhibition by AZS resulted in the accumulation of labelled glutamine γ-N, at the expense of the intensities of the α-N peak at 40.62 ppm (ratio γ-N/α-N changed from 2.2 ± 0.1 to 3.7 ± 0.1, Fig. 3C). No incorporation of [₁⁵N]NH₄⁺ was observed when PPT was added (Fig. 3B). Analysis of the free amino acid pools of the mycelium also reflects the influence of addition of inhibitors (Table 1). Levels of glutamine and alanine were influenced most by the addition of AZS or PPT. Addition of 1 mM AZS to the incubation medium increased the levels of glutamine and alanine by a factor 2 and 18, respectively. Inclusion of 5 mM PPT in the incubation medium caused a drop in the level of glutamine by a factor 10.

4. Discussion

Growth conditions of the mycelium can have a large influence on the pathway used for the assimilation of ammonium in fungi. In N. crassa it has been found that NADP-GDH and GS are responsible for ammonium assimilation when there is a large supply of nitrogen. However, when the ammonium concentration is low, assimilation is
mainly catalysed by the GS/GOGAT-pathway [4,5]. In
C. geophilum, ammonium assimilation takes place via the
concurrent activity of NADP-GDH and GS. However, in
the N-starved mycelia N-flux through GDH is higher than
the flux through GS, while in rapidly growing mycelia the
opposite situation occurs [6,24].

This study describes the use of $^{15}$N-NMR to study
ammonium assimilation in A. bisporus. The main signals
of the NMR spectra of extracts from $[^{15}$N]ammonium-
grown mycelium (Fig. 1) corresponded with $\alpha$-N of se-
veral amino acids and $\gamma$-N of glutamine. Some minor
signals were located at 32.7, 71, 83.6 and 124.7 ppm. The
resonance at 32.7 ppm presumably corresponds with $\gamma$-
aminobutyrate. Since the Krebs cycle appears to be blocked
at 2-oxoglutarate dehydrogenase, there is an active $\gamma$-
aminobutyrate shunt [25]. The resonance at 124.7 ppm
corresponds with the formation of N-acetylglucosamine.

Jennings [26] proposed that a nitrogen reserve could be
formed by polymerization of N-acetylglucosamine. This
could be the reason why the cell wall of A. bisporus
contains about 45% N-acetylglucosamine. The intensity of
the resonances at 71 and 83.6 ppm, corresponding with $\omega$,$\omega'$-N and $\delta$-N from arginine, respectively, is very low
as compared to arginine resonances in C. graminicorne [16]
and N. crassa [14]. These fungi appear to accumulate
arginine as a nitrogen reserve into their vacuoles [16,27].

Time-dependent incorporation showed that glutamine,
glutamate and alanine are labelled first (Fig. 2). Nitrogen
incorporated into glutamate is quite rapidly transaminated
to alanine. This is consistent with the high activity of
 glutamate-pyruvate transaminase found in cell-free extracts of
A. bisporus [9]. However, transamination to aspartate
was not observed, despite a high activity of glutamate
oxaloacetate transaminase in cell-free extracts [9]. The
activities of the primary enzymes of ammonium assimila-
tion have been determined by Baars et al. [9]. In ammno-
ungrown mycelium activities of GS and NADP-GDH
were 0.028 and 0.013 U · mg$^{-1}$, respectively. To deter-
mine their relative contribution in ammonium assimilation,
inhibitor studies, including effects on $^{15}$NH$_4^+$ uptake/in-
corporation and on amino acid pools, were performed
(Table 1; Fig. 3). Inhibitory effects of PPT and AZS on
actively growing mycelium were reflected in the pools of
free amino acids. Inhibition by PPT resulted in a 10-fold
decrease of the glutamine pool and a moderate increase of
the pools of glutamate and alanine. Incorporation of $^{15}$NH$_4^+$
was completely blocked. Moreover an excretion of ammno-
rium was observed. This indicates a rapid intracellular
turnover of unlabelled nitrogen-containing compounds.
Release of ammonium was also found by Lea et al. [28] upon
incubation of cyanobacteria and a number of higher plants
with PPT. Inhibition by AZS resulted in a marked accumu-
lation of alanine and a 2-fold increase of glutamine. Inhibi-
tion by AZS apparently is not absolute since labelling of
glutamate is still found. No labelled alanine was observed
which indicates that the increase in alanine must be de-
rived from the above-mentioned turnover. These results are
compatible with the presence of a $\sigma$-amidase pathway in
which unlabelled $\alpha$-N of glutamine is transaminated. Such
a pathway was described in N. crassa [29]. Based upon
the complete inhibition of $^{15}$NH$_4^+$-incorporation by PPT, a
specific inhibitor for GS from A. bisporus [23], it can be
concluded that ammonium assimilation in A. bisporus
mainly proceeds via the GS/GOGAT pathway. NADP-
GDH is not or only moderately affected by PPT and AZS,
respectively.

Ammonium assimilation in A. bisporus shares a num-er of characteristics with ammonium assimilation in the
basidiomycete fungus S. semiglobata [8]. The results of
$^{15}$N-labelling showed that, despite the high activity of
NADP-GDH, ammonium assimilation is catalysed mainly
by the action of GS/GOGAT. Martin et al. [12] concluded
from their $^{15}$N-labelling studies with ammonium-fed beech
ectomycorrhizal fungi, that ammonium assimilation mainly
occurs via the GS/GOGAT pathway and that NADP-GDH
plays little, if any, role in this process. In other fungi such
as Cenococcum graminicorne [6,24], A. nidulans [22], N.
crasa [5] and the yeasts Candida albicans and Saccha-
romyces cerevisiae [30], the concurrent operation of
NADP-GDH and GS/GOGAT is reported. Relative participa-
tion of the GS/GOGAT pathway is calculated to be
1.6% in S. cerevisiae, 78% in C. albicans [30] and about
50–60% in A. nidulans [7,22].

In the past, the elucidation of the pathway of ammo-
nium assimilation in fungi has relied heavily on the use of
mutants and on measurement of the levels and kinetic
properties of the enzymes involved, particularly NADP-
GDH and GS. In recent years $^{15}$N-NMR has been added to
to these methods of studying metabolism (for reviews see
Martin [31] and Lundberg et al. [32]). Present results have
shown a distinct role for GS/GOGAT in nitrogen assimila-
tion in A. bisporus. NADP-GDH appears to play a minor
role in the assimilation of ammonium. Studies with mutant
strains of A. bisporus may help in obtaining insight in the
precise role of this enzyme in nitrogen metabolism. More
detailed information could further be obtained from $^{15}$N-la-
labelling experiments followed by gas chromatography-mass
spectrometry (GC/MS) analysis.

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References


