Methylamine and Ammonia Transport in *Stemphylium botryosum*

By ADINA BREIMAN AND ISAAC BARASH

*Department of Botany, The George S. Wise Faculty for Life Sciences,
Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel*

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[¹⁴C]Methylamine was transported into mycelial cells of *Stemphylium botryosum* by a specific and energy-dependent transport system having an optimum at pH 6.0, a K_m of 12.5 μ M and a V_{max} of 4.1 μ mol (g dry wt)⁻¹ min⁻¹; uptake occurred against a concentration gradient. NH₄⁺ competitively inhibited methylamine transport with higher affinity towards the latter system. Sucrose and nitrate were required during transport for maximal activity. Highest transport activity developed in nitrate-grown mycelium. Nitrogen starvation decreased the activity by approximately 60 %. Preloading of mycelium with glutamine, asparagine or ammonia almost completely prevented methylamine uptake. Transport activity was inversely proportional to the intracellular concentration of the L-amides. It is postulated that ammonia uptake might be regulated by L-amides rather than by ammonia.

INTRODUCTION

Ammonia in high concentration is toxic to animals and higher plants (Warren, 1962) and may accumulate to inhibitory levels in some fungi (Cochrane, 1958; Gilpatrick, 1969). Nevertheless, the filamentous fungus *Stemphylium botryosum* can grow on high ammonia concentrations without being affected in its growth rate or in the composition of various cell constituents (Breiman, 1978). These results suggest that the intracellular ammonia concentration might be regulated by the entry of ammonia into the cells and/or its assimilation into organic nitrogen.

Ammonia transport systems in mycelial fungi have been described for *Penicillium chrysogenum* (Hackett *et al.*, 1970) and *Aspergillus nidulans* (Pateman *et al.*, 1974; Cook & Anthony, 1978*a, b*). Although the ammonia transport systems in these fungi and in yeast (Roon *et al.*, 1975) share many common properties, differences in regulation might exist. Thus ammonia transport is derepressed approximately 800-fold in *P. chrysogenum* during nitrogen starvation (Hackett *et al.*, 1970) whereas the activity of the *Saccharomyces cerevisiae* system changes very little (Roon *et al.*, 1977).

We have previously reported that the assimilation of ammonia via NADP-specific glutamate dehydrogenase in *S. botryosum* is regulated by L-asparagine and L-glutamine (Breiman & Barash, 1978). It was therefore of interest to determine whether the same factors also regulate the entry of ammonia. The object of the present study was to characterize the ammonia transport system and its possible regulation in *S. botryosum*.

METHODS

Organism and culture conditions. A wild-type strain of *Stemphylium botryosum* Wallroth (Breiman & Barash, 1976) was used throughout these studies. Mycelium was grown in Roux bottles containing 100 ml liquid medium and incubated for 5 d at 25 °C. Growth medium was made in 0.1 M-potassium phosphate buffer pH 6.5 and contained (mM): MgSO₄, 2; KCl, 6.7; K₂HPO₄, 1.2; FeCl₃, 0.06; sucrose, 30; KNO₃, 50; and chloramphenicol, 0.15. The mycelium was removed from the growth medium, washed and dispersed in

Fig. 1

Fig. 2

Fig. 1. Effect of various nitrogen sources at different concentrations on the accumulation of intracellular NH_4^+ in *S. botryosum*. NH_4^+ determinations were carried out after 1 h incubation in the presence of KNO_3 (○), L-asparagine (□), NH_4Cl (Δ) or L-glutamine (●).

Fig. 2. Effect of sucrose and nitrate on methylamine transport. The fungus was grown in nitrate medium at 25 °C for 5 d. The mycelium was then removed, washed in deionized water and divided into four parts. Each part was transferred into a different medium and used for transport assay as described in Methods. The flasks were preincubated for 10 min prior to the addition of [^{14}C]methylamine. Media used were 0.1 mM-phosphate buffer, pH 6, alone or with 10 mM- KNO_3 (□), buffer plus 15 mM-sucrose (▲) and buffer plus sucrose plus KNO_3 (○).

0.1 M-potassium phosphate buffer pH 6.0, containing 15 mM-sucrose and 10 mM- KNO_3 , as described earlier (Breiman & Barash, 1976).

Transport of methylamine. This was measured in Erlenmeyer flasks (100 ml) containing 20 ml cell suspension. The flasks were shaken on a reciprocal shaker at 30 °C for 5 min prior to the addition of the radioactive substrate and during the experiment. [^{14}C]Methylamine was used as the ammonia analogue for characterization of the NH_4^+ transport system (Hackett *et al.*, 1970). The reaction was started by a rapid addition of 30 μM -[^{14}C]methylamine (3.2×10^5 c.p.m. μmol^{-1}) to the cell suspension. Samples (4 ml, 10 mg dry wt) were taken every 30 s for at least 3 min. Uptake was terminated by filtering with suction through Whatman GF/C filter paper (2.5 cm diam.) and rinsing twice with 5 ml of cold solution containing 1 mM-methylamine. The mycelial pad was peeled off the filter and counted for radioactivity (Breiman & Barash, 1976). Results were expressed as μmol methylamine transported (g dry wt mycelium) $^{-1}$ min $^{-1}$.

Determination of the intracellular NH_4^+ concentration. The mycelium was washed thoroughly with double-distilled water and 300 mg lyophilized cells were extracted twice with 2 ml double-distilled water at 90 °C for 30 min. NH_4^+ was assayed in the supernatant by the method of Buttery & Rousell (1971), using a NADP-glutamate dehydrogenase-mediated reaction. The pool of amides and amino acids in the mycelium was estimated as previously described (Breiman & Barash, 1978). Extraction of mycelium for [^{14}C]methylamine determination was carried out as described for NH_4^+ except that the mycelium was washed in the presence of 1 mM unlabelled methylamine. Extracts were applied to thin layers of cellulose for chromatography and developed in butan-1-ol/acetic acid/water (12:3:5, by vol.) and propan-2-ol/ethanol/9 % HCl (75:75:50, by vol.). Methylamine was detected on the chromatogram using ninhydrin reagent, with amino acids, NH_4^+ and methylamine incorporated as standards.

Chemicals. [^{14}C]Methylamine hydrochloride (56 mCi mmol^{-1}) was purchased from The Radiochemical Centre, Amersham. Ethylamine and dimethylamine hydrochloride were obtained from Sigma. All other chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Effect of nitrogen source on accumulation of intracellular NH_4^+

Mycelium was starved of nitrogen by agitation in Erlenmeyer flasks containing growth medium without nitrate for 2 h at 29 °C. It was then removed by suction filtration, washed (Breiman & Barash, 1976) and transferred into the same medium containing different nitrogen sources. Ammonia was determined after shaking for a further hour. The concen-

Table 1. *Inhibition of methylamine transport by amines, amides and NH₄⁺*

The initial rate of transport was assayed under standard assay conditions, the labelled substrate and potential inhibitors (0.1 mM) being added simultaneously.

Compound added	Inhibition of transport* (%)
Methylamine	85
Dimethylamine	25
Ethylamine	14
L-Glutamine	35
L-Asparagine	35
L-Leucine	1
NH ₄ ⁺	99.8

* The transport rate in the control was 4.2 μmol methylamine (g dry wt)⁻¹ min⁻¹.

tration of intracellular NH₄⁺ in the presence of NH₄Cl and L-glutamine was about 50 % higher than in the presence of KNO₃; L-asparagine exerted an intermediate effect (Fig. 1). The concentration of intracellular NH₄⁺ was maximal after adding approximately 1 mM nitrogen source and did not increase further. These results support the view that the internal NH₄⁺ concentration must be regulated. Since external NH₄⁺ represses NH₄⁺ assimilation in *S. botryosum* (Breiman & Barash, 1978), it appears that its entry into the mycelium is the major mechanism for controlling the level of intracellular NH₄⁺.

Effect of nitrogen and carbon sources on transport of [¹⁴C]methylamine

To determine the effect of nutritional conditions on methylamine uptake, the initial transport rates were compared in the presence or absence of sucrose, nitrate or both (Fig. 2). Maximal transport was observed in the presence of sucrose and nitrate. When nitrate was omitted, the transport rate decreased by more than 50 %. Uptake of methylamine showed a strict requirement for sucrose or some other readily assimilated carbon source. The need for carbon might reflect the requirement for energy coupling in this active transport system, whereas the effect of nitrate could result from the influence of protein synthesis. These results are entirely different from those obtained with *P. chrysogenum* and *A. nidulans* in which maximal transport took place in the absence of both carbon and nitrogen sources (Hackett *et al.*, 1970). On the other hand, a similar requirement for carbon source but not nitrogen was demonstrated in yeast (Roon *et al.*, 1975).

pH, temperature and energy dependence of methylamine transport

The initial rate of methylamine uptake was maximal at pH 6.0 with approximately 50 % of the maximum activity at pH 4.5 and 8. KCl at 10 mM and above caused about 40 % inhibition of transport activity. Transport was optimal at 30 °C and had a Q_{10} value of 2 measured between 20 and 30 °C. Methylamine uptake was inhibited by more than 75 % when inhibitors of energy metabolism such as dinitrophenol, sodium azide, KCN or α -iodoacetamide were added at 1 mM.

The intracellular accumulation of [¹⁴C]methylamine against a concentration gradient was investigated by the procedures described in Methods. The mycelium was incubated in the presence of 30 μM (3×10^{-5} c.p.m. μmol^{-1}) or 3 μM (3×10^{-6} c.p.m. μmol^{-1}) methylamine for 50 min. In both treatments more than 85 % of the extracted label was identified as methylamine by thin-layer chromatography. At this time the intracellular concentration of methylamine calculated on the whole cell volume (Breiman & Barash, 1976) was 11 mM and 1 mM for the first and second treatments, respectively. This amount of methylamine represents a concentration gradient of at least 380:1 between the mycelial cells and external environment.

Fig. 3. Effect of NH_4^+ concentration on the lag in methylamine uptake. Transport was assayed as described in Methods. Initial NH_4^+ concentrations were 0.10 mM (■), 0.05 mM (●) and 0.03 mM (□); control (○).

Specificity and kinetics of methylamine transport activity

Ammonia was the strongest inhibitor of methylamine transport among a variety of amines and amides tested (Table 1). Significant inhibition was also observed with either glutamine or asparagine, whereas dimethylamine and ethylamine showed lower inhibition. Other amino acids tested only slightly decreased the initial rate of transport (results not shown). Similar inhibition by asparagine and glutamine were reported in other fungi (Hackette *et al.*, 1970; Roon *et al.*, 1975; Cook & Anthony, 1978*b*).

The time course of methylamine transport in the presence of various concentrations of NH_4^+ is shown in Fig. 3. The lag period before methylamine transport commenced was almost proportional to the initial NH_4^+ concentration. Assuming that the lag period is a measure of the time required to transport NH_4^+ into the mycelium, a rough estimation of the NH_4^+ transport rates can be made as described by Hackette *et al.* (1970). An approximate rate of $6.5 \mu\text{mol (g dry wt)}^{-1} \text{ min}^{-1}$ was calculated. This value is 66 % of that reported for mycelial cells of *P. chrysogenum* (Hackette *et al.*, 1970) and about 40 % of that reported for germinated spores of *A. nidulans* (Cook & Anthony, 1978*a*). The K_m and V_{max} values obtained for methylamine by extrapolation from a Lineweaver-Burk plot were $12.5 \mu\text{M}$ and $4.1 \mu\text{mol (g dry wt)}^{-1} \text{ min}^{-1}$, respectively. NH_4^+ competitively inhibited the entry of methylamine with a K_i of approximately $8 \mu\text{M}$.

Dependence of methylamine transport on nitrogen source

To determine the effect of the nitrogen source on the level of transport activity, the mycelium was grown on nitrate medium for 5 d, harvested, washed and transferred into various media as described in Table 2. Nitrogen starvation for 4 h decreased methylamine uptake by 60 %. These results indicate that the system for NH_4^+ transport in *S. botryosum* differs in its regulation from those in *P. chrysogenum* and germinated conidia of *A. nidulans* which required nitrogen starvation for their development or derepression (Hackette *et al.*, 1970; Cook & Anthony, 1978*b*). In yeast cells, the maximal rate of methylamine transport occurred when they were grown in the presence of NH_4^+ (Roon *et al.*, 1975), yet NH_4^+ almost completely abolished transport activity in *S. botryosum* (Table 2).

Cells incubated in the presence of nitrate showed the highest transport activity, whereas addition of glutamine, asparagine or NH_4^+ to the nitrate medium decreased the transport rate about 40-fold (Table 2). The most remarkable change in the intracellular pool following the addition of L-amides or NH_4^+ was the increase (4- to 15-fold) in the glutamine plus asparagine concentration (Table 3). In contrast to the amide pool, the intracellular NH_4^+

Table 2. *Effect of preincubation with various nitrogen sources on methylamine transport*

Mycelium was incubated in a shaker at 25 °C in nitrate medium containing various nitrogen sources (10 mM) as indicated. After 4 h it was washed and used for transport assay under standard conditions.

Nitrogen source	Transport rate [μmol (g dry wt) $^{-1}$ min $^{-1}$]
KNO ₃ (control)	4.20
L-Glutamate	2.47
L-Glutamine	0.09
L-Asparagine	0.10
NH ₄ Cl	0.09
Methylamine	1.10
Without nitrate*	1.68

* Nitrogen source was omitted from the medium.

Table 3. *Effect of different nitrogen sources on the pools of amides and NH₄⁺*

Amino acid or NH ₄ ⁺	Amino acid or NH ₄ ⁺ concn [μmol (g dry wt) $^{-1}$]				
	Nitrogen-starved mycelium*	Nitrogen source added (5 mM)†			
		KNO ₃	NH ₄ Cl	Asparagine	Glutamine
L-Glutamine + L-asparagine	3.4	6.9	40.1	101.6	27.7
Glutamate	9.17	29.5	80.1	52.0	74.7
Total amino acids	85.7	128.0	293.0	314.0	221.0
NH ₄ ⁺	9.2	13.5	20.7	17.1	22.3

* Mycelium was incubated in a shaker for 2 h in the presence of sucrose.

† Nitrogen sources were added to nitrogen-starved mycelium and incubated for 1 h.

pool was significantly more stable, increasing only 1.3- to 1.7-fold under the same conditions. Although the initial concentration of the nitrogen sources in the experiment described in Table 3 was lower (5 mM) than in Table 2 (10 mM) the level and proportion of the amides and amino acids in the intracellular pool were constant even at higher external concentrations of glutamine, asparagine or NH₄⁺ (Breiman, 1978). Consequently the two experiments can be compared and suggest that the NH₄⁺ transport activity is inversely proportional to the intracellular concentration of asparagine and glutamine.

Our results support the hypothesis that L-amides (Hackett *et al.*, 1970; Cook & Anthony, 1978*b*) rather than NH₄⁺ (Pateman *et al.*, 1974) may function as regulators of the NH₄⁺ transport system in *S. botryosum*. It should be pointed out that due to compartmentation of amino acids and amides in fungi (Pateman & Kinghorn, 1975), more direct evidence would be necessary for direct proof (Breiman & Barash, 1978). L-Amides have been demonstrated as co-repressors of NADP-specific glutamate dehydrogenase, the major enzyme for NH₄⁺ assimilation in *S. botryosum* (Breiman & Barash, 1978). It is therefore reasonable to postulate that the accumulation of L-amides, which reflects the overall nitrogen sufficiency of the cells, suppresses the uptake and assimilation of NH₄⁺. However, the requirement of nitrate for optimal development and activity implies that the present system does not function in the scavenging of low concentrations of NH₄⁺ during nitrogen starvation, as proposed for *A. nidulans* (Cook & Anthony, 1978*a*).

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