Characterization of an Ammonium Transport System in Filamentous Fungi with Methylammonium-\textsuperscript{14}C As the Substrate\textsuperscript{*}

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SUMMARY

Penicillium chrysogenum will grow on methylamine (methylammonium chloride) as its sole nitrogen source. The amine enters the mycelium by means of a transport system (permease) that satisfies several criteria for its identity as an ammonia (or ammonium ion) permease. Transport is temperature-dependent ($Q_10$ of 2.1 between 20° and 30°), pH-dependent (optimum between pH 4.5 and pH 8.5), and ionic strength-dependent (optimum and relatively constant between 0 and 10^{-2} M KCl). Methylamine transport by nitrogen-sufficient mycelium is extremely low and obeys first order kinetics up to 10^{-3} M external substrate. Nitrogen starvation causes the derepression or deinhibition (or both) of a saturable transport system ($K_m \approx 10^{-4}$ M, $V_{max} \approx 10$ μmoles per g-min). This results in an 800-fold increase in the rate of methylamine transport at external substrate concentrations below 10^{-4} M. Ethylamine transport has the same $V_{max}$, but a much higher $K_m$ value (approximately 10^{-4} M). Methylamine transport is not inhibited significantly by other amines, amino alcohols, or amino acids previously shown to be substrates of a nitrogen-regulated amino acid permease (Benko, P. V., Wood, T. C., and Segel, I. H., Arch. Biochem. Biophys., 129, 498 (1968)). The amino acid permease and the methylamine permease also do not develop coincidentally during nitrogen starvation, while the choline permease is unaffected by nitrogen starvation. Ammonia (ammonium ion) is one of the most important nitrogen sources for living cells. Although a great deal is known about the enzymology and regulation of ammonia metabolism, virtually nothing is known about the transport processes by which the ammonium ion enters living cells. This is not too surprising as most studies on transport phenomena are designed for use with radioactive substrates and no long lived radioactive isotope of nitrogen exists. Transport experiments with \textsuperscript{15}NH$_3$ would be rather expensive and time-consuming. We became interested in ammonia transport as a result of our investigation of the multiplicity and regulation of amino acid permeases in several filamentous fungi (1, 2). We found that Penicillium chrysogenum and Aspergillus nidulans possess a relatively nonspecific amino acid permease that develops as a result of nitrogen deprivation. Ammonium was the only non-L-amino acid compound that seemed to inhibit the permease (2). This result suggested that ammonia might be a regulator of the amino acid permease. However, it was also possible that ammonia was being rapidly transported into the mycelium and converted intracellularly to a feedback inhibitor of the amino acid permease. (Other results had indeed established that a brief preliminary incubation of nitrogen-starved mycelium with ammonium chloride or amino acids caused a marked decrease in subsequent amino acid transport activity.) It was important then to establish the properties of the ammonia transport systems in one existed.

We decided to determine whether methylamine-\textsuperscript{14}C could be used as an ammonia analogue and substrate for an ammonia permease. The literature on alkyl amine metabolism is not extensive. Zarlengo and Abrams (3) presented evidence that ammonia, methylamine, dimethylamine, and trimethylamine penetrate cells of Streptococcus faecalis as the free bases at pH 7.0 or below. Arst and Cove (4) reported that methylamine could be used as a nitrogen source by A. nidulans if the amine were present at 1 mM in the growth medium, but that it was toxic at 100 mM. Kung and Wagner (5) identified γ-glutamylmethylamine as a product of methylamine metabolism by Pseudomonas strain MS, while earlier Shaw, Tsai, and Stadtman (6) identified N-methylglutamate and alanine as methylamine metabolites. Amine oxidases from Pseudomonas AM1 (7) and A. nidulans (8) have been studied. An amine oxidase would produce NH$_4^+$ from methylamine and this might be the first step in methylam-

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amine utilization. However, an equivalent amount of formaldehyde would also be produced, and this is likely to be toxic.

In this paper we present evidence for the existence of a relatively specific, nitrogen regulated ammonia permease in mycelium of P. chrysogenum. The permease will accept methylamine as a substrate, but with a greatly reduced affinity.

MATERIALS AND METHODS

Growth of Mycelia—Most of the experiments described in this paper were carried out with P. chrysogenum, wild type strain DSM 75. The organism was grown aerobically, in submerged culture, at 25°C, in citrate No. 3 synthetic medium containing (per liter) glucose (40 g), Na2SO4 (1 g), (NH4)2HPO4 (10 g), (NH4)2HPO4 (6 g), K2HPO4 (18 g), and trace metals solution (10 ml) (9). The glucose was sterilized separately and added to the rest of the medium just before inoculation. The pH of the medium was about 7 at the start and remained above 5 throughout the usual 1- or 2-day growth period. The cells were grown in 500-ml Erlemeyer flasks containing 100 ml of medium, on a New Brunswick gyratory shaker operating at a speed of 250 rpm and describing a 1-inch circle. Only fine, "hairy," microfilamentous mycelium was used for transport studies. The maintenance of the organism as spore suspensions and the production of mycelia from the spores have been described earlier (1).

Growth of Mycelium on Methylamine—P. chrysogenum will utilize methylamine as sole nitrogen source, but not as well as NH4+. As in b, this rate-limited step shows up as a lag in young cultures and as a reduced linear growth rate in older cultures. Consequently, it seems unlikely that the lag represents an induction period. There are several possible explanations for the lag period. (a) Utilization of methylamine requires something that is present in dense mycelial cultures, but is not present or is insufficient in light ones. This factor could be low pH or CO2, or both. (b) Methylamine inhibits enzymes that utilize NH4+ (e.g. glutamic dehydrogenase, glutamine synthetase, carbamyl phosphate synthetase, etc.). This inhibition manifests itself as a lag period in young cultures (in which the initial increase in mycelial mass is exponential), and as a reduced linear growth rate in older cultures. (c) The methylamine contains a contaminant that is inhibitory to fungi. (d) The utilization of methylamine produces a toxic substance (e.g. formaldehyde). (e) Methylamine growth is limited by the rate at which the nitrogen can be extracted from methylamine (or the rate at which methylamine can be converted to a more readily metabolizable compound). As in b, this rate-limiting step shows up as a lag in young cultures and as a reduced linear growth rate in older ones.

Linearity of Methylamine Transport—Figs. 2 and 3 show that...
the uptake of methylamine-\(^{14}\)C is linear with time and mycelial density under our standard assay conditions. The apparent departure from linearity at high mycelial densities results from depletion of the methylamine-\(^{14}\)C from the incubation medium as well as from quenching of the light emission during scintillation counting.

**Effect of Nitrogen Source Used for Growth on Methylamine Transport Activity of Mycelium**—Table I shows the effect of varying the nitrogen source used for growth on the levels of the methylamine, methionine, and leucine transport activities of the mycelium. All of the nitrogen sources used supported good growth. The amino acid transport activities varied considerably with different nitrogen sources. However, the ratio of leucine transport activity to methionine transport activity was relatively constant at about 1.5. These results suggest that most of the methionine and leucine transport by the mycelium is mediated by a single transport system. Methylamine transport also varied with different nitrogen sources. The ratio of transport rates (methylamine-methionine and methylamine-leucine) varied from 0.05 to 27. The results strongly suggested that methylamine transport was not mediated by the same system involved in amino acid transport. However, it is apparent that the activities of both transport systems are markedly influenced by the nitrogen sufficiency of the mycelium.

**Effect of Nutrient Starvation on Transport**—Table II shows the effect of nutrient starvation on transport rates of several compounds by three fungal species. The first two columns of figures show the usual variation in transport activity observed in nutrient-sufficient mycelia of different ages. As reported earlier, sulfur starvation resulted in the development (derepression or dehilation) of specific transport systems for methionine (1), inorganic sulfate (9, 10), and choline-O-sulfate (11). Nitrogen starvation (2) or carbon starvation (12) resulted in the development of a relatively nonspecific transport system for L-\(\alpha\)-amino acids. Methylamine transport increased only after

![Figure 1](left). Growth of *Penicillium chrysogenum* in synthetic medium containing either \(\text{NH}_4^+\) or methylamine as sole nitrogen source. The inocula for both cultures were taken from a 60-hour, methylamine-grown culture. The dry weight curves were essentially identical with those shown above.

![Figure 3](left). Linearity of methylamine-\(^{14}\)C transport as a function of mycelial density in the incubation medium. The suspension contained 2 \(\times\) 10^{-2} M (0.9 amoles) methylamine-\(^{14}\)C and the indicated amounts of mycelium in 25 ml of 5 mm potassium phosphate buffer, pH 6.2.

**Fig. 4 (right).** Development of methylamine and amino acid transport activity as a result of nitrogen starvation. The mycelium was grown in citrate No. 3 synthetic medium containing an excess of \(\text{NH}_4^+\) as the sole nitrogen source. After 1 day, the mycelium was harvested, washed, and resuspended at a density of 2 g, wet weight, per 100 ml of fresh citrate No. 3-N medium. Periodically, portions of the mycelial suspension were removed, washed, and assayed for methylamine-\(^{14}\)C, amino acid-\(^{14}\)C, \(\text{KNO}_3\), and choline-O-sulfate-\(^{35}\)S transport under standard conditions at external substrate concentrations of 2 \(\times\) 10^{-2} M. Sulfate and choline-O-sulfate transport remained very low and unchanged during nitrogen starvation.

| Table I |
| --- |
| **Effect of nitrogen source used for growth on subsequent methylamine, methionine, and leucine transport activity of mycelium** |
| Mycelia of *Penicillium chrysogenum* were grown in citrate No. 3-N synthetic medium containing the indicated compounds as sole nitrogen sources. The cultures were transferred twice at 2-day intervals (10% inoculum) to dilute any \(\text{NH}_4^+\) from the original culture. |

| Nitrogen source (0.2 M) | Mycelial growth | Transport rate | Ratio of transport rates |
| --- | --- | --- | --- |
| | g, wet weight | \(\mu\) moles/g/mm | mL-methionine to methionine | mL-leucine to methionine | mL-leucine to methionine |
| \(\text{NH}_4\text{Cl}\) | 8.5 | 0.02 | 0.26 | 0.38 | 0.08 | 0.05 | 1.46 |
| \(\text{KNO}_3\) | 6.5 | 3.30 | 1.20 | 2.8 | 3.2 | 1.71 | 1.90 |
| Methylamine | 5.9 | 0.66 | 1.33 | 1.85 | 0.49 | 0.36 | 1.37 |
| L-Glutamate | 8.1 | 0.06 | 0.28 | 0.35 | 0.21 | 0.17 | 1.25 |
| L-Proline | 6.6 | 0.02 | 0.13 | 0.22 | 0.13 | 0.07 | 1.70 |
| Glycine | 7.3 | 0.01 | 0.24 | 0.43 | 0.04 | 0.02 | 1.79 |
| L-Alanine | 9.7 | 0.01 | 0.12 | 0.22 | 0.08 | 0.05 | 1.84 |
| D,L-Alanine | 6.4 | 0.02 | 0.19 | 0.53 | 0.10 | 0.06 | 1.73 |
| L-Methionine | 6.9 | 1.30 | 0.05 | 0.06 | 27.2 | 20.2 | 1.20 |
| L-Leucine | 5.5 | 2.0 | 0.18 | 0.13 | 11.1 | 15.3 | 0.72 |

* Transport rates were measured in 5 mm potassium phosphate buffer, pH 6.2, at an initial external substrate concentration of \(2 \times 10^{-2} \text{ M}\).

* At 0.4 m

* Not all of the leucine dissolved in the medium.
The organisms were grown in citrate No. 3 synthetic medium in aerobic, submerged culture at 25°C. After 24 hours, each mycelium was removed from the growth medium, washed with deionized water, and divided into five parts. One part was assayed for transport activity immediately. A second part was reincubated in complete (nutrient-sufficient) medium for 12 hours. A third part was sulfur-starved for 12 hours in citrate No. 3 medium minus a sulfur source. A fourth part was nitrogen-starved for 12 hours in citrate No. 3-N medium. The fifth part was incubated in citrate No. 3 medium minus glucose for 12 hours. The mycelia were incubated in the indicated media at a density of 2 g, wet weight, per 100 ml of medium. After the 12-hour incubation period, the mycelia were removed from the various media, washed, and resuspended at a density of 0.5 g, wet weight, per 25 ml of assay buffer. All assays were run in 0.05 M potassium phosphate buffer, pH 6.0.

### TABLE II

| Organism            | Labeled substrate (2 × 10^{-4} M) | Transport rate | μmoles/min |
|---------------------|----------------------------------|----------------|------------|
|                     | Nutrient-sufficient mycelium^a    | Nutrient-sufficient mycelium^b | Sulfur-deficient mycelium^c | Nitrogen-deficient mycelium^c | Glucose-deficient mycelium^c |
| Penicillium chrysogenum | 0.06 | 0.26 | 2.67 | 4.60 | 2.89 |
| L-Methionine        | 0.03 | 0.03 | 0.09 | 1.15 | 1.64 |
| L-Phenylalanine     | 0.01 | 0.01 | 0.01 | 1.32 | 0.01 |
| Methylamine         | <0.01 | <0.01 | 0.01 | 0.20 | 0.13 |
| Ethylamine          | 0.03 | 0.05 | 1.63 | 0.03 | <0.01 |
| Sulfate             | 0.06 | 0.04 | 1.16 | 0.30 | 0.21 |
| Choline-O-sulfate   | 2.75 | 4.25 | 2.35 | 2.37 | 2.85 |
| Choline             | 3.80 | 2.88 | 1.48 | 1.98 | 3.06 |
| Aspergillus nidulans | 0.08 | 0.41 | 2.28 | 1.88 | 0.93 |
| L-Methionine        | 0.03 | 0.06 | 0.13 | 0.47 | 0.49 |
| L-Phenylalanine     | <0.01 | <0.01 | <0.01 | 1.30 | <0.01 |
| Methylamine         | <0.01 | <0.01 | 0.06 | 0.03 | 0.03 |
| Ethylamine          | <0.01 | <0.01 | 1.66 | 0.01 | <0.01 |
| Sulfate             | 0.46 | 0.80 | 2.68 | 0.31 | 0.32 |
| Choline-O-sulfate   | 3.80 | 2.88 | 1.48 | 1.98 | 3.06 |
| Choline             | 3.85 | 3.38 | 2.20 | 2.50 | 1.64 |
| Penicillium notatum | 0.25 | 0.25 | 1.52 | 4.05 | 2.03 |
| L-Methionine        | 0.03 | 0.01 | 0.01 | 2.23 | 0.84 |
| L-Phenylalanine     | <0.01 | <0.01 | <0.01 | 2.23 | <0.01 |
| Methylamine         | <0.01 | <0.01 | 0.02 | 0.32 | 0.05 |
| Ethylamine          | 0.12 | 0.05 | 2.74 | <0.01 | 0.03 |
| Sulfate             | 0.08 | 0.03 | 0.84 | 0.27 | 0.10 |
| Choline-O-sulfate   | 8.35 | 3.38 | 2.20 | 2.50 | 1.64 |
| Choline             | 8.35 | 3.38 | 2.20 | 2.50 | 1.64 |

^a Original nutrient-sufficient rates, before reincubation at low mycelial density (2 g/100 ml) for 12 hours.

^b After reincubation for 12 hours at low mycelial density in fresh complete medium.

^c All mycelia except that in the carbon-deficient medium increased 2.5- to 3-fold in mass during the 12-hour reincubation period. The carbon-deficient mycelia remained around 2 g/100 ml.

Table III shows the requirements for the development of methylamine permease activity during nitrogen starvation. Transport activity will not develop in the absence of a carbon energy source and oxygen or in the presence of azide or actidione. The inhibition by actidione suggests that the development of transport activity requires protein synthesis, i.e., synthesis de novo of a protein component of the transport system. However, the result can be equally well explained in terms of an inhibition-deinhibition process. If the transport system were controlled by a nitrogen-containing intermediate whose major fate is incorporation into protein, then inhibiting protein synthesis would prevent removal of the regulator from the soluble pool.

**Characteristics of Methylamine Transport System—Effects of pH, Temperature, and Ionic Strength**—The pH dependence of methylamine transport is shown in Fig. 5. The decrease in transport activity on the acid side is similar to that observed for other transport systems of *P. chrysogenum* (1, 2, 10, 11). The decrease in transport rate on the alkaline side of the optimum is...
TABLE III
Requirements for development of methylamine permease during nitrogen starvation

Nitrogen-sufficient Penicillium chrysogenum was incubated in citrate No. 3-N medium under the conditions listed above at a mycelial density of 2 g, wet weight per 100 ml. The “anaerobic” mycelium was incubated in an unshaken flask. The subsequent transport rates were measured, after 10 hours of incubation, at an initial methylamine-14C concentration of $2.5 \times 10^{-6}$ M.

| Incubation conditions (minus nitrogen) | Subsequent methylamine-14C transport rate (pmoles/g-min) |
|----------------------------------------|---------------------------------------------------------|
| Complete medium (aerobic)              | 3.9                                                     |
| Minus glucose                          | 0.1                                                     |
| Minus glucose, plus 4% glycerol        | 3.6                                                     |
| Minus citrate                          | 3.8                                                     |
| Minus citrate, minus glucose           | 0.1                                                     |
| Minus citrate, minus glucose, plus 4% glycerol | 3.9                                                     |
| Minus trace metals                     | 3.9                                                     |
| Plus $10^{-4}$ M azide                 | 0.1                                                     |
| Plus $10^{-6}$ M acididione            | 0.2                                                     |
| Complete medium (“anaerobic”)          | 0.1                                                     |
| Nitrogen-sufficient control            | <0.02                                                   |

not nearly as marked as that observed for amino acids (1, 2), sulfate (9, 10), and choline O-sulfate (11). This is especially obvious in the experiments in which the pH was established immediately prior to adding the labeled methylamine. This procedure minimizes secondary effects of pH on the general metabolism of the mycelium. This result might indicate that the actual substrate of the transport system is the free (uncharged) amine, or that the free amine penetrates the mycelium by a non-carrier-mediated process.

Fig. 6 shows the temperature dependence of methylamine transport. The high Q10 value (2.1) and denaturation at temperatures above 30° are characteristic of a carrier-mediated transport process.

Fig. 7 shows the effect of ionic strength on the transport of methylamine, L-methionine, and sulfate. L-Methionine, which is a dipolar ion at the assay pH, is transported at a relatively constant rate over a wide range of ionic strength of the incubation medium. Transport of choline-O-sulfate (also a dipolar ion) showed the same ionic strength profile (11). Sulfate transport was barely detectable at very low ionic strength, but increased markedly as the ionic strength increased. Methylamine transport was maximal in suspensions of mycelium in deionized water and decreased slightly as the ionic strength increased. The significance of these differences is not clear, but they might be related to the mechanisms of transport. For example, the transport of the negatively charged sulfate ion may be coupled to the passive, simultaneous uptake of positively charged ions. A membrane-bound binding protein, with a high affinity for sulfate, might concentrate sulfate at the cell surface from a very dilute external environment (13). In order to provide an equivalent surface concentration of positively charged ions, a much higher external concentration of K+ (etc.) might be required. The mycelium may possess binding proteins and transport systems for cations, but these may be repressed to a low base level in the cation-sufficient mycelium. (The sulfate transport system was derepressed or deinhibited by sulfur starvation in the presence of the usual medium salts.) Methylammonium ion uptake might be coupled to cation excretion and consequently be relatively independent of the external ionic environment (except at high external salt concentrations at which cations might nonspecifically interfere with the binding of the methylammonium ion to the cell surface).
uncharged methylamine (free base) might be the actual species that is transported.

Energy Dependence of Methylamine Transport—Methylamine transport is inhibited by 90% or more after a brief preliminary incubation of the mycelium with 10^{-5} M azide, 2,4-dinitrophenol, p-nitrophenol, p-chloromercuribenzoate, iodoacetate, cyanide, or N-ethylmaleimide. Azide, 2,4-dinitrophenol, and cyanide act instantaneously to inhibit transport. Some of the results are shown in Fig. 8. The instantaneous action of these inhibitors suggests that cytoplasmic ATP may not be involved in transport, but rather that active transport and energy production may be intimately coupled within the cell membrane. Arsenate at 10^{-3} M (added simultaneously with the substrate or incubated with the mycelium before adding the substrate) had no inhibitory effect. These results are similar to those that we have observed earlier with other transport systems (1, 2, 11).

Accumulation and Retention of Methylamine against Concentration Gradient—Fig. 9 shows the distribution of a pulse of 3 μmoles of methylamine-^{14}C over a 50-min incubation period. By 10 min, virtually all of the methylamine was removed from the incubation medium. More than 90% of the transported label could be removed from the mycelium with hot water. Chromatography and electrophoresis of the 50-min extract showed that more than 90% of the ^{14}C was still present as unchanged methylamine-^{14}C. The remaining ^{14}C had R_P values very similar to those of glycine and serine, although positive identification of the metabolites was not made. By 5 hours, most of the methylamine had disappeared and at least three other major ^{14}C peaks appeared.

The ability of the mycelium to retain methylamine and its metabolites against a concentration gradient is shown by the data in Table IV. Furthermore, the results show that the transport system acts in a unidirectional manner; i.e., there is no apparent exchange between accumulated methylamine-^{14}C and unlabeled external methylamine. Label is lost in the presence of azide or 2,4-dinitrophenol, but at an extremely slow rate compared to the original transport rate (Figs. 2 and 9). The cumulative results suggest that transport is energy-dependent, but that retention is not (except for the energy required for maintaining the integrity of the cell membrane).

| Incubation medium | Mycelial ^{14}C content after incubation for times indicated below |
|-------------------|---------------------------------------------------------------|
|                   | Total | Insoluble, μmoles/g |
| Deionized water   | 4.3   | 4.4 | 4.3 | 4.4 | 1.7 |
| Buffer (0.005 M K^+PO_4, pH 6.2) | 4.5   | 4.5 | 4.2 | 4.1 | 1.0 |
| Plus 10^{-5} M methylamine | 4.8   | 4.9 | 4.9 | 4.9 | 0.9 |
| Plus 10^{-5} M glycine | 4.9   | 4.9 | 5.0 | 4.8 | 1.3 |
| Plus 10^{-5} M l-alanine | 4.4   | 4.4 | 4.2 | 4.1 | 1.3 |
| Plus 10^{-5} M l-methionine | 4.6   | 4.3 | 4.2 | 3.8 | 1.3 |
| Plus 10^{-5} M l-leucine | 4.6   | 4.2 | 4.1 | 4.0 | 1.5 |
| Plus 10^{-5} M l-glutamine | 4.5   | 4.2 | 4.3 | 4.1 | 1.3 |
| Plus 10^{-5} M NH_4Cl | 4.6   | 4.6 | 4.7 | 4.3 | 1.3 |
| Plus 10^{-5} M dinitrophenol | 4.0   | 3.3 | 2.0 | 2.1 | 0.8 |
| Plus 10^{-5} M azide | 4.1   | 3.5 | 1.7 | 1.1 | 0.5 |
| Plus 5% glucose | 4.4   | 4.2 | 4.1 | 3.5 | 1.7 |
Concentration Dependence of Methylamine Transport—Fig. 10 shows the effect of external methylamine concentration on the initial transport rate. In nitrogen-starved mycelium, methylamine transport obeys normal hyperbolic (Michaelis-Menten) kinetics with a $K_m$ of about $10^{-4} \text{m}$ and a $V_{\text{max}}$ of about 10 pmoles per g-min on a dry weight basis. Ethylamine transport in nitrogen-starved mycelium had the same $V_{\text{max}}$ value, but the $K_m$ value was about $10^{-4} \text{m}$. This result was further evidence that we were not observing a general amine transport system.

Methylamine transport by nitrogen-sufficient mycelium was extremely low at low external concentrations and difficult to measure. Transport seemed to be first order with respect to external concentration up to at least $10^{-3} \text{m}$ methylamine. Accurate transport rate measurements could not be made at higher concentrations because of high blank values (methylamine-14C nonspecifically adsorbed to or trapped within the mycelial mat). Methylamine uptake by nitrogen-sufficient mycelium seems to be at least partly a result of active transport rather than diffusion, as it is temperature-dependent and decreased by azide.

The data in Fig. 10 suggest that the development of transport activity after nitrogen starvation (Table II and Fig. 3) results from an increase in the affinity of the mycelium for methylamine (i.e., a decrease in the $K_m$ value). It is not clear as to exactly how the decrease in $K_m$ could arise. Two possibilities are (a) the utilization of an intracellular nitrogen-containing regulator that competes with external methylamine for a (hypothetical) mobile intramembrane carrier, and (b) the derepression of a binding protein component of the transport system. Possibility b presupposes that in the absence of a binding protein external substrate could still combine with the hypothetical carrier but that a much higher external substrate concentration is required to achieve a given substrate-carrier concentration. It is also possible that both explanations a and b may contribute to the shift in the $K_m$ value.

Effect of Potential Inhibitors on Methylamine Transport—The specificity of the methylamine transport system was investigated by observing the effects of potential inhibition on methylamine uptake. For routine assays, methylamine-14C $(10^{-4} \text{m})$ and the potential inhibitor $(10^{-3} \text{m})$ were added simultaneously to the suspension of nitrogen-starved mycelium. Methylamine transport rates were determined as usual from four aliquots taken at 30-sec intervals during the first 2 min of incubation.

Alkyl amines (dimethylamine, trimethylamine, ethylamine, propylamine, isopropylamine, and butylamine) had relatively little effect on methylamine-14C transport (16 to 28% inhibition at the 10-fold excess), while unlabeled methylamine gave an apparent inhibition of 85 to 90%. Pyridine, glutamic acid, Tris, and ethanolamine inhibited less than 15%. These results strongly suggested that we were not dealing with a general amine or amino alcohol transport system. Other simple 1-carbon and 1-nitrogen compounds such as KHCO$_3$, KNO$_3$, and urea had little or no effect. Similarly, common cations (Na$^+$, K$^+$, Mg$^{++}$, etc.) had no effect. Amino acids were relatively noninhibitory, with the exception of glycine, asparagine, and glutamine. The most potent inhibitor was NH$_4^+$ ($>97\%$).

The inhibition by glycine, asparagine, and glutamine was readily explained by the contamination of these compounds by small amounts of NH$_4^+$. The effect of NH$_4^+$ on methylamine transport is illustrated in Fig. 11. The release of inhibition after a few minutes of incubation must result from removal of NH$_4^+$ from the incubation medium by the mycelium. (At pH 6.2 it is highly improbable that NH$_3$ was volatilizing.) The length of the lag period before methylamine transport commences is almost proportional to the initial NH$_4^+$ concentration at low NH$_4^+$ concentrations. If we assume that the length of the lag period is a measure of the time required to transport NH$_4^+$ into the mycelium, we can make a rough estimate of the NH$_4^+$ transport rate. For example, it takes approximately 3 min to overcome the lag produced by $3 \times 10^{-4} \text{ m NH}_4^+$. Thus, 0.15 pmole of NH$_4^+$ (in a 5-ml aliquot) is transported by about 5 mg, dry weight, of mycelium (i.e. that amount in a 5-ml aliquot) in 3 min. This corresponds to a transport rate of about 10 pmole per g-min, which is identical with the $V_{\text{max}}$ value for methylamine. This estimate assumes (a) that the $10^{-4} \text{m}$ methylamine has negligible effect on the transport of NH$_4^+$ at $3 \times 10^{-3} \text{ m NH}_4^+$ (i.e. that the $K_m$ value for NH$_4^+$ is much lower than the $K_m$ value for methylamine) and (b) that the rate of NH$_4^+$ transport is constant over the 3-min interval (i.e. that the transport of >90% of the NH$_4^+$ starting at $3 \times 10^{-2} \text{ m NH}_4^+$ obeys zero order kinetics). Both assumptions are confirmed by the results described below.

Because NH$_4^+$ is so rapidly transported by the mycelium, it was impossible to make accurate measurements of methylamine-14C transport in the presence of a low and constant concentration of NH$_4^+$. On the other hand, because NH$_4^+$ is so powerful an inhibitor of methylamine transport, it was also very difficult to measure methylamine-14C transport rates at high external NH$_4^+$ concentrations (at which the external NH$_4^+$ concentration would remain relatively constant over the assay period), unless the substrate was also quite high in concentration. An attempt to determine the $K_i$ value for NH$_4^+$ is shown in
Fig. 11. The effect of NH$_4^+$ on methylamine-$^{14}$C transport by nitrogen-starved mycelium. The methylamine-$^{14}$C and NH$_4$Cl were added to the suspensions simultaneously.

Fig. 12. Reciprocal plot of NH$_4^+$ inhibition of methylamine-$^{14}$C transport by nitrogen-starved mycelium. (○) “plus NH$_4^+$” values; (■) “minus NH$_4^+$” control value at 10$^{-4}$ M methylamine-$^{14}$C. (The control rate would actually be 91% of the estimated V$_{max}$.)

Table V

| Methylamine concentration | NH$_4$Cl concentration | Relative velocities ($v_i/v_0$) | K$_i$ value for NH$_4^+$ (calculated) |
|---------------------------|------------------------|---------------------------------|--------------------------------------|
| 10$^{-4}$ M              | 2 × 10$^{-4}$ M        | 0.06                            | 1.7                                  |
| 10$^{-4}$ M              | 3 × 10$^{-4}$ M        | 0.07                            | 2.3                                  |
| 10$^{-4}$ M              | 4 × 10$^{-4}$ M        | 0.04                            | 2.1                                  |
| 10$^{-4}$ M              | 6 × 10$^{-4}$ M        | 0.03                            | 2.2                                  |
| 10$^{-4}$ M              | 8 × 10$^{-4}$ M        | 0.03                            | 3.1                                  |
| 10$^{-3}$ M              | 3 × 10$^{-4}$ M        | 0.34                            | 1.5                                  |
| 10$^{-3}$ M              | 5 × 10$^{-4}$ M        | 0.21                            | 1.3                                  |
| 10$^{-3}$ M              | 7 × 10$^{-4}$ M        | 0.17                            | 1.4                                  |
| 10$^{-3}$ M              | 1 × 10$^{-4}$ M        | 0.12                            | 1.4                                  |
| 10$^{-3}$ M              | 2 × 10$^{-4}$ M        | 0.10                            | 2.2                                  |
| 10$^{-3}$ M              | 5 × 10$^{-4}$ M        | 0.09                            | 4.9                                  |

Results show that NH$_4^+$ is a competitive inhibitor of methylamine transport and that the affinity of the transport system is much greater for NH$_4^+$ than for methylamine.

A second attempt to estimate the K$_i$ value for NH$_4^+$ is illustrated in Table V. In these experiments, the methylamine-$^{14}$C concentration was held constant at 10$^{-4}$ M and 10$^{-3}$ M, while the NH$_4^+$ concentration was varied. At the lowest NH$_4^+$ concentrations, initial methylamine-$^{14}$C transport rates were estimated from one or two points taken before the end of the lag period. Because the NH$_4^+$ concentrations used had to be high compared to the K$_i$ value, the usual Dixon plot (1/v against I) could not be used (the $-K_i$ intercept would fall too close to the origin). However, it was still possible to estimate the K$_i$ value by substituting the relative velocities (“plus inhibitor:minus inhibitor”) into the Michaelis-Menten equation as shown below.

$$v_i = \frac{SV_{max}}{K_m + S}$$

Multiplying numerator and denominator by K$_i$:

$$v_i = \frac{K_m + S}{K_i + K_m + K_i S}$$

Expression 2 can be simplified without introducing more than a 10% error as shown below.

If $S \gg K_m$, then $K_i S \gg K_m$, and the $K_i K_m$ term in the numerator may be neglected.
Nitrogen-starved mycelia were incubated in fresh citrate No. 3 N medium, at a density of 2 g, wet weight, per 100 ml with the indicated additions. After 1 hour the mycelia were removed from the incubation media, washed well, and assayed under standard conditions for methylamine-14C and L-methionine-35S transport. The initial external concentration of each labeled substrate was $2 \times 10^{-5}$ M. The experiment was done in several parts. Hence, the range for the control values.

| Addition (0.01 M) | Subsequent transport rate (µmoles/g-min) | Table VI |
|------------------|----------------------------------------|----------|
| None (control)   | 7.0–10.0                                |          |
| NH4Cl            | 4.8                                    |          |
| KNO3             | 11.9                                   |          |
| Methylamine-HCl  | 7.4                                    |          |
| Urea             | 11.8                                   |          |
| Choline chloride | 8.2                                    |          |
| L-Alanine        | 2.1                                    |          |
| L-Alanine        | 5.8                                    |          |
| L-Serine         | 3.5                                    |          |
| L-Lysine         | 6.5                                    |          |
| L-Aspartate      | 6.5                                    |          |
| L-Asparagine     | 0.86                                   |          |
| L-Glutamate      | 8.5                                    |          |
| L-Glutamine      | 1.9                                    |          |
| Glycine          | 5.3                                    |          |
| L-Methionine     | 0.70                                   |          |
| L-Leucine        | 0.26                                   |          |
| L-Phenylalanine  | 0.86                                   |          |
| L-Arginine       | 4.6                                    |          |
| L-α-Aminobutyrate| 0.41                                   |          |
| DL-β-Aminobutyrate| 2.9                                  |          |
| Glycinamide      | 11.8                                   |          |
| Acetamide        | 8.0                                    |          |
| Glycylglycine    | 3.3                                    |          |

\[ K_i = \frac{K_m}{K_m + K_i} \] (3)

When the relative velocity data shown in Table V were substituted into Equation 3, $K_i$ was calculated to be about $2.2 \times 10^{-7}$ M, which is in reasonably good agreement with that obtained graphically as shown in Fig. 12. A similar estimate of the $K_i$ for ethylamine gave a value of about $3.5 \times 10^{-8}$ M, which is in reasonably good agreement with the experimentally determined $K_m$ value of about $10^{-8}$ M for ethylamine-14C transport.

Feedback Inhibition of Methylamine (Ammonium) Transport System—Table VI shows the effect of refeeding substrate amounts of various nitrogen compounds on the methylamine-14C and L-methionine-35S transport rates of nitrogen-starved mycelium. A number of compounds caused a reduction in both transport activities but asparagine and glutamine had the most striking effect on methylamine transport. Leucine, methionine, α-amino butyrate, and phenylalanine, on the other hand, were more effective in reducing methionine transport. A time study established that asparagine and glutamine would "turn off" methylamine transport within a few minutes after their addition to the mycelial suspension. The reduction in transport activity could result from either a feedback inhibition of the transport system or a denaturation of one or more components of the system. The rapidity of turn off by glutamine and asparagine suggests the former. It was interesting to note that glutamine and asparagine were far more effective than NH4+ itself in reducing methylamine transport. Thus, it seems unlikely then that the amides were acting by producing NH4+ intracellularly.

**Effect of Actidione on Stability of Methylamine (Ammonium) Transport System**—As noted earlier, the addition of actidione to mycelial cultures before nitrogen starvation prevents the development of transport activity. Fig. 13 shows that the addition of actidione after a period of nitrogen starvation stimulates a decrease in previously existing transport activity. The decrease could result from a rapid turnover of a protein component of the transport system, as suggested by Wiley and Matchett (14) for the tryptophan permease. The decrease might also result from an increase in the intracellular concentration of feedback inhibitors (e.g., glutamine, asparagine) as a result of protein degradation. Grenson et al. (15) have observed a similar phenomenon in yeast and support the latter suggestion.

**Discussion**

The results presented in this paper confirm that *P. chrysogenum* possesses a highly specific membrane transport system for NH4+. It is likely that most microorganisms possess such a system. The ammonium transport system is under metabolic control. Glutamine and asparagine seem to be regulators. Glutamine plays a central role in the nitrogen metabolism of microorganisms, and, as such, its synthesis by glutamine synthetase is under feedback control by multiple products (16). During nitrogen deprivation, the cumulative feedback inhibition of glutamine synthetase is relieved. However, the steady state level of glutamine will decrease as the amide is utilized for the biosynthesis of various nitrogen-containing compounds. Consequently, the intracellular level of glutamine is a reflection of the total nitrogen sufficiency of the organism. It is not surprising then that glutamine should be a regulator of the ammonium transport system.
Our study of NH₄⁺ transport was stimulated by the observation that NH₄⁺ seemed to inhibit methionine transport via the general amino acid transport system (2). The inhibition can now be explained by the rapid uptake of NH₄⁺ and conversion intracellularly to amino acids, which are feedback inhibitors of the transport system (2). We observed a similar feedback inhibition by intracellular substrate for the choline-O-sulfate transport system of fungi (11). Recently, Ring, Gross, and Heinz (17) described the “transinhibition” of amino acid transport in Streptomyces hydrogenans.

The $K_m$ value of $2.5 \times 10^{-3}$ M for NH₄⁺ transport (assuming that the $K_m$ and $K_i$ values are identical) is one of the lowest that we have observed (1, 2, 9–12). If the true substrate is NH₃, then the actual $K_m$ value would be three orders of magnitude lower. This seems rather unlikely. The species that is transported may well be NH₃, but it seems probable that the substrate that is bound to the cell surface is NH₄⁺.

**Addendum**—Professor J. W. Eckert (Department of Plant Pathology, University of California, Riverside) has recently called to our attention an earlier publication by MacMillan (18) dealing with ammonia transport in fungi. MacMillan concluded that ammonia enters the mycelium as NH₃ by free diffusion. MacMillan’s experiments were conducted with NH₄Cl concentrations of 0.01 to 0.03 M. It is possible that at these high substrate concentrations a nonspecific permeation of NH₃ might obscure the energy-dependent, saturable transport system that we have observed. It is noteworthy, however, that the uptake rates reported by MacMillan can be calculated to be in the neighborhood of 10 μmoles per g-min, which is the $V_{max}$ value that we observe.

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