

Regulation of Nitrate Uptake in *Penicillium chrysogenum* by Ammonium Ion¹

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ABSTRACT

A nitrate uptake system is induced (along with nitrate reductase) when NH_4^+ -grown *Penicillium chrysogenum* is incubated with inorganic nitrate in synthetic medium in the absence of NH_4^+ . Nitrate uptake and nitrate reduction are probably in steady state in fully induced mycelium, but the ratios of the two activities are not constant during the induction period. Substrate concentrations of ammonium cause a rapid decay of nitrate uptake and nitrate reductase activity. The two activities are differentially inactivated (the uptake activity being more sensitive). Glutamine and asparagine are as effective as NH_4^+ in suppressing nitrate uptake activity. Glutamate and alanine were about half as effective as NH_4^+ . Cycloheximide interferes with the NH_4^+ -induced decay of nitrate uptake activity. The ammonium transport system is almost maximally deinhibited (or derepressed) in nitrate-grown mycelium.

Inorganic nitrate is an important nitrogen source for plants and soil microorganisms. The first step in the metabolism of nitrate must be its uptake into the cell. Although a considerable volume of research has been published on nitrate and nitrite reductases (the two enzymes involved in the conversion of nitrate to ammonium ion), virtually nothing is known about the mechanism of nitrate transport. To our knowledge, the work of Heimer and Filner (9) with cultured tobacco cells is the only detailed study of a distinct nitrate uptake system. In this paper, we present evidence for a nitrate uptake system in *Penicillium chrysogenum* which is induced by nitrate along with nitrate and nitrite reductases. Ammonium ion is a regulator of the uptake system.

MATERIALS AND METHODS

Organism and Media. The experiments were conducted with a wild-type strain of *Penicillium chrysogenum* (strain PS-75, ATCC 24791). The mycelium was routinely grown in submerged culture with vigorous shaking at room temperature (about 25 C) in a synthetic medium containing 15 g of sodium citrate·2H₂O, 1 g of Na₂SO₄, 11 g of NH₄Cl, 10 ml of trace metals solution (20), and 40 g of glucose per liter of 0.1 M potassium phosphate buffer, pH 7.0. The glucose was sterilized separately as a 40% (w/v) solution and added to the rest of

the medium before inoculation. In order to induce nitrate reductase and nitrate "permease," the NH_4^+ -grown mycelium was filtered, washed, and reincubated aerobically at a density of 0.5 to 1 g/100 ml in a synthetic medium containing 15 g of sodium citrate·2H₂O, 1 g of Na₂SO₄, 20 g of KNO₃, 10 ml of trace metals solution, and 40 g of glucose per liter of 0.1 M KH₂PO₄. The pH of the nitrate medium was 6.0.

Nitrate Reductase Assay. Cell-free extracts were prepared in a Bronwill eccentric motion ballistic homogenizer (8 ml of potassium phosphate buffer, pH 7.0, containing 1 mM EDTA: 4 g of 0.5 mm diameter glass beads: 2 g wet weight of blotted mycelium). After breaking the cells (2 min of treatment with continuous cooling with a stream of liquid CO₂), the homogenate was centrifuged at 48,000g in a Sorvall RC-2 centrifuge. The clarified extract generally contained 15 mg/ml protein (Biuret method against bovine serum albumin as a standard). The incubation mixture contained (final concentration): 175 μM TPNH, 10 μM FAD, 75 μM NaNO₂, and 15 μl of crude, cell-free extract in a total volume of 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The reaction was allowed to proceed for 5 and 10 min at 24 C and then stopped by adding 0.5 ml of a 1% solution of sulfanilamide in 3 M HCl. Then, 0.5 ml of 0.02% aqueous solution of N(1-naphthyl)ethylenediamine·HCl solution was added. After 10 min, the color was read on a Zeiss PMQ-II spectrophotometer at 540 nm against a blank prepared by adding the sulfanilamide-HCl solution before the enzyme. The absorbancy was compared to a standard curve prepared with known concentrations of nitrite in the standard reaction mixture.

Nitrate Uptake Assay. The uptake assay is based on the observation that nitrate in acid solution absorbs strongly at 210 nm (6). The mycelium was washed with 0.1 M potassium phosphate buffer, pH 6.0, and resuspended in the same buffer at 24 C at a density of 2 g wet weight/100 ml. The suspension was divided into two parts. To one part (30 ml), 0.3 ml of 10 mM KNO₃ was added. Periodically, 4.5-ml samples were filtered rapidly with suction. To the filtrates, 0.5 ml of concentrated HCl was added, and the absorbance was read at 210 nm on a Zeiss PMQ-II spectrophotometer against a blank prepared in the same way with no nitrate added. Samples were also taken periodically from the minus nitrate suspension to correct for leakage of UV-absorbing material from the mycelia during the 7-min uptake period. Under the above conditions, 0.1 mM NO₃⁻ has an absorbance of 0.74 in a 1-cm cuvette. Uptake rates were calculated from the rate at which the absorbance at 210 nm decreased and are reported in terms of $\mu\text{moles} \times \text{g dry weight mycelium}^{-1} \times \text{min}^{-1}$. One gram wet weight of mycelium is equivalent to 0.14 g dry weight.

Methylamine-¹⁴C Transport Assay. The specific activity of the NH_4^+ transport system was measured using methylamine-

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^{14}C as a substrate (8). Transport was measured at 24 C from 5 mM phosphate buffer, pH 6.2, by our usual 2-min uptake assay (four 5-ml samples, one every 30 sec). The initial substrate concentration was 0.1 mM. The specific activity of the methylamine- ^{14}C was 8.5×10^5 cpm/ μmole . The mycelial density in the assay medium was 0.5 g wet weight per 25 ml buffer (14 mg dry weight mycelium per 5-ml sample).

RESULTS

Growth in Nitrate. Figure 1 shows the growth of *P. chrysogenum* on NH_4^+ and NO_3^- as sole nitrogen sources. The difference in growth rates is small but significant. Nitrate assimilation involves the utilization of protons, and consequently the pH rises during growth even in the highly buffered medium. The converse is true for NH_4^+ assimilation. The difference in growth rate may be a reflection of gradual increase in pH to nonoptimal levels. Fungi in general prefer acidic environments, and *P. chrysogenum* will not grow at pH values much above 7.0. The decreased growth rate in nitrate may also result from a rate-limiting step in the conversion of NO_3^- to NH_4^+ . It is noteworthy that the NH_4^+ transport system (8) is almost maximally deinhibited or derepressed in nitrate-grown mycelium, suggesting that the cells are significantly nitrogen-deficient despite the high level of nitrate in the medium (about 0.2 M).

Nitrate Uptake Assay. Figure 2 shows the uptake of NO_3^- by half-maximally induced mycelium from a 0.1 mM solution over a 7-min period under standard assay conditions. The corrected decrease in absorbance corresponds to an uptake rate of $5.25 \mu\text{moles} \times \text{g}^{-1} \times \text{min}^{-1}$. Figure 3 shows the linearity of nitrate removal from the incubation medium as a function of mycelial density. The rate corresponds to about $5.2 \mu\text{moles} \times \text{g}^{-1} \times \text{min}^{-1}$ for all densities between 0.25 and 2 g wet weight mycelium per 100 ml of buffer. Because of the limitations of the assay and our instrumentation, we could not accurately determine nitrate uptake rates from solutions of $< 50 \mu\text{M}$. However, the nitrate uptake rate was the same at all initial

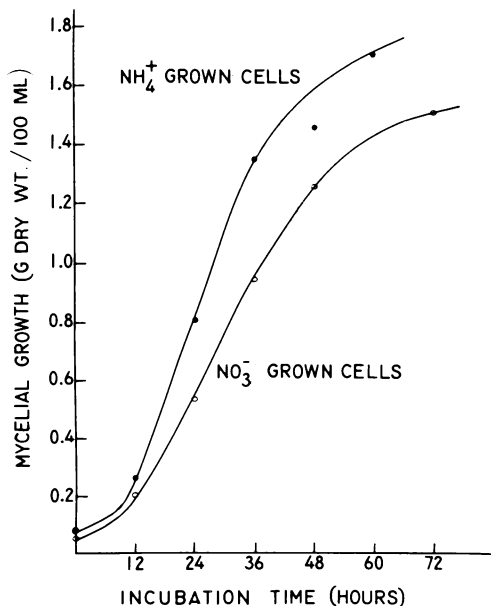


FIG. 1. Growth of *P. chrysogenum* on synthetic media containing either ammonium or nitrate as sole nitrogen source. The mycelia were cultivated in 500-ml Erlenmeyer flasks containing 100 ml of medium (see text), at 25 C, on a rotary shaker operating at approximately 250 rpm and describing a 1-inch circle.

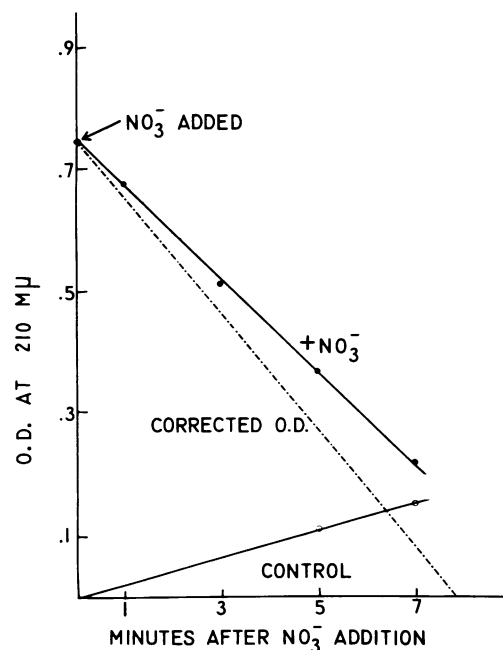


FIG. 2. Standard assay for nitrate uptake-linearity with time. The washed mycelium was resuspended at a density of 2 g wet weight/100 ml. The initial concentration of nitrate was 0.1 mM. Samples were taken at the times shown and treated as described under "Materials and Methods."

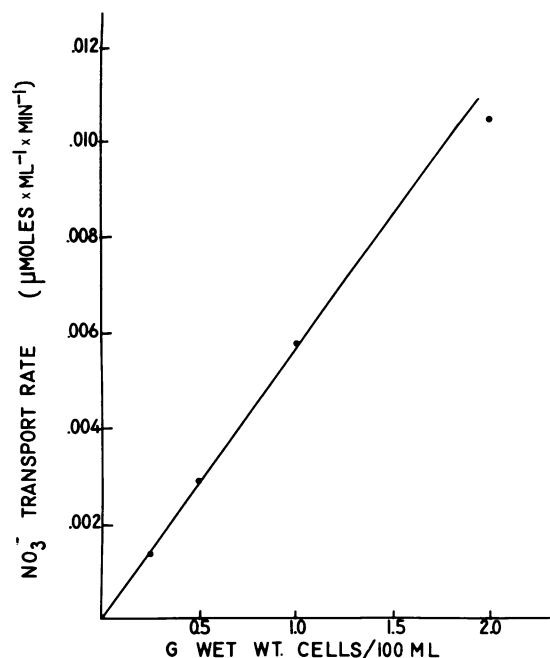


FIG. 3. Linearity of nitrate removal from 0.1 M phosphate buffer, pH 6.0, as a function of mycelial density.

nitrate concentrations between 50 μM and 1 mM. Thus, our assays were conducted at saturating nitrate concentrations and the K_m of the system must be less than 10 μM . In contrast, Heimer and Filner (9) report a K_m of 40 μM for the nitrate transport system of tobacco cells.

Induction of the Nitrate Uptake System. Nitrate uptake activity did not appear when the mycelium was nitrogen-starved

under conditions that deinhibit and derepress the transport systems for NH_4^+ (8) and amino acids (3, 4, 10, 18), nor under sulfur starvation conditions that stimulate development of the specific L-methionine (4), choline-O-sulfate (2), sulfate (5, 20), and cystine (18) transport systems. The nitrate uptake system developed only in the presence of nitrate and glucose (or another suitable carbon source, *e.g.* glycerol). The system did not

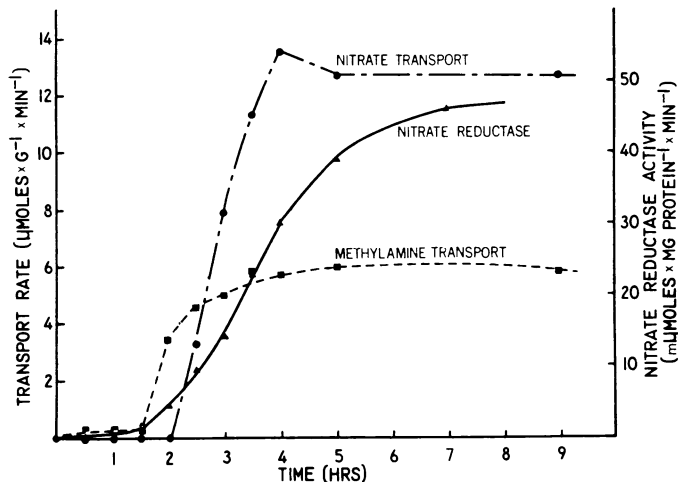


FIG. 4. Induction of nitrate reductase and nitrate uptake activity. Ammonium-grown mycelium was washed thoroughly with 0.1 M phosphate buffer, pH 6.0, and reincubated aerobically at an initial density of 0.5 g wet weight/100 ml of nitrate medium. Periodically, mycelial samples were filtered, washed with 0.1 M phosphate buffer, pH 6.0, weighed, and then resuspended as described under "Materials and Methods" for nitrate reductase and nitrate uptake measurements. The activity of the NH_4^+ transport system was also measured using methylammonium- ^{14}C as the substrate (8).

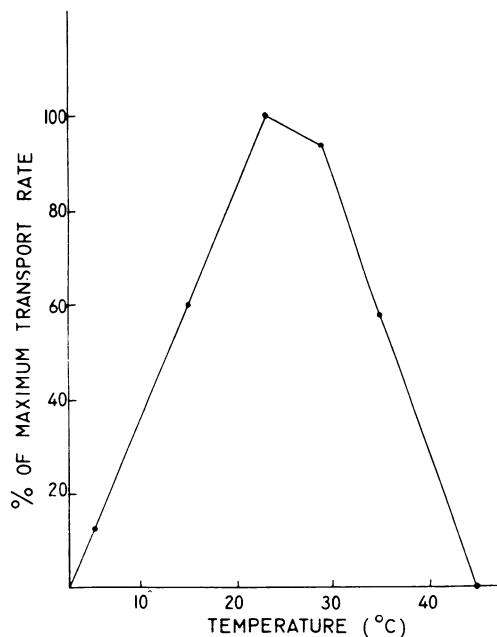


FIG. 5. Temperature dependence of nitrate uptake. The mycelium was filtered and washed at room temperature and then resuspended at 2 g/100 ml in 0.1 M phosphate buffer, pH 6.0, prewarmed or precooled to the indicated temperature. Nitrate (0.1 mM) was immediately added, and the decrease in A_{210} was measured over the next 7 min.

develop in the presence of nitrate if NH_4^+ was also present, nor in the presence of nitrate but absence of glucose. Nitrate uptake activity did not appear in the presence of 0.2 mM cycloheximide. The lack of induction in the absence of glucose cannot be explained solely on the basis of a lack of a carbon-energy source, since the general amino acid transport system derepresses and deinhibits maximally in the absence of an exogenous carbon source (10). The glucose very likely supplies the carbon skeletons for the utilization of endogenous NH_4^+ . Figure 4 shows the time course for the development of nitrate reductase and nitrate uptake activity and the activity of the NH_4^+ transport system (measured with methylammonium- ^{14}C as the substrate). Both nitrate activities appear after 1.5 to 2 hr, but their ratio is not constant during the induction period. The nonconstant ratio strongly suggests that the nitrate reductase and nitrate uptake system are not identical.

Characteristics of the Nitrate Uptake. Figure 5 shows the temperature-dependence of nitrate uptake by induced cells. Figure 6 shows the effect of pH on nitrate uptake. Since nitrate is completely ionized throughout the pH range studied, the effect of pH must be on ionizable groups of the uptake system. Nitrate uptake was relatively insensitive to the ionic strength of the incubation medium (in contrast to sulfate transport by the same organism [5]). Uptake was near maximal from 0.1 mM NO_3^- solutions in deionized water and from KCl solutions and phosphate buffers up to 0.5 M. Chlorate (0.1 mM) had no significant effect while sodium azide (0.1 mM) completely inhibited nitrate uptake. Thus, the general characteristics of the nitrate uptake system are quite similar to the other transport systems we have studied in *P. chrysogenum* (2-5, 8, 10, 18, 20).

Effect of NH_4^+ on Nitrate Uptake and Nitrate Reductase. When nitrate-induced mycelium is incubated with substrate levels of NH_4^+ (10-100 mM), nitrate reductase and nitrate uptake activities decrease (Fig. 7). The rates at which both activities decrease are much too rapid to be explained by repres-

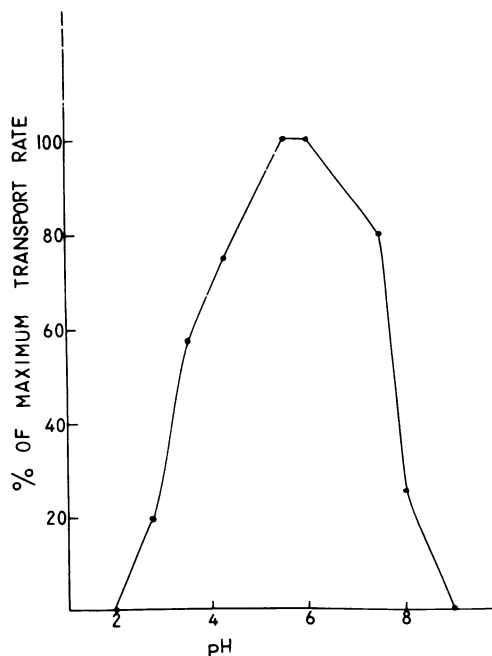


FIG. 6. pH-dependence of nitrate uptake. The mycelium was filtered and washed with the usual buffer, then resuspended at 2 g/100 ml in 0.1 M phosphate ($\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$) premixed to the desired pH.

sion and dilution of pre-existing activity by new mycelial growth. Glutamine and asparagine had the same effect as NH_4^+ in suppressing nitrate uptake. Glutamate and alanine were about half as effective as NH_4^+ . Other amino acids tested (arginine, histidine, leucine, lysine, methionine, phenylalanine, serine, and threonine) had little effect. The decrease is not a result of increasing ionic strength of the medium: 0.1 M KCl or NaCl had no effect. The NH_4^+ -promoted decrease in nitrate reductase has been reported earlier for *Aspergillus nidulans* (7), *Ustilago maydis* (13), *Chlorella* (14), *Cyanidium caldarium* (17), and *Neurospora crassa* (19). The mechanism of NH_4^+ action is unknown. Ammonium ion is not an inhibitor of nitrate reductase *in vitro*. The fact that the nitrate uptake activity decreases significantly faster than nitrate reductase supports the conclusion that the two activities are not identical. Figure 8 shows the time course for the reappearance of nitrate transport and nitrate reductase activities after the mycelium is washed free of NH_4^+ and resuspended in fresh nitrate medium. After a lag period of about 50 min, both activities increase, although not at a constant ratio. The reappearance of nitrate uptake activity is completely prevented by 0.2 mM cycloheximide. (Nitrate reductase was not checked.) No uptake activity reappeared in the absence of glucose. The rates at which the two activities reappear are much faster than the original induction rate shown in Figure 4. The difference in rates most likely reflects the difference in the intracellular pool size of NH_4^+ and glutamine. Even though the mycelium used for the experiment shown in Figure 8 had been preincubated with 0.1 M NH_4^+ for 4 hr, the total pool of regulator(s) might very well be lower than in mycelium grown for 24 hr in NH_4^+ as sole nitrogen source. In fact, if NH_4^+ -grown mycelium is nitrogen-starved for 4 to 6 hr before adding nitrate, the original induction rate of the nitrate uptake system is about the same as that shown in Figure 8. (Nitrate reductase was not measured.) When NH_4^+ -treated mycelium is washed and resuspended in the pH 6 medium with glucose, but without nitrate, uptake activity reappears, but more slowly and to only 25 to 50% of its original level. As before, cycloheximide prevents the reappearance of activity. The rate and level of recovery may be a function of the intracellular concentration of inducer (nitrate).

Effect of Carbon Starvation. When induced mycelium is washed and resuspended in fresh buffer, nitrate uptake activity decreases rapidly, in a manner identical to that shown in Figure 7. The addition of nitrate to the buffer does not prevent the decrease, but if 1% glucose is present, nitrate uptake activity remains essentially unchanged for at least 90 min, even in the absence of nitrate. The decrease in nitrate uptake activity during carbon starvation very likely results from an increase in the intracellular NH_4^+ levels when endogenous amino acids are metabolized (10). When glucose is added back to carbon-starved mycelium (in the absence of exogenous nitrate), uptake activity reappears, but only to about 25% of its pre-existing level.

Effect of Cycloheximide. Attempts were made to assess the effect of cycloheximide on the NH_4^+ -stimulated decrease in nitrate uptake activity. The results of two experiments are shown in Figure 9, A and B. The results were not as clear-cut as those obtained for nitrate reductase (13, 19) because cycloheximide alone causes a decrease in nitrate uptake activity. This is not surprising, since all transport systems that we have studied in *P. chrysogenum* decay in activity during prolonged incubation with cycloheximide (2-5, 8, 10, 18). However, we assume that under the conditions of the experiments shown in Figure 9, A and B (0.1 M phosphate, pH 6.0) cycloheximide would not significantly reduce the total amount of NH_4^+ taken up by the mycelium in 1 to 2 hr. (The total amount of methyl-

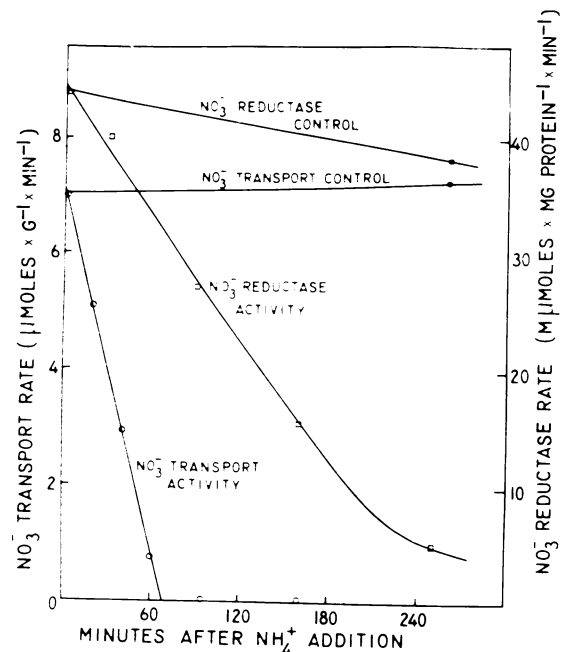


FIG. 7. Decay of nitrate uptake and nitrate reductase activities after addition of NH_4Cl (0.1 M) to fully-induced cultures growing in nitrate medium.

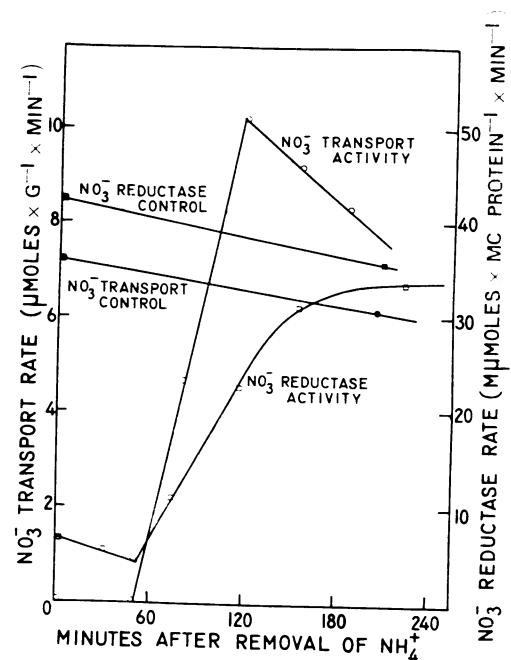


FIG. 8. Reappearance of nitrate uptake and nitrate reductase activities after mycelium preincubated with 0.1 M NH_4^+ for 4 hr is washed and resuspended in fresh nitrate medium. The controls show the activity levels over the same time period for a part of the same culture that had not been preincubated with NH_4^+ .

amine- ^{14}C taken up by nitrate-induced mycelia from 0.1 M solutions in 1 to 2 hr is not affected by cycloheximide.)

DISCUSSION

P. chrysogenum appears to possess a nitrate uptake system that is distinct from nitrate reductase. We have refrained from using the terms "nitrate permease" or "nitrate transport sys-

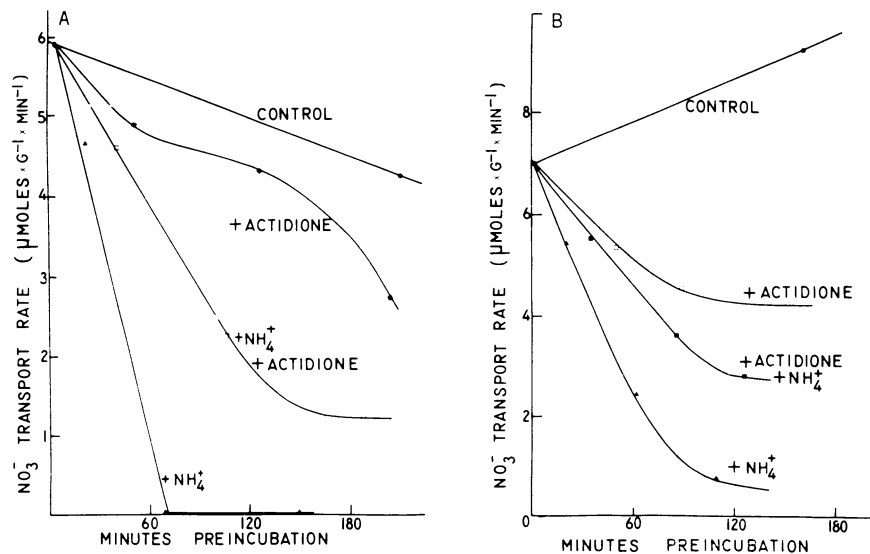


FIG. 9. Effect of cycloheximide 0.2 mM on the decay in nitrate uptake activity caused by adding 0.1 M NH_4Cl to induced mycelium growing in nitrate medium. At the times indicated mycelial samples were removed from the nitrate-cycloheximide medium and resuspended in 0.1 M phosphate buffer, pH 6.0, under standard assay conditions.

tem" because the properties of the uptake we observed may result from the combined (steady state) activities of a nitrate transport system and nitrate reductase.

The nitrate uptake system of *P. chrysogenum* is rapidly and specifically inactivated or inhibited when NH_4^+ , glutamine, or asparagine is added to induced cultures. Several explanations can be offered. (a) Nitrate uptake and nitrate reduction are in a steady state during normal growth in nitrate. Ammonium ion or a closely related metabolite (e.g. glutamine) promotes the inactivation of nitrate reductase. As a result, the intracellular nitrate builds up to a transinhibiting level. Transinhibition is the inhibition of further influx by high intracellular concentrations of the substrate of the transport system. The inhibition very likely results from the binding of the membrane carrier by intracellular substrate. The process is analogous to a Uni Uni enzyme-catalyzed reaction with product inhibition and isomerization of a stable enzyme form. In this case, the isomerization step is the return of the carrier via a partially rate-limiting step, to a conformation capable of binding external substrate (11). (b) NH_4^+ , or a metabolite thereof, is an allosteric inhibitor of the nitrate uptake system, the site of action accessible only from the intracellular side of the membrane. Hence, NH_4^+ must be transported into the mycelium in order to observe any significant effect in nitrate transport. Nitrate reductase is in a state of continual turnover within the cell, the resynthesis phase requiring the inducer (nitrate). As the transport system decays, the intracellular level of inducer drops, thereby decreasing the rate of resynthesis. Suggestion (a) is supported by the observation of Subramanian and Sorger (19) that the intracellular nitrate level is higher in NH_4^+ -treated mycelium of *N. crassa* than in control mycelium, but is not supported by our observation that NH_4^+ promotes a decay in nitrate uptake in mycelium suspended in buffer plus glucose, in the absence of exogenous nitrate.

Lewis and Fincham (13) have proposed the attractive hypothesis that NH_4^+ induces a specific protein that inactivates nitrate reductase. The suggestion is based on the observation that cycloheximide prevents the NH_4^+ -stimulated decrease in nitrate reductase (13, 19) and has a precedent in the observations of Bechet and Wiame (1) and Messenguy and Wiame (15). These workers have shown that yeast ornithine transcar-

bamylase is inhibited by the enzyme arginase, which in turn, is induced by arginine. Cycloheximide prevents the induction of arginase, and thereby, prevents the arginine-promoted decrease in ornithine transcarbamylase activity. Perhaps an analogous regulatory scheme for nitrate uptake and reduction exists in fungi. (c) Ammonium ion may induce an increased level of an NH_4^+ -utilizing enzyme (e.g. glutamic dehydrogenase, carbamyl phosphate synthetase, or glutamine synthetase) which binds to and inactivates nitrate reductase and the nitrate transport system. Alternately, the NH_4^+ -induced protein could be a specific inactivating enzyme similar in action to the adenylating or deadenylating enzyme of the glutamine synthetase system or the kinase or phosphatase of the glycogen phosphorylase system. A specific NH_4^+ -induced protease is also possible. The suggestion of an NH_4^+ -induced regulator protein is based on the assumption that cycloheximide acts only by inhibiting protein synthesis. This is undoubtedly the primary effect of the drug, but if we consider the possibility that in the absence of protein synthesis, protein degradation also ceases after a short while (12, 16), then an alternate suggestion can be made. (d) Nitrate reductase and a protein component of the nitrate uptake system are in a constant state of turnover in nitrate grown mycelium. NH_4^+ represses the resynthesis of both activities and thereby promotes their decay. Cycloheximide protects against the NH_4^+ -promoted decay by slowing up or halting the protein degradation phase of the turnover process.

It is obvious that the metabolism of nitrate is subject to a complex control system. The results reported in this paper suggest that the nitrate uptake system is under a similar control and may be intimately involved in the regulation of nitrate reductase.

ADDENDUM

Recently, Schloemer and Garrett have described a nitrate uptake system in *Neurospora crassa* that appears to be very similar to the *Penicillium chrysogenum* system (R. H. Schloemer and R. H. Garrett, 1973. Abstracts of the Annual Meeting of the American Society for Microbiology, Paper P-194).

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