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Nitrate Transport System in *Neurospora crassa*

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Nitrate uptake in *Neurospora crassa* has been investigated under various conditions of nitrogen nutrition by measuring the rate of disappearance of nitrate from the medium and by determining mycelial nitrate accumulation. The nitrate transport system is induced by either nitrate or nitrite, but is not present in mycelia grown on ammonia or Casamino Acids. The appearance of nitrate uptake activity is prevented by cycloheximide, puromycin, or 6-methyl purine. The induced nitrate transport system displays a K_m for nitrate of 0.25 mM. Nitrate uptake is inhibited by metabolic poisons such as 2,4-dinitrophenol, cyanide, and antimycin A. Furthermore, mycelia can concentrate nitrate 50-fold. Ammonia and nitrite are non-competitive inhibitors with respect to nitrate, with K_i values of 0.13 and 0.17 mM, respectively. Ammonia does not repress the formation of the nitrate transport system. In contrast, the nitrate uptake system is repressed by Casamino Acids. All amino acids individually prevent nitrate accumulation, with the exception of methionine, glutamine, and alanine. The influence of nitrate reduction and the nitrate reductase protein on nitrate transport was investigated in wild-type *Neurospora* lacking a functional nitrate reductase and in nitrate non-utilizing mutants, *nit-1*, *nit-2*, and *nit-3*. These mycelia contain an inducible nitrate transport system which displays the same characteristics as those found in the wild-type mycelia having the functional nitrate reductase. These findings suggest that nitrate transport is not dependent upon nitrate reduction and that these two processes are separate events in the assimilation of nitrate.

The pathway of nitrate assimilation represents the reduction of nitrate to ammonia (20). In *Neurospora crassa*, two cytoplasmic enzymes comprise the assimilatory nitrate pathway: nitrate reductase, which reduces nitrate to nitrite, and nitrite reductase, which catalyzes the reduction of nitrite to ammonia. The nitrate and nitrite reductases are adaptively formed in the presence of either nitrate or nitrite, but are repressed by ammonia or Casamino Acids (5, 21, 24). Ammonia and amino acids, end products of nitrate reduction, do not inhibit the in vitro activity of the nitrate reductase (7) or the nitrite reductase (M. A. Lafferty, personal communication). However, it is not known whether the entry of nitrate into the mycelia is mediated by a specific transport system.

The results of investigations of nitrate utilization in other organisms suggest that there is an inducible transport system for nitrate. When nitrogen-starved wheat seedlings are exposed to nitrate, the rate of nitrate transport is initially low but gradually increases to a constant level (17). Inhibitors of protein and ribonucleic acid (RNA) synthesis restricted the appearance of the nitrate uptake system in corn seedlings (12). Heimer and Filner (10) demonstrated that nitrate accumulation in tobacco cells was energy dependent and was a saturable process. Furthermore, tobacco cells could concentrate nitrate 80-fold, indicating an active transport mechanism for nitrate uptake.

Ammonia, amino acids, and nitrite prevented nitrate uptake in plants (4, 10, 16, 18, 30), algae (2, 28), and fungi (9, 19). However, no attempts were made to clearly demonstrate the mechanism by which these alternative nitrogen sources interfered with nitrate transport. Lyclama (16) suggested that ammonia prevents nitrate uptake indirectly by repression of the nitrate reductase which would ultimately lead to depressed nitrate uptake. However, when nitrate-grown *Spirodela* was supplied with both ammonia and nitrate, only ammonia was assimilated, although no sudden changes in the level of nitrate reductase activity were observed (3),

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indicating that ammonia may prevent nitrate uptake by some mechanism other than repression of the nitrate reductase. In *Penicillium*, nitrate transport activity is lost rapidly upon exposure to ammonia (9). This rapid loss caused by ammonium ions is partially prevented by cycloheximide, suggesting that NH_4^+ induces a protein that inhibits nitrate uptake.

This study presents evidence for a specific transport system for nitrate in *Neurospora crassa*, which is regulated by other nitrogen compounds. The results with nitrate non-utilizing mutants, *nit-1*, *nit-2*, and *nit-3*, suggest that nitrate transport is not dependent upon a functional nitrate reductase.

MATERIALS AND METHODS

Strains and media. Wild-type *N. crassa* (74A) and mutant strains *nit-1* (34547), *nit-2* (nr37), and *nit-3* (14789), deficient in the assimilatory reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductase, were grown on Fries basal medium (21) with ammonium chloride (80 mM) as the sole nitrogen source. *N. crassa* mycelia were grown at 25 C from a conidial innoculum in 800 ml of liquid medium contained in 2,800-ml flasks. After 36 h of growth on a reciprocating shaker, the mycelia were harvested, washed, and distributed among 200 ml of media containing various nitrogen sources for induction. The nitrogen sources were NH_4Cl (40 mM), $NaNO_3$ (20 mM), $NaNO_2$ (10 mM), Casamino Acid digest (5 g/liter), or NH_4NO_3 (20 mM).

Preparation of crude extracts. Mycelia were collected, washed several times with distilled water, pressed dry, weighed to determine the fresh weight, and homogenized as described previously (5).

Production of molybdenum deficiency. Mycelia were grown, as described above, in Fries medium lacking molybdenum. Where indicated, sodium tungstate or sodium vanadate (meta form) was added to the molybdate-free media (25).

EDTA treatment. Mycelia were made permeable to actinomycin D by a modification of the method of Urey and Horowitz (29). Three grams of mycelia was incubated in 100 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.1% ethylenediaminetetraacetic acid (EDTA) for 10 min with continuous shaking. The EDTA solution was replaced with 100 ml of fresh buffer containing EDTA, and incubation was continued for an additional 5 min. Mycelia were harvested and washed several times with distilled water.

Enzyme assays. NADPH-nitrate reductase and NADPH-nitrite reductase were assayed as described earlier (5, 7). One activity unit of the NADPH-nitrate reductase is expressed as the formation of 1 nmol of nitrite per min, whereas the NADPH-nitrite reductase activity is defined as the disappearance of 1 nmol of nitrite per min. Specific activity is expressed as activity units per milligram of protein. Protein was measured by the biuret method (14).

Nitrite and nitrate determinations. Nitrite concentration was determined colorimetrically by the modified diazo-coupling procedure described by Garrett and Nason (7).

Determination of nitrate was performed by the chemical reduction of nitrate to nitrite, employing the cadmium-copper reduction method (31). A 1-ml volume of 0.1 M EDTA solution containing 0.2 N NaOH was added to a sample containing 10 to 50 μmol of nitrate, followed by sufficient distilled water to give a final volume of 50 ml. The sample was then passed through a column containing copperized cadmium filings, and the final 2-ml volume was collected. Nitrite concentration was determined by using a portion of this volume. The values obtained were corrected by subtracting a blank which was obtained after passing a solution of 1 ml of the EDTA solution plus 49 ml of distilled water through the cadmium column. The efficiency of this method in reducing nitrate to nitrite was nearly constant at 98%. In instances when the presence of compounds (e.g., 2,4-dinitrophenol) made nitrate analysis by the cadmium reduction method impossible, nitrate concentration was determined enzymatically by conversion to nitrite, using a preparation of partially purified *Neurospora* nitrate reductase (6). When nitrite and nitrate were both present, nitrite concentrations were measured prior to nitrate determinations. The amount of nitrite was subtracted from the total amount of nitrate and nitrite to give the levels of nitrate present.

Nitrate content of mycelia. Crude extracts of mycelia were treated with an equal volume of 2 N HCl and centrifuged at 27,000 $\times$ *g* for 10 min. The pH of the resulting supernatants was adjusted to pH 9.5 to 10.5 with 2 N NaOH, and the solution was centrifuged at 27,000 $\times$ *g* for 10 min. Nitrate determination was performed by using the copperized cadmium reduction method.

Measurement of nitrate transport system. Mycelia growing in media containing various nitrogen sources were harvested at the desired times and washed, and 2 to 4 g (wet weight) of mycelia was placed in 200 ml (unless stated otherwise) of fresh medium containing 20 mM $NaNO_3$. After 15 to 30 min of incubation, the amount of nitrate remaining in the assay medium was determined and subtracted from the initial amount of nitrate. The value obtained represents the amount of nitrate which had been taken up by the mycelia and is expressed as micromoles of nitrate per gram of mycelia. The rate of the transport system is defined as micromoles of nitrate disappearing per gram (wet weight) per hour. Nitrate uptake was linear for at least 45 min and then gradually declined with time.

Mycelia collected at various time intervals were analyzed for nitrate content. Rate values calculated from the amount of nitrate accumulated per gram of mycelia for each time interval are expressed as the micromoles of nitrate accumulated per gram (wet weight) per hour.

Determination of ammonia and amino acids. Ammonia was measured after Conway diffusion by the phenol-hypochlorite method of Brown et al. (1), as described by Glick (8). The presence of amino acids was detected by the ninhydrin method (23).

Materials. Flavine adenine dinucleotide, actinomycin D, cycloheximide, and amino acids were supplied by the Sigma Chemical Co., St. Louis, Mo. NADPH and antimycin A were obtained from Calbiochem, Gaithersburg, Md.; sodium vanadate (*meta* form) was purchased from J. T. Baker Chemical Co., Baltimore, Md. Cadmium metal, sodium tungstate, and all other reagents were from Fisher Scientific Co., Silver Spring, Md.

RESULTS

Induction of the nitrate transport system. The effects of various nitrogen sources in the growth medium on the induction of the nitrate transport system are shown in Fig. 1. The activity of the nitrate transport system is detectable in nitrate-grown mycelia after a lag period of 30 min, reaches the maximal level in about 6 h, and remains constant. Similar kinetics of induction is observed with nitrite as the nitrogen source. The decline in the activity of the nitrate transport system at h 12 in nitrite-grown mycelia is attributed to the depletion of nitrite from the growth medium, since the addition of more nitrite to the growth medium at this time prevents this decline (not shown in the figure). Concentrations of nitrite higher than 10 mM cannot be employed because of toxicity. Mycelia grown on either ammonium chloride or Casamino Acids contained negligible amounts of nitrate transport activity. The nitrate uptake system does not appear to be derepressible since little activity is found in mycelia presented with nitrogen-free medium.

Half-life of the nitrate transport system. Cycloheximide was used to confirm the fact that the nitrate transport system in *Neurospora* is maintained by a dynamic balance between synthesis and degradation. The activity of the nitrate transport system in nitrate-grown mycelia decreased exponentially after treatment with cycloheximide (5 μg/ml) (Fig. 2). A half-life of approximately 3 h was derived from a semilogarithmic plot of the data obtained after cycloheximide treatment. Therefore, the observed plateau of maximal activity (Fig. 1) indicates a steady state where synthesis and degradation are both occurring, and does not signify the cessation of the formation of the nitrate transport system.

Active transport of nitrate. The effect of various external nitrate concentrations on the rate of nitrate uptake by the induced nitrate transport system is shown in the form of a Lineweaver-Burk plot (Fig. 3). The nitrate uptake system displays saturation kinetics, exhibiting a V_{max} of 71 μmol per g per h and an apparent K_m for nitrate of 0.25 mM.

Various metabolic inhibitors which interrupt active transport were studied to determine whether nitrate uptake is an energy-requiring process. Antimycin A, potassium cyanide, and 2,4-dinitrophenol lower nitrate uptake, thereby

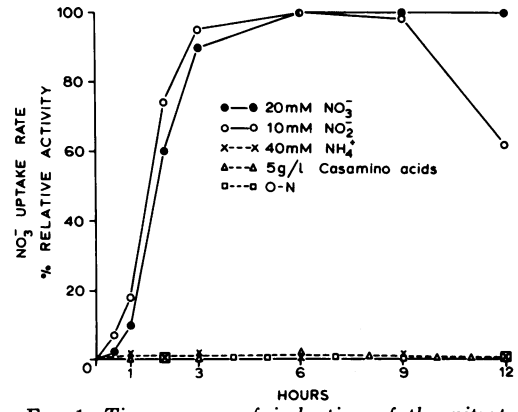

FIG. 1. *Time course of induction of the nitrate uptake system. Ammonium chloride-grown mycelia were transferred to fresh growth media containing the indicated nitrogen sources. At various time intervals, mycelia were harvested and 4-g portions were employed in the nitrate transport assay. 100% activity = 53 μmol of nitrate per g per h.*

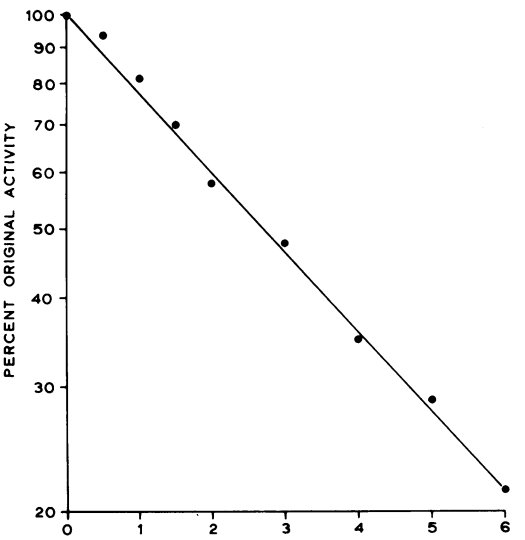

FIG. 2. *Determination of half-life of the nitrate uptake system. Mycelia induced for 5 h in nitrate medium were harvested and suspended in fresh medium containing 20 mM $NaNO_3$ plus 5 μg of cycloheximide per ml. At the indicated times, the activity of the nitrate transport system was determined (assay 1). The results are presented as a semilogarithmic plot. Mycelial protein concentration remained relatively constant at 6.5 to 7 mg/ml over the time course of this experiment.*

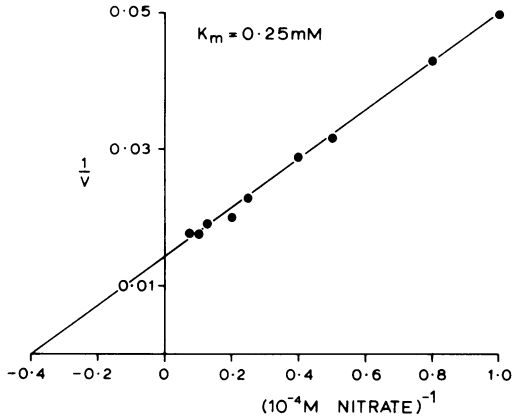

FIG. 3. *Effect of various external nitrate concentrations on the activity of the nitrate transport system. The standard nitrate transport assay procedure was employed except that the concentration of nitrate was varied and time of incubation was 5 to 10 min. In each assay, 2 to 3 g of mycelia previously induced on nitrate media for 5 h was used. Velocity is expressed as micromoles of NO_3^- per gram per hour.*

causing a progressive decrease in the rate of nitrate transport (Table 1). Further evidence that the uptake of nitrate is an active transport process is that nitrate can be transported against a concentration gradient. Mycelia can accumulate 50 times as much nitrate (micromoles per gram wet weight) as is present in the medium (micromoles per milliliter).

Effect of ammonia on nitrate transport. Preliminary studies revealed that ammonium nitrate-grown mycelia exhibit preferential assimilation of ammonia; nitrate uptake is prevented. Nitrate uptake commences only when most of the ammonia has been assimilated. Ammonium ions could interfere with nitrate uptake by repression and/or inhibition of the nitrate transport system. Figure 4 shows that the nitrate uptake system is present in ammonium nitrate-grown mycelia, follows the same course of induction, and reaches the same rate of activity as in nitrate-grown mycelia. These results obviously indicate that ammonia does not repress the development of the nitrate transport system. The effect of ammonium ions on the activity of the induced nitrate transport system is shown in Fig. 5. When ammonium ions at a concentration of 0.5 mM is present, the rate of nitrate uptake is inhibited by 75%. Ammonia exerts a non-competitive inhibition, displaying a K_i of 0.13 mM.

Effect of Casamino Acids on nitrate uptake. In addition to ammonia, Casamino Acids prevent nitrate utilization. When mycelia

TABLE 1. *Effect of metabolic poisons on the induced nitrate uptake system*[a]

Inhibitor	Time (h)	Nitrate uptake system	
		Total uptake (μmol/g)	Rate (μmol/g/h)
None	0.25	7.5	30.0
	0.5	15.1	30.2
	1.0	31.8	31.8
	1.5	44.4	29.6
Dinitrophenol (1 mM)	0.25	5.0	20.0
	0.5	7.9	11.8
	1.0	10.7	5.6
	1.5	12.4	3.4
KCN (1 mM)	0.25	6.0	24.0
	0.5	8.7	10.8
	1.0	10.1	2.8
	1.5	11.5	2.8
Antimycin A (5 μg/ml)	0.25	5.9	23.6
	0.5	8.3	9.6
	1.0	10.2	3.8
	1.5	12.1	3.8

[a] Mycelia were induced for 5 h in nitrate medium, harvested, and exposed to 100 ml of 10 mM $NaNO_3$ medium containing fixed amounts of the various inhibitors. At the indicated times, the amount of nitrate remaining in the medium was determined. From the extent of uptake a rate of nitrate uptake for the time period was calculated.

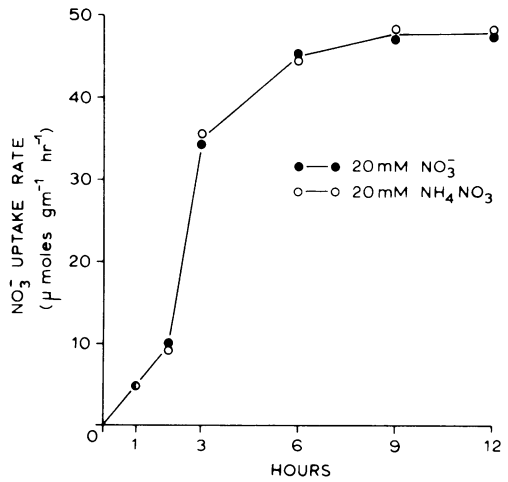

FIG. 4. *Induction of the nitrate uptake system in the presence of ammonia. Mycelia grown in Fries NH_4Cl medium were harvested, washed, and exposed at zero time to media containing 20 mM $NaNO_3$ or 20 mM NH_4NO_3. At the indicated times, mycelia were collected and assayed for nitrate uptake activity by using the standard nitrate transport assay.*

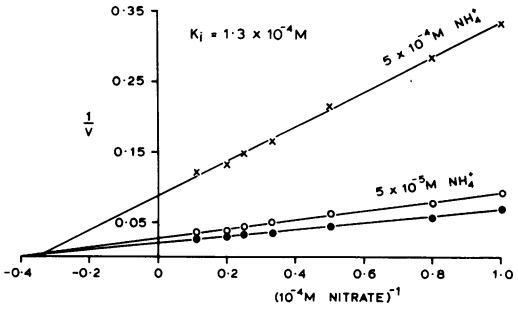

FIG. 5. *Effect of various ammonium ion concentrations on the activity of the induced nitrate uptake system. The standard assay for nitrate uptake system was followed in performing nitrate saturation curves in the presence or absence of fixed amounts of ammonia. Nitrate-induced mycelia (2 to 4 g) were incubated for either 5 or 10 min in flasks containing 100 ml of the transport assay mixture. Controls were incubated for 5 to 15 min in 200 ml of assay mixture.*

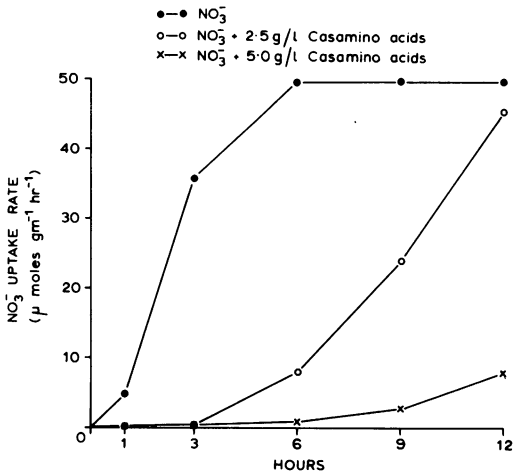

FIG. 6. *Time course of induction of nitrate uptake system in the presence of Casamino Acids. Mycelia grown in Fries NH_4Cl medium were harvested, washed, and exposed to media containing 20 mM $NaNO_3$ and the indicated amounts of Casamino Acids. At various times, mycelia were collected, washed, and used in the standard assay for the nitrate uptake system.*

grown on combined Casamino Acids plus nitrate medium were assayed for the presence of the nitrate transport system, only basal levels of nitrate uptake activity could be detected (Fig. 6). The nitrate uptake system appears in these mycelia only when all of the amino acids have been assimilated. No inhibition of the induced nitrate transport system by Casamino Acids (5.0 g/liter) or by individual amino acids (1 mM) could be demonstrated. Thus, unlike ammonia, Casamino Acids prevent nitrate uptake by repression of the nitrate transport system.

Effect of nitrite on nitrate transport. Nitrite, like nitrate, induces the nitrate uptake system (see Fig. 1). However, when ammonium-grown mycelia were transferred to medium containing both nitrite and nitrate, the mycelia utilized only nitrite. The nature of the inhibition of the nitrate transport system by nitrite was investigated with mycelia fully induced for the nitrate uptake system (Fig. 7). The K_m for nitrate in the presence of nitrite is not substantially altered, but the V_{max} is greatly reduced, indicating that nitrite inhibition of nitrate uptake is non-competitive. The K_i was found to be 0.17 mM. The non-competitive inhibition by nitrite of nitrate uptake suggests that nitrite may not be transported by the nitrate uptake system.

Effect of vanadate and tungstate on nitrate accumulation. The nitrate uptake assay based on nitrate disappearance from the medium does not yield an insight into either the effect of nitrate reduction upon the flux of nitrate into the mycelia or any possible involvement of the nitrate reductase protein in the transport of

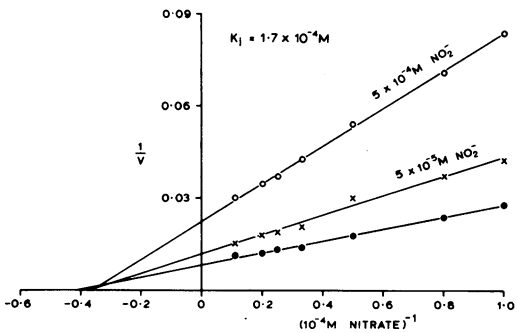

FIG. 7. *Effect of various nitrite concentrations on the activity of the induced nitrate uptake system. The standard assay for nitrate uptake was employed. Mycelia (2 g) induced for 5 h in nitrate media were incubated for 2 min in flasks containing 100 ml of the assay mixture. Controls were incubated in 200 ml of the assay mixture for 2 to 5 min.*

nitrate. To gain such insight, another approach was employed. Mycelia can be grown in medium in which molybdenum is replaced by tungstate or vanadate. Tungstate is known to prevent the formation of a functional nitrate reductase in *Neurospora* (25); vanadate exerts a similar effect (A. Nason, personal communication). Such mycelia can be used to determine the activity of the nitrate transport system without interference by subsequent nitrate reduction.

For such experiments, mycelia were grown on nitrate medium lacking molybdenum. In some instances, either tungstate or vanadate was added to the medium. Mycelia grown in the absence of molybdenum accumulate more nitrate than molybdate-grown mycelia (Table 2). When molybdenum-deficient mycelia were grown in nitrate medium supplemented with tungstate or vanadate, further increases in the level of intracellular nitrate were observed. The increased levels of internal nitrate found in mycelia treated with tungstate or vanadate are most likely attributable to the decreased levels of nitrate reductase activity.

At a concentration of 0.1 mM, vanadate is a better inhibitor of the development of the nitrate reductase activity than is tungstate (Table 2). Higher concentrations of vanadate, such as 2 mM, do not result in a further decrease in the level of the nitrate reductase or in a further increase of internal nitrate, whereas 2 mM tungstate inhibits both nitrate accumulation and nitrate reductase activity. Since high levels of tungstate can inhibit nitrate accumulation (11), vanadate was the inhibitor routinely used to prevent the development of the nitrate reductase activity.

Inhibition of nitrate accumulation by nitrogen compounds. Several parameters of the nitrate transport system were re-examined by using mycelia containing the nonfunctional nitrate reductase. Figure 8 shows the effects of various nitrogen compounds and cycloheximide on nitrate accumulation in vanadate-treated mycelia. The pattern of nitrate accumulation in nitrate-grown mycelia consists of a slow, initial uptake of nitrate lasting for approximately 2 h. This stage is then followed by a gradual linear increase in the amount of nitrate accumulated until the level of nitrate reached 48 μmol/g; then mycelia did not accumulate any more nitrate. Ammonia, Casamino Acids, and nitrite interfered with nitrate accumulation. Nitrate accumulation occurs once ammonia, nitrite, or Casamino Acids are depleted from the growth media. The accumulation of nitrate is prevented by the addition of cycloheximide (5 μg/ml) to the growth medium.

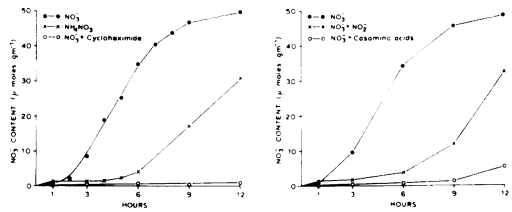

FIG. 8. *Effect of various nitrogenous compounds on nitrate accumulation. Mycelia grown in Fries NH_4Cl medium without molybdenum were harvested, washed, and transferred (8 g/flask) to a series of flasks containing molybdate-deficient media with 0.1 mM sodium vanadate and 20 mM $NaNO_3$ plus either 10 mM $NaNO_2$, 20 mM NH_4Cl, 5.0 g of Casamino Acids per liter, or 5 μg of cycloheximide per ml. At the indicated times, mycelia were collected and the intracellular nitrate content was determined.*

Individual amino acids were tested on their ability to prevent nitrate accumulation (Table 3). Any amino acid tested, at a final concentration of 1 mM, prevented nitrate transport with the exception of methionine, glutamine, and alanine. The remaining amino acids can be divided into four groups based on the extent of their inhibition of nitrate uptake. Tryptophan, arginine, valine, and threonine (group I) cause a severe inhibition of nitrate uptake ranging from 86 to 93%, whereas mycelia grown in the presence of histidine, glutamic acid, or cysteine (group II) accumulate 61 to 66% less nitrate than the untreated control. Amino acids in group III (phenylalanine, lysine, leucine, serine, and proline) are less severe in their effect on the nitrate uptake system, whereas tryosine, isoleucine, and asparagine (group IV) are only slightly inhibitory to nitrate uptake (15 to 20%).

Since the K_m values for nitrate in the nitrate transport system and in the NADPH-nitrate reductase (7) are quite similar, it is conceivable that nitrate reduction could have influenced the determination of the K_m for nitrate of the nitrate transport system. Therefore, the K_m for nitrate was re-examined in vanadate-grown mycelia by measuring the rate of nitrate accumula-

TABLE 2. *Effect of vanadate and tungstate on nitrate accumulation*[a]

Additions	NADPH-NO_3^- Reductase (U/mg of protein)	Internal nitrate (μmol/g)
None	4.6	6.9
Molybdate (0.5 μM)	8.7	4.7
Tungstate (0.1 mM)	3.2	10.1
Tungstate (2 mM)	0.7	5.1
Vanadate (0.1 mM)	0.56	17.9
Vanadate (2 mM)	0.54	18.1

[a] Ammonium-grown mycelia (5 g each) were transferred to molybdenum-deficient media containing 20 mM sodium nitrate plus either ammonium molybdate, sodium tungstate, or sodium vanadate, at the indicated concentrations. After 5 h of incubation, mycelia were harvested, and nitrate content and nitrate reductase activity were measured in mycelial extracts.

TABLE 3. *Effect of amino acids on nitrate accumulation*[a]

Group	Additions	Internal nitrate (μmol/g)	Percent of control
	None	5.0	100
I	Tryptophan	0.7	14
	Arginine	0.5	10
	Valine	0.7	14
	Threonine	0.4	8
II	Histidine	1.7	34
	Glutamic acid	1.8	36
	Cysteine	2.0	39
III	Phenylalanine	2.4	48
	Lysine	2.4	48
	Leucine	2.7	54
	Serine	2.3	46
	Proline	2.7	54
	Aspartic acid	2.8	56
IV	Tryosine	3.7	74
	Isoleucine	4.2	80
	Asparagine	3.5	70
V	Methionine	6.2	124
	Glutamine	5.1	102
	Alanine	4.9	96

[a] Mycelia grown in Fries NH_4Cl medium without molybdate were transferred to molybdenum-deficient media containing 20 mM sodium nitrate and 0.1 mM sodium vanadate plus the indicated amino acids at a final concentration of 1 mM. After 2 h of incubation, mycelia were harvested, weighed, and used in the preparation of crude extracts. Nitrate content of mycelia was determined.

tion. To prevent any inhibition of nitrate uptake by accumulated internal nitrate, mycelia were induced for the nitrate transport system by growth on nitrite. Figure 9 shows that the nitrate transport system is a saturable process, exhibiting the same rate of nitrate accumulation as rate of nitrate uptake. Nitrite and ammonia inhibit nitrate accumulation noncompetitively. The K_m for nitrate determined by nitrate accumulation is 0.3 mM. The inhibition constants for ammonia and nitrite were calculated to be 0.15 and 0.21 mM, respectively.

These results paralleled those obtained for the nitrate uptake system, as measured by nitrate disappearance, and indicate that a nitrate reductase protein, active in nitrate reduction, is not required for the activity of the nitrate transport system.

Dependence of nitrate uptake on RNA and protein synthesis. Cycloheximide and puromycin, inhibitors of protein synthesis, inhibited the induction of the nitrate transport system (Table 4). Inhibition of RNA synthesis by 6-methyl purine or actinomycin D restricted

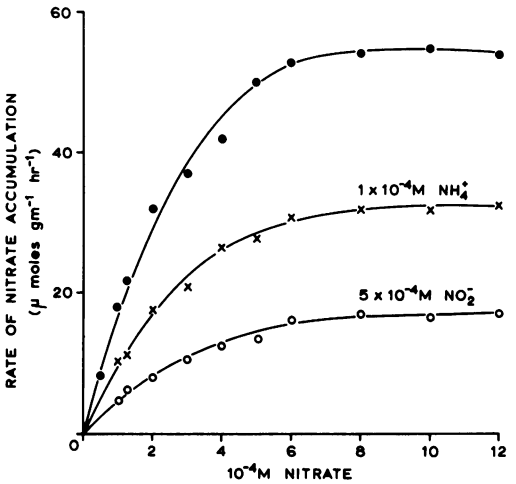

FIG. 9. *Effect of various nitrate concentrations on the rate of nitrate accumulation. Mycelia grown for 6 h in molybdate-deficient medium containing 10 mM nitrite and 0.1 mM sodium vanadate were employed in performing nitrate saturation curves in the presence or absence of the indicated amounts of ammonium or nitrite ion. The nitrate content of 3 g of mycelia incubated for 10 min in the various assay mixtures was determined, and the results are expressed as rate of nitrate accumulation.*

TABLE 4. *Effect of inhibitors of RNA and protein synthesis on nitrate accumulation and on the nitrate assimilation enzymes*[a]

Treatment	Internal nitrate (μmol/g)	Sp act (U/mg of protein)	
		NADPH-NO_3^- reductase	NADPH-NO_2^- reductase
None	24.1	10.3	8.1
Cycloheximide (5 μg/ml)	1.9	<0.1	<0.1
Puromycin (500 μg/ml)	3.8	<0.1	<0.1
6-Methyl purine (0.5 mM)	4.1	2.5	1.0
Actinomycin D (50μg/ml)	18.7	7.5	6.1
Actinomycin D (50 μg/ml) + EDTA treatment		0.3	0.6
EDTA treatment		10.6	8.4

[a] Mycelia were grown in molybdate-deficient Fries NH_4Cl medium, harvested, and distributed in 5-g portions between two series of flasks containing 20 mM sodium nitrate medium plus fixed amounts of the indicated inhibitors. One series contained 0.1 mM sodium vanadate and was used for determination of nitrate accumulation. The other series of flasks, used for measuring nitrate and nitrite reductase activities, contained ammonium molybdate (0.5 μM). A portion of mycelia was treated with 0.1% EDTA prior to exposure to actinomycin D. After a 4-h incubation period, mycelia were collected and used to prepare crude extracts. Nitrate content and enzyme activities were determined.

nitrate transport, although the inhibition by actinomycin D was slight (22%). This slight inhibition by actinomycin D might suggest that the existence of a stable messenger RNA is involved in the formation of the nitrate transport system. However, actinomycin D is known to penetrate mycelia very slowly (15), and this slow penetration is probably the reason for the slight inhibition of nitrate uptake activity. This possibility is supported by the observation that, if mycelia are pretreated with EDTA, a procedure which increases permeability of the mycelia (27, 29), actinomycin D severely restricts the synthesis of the nitrate and nitrite reductases (Table 4). In contrast, in mycelia not pretreated with EDTA, actinomycin D only partially prevents the synthesis of the two enzymes. This increased inhibition of the synthesis of the nitrate and nitrite reductases by actinomycin D in EDTA-treated mycelia is not due to EDTA itself since EDTA treatment alone does not alter the inducibility of the two enzymes. Furthermore, 0.5 mM 6-methyl purine, a concentration which inhibits the synthesis of all forms of RNA (13), has a strong depressing effect on the development of the nitrate transport system.

Nitrate reductase mutants. Mutant strains deficient in the assimilatory nitrate reductase were also examined for the presence of the nitrate transport system. Three mutants were employed. The first, *nit-1*, contains the inducible NADPH-cytochrome *c* reductase (diaphorase) activity of the nitrate reductase complex, but lacks the nitrate-specific portion of the electron transfer chain (determined by the absence of the $FADH_2$- and reduced methyl viologen-nitrate nitrate reductase activities) (22). The second mutant, *nit-3*, lacks the diaphorase portion of the electron transfer sequence (NADPH-cytochrome *c* reductase) but retains the inducible $FADH_2$- and reduced methyl viologen-nitrate reductase activities (22). *nit-2* lacks all enzymatic activities commonly associated with the NADPH-nitrate reductase (22). It was of interest to see whether the nitrate transport system in these mutants is regulated in the same manner as in the wild type. Table 5 shows that *nit-1*, *nit-2*, and *nit-3* have the ability to transport nitrate. Furthermore, nitrate accumulation in these mutants is sensitive to inhibitors of RNA and protein synthesis. Ammonia, nitrite, or Casamino Acids prevent nitrate accumulation; and, as in the wild type, nitrate accumulates once ammonia, nitrite, or Casamino Acids have been depleted from the medium. All amino acids at a concentration of 1 mM repressed the synthesis of nitrate uptake system with the exception of alanine, glutamine, and methionine. Table 6 presents the

TABLE 5. *Effect of various nitrogenous compounds on the induction of the nitrate uptake system in nitrate reductase mutants*[a]

N source	Time (h)	Internal nitrate (μmol/g)		
		nit-1	*nit-2*	*nit-3*
20 mM $NaNO_3$	1	1.7	0.5	1.0
	3	8.6	7.3	9.8
	6	28.3	31.5	34.4
	9	40.5	39.6	46.5
20 mM NH_4NO_3	1	1.6	1.0	1.3
	3	2.1	1.5	1.5
	6	3.9	4.5	4.3
	9	19.8	21.0	17.7
20 mM $NaNO_3$ + 10 mM $NaNO_2$	1	1.5	0.9	1.7
	3	2.0	1.5	1.9
	6	5.1	4.8	3.5
	9	21.8	15.9	12.0
20 mM $NaNO_3$ + 5 g of Casamino Acids per liter	1	0.8	0.6	0.9
	3	1.2	1.0	1.0
	6	1.9	1.4	1.7
	9	3.8	3.6	3.1
20 mM $NaNO_3$ + cyclohe .nide (5 μg/ml)	1	0.9	0.3	0.9
	3	1.7	0.9	0.9
	6	1.3	0.8	1.1
	9	1.9	1.1	1.3
20 mM $NaNO_3$ + 6-methyl purine (0.5 mM)	1	1.7	0.6	0.9
	3	3.1	2.5	3.5
	6	3.4	2.8	3.9
	9	3.7	2.6	3.8

[a] Mycelia of the mutants (*nit-1*, *nit-2*, and *nit-3*) initially grown in Fries NH_4Cl medium were harvested and distributed among a series of flasks containing the indicated nitrogen sources. At various times, mycelia were harvested and nitrate content was determined.

TABLE 6. *Summary of substrate affinities and inhibition constants of the nitrate uptake system in nit-1, nit-2, nit-3, and wild type*[a]

Strains	K_m (mM)	K_i of NH_4^+ (mM)	K_i of NO_2^- (mM)
nit-1	0.28	0.20	0.18
nit-2	0.35	0.15	0.25
nit-3	0.38	0.25	0.21
Wild type 74A	0.30	0.15	0.21

[a] In all cases, the K_m and K_i values were derived from Lineweaver-Burk plots of the data. Conditions were as described in the legend of Fig. 9.

results of experiments to determine the substrate affinity, in the presence and absence of ammonia and nitrite, of the nitrate uptake system in these mutants. In all three mutants, the K_m values for nitrate are similar to that found for the wild type. Furthermore, nitrite and ammonia non-competitively inhibit nitrate uptake, with K_i values close to the parental strain.

Other experiments, not reported here, indicate that the nitrate transport systems in *nit-1*, *nit-2*, and *nit-3* are similar to the nitrate transport system found in wild type with regard to its inducibility by nitrite, its repression by Casamino Acids (but not by ammonia), and its sensitivity to metabolic poisons.

DISCUSSION

The data presented here indicate that entry of nitrate into *N. crassa* is mediated by an active transport mechanism. This nitrate transport system is specifically induced only in the presence of nitrate or nitrite (Fig. 1). No nitrate uptake activity is found in mycelia grown in media lacking a nitrogen source, indicating that other inorganic anions in the growth media, i.e., sulfate, chloride, phosphate, and borate, do not affect this system. Furthermore, the activity of the nitrate transport system declines upon the depletion of the inducer from the media. Therefore, the *N. crassa* nitrate transport system, like the nitrate assimilation enzymes (5), can be characterized as an inducible rather than a derepressible system.

Nitrite, ammonia, and amino acids, intermediates and end products of the nitrate assimilation pathway, prevent nitrate uptake in *Neurospora*. Casamino Acids prevent nitrate uptake by repressing the induction of the nitrate transport system (Fig. 6). In contrast, Subramanian et al. (24) reported that amino acids had no influence on nitrate accumulation. However, these workers tested only alanine and glutamine for their effects on nitrate uptake. In the present investigation the only amino acids that did not prevent nitrate accumulation were methionine, glutamine, and alanine (Table 3). Nitrite, an inducer of the *Neurospora* nitrate transport system, also prevents the process of nitrate uptake, as previously shown in *Spirodela* (4) and tobacco cells (10). The mechanism of nitrite inhibition of nitrate uptake was determined to be non-competitive (Fig. 7).

When *N. crassa* is supplied with both nitrate and ammonia, nitrate uptake is prevented (Fig. 8). This inhibition could be due to one of several possibilities: (i) ammonia may repress the formation of the transport system; (ii) ammonia may inhibit the activity of this nitrate uptake system; or (iii) since ammonia is known to repress the nitrate reductase (5, 21), such repression would indirectly inhibit nitrate reduction and ultimately prevent nitrate uptake. The possibility of repression of the nitrate transport system by ammonia is eliminated since ammonium nitrate-grown mycelia contain the nitrate transport system and its pattern of induction in ammonium-nitrate medium parallels that seen in media containing nitrate alone (Fig. 4). Experiments reported here show that ammonia inhibits the nitrate uptake system in a non-competitive manner (Fig. 5). It is unlikely that ammonia controls nitrate uptake by repressing the nitrate reductase since, in mycelia containing high levels of nitrate reductase, ammonium ion prevents nitrate transport within 5 min (Fig. 5). No changes in the level of nitrate reductase activity were observed in this time interval (data not shown). In contrast to these results, Subramanian and co-workers observed that ammonium ions did not prevent nitrate accumulation in *N. crassa* (24, 26). Clearly these results contradict their findings and support the contention that ammonia affects the nitrate transport system principally through a non-competitive inhibitory manner.

The utilization of nitrate by organisms must be considered as two processes: transport and reduction. Attempts were made to separate these two processes and to investigate the nitrate transport system in the absence of a functional nitrate reductase. Tungstate and vanadate are known to prevent the formation of an active nitrate reductase by substituting for the necessary molybdenum moiety of this enzyme (A. Nason, personal communication). The enzyme formed in the presence of these molybdenum analogues does retain the inducible NADPH-cytochrome *c* reductase activity. In the presence of these metals, the nitrate transport system exhibits the same characteristics as the nitrate uptake system in the presence of molybdenum (and the consequent functional nitrate reductase) with regard to its inducibility by nitrate, inhibition by ammonia or nitrite, sensitivity to cycloheximide, and repression by Casamino Acids (Fig. 8 and 9). In addition, high levels of internal nitrate (48 μmol/g) may inhibit nitrate transport. This inhibition may not be physiologically significant since the high levels of intracellular nitrate were artifically obtained. These results indicate that a nitrate reductase protein, active in nitrate reduction, is not required for the activity of the nitrate transport system, since nitrate uptake occurs in

the absence of a functional nitrate reductase.

Several other lines of evidence suggest that nitrate transport and nitrate reduction are catalyzed by separate proteins. Nitrate non-utilizing mutants (*nit-1*, *nit-2*, and *nit-3*) that lack the NADPH-nitrate reductase activity contain the inducible nitrate transport system, which is similar to that in the wild type (Tables 5 and 6). Inhibitors of protein and RNA synthesis exert differential effects on the activities of the nitrate reductase and the nitrate transport system (Table 4). Puromycin and cycloheximide totally prevent synthesis of the nitrate reductase while inhibiting nitrate accumulation by 84 and 92%, respectively. 6-Methyl purine inhibits nitrate accumulation slightly more than the synthesis of the nitrate reductase. Furthermore, ammonia represses the nitrate reductase (5), but not the nitrate transport system (Fig. 4). Therefore, the nitrate transport system may be considered as a distinct part of the nitrate assimilatory pathway.

Thus, the nitrate transport system, like the nitrate assimilation enzymes in *N. crassa*, is subject to regulation by the substrates and end products of the assimilatory nitrate pathway. Nitrate and nitrite are inducers of the nitrate transport system and the enzymes of the nitrate assimilation pathway, whereas Casamino Acids exert a repressive influence on these functions. Ammonium ion regulates the pathway by at least two mechanisms: non-competitive inhibition of the nitrate transport system and repression of the nitrate assimilation enzymes. Ammonium ion also exercises a third regulatory mechanism. The nitrate reductase activity in *Neurospora* is lost rapidly when mycelia are transferred to ammonia media. This rapid decline caused by ammonium ion is prevented by inhibitors of protein synthesis (26; R. H. Garrett, unpublished observations), suggesting that ammonium is involved in an active mechanism regulating the decline of nitrate reductase activity. Nitrite, an inducer of all three components of the pathway (nitrate transport system, nitrate reductase, and nitrite reductase), noncompetitively inhibits the nitrate transport system. This mode of inhibition is perhaps significant since competitive inhibition by nitrite of nitrate uptake could conceivably result in nitrite accumulation if nitrite were also transported by this system. The accumulation would result from nitrite taken up plus the reduction of nitrate accumulated. This situation could possibly lead to nitrite toxicity. Non-competitive inhibition avoids this dilemma.

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