Developmental Regulation of Amino Acid Transport in *Neurospora crassa*

J. H. TISDALE and A. GIB DeBUSK

Genetics Group, Department of Biological Science, Florida State University, Tallahassee, Florida 32306

Received for publication 28 July 1970

Conidia of *Neurospora crassa* exhibit an ability to transport various amino acids against a concentration gradient. The conidial transport system has previously been characterized in terms of kinetics, competitions, and genetic control. This study describes the development of a new and highly active transport capability which is elaborated during the early stages of development but prior to evident germination. It has been named "postconidial" transport activity and represents as much as 20-fold greater initial rates as compared to those observed with conidia. Development of the postconidial transport activity requires protein synthesis and can be partially repressed when the substrate amino acid is present during the developmental preincubation period. A mutant has been utilized which exhibits normal conidial but fails to develop normal postconidial transport activity for any amino acid examined. Although temperature optimum and pH dependence are similar in conidial and postconidial systems, there is evidence that the new activity is not a simple amplification of an existing capability. This is reflected as a change in competition patterns between particular amino acids as development proceeds.

Systems for the active transport of metabolites, particularly sugars and amino acids, have been characterized by various investigators (1--3, 5, 6, 19, 24, 31) in both microorganisms and in higher organisms. The various methods used have included: a study of the transport kinetics of the metabolite and structural analogues (7, 20, 26); analysis of transport-deficient mutants (1, 2, 4, 8--10, 12--14, 16--18, 28, 29, 30, 32--34); and, most recently, the isolation of substrate-binding proteins believed to be involved in the transport process (11, 15, 21--23, 25).

*Neurospora crassa* has a vegetative developmental cycle beginning with conidia which germinate, develop into mycelia and then again differentiate into conidia. The study of active transport has been carried out in numerous laboratories by using different developmental stages of this organism.

DeBusk and DeBusk (7) characterized the phenylalanine transport system of the conidial or vegetative spore stage. They showed that the basic amino acids compete poorly for phenylalanine transport. Roess and DeBusk (26) used conidia to describe a system for basic amino acid transport. They found that the aromatic and neutral amino acids were poor competitors for arginine transport. Additional properties of conidial transport have been worked out (18, 34) by utilizing mutants deficient in neutral-aromatic amino acid transport. Certain of these mutants retained some residual neutral-aromatic amino acid transport which was subject to competition by the basic amino acids. Similar results have been obtained with a mutant deficient in basic amino acid transport (DeBusk, personal communication).

Kapp and Metzenberg (13) and Jacobson and Metzenberg (12) studied amino acid transport in germinated conidia. One particular mutant used in their study has been employed here. Transport studies were carried out by St. Lawerence, Malin, Altwerger, and Rachmeler (32); Stadler (29); Lester (16); and Kinsey and Stadler (14) in mycelia. Recently, Pall (20) studied amino acid transport by cultures of various ages. He found that changes, dependent on the age of the culture, occur in the degree to which tryptophan transport is inhibited by arginine. This suggests that progressive changes occur in transport as development proceeds.

It is the purpose of this report to demonstrate that a great increase in amino acid transport activity occurs during the early developmental stages before conidial germination. Development of the "postconidial" transport activity requires protein synthesis and can be partially repressed when the substrate amino acid is present during
the developmental preincubation period. A mutant with normal transport in conidia fails to develop a normal postconidial transport activity. Although the various transport systems present in conidia are amplified as development occurs, the increases are regulated since the competition patterns between amino acids are different in postconidial stages.

MATERIALS AND METHODS

Strains. Neurospora crassa wild-type strain T74A was used. This particular strain was employed for several years as a "transport standard" and is a vegetative derivative of 74-OR23-1A (FGSC 987). This strain is sensitive to the phenylalanine analogue, p-fluorophenylalanine. The mutant utilized, 55701t (FGSC 636), was isolated a number of years ago as a temperature conditional lethal. It was subsequently reported by Kappy and Metzenberg (13) to be deficient in the transport of neutral-aromatic and acidic amino acids. In this study, a stock obtained from the Fungal Genetics Stock Center, referred to by its stock F636A, exhibits a normal conidial but deficient postconidial transport activity.

Chemicals. All amino acids used were L isomers. Nondiscriminative amino acids were obtained from Calbiochem, Calif. All radioactive amino acids were 14C uniformly labeled and were obtained from either Schwarz BioResearch, Inc., N.Y., or International Chemical and Nuclear Corp., Calif.

Transport studies. All transport studies were carried out with conidia obtained by harvesting 6- to 8-day cultures into sterile distilled water (0°C) and filtered through glass wool. Dry weights were determined so that specific activity could be compared from one experiment to the next.

A typical postconidial transport assay consists of a "developmental preincubation" period of up to 240 min in the presence of a carbon source. After an appropriate preincubation period, the transport capacity of "postconidia" was determined by the addition of radioactive amino acid (and actidione except where indicated) with samples removed for counting at various time intervals. Thus, all "postconidial" experiments consisted of a preincubation and a transport phase, whereas all conidial experiments involved only the transport phase.

The developmental preincubation medium consisted of Vogel's (36) 1X salts containing 1% glucose. Conidial suspensions were used as inocula in sufficient volume to give a final concentration of 0.1 mg/ml based on prior dry-weight determinations. At the end of the developmental preincubation period, the transport was initiated by adding radioactive amino acid to a final concentration of 0.01 μC/ml, 0.1 μmole/ml, and 10 μg/ml actidione. Samples (5 ml) were removed, filtered onto membrane filters (Millipore Corp.), and washed with three portions of cold distilled water. Filters (Millipore) were glued to planchets for counting in a Beckman low Beta II proportional counter. Incubations were carried out at 25°C with shaking in a constant-temperature water bath.

Suspensions of conidia were stored in crushed ice in a refrigerator for up to 3 days in the experiments reported. No significant changes in transport properties were observed by storage of cells in this manner for as long as 5 days. This is not the case when cells are harvested into warm water and stored at 4°C.

pH studies. The pH optimum for postconidial transport was determined by using a 0.1 M potassium dibasic and 0.1 M tribasic phosphate buffer. Hydrochloric acid and potassium hydroxide were used to adjust the pH. The pH readings were taken just before the experiment and 24 hr later.

Temperature studies. Temperature dependency of postconidial transport activity was determined by carrying out transport assays with shaking in constant-temperature water baths maintained (±1.0°C) at various temperatures. The developmental preincubation period was always carried out at 25°C.

RESULTS

Conidial and postconidial transport. When wild-type conidia of N. crassa are incubated in a medium containing glucose (developmental preincubation) before the addition of phenylalanine, the initial rate of transport of this amino acid increases (Fig. 1). This increase is directly proportional to the length of preincubation time but occurs before evident germination. To obtain a direct comparison of the effect of actidione not only on transport but also on development of the postconidial transport activity, parallel experiments were carried out in the presence and absence of actidione. Since the transport time measured was short (30 min), it was felt that incorporation of amino acids into protein during this period would not measurably affect the transport rate. In any event, subsequent experiments (Fig. 3-7), in which actidione was included during the time in which transport was measured, clearly show that the uptake rates drastically differ for conidial versus postconidial transport. Figure 1 shows that when actidione (10 μg/ml, final concentration) is added to the preincubation medium, no increase in the rate of transport is observed for any of the preincubation periods. At this concentration, actidione inhibits protein synthesis in Neurospora (35). Tuveson, West, and Barratt (35) measured extractable protein from conidia incubated in a growth medium for various periods of time. Their results showed that net protein synthesis decreased within the first 0.5 hr of incubation and remained lower than the initial level for various periods of time up to 4 hr. Their labeling experiments, however, did indicate that protein turnover was taking place during this time. It can not be determined whether actidione inhibits the synthesis of a specific permease protein or some other protein involved in the early development of conidia. However, the early (first
5 hr) developmental processes of the postconidial deficient mutant appear to be normal (see below).

To determine whether an increase in transport activity after developmental preincubation is observed for amino acids other than phenylalanine, a series of similar preincubations were conducted with arginine used as the transported amino acid. Arginine belongs to the "family" of amino acids (basic) which does not significantly compete with neutral and aromatic amino acids for transport in conidia. Conidial transport of this amino acid was previously characterized by Roess and DeBusk (26) in Neurospora. Figure 2 shows an increasing rate of arginine transport with increasing preincubation time similar to that observed for phenylalanine. However, arginine transport activity differs from phenylalanine in that not all of the increase is subject to actidione inhibition. An actidione-insensitive increase (2X) does occur. This activity is fully developed by 60 min. Another wild-type strain (SY7A, FGSC 622) of Neurospora was similarly tested. This strain also exhibited both the actidione-sensitive and the actidione-insensitive increase in activity.

Postconidial transport-deficient mutant. In examining various transport mutants for conidial and postconidial transport activity, it was found that one mutant (55701 or FGSC 636A) exhibited normal conidial transport of both phenylalanine and arginine yet was deficient for postconidial transport activity. These observations are shown in Fig. 3 for phenylalanine and Fig. 4 for arginine transport. In a typical experiment, the mutant

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**Fig. 1.** Transport of phenylalanine by a wild-type strain of Neurospora crassa after various periods of preincubation. The values (0 to 5,000 counts/min) represent total transport measured at 10 min after the addition of 14C-phenylalanine subsequent to the preincubation period. Actidione (10 μg/ml) was present, where indicated, during both the preincubation and transport periods.

**Fig. 2.** Transport of arginine by a wild-type strain of Neurospora crassa after various periods of preincubation. Transport was initiated by the addition of 14C-arginine subsequent to the preincubation period. Portions were removed for counting at 10-min intervals. Actidione (10 μg/ml) was present, where indicated, during both the preincubation and transport periods. The values (0 to 10,000 counts/min) represent total transport measured at 10-min time intervals after addition of 14C-arginine.
that the strain utilized in this lab contains a mutation which was not present in the strain when employed by Kappy and Metzenberg. Our preliminary genetic analysis indicates that this may be the case. However, the opposite mating type (FGSC 81) also exhibits a deficient arginine postconidial transport and fails to develop any phenylalanine postconidial transport activity. The preliminary genetic analysis did show that the deficient postconidial transport gene segregates in a 2:4:2 ratio within an ordered tetrad. Further, this gene segregates from the temperature-sensitive gene in FGSC 636A. Tentative mapping experiments indicate that the postconidial transport gene is not on linkage group I, the assigned locus of the temperature-sensitive gene. Observations with a light microscope did not reveal the appearance of germ tubes until 5 hr

![Figure 3](http://jb.asm.org/)

**Fig. 3.** Conidial and postconidial transport activity of phenylalanine by a wild-type and a mutant strain (55701, FGSC #636) of Neurospora crassa. Transport was initiated by the addition of $^{14}$C-phenylalanine to the incubation mixture. Postconidial samples were subjected to preincubation conditions for 180 min before the transport period. Conidial samples were not preincubated before the transport period. Actidione (10 $\mu$g/ml) was present during all transport periods but not during the preincubation. The values (0 to 8,000 counts/min) represent total transport measured at 12-min time intervals after addition of $^{14}$C-phenylalanine.

exhibits greater conidial phenylalanine transport compared to the wild-type strain. However, the wild type develops a greatly increased transport after 180 min of preincubation, whereas the level of transport for the mutant remains at the conidial level. No phenylalanine postconidial transport develops. Arginine transport experiments (Fig. 4) differ from phenylalanine in that some increase in transport activity after 180 min of preincubation does occur with the mutant. Preliminary experiments indicate that this increase may represent the actidione-insensitive portion of arginine transport. Transport activity for this mutant was previously characterized by Kappy and Metzenberg (13) under different conditions, including a lower temperature (10 C). Their results showed arginine to be transported normally by the mutant. Our temperature studies do not suggest that the difference in the two results is a function of the lower temperature. It is possible

![Figure 4](http://jb.asm.org/)

**Fig. 4.** Conidial and postconidial transport activity of arginine by a wild-type and a mutant strain (55701, FGSC #636) of Neurospora crassa. Transport was initiated by the addition of $^{14}$C-arginine to the incubation mixture. Postconidial samples were subjected to preincubation conditions for 180 min before the transport period. Conidial samples were not preincubated before the transport period. Actidione (10 $\mu$g/ml) was present during all transport periods but not during the preincubation. The values (0 to 9,000 counts/min) represent total transport measured at 12-min time intervals after addition of $^{14}$C-arginine.
for the mutant and 6 hr for the wild type—both well after the time of postconidial transport measurements. However, preliminary scanning electron micrographs showed that subtle cell surface changes do occur by 3 hr of incubation in the developmental preincubation mixture (S. Sines, personal communication).

Measurements of weight increases for the mutant and the wild type during incubation periods showed that the developing postconidial transport activity can not be accounted for in such a simple way since there is no significant weight increase at 180 min of incubation. When growth of the mutant is compared to that of the wild type, FGSC 636A does exhibit a growth lag; however, as indicated above, the mutant germinates before the wild-type strain. In addition, the two temperature-insensitive tetrad segregants deficient for postconidial transport do not show a growth lag—instead they grow at the same rate as the wild-type strain. Thus, the increased activity observed in the wild type and absent in the mutant after 180 min of developmental preincubation is not due to simple growth or growth differences; however, this increase does depend on protein synthesis.

Properties of postconidial transport activity. As a means of determining possible differences between the mechanism of transport for conidial and postconidial activities, pH, temperature, and substrate competition experiments were carried out utilizing the wild-type strain. As detailed below, the experiments indicate that these properties were similar for both developmental stages. However, amino acid competition studies do indicate a difference between the two activities.

The data presented in Table 1 indicate that the pH optimum for phenylalanine postconidial transport is at or near 5.4. Conidial pH optimum for phenylalanine transport was reported by DeBusk and DeBusk (7) as 5.5.

Table 2 indicates that the pH optimum for arginine postconidial transport is at or near 5.5. Conidial pH optimum for arginine transport was reported by Roess and DeBusk (26) as about 5.6.

The effects of temperature on arginine postconidial transport are shown in Table 3. These data indicate that the temperature optimum for both the mutant and the wild-type strain is at or near 35 C. This is within the range (30 to 40 C) reported by Roess and DeBusk (26) for conidial arginine transport.

Table 4 shows the effects of temperature on phenylalanine postconidial transport. In the wild-type strain, at 12 min of transport time, there was greater postconidial transport at 35 C. However, at all other times, transport was greater at 25 C for both conidial (as shown by the mu-

### Table 1. Effect of pH on postconidial transport of phenylalanine in the wild-type strain of Neurospora crassa

<table>
<thead>
<tr>
<th>pH</th>
<th>12 min</th>
<th>24 min</th>
<th>36 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>2,149</td>
<td>3,649</td>
<td>4,701</td>
</tr>
<tr>
<td>4.3</td>
<td>3,274</td>
<td>4,661</td>
<td>5,053</td>
</tr>
<tr>
<td>5.4</td>
<td>3,550</td>
<td>5,027</td>
<td>5,844</td>
</tr>
<tr>
<td>5.7</td>
<td>2,677</td>
<td>4,090</td>
<td>4,815</td>
</tr>
<tr>
<td>6.2</td>
<td>1,694</td>
<td>2,713</td>
<td>3,370</td>
</tr>
<tr>
<td>6.7</td>
<td>1,180</td>
<td>1,793</td>
<td>2,518</td>
</tr>
<tr>
<td>7.7</td>
<td>266</td>
<td>532</td>
<td>766</td>
</tr>
<tr>
<td>9.4</td>
<td>17</td>
<td>24</td>
<td>33</td>
</tr>
</tbody>
</table>

* Value ± 0.2 pH units.

Values expressed as counts per minute per milligram (dry weight) of conidia.

### Table 2. Effect of pH on postconidial transport of arginine in the wild-type strain of Neurospora crassa

<table>
<thead>
<tr>
<th>pH</th>
<th>12 min</th>
<th>24 min</th>
<th>36 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>878</td>
<td>1,776</td>
<td>2,553</td>
</tr>
<tr>
<td>4.5</td>
<td>1,300</td>
<td>2,878</td>
<td>3,733</td>
</tr>
<tr>
<td>5.5</td>
<td>2,316</td>
<td>4,495</td>
<td>5,967</td>
</tr>
<tr>
<td>6.2</td>
<td>1,879</td>
<td>3,798</td>
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</tr>
<tr>
<td>6.2</td>
<td>1,069</td>
<td>2,025</td>
<td>2,909</td>
</tr>
<tr>
<td>6.7</td>
<td>934</td>
<td>1,808</td>
<td>2,539</td>
</tr>
<tr>
<td>7.6</td>
<td>191</td>
<td>496</td>
<td>724</td>
</tr>
<tr>
<td>9.4</td>
<td>13</td>
<td>24</td>
<td>22</td>
</tr>
</tbody>
</table>

* Value ± 0.2 pH units.

Values expressed as counts per minute per milligram (dry weight) of conidia.

### Table 3. Effect of temperature on postconidial transport of arginine in the postconidia-deficient mutant and a wild-type strain of Neurospora crassa

<table>
<thead>
<tr>
<th>Temp</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 min</td>
<td>24 min</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>159</td>
<td>187</td>
</tr>
<tr>
<td>15</td>
<td>1,660</td>
<td>2,380</td>
</tr>
<tr>
<td>25</td>
<td>2,070</td>
<td>3,571</td>
</tr>
<tr>
<td>35</td>
<td>3,648</td>
<td>5,952</td>
</tr>
<tr>
<td>50</td>
<td>1,677</td>
<td>2,139</td>
</tr>
</tbody>
</table>

* Values expressed as counts per minute per milligram (dry weight) of conidia.
TABLE 4. Effect of temperature on postconidial transport of phenylalanine in the postconidial-deficient mutant and a wild-type strain of Neurospora crassa

<table>
<thead>
<tr>
<th>Temp</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 min</td>
<td>24 min</td>
</tr>
<tr>
<td>C</td>
<td>381</td>
<td>616</td>
</tr>
<tr>
<td>0</td>
<td>1,068</td>
<td>1,816</td>
</tr>
<tr>
<td>15</td>
<td>2,400</td>
<td>3,580</td>
</tr>
<tr>
<td>25</td>
<td>2,820</td>
<td>2,976</td>
</tr>
<tr>
<td>35</td>
<td>669</td>
<td>590</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values expressed as counts per minute per milligram (dry weight) of conidia.

Control which fails to develop postconidial transport activity and postconidial transport.

Competition patterns (Fig. 5) show basic similarities between the two activities. Both exhibit a greater inhibition of transport by amino acids within a “family” (Intrafamily) than that observed by members of different “families” (Interfamily), i.e., 70 to 80% versus 5 to 40%. However, although the same relationship holds between the classes of amino acids, there is a significant increase in competition by amino acids from different “families” in the postconidial developmental stage. Specifically, phenylalanine (neutral) and arginine (basic) are more effective inhibitors of each other’s transport as the postconidial system develops. This is consistent with the development of a separate general transport system which is able to accommodate either class of amino acid. However, the basic and neutral-aromatic systems must also increase, since competition is not sufficient to indicate that only the general permease system increases. A double mutant, deficient for both the basic and the neutral-aromatic amino acid transport systems (retaining only the general transport system for these amino acids), shows approximately 90% inhibition of phenylalanine transport by arginine for conidial transport (Wolfinbarger, personal communication). This mutant was tested for postconidial competition. The results showed comparable inhibition (90 ± 2%).

Regulation of postconidial transport activity. The results reported herein show that incubating conidia in a medium containing glucose before adding an amino acid substrate results in a greatly increased transport of that amino acid (approximately 10× by 240 min). However, previous transport studies carried out by DeBusk and De-
to remove the free phenylalanine pool by dialysis of the cells. That overnight dialysis removes more than 98% of the transported amino acid was verified by measuring the loss of 14C-labeled phenylalanine and by direct pool analysis with an amino acid analyzer (B. DeBusk, unpublished data). Actidione was added at the conclusion of preincubation and during dialysis, even though the latter was carried out in the cold.

Results of such studies involving both conidial and postconidial transport, incubated in the presence and absence of the amino acid, are shown in Fig. 6 and 7. It is clear that a large portion (greater than 50% in both cases) of postconidial transport activity fails to develop (is repressed) when the developmental preincubation medium contains either phenylalanine or arginine. Addition of either amino acid to conidia followed by immediate dialysis had little effect on subsequent conidial transport. Current studies indicate that repression is specific in that phenylalanine shows a limited repression of arginine postconidial development; similarly arginine has little effect on the formation of postconidial phenylalanine activity.

**DISCUSSION**

It was demonstrated that an increased transport activity for both phenylalanine and arginine may be observed when conidia of *Neurospora crassa* are incubated in a medium which allows development to occur before the addition of an amino acid (developmental preincubation). The development of this activity is dependent on protein synthesis. It is not certain, however, whether this increased activity is due to de novo synthesis of some enzymatic component(s) of the transport systems or activation of preexisting enzymes. A further investigation into this question is in progress. Since the increased transport can be separated from conidial transport activity yet occurs before visible germination, it was termed "postconidial" transport activity.
Temperature and pH optima are approximately the same for both conidial and postconidial transport. This indicates that the nature of the two activities are similar. However, competition experiments indicate that postconidial transport does not show an equal increase of three of the transport systems present in conidia. Conidia of N. crassa have a number of transport activities. Extensive studies have been carried out by several investigators (7, 18, 34; Wollinbarger, personal communication) on the three systems utilized here in studying postconidial transport activity. These three systems are under separate genetic control. They include two family-specific systems, the neutral-aromatic permease and the basic permease. Amino acids in one "family" show little competition for transport of amino acids in the other "family." The third system is represented by a general permease in that amino acids from different "families" do significantly compete for transport. The greater inhibition of transport by members of different amino acid transport "families" observed in competition studies indicate that a greater proportion of postconidial transport as compared to conidial transport is mediated by a general permease system. This would indicate that the increased activity observed in postconidial transport is in part due to a preferential increase in a general permease-mediated system. This observation is in agreement with that of Palli (20), who postulates that more of the neutral-aromatic transport is subject to basic amino acid competition with increasing age from one-day-old through four-day-old mycelia. However, the basic and neutral-aromatic systems must also increase since competition is not sufficient to indicate that only the general permease system increases. Not only do all three systems appear to show an increased activity in postconidial transport, but, in addition, the two specific transport systems studied show a differential increase in the rate of transport, apparently reflecting a differential development of the systems during the preincubation period. Rate constants for the actidione-sensitive fractional increase in transport with preincubation time can be obtained from semilog plots of radioactivity measured for any one of the transport times versus developmental preincubation time. These constants were found to be approximately 0.014/min for phenylalanine transport and 0.018/min for arginine transport. Thus, the development of postconidial transport results in a small but measurable increase in rate favoring arginine over phenylalanine.

Additional evidence that arginine transport increases with developmental preincubation time to a greater extent than phenylalanine transport and that development of postconidial transport activity may be differentially regulated is shown by the partial development of postconidial transport activity for arginine in the mutant utilized in this study, whereas no phenylalanine transport beyond conidial level is exhibited. The two temperature-insensitive, postconidial transport-deficient tetrads segregants also show a partial development (to the same extent as its FGSC 636A parent) of postconidial transport activity for arginine with no phenylalanine transport beyond the conidial level.

The similarities (temperature and pH optima) between conidial and postconidial transport suggest a common mechanism for the transport process itself. Activity increases rapidly, extensively, and requires protein synthesis for development. The existence of a tetrad segregant which exhibits a similar mass increase and growth rate as does the wild-type strain yet fails to develop postconidial transport activity shows that the increased activity is not merely a function of simple cell growth. Thus, some form of regulation must occur as development is initiated which results in the increased activity. Further, this developmental regulation appears to differentially affect three transport systems present in conidia. The fact that the substrate amino acid partially (greater than 50%) represses the development of postconidial transport activity is additional evidence that there is developmental regulation. Regulation of this type has been observed by Rogers and Lichstein (27) in Saccharomyces cerevisiae, in which the synthesis of the biotin transport system appears to be partially repressed during growth in medium containing high concentrations of biotin.

All of the above results indicate that postconidial transport activity and developmental regulation of amino acid transport in N. crassa should be studied with mutants deficient in the different conidial transport systems before the mechanism of control can be elucidated. Such an investigation is now in progress.

ACKNOWLEDGMENTS

Facilities for preliminary studies by one of the authors (A.G.D.) were provided by the Research School of Biological Sciences, Australian National University, and are gratefully acknowledged. One of the authors (J.H.T.) is the recipient of a traineeship from the Department of Health, Education, and Welfare through a Training Grant (GM-1316) in Genetics to the Florida State University.

LITERATURE CITED

AMINO ACID TRANSPORT

acid absorption and protein synthesis in Escherichia coli