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AMINO ACID TRANSPORT IN *NEUROSPORA CRASSA*

II. PROPERTIES OF A BASIC AMINO ACID TRANSPORT SYSTEM

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SUMMARY

Studies of amino acid uptake have revealed the existence of an active transport system in *Neurospora crassa* specific for basic L-amino acids. This system, designated amino acid transport system III, has a Michaelis constant for L-lysine uptake of 4.8 μM and for L-arginine uptake of 2.4 μM. It also transports L-canavanine and L-histidine. Transport system III has little or no activity in the bat mutant, suggesting that this mutant may be missing the binding protein for system III.

The activity of transport system III is correlated with growth, being highest in rapidly growing cultures and lowest in starving cultures. Possible roles for system III in the physiology of the organism are discussed.

INTRODUCTION

Amino acid transport in *Neurospora crassa* is performed by several active transport systems, each capable of transporting a variety of amino acids. Two of these transport systems have been characterized in some detail. Transport system I is capable of transporting a variety of neutral L-amino acids[1,2]. Transport system II is a general amino acid transport system, capable of transporting a wide variety of basic, neutral, and acidic DL-amino acids[1]. In the consideration of transport system II, it was suggested that an additional transport system, probably specific for basic amino acids, was also active in Neurospora[1]. The properties of this last system, including its substrate affinities and regulation, are the subject of this report.

MATERIALS AND METHODS

Strains and chemicals

Wild type strain ST74A was used in most transport experiments. The UM535 mutant[3], which has recently been designated bat, was obtained from Dr. W. M. Thwaites. The mtr 6, ylo strain was obtained from the Fungal Genetics Stock Center.

Unlabeled amino acids and amino acid derivatives were obtained from Nutritional Biochemicals. L-[^{14}C]Arginine, L-[^{3}H]lysine, and L-[^{3}H]histidine were obtained from Schwarz Bioresearch.

Growth of cultures and uptake experiments

Growth of germinated conidia and uptake of amino acids were performed as described previously[2] with three changes in procedure. Conidia were germinated for 20 h at 25°, and harvested by centrifugation. Uptake was measured by addition of the germinated conidia to Vogel's Medium N salts containing labeled and, where appropriate, unlabeled amino acids and shaken vigorously.

The germinated conidia were washed on a filter with cold water, extracted with 5 % trichloroacetic acid and counted as described previously[1].

Nongrowing mycelial pads, starving for either carbon, nitrogen, or sulfur were grown by a procedure similar to that described previously[1]. 125-ml flasks containing 20 ml of medium were inoculated with about $1 \cdot 10^4$ conidia and grown without shaking for 48 h at 25°. Having formed mycelial pads, the cultures were gently shaken at 25° on a reciprocal shaker for 24 h. With the various media used, growth was then completed, being limited by the limiting nutrient. For the growth of carbon-starved mycelial pads, the medium used was 1X Vogel's Medium N containing 0.4 % sucrose. For nitrogen starvation, Vogel's Medium N containing only 10 % of the normal nitrogen (as NH_4NO_3) with 2 % sucrose was used. Similarly for sulfur starvation, Vogel's Medium N containing only 10 % of the normal sulfate (the remainder being replaced by chloride) with 2 % sucrose was used. The growth media ranged from pH 6.0 to 6.3.

Growing mycelial pads were produced by inoculating flasks of Vogel's Medium N containing 2 % sucrose with $1 \cdot 10^4$ conidia and growing without shaking for 2 days at 25°. The pads were washed with water and placed in 20 ml of fresh Vogel's salts immediately before uptake.

Uptake was measured by adding unlabeled amino acid (where appropriate) and labeled amino acid to the growth medium or (for growing pads) Vogel's salts and shaking vigorously at 25°. In most cases, the pads were shaken for 2 min. The mycelial pads were harvested, washed, extracted and counted as described previously[1].

For each experiment germinated conidia or mycelial pads similar to those used for the uptake were dried and weighed. The cell water was estimated by assuming it to be 3 times the dry weight of the organisms.

RESULTS

Uptake into germinated conidia

Initial studies of basic amino acid transport were confined to germinated conidia since exploratory results indicated that when grown as described above, germinated conidia of strain ST74A showed little or no activity for transport system II. Thus any uptake of basic amino acids into such germinated conidia must be due to the activity of an additional transport system (or systems).

As shown in Fig. 1, the uptake of L-lysine into germinated conidia progresses at a constant rate for periods of about 15 min. At 20 min the tritium label from the lysine was over 1000 times more concentrated in the cell water than it was in the medium. Thus, the transport involved would appear to be up a large concentration gradient. In addition NaN_3 (10 mM) or dinitrophenol (1 mM) both inhibited L-lysine uptake by over 95 %, indicating that metabolic energy is required for the

uptake. These findings support the proposition that the lysine uptake involves active transport.

Further studies were performed to characterize the amino acid transport system (or systems) responsible for the active transport of lysine. As shown in Table I, the

TABLE I

INHIBITION OF L-LYSINE UPTAKE BY VARIOUS AMINO ACIDS

The uptake of 10 μM labeled L-lysine into germinated conidia of wild type 74A was measured in the presence of 1 mM of the various amino acids or derivatives.

Unlabeled amino acid added	*Lysine uptake (% of level with no unlabeled amino acid)*
None	(100)
L-Arginine	< 1
L-Lysine	1
L-Ornithine	10
L-2,4-Diaminobutyric acid	62
L-2,3-Diaminopropionic acid	82
D-Lysine	76
N-Acetyl-L-arginine	95
Cadaverine	92
L-asparagine	106
Glycine	110
L-Leucine	91
L-Norleucine	77
D-Phenylalanine	92
L-Aspartic acid	101
L-Glutamic acid	108

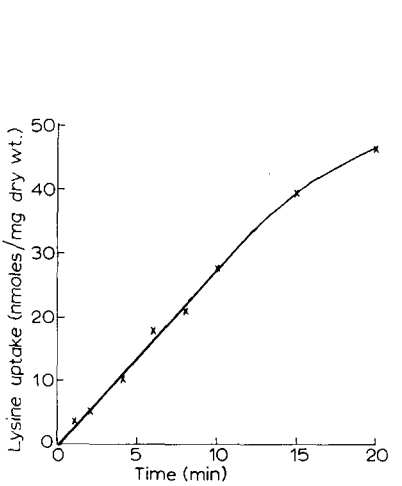


Fig. 1. Time-course of lysine uptake. The uptake of 20 μM L-lysine into germinated conidia of strain 74A was measured after the lysine had been shaken with the germinated conidia for various periods of time. v is expressed as nmoles of L-lysine taken up per mg dry weight of germinated conidia.

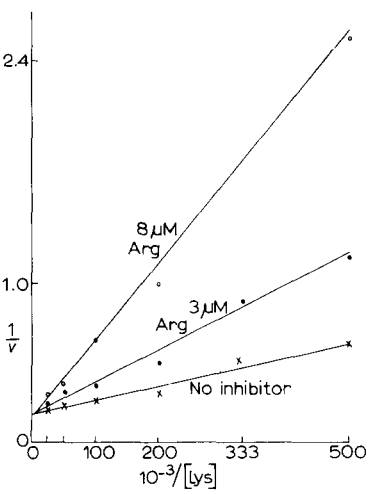


Fig. 2. Inhibition of lysine uptake by arginine. The uptake of various concentrations of L-lysine into germinated conidia of 74A was measured in the presence or absence of L-arginine. v is expressed as nmoles of L-lysine taken up per min per mg dry weight of germinated conidia.

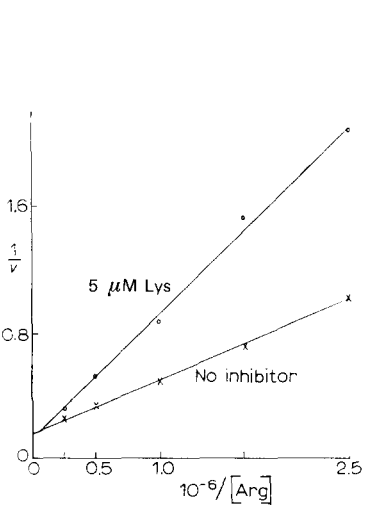
uptake of lysine is inhibited by a variety of basic amino acids but shows essentially no inhibition by neutral or acidic amino acids. This confirms the finding that transport system II shows little activity under these conditions since lysine uptake by transport system II should be inhibited by the neutral amino acids used[1]. The inhibition of the lysine uptake by several basic amino acids suggests that the uptake is due to the activity of an amino acid transport system with affinity for a variety of basic amino acids. This suggestion is confirmed by the kinetic studies performed below.

Kinetic studies of uptake

A study of the rate of lysine uptake into germinated conidia using various concentrations of L-lysine is shown in Fig. 2. The result, when plotted on a Lineweaver–Burk plot, follow Michaelis–Menten kinetics, with a Michaelis constant (K_m) for L-lysine uptake of 5 μM. It shows a maximum velocity of transport of 4.5 nmoles/mg dry weight per min. In addition, the results in Fig. 2 show that L-arginine is a competitive inhibitor of lysine uptake, having an inhibitor constant (K_i) of 2.4 μM.

Kinetic studies of the uptake of L-arginine have yielded further information on the transport of basic amino acids. In Fig. 3, the results of such a kinetic study are plotted showing that L-arginine is taken up with a Michaelis constant of 2.3 μM. L-Lysine is a competitive inhibitor of the uptake of L-arginine, showing a K_i of 4.6 μM.

The simple kinetics displayed in studies of these two amino acids provides evidence that each of these amino acids is transported by a single transport system. In this context, it should be noted, that there is excellent agreement between the K_m for lysine and its K_i when studied as an inhibitor of arginine uptake. Similarly, the K_m and K_i for arginine are essentially equal. These equalities provide substantial support for the contention that arginine and lysine are transported by the same



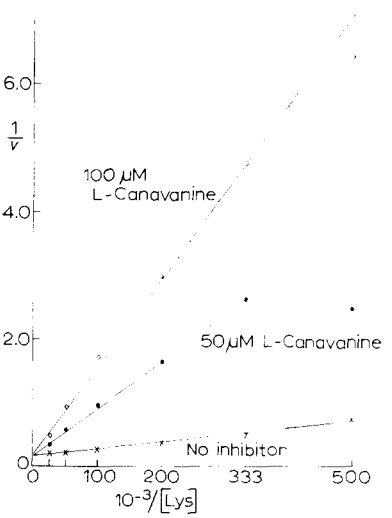


Fig. 3. Inhibition of arginine uptake by lysine. The uptake of various concentrations of L-arginine into germinated conidia of 74A was measured in the presence or absence of L-lysine. v is expressed as nmoles of L-arginine taken up per min per mg dry weight of germinated conidia.

Fig. 4. Inhibition of lysine uptake by canavanine. The uptake of various concentrations of L-lysine into germinated conidia of 74A was measured in the presence or absence of L-canavanine. v is expressed as nmoles of L-lysine taken up per min per mg dry weight of germinated conidia.

transport system. This proposed system will be designated amino acid transport system III.

Two other amino acids, L-canavanine and L-histidine were tested as inhibitors of the uptake of L-lysine. As shown in Fig. 4, canavanine is a competitive inhibitor of lysine uptake, showing a K_i of about 7 μM. The simple kinetics again support the proposition that a single amino acid transport system, system III, is responsible for the uptake involved. Although the uptake of L-canavanine itself was not studied, the results of HOROWITZ AND SRB[5] and BAUERLE AND GARNER[6], to be discussed below, suggest that canavanine is itself taken up by transport system III.

L-Histidine requires further consideration since it is a relatively poor inhibitor of system III. As shown in Fig. 5, L-histidine appears to be a competitive inhibitor of lysine uptake with a K_i of about 2.3 mM. Its poor apparent affinity, about 1000 times lower than that for arginine, raises the possibility that the inhibition involved is caused by a small amount of an impurity such as arginine or other basic amino acid present in the histidine, rather than being caused by L-histidine itself. To distinguish between these possibilities, it seemed desirable to study the uptake of L-histidine to see if it is taken up by transport system III.

L-Histidine is transported by transport systems I and II (refs. 1, 2) in addition to its possible uptake by system III. Thus in order to study histidine transport by system III, it is best to measure the uptake under conditions where the other two systems are not active. In order to do this, the mtr strain was used because it is deficient in system I (refs. 1, 7–9). The uptake was measured into germinated conidia which show little activity for system II (at least in some strains), when grown as described here. When this uptake is measured, as shown in Fig. 6, the K_m for L-histidine uptake is about 1.6 mM which is in satisfactory agreement with the K_i for L-histidine measured above. Furthermore, the uptake of 1 mM L-histidine shows essentially complete inhibition by equimolar amounts of L-arginine or L-lysine

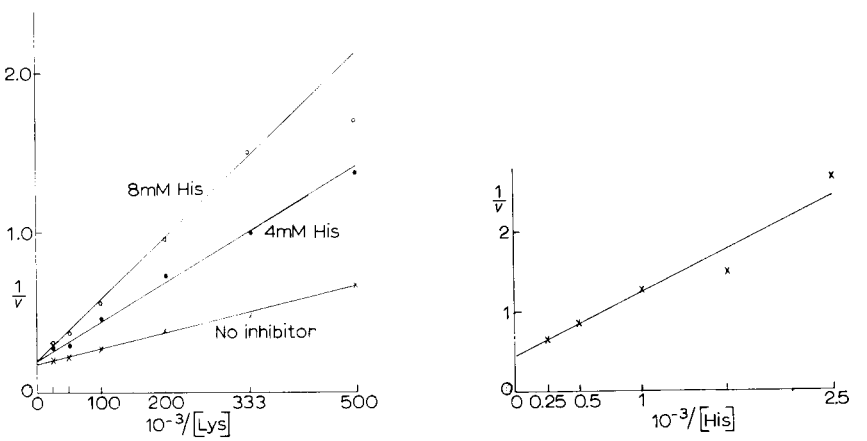


Fig. 5. Inhibition of lysine uptake by histidine. The uptake of various concentrations of L-lysine into germinated conidia of 74A was measured in the presence or absence of L-histidine. v is expressed as nmoles of L-lysine taken up per min per mg dry weight of germinated conidia.

Fig. 6. L-Histidine uptake in the mtr mutant. The uptake of L-histidine into germinated conidia of the mtr 6, ylo strain was measured. v is expressed as nmoles of L-histidine taken up per min per mg dry weight of germinated conidia.

but shows little or no inhibition by 2 mM L-alanine or L-leucine. This spectrum of inhibition is consistent with the conclusion that the histidine uptake is due to the activity of transport system III and shows that the measured uptake cannot be due to system I or II. Thus, it may be concluded that histidine has an affinity for transport system III although that affinity is much lower than those shown by the other amino acids studied.

The data in Table I provide additional information on the properties of system III since those compounds with high affinity for system III will be effective inhibitors of lysine uptake but those with little or no affinity will not be effective. In this way the effectiveness of various compounds as inhibitors can yield information on their affinities. For example D-lysine is a much poorer inhibitor than (unlabeled) L-lysine, indicating that system III has a stereospecificity favoring L-amino acids. It should be noted that as the length of the amino acid side chain decreases from lysine to ornithine to 2,4-diaminobutyric acid, the affinity decreases so that 2,3-diaminopropionic acid shows little affinity for the transport system. Finally neutral and acidic amino acids as well as certain derivatives of basic amino acids such as acetylarginine or cadaverine show little or no affinity for transport system III.

Activity of transport system III under various physiological conditions

Having established many of the properties of a transport system, it should be possible to identify the activity of that system under various physiological conditions by its particular characteristics. Since amino acid transport system II, as well as system III, is capable of transporting basic amino acids, it is necessary in general to suppress the activity of system II in order to measure the uptake of an amino acid by system III. In measuring the uptake of L-lysine, for example, high concentrations of glycine were used to competitively inhibit the uptake of lysine by system II. As shown in Fig. 7, as increasing concentrations of glycine are used to inhibit the uptake of L-lysine into carbon-starved pads, the lysine uptake approached a plateau, the residue apparently not being substantially inhibited by glycine. The inhibitable portion is presumably due to transport system II. It seemed reasonable that the residual uptake, not inhibited by glycine, is due to system III activity. In order to test this last suggestion a number of experiments were performed.

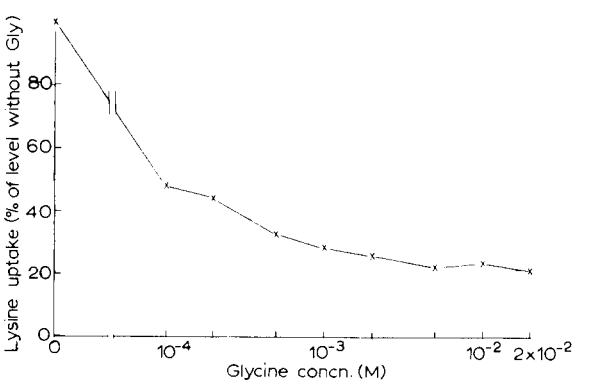


Fig. 7. Inhibition of lysine uptake into carbon-starved mycelial pads by glycine. The uptake of 10 μM L-lysine into carbon-starved pads of 74A was measured in the presence of various concentrations of glycine.

In each case the uptake of L-lysine into growing, carbon-starved, nitrogen-starved or sulfur-starved mycelial pads was measured in the presence of 50 mM glycine. The uptake of 10 μM L-lysine was measured in the presence of 1 mM of the various amino acids. As shown in Table II, arginine and ornithine inhibit the uptake of lysine but none of the other amino acids tried were effective in this inhibition. This pattern is consistent with the lysine uptake being due primarily to transport system III and is not consistent with the uptake being due to any other system previously delimited in Neurospora. The K_m for lysine uptake (in the presence of 50 mM glycine) was also measured to see if it was consistent with that found for system III*. In each of these four physiological states, the K_m measured was within 40 % of that found for lysine uptake into germinated conidia (4.8 μM). Consequently, the results again support the proposition that the uptake is due to system III. Because it is unlikely that there would be an additional transport in Neurospora similar in all these properties to system III, it may be concluded that the activity measured here is due to amino acid transport system III.

The activities of a transport system under different conditions can be compared by measuring the velocity of transport of one of the amino acids taken up by that system. For unknown reasons the maximum velocity of lysine transport by system III shows considerable variation from experiment to experiment under the various physiological conditions examined here. Nevertheless, when typical v_{max} values are compared, as shown in Table III, an interesting pattern is observed. In germinated conidia, where rapid growth is occurring, the activity of transport system III is very high. In growing mycelial pads in which the rate of growth is substantial but less than that for germinated conidia, the activity is fairly high although less than that for germinated conidia. In carbon-starved, nitrogen-starved or sulfur-starved mycelial pads in which growth has stopped, the activity is relatively low. Net protein

TABLE II

LYSINE UPTAKE INTO CULTURES OF DIFFERENT PHYSIOLOGICAL STATES: INHIBITION BY VARIOUS AMINO ACIDS

The uptake of 10 μM labeled L-lysine into various cultures of wild type 74-A was measured in the presence of 1 mM of the various amino acids. All uptake was measured in the presence of 50 mM glycine in the medium.

Unlabeled amino acid added	*Lysine uptake into different cultures (% of level with no unlabeled amino acid)*			
	Growing pads	*Sulfur-starved pads*	*Carbon-starved pads*	*Nitrogen-starved pads*
None	(100)	(100)	(100)	(100)
L-Arginine	1	3	1	10
L-Ornithine	13	16	18	23
L-2,3-Diamino-propionic acid	84	95	110	83
L-Asparagine	96	95	120	107
L-Leucine	111	107	120	94
D-Phenylalanine	115	103	121	110
L-Aspartic acid	106	94	119	99
L-Glutamic acid	106	99	110	80

* Due to high activity in growing pads, the K_m was measured at 15° instead of 25°.

TABLE III

MAXIMUM VELOCITY OF LYSINE UPTAKE BY TRANSPORT SYSTEM III

In all cases except germinated conidia, the lysine uptake was measured in the presence of 50 mM glycine. The velocity of transport is expressed in nmoles/mg dry weight per min.

Physiological state of culture	v_{max} (L-*lysine*)
Germinated conidia	4.6
Growing pads	1.2
Carbon-starved pads	0.30
Nitrogen-starved pads	0.16
Sulfur-starved pads	0.29

TABLE IV

AMINO ACID UPTAKE BY THE bat MUTANT

In each case, the lysine uptake was measured in the presence of 50 mM glycine. The arginine-insensitive component of tryptophan uptake was measured in the presence of 1 mM L-arginine. The arginine-sensitive component was measured by taking the uptake in the absence of arginine and subtracting the uptake in the presence of 1 mM L-arginine. For more information on tryptophan uptake, see ref. 1. v is expressed as nmoles/mg dry weight per min.

Physiological state	*Uptake measured*	*Transport system primarily involved*	*Uptake by bat mutant* (v)	*Uptake by 74A* (v)	*Uptake by bat mutant* (% *of 74A*)
Germinated conidia	10 μM L-lysine	III	0.0198	3.04	0.65
	100 μM L-tryptophan (arginine insensitive)	I	5.22	7.94	153
Growing pads	20 μM L-lysine	III	0.0057	1.0	0.57
	100 μM L-tryptophan (arginine insensitive)	I	2.77	2.15	129
Carbon-starved pads	10 μM L-lysine	III	0.0044	0.135	3.2
	100 μM L-tryptophan (arginine insensitive)	I	0.041	0.081	51
	100 μM L-tryptophan (arginine sensitive)	II	0.106	0.071	149
Nitrogen-starved pads	10 μM L-lysine	III	0.00589	0.0585	10
Sulfur-starved pads	10 μM L-lysine	III	0.0155	0.152	10

synthesis of course will be closely correlated with growth such that it will be highest in the germinated conidia and lowest in the starving pads. Thus the activity of system III can be seen to be regulated so that it would be quite effective in providing amino acids for net increases in protein during growth.

Properties of the bat mutant

The study of active transport is often aided by the characterization of mutant strains of an organism which are defective in their transport properties. A mutant of Neurospora, UM535, has recently been found by THWAITES AND PENDYALA[4] to be deficient in its basic amino acid transport. It was derived from an arginine-insensitive isolate of an arginine-sensitive strain[3]. Due to its transport deficiency, the mutant is now designated bat.

As shown in Table IV, the bat mutant shows little or no activity for transport system III in germinated conidia, growing mycelial pads, or carbon-, nitrogen- or sulfur-starved pads. The small amount of transport shown in the starved pads may be due to a small amount of activity of system II not inhibited by glycine since system II has high activity under these conditions. In contrast to transport system III, systems I and II show normal or approximately normal activity in the mutant. The results suggest that the bat strain may be a permeaseless mutant, lacking the specific binding protein or permease for transport system III. It should be noted that the property of the bat mutant's being deficient in lysine uptake in the presence of glycine confirms the conclusion, made above, that the lysine uptake (in the presence of glycine) is primarily due to a single transport system under all the conditions studied.

DISCUSSION AND CONCLUSIONS

Amino acid transport in Neurospora is mainly performed by several active transport systems, each of which takes up a wide variety of amino acids. Transport system I (refs. 1, 2) takes up a variety of neutral L-amino acids and system II (ref. 1) takes up basic, neutral and acidic DL-amino acids. Amino acid transport system III, described here, is a basic amino acid transport system, taking up a variety of basic L-amino acids. Neurospora differs from most bacteria, where transport systems with narrow specificities, often called permeases, seem to be most prevalent. The pattern found in other fungi[10–15,20] would appear to be intermediate between that found in Neurospora and bacteria.

Amino acid transport system III takes up several basic amino acids, showing a Michaelis constant for L-lysine estimated at 4.8 μM and for L-arginine of 2.4 μM. It shows substantial affinity for canavanine and apparently also for ornithine. It has a low but significant affinity for L-histidine with a K_m of about 2 mM*. Transport system III is specifically missing in the bat (ref. 4) mutant, suggesting that this mutant may be defective in the specific binding protein or permease for system III.

The characterization of the properties of transport systems can be of value in understanding several phenomena. For instance, the growth of biochemical mutants has been observed to be inhibited by compounds which have no effect on the growth of the wild type. In particular, arginine-requiring mutants are inhibited by lysine[16] and lysine-requiring mutants are inhibited by arginine[17]. These observations can now be understood since both amino acids are taken up by the same transport system, system III, such that each will inhibit the uptake of the other**. A strain which requires one amino acid will be prevented from taking it up by sufficient concentrations of the other amino acid, thus leading to growth inhibition.

A similar explanation is of value in understanding the properties of growth inhibition by amino acid analogues. Such inhibition is often reversed by other amino

* The low affinity of system III for histidine raises the question as to whether histidine uptake by that system would be important in supporting the growth of histidine auxotrophs; perhaps the mutants of WOODWARD *et al.*[19] will be of value in answering this question.

** Some of the uptake of arginine and lysine is also due to transport system II but here again, the uptake of one amino acid would be inhibited by the other.

acids. For example, the growth inhibition by L-canavanine can be reversed by either arginine or lysine[5]. Since canavanine is probably taken up by transport system III, its uptake would be inhibited by arginine or lysine, thus preventing it from inhibiting growth*. It should be noted that this explanation is essentially identical with that previously proposed by BAUERLE AND GARNER[6]. Much of the uptake observed by those authors was probably due to transport system III.

The characterization of discrete transport systems can be of considerable value in studies of the regulation of active transport as well as in the elucidation of phenomena such as those described above. Before the uptake of an amino acid can be concluded to be due to a single transport system, substantial numbers of kinetic and possibly also genetic studies should be performed[1]. In particular neutral amino acids in Neurospora can be taken up by systems I and II and basic amino acids can be taken up by systems II and III. Confusion may arise when the activities of the various systems are not separated. For example, the activity of a "basic amino acid permease in Neurospora" studied by ROESS AND DEBUSK[18] was probably due to both transport systems II and III since about half of the uptake they measured was inhibitable by neutral amino acids. It is possible that the CR10 mutant studied by those investigators may be allelic with the bat mutant studied here.

Once the activity of a transport system is distinguished from those of other systems present in the same organism, it is possible to study regulation of that system. In such studies it is difficult to separate out the regulation of the synthesis of the transport protein (or proteins) from direct regulation of their activity. The difficulty arises from the fact that no assay is available, in general, for the level of the transport protein or proteins. Thus generally only assays of activity *in vivo* are available. In this study, no attempt has been made to separate regulation into different levels so that the variation of activity observed could be due to regulation at either or both levels. In any case transport system III shows its highest activity under conditions of rapid growth and, thus, highest net protein synthesis. Where growth has ceased so that net protein synthesis would be expected to be negligible, system III shows its lowest activity. Thus, transport system III seems to be regulated in parallel with the net protein synthesis. The results suggest that system III may have its primary function in providing amino acids for the addition of proteins to the organism.

ACKNOWLEDGMENTS

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* Any uptake of canavanine by system II would also be inhibited by arginine or lysine.

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