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# AMINO ACID TRANSPORT IN *NEUROSPORA CRASSA*

## I. PROPERTIES OF TWO AMINO ACID TRANSPORT SYSTEMS

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SUMMARY

Kinetic studies have revealed the existence of two transport systems for amino acids in *Neurospora crassa*. Transport system I corresponds to a system previously studied by WILEY AND MATCHETT. Its activity is specifically missing in mtr mutant cultures previously described by LESTER AND STADLER. It is capable of transporting most neutral L-amino acids. Amino acid transport system II has not been described previously. It has an affinity for a wide variety of amino acids. It transports amino acids with hydrophobic and hydrophilic side chains, both basic and neutral amino acids, and D- as well as L-amino acids. Transport system II has an affinity for both $\beta$- and $\alpha$-amino acids.

Transport system I has high activity in young, rapidly growing cultures. Transport system II has little or no activity in young cultures. In older, carbon-starved cultures, however, it is more active than transport system I. This, together with the high affinities it shows for many amino acids, suggests that amino acid transport system II serves a scavenger function, removing from the medium traces of exogenous amino acids.

## INTRODUCTION

The movement of substances through membranes is one of the most important phenomena of life. Much of this movement has been found to occur through the mechanism of active transport. Amino acids constitute the largest group of compounds commonly found to be concentrated by active transport. Considerable interest has been generated in determining the stratagem used by various organisms to concentrate this important group of compounds. This report is concerned with delimiting and characterizing two of the active transport systems involved in amino acid transport in *Neurospora crassa*. Two techniques have been commonly used to delimit the range of activities of particular transport systems and to distinguish them from other transport systems which may also be active in the same organism.

Genetic studies have centered on the isolation and characterization of mutants deficient in the transport of one or more compounds. In bacteria, these mutants, often

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termed permeaseless, are generally deficient in the uptake of a small group of structurally related substrates[1–4]. The mutants often do appear to be deficient in a specific protein or permease that binds the substrate and catalyzes its transport. The determination of the transport deficiencies of such mutants is of considerable value in delimiting the range of substrate specificity of the missing transport system.

The study of permeaseless mutants is complicated, however, by the existence of other types of mutants which have their active transport affected in more general ways. Several mutants of Neurospora, for example, may have their amino acid transport activity altered through nonspecific changes in membrane structure[5,6] or possibly through otherwise altered activity of several transport systems[7]. Only in the case of the mtr mutant, whose properties are described below, does it appear likely that a mutant strain of Neurospora, deficient in amino acid transport, is defective due to a permeaseless type of deficiency[14,15]. The use of mutants to delimit transport systems is restricted, then, by the ability to distinguish permeaseless mutants from mutants unable to transport particular substances due to other types of lesions. This problem can be solved, in part, by kinetic studies on transport.

One of the original observations that led to the concept of active transport was that transport of substances across membranes often showed saturation at high substrate concentrations. Furthermore, compounds of similar structure to a transport substrate, often competitively inhibited transport of that substrate. The saturation and competition, moreover, often followed Michaelis–Menten kinetics. By studying the competitive inhibition of the transport of one substrate by other potential substrates, the range of substrate specificities of a particular transport system can be determined.

The applicability of the kinetic approach is limited by the existence of more than one transport system of overlapping specificities in a given organism. In addition, the applicability is further limited by the possible existence of noncompetitive inhibition of transport by various compounds[9].

The kinetic and genetic approaches both have their limitations. The limitations, however, lie in different areas. The two approaches, then, can complement each other in delimiting the activities of various transport systems. Although this report is involved primarily with kinetic information, the role of the mtr locus is also considered.

A number of studies of amino acid transport in Neurospora have been made where the uptake of one amino acid has been inhibited by other amino acids[10–15]. In most of these cases, kinetic analysis of the inhibition has not been done to determine whether or not the inhibition is competitive. The uptake of phenylalanine[19] and arginine[11] into Neurospora conidia is inhibitable by a large number of amino acids, basic and neutral. Furthermore, the uptake of the aromatic D-amino acids into Neurospora mycelia is also inhibitable by a wide variety of L-amino acids[12]. If these inhibitions are competitive, they imply the existence of an amino acid transport system with a very broad range of substrate specificity. Experiments discussed below show that such a transport system does exist.

WILEY AND MATCHETT[13] have studied tryptophan uptake in germinated conidia. They have shown that L-tryptophan uptake is competitively inhibited by L-phenylalanine and L-leucine but that it is not inhibited by basic or acidic amino acids. Their results imply the existence of a transport system with limited specificity responsible for the uptake of large, neutral amino acids.

Finally, the mtr mutant, briefly mentioned above, was isolated by LESTER[15] because of its resistance to 4-methyltryptophan. It was subsequently determined to be deficient in the uptake of 4-methyltryptophan, L-tryptophan, L-phenylalanine, and L-leucine, but has normal uptake of the basic amino acids[14,15]. It may be deficient, then, in the uptake of those amino acids with an affinity for the uptake system of WILEY AND MATCHETT. Thus, although the studies performed are not definitive at this point, the mtr mutant may correspond to the permeaseless mutants in bacteria. The permease controlled by the mtr locus may be responsible for the transport studied by WILEY AND MATCHETT.

There appear to be two groups of information in conflict. One group implies the existence of a transport system with broad specificity which is responsible for most of the uptake in conidia[10,11] and older mycelia[12]. Another group implies that in germinated conidia most of the uptake of neutral amino acids is catalyzed by a system of more limited specificity. This apparent conflict is resolved below.

## MATERIALS AND METHODS

### *Strain*

Wild-type strain ST74A (ref. 16) was used in most transport experiments. The D-amino acid oxidaseless strain, oxD-8, was used in transport studies of the aromatic D-amino acids[17].

The mtr mutant strain has been found to be deficient in the transport of tryptophan and several other neutral amino acids[14,15]. Transport was studied in an mtr strain carrying the ylo-1 marker which was obtained from the Fungal Genetics Stock Culture Center (FGSC No. 1117).

### *Chemicals*

All chemicals used were of reagent grade quality except as noted below. Table sugar was used as sucrose in the culture medium. Commercial grade Triton X-100 was obtained from Rohm and Haas.

L-Alanine, $\beta$-alanine, $\gamma$-aminobutyric acid, 2-aminoethanol, L-arginine, L-asparagine, L-aspartic acid, L-histidine, L-leucine, L-lysine, L-phenylalanine, pyruvic acid, and L-tryptophan were obtained from Sigma Chemical. Glycine, *N*-acetyl-L-leucine, and L-leucinamide were obtained from Nutritional Biochemicals. L-$\alpha$-Aminobutyric acid, D-phenylalanine, and L-ornithine, were obtained from Calbiochem.

[$^{14}$C]Glycine (50 mC/mmole), L-[$^{3}$H]phenylalanine (2.5 C/mmole), L-[4,5-$^{3}H_2$]-leucine (2 C/mmole), and L-[$^{14}$C]aspartic acid (113 mC/mmole) were obtained from Schwarz BioResearch. D-[1-$^{14}$C]Phenylalanine (25 mC/mmole) and L-[3-$^{14}$C]tryptophan (22 mC/mmole) were obtained from New England Nuclear. Uniformly $^{14}$C-labeled L-asparagine (102 mC/mmole) was obtained from Nuclear Chicago.

### *Maintenance and growth of Neurospora cultures*

All Neurospora strains were maintained on agar slants of HOROWITZ[18] complete medium. The liquid medium used was one-half strength VOGEL'S[19] medium N salts containing 0.5% sucrose. 125-ml erlenmeyer flasks containing 20 ml of liquid medium were inoculated with about $10^4$ conidia in aqueous suspension. The cultures were grown without shaking for 48 h at 25°. Having formed mycelial pads, the cultures were

gently shaken at 25° on a reciprocal shaker for about 24 h. The 3-day-old cultures were then used to study amino acid uptake.

The dry weight of the mycelial pads formed after 3 days growth, as described above, was about 50 mg. The medium had a pH of about 5.8 to 6.1 with the strains used.

*Uptake of amino acids*

Stock solutions were prepared of isotopically labeled amino acids diluted in specific activity with unlabeled amino acids. Aqueous stock solutions of other compounds which were to be tested as possible inhibitors of transport were prepared.

In uptake method I, pads prepared as described above had labeled amino acid from a stock solution added to the medium. The pads were shaken moderately on a reciprocal shaker at 25° for 2 min, harvested on a Buchner funnel and washed thoroughly with ice-cold distilled water. The pads were then dropped into cold 5% trichloroacetic acid. When various compounds were to be tested as inhibitors of uptake, one of two techniques was used. For some experiments, the inhibitor or inhibitors were added to the medium of the shaken cultures about 30 sec before the addition of the labeled amino acid. For other experiments, aliquots of inhibitor and labeled amino acid were mixed and added together.

Uptake method II was used when a large fraction (approx. 20%) of either the labeled amino acid or inhibitor was taken up when using uptake method I. In uptake method II, the inhibitor, if any, and 60 ml of distilled water, previously equilibrated at 25°, were added to the cultures. An aliquot of labeled amino acid was then added to the culture medium, and the cultures shaken vigorously for 1 min at 25° on a rotary shaker. The pads were harvested on a Buchner funnel and washed thoroughly with ice-cold distilled water. These pads were then dropped into cold 5% trichloroacetic acid.

*Extraction and measurement of counts taken up*

Each mycelial pad was extracted with 5 ml of cold trichloroacetic acid for at least 2 h. In most cases, two identically treated pads were pooled for the extraction. The pads were spun down. 0.5–1 ml of trichloroacetic-acid extract was neutralized with an equal volume of 0.6 M Tris. This mixture was counted in a toluene base scintillation fluid using Triton X-100 as a solubilizer[23].

## RESULTS

*Tryptophan uptake*

Active transport of amino acids can proceed very rapidly in 3-day-old pads of wild-type strain 74A. A number of amino acids can be rapidly accumulated into the mycelium to a concentration of several mg per g of cell water over their concentration in the medium. The transport of L-arginine and D-phenylalanine was shown, in exploratory experiments, to be inhibited over 95% by 0.01 M azide or 1 mM dinitrophenol. Thus, the accumulation depends on metabolic energy. Furthermore, high concentration gradients of D-phenylalanine can be produced through transport into the mutant strain oxD-8 which cannot metabolize D-amino acids[17]. These results confirm the existence of active transport of amino acids under the conditions studied.

The uptake of 10 $\mu$M L-tryptophan was shown, in preliminary experiments, to

be largely inhibitable by L-arginine. In typical 3-day-old pads of 74A, inhibition values of 70–85% were obtained with 0.1 mM L-arginine from different experiments. As shown in Fig. 1, higher amounts of arginine than this give no greater inhibition of tryptophan uptake. Similarly, 0.1 mM of the basic amino acids L-lysine, L-canavanine, and L-ornithine inhibited tryptophan uptake by 70–85%, and higher concentrations gave no increase in inhibition. These results are in contrast to the normal response to competitive inhibitors where increasing the inhibitor concentration gives increasing inhibition, asymptotically approaching complete inhibition. Two possible explanations for the above results suggest themselves: (1) Tryptophan may be taken up by two transport systems, one competitively inhibited by arginine and the other not inhibited.

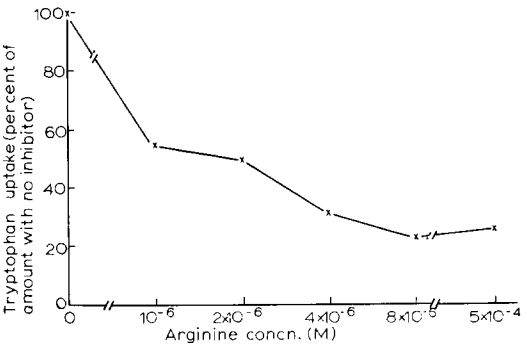


Fig. 1. L-Tryptophan uptake in the presence of various concentrations of L-arginine. 3-day-old mycelial pads of 74A had various amounts of L-arginine added to the medium. They were shaken with 10 $\mu$M tryptophan according to uptake method I. In various experiments of this type, 10 $\mu$M L-arginine appeared to give maximal inhibition of tryptophan uptake. Concentrations as high as 4 mM L-arginine gave little or no increase in inhibition.

Once the transport system which is sensitive to arginine inhibition is completely inhibited, no further inhibition can occur. (2) Alternatively, a single tryptophan transport system might be, in some way, only partially inhibited by arginine. This could occur, for example, through a common step in tryptophan and arginine transport as suggested by KOCH[33]. The inhibition, in this case, would be of some noncompetitive type. It will be shown below that the first of these explanations is consistent with the data. The second is not. Two distinct transport systems are active under the conditions studied.

If two transport systems are active here, then they can be expected to differ, in general, in their affinities for various amino acids. That is, the Michaelis constants ($K_m$) and inhibition constants ($K_i$) for a particular amino acid will probably be different for the two systems. Conversely, the demonstration of two different constants for a given amino acid is evidence for the existence of two transport systems. The following method was used to determine such constants for the proposed two transport systems.

The uptake of a particular amino acid was observed at a given concentration, both with no arginine present and with sufficient arginine to give maximum inhibition. If two transport systems are present, the uptake of the amino acid being observed in the presence of arginine should be due only to the arginine-insensitive transport system. The activity of the arginine-sensitive transport system should be equal to the

uptake in the absence of arginine *minus* the uptake in the presence of arginine. In most cases 1, 2 or 4 mM L-arginine was used to achieve maximum inhibition. By determining these activities at various concentrations of L-tryptophan and analyzing the values on a Lineweaver–Burk plot, the $K_m$'s can be determined for the two transport systems. Similar experiments can be performed for amino acids other than tryptophan which may also be transported by the two transport systems proposed.

Similarly, the inhibition constants or $K_i$'s for various amino acids can be found. The uptake of L-tryptophan can be measured in the presence of an inhibitor, such as leucine, both in the presence and absence of arginine. In this way, the effects of other inhibitors on the two proposed transport systems can be determined. The activities of the two systems at various concentrations of tryptophan can be found in the presence

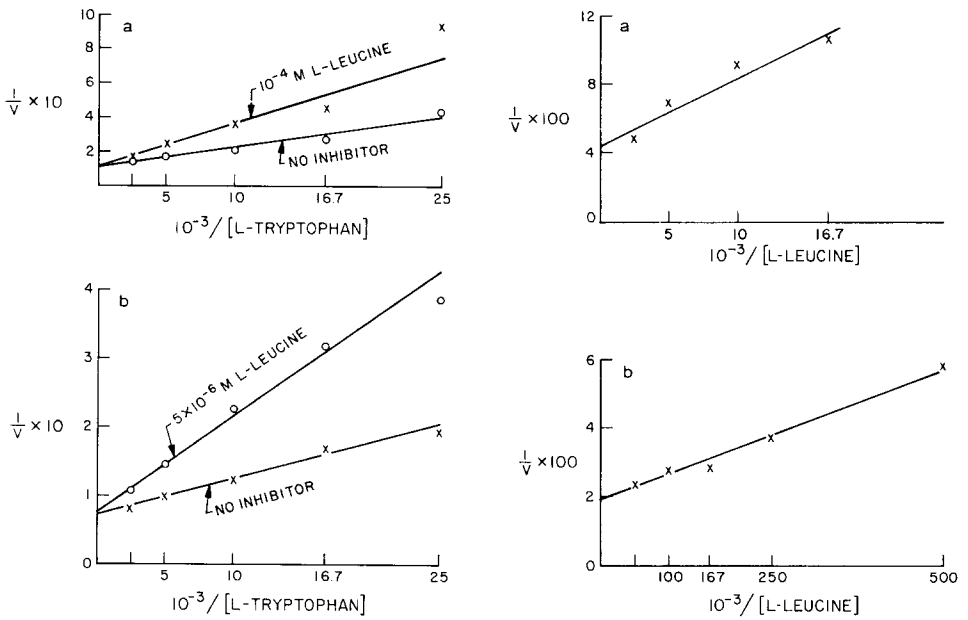


Fig. 2. L-Tryptophan uptake in the presence of L-leucine. The inhibition of the arginine-insensitive and the arginine-sensitive components of L-tryptophan uptake by L-leucine was determined as described in the text. The inhibition of the arginine-insensitive uptake of L-tryptophan by 0.1 mM L-leucine, measured in the presence of 4 mM L-arginine, is shown in (a). It was measured by using uptake method I. (b) shows the inhibition of the arginine-sensitive component of L-tryptophan uptake by L-leucine. The arginine-insensitive component was measured using 2 mM L-arginine. All values plotted in Fig. 2b were determined using uptake method II. As is noted in the text, the arginine-sensitive component of L-tryptophan uptake is substantially inhibited by much lower concentrations of L-leucine than is the arginine-insensitive component. Furthermore, for both components, the inhibition appears to be competitive. For both plots, $v$ is expressed as nmoles of L-tryptophan taken up per min per pad.

Fig. 3. L-Leucine uptake by the arginine-insensitive and arginine-sensitive transport systems (system I and II). The uptake of isotopically labeled L-leucine into 3-day-old pads of strain 74A, shown in (a), was measured in the presence of 4 mM L-arginine by uptake method I. It denotes the activity of the arginine-sensitive transport system for the transport of L-leucine. The arginine-sensitive leucine uptake into 3.5-day-old pads of strain 74A, plotted in (b), was calculated as described in the text from values obtained using uptake method II. Where L-arginine was used in the latter experiment, its concentration in the medium was 2 mM. For both graphs, $v$ is expressed as nmoles of L-leucine take up per min per pad.

or absence of an inhibitor. Analyzing these values on a Lineweaver–Burk plot should yield $K_i$'s for the two uptake systems.

Experiments of the type described above with no inhibitor present were performed to determine the Michaelis constants for L-tryptophan uptake of the two presumed uptake systems. Two typical experiments are shown in Fig. 2. The $K_m$ for the arginine-sensitive transport system is 40–50 $\mu$M. The $K_m$ for the arginine-insensitive transport system is 60 $\mu$M. Due to the inaccuracies inherent in these determinations, the values obtained are not considered to be significantly different from each other.

As is also shown in Fig. 2, L-leucine is a competitive inhibitor of L-tryptophan uptake by both transport systems. The arginine-sensitive transport system, however, requires much less leucine to be effectively inhibited than does the arginine-insensitive system. Typical $K_i$ values for L-leucine found for the two uptake systems are 3 $\mu$M and 110 $\mu$M, respectively. Moreover, as shown in Fig. 3, L-leucine itself shows an arginine-sensitive and arginine-insensitive uptake as does tryptophan. Typical $K_m$ determinations for these are 4 $\mu$M and 120 $\mu$M respectively. These are not considered to be significantly different from the $K_i$ values for leucine given above but are certainly significantly different from each other.

The values determined for leucine show that the binding sites of the arginine-sensitive and arginine-insensitive transport systems differ from each other in their affinity for L-leucine. This finding supports the proposition that two separate and distinct transport systems are active here.

L-Phenylalanine behaves very similarly to L-leucine. Early experiments showed that the arginine-sensitive transport system is effectively inhibited by much lower concentrations of L-phenylalanine than is required to inhibit effectively the arginine-insensitive system. In addition, L-phenylalanine itself demonstrates an arginine-sensitive and arginine-insensitive uptake, the Michaelis constants for which are 2 and 50 $\mu$M, respectively.

The arginine-sensitive and arginine-insensitive transport systems do show different binding properties and, therefore, do appear to be separate and distinct transport systems. Henceforth, the arginine-insensitive amino acid transport system will be called amino acid transport system I. The arginine-sensitive system will be called amino acid transport system II.

The binding constants obtained above are compared in Table I with the binding constants obtained by WILEY AND MATCHETT[13]. There is a striking similarity between the values listed for system I and those reported by WILEY AND MATCHETT. Furthermore, neither system I nor the transport studied by those authors is inhibited by lysine. System II differs from system I both in the binding constant values obtained and in its sensitivity to inhibition by lysine and other basic amino acids. It should be noted that the agreement between the affinity constants determined here for system I and those determined by WILEY AND MATCHETT eliminate the possibility that those determined here are an artifact due to the utilization of arginine in their determination, since those authors did not use arginine but did arrive at much the same values.

Amino acid transport system I, the arginine-insensitive system, would appear to be identical to the transport system previously studied by WILEY AND MATCHETT. Amino acid transport system II, however, is different from any previously described. Its sensitivity to inhibition by basic amino acids suggests that it may be the transport

TABLE I

AFFINITY CONSTANTS

All values reported here were obtained from measurements of amino acid transport into 3-day-old pads of strain 74A. The $K_m$ values were determined by measuring the uptake of isotopically labeled L-tryptophan, L-leucine, or L-phenylalanine in the presence and absence of 4 mM L-arginine. All $K_i$ values were determined by measuring the uptake of isotopically labeled L-tryptophan in the presence and absence of L-leucine or L-phenylalanine. $K_m$ and $K_i$ values for L-leucine and L-phenylalanine for transport system II were obtained using uptake method II. All other values were obtained using uptake method I. The affinity constants were calculated as described in the text.

| *Amino acid* | *Arginine-sensitive transport system (transport system II)* | | *Arginine-insensitive transport system (transport system I)* | | WILEY AND MATCHETT[21] |
|---|---|---|---|---|---|
| | $K_m$ ($\mu M$) | $K_i$ ($\mu M$) | $K_m$ ($mM$) | $K_i$ ($mM$) | $K_m$ or $K_i$ ($mM$) |
| L-Tryptophan | 40–50 | | 0.06 | | 0.05 |
| L-Leucine | 4 | 3 | 0.12 | 0.11 | 0.11 |
| L-Phenylalanine | 2 | 1.5 | 0.05 | | 0.04 |

system with affinity for a wide range of amino acids proposed in the INTRODUCTION. Experiments described below show that this is, indeed, the case.

*D-Phenylalanine uptake*

D-Phenylalanine uptake was previously found to be inhibited by a very large variety of L-amino acids[12]. It was of interest to determine if it was transported by either of the transport systems delineated above. Preliminary experiments showed that L-tryptophan uptake was inhibited by D phenylalanine but that the inhibition did not exhibit strictly competitive kinetics when analyzed on a Lineweaver–Burk plot. This was not surprising, however, in light of the above findings that tryptophan is taken up by two transport systems. If D-phenylalanine is a competitive inhibitor for one but not for the other system, then its effect on total tryptophan uptake would not appear to be competitive.

Additional work showed that D-phenylalanine is a competitive inhibitor for L-tryptophan uptake by transport system II but shows little or no affinity for transport system I. Fig. 4b shows the inhibition of the arginine-sensitive L-tryptophan uptake by D-phenylalanine. The $K_i$ measured here for D-phenylalanine is 20–30 $\mu$M. Similarly, as shown in Fig. 4a, the uptake of D-phenylalanine is competitively inhibited by L-tryptophan. This uptake is completely inhibitable by arginine and shows a $K_m$ of 20–30 $\mu$M. The $K_i$ for L-tryptophan on the uptake of D-phenylalanine is 40–50 $\mu$M.

Thus, the $K_i$ for D-phenylalanine on L-tryptophan uptake by system II is equal to the $K_m$ for D-phenylalanine. Correspondingly, the $K_i$ for L-tryptophan on D-phenylalanine uptake is equal to the $K_m$ for L-tryptophan uptake by system II (40–50 $\mu$M). These equalities and the competitive nature of the inhibitions clearly show that D-phenylalanine is transported by amino acid transport system II.

As noted above, D-phenylalanine uptake has been shown previously to be inhibited by a wide variety of L-amino acids[12]. Since D-phenylalanine is taken up by transport system II, system II may have an affinity for this same wide range of L-amino acids.

*Substrate specificity of amino acid transport system II*

A number of amino acids have been investigated to determine whether they are taken up by transport system II. The transport of all the amino acids listed in Table II is inhibited by arginine. Furthermore, the transport of L-leucine or L-tryptophan by transport system II is inhibited by each of these amino acids. The Michaelis constants listed were based on the assumption that the arginine-sensitive uptake of the individual amino acids was due to transport system II. The $K_i$'s were measured against the uptake of either L-tryptophan, L-leucine, or L-phenylalanine by transport system II. In all cases, the inhibition appeared to be competitive when analyzed on a Lineweaver–Burk plot.

Thus, a very wide range of L-amino acids demonstrates an affinity for transport system II. They include D- as well as L-amino acids, small neutral as well as large neutral amino acids, acidic as well as basic amino acids.

Two amino acids require special comment. Arginine has an extremely low $K_i$, about 0.2 $\mu$M. The low $K_i$, and therefore high affinity, together with the very high transport activity of 3-day-old pads for arginine made an accurate determination of $K_i$ difficult. 4-day-old pads, which have lowered transport activity for arginine as well

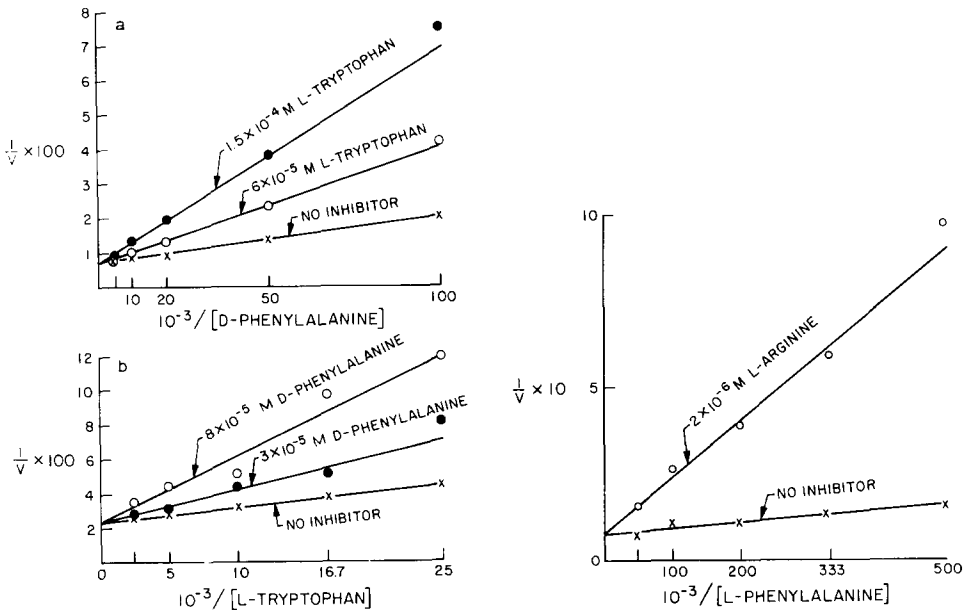


Fig. 4. Uptake of D-phenylalanine by transport system II. Fig. 4a shows the total uptake of isotopically labeled D-phenylalanine and its inhibition by two concentrations of L-tryptophan. The tryptophan inhibition appears to be competitive. Fig. 4b shows the arginine-sensitive component of L-tryptophan transport (by transport system II) and its inhibition by two concentrations of D-phenylalanine. The inhibition appears again to be competitive. Both sets of values were determined using uptake method I as described in the text. The uptake into 3-day-old pads of strain 74A was measured. $v$ is expressed as nmoles of isotopically labeled amino acid (D-phenylalanine and L-tryptophan, respectively) taken up per min per mycelial pad.

Fig. 5. Inhibition of transport system II by L-arginine. The inhibition of the arginine-sensitive uptake of L-phenylalanine into 4-day-old pads of 74A by 2 $\mu$M L-arginine is shown. The inhibition appears to be competitive. $v$ is expressed as nmoles of L-phenylalanine taken up per min per pad.

TABLE II

AMINO ACIDS TRANSPORTED BY TRANSPORT SYSTEM II

All $K_m$'s and all $K_i$'s were determined on mycelial pads of strain 74A. 3-day-old pads were used for studies on all amino acids except arginine, where 4-day-old pads were used. The inhibition constants, or $K_i$'s, were measured against the arginine-sensitive uptake of L-tryptophan, L-phenylalanine, or L-leucine. The Michaelis constants, or $K_m$'s, were determined for the arginine-sensitive uptake of each amino acid. All results were analyzed on Lineweaver–Burk plots.

| *Amino acids* | $K_m$ ($\mu M$) | $K_i$ ($\mu M$) |
|---|---|---|
| L-Tryptophan | 40–50 | |
| L-Leucine | 4 | 3 |
| L-Phenylalanine | 2 | 1.5 |
| D-Phenylalanine | 20–30 | 20–30 |
| L-Asparagine | 8 | 10 |
| Glycine | 5 | 8 |
| L-Arginine | | 0.2 |
| L-Aspartic acid | 1200 | |

as for other amino acids, were used for this determination. The inhibition of L-phenylalanine uptake by arginine is shown in Fig. 5. The very high $K_m$, and therefore low affinity of aspartic acid for system II, presents another problem. Small contaminating amounts of other amino acids with higher affinities in either the labeled or unlabeled aspartic acid could produce considerable uptake or cause inhibition of the uptake of other amino acids which was not due to the aspartic acid itself. The uptake of 0.3 mM aspartic acid into 3-day-old pads was inhibited about 75% by 1 or 4 mM L-arginine. Even when over 50% of the aspartic acid was taken up into mycelial pads in the absence of arginine, the uptake into similar pads in the presence of arginine was about 75% less. Thus, the arginine-inhibitable uptake is the uptake of the aspartic acid itself rather than of some minor impurity in the labeled aspartic acid. It is assumed that the arginine-sensitive transport was due to the activity of amino acid transport system II. It is not clear whether the high $K_m$ for transport of aspartic acid apparently demonstrated by transport system II is due to a low affinity for the negatively charged compound or due to a relatively high affinity for the small amount of uncharged aspartic acid present at the pH 6.0 of the medium. Studies at other pH's will be necessary to resolve this problem.

Further experiments were performed to determine if transport system II is inhibitable by compounds other than those tested above. The uptake of 1 $\mu$M L-phenylalanine was observed in the presence or absence of several potential inhibitors. Since the $K_m$ of L-phenylalanine for transport system II is much lower than for transport system I, the uptake of 1 $\mu$M L-phenylalanine into 3-day-old pads of 74A is primarily due to the activity of transport system II. The inhibition of this uptake by 0.1 mM of various compounds is shown in Table III.

All $\alpha$-amino acids tried were effective in inhibiting uptake. This further confirms the wide range of substrates which apparently have a good affinity for transport system II. However, none of the nonamino acids tried were effective inhibitors. Inhibition of less than 20% is not considered significantly different from 0%. Thus, in order to retain affinity, the amino group of an amino acid cannot be replaced by a hydrogen, hydroxyl group or keto group, nor can it be acetylated. Similarly, the

TABLE III

INHIBITION OF UPTAKE OF 1 $\mu$M L-PHENYLALANINE BY VARIOUS COMPOUNDS

All potential inhibitors were added to a concentration in the medium of 0.1 mM. 3-day-old pads of 74A were used to measure the uptake of 1 $\mu$M L-phenylalanine by uptake method II.

| *Compound* | *Inhibition of uptake* (%) |
|---|---|
| None | (0) |
| L-Alanine | 94 |
| $\beta$-Alanine | 80.3 |
| Lactic acid | 0 |
| Pyruvic acid | 0 |
| Propionic acid | 0 |
| $\alpha$-Amino-*n*-butyric acid | 93.6 |
| $\gamma$-Amino-*n*-butyric acid | 25.0 |
| L-Histidine | 91.8 |
| Histamine | 12.3 |
| L-Leucine | 91.9 |
| *N*-Acetyl-L-leucine | 2.3 |
| Leucinamide | 11.2 |
| L-Leucylglycine | 6.0 |
| L-Serine | 91.5 |
| 2-Aminoethanol | 0 |

carboxyl group cannot be replaced by a hydrogen or amide, nor can it be involved in a peptide bond. However, $\beta$-amino acids and probably also $\gamma$-amino acids have some affinity for transport system II.

*Control of transport system II*

Transport system II is responsible for about 75% of the tryptophan uptake in 3-day-old pads. Only the remaining 25% of the uptake is due to transport system I. However, according to WILEY AND MATCHETT[13], most, if not all, of the uptake of L-tryptophan in germinated conidia is due to amino acid transport system I. Therefore, the relative activities of the two transport systems must be in some way dependent on the physiological state of the cultures.

The relative amounts of tryptophan uptake by the two transport systems in cultures of different ages were determined by measuring the fraction of the uptake of L-tryptophan that was inhibited by arginine. The cultures of various ages were prepared as were the 3-day-old cultures except that 2-day-old cultures were not shaken before uptake, while the 4-day-old cultures were shaken for two days preceding uptake.

As shown in Fig. 6, the relative activity of transport system II increases strikingly in older cultures. The uptake of most neutral amino acids in young cultures is primarily due to transport system I, but in older cultures, system II predominates.

Older cultures under these conditions have ceased growth due to the depletion of the carbon source, sucrose, from the medium. Two to three times heavier cultures can be obtained by simply using more sucrose in the initial medium. It would appear possible, then, that the limitation of growth by carbon starvation in some way induces amino acid transport system II. It is not clear whether growth limitation due to other causes might also be effective in inducing this transport system.

*Deficiency of the mtr mutant strain*

The mtr strain, as noted above, was isolated due to its resistance to 4-methyltryptophan. It is deficient in the transport of L-tryptophan, L-leucine, and several other neutral amino acids in germinated conidia[15] and young mycelia[14]. Exploratory experiments showed that in 3-day-old cultures, however, the transport of tryptophan and leucine approached that in wild-type cultures. Subsequent experiments were performed to determine which of the two transport systems was deficient in the mutant strain.

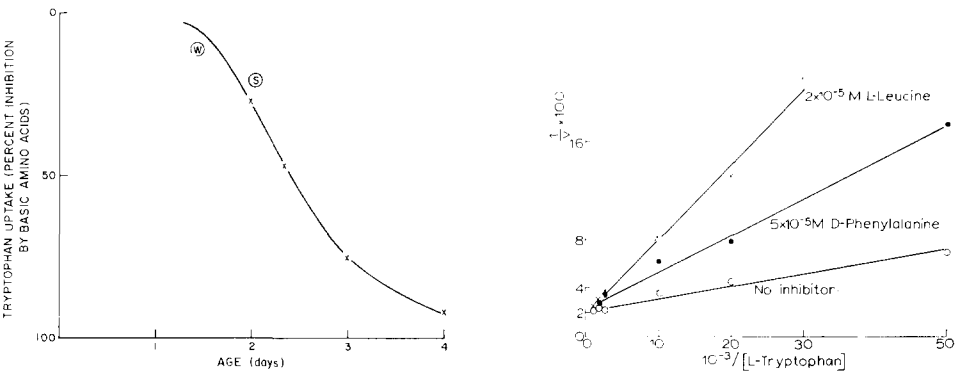


Fig. 6. Relative activities of transport system I and transport system II in Neurospora cultures of various ages. Mycelial pads of various ages of wild-type strain 74A were prepared as described in the text. The uptake of 10 $\mu$M L-tryptophan into these pads was measured in the presence and absence of 10 mM L-arginine. The inhibition of tryptophan uptake by arginine is plotted. The inhibition of tryptophan uptake by arginine reported by STADLER[14] is plotted in the position of the 'S'. The inhibition of tryptophan uptake by lysine reported by WILEY AND MATCHETT[13] is plotted in the position of the 'W'. Although the growth conditions used by those investigators differ from the procedures used in this thesis, their results appear to agree well with those reported here. The transport activity not inhibited by basic amino acids is assumed to be due to transport system I. The basic amino-acid-inhibitable transport is assumed to be due to transport system II. These assumptions have been verified, as reported above, in 3-day-old pads of 74A. The first assumption has been verified by WILEY AND MATCHETT in germinated conidia.

Fig. 7. L-Tryptophan uptake into mycelial pads of the mtr mutant. The total uptake of isotopically labeled L-tryptophan into 3-day-old pads of mtr 6, ylo was measured using uptake method II. Where noted, D-phenylalanine (50 $\mu$M) or L-leucine (20 $\mu$M) was used as inhibitor. $v$ is expressed as nmoles of L-tryptophan taken up per min per mycelial pad. As is noted in the text, the characteristics of the uptake follow those found for transport system II in the wild-type strain 74A.

In 3-day-old pads of mtr, over 99% of the uptake of 0.1 mM L-tryptophan and over 98% of the uptake of 0.4 mM L-leucine are inhibited by 4 mM L-arginine. This compares with about a 75% inhibition found for wild-type 74A. Moreover, even in 2-day-old cultures where wild type shows only 30% inhibition, over 99% of the uptake of L-tryptophan is inhibited by arginine in the mtr mutant. Thus, transport system I, the arginine-insensitive system, is specifically missing in the mtr mutant strain, but transport system II is present in normal amounts. The mtr mutant, then, behaves as a permeaseless mutant in that it is deficient in the activity of a specific transport system.

The deficiency of the mtr mutant for transport system I together with its normal activity for transport system II permits the study of the characteristics of transport system II by simpler procedures than can be used with the wild type. This is possible

since all of the uptake of L-tryptophan and L-leucine into mycelial pads of the mtr strain under the conditions used appears to be due to the activity of transport system II. Thus, inhibition by L-argin ne need not be used to distinguish between activities of systems I and II. The total uptake of L-tryptophan into pads of the mtr strain is, for example, shown in Fig. 7. The $K_m$ for L-tryptophan uptake (53 $\mu$M) and the $K_i$'s for D-phenylalanine (26 $\mu$M) and L-leucine (4.2 $\mu$M) derived from Fig. 7 are in good agreement with the affinity constants determined above for those amino acids for transport system II in wild type. This agreement confirms the characteristics of amino acid transport system II derived from wild type.

## DISCUSSION AND CONCLUSIONS

Considerable progress has been made toward the understanding of two amino acid transport systems in Neurospora. Amino acid transport system I is equivalent to that previously studied by WILEY AND MATCHETT[13]. Those authors have determined that transport system I will transport a wide range of neutral L-$\alpha$-amino acids.

Transport system I is missing in cultures of the mtr mutant, although transport system II is present at normal levels. The specificity of the defect in mtr mutant, together with the fact that only one locus has been implicated in this transport deficiency[14], makes it quite likely that the mtr locus is the structural gene for the specific transport protein, or permease, of transport system I.

Amino acid transport system II has high activity in old, carbon-starved cultures, but unlike system I, has little or no activity in young, rapidly growing cultures. It has a very broad range of substrates, transporting amino acids with hydrophobic and hydrophilic side chains, both D- and L-amino acids, and basic as well as neutral amino acids. It demonstrates an affinity for $\beta$- as well as $\alpha$-amino acids.

Previous work showing that a very broad range of amino acids inhibits the transport of L-phenylalanine[10] and L-arginine[11] into ungerminated conidia suggests that transport system II is present in conidia as well as in old mycelial cultures.

The Michaelis constants for system II of most neutral and basic amino acids investigated are quite low, the majority being between 10 and 0.1 $\mu$M. The low $K_m$'s should make this system quite efficient in removing traces of amino acids from the medium. In carbon-starved cultures, then, transport system II may serve as a scavenger, taking up any remaining exogenous amino acids.

The technique used here to separate the activities of the two transport systems studied here is derived from the suggestions of INUI AND CHRISTENSEN[34]. It may well be applicable in other systems. Any substrate which will competitively inhibit one, but not the other, of two simultaneously active transport systems can be utilized to separate their activities.

There are two previous reports of amino acid transport systems in fungi, which are similar to transport system II in having a very broad range of substrates. SURDIN *et al.*[20] have reported that most amino acids in yeast are taken up by a single transport system. Other authors[21] have disputed the conclusion of SURDIN *et al.* and have reported a number of relatively specific transport systems in yeast[21–23]. Perhaps an amino acid transport system similar to transport system II described above, which is active in yeast under some physiological conditions but not others, would aid in integrating the interpretations of the two groups.

Recently, a general amino acid transport system with affinity for a broad range of amino acids has been reported in nitrogen-starved cultures of *Penicillium chrysogenum*[24]. The dependence of the activity of this system on starvation conditions may be similar to that reported above for transport system II in Neurospora.

A number of questions about amino acid transport in Neurospora remain unresolved. There are almost certainly other transport systems than the two delimited above. Growth antagonisms of various amino acid-requiring auxotrophs of Neurospora probably occur through competition for uptake of the different exogenous amino acids added to the medium. Such antagonisms provide clues as to what transport systems remain to be studied. Arginine-requiring mutants are inhibited by lysine[25], and lysine-requiring mutants are inhibited by arginine[26]. These growth antagonisms imply the existence of a specific transport system for basic amino acids. It is proposed that this basic amino acid transport system be called transport system III. Histidine-requiring mutants are inhibited by any of a large number of neutral amino acids *plus* either arginine or lysine[27]. Thus, in young cultures, histidine is probably transported by two amino acid transport systems. These two may be transport system I and the transport system for basic amino acids postulated above. Some studies on these two transport systems are reported in a recent abstract[28].

Only a portion of the transport of L-aspartic acid, as reported above, is inhibited by L-arginine. Since aspartic acid has no affinity for transport system I (ref. 13), the residual uptake must be due to some as yet unexplored transport system. Thus, at least four amino acid transport systems are implicated in Neurospora. No evidence for additional transport systems has appeared in results obtained by the author. However, most experiments were performed at relatively low amino acid concentrations; consequently, systems having high Michaelis constants would probably not have been detected. In addition, a number of amino acids have not been studied. Furthermore, some transport systems may only be present under physiological conditions other than those studied.

Even more attention will be required to obtain an understanding of the integration of transport with the other activities of the mycelia or conidia. Clearly, the synthesis or activity of system II is under some kind of physiological control. The control of various transport systems is demonstrated through changes in several mutant strains[7,26,29,30]. Certainly, a number of useful physiological and genetic studies of the control of amino acid transport can be pursued on such mutants.

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REFERENCES

1 G. N. COHEN AND J. MONOD, *Bacteriol. Rev.*, 21 (1957) 169.
2 J. H. SCHWARTZ, W. K. MAAS AND E. J. SIMON, *Biochim. Biophys. Acta*, 32 (1959) 582.
3 M. LUBIN, *Biochim. Biophys. Acta*, 42 (1960) 535.
4 H. R. KABACK AND E. R. STADTMAN, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 920.
5 P. ST. LAWRENCE, *Genetics*, 50 (1964) 1383.
6 M. S. KAPPY AND R. L. METZENBERG, *Biochim. Biophys. Acta*, 107 (1965) 425.

7 R. H. DAVIS AND J. D. ZIMMERMAN, *Genetics*, 52 (1965) 439.
8 D. L. OXENDER AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 238 (1963) 3686.
9 A. L. KOCH, *Biochim. Biophys. Acta*, 79 (1964) 177.
10 B. G. DEBUSK AND A. G. DEBUSK, *Biochim. Biophys. Acta*, 104 (1965) 139.
11 W. B. ROESS AND A. G. DEBUSK, *Neurospora Newsletter*, 8 (1965) 8.
12 M. L. PALL, Ph. D. Thesis, Part I, California Institute of Technology, 1968, p. 23.
13 W. R. WILEY AND W. H. MATCHETT, *J. Bacteriol.*, 92 (1966) 1698.
14 D. R. STADLER, *Genetics*, 54 (1966) 677.
15 G. LESTER, *J. Bacteriol.*, 91 (1966) 677.
16 R. W. BARRATT, *Neurospora Newsletter*, 2 (1962) 24.
17 E. OHNISHI, H. MACLEOD AND N. H. HOROWITZ, *J. Biol. Chem.*, 237 (1962) 138.
18 N. H. HOROWITZ, *J. Biol. Chem.*, 171 (1947) 255.
19 H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 42.
20 Y. SURDIN, W. SLY, J. SIRE, M. BORDES AND H. DE ROBICHON-SZULMAJSTER, *Biochim. Biophys. Acta*, 107 (1965) 546.
21 M. GRENSON, M. MOUSSET, J. M. WIAME AND J. BECHET, *Biochim. Biophys. Acta*, 127 (1966) 325.
22 M. GRENSON, *Biochim. Biophys. Acta*, 127 (1966) 339.
23 J. J. GITS AND M. GRENSON, *Biochim. Biophys. Acta*, 135 (1967) 507.
24 P. V. BENKO, T. C. WOOD AND I. W. SEGAL, *Arch. Biochem. Biophys.*, 122 (1967) 783.
25 A. H. DOERMANN, *Arch. Biochem. Biophys.*, 5 (1955) 373.
26 A. M. SRB, *Genetics*, 38 (1953) 694.
27 F. HAAS, M. B. MITCHELL, B. N. AMES AND H. K. MITCHELL, *Genetics*, 37 (1952) 217.
28 C. K. WOODWARD, C. P. READ AND V. W. WOODWARD, *Genetics*, 56 (1967) 598.
29 R. H. DAVIS, *Neurospora Newsletter*, 4 (1963) 5.
30 J. A. KINSEY, *Genetics*, 56 (1967) 570.
31 D. O. WOODWARD AND K. D. MUNKRES, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 872.
32 K. D. MUNKRES AND D. O. WOODWARD, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 1217.
33 A. L. KOCH, *J. Theoret. Biol.*, 10 (1966) 301.
34 Y. INUI AND H. N. CHRISTENSEN, *J. Gen. Physiol.*, 50 (1966) 203.