BBA 76126

THE KINETICS OF L-ASPARTATE TRANSPORT IN *NEUROSPORA CRASSA* CONIDIA

L WOLFINBARGER, JR* AND A GIB DEBUSK**

Genetics Group, Department of Biological Sciences, Florida State University, Tallahassee, Fla 32306 (U S A)

(Received June 26th, 1972)

SUMMARY

Transport of the acidic amino acids by *Neurospora crassa* conidia is shown to be mediated by neutral and general amino acid transport systems. The neutral transport system is shown to possess "high affinity", but low transport capacity for the acidic amino acids. The general transport system is shown to have "low affinity" and high transport capacity for these amino acids. The non-linearity of a Lineweaver–Burk plot of aspartate transport by the wild type has been shown to result from an overlapping of two transport systems for accumulation of this amino acid. Removal, by mutation, of the neutral transport system restores the transport kinetics to a linear function.

The combined, and separate, transport activities are shown to be stereospecific, preferring the L-stereoisomeric form over the D-form, and to transport the neutrally charged species of L-aspartate and L-glutamate. The transport activity is dependent on pH, and the V, but not the $C_{\frac{1}{2}}$, constant of the general transport system is pH dependent.

Two models for amino acid transport are presented as possible mechanisms to explain the observed kinetics. One model involves three genetically distinct transport systems. The second model utilizes a single "allosteric permease" to account for the kinetic data.

INTRODUCTION

Initial studies of acidic amino acid transport in the fungi suggested that transport activity might be limited to single saturatable systems showing classical Michaelis–Menten kinetics[1–3]. The acidic amino acid transport systems described in *Neurospora crassa* mycelia[2] and *Penicillium chrysogenum* mycelia[3] appear to be similar in character. Both systems are active at pH values of 6.0 ± 0.5, appear to be specific for acidic amino acids, and develop following carbon or nitrogen starvation Because of the pH utilized it might be suggested[3] that the anionic species of aspartate and glutamate are the preferred substrates of these systems.

* Present address Departments of Biochemistry and Surgery, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

** Author to whom reprint requests should be addressed

The demonstration that the neutral and general amino acid transport systems described in *N. crassa* conidia[4] were also capable of transporting L-aspartate[5] brought the number of "acidic amino acid transport systems" in this fungus to three. Although transport of L-aspartate by the latter two systems was not extensively (kinetically) characterized, it was clearly shown that the combined transport activity was dependent on pH and resulted in the accumulation of L-aspartate against a gradient.

Because extensive kinetic analyses of aspartate and/or glutamate transport in bacterial systems had revealed the presence of two kinetically (and genetically) separable transport systems for acidic amino acids[6-8], it became of interest to determine whether or not the two (neutral and general) amino acid transport systems found in *N crassa* conidia[5] were analogous to this transport activity.

Data presented in this manuscript demonstrate that the neutrally charged species of L-aspartate and L-glutamate are the substrate species of two transport components. One component (the previously characterized neutral amino acid transport system) will be shown to correspond to a "high affinity" low transport activity system while the second component (the general amino acid transport system) corresponds to a "low affinity" high transport activity system. The presence of both systems in the wild type provides for non-linear kinetics for L-aspartate transport. Removal of the "high affinity" system by mutation results in linear aspartate transport kinetics characteristic of the "low affinity" system.

METHODS AND MATERIALS

Strains utilized

The transport kinetics of the wild type (Tatum a, $SY4f_8a$) and mutants (Pm^-N^{22}, Pm^-B^{37}, and Pm^-NB) employed in this study have been previously described[4,5]. The Pm^-N^{22} and Pm^-NB, but not the Pm^-B^{37}, mutants are reduced in transport, *e.g.* 60% reduced at pH 4.0, of L-aspartate at pH values between 2.4 and 5.8 (ref. 5). The *PmN* locus is presumed to be involved in some functional aspect of the neutral amino acid transport system such that a mutational alteration results in impaired transport activity for the neutral amino acids[4]. The presence of an altered *PmB* locus appears not to affect neutral amino acid or acidic amino acid (at lower pH values) transport[5].

Maintenance of stocks has been previously described[4].

Preparation of cells for transport studies

Conidial suspensions for transport studies were obtained from 7-day-old vegetative cultures grown at 25 °C in the light on Vogel's[9] minimal media N *plus* 2% sucrose and 2% agar. The conidia were harvested with a sterile wire needle into sterile glass distilled water (0 °C) and filtered through glass wool to remove mycelial fragments. The resultant conidial suspensions could be stored in an ice water bath for up to 3 days without observing any adverse effects on transport activity.

Conidial dry weights for the suspensions were obtained by pipetting 5-ml aliquots onto tared Millipore filters and drying overnight at 45 °C.

Measurement of amino acid transport activity

The basic composition of the incubation mixture and techniques used in the transport studies has been described[4].

Most determinations of amino acid transport velocities, unless indicated otherwise, consisted of points taken after 0.5, 4, 8, and 16 min of transport. The slope of the best fit straight line for these four points multiplied by a conversion factor gave the transport velocities reported. In those few instances where the best fit straight line for the four points was not obvious the slope was obtained by using a Decus computer program (No. FOCAL 8-63) titled CURFT. The computer program was written by D. L. Shirer at Valparaiso University and is designed to fit weighted or unweighted data to a straight line on a Cartesian, log–log or semilog plot.

The various pH values utilized in this study were obtained by substituting citric acid for sodium citrate or K_2HPO_4 for KH_2PO_4 in the basic Vogel's salts buffer and adjusting to the desired pH with HCl or KOH.

Chemicals

All radioactive amino acids utilized were uniformly labeled with ^{14}C and were of the L-isomer form. They were obtained from Schwarz (New York).

The nonradioactive amino acids utilized as "carrier" for the labeled amino acids, or for competition studies, were also of the L-isomer (except where use of the D-form is indicated) form and were obtained from Calbiochem (Calif.).

RESULTS

Earlier studies[5] had shown that (1) a neutral amino acid transport deficient mutant (*Pm*$^-$*N*^{22}) was reduced in transport of L-aspartate and (2) transport of L-aspartate was optimal in wild type and transport deficient mutants (*Pm*$^-$*N*^{22}, *Pm*$^-$*B*^{37}, and *Pm*$^-$*NB*) at pH values near 3.5. These two observations suggested that the neutrally charged (Zwitterion) species of L-aspartate, as opposed to the anionic species, might be the substrate molecule of the neutral and general amino acid transport systems.

Since these two systems had initially been characterized as "specific" for neutral amino acids it seemed reasonable to utilize L-phenylalanine transport as a measure of transport capacity. Referral to Fig. 1 shows that from pH 2.4 to pH 5.0 L-phenylalanine transport activity increases. L-Aspartate transport, however, is comparable to L-phenylalanine transport only from pH 2.4 to pH 3.5. Above pH 3.5 the ability of wild type cells to accumulate L-aspartate rapidly decreases. Since the p*K* of the γ-carboxyl group of L-aspartate is approximately 3.8, the molar concentration of the neutral species of 0.1 mM aspartate will be 0.08 mM at pH 2.8, 0.05 mM at pH 3.8 and 0.001 mM at pH 4.8 (ref. 10). Thus, if as suggested, the neutral species of L-aspartate is the substrate of the neutral and general transport systems, it would be expected that the velocity of L-aspartate transport (at a constant 10^{-4} molar concentration of L-aspartate) would decrease as the pH of the surrounding media is increased. That the transport velocity for aspartate increases between pH 2.8 and 3.5 (Fig. 1) may be explained by the observation that the transport activity (as measured with L-phenylalanine) is continually increasing. Thus, even though the molar concentration of the neutral species of L-aspartate is decreasing over this pH range, the continually increasing transport activity may compensate such that over this narrow pH range the transport velocities for L-aspartate increase.

The reduction of acidic amino acid transport activity at pH values greater and lesser than pH 3.8 suggests that two parameters may be operating to yield the apparent optimal pH for transport. Measured as a function of increasing pH (decreasing H^+ concentration), the positive slope of the pH optimum curves (Fig. 1),

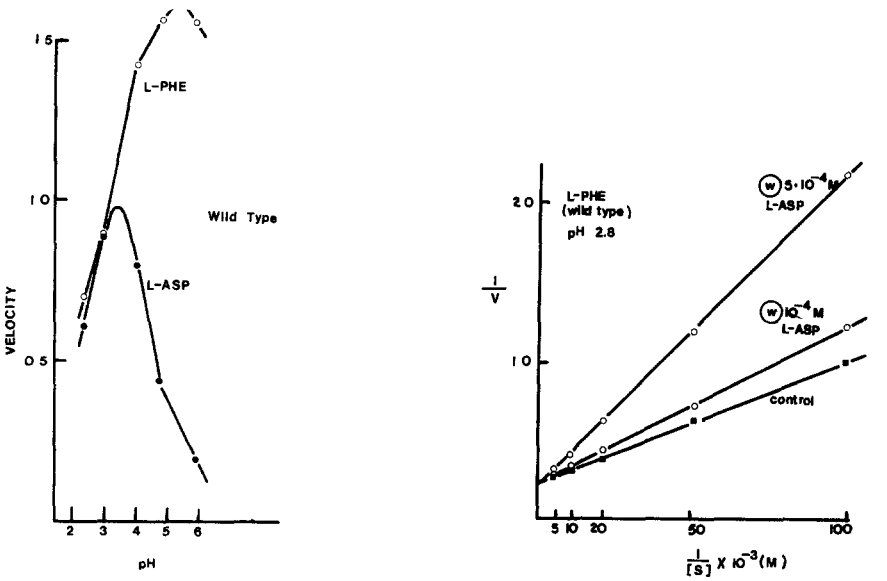

Fig 1 Velocity (nmoles/min per mg dry wt conidia) of L-phenylalanine (0 1 mM) and L-aspartate (0.1 mM) transport by the wild type as a function of pH

Fig 2 Determination of inhibition kinetics of L-aspartate for L-phenylalanine transport by the wild type at pH 2 8. The straight line designated "control" is L-phenylalanine transport without the presence of L-aspartate The velocity (v) is nmoles/min per mg dry wt conidia and the substrate concentration ($[S]$) is molar

indicates some pH sensitive component which provides for increasing transport activity while the negative slopes of the pH optimum curves indicate some pH sensitive component providing for decreasing transport activity, *e.g.* decreasing concentrations of transportable substrate molecules in the case of L-aspartate. A possible explanation for one, or both, slopes might be an irreversible inactivation of transport activity by a given range of H^+ concentration. The site of the postulated inactivation might occur at the level of the membrane, energy coupling, amino acid permease, *etc.*

This hypothesis may be tested experimentally by incubating conidia at a variety of H^+ concentrations for 16 min, filtering, washing, and resuspending at a common pH in the presence of ^{14}C-labeled L-amino acid L-aspartate). The data presented in Table I suggests that the L-aspartate transport activity in the wild type (the neutral and general amino acid transport systems) is not irreversibly inactivated by those H^+ concentrations between pH 2.8–5.8.

Figs 2 and 3 present further proof that the neutral, but not the anionic species of L-aspartate is the substrate molecule of the neutral and general transport systems. At pH 2.8, 0.1 and 0.5 mM L-aspartate competitively inhibit transport of L-phenylalanine by the wild type (Fig. 2). At this pH the neutral species of 0.1 mM and 0.5 mM

L-aspartate is approximately 0.08 and 0.4 mM, respectively[10]. However, at pH 4.8 identical concentrations (0.1 and 0.5 mM) of L-aspartate do not competively inhibit L-phenylanine transport by the wild type (Fig. 3). At pH 4.8 the molar concentration of the neutral species of 0.1 and 0.5 mM L-aspartate will approx. 0.01 and 0.05 mM, respectively[10]. It is suggested that the absence of competitive inhibition of L-phenylalanine transport at pH 4.8 by L-aspartate is the result of the low molar concentrations of the neutral species of L-aspartate at this pH. Other explanations have not been excluded, however. Thus, the anionic species of L-aspartate does not appear to compete with L-phenylalanine for transport at pH 4.8 by the wild type.

By increasing the molar concentration of L-aspartate as the pH of the surrounding media is increased, it is possible to obtain a constant molar concentration of the neutrally charged species at any pH value. Thus, a 1.0 mM concentration of L-aspartate at pH 4.8, a 0.2 mM concentration at pH 3.8, and a 0.1 mM concentration at pH 2.8 will all approximate a 0.1 mM concentration of the neutral species[10].

The data presented in Fig. 4 are the result of transport experiments at various

TABLE I

REVERSIBLE NATURE OF H^+ CONCENTRATION ON THE L-ASPARTATE TRANSPORT ACTIVITY IN THE WILD TYPE

	pH			
	2.8	*3.8*	*4.8*	*5.8*
L-Aspartate transport velocity*	0.79	0.94	0.72	0.22
L-Aspartate transport velocity* at pH 3.8**	1.03	1.00	1.12	1.10

* Transport velocity as nmoles/min per mg dry wt conidia. Specific activity of amino acid at 0.1 Ci/1.0 mole and molar concentration of 0.1 mM.

** Transport velocities measured at pH 3.8 following a 16-min preincubation at the indicated pH. See text for details.

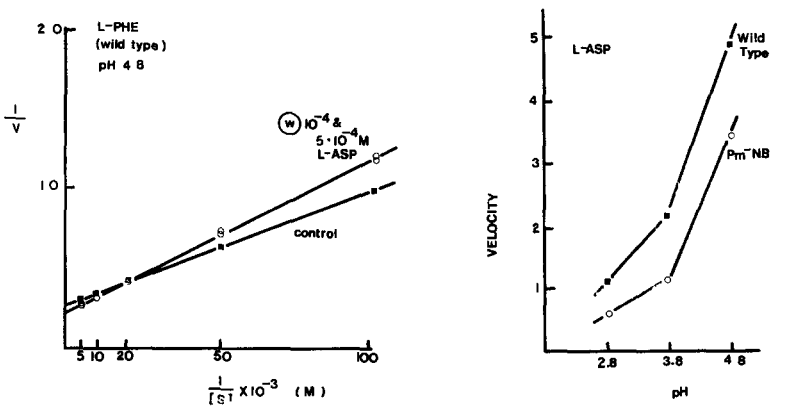

Fig. 3. Determination of inhibition kinetics of L-aspartate for L-phenylalanine transport by the wild type at pH 4.8. The straight line designated "control" is L-phenylalanine transport without the presence of L-aspartate, the velocity (v) is nmoles/min per mg dry wt conidia and the substrate concentration ($[S]$) is molar.

Fig. 4. Velocity (nmoles/min per mg dry wt conidia) of L-aspartate transport by the wild type and *Pm⁻NB* at a constant molar concentration (0.1 mM) of the neutral species of L-aspartate.

pH values and a constant 0.1 mM concentration of the neutrally charged species of L-aspartate. Transport activity for L-aspartate may be seen to increase from pH 2 8 to pH 4.8 in both the wild type and *Pm⁻NB*. This observation is contrary to that observed at a constant 0.1 mM concentration of L-aspartate at similar pH values (Fig. 1). The difference in the two observations may be readily explained if, as suggested, the neutral species of L-aspartate is the substrate of the observed transport activity. By maintaining a constant molar concentration of the neutral species of L-aspartate, the increasing transport velocities seen in Fig. 4 correspond to the increasing transport activity, with increasing pH, observed for L-phenylalanine transport in Fig. 1. If the increasing transport activity in Fig. 4 were due to transport of the anionic species it would be expected that transport of L-aspartate, with increasing pH, shown in Fig. 1 should not decrease at those pH values above 3.5.

The described dependence of acidic amino acid transport activity on pH has been attributed primarily to the neutral and general amino acid transport systems. These two systems have been reported[4, 13–15] to be specific (at pH 5 8) for the L-stereoisomeric form of the neutral and basic amino acids, respectively. The general transport system has also been reported to transport (at pH 5.8) the L- and D-forms of the acidic amino acids[14].

Table II shows that conidial transport of L-aspartate and L-glutamate (at pH 2.8) by the wild type and *Pm⁻NB* mutant is not subject to reduction by 10 × concentrations of D-aspartate or D-glutamate. The nature of this data is such that transport of the D-acidic amino acids by the neutral and general systems can not be ruled out. However, the data indicate a degree of stereospecificity for the L-forms over the D-forms by these two systems. Furthermore, preference for the L-forms must be at least 2 orders of magnitude greater than for the D-forms. A preference of lesser value would allow for a measurable reduction in transport of the L-acidic by the D-acidic amino acids.

TABLE II

DETERMINATION OF THE STEREOSPECIFICITY OF WILD TYPE AND *Pm⁻NB* TRANSPORT ACTIVITY FOR THE ACIDIC AMINO ACIDS AT pH 2 8

Unlabeled amino acid added (1 0 mM)	*Wild type*	*Pm⁻NB*
	L-[^{14}C]*Aspartate transport** (*0 1 mM*)	
None	1 30	0 52
D-Aspartate	1.27	0 55
D-Glutamate	1 33	0 59
L-Glutamate	0 65	0 50
	L-[^{14}C]*Glutamate transport** (*0 1 mM*)	
None	0 94	0 21
D-Aspartate	0 81	0 21
D-Glutamate	0 85	0 20
L-Aspartate	0 33	0 04

* Values represent velocity (nmoles/min per mg dry wt conidia) of labeled amino acid transport. The velocity is obtained from short-term ($\leq$ 16 min) uptakes

A 1.0 mM (10 ×) concentration of L-glutamate reduces transport of L-aspartate (0.1 mM) in the wild type by 50 %. This reduction in transport has been shown to be competitive in nature[10]. However, a similar concentration of L-glutamate does not reduce transport of L-aspartate by the general transport system (*Pm⁻NB*). A 10 × concentration of L-aspartate reduces transport of L-glutamate by the wild type (60 % reduced) or *Pm⁻NB* (80 % reduced). Thus, the apparent "affinities" at pH 2.8 of the neutral transport system for L-aspartate and L-glutamate may be more nearly equal than those of the general transport system for these two amino acids.

The numerous kinetic analyses of acidic amino acid transport in bacteria[6-8,12] have each reported non-linear kinetics by their respective transport activities. In *Streptomyces hydrogenans*[8], *Streptococcus faecalis*[6], and *E. coli*[7] the non-linear kinetics have been attributed to the presence of two kinetically separable transport systems. In each instance one transport system was described as having a "high affinity" for L-aspartate or L-glutamate while the second system as having a "low affinity" for these amino acids.

With the demonstration that *N. crassa* conidia had two genetically separable systems for transport of the acidic amino acids, it became of interest to determine if aspartate or glutamate transport by these two systems might result in non-linear kinetics. Further, because the observed transport activity appeared to be specific for the neutral species of L-aspartate it was decided to study (initially) transport kinetics at pH 2.8. Fig. 5 reveals that L-aspartate transport velocities by the wild type (between 0.1 and 5.0 mM concentrations of L-aspartate) do indeed yield non-linear kinetics when plotted by the method of Lineweaver and Burk[16]. If this system is analogous to the described bacterial systems, the presence of the altered *PmN* locus (in *Pm⁻NB*) should restore the "Lineweaver–Burk" plot of the transport activity in the mutant to a straight line. Referral again to Fig. 5 hows that L-aspartate transport by the *Pm⁻NB*

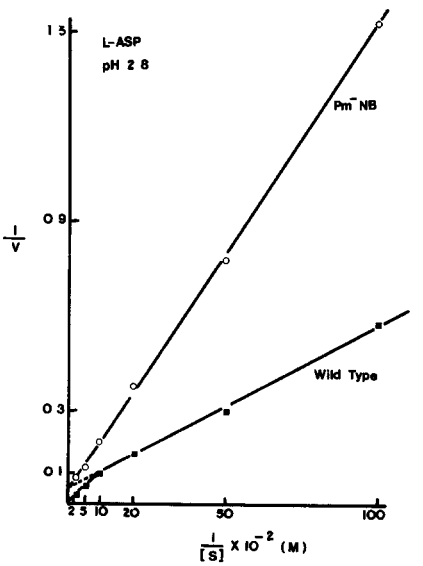

Fig. 5 Kinetics of L-aspartate transport at pH 2 8 by wild type and *Pm⁻NB* The velocity (v) is nmoles/min per mg dry wt conidia and the substrate concentration ([S]) is molar.

mutant is indeed linear, as predicted. Since it was previously shown[5] that the presence of the altered *PmB* locus did affect L-aspartate transport at these pH values it is assumed that only the altered *PmN* locus in *Pm⁻NB* is providing for the observed linear kinetics

The analysis at pH 2.8 prompted examination of the kinetics of L-aspartate transport by the wild type and *Pm⁻NB* mutant as a function of pH The kinetic constants, $C_{1/2}$ and V, of L-aspartate (and L-phenylalanine) transport are presented in Tables III and IV. (The values for L-aspartate presented in Tables III and IV were obtained by utilizing concentrations of L-aspartate less than 1.0 mM, *i.e.* where linear transport kinetics are obtained). The relationships of the $C_{1/2}$ values for L-aspartate transport by wild type and *Pm⁻NB* remain relatively constant as the pH varies, *i.e.* it requires approximately a 10-fold higher concentration of L-aspartate to 1/2 saturate the transport activity in the *Pm⁻NB* mutant than it does to 1/2 saturate the wild type transport activity at pH values 2.8, 3.8, or 4.8 (Table III) This observation suggests that non-linear kinetics for wild type L-aspartate transport might also be obtained at pH 3.8 and 4.8.

The observed transport activity in the wild type represents the combined transport activities of the neutral and general transport systems. By subtracting the *Pm⁻NB* transport velocities from the wild type transport velocities the kinetic constants for the neutral transport system may be obtained. These calculations have

TABLE III

$C_{1/2}$ CONSTANTS* FOR L-ASPARTATE AND L-PHENYLALANINE TRANSPORT AS A FUNCTION OF pH

Amino acid	*pH*			
	2.8	*3.8*	*4.8*	*5.8*
Tatum a (neutral and general amino acid transport systems)				
L-Aspartate**	0.46	0.54	1.20	***
L-Phenylalanine	0.05	—	0.03	0.02
Pm⁻NB (general amino acid transport system)				
L-Aspartate	2.34	3.40	13.9	—
L-Phenylalanine	+	—	+	0.04
(neutral amino acid transport system — by difference)++				
L-Aspartate	0.20	0.08	0.09	—

* $C_{1/2}$ constants are expressed as mM concentrations of the respective amino acid The $C_{1/2}$ constant represents that molar concentration of L-aspartate or L-phenylalanine required to achieve $^1/_2 V$. The $C_{1/2}$ constant calculated is based on the total molar concentration of L-aspartate and/or L-phenylalanine

** The $C_{1/2}$ constants for L-aspartate transport by the wild type are calculated from the linear portion of the Lineweaver–Burk plot They have been included here for their value in reconstruction of the Lineweaver–Burk plots

*** Because the $C_{1/2}$ constant is more dependent than V on the slope of the straight line, this value was not obtainable from the plotted data (see ref. 10)

+ Transport activity, in the *Pm⁻NB* mutant, for L-phenylalanine at these pH values was too low to yield accurate determinations

++ The kinetic constants for the neutral amino acid transport system are obtained by subtracting the transport velocities of *Pm⁻NB* from the wild type and plotting the differences by the method of Lineweaver and Burk

been performed and the derived constants, $C_{1/2}$ or V, have been presented in their respective tables.

One obvious consideration to be derived from Table III is the 2.34 mM $C_{1/2}$ constant for L-aspartate transport by *Pm-NB* at pH 2.8 as opposed to a value of 13.9 mM at pH 4.8. If, as earlier suggested, the neutral species of L-aspartate is the substrate of the general transport system, it would be expected that higher (roughly 10-fold) molar concentrations of L-aspartate might be required at pH 4.8 to achieve 1/2 saturation of the transport activity. Thus, the corrected (for actual molar concentration of neutral species of L-aspartate) $C_{1/2}$ value at pH 4.8 would yield a value of 1.4 mM. Over a similar pH range the $C_{1/2}$ value for L-phenylalanine transport does not change. (It should be noted here that our previously published $C_{1/2}$ values[4] for L-phenylalanine transport at pH 5.8 were incorrect by a decimal place.) That the $C_{1/2}$ value for L-aspartate transport by the neutral transport system does not significantly change with increasing pH might be attributed to (1) a corresponding decrease in molar concentration of neutral species of L-aspartate required to achieve 1/2 saturation of this system, (2) an ability to transport the anionic species of L-aspartate, or (3) the method used to calculate these values may obscure an apparent decrease in "affinity" of neutral system for L-aspartate.

The V values (Table IV) for L-phenylalanine and L-aspartate transport by the wild type appear to be relatively independent of pH changes. It should be remembered that the values for L-aspartate were calculated by using substrate concentra-

TABLE IV

V CONSTANTS* FOR L-ASPARTATE AND L-PHENYLALANINE TRANSPORT AS A FUNCTION OF pH

Amino acid	*pH*			
	2.8	*3.8*	*4.8*	*5.8*
Tatum a (neutral and general amino acid transport systems)				
L-Aspartate**	6.2	8.0	7.7	***
L-Phenylalanine	1.6	—	3.8	3.3
Pm-NB (general amino acid transport system)				
L-Aspartate	14.3	20.0	50.0	—
L-Phenylalanine	+	—	+	0.85
(neutral amino acid transport system — by difference)++				
L-Aspartate	1.54	1.50	0.54	—

* V values expressed as nmoles/min per mg dry wt conidia. The labeled amino acid is at a specific activity of 0.1 Ci/1.0 mole. The values are based on the total molar concentration of L-aspartate and L-phenylalanine.

** The V constants for L-aspartate transport by the wild type are calculated from the linear portion of the Lineweaver–Burk plot. Thus, the values should not be expected to be the additive sum of the V values of the neutral and general transport systems at a given pH. They have been included here for their value in reconstruction of the Lineweaver–Burk plots.

*** A V value for L-aspartate transport at pH 5.8 could not be calculated because of the complexity of transport kinetics.

+ Transport activity, in the *Pm-NB* mutant, for L-phenylalanine at these pH values was too low to yield accurate rate determinations.

++ The kinetic constants for the neutral amino acid transport system are obtained by subtracting the transport velocities of *Pm-NB* from the wild type and plotting the differences by the method of Lineweaver and Burk (1934).

tions which yield linear kinetics. The V values for L-aspartate transport by *Pm⁻NB*, however, are highly pH dependent. Because a plot showing how V changes with pH would describe a H^+ dissociation attributable to the permease–substrate complex (by definition no free unbound permease molecules will be present), these data further argue that the neutral species of L-aspartate is the substrate of the general transport system. The V value is independent of the decreasing molar concentration of neutral species of L-aspartate with increasing pH since by definition the transport system is saturated with substrate at all pH values. Thus, L-aspartate transport activity by the general transport system is increasing with increasing pH. It is not known at present whether the V value of 0.54 nmole/min per mg for L-aspartate transport by the neutral transport system at pH 4.8 is significantly different from the 1.54 nmoles/min per mg value obtained at pH 2.8 or 3.8.

The ability of two amino acids to competitively inhibit each others transport is a good indication that they are both transported by a common transport system(s). It was earlier shown that L-aspartate would competitively inhibit L-phenylalanine transport by the wild type at pH 2.8. (Fig. 2). However, L-phenylalanine does not appear to competitively inhibit L-asparate transport by the wild type at pH 2.8 Instead one obtains complex inhibition kinetics such that both V and $C_{1/2}$ values increase (Fig. 6). This observation was difficult to reconcile until it was found that 0.05 or 0.1 mM L-phenylalanine did not inhibit L-aspartate transport by *Pm⁻NB* at pH 2.8[10] (Fig. 6). Comparison of L-aspartate transport by *Pm⁻NB* with L-aspartate transport by the wild type (in the presence of 0.05 mM L-phenylalanine) revealed that "Lineweaver–Burk" plots of the data were identical, *i. e.* the $C_{1/2}$ and V constants were the same (Fig. 6). Thus, it was concluded that at pH 2.8, 0.05 mM L-phenylalanine blocks transport of L-aspartate by the neutral, but not the general amino acid transport system. Whether the "blockage" is competitive or not in nature is presently unknown. Similar transport reduction patterns were obtained at pH 4 8 with 0 1 mM L-phenylalanine and at pH 2.8 with 0.05 mM L-arginine[10]. That L-phenylalanine does not compete with (or reduce) L-aspartate transport by *Pm⁻NB* at pH 2.8 might have been predicted from the data presented in Table III [L-phenylalanine transport activity in the *Pm⁻NB* mutant at pH 2.8 and 4.8 was too low to obtain accurate kinetic constants (under the conditions employed), whereas that same activity was very high for L-aspartate].

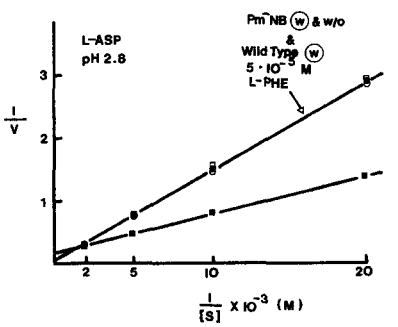

Fig 6. Determination of inhibition kinetics of L-phenylalanine for L-aspartate transport by wild type and *Pm⁻NB* at pH 2.8 The velocity (v) is nmoles/min per mg dry wt conidia and the substrate concentration ([S]) is molar

DISCUSSION

The data presented in this thesis are consistent with the hypothesis that the neutral species of L-aspartate and L-glutamate are transported in *N. crassa* conidia by two genetically separable components. One component, previously reported to be specific at pH 5.8 for the neutral amino acids, is characterized as a "high affinity" low transport capacity system. The second component, the general amino acid transport system, is described as a "low affinity" high transport capacity system. The functional presence of both transport systems in the wild type provide for non-linear kinetics of aspartate transport. Linear kinetics of aspartate transport are restored when one of the transport systems is removed by mutation.

The findings described are analogous to the reported aspartate and glutamate transport activities in *E. coli*[7], *S. faecalis*[6] and *S. hydrogenans*[8] with the exception that in *Neurospora* the neutrally charged species of L-aspartate and L-glutamate appear to be the substrate molecules. In the bacterial studies, the negatively charged (anionic) species of L-aspartate and L-glutamate are presumed to be the substrates of the transport activities. Pall[3] reported the presence of an acidic amino acid transport system in carbon or nitrogen-starved *N. crassa* mycelia. This system is also presumed to transport the negatively charged (anionic) species of glutamate and aspartate.

The occurrence of multiple transport components for accumulation of amino acids and sugars has been reported in numerous studies[4,5,17-22]. Thus, the presence of three distinct systems in *Neurospora* for transport of the acidic amino acids should not be considered as uncommon.

The relationship(s) between the acidic, neutral and general amino acid transport systems in transport of the acidic amino acids by *Neurospora* is not clear. The system described by Pall[3] is characterized by a "K_m" of 0.013 mM (at pH 6.0) and a V for aspartate transport of 0.22 nmole/min per mg dry wt mycelial pad. This system is presumed to be absent in *Neurospora* conidia[5]. Of the two systems described here, the neutral transport system has a $C_{1/2}$ of 0.2 mM (at pH 2.8) and a V for aspartate transport of 1.5 nmoles/min per mg dry wt conidia. The general transport system has a 10-fold lesser "affinity"*, *i.e.* $C_{1/2}$ of 2.34 mM at pH 2.8, and a 10-fold greater V, 14.3 nmoles/min per mg dry wt conidia.

The advantage to the cell in possessing more than one transport component for a molecule is not clear. Kay reported[7] the presence of two aspartate transport systems in *E. coli*. He was able to demonstrate that the high affinity system (ast) could serve as a transport system for aspartate as a nitrogen source and as a supply route for the C_4 acids required for anaplerosis. The second aspartate transport system, the low affinity dct system, was shown to be absolutely necessary for dicarboxylic acid catabolism. Reid *et al.*[6] on the other hand, examining the metabolic fate of glutamate transported by either of two distinct transport systems in *S. faecalis*, reported that, within the limits of resolution of their assay, there was no detectable difference in metabolism of the amino acid whether it was transported by the high affinity or low affinity system. Whether aspartate or glutamate are metabolized differently when transported by one of the three distinct transport systems in *Neurospora* has not been examined.

* For convenience in discussion the K_m ($C_{1/2}$) constant, obtained by plotting the experimental data by the method of Lineweaver and Burk (1934), is occasionally taken as a measure of the affinity (binding association–dissociation constant) of the transport system for substrate

The experimental approach employed in this study has not conclusively excluded transport of the negatively charged species of aspartate or glutamate. As discussed earlier, the use of sub-optimal pH and growth stages might be expected to exclude aspartate transport by the acidic amino acid transport system reported by Pall[3]. However, this exclusion has not been proven. The evidence presented in this manuscript strongly suggest that the transport activity available at the lower pH values is specific for the uncharged, as opposed to the charged, species of aspartate and glutamate. This conclusion must be considered as tentative, however, since none of the data totally exclude transport of the charged species Accumulation of negatively charged aspartate molecules could occur at high molar concentrations *via* (1) one or both of the two systems studied here (Heinz *et al.*[11] reported a pH optimum of 4.0 for the anionic species of L-glutamate transport in Ehrlich cells), (2) a facilitated diffusion mechanism (as found for glutamine and α-aminoisobutyrate by Reid[6]), (3) a system specific for the anionic species, or (4) increased membrane permeability to an anion Passow (as referenced by Heinz[11]) is reported to have shown an increased permeability of the red cell membrane to sulfate with decreasing pH. The effect was attributed to an increase of fixed positive charges on the membrane. Determination of whether or not the anionic species, of aspartate or glutamate are indeed transported by conidia of *N crassa* must await further more detailed studies.

The functional aspect of the positive slope in the L-aspartate (and L-phenylalanine) pH optimum curves is not presently known. The close proximity of the pH sensitive parameter, providing for increasing transport activity with increasing pH, to the isoelectric pH (pH 3.5) of eukaryotic membranes[22] should be considered in future studies of this phenomenon. The negative slope of the L-aspartate (and L-glutamate[10]) pH optimum curves has been attributed to decreasing molar concentrations of the neutral species as the pH increases.

The observed concentration dependence of the inhibition data is difficult to interpret The inhibition of L-phenylalanine transport by L-aspartate appears to be straightforward in that competitive inhibition occurs only at those pH (pH ≥ 8) values where significant concentrations of the neutral species of L-aspartate occur. However, the inhibition of L-aspartate transport by L-phenylalanine and L-arginine[10] indicates that these neutral and basic amino acids, respectively, are acting exclusively on aspartate transport by the neutral transport system. This suggestion is contrary to the proposed model of amino acid transport in *N crassa* conidia at pH 5.8. It is suggested that either the transport activities of these proposed systems are different at lower pH values or that the transport activity in *Neurospora* may be better explained by a different model

It might be suspected that the observed non-linear kinetics, as well as the complex inhibition kinetics, could be due to "allosteric" modification of a single transport system by binding of L-arginine, L-phenylalanine, or L-aspartate to a modifier site similar to that described by Halpern and Even-Shoshan[12] for glutamate transport in E. *coli*. The model of an allosteric permease may be described as containing one active site (for transport) and one or more modifier sites. These sites need not occur on the same molecule, but could reside on different subunit molecules. Binding of substrate amino acids to a modifier site could result in a change in velocity of translocation across the membrane of molecules bound at the active site. The

"affinity" of the active site for substrate may also be altered by ligand bound at a modifier site.

The proposed allosteric permease model is by no means to be considered as complete. (See ref. 10 for greater detail.) It may be used to explain the observed experimental data. However, until future experiments verify or disprove the allosteric permease concept the postulated multiple transport systems represent the most simple means to explain the data.

ACKNOWLEDGMENTS

This report is part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph. D. degree from Florida State University.

This work was supported in part by a National Institutes of Health Traineeship in Genetics to the senior author.

We would also like to acknowledge Frank Larimer and Ivan Collier for their assistance in use of the computer to analyse some of the data.

REFERENCES

1 Joiris, C R and Grenson, M (1969) *Arch Int Physiol. Biochim* 77, 154
2 Hunter, D R and Segel, I H (1971) *Arch Biochem Biophys* 144, 168
3 Pall, M L (1970) *Biochim Biophys Acta* 211, 513
4 Wolfinbarger, L , Jr and Gib DeBusk, A (1971) *Arch Biochem Biophys* 144, 503
5 Wolfinbarger, L , Jr, Jervis, H H and Gib DeBusk, A (1971) *Biochim Biophys Acta* 249, 63
6 Utech, N M , Reid, K G and Holden, J T (1970) *J Biol Chem* 245, 5273
7 Kay, W W (1971) *J Biol Chem* 246, 7373
8 Gross, W and Ring, K (1971) *Biochim Biophys Acta* 233, 652
9 Vogel, H J (1964) *Am Nat* 97, 518
10 Wolfinbarger, L , Jr (1972) Ph D Dissertation, Florida State University
11 Heinz, E , Pichler, A G and Pfeiffer, B (1965) *Biochem Z* 342, 542
12 Halpern, Y S and Even-Shoshan, A. (1967) *J Bacteriol* 93, 1009
13 Wiley, W R and Matchett, W H (1966) *J Bacteriol* 92, 1698
14 Pall, M L (1969) *Biochim Biophys Acta* 173, 113
15 Roess, W B. and Gib DeBusk, A (1968) *J Gen Microbiol* 53, 421
16 Lineweaver, H and Burk, D (1934) *J Am Chem Soc* 56, 658
17 Britten, R J and McClure, F T (1962) *Bacteriol Rev* 26, 292
18 Oxender, D L and Christensen, H N (1963) *J Biol Chem* 238, 3686
19 Ames, G F (1964) *Arch Biochim Biophys* 104, 1
20 Rotman, B and Radojkouic, J (1964) *J Biol Chem* 239, 3153
21 Grenson, M (1966) *Biochim Biophys Acta* 127, 339
22 Chez, R A , Strecker, C K , Curran, P F and Schultz, S G (1971) *Biochim Biophys. Acta* 233, 222