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Versatile Properties of a Nonsaturable, Homogeneous Transport System in *Bacillus subtilis*: Genetic, Kinetic, and Affinity Labeling Studies

(negative cooperativity/biphasic kinetics/transport mutants/whole cell transport)

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ABSTRACT **The multiphasic kinetics that characterize the transport of many amino acids into *Bacillus subtilis* suggests *a priori* at least two mechanisms; (*i*) a heterogeneous mixture of two independent transport processes, or (*ii*) a single, homogeneous system that might involve a negative cooperative mechanism. The highly specific transport system for L-tyrosine and L-phenylalanine in *B. subtilis* was studied as a case in point. The possible presence of a mixed system of independent transport systems was negated by the retention of multiphasic kinetics of transport in two types of permease mutants. Furthermore, evaluation of kinetic data obtained during transport under various uptake conditions of pH and temperature, or in the presence of metabolic inhibitors, did not reveal the heterogeneity expected of mechanism (*i*). These data, taken together with characteristics of substrate specificity and affinity labeling, provide substantial support for a negative cooperative mechanism for L-tyrosine and L-phenylalanine transport.**

In biological systems many rate functions exhibit simple kinetics, exemplified by the hyperbolic substrate saturation curve found for the familiar Michaelis–Menten type of reaction kinetics. On the other hand, examples in which activity varies over a much smaller range (positive cooperative) or a much larger range (negative cooperative) of substrate concentration are also common.

In microbial transport cellular permeases encounter extracellular nutrients whose concentrations may vary by many orders of magnitude. A homogeneous transport system for L-tyrosine and L-phenylalanine exists in *Bacillus subtilis* that probably acts through a negative cooperative (1) mechanism. The observation that other amino acids in *B. subtilis* also exhibit multiphasic transport kinetics as well as recent findings with other systems (2, 3) suggest the possible general significance of the mechanism in transport processes, especially in view of the fact that cooperativity is a characteristic feature of biomembranes (4, 5).

MATERIALS AND METHODS

B. subtilis Strains. *B. subtilis* isolate NP1 is a prototrophic derivative of strain 168 and isolate NP93 is a prototrophic derivative of strain 23 (6). Mutant SS-101 was derived from NP1 by selecting for resistance to growth inhibition of NP1 by D-tyrosine. Mutant H-36 was derived from NP-93 as a D-tyrosine-sensitive mutant. [D-Tyrosine does not inhibit the growth of strain 23 isolates (7).] Complete details of the history and characteristics of these strains have been given elsewhere (8).

Cultivation Media. Composition of minimal salts–glucose medium (9), as well as the preparation of solid media, handling of stock cultures, modification of liquid medium (for nitrogen starvation), and preparation of batches of frozen cells for use as initial inocula prior to transport experiments (10) were described as referenced.

Uptake Protocol. The details of the assay of transport activity have been given elsewhere (10, 11). Duplicate assays can be reproduced with an error of ±2% when about 10,000 cpm of radioactive substrate is taken up by 0.2 mg of cell (dry weight). At low substrate concentrations or in the presence of inhibitors the error is greater, up to ±6% when measuring 100–150 cpm. It should be noted that in a negative cooperative system the binding of inhibitors to multiple binding sites in competition with the substrates L-tyrosine and L-phenylalanine could lead to kinetic constants different from those observed for substrates alone. Since phenylalanine chloromethyl ketone ($PheCH_2Cl$) may also be a substrate (11), the K_i values observed could actually be K_m values and may not reflect variations in binding, but in rates of transport. Thus, while the K_i values for $PheCH_2Cl$ as a competitive or as an irreversible inhibitor vary from 80 to 194 μM, an average value of 146 μM (11) is taken to be representative.

Chemicals. Uniformly ^{14}C-labeled L-amino acids having the following specific activities (Ci/mol) were obtained from New England Nuclear: tyrosine (469), aspartate (207), leucine (311), arginine (311), and histidine (282). Phenylalanine (492) was obtained from Amersham/Searle. Potassium cyanide and fluoride were the products of Matheson, Coleman, and Bell. Chloramphenicol, dinitrophenol, and carbonylcyanide-*p*-chlorophenylhydrazone were purchased from Sigma. $PheCH_2Cl$ was prepared as reported (12). All other chemicals were reagent grade.

RESULTS

Kinetic Complexity of Amino Acid Transport in B. subtilis. Uptake velocities of various L-amino acids, measured over a broad range of initial extracellular concentrations, are shown in the form of double-reciprocal plots in Fig. 1. The data points can be fitted equally well to curves that are concave downward (heavy lines) or to intersecting straight lines (light lines). These data are consistent with a mixed system of independent permeases (light lines) or a single system having a complex mechanism such as negative cooperativity (heavy lines). For operational convenience, the concentration ranges on either side of the intersecting light lines (Fig. 1) are used arbitrarily to define a low-affinity phase and a high-affinity

Abbreviation: $PheCH_2Cl$, phenylalanine chloromethyl ketone.

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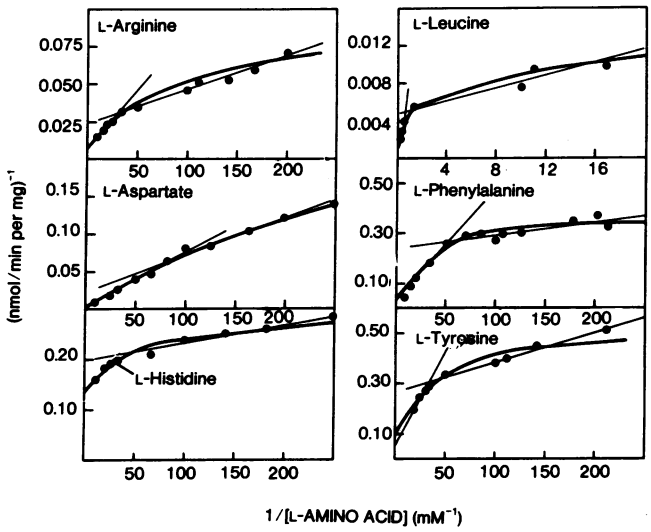

FIG. 1. Double-reciprocal plots of amino-acid uptake in *B. subtilis* strain NP1. The reciprocal of uptake velocity [nmol of L-[*U*-^{14}C]amino acid per mg (dry weight) of *B. subtilis* cell mass per min] is plotted against the reciprocal of the concentration of the transport substrate. The time interval between addition of L-[*U*-^{14}C]amino acid and determination of radioactivity taken up was 3 min with arginine, aspartate, histidine, and phenylalanine, 1 min with leucine, and 5 min in the case of tyrosine.

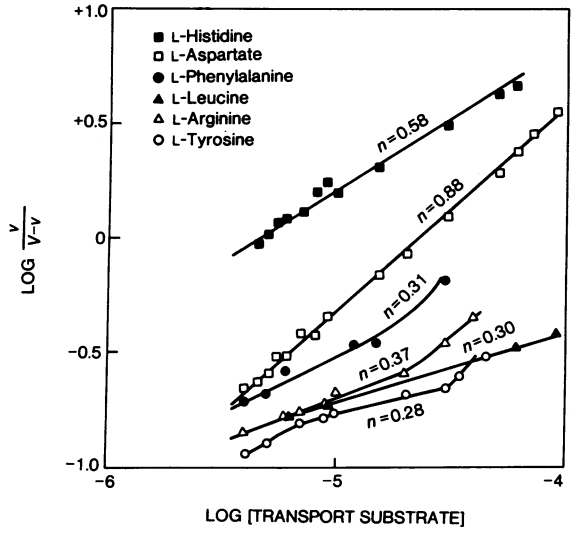

FIG. 2. Determination of interaction coefficient, n, between transport substrate binding sites. The Hill system of coordinates was used by plotting log $v/[V - v]$ against log $[S]$ where S is L-[*U*-^{14}C]amino acid, v is initial velocity of uptake, and V is maximal velocity of uptake at infinite concentration of S. The data plotted are taken from the experiments shown in Fig. 1. Values used for V in the calculation of the ordinate values were the V_{max} values given in the right-hand column of Table 1. Although Hill plots ideally yield straight lines, it is not unusual in practice to find slopes that deviate at both extremes of the concentration scale. The concentration range where linearity is observed is more compressed for some amino acids (e.g., L-tyrosine) than for others (e g., L-histidine). The deviating ends of some curves are not shown in cases where the range of linearity is largest (e.g., L-histidine and L-aspartate).

phase. K_m and V_{max} values have been approximated for each of the two near-linear portions, although the true kinetic constants may vary continuously with substrate concentrations [e.g., in a negative cooperative system (1)]. Constants derived from data taken within each concentration range (Table 1) provide a useful approximation of transport capability at any given substrate concentration. Irrespective of mechanism, it is qualitatively apparent that one "phase" has low affinity and high capacity, while the other has higher affinity and lower capacity. *A priori* the low-affinity phase of transport might reflect a second nonsaturable process such as passive diffusion. Indeed, this is consistent with some of the data wherein the imprecision is such that one segment of the double-reciprocal plot conceivably could be drawn through the origin. However, a process of passive diffusion is difficult to reconcile with the effects of mutations and with effects of metabolic inhibitors.

A plot of the data given in Fig. 1 according to the Hill system of coordinates (14) yielded interaction coefficients that were all less than 1.0 (Fig. 2). However, a mixed system of independent permeases could fortuitously yield the same results. [Our plots of literature data for transport of L-histidine in *Salmonella typhimurium* (15) and L-aspartate in *Escherichia coli* (16) give Hill coefficients less than one, and these systems are known to consist of mixtures of two genetically distinct transport systems.]

General Attempts to Detect Heterogeneous Transport Sites. The independent permeases in a variety of mixed (i.e., heterogeneous) systems described in the literature have been distinguished from one another by their differential sensitivity to pH, temperature, inhibitors, and growth conditions. The highly specific transport system for L-tyrosine and L-phenylalanine in *B. subtilis* (10) was examined in further detail in order to check for differentiating characteristics expected of a heterogeneous, mixed system.

Double-reciprocal plots of uptake data for L-tyrosine at 20, 37 and 45° (Fig. 3) exhibit nonlinear curves, and Hill coefficients of 0.20, 0.28, and 0.38, respectively, were calculated.

TABLE 1. *Summary of transport characteristics for L-amino acid uptake in B. subtilis**

Transport substrate	High-affinity phase† K_m (μM)	High-affinity phase† V_{max} (nmol/min per mg)	Low-affinity phase‡ K_m (mM)	Low-affinity phase‡ V_{max} (nmol/min per mg)
Phenylalanine	4.2	3.8	0.12	25.2
Tyrosine	2.1	3.3	0.20	17.9
Leucine	41	653	3.0	1662
Aspartate	1.2	26.6	0.50	310
Arginine	8.5	38.1	0.08	119
Histidine	1.9	5.0	0.011	6.9

* The data do not yield calculated curves fitting double-reciprocal plots of the experimental data for L-tyrosine and L-phenylalanine transport when the curve-fitting method of Neal (13) is used to determine the K_m and V_{max} values for each of the two linear portions of the curves. This method was derived to determine the true kinetic constants of each phase of transport for transport systems with two genetically distinct components.

† The kinetic parameters of the high-affinity phase were determined by ordinate and abscissa values of the straight line drawn through the ordinate-distal data points in Fig. 1.

‡ The kinetic parameters of the low-affinity phase were determined by ordinate and abscissa values of the straight line drawn through the ordinate-proximal data points in Fig. 1.

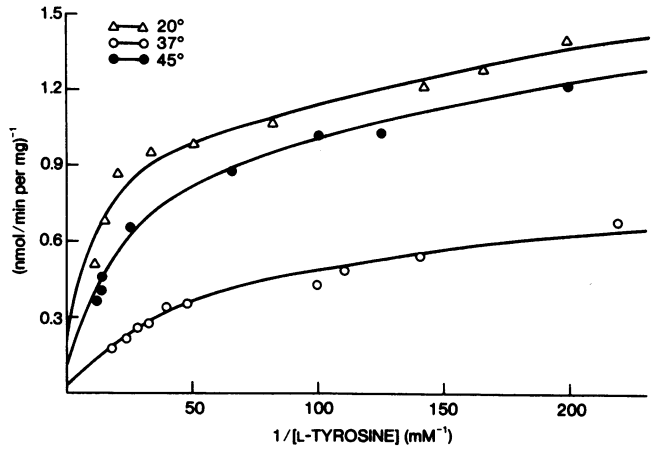

FIG. 3. Transport kinetics at various uptake temperatures. Double-reciprocal plot in which the reciprocal of the initial velocity [nmol of L-[*U*-^{14}C]tyrosine taken up per mg (dry weight) of cells per min] is plotted against the reciprocal of L-[*U*-^{14}C]-tyrosine concentration.

Secondly, L-tyrosine transport velocities were measured at pH values of 5.5, 7.0, and 8.0 (Fig. 4). Interestingly, transport capability was significantly increased as the pH deviated in either direction from the pH optimum (about neutrality) for growth. Hill coefficients calculated from these data were 0.34, 0.33, and 0.32, respectively.

Since heterogeneous components of metabolite transport often serve different metabolic functions, (e.g., refs. 15 and 16), perturbation of metabolism could reveal such heterogeneity through preferential effects upon one of the several transport components. Variation of carbon source in *B. subtilis* strongly influences endogenous L-tyrosine synthesis (17), presumably reflecting differences in relationships of metabolite flow. Nevertheless, the nature and extent of L-tyrosine and L-phenylalanine transport is unaltered (data not shown)

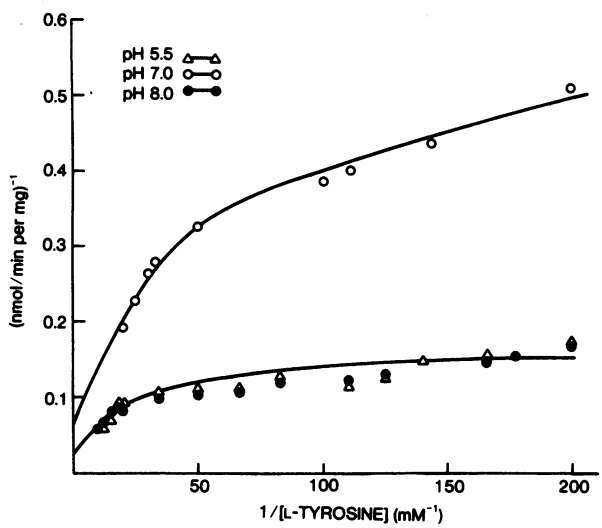

FIG. 4. Transport kinetics during uptake at different pH values. Double-reciprocal plot in which the reciprocal of the initial velocity [nmol of L-[*U*-^{14}C]tyrosine taken up per mg (dry weight) of cells per min at 37°] is plotted against the reciprocal of L-[*U*-^{14}C]tyrosine concentration. Cells grown at pH 7.0 and 37° were resuspended in minimal medium lacking nitrogen at the pH values indicated prior to assaying transport velocities.

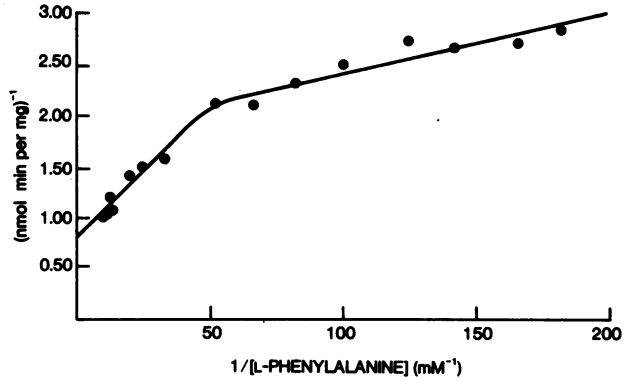

FIG. 5. Transport kinetics after irreversible inhibition of transport by $PheCH_2Cl$. Double-reciprocal plot in which the reciprocal of the initial velocity [nmol of L-[*U*-^{14}C]phenylalanine taken up per mg (dry weight) of cells per min at 37°] is plotted against the reciprocal of L-[*U*-^{14}C]phenylalanine concentration. Cells were treated with 2.5 mM $PheCH_2Cl$ for 120 min [until 9% of phenylalanine transport activity remained (11)], filtered, and resuspended in fresh transport medium. Uptake was then assayed as described elsewhere (10). Apparent values of V_{max} for L-phenylalanine entry were 0.54 and 1.23 nmol/mg (dry weight) per min for the high- and low-affinity systems, respectively. The corresponding K_m values were 3.2 and 33 μM, respectively.

when glucose, glycerol, citrate, succinate, or lactate is used as the sole source of carbon and energy during growth.

Uptake of L-phenylalanine was also studied in the presence of various metabolic inhibitors, using cells previously cultured on either glucose or glycerol as the sole source of carbon. Each phase of transport activity was similarly affected by cyanide, azide, dinitrophenol, or carbonylcyanide-*p*-chlorophenylhydrazone (Table 2). Measurements were made at widely differing extracellular concentrations of L-phenyl-

TABLE 2. *The susceptibility of* L-*phenylalanine transport to various metabolic inhibitors*

		Carbon source (0.5%)*			
		Glucose		Glycerol	
Inhibitor†	Concentration	% Inhibition ‡	§	% Inhibition ‡	§
Potassium cyanide	10 mM	68	69	57	65
	1 mM	43	55	37	40
Sodium azide	10 mM	3	10	0	10
	1 mM	0	7	1	0
Dinitrophenol	100 μM	15	7	0	10
	10 μM	0	0	0	1
Carbonylcyanide-*p*-chlorophenylhydrazone	10 μM	48	46	45	57
	1 μM	3	9	7	0

* Cells subsequently used in transport assays were grown in media containing either glucose or glycerol as the sole source of carbon and energy.

† The inhibitors were added to the assay mixtures at the same time the ^{14}C-labeled phenylalanine was introduced.

‡ External concentration of L-[*U*-^{14}C]phenylalanine was 100 μM (to measure the low-affinity phase).

§ External concentration of L-[*U*-^{14}C]phenylalanine was 1 μM (to measure the high-affinity phase).

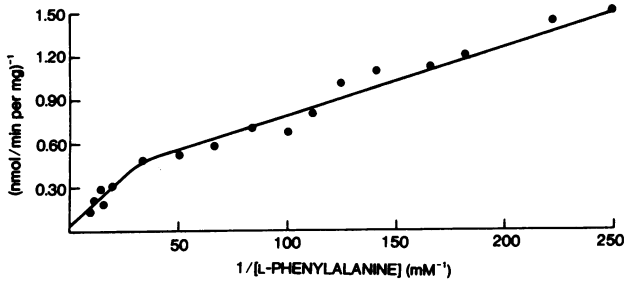

FIG. 6. Transport kinetics after irreversible inhibition of transport by $PheCH_2Cl$ in the presence of L-phenylalanine. Double reciprocal plot in which the reciprocal of the initial velocity [nmol of L-[U-^{14}C]phenylalanine taken up per mg (dry weight) of cells per min at 37°] is plotted against the reciprocal of L-[U-^{14}C]phenylalanine concentration. Cells were affinity-labeled with 2.5 mM $PheCH_2Cl$ in the presence of 2.5 mM L-phenylalanine for 120 min (11). The remaining L-leucine and L-phenylalanine transport activities were 3 and 39%, respectively. After treatment the cells were filtered, washed, and resuspended in fresh transport medium. Apparent values of V_{max} for L-phenylalanine entry were 3.03 and 22.22 nmol/mg (dry weight) per min for high- and low-affinity systems, respectively. The corresponding K_m values were 17 and 330 μM, respectively.

alanine in order to compare the two (operationally defined) kinetic phases. Again, similar effects of inhibitors in blocking transport were observed, whether mediated mainly by the high-affinity phase (1 μM transport substrate) or mainly by the low-affinity phase (100 μM transport substrate). Cyanide and the carbonylcyanide hydrazone were the most effective inhibitors of L-phenylalanine transport.

In an experiment designed to detect a possible greater sensitivity of one of the two phases of transport to affinity labeling by $PheCH_2Cl$ (11), cells were incubated with $PheCH_2Cl$ until 91% of L-phenylalanine transport activity was lost. A double-reciprocal plot of the substrate saturation data for the remaining fraction of transport activity (Fig. 5) remained nonlinear, yielding K_m values of 33 and 3.2 μM and V_{max} values of 1.23 and 0.54 nmol/mg per min for the low- and high-affinity phases, respectively. This indicates a lack of significant differential sensitivity of the two transport phases to inactivation by $PheCH_2Cl$.

$PheCH_2Cl$, in addition to being an affinity label for the L-tyrosine/L-phenylalanine transport system of *B. subtilis*, inactivates the transport of neutral, aliphatic amino acids (11). This latter inactivation is not due to affinity labeling, since no neutral, aliphatic, or aromatic amino acid affords protection against the inactivating effects of $PheCH_2Cl$. Might this reflect the existence of a specific aromatic permease and a second, independent permease for neutral, aromatic, or aliphatic amino acids? This possibility was eliminated with the following experiment (Fig. 6). Incubation of cells with $PheCH_2Cl$ in the presence of L-phenylalanine inactivated 97% of L-leucine transport, while substantial (39%) L-phenylalanine transport remained. Uptake kinetics for L-phenylalanine were nonlinear following this differential inactivation, indicating that the system transporting neutral, aliphatic amino acids does not participate significantly in L-tyrosine and L-phenylalanine uptake.

Mutations in the L-Phenylalanine/L-Tyrosine Permease. Two types of mutants were obtained. Mutant SS-101 (8) has decreased transport capacity (Fig. 7), while Mutant H-36

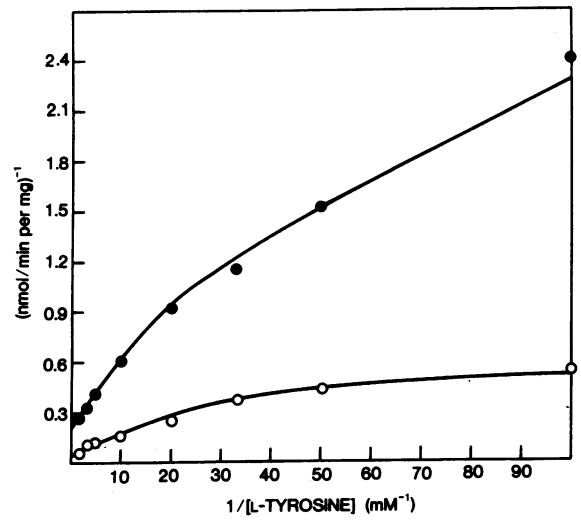

FIG. 7. Retention of nonlinear kinetics in a transport-deficient mutant. Double-reciprocal plot in which the reciprocal of initial uptake velocity [nmol of L-[U-^{14}C]tyrosine taken up per mg (dry weight) of cells per min at 45° and pH 7] is plotted against the reciprocal of L-[U-^{14}C]tyrosine concentration. Symbols: ●, uptake in mutant SS-101; and ○, uptake in wild-type NP1.

(8) exhibits increased transport capability (Fig. 8). Neither mutation abolished the nonlinear kinetics of transport, expected if a mutational deficiency abolished one component of a heterogeneous, mixed transport system. The Hill coefficient calculated from the data of Fig. 7 for mutant SS-101 was 0.39, a value similar to that of its wild-type parent (0.28). Likewise, the Hill coefficients calculated from the data of Fig. 8 for mutant H-36 and its wild-type parent were 0.64 and 0.52, respectively. Mutants lacking the L-tyrosine/L-phenylalanine transport system altogether have not yet been obtained.

DISCUSSION

The Common Duality of Transport Systems in Nature. In some mixed systems for metabolite transport (e.g., refs. 15 and 16), a high-affinity component is capable of concentrative uptake at low extracellular substrate concentration (K_m most significant), while a second low-affinity phase is well suited to the transport of large amounts of substrate at high extracellular concentration (V_{max} most significant).

In some experimental systems sufficient biochemical-genetic data exist [notably the work of Ames and Roth (18)] to establish that biphasic kinetics do indeed reflect the mixed activities of independent transport systems. Although mixed permease systems may prove to be common, numerous experimentalists [many publishing and setting a trend of interpretation prior to the Levitski–Koshland (1) hypothesis] seem not to have considered the negative cooperative mechanism as an alternative possibility. Recently, other evidence suggesting negative cooperativity in microbial transport has appeared (2, 3). The presence of a nonsaturable process with kinetic properties changing continuously throughout a wide range of substrate concentration provides an enormously attractive mechanism that seems especially appropriate for transport.

Evidence in Support of Homogeneous Transport Sites in B. subtilis. The pleiotropic effects of single, point mutations

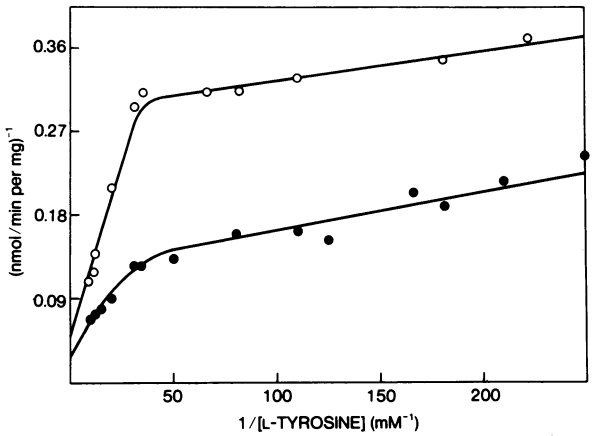

FIG. 8. Retention of nonlinear kinetics in a transport mutant having an increased uptake capability. Double-reciprocal plot in which the reciprocal of initial uptake velocity [nmol of L-[*U*-^{14}C]tyrosine taken up per mg (dry weight) of cells per min] is plotted against the reciprocal of L-[*U*-^{14}C]tyrosine concentration. Uptake was measured as described (10, 11). Symbols: ●, mutant H-36; and ○, wild-type NP93.

upon both kinetic phases of transport cannot, by definition, be reconciled with a mixture of independent transport systems. Single mutations decrease (or increase) simultaneously both kinetically-defined phases of transport. Since effects of these mutations are limited to the L-tyrosine/L-phenylalanine transport system (unpublished observation), the possibility of an active transport-uncoupling mutation or a general membrane effect is eliminated. Neither variation of pH and temperature nor the presence of a variety of metabolic inhibitors produced results suggestive of a mixture of differentially sensitive permeases. The *lack* of differential effects after testing such variables favors a negative cooperative model (although if such differential effects had been found, the negative cooperative mechanism would not necessarily have been eliminated).

Other evidence in favor of homogeneous transport sites was recently obtained through the use of $PheCH_2Cl$, an affinity labeling reagent for the L-tyrosine/L-phenylalanine transport system (11). The binding constants, K_i, for competitive inhibition of the low- and high-affinity phases of L-tyrosine (115 and 190 μM) and L-phenylalanine (80 and 121 μM) transport by $PheCH_2Cl$ were determined (short-term, 3- to 5-min exposure of cells to the irreversible inhibitor produces no significant inactivation). In addition, the binding constants, K_i for irreversible inhibition of L-tyrosine and L-phenylalanine transport, were 194 and 177 μM, respectively. These constants are all similar, as expected if $PheCH_2Cl$ were binding to the same site in each case. Furthermore, the kinetic analysis allowed the determination of the actual first-order rate constants for the chemical reaction leading to inactivation of L-tyrosine and L-phenylalanine transport: 0.0157 and 0.0122 min^{-1}, respectively. These constants are sufficiently similar to implicate a common chemical reaction.

Additional evidence for homogeneous transport sites is that a variety of analogue structures gave patterns of competitive inhibition that were similar for both the low- and high-affinity phases of transport (10). This implies the involvement of a homogeneous site at all substrate concentrations.

A Mechanism of Negative Cooperativity. Other mechanisms could be reconciled with our data [e.g., a shared protein subunit or an allosteric effect (19, 20)]. Nevertheless, a negative cooperative mechanism is most attractive for the following reasons. First, biomembrane material is intrinsically suited to such protein–protein interactions (4, 5). Secondly, a single system (therefore gene-economical) is capable of response throughout an expanded range of transport substrate concentration, exhibiting exactly the kinetic properties that are appropriate at low concentration (high affinity) or at high concentration (high capacity). A rather extensive number of observations suggest that such a range of response to concentration of transport substrate (by whatever mechanism) is of general physiological importance. A case can accordingly be made for the economic value, in terms of genes and gene products, of a single system having such flexibility. For these reasons, the mechanism may be generally distributed in nature. Re-evaluation of much of the data in the existing literature may be in order with this possibility in mind.

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