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Multiplicity of Isoleucine, Leucine, and Valine Transport Systems in *Escherichia coli* K-12

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The kinetics of isoleucine, leucine, and valine transport in *Escherichia coli* K-12 has been analyzed as a function of substrate concentration. Such analysis permits an operational definition of several transport systems having different affinities for their substrates. The identification of these transport systems was made possible by experiments on specific mutants whose isolation and characterization is described elsewhere. The transport process with highest affinity was called the "very-high-affinity"process. Isoleucine, leucine, and valine are substrates of this transport process and their apparent K_m values are either 10^{-8}, 2×10^{-8}, or 10^{-7} M, respectively. Methionine, threonine, and alanine inhibit this transport process, probably because they are also substrates. The very-high-affinity transport process is absent when bacteria are grown in the presence of methionine, and this is due to a specific repression. Methionine and alanine were also found to affect the pool size of isoleucine and valine. Another transport process is the "high-affinity" process. Isoleucine, leucine, and valine are substrates of this transport process, and their apparent K_m value is 2×10^{-6} M for all three. Methionine and alanine cause very little or no inhibition, whereas threonine appears to be a weak inhibitor. Several structural analogues of the branched-chain amino acids inhibit the very-high-affinity or the high-affinity transport process in a specific way, and this confirms their existence as two separate entities. Three different "low-affinity" transport processes, each specific for either isoleucine or leucine or valine, show apparent K_m values of 0.5×10^{-4} M. These transport processes show a very high substrate specificity since no inhibitor was found among other amino acids or among many branched-chain amino acid precursors or analogues tried. The evolutionary significance of the observed redundancy of transport systems is discussed.

The uptake of isoleucine, leucine, and valine by *Escherichia coli* was the first to be studied with radioactive precursors (3, 5). Isoleucine, leucine, and valine are virtually utilized only for protein synthesis and, when this synthesis is blocked with an inhibitor, essentially 100% of the amino acids present inside the cell are recovered unmodified from the intracellular fluid. More recent studies on isoleucine-leucine and valine uptake have established the existence in *E. coli* K-12 of a transport process specific for these amino acids (15) and the existence of binding proteins that are released in the osmotic shock fluid and which bind either isoleucine, leucine, and valine (2, 14) or leucine alone (7). The existence of more than one transport system for isoleucine, leucine, and valine was implicated by evidence from other laboratories (7, 15).

We have initiated a study on the uptake of isoleucine, leucine, and valine with the aim of obtaining genetic and kinetic information as a preliminary step to a biochemical approach with more purified materials, such as membrane vesicles (10). We believe that analysis of mutations affecting the uptake process are an essential complement of the kinetic and biochemical data. Mutations can provide convincing evidence on the existence of transport systems, their possible multiplicity, and their physiological role. Moreover, through isolation of amber and temperature-sensitive mutations, one can identify the gene product. In fact, several genes might be required for a given transport system. A system that is being analyzed with a similar approach is histidine transport (see 13 for review).

In this paper we show that isoleucine-leucine-

valine uptake (15) is composed of several different transport systems which can be identified because of the different affinities of their substrates, and in the following paper (8) we give evidence for mutations affecting each of them.

A preliminary portion of this work has been reported (J. Guardiola, M. De Felice, and M. Iaccarino, Lunteren Lectures on Molecular Genetics, Lunteren, The Netherlands, 1971).

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains used in this work.

Reagents. Reagents whose source is not mentioned were of the highest purity available. Amino acids used were all L-form, unless otherwise mentioned. Uniformly ^{14}C-labeled L-amino acids were obtained from New England Nuclear Corp., Boston, Mass. Sodium α-acetolactate was obtained by saponification, with two equivalents of NaOH, of α-acetolactic acid acetate ethyl-ester purchased from K & K laboratories, Inc., Plainview, N.Y. L-Pantoyl-lactone, D-serine, D-leucine, D-threonine, 4-aza-leucine dihydrochloride, and DL-norvaline were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. DL-Methallyl-glycine-hydrochloride hemihydrate was purchased from Cyclochemical, Division of Travenol Laboratories, Inc., Los Angeles, Calif. 4-Azaisoleucine, O-methyl-DL-threonine, sodium α,β-dihydroxy-isovalerate, disodium β-isopropyl-malate, sodium α,-β-dihydroxy-β-methyl-valerate, and thiaisoleucine hydrochloride (2-amino-3-methyl-thiobutyrate) were obtained from Reef Laboratories, Santa Paula, Calif. 5'-Trifluoroleucine was purchased from Columbia Organic Chemicals Co. Inc., Columbia, S.C. Cycloleucine, L-α-amino-n-butyric acid, and α-ketoisovaleric acid (sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. The purity of these reagents was never checked.

Transport assays. Bacteria were grown in minimal citrate medium (21), harvested by centrifugation at 4 C in mid-logarithmic phase, and washed twice with unsupplemented medium at 4 C. Determination of rate of uptake was performed by a modification of a method already described (9, 15). The washed cells were suspended in minimal medium containing 300 μg of chloramphenicol per ml and 0.4% glucose. The bacterial suspension was kept at room temperature; transport was measured after 10 min by pipetting 0.1 ml of the bacterial suspension into a test tube containing a given concentration of the ^{14}C-labeled substrate at 37 C in minimal citrate medium (the final volume is indicated below). After 0.5 min, the sample was filtered onto the center of a membrane filter (Millipore Corp., type HA, 0.45 μm, 25 mm). The cells were immediately washed on the filter with 5 ml of unsupplemented medium at room temperature. Radioactivity of the dried filters was measured as described (9). K_m and V_{max} values were evaluated by plotting $1/c$ versus $1/v$, where c is the concentration of the substrate expressed in micromoles per liter and v is the rate of uptake expressed in micromoles of ^{14}C-labeled substrate incorporated per 0.5 min per gram of cells (dry weight).

Bacterial concentration, total amount of assay mixture, and specific activities of ^{14}C-labeled amino acids were varied so that: (i) the radioactivity taken up by the cells never exceeded 10 to 15% of the total radioactivity in the tube; (ii) filtration of bacteria was complete within 5 s; and (iii) the counts per minute actually measured were at least three times greater than the background (a tube containing no bacteria). When very-high-affinity transport was measured, the total volume in the assay tubes was 5 ml, the specific activity of ^{14}C-labeled amino acids was 2×10^8 counts per min per μmol, the background was 30 to 300 counts/min, and from 0.06 mg to 0.18 mg of cells per tube were used. When high-affinity or low-affinity transport was measured, the total volume in the assay tubes was 1 ml, the specific activity of ^{14}C-labeled amino acids was 3×10^7 to 7×10^7 counts per min per

TABLE 1. *Bacterial strains*[a]

Strains	Genotype	Origin
X478	*thi, leuB, purE, proC, lysA, trp, metE, ara, lacZ, xyl, azi, str, tonA, tsx*, F$^-$	From P. Berg
PB154	*argH, trpA36*, F$^-$	From P. Berg
MI2	*argH, trpA36, ilvA601*, F$^-$	From PB154 (ultraviolet mutagenesis)
MI148a	*thi, purE, proC, lysA, trp, metE, ara, lacZ, xyl, str, tonA, tsx*, F$^-$	Leu$^+$, Azi^s, Ara$^-$ transductant of X478 with P1 grown on on a wild-type strain
MI148d	*thi, purE, proC, lysA, trp, ara, lacZ, xyl, str, tonA, tsx*, F$^-$	Met$^+$ transductant of MI148a with P1 grown on a wild-type strain
MI237	*thi, purE, trp, lysA, metE, ara, xyl, str, tonA, tsx, brnQ2, brnR3*, F$^-$	Spontaneous valine-resistant organism from strain MI174b (7 and 8)
MI237a	*thi, purE, trp, lysA, ara, xyl, str, tonA, tsx, brnQ2, brnR3*, F$^-$	Met$^+$ transductant of MI237 with P1 grown on a wild-type strain
MI237b	*thi, purE, trp, lysA, ilvA,*^{601} *ara, xyl, str, tonA, tsx, brnQ2, brnR3*, F$^-$	Met$^+$, Ile$^-$ transductant of MI237 with P1 grown on MI2

[a] Symbols for genetic markers are those used by Taylor and Trotter (19); the *brn* symbol describes genes for transport of the branched-chain amino acids; the *brnQ* marker has already been described (9); the *brnR* marker has been described (8).

μmol, the background was from 100 to 500 counts/min, and from 0.5 to 1.0 mg of cells per tube were used. Counting efficiency was 80% (9).

RESULTS

The kinetic analysis of active transport for branched-chain amino acids described in this paper shows that more than one transport system for these amino acids are present. As shown below, when the rate of uptake is measured as a function of substrate concentration, components with different affinities are observed. The most likely interpretation of the experiments reported is that there is (i) a "very-high-affinity" transport process due to at least one transport system, (ii) a "high-affinity" transport process which is shown to be due to two different transport systems (8), and (iii) a "low-affinity" transport process which is due to at least three transport systems. The experimental evidence has been divided accordingly.

In this and the accompanying paper (8), the term "uptake" will be used in an operational sense whenever a compound is removed from the medium by the cells. The term "transport process" will be used to define uptake showing Michaelis-Menten kinetics and measurable apparent kinetic constants (K_m and V_{max}). The term "transport system" will be used to define a specific apparatus that permits transport of a given substrate and which can be abolished by mutation. Therefore, the finding of uptake with different kinetic constants implies different transport processes performed by different transport systems. On the other hand, a specific transport process might be performed by more than one transport systems, as will be shown to be the case of high-affinity transport (8).

Very-high-affinity transport process. When radioactive isoleucine, leucine, or valine is used as a substrate for transport at concentrations within the 10^{-8} M range, uptake is measured as shown in Fig. 1. Strain PB154 takes up isoleucine, leucine, and valine with apparent K_m values of 10^{-8}, 2×10^{-8}, and 10^{-7} M, while V_{max} values are 0.5, 0.4, and 2.1 μmol per 0.5 min per g of cells, respectively. The double reciprocal plot is linear, but at the highest substrate concentrations a deviation from linearity is observed. When this part of the plot is expanded and more experimental points (not shown in the figure) are used, this portion is also linear and extrapolates to an apparent K_m value of 2×10^{-6} for all three amino acids. These data therefore show that, besides the transport system(s) for branched-chain amino acids with a K_m into the 10^{-8} M range, which we will call

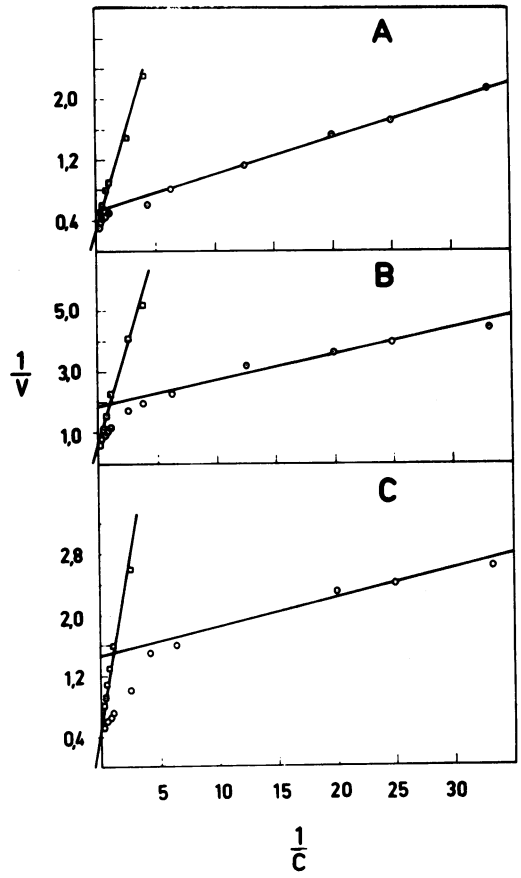

FIG. 1. *Dependence of the rate of uptake upon the concentration of valine (A), leucine (B), and isoleucine (C) in strains MI148a (□) and PB154 (○). Plot of 1/v versus 1/c, where v is the rate of uptake (micromoles per 0.5 min per g of cells) and c is the micromolar concentration of amino acid. Values are average of three determinations. Specific activities, counting efficiencies, and background values are reported in Materials and Methods.*

hereafter the very-high-affinity transport process, there is also a second transport process with lower affinity which will be called the high-affinity transport process.

Several *E. coli* K-12 strains from our collection do not show the very-high-affinity transport process. One of these strains is MI148a, for which uptake determinations are reported in Fig. 1. Strain MI148a has a growth requirement for methionine, and appropriate experiments demonstrated that the absence of the very-high-affinity transport process in strain MI148a was due to the presence of methionine in the growth medium. When strain PB154 is grown in the presence of methionine and the uptake rate is

measured as a function of substrate ([^{14}C]isoleucine, -leucine, or -valine) concentration, some uptake remains, but on a double reciprocal plot the experimental points fall on the same line drawn for strain MI148a in Fig. 1. The remaining uptake is therefore high-affinity transport. This conclusion is confirmed by the experiments of Fig. 2. Figure 2A shows the rate of valine uptake versus concentration in strains PB154 and MI148a. The difference between the two curves is due to the absence of uptake through the very-high-affinity transport, as shown in Fig. 1A where the same data are reported in a double reciprocal plot. Figure 2B shows an analogous experiment on strain MI237a, which lacks the high-affinity transport (see reference 8), grown in the absence or in the presence of methionine. In this experiment both curves are lower because the high-affinity transport process is missing, but the difference between the two curves, representing the very-high-affinity transport process, is equal to the difference in Fig. 2A. When a double reciprocal plot is made with the data of the upper curve (see insert to Fig. 2B) a K_m value of 10^{-7} M is observed. In conclusion, when a strain is grown in the presence of methionine, it shows very little, if any, of the very-high-affinity transport process. This result could be due to inhibition of uptake by the methionine that is carried with

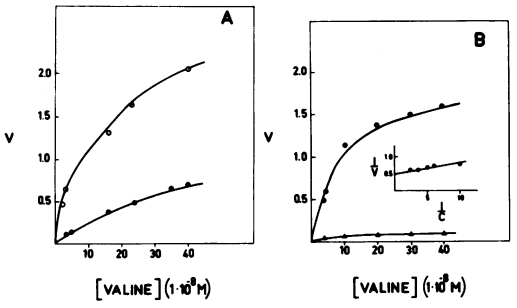

FIG. 2. *(A) Dependence of the rate of uptake upon concentration of valine in strain PB154 (○) and strain MI148a (●). Plot of v versus c, where v is the rate of uptake (micromoles per 0.5 min per g of cells) and c is the 10^{-8} M concentration of valine. Specific activity, counting efficiency, and background values are reported in Materials and Methods. (B) Dependence of the rate of uptake upon concentration of valine in strain MI237b grown with methionine (▲) and without methionine (●). Plot of v versus c where v is the rate of uptake (micromoles per 0.5 min per g of cells) and c is the 10^{-8} M concentration of valine. In the insert, a double reciprocal plot of the date of the upper curve is reported. Specific activity, counting efficiency, and background values are reported in Materials and Methods.*

cells into the assay tube (this is unlikely, however, because methionine is diluted during centrifugation and resuspension of bacteria). An alternative explanation is that methionine represses the very-high-affinity transport process. An experiment was devised to distinguish between these hypotheses. The very-high-affinity transport was determined in strain MI237a (a *met*$^+$ derivative of strain MI237) pregrown either in minimal medium or in a medium supplemented with methionine or glycylmethionine (Table 2). Since dipeptides and amino acids are taken up through different transport systems (see reference 6 and those quoted within) glycylmethionine might not inhibit the very-high-affinity transport process, whereas it should behave just like methionine if it causes repression. The results of Table 2 show that methionine and glycylmethionine are equally effective in decreasing the uptake of branched-chain amino acids through the very-high-affinity transport process when bacteria are pregrown in their presence. In addition to causing repression, however, methionine is also an inhibitor of this transport process. The results of Table 2 exclude the possibility that methionine inhibits from within the cell (transinhibition; see 17); in fact, if it is so, preloading of the cells with 4×10^{-5} M methionine should increase uptake inhibition, but, as shown in the table, this does not happen. In conclusion, the data are in agreement with the hypothesis that methionine causes repression of the very-high-affinity transport process. It is possible that threonine, alanine, and the substrates of this transport process (see below) also cause repression, but we did not pursue this point.

The specificity of the very-high-affinity transport process was analyzed by an experiment shown in Table 3. Under the conditions used in this experiment, only about 70% of the uptake occurs through the very-high-affinity transport process, the remaining 30% being through the high-affinity one (when strain PB154 is grown in the presence of methionine, the uptake of 4×10^{-8} M substrate is 30% that of the same strain grown without methionine). It is apparent from the data of this table that isoleucine, leucine, and valine inhibit most of the uptake, and this proves that there is either only one very-high-affinity transport system for all three branched-chain amino acids or, if more than one, each one can serve for all three amino acids. The uptake left after inhibition by isoleucine, leucine, and valine is less than 30% because these amino acids inhibit not only the very-high-affinity transport process but also the high-affinity one

TABLE 2. *Very-high-affinity transport of isoleucine,[a] leucine, and valine in strain MI237a grown under different conditions*

Growth medium[b]	Inhibitor in the assay (4×10^{-5} M)	Transport of substrates:		
		Isoleucine[c]	Leucine[c]	Valine[c]
Minimal		0.230	0.125	0.186
	Methionine	0.108	0.062	0.068
	Glycylmethionine	0.165	0.099	0.140
	Methionine (preloaded)[d]	0.107	0.072	0.071
Methionine		0.038	0.027	0.007
	Methionine	0.023	0.016	0.005
	Glycylmethionine	0.029	0.023	0.008
Glycylmethionine		0.045	0.023	0.012
	Methionine	0.019	0.010	0.005
	Glycylmethionine	0.029	0.019	0.005

[a] [^{14}C]isoleucine, -leucine, or -valine concentrations were 4×10^{-8} M.

[b] Minimal medium supplemented with substances needed by this strain for growth. When methionine or glycylmethionine were also added, as indicated, their concentration was 50 and 75 μg/ml, respectively. For other details see Materials and Methods.

[c] Micromoles of amino acid per 0.5 min per g of cells.

[d] These cells were preincubated with 4×10^{-5} M methionine for 40 min after addition of chloramphenicol. During this time, cells incubated without methionine lose about 25% of the initial activity, and the values reported in the table are corrected for the inactivation which was measured in each case. Methionine (4×10^{-5} M) was present also in the assay.

TABLE 3. *Inhibition of very-high-affinity transport of isoleucine,[a] leucine, and valine in strain PB154 by different amino acids*

Inhibitors (4×10^{-5} M)	Isoleucine (%)	Leucine (%)	Valine (%)
None	100 (0.3)[b]	100 (0.220)[b]	100 (0.160)[b]
Isoleucine		7.2	0.1
Leucine	0.1		2
Valine	0.6	10	
Threonine	9	20	7
Methionine	30	28	21
Alanine	29	28	22
Arginine	90	71	79
Lysine	101	70	79
Cysteine	89	68	79
Glycine	104	70	80
Serine + histidine + tryptophan + phenylalanine[c]	63	65	50
Aspartate + asparagine + glutamate + glutamine + proline[c]	95	93	90

[a] [^{14}C]isoleucine, -leucine, or -valine concentrations were 4×10^{-8} M. For other details see Materials and Methods.

[b] Micromoles of amino acid per 0.5 min per g of cells.

[c] All these amino acids were present each at a final concentration of 4×10^{-5} M.

(see below). Methionine and alanine appear to be more specific inhibitors of the very-high-affinity transport process although they might inhibit, more weakly, also the high-affinity one (see Table 5). The inhibition caused by threonine is more efficient than that caused by methionine and alanine because part of the high-affinity transport process is also inhibited by threonine. Isoleucine, leucine, and valine, used as inhibitors, inhibit 99% of isoleucine and valine uptake. On the other hand, isoleucine and valine inhibit 90% of leucine uptake. The significance of this finding will be discussed below. Inhibition of the very-high-affinity

transport process by several analogues of the branched-chain amino acids is shown in Table 6 and will be discussed below.

The growth of *E. coli* K-12 is inhibited by valine (12). If the very-high-affinity transport process affects the pool size of valine, valine inhibition of the growth should be decreased by the amino acids that inhibit valine uptake through this transport process, like methionine or alanine. Experiments shown in Fig. 3A and 3B support this hypothesis. In the experiment of Fig. 3A, a small inoculum (2×10^5 cells) of strain MI148d (*brn*$^+$, *met*$^+$, Val^s) was pipetted into 5 ml of supplemented minimal medium containing different concentrations of valine, and the growth yield of the cultures was determined. A 0.1-μg amount of valine per ml is sufficient to inhibit growth completely, whereas, if 100 μg of methionine per ml is also present, complete inhibition is only achieved at 1.4 μg of valine per ml. Alanine (100 μg/ml) gave results similar to that observed with methionine, whereas histidine (100 μg/ml) had no effect. Another experiment was performed with a strain requiring isoleucine for growth and having no high-affinity transport process (strain MI237b, *ilvA601*, *brnQ2*, *brnR3*) (Fig. 3B). Growth of this strain on low concentrations of isoleucine is inhibited by methionine, and this inhibition is not observed when glycylisoleucine is used (data not reported).

In conclusion, the very-high-affinity transport process permits the uptake of isoleucine, leucine, and valine; is repressed by methionine; and is inhibited by threonine, methionine, and alanine. Assuming that this inhibition is due to the fact that these amino acids are taken up through the same transport system, the very-high-affinity transport system would permit the uptake of isoleucine, leucine, valine, threonine, methionine, and alanine. We did not try to confirm this assumption by direct measurement of threonine, methionine, and alanine transport because, as in the case of threonine (see below), probably multiple transport systems for these amino acids do exist and therefore only a small uptake inhibition by isoleucine, leucine, or valine should be observed.

High-affinity transport process. The experiments described in this section are all performed with strain X478 or its derivatives which, because of the presence of methionine in the growth medium, do not show the very-high-affinity transport process.

A double reciprocal plot of the initial rate of uptake versus amino acid concentration is shown in Fig. 4. This figure shows that for either

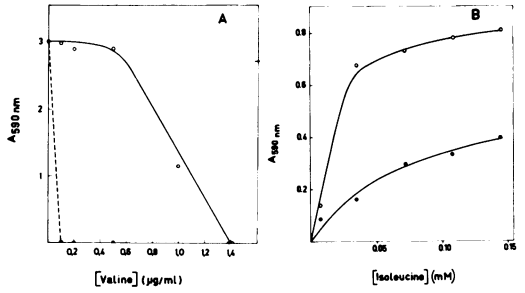

FIG. 3. *(A) Dependence of the growth yield of strain MI148d as a function of valine concentration in the medium in the presence (●) or in the absence (○) of 100 μg of methionine per ml. The growth was determined as absorbance at 590 nm after overnight incubation at 37 C. (B) Dependence of growth yield of strain MI237b as a function of isoleucine concentration in the medium in the presence (○) or in the absence (●) of 100 μg of methionine per ml. The growth was determined as absorbance at 590 nm after overnight incubation.*

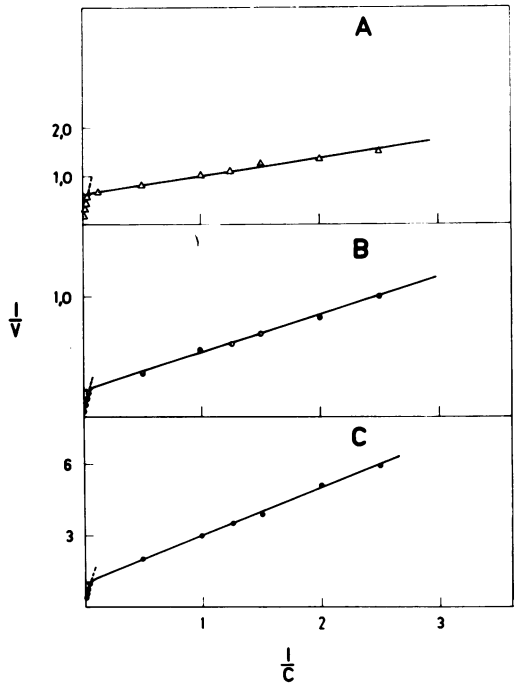

FIG. 4. *Dependence of the rate of uptake in strain MI148a as a function of valine (A), leucine (B), and isoleucine (C) concentration in the presence of methionine. Plot of 1/v versus 1/c, where v is the rate of uptake expressed as micromoles per 0.5 min per g of cells and c is the micromolar concentration of amino acid. Values are average of two determinations. Specific activities, counting efficiencies, and background values are reported in Materials and Methods.*

isoleucine, leucine, or valine there is a sharp break in the curve at a concentration of about 10^{-5} M. This break proves the presence of two transport processes, one with higher affinity and the other with lower affinity (see below); the experimental points referring to strain MI148a in Fig. 1 are coincident with the experimental points of the transport process with higher affinity in Fig. 4. Apparent K_m and V_{max} values were obtained from plots with more appropriate scales in which more numerous experimental points (not shown in the figure) were used in each section of the plot. No correction was made for the contribution of the other transport process to the activity that was being taken into consideration. The apparent kinetic constants are shown in Table 4; it can be seen that the apparent K_m values are 30- to 55-fold different, in agreement with the striking difference observed in the slope of the curves of Fig. 4, whereas the apparent V_{max} is 3- to 5-fold higher. Also in other cases (1, 11) a higher V_{max} has been reported for the lower-affinity transport process. It should be pointed out, however, that not only the low-affinity transport process contributes to this V_{max} but also the high-affinity one. A lower V_{max} for the low-affinity transport process is found when no high-affinity transport process is present (see below and Table 8).

The high-affinity transport process of isoleucine, leucine, or valine is inhibited by the other two amino acids (see below and Table 7); this shows that they are all substrates of the same transport process. In the following paper, however, data are presented that show that there are two different high-affinity transport systems and that isoleucine, leucine, and valine are substrates for both of them (8).

The specificity of the high-affinity transport process was analyzed in the experiment shown in Table 5, where the inhibition of the initial rate of uptake of 2×10^{-6} M valine by different amino acids is shown. At this concentration,

TABLE 4. *Apparent kinetic constants for isoleucine, leucine, and valine high-affinity transport in strain MI148a*[c]

Substrate	High-affinity transport		Low-affinity transport	
	K_m	V_{max}	K_m	V_{max}
Isoleucine	1.5	0.5	50	2
Leucine	1.5	0.5	65	1.5
Valine	1.0	0.5	55	2.8

[a] K_m, micromolar; V_{max}, micromoles of amino acid per 0.5 min per g of cells. For other details see Materials and Methods.

TABLE 5. *Transport inhibition of 2×10^{-6} M valine in strain MI148a by different amino acids*

Inhibitors[a] (2×10^{-4} M)	μmol of valine per 0.5 min per g of cells)
None	0.97
Isoleucine	0.03
Leucine	0.06
Threonine	0.70
Alanine	0.80
Methionine	0.56

[a] Arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, lysine, phenylalanine, proline, serine, tyrosine, and tryptophan had very little effect, if any, on valine uptake in this strain. For other details see Materials and Methods.

valine is taken up mainly through the high-affinity transport process, and only about 5% is taken up through the transport system with 0.5 $\times 10^{-4}$ M K_m. (Because of the presence of methionine the very-high-affinity transport process is not active.) Data of Table 5 show that only isoleucine and leucine completely inhibit uptake of ^{14}C-labeled valine through the high-affinity transport process. Among other amino acids, methionine and threonine give about 50% inhibition and alanine gives about 30% inhibition. The effect of methionine and alanine might be due to the inhibition of a fraction of the uptake through the very-high-affinity transport process remaining. In fact, the V_{max} for valine of the very-high-affinity transport process is 2.1 as compared to the value of 0.5 for the high-affinity one (see above). Since at the concentration of valine used in the experiment of Table 5 the system(s) contributing to the high-affinity transport process is not saturated, a 10% fraction of the uptake through the very-high-affinity transport process would be enough to explain the inhibition. The inhibition caused by threonine is actually complete inhibition of one of the two high-affinity transport systems, as shown in the accompanying paper (8). Also, several analogues of the branched chain amino acids were tested—data of Table 6 show that 2×10^{-4} M pantoly lactone, D-serine, and D-leucine, 4×10^{-4} M DL-methallyl-glycine, azaisoleucine, and azaleucine, and 10^{-3} M or 10^{-2} M D-threonine give no inhibition of the high-affinity transport process of valine; 4×10^{-4} M DL-norvaline, trifluoroleucine, and thiaisoleucine give about 90% inhibition (i.e., about 95% inhibition of the high-affinity transport); and 4×10^{-4} M *O*-methyl-DL-threonine, and 2×10^{-4} M cycloleucine give 75 and 40% inhibition, respectively. Inhibition of the very-high-

TABLE 6. *Inhibition of the very-high-affinity transport and high-affinity transport for valine by various substances*

Inhibitors	Very-high-affinity[a] transport (%)	High-affinity[b] transport (%)
None	100	100
Cycloleucine[c]	35	58
DL-5'-Trifluoroleucine[d]	7	8
L-Pantoyl lactone[c]	89	95
O-methyl-DL-threonine[d]	2	26
DL-4-Thiaisoleucine[d]	20	15
DL-4-Azaisoleucine[d]	25	89
D-Serine[c]	40	90
D-Leucine[c]	19	84
DL-Norvaline[d]	1	12
DL-Methylallyl glycine[d]	20	95
4-Azaleucine[d]		83
D-Threonine[e]		100

[a] Transport of 4×10^{-8} M [^{14}C]valine was measured in strain MI237a. The noninhibited value was 0.23 μmol per 0.5 min per g of cells. For other details see Materials and Methods.

[b] Transport of 2×10^{-6} M [^{14}C]valine was measured in strain MI148a. The noninhibited value was 0.65 μmol per 0.5 min per g of cells.

[c] Concentration of these inhibitors was 4×10^{-5} M when very-high-affinity transport was measured and 2×10^{-4} M when high-affinity transport was measured.

[d] Concentration of these inhibitors was 8×10^{-5} M when very-high-affinity transport was measured and 4×10^{-4} M when high-affinity transport was measured.

[e] Concentration of D-threonine was 10^{-3} M, and the same result was obtained when it was 10^{-2} M.

affinity transport process was in some cases (azaisoleucine, D-serine, D-leucine, and methallyl glycine) strikingly different from that observed for the high-affinity one, and this confirms that they are separate functions. These compounds appear to be specific inhibitors of the very-high-affinity transport. They should prove useful to measure this transport process under specific experimental conditions or to isolate mutants in which it is abolished.

Low-affinity transport process. The double reciprocal plot of initial rate of uptake versus substrate concentration for isoleucine, leucine, and valine uptake shows a sharp break at a concentration of about 10^{-5} M (Fig. 4). This suggested the presence of a transport system(s) with lower affinity, whose apparent kinetic constants, measured in an experiment similar to that of Fig. 4, are reported in Table 4. Another example of transport processes with different affinities has been reported by Ames (1) in 1964 and later by Brown (4), in the case of aromatic amino acids. These authors show that, besides the transport system with an apparent K_m value of 0.5×10^{-6} M, there are also more transport systems with lower affinity (K_m value of 2×10^{-6} M), each one being specific for one of the aromatic amino acids. By analogy, we made the hypothesis that the transport process with lower affinity might be more specific, i.e., there might be three different transport systems, each one being specific for one of the three branched-chain amino acids. In agreement with this hypothesis, the data reported in Table 7 show that some residual uptake remains when an excess of a branched-chain amino acid is used to inhibit uptake of a 2×10^{-6} M substrate. We therefore constructed a series of double reciprocal plots of initial uptake rates of isoleucine, leucine, or valine versus their concentration in the presence of excess inhibitor (one of the other two branched-chain amino acids). The data are summarized in Table 8 and confirm the presence of three different transport processes with an apparent K_m value of 0.5×10^{-4} M, each one being specific either for isoleucine, leucine, or valine. In the experiments of Table 8 there is no contribution of the high-affinity transport process to the V_{max}. In agreement with this, the V_{max} values of Table 8 are lower than those of Table 4.

The low-affinity transport systems for isoleucine, leucine, and valine, although specific for one of these amino acids, might also transport some other compound. To test this hypothesis, we performed the experiment summarized in Table 9. In this table it is shown that all the amino acids tested for inhibition are inactive, and the same is true for some isoleucine, leu-

TABLE 7. *Inhibition of the high-affinity transport of one branched-chain amino acid by an excess of the other two*[a]

Inhibitor	Inhibition of transport of substrates[b]		
	Isoleucine	Leucine	Valine
None	1.02	0.4	0.90
2×10^{-4} M isoleucine		0.09	0.04
2×10^{-3} M isoleucine		0.06	0.03
2×10^{-4} M leucine	0.06		0.03
2×10^{-3} M leucine	0.06		0.03
2×10^{-4} M valine	0.04	0.10	
2×10^{-3} M valine	0.05	0.10	

[a] The strain used was MI148a. [^{14}C]isoleucine, -leucine, or -valine concentration was 2×10^{-6} M. For other details see Materials and Methods.

[b] Micromoles per 0.5 min per g of cells.

TABLE 8. *Apparent kinetic constants for isoleucine, leucine, and valine transport in the absence or in the presence of competing amino acids[a] in strain MI148a*

Substrate	Inhibitors[b]							
	None		Isoleucine		Leucine		Valine	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Isoleucine ..	2.1	0.5			50	1	50	0.5
Leucine	2.1	0.5	50	0.2			50	0.5
Valine	2	0.3	50	1	50	1		

[a] K_m, micromolar; V_{max}, micromoles of amino acids per 0.5 min per g of cells. For other details see Materials and Methods.

[b] Concentration of inhibitors was 2×10^{-4} M.

cine, or valine precursors or analogues. This extreme, apparent specificity might suggest that the low-affinity transport systems are kinetic artifacts, although this is unlikely because they show Michaelis-Menten type of kinetics. In the following paper we show that a specific mutation abolishes one of the low-affinity transport systems (8).

DISCUSSION

In this paper several different transport processes for branched-chain amino acids are described. There is no discrepancy between our findings and the conclusion reached by previous workers, namely, that one transport system exists for isoleucine, leucine, and valine (15). In fact, the conditions used by other workers were such that the very-high-affinity and the high-affinity transport processes were assayed. Since isoleucine, leucine, and valine are substrates of these transport processes, cross-inhibition experiments with these three amino acids indicate the presence of only one transport process. Inhibition by other amino acids can help in distinguishing these transport processes only if the inhibitor is used at the proper substrate concentration. For example, alanine inhibition of uptake of branched-chain amino acids has already been reported (18). These authors were measuring not only the very-high-affinity but also the high-affinity transport process, and therefore the inhibition reported by them was less pronounced than that reported in Table 3. In agreement with our data, preliminary kinetic evidence for transport systems with a K_m value lower than 10^{-6} M has been reported (7, 15, 20).

We cannot exclude that there are more transport systems besides those for which we give evidence. Indeed, the data of Table 3 suggest that there might be a transport system specific for leucine with an apparent K_m value lower than 0.5×10^{-4} M (the K_m value of the low-affinity transport system for leucine). An analogous suggestion for the existence of such a transport system comes also from the description of a binding protein isolated from the osmotic shock fluid of a derivative of *E. coli* K-12 that binds only leucine (7). However, we have not been able to find a leucine-specific high-affinity transport system, so that a possi-

TABLE 9. *Inhibition of low-affinity transport of isoleucine,[a] leucine, or valine in strain MI148a by different substances*

Inhibitor	Inhibition of transport of substrates[b]:		
	Isoleucine	Leucine	Valine
None	0.268	0.305	0.267
Arginine + lysine + cysteine + methionine[c]	0.184	0.200	0.187
Glycine + alanine + serine + threonine[c]	0.202	0.280	0.243
Histidine + tryptophan + phenylalanine[c]	0.195	0.194	0.252
Aspartate + asparagine + glutamate + glutamine + proline	0.181	0.260	0.237
DL-Norvaline[d]	0.260	0.318	0.215
L-cycloleucine[c]	0.259	0.354	0.261
DL-*O*-methylthreonine[d]	0.212	0.305	0.175
DL-Aza-leucine[d]	0.281	0.346	0.201
L-Amino butyric acid[c]	0.264	0.310	0.207
Thiaisoleucine[d]	0.185	0.296	0.175
Trifluoroleucine[d]	0.237	0.233	0.208
Sodium-α-acetolactate (1 mM).	0.237	0.337	0.203
Sodium-α,β-dihydroxyisovalerate[d]	0.339	0.349	0.212
α-Ketoisovalerate[d]	0.235	0.321	0.140
β-Isopropyl malate[d]	0.243	0.348	0.208
Sodium-α,β-dihydroxy-β-methylisovalerate	0.235	0.413	0.188

[a] [^{14}C]isoleucine, -leucine, or -valine concentration was 2×10^{-4} M. When isoleucine transport was measured, 2×10^{-3} M nonradioactive leucine and valine were present; when leucine transport was measured, 2×10^{-3} M isoleucine and valine were present; when valine transport was measured, 2×10^{-3} M isoleucine and leucine were present. For other details see Materials and Methods.

[b] Values reported are micromoles (of either [^{14}C]isoleucine, [^{14}C]leucine, or [^{14}C]valine) per 0.5 min per g of cells.

[c] Concentration of these inhibitors was 2 mM (final concentration for each one of them when present together).

[d] Concentration of these inhibitors was 4 mM.

ble alternative explanation for the data of Table 3 should be sought. The leucine-binding protein described by Furlong and Weiner (7) might be required for one of the other transport systems; for example, it might be required for leucine uptake through the very-high-affinity transport system or it might serve another function. We have isolated and purified both the isoleucine-leucine-valine-binding protein and the leucine-binding protein from strain MI148a. Their dissociation constants and inhibition of binding by trifluoroleucine are in agreement with the published values (7, 14) (experiments not reported).

If it is possible that another transport system for leucine exists, this is unlikely in the case of isoleucine. In fact, when the very-high-affinity transport process is inhibited by methionine and the other transport processes (high affinity and low affinity for isoleucine) are abolished by mutation, an isoleucine-requiring (*ilvA*) strain will not utilize isoleucine for growth (8).

Data of this paper also define conditions under which a specific transport system can be analyzed. To assay the very-high-affinity transport process, 10^{-8} to 4×10^{-8} M ^{14}C-labeled substrate concentrations should be used, and the fraction of uptake through the high-affinity transport process can be estimated by measuring the fraction resistant to methionine inhibition or repression. If a mutant lacking the high-affinity transport process is used, the methionine-resistant fraction will disappear. To assay the low-affinity transport process, a higher ^{14}C-labeled substrate concentration should be used, whereas the transport systems with higher affinity can either be inhibited with the two branched-chain amino acids not used as substrates or abolished by mutation.

The multiplicity of transport systems for a given substance or group of substances has already been reported in several cases, as, for example, for histidine, aromatic amino acids, and tryptophan (see 13 for references). It is clear, therefore, that *E. coli* uses a substantial fraction of its genome for transport. The usefulness of multiplicity from an evolutionary point of view is not easy to understand since most other genes are not duplicated. It is probable, therefore, that these different transport systems serve different functions. We do not know yet what these functions might be, but it is now easy to construct strains missing one or more transport systems and analyze different parameters such as pool size, excretion, and regulation of amino acid biosynthesis. We already reported (9), with one of these mutant strains, an observation so far unexplained. The isoleucine-valine biosynthetic enzymes are not fully repressed when a strain missing one of the two high-affinity transport systems (strain MI174b) is grown in rich medium.

Previous authors (14, 16) have given evidence for repressibility of leucine uptake in *E. coli*. This would lead to a paradox because less uptake would be present in a cell which, because of growth in a medium supplemented with branched-chain amino acids, contains also a repressed level of the specific biosynthetic enzymes and therefore needs more external branched-chain amino acids. The multiplicity of transport systems described in this paper partially explains the paradox. In fact, we show that methionine causes repression only of the very-high-affinity transport process, and it is likely that the previous observation on repressibility of leucine uptake concerned the same transport process. Therefore, growth in the presence of methionine causes repression of only a fraction of total uptake. The physiological meaning of this repression is not, however, easily understandable. In fact, if a very-high-affinity transport process for branched-chain amino acids is not needed when these are abundant in the growth medium since the other transport systems would be equally efficient, it would certainly be needed in a medium containing methionine and very low concentrations of branched-chain amino acids. It is likely, therefore, that either regulation of the very-high-affinity transport process is more complex than what appears from the data reported in this paper, or the very-high-affinity transport system(s) serves another function(s) besides simple transfer of the amino acids from the medium into the cells.

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