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Transport of L-4-Azaleucine in *Escherichia coli*

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The uptake of L-4-azaleucine was examined in *Escherichia coli* K-12 strains to determine the systems that serve for its accumulation. L-4-Azaleucine in radiolabeled form was synthesized and resolved by the action of hog kidney *N*-acyl-amino-acid amidohydrolase (EC 3.5.1.b) on the racemic α-*N*-acetyl derivative of DL-[*dimethyl*-^{14}C]4-azaleucine. L-4-Azaleucine is taken up in *E. coli* by energy-dependent processes that are sensitive to changes in the pH and to inhibition by leucine and the aromatic amino acids. Although a single set of kinetic parameters was obtained by kinetic experiments, other evidence indicates that transport systems for both the aromatic and the branched-chain amino acids serve for azaleucine. Azaleucine uptake in strain EO317, with a mutation leading to derepression and constitutive expression of branched-chain amino acid (LIV) transport and binding proteins, was not repressed by growth with leucine as it was in parental strain EO300. Lesions in the aromatic amino acid transport system, *aroP*, also led to changes in the regulation of azaleucine uptake activity when cells were grown on phenylalanine. Experiments on the specificity of azaleucine uptake and exchange experiments with leucine and phenylalanine support the hypothesis that both LIV and *aroP* systems transport azaleucine. The ability of external azaleucine to exchange rapidly with intracellular leucine may be an important contributor to azaleucine toxicity. We conclude from these and other studies that at least four other processes may affect azaleucine sensitivity: the level of branched-chain amino acid biosynthetic enzymes; the level of leucine, isoleucine, and valine transport systems; the level of the aromatic amino acid, *aroP*, uptake system; and, possibly, the ability of the cell to racemize D and L amino acids. The relative importance of these processes in azaleucine sensitivity under various conditions is not known precisely.

DL-4-Azaleucine was first synthesized in 1963 as an analogue of leucine (24) and subsequently isolated as a metabolic product of *Streptomyces neocaliberis* (5). It is a potent growth inhibitor of *Escherichia coli* (20, 24), *Salmonella typhimurium* (25), *Pseudomonas mildenbergii* (5), *Proteus rettgeri* (5), *Sarcina lutea* (5), *Leuconostoc dextranicum* (5), and blue-green algae (1). The toxicity of azaleucine has been associated with its incorporation into protein in place of leucine, resulting in inactive enzymes (4, 20), and with its repression of the biosynthetic enzymes for leucine, isoleucine, and valine (6, 20, 28). In *E. coli*, L-leucine prevents the inhibition of growth by azaleucine, whereas isoleucine or valine fails to prevent inhibition (24). Of the 20 naturally occurring amino acids tested for reversal of the growth inhibition caused by azaleucine in *S. typhimurium* (25), only leucine completely reversed the inhibition. The amino acids phenylalanine, tyrosine, and tryptophan partially overcame the inhibition, whereas all other amino acids were ineffective. These results led Stieglitz and Calvo (25) to suggest that the uptake of azaleucine occurred by both a general tranport system for aromatic amino acids and by a leucine transport system.

Resistance to the growth inhibitory effects of azaleucine has often been used as a method to select mutants derepressed in the biosynthetic enzymes for the branched-chain amino acids (18). Resistance to azaleucine has also been used to select mutants that presumably have lost transport systems for azaleucine. When amino acid uptake is measured in some of these strains, either a specific defect in branched-chain amino acid transport or a general membrane defect involving the uptake of several structurally unrelated amino acids and sugars has been found (S. C. Quay, M. Rahmanian, and D. L. Oxender, Fed. Proc. **33**:1394, 1974).

The purpose of this report is to describe more completely the transport systems serving for

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azaleucine and its growth inhibitory properties in *E. coli* strain K-12. The resolution of DL-4-azaleucine by the action of renal aminoacylase I (*N*-acylamino-acid amidohydrolase, EC 3.5.1.b) on the *N*-acetyl derivative of the racemic amino acid mixture permitted, for the first time, transport studies with L-4-azaleucine. Our results indicate that L-4-azaleucine uptake occurs by an energy-dependent process and is inhibited by leucine and the aromatic amino acids. The uptake of L-azaleucine in mutants altered in the shock-sensitive, branched-chain amino acid (LIV-I) transport system or in the aromatic amino acid (*aroP*) transport system suggests that both systems serve for the uptake of L-azaleucine by *E. coli*.

MATERIALS AND METHODS

Bacteria and medium. The *E. coli* K-12 strains used in these experiments are listed in Table 1. All transductions were performed with P1 phage as described previously (15). Strain EO346 was constructed by transducing strain EO302 to leucine independence with strain EO220 (strain AB1157, with an aromatic amino acid transport, *aroP*, mutation). Co-transduction of the *aroP* marker with *leu* was established by resistance to 40 mg of β-thienylalanine per liter. Cultures were grown to mid-logarithmic growth on Vogel-Bonner minimal medium E (27) supplemented with 0.2% D-glucose as the carbon source unless stated otherwise in the text. Amino acid supplements were 25 mg/liter unless stated otherwise. Cells were harvested by centrifugation at 6 C and washed in the growth medium minus glucose and containing 80 mg of chloramphenicol per liter.

TABLE 1. *E. coli strains*

Strain	Genotype	Relevant distinctive characteristics or source
EO300	Wild type K-12	ATCC 14948
EO301	*leu, trp*	Parent of EO300 series; R. Somerville
EO302	*leu*	By transduction of EO301
EO311	*leu, trp*	D-Leucine utilizations (Dlu); constitutively derepressed branched-chain amino acid transport and branched-chain amino acid-binding proteins (22)
EO312	*trp*	Dlu; by transduction of EO311
EO317		Dlu; by transduction of EO312
EO324	*leu, trp*	Dlu; R-10; by penicillin selection for nonutilization of D-leucine in strain EO311; defective LIV-I transport system not affecting the LIV-binding proteins (21)
EO325	*leu*	By transduction of EO324
EO345		By transduction of EO325
EO346	*aroP*	By co-transduction of EO302 to leucine independence and β-thienylalanine resistance from strain EO220 (strain AB1157, *leu*$^+$, *aroP*)

Transport assay. Procedures for measuring transport were similar to those reported earlier (21). The rate of azaleucine uptake was sufficiently linear with time at the concentrations used in this study so that 1- and 2-min observations approximated the initial rate of entry. The incubation mixture at 37 C consisted of Vogel-Bonner buffered salts (pH 7), the radioactive amino acid, and 40 mg of chloramphenicol per liter. All experiments were made in duplicate. The quantity of cells used was estimated by the absorbance at 600 nm, using a previously determined standard curve relating absorbance and dry weight.

Zone-of-inhibition assay. Approximately 10^7 cells were plated on minimal medium agar plates containing 0.2% D-glucose and a specific amino acid in the required cases. Sterile 14-mm Whatman no. 3 filter disks containing the desired concentration of azaleucine were placed in the middle of the plates, and the diameters of the clear zones without growth surrounding the disk were measured after 24 h at 37 C, the diameters of the disk excluded.

Synthesis and resolution of L-[dimethyl-^{14}C]4-azaleucine, 2-amino-3-[dimethyl-^{14}C]aminopropionic acid, as its 2-N-acetyl derivative. Acetamidoacrylic acid (200 mg) was reacted for 72 h at 40 C with 1.23 ml of 32% aqueous dimethylamine, containing 0.1 mCi of the ^{14}C-labeled form as the hydrochloride, which we had purchased from Mallinckrodt Chemical Co. The solution was dried in a desiccator over concentrated H_2SO_4. The residue was taken up in 1.5 ml of absolute ethanol, seeded, and permitted to crystallize at 5 C (24).

Resolution. A 210-mg amount of another preparation of 2-acetoamido-3-[*dimethyl*-^{14}C]aminopropionic acid hydrochloride was dissolved in 3 ml of water. A drop of phenol red was added, and about 1 ml of N LiOH was added to obtain a pH of about 7.5. A 25-mg amount of hog kidney acylase I, *N*-acylamino-acid amidohydrolase (EC 3.5.1.b; Schwarz/Mann, 4,010 U/mg), was added, and the solution was incubated at 37 C. LiOH was added dropwise to maintain the pH. After 4 h, an additional 3 mg of acylase was added. After 6 h the pH was lowered to about 5 with acetic acid and, to remove proteins, the solution was shaken vigorously and repeatedly with mixtures of chloroform (1.2 ml) and *n*-amyl alcohol (0.12 ml) until no further precipitate formed during 2 h of shaking. The aqueous solutions and washings were combined and taken to dryness. After redissolving, the product was treated with 50 mg of activated charcoal. The filtrate was then placed on an Amberlite IRC-50 column (30 by 1 cm), and water was passed through slowly to remove the unsplit acetamido acid. The free L amino acid was then eluted from the column with 0.5 N HCl. This eluate was taken to dryness and taken up in absolute

alcohol plus a few drops of water as needed. Crystallization then followed at 5 C. A nonradioactive preparation showed $[\alpha]_D^{25} = +22°$ (C = 2 in 5 N HCl). A small sample of the labeled compound on a paper chromatogram, developed with ethanol-14 M aqueous NH_3- water (80:5:15), showed over 98% of the counts at an R_f of 0.53 and less than 0.2% at 0.64, the position taken by the acetamido acid. The material was subjected to purification by paper chromatography before use. Tritiation by the Wilzbach procedure, either after resolution or at the stage of the acetamido acid, led to a heterogeneous final product from which satisfactory samples were not recovered.

Chemicals. DL-Azaleucine was synthesized and also obtained from Calbiochem; acetamidoacrylic acid and dimethylamine were purchased from Aldrich Chemical Co. Chloramphenicol was purchased from Sigma Chemical Co. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone was the generous gift of P. G. Heytler, E. I. du Pont de Nemours and Company. Radioactive compounds not synthesized in this laboratory were obtained from New England Nuclear. Other chemicals were of reagent grade. Optically active amino acids were in the L form unless specified otherwise.

RESULTS

Characterization of L-4-azaleucine transport. The uptake of L-azaleucine by *E. coli* K-12 was studied in four isogenic strains derived from *E. coli* wild-type EO300. Two strains have previously characterized mutations (21, 22) in branched-chain amino acid transport. Strain EO312 is constitutively derepressed for leucine, isoleucine, and valine transport by a shock-sensitive (LIV-I) transport system. Strain EO345 was derived from strain EO312 and has lost uptake by the LIV-I system while retaining the membrane-bound (LIV-II) system for branched-chain amino acids. Strain EO346 has lost the transport system for the aromatic amino acids (*aroP*) (2, 3). The uptake of azaleucine at 50 μM in each of these strains was examined at 37 C and was found to be linear for at least 2 min. A 3- to 20-fold steady-state concentration of azaleucine was reached in about 8 min. Inhibitors of energy conservation processes were tested for inhibition of transport to confirm that azaleucine uptake requires metabolic energy. Transport was reduced by greater than 90% with 10 mM 2,4-dinitrophenol, sodium cyanide, or sodium arsenate (pH 7), or by 60 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone. The addition of 10 mM D-glucose or 10 mM DL-lactate stimulated azaleucine uptake by 15 and 91%, respectively.

The concentration dependence of azaleucine uptake was examined next in these strains. When the azaleucine level was varied from 0.01 to 0.5 mM, behavior characteristic of a single, saturable uptake process was observed for the four strains. The K_m and V_{max} for azaleucine uptake in strains EO300, EO317, and EO346 were 0.1 mM and 0.5 mmol/kg of cells (dry weight) per min, respectively. Only strain EO345, with a defect in the LIV-I uptake system, had a change in the kinetic parameters of azaleucine transport. This strain showed a K_m of 0.65 mM and a V_{max} of 1.0 mmol/kg of cells (dry weight) per min.

These kinetic parameters were determined at pH 7.0. Since azaleucine has a pK_{2a} of 6.8 (8), one would expect the transport activity to vary with pH under physiological conditions if the state of protonation of this group is important for uptake. The uptake of 77 μM azaleucine in strain EO300 as a function of pH is described in Fig. 1. These results indicate that one of the dipolar ionic forms has the greatest reactivity for uptake. Even though the α,β-dipolar ion was shown to predominate in aqueous solution, we assume as for other cells and other substrates that the less abundant α,α-dipolar ion actually serves as the substrate.

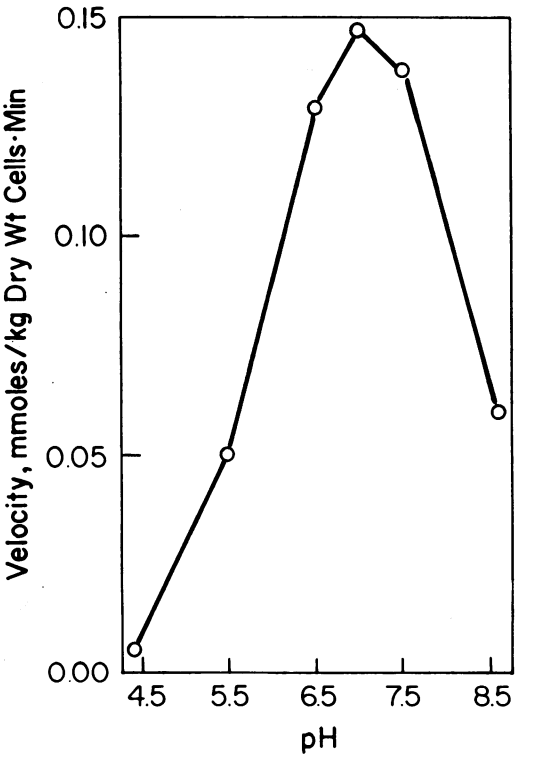

FIG. 1. *Uptake of L-azaleucine as a function of pH. Cells were harvested in Vogel-Bonner buffer titrated to the required pH and containing 80 mg of chloramphenicol per liter. The uptake of 77 μM L-azaleucine was measured during a 2-min interval. The medium pH was measured before and after the experiment and did not change significantly.*

These preliminary experiments indicate that azaleucine is transported as a neutral amino acid by a saturable process which requires metabolic energy and that strains with mutations in one of the LIV transport systems have a corresponding defect in azaleucine uptake.

Regulation of L-azaleucine transport. The regulation of transport systems for leucine, azaleucine, and phenylalanine was examined by growing strains in minimal medium supplemented with either leucine or phenylalanine, or by growing strains in L-broth (16) and then examining the transport of leucine, phenylalanine, and azaleucine (Table 2).

The repression of leucine transport by growth on leucine confirmed earlier work (17, 21, 26). As shown in the table, leucine transport in wild-type EO300 and the *aroP* mutant EO346 was repressed by growth with leucine or in L-broth. Strain EO317 had been shown previously to have a constitutively derepressed LIV-I transport activity (21, 22). This conclusion was confirmed in these experiments. Strain EO345 has a mutation (*dluR-10*) leading to loss of the LIV-I system (21) and shows the nonrepressible and low-capacity leucine uptake attributed to the LIV-II system, which is unaffected in this mutant.

Phenylalanine transport and regulation by growth on phenylalanine were similar in the parental strain EO300 to that in the strains with mutations in branched-chain amino acid transport, EO317 and EO345. The *aroP* mutation in strain EO346 caused a large decrease in phenylalanine uptake in cells grown in minimal medium. The low level of transport was not repressed by growth on phenylalanine.

The regulation of azaleucine transport was examined next to identify the pattern of its repression in these strains. Azaleucine uptake was repressed by growth of parental strain EO300 with either leucine or phenylalanine. Azaleucine transport in the derepressed, constitutive LIV-I transport mutant EO317 was not repressed by growth with leucine, although phenylalanine repressed the level to less than one-sixth. This pattern of regulation was also observed in strain EO345, which has intact the *aroP* and LIV-II transport systems, but not the LIV-I system. A regulation pattern similar to that for leucine uptake was observed for azaleucine transport in the *aroP* mutant EO346. Although quantitative interpretations must be made with caution because of the low specific activity of the L-[^{14}C]4-azaleucine, the patterns of regulation of azaleucine uptake in these strains indicates that the general branched-chain amino acid transport systems LIV-I and LIV-II and the aromatic amino acid system *aroP* serve for the intracellular accumulation of azaleucine.

Specificity of L-azaleucine transport. The specificity of L-azaleucine uptake was determined by examining the rate of its transport at a level of 0.1 mM with potential inhibitors at a level of 1 mM. The results of such experiments (Table 3) indicate that leucine and the aromatic amino acids phenylalanine, tyrosine, and tryptophan are the best inhibitors of azaleucine uptake. When phenylalanine and leucine were present together (data not shown), the uptake of azaleucine was inhibited completely, demonstrating the additive action of these compounds as inhibitors of the transport of azaleucine. Histidine, a substrate of the aromatic transport system (2, 3), was also a good inhibitor. Substrates or inhibitors of branched-chain amino acid transport systems such as methionine

TABLE 2. *Regulation of leucine (Leu), phenylalanine (Phe), and azaleucine (Aza) transport*

Growth conditions[a]	Transport activity[b]											
	EO300			EO317			EO345			EO346		
	Leu	Phe	Aza	Leu	Phe	Aza	Leu	Phe	Aza	Leu	Phe	Aza
Minimal medium	44	79	190	146	63	75	6	72	75	75	11	105
Same + leucine	4	77	100	162	68	65	5	53	75	4	11	15
Same + phenylalanine	38	15	50	187	21	10	6	12	25	68	9	50
L-broth	4	10	10	151	11	10	6	8	10	5	5	2

[a] Cells were grown at 37 C with shaking of Vogel-Bonner minimal medium E with 0.2% D-glucose as carbon source and with 0.4 mM leucine or 1 mM phenylalanine present as indicated, or in L-broth.

[b] Transport activity was measured with leucine at 0.19 μM, phenylalanine at 0.38 μM, or L-azaleucine at 77 μM. The specific activity is expressed as micromoles of substrate taken up per kilogram of cells (dry weight) per

Corrections for p. 958:

(13, 26), valine, isoleucine, norleucine (J. M. Wood, Fed. Proc. **33**:1394, 1974), and homoserine (26) were modest inhibitors. The slight inhibition by arginine and lysine may reflect the degree to which azaleucine is transported as a cationic amino acid. Table 4 shows that the inhibition of 77 μM azaleucine uptake by 1 mM arginine or lysine increased as the pH decreased. It seems probable, therefore, that at acidic pH values transport of the cationic form of azaleucine can occur by the systems for the basic amino acids. Leucine or phenylalanine at 1 mM reduced the uptake of azaleucine by 60% or more at all hydrogen ion concentrations tested, however, indicating the neutral species to be the major form of azaleucine transported even at these lower pH values.

The inhibition of the uptake of azaleucine by leucine and phenylalanine was studied in more detail by determining the K_i values for these two amino acids on azaleucine uptake (Table 5). In the parental strain, leucine showed a much higher K_i than did phenylalanine. The mutations in strain EO317 led to an improvement in the ability of leucine to inhibit azaleucine uptake while making inhibition of azaleucine transport by phenylalanine too small to observe readily. The loss of the LIV-I transport system in strain EO345 and the loss of the aromatic system in strain EO346 produced the expected changes in the ability of leucine or phenylalanine, respectively, to decrease azaleucine accumulation. These results support the conclusion that both the aromatic amino acid and the branched-chain amino acid transport systems serve for azaleucine uptake. Experiments showed that the inhibition of azaleucine transport by leucine or phenylalanine was accompanied by the uptake of these amino acids. The uptake of leucine or phenylalanine each at a 0.1 μM level was measured in the presence or absence of 1 mM azaleucine or other inhibitors. Table 6 contains the results and shows that the uptake of both leucine and phenylalanine is significantly lowered by azaleucine. As ex-

TABLE 3. *Specificity of* L-*azaleucine uptake in strain EO300*

Inhibitor[a] (1 mM)	Inhibition[b] (%)
Leucine, tryptophan, tyrosine, phenylalanine	81–88
Histidine	70
D-Phenylalanine	67
Methionine	55
Azaleucine, valine, homoserine	40–49
Isoleucine, norvaline	33–36
D-Leucine, threonine, arginine, lysine	19–25
Glycine, alanine, proline, glutamine, ornithine, homoarginine, serine, *trans*- and *cis*-hydroxyproline	<8

[a] Optically active amino acids are of the L configuration unless stated otherwise.

[b] The uptake of L-azaleucine at a level of 0.1 mM was measured for 1 min to approximate initial rates.

TABLE 4. *Effect of pH changes on the inhibition of* L-*azaleucine uptake*

pH	Inhibition of azaleucine uptake[a] (%)			
	Arginine	Lysine	Leucine	Phenylalanine
5.5	20	28	74	59
6.5	7	19	91	62
7.5	9	0	76	68
8.6	0	0	59	77

[a] The inhibition of the uptake of 77 μM L-azaleucine was measured with arginine, lysine, leucine, or phenylalanine (1 mM) as inhibitor. The control rates of azaleucine uptake in the absence of inhibitors were 0.05, 0.13, 0.14, 0.16 mmol/kg of cells (dry weight) per min at pH 5.5, 6.5, 7.5, and 8.6, respectively.

TABLE 5. *Inhibition constants of leucine and phenylalanine on* L-*azaleucine uptake in strain EO300*

Strains	Inhibition constants (K_i)[a]	
	Leucine	Phenylalanine
EO300	135	3
EO317	50	>200
EO345	500	3
EO346	90	>200

[a] The azaleucine concentrations in these experiments ranged from 25 to 100 μM. Uptake was observed for 2 min. The values of K_i (micromolar) were estimated by the method of Dixon and Webb (12).

TABLE 6. *Inhibitors of leucine and phenylalanine uptake in strain EO300*

Inhibitor (1 mM)	Leucine uptake[a]	Phenylalanine uptake
None	9.6	10.6
Azaleucine	2.1	3.5
Leucine	0.1	3.8
Isoleucine	0.3	10.6
Valine	0.4	9.8
Phenylalanine	7.2	0.1
Tryptophan	9.0	2.2
Tyrosine	6.2	0.2

[a] Uptake of 0.1 μM leucine or phenylalanine was measured for 30 s. The specific activity is expressed as micromoles taken up per kilogram of cells (dry weight) per minute.

pected, the branched-chain amino acids were strong inhibitors of leucine transport, whereas aromatic amino acids had little effect. Phenylalanine accumulation was strongly inhibited by the aromatic amino acids as expected, whereas the strong inhibitory effect of leucine, not shared by isoleucine or valine, was somewhat paradoxical. This inhibition may reflect the reaction of leucine with the *aroP* system at the high (1 mM) leucine levels used in these experiments.

Stimulation of exchange of intracellular and extracellular amino acids. The ability of externally added amino acids to stimulate the release of intracellular amino acids has been used to establish a common transport system for the compounds in question (29). To support our findings on the systems that serve for the uptake of azaleucine, the ability of internal azaleucine to exchange for external leucine or phenylalanine was tested. Figure 2A shows the rates of exit when parental strain EO300 was loaded from a 100 μM azaleucine solution. As had been shown earlier for other amino acids (17), little exit was observed in the absence of an external amino acid. Adding 1 mM cold DL-azaleucine or 0.1 mM leucine or phenylalanine caused the loss of half of the internal azaleucine during the first 2 min of incubation. External azaleucine stimulated only weakly the exodus of labeled azaleucine. When the cells were preloaded with radiolabeled leucine (Fig. 2B), azaleucine and leucine rapidly exchanged for the cellular leucine, whereas external phenylalanine was ineffective in exchanging for leucine. The exodus of phenylalanine (Fig. 2C) was stimulated by azaleucine and phenylalanine as well as by leucine, although the steady-state level reached was somewhat lower with azaleucine and phenylalanine than with leucine. These results are consistent with the earlier observation (Table 6) that phenylalanine uptake was inhibited by leucine. Kinetic relations that could account for the observation that external leucine stimulated the exodus of internal phenylalanine, whereas phenylalanine did not stimulate the exit of internal leucine, have been discussed earlier (9). Brown (7) has noted that external leucine and azaleucine could displace 26 and 40%, respectively, of the cellular tyrosine. He attributed this finding to a small degree of reactivity of leucine with the *aroP* system. Our results support this conclusion.

Growth inhibition by azaleucine in strains with alterations in transport systems. The ability of the racemic compound DL-azaleucine to inhibit growth was examined in these strains. The effect on azaleucine sensitivity of the transport mutations in these strains was estimated

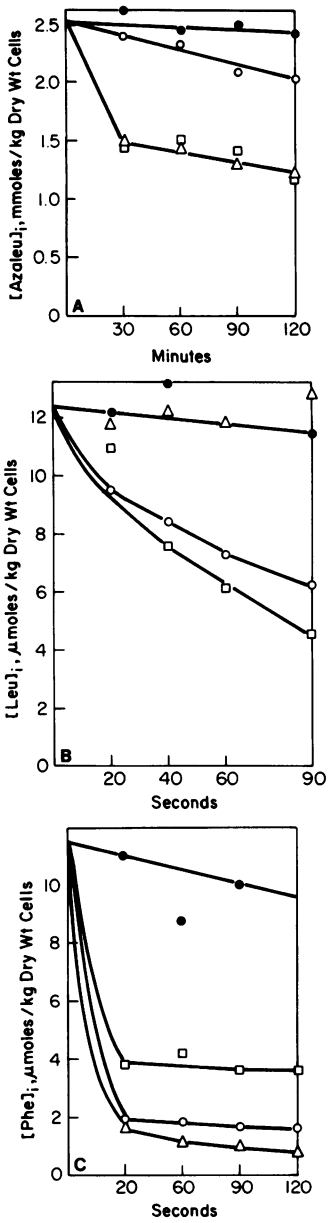

FIG. 2. *(A) Exchange exodus of azaleucine in strain EO300. Cells were loaded from 100 μM [^{14}C]-azaleucine for 15 min. They were then collected by centrifugation and suspended in either amino acid-free buffer (●), 1 mM DL-azaleucine (○), 100 μM leucine (□) or 100 μM phenylalanine (△). Samples were removed at the indicated time intervals and filtered, and intracellular [^{14}C]azaleucine was determined by liquid scintillation spectrometry. (B) Exchange exodus of leucine in strain EO300. Cells were loaded from 0.1 μM L-[^{3}H]leucine for 5 min. Other procedures and symbols as above. (C) Exchange exodus of phenylalanine in strain EO300. Cells were loaded from 0.1 μM L-[^{3}H]phenylalanine for 5 min. Other procedures and symbols as above.*

by measuring the size of the zone of inhibition on minimal medium agar plates (Table 7). The strain with derepressed leucine transport (EO317) showed the greatest sensitivity to azaleucine. The sensitivity to the analogue of strains EO300 and EO345 was similar. Strain EO346, the *aroP* mutant, was remarkably resistant to azaleucine even at a concentration of 6 mg, over two orders of magnitude higher than the level effective in strain EO317.

The ability of various compounds to prevent growth inhibition by azaleucine was examined next by placing potential antagonists at 0.5 mM levels in the minimal medium agar plates (Table 8). Only leucine could antagonize completely the inhibition of growth by azaleucine. Substrates of the aromatic transport system were poor antagonists in strain EO317. Methionine has been shown previously to inhibit branched-chain amino acid transport (13), whereas growth on methionine represses the LIV-I transport system (13) and may increase the intracellular level of leucine (14). The strong antagonism of azaleucine toxicity by methionine in strain EO317 probably does not arise from a repression of transport, since this strain has nonrepressible LIV-I transport. The lack of sparing ability by methionine in strain EO345 suggests that the ability of methionine to enlarge the leucine pool may not be an important contribution to azaleucine resistance. These results taken together support the idea that the antagonism of azaleucine toxicity by methionine occurs through its inhibition of azaleucine transport.

DISCUSSION

L-4-Azaleucine uptake systems. The synthesis and resolution of L-4-azaleucine in radioactive form permitted the examination of azaleucine transport in *E. coli*. Preliminary experiments showed the uptake of azaleucine to be saturable and sensitive to inhibition by cyanide, dinitrophenol, arsenate, and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone. Although kinetic studies gave a single K_m and a single V_{max}, other evidence indicates that more than one system serves for azaleucine transport. Azaleucine uptake in strains with mutations in the aromatic (*aroP*) and branched-chain (LIV-I and LIV-II) amino acid transport systems led us to conclude that these systems serve for azaleucine accumulation. Experiments on the specificity of azaleucine uptake and exchange experiments with phenylalanine or leucine support this conclusion. The pattern of repression of azaleucine uptake by the wild-type and mutant strains grown on leucine and phenylalanine also support a role for both transport systems in azaleucine uptake, although the major route of entry seems to be that shared with phenylalanine.

The initial rate of azaleucine uptake observed under a given set of conditions should be a sum of the initial rates attributable to the participating transport systems if these operate independently. The steady state attainable with time will not, however, be any such sum, as one of us has pointed out elsewhere (10, 11). If

TABLE 8. *Antagonism of azaleucine growth inhibition by selected amino acids*[a]

Antagonist (0.5 mM)	Zone of growth inhibition	
	EO317[b]	EO345
Leucine	0	0
Methionine	13	22
Isoleucine	24	20
Tyrosine	24	14
Phenylalanine	26	14
Tryptophan	26	17
Histidine	29	19
None	31	22

[a] The ability of selected amino acids to antagonize the growth inhibition by azaleucine was assayed by the zone-of-inhibition assay. The potential antagonist was placed in minimal medium plates at a level of 0.5 mM.

[b] Strains EO317 and EO345 were grown on plates with 0.3 and 3.0 mg of DL-azaleucine, respectively, placed on the sterile filter disks.

TABLE 7. *Growth inhibition by* D L-*azaleucine*

Strain	Zone of growth inhibition[a]				
	0.04 mg	0.2 mg	1.0 mg	3.0 mg	6.0 mg
EO300				21	
EO317	7	31	86[b]	86[b]	
EO345	0	6	18	21	
EO346	0	0	0	0	2

[a] The zone of inhibition (millimeters) was measured after 24 h. The concentrations represent the amount of azaleucine added to the 14-mm disk.

[b] Complete inhibition of growth.

one of the systems operates more steeply uphill than the others, the steady state may well be composed principally of entry by that system and exodus by reversal of the least steeply uphill system. Therefore, it becomes possible that the chemical reaction driving one of these transport systems comes to drive also the reversal of the reaction driving another transport system, through a chemiosmotic-chemical coupling.

Azaleucine in solution at pH 7 exists both as a cationic and as an α,β-dipolar ionic amino acid. Ornithine and homoarginine, substrates of the lysine-, arginine-, and ornithine-specific and the arginine-specific uptake systems (19, 23), respectively, failed to diminish azaleucine uptake significantly. These results, taken together with the strong inhibition seen with leucine or phenylalanine, suggest a preference by the cell for a dipolar ionic form. One of us has noted a similar preference for a dipolar ionic form for azaleucine transport in the Ehrlich ascites tumor cell (8). The loss of transport reactivity on lowering the pH indicates that the apparent pK_{2a} applying in the membrane environment is displaced to lower pH values. A displacement of about one unit is suggested by the experiments depicted in Fig. 1. The loss of transport reactivity at alkaline pH values indicates the importance of a second titratable group in this pH range, presumably membrane-bound. The rather high K_m observed for azaleucine uptake could be consistent with the small amount of the α,α-dipolar ionic form present, if we assume that only this form is reactive for transport.

DL-4-Azaleucine growth inhibition. The ability of DL-azaleucine to inhibit growth was not always correlated with the rate or capacity of a strain to take up L-azaleucine. For example, strain EO317 was over 100 times more sensitive to inhibition by DL-azaleucine than strain EO346, although the kinetic parameters for uptake in these strains were indistinguishable. The observation that extracellular DL-azaleucine rapidly exchanges for the cellular leucine, but not vice versa, is important for understanding inhibition by azaleucine. We imagine that the sequence of events on exposure of a strain to azaleucine may be as follows. Azaleucine rapidly exchanges for the intracellular leucine; the decrease in intracellular leucine leads to a leucine-limiting signal for the derepression of the branched-chain amino acid biosynthetic enzymes; presumably intracellular azaleucine does not substitute for leucine in preventing this derepression. The substitution of azaleucine for leucine would lead to synthesis of partially active or inactive proteins. Azaleucine resistance could then result if the level of leucine biosynthetic enzymes were high enough before exposure to azaleucine to compensate for loss of leucine by exchange for azaleucine. This sequence could explain the observation that cells grown initially on complex medium show greater sensitivity to azaleucine than cells grown on minimal media, since the biosynthetic enzymes are initially fully repressed in the first case. The observation that strain EO303 (a Trp^- derivative of strain EO300) had 25 to 35% more *ilvB* and *leuB* gene product activities when grown in minimal medium than strain EO312 under similar growth conditions (S. C. Quay, D. L. Oxender, S. Tsuyumu, and H. E. Umbarger, personal communication) is also consistent with this hypothesis. However, the finding of a D-leucine-utilizing phenotype in a Leu^- derivative of strain EO317 not related to transport complicates these conclusions. This locus may be involved in conversion of D-leucine to L-leucine and could alter DL-azaleucine sensitivity by a similar action on this racemate (J. J. Anderson and D. L. Oxender, unpublished data).

Wasmuth and Umbarger (28) reported that azaleucine could replace leucine in multivalent repression of threonine deaminase-forming potential. In those experiments, leucine was added to a culture containing azaleucine, and enzyme activities were measured soon after, with the assumption that the added leucine permits normal protein synthesis. Our finding that about 60% of the intracellular azaleucine was not exchangeable for external leucine may, in fact, indicate that an assumption implicit in the experiments of Wasmuth and Umbarger is incorrect.

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