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Transport of Biosynthetic Intermediates: Regulation of Homoserine and Threonine Uptake in *Escherichia coli*

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Homoserine is transported by a single system that it shares with alanine, isoleucine, leucine, phenylalanine, threonine, valine and perhaps cysteine, methionine, serine, and tyrosine. We investigated the regulation of this transport system and found that alanine, isoleucine, leucine, methionine, and valine each repress the homoserine-transporting system. From the concentration resulting in 50% repression of this transport system and the maximal amount of repression, we ranked the amino acids according to their effectiveness in repressing homoserine transport (in decreasing order): leucine > methionine > alanine- > valine > isoleucine. The exponential rate of decrease in transport capacity after leucine addition equals the exponential growth rate of the culture, and protein synthesis is necessary for the derepression seen when leucine is removed. Threonine, in addition to using the above system, is transported by a second system shared with serine. We present further evidence for this serine-threonine transport system and show that it is not regulated like the homoserine-transporting system.

Although amino acid transport has been studied extensively, little is known concerning the transport of those amino acids that are biosynthetic intermediates or have multiple fates within the cell. The uptake of homoserine and threonine by *Escherichia coli* B/r was studied as an example of this phenomenon (39). It was found that homoserine is transported by a system shared with alanine, isoleucine, leucine, phenylalanine, threonine, and valine. Evidence for a common system derives from (i) competition studies, (ii) simultaneous reduction of the transport of these amino acids in cells grown with leucine, and (iii) simultaneous reduction of the transport of these amino acids in a strain selected only for lack of homoserine transport. Competition studies also suggest that cysteine, methionine, serine, and tyrosine might be transported by this system. Thus, the system that transports homoserine is similar to the LIV-I system described by Rahmanian et al. (33) and the "very-high-affinity" system described by Guardiola et al. (13).

Threonine, in addition to sharing the above system with homoserine, is transported by a second system shared with serine. The evidence for this second system consists of (i) incomplete inhibition of threonine uptake by any single amino acid, (ii) only partial loss of threonine uptake in the mutant unable to transport homoserine, and (iii) only partial reduction of threonine uptake when cells are grown in millimolar concentrations of leucine. In this last case, the remaining threonine uptake can be inhibited only by serine, and the inhibition is complete.

Early work had indicated that the transport of leucine was repressed by leucine, cycloleucine, and methionine (17). The broad specificity of the homoserine-transporting system (39) suggested that additional regulatory effectors might exist. In this paper we show that leucine, isoleucine, valine, alanine, and methionine can repress the transport system. The concentration ranges where these amino acids repress the transport system, as well as the time course for leucine repression and derepression, were determined. Additional evidence presented in this paper confirms the existence of a serine-threonine system and indicates that it is not regulated like the homoserine-transporting system.

MATERIALS AND METHODS

Organisms and growth conditions. This work was done with *E. coli* B/r and a mutant derived from it, strain Hr14, that lacks the homoserine-transporting system (39). The cells were grown in glucose-minimal medium (9) according to the methods described earlier (39). The medium was supplemented with amino acids where stated.

Transport assay in resting cells. Resting cells were prepared as described previously (39), except that NH_4^+ was omitted throughout. For the assay a portion of the cell suspension was diluted 10-fold

into glucose-minimal medium lacking NH_4^+ and incubated for 10 min at 37 C. At time zero part of this dilute cell suspension was added to the radioactive amino acid and the unlabeled additions, if any. A wide-bore pipette was used to ensure rapid delivery of the cells (less than 1 s). At the appropriate times, 1-ml samples were withdrawn, filtered, and counted as described previously (39). In all cases the initial rate of uptake was taken as a measure of the transport capacity of the cells. For homoserine, the initial rate was obtained by withdrawing samples at 30 and 60 s; in the case of threonine, samples were withdrawn at 15 and 30 s. All transport assays were done in duplicate.

Time course for repression. The cells were grown for more than four generations in glucose-minimal medium to a density of approximately 2×10^8 cells per ml. A portion of the culture was removed and immediately added to a tube containing [^{14}C]homoserine. Samples (1 ml) were withdrawn from the tube at 30 and 60 s, filtered, and later counted to determine the uptake of homoserine by cells grown in the absence of homoserine. The original culture was then diluted twofold with prewarmed, fresh medium plus leucine such that the final concentration of leucine was 1 mM. After mixing, the culture was maintained in exponential growth throughout the experiment by the appropriate dilutions with fresh, prewarmed medium. During incubation, portions were removed at appropriate times, centrifuged, and washed once with ice-cold minimal medium lacking glucose, NH_4^+, and leucine. The final pellet in each case was suspended in the original sample volume of fresh, prewarmed glucose-minimal medium lacking NH_4^+ and leucine. After 10 min at 37 C, the cells were assayed for the ability to take up [^{14}C]homoserine. Control experiments with cells grown in glucose-minimal medium indicated that there is no difference in the uptake of homoserine between growing cells and resting cells prepared as outlined above.

Time course for derepression. Derepression was initiated when cells previously grown with leucine were centrifuged, washed with ice-cold minimal medium lacking leucine and glucose, and suspended in the original volume of prewarmed glucose-minimal medium lacking leucine. At various times, samples were removed from the culture, and the growing cells were assayed for the uptake of [^{14}C]homoserine. As before, the culture was maintained in exponential growth by suitable dilutions with fresh, prewarmed medium. In a separate experiment, the culture was divided into two portions after the removal of leucine. One was treated as just described, whereas chloramphenicol (final concentration 50 μg/ml) was added to the other. At various times samples were removed from the culture containing chloramphenicol and treated, as described in the previous paragraph, to remove the chloramphenicol, and the cells were assayed for the uptake of [^{14}C]homoserine. Fresh medium was not added to the culture containing chloramphenicol when samples were removed.

It was necessary to remove the chloramphenicol prior to the transport assay because we found that homoserine is transported poorly in the presence of 50 μg of chloramphenicol per ml. However, if cells derepressed for the transport of homoserine were treated briefly with chloramphenicol, centrifuged, washed, and resuspended in fresh medium lacking chloramphenicol, then the uptake of homoserine proceeded reproducibly at half the rate observed with untreated cells. Reduction of protein synthesis seems to be involved rather than a direct inhibition of homoserine entry by chloramphenicol. Chloramphenicol at concentrations low enough not to inhibit protein synthesis, yet up to 30-fold greater than the homoserine concentration, did not compete with homoserine for uptake. Also, inhibitors of protein synthesis other than chloramphenicol reduced homoserine uptake.

Protein determination. The protein content of all cultures was determined by the method of Lowry et al. (26), with bovine serum albumin as a standard.

Materials. L-[U-^{14}C]homoserine (21 Ci/mol) and L-[U-^{14}C]serine (159 Ci/mol) were purchased from Amersham/Searle. L-[U-^{14}C]threonine (208 Ci/mol) was obtained from Schwarz/Mann. The chloramphenicol and all of the unlabeled amino acids were obtained from Sigma Chemical Co. Only the L-isomers of the amino acids were used.

RESULTS

Regulation of the homoserine-transporting system. The data presented previously (39) indicate that homoserine is transported by a system that has a broad specificity and is repressed by leucine. These results prompted us to ascertain whether other amino acids also might repress the transport of homoserine. Figure 1A summarizes these data. Most of the amino acids that compete with homoserine for uptake also repress the uptake of homoserine. At the concentration used, methionine represses the transport of homoserine almost as well as leucine; isoleucine, alanine, and valine repress the uptake of homoserine by at least 50%. Phenylalanine, tyrosine, and homoserine also seem to have some effect.

Additional experiments were done with alanine, isoleucine, leucine, methionine, and valine to determine at what concentrations these amino acids could repress the uptake of homoserine (Fig. 2). From these curves one can determine the maximal amount of repression and the concentration that causes 50% of the maximal repression. It is patent that these amino acids can be ranked in order of their effectiveness in repressing the uptake of homoserine (in decreasing order): leucine>methionine>alanine>valine>isoleucine.

Because repression caused by leucine was most effective, it was studied in greater detail. The time course of repression by leucine and derepression following the removal of leucine is shown in Fig. 3. A semilogarithmic plot of homoserine transport as a function of time

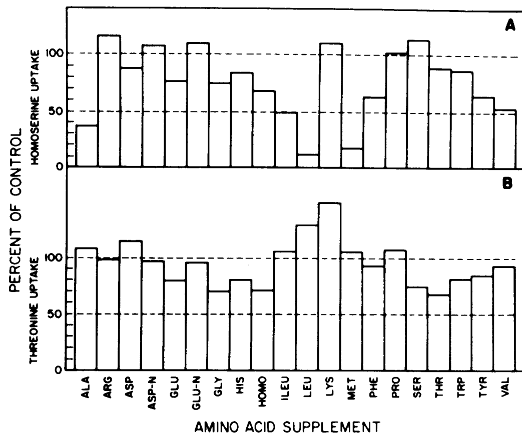

FIG. 1. *Regulation of homoserine or threonine uptake by various amino acids. The cells were grown exponentially for more than four generations, and their transport capacity was assayed. The growth media were supplemented with the indicated amino acid at a final concentration of 10^{-3} M. (However, 10^{-4} M tyrosine was used because of its low solubility, and cysteine, an amino acid that competes with homoserine for transport, was not studied because it is rapidly oxidized to cystine.) The transport capacity of cells grown in the presence of an amino acid is reported as a percentage of the transport capacity of cells grown in unsupplemented medium. The mean of at least three experiments is represented by each bar. The average percent variation about the means is 15 ± 7. Therefore, a change of twofold or more is considered significant. (A) The uptake of 1.57×10^{-6} M L-[^{14}C]homoserine by E. coli B/r. (B) The uptake of 1.3×10^{-7} M L-[^{14}C]threonine by strain Hr14, the mutant that lacks the homoserine-transporting system.*

following the addition of leucine shows that the rate of decay is equal to the growth rate of the culture (Fig. 4). This relationship suggests that the transport system is diluted out by growth. The data in Fig. 3 demonstrate that protein synthesis is required for derepression.

Serine-threonine transport system. Our earlier work showed that threonine uptake in leucine-grown cells is inhibited only by serine. Under these conditions, the average K_m for threonine uptake is 2×10^{-6} M and the K_i for serine inhibition of threonine uptake is 1.4×10^{-6} M. These data suggested that serine and threonine share a transport system (39). We now have data for the uptake of serine that confirms the existence of such a system.

The time course of serine uptake is depicted in Fig. 5. Robbins and Oxender (34) noted that serine uptake is difficult to measure because serine is so rapidly metabolized. To ensure the accuracy of our measurements we withdrew duplicate samples at 15, 30, 45, 60 s and used the slope of the line drawn through these points and the origin as a measure of the initial rate of serine uptake. The double-reciprocal plot of serine uptake as a function of concentration is linear over the concentration range 10^{-7} to 10^{-5} M. However, at concentrations above 10^{-5} M the double-reciprocal plot appears biphasic

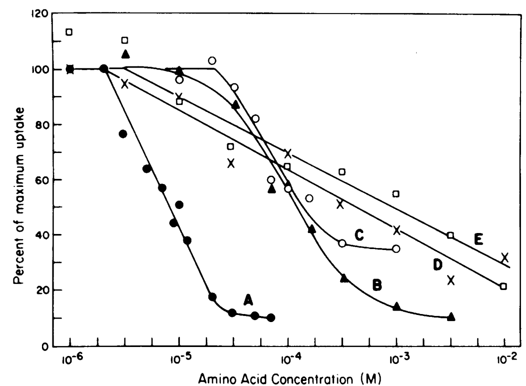

FIG. 2. *Repression characteristics for those amino acids that repress the uptake of homoserine. Wild-type cells were grown exponentially for more than four generations, and their ability to transport homoserine was assayed. The growth media were supplemented with amino acids at the concentrations shown. The maximal uptake is the amount of homoserine transported by cells grown with unsupplemented medium. The concentration of [^{14}C]homoserine in the transport assay was 1.57×10^{-6} M. Amino acid supplement in the growth medium: (A) leucine, (B) methionine, (C) alanine, (D) valine, and (E) isoleucine.*

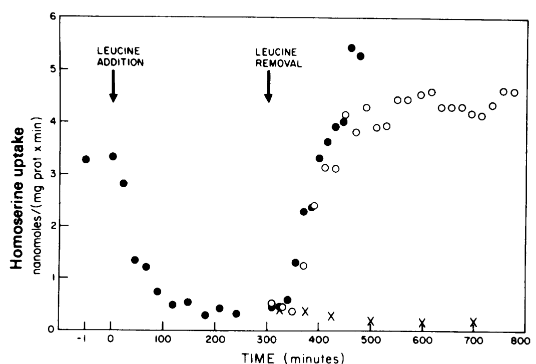

FIG. 3. *Time course for the repression of homoserine uptake by leucine and the subsequent derepression after the removal of leucine. Wild-type cells were prepared and assayed as described. The concentration of L-[^{14}C]homoserine used in the transport assay was 1.57×10^{-6} M. Symbols: ●, ○, minus chloramphenicol; ×, plus 50 μg of chloramphenicol per ml.*

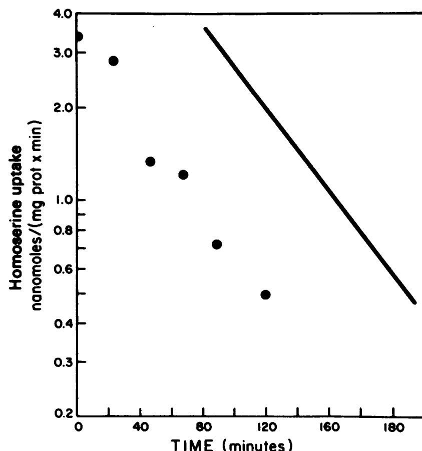

FIG. 4. *Repression time course plotted on semilog coordinates. The data (●) are replotted from Fig. 3. The solid line represents an exponentially decaying function with half-life equal to the doubling time of the culture (~46 min).*

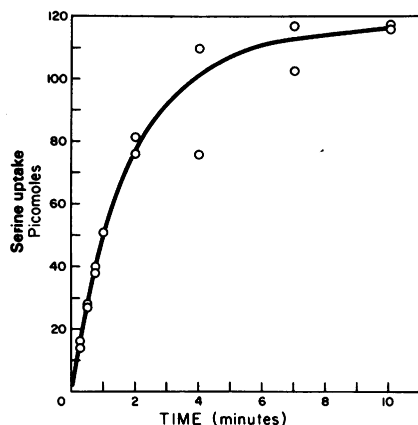

FIG. 5. *Time course for the uptake of serine. Wild-type cells were grown and assayed for their ability to transport serine. The transport assay was started by adding 20 μliters of L-[^{14}C]serine to 10 ml of cells. The final concentration of serine in the assay was 2 × 10^{-7} M.*

(Fig. 6). This nonlinearity is apparent when cells are grown in the presence and absence of leucine, so it is unlikely that it reflects the homoserine-transporting system. It is not known whether the nonlinearity is caused by another transport system for serine, the involvement of serine in cell wall biosynthesis, or negative cooperativity (24). The apparent K_m for serine uptake by the "high-affinity system" is 1.3 × 10^{-6} M.

Competition studies (data not shown) were done to enumerate the amino acids that could compete with serine for uptake. Of the 21 amino acids tested at concentrations 500 times the serine concentration, threonine was the only amino acid to inhibit the uptake of serine strongly. Threonine inhibition was approximately 85%. This inhibition pattern occurred whether the cells were grown in the presence or absence of leucine. The K_i for threonine inhibition of serine uptake is 1.8 × 10^{-6} M and the inhibition is competitive as shown in Fig. 7. When the serine K_m and the threonine K_i are compared with the threonine K_m and the serine K_i measured in leucine-grown cells, it is evident that the apparent K_m for [^{14}C]serine uptake is the same as the K_i for unlabeled serine inhibition of [^{14}C]threonine uptake and that a similar relation holds for threonine. These data provide

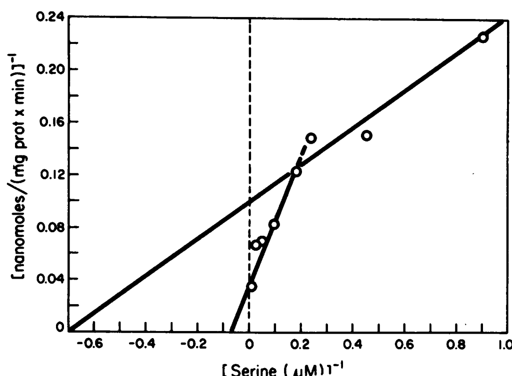

FIG. 6. *Double-reciprocal plot for serine uptake by E. coli B/r. The cells were grown, and their transport activity was measured. Initial rates were obtained as described in the text.*

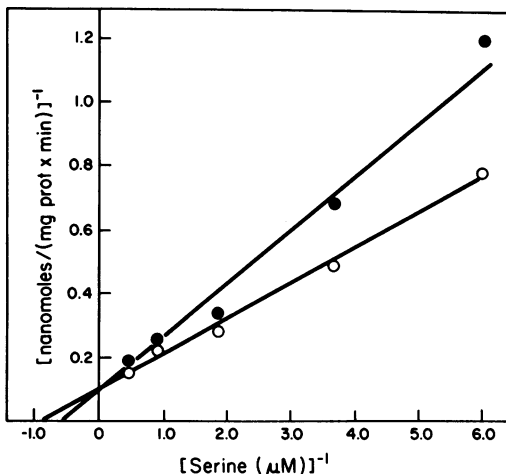

FIG. 7. *Double-reciprocal plots for the uptake of serine at low concentrations and the inhibition of serine uptake by 10^{-6} M threonine. Symbols: ○, minus threonine; ●, plus threonine.*

strong evidence for a serine-threonine transport system.

Regulation of the serine-threonine transport system. Experiments were done to determine whether the serine-threonine transport system is regulated like the homoserine-transporting system. Threonine uptake was used to monitor the activity of the serine-threonine system. To eliminate the contribution of the homoserine-transporting system, threonine uptake was measure in the mutant strain Hr14, which is unable to transport homoserine. A survey was done to ascertain whether growth on any amino acid would affect the transport of threonine (Fig. 1B). It is evident that none of the amino acids tested causes a twofold change in the uptake of threonine. Thus, not only does the serine-threonine transport system have a much narrower range of substrates than the homoserine-transporting system, but also it is not regulated like the homoserine-transporting system.

DISCUSSION

It has been known for some time that the transport systems for many of the sugars are inducible (31). On the other hand, the transport systems for the amino acids originally were thought to be constitutive (8, 31). Inducible transport systems for aspartate (22), cystine (4), glutamate (16, 27), proline (21), phenylalanine (14, 15), and tryptophan (5, 6, 37) have now been demostrated in bacteria, whereas repressible transport systems have been found for cystine-diaminopimelate (4), glutamate (28, 29), glutamine (40), lysine-arginine-ornithine (7, 30, 35, 36), and leucine-isoleucine-valine (1, 2, 10, 13, 17, 33).

Difficulties in detecting the regulation of transport systems arise from the multiplicity of systems that can transport a given compound. For example, even though one system is completely repressed, the uptake of a compound might be reduced only a small amount if another system for its transport exists. The uptake of threonine by *E. coli* illustrates this possibility. Threonine and homoserine are transported by a common system; yet the uptake of threonine is reduced only 40% in cells grown with leucine whereas the uptake of homoserine is virtually eliminated (39). This indicates an additional transport system for threonine. Additional complications are encountered if one transport system is induced under conditions that repress the other transport system. In this case, it is conceivable that no change in the rate of uptake of the compound would be observed. The uptake of alanine provides an example of this phenomenon (34). Alanine is transported by both the LIV-I and glycine transport systems in *E. coli* K-12. Under conditions that repress the LIV-I transport system, the uptake of glycine is doubled and the uptake of alanine remains unchanged. These difficulties would not occur if specific probes were available to independently monitor the separate systems.

By using homoserine as a specific probe for the transport system with broad specificity, some of the system's regulatory properties were delineated. Almost all of the amino acids that compete with homoserine for uptake repress the transport of homoserine to some extent (Fig. 1A). The exceptions are serine and threonine. The data in Fig. 1A are in agreement with earlier work indicating that leucine (1, 13, 17, 33), methionine (13, 17), and isoleucine (2) repress the transport system for the branched-chain amino acids. However, the concentration of leucine that causes half-maximal repression in *E. coli* K-12 (obtained by replotting Fig. 10 from reference 32) is 10 times higher than the value in Fig. 2. The reason for this difference is unknown. The results presented in Fig. 1A for isoleucine and valine are contrary to those previously reported (2, 19, 32). Penrose et al. (32) indicated that neither isoleucine nor valine repressed the uptake of leucine by *E. coli* K-12. The discrepancies are most likely due to the existence of multiple systems for leucine transport but only one for homoserine transport. It is also possible that strain differences are involved.

Contrary to the data in Fig. 1A, Anraku and his collaborators (2, 19) reported that valine induced the transport system for the branched-chain amino acids in *E. coli*. Because both K_m and V_{max} values for the three amino acids were altered (2), their data indicate that simple induction is not occurring. Moreover, they reported that the amount of binding protein released by osmotic shock was less from valine-grown cells than from cells grown without valine (2). These data suggest that perhaps valine represses one system but induces another.

The number of compounds that regulate this transport system is unusually large. Guroff and Bromwell (14) reported that both phenylalanine and tyrosine appear to induce a transport system for the aromatic amino acids in *Comamonas* sp. 11299a. Similarly, it has been reported that both arginine and ornithine repress the transport of the basic amino acids in *E. coli* (7, 36). In most instances, however, only one regulatory effector is reported.

The amino acids can be ranked according to their effectiveness in repressing homoserine transport. In decreasing order they are: leucine- > methionine > alanine > valine > isoleucine. For these amino acids to repress the uptake of homoserine, they must enter the cell, perhaps be metabolized, and possibly combine with a regulatory protein that eventually interacts with the deoxyribonucleic acid. One hypothesis to explain the order of effectiveness of the amino acids is their relative affinity for the transport system. However, by using the K_i values measured against threonine uptake as the criterion of affinity, the decreasing order is: isoleucine- > leucine > valine > alanine > methionine (39). This is not the same order obtained by ranking the amino acids according to their effectiveness in repressing homoserine transport. Also, the system has a greater affinity for threonine than for valine, alanine, or methionine, but threonine does not repress the uptake of homoserine. Therefore, it is unlikely that the affinity of the transport system for an amino acid is the sole criterion that determines a compound's effectiveness in repressing homoserine transport. Additional work will be necessary to delineate those steps in the sequence of events leading to repression that are crucial in determining the effectiveness of these amino acid effectors.

Our competition data (39) indicate that methionine, one of the amino acids causing the strongest repression of homoserine transport, might be transported by the homoserine-transporting system. Kadner (18) has studied more extensively the transport of methionine in *E. coli* K-12 and found two distinct transport systems. Coincidentally, his K_m for the "low-affinity" methionine transport system is 40×10^{-6} M, whereas our K_i for methionine inhibition of the homoserine-transporting system is 36×10^{-6} M (39), which might suggest that these systems are related. On the other hand, Kadner reported that the methionine transport systems are quite specific (e.g., isoleucine, leucine, and valine do not compete with methionine for uptake) and not regulated to any significant degree by methionine (18). Furthermore, all of Kadner's transport assays were done in the presence of 100 μg of chloramphenicol per ml, which, according to our results, probably eliminated any contribution of the homoserine-transporting system to methionine transport. Thus, it seems unlikely that the low-affinity methionine transport system of Kadner is identical with the homoserine-transporting system.

In addition to being transported by the homoserine system, threonine is transported by another system shared with serine. The following data strongly support the concept of a serine-threonine transport system. (i) Under conditions where the homoserine-transporting system is repressed, serine is the only inhibitor of threonine uptake and the inhibition is complete (39). (ii) Threonine is the only strong inhibitor of serine uptake under all conditions tested. (iii) The apparent K_m for [^{14}C]serine uptake equals the K_i for unlabeled serine inhibition of [^{14}C]threonine uptake, and the apparent K_m for [^{14}C]threonine uptake equals the K_i for unlabeled threonine inhibition of serine uptake. (The uptake of threonine was measured in leucine-grown cells [39].) The transport of serine and threonine by a common system also has been demonstrated in membrane vesicles prepared from *E. coli* (25), *Staphylococcus aureus* (38), and *Bacillus subtilis* (23). There is no apparent regulation of the serine-threonine transport system, which is in contrast to the variety of amino acids that regulate the homoserine-transporting system (Fig. 1A and 1B). However, it is possible that by using different carbon and/or nitrogen sources regulation would be detected. There is precedent for this. In fungi (3, 11, 12) there exists a transport system for amino acids that has broad specificity and is affected by the presence of NH_4^+. Similarly, the use of glucose as a carbon source prevents the induction of the transport systems for tryptophan (6) and the C_4-labeled dicarboxylic acids (22). Moreover, Kanzaki and Anraku (19) demonstrated an increase in the uptake of isoleucine, leucine, and valine by *E. coli* when pyruvate is the carbon source instead of glycerol. Kay (20) reported similar results for aspartate transport. Thus, it is possible that by using carbon sources other than glucose and/or nitrogen sources other than NH_4^+, additional regulatory properties would be uncovered for the serine-threonine transport system.

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