Resolution of the Multiplicity of the Glutamate and Aspartate Transport Systems of *Escherichia coli* *

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Mutants of *Escherichia coli* D,W with altered levels of glutamate and aspartate transport were used to resolve the following five separate transport systems for these two amino acids: (a) a binding protein-dependent, sodium-independent, cysteate-inhibitable glutamate-aspartate system; (b) a binding protein-independent, sodium-independent, glutamate-aspartate system which is inhibitable by β-hydroxyaspartate or cysteate; (c) a binding protein-independent, sodium-dependent, α-methylglutamate-inhibitable glutamate-specific system (Miner, K. M., and Frank, L. (1974) *J. Bacteriol.* 117, 1093-1098; Halpern, Y. S., Barash, H., Dover, S., and Druck, K. (1973) *J. Bacteriol.* 114, 53-58); (d) a binding protein-independent aspartate-specific system (Kay, W. W. (1971) *J. Biol. Chem.* 246, 7373-7382); and (e) a dicarboxylic acid transport (det) system which transports succinate, maleate, fumarate, and aspartate (Kay, W. W., and Kornberg, H. L. (1971) *Eur. J. Biochem.* 18, 274-281).

Mutants were generated which have specific alterations in the level of each system, and through the use of these mutants *K* < sub > m </ sub > values were obtained for three of these systems as well as estimates for the contribution of four of these systems to the total glutamate and aspartate transport in *E. coli* D,W measured in the presence of 40 mM sodium ion and at substrate concentrations of 28 μM. The binding protein-dependent system has *K* < sub > m </ sub > values of 0.5 μM for glutamate and 0.5 μM for aspartate, and it is responsible for 15% of the glutamate and 20% of the aspartate transport. The β-hydroxyaspartate-inhibitable glutamate-aspartate transport system has *K* < sub > m </ sub > values of 5 μM for glutamate and 4 μM for aspartate, and it is responsible for 60% of the total glutamate and 52% of the total aspartate transport. The sodium-dependent glutamate-specific transport system has a *K* < sub > m </ sub > value of 1.5 μM for glutamate and accounts for 25% of the total glutamate transport. The aspartate-specific system is responsible for 23% of the total aspartate transport. The amount of aspartate transported through the dicarboxylic acid system was negligible under the conditions of these experiments.

The transport of glutamate and aspartate by *Escherichia coli* appears to be mediated by several overlapping transport systems. Kay and Kornberg (2) and Kay (3) have reported aspartate transport in whole cells to be mediated by two systems: a low affinity system shared by succinate, fumarate, malate, and aspartate, and a high affinity aspartate-specific system which is weakly inhibited by glutamate. The high affinity aspartate-specific system was found to be retained in membrane vesicles (3). Other investigators, working with mutants derived from *E. coli* B (4) and K 12 (5) having derepressed levels of glutamate transport, have partially characterized a sodium-stimulated glutamate transport system which is also retained in membrane vesicles. Using this type of mutant the *K* < sub > m </ sub > of glutamate transport in whole cells and in membrane vesicles was found to be strongly dependent on sodium ion, decreasing 10-fold in the presence of 15 mM sodium ion (6, 7). This sodium-stimulated system appears to be specific for glutamate since aspartate does not inhibit glutamate transport in membrane vesicles prepared from *E. coli* K 12 (8). However, Lombardi and Kaback (9), using membrane vesicles prepared from *E. coli* ML308-225, have shown that these two amino acids share a common transport system. Miner and Frank (4) have also reported the isolation of mutants of *E. coli* B which have elevated glutamate transport which is only partially stimulated by sodium in either whole cells or in membrane vesicles.

Willis and Furlong (10) have recently purified to homogeneity an osmotic shock-releasable protein from *E. coli* W which binds either glutamate (*K* < sub > d </ sub > = 0.7 μM) or aspartate (*K* < sub > d </ sub > = 1.2 μM). Glutamate-aspartate binding proteins have also been purified from *E. coli* K 12 by Barash and Halpern (11) and from *Salmonella typhimurium* by Aksamit et al. (12). Similar proteins capable of binding other substrates have been shown to serve as recognition components of the specific transport systems (13-15) as well as the receptors for chemo- tactic responses toward the respective substrates (16, 17). Until now the role of the glutamate-aspartate binding protein in transport was not clearly established. Barash and Halpern have reported that mutants with elevated levels of the sodium-stimulated glutamate transport system also have elevated levels of the glutamate-aspartate binding protein (11). However, they have also found that the binding protein and this

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transport system are not co-regulated under different culture conditions (18). In addition, sodium does not affect the in vitro binding of glutamate or aspartate to the binding protein (10). Thus, the binding protein does not appear to be the recognition component of a sodium-dependent transport system. Miner and Frank have suggested the presence of two glutamate transport systems, an osmotic shock-resistant, sodium-stimulated, membrane-bound system and an osmotic shock-sensitive system (4). Willis and Furlong postulated that the glutamate transport system of E. coli was conditionally dependent on the glutamate-aspartate binding protein (19).

In this paper we report the kinetic and physiological consequences of a series of specific mutations in the different transport systems for glutamate and aspartate. A model (Fig. 1) is presented for the multiplicty of transport of these two amino acids which is consistent with the data reported here as well as with observations of other workers. The glutamate-aspartate binding protein serves as the substrate recognition component for only one of five separate transport systems. These results also explain why so many conflicting observations regarding the mechanism of uptake for those two amino acids have appeared in the literature.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-[U-¹⁴C]Glutamate (238 mCi/mmol), L-[U-¹⁴C]aspartate (207 mCi/mmol), L-[U-¹⁴C]proline (361 mCi/mmol), L-[U-¹⁴C]glutamine (252 mCi/mmol), L-[U-¹⁴C]leucine (288 mCi/mmol), and L-[U-¹⁴C]succinate (13 mCi/mmol) were from New England Nuclear. dL-α-Methylglutamic acid, N,N′-methylenebisacrylamide, βL-α-methylaspartic acid, and di-α-tartrate were from Sigma Chemical Co. dl-threo-β-Hydroxyaspartate and L-cysteic acid were purchased from Calbiochem, and L-glutamate-γ-ethyl ester and Celulon phosphate were from Nutritional Biochemicals Corporation. Acrylamide was obtained from Bio-Rad Laboratories, and L-glutamate-γ-ethyl ester was found to contain approximately 1% acetic acid revealed no detectable glutamate or aspartate (less than 0.1%). The acrylamide and N,N′-methylenebisacrylamide were re-crystallized before use (20). Arnel-coated ion exchange resins and inhibitors used in the transport assays were prepared as previously described (19).

**Methods**

**Bacterial Strains**—All mutants used in this study were derived from *Escherichia coli* W strain D,W which was obtained from Dr. L. Leive, National Institutes of Health, Bethesda, Md.

**Media and Culture Conditions**—The minimal salts medium contained per liter: 7.35 g of KH₂PO₄, 5 g of KH₂PO₃, 0.55 g of Na₂SO₄, 0.2 g of MgCl₂·6 H₂O, and 1 ml of trace elements (23). Ammonium chloride (2.67 g/liter) was added as the nitrogen source and 1% sodium lactate as the carbon source unless otherwise indicated.

**Cultures for transport assays and small scale osmotic shocks** were grown at 37° in a rotary water-bath shaker. Large scale cultures for protein purification were grown as previously described (24) using the above minimal salts medium with ammonium chloride as the nitrogen source and 1% potassium succinate as the carbon source. Additional ammonium chloride was added as needed and the pH was maintained at 7.0 by the addition of 20% potassium succinate, pH 4.5. Cells were subcultured in a 10-liter New Brunswick Microfermentor, transferred to a 70-liter Fermentation Designs Inc. fermentor for growth, and harvested at early stationary phase with a continuous flow Cepa centrifuge.

**Mutant Selection**—Mutants resistant to β-hydroxyaspartate were isolated by placing 0.1 ml of a 0.23 mM potassium β-hydroxyaspartate solution in a well (6 mm in diameter) punched in a minimal salts agar culture plate containing 1% glucose as the carbon source. Mutants with an elevated level of β-hydroxyaspartate-inhibitable glutamate-aspartate transport system were selected by the same procedure with the β-hydroxyaspartate concentration increased to approximately 0.05%.

**Electrophoresis**—Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Ames (27) using a Studier type apparatus (28, 29) and the discontinuous buffer system of Laemmli (30). The gels were stained in 25% 2-propanol, 10% glacial acetic acid in water (v/v/v) with 0.1% Coomassie brilliant blue (w/v) for 1 h. Gels were destained by diffusion in the above solution without the dye.

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**Electrophoretic Purification**—The initial steps in the purification of the glutamate-aspartate binding protein (osmotic shock, removal of material insoluble at pH 4.5, CM-cellulose chromatography, and DEAE-cellulose chromatography) were performed as previously described (10). The pass-through fraction from the DEAE-cellulose column which contained the glutamate-aspartate binding protein was dialyzed against deionized water and sodium acetate (pH 5.5) was added to a final concentration of 10 mM. Part of this material (74 mg) was loaded onto a phosphocellulose column with a resin bed.
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volume of 46 ml (30 cm in length, 1.4 cm in diameter) which had been equilibrated with 10 mM sodium acetate at pH 5.5. The column was was washed with 5 column volumes of equilibration buffer followed by a linear sodium chloride gradient of 0 to 0.19 M salt in 20 column volumes of equilibration buffer. The gradient was controlled by an Ultrargrad Gradient Maker (LKB) which held the salt concentration constant whenever the column effluent had an absorption at 280 nm greater than 0.3. These modifications resulted in a more efficient purification than previously reported (10-12). The glutamate-aspartate binding protein peak was eluted between 0.1 and 0.11 M sodium chloride. The specific activity of the pooled fractions was 25 nmol of glutamate bonding of protein. During the purification of this protein, the glutamate and aspartate binding activity co-chromatographed on CM-cellulose, DEAE-cellulose, and phosphocellulose ion exchange columns and no other glutamate or aspartate binding activities were detected.

Binding Assays - Ligand binding to the glutamate-aspartate and glutamine binding protein was measured as described previously (10, 31).

Immunodiffusion Assay - The relative levels of glutamate-aspartate binding protein present in osmotic shock fluids were determined by an Ouchterlony double diffusion immunoprecipitation assay in agar plates. Aliquots (5 µl) of serially diluted (1:2) concentrated osmotic shock fluid (10 mg/ml) were allowed to diffuse against antisera (5 µl) prepared against the glutamate-aspartate binding protein (10). The quantity of binding protein present was estimated from the lowest dilution that gave an observable immunoprecipitation reaction.

Protein Determinations - Protein concentrations were determined by the method of Lowry et al. (32) using bovine serum albumin (33) as a standard.

RESULTS

Transport Properties of Mutants with Altered Levels of Aspartate-specific and D-Hydroxyaspartate-inhibitable Glutamate Aspartate Transport Systems - Mutants of Escherichia coli strain D2W with decreased levels of transport through the aspartate-specific transport system and the D-hydroxyaspartate-inhibitable glutamate-aspartate transport system (Fig. 1) were selected as colonies resistant to D-hydroxyaspartate as described by Kay (3) (Table I). The properties of 19 separately isolated mutants were examined and two classes of mutants were observed. Mutants such as HA9, isolated from small colonies appearing in the zone of growth inhibition (14 separate isolates), were found to be partially resistant to this analogue when examined by the radial streak method (Table II). Mutants isolated from large colonies (5 separate isolates such as mutant HA12) were fully resistant to growth inhibition by D-hydroxyaspartate (Table II). Both types of mutants were able to grow on succinate as sole carbon source and remained fully sensitive to D(-)-tartrate indicating that the dicarboxylic acid transport system (2) was still functional (Table II).

The initial rates of glutamate and aspartate transport in the parent strain and in the transport-deficient mutant HA9 are shown in Table II. Mutant HA9 which is partially resistant to D-hydroxyaspartate has 75% of the wild type aspartate transport and no significant change in glutamate transport. Further, Fig. 2 shows that the glutamate-resistant component of aspartate uptake was lost in mutant HA9. This mutant therefore appears to be devoid of the aspartate-specific system described by Kay (3). Mutant HA9 still retains a component of glutamate and aspartate uptake which is inhibitable by D-hydroxyaspartate (Fig. 3). Mutant HA12, on the other hand, is fully resistant to D-hydroxyaspartate (Table II). In contrast to mutant HA9, glutamate transport in mutant HA12 has been reduced by 55% and aspartate transport by 75% compared with the parent strain E. coli D2W (Table II). Further, since the remaining aspartate transport is effectively inhibited by glutamate (Fig. 4) and not by D-hydroxyaspartate (Fig. 3A), these results would suggest that this mutant lacks the aspartate-specific system as well as a system which transports both glutamate and aspartate, and which is also inhibited by D-hydroxyaspartate.

A mutant with increased levels of a D-hydroxyaspartate-inhibitable glutamate-aspartate transport system was isolated by a selection procedure based on the following rationale. Mutants with increased levels of this transport system should be able to utilize aspartate as a nitrogen source more efficiently than the parent strain and at the same time be more sensitive to growth inhibition by D-hydroxyaspartate. In order to avoid complications of aspartate entering through the

| Strain or mutant | Parent | Selection methoda | Aspartate-specific | D-Hydroxyaspartate-inhibitable glutamate-aspartate | Glutamate-specific | Glutamate-aspartate binding protein-dependent | Dicarboxylic acid (ict) |
|-----------------|--------|------------------|-------------------|-----------------------------------------------|-----------------|---------------------------------------------|----------------------|
| D2W             | D2W    | Resistance to D-hydroxyaspartate | +                  | +                                             | +               | +                                           | -                    |
| HA9             | D2W    | Resistance to D-hydroxyaspartate | -                  | -                                             | -               | -                                           | +                    |
| HA12            | HA9    | Increased growth on glutamate | -                  | -                                             | 10X             | -                                           | +                    |
| HA12-MG1        | HA12-MG1 | Increased growth on glutamate | -                  | -                                             | -               | +                                           | -                    |
| HA12-STA1      | HA12   | Resistance to D- (+)-tartrate | -                  | -                                             | -               | -                                           | -                    |
| HA12-GA7       | HA12   | Increased growth on glutamate | -                  | -                                             | -               | +                                           | 5-9X                 |
| IT52            | D2W    | Resistance to D- (+)-tartrate | +                  | +                                             | +               | +                                           | -                    |
| IT52:GA6       | IT52   | Increased growth on aspartate | +                  | 3-4X                                         | +               | +                                           | -                    |

a Details of the selection methods are outlined under "Methods." Symbols indicate either the presence (+) or absence (-) of the indicated transport system. Mutants with elevated levels of a transport system are indicated by a number reflecting the fold increase followed by an X.

b Mutants HA12 and IT52 were isolated from cultures which had been mutagenized with ICR-191 by the method described under "Methods."
Table II

Analogue sensitivities and specific activities of uptake of Escherichia coli D,W and mutants used in this study

| Strain or mutant | Analogue sensitivity | Transport activity |
|------------------|----------------------|-------------------|
|                  | \( \beta \)-Hydroxyaspartate | \( \alpha \)-Methylglutamate | \( \beta \)-Tartrate | Growth on succinate | Glutamate | Aspartate | Succinate | Proline |
| D,W              | S                    | R                  | S                  | +                    | 21        | 22        | 10        | 12       |
| HA9              | P                    | R                  | S                  | +                    | 22        | 17        | 9         | 12       |
| HA12             | R                    | R                  | S                  | +                    | 8.1       | 5.6       | 11        | 11       |
| HA12-MG1         | R                    | R                  | S                  | +                    | 53        | 5.6       | 13        | 11       |
| HA12-STR51       | R                    | R                  | R                  | –                    | N.D.      | 5.0       | 0.5       | N.D.     |
| HA12-GA7         | P                    | R                  | R                  | –                    | 33        | 21        | N.D.      | 12       |
| IT52             | S                    | R                  | R                  | –                    | 20        | 19        | 1.5       | 12       |
| IT52-SAsp6       | SS                   | R                  | R                  | –                    | \( \uparrow \) 4 | \( \uparrow \) 9 | 1.7       | 11       |

Analogue sensitivities were determined by the radial streak method described under "Methods." Abbreviations are: S, sensitive; R, resistant; P, partially resistant; SS, more sensitive than the parent.

* Cells were grown and assayed as described under "Experimental Procedures." Glutamate, aspartate, and proline assays contained 40 mM sodium chloride and either 28 \( \mu \)M \[^{14} \text{C}\]glutamate, 28 \( \mu \)M \[^{14} \text{C}\]aspartate, or 28 \( \mu \)M \[^{14} \text{C}\]proline. Succinate assays contained 80 \( \mu \)M \[^{14} \text{C}\]succinate.

† Growth on succinate was determined by the radial streak method using minimal salts culture plates with 1% potassium succinate in a 6-mm well in the center of the plate as the sole carbon source.

‡ Cultures for succinate transport assays were grown on minimal salts media with 1% sodium lactate and 20 mM potassium succinate as carbon sources.

N.D. indicates value not determined.

dicarboxylic acid transport (dct) system, a dct-negative mutant (IT52) was used as the parent (Table I). The mutants were selected by plating a washed culture of mutant IT52 on a minimal media culture plate containing 1% glycerol as the carbon source and 5 mM aspartate as the sole source of nitrogen. Second step mutants appeared as small colonies on a faint lawn of the parent strain. Succinate was included in the culture media to prevent revertants of IT52 to a dicarboxylate transport positive phenotype. (Since the dct system is capable of transporting aspartate, dct-positive revertants would be able to efficiently utilize aspartate as a nitrogen source. However, the presence of excess succinate would keep the dct transport system saturated and thus prevent aspartate from entering the cell through this transport system.)

Mutant IT52-SAsp6 was selected by this procedure and, as noted in Table II, is more sensitive to \( \beta \)-hydroxyaspartate than its parent IT52 or the parent of IT52 (E. coli D,W).

The transport properties of mutant IT52-SAsp6 are shown in Table III. Both glutamate and aspartate transport activities are increased in this mutant compared to its parent IT52 or the parent of IT52 (E. coli D,W) (Table II), while no change in the transport of succinate, proline (Table II), leucine, or glutamine, was observed (data not shown). Further, the increased activity is inhibited by \( \beta \)-hydroxyaspartate (Fig. 3). Using the portion of uptake that is inhibitable by \( \beta \)-hydroxyaspartate as a measure of the level of the \( \beta \)-hydroxyaspartate-inhibitable glutamate-aspartate transport system, this mutant has a 3.8-fold increase in glutamate transport and a 3.1-
FIG. 4 (left). Inhibition of aspartate uptake by glutamate and cysteate in mutants HA12 and HA12-MGl. Cells were grown and assayed as described under "Methods." Transport assays contained 28 μM \[^{14}C\]aspartate, 40 mM sodium chloride, and potassium salts of the inhibitors at the concentrations indicated. □, mutant HA12; □, mutant HA12-MGl.

FIG. 5 (center). Inhibition of glutamate uptake by α-methylglutamate, cysteate, and aspartate in mutant HA12. Cells were grown and assayed as described under "Methods." Transport assays contained 28 μM \[^{14}C\]glutamate, potassium salts of the inhibitors at the concentrations indicated, and either sodium (□) or potassium (■) chloride at a final concentration of 40 mM.

FIG. 6 (right). Inhibition of glutamate uptake by α-methylglutamate, cysteate, and aspartate in mutant HA12-MGl. Cells were grown and assayed as described under "Methods." Transport assays contained 28 μM \[^{14}C\]glutamate, potassium salts of the inhibitors at the concentrations indicated, and either sodium (□) or potassium (■) chloride at a final concentration of 40 mM.

TABLE III

| Inhibition of glutamate and aspartate uptake in mutants IT52 and IT52-Asp6 |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Inhibitor                      | Mutant IT52     | Mutant IT52-Asp6 |
|                                | Inhibitor       | \[^{14}C\]Glu-  | \[^{14}C\]Asp-  | \[^{14}C\]Glu-  | \[^{14}C\]Asp-  |
|                                | concentration   | tamate uptake   | tamate uptake   | tamate uptake   | tamate uptake   |
|                                | mM              | (nmol/min/mg)   | (nmol/min/mg)   | (nmol/min/mg)   | (nmol/min/mg)   |
| None                           |                 | 21              | 25              | 61              | 51              |
| β-Hydroxyaspartate             | 6.7             | 6.4             | 12              | 6.1             | 11              |
| α-Methylglutamate              | 84              | 4.3             | 12              | 13.8            | 30              |
| Aspartate                      | 3               | 0.6             | 1.7             | 0.8             | 1.7             |
| Cysteate                       | 60              | 0.5             | 7.3             | 0.6             | 4.8             |
| Glutamate                      | 3               | 0.6             | 10              | 1.0             | 6.7             |

The increased sensitivity to β-hydroxyaspartate is also consistent with the observed increase in activity of this system (Table II). Cysteate also effectively inhibits the elevated glutamate and aspartate uptake in this mutant (Table III).

The specificity of the glutamate and aspartate transport activity in mutant HA12 was examined. Aspartate uptake is effectively inhibited by either glutamate or cysteate (Fig. 4). Moreover, the inhibition pattern of glutamate uptake is altered by the presence of sodium ion (Fig. 5). In the absence of sodium ion (replaced by 40 mM potassium chloride) either aspartate or cysteate are effective inhibitors of glutamate transport while α-methylglutamate only weakly inhibits glutamate transport. However, in the presence of 40 mM sodium ion the glutamate transport activity increases 2.5-fold and the increased increment of transport is more sensitive to inhibition by α-methylglutamate. Aspartate and cysteate inhibit approximately the same absolute amount of glutamate transport in the presence or absence of sodium ion. However, since the total glutamate uptake is increased by sodium ion, inhibition by aspartate or cysteate plateaus after inhibiting only about 40% of the total glutamate transport. Various combinations of the three inhibitors were used to inhibit glutamate uptake. As seen in Table IV, cysteate and aspartate appear to inhibit the same component of glutamate transport. Either analogue alone inhibits 35 to 40% of the glutamate transport while both analogues present together produce no further inhibition. The inhibition by either aspartate or cysteate, on the other hand, is additive with the α-methylglutamate inhibition. The presence of α-methylglutamate with either aspartate or cysteate inhibits approximately 90% of glutamate transport. The same results were obtained with mutant HA12-ST51, a double mutant missing the dct system, the β-hydroxyaspartate-inhibitable glutamate-aspartate transport system, and the aspartate-specific transport system (data not shown).

These results indicate in mutant HA12 at least two glutamate transport systems remain, a sodium-independent system which is inhibitable by either aspartate or cysteate, and a sodium-dependent system which is inhibitable by α-methylglutamate (Fig. 1).

Selection of Mutants with Increased Levels of Glutamate-specific and Glutamate-Aspartate Binding Protein-dependent Systems – Mutants with specific increases in the glutamate-specific transport system and the glutamate aspartate binding protein-dependent were isolated by plating washed cultures of mutant HA12 which is devoid of the β-hydroxyaspartate-inhibitable glutamate-aspartate transport system and the aspartate-specific system on minimal media plates containing 10 mM potassium glutamate as the sole carbon and
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Cells were grown and assayed as described under "Experimental Procedures." Transport assays contained 40 mM sodium chloride, 25 μM [14C]glutamate, and inhibitors at the concentrations indicated.

| Addition       | Inhibitor concentration (mM) | Glutamate transport (% control) |
|----------------|-----------------------------|---------------------------------|
| None           |                             | 100                             |
| Aspartate      | 3                           | 61                              |
| Cysteate       | 60                          | 67                              |
| α-Methylglutamate | 84                      | 30                              |
| Aspartate+     | 3                           | 66                              |
| Cysteate+      | 60                          | 12                              |
| α-Methylglutamate | 84                      | 7                               |

The transport properties of the α-methylglutamate-sensitive mutant, HA12-MGl, are shown in Tables II and VI and in Figs. 3 and 6. Glutamate uptake in the presence of 40 mM sodium chloride in mutant HA12-MGl is 6.5-fold higher than in the parent strain, HA12 (Table II), whereas in the presence of 40 mM potassium chloride, glutamate transport in this mutant is only slightly higher than in strain HA12 (Fig. 6). The sodium-stimulated glutamate transport in mutant HA12-MGl is inhibited by α-methylglutamate but not by β-hydroxyaspartate, aspartate, cysteate (Figs. 3B and 6), or α-methylaspartate (data not shown). Aspartate transport is not altered in this mutant (Table II) and remains inhibitable by glutamate and cysteate (Fig. 4) but not by β-hydroxyaspartate (Fig. 3A) or α-methylaspartate (data not shown).

These results indicate that a sodium-stimulated glutamate transport system which does not recognize aspartate has been elevated in mutant HA12-MGl (Fig. 1). The specific activity of succinate, proline (Table II), leucine, or glutamine uptake was not changed by this mutation (data not shown).

A second mutant, HA12-GA7, grows well on glutamate but, unlike HA12-MGl, is not sensitive to growth inhibition by α-methylglutamate (Table II). The transport properties of this mutant are summarized in Table V. The total level of glutamate and aspartate uptake in the absence of sodium ion increased 9-fold and 5-fold, respectively, in the mutant relative to the parent strain (Table V) while the uptake of proline (Table II), leucine, or glutamine was not altered (data not shown). The transport system elevated in this mutant does not correspond to the β-hydroxyaspartate-inhibitable glutamate-aspartate transport system since β-hydroxyaspartate does not inhibit either the elevated glutamate or aspartate transport (Fig. 3). Nor does this elevated transport correspond to the sodium-stimulated glutamate-specific system since the glutamate transport in this mutant is inhibited by cysteate and aspartate and is not dependent on the presence of sodium ion (Tables V and VI). In addition, aspartate, which is not a substrate of the glutamate-specific system, is transported by the system elevated in this mutant. The specificity of the glutamate and aspartate transport elevated in HA12-GA7 does correspond to cysteate-inhibitable sodium-independent glutamate and aspartate transport observed in the parent strain (Fig. 4 and 5) which is attributed to the glutamate-aspartate binding protein-dependent transport system (Fig. 1).

Comparison of Binding Protein Levels in Parent and Mutants—The level of the glutamate-aspartate binding protein in osmotic shock fluids derived from E. coli strain D/W and mutants HA12 and HA12-GA7 was assayed by immunodiffusion as described under "Experimental Procedures." Shock fluids derived from mutant HA12-GA7 contain approximately eight times more binding protein than either its parent, HA12, or the parent of HA12, E. coli D/W. Slab gel electrophoresis of osmotic shock fluid proteins in the presence of sodium dodecyl sulfate (Fig. 7) shows that of the shock-releasable proteins, only the glutamate-aspartate binding protein appears to be elevated in mutant HA12-GA7, and further, the binding protein level is not significantly altered in any of the other mutants shown in this figure.

Specificity of Glutamate Aspartate Binding Protein Discrepancies in the reported specificity of the glutamate-aspartate binding protein led us to reexamine the kinetic properties of this protein. Willis and Furlong (10) and Barash and Halpern (11) reported that the γ-ethyl esters of glutamate were inhibitors of the glutamate-aspartate binding protein, however, there was a significant difference in the reported Ks values. Further Aksamit, et al. reported that cysteate inhibited glutamate binding to a glutamate-aspartate binding protein purified from Salmonella typhimurium (12), an observation that was interesting in light of our studies on the specificity of glutamate and aspartate transport in E. coli.

The glutamate-aspartate binding protein and the amino acid analogues were purified as described under "Experimental Procedures," l-Cysteate inhibits glutamate binding to the glutamate-aspartate binding protein with a Ks of 25 μM (Fig. 8). The same data plotted by the method of Cornish-Bowden (34) (data not shown) indicate that the inhibition is competitive. Cysteate also inhibits aspartate binding (data not shown). Glutamate binding was not significantly inhibited by a 300-fold excess of β-α-methylglutamate, an inhibitor of the sodium-dependent glutamate transport system (Fig. 1), or by a 100-fold excess of β-hydroxyaspartate, an inhibitor of the β-
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**Table VI**

| Transport system | $K_a$ | $V_a$ | Strain used for determination of $K_a$ and/or $V_a$ | Contribution to the total wild type transport velocity |
|------------------|-------|-------|-----------------------------------------------|-----------------------------------------------|
| Glutamate-aspartate binding protein-dependent system | NaCl | KCl | µM | Glutamate | Aspartate | Glutamate | % |
| Glutamate-aspartate binding protein-dependent system | 0.5 | 0.5 | 0.5 | 0.6 | 21 | 20 | 31 | 29 | HA12-GAT<sup>6</sup> | 25<sup>6</sup> | 15<sup>6</sup> |
| β-Hydroxyaspartate-inhibitable glutamate-aspartate system | 4 | 5 | 5 | 6 | 59 | 57 | 67 | 68 | IT52-Asp<sup>6</sup> | 52<sup>6</sup> | 60<sup>6</sup> |
| Glutamate-specific system (sodium-dependent) | &ndash; | &ndash; | 1.5 | N.D.<sup>4</sup> | 0 | 0 | 50 | N.D. | HA12-MGl<sup>1</sup> | 0 | 25<sup>1</sup> |
| Aspartate-specific system | N.D. | N.D. | 5 | 5 | 0 | 0 | D-W, HA9 | 23<sup>0</sup> | 0 |

<sup>4</sup> Cells for $K_a$ determinations were grown and assayed as described under "Methods." Transport assays contained either 10 mM sodium or potassium chloride and either [14C]glutamate or [14C]aspartate at concentrations between 0.1 &mu;M and 50 &mu;M. The $K_a$ values were determined from reciprocal linear transformation plots (s/s versus s) of the velocity versus substrate data (35).

<sup>6</sup> The $V_a$ values were determined from linear transformation of the velocity versus substrate data and reflect the sum of all of the transport systems present in the indicated mutant.

<sup>1</sup> The percentage of contribution of each system to the total is that determined in the presence of 40 mM sodium chloride.

<sup>4</sup> In mutant HA12-GAT in the presence of potassium chloride, the binding protein-dependent glutamate-aspartate transport system accounts for 93% of the glutamate transport and 90% of the aspartate transport as indicated by the portion of the glutamate or aspartate transport in this mutant which is inhibitable by cysteate (Table VI). In the presence of sodium ion, this system accounts for 88% of the glutamate and 95% of the aspartate transport as indicated by the portion of the glutamate or aspartate transport activity in this mutant which is inhibitable by cysteate in the presence of 40 mM sodium chloride (data not shown).

<sup>6</sup> The contribution of the glutamate-aspartate binding protein-dependent system to the wild type uptake of glutamate is estimated to be the portion of the glutamate transport in mutant HA12 inhibited by aspartate or cysteate (3.2 nmol/min/mg) (Fig. 5). The contribution of this system to the total aspartate uptake is estimated to be the aspartate transport remaining in mutant HA12 (5.6 nmol/min/mg) (Table II) after genetic loss of the aspartate-specific system and the β-hydroxyaspartate-inhibitable glutamate-aspartate system.

<sup>0</sup> In mutant HA12-MGl with 50% of the aspartate transport and 75% of the aspartate transport in mutant IT52-Asp<sup>6</sup> is mediated by the β-hydroxyaspartate-inhibitable glutamate-aspartate system as determined by the portion of the glutamate and aspartate uptake inhibitable by β-hydroxyaspartate (Fig. 3). In the absence of sodium, 99% of the glutamate uptake and 78% of the aspartate uptake is inhibitable by β-hydroxyaspartate (Table III). In the presence of sodium ion (Table VI) inhibitable by β-hydroxyaspartate (12.6 nmol/min/mg) (Fig. 3).

<sup>1</sup> The glutamate-specific transport system accounts for 85% of the glutamate uptake in the presence of potassium ion in mutant HA12-MGl with the remaining uptake being through the glutamate-aspartate binding protein-dependent system. The velocity contribution of the glutamate-specific system in this mutant was determined by subtracting the velocity of the glutamate-aspartate binding protein-dependent system (8 nmol/min/mg) from the total glutamate transport activity of mutant HA12-MGl (53 nmol/min/mg, Table III). The activity of the binding protein-dependent system is estimated as the portion of the glutamate transport (in the absence of sodium ion) inhibitable by aspartate (Fig. 6).

<sup>6</sup> The glutamate-specific transport system velocity is estimated from the portion of the sodium-stimulated glutamate transport in mutant HA12 resistant to inhibition by aspartate or cysteate (5.3 nmol/min/mg) (Fig. 5).

<sup>1</sup> The contribution of the aspartate-specific system to the total aspartate transport is taken as the amount of aspartate transport lost when this system is genetically removed in mutant HA9 (6 nmol/min/mg) (Table II).

In the presence of sodium ion, 65% of the glutamate transport and 75% of the aspartate transport in mutant IT52-Asp<sup>6</sup> is mediated by the β-hydroxyaspartate-inhibitable glutamate-aspartate transport system. Three times recrystallized l-glutamate-γ-ethyl ester inhibited binding with a $K_i$ of 500 &mu;M, a value compatible with the inhibition that would be caused by the residual 0.05% glutamate still present in this preparation. The $K_i$ appears to increase in proportion with the purity of the ester.

**Kinetic Parameters of Glutamate and Aspartate Transport**

The $K_a$ values of transport were determined by using three mutants (IT52-Asp<sup>6</sup>, HA12-MGl, and HA12-GAT) which have elevated levels of the β-hydroxyaspartate-inhibitable glutamate-aspartate system, the glutamate-specific system and the binding protein-dependent glutamate-aspartate system, respectively. In each of these three mutants, the transport system which has been specifically elevated accounts for 75% or more of the total glutamate and/or aspartate transport (Table VI). This allows for the kinetic characterization of these three systems in the absence of significant velocity contributions by the other glutamate and aspartate transport systems. The reciprocal plots used to determine $K_a$ values (35) presented in Table VI were linear in all cases.
Fig. 7. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of osmotic shock-releasable proteins from strain D,W and mutants HA12, HA12-MG1, and HA12-GA7. The resolving gel contained 11% acrylamide and 0.29% N,N'-methylenebisacrylamide. The osmotic shock fluids were concentrated 20-fold by lyophilization. A to D, osmotic shock proteins (20 pg/lane) from strain D,W and mutants HA12, HA12-MG1, and HA12-GA7, respectively; E, purified glutamate-aspartate binding protein (1.3 pg); F, purified glutamine-binding protein (1.3 pg).

Fig. 8. Dixon plot of cysteate inhibition of glutamate binding to the glutamate-aspartate binding protein. The assay mixture contained 40 mM potassium phosphate buffer (pH 6.9), 20 mM magnesium chloride, cysteate at the indicated concentrations, 0.8 µg of binding protein, and either 6.6 µM (○) or 0.8 µM (●) glutamate in 40 µL. Of this volume, 30 µL were applied to a nitrocellulose filter and washed with 250 µL of 0.2 M magnesium chloride.

The combination of the inhibition data and the specific activities of transport in the mutants allows for the estimation of the contributions of four of the transport systems to the total glutamate and aspartate transport in the wild type strain D,W (Table VI). Under the conditions of these assays, the dct system does not appear to catalyze a significant percentage of the total aspartate transport since the genetic removal of this system (mutant HA12-ST51) does not result in a significant change in the level of aspartate transport (Table II). Under the assay conditions used, 60% of the glutamate transport is mediated through the β-hydroxyaspartate-inhibitable glutamate-aspartate transport system, 25% through the sodium-dependent glutamate-specific system, and 15% through the binding protein-dependent system. For aspartate, 52% of the uptake is mediated by the β-hydroxyaspartate-inhibitable glutamate-aspartate system, 23% by the aspartate-specific system, and 25% by the binding protein-dependent system.

**DISCUSSION**

The studies presented in this paper were undertaken to characterize the glutamate and aspartate transport systems of *Escherichia coli* and to assess the role of the glutamate-aspartate binding protein in transport. Previous studies in our laboratory (10, 19) as well as those by Barash and Halpern (11) and Kahane et al. (18) have suggested that the glutamate-aspartate binding protein is probably involved in the transport of glutamate and aspartate; however, the exact role of this protein had remained elusive. Our attempts to resolve this question have resulted in the work reported here where we have found that the binding protein appears to serve as the substrate recognition component for one of five separate transport systems which operate by at least three different mechanisms (i.e. sodium-independent membrane-bound, sodium-dependent membrane-bound, and binding protein-dependent). Earlier work resulted in the partial characterization of three of the five systems (2, 3, 5, 7); however, in many cases the data were difficult to interpret due to the unresolved multiplicity of the transport systems which accumulate these two amino acids. The five separate transport systems for these two amino acids are: the membrane-bound aspartate-specific system (3), a previously uncharacterized binding protein-independent β-hydroxyaspartate-inhibitable system shared by glutamate and aspartate, a sodium-dependent, membrane-bound glutamate-specific system (7), a binding protein-dependent glutamate-aspartate system, and the dicarboxylic acid transport system (2, 3) which recognizes succinate, fumarate, malate, and aspartate (Fig. 1).

Genetic removal of the aspartate-specific system (mutant HA9) results in the loss of a component of aspartate transport which is resistant to inhibition by glutamate or cysteate (Fig. 2) while glutamate transport is not altered (Table II). The system lost in this mutant appears to be the same aspartate-specific system previously described by Kay (3). In addition to aspartate, this system probably also recognizes β-hydroxyaspartate since mutant HA9 was isolated by virtue of its resistance to this analogue (Table I). Kay has also reported that β-hydroxyaspartate inhibits aspartate transport through the aspartate-specific system (3), although the ratio of inhibitor to substrate needed to demonstrate significant inhibition was much higher than the ratio used in this study (Fig. 3). Kay's studies indicate that in whole cells the aspartate-specific system has a Kₐ for aspartate transport of 3.7 µM and that this system is retained in membrane vesicles. Willis and Furlong (19) have also reported aspartate transport to be retained in membrane vesicles prepared from cultures of *E. coli* D,W grown on succinate as the sole carbon source. The
aspartate transport in these preparations had a $K_m$ of 2 μM and was not inhibited by glutamate, suggesting that the activity that they observed probably corresponds to the aspartate-specific system described in this paper and by Kay.

The β-hydroxyaspartate-inhibitable glutamate-aspartate transport system was characterized by generating a mutant (IT52-SAasp6) with a specific 3- to 4-fold increase in the level of this system (Fig. 3, Table III). The aspartate-specific system does not appear to be elevated in this mutant since the glutamate-resistant component of aspartate transport remains essentially the same in the mutant IT52-SAsp6 as in its parent. The $K_m$ values for glutamate and aspartate uptake were 5 μM and 4 μM, respectively (Table VI). Neither the maximal velocities nor the $K_m$ values of this system were affected by sodium ion. Preliminary data indicate that membrane vesicles prepared from E. coli D,W cultured as described in this paper retain a shared glutamate-aspartate transport system which is inhibitable by β-hydroxyaspartate and cysteate. Lombardi and Kaback have also observed a shared glutamate-aspartate transport system in membrane vesicles prepared from glucose-cultured E. coli ML 308-225 (9).

Mutant HA12 is devoid of the aspartate-specific system since all of the aspartate transport remaining is inhibitable by glutamate (Fig. 4). This mutant also lacks the β-hydroxyaspartate-inhibitable glutamate-aspartate system since β-hydroxyaspartate does not inhibit either the glutamate or aspartate transport in this mutant (Fig. 3). The fact that mutant HA12 was derived from a single step selection using mutagenized cells suggests the possibility that these two systems are genetically linked or perhaps share a common component, although a double mutation has not yet been ruled out. Kinetic analysis of glutamate and aspartate transport in this mutant indicates that in the presence of sodium ion, there are at least two remaining components of glutamate transport (Fig. 5, Table IV) and one for aspartate transport (Fig. 4). The amount of glutamate transport lost in mutant HA12 is the same as that inhibited by β-hydroxyaspartate in the parent strain E. coli D,W (assayed in the presence of sodium ion), indicating that the mutation did not affect the level of the two remaining glutamate transport systems.

The transport systems remaining in mutant HA12 were characterized by examining the properties of two different types of second step mutants which were isolated on the basis of their ability to utilize glutamate as a sole carbon and nitrogen source more efficiently than the parent, mutant HA12. The first type of mutant (HA12-MGl) has an increase in only the sodium-dependent glutamate-specific transport system. This increase results in sensitivity to the analogue α-methylglutamate (Table I) as reported earlier by Halpern and Umbarger (36). In agreement with Frank and Hopkins (6) we have also found that mutant HA12-MGl is more sensitive to growth inhibition by L-allo-γ-hydroxyglutamate than HA12 indicating that the glutamate-specific system probably recognizes this analogue as well. The maximal velocity of glutamate transport (45 nmol/min/mg) through this system in the derepressed mutant HA12-MGl accounts for 85% of the glutamate transport in the presence of sodium ion; the remaining 15% goes through the glutamate-aspartate binding protein-dependent transport system (Table VI). In the presence of sodium ion, this glutamate-specific system is a high affinity system with a $K_m$ of 1.5 μM for glutamate transport (Table VI). The recent work by Kahane et al. indicates that the sodium-dependent glutamate-specific system is active in membrane vesicle preparations (37). The reported noncompetitive inhibition of glutamate transport by aspartate in whole cells (19, 38) is more than likely due to aspartate inhibition of the binding protein-dependent system and/or the β-hydroxyaspartate-inhibitable glutamate-aspartate system since there is little or no inhibition by aspartate of the sodium dependent glutamate-specific system (Fig. 6). In cases where multiple transport systems are operative, it is difficult to assess the nature of inhibition by a substrate analogue since the inhibitor may be affecting several systems with differing inhibition constants while having no effect on the activity of other systems.

The other type of second step mutant (HA12-GA7) capable of utilizing glutamate as a carbon and nitrogen source exhibits an increase in the glutamate-aspartate binding protein-dependent transport system. The 5- to 9-fold increase in the cysteate-inhibitable glutamate-aspartate transport (Table V) corresponds well with the approximate 8-fold increase in the glutamate-aspartate binding protein. More than 90% of the glutamate and aspartate transport in this mutant (measured in the absence of sodium ion) is mediated by the binding protein-dependent system since the glutamate-specific system, which is also present in this mutant, is strongly dependent on sodium ion (Table VI). Mutant HA12-GA7, like its parent, mutant HA12, is missing the aspartate-specific system and the β-hydroxyaspartate-inhibitable glutamate-aspartate system (Fig. 3 and Table V). The binding protein-dependent system has the highest substrate affinity of all the observed glutamate and aspartate transport systems with $K_m$ values of 0.5 μM for both glutamate and aspartate (Table VI). The $K_m$ value determined for glutamate transport in mutant HA12 in the absence of sodium ion was also 0.5 μM (data not shown) indicating that the mutation to HA12-GA7 resulted only in an increase in the maximal velocity of the binding protein-dependent system. The affinity of the transport system correlates well with the reported $K_m$ values of 0.7 μM for glutamate and 1.2 μM for aspartate binding to the isolated glutamate-aspartate binding protein (10). The $K_m$ values of whole cell glutamate and aspartate transport through the binding protein-dependent system (Table VI) and the $K_m$ values of binding to the purified glutamate-aspartate binding protein are not affected by the presence of sodium ion (10).

The disc gel electrophoretic analysis of the osmotic shock-releasable proteins (Fig. 7) shows that only one of this class of proteins, the glutamate-aspartate binding protein, is derepressed in mutant HA12-GA7. An important point with respect to the disc gel electrophoretic analysis is that the change in protein profiles is sufficiently dramatic that in a similar situation where the gene product had not previously been identified and purified, it would be possible to determine which protein is derepressed simply by observing the gel profile.

Of the mutants described in this paper, only those selected by resistance to D(-)-lactate (HA12-ST51 and IT52 and its derivative IT52-SAasp6) have altered succinate transport and thus have reduced levels of or completely lack the dct system. Further, the loss of the dct system in mutant HA12-ST51 does not affect glutamate or aspartate transport as expressed in lactate grown cells at the substrate concentrations used in this study (28 μM) (Table II). These results demonstrate that the dct system is distinct from the four other transport systems described in this paper (Fig. 1).

The possibility of the involvement in chemotaxis of the
differences which have contributed to the confusion in the literature so much as the overlapping specificities of the five expression of these systems under different culture conditions.

independent transport systems as well as the differential made in S. typhimurium (12), it is probably not strain as well as preliminary observations of aspartate transport observations made in a number of different strains of E. coli (39). Similarly, succinate, malate, and fumarate, sub-

ually regulated with differing responses to catabolite repres-

itive mutant of E. coli which they suggest lacks a common

ibov and Adler have isolated an aspartate chemotaxis nega-

that chemotaxis toward a-methylglutamate is increased in

of S. typhimurium (12). Preliminary experiments indicate

bound aspartate binding activity was recently investigated by

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