*Biochimica et Biophysica Acta*, 1025 (1990) 225–233
Elsevier



BBAMEM 74898

# Development of system $B^{0,+}$ and a broad-scope $Na^+$-dependent transporter of zwitterionic amino acids in preimplantation mouse conceptuses

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(Received 27 November 1989)

Key words: Amino acid transport; System B; System $B^{0,+}$; Development; Preimplantation conceptus; (Mouse egg); (Mouse embryo)

**The nature and ontogeny of $Na^+$-dependent L-alanine transport was examined in mouse eggs and preimplantation conceptuses. Mediated L-alanine uptake was not detected in fertilized or unfertilized eggs, but a small amount of $Na^+$-dependent L-alanine transport was detected in 2-cell conceptuses. $Na^+$-dependent alanine transport was more rapid at the 8-cell stage of development, and more than 10-fold faster in blastocysts than in 8-cell conceptuses. Analog inhibition analyses were consistent with the interpretation that L-lysine-sensitive and L-lysine-resistant components of transport were present at the 2-cell, 8-cell and blastocyst stages of development. The range of amino acids and their analogs that inhibited the most conspicuous component of alanine transport in blastocysts was consistent with the conclusion that system $B^{0,+}$ is largely responsible for L-alanine uptake in these conceptuses. Moreover, system $B^{0,+}$, but not other known systems in blastocysts, became susceptible to activation as these conceptuses approached the time of implantation, so this activation could be involved in implantation. Although the data are consistent with the possibility that system $B^{0,+}$ is also present in 2-cell and 8-cell conceptuses, the relatively slow L-alanine transport in conceptuses at these earlier stages of development precluded more detailed study of their ability to take up alanine. Similarly, the less conspicuous L-lysine-resistant component of L-alanine transport in blastocysts also may be present in conceptuses as early as the 2-cell stage. The L-lysine-resistant component of L-alanine transport could not be attributed to residual system $B^{0,+}$ activity, however, because it was inhibited more strongly by *trans*-OH-L-proline than L-arginine, whereas the reverse was the case for system $B^{0,+}$. Similarly, L-tryptophan and L-leucine each inhibited system $B^{0,+}$ more strongly than L-serine or L-cysteine, whereas all four of these amino acids inhibited the L-lysine-resistant component equally well. Moreover, a Hofstee plot for L-alanine influx was consistent with the interpretation that at least two mediated components of $Na^+$-dependent L-alanine transport are present in blastocysts. The less conspicuous component of L-alanine transport in blastocysts was relatively susceptible to inhibition by L-leucine and L-tryptophan, but it resisted inhibition by the 'model' system A substrate, MeAIB, and the system ASC inhibitors, L-penicillamine and cationic amino acids. Therefore, the zwitterion-preferring $Na^+$-dependent transport process in blastocysts appears to be sufficiently distinct from systems A and ASC as well as from $B^{0,+}$ to warrant its own provisional designation as system B.**

## Introduction

Fertilized and unfertilized mouse eggs and 2-cell conceptuses take up amino acids via several system-mediated processes. These processes include the $Na^+$-dependent transport system Gly [1,2] and the $Na^+$-independent systems L, $b^{0,+}$ and $b^+$ (Ref. 3, and unpublished data). System Gly prefers glycine and sarcosine as substrates, whereas system L selects for bulkier zwitterionic amino acids. System $b^{0,+}$ accepts some cationic and zwitterionic amino acids about equally well as substrates, although it prefers bulky amino acids whose carbon skeletons do not branch at the $\alpha$- or $\beta$-positions [3,5]. Like the better-known system $y^+$, system $b^+$ selects for cationic amino acids in the absence of $Na^+$; but unlike system $y^+$, system $b^+$ does not interact with

Abbreviations: BCH, 2-amino-*endo*-bycyclo[2.2.1]heptane-2-carboxylic acid; BCO, 3-amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid; HCG, human chorionic gonadotropin; MeAIB, 2-(methylamino)isobutyrate.

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certain zwitterionic amino acids, such as homoserine, in the presence of $Na^+$ (Ref. 5, and unpublished data). Therefore, except for system Gly, the mediated amino acid transport that has been characterized so far in eggs and 2-cell conceptuses appears to be entirely $Na^+$-independent [3]. Nevertheless, a small amount of otherwise uncharacterized $Na^+$-dependent L-alanine uptake was detected repeatedly in conceptuses at the 2-cell stage of development [6], and $Na^+$-dependent L-alanine uptake also has been detected in 4-cell conceptuses [7]. Therefore, one goal of the present study was to examine the nature and ontogeny of this $Na^+$-dependent component of L-alanine transport.

$Na^+$-dependent uptake of amino acids increases dramatically in mouse conceptuses between the 2-cell and blastocyst stages of development [1,2,7–10], and this increase apparently results from an increase in the activity of system $B^{0,+}$ [1,11]. System $B^{0,+}$ accepts a very broad scope of both cationic and zwitterionic amino acids as substrates, although it rejects amino acids that have an $\alpha$-N-methyl group [1,11]. $Na^+$-dependent L-alanine uptake has been studied in blastocysts activated from ovariectomy-induced delay of implantation in situ, and it is transported by system $B^{0,+}$ in these conceptuses [11]. Nevertheless, except for reports that $Na^+$-dependent L-alanine uptake appears to depend on $Na^+/K^+$-ATPase activity [1,12], alanine transport has not been studied in blastocysts from intact mice. We wanted not only to characterize further L-alanine transport via system $B^{0,+}$, but also to determine if blastocysts contain other $Na^+$-dependent transport systems for L-alanine uptake. More than one $Na^+$-dependent transport system for glycine uptake has been observed in 8-cell conceptuses and blastocysts, although only system Gly was detected in 2-cell conceptuses and fertilized or unfertilized eggs [1]. Furthermore, system $B^{0,+}$ increases in activity 3-fold within 20 min when blastocysts activated from delay of implantation are disturbed (e.g., flushed from uteri in culture medium) near the time of implantation, but such is not the case for blastocysts during delay of implantation [13]. The ability of blastocysts and the uterus to rapidly change the catalytic activities of the enzymes and transport processes they contain while they are receptive to attachment to each other, could be an important part of the mechanism of implantation [8,13]. Therefore, we determined if uptake of amino acids via system $B^{0,+}$ and other amino acid transport processes also changes in blastocysts from intact mice when these conceptuses are disturbed near the time of implantation.

## Materials and Methods

Several descriptions of the methods for obtaining eggs and conceptuses and measuring their abilities to take up amino acids have been published recently [1,3,5,11,13]. Sexually mature, 8–11 week old Swiss ICR mice (Harlan Sprague Dawley, Inc.) that had been acclimated to a 14 h light: 10 h dark cycle for at least two weeks in our animal facility, were treated with gonadotropins to induce them to ovulate [14]. In most experiments, unfertilized eggs were removed from oviducts in Brinster's medium [15] approximately 17 h after administration of human chorionic gonadotropin (HCG) at about 1600 h the preceding day. Eggs were freed from cumulus cells by exposing them to 145 IU of hyaluronidase (Sigma Chemical Co.) in 1.0 ml of Brinster's medium for less than 5 min. In a few experiments unfertilized and fertilized eggs were isolated and used in experiments within less than 16 h after administration of HCG, but the results of these studies were indistinguishable from results obtained with eggs obtained about 17 h after injection of HCG (data not shown). Conceptuses were removed from oviducts about 17 (1-cell stage), 41 (2-cell stage), and 66 (8-cell stage) h after HCG administration or from uteri about 94 or 114 h after administration of this hormone (blastocysts). No attempt was made in the studies reported here to distinguish between 8-cell conceptuses before and after compaction. Detection of a copulatory plug the morning after injection of HCG and observation of sperm were the only criteria used to designate eggs as fertilized, so some eggs that we assumed to be fertilized probably were not. Nevertheless, since most such eggs develop in situ (unpublished observation), most eggs were probably fertilized after mice mated. Eggs and conceptuses were washed and stored for less than 6 h in Brinster's medium in a humidified atmosphere of 5% $CO_2$ in air at 37°C (pH 7.4). Except for blastocysts obtained near the time of implantation (see results), transport was not observed to change in eggs or conceptuses during incubation for 6 h in vitro.

Eggs or conceptuses were incubated with a $^3H$-labeled form of L-alanine, L-leucine, or L-lysine (20–60 Ci/mmol; ICN Pharmaceuticals or Amersham) and various concentrations of nonradioactive amino acids as indicated in figures and tables. Amino acids were dissolved in a modification of Brinster's medium ($NaHCO_3$ replaced with $KHCO_3$ and sodium salts of pyruvate and lactate replaced with NaCl), a modification of Spindle's [16] flushing medium-I ($Na^+$ salts of lactate and pyruvate replaced with NaCl, $Na_2HPO_2$ replaced with $K_2HPO_4$, and Phenol red deleted) or phosphate-buffered NaCl (pH 7.1; Refs. 1, 3, 5, 11, 13). Amino acid uptake by eggs and conceptuses was measured over time periods that approximated initial velocities (i.e., 5 or 20 min) as indicated in the legends of the figures and table (Refs. 1, 3, 11 and unpublished data). Uptake was about the same when it was measured in Brinster's medium, modified Brinster's medium, modified flush medium-I or phosphate-buffered NaCl (Refs. 1, 3–5, 11 and unpublished data), but modified flush medium-I

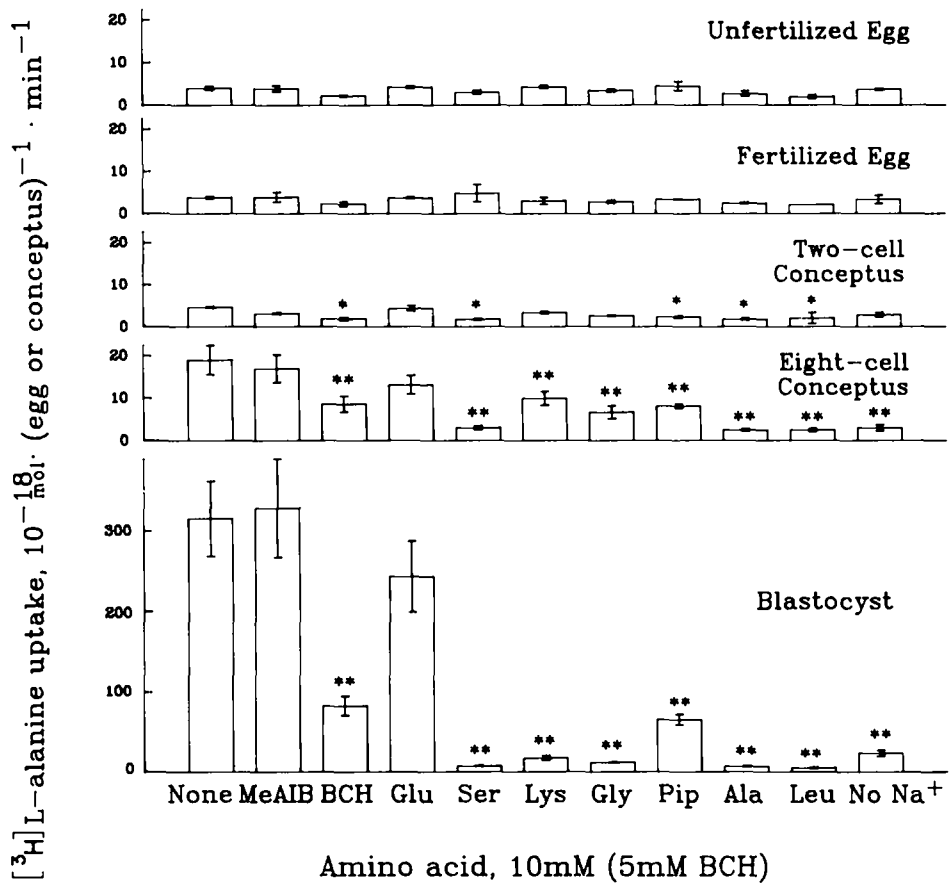


Fig. 1. Effect of various amino acids on uptake of L-[$^3$H]alanine by eggs and preimplantation conceptuses (10 mM BCO replaced 5 mM BCH in experiments with eggs). Eggs or conceptuses were incubated with 1.0 $\mu$M L-[$^3$H]alanine (50 Ci/mmol) for 20 min in modified Brinster's medium or this medium in which $Li^+$ was substituted for $Na^+$. The mean uptake $\pm$ S.E. was calculated from four replicate determinations (approximately five eggs or conceptuses/determination) obtained in two independent experiments. Uptake of radiolabel by blastocysts, 8-cell conceptuses and eggs or 2-cell conceptuses in the absence of added, nonradioactive amino acids was about 5400%, 315% and 78%, respectively, above the approx. 30 counts per min detected in samples of the final wash medium. Statistically significant inhibition is indicated with single ($P < 0.05$) or double ($P < 0.01$) asterisks as determined with analysis of variance. Analysis of variance could not be used to show statistically significant inhibition by some of the strongest inhibitors because some of the relevant variances were unequal, but in these cases it was clear without analysis that inhibition was significant (e.g., inhibition by L-serine, glycine, L-alanine, and L-leucine in blastocysts). Although uptake by 2-cell conceptuses in medium that contained no added $Na^+$ was not found to differ statistically from uptake in the presence of $Na^+$, alanine uptake can be shown to be $Na^+$-stimulated when enough replicate determinations are obtained ($P < 0.01$). Blastocysts were obtained from uteri about 94 h after HCG injection. MeAIB, 2-(methylamino)isobutyrate; Pip, L-pipecolate.

and phosphate-buffered NaCl are more convenient for maintaining the desired pH during relatively short (e.g., 5-min) incubation periods [1,3,5]. In some cases $Na^+$ in the medium was replaced with choline or $Li^+$ during labeling in order to measure $Na^+$-independent amino acid uptake.

In many experiments amino acid uptake was examined at substrate concentrations near 1 $\mu$M for two principal reasons. First, this approach increases the probability that low-$K_m$, low-capacity transport systems will be detected in the presence of higher-$K_m$, higher-capacity systems [17], without hampering the ability to detect higher-$K_m$ transport activities. Second, amino acid transport systems with $K_m$ values for some substrates near 1 $\mu$M have been detected in preimplantation conceptuses [1,3]. In contrast, initial inhibition studies were conducted with concentrations of potentially inhibitory amino acids of about 10 mM in order nearly to maximize any effect they may have (such concentrations of potential inhibitors are commonly used in transport studies, e.g., Refs. 17–19). The experimental approach of testing the effects of potential inhibitors on uptake at relatively low substrate concentrations does not increase the possibility that contaminants rather than inhibitors will affect uptake. As for potential inhibitors themselves, the possible effects of contaminants depend primarily on the $K_i$ values of possible contaminants regardless of whether the substrate concentration is at or far below the $K_m$ value for substrate uptake. Finally, we have shown that uptake of alanine and other amino acids at concentrations near 1 $\mu$M cannot be attributed to binding of the amino acids to the plasma membrane rather than to uptake; virtually no uptake or binding could be detected in eggs and

conceptuses that had been killed by exposing them to ultrasound in a Bransonic 12 ultrasonic cleaner (SmithKline Co.) for 5–25 min at 25–30 °C in 150 mosmolar phosphate-buffered NaCl also containing 10 mM KCN (data not shown). Although the latter treatment disrupted most conceptuses inside their zona pellucidae, it was not excessively harsh since some conceptuses survived the treatment as determined visually and by their ability to accumulate radiolabeled amino acids.

2-(Methylamino)isobutyrate (MeAIB), L-glutamate, L-serine, L-lysine, taurine, L-phenylalanine, L-asparagine, *trans*-4-OH-L-proline, *cis*-4-OH-L-proline, sarcosine, L-penicillamine, L-cysteine, glycine, L-pipecolate, L-proline, L-leucine, L-valine, L-tryptophan, L-alanine and L-arginine were purchased from Sigma, and 2-amino-*endo*-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH) was purchased from Behring Diagnostics. 3-Amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid (BCO) was a gift from Professor Carmen Avendaño [20]. The concentrations of radiolabeled and nonlabeled amino acids in the medium did not change significantly in the presence of eggs and conceptuses as discussed previously [1,5]. After incubation with amino acids, eggs or conceptuses were processed [1,3,5,11,13] to determine how much of the substrate they had taken up. Samples of the final wash medium, equal to the volume of medium in which eggs or conceptuses were finally collected, contained near background levels of radioactivity except for data reported in Fig. 1. In the latter case, samples of media contained 30 counts per min instead of the approx. 20 counts per min detected as background. Various statistical methods, such as analysis of variance [21,22] were used to assess the data as indicated in the legends of the figures and tables.

## Results

### *L-Alanine uptake by eggs and cleavage stage conceptuses*

Little or no substrate-saturable L-alanine uptake was detected in unfertilized or fertilized eggs (Fig. 1). When enough replicate determinations were accumulated for alanine uptake by 2-cell conceptuses, however, a small saturable, and apparently $Na^+$-dependent component has been detected consistently (Fig. 2, data not shown, and Ref. 6). L-Alanine uptake in 2-cell conceptuses was inhibited by BCH, L-serine, L-pipecolate (an analog of proline), L-leucine, L-lysine, sarcosine and glycine (Figs. 1 and 2). $Na^+$-dependent L-alanine transport was more conspicuous in 8-cell conceptuses (Figs. 1 and 3), and it was inhibited better by *trans*-4-OH-L-proline than by the corresponding *cis* isomer or by L-lysine (Fig. 3). BCH, L-serine, glycine, L-pipecolate and L-leucine also inhibited L-alanine uptake by 8-cell conceptuses (Fig. 1). Moreover, a component of $Na^+$-dependent L-alanine transport in 2-cell and 8-cell conceptuses appeared to resist inhibition by L-lysine (Fig. 3). L-Alanine uptake by 2-cell and 8-cell conceptuses was not inhibited by MeAIB or L-glutamate (Fig. 1).

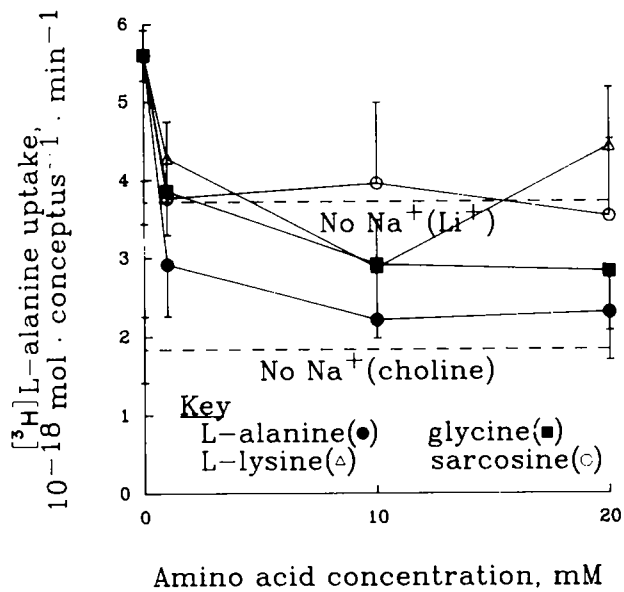


Fig. 2. Effect of sarcosine, glycine and L-lysine on L-[$^3$H]alanine uptake by 2-cell conceptuses. Conceptuses were incubated with 1.1 μM L-[$^3$H]alanine (47 Ci/mmol) and the indicated concentrations of nonradioactive amino acids for 5 min in phosphate-buffered NaCl. The mean uptake ± S.E. was calculated from six replicate determinations (approximately seven conceptuses/determination) obtained in three independent experiments. (Uptake in phosphate-buffered LiCl or choline chloride is indicated by the dashed lines.) Uptake of radiolabel in the absence of added, nonradioactive amino acids was about 38% above background. Uptake of L-[$^3$H]alanine was significantly slower in the presence of sarcosine, glycine, or L-lysine ($P < 0.05$) or nonradioactive L-alanine ($P < 0.01$), and it was apparently $Na^+$-stimulated ($P < 0.01$) as determined with analysis of variance.

### *Transport of L-alanine in blastocysts*

L-Alanine uptake was predominantly $Na^+$-dependent in blastocysts and inhibited by BCH, L-serine, L-lysine, glycine, L-pipecolate, L-leucine, L-tryptophan, L-cysteine, L-arginine and *trans*-OH-L-proline but not *cis*-OH-L-proline, L-penicillamine, MeAIB and L-glutamate (Figs. 1 and 4). A relatively inconspicuous, $Na^+$-dependent component of L-alanine transport which resisted inhibition by L-lysine and L-arginine was also detected in blastocysts (Fig. 4). The latter component of L-alanine transport appeared to have a slightly more restricted ability to interact with zwitterionic amino acids than the predominant component of uptake (i.e., system $B^{0,+}$), as indicated by its resistance to inhibition by BCH and L-proline (Fig. 5). Moreover, the less conspicuous component of alanine transport could not be attributed to the same transport process as the predominant component of alanine uptake because the former component was more susceptible to inhibition by *trans*-OH-L-proline than by arginine, whereas the reverse was true for the predominant component (Fig. 4). Similarly, L-tryp-

tophan and L-leucine were better inhibitors of total L-alanine uptake than either L-serine or L-cysteine, whereas all four of these amino acids inhibited the L-lysine-resistant component of $Na^+$-dependent alanine uptake equally well (Fig. 4). In addition, a Hofstee plot for influx was consistent with the presence of at least two components of $Na^+$-dependent L-alanine transport in blastocysts (Fig. 6). One system, corresponding to the less conspicuous component of transport discussed above, had an apparent $K_m$ value of approx. 380 $\mu$M and a $V_{max}$ value of about 25 fmol · blastocyst$^{-1}$ · min$^{-1}$, whereas these values for uptake via system $B^{0,+}$ were about 26 $\mu$M and 16 fmol · blastocyst$^{-1}$ · min$^{-1}$, respectively, as determined with the method described by Spears et al. [23]. Inhibition of the less conspicuous component of L-alanine uptake in blastocysts by some amino acids cannot be attributed to an osmotic or another non-specific effect of up to 36 mM of added amino acids, since no inhibition was detected in the presence of other amino acids such as *cis*-4-OH-L-proline, L-arginine, MeAIB, L-glutamate and taurine (Figs. 4b and 5).

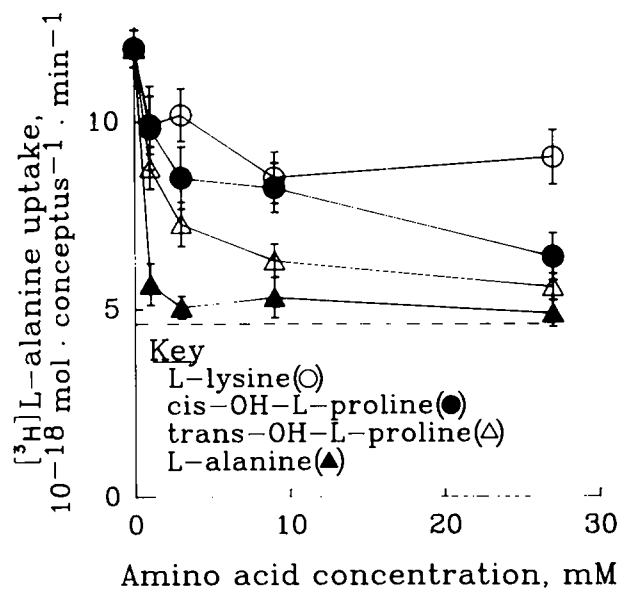


Fig. 3. Effect of *cis*- or *trans*-4-OH-L-proline and L-lysine on L-[$^3$H]alanine uptake by 8-cell conceptuses. Conceptuses were incubated with 1.0 $\mu$M L-[$^3$H]alanine (50 Ci/mmol) and the indicated concentrations of nonradioactive amino acids for 20 min in modified Brinster's medium or this medium in which choline was substituted for $Na^+$. The mean uptake ± S.E. was calculated from eight replicate determinations (approximately eight conceptuses/determination) obtained in five independent experiments. (Uptake in $Na^+$-depleted medium is indicated by the dashed line.) Uptake of radiolabel in the absence of added, nonradioactive amino acids was more than 400% above background. Inhibition by each amino acid was statistically significant ($P < 0.01$; analysis of variance), and *trans*-4-OH-L-proline was a more effective inhibitor than the *cis* isomer of this amino acid ($P < 0.01$; '*t*'-test).

TABLE I

*Effect of incubation in vitro on amino acid uptake by mouse blastocysts*

Approximately half of the blastocysts from 1–3 mice were incubated with 0.4 $\mu$M L-[$^3$H]alanine, 0.4 $\mu$M L-[$^3$H]lysine, or 0.8 $\mu$M L[$^3$H]leucine (20–60 Ci/mmol) beginning within 5 min after the mice were killed (5.22 ± 0.16 blastocysts were utilized per determination). The other half of the blastocysts were incubated for 1 h in Brinster's medium prior to incubation with the same $^3$H-labeled amino acid in phosphate-buffered NaCl or LiCl for 5 min. The number of replicate experiments for each row is indicated under column '*n*'.

| Type of blastocyst | Transport processes | Substrate | *n* | Uptake before (0 h) or after 1 h incubation [a] | |
|---|---|---|---|---|---|
| | | | | 0 h | 1 h |
| Early [b] | $B^{0,+}$ and [c] | L-alanine | 7 | 98 ± 17 | 96 ± 10 |
| Late [d] | $B^{0,+}$ and [c] | L-alanine | 44 | 153 ± 22 ** | 498 ± 34 |
| | $b^{0,+}$ and [e] | L-lysine | 21 | 141 ± 12 | 136 ± 15 |
| | [e] | L-lysine | 23 | 24 ± 2 * | 18 ± 2 |
| | $b^{0,+}$ and [f] | L-leucine | 16 | 210 ± 18 | 272 ± 31 |
| | [f] | L-leucine | 16 | 89 ± 18 | 112 ± 29 |
| Implanting [g] | $B^{0,+}$ [h] | L-alanine | 38 | 150 ± 20 ** | 1140 ± 90 |
| | $b^{0,+}$ and [e] | L-lysine | 10 | 110 ± 9 | 101 ± 11 |
| | [e] | L-lysine | 10 | 29 ± 3 ** | 15 ± 1 |

[a] Mean ± S.E. ($10^{-18}$ mol · blastocyst$^{-1}$ · min$^{-1}$). Statistically significant differences between 0 h and 1 h are indicated with single ($P < 0.02$; pairing design '*t*'-test) or double ($P < 0.01$; group comparison '*t*'-test) asterisks.

[b] Early blastocysts were obtained from uteri about 94 h after administration of HCG (i.e., about 82 h post coitum and 26 h before implantation).

[c] L-Alanine uptake (approx. 5% of the total at 0.4 $\mu$M exogenous alanine) via the combination of the L-lysine-resistant saturable component and the nonsaturable component.

[d] Late blastocysts were obtained from uteri about 114 h after administration of HCG (i.e., about 102 h post coitum and 6 h before implantation).

[e] Lysine uptake via the combination of system $b^+$, which is the cation-preferring component in Ref. 5, and the nonsaturable component (i.e., total $Na^+$-independent uptake in the presence of 20 mM L-leucine).

[f] Leucine uptake via the combination of system L, which is the zwitterion-preferring component in Ref. 5, and the nonsaturable component (i.e., total $Na^+$-independent uptake in the presence of 20 mM L-lysine).

[g] Implanting blastocysts were obtained from the uterus 20–25 h after administration of estrogen to mice that had been ovariectomized and treated with progesterone as described previously (Refs. 11, 13).

[h] Data for this row is from Table II of Ref. 13.

*Changes in amino acid transport in blastocysts as they approach the time of implantation*

We have not detected qualitative changes in the amino acid transport systems present in blastocysts between 26 and 6 h prior to implantation (Refs. 8 and 13 and data not shown). Blastocysts did differ at these two times of development, however, in the relative quantities of the major transport activities they could express, and in whether the quantities of these activities changed quickly when blastocysts were removed from uteri. The $V_{max}$ values for uptake of L-leucine, L-alanine, and L-lysine via system $B^{0,+}$ increased 2–3-fold during

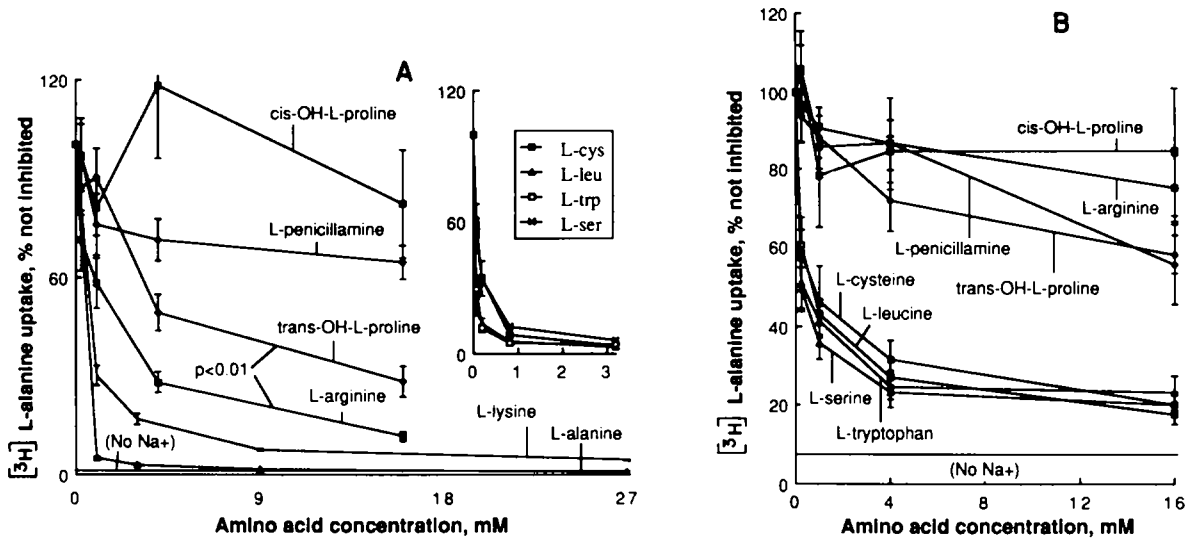


Fig. 4. Effect of various concentrations of several amino acids on (A) total L-[$^3$H]alanine uptake (primarily system $B^{0,+}$) and (B) L-lysine-resistant L-[$^3$H]alanine uptake by blastocysts. Conceptuses were obtained from uteri about 94 h after HCG injection and incubated with 0.85 (A) or 2.1 (B) $\mu$M L-[$^3$H]alanine (47 Ci/mmol) for 5 min in phosphate-buffered NaCl. The effects of L-penicillamine, L-cysteine, L-leucine and *cis*-OH-L-proline on alanine uptake were determined in the presence of 10 mM dithiothreitol, and dithiothreitol appeared to have no effect on alanine uptake. The mean $\pm$ S.E. uptake was calculated from three to seven replicate determinations (approximately five blastocysts/determination) obtained in two to four independent experiments. Uptake in phosphate-buffered LiCl is indicated by the lines labeled 'No $Na^+$'. Uptake of radiolabel in the absence of added, nonradioactive amino acids was more than 1500% above background in panel A and about 260% above background in panel B. Total L-[$^3$H]alanine uptake (A) was significantly slower in the presence of L-arginine, trans-OH-L-proline, L-cysteine, L-leucine, L-lysine, L-alanine, L-tryptophan and L-serine ($P < 0.01$). [$^3$H]Alanine uptake in the presence of 20 mM L-lysine (B) was inhibited significantly by *trans*-OH-L-proline, L-cysteine, L-leucine, L-tryptophan and L-serine ($P < 0.01$). In some cases where inhibition was strongest, analysis of variance could not be used to compare the means because the relevant variances were unequal; but in those cases it was clear without analysis that inhibition was significant (e.g., inhibition by L-cysteine, L-serine, L-tryptophan and L-leucine). Inhibition by *trans*-OH-L-proline or L-arginine was tested at the same time in the same experiments and these two groups were significantly different in the presence (B) and absence (A) of 20 mM L-lysine ($P < 0.01$; '$t$' tests). Similarly, inhibition by L-tryptophan or L-leucine, respectively, was greater than by L-serine or L-cysteine in the absence of 20 mM L-lysine ($P < 0.01$).

this period of development, while the $V_{max}$ values for L-leucine and L-lysine transport by system $b^{0,+}$ remained about the same [8]. More interestingly, uptake of alanine via system $B^{0,+}$ increased 3-fold in blastocysts obtained about 6 h prior to implantation (late blastocysts) when they were simply incubated for 1 h

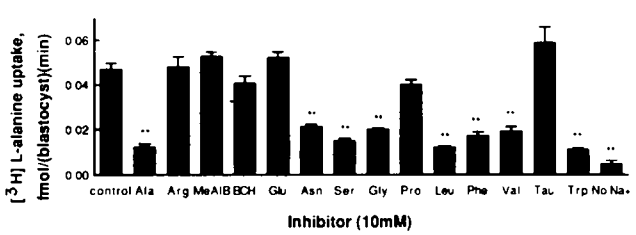


Fig. 5. Effect of various amino acids on L-lysine-resistant L-[$^3$H]alanine uptake by blastocysts. Conceptuses were obtained about 94 h after HCG injection and incubated with 2.1 $\mu$M L-[$^3$H]alanine and 20 mM L-lysine for 5 min in phosphate-buffered NaCl or this medium in which $Li^+$ was substituted for $Na^+$. The mean $\pm$ S.E. uptake was calculated from five replicate determinations (approximately eight blastocysts/determination) obtained in four independent experiments. Uptake of radiolabel in the absence of added, nonradioactive amino acids was about 450% above background. Statistically significant inhibition is indicated with double asterisks ($P < 0.01$) as determined with analysis of variance. MeAIB, 2-(methylamino)isobutyrate; Tau, taurine.

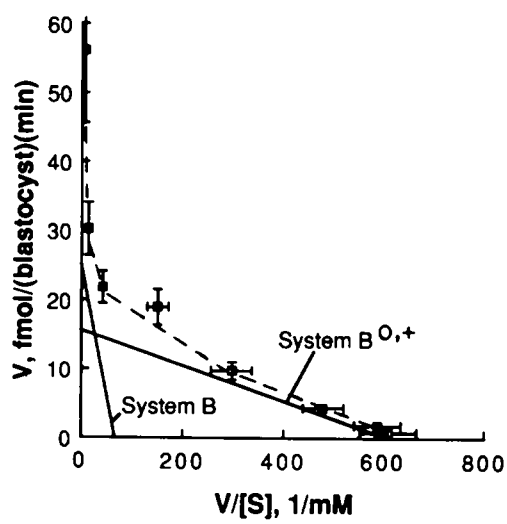


Fig. 6. Hofstee plot of $Na^+$-dependent L-alanine uptake by blastocysts. Conceptuses were obtained from uteri about 94 h after HCG injection and incubated with the indicated concentrations of L-alanine (1.1 $\mu$M L-[$^3$H]alanine; 47 Ci/mmol) for 5 min in modified flush medium-I (see Materials and Methods) to determine the mean uptake of eight replicate determinations (approximately five blastocysts/determination) obtained in three independent experiments. $Na^+$-independent uptake ($Li^+$ substituted for $Na^+$) was subtracted from total uptake to produce the data presented. At least two components of transport appeared to be present, and the values of $K_m$ and $V_{max}$ were estimated utilizing the method of Spears et al. [23] to be 26 and 380 $\mu$M and 16 and 25 fmol·blastocysts$^{-1}$·min$^{-1}$, respectively.

(or more) in vitro, whereas no similar increase occurred in blastocysts obtained 26 h before implantation (early blastocysts in Table I). Moreover, uptake of L-lysine by system $b^+$ (in combination with nonsaturable uptake) decreased when late blastocysts were incubated for 1 h, while no changes in uptake of L-lysine or L-leucine by system $b^{0,+}$ or of L-leucine by system L were observed during the same incubation period (Table I). Similar quantitative changes occurred in the transport activities of systems $B^{0,+}$ and $b^+$ in blastocysts that had been activated from delay of implantation with estrogen 20–25 h earlier (implanting blastocysts; Table I), but no such changes have been observed in blastocysts maintained during delay of implantation with progesterone [13].

## Discussion

*$Na^+$-dependent amino acid transport prior to blastocyst formation*

Other than system Gly [1], we have not detected statistically significant $Na^+$-dependent amino acid transport in conceptuses prior to the 2-cell stage of development. At the 2-cell stage, $Na^+$-dependent L-leucine, L-lysine or L-arginine uptake has not been detected consistently in conceptuses when either choline or $Li^+$ has been substituted for $Na^+$ in medium [3], so $Na^+$-dependent uptake of these amino acids is either negligible or obscured by larger components of mediated $Na^+$-independent transport. Although barely perceptible, the pattern of inhibition of $Na^+$-dependent L-alanine uptake in 2-cell conceptuses (Figs. 1 and 2) is consistent with the possibility that this amino acid is taken up by: (a) system $B^{0,+}$ (which can be recognized because it is inhibited by L-lysine and BCH), (b) system Gly (which is inhibited by sarcosine), (c) a L-lysine-resistant component other than system Gly (see next section), or (d) any combination of these transport processes at this stage of development. We have not detected a sarcosine resistant component of glycine transport in conceptuses at the 2-cell stage [1], and this finding supports the hypothesis that these conceptuses take up L-alanine through a weak interaction of this amino acid with system Gly. It is also possible, however, that sarcosine and glycine react relatively weakly with the 'sarcosine-resistant' component of transport [1] which begins to appear at the 2-cell stage, and that 20 mM sarcosine obscures detection of this small component in 2-cell conceptuses when glycine is the substrate. Alanine uptake may also be $Li^+$-stimulated in 2-cell conceptuses, although the difference between uptake in choline- and $Li^+$-containing media could not be distinguished statistically (Fig. 2). If it is nevertheless the case that $Li^+$ can partially replace $Na^+$ to stimulate the small amount of mediated alanine uptake in 2-cell conceptuses, then this transport activity would be distinct from system Gly which, in 2-cell conceptuses [1] and in pigeon erythrocytes [24], is not stimulated by $Li^+$. A substantial component of $Na^+$-dependent L-alanine uptake has been detected in 4-cell conceptuses [7], and we show here that $Na^+$-dependent alanine uptake becomes relatively conspicuous in conceptuses by the 8-cell stage of development (Figs. 1 and 3). We tentatively attribute the L-lysine sensitive portion of this transport to system $B^{0,+}$, as we did when glycine was the substrate [1]. Similarly, as for our studies with glycine [1], a L-lysine-resistant component of L-alanine uptake is also present at this stage (Fig. 3) as well as in blastocysts (Fig. 4). The somewhat greater inhibition of the L-lysine-resistant component of L-alanine uptake by *trans*-4-OH-L-proline than by the *cis* isomer of this amino acid in 8-cell conceptuses (Fig. 3) is consistent with the possibility that it is the same as the less conspicuous of two $Na^+$-dependent systems in blastocysts. These systems may appear first in conceptuses at the 2-cell stage of development, but it is at the blastocyst stage that the systems are most conspicuous.

*Two novel components of $NA^+$-dependent L-alanine transport in blastocysts*

Relatively strong inhibition of L-alanine uptake by L-leucine, BCH, L-lysine and L-arginine (Figs. 1 and 4) is consistent with the interpretation that the novel system $B^{0,+}$ [11,13] is primarily responsible for L-alanine uptake by blastocysts obtained from intact mice. A less conspicuous, L-lysine-resistant, $Na^+$-dependent component of L-alanine transport was also detected in blastocysts (Fig. 4) as reported previously for glycine transport [1]. The L-lysine resistant component of L-alanine transport cannot be attributed to residual system $B^{0,+}$ activity because it is inhibited more strongly by *trans*-OH-L-proline than by L-arginine, whereas the reverse is true for inhibition of system $B^{0,+}$ (Fig. 4). Similarly, L-tryptophan and L-leucine inhibit system $B^{0,+}$ more strongly than do L-serine and L-cysteine, but all four of these amino acids inhibit the L-lysine-resistant component equally well (Fig. 4). Furthermore, the results of kinetic analysis are consistent with the presence of at least two $Na^+$-dependent L-alanine transporters in blastocysts (Fig. 6); a higher affinity system ($K_m \cong 26$ $\mu$M and $V_{max} \cong 16$ fmol · blastocysts$^{-1}$ · min$^{-1}$) which appears to be system $B^{0,+}$ [11] and a lower-affinity system ($K_m \cong 380$ $\mu$M and $V_{max} \cong 25$ fmol · blastocysts$^{-1}$ · min$^{-1}$) which, like $B^{0,+}$, appears to be novel.

The lower-affinity system for L-alanine transport in blastocysts can be distinguished from better-known, broad scope, $Na^+$-dependent systems for zwitterionic amino acid transport in several ways. System A accepts $\alpha$-N-methylated amino acids as substrates [8,17–19], but the 'model' system A substrate, MeAIB, failed to inhibit alanine uptake by blastocysts (Fig. 5). Moreover,

bulky amino acids, such as L-leucine and L-tryptophan, interact with the L-lysine-resistant, zwitterion-preferring L-alanine transporter in blastocysts relatively strongly (Figs. 4 and 5), whereas these amino acids usually interact relatively weakly with systems A and ASC. Moreover, penicillamine is a strong inhibitor of system ASC in hepatocytes [25], but it inhibits alanine uptake by blastocysts weakly if at all (Fig. 4). Finally system ASC is susceptible to inhibition by L-lysine and L-arginine, and this inhibition has been used to infer the structure of the amino acid receptor site of system ASC [26]. The $Na^+$-dependent, zwitterion-preferring system in blastocysts was detected because it resists inhibition by cationic amino acids, however (Fig. 4 and Ref. 1). The latter observation is consistent with the theory that the structure of the amino acid receptor site of the system in blastocysts is different from the structure of system ASC's receptor site. More detailed kinetic studies, such as 'ABC' testing [8,17–19], were not attempted for the L-lysine-resistant component of L-alanine transport. The latter component is relatively inconspicuous, and, even in the presence of 20 mM L-lysine to inhibit most of the system $B^{0,+}$ activity, system $B^{0,+}$ would contribute substantially to uptake at L-alanine concentrations above the $K_m$ value of the L-lysine-resistant component. Nevertheless, we suggest that the L-lysine-resistant, zwitterion-preferring $Na^+$-dependent component of L-alanine and glycine transport in blastocysts is sufficiently distinct from systems A, ASC and $B^{0,+}$ to warrant its separate provisional designation as system B. Although its characterization is incomplete, system B seems to resemble broad scope transport activities in the brush borders of bovine kidney [27] and rabbit intestine [28] more than does system $B^{0,+}$, because system B and the broad scope activities in the kidney and intestine each interact relatively poorly with cationic amino acids.

*Possible significance of changes in amino acid transport in blastocysts during the day preceding implantation*

Although the quantity of system $B^{0,+}$ activity that blastocysts can express increases by about two to 3-fold between 26 and 6 h before implantation [8], the amount of this activity that blastocysts actually express in situ near the time of implantation may not be as high as the level of activity they are capable of exhibiting. The level of $B^{0,+}$ activity expressed by blastocysts obtained from intact mice about 6 h prior to implantation increases about 3-fold within 1 h after they are disturbed (e.g., flushed from uteri in culture medium; Table I). A similar increase is attributable mainly to an increase in the $V_{max}$ value for L-alanine uptake in implanting blastocysts obtained 20–25 h after administration of estrogen to their ovariectomized mothers, and the increase requires only 20 min to complete [13]. No such increase occurs when early blastocysts from intact mice (Table I) or diapausing conceptuses from ovariectomized, progesterone-treated mice [13] are disturbed in similar ways. Moreover, system $b^+$ activity decreases when late normal and implanting blastocysts are removed from the uterine lumen (Table I and Ref. 13). The extent of the latter decrease may be greater than the data indicate, unless nonsaturable uptake also decreases. For technical reasons associated with quickly obtaining and manipulating a large enough number of conceptuses, the nonsaturable component of uptake has not been subtracted from the uptake listed in Table I. In contrast, no changes were observed in the capacities of blastocysts to take up amino acids by systems L or $b^{0,+}$ when blastocysts were disturbed near the time of implantation. Therefore, it seems unlikely that the changes in transport that occur when either late normal or implanting blastocysts are removed from the uterus (i.e., disturbed) can be attributed to damage to the conceptuses or another nonspecific effect of the isolation procedure on amino acid transport in blastocysts. In fact, apparently the same increase in system $B^{0,+}$ activity can be elicited in implanting blastocysts in situ by gently massaging the uterus with a blunt instrument [13]. The latter treatment is known to cause a rapid increase in the levels of adenyl cyclase activity and cAMP in the rodent uterus, but only while it is receptive to implantation [29–32]. We suggested [13] that rapid changes in the activities of amino acid transport systems may reflect a more general susceptibility of blastocysts to metabolic perturbation near the time of implantation. Such sensitivity of the metabolism of both the uterus and blastocyst may be needed for communication between them, perhaps to coordinate the implantation process.

We also suggested previously that the increase in system $B^{0,+}$ activity that occurs in blastocysts when they are disturbed near the time of implantation could be secondary to an increase in the intracellular $[K^+]$ and a decrease in the $[Na^+]$ [13]. The present studies indicate, however, that if these changes in ion concentrations occur, they do not lead to an increase in membrane potential. If membrane potential increases then uptake of cationic amino acids via cation-preferring transport processes such as system $b^+$ (or via nonsaturable routes) should also increase, not decrease (Table I). In fact, the velocity of L-arginine uptake via system $y^+$ in human fibroblasts correlates directly with the magnitude of the membrane potential [33]. Much remains to be learned about the mechanisms by which changes in amino acid transport activities occur when blastocysts are disturbed near the time of implantation. Nevertheless, if we can determine how system $B^{0,+}$ activity increases while system $b^+$ activity (and perhaps nonsaturable uptake) decreases in the latter conceptuses, then we may gain insight not only into how blastocysts communicate with

the uterus, but also into biochemical mechanisms of amino acid transport.

**Acknowledgements**

We thank Barbara Le Breton, Mr. Howard Wasserlauf and Mr. Brian Weimer for helping us prepare the manuscript. This work was supported by NICHD grant HD21801.

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