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Transport of benzenoid amino acids by system T and four broad scope systems in preimplantation mouse conceptuses

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We have studied transport of L-tryptophan, L-tyrosine and L-phenylalanine as factors contributing to homeostasis of these amino acids in preimplantation mouse conceptuses. Benzenoid amino acids were transported by the Na^+-independent systems L and $b^{0,+}$ in 1-cell conceptuses, and by these systems plus the Na^+-dependent systems $B^{0,+}$ and B in blastocysts. In addition, a component of Na^+-independent tryptophan, tyrosine and phenylalanine transport in 1-cell and 2-cell conceptuses and in blastocysts resisted inhibition by L-leucine. The latter component of transport not only preferred benzenoid amino acids and in particular tryptophan as substrates, but it also was inhibited strongly and competitively by α-*N*-methyl-L-tryptophan. The leucine-resistant component of tryptophan transport also was inhibited strongly by *N*-ethylmaleimide and D-tryptophan, and it appeared to be inhibited weakly by 3-amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid (BCO) but not by other amino acids tested as inhibitors. By these criteria, the leucine-resistant component of transport of benzenoid amino acids resembled system T in human red blood cells and rat hepatocytes. It is not entirely clear why preimplantation blastocysts have five good systems for transport of tryptophan. It is possible, however, that tryptophan homeostasis is particularly important during preimplantation development since it has been shown elsewhere that tryptophan availability in blood increases within one day after rat eggs are fertilized.

Introduction

The concentrations of both free and bound tryptophan increase in the rat circulatory system within one day after rat eggs are fertilized, and the available tryptophan remains elevated until at least day 12 of pregnancy [1]. The increase in circulating tryptophan correlates with inhibition of rat liver tryptophan 2,3-dioxygenase activity [1], so the increase may result from a decrease in tryptophan catabolism via the kynurenine-anthranilate pathway. Since rat and mouse conceptuses do not appear to grow for at least the first four days of their development (e.g., Ref. 2), the increase in tryptophan availability may have functions other than to support protein accumulation in preimplantation conceptuses. In this regard, even a quantitatively minor metabolite of tryptophan, picolinate, improves development of preimplantation mouse conceptuses when it is consumed by mice deprived of dietary tryptophan, niacin and vitamin B_6 during the first three days of pregnancy [3]. Unfortunately studies such as the latter one do not distinguish the role of the mother from that of the conceptus in tryptophan homeostasis. For this reason, we have begun to study tryptophan homeostasis in preimplantation mouse conceptuses by studying membrane transport of this and other benzenoid amino acids.

Several amino acid transport processes have been detected in mouse conceptuses [4–12], and three out of the five systems that accept zwitterionic amino acids as substrates are novel. In blastocysts, system $B^{0,+}$ is the predominant Na^+-dependent transporter of a broad scope of zwitterionic and cationic amino acids [4]. Similarly, system $b^{0,+}$ is the predominant mediated Na^+-independent transport process in blastocysts, although its substrate selectivity within each of the cationic and zwitterionic amino acid categories differs considerably from that of system $B^{0,+}$ [5]. A less conspicuous Na^+-dependent transporter of zwitterionic amino acids, provisionally termed system B, is also present in blastocysts [6], as is system L [5,7], which is a Na^+-independent transporter of bulky zwitterionic substrates in numerous tissues [8,13]. Prior to formation of blastocysts, system

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L predominates in conceptuses [7] along with system Gly [9,14], which may be the only Na^+-dependent system present in mouse conceptuses until after the 2-cell stage of development [6,9]. Except for system Gly, each of the four other systems in mouse conceptuses should transport benzenoid amino acids, although experiments described here are the first to test this hypothesis directly.

It is also possible that additional systems are present in preimplantation mouse conceptuses to mediate transport of tryptophan and other benzenoid amino acids. As for the systems discussed above [6–11], the activities of these additional systems could be developmentally regulated. Since most of the relatively broad scope transporters of zwitterionic amino acids probably have already been detected in conceptuses [4–9], we anticipate that the substrate selectivities of any additional systems are more limited. One such transport system in human red blood cells [15–17] and rat hepatocytes [18] is system T, which selects for benzenoid amino acids. We report here the results of experiments designed to determine whether a system, such as T, or related systems are present in preimplantation mouse conceptuses.

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 [3–12]. 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 [19]. Eggs or conceptuses were removed from oviducts in Brinster's medium [20] about 17 (1-cell stage), 41 (2-cell stage), and 66 (8-cell stage) h after administration of human chorionic gonadotropin (HCG) or from uteri about 94 h after administration of this hormone (blastocysts). 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 six h in Brinster's medium in a humidified atmosphere of 5% CO_2 in air at 37 °C (pH 7.4). Transport was not observed to change in eggs or conceptuses at these stages of development during incubation for 6 h in vitro.

Amino acid uptake increased linearly with time for at least 5 min at 37 °C, and virtually no uptake could be attributed to binding (Refs. 4, 6, 7, 9, 12 and data not shown). Therefore, initial velocities of transport were estimated by incubating eggs or conceptuses for 5 min with a ^3H-labeled form of L-alanine, L-leucine, L-tryptophan, L-tyrosine, or L-phenylalanine (5–130 Ci/mmol; ICN Pharmaceuticals or Amersham) and various concentrations of nonradioactive amino acids [4–12]. Radiolabeled preparations were evaporated to dryness with a stream of N_2 at room temperature before use in experiments. Amino acids were dissolved in phosphate-buffered NaCl (pH 7.1; Refs. 4–12), or a modification of Spindle's [21] flushing medium-I (Na^+ salts of lactate and pyruvate replaced with NaCl, Na_2HPO_4 replaced with K_2HPO_4, and phenol red deleted; Ref. 6). In some cases, Na^+ in the medium was replaced with choline or Li^+ during labeling in order to measure Na^+-independent amino acid uptake. Although bovine serum albumin binds tryptophan, we included a low concentration of this protein in media unless specified otherwise. Conceptuses are considerably easier to manipulate rapidly when albumin is present in the medium. Moreover, the concentration of albumin we used (1 mg/ml or 15 μM) would bind less than 25% of the tryptophan present in the medium even at a total tryptophan concentration as low as 1.0 μM and a K_a value as high as $20 \cdot 10^3$ M^{-1} [22]. In comparative studies, albumin did not appear to alter our results significantly (see Results and Discussion).

In many experiments amino acid uptake was examined at substrate concentrations near 1 μM for two principal reasons as discussed and justified in detail elsewhere [6,12]. Briefly, 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 [23], 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 μM have been detected in preimplantation conceptuses [7,9,12]. Except for 2-amino-*endo*-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH) (Behring Diagnostics) and 3-amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid (BCO) (gift from Professor Carmen Avendaño, Ref. 24), chemicals were purchased from Sigma Chemical Co. The concentrations of radiolabeled and non-labeled amino acids in the medium did not change significantly in the presence of eggs and conceptuses as discussed previously [5,9]. After incubation with amino acids, eggs or conceptuses were processed [4,6,11] to determine how much of the substrate they had taken up. Samples of the final wash medium, equal in volume to the volume in which conceptuses were collected, contained near background levels of radioactivity and were subtracted from the radioactivity in samples containing conceptuses. Parametric [25,26] and non-parametric [27–31] statistical methods were used to perform analysis of variance and to calculate the variability in the values of kinetic parameters, respectively, as discussed previously [7,12].

Results and Discussion

L-Leucine-resistant uptake of benzenoid amino acids

Initial experiments were designed to determine the effects of 10 mM BCH, L-arginine, 2-(methylamino)isobutyrate (MeAIB), L-glutamate, L-phenylalanine, L-leucine, L-glutamine, L-methionine, L-alanine and L-tryptophan on uptake of L-[^{3}H]tyrosine from a 0.78 μM solution by unfertilized and fertilized eggs, 2- and 8-cell conceptuses, and blastocysts. The results of these initial experiments (not shown) and prior studies [5,7] supported the conclusion that tyrosine was transported mainly by system L in preimplantation conceptuses before they form blastocysts and primarily by system

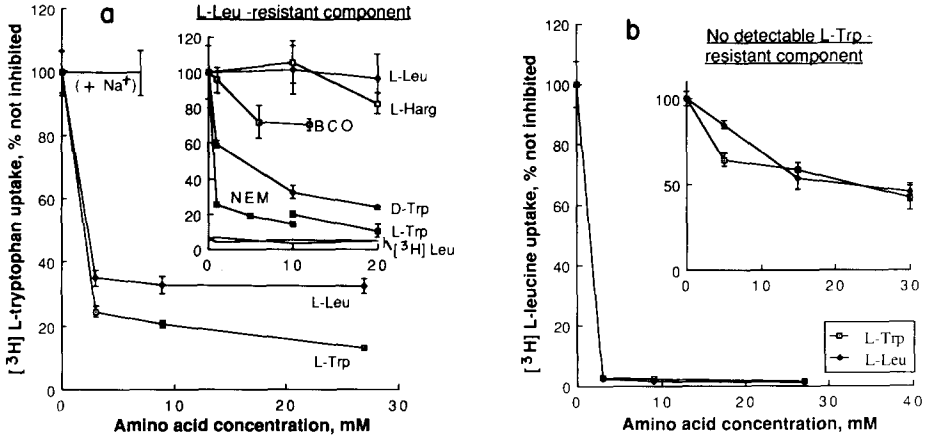

Fig. 1. L-Leucine-resistant L-tryptophan uptake (panel a) but no L-tryptophan-resistant L-leucine uptake (panel b) in 1-cell mouse conceptuses. Conceptuses were incubated with (a) 50 μCi/ml [^{3}H]tryptophan (5.1–8.6 μM) or (b) 50 or 200 (inset) μCi/ml [^{3}H]leucine (0.4–1.5 μM) and various concentrations of the indicated nonradioactive amino acids for 5 min in phosphate-buffered LiCl or choline Cl. For data presented in insets, 10 mM leucine and 3 mM L-arginine (a) or 10 mM tryptophan and 3 mM L-homoarginine (b) were present in addition to the indicated amino acids. Data obtained in LiCl and choline Cl solutions were similar and they were combined. The mean uptake ± S.E. was calculated from 3–6 replicate determinations (approximately 10 conceptuses/determination) obtained in one (b) or two (a) independent experiments. Uptake in phosphate-buffered NaCl is indicated by the horizontal line marked '+Na^+'. Results similar to those presented in the inset of 'b' also were obtained with 2-cell conceptuses (data not shown). The lines without symbols for points or error bars at about 5% in the inset of 'a' were for [^{3}H]leucine utpake as a % of [^{3}H]tryptophan uptake under similar conditions and in the presence of various concentrations of nonradioactive leucine or tryptophan (not distinguished). Harg, homoarginine.

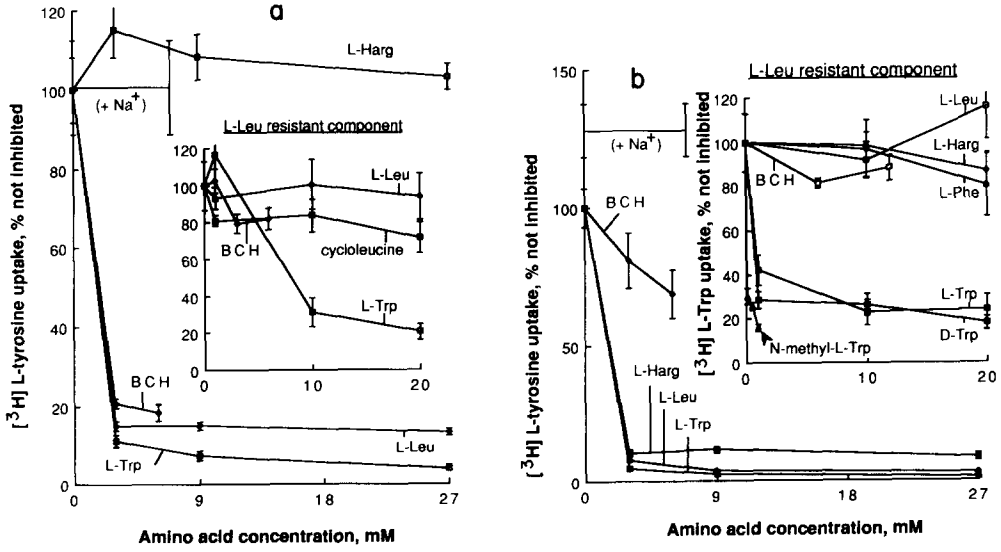

Fig. 2. L-Leucine-resistant L-tyrosine and L-tryptophan uptake by 2-cell conceptuses (panel a) and blastocysts (panel b). Conceptuses were incubated with 40 or 50 μCi/ml [^{3}H]tyrosine (1.0 μM) or [^{3}H]tryptophan (5.1–8.6 μM) and various concentrations of the indicated nonradioactive amino acids for 5 min in phosphate-buffered LiCl. For data presented in insets, 10 mM leucine and 3 mM L-arginine (or homoarginine) were present in addition to the indicated amino acids, and albumin was not present in the medium used in experiments reported in the inset of 'b'. The mean uptake ± S.E. was calculated from 4–9 determinations (about 5–10 conceptuses/determination) obtained in 2–5 independent experiments (one experiment and three determinations for two of the three concentrations of D-tryptophan and *N*-methyl-L-tryptophan). Uptake in phosphate-buffered NaCl is indicated by the horizontal lines marked '+Na^+'.

$b^{0,+}$ thereafter. Nevertheless, slightly greater inhibition of tyrosine uptake by tryptophan or phenylalanine than by leucine or methionine was consistent with the interpretation that a small component of tyrosine uptake occurred via an L-leucine-resistant, benzenoid amino acid-preferring transport process in eggs and preimplantation conceptuses.

To further test the latter possibility, we examined the effects of various concentrations of nonradioactive L-leucine and L-tryptophan on uptake of ^{3}H-labeled L-tyrosine, L-tryptophan, L-phenylalanine and L-leucine by 1- and 2-cell conceptuses and by blastocysts. An inconspicuous component of tryptophan uptake by 1-cell conceptuses resisted inhibition by leucine (Fig. 1a), and such was also the case for transport of tyrosine and phenylalanine ($P < 0.01$; data not shown). Similarly, 2-cell conceptuses contained a leucine-resistant component of tyrosine uptake (Fig. 2a), and blastocysts also exhibited tyrosine and tryptophan transport that was not inhibited by leucine (Fig. 2b). The leucine-resistant component of tryptophan transport was inhibited relatively strongly by nonradioactive L-tryptophan, α-*N*-methyl-L-tryptophan, D-tryptophan and *N*-ethylmaleimide, but not by most other substances that were tested as inhibitors (insets of Figs. 1a and 2; Table I). No residual, L-tryptophan-sensitive L-[^{3}H]leucine uptake was detected in 1-cell conceptuses in the presence of an excess of nonradioactive L-leucine (Fig. 1a, inset), so detection of a leucine-resistant, tryptophan-sensitive component of uptake of radiolabeled L-tryptophan, L-tyrosine or L-phenylalanine does not appear to have been an artifact of the assay system. Similarly, tryptophan-resistant [^{3}H]leucine uptake was not detected in 1-cell conceptuses (Fig. 1b).

TABLE I

Effect of various substances on L-leucine-resistant L-tryptophan uptake (system T activity) in 1-cell conceptuses

Conceptuses were incubated with 8.6 μM [^{3}H]tryptophan, 10 mM L-leucine, 10 mM L-arginine and the indicated concentrations of various substances for 5 min in phosphate-buffered LiCl. The mean uptake ± S.E. was calcualted from four replicate determinations (12 conceptuses per determination) obtained in two independent experiments (two determinations and one experiment for dopamine, 5-OH-L-tryptophan, serotonin and *N*-methylserotonin). Inhibition to less than about 72% of the 'None' group was statistically significant ($P < 0.01$; analysis of variance).

Inhibitor	% of [^{3}H]tryptophan uptake not inhibited
None	100.0 ± 6.9
2 mM L-phenylalanine	90.2 ± 8.2
2 mM L-tyrosine	92.0 ± 6.9
2 mM L-DOPA	86.5 ± 1.3
2 mM dopamine	74.8 ± 5.9
2 mM norepinephrine	87.2 ± 5.9
2 mM epinephrine	94.2 ± 3.6
2 mM L-tryptophan	49.5 ± 2.6
2 mM *N*-methyl-L-tryptophan	52.1 ± 2.2
2 mM 5-OH-L-tryptophan	82.1 ± 1.5
20 mM *N*-methyl-L-phenylalanine	71.5 ± 8.2
20 mM *N*-acetyl-L-phenylalanine	83.2 ± 3.9
20 mM L-phenylalanine	47.3 ± 2.6
20 mM L-tryptophan	17.2 ± 0.9
20 mM serotonin	65.2 ± 5.1
20 mM *N*-methylserotonin	49.1 ± 3.7
20 mM L-valine	120.0 ± 7.9
20 mM L-leucine	114.0 ± 8.1

Three components of Na^+-independent L-tryptophan transport in 1-cell conceptuses and blastocysts

From the preceding experiments and studies reported previously [5,7], tryptophan appears to be transported by at least three Na^+-independent systems in both 1-cell conceptuses and blastocysts. They are systems L and $b^{0,+}$ and a process that seems to prefer benzenoid amino acids as substrates. For convenience, the latter process will be referred to as system T; its similarities to this system are discussed in a subsequent subsection. Since each of these transport processes appears to be selectively inhibited by some amino acids that do not interact strongly with the other two systems, we largely 'isolated' each transport activity by selectively inhibiting the other two mediated transport processes. (See above and Refs. 5 and 7 for evidence for the selective inhibition described below.)

When 1-cell conceptuses were incubated in the presence of 10 mM L-arginine and *N*-ethylmaleimide to inhibit systems $b^{0,+}$ and T, respectively, [^{3}H]tryptophan uptake was inhibited strongly by nonradioactive L-tryptophan and the system L substrates, BCO and BCH, but not by the system $b^{0,+}$ inhibitor, L-homoarginine, or the system T inhibitor, *N*-methyl-L-tryptophan (Fig. 3a). Similarly, in the presence of 10 mM BCH and L-arginine to inhibit systems L and $b^{0,+}$, respectively, [^{3}H]tryptophan transport by 1-cell conceptuses was inhibited strongly by nonradioactive L-tryptophan, *N*-ethylmaleimide and the system T inhibitor, *N*-methyl-L-tryptophan, but not in a statistically significant manner by the system $b^{0,+}$ inhibitor, L-homoarginine, or the system L substrate, BCO (Fig. 3b). Likewise, in the presence of 10 mM BCH and 2 mM *N*-methyl-L-tryptophan to inhibit systems L and T, respectively, uptake of [^{3}H]tryptophan by 1-cell conceptuses was inhibited strongly by nonradioactive L-tryptophan, *N*-ethylmaleimide, and the system $b^{0,+}$ substrates L-arginine and L-homoarginine, but not by the system L substrate, BCO (Fig. 3c). In the latter case, we attribute arginine- and homoarginine-resistant uptake mainly to residual system T activity which was not inhibited completely by 2 mM *N*-methyl-L-tryptophan. (The maximum solubility of *N*-methyl-L-tryptophan in our media appeared to be about 2 mM.) D-Tryptophan was a moderately strong inhibitor of systems $b^{0,+}$ and L (data not shown), as well as a strong inhibitor of system T (Figs. 1a and 2b,

insets), and *N*-ethylmaleimide inhibited system $b^{0,+}$ as well as system T (Figs. 3b and c). Therefore, neither D-tryptophan nor *N*-ethylmaleimide could be used to help isolate the small amount of system $b^{0,+}$ in 1-cell conceptuses [7]. Nevertheless, the effect of the system $b^{0,+}$ substrate, arginine, and the system T inhibitor, *N*-methyl-L-tryptophan, together appeared to be greater than the inhibition that could be achieved by either substance alone when [^{3}H]tryptophan uptake was measured in the presence of 10 mM BCH to inhibit system L (Figs. 3c and d). For these reasons, system $b^{0,+}$ appeared to transport tryptophan in 1-cell conceptuses.

Three components of Na^+-independent L-tryptophan transport also were detected and largely isolated in blastocysts. In the presence of 10 mM *N*-ethylmaleimide and L-arginine to inhibit systems T and $b^{0,+}$, respectively, the system L substrates, BCO and BCH, inhibited [^{3}H]tryptophan uptake by blastocysts almost as strongly as nonradioactive L-tryptophan did (Fig. 4a). In the latter case, *N*-methyl-L-tryptophan and L-homoarginine also were weak inhibitors ($P < 0.05$), so the latter amino acids may interact weakly with system L, which is the case at least for cationic amino acids for other instances of system L [13]. Similarly, when system T activity was isolated by incubating blastocysts in 10 mM BCH and L-arginine to inhibit systems L and $b^{0,+}$, respectively, *N*-methyl-L-tryptophan and *N*-ethylmaleimide inhibited [^{3}H]tryptophan uptake strongly, whereas BCO was a weak inhibitor and L-homoarginine inhibited uptake weakly or not at all (Fig. 4b). BCO may

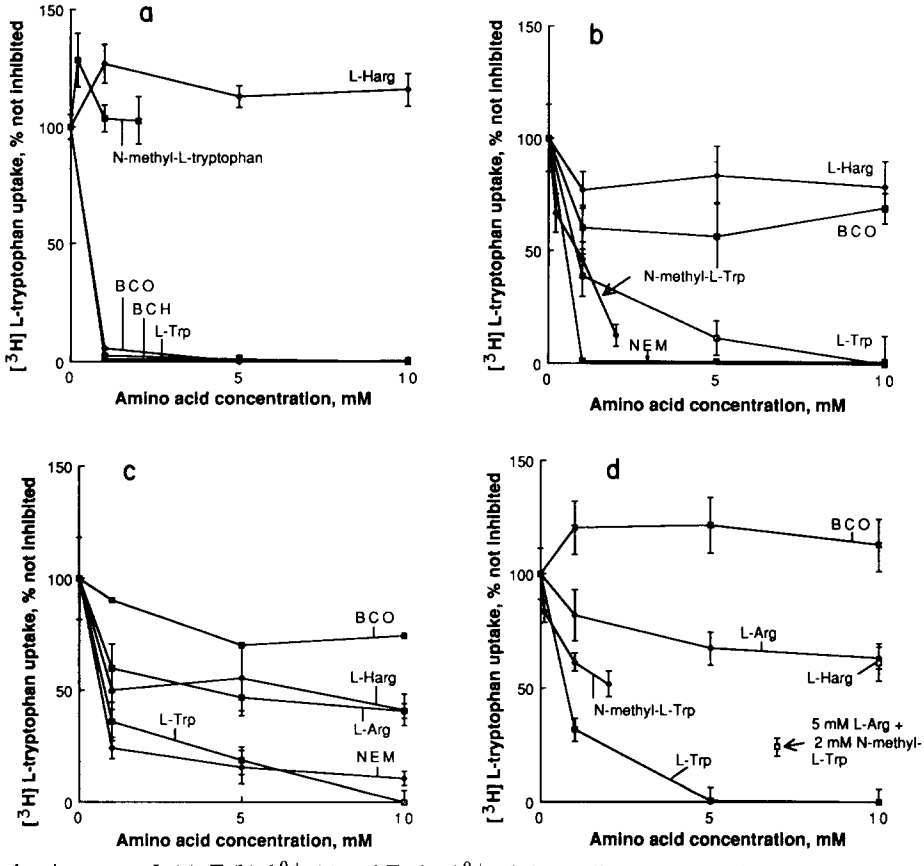

Fig. 3. L-Tryptophan uptake via systems L (a), T (b), $b^{0,+}$ (c) and T plus $b^{0,+}$ (d) in 1-cell conceptuses. Conceptuses were incubated with 20 (a) or 50 (b, c, d) μCi/ml [^{3}H]tryptophan (1.8–8.6 μM) and various concentrations of the indicated nonradioactive amino acids for 5 min in phosphate-buffered LiCl. Experimental results were obtained in the presence (panel a) of 10 mM L-arginine plus 10 mM *N*-ethylmaleimide (NEM) (to largely isolate system L); in the presence (panel b) of 10 mM BCH plus 10 mM L-arginine (to largely isolate system T); in the presence (panel c) of 10 mM BCH plus 2 mM *N*-methyl-L-tryptophan (to largely isolate system $b^{0,+}$); or in the presence (panel d) of 10 mM BCH to largely isolate systems T plus $b^{0,+}$ (see text and Refs. 5 and 7 for discussion of inhibitor selectivities). The mean uptake $\pm$ S.E. was calculated from 4–12 replicate determinations (3–8 conceptuses/determination) obtained in 2–4 independent experiments (two determinations and one experiment for BCO in 'c'; no S.E. values calculated). All values were adjusted assuming 100% inhibition by 10 mM nonradioactive L-tryptophan (i.e., nonsaturable uptake and < 26% of saturable uptake are not represented). In 'b', inhibition by NEM was actually somewhat greater than is indicated. 100% Uptake of [^{3}H]tryptophan represents 237, 22 and $12 \cdot 10^{-18}$ mol·conceptuses^{-1}·min^{-1}·(μM [^{3}H]tryptophan)$^{-1}$ in 'a', 'b' and 'c', respectively. The quantity of radioactivity actually detected at 100% uptake in 'c' was about 100% above background. Analysis of variance was used to determine if inhibition was statistically significant ($P < 0.01$) unless the variances were unequal in which cases inhibition was obvious without formal statistical analysis (e.g., inhibition by BCH and BCO in 'a'). By these criteria, BCH, BCO and nonradioactive L-tryptophan inhibited uptake in 'a', *N*-methyl-L-tryptophan, NEM and nonradioactive tryptophan inhibited uptake in 'b', and NEM, L-arginine, L-homoarginine, and nonradioactive tryptophan inhibited uptake in both 'c' and 'd' (NEM is not shown in 'd'). *N*-Methyl-L-tryptophan also inhibited uptake in 'd', and inhibition by 5 mM arginine plus 2 mM *N*-methyl-L-tryptophan was greater than by either of these substances alone in 'd' ($P < 0.05$).

only appear to inhibit system T in blastocysts because system L activity was not inhibited completely by 10 mM BCH (Fig. 4b), or BCO may interact weakly with system T as it does in human red blood cells [17]. System $b^{0,+}$ is the predominant Na^+-independent transport system in blastocysts [5,7], whereas uptake of benzenoid amino acids by system T is relatively inconspicuous at substrate concentrations near 1 μM (Fig. 2b). Therefore, uptake of [^{3}H]tryptophan in the presence of 10 mM BCH to inhibit system L was almost completely inhibited by the system $b^{0,+}$ inhibitors, L-arginine, L-homoarginine and *N*-ethylmaleimide (Fig. 4c). As anticipated, neither the system T inhibitor, *N*-methyl-L-tryptophan, nor the system L substrate, BCO, significantly influenced [^{3}H]tryptophan uptake in the presence of 10 mM BCH (Fig. 4c).

Each of the three Na^+-independent systems for transport of benzenoid amino acids in blastocysts and two of the three systems in 1-cell conceptuses also were isolated for kinetic studies in a manner similar to that described above. We did not attempt to isolate system $b^{0,+}$ in 1-cell conceptuses because uptake of [^{3}H]tryptophan by this system was barely detectable at this stage of development. In fact, system $b^{0,+}$ seemed to be partially obscured by residual system T activity and, perhaps, by system L activity even when the latter two transport processes had been largely inhibited (Fig. 3c).

As we reported previously for L-leucine transport [5,7], the V_{max} value for tryptophan transport via system L was lower in blastocysts than in 1-cell conceptuses, and BCH competitively inhibited the system at both stages of development (Table II). Similarly, system T activity decreased somewhat in conceptuses between the 1-cell and blastocyst stages of development, and *N*-methyl-L-tryptophan competitively inhibited system T equally well at both stages (Table II). In contrast, system $b^{0,+}$ was the most conspicuous Na^+-independent transporter of tryptophan in blastocysts, whereas it was scarcely detectable in 1-cell conceptuses (legends of Figs. 3 and 4; Table II) as is the case for transport of other $b^{0,+}$ substrates [5,7]. L-Arginine was a particularly strong competitive inhibitor of system $b^{0,+}$ in blastocysts (Table II), as anticipated from its K_m value of about 2 μM for transport via system $b^{0,+}$ in 1-cell conceptuses [7]. The variabilities in the values of V_{max} and K_m we report (Table II) may have been over-estimated by about 2-fold [12,31]. However, as discussed previously [12], we think that the method we use to calculate this variability is statistically more acceptable than many other methods.

The presence of system T as well as system L in 1-cell conceptuses also was demonstrated kinetically under conditions where both systems were fully active. Under such conditions and in the absence or presence of 20 mM L-lysine to inhibit system $b^{0,+}$, Hofstee plots of mediated Na^+-independent tryptophan uptake were curved, thus supporting the conclusion that at least two systems were transporting tryptophan (Fig. 5). As anticipated (see Materials and Methods), similar results were obtained in the absence or presence of albumin at a

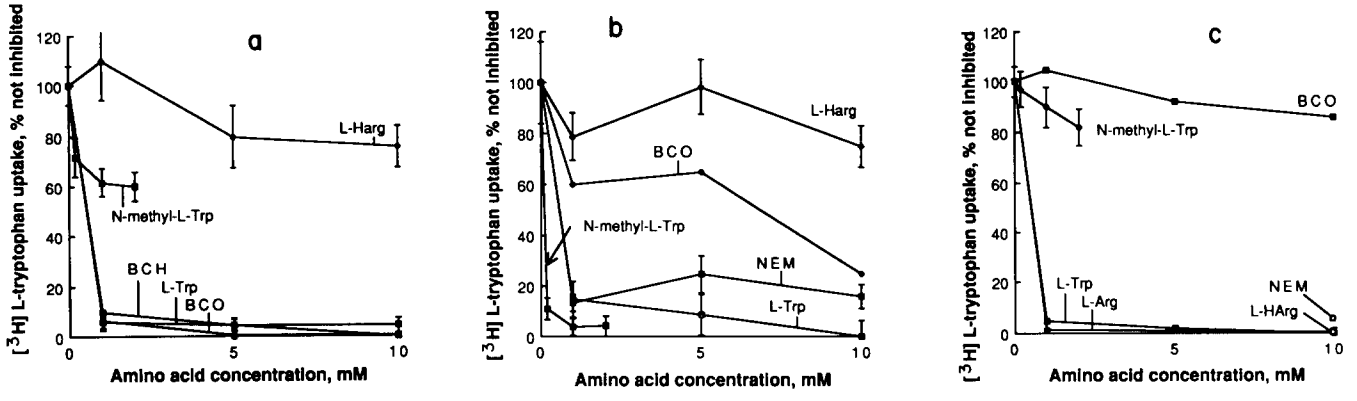

Fig. 4. L-Tryptophan uptake via systems L (a), T (b) and $b^{0,+}$ (c) in blastocysts. Conceptuses were incubated with 20 (c) or 50 (a and b) μCi/ml [^{3}H]tryptophan (1.8–8.6 μM) and various concentrations of the indicated nonradioactive amino acids for 5 min in phosphate-buffered LiCl. (Albumin was absent from the medium in 'b'.) Experimental results were obtained in the presence (panel a) of 10 mM L-arginine plus 10 mM *N*-ethylmaleimide (NEM) (to largely isolate system L); in the presence (panel b) of 10 mM BCH plus 10 mM L-arginine (to largely isolate system T); or in the presence (panel c) of 10 mM BCH (to largely isolate system $b^{0,+}$) (see text and Refs. 5 and 7 for discussion of inhibitor selectivities). The mean ± S.E. was calculated from 3–13 replicate determinations (3–6 conceptuses/determination) obtained in 2–5 independent experiments (two determinations and one experiment for BCO in 'b' and 'c'; no S.E. values calculated). All mean values were adjusted assuming 100% inhibition by 10 mM nonradioactive L-tryptophan (i.e., nonsaturable uptake and < 15% of saturable uptake are not represented). 100% Uptake of [^{3}H]tryptophan represents $69 \cdot 10^{-18}$, $23 \cdot 10^{-18}$ and $395 \cdot 10^{-18}$ mol·conceptus^{-1}·min^{-1}·(μM [^{3}H]tryptophan)$^{-1}$ in 'a', 'b' and 'c', respectively. The quantity of radioactivity actually detected at 100% uptake in 'b' was about 200% above background. Analysis of variance was used to determine if inhibition was statistically significant unless the variances were unequal in which cases inhibition was obvious without formal statistical analysis (e.g., inhibition by NEM, L-arginine and L-homoarginine in 'c'). By these criteria, BCH, BCO and nonradioactive L-tryptophan ($P < 0.01$) and L-homoarginine and *N*-methyl-L-tryptophan ($P < 0.05$) inhibited uptake in 'a'; NEM, *N*-methyl-L-tryptophan and nonradioactive L-tryptophan ($P < 0.01$) and BCO ($P < 0.05$) inhibited uptake in 'b'; and NEM, L-arginine, L-homoarginine and nonradioactive L-tryptophan ($P < 0.01$) inhibited uptake in 'c'.

concentration of 1.0 mg/ml (Fig. 5). Therefore, this protein did not appear to interfere significantly with our assay of tryptophan transport. Hofstee plots of mediated, Na^+-independent tyrosine transport in the presence

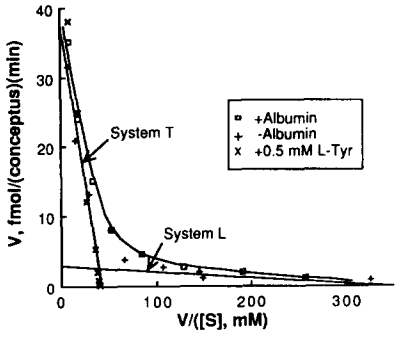

Fig. 5. Hofstee plots of Na^+-independent L-tryptophan transport in 1-cell conceptuses. Conceptuses were incubated with the indicated concentrations of tryptophan for 5 min in phosphate-buffered LiCl containing 1.0 mg/ml albumin (squares) or this medium without albumin but with 20 mM L-lysine (+) to determine the mean uptake of four replicate determinations at each concentration (6–10 conceptuses/determination) obtained in two independent experiments. Nonsaturable uptake was subtracted from total uptake to produce the data presented. At least two components of transport appeared to be present in both cases, and an approximate representation for the two components detected in the presence of albumin are labeled systems T and L (calculated utilizing the method described in Ref. 35). The curved line represents the combination of these two straight lines. The effect of 0.5 mM L-tyrosine on tryptophan transport is also shown (×).

or absence of lysine and albumin also were curved, so it too appeared to be transported simultaneously by systems T and L in 1-cell conceptuses (data not shown). In contrast, Hofstee plots of mediated, Na^+-independent leucine transport were not curved, so leucine seemed to be taken up mainly by system L in 1-cell conceptuses (data not shown; the V_{max} value of system $b^{0,+}$ is too low and its K_m value is probably too close to that of system L to resolve these two systems kinetically in 1-cell conceptuses, Ref. 23). In the latter case, L-tryptophan was a strong competitive inhibitor of leucine uptake with a K_i value (19 μM) which was nearly the same as the value of its K_m for transport via system L (Table II).

Na^+-dependent L-tryptophan transport by blastocysts

Blastocysts had a small, Na^+-stimulated component of benzenoid amino acid transport ($P < 0.05$) that was not detected in 1- and 2-cell conceptuses (Figs. 1 and 2). Unfortunately, all known inhibitors of systems $b^{0,+}$ and L also inhibit system $B^{0,+}$ [4–9]. Therefore, it was not

TABLE II

Values of kinetic parameters for transport and inhibition of transport of L-tryptophan in 1-cell conceptuses and blastocysts

Conceptuses were incubated with various concentrations of L-tryptophan for 5 min in phosphate-buffered LiCl or NaCl. Substrate concentrations were selected to be within about an order of magnitude above and below the anticipated K_m values (Ref. 36) as determined in preliminary studies. Means of 6–7 replicate determinations (3–9 conceptuses per determination) obtained in three independent experiments (two experiments and four determinations for system L in 1-cell conceptuses) were calculated at each concentration to produce Hofstee plots and calculate the values of kinetic parameters and their 73–77% confidence intervals [27–31]. These confidence intervals were selected because they are somewhat larger than the S.E. values of the mean values of kinetic parameters which are often reported in the literature. The nonparametric method we used to estimate variability in the values of kinetic parameters over estimates this variability by about 2-fold when the means at five or six substrate concentrations are assessed together [31]. Nevertheless, we think the method we use is statistically more acceptable than other methods used to estimate this variability [12]. Systems L, T and $b^{0,+}$ were isolated by measuring Na^+-independent tryptophan uptake in the presence of an excess of amino acids selected to inhibit the other two systems (e.g., see Figs. 1–4). Nonsaturable uptake was subtracted from total uptake before kinetic parameters were calculated. System $B^{0,+}$ activity was assessed by subtracting uptake in the absence of Na^+ (Na^+ replaced with Li^+) from uptake in the presence of Na^+. Only rough estimates of the values of kinetic parameters were obtained with the latter procedure for tryptophan because the Na^+-independent component of tryptophan uptake was relatively large. Since uptake via system B in blastocysts was negligible at concentrations of amino acids needed to estimate kinetic parameters for system $B^{0,+}$ [6,9], it was not necessary to deduct uptake via system B from total Na^+-dependent uptake to calculate the values of kinetic parameters for system $B^{0,+}$. K_i values were calculated from the effects of inhibitory amino acids on the K_m values of substrates except for inhibition of tryptophan uptake by alanine via system $B^{0,+}$. In the latter case, the K_i value was calculated from the ability of 90 μM alanine to inhibit Na^+-dependent uptake of 2.5 μM tryptophan utilizing the formula in Ref. 11.

System and stage of development	Values of kinetic parameters (73–77% confidence interval)		
	V_{max} [a]	K_m [b]	K_i [b] (inhibitor)
1-cell			
L	4.9 (4.6–5.5)	24 (21–38)	63 (BCH)
T	76 (75–112)	3500 (3500–5500)	870 (*N*-methyl-L-Trp)
$b^{0,+}$	[c]	[c]	
Blastocyst			
L	1.6 (1.3–2.4)	24 (13–47)	≈ 58 (BCH)
T	47 (36–58)	1700 (1200–2400)	780 (*N*-methyl-L-Trp)
$b^{0,+}$	25 (24–26)	55 (48–64)	3.8 (L-Arg)
B	[c]	[c]	
$B^{0,+}$	≈ 10	≈ 8	≈ 20 (L-Ala)
$B^{0,+}$ (Ala) [d]	25 (22–27)	32 (28–37)	13 (L-Trp)

Notes to Table II:

[a] fmol·conceptus^{-1}·min^{-1}.

[b] μM.

[c] Uptake via the indicated component of transport was too slow relative to uptake via other components to accurately estimate kinetic parameters.

[d] L-Alanine transport was measured in the presence or absence of Na^+ (Li^+ used to replace Na^+) in modifications of Spindle's flush medium-I (see Materials and Methods).

possible to isolate and characterize the Na^+-dependent component of tryptophan transport in blastocysts, which appeared to be mainly system $B^{0,+}$. Nevertheless, rough estimates of the values of kinetic parameters for Na^+-dependent L-tryptophan uptake were obtained by subtracting the relatively large Na^+-independent component of tryptophan transport from total transport (Table II). Moreover, inhibition of Na^+-dependent tryptophan transport by L-alanine was probably competitive because 90 μM alanine inhibited Na^+-dependent uptake of 2.5 μM ($P < 0.01$) but not 182 μM tryptophan (Table II). Likewise, tryptophan competitively inhibited Na^+-dependent L-alanine uptake (Table II), and alanine is known to be transported mainly by system $B^{0,+}$ in blastocysts [4,6,11]. Furthermore, the K_i value for inhibition of alanine transport by tryptophan was close to the approximate value of its K_m for Na^+-dependent uptake, and the K_m value for alanine transport was about the same as its approximate K_i value for inhibition of tryptophan uptake (Table II; 'AB' portion of the 'ABC' test, Refs. 4, 5, 8, 23). Therefore, the major route of Na^+-dependent transport of both alanine and tryptophan in blastocysts is probably system $B^{0,+}$.

A component of Na^+-dependent L-[^{3}H]tryptophan transport, that was much smaller than system $B^{0,+}$, was also detected in the presence of an excess of L-lysine, BCH and *N*-methyl-L-tryptophan to inhibit system $B^{0,+}$ and the three Na^+-independent systems for tryptophan transport in blastocysts (Fig. 6). This inconspicuous Na^+-dependent component of [^{3}H]tryptophan uptake

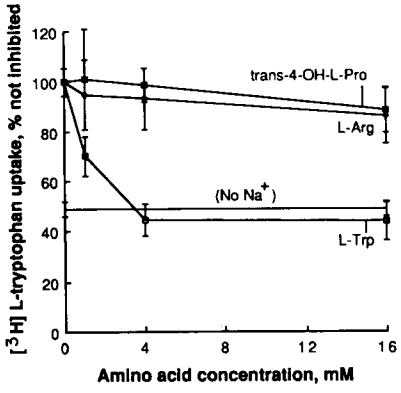

Fig. 6. Effect of various concentrations of L-arginine, *trans*-4-OH-L-proline and nonradioactive L-tryptophan on BCH- and L-lysine-resistant Na^+-dependent [^{3}H]tryptophan uptake (System B activity) in blastocysts. Conceptuses were incubated with 1.8 or 3.6 μM [^{3}H]tryptophan, 20 mM L-lysine (to inhibit systems $B^{0,+}$ and $b^{0,+}$), 10 mM BCH (to inhibit systems $B^{0,+}$ and L), 2 mM *N*-methyl-L-tryptophan (to inhibit system T), and various concentrations of the indicated amino acids for 5 min in phosphate-buffered NaCl (see Refs. 4–9 and text for discussion of inhibitors). The mean uptake ± S.E. was calculated from 6–7 replicate determinations (5–8 conceptuses/determination) obtained in four independent experiments. Uptake in phosphate-buffered LiCl is indicated by the horizontal line labeled 'No Na^+'. Uptake was significantly slower in the absence of Na^+ ($P < 0.01$) or in the presence of nonradioactive tryptophan ($P < 0.01$; analysis of variance).

was inhibited by nonradioactive L-tryptophan but not in a statistically significant manner by L-arginine or *trans*-4-OH-L-proline (Fig. 6). Therefore, it was probably due to the activity of system B not to residual system $B^{0,+}$ activity or to an osmotic or some other artifact of uptake in the presence of high amounts of excess amino acids [6]. The latter conclusion is also supported by the finding that tryptophan strongly inhibits uptake of L-alanine by system B in blastocysts [6]. Although *trans*-OH-L-proline is a weak inhibitor of alanine uptake via system B [6], its inhibition of tryptophan transport was apparently too weak to detect when 20 mM lysine, 10 mM BCH and 2 mM *N*-methyl-L-tryptophan were also present (Fig. 6). As for alanine transport [6], uptake of L-tryptophan by system B was too slow under the conditions needed to detect it to characterize it further with more detailed kinetic experiments.

Comparison of the system for benzenoid amino acid transport in preimplantation mouse conceptuses to system T in human red blood cells and rat hepatocytes

As for system T in other types of cells [15–18], system T in mouse conceptuses is Na^+-independent and interacts preferentially with benzenoid amino acids. In conceptuses, as in red cells [15,16], system T interacts more strongly with tryptophan than with tyrosine or phenylalanine (e.g., Table I). Similarly, uptake of tryptophan by system T in both conceptuses (e.g., Fig. 4b) and red cells [17] is inhibited strongly by *N*-ethylmaleimide and, perhaps, weakly by BCO. Moreover, system T in conceptuses (e.g., Fig. 2b, inset) and other cells [15,16,18] interacts about equally strongly with D- and L-tryptophan, although D-tryptophan apparently is not transported by the system in human red cells [16]. Finally, α-*N*-methylation of L-tryptophan may increase its reactivity with system T somewhat in 1-cell conceptuses and blastocysts, as indicated by the K_i and K_m values for these substances (Table II), and such is also the case for system T in red cells [16]. For all of these reasons, the system which prefers benzenoid substrates in preimplantation mouse conceptuses appears to be nearly identical to system T in human red blood cells and rat hepatocytes [15–18]. To our knowledge, this is the first report to show that system T is present in other than human red cells and rat hepatocytes. In some cases, however, this lack of detection of system T in other cell types may be the result of insufficient attempts to detect it rather than because the system is absent from all other types of cells. Moreover, a transport process in the lysosomes of fibroblasts has been provisionally termed system t because it resembles system T in red cells and hepatocytes [32]. In a previous paper [5], we suggested that system T may be a member of an extended system L family the members of which are Na^+-independent transporters of bulky zwitterionic

amino acids. Contrary to the latter hypothesis, the ability of system T to interact strongly with *N*-methyl-L-tryptophan, as reported originally for red cells [16] and substantiated here for conceptuses (e.g., Table II), supports the conclusion that system T is not simply a form of system L with a somewhat more limited substrate specificity. As for other instances of system L (e.g., Ref. 33), system L in preimplantation mouse conceptuses does not interact strongly with the α-*N*-methyl derivatives of L-tryptophan or L-leucine (Figs. 3a and 4a; data not shown). Therefore, it seems unlikely that system T is the product of a dispensable duplicate of a gene for system L, although functional dispensable duplicate genes for some proteins appear to be present in the genomes of vertebrates [34].

Why do preimplantation mouse blastocysts have system T in addition to four other distinct substrate-saturable systems for tryptophan transport?

Tryptophan and other benzenoid amino acids are transported primarily via systems T and L in human red blood cells [15–17] and rat hepatocytes [18], and such is also the case in mouse conceptuses before they form blastocysts (e.g., Fig. 3). In conceptuses, however, system L has a relatively low K_m value for tryptophan (Table II), whereas the forms of system L in red cells and freshly isolated hepatocytes have K_m values for tryptophan that are about an order of magnitude above those of system T [15,18]. Moreover, in blastocysts, at least five systems appear to contribute to transport of benzenoid amino acids. Except for system $b^{0,+}$, each of these systems interacts at least as strongly with tryptophan as with other good substrates of the systems (Table II and Ref. 6). Even in the case of system $b^{0,+}$, which seems to have a preference for arginine over most other amino acids (Table II and Ref. 7), interaction of tryptophan with the system appears to be strong enough to be of potential physiological significance. The effective concentrations of tryptophan in blood [22] and other such fluids are probably much less than 100 μM, so it is somewhat surprising that system T has a K_m value for this amino acid above one mM (Table II). In the presence of the mixture of competing amino acids usually encountered by mammalian cells in situ, however, the apparent K_m values for most instances of Na^+-independent transport of zwitterionic amino acids are probably on the order of at least one mM. Therefore, by the latter functionally important criterion, system T appears to resemble other mediated routes of benzenoid amino acid transport. The possibility that tryptophan transport is particularly important in preimplantation conceptuses is supported by the observation that tryptophan availability in blood increases within one day after rat eggs are fertilized [1]. We are currently attempting to determine if the availabilities of other amino acids as well as tryptophan change when mouse eggs are fertilized, since other amino acids could affect transport and homeostasis of benzenoid amino acids in preimplantation conceptuses.

It is also possible that interaction of benzenoid amino acids with system T is incidental to the actual physiological function of the system. We have not, however, discovered a substance which interacts more effectively with system T than do tryptophan and its α-*N*-methyl derivative (e.g., Table I). Possible physiological functions of system T could involve, but need not be limited to, interaction with or transport of substances that are partially similar in structure to benzenoid amino acids including peptides or macromolecules. Regardless of its function, because system T is present in preimplantation mouse conceptuses as well as in human red blood cells and rat hepatocytes, we suggest that system T may be more widely distributed in mammalian cells than is obvious from existing data.

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