Characterization of tryptophan transport in human placental brush-border membrane vesicles

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The characteristics of tryptophan uptake in isolated human placental brush-border membrane vesicles were investigated. Tryptophan uptake in these vesicles was predominantly Na⁺-independent. Uptake of tryptophan as measured with short incubations occurred exclusively by a carrier-mediated process, but significant binding of this amino acid to the membrane vesicles was observed with longer incubations. The carrier-mediated system obeyed Michaelis–Menten kinetics, with an apparent affinity constant of $12.7 \pm 1.0 \mu\text{M}$ and a maximal velocity of $91 \pm 5 \text{ pmol/15 s per mg of protein}$. The kinetic constants were similar in the presence and absence of a Na⁺ gradient. Competition experiments showed that tryptophan uptake was effectively inhibited by many neutral amino acids except proline, hydroxyproline and 2-(methylamino)isobutyric acid. The inhibitory amino acids included aromatic amino acids as well as other system-l-specific amino acids (system l refers to the classical L system, according to the most recent nomenclature of amino acid transport systems). The transport system showed very low affinity for D-isomers, was not affected by phloretin or glucose but was inhibited by *p*-azidophenylalanine and *N*-ethylmaleimide. The uptake rates were only minimally affected by change in pH over the range 4.5–8.0. Tryptophan uptake markedly responded to *trans*-stimulation, and the amino acids capable of causing *trans*-stimulation included all amino acids with system-l-specificity. The patterns of inhibition of uptake of tryptophan and leucine by various amino acids were very similar. We conclude that system t, which is specific for aromatic amino acids, is absent from human placenta and that tryptophan transport in this tissue occurs via system l, which has very broad specificity.

INTRODUCTION

Placental transfer of nutrients from mother to fetus plays a crucial role in fetal growth and development. The brush-border membrane of the placenta possesses specific carriers which are responsible for the transport of these nutrients, including amino acids. Most studies on amino acid transport in the placenta have been done with either placental slices or perfused whole placenta. These approaches have limitations, such as tissue metabolism, which make it difficult to define specific transport phenomena. In recent years, there have been a few studies on amino acid transport with isolated brush-border membrane vesicles from human placenta (for reviews, see Bissonnette, 1982; Yudilevich & Sweiry, 1985). Isolated membrane vesicles offer a convenient system to study placental transport, but this system has been used only in a limited way, with very few amino acids as test substrates. In view of the diversity of amino acid transport systems known to be present in different cell types (Christensen, 1985), more work is needed on amino acid transport by using human placental brush-border membrane vesicles in order to characterize fully various aspects of this important phenomenon.

We describe in this paper the characteristics of tryptophan transport in brush-border membrane vesicles isolated from normal term human placentas. Tryptophan

is an essential amino acid which, apart from its role in protein synthesis, is a precursor for a number of biologically active compounds, including 5-hydroxytryptamine. But virtually nothing is known about placental transport of this amino acid. Moreover, a new transport system, designated as t, has been identified in human erythrocytes and in rat liver cells, which exclusively mediates the transport of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Rosenberg *et al.*, 1980; Vadgama & Christensen, 1985; Lopez-Burillo *et al.*, 1985; Salter *et al.*, 1986). The system t is Na⁺-independent, but is distinct in many respects from the classical Na⁺-independent l system, which also accepts tryptophan as a substrate in many cell types. The purpose of this paper was therefore to characterize tryptophan transport in human placental brush-border membrane vesicles with respect to Na⁺-dependence, kinetics and inhibition by other amino acids, and also to determine the number of different transport systems available for this amino acid in the human placental brush-border membrane.

EXPERIMENTAL

Materials

L-[5-³H]Tryptophan (sp. radioactivity 28.7 Ci/mmol) and L-[5-³H]proline (sp. radioactivity 9.9 Ci/mmol) were

Abbreviations used: BCH, 2-*endo*-aminobicyclo-[2.2.1]heptane-2-carboxylic acid; MeAIB, 2-(methylamino)isobutyric acid. Lower-case designations are used for Na⁺-independent amino acid transport systems, as recommended by Bannai *et al.* (1984). System l refers to the Na⁺-independent transport system that exhibits a wide-scope substrate specificity but prefers bulkier amino acids, both aromatic and aliphatic. System t refers to the Na⁺-independent transport system that is selective for aromatic amino acids.

obtained from New England Nuclear, Boston, MA, U.S.A. L-[4,5-³H]Leucine (sp. radioactivity 50 Ci/mmol) was from ICN Radiochemicals, Irvine, CA, U.S.A. 2-(Methylamino)isobutyric acid (MeAIB), L-tryptophan, D-tryptophan, L-leucine, L-glutamine and L-cysteine were purchased from Sigma, St. Louis, MO, U.S.A., and all other amino acids were from Calbiochem, La Jolla, CA, U.S.A. Glycylproline *p*-nitroanilide was generously given by Professor A. Barth, Martin Luther Universität, Halle-Wittenberg, German Democratic Republic. 2-(*t*-Butoxycarbonyloxyimino)-2-phenylacetoneitrile was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Methods

Preparation of membrane vesicles. The syncytiotrophoblast brush-border membrane vesicles from normal term human placentas were prepared by using a Ca²⁺-precipitation technique as described by Ganapathy *et al.* (1985). The intravesicular volume of these vesicles was $2.7 \pm 0.7 \mu\text{l}/\text{mg}$ of protein, as calculated from the intravesicular content at equilibrium of three different solutes (proline, carnosine and glycylsarcosine) which exhibit minimal binding to the vesicles. The purity of brush-border membrane preparations was assessed by measuring alkaline phosphatase and 5'-nucleotidase activities. These enzymes have been shown to be specifically associated with the brush-border membrane in the human placenta (Jones & Fox, 1976; Whitsett & Lessard, 1978; Booth *et al.*, 1980). As shown in Table 1, the marker enzymes alkaline phosphatase and 5'-nucleotidase were enriched about 20-fold in the purified brush-border membranes compared with the original homogenate. We also assayed dipeptidyl peptidase IV in these preparations. This enzyme is exclusively present in the brush-border membrane of the absorptive cells of small intestine and kidney. The results in Table 1, however, show that placental brush-border membrane contains very little, if any, of this enzyme, because the enrichment of this enzyme is small compared with that of alkaline phosphatase and 5'-nucleotidase.

Uptake studies. Uptake measurements were made by a rapid-filtration technique as described by Ganapathy *et al.* (1981), with Millipore filters (pore size $0.65 \mu\text{m}$). The experiments were regularly done in duplicate or triplicate, and the variation was always less than $\pm 10\%$ of the mean value.

Synthesis of *p*-azidophenylalanine. *p*-Azido-L-phenylalanine hydrochloride was synthesized by the method described by Schwyzer & Caviezel (1971), except that 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetoneitrile was used instead of *t*-butoxycarbonylazide in the preparation of α -*t*-butoxycarbonyl-*p*-nitro-L-phenylalanine. All intermediates gave melting points and i.r. spectra in agreement with reported values.

Enzyme assays. Alkaline phosphatase (EC 3.1.3.1) was determined by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate in 50 mM-glycine/NaOH buffer (pH 10.5) in the presence of 0.5 mM-MgCl₂. 5'-Nucleotidase (EC 3.1.3.5) was assayed with AMP as the substrate in 50 mM-Tris/HCl buffer, pH 8.5, and the liberated phosphate was determined, after trichloroacetic acid precipitation of proteins, by the method of Fiske & Subbarow (1925). Dipeptidyl peptidase IV (EC 3.4.14.5) was measured with glycylproline *p*-nitroanilide as the chromogenic substrate, by the method of Hama *et al.* (1982).

RESULTS

Effect of ion gradients on tryptophan uptake

Fig. 1 shows the time course of the uptake of $0.1 \mu\text{M}$ -tryptophan measured in the presence of a NaCl or a KCl gradient (extravesicular > intravesicular). Tryptophan uptake in the presence of a Na⁺ gradient was slightly greater than that in the presence of a K⁺ gradient. However, the uptake observed in the presence of a K⁺ gradient was also very rapid, and therefore it cannot completely be due to simple diffusion. To check the possibility that K⁺ may substitute for Na⁺ to some extent as the co-transported cation, we measured initial uptake rates of tryptophan in the presence of various ion gradients and compared these rates with the uptake in the absence of any ion gradient (mannitol alone). The uptake rates in all cases were similar (Table 2). These results strongly argue against a specific role for Na⁺ in the translocation of tryptophan across human placental brush-border membrane.

Whether this pathway is a carrier-mediated process or is due to simple diffusion was examined by studying the effect of increasing concentrations of unlabelled tryptophan on the uptake of labelled tryptophan. The experiment was done in both the presence and the absence of a Na⁺ gradient. Addition of unlabelled tryptophan to the incubation medium inhibited the

Table 1. Specific activities of marker enzymes in the placental brush-border membrane and in the homogenate

Marker enzymes were assayed in the brush-border membrane preparations and in the homogenate of washed placental tissue. The specific activity in all cases is expressed as nmol/min per mg of protein. Recovery represents the percentage of the enzyme activity present in the brush-border membrane preparations compared to the original homogenate. The results are given as means $\pm$ S.D. for six to eight determinations.

Enzyme	Homogenate	Brush-border membrane	Enrichment	Recovery (%)
Alkaline phosphatase	0.31 ± 0.09	5.92 ± 1.95	19.1 ± 2.2	3.0 ± 0.7
5'-Nucleotidase	0.12 ± 0.02	2.26 ± 0.56	18.5 ± 2.8	3.0 ± 0.5
Dipeptidyl peptidase IV	0.037 ± 0.004	0.13 ± 0.03	3.6 ± 1.1	0.6 ± 0.2

Fig. 1. Effect of a Na^+ gradient on tryptophan uptake

The membrane vesicles were suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake was measured in 10 mM-Hepes/Tris buffer, pH 7.5, containing 80 mM-mannitol and either 100 mM-NaCl or 100 mM-KCl. Uptake was initiated by mixing 50 μl of membrane suspension (0.3 mg of protein) with 200 μl of uptake buffer containing labelled tryptophan. Final concentration of tryptophan in the incubation medium was 100 nM. ●, Na^+ gradient; ○, K^+ gradient.

Table 2. Effect of different cation and anion gradients on tryptophan uptake

Membrane vesicles were preloaded with 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake buffer contained labelled tryptophan, mannitol and salt (total osmolality = 280 mosm) in 10 mM-Hepes/Tris buffer, pH 7.5. Uptake was initiated by mixing 50 μl of membrane suspension (0.3 mg of protein) with 200 μl of uptake buffer. Final concentration of tryptophan was 50 nM, and the time of incubation was 10 s. The data represent means $\pm$ S.D. for triplicates.

Salt gradient	Tryptophan uptake (pmol/mg of protein)
Mannitol	0.36 ± 0.03
NaCl	0.50 ± 0.02
NaSCN	0.65 ± 0.04
Na_2SO_4	0.46 ± 0.03
KCl	0.41 ± 0.02
KSCN	0.47 ± 0.01
K_2SO_4	0.41 ± 0.02
LiCl	0.51 ± 0.01
Li_2SO_4	0.44 ± 0.01
RbCl	0.48 ± 0.03
CsCl	0.50 ± 0.02
Tetraethylammonium chloride	0.45 ± 0.01

Fig. 2. Competition between labelled and unlabelled tryptophan for uptake

The membrane vesicles were suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake was measured in both the presence (●) and the absence (○) of a Na^+ gradient. Uptake was initiated by mixing 50 μl of membrane suspension (0.3 mg of protein) with 200 μl of uptake buffer containing labelled tryptophan (final concn. 50 nM) and various concentrations of unlabelled tryptophan (final concns. 100 nM–10 mM). Incubation time was 15 s.

uptake of labelled tryptophan in both cases, indicating competition and hence the presence of a carrier-mediated process (Fig. 2). The uptake of radiolabel was almost completely abolished in the presence of 10 mM unlabelled tryptophan. These results, taken collectively, demonstrate that tryptophan uptake in human placental brush-border membrane vesicles is a Na^+ -independent carrier-mediated process, and the contribution of a diffusional component, if any, to the total uptake is negligible.

Binding of tryptophan to the membrane vesicles

The integrity of membrane vesicles was examined by studying the effect of osmolality of the medium on the equilibrium uptake of tryptophan (Fig. 3). The equilibrium concentration of tryptophan in the vesicles changed only minimally over an osmolality range of 200–667 mosm, indicating significant binding of the amino acid to the membrane vesicles. The binding component accounted for approx. 60% of the uptake observed under standard conditions. On the contrary, the equilibrium concentration of proline was directly proportional to the reciprocal of osmolality of the medium. Therefore the membrane preparation did consist of vesicles with an osmotically responsive intravesicular space. For several reasons, the binding of tryptophan to the membrane vesicles could not be the result of non-specific interaction of the amino acid with the membranes. Firstly, as shown in Fig. 2, tryptophan

Fig. 3. Effect of osmolality of the medium on the uptake of tryptophan (●) and proline (○)

The membrane vesicles were suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Equilibrium uptake was measured by incubating 50 μ l of membrane suspension (0.3 mg of protein) with 200 μ l of 10 mM-Hepes/Tris buffer, pH 7.5, containing labelled tryptophan or labelled proline and various concentrations of mannitol. Final concentrations of tryptophan and proline were 50 nM and 500 nM respectively.

uptake measured at 15 s incubation represents carrier-mediated transport because of the observed competition between labelled and unlabelled tryptophan. Secondly, many neutral amino acids inhibit tryptophan uptake, but imino and *N*-methylated amino acids have no effect (see below), indicating again interaction of tryptophan with specific binding sites in the membrane. Thirdly, preloading the vesicles with many neutral amino acids markedly stimulates tryptophan uptake (see below). If the observed tryptophan uptake were due to non-specific binding, the process would not have exhibited this *trans*-stimulation phenomenon. All of these results indicate that tryptophan is in fact transported across the membrane into the intravesicular space, and that the minimal response of equilibrium uptake of tryptophan to osmolality of the medium is probably due to binding of this amino acid to its carrier on the internal surface of the membrane.

Kinetics of tryptophan uptake

The kinetics of tryptophan uptake were investigated in the presence of a Na⁺ gradient as well as in mannitol alone. Initial uptake rates (15 s incubation) were obtained over the concentration range 1–100 μ M-tryptophan. The kinetic constants were calculated from the data by linear-regression analysis. In the presence of

a Na⁺ gradient, the apparent affinity constant for the uptake process was 11.9 ± 0.4 μ M and the maximal velocity was 101 ± 4 pmol/15 s per mg of protein. In the absence of a Na⁺ gradient, the apparent affinity constant and the maximal velocity for the process were 12.7 ± 1.0 μ M and 91 ± 5 pmol/15 s per mg of protein respectively. It is obvious from these results that Na⁺ does not play a significant role in tryptophan transport in human placental brush-border membrane vesicles, because the kinetic parameters for the uptake process were essentially the same in the presence and in the absence of Na⁺. Therefore, all further experiments were carried out in the absence of Na⁺.

Inhibition of tryptophan uptake by other amino acids

Table 3 shows the relative effectiveness of several amino acids and other compounds such as glucose and phloretin as inhibitors of tryptophan uptake in human placental brush-border membrane vesicles in the absence of Na⁺. The concentration of labelled tryptophan in these experiments was 50 nM and the uptake was measured in the presence of two concentrations (10 μ M and 500 μ M) of inhibitor. At a concentration of 500 μ M (10000-fold excess), all neutral amino acids except proline, hydroxyproline and MeAIB effectively inhibited tryptophan uptake, whereas glucose and phloretin had no effect. Essentially similar results were obtained at a concentration of 10 μ M (200-fold excess), except that glycine and D-tryptophan were no longer inhibitory at this low concentration. The K_i values for the inhibitory amino acids were calculated by the method of Hajjar & Curran (1970), by using 12.7 μ M as the apparent affinity constant for tryptophan uptake. These values are also given in Table 3. It can be concluded from these data that the system responsible for tryptophan uptake in placental brush-border membrane vesicles shows higher affinity for aromatic as well as aliphatic amino acids with bulky side chains than for amino acids with small side chains. The system has very little affinity for D-tryptophan and glycine and no affinity for imino and *N*-methylated amino acids. Thus the substrate specificity of the tryptophan uptake system in human placenta is similar to that of system l rather than that of system t.

Trans-stimulation of tryptophan uptake

The ability of a variety of neutral amino acids to cause *trans*-stimulation of tryptophan uptake was examined by equilibrating the membrane vesicles with unlabelled amino acids for 1 h at room temperature and then determining the uptake of labelled tryptophan. The results are given in Table 4. Preloading the vesicles with aromatic amino acids as well as with many aliphatic, system-l-specific, amino acids stimulated tryptophan uptake, and the stimulation varied from 1.6- to 6.9-fold. These data strengthen the view that tryptophan uptake in human placental brush-border membrane vesicles occurs via the l system. D-Tryptophan, proline and MeAIB, which show no affinity for the l system, had little effect on tryptophan uptake. It is noteworthy that the amino acids that failed to cause *trans*-stimulation were also those that produced little *cis*-inhibition. Among the amino acids capable of causing *trans*-stimulation, except tryptophan and tyrosine, there was a significant correlation between the degree of *cis*-inhibition and *trans*-stimulation ($r^2 = 0.75$). Tryptophan and tyrosine, though very good inhibitors of tryptophan uptake,

Table 3. Inhibition of tryptophan uptake by other amino acids

Membrane vesicles were preloaded with 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake (15 s) was measured in the same buffer. Final concentration of labelled tryptophan was 50 nM. The concentration of inhibitory amino acids was either 10 μ M (B) or 500 μ M (A). Abbreviation: ND, not determined. The data represent means $\pm$ s.d. for triplicates.

Addition	Tryptophan uptake				Inhibition constant (K_i) (μ M)
	A		B		
	(pmol/mg of protein)	(%)	(pmol/mg of protein)	(%)	
None	0.41 $\pm$ 0.04	100	0.43 $\pm$ 0.04	100	
Tryptophan	0.03 $\pm$ 0.01	7	0.15	35	—
Tyrosine	0.06 $\pm$ 0.01	15	0.22	50	10.0
Histidine	0.03 $\pm$ 0.01	7	0.23	53	11.2
Glutamine	0.05 $\pm$ 0.01	12	0.30	70	23.2
Cycloleucine	0.06 $\pm$ 0.01	15	0.32	73	26.9
Serine	0.09 $\pm$ 0.01	22	0.30	69	22.2
Threonine	0.06 $\pm$ 0.01	15	0.27	63	17.0
Phenylalanine	0.15 $\pm$ 0.01	37	0.20	47	8.2
Leucine	0.18 $\pm$ 0.01	44	0.20	47	8.2
Alanine	0.14 $\pm$ 0.01	34	0.38	87	51.0
Valine	0.15 $\pm$ 0.01	37	0.26	60	14.9
Isoleucine	0.16 $\pm$ 0.02	39	0.21	50	10.0
Methionine	0.16 $\pm$ 0.01	39	ND	ND	ND
BCH	0.10 $\pm$ 0.01	24	0.27	63	17.0
D-Tryptophan	0.07 $\pm$ 0.03	17	0.46	107	102.0
Glycine	0.26 $\pm$ 0.01	63	0.48	112	848.0
Proline	0.42 $\pm$ 0.02	102	ND	ND	ND
Hydroxyproline	0.40 $\pm$ 0.01	98	ND	ND	ND
MeAIB	0.44 $\pm$ 0.02	107	ND	ND	ND
Glucose	0.43 $\pm$ 0.01	105	ND	ND	ND
Phloretin	0.55 $\pm$ 0.04	134	ND	ND	ND

Table 4. Effect of amino acid preloading on tryptophan uptake

Membrane vesicles were suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. These vesicles were incubated at room temperature for 1 h in the same buffer without (control) or with (experimental) 50 μ M amino acid. In the control experiments, 50 μ l of control vesicle suspension was added to 950 μ l of buffered 280 mM-mannitol containing labelled tryptophan and 2.5 μ M unlabelled amino acid. In the *trans*-stimulation assay, 50 μ l of experimental vesicle suspension was added to 950 μ l of buffered 280 mM-mannitol containing only labelled tryptophan. The final concentration of labelled tryptophan in the incubation medium was 100 nM. The time of incubation in the uptake assay was 15s. The data represent means $\pm$ s.d. for triplicates.

Amino acid	Uptake (pmol/mg of protein)		Stimulation (-fold)
	Control	<i>Trans</i> -stimulation	
Tyrosine	0.26 $\pm$ 0.09	1.78 $\pm$ 0.05	6.85
Tryptophan	0.30 $\pm$ 0.02	1.69 $\pm$ 0.04	5.66
Histidine	0.43 $\pm$ 0.01	1.59 $\pm$ 0.03	3.70
Methionine	0.30 $\pm$ 0.05	0.86 $\pm$ 0.08	2.87
Phenylalanine	0.18 $\pm$ 0.02	0.50 $\pm$ 0.03	2.78
Isoleucine	0.27 $\pm$ 0.01	0.75 $\pm$ 0.01	2.78
BCH	0.47 $\pm$ 0.02	1.15 $\pm$ 0.02	2.47
Leucine	0.23 $\pm$ 0.02	0.45 $\pm$ 0.02	1.93
Serine	0.49 $\pm$ 0.04	0.90 $\pm$ 0.03	1.86
Glutamine	0.52 $\pm$ 0.03	0.81 $\pm$ 0.03	1.56
D-Tryptophan	0.66 $\pm$ 0.03	0.80 $\pm$ 0.01	1.21
Alanine	0.67 $\pm$ 0.02	0.79 $\pm$ 0.05	1.18
Proline	0.75 $\pm$ 0.03	0.78 $\pm$ 0.02	1.04
MeAIB	0.71 $\pm$ 0.01	0.72 $\pm$ 0.02	1.01

Table 5. Inhibition of leucine uptake by other amino acids

Membrane vesicles were preloaded with 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake (30 s) was measured in the same buffer. Final concentration of labelled leucine was 50 nM. The concentration of inhibitory amino acids was 5 μ M (C), 50 μ M (B) or 500 μ M (A). Leucine uptake in the absence of inhibitory amino acids was 0.22 ± 0.04 pmol/mg of protein; the uptake in the presence of inhibitors is given as a percentage of this control value.

Amino acid	Leucine uptake (%)		
	A	B	C
Control	100	100	100
Tryptophan	5	11	22
Tyrosine	4	6	22
Phenylalanine	20	15	21
Leucine	21	15	17
Alanine	11	18	35
Valine	13	10	22
Isoleucine	10	17	27
Histidine	9	18	41
Methionine	14	17	30
Glycine	22	31	60
Proline	56	80	99

showed disproportionately high *trans*-stimulation compared with other inhibitory amino acids. The reasons for this are not clear from our current data, but it is possible that the system responsible for tryptophan transport in human placenta may represent a variant of the l system for which tyrosine and tryptophan show disproportionately high *trans*-stimulation compared with their ability to cause *cis*-inhibition.

Effect of pH on tryptophan uptake

We also studied the effect of extravesicular pH on the initial rates of tryptophan uptake. The pH was varied from 4.5 to 8.0. The uptake rates were little affected over this pH range (results not shown).

Inhibition of leucine uptake by other amino acids

The ability of various amino acids to inhibit the Na⁺-independent uptake of leucine was then studied (Table 5). Many amino acids, including tryptophan, tyrosine and phenylalanine, effectively inhibited leucine uptake. Proline and glycine caused significant inhibition when they were present at very high concentrations, but the inhibition decreased as their concentrations were lowered. For example, proline failed to inhibit leucine uptake when its concentration was 100-fold in excess of labelled leucine, whereas at 10000-fold excess concentration it caused 45% inhibition. Therefore glycine and proline have low or little affinity for this transport system.

Inhibition by *N*-ethylmaleimide

The effect of pretreatment of membrane vesicles with the thiol reagent *N*-ethylmaleimide on the Na⁺-independent uptake of leucine and tryptophan and on the Na⁺-dependent uptake of alanine and proline was studied, and the data are presented in Table 6. System A (Na⁺-dependent proline uptake) and system ASC

Table 6. Effect of *N*-ethylmaleimide on amino acid uptake

Membrane vesicles suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol were incubated at room temperature for 1 h without (control) or with 5 mM-*N*-ethylmaleimide. Reagent that had not reacted was removed by centrifugation, and the vesicles were washed once with the preloading buffer (10 mM-Hepes/Tris, pH 7.5, 280 mM-mannitol) and suspended in the same buffer. Uptake of leucine and tryptophan was measured in the absence of Na⁺, whereas uptake of alanine and proline was measured in the presence of Na⁺. Final concentrations of leucine, tryptophan, alanine and proline in the incubation medium were 50 nM, 100 nM, 8 μ M and 1 μ M respectively. The time of incubation in the uptake assay in all cases was 30 s. The results represent means $\pm$ S.D. for triplicates. The values in parentheses represent percentages of the control uptake.

Amino acid	Amino acid uptake (pmol/mg of protein)	
	Control	<i>N</i> -Ethylmaleimide treatment
Leucine (Na ⁺ -independent)	0.18 ± 0.03	0.15 ± 0.01 (83)
Tryptophan (Na ⁺ -independent)	0.74 ± 0.04	0.36 ± 0.01 (49)
Alanine (Na ⁺ -dependent)	85.8 ± 1.2	23.5 ± 0.7 (27)
Proline (Na ⁺ -dependent)	1.44 ± 0.04	0.68 ± 0.09 (47)

(Na⁺-dependent alanine uptake) were inhibited by *N*-ethylmaleimide. Na⁺-independent uptake of tryptophan and leucine (system l) were also significantly inhibited by the thiol reagent, though there were differences in the degrees of inhibition.

Inhibition by *p*-azidophenylalanine

Studies with human erythrocytes have suggested that *p*-azidophenylalanine, a photolabile phenylalanine analogue, may be a specific inhibitor of system t, with no affinity for system l (Vadgama & Christensen, 1985). Therefore we investigated the effects of *p*-azidophenylalanine on the Na⁺-independent uptake of leucine and tryptophan in human placental brush-border membrane vesicles (Fig. 4). The uptake of tryptophan and that of leucine were markedly inhibited by *p*-azidophenylalanine, and the apparent inhibition constant in both cases was approx. 10 μ M. These results differ from those obtained in human erythrocytes, where *p*-azidophenylalanine inhibited tryptophan uptake, but had little effect on leucine uptake. Thus this phenylalanine analogue cannot be considered as a specific inhibitor of system t. In fact, *p*-azidophenylalanine has been shown to be actively taken up by pig intestinal brush-border membrane vesicles via a Na⁺-dependent mechanism, and the uptake was markedly inhibited by alanine and phenylalanine (Noren *et al.*, 1983). Therefore it appears that when both system t and system l are present, as in human erythrocytes, the substrate specificity of system l is restricted to non-aromatic neutral amino acids with bulky side chains and does not accept *p*-azidophenylalanine as substrate. However, when system t is absent, as in small intestine and placenta, system l exhibits broader substrate specificity, with high affinity for bulky aliphatic amino acids as well as for aromatic amino acids including the phenylalanine analogue, *p*-azidophenylalanine.

Fig. 4. Inhibition of Na^+ -independent uptake of leucine (○) and tryptophan (●) by *p*-azidophenylalanine

The membrane vesicles were suspended in 10 mM-Hepes/Tris buffer, pH 7.5, containing 280 mM-mannitol. Uptake was initiated by mixing 50 μl of membrane suspension (0.3 mg of protein) with 200 μl of uptake buffer containing labelled tryptophan or labelled leucine. Final concentrations of tryptophan and leucine were 50 nM and 65 nM respectively, and that of *p*-azidophenylalanine was varied from 100 nM to 1 mM. Time of incubation was 15 s for tryptophan uptake and 30 s for leucine uptake.

DISCUSSION

Amino acid transport in human placenta has been studied in membrane vesicles (Ruzycki *et al.*, 1978; Boyd & Lund, 1981; Montgomery & Young, 1982), but all these studies used amino acid substrates that are specific for the Na^+ -dependent transport systems A or ASC. The present paper describes for the first time the characteristics of Na^+ -independent amino acid transport in human placental brush-border membrane vesicles. The presence of the Na^+ -independent l system in human placenta was originally suggested on the basis of experiments performed on BCH uptake in placental villous fragments (Enders *et al.*, 1976). Later studies have supported this suggestion by observing that a portion of transport of neutral amino acids with bulky side chains in placental tissue fragments was not susceptible to inhibition by α -aminoisobutyric acid (Henderson *et al.*, 1981; Eaton *et al.*, 1982). Since all these studies were performed in intact villous fragments, the experiments were done in the presence of Na^+ . Moreover, BCH is not an exclusive substrate for the l system (McClellan & Schafer, 1973; Ullrich *et al.*, 1974; Christensen, 1979). Therefore the

conclusions on the characteristics of the Na^+ -independent l system from those studies were at best indirect.

The present paper provides direct evidence for the presence of a Na^+ -independent amino acid transport system in human placenta and describes its characteristics such as substrate specificity and kinetics in detail. Tryptophan was used as the test substrate in these experiments. Uptake of tryptophan in isolated placental brush-border membrane vesicles is predominantly Na^+ -independent, but occurs via a carrier-mediated process which exhibits Michaelis-Menten saturation kinetics. This amino acid, owing to its bulky and hydrophobic side chain, is a preferred substrate for the l system in many cell types. However, the presence of a new amino acid transport system (system t) specific for aromatic amino acids has been reported in certain cell types (Rosenberg *et al.*, 1980; Vadgama & Christensen, 1985; Salter *et al.*, 1986). Therefore the main emphasis of the present paper was to identify the transport system responsible for Na^+ -independent tryptophan uptake in human placenta.

Even though some of the properties, such as Na^+ -independence, weak interaction with imino and *N*-methylated amino acids, inhibition by *N*-ethylmaleimide and pH profile, are similar for system t and for tryptophan transport in human placenta, there are also many important differences between them. System t is specific for aromatic amino acids and exhibits high affinity for their D-isomers, whereas the system responsible for tryptophan uptake in human placenta accepts a wide range of neutral amino acids as substrates but has very low affinity for D-isomers. Phloretin has been shown to inhibit system t (Rosenberg *et al.*, 1980), but it has no effect on placental tryptophan uptake. The response of system t to *trans*-stimulation is questionable. Vadgama & Christensen (1985) concluded that system t shows very little, if any, response to *trans*-stimulation, whereas Lopez-Burillo *et al.* (1985) demonstrated strong *trans*-stimulation, although both studies were done in human erythrocytes. Tryptophan uptake in human placental brush-border membrane vesicles was stimulated many-fold by preloading the vesicles with aromatic amino acids as well as with many system-l-specific neutral amino acids. Thus the properties of placental tryptophan uptake closely resemble those of system l and differ in many respects from those of system t. We therefore conclude that system t is absent in human placenta and tryptophan transport in this tissue occurs via system l.

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