Heterogeneity of L-alanine transport systems in brush-border membrane vesicles from rat placenta during late gestation

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The placental uptake of L-alanine was studied by using purified brush-border membrane vesicles from rat trophoblasts. Saturation curves were carried out at 37 °C in buffers containing 100 mM (zero-*trans*) -NaSCN, -NaCl, -KSCN, -KCl, or -N-methyl-D-glucamine gluconate. The uncorrected uptake results were fitted by non-linear regression analysis to an equation involving one diffusional component and either one or two saturable Michaelian transport terms. In the presence of NaCl, two distinct L-alanine transport systems were distinguished, named respectively System 1 (S-1; V_{m1} about 760 pmol/s per mg of protein; K_{T1} = 0.5 mM) and System 2 (S-2; V_{m2} about 1700 pmol/s per mg; K_{T2} = 9 mM). In contrast, in the presence of K⁺ (KCl = KSCN) or in the absence of any alkali-metal ions (N-methyl-D-glucamine gluconate), only one saturable system was apparent, which we identify as S-2. When Na⁺ is present, S-1, but not S-2, appears to be rheogenic, since its maximal transport capacity significantly increases in the presence of an inside-negative membrane potential, created either by replacing Cl⁻ with the permeant anion thiocyanate (NaSCN > NaCl) or by applying an appropriate K⁺ gradient and valinomycin. α -(Methylamino)isobutyrate (methyl-AIB) appears to be a substrate of S-1, but not of S-2. For reasons that remain to be explained, however, methyl-AIB inhibits S-2. We conclude that S-1 represents a truly Na⁺-dependent mechanism, where Na⁺ behaves as an obligatory activator, whereas S-2 cannot discriminate between Na⁺ and K⁺, although its activity is higher in the presence of alkali-metal ions than in their absence (Na⁺ = K⁺ > N-methyl-D-glucammonium ion). S-2 appears to be fully developed 2 days before birth, whereas S-1 undergoes a capacity-type activation between days 19.5 and 21.5 of gestation, i.e. its apparent V_{max} nearly doubles, whereas its K_T remains constant.

INTRODUCTION

During intrauterine life, rapid fetal growth depends on the efficient transport of maternal amino acids across the placenta (Palou *et al.*, 1977; Phillips *et al.*, 1978). The amino acids taken up by the fetus are used to maintain both the high metabolic functions of the trophoblast and the fetal metabolic requirements (Battaglia & Meschia, 1978; Remesar *et al.*, 1980; Carroll & Young, 1983). L-Alanine is an important metabolic fuel for the fetus. Its concentration is higher in the fetal circulation than in maternal blood. Because its origin is essentially maternal, placental L-alanine transfer must occur against the concentration gradient (Enders *et al.*, 1976).

There are several reports (Enders *et al.*, 1976; Ruzycski *et al.*, 1978; Boyd & Lund, 1981; Asai *et al.*, 1982; Ganapathy *et al.*, 1986; Kudo *et al.*, 1987; Johnson & Smith, 1988) showing that neutral amino acids are taken up by the human placenta through both Na⁺-dependent and Na⁺-independent transport pathways of overlapping specificities. Even though there are several reports concerning various general aspects of protein metabolism in pregnant rats and rat placenta (Palou *et al.*, 1977; Remesar *et al.*, 1980), much remains to be known about the placental transport systems of neutral amino acids (Yamaguchi *et al.*, 1978; Schlepphorst *et al.*, 1980). For these reasons, an isolated membrane preparation from the maternal surface of the syncytiotrophoblast rat placenta should be useful to characterize the development of placental amino acid transport systems at the end of gestation.

In the present study we describe the characterization of L-alanine transport systems in brush-border membrane vesicles from trophoblast rat placenta. Our results show the existence in these vesicles of at least one truly Na⁺-dependent rheogenic

transport system shared by L-alanine and α -(methylamino)-isobutyrate (methyl-AIB) (we call it System 1; S-1), plus a second system, L-alanine-specific, non-rheogenic and unable to discriminate between Na⁺ and K⁺ (System 2; S-2).

S-2 appears to be fully mature 2 days before gestation. In contrast, during the last 2 days of gestation there appears to be a specific increase in the apparent V_m of S-1, with no significant change in its K_T .

MATERIALS AND METHODS

Materials

L-[U-¹⁴C]Alanine, L-[1-¹⁴C]methyl-AIB and D-[1-¹⁴C]mannitol were from New England Nuclear (Itisa, Madrid, Spain). Valinomycin, L-amino acids, D-mannitol and BSA (fraction V) were from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of microvillous membranes

This was done from albino Wistar rats of known gestational age (19.5–21.5 days) as described by Alonso de la Torre *et al.* (1991). The final cream-coloured pellet (P3) was resuspended in a small volume of buffer B (500 mM-sorbitol in 20 mM-Tris/20 mM-Mes buffer, pH 7.4) and homogenized by passing it six times through a 25-gauge hypodermic needle. Membrane vesicles were adjusted to about 15 mg of protein/ml and stored in liquid nitrogen until use. To obtain K⁺-loaded vesicles, two distinct procedures were used with similar results, namely entrapment during vesicle formation or application of two cycles of freezing and thawing in the presence of the desired medium. As compared with the homogenate, the microvillous-membrane

Abbreviations used: methyl-AIB, α -(methylamino)isobutyrate; S-1 and S-2, L-alanine transport systems 1 and 2.

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vesicle preparation was enriched 6-fold in alkaline phosphatase (EC 3.13.1; method of Bretauiere & Spilman, 1984). Basolateral-membrane contamination was assessed by measuring the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (EC 3.6.1.37; Scharschmidt *et al.*, 1979) which, as compared with the homogenate, was enriched only 1.5-fold in the brush-border membrane vesicle preparation. Protein concentration was measured as described by Markwell *et al.* (1978) with BSA as standard.

Uptake assays

Substrate uptake was quantified by using a rapid filtration technique (Hopfer *et al.*, 1973; Brot-Laroche *et al.*, 1986). In standard experiments, incubations at 37 °C were started by mixing 10 μl of the vesicle preparation in buffer B with 90 μl of the same buffer containing ^{14}C -labelled substrate and the appropriate salts and effectors. Sorbitol was added as needed to obtain a constant net osmotic gradient (out)/(in) = 600/500 mosm. Incubations were stopped with 4 ml of ice-cold stop solution containing 350 mM-KCl and 25 mM-MgSO₄ in 20 mM-Tris/20 mM-Mes buffer, pH 7.4. The diluted samples were rapidly filtered under negative pressure through wetted cellulose nitrate filters (0.65 μm pore; Millipore Ibérica, Madrid, Spain), which were then rinsed with 4 $\times$ 4 ml of stop solution. The entire washing procedure lasted less than 15 s. Appropriate blanks were made to correct for non-specific marker retention on the filters. After dissolving the filters in 4 ml of scintillation fluid (Ready Safe, Beckman), radioactivity was measured in a scintillation spectrometer. Additional details are specified in the legends to Figures and Tables. Initial uptake rates were determined by using a Short-Time Incubation apparatus (Innovativ Labor A.G., Zürich, Switzerland).

Expression of results and statistical analyses

Results are expressed (Brot-Laroche *et al.*, 1986) as absolute uptakes (pmol/mg of protein), absolute velocities (pmol/s per mg of protein), or relative velocities (nl/s per mg of protein). The data shown are usually the means from several experiments performed with at least two different membrane preparations, each at least in triplicate. Occasionally, representative results obtained with a single membrane preparation are presented. Data were compared by using a one-way analysis of variance (Snedecor & Cochran, 1967).

Fitting of the transport results to the Michaelis-Menten equation

Uncorrected absolute initial-velocity data as a function of the substrate concentration were fitted by iteration to an equation involving the sum of one non-saturable diffusional component, and either one or two Michaelian saturable transport terms. Briefly, we followed the procedure of Fletcher & Powell (1964) as modified by van Melle & Robinson (1981) and Robinson & van Melle (1983). The regression functions were fitted in a single run to each data set by minimizing the sum of squares of errors. Comparison of a 'lack of fit' component with a 'pure error' one was accomplished to obtain an *F* value that provides a quantitative assessment of the goodness of fit. Statistical comparison between the various estimated kinetic parameters was done by applying the van Melle & Robinson (1981) *F'* test. All calculations were performed with an Apple-Macintosh SE microcomputer.

RESULTS AND DISCUSSION

L-Alanine transport experiments

Absence of non-specific binding of L-alanine. The equilibrium uptake of 0.1 mM-L-alanine was determined after 2 h of incubation under standard conditions as a function of the external

Fig. 1. Effect of cations and anions on the time course of L-alanine and methyl-AIB uptake by microvillous membrane vesicles from rat placenta

Vesicles in 20 mM-Tris/20 mM-Mes buffer, pH 7.4, containing 500 mM-sorbitol were used. Incubations were performed for the indicated time periods in the same buffer containing 0.1 mM of the indicated ^{14}C -labelled substrates (a, L-alanine; b, methyl-AIB) and 100 mM-NaSCN (■), -NaCl (□) or -KCl, -KSCN or -glucamine gluconate (◆). The results are presented as mean absolute uptakes $\pm$ S.E.M. ($n = 3$ per point), except for the results in the absence of Na⁺, which, to simplify the Figure, have been pooled and are illustrated under the same symbol ($n = 9$).

osmolarity, which was varied by adding increasing concentrations of the non-permeant polyol, D-sorbitol. The results (not shown) indicate that the uptake approaches values indistinguishable from zero when the external osmolarity tends to infinity. We conclude that L-alanine uptake involves entrapment into an osmotically active space and that non-specific binding to either intra- or extra-vesicular structures is negligibly small. Similar results were obtained by Johnson & Smith (1988), who used human placental microvilli. Further evidence for the absence of binding to the external side of the membrane is given below.

Comparative effect of cations and anions on the time course of L-alanine uptake. To establish the L-alanine-transport requirement for cations, the uptake of 0.1 mM-L-alanine as a function of time was studied in the presence of inward-directed gradients (out/in = 100 mM/0) of NaSCN, NaCl, KSCN, KCl or methylglucamine gluconate. In the presence of a Na⁺ gradient, L-alanine uptake exhibited a typical overshoot, which was larger when the accompanying anion was thiocyanate rather than Cl⁻ (Fig. 1a). In contrast, uptake took place downhill in the absence of Na⁺, the results being indistinguishable when the replacing salt was KSCN, KCl or methylglucamine gluconate (lower curve, Fig. 1a).

As to the initial velocities, L-alanine uptake was 10 times larger in the presence of NaSCN (247 pmol/s per mg of protein) than in KSCN, KCl or methylglucamine gluconate (24 pmol/s per mg of protein). The initial rate in the presence of NaCl was intermediate (160 pmol/s per mg of protein). However, under any and all of the above conditions, the L-alanine entry rate was much larger than that of the classical diffusion marker, D-mannitol (2.9 pmol/s per mg of protein), indicating that carrier-mediated L-alanine uptake can occur in the complete absence of Na⁺.

Effect of an outward-directed K⁺ gradient and valinomycin on L-alanine transport. The observation that NaSCN is significantly better than NaCl in increasing both the initial entry rate and the magnitude of the overshoots observed with 0.1 mM-L-alanine strongly indicates that, in these vesicles, there is a Na⁺-dependent L-alanine uptake that is rheogenic, i.e. involves transfer of the net positive charge of Na⁺ across the membrane.

To confirm this conclusion, we performed uptake experiments in the presence of zero *trans*, 100 mM-NaCl and an outward-directed K⁺ gradient ([K⁺]_{out}/[K⁺]_{in} = 10 mM/100 mM; Fig. 2). By itself, K⁺ did not modify the uptake, since, in the absence of valinomycin, the L-alanine overshoots did not differ significantly from those obtained with NaCl and no K⁺ (compare with the middle curve, Fig. 1*a*). However, the presence of valinomycin significantly increased both the size of the overshoot and the initial L-alanine uptake rate, strongly indicating that: (i) the intrinsic K⁺ permeability characterizing these vesicles is negligibly small in the absence of valinomycin; and (ii) the L-alanine/Na⁺ co-transport is rheogenic, fully confirming the anion-replacement results previously discussed.

The relative velocity test. Since evidence for Na⁺-dependent rheogenic transport had been obtained by using 0.1 mM-L-alanine, the obvious question became: what would be the result if the substrate concentration were varied? An initial attempt to answer this question is summarized in Table 1. Here, the L-alanine and D-mannitol relative uptakes are compared in the presence of the same salts used in the preceding experiments. All alanine uptakes are significantly higher than those of the diffusion marker D-mannitol, indicating that, even in the complete absence of Na⁺, L-alanine uptake involves some specific transport mechanism. Furthermore, L-alanine uptake is saturable even in the absence of Na⁺, as indicated by the significant fall in the relative uptake values elicited by increasing the substrate concentration from 0.1 to 10 mM. Because such findings strongly suggest the existence of heterogeneity, we next undertook a kinetic analysis of L-alanine uptake to establish the number and nature of the transport systems involved.

Concentration-dependence of L-alanine uptake. L-Alanine uptake as a function of its concentration was measured in the presence of 100 mM (zero *trans*) concentrations of NaSCN, NaCl, KSCN or methylglucamine gluconate. Previously, we had established that L-alanine uptake is linear for up to 3 s at any of the concentrations used (results not shown). To estimate the kinetic parameters for transport, the uncorrected total uptake rates were subjected to non-linear regression analysis (see Table 2*a*). In the presence of Na⁺, the results indicate quite clearly the existence of at least two distinct transport systems plus a rather high diffusional component. On the contrary, in the absence of Na⁺ only one transport system was apparent. The diffusion component remained unchanged.

To interpret these results, let us begin with the Na⁺ data. We first distinguish (columns 2 and 3, Table 2*a*) a high-affinity system with a *K*_{T1} of about 0.5 mM, which we call S-1. The apparent *V*_{m1} of this system in the presence of SCN⁻ is about double that in the presence of Cl⁻, which begs the conclusion that this is the rheogenic transport system evinced by the experiment

Fig. 2. Effect of an inside-negative membrane potential on L-alanine uptake

Experimental conditions and expression of results were as in Fig. 1, except that the vesicles had been charged with 100 mM-KCl. The external media contained 0.1 mM-L-[¹⁴C]alanine and 100 mM-NaCl but no K⁺, to give, after vesicle addition, an initial [K⁺]_{out}/[K⁺]_{in} = 10 mM/100 mM gradient. Incubations were performed for the indicated time periods in either the presence or the absence of valinomycin (Val). The initial uptake rates (not clearly evident because of the scale used) were statistically different by a one-way analysis of variance (*P* < 0.005).

Table 1. Relative uptake of L-alanine, methyl-AIB and D-mannitol in the presence and absence of alkali-metal ions

Experimental conditions were as in Fig. 1, except that only two substrate concentrations were used and, to measure the initial uptake rates, a 2.7 s incubation time. The results are shown as relative velocities. Identical lower-case letters in parentheses identify results that are statistically indistinguishable by a one-way analysis of variance. Abbreviation: GLU, signifies *N*-methyl-D-glucamine gluconate.

Substrate	Salt	[Substrate] (mM)	Relative velocity (nl/s per mg of protein)	S.E.M.	<i>n</i>
L-Alanine	NaSCN	0.1	2550	277	7
	NaSCN	10	267	9	9
	KSCN	0.1	274 (a)	5	8
	KSCN	10	125 (b)	4	9
	GLU	0.1	245 (a)	10	5
	GLU	10	107 (b)	4	6
Methyl-AIB	NaSCN	0.1	676	35	8
	NaSCN	10	132	6	9
	KSCN	0.1	43 (c)	3	6
	KSCN	10	46 (c)	2	8
	GLU	0.1	43 (c)	1	3
	GLU	10	36 (c)	2	3
D-Mannitol	NaCl*	0.1	29 (d)	0.1	6

* Identical results were found when either NaCl or *N*-methylglucamine gluconate was used.

in Fig. 1*a*). We therefore conclude that S-1 requires Na⁺ as an obligatory activator (for definition see Alvarado & Lherminier, 1982), and this is why no transport activity can be found when the Na⁺ is fully replaced by either K⁺ or the methylglucammonium ion.

The second system, S-2 (columns 4 and 5, Table 2*a*) has a lower but not negligible apparent affinity for alanine in the presence of NaSCN, with a *K*_{T2} of about 8 mM, although its capacity is of the same order of magnitude as that of S-1. Here, however, no significant difference between SCN⁻ and Cl⁻ exists, indicating that S-2 is not rheogenic. In further contrast with S-1,

Table 2. Kinetic parameters for L-alanine and methyl-AIB transport by microvillous membrane vesicles from rat placenta

Vesicles were prepared at 21.5 days of gestation in 20 mM-Tris/20 mM-Mes buffer, pH 7.4, containing 500 mM-sorbitol. Incubations were performed under standard conditions for 2.7 s in the same buffer containing the indicated substrates (concentration range 0.05–100 mM) and 100 mM (zero *trans*) salts. The kinetic parameters ($\pm$ S.E.M.) listed were estimated from the uncorrected absolute uptake data by non-linear regression analysis as described in the Materials and methods section. Units: V_m , pmol/s per mg of protein; K_T , mM; K_d , nl/s per mg of protein. For the *F* test, the degrees of freedom (df) for lack of fit and for pure error, in that order, are indicated in brackets. Further details are given in the text. GLU signifies *N*-methyl-D-glucamine gluconate; – means that the corresponding parameter was undetectable.

Substrate	Salt	V_{m1}	K_{T1}	V_{m2}	K_{T2}	K_d	<i>F</i> , [df]
(a) L-Alanine	NaSCN	1373 $\pm$ 114	0.44 $\pm$ 0.07	1242 $\pm$ 211	7.6 $\pm$ 1.3	39.5 $\pm$ 6.8	0.218 [7,55]
	NaCl	758 $\pm$ 8.0	0.52 $\pm$ 0.01	1714 $\pm$ 252	9.3 $\pm$ 1.5	38.0 $\pm$ 6.1	0.251 [7,55]
	KSCN	–	–	1088 $\pm$ 1.2	5.2 $\pm$ 0.6	42.0 $\pm$ 2.6	1.039 [6,62]
	GLU	–	–	643 $\pm$ 6.5	3.1 $\pm$ 0.4	41.0 $\pm$ 5.2	0.595 [8,37]
(b) Methyl-AIB	NaSCN	1185 $\pm$ 133	1.8 $\pm$ 0.3	–	–	36.0 $\pm$ 4.6	0.561 [8,46]
	NaCl	658 $\pm$ 7.5	1.8 $\pm$ 0.2	–	–	37.0 $\pm$ 3.3	0.617 [8,62]
	KSCN	–	–	–	–	44.0 $\pm$ 0.9	1.378 [7,25]
	GLU	–	–	–	–	41.0 $\pm$ 0.8	1.014 [9,16]
(c) L-Alanine + + 10 mM-methyl-AIB	NaSCN	877 $\pm$ 82	1.79 $\pm$ 0.17	2372 $\pm$ 221	15.4 $\pm$ 1.4	19.3 $\pm$ 1.8	1.035 [7,53]

saturable L-alanine transport does not occur in the absence of Na^+ , and the estimated kinetic parameters are of the same order of magnitude as those observed in its presence. Thus the V_{m2} values can be arranged in the order Na^+ (100%) = K^+ (75%) > glucammonium ion (40%). The K_{T2} parameter is smaller in the absence of alkali-metal ions than in their presence, but these differences are small and barely significant.

To sum up, the results just described suggest, but do not prove, that all of the L-alanine uptakes evidenced by the results in columns 4 and 5 of Table 2(a) involve a single transport system, S-2, that exhibits a low cationic specificity. Independently of whether S-2 is a homogeneous transport system or not, the existence of S-2 explains the relative-velocity results presented in Table 1. We conclude that S-2 cannot distinguish readily between Na^+ and K^+ , but works significantly faster when an alkali-metal ion is present. Therefore, it is not warranted to say that S-2 is either ' Na^+ -dependent' or ' Na^+ -independent'.

The K_T values observed for the two L-alanine transport systems defined here are similar to those found by Asai *et al.* (1982) in human placenta membrane vesicles, although those authors did not enter the cationic-dependence question. Johnson & Smith (1988) also found two L-alanine uptake systems in human placenta, which they described as, respectively, Na^+ -dependent and Na^+ -independent. The K_T value found by those authors for the first system agrees well with that estimated by us for S-1 (K_T about 0.4 mM in either case), but the K_T of their so-called Na^+ -independent system (0.8 mM) is 10 times smaller than that estimated by us for S-2 (K_{T2} = 8 mM). Apart from involving different species, this discrepancy could perhaps be accounted for by the fact that Johnson & Smith (1988) worked at 22 °C, whereas we did so at 37 °C. It is known that certain transport systems exhibit significant changes in their kinetic behaviour as the temperature is varied (Young *et al.*, 1983; Brot-Laroche *et al.*, 1986; Fincham *et al.*, 1986). Johnson & Smith (1988) suggested that their Na^+ -independent L-alanine uptake involves the asc system, which is highly sensitive to temperature (Fincham *et al.*, 1986). The asc system, first described for the erythrocyte, has been defined as having the characteristics of ASC, except that it is Na^+ -independent (Vadgama & Christensen, 1985). According to our findings, however, S-2 cannot be said to be either Na^+ -dependent or Na^+ -independent. More work will be required to reconcile our results and those of Johnson & Smith (1988).

As concerns K_d (column 6, Table 2a), it indeed appears to be very high and independent of the nature of the salt present.

Table 3. Inhibitory effect of amino acids on L-alanine and methyl-AIB uptake by microvillous membrane vesicles from rat placenta

The vesicles were preloaded with 500 mM-sorbitol in 20 mM-Tris/20 mM-Mes buffer, pH 7.4. Uptake of either L-alanine or methyl-AIB (0.1 mM each) was measured for 2.7 s under standard conditions by incubating the vesicles in the same buffer supplemented with 100 mM (zero *trans*) -NaSCN or -KSCN, plus the indicated inhibitors (10 mM). The results represent means $\pm$ S.E.M. of three to five determinations per point. Values in parentheses indicate complete absence of inhibition, the negative sign meaning non-significant activation. Abbreviation: AIB, α -aminoisobutyrate.

Substrate...	L-Alanine uptake (% inhibition)		Methyl-AIB uptake (% inhibition)
	NaSCN	KSCN	NaSCN
L-Alanine	98 $\pm$ 0.3	75 $\pm$ 5.2	92 $\pm$ 3.1
Methyl-AIB	50 $\pm$ 0.5	(–9 $\pm$ 0.4)	84 $\pm$ 5.2
AIB	72 $\pm$ 3.5	53 $\pm$ 3.5	80 $\pm$ 1.3
L-Leucine	59 $\pm$ 0.3	77 $\pm$ 0.4	44 $\pm$ 1.1
L-Isoleucine	18 $\pm$ 1.8	67 $\pm$ 4.7	3 $\pm$ 0.1
Cycloleucine	72 $\pm$ 0.5	70 $\pm$ 1.2	77 $\pm$ 0.6
L-Phenylalanine	13 $\pm$ 1.3	79 $\pm$ 1.4	24 $\pm$ 1.3
L-Proline	45 $\pm$ 1.2	(–2 $\pm$ 0.1)	55 $\pm$ 2.3
L-Threonine	55 $\pm$ 0.3	71 $\pm$ 0.5	36 $\pm$ 0.1

Because of its theoretical and practical interest, this point will be discussed in greater detail below.

Substrate specificity of L-alanine transport systems. The inhibition of L-alanine transport was studied by using a single 0.1 mM substrate concentration and a 100-fold excess of nine neutral amino acids, including L-alanine itself (Table 3). In the presence of NaSCN, most (94 %) of the L-alanine uptake involves S-1. Uptake was strongly inhibited (nearly 100 %) by L-alanine itself and by cycloleucine and α -aminoisobutyrate (about 72 %). Incomplete inhibition was observed with L-leucine and L-threonine (55–60 %) and with methyl-AIB and L-proline (45–50 %). No significant inhibition (< 20 %) was obtained with either L-isoleucine or L-phenylalanine. This inhibition pattern is similar to that found by Johnson & Smith (1988) in human placenta, except that those workers observed lower inhibitions with methyl-AIB, L-proline and L-phenylalanine.

In the absence of Na^+ , L-alanine uptake was inhibited by all

Table 4. Kinetic parameters for L-alanine uptake in microvillous membrane vesicles from rat placenta during the last 3 days of gestation

Brush-border membrane vesicles from rat placenta were prepared at the indicated days of gestation in 20 mM-Tris/20-Mes buffer, pH 7.4, containing 500 mM-sorbitol. Incubations were performed under standard conditions for 2.7 s in the same buffer containing L-alanine concentrations in the range 0.1–100 mM and 100 mM (zero *trans*) NaSCN. The kinetic parameters shown were estimated and are represented in the same units as in Table 2.

Stage of gestation (days)	V_{m1}	K_{T1}	V_{m2}	K_{T2}	K_d	F , [df]
19.5	793 ± 32	0.52 ± 0.02	1479 ± 89	7.0 ± 0.4	27.8 ± 1.7	1.045 [7,59]
20.5	1281 ± 124	0.59 ± 0.06	2463 ± 239	10.6 ± 1.0	22.8 ± 2.2	0.898 [7,47]
21.5	1373 ± 114	0.44 ± 0.07	1242 ± 211	7.6 ± 1.3	39.5 ± 6.8	0.218 [7,55]

the amino acids tested, except L-proline and methyl-AIB, once more in agreement with the results for human placenta. The lack of effect of methyl-AIB in the absence of Na^+ strongly suggests that this amino acid is a substrate for S-1, but not for S-2. This idea led us to study both the kinetics of methyl-AIB interaction with L-alanine transport and the transport of methyl-AIB itself.

Methyl-AIB transport experiments

Effect of cations and anions on the time course of methyl-AIB uptake. Methyl-AIB uptake as a function of incubation time was studied in the presence of 100 mM (zero *trans*) -NaSCN, -NaCl, -KSCN, -KCl or -methylglucamine gluconate (Fig. 1b). The results are qualitatively similar to those found for L-alanine (Fig. 1a). Thus uptake exhibited a typical overshoot in the presence of Na^+ , and both the initial entry rate and the extent of the overshoots were larger when the accompanying anion was SCN^- rather than Cl^- . Quantitatively, however, methyl-AIB exhibited considerably slower entry rates and lower overshoots, which can be explained in terms of a smaller affinity of methyl-AIB for S-1, the rheogenic carrier involved in L-alanine transport.

Also similarly to L-alanine, methyl-AIB uptake in the absence of Na^+ was slow and no overshoot could be observed; KSCN, KCl and methylglucamine gluconate gave essentially identical results (lower curve, Fig. 1b). Nevertheless, methyl-AIB uptake under these conditions seems to involve a mediated mechanism since, as shown in Table 1, the relative uptakes in the presence of either K^+ or the glucammonium ion are significantly higher than those exhibited by D-mannitol. The absence of saturation evinced by the methyl-AIB results (the relative uptake did not change when the substrate concentration was raised from 0.1 to 10 mM) probably derives from the low affinity of this amino acid for still another transport system, as we discuss below.

Concentration-dependence of methyl-AIB uptake. In order to quantify better the observed differences between methyl-AIB and L-alanine transport, saturation experiments were carried out along the lines previously described for L-alanine. The most striking results (Table 2b) are that: (i) methyl-AIB is not transported in the absence of Na^+ ; (ii) in the presence of Na^+ , methyl-AIB is taken up by a single system; and (iii) V_{m1} about doubles when the Cl^- ion is fully replaced by SCN^- . This evidence permits the conclusion that methyl-AIB shares S-1 with L-alanine, both amino acids exhibiting the key properties of obligatory Na^+ -dependence and rheogenicity. However, the K_{T1} ratio methyl-AIB/L-alanine equals 3.8, indicating that the apparent affinity of methyl-AIB for S-1 is nearly 4 times smaller than that of L-alanine. Such a result explains the quantitative differences in transport, illustrated in Fig. 1. Working with human placenta microvilli, Kudo *et al.* (1987) also found that methyl-AIB transport requires Na^+ and is rheogenic.

Interestingly, the apparent diffusion constant, K_d , for methyl-AIB is very large and indeed essentially identical with that found

for L-alanine (column 6, Tables 2a and 2b). This important fact will be reconsidered below.

Substrate specificity of methyl-AIB transport. The inhibition of 0.1 mM-methyl-AIB uptake by a 100-fold excess of various amino acids was studied in the presence of 100 mM- (zero *trans*) NaSCN (see Table 3).

The inhibition pattern was in general similar to that found for L-alanine. Thus, for instance, the inhibition caused by L-isoleucine and L-phenylalanine was in both cases negligible. As would have been expected, methyl-AIB strongly inhibited its own uptake, and nearly complete inhibitions were also caused by L-alanine, AIB and cycloleucine. L-Threonine and L-leucine inhibited slightly less than they inhibited L-alanine uptake.

Kinetics of L-alanine transport in the presence of methyl-AIB

Preliminary studies (results not shown) on the effect of increasing methyl-AIB concentrations at constant (0.1 mM) L-alanine indicated that a 50 %-maximal inhibition plateau was reached at or above 10 mM-methyl-AIB. Such a result strongly indicates the existence of at least two L-alanine transport systems: one sensitive, the other insensitive, to methyl-AIB inhibition.

L-Alanine saturation curves were performed in the presence of 100 mM (zero *trans*)-NaSCN and 10 mM-methyl-AIB. Our findings go beyond the expectation that methyl-AIB would simply compete with L-alanine for the Na^+ -dependent rheogenic transport system, S-1. In fact, the results obtained (Table 2c) indicate that methyl-AIB affects L-alanine transport at all possible levels. Namely, it: (i) increased K_{T1} and K_{T2} by factors of 4 and 2 respectively; (ii) decreased V_{m1} but increased V_{m2} by factors of $\frac{2}{3}$ and 2 respectively; and finally (iii) significantly decreased K_d to half the control value. Most of these effects can be said to be surprising.

First, the effect on K_{T1} agrees qualitatively with the hypothesis that methyl-AIB fully competitively inhibits L-alanine transport. Quantitatively, however, the agreement with theory is not as perfect, because the results yield for methyl-AIB a K_i of about 3.6 mM, double the K_{T1} of 1.8 mM estimated from saturation curves (Table 2b). The situation is further complicated by the existence of a highly significant fall in the apparent value of V_{m1} , which suggests that an additional non-competitive effect occurs. Such a result is reminiscent of the fully non-competitive inhibition of intestinal L-methionine transport by D-methionine, which has been described but remains to be explained (Brachet *et al.*, 1987).

The effects on both K_{T2} and V_{m2} were also unexpected since, as mentioned above, methyl-AIB is not a substrate for S-2. The effect on K_d will be discussed below.

Biological significance of the high diffusion constant characterizing L-alanine and methyl-AIB transport in placental microvillous vesicles

The high diffusion constant exhibited by both L-alanine and

methyl-AIB, with an average value of $K_d = 39.8 \pm 1.7$ nl/s per mg (column 6, Tables 2a and b), deserves close scrutiny. There are two main possible interpretations for this finding: either (i) the intrinsic permeability of these amino acids is very high in these vesicles, or (ii) there is a third transport system having a low cationic selectivity and exhibiting an exceedingly low affinity for either amino acid, which would explain the apparently diffusional kinetics observed.

That non-specific amino acid binding cannot explain our K_d results can be deduced from separate measurements of the initial rates of uptake (results not shown). For both amino acids, the uptake curves can be extrapolated to pass through the origin, which negates the existence of quantitatively significant binding to external membrane receptors.

The passive diffusibility of zwitterionic amino acids across biological membranes is believed to be negligible (Christensen, 1984), and there is no reason to believe that the placenta is an exception to this rule. It therefore seems legitimate to assume that any uptake taking place above the threshold level of the diffusion marker, mannitol, constitutes evidence for mediated transport.

Taken together, the preceding arguments favour the interpretation that the high K_d values observed reflect the existence in the placenta of an additional transport system (or systems) of very low affinity. If such systems are characterized by exceedingly high K_T values, then uptake will necessarily exhibit linear diffusion-like kinetics, saturation being impossible or at least difficult to approach under the conditions of 'regular' experiments. Apart from the strong theoretical weight of this argument (see Wilbrandt & Rosenberg, 1961), we can mention here that recent evidence (V. Sorribas & F. Alvarado, unpublished work) indicates the existence of similar extremely low-affinity systems for both sugars and amino acids in the enterocytic apical membrane. Thus the phenomenon here discussed is not unique to the placenta.

Additional evidence that the high K_d parameter estimated for L-alanine and methyl-AIB in the present work involves a specific transport system (or systems) is provided by the kinetic inhibition experiment discussed in the preceding section. In effect, methyl-AIB significantly decreased by 50 % the L-alanine K_d parameter (Table 2c), a result never to be expected were this K_d to represent a true diffusion constant. It is important to emphasize that such an inhibition in no way suggests competition. Quite a few examples exist in the literature indicating that certain amino acids and other organic substances can inhibit systems for which they are not substrates (see, for instance, Kilberg *et al.*, 1981; Brachet *et al.*, 1987; Balkovetz *et al.*, 1988).

Development of L-alanine transport systems during the last 3 days of gestation

The variation of amino acid uptake in placenta as a function of gestational development has not been studied in depth. During gestation, the placenta increases in weight, and it is widely accepted that the transfer of nutrients is proportional to placental size (Butterstein & Leatham, 1974). Furthermore, it is at the end of gestation when the greatest increase in fetal weight occurs, such that an increase in nutrient supply becomes necessary. In view of this, we investigated the kinetics of L-alanine transport in rat placenta during the last 3 days of gestation.

Saturation curves for L-alanine uptake were carried out with brush-border membrane vesicles from rat placenta of 19.5, 20.5 and 21.5 days of gestation. In either case, the kinetic parameters estimated by iteration (listed in Table 4) revealed the same pattern described above for the placenta at term (21.5 days; results of Table 2a, reproduced in Table 4 to permit a direct comparison). Thus two distinct L-alanine transport systems and

a high K_d value were observed at either age. Some of the parameters exhibited a high stability, in particular K_{T1} ; others revealed fluctuations that do not seem to be systematic and indeed are not significant statistically (e.g. V_{m2} , K_{T2}).

It seems interesting that, at 19.5 and 20.5 days of gestation, the K_d parameter was smaller (about 64 %) than that estimated at term. However, these differences were barely significant and do not justify drawing the conclusion that the 'third system' mentioned in the preceding section increases in activity during the last days of gestation. More work will be required to confirm this intriguing question.

The only difference found to be statistically significant concerns V_{m1} , which was lowest (60 %) at 19.5 days and reached its maximum stable value between 20.5 and 21.5 days. Such a result is consistent with the observation that significant increases in methyl-AIB uptake occur between days 16 and 20 of gestation in rat placenta (Schlepphorst *et al.*, 1980). Moreover, in brush-border membrane vesicles from human placenta towards the end of gestation, a 5-fold increase has been found in the V_m of L-alanine uptake, as compared with that found in brush-border membrane vesicles from placenta at the beginning of gestation (Iioka *et al.*, 1986). According to our present findings, the increase in placental transfer of L-alanine observed by others as a function of gestational age probably represents the specific induction of S-1.

A comment on statistics

As mentioned in the Materials and methods section, our kinetic results were evaluated statistically by applying the F and F' tests of van Melle & Robinson (1981). Given the large number of comparisons performed, however, it appears prohibitive to present here all the details. Specific examples of the statistical evaluation of kinetic data, similar to those performed by us here, have been published (van Melle & Robinson, 1981; Vasseur *et al.*, 1992).

Concluding remarks

It is tempting to speculate that what we call here 'system 1' corresponds to the 'system A' of Christensen and associates. We, however, have refrained from adopting such a nomenclature, for the following reasons.

Although accomplishing a classification of the amino acid transport systems of mammalian cells is highly desirable, we feel that the information at present available on individual transport systems in distinct cells is too incomplete to warrant making too narrow definitions. Current research aims at defining transport systems in molecular terms, but the fact is that it is too early to presuppose the existence of homology between systems differing in cellular origin. Before new knowledge warrants it, therefore, it seems to us better to adopt a non-committal nomenclature like the one used in the present paper. Our S-2 is a good example in this regard, since it does not fit any of the transport systems described in other cells or tissues up to the present.

Taken together, our results illustrate the complexity of the heterologous interactions often seen to occur between transport systems thought to be distinct (for review, see Robinson & Alvarado, 1977). To explain the results in Table 3 would require an amount of work vastly exceeding the limits of the present paper. Discrimination between amino acid transport systems is too frequently based on incomplete observations such as those listed in Table 3, where amino acid A is simply found to inhibit transport of amino acid B. Regrettably, in the absence of little more information, the assumption is often made or implied that direct competition for a common carrier system explains such results. Few laboratories, if any, have carried out the rigorous kinetic work needed to prove beyond reasonable doubt whether

or not a given amino acid pair (much less, an amino acid group), indeed compete with one another for a single site in a common carrier system. In concluding, let us just mention one example that directly concerns the placenta. The L-lactate/H⁺ symporter of the apical placental membrane is inhibited by L-succinate, a dicarboxylic acid not transported by this particular transport system (Balkovetz *et al.*, 1988). Evidence for inhibition should not be freely equated with evidence for the sharing of a common transport pathway.

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