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Characterization of a novel Na^+-independent amino acid transporter in horse erythrocytes

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Horse erythrocytes are polymorphic with respect to L-alanine permeability. The present investigation compared the specificity, kinetics and cation-dependence of erythrocyte amino acid transport in two groups of thoroughbred horses, those with erythrocyte L-alanine permeabilities in the range 5–15 μmol/h per litre of cells (0.2 mM extracellular L-alanine, 37°C) (transport-negative type) and those with L-alanine permeabilities in the range 450–700 μmol/h per litre of cells (transport-positive type). Transport-positive cells are shown to possess a novel high-affinity, stereospecific, Na^+-independent transporter selective for neutral amino acids of intermediate size. This carrier system (provisional designation *asc*) operates preferentially in an exchange mode and is functionally absent from erythrocytes of transport-negative-type horses.

Transport of amino acids across the erythrocyte membrane contributes to cell viability by providing precursors for intracellular GSH biosynthesis (Young, 1983; Young & Tucker, 1983). Erythrocyte amino acid transporters may also provide an efflux route for amino acids produced during reticulocyte maturation (Tucker & Young, 1980). Some sheep, for example, exhibit defective erythrocyte amino acid transport (Young *et al.*, 1975). The lesion is inherited, and results in partial GSH deficiency, the intracellular accumulation of amino acids, a markedly diminished erythrocyte potential lifespan and an increased susceptibility to oxidative stress (Ellory *et al.*, 1972; Tucker, 1974; Young *et al.*, 1976, 1982; Tucker *et al.*, 1981). Amino acid transport in normal sheep erythrocytes is mediated by system *C*, a low-affinity, Na^+-independent transporter that is stereospecific and selective for L-cysteine and other neutral amino acids of intermediate size (Young *et al.*, 1976, 1980; Young & Ellory, 1977). The equivalent amino acid transporter in human erythrocytes (system *ASC*) exhibits a similar substrate specificity, but is Na^+-dependent and has a high affinity for its optimal substrates (Young *et al.*, 1980, 1983; Al-Saleh & Wheeler, 1982).

We have recently demonstrated that erythrocytes from thoroughbred horses exhibit a wide range of amino acid permeabilities (Fincham & Young, 1983; Fincham *et al.*, 1985*a*). Initial rates of L-alanine uptake in cells from different animals ranged over two orders of magnitude, with erythrocytes from approx. 30% of the horses tested being amino-acid-transport-deficient (5–15 μmol/h per litre of cells at 0.2 mM extracellular L-alanine, 37°C). The remaining transport-positive-type animals were divisible into four distinct subgroups on the basis of L-alanine permeability, the highest permeability grouping giving L-alanine transport rates in the range 450–700 μmol/h per litre of cells (0.2 mM-L-alanine). The objective of the present study was to compare the specificity, kinetics and cation-dependence of amino acid transport in these highly permeable cells with that in amino-acid-transport-deficient horse erythrocytes. Our results demonstrate that the former cell type possesses a novel high-affinity, Na^+-independent *ASC*-like transporter that is functionally absent from transport-deficient horse erythrocytes. We discuss this finding in the context of possible evolutionary relationships between different erythrocyte amino-acid-transport systems.

Abbreviations used: GSH, reduced glutathione.

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Materials and methods

Whole blood was obtained from thoroughbred horses of English origin maintained under standard husbandry conditions in the stables of the Royal Hong Kong Jockey Club. Blood was collected by venepuncture into heparinized tubes and the erythrocytes washed three times with 20vol. of incubation medium containing 150mM-NaCl, 5mM-glucose and 15mM-Mops, pH7.5 at 37°C. The buffy coat was removed by aspiration. Animals were classified as to transport type by measuring their erythrocyte permeability to L-alanine (0.2mM extracellular concentration, 37°C) (Fincham & Young, 1983; Fincham *et al.*, 1985*a*). Cells were used within 24h of sampling.

Materials

Uniformly ^{14}C-labelled D- and L-amino acids and ^3H_2O were obtained from Amersham International, Amersham, Bucks., U.K., and used without further purification. Non-radioactive amino acids and other reagents were purchased from Sigma.

Amino-acid-uptake studies

For time-course experiments, amino acid uptake was initiated by mixing equal volumes of pre-warmed cell suspension (37°C, 20% haematocrit) and ^{14}C-labelled amino acid (0.4mM, 1μCi/0.4μmol). Aliquots (0.2ml) were removed at predetermined time intervals, added to 1.5ml microcentrifuge tubes containing 0.8ml of ice-cold washing medium (107mM-$MgCl_2$/10mM-Tris/HCl, pH7.9 at 20°C) layered on top of 0.5ml of ice-cold n-dibutyl phthalate, and the tubes immediately centrifuged at 15000*g* for 10s in an Eppendorf 5414 microcentrifuge. The aqueous and n-dibutyl phthalate layers were removed by aspiration, leaving the cell pellets with small coverings of oil at the bottoms of the tubes. The insides of the tubes were then carefully wiped with absorbent tissue paper. Cell pellets were lysed by the addition of 0.5ml of 0.1% (v/v) Triton X-100 in water, then deproteinized with 0.5ml of 5% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation (15000*g*, 2min). Radioactivity present in the protein-free supernatants was measured by liquid-scintillation spectroscopy with appropriate quench correction. Blank values, corresponding to radioactivity trapped in the extracellular space, were obtained by mixing ice-cold cell suspensions and radioactive permeant at 0°C, followed by immediate centrifugation through n-dibutyl phthalate. Amino-acid-uptake values were calculated after subtraction of these blanks.

An alternative cold-start, washing–centrifugation procedure was used to measure initial rates of amino acid uptake (Young & Ellory, 1982). Briefly, equal volumes (0.2ml) of pre-cooled, ice-cold cell suspension (20% haematocrit) and radioactive amino acid solution were mixed in microcentrifuge tubes and the incubations initiated by transferring the tubes into a 37°C water bath. Incubations (15 min) were terminated by transferring the tubes back into an ice bath for a further 15min. Cells were then rapidly washed free of extracellular radioactivity by repeated centrifugation (15000*g*, 10s) and resuspended in 4 × 1ml aliquots of ice-cold washing medium. Cell pellets were processed for liquid-scintillation counting as described above. Na^+-free solutions were made by replacing Na^+ with the appropriate cation. Incubations containing cysteine also included dithiothreitol at a final concentration of 10mM to prevent oxidation of the amino acid.

The haemoglobin content of cell suspensions was measured as cyanmethaemoglobin and converted into haematocrit by using an experimentally determined value for the haemoglobin content of the packed horse erythrocytes (0.242g/ml). Cells in incubation medium contained 70% water by volume, measured by using ^3H_2O.

Amino acid analysis

Cell pellets were deproteinized with 5% (w/v) sulphosalicyclic acid and analysed on an LKB 4400 automated amino acid analyser with the manufacturer's recommended lithium buffer system for physiological fluids.

Results

Measured L-alanine-uptake rates (0.2mM extracellular concentration, 37°C) in horse erythrocytes range between 5 and 700μmol/h per litre of cells, individual animals belonging to one of five distinct permeability subgroupings (Fincham & Young, 1983; Fincham *et al.*, 1985*a*). Horses for the present study were selected from the two extreme transport groups, those with L-alanine uptake rates between 5 and 15μmol/h per litre of cells and those with high L-alanine permeabilities in the range 450–700μmol/h per litre of cells. For simplicity, we refer to these two groups of horses as 'transport-negative type' and 'transport-positive type' respectively.

Time course of amino acid uptake

The time course of uptake of a series of ten representative amino acids (extracellular concn. 0.2mM) were measured in both transport-positive and transport-negative cells. Fig. 1(*a*) shows that transport-positive erythrocytes are most permeable to L-cysteine, L-serine, L-alanine and L-valine. All four amino acids entered the cells rapidly, reaching values of 0.21–0.28mmol/litre of cells

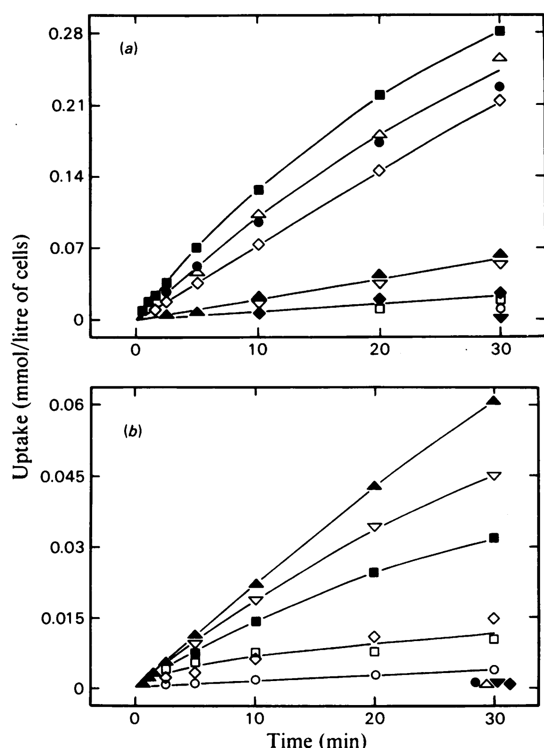

Fig. 1. *Time courses of amino acid uptake by (a) transport-positive and (b) transport-negative horse erythrocytes*
Amino acid uptake (extracellular concentration 0.2mM, 37°C) was measured by the n-dibutyl phthalate method described in the text. The L-cysteine incubations contained 10mM-dithiothreitol. Symbols: ■, L-cysteine; △, L-serine; ●, L-alanine; ◇, L-valine; ▲, L-phenylalanine; ▽, L-leucine; ◆, L-lysine; □, glycine; ○, D-alanine; ▼, L-glutamate.

after 30min (0.3–0.4mmol/litre of cell water). In contrast, the large neutral amino acids L-phenylalanine and L-leucine entered the cells considerably more slowly. Permeabilities to the other amino acids tested were even lower (L-lysine, glycine > D-alanine > L-glutamate).

Transport-negative erythrocytes (Fig. 1*b*) were most permeable to L-phenylalanine and L-leucine, the measured influx rates being similar in magnitude to those obtained for these amino acids in transport-positive cells. L-Cysteine uptake was the next fastest, followed by that of L-valine and glycine. Uptake of D- and L-alanine, L-serine and L-lysine was small, with negligible transport of L-glutamate.

Unless otherwise stated, subsequent values for amino acid permeability refer to initial uptake rates measured by using a 15min incubation period.

Specificity of amino acid uptake

To investigate the specificity of amino acid uptake in the two cell types in more detail, we measured initial rates of uptake for 22 naturally occurring amino acids. Mean results for three animals of each transport type are summarized in Fig. 2. The most striking feature of the data is the high permeability of transport-positive erythrocytes to neutral amino acids of intermediate size, the amino acids showing the largest differences in uptake rate between the two cell types. Values for the ratio of initial uptake rates in the two cell types (transport-positive/transport-negative) are L-alanine (76.5), L-serine (57.8), L-threonine (26.0), L-valine (17.6), L-cysteine (10.3), L-tyrosine (4.6), D-alanine (2.8), glycine (1.5), L-isoleucine (1.2), and L-leucine, L-phenylalanine, L-proline, L-methionine and L-tryptophan (1.1). There was a 20-fold difference in the uptake rates for L- and D-alanine in transport-positive erythrocytes, in contrast with transport-negative cells, where the two isomers entered the cell at the same slow rate.

These data strongly suggest that transport-positive horse erythrocytes possess a stereospecific amino acid transporter selective for intermediate-sized neutral amino acids, this transporter being functionally absent from transport-negative cells. Interestingly, transport-positive cells were also significantly more permeable than transport-negative cells to L-lysine (ratio 12.0) and L-ornithine (2.7) and, to a lesser extent, to L-arginine and L-histidine (1.6). Both cell types were essentially impermeable to both L-glutamate and L-aspartate, in agreement with previous studies in human and sheep erythrocytes (Young *et al.*, 1980). L-Asparagine and L-glutamine gave ratios of 2.0 and 1.7 respectively.

Concentration-dependence of amino acid uptake

Fig. 3(*a*) compares the concentration-dependence of L-alanine uptake by transport-positive and transport-negative erythrocytes over an extracellular L-alanine concentration range of 0.05–5mM. Uptake by transport-positive cells was saturable. In contrast, L-alanine uptake by transport-negative erythrocytes was considerably slower and linear with respect to concentration (0.2mmol/h per litre of cells at 5mM extracellular L-alanine). Correction of the transport-positive-cell data for the linear transport-negative-cell flux yielded a simple hyperbolic relationship between initial rate of transport (v) and extracellular substrate concentration (s). Linear-regression analysis of a plot of s/v against s gave an apparent K_m value of 0.34mM and a $V_{max.}$ of 2.0mmol/h per litre of cells. The comparable experiment for D-alanine uptake by the two cell types is shown in

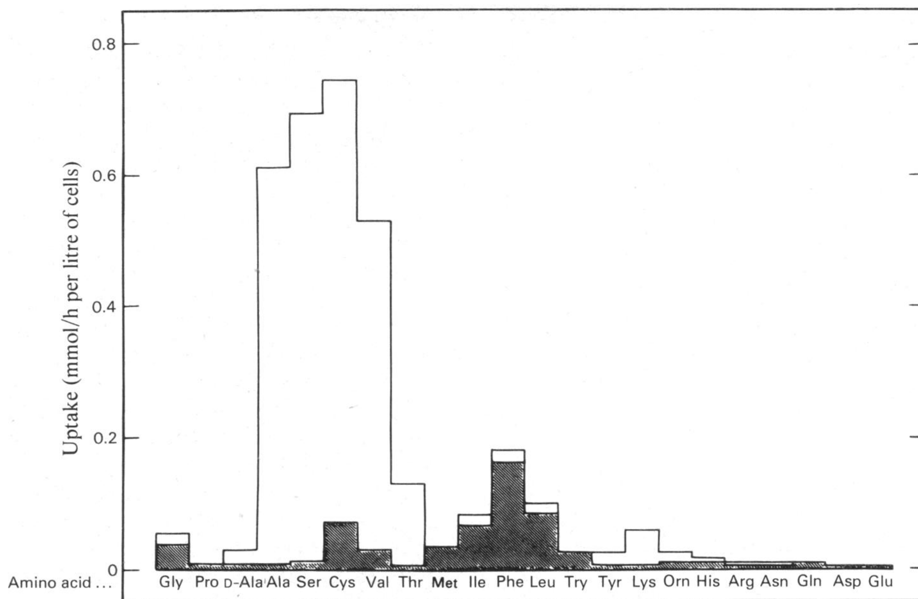

Fig. 2. *Substrate specificity of amino acid uptake in transport-positive and transport-negative horse erythrocytes*
Initial rates of amino acid uptake were determined in transport-positive (□) and transport-negative (▧) cells at 37°C and an initial extracellular concentration of 0.2mM by the washing–centrifugation method described in the text. Data are for three animals of each transport type. S.E.M. values (not shown) were typically less than 10% of the means.

Fig. 3(*b*). D-Alanine uptake by transport-positive cells was greater than that by transport-negative cells (see also Figs. 1 and 2), but was non-saturable, even at 5mM extracellular amino acid.

Figs. 4(*a*) and 4(*b*) compare the concentration-dependence of L-cysteine (0.05–5mM) and L-leucine (1.25–50mM) transport in the two types of horse erythrocyte. Transport of L-cysteine into transport-positive cells was resolved into saturable and non-saturable components of uptake, the former component of transport being absent from transport-negative cells. The apparent K_m and $V_{max.}$ values for the saturable component of transport were 0.15mM and 1.0mmol/h per litre of cells respectively. The non-saturable uptake of L-cysteine by the two cell types (1.0mmol/h per litre of cells at 5mM extracellular amino acid) was considerably greater than that for L- or D-alanine (~0.2mmol/h per litre of cells, 5mM amino acid) (see also Fig. 2). L-Leucine was one of the amino acids showing no significant difference in transport rate (0.2mM) between the two types of horse erythrocyte (Fig. 2). As shown in Fig. 4(*b*), L-leucine uptake over the concentration range 1.25–50mM was similar in the two types of horse erythrocyte, with no evidence of saturation even at high extracellular L-leucine concentrations. This contrasts with the situation in human erythrocytes, where L-leucine transport is mediated by a high-capacity, low-affinity (apparent K_m 6mM) transporter (system *L*) (Young *et al.*, 1980).

Effect of L-cysteine on L-alanine transport

To investigate whether L-cysteine and L-alanine share a common transport system, the uptake of L-alanine (0.1–0.75mM) by transport-positive horse erythrocytes was measured in the presence of various concentrations of L-cysteine (0.4–2.0mM). L-Cysteine was found to be an effective inhibitor of L-alanine transport. Dixon (1953) analysis of the data confirmed that the inhibition was competitive, with an apparent K_i value of 0.15mM (Fig. 5). Thus the apparent K_i value for L-cysteine inhibition of L-alanine transport is the same as the apparent K_m value for L-cysteine uptake.

Na^+-dependence of amino acid uptake

To test for Na^+-dependent amino acid transport in horse erythrocytes, the uptake of L-alanine (0.2mM extracellular concentration) by the two cell types was measured in different media containing either Na^+, K^+, Li^+, Mg^{2+} or choline chloride (Table 1). No evidence of Na^+-dependence was found. In contrast, L-alanine transport in human erythrocytes under the same experimental conditions was found to be markedly Na^+-dependent (see also Young *et al.*, 1983).

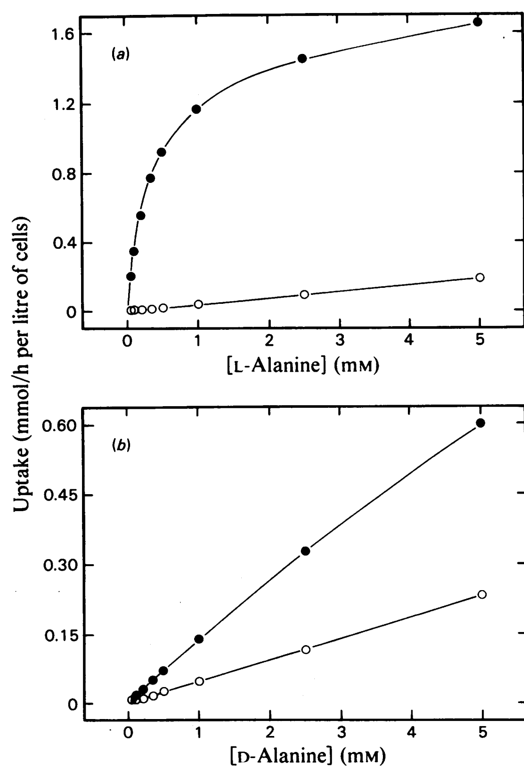

Fig. 3. *Concentration-dependence of (a) L-alanine uptake and (b) D-alanine uptake by transport-positive and transport-negative horse erythrocytes*

Initial rates of L-alanine uptake were measured at 37°C in the presence of Na^+. ●, Transport-positive cells; ○, transport-negative cells. Values are means of triplicate estimates. See the text and the legend to Fig. 2 for other experimental details.

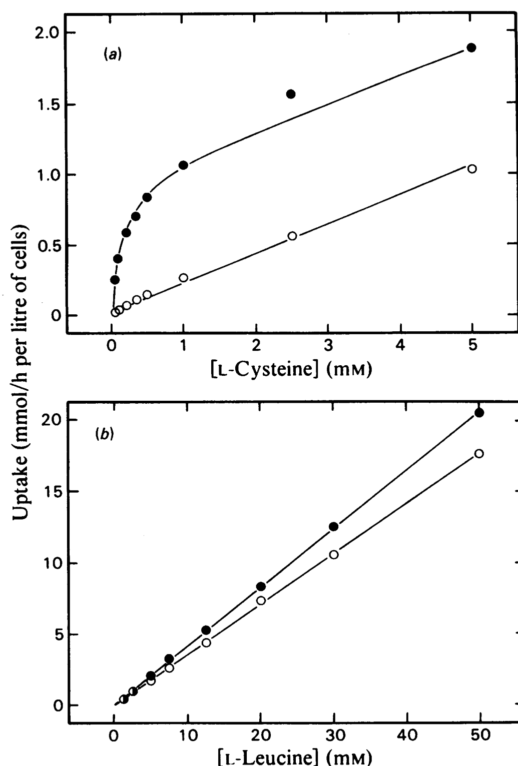

Fig. 4. *Concentration-dependence of (a) L-cysteine uptake and (b) L-leucine uptake by transport-positive and transport-negative horse erythrocytes*

Initial rates of L-cysteine and L-leucine uptake were measured at 37°C in the presence of Na^+ as described in the text and the legend to Fig. 2. The L-cysteine incubations contained 10mM-dithiothreitol. Values are means of triplicate estimates. ●, Transport-positive cells; ○, transport-negative cells.

Table 1. *Effects of different cations on L-alanine uptake in horse erythrocytes*

Initial L-alanine-uptake rates (0.2mM extracellular concentration, 37°C) were determined in the presence of different cations as described in the text. Data are mean values (±S.E.M.) for erythrocytes from three transport-positive-type horses and two transport-negative-type horses. Under the same experimental conditions, L-alanine uptake by human erythrocytes was decreased by 70, 52, 52 and 69% in K^+, Mg^{2+}, Li^+ and choline chloride media respectively, relative to uptake in NaCl medium.

	L-Alanine uptake (μmol/h per litre of cells)	
Cation	Transport-positive cells	Transport-negative cells
Na^+	624 ± 14	8.6 (8.2, 8.9)
K^+	590 ± 19	8.2 (7.7, 8.7)
Li^+	626 ± 13	7.2 (6.9, 7.4)
Mg^+	470 ± 20	6.2 (6.3, 6.1)
Choline	688 ± 24	9.2 (8.7, 9.7)

Exchange versus net transport of amino acid

Analysis of freshly washed transport-positive erythrocytes revealed the presence of significant intracellular concentrations of amino acids, including alanine, serine, threonine and valine. These latter four amino acids are substrates for the transporter (Fig. 2), but were retained by cells even during prolonged incubation at 37°C. Extracellular L-alanine induced the selective efflux of these amino acids, presumably by homo- and hetero-exchange. The question of exchange versus net transport was studied quantitatively by correlating L-[^{14}C]alanine uptake with intracellular levels of these amino acids. An incubation period of 6h was used to maximize the possibility of net amino acid transport. The results of an experiment performed at different initial extracellular concentrations of L-[^{14}C]alanine (0.2–10mM) are presented in Table 2.

Table 2. *Exchange of intracellular amino acids with extracellular* L-*alanine*

Transport-positive erythrocytes were incubated with various concentrations of extracellular L-[^{14}C]alanine for 6 h at 37°C, then analysed for intracellular levels of alanine (total and ^{14}C-labelled), serine, threonine and valine. Amino acid levels in cells at the start of the incubation were: alanine, 0.43 mmol/litre of cells; (serine + threonine + valine), 1.19 mmol/litre of cells. In a parallel experiment, L-[^{14}C]alanine uptake was measured in transport-negative cells. Uptake of radioactive L-alanine was 0.03 (0.2 mM), 0.06 (0.5 mM), 0.09 (1.0 mM) 0.19 (2.5 mM) 0.35 (5 mM) and 0.70 (10 mM) mmol/litre of cells after 6 h. Values are means of duplicate estimates.

		Amino acid concentration (mmol/litre of cells)	
Extracellular L-[^{14}C]alanine (mM)	L-[^{14}C]Alanine uptake (mmol/litre of cells)	Alanine	Serine + threonine + valine
0	—	0.40	0.84
0.2	0.36	0.74	0.62
0.5	0.72	1.05	0.56
1.0	1.01	1.10	0.35
2.5	1.40	1.58	0.25
5.0	1.79	1.74	0.17
10.0	2.45	2.32	0.20

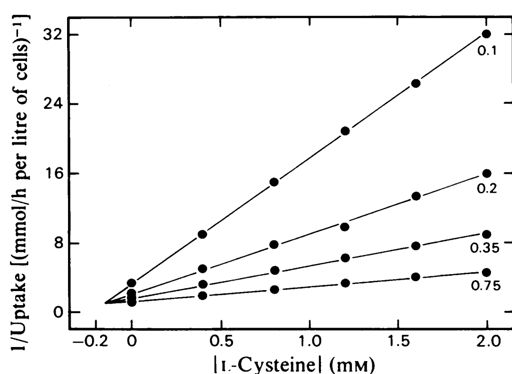

Fig. 5. *Effect of* L-*cysteine on* L-*alanine uptake by transport-positive horse erythrocytes*

The inhibitory amino acid was added to the cells at the same time as the permeant. Initial rates of L-alanine uptake were measured at 37°C. The incubations contained 10 mM-dithiothreitol to prevent L-cysteine oxidation. Values are means of triplicate estimates. The estimated apparent K_i value is 0.15 mM.

The data suggest that carrier-mediated amino acid uptake occurs largely by exchange. The net uptake of amino acid observed at high L-alanine concentrations can be attributed in part to passive L-alanine influx as judged by parallel measurements of L-[^{14}C]alanine uptake in transport-negative cells (see also Table 2). Further evidence of amino acid exchange was found when we measured extended progress curves for L-[^{14}C]alanine uptake in transport-positive erythrocytes (8 h incubation). Uptake of 0.2 mM-L-alanine reached a maximum (0.49 mmol/litre of cells) after 2 h, corresponding to a distribution ratio (intracellular concentration/extracellular concentration) of 4.2. This accumulation must have occured at the expense of intracellular amino acids. A similar overshoot was observed for 0.5 mM-L-alanine after 3 h and was followed by progressive loss of radioactivity from the cells.

Discussion

Experiments from this laboratory have previously shown that amino acid transport by normal sheep erythrocytes is mediated by system *C*, a low-affinity, Na^+-independent transporter selective for neutral amino acids of intermediate size (Young *et al.*, 1975, 1976; Young & Ellory, 1977). Erythrocytes from transport-negative type sheep lack system *C* activity, causing GSH deficiency (Tucker *et al.*, 1981; Young *et al.*, 1982). The substrate specificity of this carrier closely resembles that of system *ASC* defined by Christensen (1969, 1975) in a variety of cell types. However, system *ASC*, which has recently been shown to occur in mature human erythrocytes (Young *et al.*, 1980, 1983; Rosenberg, 1982; Al-Saleh & Wheeler, 1982), is Na^+-dependent and has a high affinity for its optimal substrates. It has been proposed that system *C* is a low-affinity *ASC* variant that has lost its Na^+-dependence (Young & Ellory, 1977). We have considered the possibility that this may occur physiologically during maturation of sheep reticulocytes (Tucker & Young, 1980).

In the present study we have shown that the substrate specificity of amino acid transport by

transport-positive horse erythrocytes is very similar to that of systems *C* and *ASC*. Thus transport-positive horse erythrocytes are most permeable to L-alanine, L-serine and L-cysteine. These amino acids, together with L-valine and L-threonine (also L-lysine), show the largest differences in uptake between transport-positive and transport-negative cells. The 20-fold difference between L- and D-alanine uptake in transport-positive horse cells demonstrates a high degree of stereoselectivity in transport. By contrast, L- and D-alanine were found to enter transport-negative horse erythrocytes at the same slow rate, suggesting the absence of carrier-mediated alanine transport in these cells. This is confirmed by the linear concentration-dependence of L-alanine uptake by transport-negative erythrocytes. The amino acid carrier present in transport-positive cells was shown to have a high affinity for L-alanine and L-cysteine (like system *ASC*), but was Na^+-independent (like system *C*). Our results further suggest that the horse erythrocyte transporter operates preferentially (but perhaps not exclusively) in an exchange mode. This is also the case for system *ASC* (Christensen, 1979), except in human erythrocytes (Al-Saleh & Wheeler, 1982).

Another link between the sheep and horse erythrocyte amino acid transporters and system *ASC* is the finding that the dibasic amino acids L-lysine and L-ornithine showed major differences in uptake between transport-positive and transport-negative horse erythrocytes, as observed previously with transport-positive and transport-negative sheep cells (Young *et al.*, 1976). In the case of sheep erythrocytes, it has been demonstrated that L-lysine uptake by transport-positive cells can be inhibited by L-alanine. The transport of dibasic amino acids by a carrier system selective for neutral amino acids is not implausible. Thus it has been shown that dibasic amino acids can act as inhibitors of the pigeon erythrocyte system *ASC* by interaction of the side-chain amino group with the Na^+-binding site (Christensen *et al.*, 1969; Thomas & Christensen, 1970, 1971; Thomas *et al.*, 1971). The sheep and horse erythrocyte transporters may have residual or modified Na^+-binding sites that permit the binding and transport of dibasic amino acids, but no longer accept or transport Na^+. The apparent K_m value for L-lysine uptake by the horse erythrocyte transporter is approx. 9mM (Fincham *et al.*, 1985*b*). As in sheep, the functional absence of carrier-mediated dibasic amino acid transport from transport-deficient horse erythrocytes results in the intracellular accumulation of high concentrations of lysine and either ornithine or arginine, depending on whether or not the cell is also arginase-deficient (Ellory *et al.*, 1972; Tucker *et al.*, 1977).

In conclusion, the present results demonstrate that transport-positive horse erythrocytes possess a novel high-affinity, Na^+-independent transporter selective for neutral amino acids of intermediate size. This carrier mechanism is functionally absent from the erythrocytes of transport-negative type horses. The properties of this transporter are intermediate between those of system *C* and system *ASC*, providing evidence that the three transporters may be variants of the same carrier system. Recent independent experiments by Vadgama & Christensen (1985) suggest the presence of a comparable Na^+-independent transporter in pigeon erythrocytes. These authors join us in proposing the provisional designation system *asc* for the new transporters. By doing so we emphasize the similarities between the horse (and pigeon) erythrocyte transporter and system *ASC*, while at the same time recognizing the convention of using lower-case letters for Na^+-independent systems (Bannai *et al.*, 1984).

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