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HETEROGENEITY OF AMINO ACID TRANSPORT IN HORSE ERYTHROCYTES: A DETAILED KINETIC ANALYSIS OF INHERITED TRANSPORT VARIATION

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SUMMARY

1. Thoroughbred horses were divisible into five distinct amino acid transport subgroups on the basis of their erythrocyte permeability to L-alanine, measured uptake rates ranging from 5 to 625 μmol l cells^{-1} h^{-1} (0·2 mM-extracellular L-alanine, 37 °C).

2. Erythrocytes from animals belonging to the lowest L-alanine permeability subgroup (5–15 μmol l cells^{-1} h^{-1}) (transport-deficient type) exhibited slow non-saturable transport of this amino acid. In contrast, cells from horses of the four transport-positive subgroups possessed additional high-affinity (apparent L-alanine K_m (Michaelis constant) $\simeq$ 0·3 mM) and/or low-affinity (apparent L-alanine $K_m \simeq$ 13 mM) Na^+-independent transport routes selective for L-neutral amino acids of intermediate size. The two transporters, designated systems asc_1 and asc_2, respectively, also possessed a significant affinity for dibasic amino acids.

3. Amino acid transport activity in horse erythrocytes behaved as if controlled by three co-dominant alleles (s, h and l), where s is a silent allele, and h and l code for the functional presence of systems asc_1 and asc_2, respectively.

4. At physiological temperature, system asc_1 operated preferentially in an exchange mode. In contrast, system asc_2 did not participate in exchange reactions at 37 °C, but did exhibit significant *trans*-acceleration at 25 °C.

5. Reduction of the incubation temperature also resulted in dramatic decreases in apparent K_m and V_{max} for L-alanine uptake by system asc_2, whereas the effects of temperature on system asc_1 were much less marked. At 5 °C the two transporters exhibited equivalent kinetic constants for L-alanine influx. L-Alanine uptake by transport-deficient cells was relatively insensitive to temperature. Influx by this route may represent the ground-state permeability of the lipid bilayer.

6. The effects of low temperature on system asc_2 suggest a preferential impairment of the mobility of the unloaded carrier relative to that of the loaded transporter. Similarly, the different kinetic properties of systems asc_1 and asc_2 at physiological temperature are attributed to a difference in the mobilities of the empty carriers, this difference being minimized at 5 °C.

INTRODUCTION

The erythrocyte has an established reputation as a convenient and readily accessible cell system for the characterization of membrane transport processes (see for example, Ellory & Lew, 1977). In the case of amino acids, kinetic studies have identified eight functionally distinct amino acid transporters in mammalian erythrocytes, each with its own characteristic species distribution, ion requirements and substrate specificity (Winter & Christensen, 1964, 1965; Gardner & Levy, 1972; Hoare, 1972*a*, *b*; Young, Ellory & Tucker, 1975, 1976; Young & Ellory, 1977*a*; Young, Jones & Ellory, 1980; Young, Wolowyk, Jones & Ellory, 1979; Rosenberg, Young & Ellory, 1980; Ellory, Jones & Young, 1981*b*; Ellory, Jones, Preston & Young, 1981*a*; Rosenberg, 1981*a*, *b*, 1982; Al-Saleh & Wheeler, 1982; Young, Wolowyk, Jones & Ellory, 1983; Inaba & Maede, 1984; Fincham, Mason & Young, 1985*a*). Many of these systems are present in a wide variety of other cell types and tissues (Christensen, 1979, 1984). In addition, the erythrocyte band 3 anion-exchange transporter has a significant affinity for glycine and some other amino acids (Young, Jones & Ellory, 1981; Ellory *et al.* 1981*b*). Physiologically, erythrocyte amino acid transporters provide the amino acid precursors (L-cysteine, L-glutamate, glycine) for intracellular glutathione (GSH) biosynthesis (Young *et al.* 1975, 1976; Young, Tucker & Kilgour, 1982; Maede, Inaba & Taniguchi, 1983; Inaba & Maede, 1984) and may also participate in the efflux of amino acids produced by protein degradation during reticulocyte maturation (Tucker & Young, 1980). It has also been suggested that amino acid transporters are responsible for the efflux of intracellular amino acids arising from erythrocyte hydrolysis of plasma peptides (King, York, Chapman & Kuchel, 1983; King & Kuchel, 1984, 1985; Vandenberg, King & Kuchel, 1985).

Two well-characterized erythrocyte amino acid transporters are of particular interest (systems ASC and C). System ASC, which is found in human (and avian) erythrocytes and rabbit reticulocytes, is a high-affinity Na^+-dependent system selective for neutral amino acids of intermediate size, including the GSH precursor L-cysteine (Thomas & Christensen, 1970, 1971; Young *et al.* 1979, 1980; Al-Saleh & Wheeler, 1982; Young *et al.* 1983). System C is found in sheep erythrocytes and has a similar substrate specificity to system ASC (Young *et al.* 1976, 1980; Young & Ellory, 1977*a*). System C, however, has a relatively low apparent affinity for its optimal substrates and is Na^+ independent. An inherited deficiency of system C in the erythrocytes of some sheep leads to GSH deficiency and the intracellular accumulation of amino acids (Ellory, Tucker & Deverson, 1972; Young *et al.* 1975, 1976). Such cells have a markedly diminished potential lifespan and an increased susceptibility to oxidative stress (Tucker, 1974; Tucker, Young & Crowley, 1981). System ASC from avian erythrocytes and rabbit reticulocytes has been shown to interact with dibasic amino acids, the positive charge on the amino acid side-chain supposedly occupying the Na^+ binding site of the transporter (Thomas & Christensen, 1970, 1971). Similarly, system C has a significant affinity for dibasic amino acids (Young *et al.* 1976, 1980; Young & Ellory, 1977*a*), lysine and ornithine (or arginine, in the case of arginase-deficient cells) accumulating to high concentrations in transport-deficient erythrocytes (Ellory *et al.* 1972; Tucker, Wright & Young, 1977). These observations have led to the suggestion that system C may be an Na^+-independent variant of system ASC (Young & Ellory, 1977*a*).

We have recently shown that horse erythrocytes are also polymorphic with respect to amino acid transport, 30 % of thoroughbred horses having erythrocytes which are amino acid transport deficient (Fincham, Young, Mason, Collins & Snow, 1985*c*). We also identified the presence of a novel amino acid transporter in the erythrocytes of some transport-positive type horses (Fincham *et al.* 1985*a*). This system (designated asc) has properties intermediate between those of systems ASC and C, resembling system ASC by having a high apparent affinity for L-cysteine and L-alanine but resembling system C by being Na^+ independent. The objective of the present investigation was to undertake an in-depth kinetic analysis of amino acid transport variation in horse erythrocytes. Data are presented which demonstrate that both high-affinity and low-affinity Na^+ independent transporters are present in erythrocytes of this species. The two systems are shown to represent allelic variants of the same transport mechanism, functional differences between the two transporters arising from differences in the mobilities of the unloaded carriers.

METHODS

Animals

Thoroughbred horses of English origin were maintained under standard husbandry conditions in the stables of the Royal Hong Kong Jockey Club. Whole blood samples were obtained by jugular venepuncture into heparinized tubes. Cells were used within 24 h of sampling.

Materials

D- and L-[U-^{14}C]-labelled amino acids and ^3H_2O were purchased from Amersham International plc, Amersham, Bucks., U.K. Non-radioactive amino acids were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. *n*-Dibutylphthalate was obtained from E. Merck, Darmstadt, F.R.G. All other reagents were of analytical grade.

Erythrocytes

Cells were prepared for transport experiments by washing three times with 20 vol. of an incubation medium containing 150 mM-NaCl, 15 mM-MOPS (morpholine propane sulphonate) and 5 mM-glucose, the pH adjusted to 7·5 (37 °C) with KOH. The buffy coat (white cells and platelets) was discarded. Cell suspensions were made to a haematocrit of approximately 20 % in incubation medium.

Haemoglobin

The haemoglobin content of cell suspensions was measured as cyanmethaemoglobin at 540 nm (1:100 dilution in 'Drabkin's' solution; 0·1 g NaCN, 0·3 g $K_3Fe(CN)_6$ l distilled water^{-1}) and converted to haematocrit using an experimentally determined value for the haemoglobin content of packed horse erythrocytes of 242 g l^{-1}.

Amino acid transport studies

Influx. To measure the time course of amino acid uptake in horse erythrocytes, equal volumes of pre-warmed (37 °C) washed cell suspension and pre-warmed incubation medium containing the appropriate concentration of radioactive permeant (1 μCi ml^{-1}) were mixed together. Iso-osmolality at various amino acid concentrations was maintained by adjusting the NaCl composition of the incubation medium. Incubations were stopped at predetermined time intervals (30 s–8 h) by transferring 0·2 ml of the cell suspension (10 % haematocrit) to an Eppendorf microcentrifuge tube (volume 1·5 ml) containing 0·8 ml of ice-cold incubation medium layered on top of 0·5 ml ice-cold *n*-dibutylphthalate. The tube was immediately centrifuged at 15000 *g* for 10 s using an Eppendorf 5414 microcentrifuge. The aqueous medium and *n*-dibutylphthalate layers were removed by suction, leaving the cell pellet at the bottom of the tube. After carefully wiping the inside of the centrifuge tube with tissue paper, the cell pellet was lysed with 0·5 ml 0·5 % (v/v) Triton X-100 in water and 0·5 ml 5 % (w/v) trichloroacetic acid was added. The precipitate was removed by

centrifugation (2 min, 15000 *g*) and 0·9 ml of the supernatant was counted for radioactivity in an LKB Rackbeta Scintillation Counter with quench correction. Blank values were obtained by processing cell samples which had been mixed with radioactive permeant at 0 °C and immediately centrifuged. Uptake values were calculated after subtraction of these blanks.

In experiments where initial rates of amino acid uptake were determined, an alternative method of separating cells from extracellular medium was used. Here, equal volumes (0·2 ml) of pre-cooled (ice-cold) cell suspension and radioactive amino acid solution were mixed in microcentrifuge tubes and the incubations initiated by transferring the tubes into a water-bath at 37 °C. Incubations (15 min) were stopped by transferring the tubes back into the ice-bath for a further 15 min. The cells were rapidly washed four times with 1 ml portions of ice-cold incubation medium (10 s, 15000 *g*). The washed cell pellets were then treated as above. For erythrocytes from some horses (groups 4 and 5, Figs. 1 and 2) it was necessary to use a 5 min incubation period to measure initial rates of transport. In these cases, pre-warmed cells (0·2 ml) were mixed with an equal volume of pre-warmed amino acid solution and the incubation terminated by the addition of 1 ml ice-cold incubation medium. The cell pellets were washed and deproteinized as described above.

Efflux. Washed erythrocytes were incubated at a haematocrit of 30% for 2–4 h at 37 °C in incubation medium containing 0·2–40 mM-L-[U-^{14}C]alanine. The cells were rapidly washed (6 × 10 vol. of ice-cold incubation medium) by centrifugation and resuspended in ice-cold medium to a haematocrit of 20%. L-Alanine efflux at 37 or 25 °C was measured by mixing 0·5 ml of cell suspension with 4·5 ml of pre-warmed incubation medium containing the appropriate non-radioactive amino acid. At predetermined time intervals (2·5, 5, 10 and 15 min), 1 ml portions of cell suspension (final haematocrit approximately 2%) were removed into ice-cold microcentrifuge tubes containing 0·2 ml ice-cold *n*-dibutylphthalate and the cells immediately sedimented below the oil by centrifugation at 15000 *g* for 20 s. A sample (0·7 ml) of supernatant was removed for scintillation counting. The initial intracellular L-[^{14}C]alanine concentration was measured by processing an aliquot of the 20% 'loaded' cell suspension as described for L-alanine influx. Efflux rate constants were estimated by fitting straight lines to graphs of log [1 − (supernatant counts/total counts)] against time. Efflux rates (mmol l cells^{-1} h^{-1}) were obtained by multiplying the efflux rate constant (h^{-1}) by the intracellular amino acid concentration.

In control experiments, samples of efflux medium from group 5 cells (15 min incubation) were subjected to thin-layer chromatography (Silica gel G, ethanol/water 7:3) to determine the integrity of the ^{14}C-labelled alanine during efflux experiments. The ^{14}C-tracer was found to run in an equivalent position to non-radioactive L-alanine.

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.

Computational analysis of influx data

The maximum likelihood method (the program MLP of G. J. S. Ross, copyright 1978, Lawes Agricultural Trust, Rothamstead Experimental Research Station, Harpenden, U.K.), was used initially to test three different models of amino acid uptake by transport-positive cells.

(i) One saturable uptake system plus diffusive entry

$$v = \frac{V_{max}[S]}{[S]+K_m} + K_D[S].$$

(ii) Two saturable uptake systems, no diffusion

$$v = \frac{V_{max,1}[S]}{[S]+K_{m,1}} + \frac{V_{max,2}[S]}{[S]+K_{m,2}}.$$

(iii) Two saturable uptake systems plus diffusive entry

$$v = \frac{V_{max,1}[S]}{[S]+K_{m,1}} + \frac{V_{max,2}[S]}{[S]+K_{m,2}} + K_D[S].$$

For each model, the curve-fitting program estimated values for K_m (Michaelis constant), V_{max} and K_D (apparent diffusion constant). [S] represents substrate concentration. A FORTRAN 77

subroutine was used to calculate the uptake ($\hat{v}$) to fit a given model at each of the experimental substrate concentrations. The logarithmic sum of squares was derived from the calculated ($\hat{v}$) and the measured (v) uptake values for each substrate concentration, thus

$$\Sigma(\ln(v) - \ln(\hat{v}))^2,$$

and then minimized by the subroutines E04HBF and E04JBF of the NAG subroutine library (NAG MK10, Numerical Algorithms Group, Oxford, U.K.) to obtain the best fit. The kinetic constants reported in this paper were obtained using E04JBF, which allows constrained minimization, and the parameters were subject to a lower bound of zero and an upper bound of 0·1 for K_D and 100 for K_m and V_{max}. No standard errors could be obtained if the minimization terminated while a parameter was at a boundary value. With a normal termination, a variance–covariance matrix for the parameters was obtained from the residual variance and the inverse of the Hessian matrix (provided by E04JBF).

For a given data set, a comparison of the fit obtained with different models was made by usual analysis of variance.

RESULTS

Amino acid permeability of horse erythrocytes

Distribution of L-alanine transport activity. Fig. 1 shows the initial rates of L-alanine uptake (0·2 mM, 37 °C) by erythrocytes from 100 thoroughbred horses. Transport rates ranged from 5 to 625 μmol l cells^{-1} h^{-1}, animals falling into five distinct groups on the basis of their erythrocyte permeability to amino acid. Thus, L-alanine transport activities in the upper panel (*A*) of Fig. 1 were divisible into three separate subgroups: 5–15 (group 1), 25–55 (group 2) and 70–110 (group 3) μmol l cells^{-1} h^{-1}, representing 26, 29 and 26 % of the animals tested, respectively. The distribution of animals with erythrocytes in the high-permeability range (lower panel (*B*) of Fig. 1) suggests two further subgroups: 305–430 (group 4) and 485–625 (group 5) μmol l cells^{-1} h^{-1}, accounting for 12 and 7 % of the animals tested, respectively.

The permeability characteristics of erythrocytes from individual animals representative of each of the five groups remained constant during repeated analyses over a period of 3 years. No relationship was apparent between erythrocyte amino acid transport activity and animal age or sex. Inheritance data (D. A. Fincham, D. K. Mason & J. D. Young, unpublished observations) confirm that the transport variation is under genetic control (see Discussion). The horses with erythrocyte L-alanine permeabilities indicated by the open symbols in Fig. 1 were selected for further detailed kinetic analysis.

Time course of L-alanine uptake. Typical time courses of L-alanine uptake (0·2 mM, 37 °C) by erythrocytes from animals representative of each of the five subgroups categorized in the previous section are shown in Fig. 2.

Uptake in group 1 cells was slow and linear with time. In contrast, group 4 and 5 cells showed rapid transport, intracellular L-[^{14}C]alanine levels reaching apparent steady states in excess of equilibration values within the experimental period of 3 h. Assuming that horse erythrocytes contain 70 % water by volume (see below), all of which is available to amino acid, the theoretical equilibration value would be 0·14 mmol amino acid l cells^{-1} at 0·2 mM-extracellular L-alanine. From the data in Fig. 2 it can be seen that intracellular L-alanine in group 4 and 5 cells reached levels of 0·31 and 0·69 mmol l cell water^{-1}, representing concentration ratios of 1·6 and 3·5

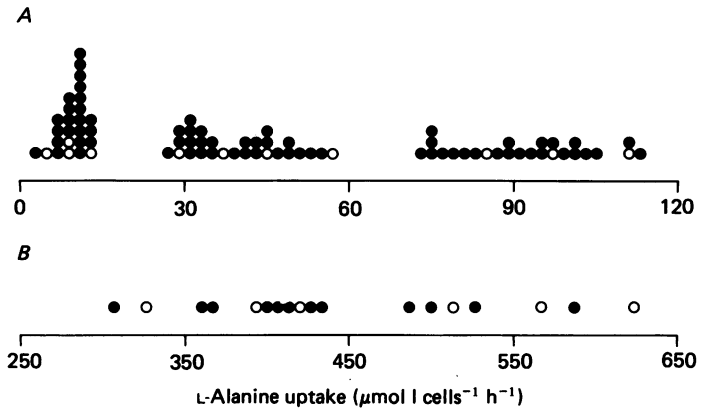

Fig. 1. Distribution of L-alanine transport activity in thoroughbred horse erythrocytes. Initial rates of L-[U-^{14}C]alanine influx (0·2 mM, 37 °C) were determined as described in the text. Each point (●) represents the amino acid permeability of cells from an individual animal. Open symbols (○) represent animals chosen for detailed kinetic analysis.

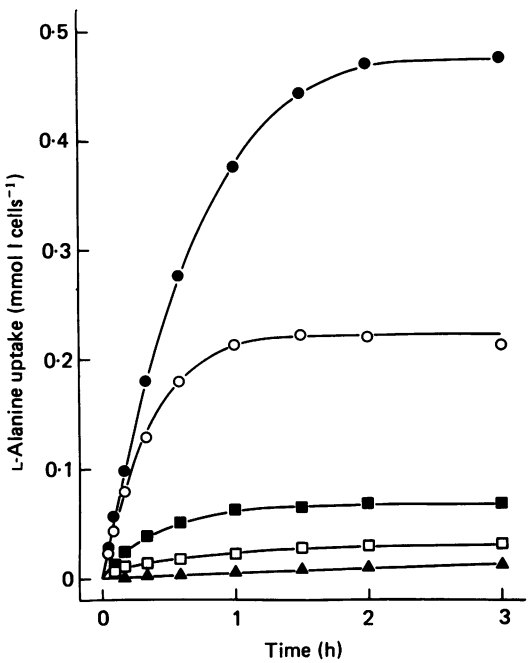

Fig. 2. Time course of L-alanine uptake by transport-deficient and transport-positive horse erythrocytes. The L-alanine concentration was 0·2 mM, 37 °C. Uptake measured in either NaCl or KCl media was found to co-plot for each of the subgroups 1 to 5. ▲, group 1 cells; □, group 2 cells; ■, group 3 cells; ○, group 4 cells; ●, group 5 cells.

above equilibration, respectively. In contrast, L-alanine uptake by cells from the intermediate transport-positive groups 2 and 3 reached plateaux below the level expected for equilibration with cell water (0·04 and 0·10 mmol l cell water^{-1}, representing distribution ratios significantly below one, of 0·2 and 0·5, respectively). No obvious difference in erythrocyte water space was observed between the different

subgroups. Mean values for two animals of each type were 67·6, 68·4, 69·9, 70·6 and 70·0 % (v/v) for groups 1–5, respectively, measured using ^3H_2O. Removal of Na^+ from the incubation medium (using K^+ as the Na^+ substitute) had no effect on the time courses of uptake of L-alanine in cells from the different subgroups (see also Fincham *et al.* 1985*a*).

Figs. 3–5 show prolonged time courses (up to 8 h, 37 °C) for L-alanine uptake by cells from groups 1, 3 and 5 using different concentrations of extracellular L-alanine in the range 0·2 to 10 mM. For group 5 cells (Fig. 3) it can be seen that the apparent

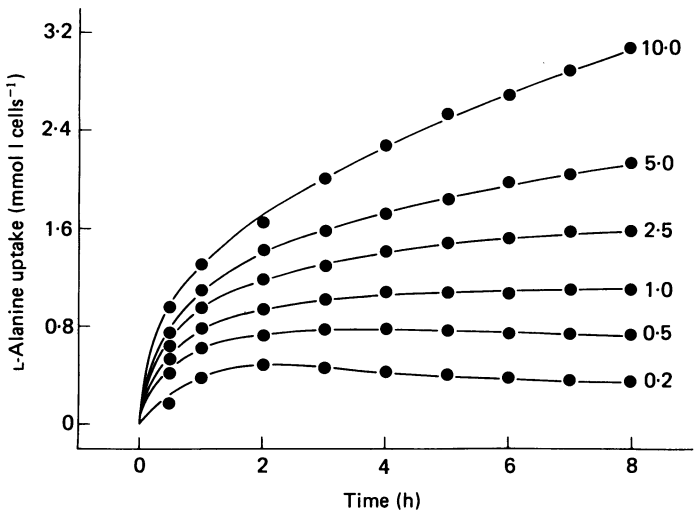

Fig. 3. Time course of uptake of various concentrations of L-alanine by transport-positive (group 5) horse erythrocytes. Cells were incubated over a period of 8 h at 37 °C with L-[U-^{14}C]alanine (0·2–10 mM extracellular concentration, indicated to right of Figure). See text for other experimental details.

accumulation of L-alanine inside the cells is only observed at low extracellular L-alanine concentrations and that it represents an overshoot phenomenon. At initial extracellular L-alanine concentrations of 0·2 and 0·5 mM, maximum intracellular values were reached at 2 and 3 h, respectively. Following these peaks, there was a progressive loss of radioactive L-alanine from the cells. Increasing the extracellular L-alanine concentration (1 and 2·5 mM) resulted in hyperbolic progress curves approaching expected equilibration values. Higher extracellular L-alanine concentrations (5 and 10 mM) produced biphasic progress curves, with an initial rapid component of influx followed by a slower uptake phase. A similar experiment with group 3 erythrocytes (Fig. 4) shows that the partial-equilibration phenomenon observed in these cells at low L-alanine concentrations (0·2 and 0·5 mM) progressively disappears as the extracellular L-alanine concentration is increased, until normal equilibration values are approached at 5 and 10 mM-external amino acid. In group 1 cells, uptake was linear with time at each of the concentrations studied (Fig. 5). The magnitude of this influx at 10 mM-extracellular L-alanine was approximately the same as that of the slow linear component of L-alanine uptake observed in group 5 cells at this concentration.

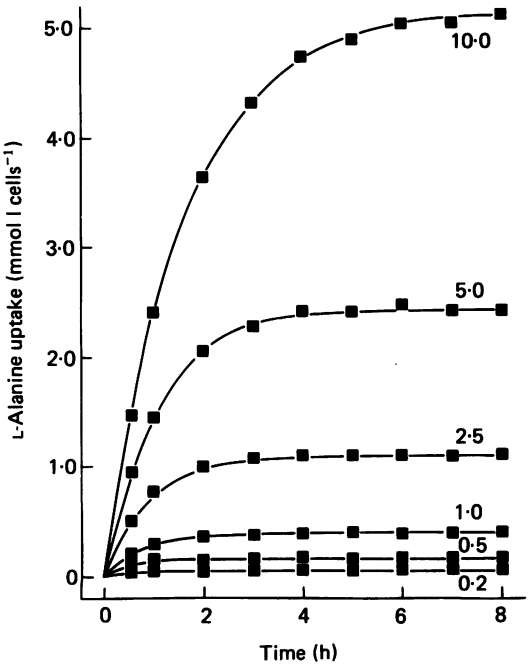

Fig. 4. Time course of uptake of various concentrations of L-alanine by transport-positive (group 3) horse erythrocytes. Cells were incubated over a period of 8 h at 37 °C with L-[U-^{14}C]alanine (0·2–10 mM extracellular concentration, indicated to right of Figure). Intracellular L-[^{14}C]alanine levels were determined by liquid scintillation counting as described in Methods. In a parallel experiment, samples of erythrocytes were subjected to analysis on an LKB 4400 amino acid analyser to determine intracellular levels of alanine (total), serine, threonine and valine. Amino acid levels at the start of the incubation were, alanine 0·12 mmol l cells^{-1} and serine + threonine + valine 0·40 mmol l cells^{-1}. After 6 h, measured intracellular alanine and serine + threonine + valine levels (mmol l cells^{-1}) were 0·14 and 0·13 (0·2 mM-extracellular L-[^{14}C]alanine), 0·26 and 0·13 (0·5 mM), 0·52 and 0·13 (1·0 mM), 1·1 and 0·13 (2·5 mM), 2·7 and 0·13 (5 mM) and 4·8 and 0·13 (10 mM), respectively. These values compare with intracellular alanine and serine + threonine + valine levels of 0·10 and 0·12 mmol l cells^{-1} after 6 h in cells incubated in the absence of extracellular L-[^{14}C]alanine.

Specificity of amino acid uptake. To investigate the substrate specificity of amino acid uptake by horse erythrocytes, initial rates of uptake of twenty-two naturally occurring amino acids were measured (0·2 mM, 37 °C) in cells of three animals from each of the five groups described in the preceding sections. The results are summarized in Table 1. The first column of Table 1 presents data for group 1 cells. The low permeability to L-alanine previously shown by these cells was confirmed. Furthermore, there was no indication of stereoselectivity between the L- and D-isomers of this amino acid. Other neutral amino acids which also exhibited low uptake rates were L-serine > L-proline > L-threonine. Increasing the size of the neutral amino acid side chain resulted in an increase in amino acid permeability. This is reflected in the uptake of L-valine < L-methionine < L-isoleucine < L-leucine < L-phenylalanine, the latter having the highest permeability of all the amino acids tested in group 1 cells. Uptake

rates for L-tryptophan and L-tyrosine were lower than for L-valine. Uptake of glycine was also low, but significantly higher than L-alanine, with L-cysteine entering twice as fast as glycine. Other amino acids to exhibit very low permeabilities were the dibasic amino acids L-lysine < L-arginine < L-ornithine < L-histidine. L-Glutamine transport was also slow but twice that of L-asparagine, while the acidic amino acids L-aspartate and L-glutamate showed the lowest permeabilities.

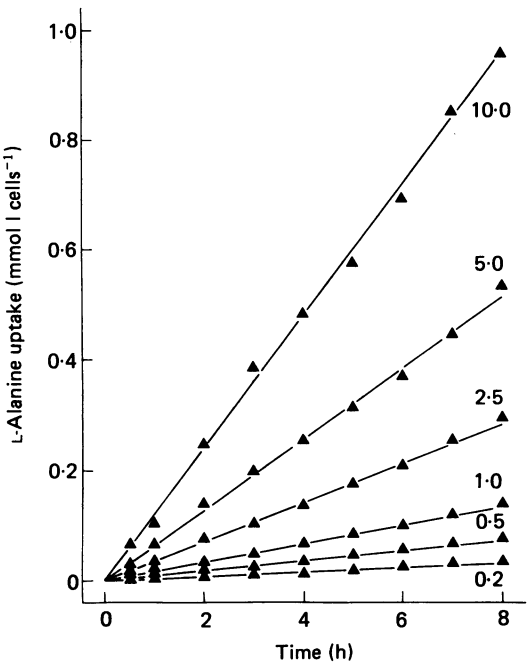

Fig. 5. Time course of uptake of various concentrations of L-alanine by transport-deficient (group 1) horse erythrocytes. Cells were incubated over a period of 8 h at 37 °C with L-[U-^{14}C]alanine (0·2–10 mM extracellular concentration, indicated to the right of the Figure). See text for other experimental details.

In contrast, the cells most permeable to L-alanine (group 5, last column in Table 1) showed a 19·7-fold selectivity for the L- over the D-isomer. Unlike group 1 erythrocytes, these cells also rapidly transported other neutral amino acids of intermediate size (L-cysteine > L-serine > L-valine > L-threonine). Larger neutral amino acids showed uptake rates in these cells comparable to group 1 erythrocytes with L-phenylalanine > L-leucine > L-isoleucine > L-methionine > L-tryptophan > L-tyrosine. Glycine and L-asparagine transport were 1·5 and 2·0 times higher in group 5 cells compared to group 1 cells. Amino acids showing similar very slow uptake rates in the two cell types were L-proline, L-glutamine, L-glutamate and L-aspartate. An interesting feature of the data presented in Table 1 is the difference in uptake of dibasic amino acids between group 1 and group 5 cells. The latter have a significant permeability to L-lysine > L-ornithine > L-histidine > L-arginine compared to group 1 cells. These results suggest that group 5 erythrocytes possess stereospecific

TABLE 1. Substrate specificity of amino acid uptake by horse erythrocytes

Amino acid	Transport subgroup				
	Group 1	Group 2	Group 3	Group 4	Group 5
Glycine	37±2	33±4	45±7	47±9	56±3
L-Proline	8±0·3	8±0·1	8±0·1	9±0·3	9±0·5
D-Alanine	11±0·5	21±5	29±1	40±2	31±2
L-Alanine	8±1	42±6	88±12	368±33	612±7
L-Serine	12±0·1	56±8	116±10	486±38	694±13
L-Cysteine	72±8	106±14	192±20	500±28	744±45
L-Valine	30±2	60±4	100±6	386±43	529±8
L-Threonine	5±0·3	12±3	21±1	115±13	130±2
L-Methionine	35±2	33±1	32±1	32±1	36±2
L-Isoleucine	67±4	77±5	72±4	81±6	83±5
L-Leucine	86±3	89±7	88±8	99±11	98±8
L-Phenylalanine	162±10	170±11	154±9	174±16	183±9
L-Tryptophan	24±2	28±2	25±2	29±0·6	26±1
L-Tyrosine	5±0·1	7±0·6	11±0·6	23±3	23±0·6
L-Lysine	5±1	9±1	18±2	64±9	60±2
L-Ornithine	9±2	9±0·3	12±0·6	24±3	24±2
L-Histidine	10±0·6	10±0·6	12±0·3	18±1	16±0·6
L-Arginine	6±1	8±2	8±0·6	13±2	10±1
L-Asparagine	4±0·3	5±2	6±0·6	10±2	8±0·3
L-Glutamine	10±0·3	12±4	13±2	17±3	8±1
L-Aspartate	4±0·3	5±0·6	5±0·6	6±0·3	4±0·3
L-Glutamate	3±0·1	5±0·3	6±0·3	5±0·6	3±0·1

Initial rates of amino acid uptake at 37 °C were determined in cells from each of the five transport subgroups identified in Fig. 1 at an initial extracellular concentration of 0·2 mM. Data are mean values (±S.E of mean) for three animals of each transport type. Transport rates are expressed as μmol l cells^{-1} h^{-1}.

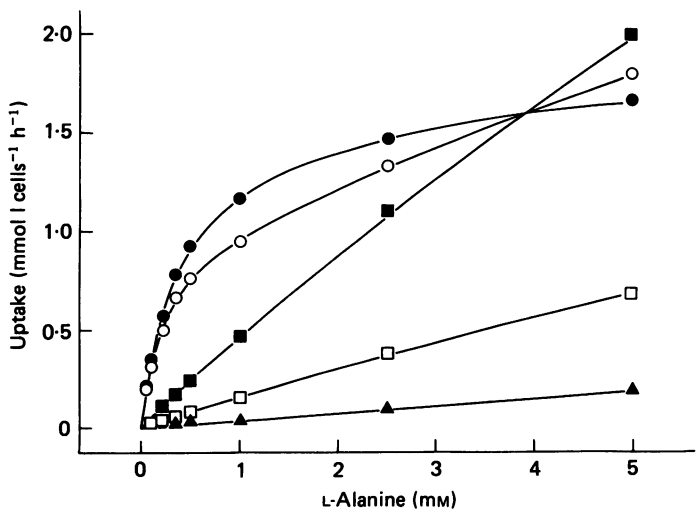

Fig. 6. Concentration dependence of L-alanine uptake (low-concentration range) by transport-deficient and transport-positive horse erythrocytes. Initial rates of L-alanine uptake (0·05–5 mM extracellular concentration) were measured at 37 °C in NaCl medium. ▲, group 1 cells; □, group 2 cells; ■, group 3 cells; ○, group 4 cells; ●, group 5 cells.

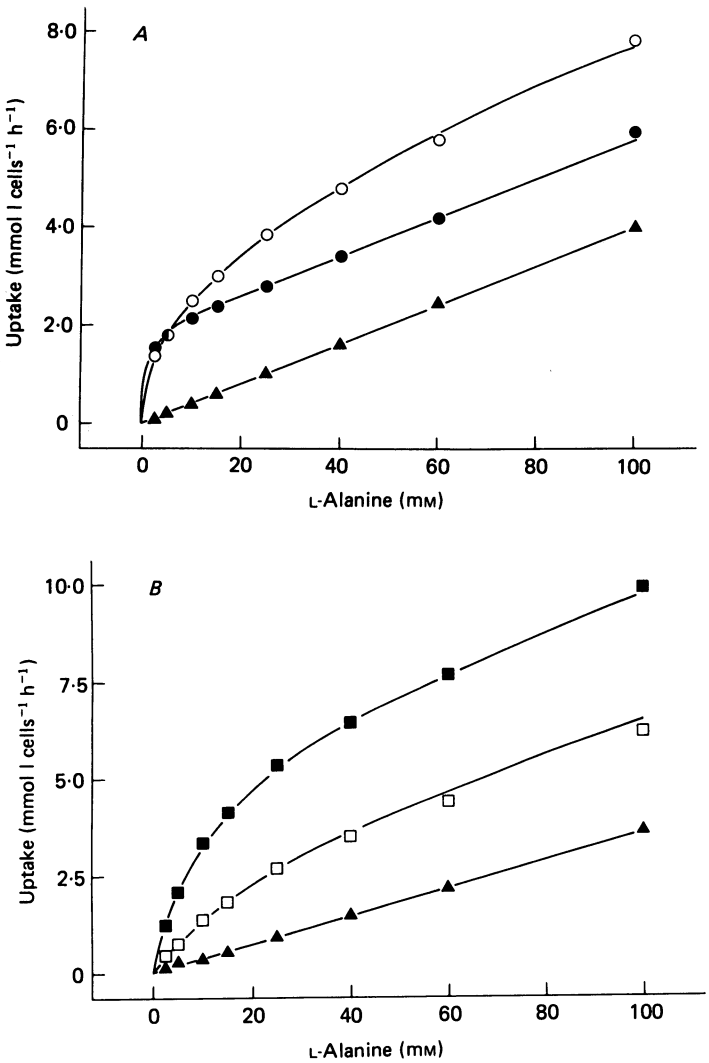

Fig. 7. Concentration dependence of L-alanine uptake (high-concentration range) by transport-deficient and transport-positive horse erythrocytes. Initial rates of L-alanine uptake (2·5–100 mM) were measured at 37 °C in NaCl medium. *A*, ▲, group 1 cells; ○, group 4 cells; ●, group 5 cells. *B*, ▲, group 1 cells; □, group 2 cells; ■, group 3 cells.

transport activity selective for neutral amino acids of intermediate size (and with a significant affinity for dibasic amino acids), the system being functionally absent from group 1 cells.

The intermediate transport-positive subgroups 2, 3 and 4 all showed similar amino acid permeability profiles to group 5 cells, the difference between the subgroups being in the rates of mediated transport (group 5 > group 4 > group 3 > group 2).

Kinetics of amino acid transport in horse erythrocytes

Concentration dependence of L-*alanine influx.* Figs. 6 and 7 compare the concentration dependence of L-alanine uptake by erythrocytes representative of the five amino acid transport subgroups over extracellular L-alanine concentration ranges of 0·05–5 and 2·5–100 mM. The results suggest the presence of three separate components of L-alanine transport in horse erythrocytes: (a) a non-saturable component present in all cell types, (b) a high-affinity saturable transport route present in group 4 and group 5 cells (apparent $K_m \simeq 0{\cdot}3$ mM) and (c) a low-affinity saturable route present in group 2, 3 and 4 erythrocytes (apparent $K_m \simeq 15$ mM).

TABLE 2. Kinetic constants for L-alanine uptake by horse erythrocytes

Horse	Amino acid transport subgroup	Presumed genotype	Uptake at 0·2 mM-L-alanine: Measured (μmol l cells^{-1} h^{-1})	Uptake at 0·2 mM-L-alanine: Calculated (μmol l cells^{-1} h^{-1})	Kinetic parameters: $K_{m,l}$ (mM)	$K_{m,h}$ (mM)	$V_{max,l}$ (mmol l cells^{-1} h^{-1})	$V_{max,h}$ (mmol l cells^{-1} h^{-1})	K_D (h^{-1})
S90	1	s, s	8	6	—	—	—	—	0·0306
S134	1	s, s	8	6	—	—	—	—	0·0324
S138	1	s, s	9	7	—	—	—	—	0·0351
S153	1	s, s	10	8	—	—	—	—	0·0417
T78	2	l, s	31	37	20·21	—	2·91	—	0·0380
T139	2	l, s	36	33	16·13	—	2·17	—	0·0336
M184	2	l, s	39	38	7·69	—	1·30	—	0·0241
T63	2	l, s	54	61	11·48	—	3·16	—	0·0331
S160	3	l, l	85	106	13·82	—	6·90	—	0·0360
T72	3	l, l	96	106	12·63	—	6·50	—	0·0287
N120	3	l, l	110	107	13·68	—	6·96	—	0·0305
T62	4	h, l	317	327	8·27	0·153	3·64	0·415	0·0311
P132	4	h, l	400	388	15·71	0·225	1·98	0·757	0·0325
L123	4	h, l	421	494	14·97	0·206	2·91	0·908	0·0434
T80	5	h, s	520	486	—	0·360	—	1·36	0·0256
P117	5	h, s	563	565	—	0·377	—	1·61	0·0448
M173	5	h, s	625	618	—	0·418	—	1·89	0·0210

The kinetic constants K_m and V_{max} (37 °C) are shown with subscripts l and h, referring to the low-affinity saturable and high-affinity saturable components of transport, respectively. Non-saturable uptake is represented by K_D. Standard errors on individual K_m, V_{max} and K_D estimates were typically < 8 %. Values for initial rates of L-alanine uptake at 0·2 mM extracellular concentration were either measured directly or calculated from the kinetic constants. See text for other experimental details and genotype classification.

Table 2 summarizes the computed kinetic parameters for L-alanine transport in erythrocytes of the seventeen horses chosen for detailed study as indicated by the open symbols in Fig. 1. The animals were ranked according to their erythrocyte L-alanine uptake rates measured at 0·2 mM-extracellular amino acid (37 °C) and comprised four individuals from amino acid transport group 1, four from group 2 and three each from groups 3, 4 and 5. The concentration dependence of L-alanine uptake by erythrocytes from each animal was measured over high- and low-concentration ranges as shown in Figs. 6 and 7. The data for the thirteen transport-positive-type horses were fitted with functions containing either three, four or five parameters

(models i, ii or iii, respectively) as detailed in Methods. A given model was accepted or rejected based on analysis of variance of the change in residual sum of squares (Δ r.s.s.) upon changing from the three- to the four- to the five-parameter model. In no case did the four-parameter model (ii, with no allowance for the parameter K_D) give as good a fit as i or iii. This may be taken as substantial evidence for the presence of non-mediated uptake in each of the transport-positive cell types. The three-parameter model (i) was accepted when Δ r.s.s. upon changing to the five-parameter model (iii) was not significant, while the latter was taken as the best fit if Δ r.s.s. was significant.

In amino acid transport group 1 cells (transport-deficient) the only detectable component of uptake was the non-saturable route. Analysis (by Rankit plot; Bliss, 1967; Atkins, 1976) of the values for K_D estimated in the most appropriate model (i or iii) for each transport-positive-type horse and for the four transport-deficient animals (K_D measured by linear regression through the origin) revealed K_D to be normally distributed about a mean value of $0{\cdot}0318 \pm 0{\cdot}0020$ (S.E of mean, $n = 17$) h^{-1} i.e. $0{\cdot}0455 \pm 0{\cdot}003$ h^{-1} (l water l cells^{-1}). The different amino acid transport subgroups were evenly distributed in the range of K_D.

In both group 2 and group 3 cells, the most appropriate model for the data was the three-parameter model. The saturable component present in these cell types had a low affinity for L-alanine, the estimated apparent K_m values for erythrocytes from different horses exhibiting close similarity. This component of uptake was also detected in erythrocytes from amino acid transport subgroup 4. Again, the apparent K_m values in this cell type were in the range observed for group 2 and group 3 cells. Analysis of the data (by Rankit plot) revealed the apparent K_m of this transport in subgroups 2, 3 and 4 cells to be normally distributed around a mean value of $13{\cdot}2 \pm 1{\cdot}2$ mM ($n = 10$). In addition to the low-affinity saturable transport route, group 4 cells were found to possess a high-affinity saturable transport component. Thus, the five-parameter model was found to provide the best fit to the data. In contrast, group 5 cells were fitted best by the three-parameter model. The single saturable route identified in these erythrocytes also had a high affinity for L-alanine. Mean apparent K_m values for the high-affinity transport route in group 4 and group 5 cells were found to be $0{\cdot}195 \pm 0{\cdot}021$ ($n = 3$) and $0{\cdot}385 \pm 0{\cdot}017$ ($n = 3$) mM, respectively, approximately 50-fold lower than the apparent K_m of the low-affinity transport component. Thus, the use of curve-fitting procedures confirmed the existence of three separate transport routes for L-alanine in horse erythrocytes.

In contrast to the constancy in values for apparent K_m observed for the low-affinity transport system, significant differences were apparent in V_{max} among the different transport subgroups. Thus, the mean V_{max} of the low-affinity transporter in group 2 cells was 35 % of the mean V_{max} for group 3 cells. The latter was also 2·4-fold higher than V_{max} for the low-affinity saturable component of transport in group 4 cells. Rankit analysis of V_{max} for the low-affinity transporter in group 2 and group 4 cells showed V_{max} to be normally distributed about a mean value of $2{\cdot}59 \pm 0{\cdot}30$ mmol l cells^{-1} h^{-1} ($n = 7$). In contrast, a mean V_{max} of $6{\cdot}79 \pm 0{\cdot}14$ mmol l cells^{-1} h^{-1} ($n = 3$) was calculated for group 3 cells. Differences in V_{max} were also apparent for the high-affinity saturable component of transport. In group 5 cells the mean V_{max} value was 2·4-fold higher than that for the high-affinity component identified in group 4

cells ($1{\cdot}62 \pm 0{\cdot}15$ ($n = 3$) and $0{\cdot}69 \pm 0{\cdot}14$ ($n = 3$) mmol l cells^{-1} h^{-1}, respectively) (Table 2).

Stereospecificity of alanine uptake. The ability of transport-positive horse erythrocytes to discriminate between L- and D-alanine (Table 1) was further investigated by measuring the kinetics of D-alanine influx in horse erythrocytes of transport groups 1, 3 and 5 over the concentration range 2·5–100 mM. Calculated apparent K_m values for D-alanine transport in group 5 and group 3 cells (corrected for uptake by group 1 cells) were 30 mM (V_{max} 0·43 mmol l cells^{-1} h^{-1}) and > 100 mM, respectively. As detailed in the preceding section, apparent K_m values for saturable L-alanine transport in these cells were in the region of 0·3 and 13 mM, respectively.

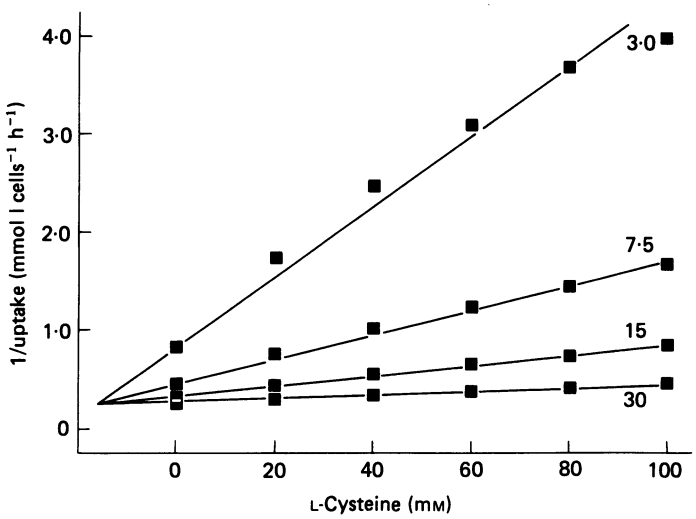

Fig. 8. Effect of L-cysteine on L-alanine uptake by transport-positive (group 3) horse erythrocytes. Initial rates of L-alanine uptake (3–30 mM extracellular concentration, indicated to the right of Figure) were measured at 37 °C. The inhibitory amino acid was added to the cells at the same time as the permeant. Incubations contained 10 mM-dithiothreitol to prevent L-cysteine oxidation. The estimated apparent K_I value is 16 mM.

Competitive inhibition of L-alanine uptake. Previous experiments have established that the high-affinity saturable component of L-alanine uptake in horse erythrocytes is competitively inhibited by L-cysteine with an apparent K_I (inhibition constant) value of 0·15 mM (Fincham *et al.* 1985*a*). To test whether these two amino acids share the low-affinity saturable system in horse erythrocytes, the uptake of L-alanine (3–30 mM) by group 3 cells was measured in the presence of varying concentrations of L-cysteine (20–100 mM). L-Cysteine was found to be an effective inhibitor of L-alanine transport by this system. Dixon (1953) analysis of the data (Fig. 8) confirmed that inhibition was competitive with an apparent K_I value of 16 mM. Concentration dependence studies with L-cysteine in group 3 and group 1 cells over the range 2·5–100 mM-extracellular amino acid estimated the apparent K_m for L-cysteine influx by the low-affinity transporter to be 16 mM (data not shown). 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.

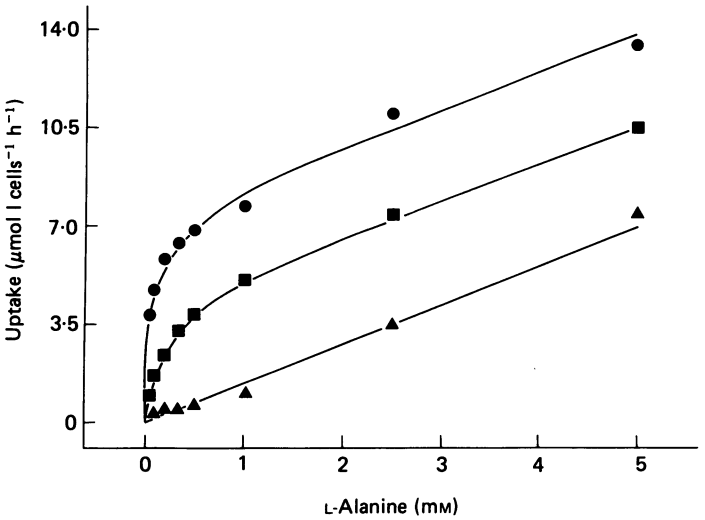

Fig. 9. Concentration dependence of L-alanine uptake by transport-deficient and transport-positive horse erythrocytes at 5 °C. Initial rates of L-alanine uptake were measured in NaCl medium. ▲, group 1 cells; ■, group 3 cells; ●, group 5 cells.

TABLE 3. Effects of temperature on the kinetic constants of L-alanine uptake by horse erythrocytes

	Kinetic parameters				
	High-affinity system		Low-affinity system		
Temperature (°C)	K_m (mM)	V_{max} (mmol l cells^{-1} h^{-1})	K_m (mM)	V_{max} (mmol l cells^{-1} h^{-1})	K_D (h^{-1})
37	0·35	1·69	14	6·90	0·036
25	0·25	0·35	5	1·25	0·0088
5	0·10	0·008	0·2	0·004	0·0015

Kinetic parameters for the high-affinity and low-affinity transporters were determined using group 5 and group 3 cells, respectively. K_D determinations were made using group 1 erythrocytes. See text for other experimental details.

Temperature dependence of L-alanine influx. Marked temperature-dependent changes in the kinetic parameters of carrier-mediated L-alanine transport have been observed in sheep erythrocytes (Young *et al.* 1976) and in human red blood cells (Young *et al.* 1983). Fig. 9 shows the concentration dependence of L-alanine uptake by group 1, 3 and 5 cells at 5 °C, while Table 3 summarizes kinetic constants for L-alanine transport at 37, 25 and 5 °C. Decreasing the incubation temperature from 37 to 25 °C resulted in comparable decreases in the apparent K_m and V_{max} values of the two saturable uptake mechanisms in horse erythrocytes (K_m ratios 1·4 and 2·8; V_{max} ratios 4·8 and 5·5 for the high-affinity and low-affinity transporters, respectively). However, over the temperature range 25–5 °C the two systems responded differently. Thus, for the high-affinity transporter K_m and V_{max} ratios were 2·5 and 43·8, respectively, whereas for the low-affinity system the changes in the kinetic

constants were greater (K_m ratio 25; V_{max} ratio 313). It is clear, therefore, that the low-affinity transporter is considerably more sensitive to temperature than the high-affinity system, and that the large apparent difference in K_m between the systems at 37 °C is not observed at low temperature (Fig. 9). The temperature sensitivity of non-saturable L-alanine transport in group 1 cells (Table 3) was comparable to changes in V_{max} for the two saturable systems over the range 37–25 °C (K_D ratio 4·1). In contrast, this component of transport was relatively insensitive to changes in incubation temperature from 25 to 5 °C compared to the saturable systems (K_D ratio 5·9).

Concentration dependence of L-leucine influx. The relatively high uptake of L-leucine and L-phenylalanine by transport-deficient (group 1) cells compared with D- and L-alanine (Table 1) is consistent with the ground-state permeability of lipid bilayers to these amino acids (Young & Ellory, 1977*b*), but might also indicate the presence of transport system L activity in the cells (Young *et al.* 1980). Concentration dependence studies over an L-leucine concentration range 0·05–50 mM showed uptake that was non-saturable in group 1, 3 and 5 erythrocytes (data not shown). Thus, no kinetic evidence was found for a separate transporter selective for large neutral amino acids in horse erythrocytes.

L-Alanine efflux

Previous experiments (Fincham *et al.* 1985*a*) have provided evidence that the high-affinity amino acid transporter in horse erythrocytes operates preferentially in an exchange mode at 37 °C. In contrast, no evidence of exchange by the low-affinity transporter was found when L-[U-^{14}C]alanine influx in group 3 erythrocytes was correlated quantitatively with intracellular levels of alanine, serine, threonine and valine (see legend to Fig. 4). These latter amino acids are all substrates of the low-affinity system (Table 1). L-[U-^{14}C]Alanine efflux experiments were undertaken to study the contrasting exchange behaviour of the two systems in more detail.

Fig. 10*A* and *B* shows the time courses of L-alanine efflux (37 °C) from cells of amino acid transport subgroups 5 and 3, respectively. In both cases, plots of log [intracellular alanine] *versus* time were linear during the experimental period of 40 min. Also apparent from Fig. 10*A* is the ability of external non-radioactive L-alanine but not D-alanine (0·3 mM extracellular concentration) to stimulate radiolabelled L-alanine efflux from group 5 cells. In contrast, extracellular L-alanine (15 mM) did not stimulate tracer efflux from group 3 cells (Fig. 10*B*). In another experiment, L-alanine efflux was measured in the presence of extracellular L-, and D-alanine concentrations of 0·25–10 mM for group 5 cells and L-alanine concentrations of 2·5–100 mM for group 3 cells (Fig. 11). With the former cell type, stimulation of L-[U-^{14}C] alanine efflux by extracellular L-alanine was saturable, the maximum stimulation under these experimental conditions representing a 4·75-fold increase in efflux rate over the control value (efflux in the absence of extracellular L-alanine). The external L-alanine concentration required to give half-maximal stimulation of efflux was approximately 0·5 mM. High concentrations of extracellular D-alanine were also able to stimulate L-alanine efflux. However, stimulation was linear with respect to extracellular D-alanine concentration and less than that given by the L-isomer (3·25-fold increase at 10 mM-extracellular D-alanine). In group 3 cells, extracellular L-alanine concen-

trations as high as 100 mM had no detectable effect on L-alanine efflux. These experiments confirm the strong exchange characteristics of the high-affinity transporter and support the earlier evidence suggesting lack of exchange by the low-affinity system. The latter transporter did however exhibit *trans*-acceleration when the incubation temperature was reduced from 37 to 25 °C, 50 mM-extracellular L-alanine causing a 91 % increase in L-[U-^{14}C]alanine efflux (initial intracellular L-[U-^{14}C]alanine

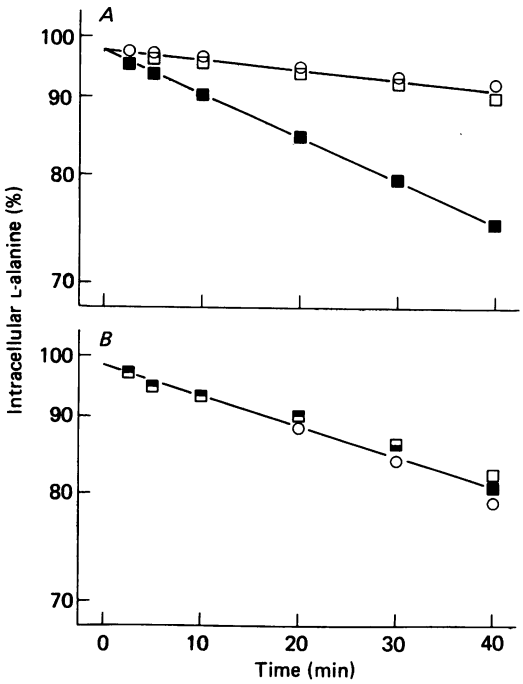

Fig. 10. Time course of L-alanine efflux from transport-positive horse erythrocytes. Cells from group 5 (*A*) and group 3 (*B*) animals were loaded with L-[U-^{14}C]alanine to intracellular concentrations of 0·16 and 10·2 mmol l cells^{-1}, respectively. L-Alanine efflux into tracer-free incubation medium was measured at 37 °C in the absence (○) or in the presence of extracellular L- (■) or D- (□) alanine (0·3 mM, *A*; 15 mM, *B*).

concentration 8·8 mmol l cells^{-1}). The sheep erythrocyte system C also exhibits significant *trans*-acceleration at 25 °C (Young & Ellory, 1977*a*). To investigate the exchange properties of system C at 37 °C, sheep erythrocytes were loaded with radiolabelled L-alanine (18·1 mmol l cells^{-1}) and efflux measured into incubation medium in the absence (control) and in the presence of 50 mM-extracellular non-radioactive L-alanine. Stimulation of L-[U-^{14}C]alanine efflux by external L-alanine was very weak (21 % above control) at physiological temperature.

The ability of the high-affinity transporter to participate in exchange reactions was exploited in Table 4 to further investigate the substrate specificity of the system for neutral amino acids. Relatively high concentrations of extracellular amino acid (5 mM) were used in this series of experiments in an attempt to detect amino acids with low, but significant affinities for the transport system. Amino acids which most

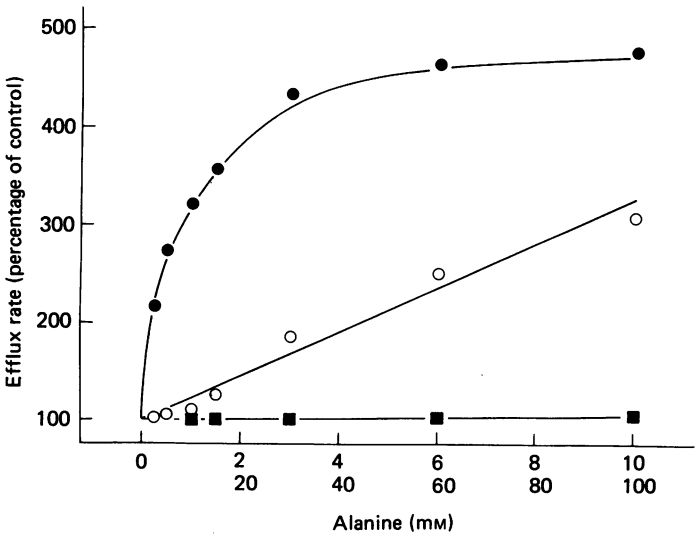

Fig. 11. Stimulation of L-alanine efflux from transport-positive horse erythrocytes by extracellular alanine. Initial rates of L-alanine efflux from group 5 cells at 37 °C were measured in the presence of various concentrations of extracellular L- (●) and D- (○) alanine (0·25–10 mM). Also shown is the effect of extracellular L-alanine (■, 2·5–100 mM) on L-[U-^{14}C] alanine efflux from group 3 cells. Results are expressed as a percentage of the control efflux rate measured in the absence of extracellular amino acid. The initial intracellular L-[^{14}C]alanine concentrations were 0·14 mmol l cells^{-1} for group 5 cells and 7·9 mmol l cells^{-1} for group 3 cells.

TABLE 4. Effects of amino acids on L-alanine efflux from transport-positive (group 5) horse erythrocytes

Amino acid	Efflux (percentage of control)	Amino acid	Efflux (percentage of control)
Glycine	121	L-Valine	388
Sarcosine	105	L-Norleucine	106
Taurine	98	L-Leucine	94
L-Proline	88	L-Isoleucine	100
L-Alanine	530	L-Phenylalanine	97
D-Alanine	174	L-Cysteine	329
L-α-Amino-*n*-butyrate	543	L-Serine	619
D-α-Amino-*n*-butyrate	121	L-Threonine	403
γ-Amino-*n*-butyrate	98	L-Methionine	100
α-Aminoisobutyrate	254	L-Asparagine	111
L-Norvaline	276	L-Glutamine	101

The initial intracellular L-[^{14}C]alanine concentration was 0·14 mmol l cells^{-1}. Extracellular amino acids were present at a concentration of 5 mM. Results are expressed as a percentage of the control efflux rate measured at 37 °C in the absence of extracellular amino acid.

readily stimulated L-alanine efflux from group 5 cells were; L-serine > L-α-amino-*n*-butyrate > L-alanine > L-threonine > L-valine > L-cysteine > L-norvaline > L-α-aminoisobutyrate. The relatively poor stimulation of L-alanine efflux by D-alanine and D-α-amino-*n*-butyrate emphasizes the stereoselectivity of the system. Stimulation of efflux by glycine was weak, but greater than with sarcosine,

demonstrating intolerance of N-methylation by the system. The importance of the α-amino group was demonstrated by the lack of stimulation of L-alanine efflux by γ-amino-*n*-butyrate. Increasing the length or bulk of the amino acid side-chain was also not tolerated. Thus, L-leucine, L-isoleucine, the straight-chain analogue L-norleucine, L-methionine and L-phenylalanine produced little or no stimulation of L-alanine efflux from group 5 erythrocytes. The side-chain of L-asparagine was poorly tolerated by the system, but produced a greater stimulation of L-alanine efflux than L-glutamine. Taurine was ineffective.

DISCUSSION

Erythrocytes from different human subjects exhibit relatively uniform rates of L-alanine uptake (Young *et al.* 1983). In contrast, red cells from thoroughbred horses are highly variable with respect to L-alanine transport activity, differing by up to two orders of magnitude in the range 5–625 μmol l cells^{-1} h^{-1} (0·2 mM-extracellular amino acid, 37 °C) (Fincham *et al.* 1985*c*). As illustrated in Fig. 1, horses can be divided into five distinct permeability groups on the basis of their erythrocyte permeability to this amino acid. The results presented here demonstrate that these permeability groupings correspond to distinct kinetic phenotypes characterized by the functional presence or absence of two saturable amino acid transporters; one with a high apparent affinity for L-alanine (apparent $K_m \simeq 0{\cdot}3$ mM), the other with a relatively low apparent affinity for this amino acid (apparent $K_m \simeq 13$ mM). In addition to these two transport routes, erythrocytes from all horses were shown to possess a non-saturable component of L-alanine influx. The relatively low temperature sensitivity of this uptake route, its lack of stereoselectivity and its preference for large hydrophobic amino acids suggest that it represents non-mediated diffusion of amino acid across the lipid bilayer. Measured rates of L-alanine transport in phospholipid vesicles are consistent with this conclusion (Young & Ellory, 1977*b*). Animals exhibiting only this component of transport are termed amino acid transport deficient. Rates of erythrocyte L-alanine transport in these animals (group 1) were comparable to those observed previously for transport-deficient sheep erythrocytes (Young *et al.* 1976).

Kinetic analyses of the different transport phenotypes in horse erythrocytes are consistent with inheritance data implicating the involvement of three allelelomorphic genes in the regulation of amino acid transport activity, separate co-dominant genes coding for high-affinity transport activity, low-affinity transport activity and transport deficiency (h, l and s, respectively) (Table 2). Some important observations relating to the genetics of amino acid transport variation in thoroughbred horse erythrocytes are (a) that crosses between transport-deficient animals always produce transport-deficient progeny, indicating that these horses are homozygous for the s gene, (b) that crosses involving group 2 or group 5 transport-positive-type animals can give rise to transport-deficient progeny, indicating that these horses are heterozygous for the s gene (genotypes l, s and h, s, respectively) and (c) that crosses involving group 3 and group 4 transport-positive-type horses do not give rise to transport-deficient offspring (genotypes l, l and h, l, respectively) (D. A. Fincham, D. K. Mason & J. D. Young, unpublished observations). As expected from these genotype classifications, the low-affinity transport activity in erythrocytes from group 3 horses had twice the V_{max} of that in cells from group 2 and group 4 animals.

Of the six predicted phenotypes, only five were detected in thoroughbred horses. The missing (and rarest) phenotype (animals homozygous for h; expected frequency 0·7 %) is likely to occur most commonly in horse breeds and species where s and l are less widely distributed. For example, erythrocytes from some cross-bred ponies exhibit L-alanine uptake rates (0·2 mM extracellular concentration, 37 °C) as high as 1000 μmol l cells^{-1} h^{-1} compared with a mean transport rate in group 5 thoroughbred horse red cells of approximately 500 μmol l cells^{-1} h^{-1} (Fig. 1 and Fincham *et al.* 1985*c*). Kinetic analysis of one such animal gave an apparent L-alanine K_m of 0·43 mM and a V_{max} of 3·27 mmol l cells^{-1} h^{-1}, a value 2-fold higher than the mean V_{max} of group 5 erythrocytes (Table 2).

Physiologically, the absence of carrier-mediated amino acid transport in erythrocytes of transport-deficient-type horses predisposes the cells to GSH deficiency and is associated with the intracellular accumulation of amino acids, particularly lysine and, in arginase-positive cells, ornithine (D. A. Fincham, D. K. Mason & J. D. Young, unpublished observations). Arginase-negative horse erythrocytes (Fincham, Mason & Young, 1985*b*) contain millimolar concentrations of arginine even if they possess amino acid transport activity. This is because L-arginine is a poorly transported substrate of the two horse erythrocyte carrier systems (Table 1). As mentioned in the Introduction, high intracellular levels of dibasic amino acids and GSH deficiency also occur in sheep erythrocytes which lack the system C amino acid transporter (Ellory *et al.* 1972; Young *et al.* 1975). Inheritance studies have shown that amino acid transport activity in sheep erythrocytes behaves as if controlled by two co-dominant autosomal alleles, giving rise to one transport-deficient and two transport-positive phenotypes (Young *et al.* 1982).

The substrate specificities of the Na^+-independent horse and sheep erythrocyte amino acid transporters for neutral amino acids are remarkably similar both to each other and to the Na^+-dependent system ASC, all four systems sharing a common selectivity for neutral amino acids of intermediate size. Thus, GSH deficiency in amino acid transport-deficient sheep (and presumably horse) erythrocytes is primarily a consequence of impaired L-cysteine transport (Young & Tucker, 1983). System ASC from human erythrocytes has a high apparent affinity for this amino acid (Young *et al.* 1979). Other neutral amino acids apart from L-alanine and L-cysteine which are common substrates for the four systems include L-serine and L-threonine. The horse and sheep erythrocyte systems also show a high degree of stereoselectivity, a characteristic feature of system ASC. A further point of similarity is that system ASC as defined in a number of cell types operates preferentially in an exchange mode (see Christensen, 1979, 1984, for examples). This is also the case for the high-affinity transporter in horse erythrocytes, while the low-affinity transport systems in both horse and sheep erythrocytes exhibit *trans*-acceleration at 25 °C (Young & Ellory, 1977*a*; Fincham *et al.* 1985*a*).

The strong exchange properties of the high-affinity transporter in horse erythrocytes account for the ability of group 4 and group 5 cells to accumulate extracellular L-[^{14}C]alanine (Figs. 2 and 3) and, in addition, offer an explanation for the higher V_{max} and K_m of the system in group 5 erythrocytes compared with group 4 cells (Table 2). Mean intracellular (alanine + serine + threonine + valine) levels in group 5 erythrocytes ($\simeq$ 1·1 mmol l cells^{-1}) are higher than in group 4 erythrocytes

($\simeq$ 0·63 mmol l cells^{-1}). This allows the high-affinity transporter in group 5 cells to participate more fully in exchange reactions. By analogy with the kinetic behaviour of other transport systems (see Hoare, 1972*a*, *b*; Cabantchik & Ginsburg, 1977; and Naftalin & Holman, 1977, for examples), this approach towards equilibrium exchange conditions might be expected to increase both K_m and V_{max} for influx. As predicted by the simple carrier model of Lieb & Stein (1974) there was no significant difference in the mean V_{max}/K_m ratio for system asc_1 in group 4 *versus* group 5 horse erythrocytes. The different levels of amino acid substrates in the two cell types can in turn be attributed to the presence of the low-affinity transporter in group 4 erythrocytes, providing a route for the net loss of intracellular amino acids.

In our initial study of amino acid transport in group 5 horse erythrocytes (Fincham *et al.* 1985*a*), the high-affinity transporter was provisionally assigned the symbol asc in recognition of the system's close similarity to system ASC. Lower case letters were used in accordance with a recent proposal on the nomenclature of Na^+-independent amino acid transport systems (Bannai, Christensen, Vadgama, Ellory, Englesberg, Guidotti, Gazzola, Kilberg, Lajtha, Sacktor, Sepulveda, Young, Yudilevich & Mann, 1984). The symbol asc was also used to emphasize the possibility that the Na^+-independent transporter in horse erythrocytes might represent a physiological, genetic or evolutionary variant of system ASC, a suggestion also made previously for the sheep erythrocyte system C (Young & Ellory, 1977*a*). The use of asc is retained here for horse erythrocytes, the high-affinity system being designated asc_1 and the low-affinity transporter system asc_2. According to this nomenclature, system C is the sheep equivalent of asc_2.

An independent study has also reported the presence of an asc system in pigeon erythrocytes (Vadgama & Christensen, 1985*a*). However, it is not immediately clear to what extent its properties resemble those of the horse (and sheep) transporters described here. The avian system was distinguished from the other amino acid transporters in pigeon erythrocytes (notably systems ASC and L) primarily on the basis of cross-inhibition studies in the absence of Na^+. Thus, the relative affinities of the system for its preferred substrates (L-alanine, L-serine, L-threonine and L-valine) are not known. The kinetic complexity of amino acid transport in these cells contrasts markedly with the situation in horse erythrocytes where only the asc system(s) are present. Vadgama & Christensen (1985*a*) suggest that system asc is unrelated to system ASC. They argue that binding of Na^+ with substrate defines system ASC selectivity to an extent that major changes in substrate specificity would result from loss of co-substrate action by Na^+.

Critical evidence in favour of possible structural homology between the amino acid transporters in horse and sheep erythrocytes, and system ASC concerns their interaction with dibasic amino acids. As detailed earlier, transport deficiency in both horse and sheep erythrocytes results in the intracellular accumulation of high concentrations of dibasic amino acids. Previous studies with sheep erythrocytes (Young *et al.* 1976, 1980; Young & Ellory, 1977*a*) and data presented in Table 1 indicate that the horse and sheep transporters are major routes for L-lysine and L-ornithine transport in erythrocytes from these species. Differences in L-arginine uptake between transport-positive and transport-negative horse erythrocytes are less marked than for the other dibasic amino acids (Table 1). Initial kinetic studies with

this amino acid have established that it interacts atypically with system asc_2, having the same apparent K_m as L-alanine, but a 77-fold lower V_{max} (Fincham, Mason & Young, 1984). Relative to L-alanine, L-arginine is effectively a non-transported substrate of system asc_2. In consequence, L-arginine functions as a *trans*-inhibitor of the system (Fincham, Young & Mason, 1986). Such *trans*-inhibition is likely to occur physiologically in horse erythrocytes, most of which are arginase deficient and contain millimolar concentrations of this amino acid (see earlier). Indeed, *trans*-inhibition effects may have contributed to the anomalous partial equilibration of L-alanine across the membranes of group 2 and group 3 horse erythrocytes observed in Figs. 2 and 4.

This unusual and unexpected interaction of L-arginine with system asc_2 closely resembles the interaction of dibasic amino acids with system ASC in pigeon erythrocytes and rabbit reticulocytes where it has been proposed that the positively-charged amino or guanidino groups on the amino acid side-chain interact with the Na^+ binding site of the transporter (Thomas & Christensen, 1970, 1971). Thus, the horse and sheep erythrocyte amino acid transporters may have residual or modified Na^+ binding sites that no longer accept or transport Na^+, but which permit the binding and transport of dibasic amino acids. In this respect it is interesting that the Na^+-independent system asc of pigeon erythrocytes fails to interact with dibasic amino acids (Vadgama & Christensen, 1985*b*).

A final important question to be considered is the origin of the kinetic differences between systems asc_1 and asc_2. At physiological temperature, the major difference observed between the two transporters is in their measured apparent K_m values for amino acid influx. Apparent K_m does not represent a true measure of carrier affinity, but is a complex group of rate constants involving carrier movement across the membrane as well as those governing permeant binding and dissociation. The apparent K_m value for L-alanine influx by system asc_2 was dramatically reduced upon lowering the incubation temperature, whereas the corresponding effect on system asc_1 was less marked. At 5 °C, the differences between the two systems in both K_m and V_{max} were minimized. A further kinetic difference between the two transporters at 37 °C is that system asc_1 shows strong exchange characteristics, a feature not observed with system asc_2. However, as the temperature is lowered, the asc_2 transporter begins to exhibit *trans*-acceleration. These effects on system asc_2 are consistent with a preferential impairment of the mobility of the unloaded carrier relative to that of the loaded transporter at low temperature. Similarly, the different kinetic properties of systems asc_1 and asc_2 at physiological temperature may be largely attributable to a difference in the mobilities of the empty carriers, this difference being minimized at 5 °C. Studies with other erythrocyte membrane transport systems have found results consistent with low temperature affecting the mobility of the empty carrier in broadly similar ways (Hoare, 1972*a*, *b*; Plagemann & Wohlheuter, 1984; Jarvis & Martin, 1986).

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REFERENCES

AL-SALEH, E. A. S. & WHEELER, K. P. (1982). Transport of neutral amino acids by human erythrocytes. *Biochimica et biophysica acta* **684**, 157–171.

ATKINS, G. L. (1976). Tests for the goodness of fit of models. *Biochemical Society Transactions* **4**, 357–361.

BANNAI, S., CHRISTENSEN, H. N., VADGAMA, J. V., ELLORY, J. C., ENGLESBERG, E., GUIDOTTI, G. G., GAZZOLA, G. C., KILBERG, M. S., LAJTHA, A., SACKTOR, B., SEPULVEDA, F. V., YOUNG, J. D., YUDILEVICH, D. & MANN, G. (1984). Amino acid transport systems. *Nature* **311**, 308.

BLISS, C. I. (1967). *Statistics in Biology*, vol. 1, pp. 108–110. New York: McGraw-Hill.

CABANTCHIK, Z. I. & GINSBURG, H. (1977). Transport of uridine in human red blood cells. Demonstration of a simple carrier mediated process. *Journal of General Physiology* **69**, 75–96.

CHRISTENSEN, H. N. (1979). Exploiting amino acid structure to learn about membrane transport. *Advances in Enzymology* **49**, 41–101.

CHRISTENSEN, H. N. (1984). Organic ion transport during seven decades. The amino acids. *Biochimica et biophysica acta* **779**, 255–269.

DIXON, M. (1953). The determination of enzyme inhibitor constants. *Biochemical Journal* **55**, 170–171.

ELLORY, J. C., JONES, S. E. M., PRESTON, R. L. & YOUNG, J. D. (1981*a*). A high-affinity sodium-dependent transport system for glutamate in dog red cells. *Journal of Physiology* **320**, 99*P*.

ELLORY, J. C., JONES, S. E. M. & YOUNG, J. D. (1981*b*). Glycine transport in human erythrocytes. *Journal of Physiology* **320**, 403–422.

ELLORY, J. C. & LEW, V. L. (1977). *Membrane Transport in Red Cells*. London: Academic Press.

ELLORY, J. C., TUCKER, E. M. & DEVERSON, E. V. (1972). The identification of ornithine and lysine at high concentrations in the red cells of sheep with an inherited deficiency of glutathione. *Biochimica et biophysica acta* **279**, 481–483.

FINCHAM, D. A., MASON, D. K. & YOUNG, J. D. (1984). Dibasic amino acid transport in horse erythrocytes. *Journal of Physiology* **358**, 92*P*.

FINCHAM, D. A., MASON, D. K. & YOUNG, J. D. (1985*a*). Characterization of a novel Na^+-independent amino acid transporter in horse erythrocytes. *Biochemical Journal* **227**, 13–20.

FINCHAM, D. A., MASON, D. K. & YOUNG, J. D. (1985*b*). Red cell amino acid transport and arginase. Two new blood group markers in horses. *Animal Blood Groups Biochemical Genetics* **16**, suppl. 1, 37–38.

FINCHAM, D. A., YOUNG, J. D. & MASON, D. K. (1986). Arginine modulates neutral amino acid transport in horse erythrocytes. *Biochemical Society Transactions* **14**, 294.

FINCHAM, D. A., YOUNG, J. D., MASON, D. K., COLLINS, E. A. & SNOW, D. H. (1985*c*). Breed and species comparison of amino acid transport variation in equine erythrocytes. *Research in Veterinary Science* **38**, 346–351.

GARDNER, J. D. & LEVY, A. G. (1972). Transport of dibasic amino acids by human erythrocytes. *Metabolism Clinical and Experimental* **21**, 413–431.

HOARE, D. G. (1972*a*). The transport of L-leucine in human erythrocytes: a new kinetic analysis. *Journal of Physiology* **221**, 311–329.

HOARE, D. G. (1972*b*). The temperature dependence of the transport of L-leucine in human erythrocytes. *Journal of Physiology* **221**, 331–348.

INABA, M. & MAEDE, Y. (1984). Increase of Na^+ gradient-dependent L-glutamate and L-aspartate transport in high K^+ dog erythrocytes associated with high activity of (Na^+, K^+)-ATPase. *Journal of Biological Chemistry* **259**, 312–317.

JARVIS, S. M. & MARTIN, B. W. (1986). Effects of temperature on the transport of nucleosides in guinea pig erythrocytes. *Canadian Journal of Physiology and Pharmacology* **64**, 193–198.

KING, G. F. & KUCHEL, P. W. (1984). A proton NMR study of iminodipeptide transport and hydrolysis in the human erythrocyte. Possible physiological roles of the coupled system. *Biochemical Journal* **220**, 553–560.

KING, G. F. & KUCHEL, P. W. (1985). Assimilation of α-glutamyl-peptides by human erythrocytes. A possible means of glutamate supply for glutathione synthesis. *Biochemical Journal* **227**, 833–842.

KING, G. F., YORK, M. J., CHAPMAN, B. E. & KUCHEL, P. W. (1983). Proton NMR spectroscopic studies of dipeptidase in human erythrocytes. *Biochemical and Biophysical Research Communications* **110**, 305–312.

LIEB, W. R. & STEIN, W. D. (1974). Testing and characterizing the simple carrier. *Biochimica et biophysica acta* **373**, 178–196.

MAEDE, Y., INABA, M. & TANIGUCHI, N. (1983). Increase of Na–K-ATPase activity, glutamate and aspartate uptake in dog erythrocytes associated with hereditary high accumulation of GSH, glutamate, glutamine and aspartate. *Blood* **61**, 493–499.

NAFTALIN, R. J. & HOLMAN, G. D. (1977). Transport of sugars in human red cells. In *Membrane Transport in Red Cells*, ed. ELLORY, J. C. & LEW, V. L., pp. 257–300. London: Academic Press.

PLAGEMANN, P. G. W. & WOHLHUETER, R. M. (1984). Effect of temperature on kinetics and differential mobility of empty and loaded nucleoside transporter of human erythrocytes. *Journal of Biological Chemistry* **259**, 9024–9027.

ROSENBERG, R. (1981*a*). L-Leucine transport in human red blood cells: a detailed kinetic analysis. *Journal of Membrane Biology* **62**, 79–93.

ROSENBERG, R. (1981*b*). A kinetic analysis of L-tryptophan transport in human red blood cells. *Biochimica et biophysica acta* **649**, 262–268.

ROSENBERG, R. (1982). Na^+-independent and Na^+-dependent transport of neutral amino acids in the human red blood cell. *Acta physiologica scandinavica* **116**, 321–330.

ROSENBERG, R., YOUNG, J. D. & ELLORY, J. C. (1980). Tryptophan transport in human red blood cells. *Biochimica et biophysica acta* **598**, 375–384.

THOMAS, E. L. & CHRISTENSEN, H. N. (1970). Indication of spatial relations among structures recognising amino acids and Na^+ at a transport receptor site. *Biochemical and Biophysical Research Communications* **40**, 277–283.

THOMAS, E. L. & CHRISTENSEN, H. N. (1971). Nature of the cosubstrate action of Na^+ and neutral amino acid in a transport system. *Journal of Biological Chemistry* **246**, 1682–1688.

TUCKER, E. M. (1974). A shortened life-span of sheep red cells with a glutathione deficiency. *Research in Veterinary Science* **16**, 19–22.

TUCKER, E. M., WRIGHT, P. C. & YOUNG, J. D. (1977). The influence of arginase deficiency on amino acid concentrations in sheep erythrocytes with a normal and a defective transport system for amino acids. *Journal of Physiology* **271**, 47–48*P*.

TUCKER, E. M. & YOUNG, J. D. (1980). Biochemical changes during reticulocyte maturation in culture: a comparison of genetically different sheep erythrocytes. *Biochemical Journal* **192**, 33–39.

TUCKER, E. M., YOUNG, J. D. & CROWLEY, C. (1981). Red cell glutathione deficiency: clinical and biochemical investigations using sheep as an experimental model system. *British Journal of Haematology* **48**, 403–415.

VADGAMA, J. V. & CHRISTENSEN, H. N. (1985*a*). Discrimination of Na^+-independent transport systems *L*, *T* and *asc* in erythrocytes. Na^+-independence of the latter a consequence of cell maturation? *Journal of Biological Chemistry* **260**, 2912–2921.

VADGAMA, J. V. & CHRISTENSEN, H. N. (1985*b*). A new Na^+-independent transport system for dipolar amino acids apparently corresponding to systems persisting after erythrocyte maturation in some mammalian genotypes. *Annals of the New York Academy of Sciences* **456**, 454–456.

VANDENBERG, J. I., KING, G. F. & KUCHEL, P. W. (1985). The assimilation of tri- and tetra-peptides by human erythrocytes. *Biochimica et biophysica acta* **846**, 127–134.

WINTER, C. G. & CHRISTENSEN, H. N. (1964). Migration of amino acids across the membrane of the human erythrocyte. *Journal of Biological Chemistry* **239**, 872–878.

WINTER, C. G. & CHRISTENSEN, H. N. (1965). Contrasts in neutral amino acid transport by rabbit erythrocytes and reticulocytes. *Journal of Biological Chemistry* **240**, 3594–3600.

YOUNG, J. D. & ELLORY, J. C. (1977*a*). Substrate specificity of amino acid transport in sheep erythrocytes. *Biochemical Journal* **162**, 33–38.

YOUNG, J. D. & ELLORY, J. C. (1977*b*). Red cell amino acid transport. In *Membrane Transport in Red Cells*, ed. ELLORY, J. C. & LEW, V. L., pp. 301–325. London: Academic Press.

YOUNG, J. D., ELLORY, J. C. & TUCKER, E. M. (1975). Amino acid transport defect in glutathione-deficient sheep erythrocytes. *Nature* **254**, 156–157.

YOUNG, J. D., ELLORY, J. C. & TUCKER, E. M. (1976). Amino acid transport in normal and glutathione-deficient sheep erythrocytes. *Biochemical Journal* **154**, 43–48.

YOUNG, J. D., JONES, S. E. M. & ELLORY, J. C. (1980). Amino acid transport in human and in sheep erythrocytes. *Proceedings of the Royal Society* B **209**, 355–375.

YOUNG, J. D., JONES, S. E. M. & ELLORY, J. C. (1981). Amino acid transport via the red cell anion transport system. *Biochimica et biophysica acta* **645**, 157–160.

YOUNG, J. D. & TUCKER, E. M. (1983). Erythrocyte glutathione deficiency in sheep. In *Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects*, ed. LARSSON, A., ORRENIUS, S., HOLMGREN, A. & MANNERVIK, B., pp. 373–384. New York: Raven Press.

YOUNG, J. D., TUCKER, E. M. & KILGOUR, L. (1982). Genetic control of amino acid transport in sheep erythrocytes. *Biochemical Genetics* **20**, 723–731.

YOUNG, J. D., WOLOWYK, M. W., JONES, S. E. M. & ELLORY, J. C. (1979). Sodium-dependent cysteine transport in human red blood cells. *Nature* **279**, 800–802.

YOUNG, J. D., WOLOWYK, M. W., JONES, S. E. M. & ELLORY, J. C. (1983). Red cell amino acid transport. Evidence for the presence of system *ASC* in mature human red blood cells. *Biochemical Journal* **216**, 349–357.