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Red-cell amino acid transport

Evidence for the presence of system ASC in mature human red blood cells

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The properties of Na^+-dependent L-alanine transport in human erythrocytes were investigated using K^+ as the Na^+ substitute. Initial rates of Na^+-dependent L-alanine uptake (0.2 mM extracellular amino acid) for erythrocytes from 22 donors ranged from 40 to 180 μmol/litre of cells per h at 37°C. Amino acid uptake over the concentration range 0.1–8 mM was consistent with a single saturable component of Na^+-dependent L-alanine transport. Apparent K_m and $V_{max.}$ values at 37 and 5°C measured in erythrocytes from the same donor were 0.27 and 0.085 mM respectively, and 270 and 8.5 μmol/litre of cells per h respectively. The transporter responsible for this uptake was identified as system ASC on the basis of cross-inhibition studies with a series of 42 amino acids and amino acid analogues. Apparent K_i values for glycine, L-α-amino-n-butyrate, L-serine and L-leucine as inhibitors of Na^+-dependent L-alanine uptake at 37°C were 4.2, 0.12, 0.16 and 0.70 mM respectively. Reticulocytes from a patient with inherited pyruvate kinase deficiency were found to have a 10-fold elevated activity of Na^+-dependent L-alanine uptake compared with erythrocytes from normal donors. Separation of erythrocytes according to cell density (cell age) established that even the oldest mature erythrocytes retained significant Na^+-dependent L-alanine transport activity. Amino acid transport was, however, a more sensitive indicator of cell age than acetylcholinesterase activity. Erythrocytes were found to accumulate L-alanine against its concentration gradient (distribution ratio approx. 1.5 after 4 h incubation), an effect that was abolished in Na^+-free media. Na^+-dependent L-alanine uptake was shown to be associated with L-alanine-dependent Na^+ influx, the measured coupling ratio being 1:1.

Transport of amino acids across the red-blood-cell membrane is required for normal erythrocyte function and viability. In sheep, for example, defective amino acid transport results in glutathione deficiency, the intracellular accumulation of amino acids, particularly ornithine and lysine, and a markedly diminished red-cell potential life-span (Young *et al.*, 1976). Glutathione deficiency is caused primarily by a reduced intracellular availability of cysteine for glutathione biosynthesis, whereas the accumulation of amino acids inside the cell presumably reflects the inability of transport-deficient erythrocytes to lose amino acids produced during reticulocyte maturation (Tucker *et al.*, 1977; Tucker & Young, 1980; Young & Tucker, 1983). The amino acid transporter normally responsible for cysteine and dibasic amino acid transport in sheep red cells does not require Na^+ for activity and is abbreviated system C in recognition of its physiological role in cysteine uptake (Young & Ellory, 1977; Young *et al.*, 1979*a*). The deletion of system C activity from the red cells of some sheep is genetically controlled (Young *et al.*, 1982).

In view of the importance of system C to the sheep red blood cell, it might reasonably be expected to find system C in erythrocytes from all species. This does not appear to be the case, and careful

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kinetic studies have failed to detect system C activity in human erythrocytes (Young *et al.*, 1979*a*). Instead, human cells possess a number of amino acid transporters not found in sheep erythrocytes. Three of these systems (L, T and Ly^+) appear to function as non-concentrative facilitated-diffusion carriers with no measurable cation or anion requirement, and one requires both Na^+ and Cl^- for activity (system Gly) (Winter & Christensen, 1964; Gardner & Levy, 1972; Hoare, 1972*a*,*b*; Young *et al.*, 1979*a*; Rosenberg *et al.*, 1980; Ellory *et al.*, 1981*a*; Rosenberg, 1981; Al-Saleh & Wheeler, 1982). Experiments from this laboratory (Young *et al.*, 1979*a*,*b*) and by Al-Saleh & Wheeler (1982) suggest that human red cells possess a second Na^+-dependent amino acid uptake route with a similar substrate specificity to system C. In the present paper we describe detailed investigations of the properties of this latter system, using L-alanine as the test substrate. In agreement with the conclusions of Al-Saleh & Wheeler (1982), our results suggest that the human red-cell transporter is equivalent to the well-defined system ASC found extensively in other cell types (see, for example, Christensen, 1979). We further demonstrate that Na^+-dependent L-alanine transport is coupled to L-alanine-dependent Na^+ uptake in a 1:1 stoichiometry. System ASC is most active in reticulocytes, but present in mature human erythrocytes of all cell ages.

Materials and methods

Whole blood samples were collected into heparinized tubes. Erythrocytes were washed three times with 20 vol. of incubation medium. The buffy coat was discarded. Reticulocyte counts were typically less than 2%.

Materials

L-Amino acids uniformly labelled with ^{14}C or ^3H and ^{24}Na were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and used without further purification. Non-radioactive amino acids, amino acid analogues and other reagents were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Amino acid uptake studies

^{14}C-labelled-amino acid uptake was measured by the washing method described in detail by Young & Ellory (1982). Unless otherwise stated, all values refer to initial uptake rates measured at 37°C using incubation times of between 5 and 15 min. The incubation medium contained 150 mM-NaCl, 5 mM-glucose and 10 mM-Tris/HCl (pH 7.4 at 37°C or 5°C, as appropriate). Na^+-free solutions were made by replacing NaCl with KCl. Unlike choline chloride, KCl only induced modest cell-volume changes (Fig. 3). Inhibitory amino acids and amino acid analogues were added to cells at the same time as the permeant amino acid.

Red cells were separated according to cell age by the Percoll-density-gradient-centrifugation technique of Wolowyk (1982). Acetylcholinesterase was assayed by the method of Chow & Islam (1970) with acetylthiocholine as substrate.

Na^+/amino acid coupling ratio

To measure the Na^+/alanine coupling ratio, ouabain-treated human erythrocytes were incubated at 37°C for 8 min with various concentrations of $^{24}NaCl$ (0–82 mM) in the presence and absence of 0.6 mM-L-[^{14}C]alanine. The incubation medium contained 1 mM-ouabain and 0.5 mM-bumetanide to reduce Na^+-influx via the Na^+-pump and the Na^+/K^+-cotransport system respectively, and K^+ was used as the Na^+ substitute. Control experiments confirmed that ouabain had no significant effect on Na^+-dependent L-alanine transport. At the concentration used in these experiments (0.5 mM), bumetanide inhibited Na^+-dependent L-alanine uptake by less than 10% (see also Wolowyk *et al.*, 1983). After incubation, cells were washed free of extracellular radioactivity in the usual way and lysed with 0.5% (w/v) Triton X-100. ^{24}Na radioactivity in the haemolysates was measured in a γ-counter. Samples were subsequently deproteinized with trichloroacetic acid, and allowed to decay for 2 weeks before ^{14}C determination by liquid-scintillation counting. L-Alanine-dependent Na^+-uptake was calculated as the difference between ^{24}Na transport in the presence and absence of L-alanine, and Na^+-dependent L-alanine uptake was calculated as the difference between L-[^{14}C]alanine uptake in the absence and in the presence of various concentrations of Na^+. Each determination was performed in triplicate.

Results

Concentration- and temperature-dependence of L-alanine uptake

Fig. 1 shows the concentration-dependence of L-alanine uptake by human red cells measured at 37°C and 5°C in the presence and absence of Na^+, using K^+ as the Na^+ substitute. The extracellular L-alanine concentration ranged from 0.05 to 1.0 mM. In agreement with previous results from this laboratory, and those of Al-Saleh & Wheeler (1982), Na^+-dependent L-alanine uptake (measured as the difference between the initial rates of transport in Na^+ and K^+ media) was saturable and conformed to simple Michaelis–Menten kinetics, giving apparent K_m and $V_{max.}$ estimates of 0.27 mM and 0.27 mmol/litre of cells per h respectively at 37°C. Reducing the incubation temperature from 37°C to 5°C caused a

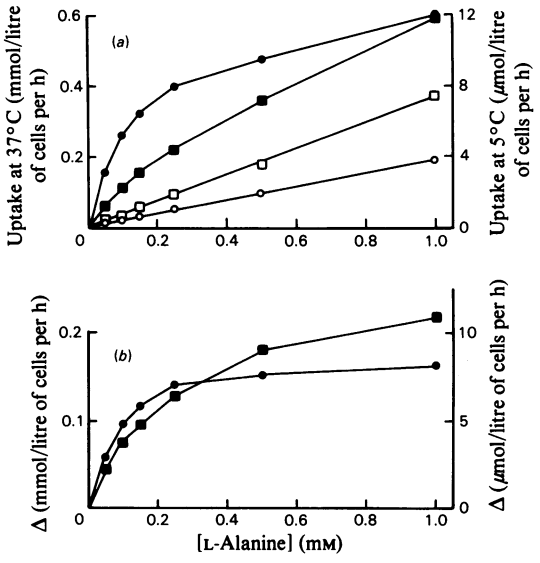

Fig. 1. *Concentration- and temperature-dependence of* L-*alanine uptake by human erythrocytes*
(*a*) Initial rates of L-alanine uptake were measured in NaCl (● and ■) and KCl media (○ and □) at 37°C (■ and □) and at 5°C (● and ○). (*b*) Δ is the difference between NaCl and KCl uptake rates (■, at 37°C; ●, at 5°C).

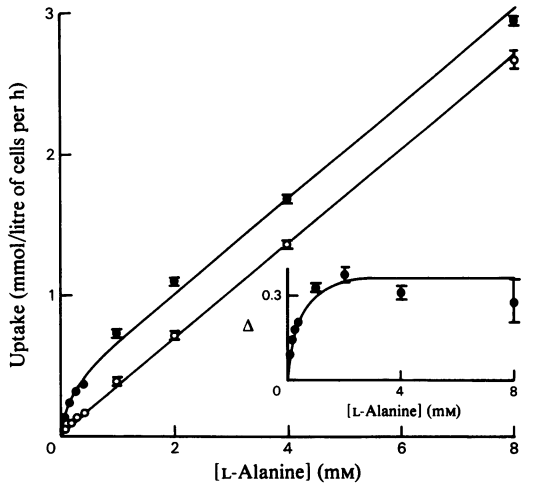

Fig. 2. *Concentration-dependence of* L-*alanine uptake by human erythrocytes (high concentration range)*
Initial L-alanine uptake rates were measured at 37°C in the presence (●) and in the absence of Na^+ (○). Δ is the difference between NaCl and KCl uptake rates. Data are fitted as $v = [0.27/(1 + 0.27/s)] + 0.34s$, where v is the initial rate of transport and s is the extracellular amino acid concentration. Values are means (±S.E.M.) of triplicate determinations.

3.2-fold reduction in apparent K_m and a 32-fold reduction in $V_{max.}$ (0.085 mM and 8.5 μmol/litre of cells per h respectively). The results presented in Fig. 1 also demonstrate that Na^+-dependent L-alanine uptake is less temperature-sensitive than Na^+-independent alanine transport. For example, at an L-alanine concentration equal to the apparent K_m for Na^+-dependent transport (0.27 mM), Na^+-independent L-alanine uptake accounted for 40% of the total flux at 37°C compared with 12% at 5°C. At 0.085 mM, Na^+-independent uptake accounted for only 8% of the transport at 5°C.

In Fig. 2, we show the initial rate of L-alanine uptake (±Na^+) at 37°C over the extended concentration range 0.1–8.0 mM. Within experimental error, the results are consistent with a single saturable component of Na^+-dependent transport. At the higher L-alanine concentrations, the Na^+-dependent component of transport represents only a small fraction of total uptake (9% at 8 mM). The estimates of apparent K_m and $V_{max.}$ from this experiment are 0.25 mM and 0.36 mmol/litre of cells per h respectively.

To investigate whether human red cells can concentrate L-alanine, we measured intracellular radioactivity during prolonged incubation of cells with 0.2 mM extracellular ^{14}C-labelled amino acid. Fig. 3 shows the results of such an experiment

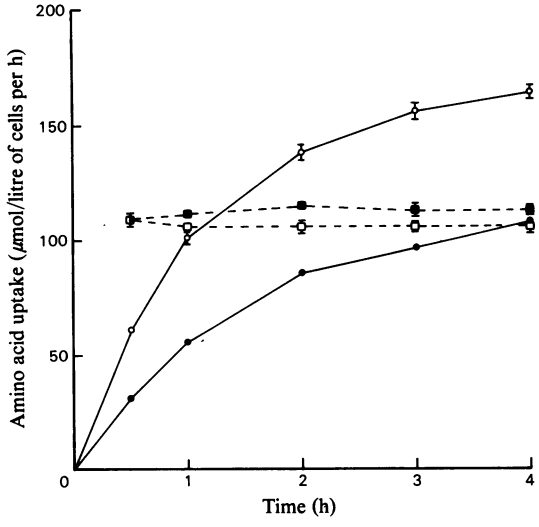

Fig. 3. *Time-course of* L-*alanine uptake by human erythrocytes in the presence and in the absence of* Na^+
L-[^{14}C]Alanine (● and ○) and [G-^3H]leucine (■ and □) uptake (initial extracellular concentrations 0.2 mM) were measured in the presence (○ and □) and in the absence (● and ■) of Na^+, using KCl as the NaCl substitute. See the text for other experimental details. Values are means (±S.E.M.) of triplicate determinations.

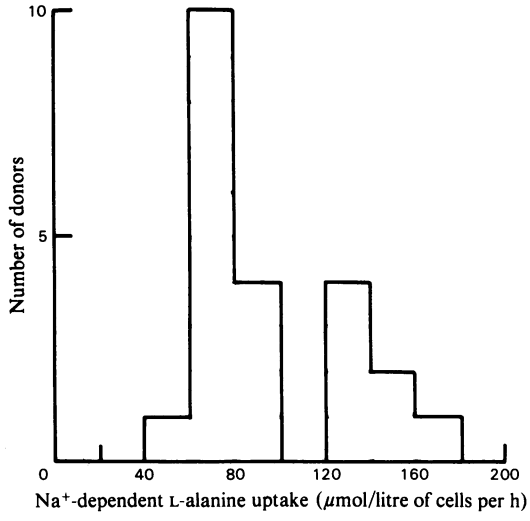

Fig. 4. *Variation in Na^+-dependent L-alanine transport in erythrocytes from different donors*
Initial rates of L-alanine transport were measured in red cells from different subjects at 37°C and with an extracellular L-alanine concentration of 0.2mM. Values are means of triplicate determinations for each donor.

performed in both NaCl and KCl media. In this experiment, the incubation medium contained both L-[^{14}C]alanine and L-[^{3}H]leucine, the intention being to monitor the L-leucine intracellular space as a control for possible volume changes during prolonged incubation in KCl medium. L-Leucine uptake by the L-system is rapid ($t_{\frac{1}{2}} < 1$ min at 37°C). It can be seen that over 4h there was a small (approx. 6%) difference in the L-leucine uptake (± Na^+) expressed per litre of cells based on haemoglobin determinations (cyanmethaemoglobin assay). In contrast, L-alanine showed a large Na^+-dependent uptake, reaching a higher value (180μmol/litre of cells) in NaCl medium than did L-leucine. The L-alanine uptake after 4h in NaCl medium was equivalent to approx. 300μmol/litre of cell water, compared with approx. 200μmol in the KCl medium and externally. L-Leucine (NaCl and KCl media) reached similar final values to those for L-alanine in KCl medium. Although L-alanine does not give a large concentration difference, and the experiment can be criticized in terms of not reaching equilibrium, there is a marked difference between L-leucine and L-alanine transport, suggestive of the latter showing significant accumulation. Previous experiments performed with human and sheep red cells suggest that L-alanine is not significantly metabolized in erythrocytes (Young & Ellory, 1977; Al-Saleh & Wheeler, 1982).

Table 1. *Na^+-dependent L-alanine transport in normal and reticulocyte-rich human blood*
Initial rates of L-alanine uptake by red cells from normal human peripheral blood and by cells from a patient with severe reticulocytosis (70%) induced by inherited pyruvate kinase deficiency were measured in NaCl and KCl media as described in detail by Young & Ellory (1982). Values are means (+ S.E.M.) of triplicate determinations.

	Alanine uptake (μmol/litre of cells per h)		
	Total uptake (*a*)	Na^+-insensitive uptake (*b*)	Na^+-dependent uptake (*a*−*b*)
Reticulocyte-rich blood (*c*)	1351 ± 68	130 ± 3	1221 ± 68
Control blood (*d*)	256 ± 1	128 ± 2	128 ± 2
Ratio of (*c*)/(*d*)	5.3	1.0	9.5

Population distribution of Na^+-dependent alanine uptake

To determine the variation in values for Na^+-dependent alanine uptake in red cells from different individuals, flux measurements were made on samples from 22 donors (Fig. 4). The results showed a 4-fold variation in flux that did not correlate with either age or sex. To establish the reproducibility of measurements, one individual (J.C.E.) was measured 11 times over a period of 5 years. The mean value (±S.D.) for uptake (at 0.2mM alanine) was 110.3 ± 18.9 (range 91–140)μmol/litre of cells per h and showed no consistent variation over this period. Similar comparisons but with fewer determinations confirmed this constancy in four other individuals.

Effect of cell age on Na^+-dependent L-alanine transport activity

Table 1 compares the L-alanine transport activity of normal human blood with that of a reticulocyte-rich blood sample obtained from a patient with inherited pyruvate kinase deficiency (reticulocyte count 70%). Both samples gave an uptake rate of 130μmol/litre of cells per h in the absence of Na^+. In contrast, Na^+-dependent amino acid uptake was 10-fold higher in the reticulocyte-rich sample. The age-dependence of L-alanine transport was studied further by fractionating cells from normal donors according to red-cell age on iso-osmotic Percoll density gradients as described previously (Wolowyk, 1982). Acetylcholinesterase, a red-cell enzyme known to decrease with cell age, was used as an internal control. Results from a representative experiment are presented in Fig. 5. Both acetylcholinesterase activity and Na^+-dependent L-alanine

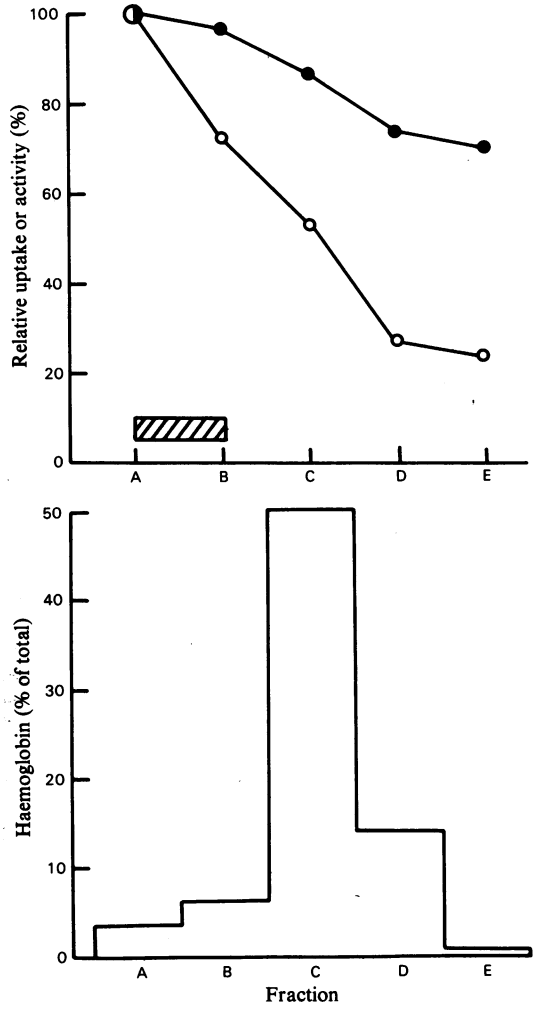

Fig. 5. *Effect of cell age on Na^+-dependent L-alanine transport in human erythrocytes*

Na^+-dependent L-alanine uptake (0.2 mM extracellular concentration) and acetylcholinesterase activity were measured as described in the text. Cells were separated into five fractions on a Percoll density gradient with fraction A (top of gradient) corresponding to the youngest cells. The upper Figure compares the Na^+-dependent alanine uptake (○) and acetylcholinesterase (●) activities of the fractions. The hatched area corresponds to fractions containing reticulocytes. The lower Figure shows the haemoglobin distribution in the Percoll gradient.

uptake decreased with increasing cell density. Fraction A represents the youngest (least dense) cells and fraction E contains the oldest cells. The decrease in Na^+-dependent L-alanine transport activity was substantially greater than that of acetylcholinesterase (ratio of fraction A/fraction E 4.03 and 1.41 respectively). Reticulocytes were confined to the first two fractions of the gradient. In contrast with Na^+-dependent L-alanine transport, Na^+-independent L-alanine uptake changed little with cell age (results not shown).

Amino acid competition studies

To investigate the substrate specificity of the amino acid carrier responsible for Na^+-dependent L-alanine uptake, we tested a series of non-radioactive amino acids and amino acid analogues as inhibitors of Na^+-dependent L-[^{14}C]alanine uptake. The results are presented in Table 2. Unless otherwise stated, L-[^{14}C]alanine was present at a concentration of 0.2 mM and the inhibitor at 1.0 mM.

D-Alanine was a poor inhibitor of Na^+-dependent L-alanine uptake, demonstrating that the carrier is stereospecific. Stereospecific inhibition of transport by α-amino-n-butyrate and leucine was also observed. The presence of an α-amino group was critical for transport inhibition as judged by the lack of significant inhibition by β-alanine, γ-amino-n-butyrate, DL-β-aminoisobutyrate and DL-isoserine. Acetylation or methylation of the α-amino group similarly abolished transport inhibition (*N*-acetyl-L-alanine and *N*-methyl-DL-alanine). Interestingly, the methyl ester of DL-α-amino-n-butyrate inhibited transport by 33%. Replacement of the α-hydrogen atom of L-alanine by a methyl group (α-amino-isobutyrate) also largely abolished the ability of the amino acid to inhibit L-alanine transport. The cyclic amino acids L-proline and cycloleucine were poor inhibitors of transport (28% inhibition and no detectable inhibition respectively).

The amino acid transporter was more tolerant to changes in the structure of the amino acid side chain. Thus, increasing the length of the side chain from one to two (L-α-amino-n-butyrate) or three carbon atoms (L-norvaline) or the presence of thiol (L-cysteine, DL-homocysteine), hydroxy (L-serine, L-homoserine, L-threonine), cyano (β-cyano-L-alanine) or chloro (β-chloro-L-alanine) groups had no deleterious effect on transport inhibition. Further increases in the length or bulk of the amino acid side chain did, however, reduce the amount of inhibition observed. The comparison between L-valine and L-norvaline suggests a preference for straight-chain molecules (23 and 63% inhibition respectively). At the other extreme, glycine, the smallest amino acid, was a significant, but poor, inhibitor of Na^+-dependent L-alanine uptake. The basic amino acids, L-glutamate and *O*-phospho-L-serine were also poor inhibitors of L-alanine transport.

The kinetics of inhibition by four amino acids (glycine, L-α-amino-n-butyrate, L-serine and L-leucine) were investigated in more detail by measuring Na^+-dependent L-alanine uptake at two L-alanine concentrations in the presence of various con-

Table 2. *Inhibition of Na^+-dependent L-alanine uptake by non-radioactive amino acids and amino acid analogues*

Na^+-dependent L-alanine uptake was measured as the difference between initial rates of transport in NaCl and KCl media (L-alanine concentration 0.2 mM). Inhibitors (1 mM) were added to cells at the same time as the permeant. Values are means of duplicate determinations.

	Na^+-dependent L-alanine uptake (% inhibition)
Glycine	28*
L-Proline	28
L-Allylglycine (2-aminopent-4-enoic acid)	67
Glycylglycine	0
L-Alanine	68
D-Alanine	9
β-Chloro-L-alanine	80
β-Cyano-L-alanine	74
N-Acetyl-L-alanine	5
β-Alanine	0
N-Methyl-DL-alanine	0
L-α-Amino-n-butyrate	79
D-α-Amino-n-butyrate	11
DL-α-Amino-n-butyric acid methyl ester	33
γ-Amino-n-butyrate	11
α-Aminoisobutyrate	7
DL-β-Aminoisobutyrate	0
L-Cysteine	94
DL-Homocysteine	74
S-Ethyl-L-cysteine	27
L-Serine	82*
DL-Isoserine (DL-β-amino-α-hydroxy-propionate)	5
L-Homoserine	89
O-Phospho-L-serine	0
L(+)-Penicillamine	17
L-Threonine	83*
L-Valine	23
L-Norvaline	63
L-Leucine	37
D-Leucine	0
L-Norleucine	30
L-Isoleucine	31
Cycloleucine (1-aminocyclopentane-1-carboxylic acid)	0
L-Methionine	47
L-Tryptophan	26
L-2,4-Diamino-n-butyrate	0
L-Ornithine	9
L-Lysine	8
L-Arginine	9
S-2-Aminoethyl-L-cysteine	28
L-Histidine	4
L-Glutamate	2

* 0.1 mM-L-[^{14}C]alanine, 1 mM inhibitory amino acid.

centrations of the appropriate inhibitory amino acid (Young & Ellory, 1977). Estimated apparent K_i values from these experiments are summarized in Table 3. L-α-Amino-n-butyrate was the most effective inhibitor of Na^+-dependent L-alanine transport, followed by L-serine, L-leucine and finally glycine. There was a 35-fold difference in apparent K_i value between glycine and L-α-amino-n-butyrate and a 6-fold difference between L-α-amino-n-butyrate and L-leucine. L-Serine gave an apparent K_i value slightly lower than the apparent K_m for Na^+-dependent L-alanine transport. Direct measurements of L-[^{14}C]serine uptake by human red cells in the presence and absence of Na^+ revealed a large saturable component of Na^+-dependent L-serine transport representing most of the total L-serine uptake. The apparent K_m value for this uptake was 0.14 mM, a value close to the apparent K_i for L-serine inhibition of Na^+-dependent L-alanine transport. The $V_{max.}$ for Na^+-dependent L-serine uptake was 280 μmol/litre of cells per h while Na^+-independent L-serine transport accounted for only 10% of the total flux at 0.14 mM extracellular L-serine. Uptake by this latter route exhibited a linear concentration dependence.

Table 3. *Amino acid inhibition of Na^+-dependent L-alanine transport*

Apparent K_i values were determined as described in the text. Values in parentheses refer to determinations from individual experiments. The L-α-amino-n-butyrate apparent K_i is derived from a single experiment.

	Apparent K_i (mM)
Glycine	4.2 (3.5, 4.8)
L-α-Amino-n-butyrate	0.12
L-Serine	0.16 (0.10, 0.15, 0.22)
L-Leucine	0.70 (0.80, 0.60)

Species variation in Na^+-dependent L-alanine transport activity

We have previously shown that sheep red cells do not exhibit Na^+-dependent L-alanine transport activity. In the present investigation we extended this study to four other species (horse, pig, guinea-pig and hedgehog). The hedgehog was the only species to exhibit a marked Na^+-dependent component of L-alanine uptake [65% of the total flux; 134 ± 2 ($n = 3$) and 48 ± 3 ($n = 3$) μmol/litre of cells per h (means ± S.E.M.) in the presence and in the absence of Na^+ at 0.2 mM amino acid].

Na^+/amino acid coupling ratio

Concentrations of L-alanine sufficient to saturate the Na^+-dependent uptake mechanism were found to increase significantly the initial rate of $^{24}Na^+$-uptake measured in the presence of the Na^+-transport inhibitors ouabain and bumetanide. In Fig. 6, the difference between $^{24}Na^+$ uptake in the presence and in the absence of 0.6 mM-L-alanine is plotted against

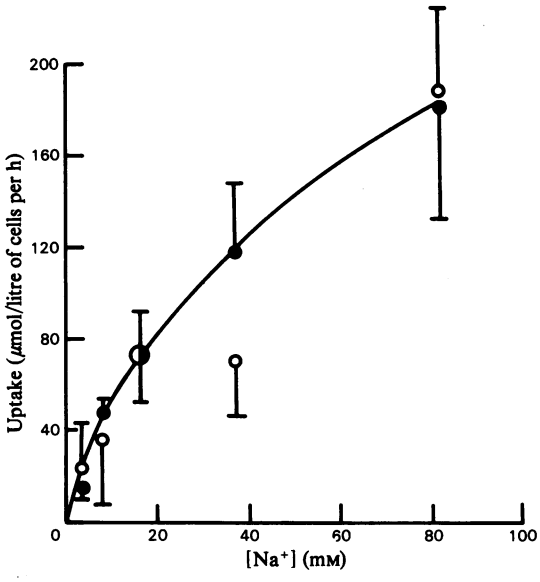

Fig. 6. *Coupling ratios for Na^+-dependent L-alanine transport in human erythrocytes*

L-Alanine-dependent $^{24}Na^+$-uptake (●) and Na^+-dependent L-[^{14}C]alanine uptake (○) were simultaneously measured in the same incubations in the presence of ouabain and bumetanide as described in the text. Values are means (± S.E.M.) of triplicate determinations.

the extracellular Na^+ concentration and compared with parallel estimates of the Na^+-dependent L-alanine uptake occurring in the same incubations. Both L-alanine-dependent Na^+-uptake and Na^+-dependent L-alanine uptake increased with increasing extracellular Na^+ concentration, with some evidence of saturation at higher Na^+ levels. Within experimental error, the results are consistent with an Na^+/alanine coupling ratio of 1:1. Results for 16 experiments performed with extracellular Na^+ concentrations ranging from 4 to 150mM and L-alanine concentrations between 0.2 and 1.0mM gave a mean (±S.E.M.) Na^+/amino acid coupling ratio of 1.18 ± 0.09.

Discussion

Until recently, it was generally supposed that mature mammalian red cells were incapable of Na^+-dependent amino acid transport. The results reported in the present paper confirm and extend our previous observations and those of Al-Saleh & Wheeler (1982) that normal human blood exhibits substantial Na^+-dependent L-alanine transport activity. Concentration-dependence studies over the range 0.05–8.0mM are consistent with a single saturable route for Na^+-dependent L-alanine uptake (apparent K_m approx. 0.3mM at 37°C). This transport function shows a marked decline during reticulocyte maturation and subsequent red-cell aging, but even the oldest circulating red cells seem to retain detectable Na^+-dependent L-alanine transport activity. This contrasts with the situation in sheep, where reticulocyte maturation is associated with the complete loss of Na^+-dependent L-alanine transport (Young *et al.*, 1979*a*; Tucker & Young, 1980). In pig, horse and guinea-pig red cells, Na^+-dependent L-alanine transport activity represented less than 5% of the total uptake, in contrast with hedgehog red cells, where most of the L-alanine uptake was Na^+-dependent (65% at 0.2mM). A small Na^+-dependent component of L-alanine uptake has been reported in rat and rabbit red cells (Yunis & Arimura, 1965; Wheeler & Christensen, 1967). It is not clear to what extent this might simply reflect reticulocyte, white-cell or platelet contamination. Human red-cell suspensions that have been passed through α-cellulose/microcrystalline cellulose columns to remove white cells and platelets still retain Na^+-dependent L-alanine transport activity (D. A. Fincham & J. D. Young, unpublished work). In our separation experiments with human red cells on the basis of age we found that L-alanine transport activity decreased more rapidly with red-cell age than did acetylcholinesterase activity, suggesting that Na^+-dependent amino acid transport may be a more sensitive indicator of red-cell age than some of the commonly used red-cell enzyme markers (see, e.g., Rennie *et al.*, 1979).

We have also been able to show that Na^+-dependent L-alanine uptake by human erythrocytes is associated with a parallel L-alanine-dependent Na^+-uptake, the measured coupling ratio being 1:1. This represents the first direct demonstration of Na^+/amino acid coupling in mammalian red cells and establishes that Na^+-dependent L-alanine transport in human red cells is mediated by an authentic Na^+/amino acid co-transport system. Prolonged incubation of cells with L-alanine in the presence of Na^+ resulted in amino acid distribution ratios (concentration in cell water/concentration in extracellular medium) significantly greater than 1, suggesting that the transporter is capable of modest intracellular accumulation of L-alanine. High steady-state distribution ratios of amino acid would not be expected in human red cells in view of the large Na^+-independent L-alanine 'leak flux' (Figs. 1 and 2).

The substrate specificity of the transporter responsible for Na^+-dependent L-alanine transport was investigated indirectly by studying the inhibitory effects of a series of 42 different amino acids and amino acid derivatives. The list of compounds tested (Table 2) contains 22 amino acids previously tested as inhibitors of Na^+-dependent L-alanine uptake by

Al-Saleh & Wheeler (1982) under different experimental conditions, but in addition includes 20 compounds not previously studied. Apparent K_i values were also determined for four selected amino acids. The overall inhibition pattern observed in the present experiments closely resembles that expected of an ASC-type system as defined in a variety of cell types, including avian red cells and mammalian reticulocytes, by Christensen and co-workers (see, for example, Christensen, 1979). The apparent K_i (K_m) values obtained in the present work with serine, alanine and glycine agree well with those given in Table 5 of Al-Saleh & Wheeler (1982), who used serine rather than alanine as the primary substrate. We have previously demonstrated that human red cells also possess an Na^+-dependent amino acid transporter selective for glycine and sarcosine (system Gly) (Ellory *et al.*, 1981*a*). Several lines of evidence justify the separation of system ASC and Gly transport activities in human red cells. First, system Gly requires Cl^- for activity, whereas system ASC has no such anion dependence. Secondly, the apparent K_m for glycine uptake by system Gly is 25 μM in contrast with an apparent K_i of 4.2 mM for glycine inhibition of L-alanine uptake by system ASC. Thirdly, the substrate specificity of amino acid uptake by the two routes as measured in competition experiments is very different. For glycine uptake by human red cells, two discrete Na^+-dependent entry routes can be resolved: system Gly and a parallel low-affinity (apparent $K_m > 2$ mM) uptake that is inhibited by L-alanine and that does not require Cl^- for activity (Ellory *et al.*, 1981*a*). This latter component of transport presumably reflects glycine uptake by system ASC.

The amino acid transporter (system C) found in mature sheep red cells has a similar substrate specificity to system ASC but lacks its Na^+-dependence and has a relatively low affinity for its optimal substrates (L-alanine and L-cysteine apparent K_m values 16 and 14 mM respectively) (Young & Ellory, 1977; Young *et al.*, 1979*a*). We have previously shown that a genetically controlled deletion of system C in the red cells of some sheep results in an inability to transport L-cysteine (and to a lesser extent, glycine) and hence leads to glutathione deficiency (Young *et al.*, 1976). The substrate-specificity studies presented here suggest that system ASC in human cells may fulfil a similar physiological role. Human red cells have been shown to exhibit saturable Na^+-dependent L-cysteine uptake (Young *et al.*, 1979*b*). The measured apparent K_m value of 18 μM was low and comparable with physiological plasma L-cysteine levels. Uptake of L-cysteine by this route was inhibited by L-alanine and other ASC substrates (Young *et al.*, 1979*a*). Na^+-independent L-cysteine and L-alanine transport in human cells are likely to be mediated by system L, a high-capacity carrier selective for large neutral amino acids.

The similarity of the human red-cell system ASC and the sheep system C in terms of their substrate specificity suggests to us that these two transporters are species variants of the same carrier mechanism. This possibility is supported by recent studies of amino acid transport in horse red cells where a novel high-affinity Na^+-independent ASC-type system has been found in the red cells of some animals (Fincham & Young, 1983). These observations have interesting and important implications for our understanding of the structure, function and molecular evolution of membrane transport systems in higher organisms. As in sheep, horses exhibit genetically controlled amino acid transport variation, initial rates of L-alanine uptake in cells from different animals differing by up to two orders of magnitude. Red cells from 30% of thoroughbred horses are amino acid-transport-deficient (Fincham & Young, 1983; D. A. Fincham & J. D. Young, unpublished work). Results presented in Fig. 4 suggest that human red cells from different subjects exhibit significant, if more modest, variations in Na^+-dependent L-alanine transport activity.

In conclusion, two distinct Na^+-dependent amino acid transporters have now been demonstrated in human red cells (systems ASC and Gly). Their presence in these cells opens up new possibilities for detailed kinetic and molecular studies of cation- (and anion-) dependent solute transport. Carnivore red cells have been shown to possess an Na^+-dependent carrier system selective for acidic amino acids (system Glu) (Ellory *et al.*, 1981*b*).

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