Substrate Specificity of Amino Acid Transport in Sheep Erythrocytes

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The specificity of amino acid transport in normal (high-glutathione) sheep erythrocytes was investigated by studying the interaction of various neutral and dibasic amino acids in both competition and exchange experiments. Apparent K_i values were obtained for amino acids as inhibitors of L-alanine influx. Amino acids previously found to be transported by high-glutathione cells at fast rates (L-cysteine, L- α -amino-*n*-butyrate) were the most effective inhibitors. D-Alanine and D- α -amino-*n*-butyrate were without effect. Of the remaining amino acids studied, only L-norvaline, L-valine, L-norleucine, L-serine and L-2,4-diamino-*n*-butyrate significantly inhibited L-alanine uptake. L-Alanine efflux from pre-loaded cells was markedly stimulated by extracellular L-alanine. Those amino acids that inhibited L-alanine influx also stimulated L-alanine efflux. In addition, D-alanine, D- α -amino-*n*-butyrate, L-threonine, L-asparagine, L- α , β -diaminopropionate, L-ornithine, L-lysine and S-2-aminoethyl-L-cysteine also significantly stimulated L-alanine efflux. L-Lysine uptake was inhibited by L-alanine but not by D-alanine, and the inhibitory potency of L-alanine was not influenced by the replacement of Na⁺ in the incubation medium with choline. L-Lysine efflux from pre-loaded cells was stimulated by L-alanine but not by D-alanine. It is concluded that these cells possess a highly selective stereospecific amino acid-transport system. Although the optimum substrates are small neutral amino acids, this system also has a significant affinity for dibasic amino acids.

Erythrocytes from Finnish Landrace sheep with an inherited deficiency of GSH (reduced glutathione) contain high intracellular concentrations of certain amino acids, notably ornithine and lysine (Ellory *et al.*, 1972). These cells differ markedly from normal sheep erythrocytes in their permeability to some neutral and dibasic amino acids (Young *et al.*, 1975). The decreased amino acid permeability is the result of a membrane-transport defect, and the extent of this transport lesion has been established by measuring the relative uptake rates of a series of natural amino acids by both normal (high-GSH) and GSH-deficient (low-GSH) erythrocytes (Young *et al.*, 1976). These studies strongly suggest that high-GSH sheep erythrocytes have a stereospecific transport system for neutral amino acids of intermediate size (the cysteine system), which is lacking in low-GSH cells. The permeability to the dibasic amino acids ornithine and lysine was also significantly different in the two types of erythrocyte. Since lysine uptake by high-GSH cells was markedly inhibited by L-alanine and L- α -amino-*n*-butyrate, but not by D-alanine, it was suggested that these amino acids were also transported by the cysteine system. The present investigation studies the interaction of different amino acids in both competition and exchange experiments to characterize further neutral and dibasic amino acid

transport in sheep erythrocytes. Evidence is presented which confirms the original findings that both neutral and dibasic amino acids share a common transport system in these cells. A preliminary report of some of these results has already appeared (Ellory *et al.*, 1976).

Materials and Methods

Whole blood was obtained from Finnish Landrace sheep by jugular venepuncture into heparinized containers. Animals were classified as to GSH-type on the basis of erythrocyte GSH and amino acid concentrations (Tucker & Kilgour, 1970; Ellory *et al.*, 1972).

Materials

¹⁴C-labelled amino acids were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive amino acids were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., except for L-alanine, L-cysteine, L-isoleucine, L-lysine, L-ornithine, L-threonine and L-serine (BDH Chemicals Ltd., Poole, Dorset, U.K.) and S-2-aminoethyl-L-cysteine and L- α , β -diaminopropionic acid (Calbiochem Ltd., Hereford, U.K.). When necessary, amino acid solutions were adjusted to the required pH with NaOH or HCl.

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Amino acid-uptake studies

Amino acid uptake was measured as previously described (Young *et al.*, 1976). All fluxes were carried out at 25°C unless otherwise stated; 25°C was chosen as a convenient low temperature for demonstrating kinetic effects, since lowering the temperature results in a marked increase in the apparent affinity of erythrocyte amino acid-transport systems (Hoare, 1972*b*; Young *et al.*, 1976). Iso-osmolality at various amino acid concentrations was maintained by adjusting the concentration of NaCl in the incubation medium. Choline replaced Na⁺ in the Na⁺-dependence experiments.

Amino acid-efflux studies

Washed erythrocytes were incubated at a haematocrit of 30% for 2 h at 37°C in a medium (Young *et al.*, 1976) containing 40 mM-L-[U-¹⁴C]alanine (0.025 μ Ci/ μ mol). The cells were rapidly washed in 4 $\times$ 10 vol. of ice-cold incubation medium by using an Eppendorf 3200 micro-centrifuge (15000*g*, 10 s) and then resuspended to give a haematocrit of 4%. L-Alanine efflux at 25°C was measured by mixing 0.5 ml of cell suspension with 0.5 ml of incubation medium containing the appropriate non-radioactive amino acid. At pre-determined time-intervals, incubations were stopped by rapidly sedimenting the cells (15000*g*, 20 s). A sample (0.7 ml) of supernatant was transferred to 7 ml of Unisolve (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and counted for radioactivity in a β -scintillation spectrometer with quench correction. The initial intracellular L-alanine concentration was measured by adding 0.25 ml of the 4% cell suspension to 0.5 ml of 0.5% (v/v) Triton

X-100, and deproteinizing the resulting lysate with 0.25 ml of 66% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation (15000*g*, 30 s), and 0.9 ml of the supernatant was added to 7 ml of Unisolve and counted for radioactivity with quench correction. Samples of the trichloroacetic acid extracts were also subjected to paper chromatography (Winter & Christensen, 1965) to determine the extent of conversion of L-[U-¹⁴C]alanine into other soluble radioactive compounds. No metabolite of L-alanine was detected.

Results

Effects of amino acids on L-alanine uptake by high-GSH erythrocytes

We have previously shown that the concentration-dependence of L-alanine uptake by normal (high-GSH) erythrocytes is consistent with a single component conforming to simple Michaelis-Menten kinetics with an apparent K_m of 6.0 mM and a V_{max} of 2.85 mmol/h per litre of cells at 25°C (Young *et al.*, 1976). In the present series of inhibition studies L-alanine uptake was measured at two concentrations (5 and 10 mM) in the presence of another amino acid (5–25 mM). These data were analysed by the Dixon (1953) procedure to estimate apparent K_i values. Fig. 1 shows two typical amino acid inhibition patterns (L- α -amino-*n*-butyrate, apparent K_i 6.2 mM; and L-methionine, no inhibition measurable). To confirm that the inhibition was of a simple competitive type, the effect of L- α -amino-*n*-butyrate on L-alanine uptake was investigated over a wider range of L-alanine concentrations, and the data were analysed by the Dixon (1953) method and by the alternative

Fig. 1. Effects of L- α -amino-*n*-butyrate (a) and L-methionine (b) on L-alanine uptake by high-GSH erythrocytes. Experimental details are described in the text. ●, 5 mM-L-alanine; ○, 10 mM-L-alanine.

Fig. 2. Effect of L- α -amino-n-butyrate on L-alanine uptake by high-GSH erythrocytes

The L- α -amino-n-butyrate concentrations used were 0 (■), 5 (○), 10 (●), 15 (□) and 25 (▲) mM. See text for other experimental details. Analysis of these data by the Dixon (1953) procedure gave an apparent K_i value for L- α -amino-n-butyrate of 6.5 mM.

Lineweaver-Burk procedure (Fig. 2). The results were consistent with simple competitive inhibition with an apparent K_i value of 6.5 mM at 25°C.

Table 1 lists the apparent K_i values given by a variety of amino acids as inhibitors of L-alanine uptake. The amino acids previously found to be transported by high-GSH cells at high rates (e.g. L-cysteine and L- α -amino-n-butyrate) were the most effective inhibitors. Decreasing or increasing the length of the amino acid side chain, branching of the side chain, or the introduction of an aromatic ring all markedly decreased the degree of inhibition. The lack of inhibition by D-alanine, D- α -amino-n-butyrate and α -aminoisobutyrate emphasized the structural requirements at the α -carbon atom. Similarly, the lack of inhibition by γ -amino-n-butyrate or butyrate confirmed the importance of the α -amino group. L-Cysteine and L-serine as structural analogues of L- α -amino-n-butyrate were both inhibitors of L-alanine uptake. Cysteine was the more effective and this may be associated with the presence of the relatively bulky -SH group (cf. -OH group of serine). The presence of the sulphur atom in L-methionine resulted in a dramatic decrease in inhibitory effectiveness compared with the corresponding straight-chain amino acid (L-norleucine).

A variety of dibasic amino acids were tested for their effects on L-alanine uptake. L-2,4-Diamino-n-butyrate was a surprisingly effective inhibitor, and results for the homologous series of dibasic amino acids paralleled the data for straight-chain neutral amino acids. Thus decreasing (L- α , β -diamino-

Table 1. Effects of amino acids on L-alanine influx and efflux in high-GSH erythrocytes

Apparent K_i values and initial L-alanine efflux rates were determined as described in the text. In the efflux experiments the initial intracellular L-alanine concentration was 9.2 mmol/litre of cells, and extracellular amino acids were present at a concentration of 50 mM; values are means $\pm$ S.E.M. of these observations.

	Apparent K_i value (mM)	Stimulation of L-alanine efflux (%)
Glycine	*	*
Proline	*	*
L-Alanine	†	167 $\pm$ 4
D-Alanine	*	13 $\pm$ 4
L- α -Amino-n-butyrate	6.5	125 $\pm$ 1
D- α -Amino-n-butyrate	*	19 $\pm$ 2
γ -Amino-n-butyrate	*	*
α -Aminoisobutyrate	*	*
Sodium butyrate	*	*
L-Norvaline	8.5	140 $\pm$ 2
L-Valine	41.5	69 $\pm$ 3
L-Norleucine	40.0	22 $\pm$ 1
L-Leucine	*	*
L-Isoleucine	*	*
L-Cycloleucine	*	*
L-Phenylalanine	*	*
L-Cysteine	7.0	143 $\pm$ 4
L-Serine	38.0	89 $\pm$ 1
L-Threonine	*	23 $\pm$ 2
L-Methionine	*	*
L-Asparagine	*	22 $\pm$ 2
L-Glutamine	*	*
L- α , β -Diaminopropionate	*	60 $\pm$ 1
L-2,4-Diamino-n-butyrate	23.0	116 $\pm$ 1
L-Ornithine	*	57 $\pm$ 3
L-Lysine	*	42 $\pm$ 1
D-Lysine	*	*
S-2-Aminoethyl-L-cysteine	*	54 $\pm$ 2
L-Arginine	*	*
Cadaverine	*	*

* No inhibition of influx or stimulation of efflux measurable.

† Under these experimental conditions the apparent K_m value for L-alanine influx was 6.0 mM.

propionate) or increasing (L-ornithine, L-lysine and S-2-aminoethyl-L-cysteine) the length of the amino acid side chain resulted in a loss of inhibition compared with L-2,4-diamino-n-butyrate.

Effects of amino acids on L-alanine efflux from high-GSH erythrocytes

Competition experiments suffer from the limitation that an analogue that inhibits the transport of another is not necessarily transported by the same system (see, e.g., Christensen, 1975a). This section

Fig. 3. Time-course of L-alanine efflux from high-GSH erythrocytes in the presence and absence of extracellular amino acids

L-Alanine efflux from pre-loaded cells was measured as described in the text. Extracellular amino acids were present at a concentration of 20mM. ●, Control; ▲, L-alanine; ▼, D-alanine; ■, L- α -amino-*n*-butyrate. The initial intracellular L-alanine concentration was 8.8mmol/litre of cells.

describes an alternative approach to the investigation of the structural specificity of the cysteine transport system.

When certain amino acids were present in the external medium, there was a marked stimulation of L-alanine efflux. Fig. 3 shows the time-course of L-alanine efflux in the presence of extracellular D- and L-alanine and L- α -amino-*n*-butyrate. Efflux in the presence of D-alanine was slightly greater than in the control, and was considerably less than that observed in the presence of extracellular L-alanine and L- α -amino-*n*-butyrate. The relationship between the initial rate of L-alanine efflux and the extracellular L-alanine concentration is shown in Fig. 4. The external L-alanine concentration required to give half-maximal stimulation of efflux was 5.2mM. Since initial rates of L-alanine efflux were measured in these experiments, and since the incubation haematocrit was only 2%, the observed stimulation of efflux is not the result of inhibition of [14 C]alanine back-flux by extracellular amino acid but is an example of accelerative exchange diffusion (Stein, 1967; Christensen, 1975a). In these circumstances the stimulation of efflux is attributed to an influx of external amino acid by the same transport system, resulting in a more rapid reorientation of the carrier than would otherwise occur. A similar effect has been observed for leucine transport by human erythrocytes (Hoare, 1972a). In a control experiment L-[U- 14 C]alanine uptake (external concentration 10mM) was measured in cells pre-loaded with non-radioactive L-alanine (13.4mmol/litre of cells) and compared with cells

Fig. 4. Stimulation of L-alanine efflux from high-GSH erythrocytes by extracellular L-alanine

Initial L-alanine efflux rates were measured in the presence of various concentrations of extracellular L-alanine. The initial intracellular L-alanine concentration was 10.6mmol/litre of cells. See the text for methods.

preincubated in the absence of amino acid. There was a 71% increase in the initial influx rate in the L-alanine-loaded cells, demonstrating the symmetry of the transport system in this respect.

Table 1 also shows the ability of various amino acids to stimulate L-alanine efflux. A relatively high extracellular amino acid concentration (50mM) was used in this series of experiments in an attempt to detect amino acids with very low, but still significant, affinities for the transport system. Amino acids in this category could not be detected in the competition experiments. The amino acids that inhibited L-alanine influx also markedly stimulated L-alanine efflux, and the degree of stimulation strongly correlated with the estimated K_i values, amino acids with low apparent K_i values giving the largest L-alanine efflux rates. Eight additional amino acids, namely D-alanine, D- α -amino-*n*-butyrate, L-threonine, L-asparagine, L- α,β -diaminopropionate, L-ornithine, L-lysine and *S*-2-aminoethyl-L-cysteine, also induced a significant stimulation of L-alanine efflux, demonstrating that these amino acids also interact with the cysteine system. The lack of effect of these amino acids on L-alanine uptake (Table 1) probably reflects their low affinity for the transport system. For example the apparent K_m value for L-lysine uptake by high-GSH erythrocytes has been estimated to be over 100mM at 37°C (Young *et al.*, 1976). The remaining amino acids did not significantly stimulate L-alanine efflux.

These efflux data reinforce the conclusions reached in the previous section about the structural specificity of the cysteine transport system. They also demonstrate that dibasic amino acids other than L-2,4-diamino-*n*-butyrate interact with this system. It is significant that whereas L-lysine stimulated L-alanine

Fig. 5. Time-course of L-lysine efflux from high-GSH erythrocytes in the presence and absence of extracellular amino acids

Erythrocytes were incubated at a haematocrit of 30% for 5 h at 37°C with 40 mM-L-[U- 14 C]lysine (0.025 μ Ci/ μ mol). Lysine efflux was measured by the method described in the text for L-alanine. Extracellular amino acids were present at a concentration of 20 mM. ●, Control; ▲, L-alanine; ▼, D-alanine. The initial intracellular L-lysine concentration was 10.4 mmol/litre of cells.

efflux, D-lysine was without effect. This agrees with the stereospecificity observed for the neutral amino acids. In addition the efflux results suggest that L-threonine and L-asparagine may also be transported by the cysteine system. Each of these amino acids showed a threefold difference in uptake rate between high- and low-GSH cells (Young *et al.*, 1976). Indeed there was very good agreement between the group of amino acids postulated to share the cysteine system on the basis of the competition and efflux experiments, and those postulated to be transported by this system on the basis of permeability differences between high- and low-GSH erythrocytes.

Effects of D- and L-alanine on L-lysine efflux from high-GSH erythrocytes

L-Lysine but not D-lysine stimulated L-alanine efflux from cells pre-loaded with L-alanine. Fig. 5 shows the effects of D- and L-alanine on L-[U- 14 C]-lysine efflux from erythrocytes pre-loaded with L-lysine. L-Alanine but not D-alanine produced a marked stimulation of efflux.

L-Alanine inhibition of L-lysine uptake by high-GSH erythrocytes: Na^+ -dependence

We have shown that L-alanine, L- α -amino-*n*-butyrate but not D-alanine markedly inhibit L-lysine influx into high-GSH cells (Young *et al.*, 1976). Christensen (1975a,b) has developed the concept that (neutral amino acid + Na^+) can sometimes be

Table 2. Effects of D-alanine and L-alanine on L-lysine uptake by high-GSH erythrocytes in the presence and absence of Na^+

L-Lysine uptake (extracellular concentration 0.2 mM) was measured at 37°C by the method described in the text for L-alanine. Values are means $\pm$ S.E.M. of three determinations.

	L-Lysine uptake (μ mol/h per litre of cells)	
	135 mM- Na^+	0.2 mM- Na^+
Control	15.9 $\pm$ 0.5	16.1 $\pm$ 0.1
+25 mM-D-alanine	14.1 $\pm$ 0.6	15.3 $\pm$ 0.1
+25 mM-L-alanine	8.8 $\pm$ 0.1	8.4 $\pm$ 0.2

equivalent to a dibasic amino acid. To test whether this occurred in the present system, the effect of L-alanine on L-[U- 14 C]lysine uptake by high-GSH cells was measured in both high- and low- Na^+ media. No difference in inhibitory potency was observed when the Na^+ concentration in the incubation medium was lowered from 135 mM to 0.2 mM (Table 2).

Discussion

It is apparent from the present results that normal sheep erythrocytes possess a highly selective stereospecific transport system for amino acids. Although the optimum substrates are small neutral amino acids such as L-alanine, L- α -amino-*n*-butyrate and L-cysteine, it is clear that this system (the cysteine system) also has a significant affinity for a variety of dibasic amino acids. The absence of this transport system from GSH-deficient erythrocytes is presumably responsible for the low GSH concentration, and may also account for the presence of high concentrations of ornithine and lysine in these cells, since a comparison of the relative uptake rates of L-lysine and L-ornithine by normal and GSH-deficient erythrocytes (Young *et al.*, 1976) suggests that the cysteine-transport system is a major route for the transport of these dibasic amino acids in normal sheep erythrocytes. In this connexion it is noteworthy that whereas L-alanine and L- α -amino-*n*-butyrate but not D-alanine markedly inhibit L-lysine influx into high-GSH cells (Young *et al.*, 1976), the residual L-lysine uptake by GSH-deficient erythrocytes is insensitive to both L-alanine and L- α -amino-*n*-butyrate (J. D. Young & J. C. Ellory, unpublished work).

When the kinetic parameters of the cysteine-transport system were investigated, amino acid influx was found to conform to simple Michaelis-Menten kinetics, with the apparent K_m and V_{max} values showing a marked temperature-dependence (Young *et al.*, 1976). The system also exhibited the phenomenon of accelerative exchange diffusion in

both influx and efflux experiments, demonstrating the symmetry of the transport mechanism in this respect. Although not investigated in such detail, the kinetic characteristics of this transport system in sheep erythrocytes are superficially similar to those of the leucine-transport system in human erythrocytes which has been investigated in some depth by Hoare (1972a,b).

Several conclusions can be made about the specificity of the cysteine-transport system. It is highly stereospecific. The presence of the α -amino and α -carboxyl groups are essential for activity, and the replacement of the α -hydrogen atom with a methyl group similarly results in a loss of activity. In addition, the transport system prefers straight-chain amino acids, and branching of the side chain or the presence of an aromatic ring is not readily tolerated. Further, the length of the amino acid side chain is of critical importance. Thus L- α -amino-*n*-butyrate is a much better substrate than either glycine or L-norleucine. The transport system has the ability to distinguish between L-cysteine and L-serine on the one hand, and L-methionine and L-norleucine on the other, demonstrating a high degree of selectivity at two positions along the amino acid side chain. In contrast, the transport system accepts the presence of a terminal amino group on the side chain. The bulkier guanidinium group of arginine is not, however, tolerated. The acidic amino acids L-aspartate and L-glutamate are not transported.

For a system with such a high substrate selectivity in other respects, it is perhaps surprising that dibasic amino acids are also transported. Several other examples of dibasic and neutral amino acids sharing a transport system have been described (see, e.g., Christensen, 1975a). Christensen and co-workers have developed the attractive idea that (Na^+ +neutral amino acid) may substitute for a dibasic amino acid in the cationic amino acid transport system of rabbit reticulocytes and Ehrlich ascites-tumour cells (Christensen *et al.*, 1969; Thomas *et al.*, 1971). Interestingly, in the present case, such a mechanism cannot be operating, since no Na^+ -dependence of neutral amino acid uptake can be demonstrated. In addition, L-alanine inhibition of L-lysine transport was not influenced by the absence of Na^+ from the incubation medium. Another possibility to be considered is the transport of dibasic amino acids as neutral species. However, the high pK of the lysine ϵ -amino group makes only 0.1% of the amino acid available in the uncharged form under our experimental conditions. Therefore, unless the amino acid-binding site has the ability to suppress dramatically protonation of the ϵ -amino group, the dibasic amino acids must be transported in the ionized form.

The amino acid specificity of the cysteine system is unlike that of previously described amino acid-

transport systems in mammalian erythrocytes (Winter & Christensen, 1964, 1965; Antonioli & Christensen, 1969; Christensen & Antonioli, 1969; Gardner & Levy, 1972). In this respect it closely resembles the alanine-serine-cysteine (ASC) system defined by Christensen and co-workers (Christensen, 1969, 1975a,b). However, the 'ASC' system, which is present in rabbit reticulocytes but which has not been found in mature mammalian erythrocytes, differs from the present system in its Na^+ -dependence and in its apparent limitation to transport by exchange. It is intriguing to speculate that the present erythrocyte system may represent a modified component of a reticulocyte mechanism that has lost its Na^+ -dependence and has altered exchange characteristics. Further experiments to characterize amino acid transport in reticulocytes from high- and low-GSH sheep may allow this point to be resolved.

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