

## CHARACTERISATION OF AMINO ACID TRANSPORT IN RED BLOOD CELLS OF A PRIMITIVE VERTEBRATE, THE PACIFIC HAGFISH (*EPTATRETUS STOUTI*)

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*Accepted 13 June 1990*

### Summary

Intracellular amino acid levels and the characteristics of amino acid transport were investigated in red blood cells of a primitive vertebrate, the Pacific hagfish (*Eptatretus stouti* Lockington). In contrast to red cells from euryhaline teleosts and elasmobranchs, which contain high concentrations of  $\beta$ -amino acids, those from hagfish exhibited an intracellular amino acid pool (approx.  $100 \text{ mmol l}^{-1}$  cell water) composed almost entirely of conventional  $\alpha$ -amino acids. Red cell:plasma distribution ratios for individual amino acids ranged from 219, 203 and 173 for alanine,  $\alpha$ -amino-*n*-butyrate and proline, respectively, to 11 and 13 for lysine and arginine. Corresponding distribution ratios for  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  were 0.043, 21 and 0.32, respectively. The cellular uptake of amino acids, with the exception of L-proline and glycine, was  $\text{Na}^+$ -independent. Compared with mammalian and avian red cells, those from hagfish exhibited  $10^4$ -fold higher rates of L-alanine transport. Uptake of this amino acid from the extracellular medium was concentrative, but occurred as a 1:1 exchange with intracellular amino acids. The L-alanine transport mechanism was identified as an *asc*-type system on the basis of its  $\text{Na}^+$ -independence and selectivity for neutral amino acids of intermediate size. A volume-sensitive amino acid channel, which is found in both euryhaline teleosts and in elasmobranchs, is absent from hagfish red cells.

### Introduction

A wide range of different amino acid transport systems has been identified in mammalian and avian red blood cells, including  $\text{Na}^+$ -dependent systems, *ASC*, *N*, *Gly*,  $x^-$  and  $\beta$ , and  $\text{Na}^+$ -independent systems, *asc*,  $y^+$ , *L* and *T* (Christensen, 1979; Young, 1983; Harvey and Ellory, 1989). One function of amino acid

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Key words: Pacific hagfish, *Eptatretus stouti*, red blood cell, amino acid transport.

transport in these cells is to provide intracellular precursors for glutathione (GSH) biosynthesis (Young and Tucker, 1983). In human red cells, cysteine transport for this purpose is mediated largely by the classical  $\text{Na}^+$ -dependent system *ASC* (Young *et al.* 1979, 1983; Al-Saleh and Wheeler, 1982). In sheep and horse red cells, in contrast, cellular uptake of this amino acid occurs *via* a novel group of transporters designated *asc(C)* (Young *et al.* 1975, 1976; Young and Ellory, 1977; Fincham *et al.* 1985a, 1987a). These transporters share system *ASC*'s selectivity for neutral amino acids of intermediate size, are inhibited by the  $\text{Na}^+$  site inhibitor harmaline, but do not require cations for activity (Young *et al.* 1988). Inherited deficiencies of *asc* are relatively common in some horse and sheep breeds and lead to GSH deficiency and decreased red cell viability (Tucker *et al.* 1981; Fincham *et al.* 1985a,b; Fisher *et al.* 1986).

In fish, studies of red cell amino acid transport have been limited largely to taurine,  $\gamma$ -amino-*n*-butyrate (GABA) and  $\beta$ -alanine.  $\beta$ -Amino acids are found in high concentration in both euryhaline teleost and elasmobranch red cells, where they contribute to cell volume regulation (Fugelli and Thoroed, 1986; Fincham *et al.* 1987b; Goldstein and Kleinzeller, 1987). In both eel and flounder red cells, taurine uptake from plasma is mediated by a specific  $\beta$ -system with an apparent  $\text{Na}^+/\text{Cl}^-$ /taurine coupling ratio of 2:1:1 (Fincham *et al.* 1987b). A reduction in extracellular osmolarity, leading to an increase in cell volume, reversibly decreases the activity of the transporter. In parallel with this, low external osmolarity stimulates the activity of a  $\text{Na}^+$ -independent taurine permeability pathway, producing net efflux of amino acid from the cells and a return towards the original cell volume (Fugelli and Thoroed, 1986; Fincham *et al.* 1987b). This transport route has the properties of a channel and accepts a range of  $\alpha$ -amino acids and sugars in addition to  $\beta$ -amino acids. It is down-regulated by catecholamines and inhibited by loop diuretics (Wolowyk *et al.* 1989). Similar transport mechanisms are present in skate red cells (Goldstein and Kleinzeller, 1987). Recent studies implicate protein kinase C in the volume regulatory response (McConnell and Goldstein, 1988).

In this report we describe the first investigation of amino acid levels and amino acid transport in red cells of the Pacific hagfish (*Eptatretus stouti* Lockington), generally considered as amongst the most primitive of living vertebrates. In contrast to other fish species, hagfish red cells were found to contain high intracellular levels of conventional  $\alpha$ -amino acids. The cells possessed uniquely high *asc*-type transport activity, but lacked the volume-sensitive amino acid channel found in other fish species.

## Materials and methods

### *Fish*

Hagfish (*Eptatretus stouti*) were trapped at 165–201 m (90–110 fathoms) in Trevor Channel, Barkley Sound, Bamfield, British Columbia, and maintained in running sea water until bled from the subcutaneous sinus into heparinised tubes.

### Materials

D- and L-[U- $^{14}\text{C}$ ]-labelled amino acids, [U- $^{14}\text{C}$ ]sucrose and  $^3\text{H}_2\text{O}$  were purchased from Amersham International plc, Amersham, Bucks, UK. Non-radioactive amino acids were obtained from Sigma Chemical Co. Ltd, Poole, Dorset, UK. When necessary, amino acid solutions were adjusted to the required pH with KOH or HCl. *n*-Dibutylphthalate was purchased from E. Merck, Darmstadt, FRG. All other reagents were of analytical grade.

### Red cells

Cells were prepared for transport experiments by washing three times with 20 vols of an incubation medium containing  $500\text{ mmol l}^{-1}$  NaCl,  $5\text{ mmol l}^{-1}$  glucose and  $15\text{ mmol l}^{-1}$  Mops (titrated to pH 7.5 at  $10^\circ\text{C}$  with KOH). The buffy coat was discarded and the washed red cells resuspended to a haematocrit of 20 % in incubation medium. The water content of hagfish red cells, determined using  $^3\text{H}_2\text{O}$  with [ $^{14}\text{C}$ ]sucrose as extracellular space marker, was 68.6 % (v/v).

### Amino acid transport

Uptake of [ $^{14}\text{C}$ ]-labelled amino acid ( $10^\circ\text{C}$ ) was initiated by mixing cell suspension at  $10^\circ\text{C}$  with an equal volume of incubation medium at the same temperature containing the appropriate concentration of radioactive permeant (typically  $1\text{ }\mu\text{Ci ml}^{-1}$ ). Incubations were stopped at pre-determined time intervals (5 s to 3 h) by transferring 0.2 ml of the cell suspension (10 % haematocrit) to a microfuge tube (volume 1.5 ml) containing 0.8 ml of ice-cold incubation medium layered on top of 0.5 ml of ice-cold *n*-dibutylphthalate (an 'oil tube'). The oil tube, which was positioned in the rotor of an Eppendorf 5414 microfuge, was immediately centrifuged at  $15\,000\text{ g}$  for 10 s. 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 absorbent dental roll, the cell pellet was lysed with 0.5 ml of 0.5 % (v/v) Triton X-100 in water, and 0.5 ml of 5 % (v/v) trichloroacetic acid was added. The precipitate was removed by centrifugation ( $15\,000\text{ g}$  for 2 min) and 0.9 ml of the protein-free supernatant was counted for radioactivity by liquid scintillation spectroscopy with appropriate quench correction. Correction for radioactivity trapped in the extracellular space was made by processing cell samples which had been mixed at  $1^\circ\text{C}$  with either [ $^{14}\text{C}$ ]sucrose or the impermeant amino acid [ $^{14}\text{C}$ ]taurine (see Fig. 1) and immediately centrifuged. Uptake values were calculated after subtraction of these 'blank' estimates. Back extrapolation of L-alanine ( $0.2\text{ mmol l}^{-1}$  extracellular concentration) time courses established that the 'dead time' between addition of cell suspension to the oil tube and termination of the flux was 1 s. Initial rates of amino acid uptake, determined from 5 s incubations and expressed as  $\mu\text{mol l}^{-1}\text{ cell water h}^{-1}$ , were calculated to include this 'dead time'. Incubation times were measured manually using a conventional laboratory timer.

To measure efflux, washed hagfish red cells were incubated at a haematocrit of 10 % for 10 min at 10°C in incubation medium containing 0.2 mmol l<sup>-1</sup> L-[<sup>14</sup>C]alanine. The cells were rapidly washed free of extracellular radioactivity (6×20 vols of ice-cold incubation medium) by centrifugation, and resuspended in ice-cold medium to a haematocrit of 20 %. Efflux of radiolabelled amino acid was initiated by mixing 1 vol. of the ice-cold cell suspension with 19 vols of pre-warmed (10°C) incubation medium or medium containing the appropriate concentration of non-radioactive amino acid. At pre-determined time intervals (10–60 s), 1 ml portions of cell suspension (final haematocrit 1 %) were removed into ice-cold microcentrifuge tubes containing 0.2 ml of ice-cold *n*-dibutylphthalate and the cells were immediately sedimented below the oil (15 000 *g* for 20 s). A 0.5 ml sample of cell-free supernatant was removed for scintillation counting. The starting intracellular tracer level was measured by processing a sample of the 20 % 'loaded' cell suspension as described for amino acid influx.

#### *Amino acid determinations*

Plasma and red cell samples were deproteinised with 5 % (v/v) sulphosalicylic acid and analysed on an LKB 4400 automated amino acid analyser using the manufacturer's recommended lithium buffer system for physiological fluids.

#### *Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> determinations*

Na<sup>+</sup> and K<sup>+</sup> determinations were made by flame photometry using an EEL flame photometer with appropriate filters and standards. Plasma samples were diluted with deionised water, and cells were pre-washed four times with an excess of ice-cold Na<sup>+</sup>- and K<sup>+</sup>-free solution (333 mmol l<sup>-1</sup> MgCl<sub>2</sub> buffered with 10 mmol l<sup>-1</sup> Mops/Tris, pH 7.5). Cl<sup>-</sup> determinations of plasma or red cells pre-washed four times with an excess of ice-cold Cl<sup>-</sup>-free solution (500 mmol l<sup>-1</sup> sodium methylsulphate buffered with 10 mmol l<sup>-1</sup> Mops/Tris, pH 7.5) were carried out by coulometric titration using a Corning model 920M chloride meter.

### **Results**

#### *Plasma and red cell amino acid, cation and chloride levels*

Concentrations of amino acids in hagfish plasma were within the range normally encountered in mammalian and other higher vertebrate species (Table 1). In marked contrast, the mean red cell content of amino acids in three fish was approximately 100 mmol l<sup>-1</sup> cell water. The intracellular amino acid pool was composed largely of neutral amino acids (87 %), with only minor contributions from acidic (5 %) or dibasic amino acids (8 %). Cell:plasma distribution ratios for individual amino acids ranged from 219, 209 and 173 for alanine,  $\alpha$ -amino-*n*-butyrate and proline, respectively, to 11 and 13 for lysine and arginine. Unlike

Table 1. Amino acid levels in hagfish red blood cells and plasma

Amino acid	Red blood cells (mmol l <sup>-1</sup> cell water)	Plasma (mmol l <sup>-1</sup> )	Ratio (rbc:plasma)
GABA	1.09±0.06	0.020±0.002	55
Proline	16.8±1.8	0.097±0.029	173
Glycine	3.72±1.02	0.115±0.037	32
Alanine	8.99±0.64	0.041±0.008	219
α-Amino-n-butyrate	2.23±0.13	0.011±0.004	203
Serine	4.15±0.50	0.092±0.0165	45
Threonine	7.58±0.70	0.113±0.080	67
Asparagine	1.79±0.09	0.067±0.005	27
Glutamine	6.93±0.47	0.108±0.023	64
Valine	6.27±0.30	0.106±0.042	59
Methionine	2.72±0.38	0.053±0.024	51
Leucine	8.76±1.36	0.159±0.064	55
Isoleucine	3.18±0.24	0.059±0.023	54
Phenylalanine	4.73±1.15	0.075±0.044	63
Tyrosine	4.16±0.67	0.058±0.032	72
Histidine	1.22±0.24	0.014±0.011	87
Lysine	4.00±0.90	0.352±0.135	11
Arginine	3.36±1.00	0.265±0.136	13
Aspartate	1.63±0.49	0.049±0.018	33
Glutamate	3.00±0.48	0.064±0.045	47
Total	96.3	1.92	50

Samples of freshly collected whole blood were centrifuged at 15 000 *g* for 5 min and the separated plasma and cell pellets were extracted with sulphosalicylic acid as detailed in Materials and methods.

Residual plasma trapped in the cell pellets (5 % of total pellet volume) did not contribute significantly to measured intracellular amino acid levels.

Values are means±s.e.m. for three fish.

Only those amino acids present in detectable concentrations (>10 μmol l<sup>-1</sup>) are listed.  
rbc, red blood cell.

teleost and elasmobranch red cells, those from the hagfish contained only small amounts of GABA and no detectable taurine or β-alanine.

Hagfish plasma contained 400 mmol l<sup>-1</sup> Na<sup>+</sup> and 7 mmol l<sup>-1</sup> K<sup>+</sup>, compared with intracellular levels of 17 and 148 mmol l<sup>-1</sup> cell water, respectively (Table 2). These concentrations give almost identical transmembrane ratios of 23 (out:in) for Na<sup>+</sup>, and 21 (in:out) for K<sup>+</sup>. In contrast to red blood cells from higher vertebrate species, a large (threefold) transmembrane Cl<sup>-</sup> gradient was also present.

#### *Amino acid permeability of hagfish red cells*

Fig. 1 shows time courses of uptake by hagfish red cells of a representative series of [<sup>14</sup>C]-labelled amino acids, measured at 10°C in NaCl medium and at an initial extracellular concentration of 0.2 mmol l<sup>-1</sup>. Cells were most permeable to L-alanine and L-leucine, uptake within the first 3 min of the incubation period

Table 2. Cation and chloride levels in hagfish red blood cells and plasma

Ion	Red blood cells (mmol l <sup>-1</sup> cell water)	Plasma (mmol l <sup>-1</sup> )	Ratio (rbc:plasma)
Na <sup>+</sup>	17.2±0.5	400.3±7.4	0.043
K <sup>+</sup>	148.2±6.1	7.0±0.3	21
Cl <sup>-</sup>	150.6±2.6	467.8±4.3	0.32

Values are means±S.E.M. for six fish.  
rbc, red blood cell.

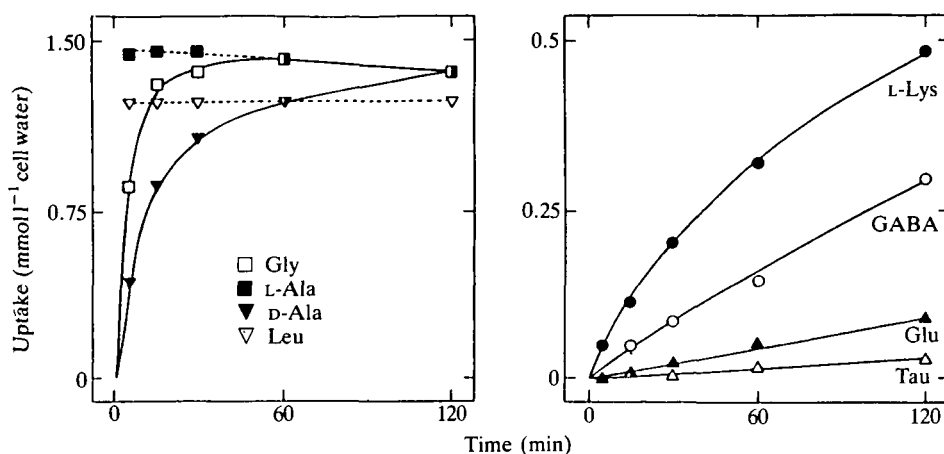


Fig. 1. Time courses of amino acid uptake (initial extracellular [<sup>14</sup>C]amino acid concentration, 0.2 mmol l<sup>-1</sup>) at 10°C in NaCl medium were determined as described in the text. Values are means of duplicate determinations. Lines fitted by eye.

corresponding to the majority of available extracellular amino acid. Alanine transport was partially stereospecific, the D-isomer reaching the same final intracellular level as L-alanine, but with a slower time course. Uptake rates for the other amino acids tested were in the order glycine>L-lysine>GABA>L-glutamate>taurine. After 2 h of incubation, uptake values for the first three of these amino acids were significantly above the starting extracellular concentration of 0.2 mmol l<sup>-1</sup>. Detailed time courses of L-[<sup>14</sup>C]alanine uptake measured at three different initial extracellular concentrations (0.1, 1.0 and 10 mmol l<sup>-1</sup>) are presented in Fig. 2. At the lowest permeant concentration, uptake proceeded rapidly, and at 15 s there was a one-third depletion of extracellular radioactivity. At steady state, the cell:medium [<sup>14</sup>C] distribution ratio was 320. Corresponding distribution ratios at the two higher L-alanine concentrations were 96 (1.0 mmol l<sup>-1</sup>) and 13 (10 mmol l<sup>-1</sup>), with L-[<sup>14</sup>C]alanine uptake values of 12.6 and 66 mmol l<sup>-1</sup> cell water, respectively.

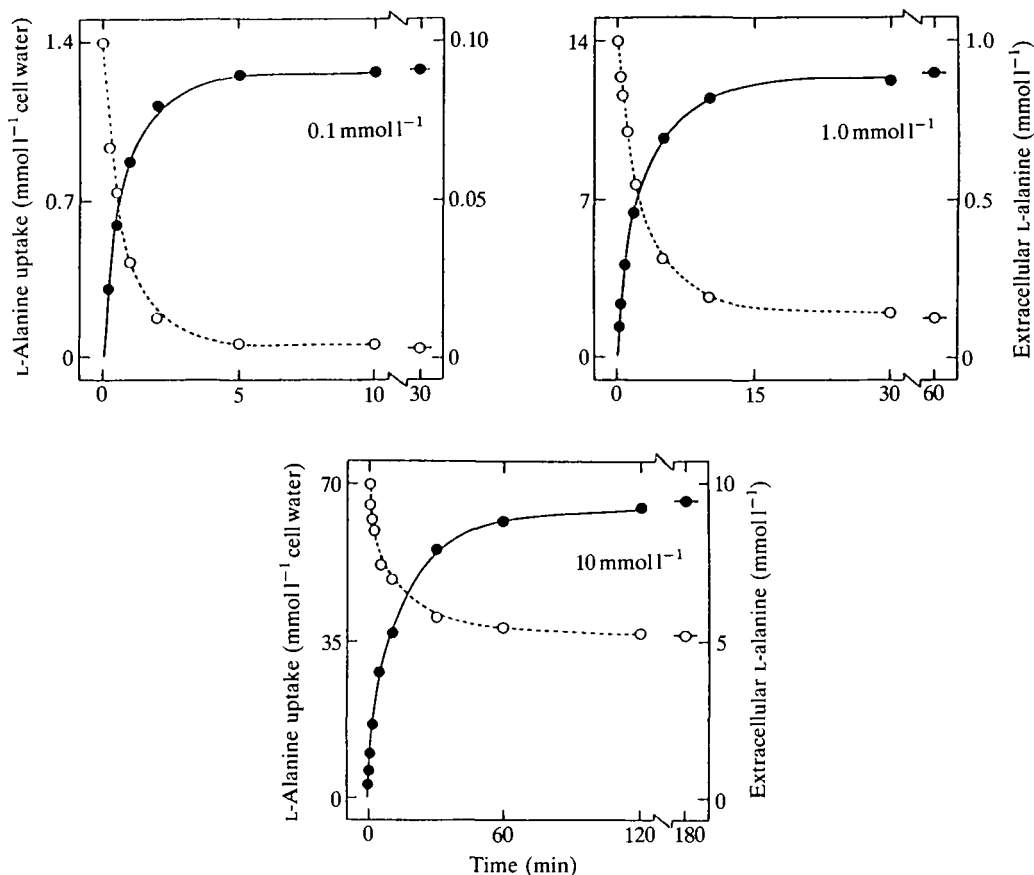


Fig. 2. Time courses of L-[<sup>14</sup>C]alanine uptake by hagfish red cells (●) and depletion of radioactivity from the extracellular medium (○) were determined at 10°C and three different initial extracellular L-[<sup>14</sup>C]alanine concentrations (0.1, 1.0 and 10 mmol l<sup>-1</sup>), as described in Materials and methods. Values are means of duplicate determinations. Lines fitted by eye.

#### *Cation-dependence of amino acid uptake*

To test for Na<sup>+</sup>-dependence of amino acid uptake, the initial rate (5 s flux) and 10 min uptake values for selected amino acids (0.2 mmol l<sup>-1</sup> initial extracellular concentration) were measured both in NaCl medium and in medium where NaCl was iso-osmotically replaced by choline chloride (Table 3). Initial rates of transport for L-alanine, L-glutamine, L-leucine and L-histidine were in the range 66–153 mmol l<sup>-1</sup> cell water h<sup>-1</sup>, with no indication of Na<sup>+</sup>-dependence. In agreement with the results presented in Fig. 1, glycine exhibited a lower rate of transport. This was significantly decreased (24 %) in choline chloride medium, but choline substitution did not affect the 10 min uptake value. L-Proline transport was slow and highly Na<sup>+</sup>-dependent, uptake in NaCl medium being 36 times greater than in choline chloride medium.

Table 3. *Na<sup>+</sup>-dependence of amino acid uptake by hagfish red blood cells*

Amino acid	5 s incubation		10 min incubation	
	NaCl (mmol l <sup>-1</sup> cell water h <sup>-1</sup> )	Choline chloride (mmol l <sup>-1</sup> cell water h <sup>-1</sup> )	NaCl (mmol l <sup>-1</sup> cell water)	Choline chloride (mmol l <sup>-1</sup> cell water)
L-Proline			0.321±0.014	0.009±0.001*
Glycine	17.4±0.6	14.4±0.3*	0.860±0.010	0.860±0.009
L-Alanine	98.0±3.4	98.1±0.3	1.65±0.01	1.65±0.01
L-Glutamine	66.0±4.4	57.6±2.9	1.46±0.01	1.47±0.02
L-Leucine	153±3	150±6	1.60±0.01	1.68±0.01
L-Histidine	141±7	141±7	1.34±0.02	1.40±0.01

Initial rates of L-[<sup>14</sup>C]amino acid uptake (5 s incubation) and 10 min uptake values were measured at 10°C and an initial extracellular concentration of 0.2 mmol l<sup>-1</sup>.

Values are means±S.E.M. of quadruplicate (5 s incubation) or triplicate (10 min incubation) determinations.

Significant differences between NaCl and choline chloride values are indicated by \* (*P*<0.05).

Proline uptake in 5 s was too low to be measured accurately.

For L-alanine, we also investigated transport in media where NaCl was replaced by LiCl or *N*-methyl-D-glucamine chloride and under cation-free conditions (NaCl replaced by iso-osmotic mannitol). None of these substitutions significantly influenced the ability of hagfish red cells to accumulate L-alanine (data not shown). We also considered the possibility that L-alanine uptake by the cells might be proton-dependent. Pre-treatment of cells in NaCl medium for 1 min at 10°C with the proton conductors CCCP (carbonylcyanide-*m*-chlorophenylhydrazone) and FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) (10 μmol l<sup>-1</sup>, 10 % haematocrit) had no significant effect on 5 s or 10 min L-alanine uptake values (data not shown).

Subsequent experiments in the present study focused on the properties of the L-alanine transport mechanism(s) in hagfish red cells.

#### *L-Alanine influx by homo- and heteroexchange*

The remarkable ability of hagfish red cells to achieve cation-independent accumulation of L-alanine from the extracellular medium was confirmed by direct amino acid analysis. In the experiment summarised in Table 4, cells were incubated in the presence and in the absence of 10 mmol l<sup>-1</sup> extracellular L-[<sup>14</sup>C]alanine and the cellular content of individual amino acids and radiolabelled L-alanine were monitored as a function of time. In cells incubated with extracellular L-[<sup>14</sup>C]alanine, cellular [alanine] rose from 5.7 mmol l<sup>-1</sup> cell water at time zero to 59.3 mmol l<sup>-1</sup> cell water after 1 h, an increase of 53.6 mmol l<sup>-1</sup> cell water. The corresponding uptake of L-[<sup>14</sup>C]alanine was 58.5 mmol l<sup>-1</sup> cell water. As shown in Table 4, the large net uptake of alanine was accompanied by a corresponding efflux of other amino acids from the cells, such that the total intracellular amino acid content of 117 mmol l<sup>-1</sup> cell water remained unchanged during the 1 h

Table 4. Exchange of intracellular amino acids with extracellular L-alanine

Time (min)	L-[ <sup>14</sup> C]alanine uptake (mmol l <sup>-1</sup> cell water)	Amino acid concentration (mmol l <sup>-1</sup> cell water)		
		Alanine	Others	Total
0	—	5.7	111	117
2	16.8	20.4	94.9	115
10	39.6	42.1	72.9	115
60	58.5	59.3	57.6	117
60*	—	5.5	103	109

Cells were incubated in NaCl medium at 10°C with 10 mmol l<sup>-1</sup> extracellular L-[<sup>14</sup>C]alanine for 2–60 min and then analysed for intracellular levels of alanine (<sup>14</sup>C-labelled and total) and other amino acids.

\*Control cell incubated in the absence of extracellular L-[<sup>14</sup>C]alanine.

Values are means of duplicate determinations.

Amounts of individual intracellular amino acids (mmol l<sup>-1</sup> cells) exchanged for extracellular L-alanine at 2, 10 and 60 min are given in Fig. 3.

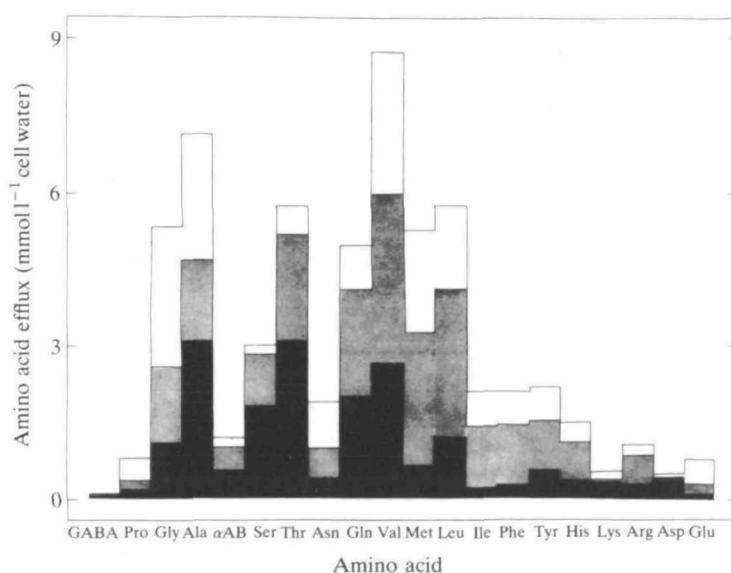


Fig. 3. Values refer to amounts of individual intracellular amino acids exchanged for 10 mmol l<sup>-1</sup> extracellular L-alanine at 2 min (■), 10 min (□) and 60 min of incubation (▒). Please see Table 4 and text for experimental details. αAB, α-amino-*n*-butyrate.

incubation period. During the initial phase of L-alanine uptake, the exchanged amino acids were largely made up of threonine, serine, glutamine, valine and alanine (Fig. 3). Additional amino acids exchanged for extracellular L-alanine during the later stages of the incubation included glycine, methionine and leucine.

Cells incubated for 1 h in the absence of extracellular L-alanine retained 97 % of their intracellular amino acid pool.

#### *L-Alanine efflux*

Efflux of L-[ $^{14}$ C]alanine from pre-loaded cells was used as an alternative way to examine the properties of L-alanine transport in hagfish red cells. Fig. 4 shows that efflux of tracer, measured at 10°C in NaCl medium, was very slow in the absence of extracellular amino acid, but rapid in the presence of external non-radioactive alanine (10 mmol l $^{-1}$  extracellular concentration), the L-isomer causing a greater stimulation of transport than the D-isomer. In another experiment, the initial rate of L-alanine efflux (30 s flux) was measured in the presence of different extracellular concentrations of L- and D-alanine in the range 0.05–10 mmol l $^{-1}$  (Fig. 5). Stimulation of L-[ $^{14}$ C]alanine efflux by extracellular L-alanine was saturable, the maximum response representing a 9.3-fold increase in efflux rate over the control value (efflux measured in the absence of extracellular alanine). The external L-alanine concentration required to give half-maximal stimulation of efflux was 0.25 mmol l $^{-1}$ . Stimulation of L-[ $^{14}$ C]alanine efflux by D-alanine was less effective, with a considerably lower apparent affinity compared with the L-isomer.

The ability of hagfish red cells to participate in exchange reactions was exploited to investigate the substrate specificity of the L-alanine transporter in these cells (Table 5). Extracellular amino acid concentrations of 1 mmol l $^{-1}$  were used. In certain cases, a higher concentration of 10 mmol l $^{-1}$  was employed in attempts to detect amino acids with low, but significant, transporter affinities. Neutral amino acids most effective in stimulating L-alanine efflux (30 s incubation) were L-serine, the L-cysteine analogue L- $\alpha$ -amino-*n*-butyrate and L-alanine followed, in order of

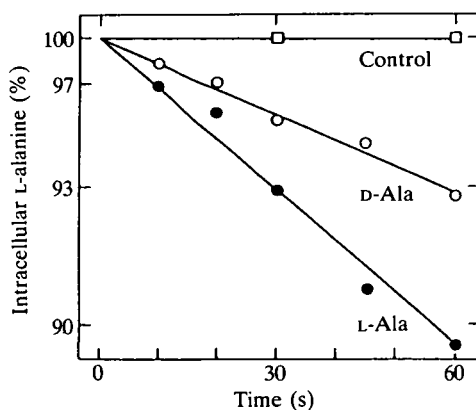


Fig. 4. Cells were preloaded with L-[ $^{14}$ C]alanine as described in Materials and methods. Efflux at 10°C was into amino-acid-free NaCl medium (control) or into medium containing 10 mmol l $^{-1}$  extracellular non-radioactive L- or D-alanine. Values are means of duplicate determinations and are expressed as a percentage of the time zero intracellular [ $^{14}$ C]alanine concentration. Lines fitted by eye.

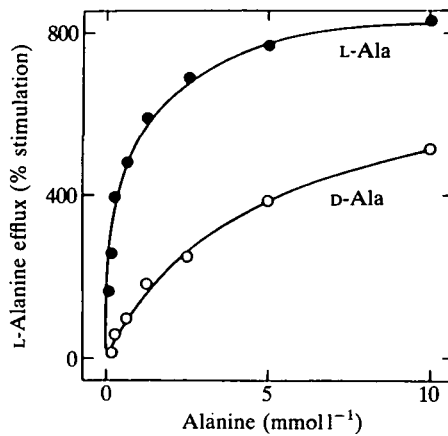


Fig. 5. Cells were preloaded with L-[ $^{14}$ C]alanine as described in Materials and methods. Efflux rates at 10°C (30 s flux) into media containing different concentrations of non-radioactive L- and D-alanine are expressed as percentage increases over the control efflux rate measured in the absence of extracellular amino acid. Values are means of duplicate determinations. Lines fitted by eye.

Table 5. Effects of amino acids on L-alanine efflux from hagfish red blood cells

Amino acid	Efflux rate (% control)	Amino acid	Efflux rate (% control)
L-Proline	(110)	L-Valine	339
Glycine	285 (612)	L-Methionine	164
Sarcosine	(118)	L-Leucine	194 (406)
L-Alanine	562 (830)		
D-Alanine	230	Taurine	88
$\beta$ -Alanine	95		
L- $\alpha$ -Amino- <i>n</i> -butyrate	566	L- $\alpha,\beta$ -Diaminopropionate	335
D- $\alpha$ -Amino- <i>n</i> -butyrate	203	L-2,4-Diaminobutyrate	109
$\alpha$ -Amino- <i>iso</i> -butyrate	162	L-Lysine	(118)
GABA	98	L-Arginine	109 (160)
L-Serine	563 (789)		
L--Glutamine	370	L-Glutamate	93

Cells were preloaded with L-[ $^{14}$ C]alanine as described in Materials and methods.

Extracellular amino acids were present at 1 mmol l $^{-1}$  (or 10 mmol l $^{-1}$ , data shown in parentheses). Results (means of duplicate determinations) are expressed as a percentage of the control efflux rate (30 s flux, 10°C) measured in the absence of extracellular amino acid.

effectiveness, by L-glutamine, L-valine, glycine, D-alanine, D- $\alpha$ -amino-*n*-butyrate, L-leucine, L-methionine and  $\alpha$ -amino-*iso*-butyrate. A modest stimulatory effect (60 % at 10 mmol l $^{-1}$  extracellular concentration) was also observed for the dibasic amino acid L-arginine.  $\alpha,\beta$ -Diaminopropionate, in contrast, caused marked stimulation of L-alanine efflux. At the experimental pH of 7.5, this amino acid

( $pK_2=6.7$ ) is likely to act largely (86 %) as a neutral substrate. The acidic amino acid L-glutamate did not stimulate L-alanine efflux.

To test for the presence of a volume-sensitive amino acid channel in hagfish red cells, cells were pre-loaded with radioactive L-alanine, as described in Materials and methods, and incubated at 10°C for either 30 s or 10 min in 500 mmol l<sup>-1</sup> isotonic NaCl incubation medium or in a series of hypotonic media prepared by 10–50 % (v/v) dilution of 500 mmol l<sup>-1</sup> NaCl medium with water. In contrast to the large and progressive increase in  $\alpha$ - and  $\beta$ -amino acid permeability seen in other fish species, L-alanine efflux from hagfish red cells was unresponsive to changes in medium osmolarity. For example, at an intermediate medium dilution of 25 % (relative cell volume 1.19), L-alanine effluxes were 92 % (30 s incubation) and 103 % (10 min incubation) of control (efflux under isotonic conditions, mean of duplicate determinations). Corresponding values at the maximum medium dilution of 50 % were: relative cell volume 1.45, L-alanine effluxes 98 and 112 % of control, respectively. In separate experiments, it was found that hypotonic cell swelling had no significant effect on the low taurine permeability of hagfish red cells seen in Fig. 1 (data not shown).

### Discussion

In the present series of experiments, we have demonstrated the occurrence of exceptionally high neutral amino acid transport activity and high intracellular levels of amino acids in red cells of the Pacific hagfish (*Eptatretus stouti*), one of the most primitive of living vertebrates. The total intracellular amino acid pool (approx. 100 mmol l<sup>-1</sup> cell water) was 50-fold greater than that of plasma, but in the same range as reported previously for parietal muscle from the closely related Atlantic hagfish *Myxine glutinosa* (Cholette and Gagnon, 1973). In both hagfish cell types, the dominant intracellular amino acid was proline, followed by the other neutral amino acids alanine, threonine, valine and leucine. Plasma:red cell distribution ratios were highest for proline, alanine and  $\alpha$ -amino-*n*-butyrate. Of the amino acids tested, only L-proline and glycine exhibited evidence of Na<sup>+</sup>-dependent transport activity. In other vertebrate species, cation-dependent uptake of these amino acids is mediated by the Na<sup>+</sup>-dependent system ASC and by the Na<sup>+</sup>- and Cl<sup>-</sup>-dependent system Gly (Christensen, 1979; Young, 1983; Harvey and Ellory, 1989). Hagfish red cells have a large (20-fold) inwardly directed transmembrane Na<sup>+</sup> gradient and also a threefold transmembrane Cl<sup>-</sup> gradient (Table 2).

For alanine, uptake by the hagfish red cell was stereoselective for the L-isomer and tightly coupled to amino acid efflux (1:1 stoichiometry). L-Alanine homo-exchange efflux proceeded as expected from simple Michaelis–Menten kinetics, half-maximal stimulation of tracer L-alanine efflux occurring at an extracellular L-alanine concentration of 0.25 mmol l<sup>-1</sup>. As judged by *trans*-acceleration experiments, the L-alanine transport mechanism exhibited a substrate preference for neutral amino acids of intermediate size. This feature, together with the system's

lack of  $\text{Na}^+$ -dependence, identifies it as an *asc*-type transporter similar to systems characterised initially by us in horse and sheep red cells (Young and Ellory, 1977; Fincham *et al.* 1985a, 1987a) and subsequently by others in avian red cells (Vadgama and Christensen, 1985a), mammalian exocrine pancreas (Mann and Peran, 1986) and erythroid cells from foetal rat liver (Vadgama *et al.* 1987).

It has been established that  $\text{Na}^+$  binding to the human red cell *ASC*-system is competitively inhibited by the hallucinogenic alkaloid harmaline and that a topographically equivalent harmaline binding site is also present on horse red cell *asc* (Young *et al.* 1988). In contrast to *ASC*, however, the *asc* site exhibits no measurable affinity for  $\text{Na}^+$ . In preliminary inhibition studies, we have confirmed that harmaline blocks L-alanine uptake ( $0.2 \text{ mmol l}^{-1}$  extracellular concentration,  $10^\circ\text{C}$ ) by hagfish red cells, the concentration required for 50% inhibition ( $3.5 \text{ mmol l}^{-1}$ ) comparing favourably with  $K_i$  values in the range  $1\text{--}3 \text{ mmol l}^{-1}$  for harmaline inhibition of human red cell *ASC* and horse red cell *asc* (Young *et al.* 1988). For *ASC*,  $\text{Na}^+$  and amino acid bind in close juxtaposition, such that the combined ( $\text{Na}^+$ /neutral amino acid) permeation site can be occupied by a dibasic amino acid (Thomas and Christensen, 1970, 1971). Parallel interactions of dibasic amino acids with mammalian *asc* transporters have also been observed (Young *et al.* 1976; Young and Ellory, 1977; Fincham *et al.* 1988). In contrast to harmaline inhibition, this property is poorly expressed in hagfish, as judged by the relative rates of L-alanine and L-lysine uptake by the cells (Fig. 1, Table 3) and by the small effects of dibasic amino acids on L-alanine efflux (Table 5). Studies reported by Vadgama and Christensen (1985b) indicate that dibasic amino acids also interact weakly with the avian red cell *asc*-type transport mechanism.

Based upon the initial rate of L-alanine uptake in Table 3, *asc*-type transport activity in hagfish red cells is  $10^4$ -fold higher than that found in horse and sheep red cells at the same temperature and is correspondingly higher than the *ASC* transport capacity of human and avian red cells (Eavenson and Christensen, 1967; Young *et al.* 1976, 1983; Fincham *et al.* 1987a). Uptake rates for L-leucine and L-histidine were even higher (Table 3). Studies to be published elsewhere indicate that these amino acids are transported by a separate high-affinity *L*-type exchange mechanism. We consider it likely, therefore, that amino acid transporters represent major intrinsic membrane components of hagfish red cell membranes. Since hagfish red cells have minimal numbers of the band 3 anion-exchange transporter (Ellory *et al.* 1987), normally the most abundant red cell membrane protein, these cells represent an ideal model system in which to explore molecular aspects of  $\text{Na}^+$ -independent amino acid transporters. As a first step in this direction, we have established that the hagfish L-alanine transporter, in common with the sheep *asc* system (Young, 1980), possesses a *p*-chloromercuribenzenesulphonate (PCMBs)-reactive thiol group(s) located within its exofacial permeant binding site (D. A. Fincham, M. W. Wolowyk and J. D. Young, unpublished observation). It is anticipated that this cysteine residue will facilitate identification and subsequent isolation of the transporter.

Hagfish plasma is in osmotic equilibrium with the external environment under a

wide range of osmotic pressures (Cholette and Gagnon, 1973). In fish adapted to different salinities, intracellular isosmotic regulation in muscle, and presumably also in red cells, relies, in part, on compensating changes in the intracellular amino acid pool (Cholette and Gagnon, 1973). As detailed in the Introduction, intracellular amino acids also participate in cell volume regulation in elasmobranchs and euryhaline teleosts, but the mechanisms involved appear to differ. For example, the dominant amino acids in elasmobranch (Goldstein and Klein-zeller, 1987) and teleost red cells (Fugelli and Thoroed, 1986; Fincham *et al.* 1987*b*) are  $\beta$ -amino acids, compared with conventional neutral amino acids in hagfish. Also, as reported here, a volume-activated amino acid channel present in these species does not appear to be present in hagfish red cells, exposure to hypotonic medium having no measureable effect on either taurine or L-alanine permeability.

With regard to cellular accumulation of amino acids and their participation in cell volume regulation, it is important to emphasise that both the hagfish *asc* and *L* transporters are  $\text{Na}^+$ -independent and function predominantly in an exchange mode. Thus, the dramatic ability of the hagfish *asc* system to accumulate L-alanine from the extracellular medium occurs at the expense of intracellular amino acids. Possible sources of these amino acids include  $\text{Na}^+$ -dependent uptake from plasma (see above) and, as occurs in invertebrates, intracellular metabolism (Pierce, 1982). In preliminary experiments, we have found that  $\text{Na}^+$ -dependent uptake of L-[ $^{14}\text{C}$ ]proline from the extracellular medium is highly concentrative; the cells achieve a 50-fold transmembrane distribution ratio over a 5 h incubation period (initial extracellular L-proline concentration  $0.2 \text{ mmol l}^{-1}$ ). Competition experiments identified the transport mechanism as an ASC-type system with an unusually high affinity for imino acids. To exclude the unlikely possibility that hagfish red cells accumulate amino acids in exchange for non-amino-acid organic solutes such as sugars, keto acids or nucleosides, we have tested a wide range of more than 50 membrane transport inhibitors for their ability to block L-alanine uptake. With the exception of thiol reagents (see above), none was an effective inhibitor of L-alanine transport (D. A. Fincham, M. W. Wolowyk and J. D. Young, unpublished data). Both  $\text{Na}^+$ -dependent uptake from plasma and intracellular amino acid production/degradation are potential sites for the regulation of intracellular amino acid levels in response to changes in extracellular osmolarity. In this context, *asc* and *L* would facilitate the rapid exchange of amino acids between cells and plasma, serving to modulate the amino acid composition of the intracellular pool and perhaps allowing red cells to participate in inter-organ transport of amino acids (Christensen, 1982). As occurs in mammalian red cells (Young *et al.* 1975; Fisher *et al.* 1986; Fincham *et al.* 1987*a*), the hagfish red cell *asc* transporter may also function to make available plasma cysteine for intracellular glutathione biosynthesis.

In summary, the present series of experiments demonstrates the presence of high concentrations of neutral amino acids and correspondingly high rates of amino acid transport in red cells of the Pacific hagfish. For L-alanine, transport was

mediated by a  $\text{Na}^+$ -independent *asc*-type mechanism, indicating that transporters of this type pre-date, or arose at an early stage of, vertebrate evolution. A volume-sensitive amino acid channel found in higher fish species was not present in hagfish red cells.

We are grateful to the staff of the Bamfield Marine Station for providing laboratory and other facilities. This research was funded by project grants from the Natural Sciences and Engineering Research Council of Canada, The Royal Hong Kong Jockey Club and the University and Polytechnics Grants Committee, Hong Kong. In 1988, DAF was supported as a Research Associate of the Western Canadian Universities Marine Biological Society. JDY is a Heritage Medical Scientist.

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