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TRANSPORT OF NEUTRAL AMINO ACIDS BY HUMAN ERYTHROCYTES

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The transport of several neutral amino acids by human erythrocytes in vitro was studied. The measurements made included steady-state distributions, kinetics of initial rates of uptake, effects of monovalent cations and anions, general mutual inhibitory interactions, kinetics of inhibitions, effluxes, ability to produce accelerative exchange diffusion, and the inhibitory action of the thiol reagent *N*-ethylmaleimide. The results are interpreted as showing that the human erythrocyte membrane possesses several distinct transport systems for these amino acids, including one Na^+-dependent system and one dependent on both Na^+ and a suitable anion, that are qualitatively similar to those systems previously described in pigeon erythrocytes and mammalian reticulocytes. Quantitatively, however, the systems differ among the different kinds of red cell and a major difference lies in their abilities to produce accelerative exchange diffusion.

Introduction

In a recent review of amino acid transport in red blood cells Young and Ellory [1] pointed out that information on the permeability of red cells to amino acids was surprisingly limited and fragmentary. This situation is emphasised if only human red blood cells are considered and is in marked contrast to the extensive studies of their permeability to both cations and anions and to monosaccharides. At the time of the review the only well-known studies of amino acid transport by human erythrocytes were those of Winter and Christensen [2], Yunis and Arimura [3], Gardner and Levy [4] and Hoare [5,6]. Most of those studies were not intended to be comprehensive in their coverage but they had revealed some apparent discrepancies and had suggested that erythrocytes possessed fewer transport systems than other cells [1]. Young and Ellory noted in particular that "with the possible exception of glycine and L-alanine transport by both human and rabbit red cells... no Na^+ dependence has been observed. The Na^+ dependence in these exceptional cases was only partial,... so that effects other than a direct Na^+ dependence of amino acid transport may have been involved" [1]. Shortly after that review was published we accidentally discovered that clearly Na^+-dependent uptake of some neutral amino acids did occur in human erythrocytes, so that it seemed worthwhile to undertake a more detailed study of amino acid transport by these cells, with particular emphasis on the effects of extracellular Na^+. Since then several, mostly short, papers dealing in part with the same topic have been published. Where they overlap the results presented here are generally in agreement with the findings

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Abbreviation: ICW, intracellular water.

When amino acid names are used without a prefix only the L isomer of optical isomers is referred to.

reported in those papers, which will be considered further in the discussion section.

Methods

Materials. Human blood with standard 'acid-citrate-dextrose' solution added was kindly provided by the haematology department of a hospital; it had been stored at 4°C for 1 to 20 days before use. The erythrocytes were sedimented by centrifugation at about $3000 \times g$ and the plasma and buffy coat removed by aspiration. The cells were washed 3 times by resuspension and sedimentation in a cold isotonic solution, usually 155 mM KCl, and finally suspended in the ratio 1 volume of packed cells plus 0.5–1 volume of solution. The suspension was kept on ice until used. (U-^{14}C)-labelled L-amino acids and [U-^{14}C]sucrose were purchased from The Radiochemical Centre, Amersham, Bucks. Sodium and potassium methylsulphates were obtained from Hopkins and Williams, Romford, Essex. All other chemicals were supplied by the Sigma Chemical (London) Co. Ltd., Poole, Dorset, or by British Drug Houses Ltd., Poole, Dorset, and were of Analar grade whenever possible. All solutions were prepared with water that had been distilled twice from a glass still.

Incubations. Two standard media were used throughout, an Na^+ medium and a K^+ medium. Both contained 2 mM $MgSO_4$ and 15 mM Tris, and were adjusted to pH 7.5 at about 20°C by titration with HCl. In addition the Na^+-medium contained 140 mM NaCl plus 5 mM KCl, whereas the K^+ medium contained 145 mM KCl. For some experiments the NaCl and KCl were replaced by the chloride salt of another monovalent cation, and in others the chloride was replaced by another anion; details are noted in the legends to tables and figures. Amino acids were also added as required, giving final concentrations in the range 0.01–10 mM. The appropriate ^{14}C-labelled amino acids were added to give final concentrations of 0.3–1 μCi/ml.

Two slightly different procedures were used to measure amino acid uptake by the cells. The first was based on that used by Eavenson and Christensen [7] for pigeon erythrocytes and the second was similar to that originally used for sheep erythrocytes by Young et al. [8]. In the former method 0.3 ml of cell suspension was added to 3 ml of incubation medium in a 25-ml conical flask maintained at the required temperature (37°C or 20°C) in a water bath and the mixture was shaken mechanically throughout the incubation. The latter was stopped by pouring the contents of the flask into 4 ml of ice-cold isotonic solution (usually 155 mM KCl) in a previously weighed glass centrifuge tube, with a tapered end. The cells were immediately sedimented by centrifugation at about $3000 \times g$ and 4°C for 1 min. The supernatant fluid was quickly removed by aspiration, the cells were resuspended in 7 ml of ice-cold isotonic solution and then the tube was centrifuged as before, but for exactly 10 min. A sample of the supernatant solution was taken for counting and the rest quickly removed by aspiration and, finally, by absorption into pointed strips of filter paper. The inside of the tube was wiped with tissue paper and then it was weighed again. Then 1.5 ml of cold 5% (w/v) trichloroacetic acid was added, with vigorous mixing, and the denatured material was sedimented by centrifugation. The clear acid extract was removed and a sample taken for counting. Finally the contents of the tube were dried at 110°C overnight and the tube again weighed. From the wet and dry weights of the cells the total water content per g wet weight of cells was calculated. The volume of extracellular water per g wet weight of cells was determined with the use of [^{14}C]sucrose. A 0.3-ml sample of the red cell suspension was added to 7 ml of incubation medium containing 4 mM sucrose (0.2 μCi) in a weighed centrifuge tube, the contents were mixed and then the tube was centrifuged for 10 min and processed exactly as described above. All the radioactivity in the acid extract was assumed to have been extracellular. From the measurements of total water and extracellular water, the volume of intracellular water (ICW) per g wet weight of cells was calculated. The use of special racks to hold syringes, flasks and tubes enabled five simultaneous incubations to be made.

In the second method a 0.1-ml sample of the erythrocyte suspension was added to 1 ml of incubation medium in a 1.5-ml capacity polypropylene conical centrifuge tube. The contents of the tube were mixed initially but only occasionally during the incubation. The latter was stopped by

transferring the tube from the water bath to a mixture of ice and water and then centrifuging it for 20 s in an Eppendorf Microcentrifuge. The supernatant fluid was removed by aspiration and the cells were rapidly washed four times by resuspension and sedimentation in 1 ml of ice-cold isotonic solution (usually 155 mM KCl). After the final centrifugation the supernatant fluid was removed by aspiration. They were then lysed by addition of 0.3 ml of 1% (v/v) Triton X-100 and denatured by mixing with 0.3 ml of 10% (w/v) trichloroacetic acid. The volumes of extra- and intra-cellular water per g wet weight of cells were determined as described above. Trial experiments showed that results obtained from these two procedures were identical.

A modification of the second method was used for incubations of less than 1 min. This consisted essentially of separating the cells from the incubation medium by sedimenting them through a layer of oil [9]. Incubations (at 20°C) were stopped by transferring 0.5 ml of the incubation mixture to an Eppendorf Microfuge tube (vol 1.5 ml) containing 0.5 ml ice-cold isotonic solution layered on top of 0.5 ml of ice-cold dibutylphthalate. The tube was immediately centrifuged at about $10000 \times g$ for 20 s and then the supernatant fluids were removed by aspiration. After the inside of the tube had been wiped with tissue paper the pelleted cells were haemolysed and deproteinised as described above.

The amounts of radioactivity in the various samples were measured by liquid scintillation counting. Where necessary trichloroacetic acid was added to samples such that all samples contained the same final concentration of the acid; quenching was then constant in all samples.

For measurements of the efflux of amino acids from the cells the latter were first incubated with the labelled amino acid in Na^+ medium at 37°C for up to 2 h, then sedimented by centrifugation at 4°C and washed twice in ice-cold isotonic solution (usually 155 mM KCl) to remove extracellular radioactivity. The intracellular radioactivity was measured as described above and efflux was monitored by measurement of the decrease in the intracellular concentration during timed incubations.

Results

Preliminary tests

A series of preliminary experiments were carried out to establish the conditions required for the measurement of amino acid fluxes through the human erythrocyte membrane. The results are summarized here without the supporting data, which are recorded elsewhere [10].

Distinction between uptake and binding. Samples of erythrocytes were incubated with ^{14}C-labelled amino acid in the usual manner and then sedimented and washed by centrifugation. One sample was immediately extracted with trichloroacetic acid and the amount of radioactivity in the acid extract measured. A duplicate sample of cells was lysed with water and the cell membranes sedimented by centrifugation. The supernatant was removed and a known volume extracted with trichloroacetic acid, after which the radioactivity in the acid extract was measured. Comparison of the two samples processed in this way showed that no significant radioactivity was associated with the sedimented membranes. Hence, unless water alone reversed binding, the radioactivity must have come from inside the cells.

Identification of the radioactive material. Acid extracts of erythrocytes that had been incubated separately for 2 h with each of the radioactive amino acids used were analysed by thin-layer chromatography. Each sample produced a single peak of radioactivity and the R_f values of the peaks corresponded to those of the original radioactive amino acids chromatographed at the same time.

Metabolic state of the erythrocytes. In an attempt to ensure that the different batches of human erythrocytes were in a similar metabolic state the washed cells were initially always incubated for 1 h at 37°C in Na^+ medium containing 10 mM adenosine [11]. However, when the uptakes of alanine and leucine were measured with cells treated in this way and compared with the uptake by control, untreated, cells, no differences were detected. Hence the preliminary incubation with adenosine was omitted in later experiments.

Comparison of old and fresh erythrocytes. In a single experiment a sample of fresh erythrocytes was used to compare the uptakes of serine and glycine with that by cold-stored cells. No signifi-

cant differences were detected and cold-stored cells were used for all other experiments.

Initial rate measurements. Time courses for the influx of glycine, alanine, serine and threonine during incubation in Na^+ medium at 37°C showed that good estimates of the initial rates of uptake could be obtained with incubation of 10–20 min when the amino acid concentration was 0.1 mM, and of 5–10 min when the concentration was 4 mM. Hence incubations of 10–20 min depending on the amino acid and its concentration, were used to estimate initial rates of uptake of these amino acids. For valine, leucine and phenylalanine the uptake was linear with incubation times only up to about 40 s at 20°C when the amino acid concentration was 0.2 mM. With 1 mM concentrations it was more difficult to estimate the initial rates, and incubation times of less than 15 s were found to be unreliable whatever technique was used. As a routine, therefore, incubations of 30 s at 20°C were used for these three amino acids.

Na^+-dependent uptake of amino acids

With extracellular concentrations in the range 0.2 to 0.5 mM, influxes of glycine, alanine, serine and threonine were always higher when the erythrocytes were incubated in Na^+ medium than when K^+ medium was used. In contrast, the rates of uptake of valine, leucine, phenylalanine and proline were not significantly affected when K^+ medium was used in place of Na^+ medium. To see whether the observed differences could be attributed to stimulation by Na^+ or inhibition by K^+, the influxes of selected amino acids were measured during incubation in media containing various different cations in place of the Na^+ of K^+. The results in Table I show clearly that the differences were caused by the extracellular Na^+ stimulating influx. The difference in the rates measured in the presence and absence of extracellular Na^+ has therfore been termed Na^+-dependent influx.

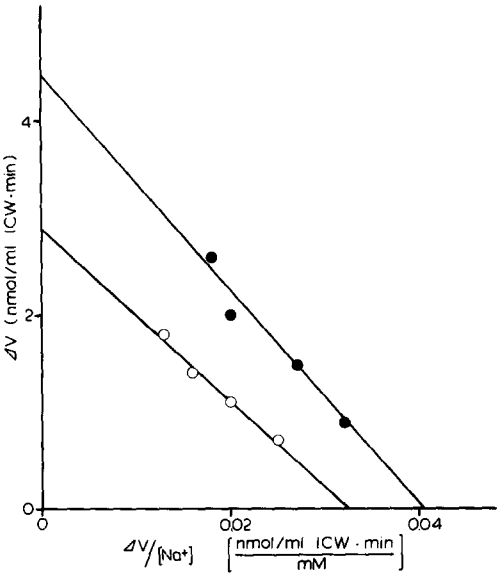

Fig. 1. Variation of influxes of alanine and serine with extracellular Na^+ concentration. The initial rates of uptake of alanine (○) and serine (●) were measured at 37°C in the presence of different Na^+ concentrations as described in the text. Incubation media were prepared by mixing appropriate volumes of Na^+ medium and K^+ medium. The concentration of the amino acids was 0.2 mM. Na^+-dependent influx (ΔV) was calculated by subtraction of values obtained in the absence of Na^+ from those obtained in the presence of Na^+.

TABLE I

EFFECT OF EXTRACELLULAR Na^+ ON AMINO ACID INFLUXES

The initial rates of uptake of glycine (0.5 mM), alanine (0.2 mM) and serine (0.5 mM) were measured at 37°C, as described in the text, but in the indicated media. The initial rate of uptake of leucine (0.2 mM) was measured at 20°C. Each medium contained 2 mM $MgSO_4$ and 15 mM Tris-HCl (pH 7.5 at 20°C). In addition they contained 140 mM NaCl plus 5 mM KCl (Na^+ medium); 145 mM KCl (K^+ medium); 145 mM LiCl (Li^+ medium); 145 mM choline chloride (choline medium); or 175 mM Tris-HCl (pH 7.5) (Tris medium). Mean values from two experiments are given.

Incubation Medium	Influx of amino acid (nmol/ml ICW per min)			
	Gly	Ala	Ser	Leu
Sodium	1.8	3.0	4.8	76
Potassium	1.0	0.8	0.8	78
Lithium	1.0	1.0	1.0	76
Choline	1.2	0.8	0.9	78
Tris	1.2	1.0	0.9	76

Effect of the concentration of Na^+. The Na^+-dependent influxes of alanine and serine were hyperbolic functions of the extracellular Na^+ concentration and the analysis illustrated in Fig. 1 gave values of about 90 mM and 110 mM, respectively, for the concentration of Na^+ necessary to give half maximum fluxes. However, the Na^+-

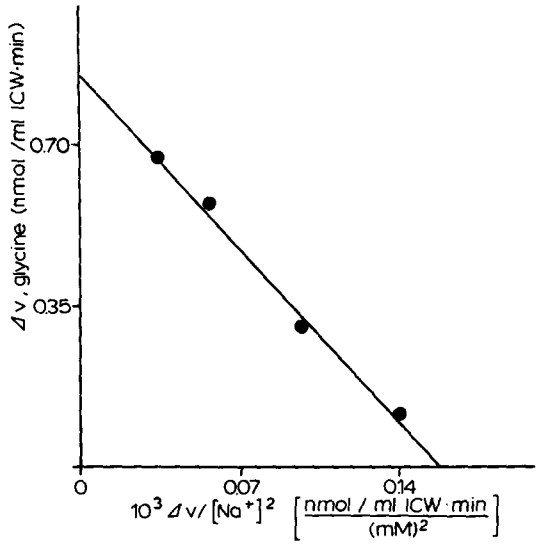

Fig. 2. Variation of glycine influx as a function of the square of the extracellular Na^+ concentration. The experimental details were exactly as described in the legend to Fig. 1, except that glycine uptake was measured.

dependent influx of glycine varied in a sigmoid manner as a function of the Na^+ concentration, and the plot in Fig. 2 shows that the flux was a hyperbolic function of the square of the Na^+ concentration. Half maximum influx required about 75 mM Na^+.

Effects of anions on amino acid fluxes

The influxes of glycine, alanine, serine and leucine were measured during incubations in media containing various anions in place of chloride. No significant effects were detected with alanine, serine or leucine when the chloride in the Na^+ medium was replaced by other anions. Nor were these fluxes affected by changes of the anion in the K^+ medium, except when fluoride was used. The combination of high concentrations of K^+ and F^- produced dramatic increases in the influxes of alanine and serine, but had no effect on leucine influx. In contrast, glycine influx was markedly dependent on the nature of the anion present. All the anions tested except bromide decreased the Na^+-dependent glycine influx and some also caused statistically significant decreases in the Na^+-independent uptake. In the presence of acetate or methylsulphate the Na^+-dependent influx of glycine was very low (Table II). Again the combination of K^+ and F^- had a very large effect.

Kinetics of amino acid influxes

Na^+-independent fluxes. The dependence of the initial rates of uptake of selected amino acids on their extracellular concentrations was tested first in K^+ medium. These rates were low for

TABLE II

EFFECTS OF ANIONS ON GLYCINE INFLUX

Erythrocytes were incubated at 37°C for 30 min in the appropriate media to exchange intracellular Cl^- for the anions to be tested. The media contained the indicated anion in place of the usual Cl^-, except for the buffer which was 15 mM Tris acetate, pH 7.5 at 20°C. The initial rate of uptake of glycine (0.2 mM) was then measured in each medium as described in the text. The mean values given are expressed as a percentage of the influx in the standard Na^+ medium containing Cl^-. Unless otherwise indicated, each value is significantly different from that measured in the Cl^--medium ($P<0.01$).

Main anion	Relative influx of glycine (%)			Number of expts.
	Na^+ medium (A)	K^+ medium (B)	Na^+-dependent (A − B)	
Cl^-	100	37 ± 1	63 ± 1	8
Br^-	81 ± 11	31 ± 1	50 ± 10[a]	3
NO_3^-	51 ± 4	22 ± 2	29 ± 2	3
SCN^-	52[b]	25[b]	27[b]	2
I^-	48 ± 6	23 ± 2	25 ± 4	4
SO_4^{2-}	57 ± 7	40 ± 3	17 ± 1	3
$CH_3SO_4^-$	44 ± 1	31 ± 1	13 ± 1	3
CH_3COO^-	37 ± 2	26 ± 1	11 ± 1	3
F^-	125 ± 7	115 ± 5	10 ± 2	3

[a] Not significantly different from value in Cl^- medium.

[b] Significance not measurable.

TABLE III

KINETIC CONSTANTS FOR THE UPTAKE OF AMINO ACIDS BY HUMAN ERYTHROCYTES

The values were obtained graphically from initial rate measurements, as described in the text. For glycine, alanine, serine and threonine the measurements were made at 37°C and the K_m and V values refer to the Na^+-dependent components of influx, whilst the P values refer to the Na^+-independent components. The measurements for valine and phenylalanine were made at 20°C. Mean values from three experiments are given for alanine, serine and threonine; the rest are from single experiments.

Amino acid	K_m (mM)	V (nmol/ml ICW per min)	P (min^{-1})
Glycine	0.06	0.9	$2.3 \cdot 10^{-3}$
Alanine	0.4	7.0	$6.8 \cdot 10^{-3}$
Serine	0.3	8.3	$3.2 \cdot 10^{-3}$
Threonine	0.09	3.5	$8.1 \cdot 10^{-3}$
Valine	7.1	560	-
Phenylalanine	2.9	690	-

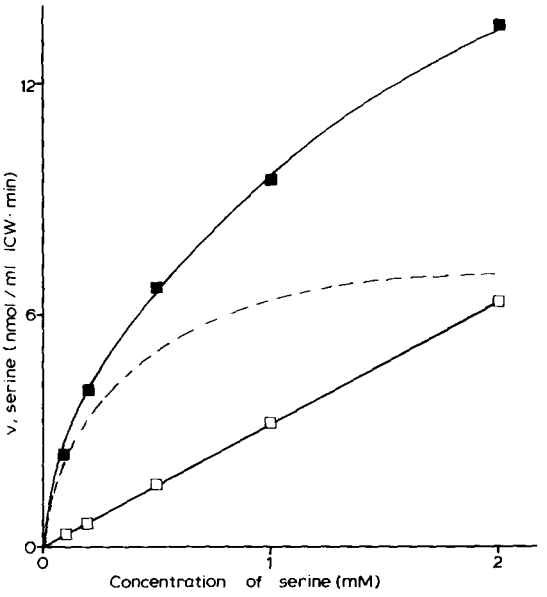

Fig. 4. Concentration dependence of serine influx. The initial rate of serine uptake at 37°C was measured from the indicated concentrations of serine during incubation in Na^+ medium (■) or K^+ medium (□). The Na^+-dependent component of influx is indicated by the broken line.

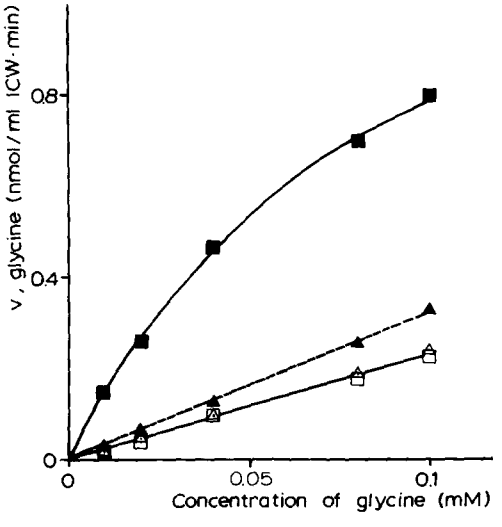

Fig. 3. Concentration dependence of glycine influx. Samples of erythrocytes were separately washed in isotonic solutions of KCl of CH_3COOK and then incubated for 30 min at 37°C in NaCl medium or CH_3COONa medium, respectively. The cells were washed twice and the influx of glycine from the indicated concentrations was measured during incubation in the appropriate media. ■, NaCl medium; □, KCl medium; ▲, CH_3COONa medium; △, CH_3COOK medium.

glycine, alanine, serine and threonine, and they increased linearly with amino acid concentration. Hence they could be described as $V_0 = P$ [A], where V_0 is the initial rate of uptake of the amino acid present at concentration [A] and P is a constant with values in the range 0.002–0.008 min^{-1} (Table III). The influxes of both valine and phenylalanine were much higher and were related to their concentrations in a hyperbolic manner, with no sign of a linear component. Thus these fluxes consisted of a single saturable component which could be described by $V_0 = V$ [A]/(K_m + [A]), where V is the maximum influx at saturating concentrations of A and K_m is the concentration of A required for half-maximum influx. Values of these constants for the uptake of valine and phenylalanine were obtained by the usual graphical analysis and are listed in Table III.

Na^+-dependent fluxes. The influxes of glycine, alanine, serine and threonine from Na^+ medium were not linear functions of their concentrations. Instead the Na^+-dependent components showed simple saturation kinetics, so that the total uptake could be described by: $V_0 = P$[A] + V[A]/(K_m +

[A]). The results for glycine and serine are illustrated in Figs. 3 and 4, respectively, and the values of all the kinetic constants measured in this way are given in Table III. Fig. 3 also shows the kinetics of glycine uptake in the presence of acetate instead of chloride. There was a small Na^+-dependent component of uptake in the presence of acetate that increased linearly with glycine con-

TABLE IV

INHIBITORY INTERACTIONS AMONG AMINO ACIDS

Na^+-dependent influxes of glycine, L-alanine and L-serine were calculated from measurements of the initial rates of uptake of ^{14}C-labelled amino acids (0.2 mM) in both Na^+- and K^+-media at 37°C. The measurements were repeated in the presence of each of the indicated unlabelled amino acids (10 mM) and the fluxes expressed as a percentage of the corresponding control values. The influxes of L-leucine and L-phenylalanine were measured similarly, but only in Na^+ medium and at 20°C. Mean values from three to seven experiments are given for the Na-dependent fluxes but for clarity the S.E. have been omitted. For these three amino acids values marked * are not significantly different from the control, all other values being significantly different ($P<0.05$, or better, by the Student's *t*-test). The values for leucine and phenylalanine fluxes are means from two experiments. Full details of the results and statistical analysis are given in Ref. 10. AIB, α-aminoisobutyric acid.

Inhibitor (10 mM)	Relative influx (%) of				
	Gly	L-Ala	L-Ser	L-Leu	L-Phe
None	100	100	100	100	100
Glycine	18	47	52	100	100
Sarcosine	23	70	85	102	110
N-Acetylglycine	56	101 *	94 *	94	106
L-Alanine	82 *	9	3	95	115
D-Alanine	76 *	66	93 *	103	108
β-Alanine	89 *	99 *	89	112	117
N-Methyl-DL-alanine	38	82	98 *	97	108
N-Acetyl-L-alanine	56	77 *	80	88	106
β-Chloro-L-alanine	61	3	3	106	93
L-Serine	80 *	6	3	95	96
D-Serine	61	39	64	100	107
L-Cysteine	71	3	2	71	76
AIB	92 *	82	101 *	93	96
N-(Methylamino) AIB	79	87 *	99 *	103	100
L-Threonine	77	3	6	86	88
D-Threonine	56	34	45	120	109
L-Homoserine	53	1	5	89	103
L-Valine	89 *	26	34	46	37
D-Valine	97 *	75	87	88	92
L-Norvaline	59	3	17	35	26
L-Methionine	64	5	22	42	37
D-Methionine	67 *	58	80	91	84
L-Proline	64	43	39	117	103
D-Proline	61	82	108 *	79	109
L-Hydroxyproline	59	12	13	105	98
L-Leucine	73	16	22	24	25
D-Leucine	92 *	73	92 *	49	55
L-Isoleucine	82	25	38	29	29
D-Isoleucine	62	73	80	96	97
L-Norleucine	52	13	9	–	–
D-Norleucine	89 *	79 *	110 *	94	78
N-Methyl-L-leucine	82 *	82	98 *	–	67
N-Acetyl-L-leucine	76 *	105 *	103 *	110	86
L-Histidine	65	65	85 *	113	103
L-Phenylalanine	68	35	52	19	16
D-Phenylalanine	88 *	64	80	83	82
L-Tryptophan	45	21	30	93	87

centration. A separate experiment (not shown) indicated that this component remained a linear function of glycine concentration up to at least 6 mM glycine.

Inhibitory interactions among amino acids

The results of an extensive series of inhibition experiments are summarized in Table IV. Only the Na^+-dependent fluxes of glycine, alanine and serine are considered because no significant changes in their Na^+-independent fluxes were observed. Although these data show that the uptake of each amino acid was subject to inhibition by a considerable number of other amino acids, so that no absolutely clear-cut divisions occurred, some general trends are discernable and the obvious ones will be pointed out here.

Glycine influx was markedly inhibited only by sarcosine but was considerably inhibited by several other amino acids which do not constitute any obvious group in terms of size, polarity or optical isomers. However, it is noteworthy that *N*-acetylglycine, *N*-methyl-DL-alanine, *N*-acetyl-L-leucine and *N*-(methylamino)isobutyric acid each produced substantial inhibition of glycine influx but had no or very little effect on the uptakes of the other amino acids tested.

The influxes of both alanine and serine were significantly inhibited by most of the other amino acids, the exceptions being the *N*-acetyl and *N*-methyl derivatives, α-aminoisobutyric acid, D-proline and D-norleucine. Stereospecificity was quite marked, the L isomers always causing more inhibition than the corresponding D-isomers. It is also apparent tha the presence of a polar group, such as -OH, -SH, -Cl or -S-, in the inhibiting amino acid enhanced its effect. This is shown particularly by comparison of the inhibitions caused by the D isomers (e.g. D-alanine and D-serine) and by L-proline and L-hydroxyproline.

Leucine and phenylalanine influxes were not inhibited by as many amino acids as were the fluxes of alanine and serine. As with the latter, the *N*-methyl and *N*-acetyl derivatives had virtually no effect. On the other hand, those amino acids that caused most inhibition of alanine and serine fluxes generally caused little inhibition of leucine and phenylalanine fluxes. There were, however, a few amino acids that inhibited the influxes of all four of these to considerable and similar extents, the main ones being L-valine, L-norvaline, L-methionine, L-leucine, L-isoleucine and L-phenylalanine. Stereo specificity was again clearly evident, with the L-isomers being much more effective inhibitors than the corresponding D-forms.

The relative inhibitory effects of all the amino acids tested on the influxes of pairs of amino acids are illustrated in Fig. 5. The influxes of alanine

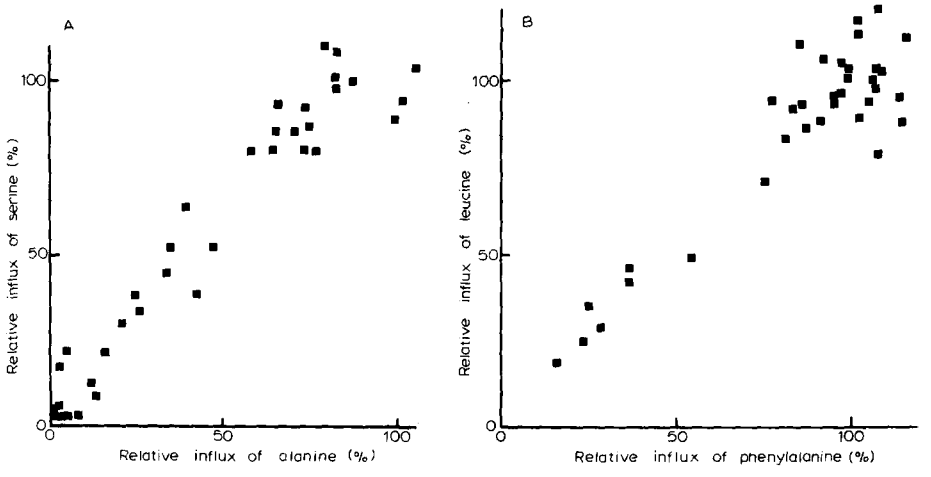

Fig. 5. Correlations between the relative influxes of alanine and serine and of leucine and phenylalanine. The data in Table IV for these two pairs of amino acids are plotted to illustrate the correlations. Linear regression analysis gave correlation coefficients of 0.96 for (A) and 0.93 for (B). There was no such general correlation between the fluxes of any other pair from the five tested.

and serine were closely correlated, as were those of leucine and phenylalanine; but no general correlations were apparent for any other pair from the five studied. (The correlation coefficients for the pairs glycine–serine, glycine–phenylalanine and serine–phenylalanine were 0.15, 0.26 and 0.28, respectively).

Kinetics of inhibitory interactions. The inhibitory actions of selected amino acids on the influx of L-phenylalanine and the Na^+-dependent influx of L-serine were examined kinetically in two series of experiments. In one the influxes of the latter amino acids from two different concentrations were measured in the presence of various concentrations of the inhibiting amino acid, the results being plotted according to Dixon [12]. In the other experiments the concentrations of serine and phenylalanine were varied in the presence and absence of the inhibiting amino acid and the data were analysed by the Eadie-Hofstee method [13]. The results obtained are summarized in Table V. Phenylalanine influx was inhibited competitively by L-methionine, L-valine and L-leucine. For both methionine and leucine the apparent K_i values of 4.1 mM and 2.6 mM, respectively, are close to their K_m values for uptake of 5.2 mM [2] and 2.0 mM [6] reported in the literature. The apparent K_i value of 12.9 mM for valine is, however, somewhat higher than its K_m value of 7.1 mM (Table III). Serine influx was inhibited competitively by glycine, L-alanine, L-threonine, L-methionine, L-proline and L-hydroxyproline. The apparent K_i values for alanine and threonine were close to their K_m values for uptake (Tables V and III); but the apparent K_i value for glycine was much higher than its K_m value, whilst the apparent K_i value for methionine was much lower than its K_m value (Tables III and V, and Ref. 2). No K_m values are available for proline and hydroxyproline but the lower apparent K_i for hydroxyproline, compared with that for proline (Table V) again shows that the presence of the polar -OH group enhances interaction with this transport system.

The data for phenylalanine and leucine as inhibitors of serine uptake indicated that these inhibitions were kinetically complex. For leucine both plots gave non-linear lines, whereas for phenylalanine only the Dixon plot was curved. The Eadie-Hofstee plot for phenylalanine indicated a mixed type of inhibition that decreased the V_m and

TABLE V

SUMMARY OF KINETIC ANALYSIS OF INHIBITORY INTERACTIONS

The inhibitory actions of the indicated amino acids on the Na^+-dependent influx of L-serine and the influx of L-phenylalanine were analysed kinetically as described in the text.

Influx of	Inhibitor	Type of inhibition	Apparent K_i (mM)
L-Ser	Gly	Competitive	2.5
L-Ser	L-Ala	Competitive	0.2–0.3
L-Ser	L-Ser	Competitive	0.1–0.3
L-Ser	L-Thr	Competitive	0.2
L-Ser	L-Met	Competitive	0.8
L-Ser	L-Pro	Competitive	5.4
L-Ser	L-Hyp	Competitive	2.5
L-Ser	L-Val	Mixed [a]	(2.2) [a]
L-Ser	L-Leu	Mixed	–
L-Ser	L-Phe	Mixed	–
L-Phe	L-Met	Competitive	4.1
L-Phe	L-Val	Competitive	12.9
L-Phe	L-Leu	Competitive	2.6

[a] One method indicated competitive inhibition, see text.

TABLE VI

EFFECTS OF EXTRACELLULAR AMINO ACIDS ON THE EFFLUXES OF GLYCINE, ALANINE AND SERINE

Washed erythrocytes were incubated with ^{14}C-labelled glycine (1.1 mM), alanine (2 mM) or serine (1.1 mM) for 2 h in Na^+ medium. The cells were separated from the medium and washed twice by resuspension and centrifugation before being incubated in Na^+ medium or in Na^+ medium containing the indicated unlabelled amino acid at 10 mM. Effluxes were calculated from the concentrations of labelled amino acids remaining in the cells after 30 min incubation at 37°C. The initial intracellular concentrations were: glycine, 0.16; alanine, 0.95 mM; serine, 0.41 mM.

Extracellular amino acid	Efflux (nmol/ml ICW per min) of		
	Glycine	Alanine	Serine
None	0	7.0	0
Glycine	0	7.0	–
Alanine	–	8.3	–
Serine	–	10.0	3.3
Valine	0	6.0	2.5
Leucine	–	5.0	–
Phenylalanine	–	2.7	–

TABLE VII

EFFECTS OF EXTRACELLULAR AMINO ACIDS ON THE EFFLUXES OF VALINE, LEUCINE AND PHENYLALANINE

The experiments were performed as described in Table VI, except that the initial incubations were for 1 h with labelled valine (1.1 mM), leucine (1.1 mM) or phenylalanine (2 mM) and the effluxes were calculated from the concentrations remaining in the cells after incubation at 20°C for 2 min (leucine) or 5 min (valine and phenylalanine). The initial intracellular concentrations were: valine, 0.83 mM; leucine, 0.2 mM; phenylalanine, 1.7 mM.

Extracellular amino acid	Efflux (nmol/ml ICW per min) of		
	Valine	Leucine	Phenylalanine
None	26	18	80
Glycine	26	–	–
Alanine	38	–	60
Methionine	62	–	120
Valine	52	–	120
Leucine	60	36	120
Phenylalanine	54	–	100

increased the apparent K_m for serine influx. With valine, although the Dixon plot was consistent with competitive inhibition, giving an apparent K_i of about 2.2 mM, the Eadie-Hofstee plot was similar to that for phenylalanine, indicating a mixed type of inhibition.

Exchange fluxes

Accelerative exchange diffusion, as defined by Stein [14], characterizes most of the Na^+-dependent fluxes of amino acids in pigeon erythrocytes [7,15] and most of the Na^+-independent fluxes of amino acids in Ehrlich ascites tumour cells [16]. To see if this phenomenon occurs in human erythrocytes experiments were carried out with selected amino acids under conditions that should have been optimal for demonstrating it. The effluxes of ^{14}C-labelled amino acids in the presence and absence of relatively high concentrations of extracellular unlabelled amino acids were measured. The results for those amino acids showing Na^+-dependent uptake are shown in Table VI and those for amino acids whose uptake was Na^+-independent in Table VII.

In the absence of extracellular amino acids no efflux of either glycine or serine could be detected in Na^+- or K^+-media during incubations of up to 1 h. However, a significant efflux of alanine was measurable after 30 min incubation in Na^+ medium, and it continued for at least 2 h. The addition of extracellular amino acids did not induce any efflux of glycine; but both serine and valine stimulated serine efflux in the presence of extracellular Na^+ (Table VI) but not in its absence (results not shown). Alanine efflux into Na^+ medium was similarly increased by extracellular serine, and slightly by alanine, but not by glycine. In contrast, extracellular phenylalanine clearly inhibited alanine efflux, and both leucine and valine appeared to cause slight inhibition.

The effluxes of valine, leucine and phenylalanine, like their influxes, were much more rapid (Table VII). Valine efflux was clearly stimulated by the presence of extracellular methionine, valine, leucine and phenylalanine, but glycine had no effect and alanine elicited only a marginal effect. Parallel results were observed for phenylalanine efflux, though the percentage increases were much lower. Extracellular leucine doubled the efflux of leucine.

Effects of N-ethylmaleimide on amino acid influxes

Exposure of erythrocytes to the thiol reagent *N*-ethylmaleimide before measuring amino acid influx in the absence of free *N*-methylmaleimide resulted in inhibition of the uptakes of glycine, serine and phenylalanine. There was, however, a marked difference in the inhibitions of these three amino acid fluxes. Although glycine and serine were similar in that only their Na^+-dependent components of uptake were inhibited, the effect of the concentration of *N*-ethylmaleimide on these two fluxes was quite different. Na^+-dependent glycine influx was inhibited maximally about 80% by treatment of the cells with 0.2 mM *N*-ethylmaleimide, and half-maximum inhibition required only about 0.12 mM (Fig. 6A). In contrast, Na^+-dependent serine influx was inhibited only about 67% by 5 mM *N*-ethylmaleimide (Fig. 6B). Extrapolation of the curve in Fig. 6B indicates that 100% inhibition would result with a sufficiently high concentration of *N*-ethylmaleimide and that about 2 mM would give half-maximum inhibition. With phenylalanine, 5 mM *N*-ethylmaleimide caused about 55% inhibition and the curve showed

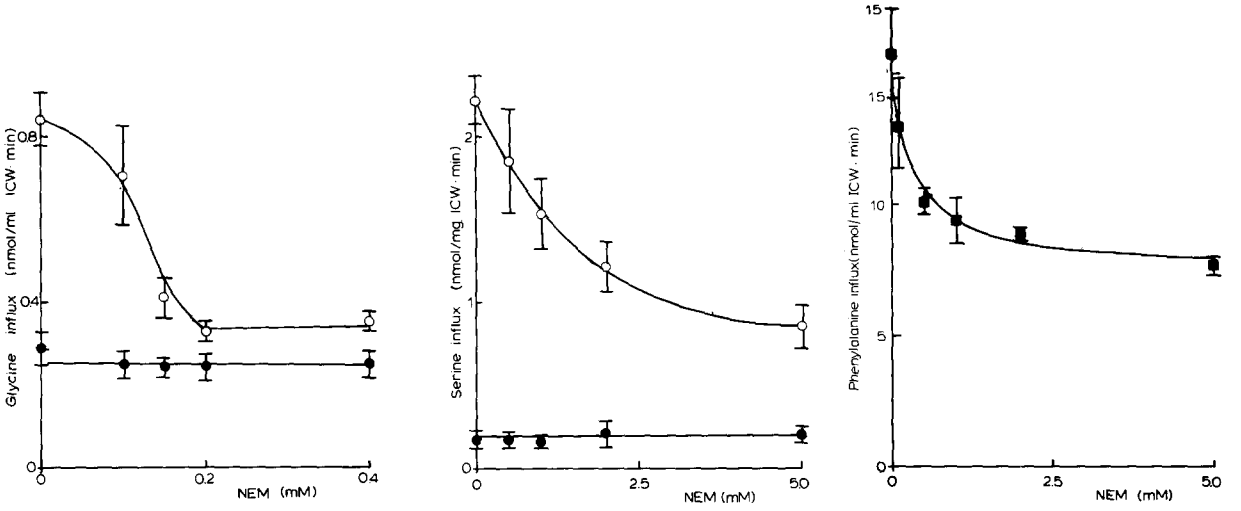

Fig. 6. Inhibition of amino acid influxes by *N*-ethylmaleimide (NEM). Erythrocytes were incubated at 37°C for 60 min in Na^+ medium containing the indicated concentrations of NEM. The cells were separated from the medium and washed twice in Na^+ or K^+ medium by resuspension and centrifugation. The influxes of (A) 0.1 mM glycine and (B) 0.1 mM serine were then measured during incubations at 37°C in Na^+ medium (○) and K^+ medium (●). Phenylalanine influx (C) was measured from a 0.1 mM concentration in Na^+ medium at 20°C. Mean values (±S.E.) are given from four experiments.

that this was almost the maximum inhibition that could be obtained (Fig. 6C). Half-maximum inhibition was given by about 0.25 mM *N*-ethylmaleimide.

To see if the amino acids could protect against the action of *N*-ethylmaleimide, 10 mM glycine was added to the medium containing 0.2 mM *N*-ethylmaleimide during the preliminary 60-min incubation in one experiment and 10 mM serine was added with 1 mM *N*-ethylmaleimide in another. Neither amino acid prevented the inhibitory effect of the thiol reagent. Similarly, the action of the reagent on the Na^+-dependent uptake of glycine was the same regardless of whether the cells were exposed to the *N*-ethylmaleimide in Na^+ medium or K^+ medium.

Steady-state intracellular concentrations

Uptake of glycine from Na^+-medium at 37°C increased linearly with time for at least 3 h, so that there was no sign of a steady-state concentration being reached inside the cells. With 0.5 mM extracellular amino acid the internal concentration reached only about 0.25 mM. Under the same conditions the uptakes of both serine and threonine began to level off after 2 h incubation, giving intracellular concentrations of about 0.3 mM. The rate of alanine uptake decreased only slightly between 2 and 3 h incubation, reaching about 0.35 mM inside the cells. In contrast, steady-state intracellular concentrations of valine, leucine and phenylalanine were attained after about 1 h incubation at 20°C, and with extracellular concentrations of 0.2 mM the intracellular concentrations reached about 0.3 mM.

Discussion

Effects of extracellular Na^+

Qualitatively the observed effects of extracellular Na^+ on the uptake of neutral amino acids by human erythrocytes were remarkably similar to those reported for both mammalian reticulocytes and pigeon erythrocytes. Various different mammalian reticulocytes show Na^+-dependent uptake of glycine [3,17,18] and alanine [3,17,19], whilst pigeon erythrocytes take up several amino acids in a Na^+-dependent manner, including glycine [7,15,20–22], alanine [7,15,21,22], serine [7,15,22–24] and threonine [22,24]. This similarity also extends to the nature of the responses to the concentration of Na^+, with glycine influx varying in a sigmoid manner as a function of Na^+ concentration (Fig. 2 and Refs, 15, 20 and 22) but the other

amino acid fluxes in a hyperbolic fashion (Fig. 1 and refs. 7, 15, 17, 22 and 23). Quantitatively, however, the Na^+-dependent fluxes of these amino acids in human erythrocytes differed from those in reticulocytes and pigeon erythrocytes. First, under similar conditions, the concentration of Na^+ required for half-maximum stimulation of the fluxes was generally higher in the human erythrocytes. For alanine and serine the difference was 4- to 5-fold (Fig. 1 and Refs. 7, 15 and 17) while for glycine it was 1.5- to 1.9-fold (Fig. 2 and Refs. 15 and 17). Second, the absolute values of the fluxes were lower in human erythrocytes. Thus, again under similar conditions, V values for Na^+-dependent uptake of glycine, alanine and serine were 10 to 30 times lower in human erythrocytes (Table III) than those in rabbit reticulocytes [17] and pigeon erythrocytes [7]. Third, the ratio of Na^+-dependent to Na^+-independent fluxes was lower in human erythrocytes. This difference arose because the Na^+-independent fluxes in human erythrocytes were only 3- to 10-times lower than those in the other cells (Table III and Refs. 7 and 17). The combined effect of these quantitative differences in response to extracellular Na^+ is to make Na^+-dependent fluxes of amino acids much more difficult to detect and to quantify in the mature human red cells than in reticulocytes or pigeon erythrocytes, especially if the concentrations of the amino acids used are much above the K_m values for the Na^+-dependent fluxes. Hence failure to detect significant Na^+-dependent amino acid transport in mature mammalian red cells could easily result from failure to choose the appropriate experimental conditions, as well as from use of an inappropriate amino acid. Thus it is noteworthy that Wise [25], using an amino acid concentration of 1 mM, could not detect Na^+-dependent transport of alanine or α-aminoisobutyric acid by rat erythrocytes, whereas Yunis and Arimura [3], using a concentration of 0.1 mM, did detect Na^+-dependent uptake of glycine, alanine and α-aminoisobutyric acid by rat erythrocytes, and of glycine and alanine by human erythrocytes. A similar small Na^+-dependent component of uptake of both glycine and alanine by mature rabbit red cells was observed by Wheeler and Christensen [17], who used an amino acid concentration of 0.2 mM.

Comparisons of the transport of amino acids by both reticulocytes and erythrocytes from rat [3,25], human [3], rabbit [17,19,26] and sheep [18] have emphasized the notion that Na^+-dependent transport is a characteristic property of the immature cells, and is lost as the cells mature [18,26]. Hence Na^+-dependent transport of amino acids by mature mammalian red cells was considered to be either non-existent or relatively unimportant [1]. However, the results described above for human erythrocytes support and extend the more recent findings of Ellory and his colleagues, who have shown that these cells transport alanine [27,28], cysteine [28,29] and glycine [30] in a Na^+-dependent manner. Since these Na^+-dependent components of uptake are significant when amino acid concentrations typical of physiological levels are used [27,28], it seems that they should not be dismissed as unimportant. Moreover, the well-known advantages of human erythrocytes, compared with reticulocytes and avian red cells as well as all other cells, for all kinds of studies of membrane structure and function are further enhanced by these findings.

Effects of extracellular anions

The sensitivity of Na^+-dependent glycine uptake by human erythrocytes to the nature of the anion in the extracellular medium (Table II) confirms the recent findings of Ellory et al. [30] and parallels results obtained with pigeon erythrocytes [31,37]. The relative efficacies of the various anions tested were also similar in both kinds of cell. Similarly, the lack of effect of anions on Na^+-dependent alanine and serine uptake by human erythrocytes parallels both indirect [31] and direct [37] evidence for the anion-independence of these fluxes in pigeon erythrocytes. The increased permeability of human erythrocytes to K^+ in the presence of high concentrations of F^- is well documented and the underlying mechanism of the F^- effect is both complex and unclear [11]. Our findings show that F^- increases the membrane's permeability to amino acids as well as to K^+, and a separate experiment also showed that permeability to sucrose was increased. The lack of effect of F^- on leucine influx can be attributed to the short incubations (30 s) and the lower temperature (20°C) used for these experiments.

Interpretation in terms of distinct transport systems

Taken together the results presented here provide good evidence for the presence of a number of distinct amino acid transport systems in the human erythrocyte membrane. Also, the characteristics of these systems correspond closely with the general concepts developed by Christensen and his colleagues for the identification of separate permeation routes in a variety of cells and tissues [32,33] so hat the same terminology may be applied.

ASC system. This transports amino acids such as alanine, serine, threonine and cysteine and is characterized by its absolute requirement for Na^+, its insensitivity to the nature of the accompanying anion, its preference for L isomers and its inability to transport *N*-methylated amino acids. The first two characteristics were shown directly and the last two may be inferred from the inhibition data in Table IV. As pointed out above, the differences between this ASC system and those in pigeon erythrocytes and mammalian reticulocytes are mainly quantitative, rather than qualitative; but the major difference is that of exchange. Accelerative exchange diffusion via the human erythrocyte system is so limited (Table VI) that it can be of little or no importance, whereas such exchange characterizes the ASC systems of the reticulocyte [17] and the pigeon erythrocyte [7,15,22,24]. In fact, the latter system might well operate solely as an exchanging system [34,37]. It is also notable that no transport of proline, valine, leucine or phenylalanine via the ASC system could be detected in the human erythrocytes, although each of these inhibited Na^+-dependent serine transport (Tables IV and V), in contrast to the pigeon cells, where some Na^+-dependent uptake of these amino acids was observed [7]. However, the very low capacity of the ASC system in the human cells compared with that of the L system (see below) could render Na^+-dependent uptake of, for example, valine too small a fraction of its total uptake to be detected as significant. The ability of valine to produce a small stimulation of serine efflux (Table VI) does suggest that valine can interact with the ASC system in more than an inhibitory fashion.

Gly system. This is considered to be specific for glycine and is characterized by its absolute requirement for both Na^+ and a suitable anion, its ability to transport, or interact with, sarcosine and *N*-ethylglycine, and its inability to produce accelerative exchange diffusion. Although these characteristics were fulfilled in general terms by the results described above, we did also observe some statistically significant inhibitions of Na^+-dependent glycine influx by several other amino acids (Table IV). One possible explanation for some of those observations is that small quantities of glycine were present as contaminants of the 'inhibitory' amino acids. For example, from the data in Table III and the conditions used for the inhibition experiments, an inhibition of 10% would be expected if the 'inhibitory' amino acid contained only 0.3% glycine, a possibility we could not assess. Another source of limited inhibition is the possibility that Na^+-dependent uptake of glycine in the presence of acetate represented uptake via the ASC system, so that solutes such as alanine or serine would be expected to inhibit that component, which would also be present with chloride as the main anion. In agreement with this interpretation was the finding that with 0.02 mM glycine all of the Na^+-dependent influx in the presence of acetate was inhibited by the addition of alanine or serine, but sarcosine had no effect. This interpretation also seems to be in keeping with the competitive inhibition of serine influx by glycine, with a K_i value of 2.5 mM (Table V). Similar results and the same interpretation have recently been recorded by Ellory et al. [30]. However, an apparent anomaly persists in that the Na^+-dependent glycine influx in the presence of acetate was a linear function of glycine concentration up to at least 6 mM glycine (Fig. 3 and text), which is not in keeping with an apparent K_m value of about 2.5 mM. At present the only explanation is that this component of glycine uptake was too small for departure from linearity to be detected.

L system. This transports amino acids such as valine, leucine and phenylalanine and is characterized by its lack of requirement for Na^+, its insensitivity to the nature of the anion present and its stereospecificity. Again the quantitative properties of this system differ from the corresponding system in pigeon erythrocytes. Both the K_m and

the V values (table III) were very much larger than those recorded for the pigeon cell [7], which were similar to those for the Gly and ASC systems in human cells. Another difference concerned the ability of this system to cause accelerative exchange diffusion. No such exchange was detected in pigeon erythrocytes [7] but Na^+-independent accelerative exchange of valine was shown by rabbit reticulocytes [17,19]. The results in Table VII indicate that the L system of human erythrocytes can produce accelerative exchange diffusion, which is in keeping with Hoare's [5,6] demonstration that V for exchange of leucine was greater than V for leucine entry. It also appears that the inhibition data in Table IV and Fig. 5 that the L system in human erythrocytes is somewhat more selective than L systems in other cells and tissues [32] and the failure of tryptophan to produce much inhibition of the uptakes of leucine of phenylalanine can be explained by the recent demonstration of the existence of a specific 'T' system in these cells [35].

Apparently non-saturable uptakes

The Na^+-independent uptakes of glycine, alanine, serine and threonine could not be distinguished from simple diffusion under the conditions used here. However, the results obtained by Young et al. [28] using high concentrations of alanine indicate that these fluxes are mediated and probably represent uptake via the L system, for which these amino acids have extremely high K_m values (> 100 mM).

Effects of N-ethylmaleimide

The differential inhibitory effects of *N*-ethylmaleimide on the uptakes of glycine, serine and phenylalanine (Fig. 6) provide further support for the existence of distinct Gly, ASC and L systems. (The particularly high sensitivity of the Gly system to this thiol reagent could prove valuable in helping to characterize the system in terms of membrane protein components.) However, the fact that concentrations of *N*-ethylmaleimide sufficient to inhibit phenylalanine influx by 50% had no effect on the Na^+-independent uptakes of either glycine or alanine is difficult to reconcile with these components representing uptake via the L system, so that a more extensive study of the action of thiol reagent on these systems could be useful.

Although mature erythrocytes require an adequate supply of cysteine [29] it is not obvious why they should possess transport systems for most amino acids and the simplest explanation for their continued existence is that their rates of degradation are slow compared with the average life span of the cells. However, it is now clear that there is considerable variation among the amino acid transport systems of red cells from different species [18,28,36] so that it remains possible that the degradation of these systems is in some way controlled. It is also possible that red blood cells always serve to provide a circulating pool of amino acids, absorbing then when the plasma concentrations are high and releasing them when the plasma concentration falls, so acting as an amino acid 'buffer' system.

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