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Characterization of peptide fluxes into human erythrocytes

A proton-n.m.r. study

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A new protocol for measuring cellular uptake of dipeptides was developed in which the problem of peptide hydrolysis is obviated by introduction into the cell suspension of a membrane-permeant peptidase inhibitor. The uptake of unlabelled dipeptide is readily monitored so long as some analytical technique is available for measuring the intracellular peptide concentration; in this study we used n.m.r. spectroscopy. Using this protocol, we demonstrated that dipeptide uptake by human erythrocytes occurs by simple diffusion through the lipid bilayer and not via a high-capacity protein-mediated transport system. Substantiating evidence includes demonstration that: (*a*) the fluxes are slow compared with known protein-mediated transport processes in human erythrocytes; (*b*) the uptake is not stereospecific; (*c*) the uptake does not display saturation kinetics; (*d*) the fluxes are significantly enhanced by butanol; (*e*) a distinct correlation exists between the size-corrected permeability coefficients of the dipeptides and their calculated n-octanol/water partition coefficients. It is calculated that under normal physiological conditions the diffusive fluxes of circulating plasma peptides into human erythrocytes are too small for these cells to play a significant role in dipeptide catabolism.

INTRODUCTION

The existence of saturable membrane transport systems for the uptake of di- and tri-peptides has been proven for several mammalian tissues, including intestinal epithelium (Boyd & Ward, 1982), kidney (Ganapathy & Leibach, 1986) and placenta (Ganapathy *et al.*, 1985). However, the question whether mammalian erythrocytes are capable of facilitated peptide transport remains unresolved. Early n.m.r. studies on concentrated suspensions of human erythrocytes, in which the (apparent) intracellular rate of peptide hydrolysis was used as an indirect measure of the rate of peptide uptake, seemed to indicate that these cells possess low-affinity high-capacity peptide-transport systems (King & Kuchel, 1984, 1985; Vandenberg *et al.*, 1985). However, conventional flux experiments employing low-haematocrit erythrocyte suspensions failed to confirm these findings (Young *et al.*, 1987); it was suggested in the latter work that the peptide-uptake rates measured by n.m.r. spectroscopy were artifactually high as a consequence of cell lysis resulting from the high haematocrits and extended incubation times used in these experiments. This caveat was highlighted in a subsequent n.m.r. study (Kuchel *et al.*, 1987).

The present paper aims to clarify the nature of dipeptide uptake by human erythrocytes. Clearly, unequivocal demonstration of the existence of peptide-transport systems in these cells would have important implications for our understanding of their physiology and for providing potential pharmacological access to their intracellular environs. We have combined n.m.r. spectroscopy with conventional flux methodologies to measure the rate of uptake of dipeptides by human erythrocytes. These results have enabled the mechanism of uptake to be established and the role of erythrocytes in peptide turnover to be assessed.

EXPERIMENTAL

Materials

3-Trimethylsilyl[2,2,3,3-^{2}H]propionate was from Fluka A.G. (Buchs, Switzerland). [2,3,4,6,6-^{2}H]Glucose was supplied by Merck, Sharp and Dohme (Pointe Claire-Dorval, Que., Canada). Amastatin, bestatin and all dipeptides were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were analytical-reagent grade. In-date plasma-depleted blood (stored in citrate/phosphate/dextrose preservative solution) was supplied by the John Radcliffe Hospital, Oxford, U.K.

Preparation of erythrocyte suspensions

Human erythrocytes were prepared from plasma-depleted whole blood by repeated centrifugal washing ($\times 2$, 1500 *g*) with 3 vol. of ice-cold 0.9% NaCl followed by three similar treatments with ice-cold Krebs bicarbonate buffer (Krebs & Henseleit, 1932). Care was taken to remove the buffy coat, containing most of the leucocytes and platelets, after the first centrifugation. Preliminary experiments indicated that complete removal of leucocytes by filtering through a cotton-wool column (Beutler *et al.*, 1976) made no difference to the measured rates of peptide uptake or hydrolysis: this step was subsequently omitted. The final packed cell suspension (haematocrit approx. 0.8) was used directly for peptide-uptake experiments. When required, haemolysates were prepared from the packed erythrocyte suspension by a single freeze–thaw cycle (−12 °C/20 °C).

Preparation of re-sealed ghosts

Re-sealed ghosts of human erythrocytes were prepared by a modification of the procedure of Richards & Eisner (1982). Briefly, packed erythrocytes were lysed by dilution in 100 times their own volume of a hypotonic solution containing 10 mM-Mops, 3 mM-Na_3ATP and 3 mM-$MgCl_2$. Reagents to be trapped inside the re-sealed ghosts were then added before the introduction of sufficient NaCl to restore isotonicity. The suspension was then incubated at 37 °C for 20 min with constant stirring. The slightly pinkish re-sealed ghosts were recovered by centrifugation at 15000 *g* for 20 min.

Haemoglobin assay

Haemoglobin concentrations were assayed by using the tetramethylbenzidine procedure (Geissler & Stith, 1978). Absorbances were measured on a Uvikon 810P spectrophotometer, and

Abbreviation used: Cbz, benzyloxycarbonyl.

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dilutions of fresh venous blood, assumed to have a haemoglobin concentration of 330 mg/ml, were used for calibration. The molar absorption coefficient of the haemoglobin adduct was calculated to be 63400 $M^{-1} \cdot cm^{-1}$.

Peptidase-inhibition studies

The inhibitory effect of amastatin and bestatin on dipeptide hydrolysis by human erythrocytes was determined as follows. To a 5 mm-outer-diameter n.m.r. tube was added 400 μl of haemolysate and the required concentration of inhibitor. After incubation of the tube at 37 °C for 30 min, dipeptide was added from an approximately isotonic 300 mM stock solution, pH 7.2±0.1. The samples was then rapidly placed in the n.m.r. probe, and 1–2 min was allowed for thermal equilibration before the rate of peptide hydrolysis was monitored by using proton spin-echo n.m.r. spectroscopy as described previously (King *et al.*, 1983; King & Kuchel, 1984, 1985). Reaction time was calculated as the period from peptide addition to the midpoint of each spectral acquisition. Peptide and amino acid concentrations were calculated as described previously (King *et al.*, 1986) by multiplying the peak amplitudes of relevant resonances by their best-fit n.m.r. extinction coefficients.

Peptide-uptake studies

A 600 μl suspension of human erythrocytes containing 10 mM-glucose, 200 μM-bestatin and, when required, 100 mM-butanol was added to a microcentrifuge tube: the mixture was then incubated at 37 °C for 45 min to facilitate equilibration of bestatin across the erythrocyte membrane (see the Results section). In cases where the n.m.r. resonances of fully protonated glucose would have obscured those of the dipeptide being studied (e.g. Gly-Gly), [2,3,4,6,6-^{2}H]glucose replaced the fully protonated form. Uptake time courses were initated by the addition of dipeptide; when solubility permitted, this was from an approximately isotonic 300 mM stock solution, pH 7.2±0.1. After incubation at 37 °C for 0, 1, 2, 3 or 4 h, the cell suspensions were washed (×5) by centrifugation for 10 s at 12000 *g* (Eppendorf Microcentrifuge 5415) after dilution in 1.0 ml of ice-cold Krebs bicarbonate buffer containing 200 μM-bestatin. Washing was initially performed at 4 °C to minimize the possibility of dipeptide efflux, but uptake was found to be slow enough to assume that this would be negligible even at room temperature. The peptide and amino acid contents of the washed erythrocytes were then determined in one of two ways.

(i) Cells were lysed by a single freeze–thaw cycle and proton spin-echo n.m.r. spectra were acquired of the resulting haemolysate.

(ii) An equal volume of ice-cold 8% (v/v) $HClO_4$ was added to the cells. Debris was removed by centrifugation at 12000 *g* (Eppendorf Microcentrifuge 5415) for 10 min, and the resulting supernatant was neutralized by addition of 5 M-KOH. The precipitated $KClO_4$ was removed by centrifugation (12000 *g* for 5 min) and the supernatant was adjusted to pH 7.2±0.1 before analysis by means of single-pulse n.m.r. spectroscopy.

The use of $HClO_4$ extracts is time-consuming, but it obviates the n.m.r. problems associated with paramagnetic deoxyhaemoglobin, which accumulates during long-term storage of haemolysates. Peptide and amino acid concentrations in $HClO_4$ extracts were determined by the use of suitably prepared calibration curves (Beilharz *et al.*, 1984).

Prolidase (proline dipeptidase, EC 3.4.13.9) is exclusively responsible for the hydrolysis of *C*-terminal prolyl dipeptides (Endo *et al.*, 1982); bestatin has no inhibitory effect on the erythrocyte enzyme (G. F. King, unpublished work). Thus, for uptake studies with Ala-Pro, the 200 μM-bestatin in the cell suspensions and wash solution was replaced by 20 mM-*N*-benzyloxycarbonyl-L-proline (Cbz-Pro); this compound is a reasonably potent inhibitor of prolidase (K_i approx. 90 μM) and millimolar concentrations equilibrate across the human erythrocyte membrane in a few minutes (King *et al.*, 1989). Because of its rapid permeation of erythrocytes, only 15 min incubation at 37 °C (cf. 45 min for bestatin) was allowed to facilitate its transmembrane equilibration.

Proton-n.m.r. measurements

All spectra were recorded at 500 MHz on a Bruker AM 500 spectrometer operating at 37 °C in the Fourier mode. Spectra of haemolysates were acquired by using the spin-echo pulse sequence with a τ value of 65 ms and pre-irradiation of the water resonance as described previously (King & Kuchel, 1984). The spin-echo pulse sequence enables resonances from large immobile biomolecules such as haemoglobin and membrane phospholipids to be selectively excluded on the basis of their shorter transverse relaxation times, leaving an edited spectrum containing only resonances from small mobile metabolites such as the dipeptides and amino acids of interest in the present study (Brown *et al.*, 1977). Spectra of $HClO_4$ extracts of erythrocytes were obtained by using a simple single-pulse sequence with pre-irradiation of the water resonance.

The resonance of 3-trimethylsilyl[2,2,3,3-^{2}H]propionate, contained in a coaxial insert within the n.m.r. tube as a 0.025% solution in ^2H_2O, was used as the chemical-shift resonance at 0.00 p.p.m. in all experiments. The ^2H_2O in the insert was used for field/frequency locking. Resonance amplitudes were normalized with respect to the *N*-methyl resonance of ergothioneine, an endogenous metabolite whose concentration is time-invariant (Isab & Rabenstein, 1979).

Calculation of partition coefficients and molecular volumes

Dipeptide n-octanol/water partition coefficients ($K_{n\text{-octanol}}$) were calculated *ab initio* by using the FRAGMENT method of Hansch & Leo (1979). Briefly, this procedure assigns constant values to a small number of fundamental functionalities and then attempts to incorporate factors resulting from particular structural features (e.g. unsaturation, interacting hydrogen-bonding groups) that are considered to affect the partitioning in a predictable manner. The final $K_{n\text{-octanol}}$ value may thus be thought of as arising from an equation of the form:

$$\log K_{n\text{-octanol}} = \sum_{1}^{n} a_n f_n + \sum_{1}^{m} b_m F_m + \sum_{1}^{r} SF_1 \qquad (1)$$

where a is the number of occurrences of the fundamental fragment f of type n, b is the number of occurrences of factors F of type m that influence the partitioning, and SF are special 'once-only' factors applied for special structural features such as aliphatic chain branching. Hansch & Leo (1979) have shown that this procedure yields accurate $K_{n\text{-octanol}}$ values for a wide range of organic molecules, including ions.

The volume of a dipeptide that is impenetrable to thermal collision (often referred to as the 'van der Waals volume') was calculated by using the equations given by Bondi (1964); however, we found that less than 1% error was introduced by simply using the tables of functionality volumes given by the author.

RESULTS

Inhibition of human erythrocyte dipeptidase activity

In theory, indirect measurement of dipeptide uptake by human erythrocytes by observation of the rate of appearance of n.m.r. resonances from their hydrolytic products is an elegant technique (see, e.g., King & Kuchel, 1984). In practice, it suffers from a

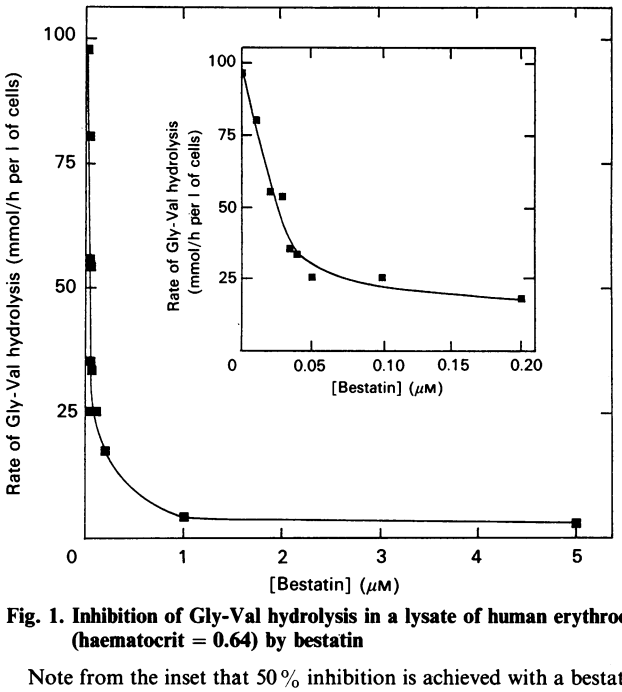

Fig. 1. Inhibition of Gly-Val hydrolysis in a lysate of human erythrocytes (haematocrit = 0.64) by bestatin

Note from the inset that 50% inhibition is achieved with a bestatin concentration of approx. 0.03 μM. The continuous lines are visual fits to the data.

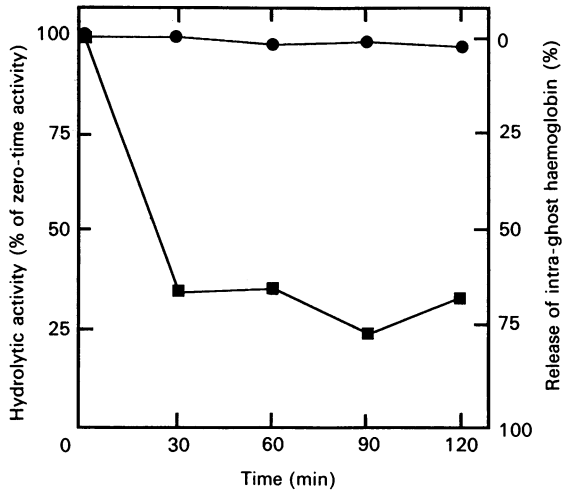

Fig. 2. Efflux of bestatin (1 μM) from re-sealed ghosts of human erythrocytes

The Gly-Val-hydrolysing activity of a haemolysate to which extra-ghost medium had been added, presented here as a percentage of the activity obtained at zero time (■), was used as a measure of bestatin efflux. The percentage release of intra-ghost haemoglobin (●) served to indicate the extent of ghost lysis. Note that bestatin appears to have equilibrated across the ghost membrane within 30 min.

potential artifactual contribution to the measured flux rate from haemolytically released peptidases (Young *et al.*, 1987). That is, if peptide degradation also occurs outside the cell, then the rate of hydrolysis no longer reflects the transport rate. An impermeant inhibitor(s) of erythrocyte peptidases would obviate the potential problem (Odoom *et al.*, 1988). We chose to assess the microbial peptidase inhibitors amastatin and bestatin for this task since, on the basis of charge and size considerations, it appeared unlikely that human erythrocytes would be highly permeable to them.

Since amastatin and bestatin are both slow tight-binding inhibitors (Rich *et al.*, 1984), it was expected that a preincubation of the inhibitor with haemolysate before addition of dipeptide would be required to achieve maximum inhibitory potency. This notion was tested by examining the effect of bestatin on Gly-Leu and Gly-Val hydrolysis in a haemolysate after preincubation periods ranging from 0 to 3 h. The inhibitory potency was found to be independent of the preincubation period (results not shown); however, to ensure that maximum inhibition was achieved, a preincubation period of 30 min was used in all experiments.

Fig. 1 illustrates the potent inhibitory effect of bestatin on the hydrolysis of Gly-Val by a haemolysate; the IC_{50} (concentration causing 50% inhibition) is approx. 0.03 μM and it is clear that bestatin concentrations greater than about 1 μM lead to almost complete inhibition of dipeptide hydrolysis. Amastatin was a much less potent inhibitor of erythrocyte dipeptidase activity. For example, the IC_{50} with Gly-Gly, a substrate that is poorly hydrolysed by erythrocytes, was only approx. 55 μM. Thus amastatin was not considered to be sufficiently potent to overcome the problem of haemolytically released peptidases in n.m.r. time courses of peptide uptake, and was not considered any further in this study.

Permeability of human erythrocytes to bestatin

The lack of suitably radiolabelled bestatin makes difficult the direct observation of its uptake by human erythrocytes at micromolar extracellular concentrations. The following indirect approach was thus used to examine the permeability of erythrocytes to bestatin. Re-sealed ghosts of human erythrocytes containing 1 μM-bestatin were prepared and immediately incubated at 37 °C. At 30 min intervals over a period of 2 h, samples of the supernatant were taken and examined for their ability to inhibit the hydrolysis of Gly-Val in a lysate of human erythrocytes. From the IC_{50} curve (see Fig. 1), and taking into account various dilution factors, the extent of bestatin efflux from the ghosts could be assessed. The loss of intra-ghost haemoglobin, which is impermeant to erythrocyte membranes, provided a suitable endogenous marker of ghost lysis.

Fig. 2 shows the hydrolytic activity of a haemolysate, to which ghost supernatant has been added, as a function of the ghost incubation time; the hydrolytic activity has been normalized by setting the value obtained by addition of the 0 min supernatant to 100%. Addition of the supernatant from a 30 min ghost incubation causes an approx. 70% decrease in hydrolytic activity, indicating substantial release of intra-ghost bestatin. Over the same time period, as indicated in Fig. 2, the release of intra-ghost haemoglobin is minimal, indicating that the efflux of bestatin from the ghosts is a true permeability phenomenon and not the result of random ghost lysis. Since the inhibitory potency of ghost supernatants does not increase significantly with longer incubation times, it appears that micromolar concentrations of bestatin are capable of equilibrating across the human erythrocyte membrane within 30 min. A reasonably high $K_{n\text{-octanol}}$ value of 0.11 was calculated for bestatin, suggesting that the rapid flux of this molecule into human erythrocytes may be due to simple passive diffusion through the lipid bilayer.

Uptake of dipeptides by human erythrocytes

Owing to its rapid permeation of human erythrocytes, bestatin cannot be used as an exclusively extracellular inhibitor of erythrocyte dipeptidase activity and therefore cannot be employed to negate the problem of haemolytically released peptidases in direct n.m.r. time courses of erythrocyte peptide uptake. However, its potent inhibition of erythrocyte dipeptidase activity, in combination with its rapid transmembrane equilibration, indicates that it can be used in conventional 'bench'

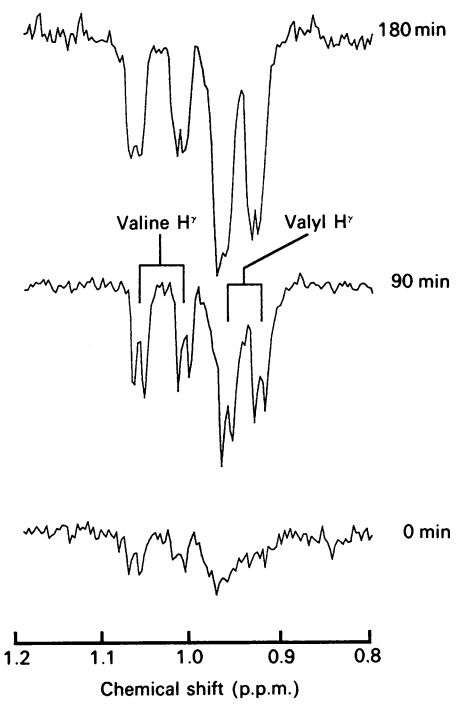

Fig. 3. Proton spin-echo n.m.r. spectra (0.8–1.2 p.p.m. region) of haemolysates from an experiment in which human erythrocytes had been incubated with 15 mM-Gly-Val

The only time-dependent spectral changes are the appearance of dipeptide-valyl H$^{\gamma}$ resonances and an increase in intensity of the valine H$^{\gamma}$ resonances, indicating accumulation of Gly-Val by the erythrocytes and subsequent intracellular dipeptide hydrolysis. Data from the dipeptide-glycyl H$^{\alpha}$ and glycine H$^{\alpha}$ resonances were less reliable because of the proximity of numerous glucose resonances, and consequently they were not used in the calculation of flux rates.

experiments to inhibit fully both intra- and extra-cellular dipeptide hydrolysis. Thus the rate of uptake of an unlabelled dipeptide by erythrocytes can be readily measured so long as some analytical technique is available for monitoring the intracellular dipeptide concentration as a function of time. Clearly, this methodology is not as elegant as the 'on-line' n.m.r. technique, but it does enable measurement of the uptake of unlabelled substrates without the complications of hydrolysis.

We developed a protocol in which 200 μM-bestatin was allowed to equilibrate across the membrane of human erythrocytes before measurement of the uptake of dipeptides by these cells (see under 'Peptide-uptake studies' in the Experimental section); the dipeptide-uptake rate was calculated by using n.m.r. spectroscopy to analyse the intracellular peptide concentration. Although n.m.r. is intrinsically insensitive (only intracellular dipeptide concentrations greater than about 50 μM can be detected), it has the advantage of allowing simultaneous observation of amino acid concentrations (to assess the potency of bestatin or Cbz-Pro) and the metabolic status of the cell (e.g. glucose, lactate and GSH concentrations).

Fig. 3 shows a small region of a typical series of spin-echo n.m.r. spectra of haemolysates obtained from an experiment monitoring the uptake of 15 mM-Gly-Val; only three of the six time points used in the flux calculation are shown. Accumulation of dipeptide by the erythrocytes is evidenced by the appearance with time of dipeptide-valyl H$^{\gamma}$ resonances at 0.96 and 0.93 p.p.m. At zero time there are valine H$^{\gamma}$ resonances at 1.06 and 1.01 p.p.m. due to endogenous concentration of this amino acid; however, the amplitude of these resonances increases with time, indicating that intracellular hydrolysis has not been completely inhibited by bestatin (analysis of supernatants indicated that extracellular hydrolysis was completely inhibited during the flux experiment). The increase over the endogenous concentration of intracellular amino acid appeared to result from dipeptide degradation during prolonged storage after the flux experiment was completed, as it could be minimized by analysing haemolysates as soon as possible after the 'bench' experiment and by storing them at −20 °C in the intervening period.

The methodology just described was used to measure the rate of uptake of a variety of dipeptides by human erythrocytes. These rates are summarized along with the calculated $K_{n\text{-octanol}}$ and molecular volumes for these dipeptides in Table 1; also listed are the flux rates for some dipeptides in the presence of 100 mM-butanol. The measured rate of uptake of Gly-Gly (see Table 1) is slightly lower than the value of 70 μmol/h per l of cells obtained previously by Young *et al.* (1987) using an extracellular

Table 1. Molecular volumes, $\log K_{n\text{-octanol}}$ values and rates of uptake by human erythrocytes for various dipeptides

Dipeptide	Rate of uptake by human erythrocytes (μmol/h per l of cells)* No butanol	Rate of uptake by human erythrocytes (μmol/h per l of cells)* +100 mM-butanol	$\log K_{n\text{-octanol}}$†	Molecular volume‡ (cm^3·mol^{-1})
Gly-Phe	252±45	–	−3.03	122.1
Gly-Leu	156±11	–	−3.09	110.5
Gly-D-Leu	153±11	–	−3.09	110.5
Ala-Pro	106±20	171±16	−3.52	102.3§
Gly-Val	102±15	145±22	−3.62	100.3
Gly-Gly	56±10	73±19	−5.12	69.5
Ala-Glu	27±9	45±19	−7.49	116.6
Gly-Glu	< 10	24±8	−7.75	106.4
Gly-Lys	< 10	–	−7.86	117.7

* At 37 °C and 15 mM extracellular dipeptide concentration.
† Calculated by using the FRAGMENT method of Hansch & Leo (1979).
‡ Calculated by using the equations given by Bondi (1964).
§ Includes a correction for pyrrolidine-ring crowding of −1.1 cm^3·mol^{-1}.

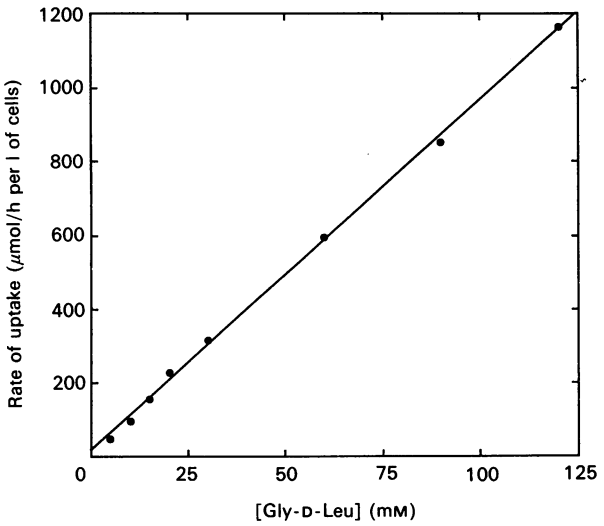

Fig. 4. Concentration-dependence of the rate of uptake of Gly-D-Leu by human erythrocytes

The continuous line represents the least-squares best fit of a straight line to the dataset, which yielded a slope of 9.52 and a correlation coefficient of 0.999.

dipeptide concentration of 10 mM; the small discrepancy may be due to the activity of haemolytically released peptidases in the latter study.

Concentration-dependence of dipeptide uptake by human erythrocytes

In a separate series of experiments, the initial Gly-D-Leu concentration was varied in order to determine the concentration-dependence of the rate of uptake of this dipeptide by human erythrocytes; these data are shown in Fig. 4. Least-squares regression of a straight line on to the dataset clearly indicates a linear relationship between flux and extracellular dipeptide concentration.

DISCUSSION

The existence or otherwise of peptide-transport systems in human erythrocytes has never been demonstrated conclusively. However, the peptide-uptake rates presented here, which were obtained with a new protocol that is essentially free from any artifactual contributions, provide strong evidence that the human erythrocyte does not possess a high-capacity peptide-transport system. We propose that most of the peptide uptake by human erythrocytes occurs by simple passive diffusion for the following reasons.

(i) The measured dipeptide flux rates (Table 1) are much smaller than the rate constants for known protein-mediated transport processes in human erythrocytes. For example, glycine is transported into the human erythrocyte more slowly than any other amino acid and yet its flux into these cells (Young & Ellory, 1977) is about 15 times faster than that of Gly-Phe, which has the highest uptake rate of any of the dipeptides we examined.

(ii) The dipeptide fluxes are non-stereospecific, as evidenced by identical uptakes rates for the D- and L-isomers of Gly-Leu (Table 1). This contrasts dramatically with the marked stereospecificity of intestinal dipeptide-transport systems (e.g., Boyd & Ward, 1982).

(iii) Butanol is an anaesthetic-like molecule that is capable of increasing membrane fluidity, thereby enhancing passive diffusion of molecules while usually inhibiting or sometimes having no effect on protein-mediated transport processes (Lieb & Stein, 1986). Butanol was found to increase substantially the permeability of human erythrocytes to all dipeptides on which it was tested (Table 1), providing direct evidence for the passive nature of the dipeptide fluxes.

(iv) The rate of uptake of Gly-D-Leu was found to be linearly dependent on the extracellular dipeptide concentration over the entire range tested, 0–120 mM (Fig. 4). The absence of saturation kinetics seems to preclude the existence of a specific protein-mediated transport system; this would only be possible if the K_m were so large ($\gg$ 120 mM) as to question any physiological relevance.

There is one final line of evidence that argues strongly in favour of the passive nature of dipeptide uptake by human erythrocytes and simultaneously may provide some insight into the mechanism of diffusion through the lipid bilayer. If dipeptide uptake by human erythrocytes occurs solely by diffusion, one would expect there to be a linear correlation between the logarithm of the dipeptide permeability coefficients and the logarithm of their $K_{\text{n-octanol}}$ values. Lieb & Stein (1986) have demonstrated this relationship for a number of small neutral molecules that are capable of diffusing across the human erythrocyte membrane. This correlation was substantially improved if the permeability coefficients were corrected to account for the size of the diffusant; this procedure is based on the premise that the rates of passive diffusion across cell membranes should be explicable by consideration of both the size and hydrophobicity of the permeant. By using the theoretical treatment proposed by Lieb & Stein (1986), the size-corrected permeability coefficient, $P^{v=0}$, is given by:

$$P^{v=0} = P \cdot 10^{m_v \cdot v} \quad (2)$$

where P is the experimentally measured permeability coefficient ($cm^2 \cdot s^{-1}$), v is the molecular (van der Waals) volume ($cm^3 \cdot mol^{-1}$) and m_v ($cm^{-3} \cdot mol$) is a measure of the size selectivity for diffusion in the chosen solvent; the latter can be calculated as the negative slope of a plot of the logarithm of the diffusion coefficients for the molecules in the chosen solvent versus v.

Lieb & Stein (1986) found that the correlation with size-corrected permeability coefficients was better for $K_{\text{hexadecane}}$ than for $K_{\text{n-octanol}}$. However, since the $K_{\text{hexadecane}}$ values for the dipeptides in the present study are too small to be accurately measured without the use of suitable radiolabels, we chose to use $K_{\text{n-octanol}}$ values in the present study because various methods exist for calculating these values *ab initio* (see under 'Calculation of partition coefficients and molecular volumes' in the Experimental section). Thus Fig. 5 shows a plot of $\log P^{v=0}$ versus $\log K_{\text{n-octanol}}$ in which the ● symbols represent the dipeptides examined in the present study and the ■ symbols represent diffusants investigated in previous work (see Table 2.1 in Lieb & Stein, 1986).

Ignoring the Ala-Glu datum point temporarily, it is clear that there is a significant positive correlation between $\log P^{v=0}$ and $\log K_{\text{n-octanol}}$ for the dipeptides; linear-regression analysis yields a correlation coefficient (r) of 0.97. Furthermore, the dipeptide data lie close to the line describing the relationship between $\log P^{v=0}$ and $\log K_{\text{n-octanol}}$ for the previously studied diffusants; this line, which is not shown for the sake of clarity, has a slope of 1.33, an ordinate intercept of −0.68 and $r = 0.96$. Linear-regression analysis of the entire dataset, including the dipeptides, yields the line shown in Fig. 5, which has a slope of 1.21, an ordinate intercept of −0.68 and $r = 0.97$. This strongly argues that dipeptide uptake by human erythrocytes occurs by simple passive diffusion through the bilayer, and simultaneously illustrates that the theoretical treatment by Lieb & Stein (1986) of the relationship between the passive permeabilities of small

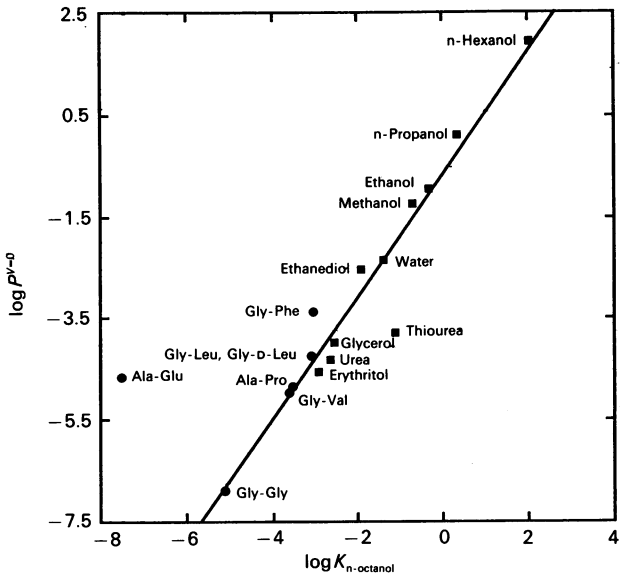

Fig. 5. Correlation between $\log P^{V=0}$ and $\log K_{n\text{-octanol}}$ values for (●) the dipeptides investigated in this study, and (■) permeants previously examined by Lieb & Stein (1986)

Permeability coefficients (P) for the dipeptides were calculated by using the equation:

$$P = \frac{f \cdot V_{out}}{[S]_0 \cdot SA_{total}}$$

where f is the flux rate given in Table 1, $[S]_0$ is the extracellular dipeptide concentration in the flux experiments (15 mM), V_{out} is the volume of the external compartment, and SA_{total} is the total erythrocyte membrane area, which is obtained by multiplying the iso-osmotic surface area of a single erythrocyte (1.43×10^{-6} cm^2; Kirch & Kuchel, 1986) by the total number of cells in the suspension. $P^{V=0}$ values were calculated by using eqn. (2) and the m_v value of 0.0546 cm^{-3}·mol calculated by Lieb & Stein (1986) for n-octanol. The continuous line represents the least-squares best fit of a straight line to the entire dataset with Ala-Glu excluded.

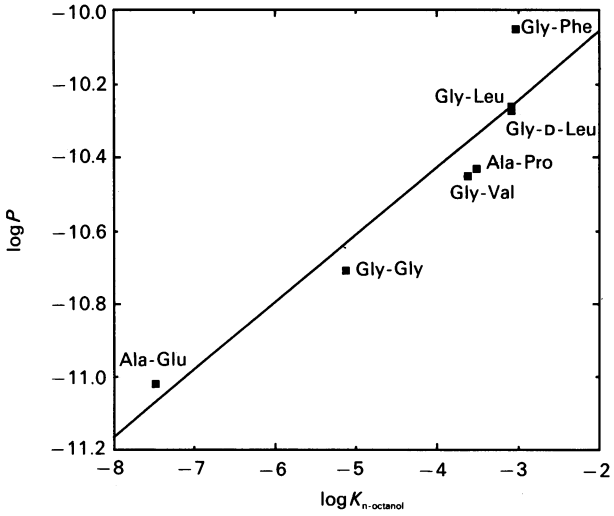

Fig. 6. Correlation between $\log P$ and $\log K_{n\text{-octanol}}$ for the dipeptides investigated in this study

The continuous line represents the least-squares best fit of a straight line to the entire dataset.

neutral diffusants and their hydrophobicity and size can be readily extended to include larger organic ions.

The anomalously high permeation of Ala-Glu, however, still needs to be explained. One possibility is that Ala-Glu uptake by human erythrocytes may consist of protein-mediated processes in addition to the basal rate of diffusion; possible candidates for the uptake of Ala-Glu include capnophorin (also known as band 3 or the anion transporter; Knauf, 1979) and the dicarboxylic acid carrier (Halestrap, 1976). However, an alternative explanation results from consideration of Fig. 6, which is a plot of the logarithm of the uncorrected permeability coefficients versus $\log K_{n\text{-octanol}}$ for the dipeptides. A good correlation between these variables is obtained ($r = 0.95$) and the Ala-Glu datum point no longer seems anomalous.

This surprising result, that correction for the size of the diffusant seems almost unnecessary in describing the relationship between its permeability and partitioning in the organic solvent, may simply be due to the small range of molecular volumes that the dipeptides encompass. That is, the dataset of Lieb & Stein (1986) consists of ten disparate molecules whose molecular volumes vary over a 7-fold range from 10.6 to 72.9 cm^3·mol^{-1}, whereas the volumes of the dipeptides examined in the present study vary only over a 2-fold range from 69.5 to 122.1 cm^3·mol^{-1} (Table 1); hence correction for molecular size is less important in the present work.

However, the absence of the requirement to size-correct the permeability coefficients may also suggest that reptation (De Gennes, 1971) plays a role in diffusion of dipeptides through the erythrocyte bilayer. That is, although it is probably safe to assume that the probability of finding transient 'pores' in the membrane sufficient to accommodate the smaller molecules examined by Lieb & Stein (1986) will be a function of their total molecular volume, this may not be true for larger molecules such as dipeptides with distinct structural asymmetry. For example, the long axis of a dipeptide (i.e. the backbone) may snake or reptate across the membrane, and relative permeabilities would be more likely to be a function of the volume and hydrophobicity of the side chain rather than the overall molecular volume (implicit in this argument is the notion that there will be a greater probability of finding adjacent 'pores' in the membrane that fit the short rather than the long dimension of the diffusant). However, it should be noted that there is little evidence so far that reptation plays a significant role in diffusion within biological membranes (Lieb & Stein, 1986).

Regardless of the explanation for the anomalously high $P^{V=0}$ value for Ala-Glu, the distinct positive correlation for the other dipeptides between their measured permeabilities and calculated $K_{n\text{-octanol}}$ values provides strong evidence that uptake of these molecules by human erythrocytes occurs by passive diffusion and not by a protein-mediated peptide-transport system. Thus the apparent diffusion constants (K_D) for these dipeptides are readily obtained by dividing the measured flux rate by the extracellular peptide concentration (15 mM); this yields values ranging from 0.0037 h^{-1} (Gly-Gly) to 0.017 h^{-1} (Gly-Phe). Plasma dipeptide concentrations, even after a high-protein meal, are usually in the micromolar range (Adibi, 1975), and thus the diffusive fluxes of dipeptides into human erythrocytes (i.e. $K_D \cdot [S]_0$) under normal physiological conditions will only be of the order of nmol/h per l of cells. This is very low compared with the rates of dipeptide uptake by renal transport systems (see, e.g., Ganapathy *et al.*, 1981) and degradation by plasma peptidases (see, e.g., Myara *et al.*, 1984), and we therefore suggest that human erythrocytes do not play a significant role in the catabolism of circulating plasma peptides. However, an unequivocal conclusion must await an examination of the possibility that these cells possess a low-capacity high-affinity peptide transporter, which will probably require the use of radiolabelled peptides.

The latter point is especially important with respect to the proposal by King & Kuchel (1985) that α-glutamyl-dipeptide uptake may provide a source of glutamate for human erythrocytes, which do not possess a transport system for this amino acid (Ellory, 1988). That is, if Ala-Glu uptake by human erythrocytes is totally diffusive, then the uptake rate measured in this study with an extracellular dipeptide concentration of 15 mM would translate into a very small diffusive flux at physiological plasma concentrations. However, even though Young *et al.* (1987) obtained an Ala-Glu flux rate that was lower than in the present study (approx. 9 μmol/h per l of cells, correcting for the small difference in extracellular dipeptide concentrations), this would still give a spurious $P^{V=0}$ value on the plot shown in Fig. 5; a flux rate of $\ll 1$ μmol/h per l of cells would be required for the Ala-Glu point to lie near the line of best-fit for the other permeants. Thus, as mentioned above, the anomalously high permeability of the membrane to Ala-Glu may suggest that its uptake is at least partially due to protein-mediated transport. Consequently, the extent to which α-glutamyl-dipeptide uptake is capable of meeting the human erythrocyte's glutamate requirement of approx. 12 μmol/h per l of cells (King & Kuchel, 1985) will primarily depend on the affinity of the transporter(s) for all of the different dipeptides in this family, since, despite the observation that they are highly resistant to hydrolysis by plasma peptidases (King, 1985), their plasma concentration is still likely to be in the micromolar range.

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REFERENCES

Adibi, S. A. (1975) in Peptide Transport in Protein Nutrition (Matthews, D. M. & Payne, W., eds.), pp. 147–166, North-Holland, Amsterdam
Beilharz, G. R., Middlehurst, C. R., Kuchel, P. W., Hunt, G. E. & Johnson, G. F. S. (1984) Anal. Biochem. **137**, 324–329
Beutler, E., West, C. & Blume, K.-G. (1976) J. Lab. Clin. Med. **88**, 328–333
Bondi, A. (1964) J. Chem. Phys. **68**, 441–451
Boyd, C. A. R. & Ward, M. R. (1982) J. Physiol. (London) **324**, 411–428
Brown, F. F., Campbell, I. D., Kuchel, P. W. & Rabenstein, D. L. (1977) FEBS Lett. **82**, 12–16
De Gennes, P. G. (1971) J. Chem. Phys. **55**, 572–579
Ellory, J. C. (1988) in Amino Acid Transport in Animal Cells (Yudilevich, D. & Boyd, C. A. R., eds.), pp. 106–119, University of Manchester Press, Manchester
Endo, F., Matsuda, I., Ogata, A. & Tanaka, S. (1982) Pediatr. Res. **16**, 227–231
Ganapathy, M. E., Mahesh, V. B., Devoe, L. D., Leibach, F. H. & Ganapathy, V. (1985) Am. J. Obstet. Gynecol. **153**, 83–86
Ganapathy, V. & Leibach, F. H. (1986) Am. J. Physiol. **251**, F945–F953
Ganapathy, V., Mendicino, J. & Leibach, F. H. (1981) Biochim. Biophys. Acta **642**, 381–391
Geissler, U. C. & Stith, W. J. (1978) Transfusion **18**, 328–332
Halestrap, A. P. (1976) Biochem. J. **156**, 193–207
Hansch, C. H. & Leo, A. (1979) Substituent Constants for Correlation Analysis in Chemistry and Biology, pp. 18–43, Wiley–Interscience, New York
Isab, A. A. & Rabenstein, D. L. (1979) FEBS Lett. **106**, 325–329
King, G. F. (1985) Ph.D. Thesis, University of Sydney
King, G. F. & Kuchel, P. W. (1984) Biochem. J. **220**, 553–560
King, G. F. & Kuchel, P. W. (1985) Biochem. J. **227**, 833–842
King, G. F., Chapman, B. E., York, M. J. & Kuchel, P. W. (1983) Biochem. Biophys. Res. Commun. **110**, 305–312
King, G. F., Middlehurst, C. R. & Kuchel, P. W. (1986) Biochemistry **25**, 1054–1062
King, G. F., Crossley, M. J. & Kuchel, P. W. (1989) Eur. J. Biochem. **180**, 377–384
Kirk, K. & Kuchel, P. W. (1986) J. Magn. Reson. **68**, 311–318
Knauf, P. A. (1979) Curr. Top. Membr. Transp. **12**, 249–363
Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–66
Kuchel, P. W., Chapman, B. E. & King, G. F. (1987) Biochem. J. **242**, 311–312
Lieb, W. R. & Stein, W. D. (1986) Transport and Diffusion across Cell Membranes, pp. 69–112, Academic Press, London
Myara, I., Myara, A., Mangeot, M., Fabre, M., Charpentier, C. & Lemmonier, A. (1984) Clin. Chem. **30**, 211–215
Odoom, J. E., Campbell, I. D., Ellory, J. C. & King, G. F. (1988) Biochem. Soc. Trans. **16**, 635–636
Rich, D. H., Moon, B. J. & Harbeson, S. (1984) J. Med. Chem. **27**, 417–422
Richards, D. E. & Eisner, D. A. (1982) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 165–177, Academic Press, London
Vandenberg, J. I., King, G. F. & Kuchel, P. W. (1985) Biochim. Biophys. Acta **846**, 719–726
Young, J. D. & Ellory, J. C. (1977) in Membrane Transport in Red Cells (Ellory, J. C. & Lew, V. I., eds.), pp. 301–325, Academic Press, London
Young, J. D., Wolowyk, M. W., Fincham, D. A., Cheeseman, C. I., Rabenstein, D. L. & Ellory, J. C. (1987) Biochem. J. **242**, 309–311

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