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# Assimilation of α-glutamyl-peptides by human erythrocytes

## A possible means of glutamate supply for glutathione synthesis

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Human erythrocytes are essentially impermeable to glutamate and yet there is a continual requirement for the amino acid for glutathione synthesis. In addition, the intracellular glutamate concentration is approximately five times that of plasma. We present evidence that glutamate enters the red cell as small peptides which are rapidly hydrolysed by cytoplasmic peptidase(s) and that with the estimated physiological levels of plasma glutamyl-peptides the rate of inward flux would be adequate to maintain the glutamate pool at its observed level. Experimentally, we used $^1H$ spin-echo n.m.r. spectroscopy to follow peptide hydrolysis, since peptide spectra are different from those of the free amino acids and the spin-echo sequence enables the monitoring of reactions in concentrated lysates and whole cell suspensions. Thus, the system was studied under near-physiological conditions. Weighted non-linear regression analysis of progress curves using the integrated Michaelis–Menten equation was used to obtain estimates of $K_m$ and $V_{max.}$ for the hydrolysis of α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate in lysates and whole cell suspensions; the values for lysates were $K_m = 3.60 \pm 0.29$ and $5.4 \pm 0.4$ mmol/l and $V_{max.} = 120 \pm 4$ and $46.7 \pm 1.7$ mmol/h per l of packed cells respectively. In whole cell suspensions the rate of peptide hydrolysis was much slower and dominated by the transmembrane flux-rate. The estimates of the steady-state kinetic parameters for the transport were $K_t = 2.35 \pm 0.41$ and $11.2 \pm 1.0$ mmol/l and $V_{max.} = 3.26 \pm 0.13$ and $19.7 \pm 0.7$ mmol/h per l of packed cells respectively for the previously mentioned peptides. Using the n.m.r. procedure we failed to detect any glutaminase activity in whole cells or lysates; thus, we exclude the possibility that glutamate gains entry to the cell as glutamine which is subsequently hydrolysed by glutaminase.

The biologically ubiquitous tripeptide glutathione (γ-glutamylcysteinylglycine; GSH) is present at a relatively constant concentration of 2–3 mmol/l in human erythrocytes (Beutler, 1975) but it is in dynamic equilibrium with a half-life of approx. 4 days (Dimant *et al.*, 1955). It now appears that GSH degradation is initiated by translocation of intracellular GSH to the plasma (Meister & Anderson, 1983) rather than active efflux of oxidized glutathione (GSSG) as implied from earlier studies (Kondo *et al.*, 1980).

GSH is synthesized in erythrocytes from its constituent amino acids via consecutive reactions catalysed by γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). Membrane-bound γ-glutamyl transpeptidase may facilitate entry of the requisite amino acids in tissues where it exists (e.g. kidney, liver). This enzyme catalyses a transpeptidation reaction, at the extracellular membrane surface, between plasma amino acids and GSH. The resulting γ-glutamyl-amino acid dipeptides may subsequently be absorbed and cleaved intracellularly by γ-glutamyl cyclotransferase (EC 2.3.2.4) to yield 5-oxoproline and free amino acids. Since the erythrocyte membrane lacks γ-glutamyl transpeptidase activity (Board & Smith, 1977; Srivastava, 1977) then provision of the necessary amino acids to these cells must be by some other mechanism.

The red cell membrane possesses well-characterized transport systems for glycine and cysteine

Abbreviations used: DSS, sodium 2,2-dimethyl-2-silapentane 5-sulphonate; FeDx, $Fe^{3+}$–desferrioxamine complex; GSH, glutathione; GSSG, oxidized glutathione.

(Young & Ellory, 1977) which can supply these amino acids for GSH synthesis. However human erythrocytes are almost completely impermeable to glutamate (Winter & Christensen, 1964; Young, 1983). This observation has led to various proposals as to how human red cells maintain an intracellular glutamate pool of approx. 300 $\mu$mol/l (Heinle *et al.*, 1976) compared with the plasma concentration of approx. 60 $\mu$mol/l (Dickinson *et al.*, 1965); these include uptake of 2-oxoglutarate followed by transamination (Sass, 1963) and/or uptake of glutamine with subsequent glutaminase-catalysed release of glutamate (Ellory *et al.*, 1983).

In view of recent evidence indicating that the mature human erythrocyte possesses peptide transport system(s) (King *et al.*, 1983; King & Kuchel, 1984) we have investigated the hypothesis that absorption and subsequent hydrolysis of α-glutamyl-peptides provides the major source of glutamate for these cells. Proton spin-echo n.m.r. spectroscopy was used in these studies because of its now well-documented ability to monitor the kinetics of peptide transport and hydrolysis *in situ* (King *et al.*, 1983; King & Kuchel, 1984; Middlehurst *et al.*, 1984).

## Experimental

### *Materials*

DSS was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. L-Glutamate and L-glutamine were from BDH Chemicals Ltd., Poole, Dorset, U.K. Desferal (desferrioxamine mesylate) was obtained from Ciba-Geigy, Lane Cove, N.S.W., Australia. [2,3,4,6,6-$^2$H]Glucose was purchased from Merck, Sharp and Dohme, Point Claire-Dorval, Que., Canada. L-Alanyl-α-L-glutamate and α-L-glutamyl-L-alanine were obtained from Vega Biochemicals, Tucson, AZ, U.S.A. All other reagents were AR grade. Pyrogen-free cotton wool was supplied by Tuta Laboratories Pty. Ltd., Lane Cove, N.S.W., Australia; Carbogen was from Commonwealth Industrial Gases, Alexandria, N.S.W., Australia.

### *Preparation of erythrocytes*

Leucocyte-free human and rabbit erythrocyte suspensions were prepared from freshly drawn venous blood as described previously (King & Kuchel, 1984). Rabbits (mature, male Castle Hill Laboratory Whites) were bled from the marginal ear vein. The haematocrits of these suspensions, after washing, varied between 0.60 and 0.90. Haemolysates were prepared by a single freeze–thaw cycle (−12°C/25°C).

Immediately prior to $^1$H-n.m.r. measurements, cell suspensions (440 $\mu$l) or haemolysates (430 $\mu$l) were added to 5mm-outer-diameter n.m.r. tubes (527-PP, Wilmad, Beuna, NJ, U.S.A.) and heated to 37°C. Addition of [2,3,4,6,6-$^2$H]glucose (to maintain cellular energy requirements), nicotinamide (to prevent depletion of nicotinamide nucleotides in haemolysates) and peptide substrate was performed as described previously, with each tube having a final volume of 500 $\mu$l (King & Kuchel, 1984). For experiments involving cell suspensions the osmolarities of all solutions were adjusted to lie in the range 270–310 mosM/kg. Addition of solutions which are not iso-osmolar with the intracellular red cell milieu (approx. 290 mosM/kg) will induce changes in red cell shape which affect the amplitudes of $^1$H-n.m.r. spin-echo signals by rendering inhomogeneous the magnetic field in the vicinity of the cell (Endre *et al.*, 1984). Osmotic pressures were measured by using a vapour pressure osmometer (model 5100B; Wescor Instruments, Logan, UT, U.S.A.).

Zero reaction time was taken as the time of substrate addition and n.m.r. measurements were normally begun within 2.5min for lysate experiments or 5.0min for cell experiments.

### *N.m.r. measurements*

400MHz $^1$H-n.m.r. spectra were acquired at 37°C with a Bruker WM400 spectrometer and processed as described previously (King & Kuchel, 1984); the HGSE pulse sequence was used with 60ms delay times and a water saturation pulse of 0.5s duration for cell suspensions and 0.4s for haemolysates. DSS (0.5% in $^2H_2O$), which was present in a small coaxial capillary within the n.m.r. tube, was used as the chemical shift ($\delta$) reference at 0.000p.p.m. The $^2H_2O$ in the capillary was used for field-frequency locking.

For studies of initial velocities, alanine concentrations were determined from the intensity ratio of the alanine $H^\beta$ resonance to that of DSS by using calibration curves; these were prepared as described by Beilharz *et al.* (1984).

### *Preparation of $Fe^{3+}$-desferrioxamine (FeDx) complex*

The paramagnetic ion $Fe^{3+}$ was liganded to the chelating agent desferrioxamine by addition of $FeCl_3$ to a solution containing a slight molar excess of desferrioxamine mesylate. After adjusting its pH to lie in the range 7.2–7.4, the final FeDx solution was filtered several times through a 0.2 $\mu$m Millipore filter system.

### *Numerical methods*

Non-linear least-squares regression of the Michaelis–Menten equation onto initial-velocity-versus-concentration data or of the integrated form of the Michaelis–Menten equation onto substrate- or product-versus-time data (see the

Results section) was performed on a CYBER 720 computer using a program developed by Miller (1981) which is based on a modification of the Levenberg–Morrison–Marquardt algorithm (Osborne, 1976).

## Results

*Interpretation of $^1$H-n.m.r. spectra*

Figs. 1(*a*)–1(*c*) show HGSE $^1$H-n.m.r. spectra of aqueous solutions of an equimolar mixture of the free amino acids alanine and glutamate, and the dipeptides α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate, respectively. The peptide spectra are dominated by the glutamyl $H^{\gamma}$ resonances (a′ at 2.351 p.p.m. and a″ at 2.209 p.p.m.) and the alanyl $H^{\beta}$ resonances (c′ at 1.327 p.p.m. and c″ at 1.484 p.p.m.). Note that the chemical shifts of these resonances depend on the position of each residue in the dipeptide. Also note that the free glutamate $H^{\gamma}$ (a at 2.329 p.p.m.) and free alanine $H^{\beta}$ (c at 1.452 p.p.m.) protons resonate at chemical shifts different from the corresponding protons of the dipeptides. Hydrolysis of these dipeptides can thus be monitored by the decrease in amplitude of the glutamyl $H^{\gamma}$ and alanyl $H^{\beta}$ resonances and the simultaneous increase in amplitude of the free glutamate $H^{\gamma}$ and free alanine $H^{\beta}$ resonances. Resonances from the inequivalent and highly coupled glutamyl $H^{\beta}$ protons (b, b′ and b″) have negligible amplitude due to phase modulation during the time of the spin-echo pulse sequence and thus cannot be used for kinetic analysis.

Fig. 2(*a*) shows a typical series of $^1$H-n.m.r. HGSE spectra obtained when a lysate of human erythrocytes (final haematocrit 0.55) was incubated with α-L-glutamyl-L-alanine (11.6 mmol/l). The changes in peptide and amino acid peak amplitudes, as described above, are evidence of dipeptide hydrolysis. Fig. 2(*b*) shows for comparison a similar series of $^1$H-n.m.r. HGSE spectra obtained when a haemolysate (final haematocrit 0.77) was incubated with the reverse-orientation dipeptide, L-alanyl-α-L-glutamate (13.0 mmol/l). It is clear that the hydrolysis of L-alanyl-α-L-glutamate by the cytosolic erythrocyte peptidases is considerably slower than the hydrolysis of α-L-glutamyl-L-alanine; at $t = 15.9$ min the former reaction is not yet half-finished whereas the latter reaction is almost complete. (This observation can be made most readily by comparison of the relative heights of the alanine $H^{\beta}$ and alanyl $H^{\beta}$ resonances in each time course at $t = 15.9$ min).

A typical series of $^1$H-n.m.r. HGSE spectra depicting the hydrolysis of α-L-glutamyl-L-alanine (11.8 mmol/l of extracellular fluid) by a suspension of intact cells (final haematocrit 0.79) is shown in Fig. 3(*a*). Note that the cell spectra are similar to

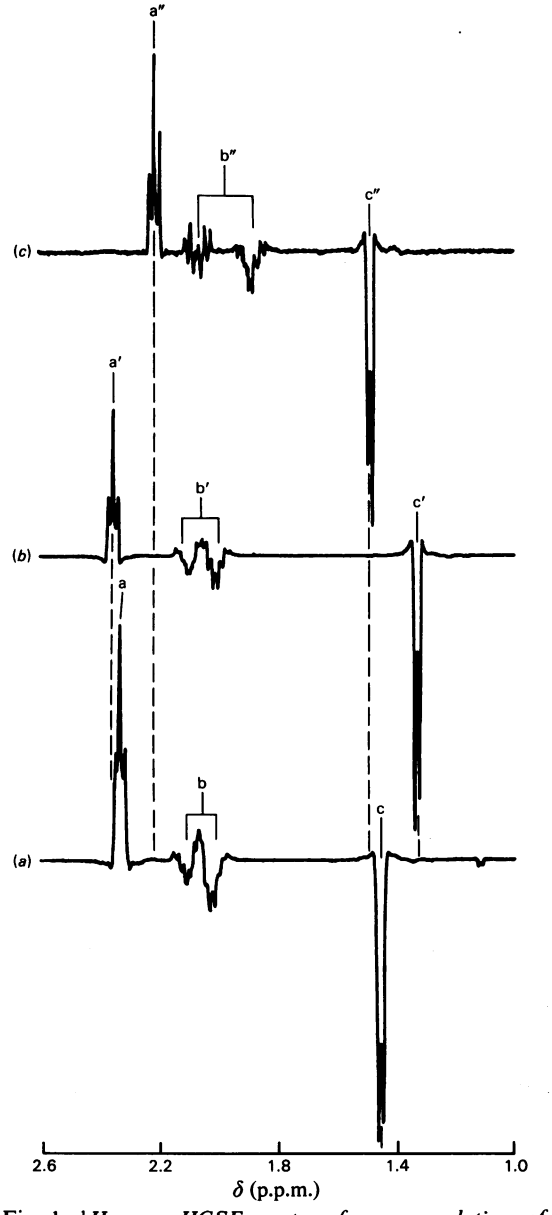


Fig. 1. *$^1$H-n.m.r. HGSE spectra of aqueous solutions of (a) an equimolar mixture of* L-*alanine and* L-*glutamate, (b)* α-L-*glutamyl-*L-*alanine and (c)* L-*alanyl-*α-L-*glutamate*

N.m.r.: 128 transients/spectrum in 16 kilobyte data locations, 0.5 s saturation pulse at the water frequency. Spectral assignments: a, a′ and a″, resonances from the $H^{\gamma}$ protons of free glutamate and the glutamate residues of α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate respectively; b, b′ and b″, resonances from the $H^{\beta}$ protons of free glutamate and the glutamate residues of α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate respectively (note that these protons are magnetically inequivalent in all three situations, thereby giving rise to two distinct sets of resonances); c, c′ and c″, resonances from the $H^{\beta}$ protons of free alanine and the alanine residues of α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate respectively.

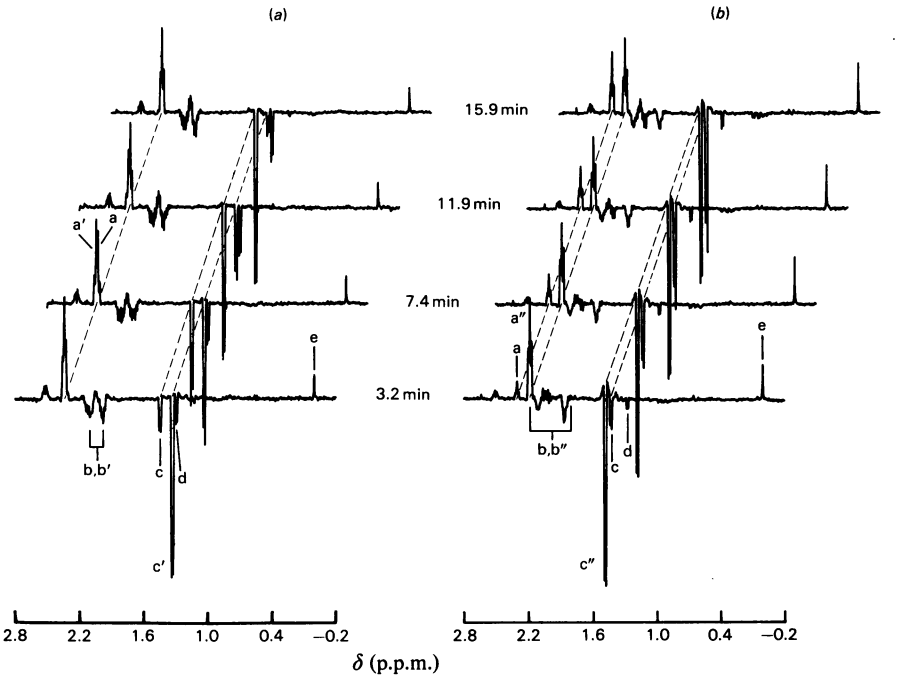


Fig. 2. *Time series of* $^1$*H-n.m.r. HGSE spectra of* (*a*) *a haemolysate* (*final haematocrit 0.55*) *to which* α-L-*glutamyl*-L-*alanine was added* (*final concn. 11.6mmol/l*) *at time zero, and* (*b*) *a haemolysate* (*final haematocrit 0.77*) *to which* L-*alanyl*-α-L-*glutamate was added* (*final concn. 13.0mmol/l*) *at time zero*

Other experimental conditions were as explained in the text. Spectral assignments are as in Fig. 1 with the additions of: d, methyl protons of lactate; e, methyl protons of DSS.

those from haemolysates except that the resonances are somewhat broader (due to increased magnetic field inhomogeneity caused by compartmentation) and that the lactate methyl resonance (d in Fig. 3 at 1.256p.p.m.) becomes considerably more intense due to the longer time period available for glycolysis during cell experiments. For comparison, Fig. 3(*b*) shows a time course of $^1$H-n.m.r. HGSE spectra obtained when a suspension of intact cells (final haematocrit 0.79) was incubated with L-alanyl-α-L-glutamate (29.6mmol/l of extracellular fluid). The rate of spectral changes indicates that L-alanyl-α-L-glutamate is transported into human erythrocytes considerably faster than α-L-glutamyl-L-alanine, since the coupled transport–hydrolysis process has proceeded to a greater extent-of-reaction in time courses of the former dipeptide despite an initial concentration that was 2.5 times higher. However it should be noted from the much-extended time courses in the cell experiments, as compared with those involving haemolysates (Fig. 2), that hydrolysis of both dipeptides is considerably slower in suspensions of intact cells, thus indicating that transport of each dipeptide across the membrane is the rate-limiting step in the linked transport–hydrolysis process. The decline in amplitude of the peptide resonances and the concomitant increase in amplitude of the amino acid resonances in the spectra from intact cell suspensions thus give good approximations to the rate of substrate influx. This argument has been developed previously and validated for the transport of glycyl-L-proline into human erythrocytes (King & Kuchel, 1984).

*Kinetic analysis of results*

Initial velocities of α-L-glutamyl-L-alanine hydrolysis were estimated from plots of the amplitude of the free alanine $H^{\beta}$ resonance versus time. The most appropriate low-degree polynomial was regressed onto the product-versus-time data and the initial velocity was subsequently obtained from the first derivative of the polynomial at time zero. Fig. 4 shows the dependence of the initial reaction velocity on the α-L-glutamyl-L-alanine concentration in haemolysates. Steady-state kinetic parameters for this peptidase-catalysed reaction were obtained by weighted non-linear regression of the Michaelis–Menten equation onto the initial velocity-versus-concentration data.

Kinetic parameters for all other hydrolytic reactions (involving cells or haemolysates) were ob-

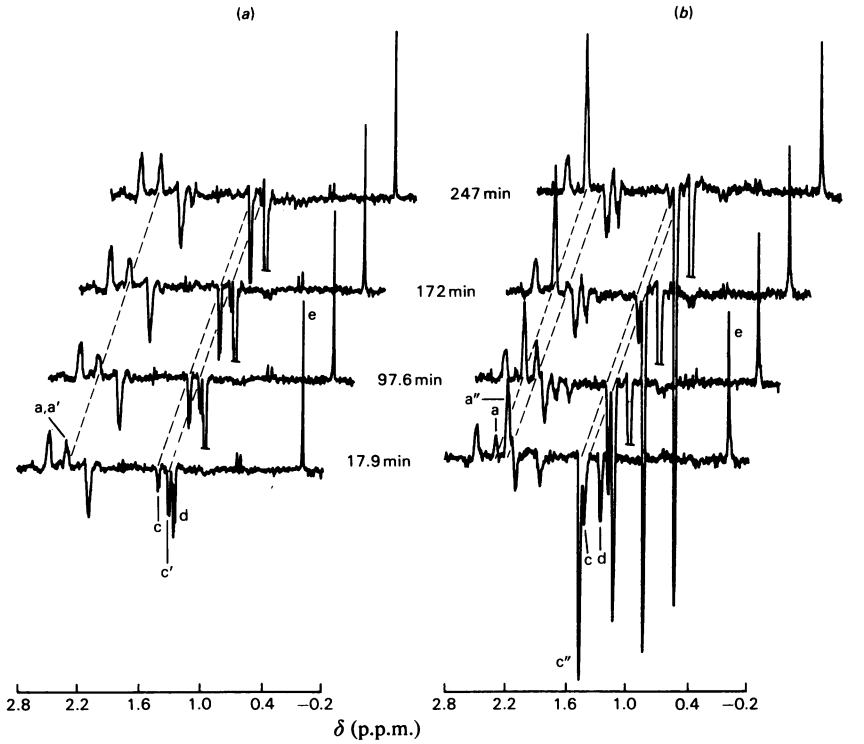


Fig. 3. *Time series of* $^1$*H-n.m.r. spectra of* (*a*) *a suspension of intact erythrocytes* (*final haematocrit 0.79*) *to which* α-L-*glutamyl-*L-*alanine was added* (*final concn. 11.8mmol/l of extracellular fluid*) *at time zero, and* (*b*) *a suspension of intact erythrocytes* (*final haematocrit 0.79*) *to which* L-*alanyl-*α-L-*glutamate was added* (*final concn. 29.6mmol/l of extracellular fluid*) *at time zero*

Spectral assignments are as in the time courses of Fig. 2; however, note in comparison the much more protracted time courses in this Figure.

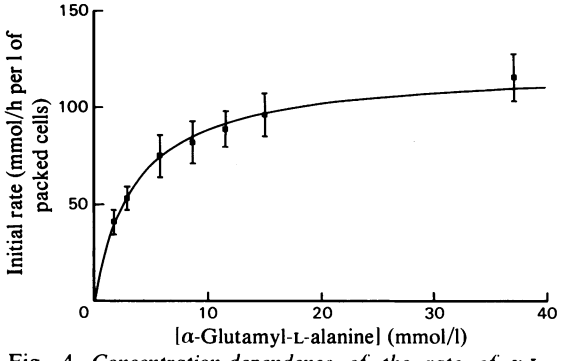


Fig. 4. *Concentration-dependence of the rate of* α-L-*glutamyl-*L-*alanine hydrolysis by a lysate of human erythrocytes at 37°C*

The continuous line was obtained by non-linear regression of the Michaelis–Menten equation onto the data; this yielded the values of $K_m$ and $V_{max.}$ shown in Table 1. Error bars denote ± 1 S.D.

tained via progress curve analysis. This involved non-linear regression of the appropriate integrated form of the Michaelis–Menten expression onto substrate- or product-versus-time data. Eqn. (1) is the integrated equation for a non-product-inhibited Michaelis–Menten enzyme, expressed in terms of substrate concentration: $t$ is the time of reaction, [S] is the substrate concentration at time $t$, $[S]_0$ is the initial substrate concentration, and $K_m$ and $V_{max.}$ are the Michaelis–Menten parameters:

$$t = 1/V_{max.} \cdot \{[S]_0 - [S] - K_m \cdot \ln([S]/[S]_0)\} \quad (1)$$

Thus, the corresponding equation for product can be obtained by replacing [S] with $([S]_0 - [P])$. Eqn. (1) is an explicit function $t$ of [S] (or [P]) and as such it may be used in non-linear regression of an $n$-set of ($[S]_i$; $i = 1, \ldots, n$) data pairs by transposing the dependent, [S], and independent, $t$, variables. However we applied here the important modification (Darvey *et al.*, 1975) that each transposed data pair is weighted by the value of $d[S]_i/dt$, the velocity estimate at $[S]_i$ obtained from the differential Michaelis–Menten expression. Analysis of both synthetic and experimental progress curves by using this method (without the weighting) has been described previously (Atkins & Nimmo, 1973; Kuchel *et al.*, 1984). An example of a fit of eqn. (1), weighted as above, to substrate-versus-time data is

shown in Fig. 5. Concentrations of substrates and products were calculated from their $^1$H-n.m.r. peak amplitudes in conjunction with the conservation of mass conditions.

The $K_m$ and $V_{max.}$ values reported for the various reactions in Table 1 are the weighted means of $n$ integrated fits at various initial substrate concentrations except for those for α-L-glutamyl-L-alanine hydrolysis in haemolysates, which are from the initial velocity analysis.

*The contribution of haemolysis to the inferred rates of transport*

Given that the maximal velocity of α-glutamyl dipeptide hydrolysis is, in general, considerably larger for haemolysates than whole cell suspensions (see Table 1), then it is clear that a small proportion of haemolysed cells could contribute significantly to the observed rate of hydrolysis in the latter. However, in a recent study in which the rate of influx of arginine into human erythrocytes was monitored by a similar indirect n.m.r. method employing the intracellular arginase activity of the cells, it was found that the estimates of the steady-state transport parameters were similar to those derived from conventional direct transport measurements (Kuchel *et al.*, 1984); this indicated that over the time period of these experiments (1.25h), at least, haemolysis made a negligible artefactual contribution to estimates of the rate of transport.

Furthermore, a previous study has demonstrated the rapid appearance of glycine in an intact erythrocyte suspension incubated with glycylglycine over a period of 2–4h in which all extracellular resonances were suppressed by the addition of the impermeant paramagnetic complex FeDx (King *et al.*, 1983); if haemolysis resulted in a major contribution to the inferred rate of glycylglycine transport then, given that glycine is transported very slowly into human erythrocytes (Young, 1983), there would have been a negligible increase in the intensity of the glycine resonance with time since the newly-formed glycine would have been extracellular and hence its n.m.r. signal suppressed. The increasing intensity of the (intracellular) glycine resonance in the spectra thus indicated a transport phenomenon and not an artefact of cell lysis.

However, to obtain unequivocal assurance that haemolysed cells were not contributing significantly to the observed rate of hydrolysis of α-glutamyl dipeptides by suspensions of intact cells, a control experiment similar to that described above was undertaken. Fig. 6(*a*) shows the $^1$H-n.m.r. HGSE spectrum of a suspension of intact erythrocytes (final haematocrit 0.70) to which α-L-glutamyl-L-alanine (26.8mmol/l of extracellular fluid) was added at $t = 0$min. Fig. 6(*b*) shows the spectrum of the same sample after addition of FeDx (1.0mmol/l of extracellular fluid); this resulted in the glutamyl $H^{\gamma}$ and alanyl $H^{\beta}$ resonances of the peptide (b and g respectively) being reduced to approx. 30% of their original amplitude relative to the intracellular reference ergothioneine (a). Addition of sufficient FeDx to suppress totally the

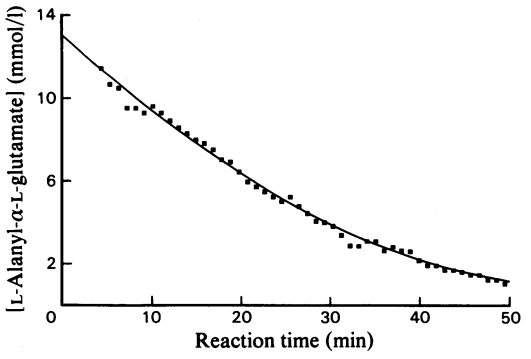


Fig. 5. *Substrate-versus-time progress curve for the hydrolysis of* L-*alanyl-*α-L-*glutamate by a haemolysate (same experiment as depicted in Fig. 2a) obtained by analysis of the peak amplitudes of the glutamyl $H^{\gamma}$ resonance*

The continuous line is from non-linear regression of eqn. (1), weighted with d[S]/d$t$, onto the data, which yielded $K_m = 7.5 \pm 1.0$mmol/l and $V_{max.} = 47.5 \pm 3.0$mmol/h per l of packed cells.

Table 1. *Michaelis–Menten kinetic parameters for the hydrolysis of* α-L-*glutamyl-*L-*alanine and* L-*alanyl-*α-L-*glutamate by human erythrocyte lysates and whole cells*

Values are means ± 1S.D.

| Substrate | $V_{max.}$ (mmol/h per l of cells) | Michaelis constant (mmol/l) | $n$ |
|---|---|---|---|
| α-L-Glutamyl-L-alanine | | | |
| (*a*) Haemolysates | 120 ± 4 | 3.60 ± 0.29 | – |
| (*b*) Intact erythrocytes | 3.26 ± 0.13 | 2.35 ± 0.41 | 2 |
| L-Alanyl-α-L-glutamate | | | |
| (*a*) Haemolysates | 46.7 ± 1.7 | 5.4 ± 0.4 | 6 |
| (*b*) Intact erythrocytes | 19.7 ± 0.7 | 11.2 ± 1.0 | 3 |

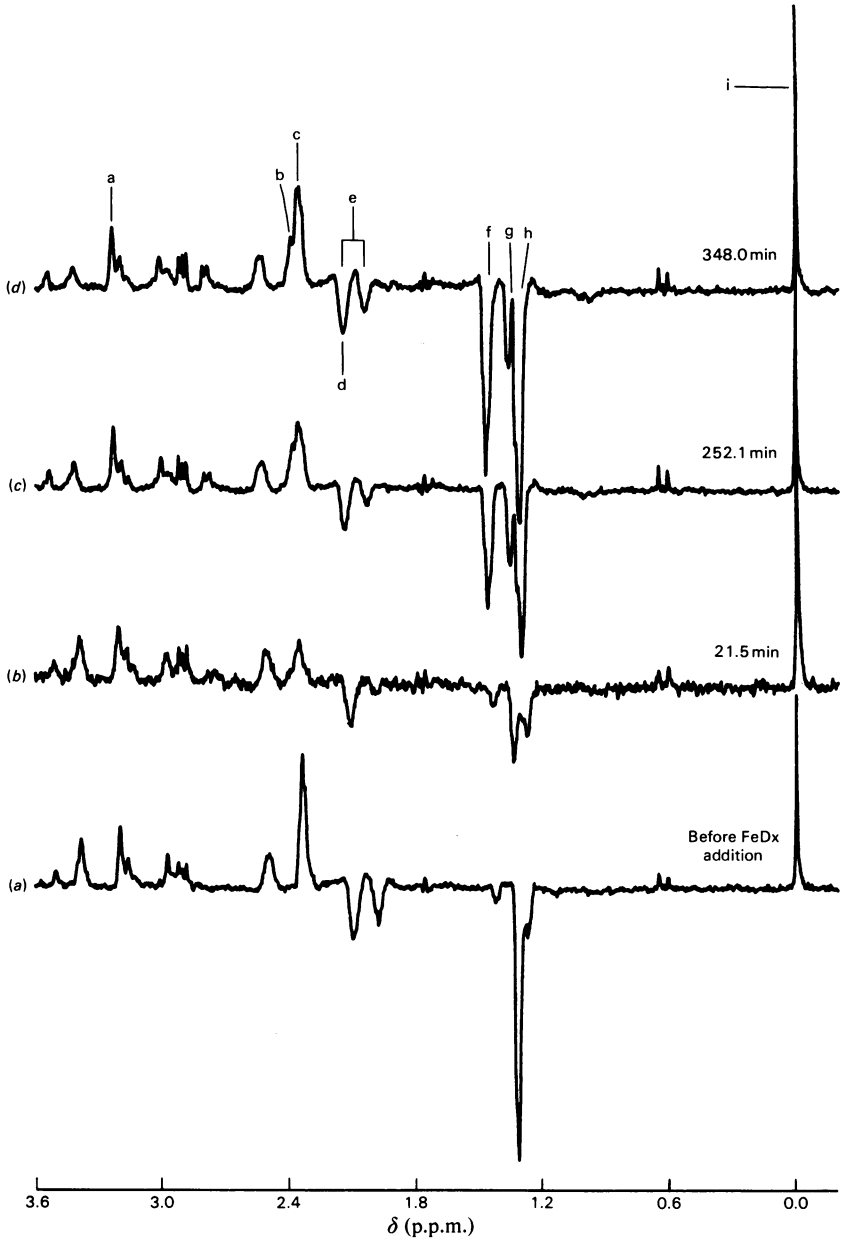


Fig. 6. *(a), $^1$H-n.m.r. HGSE spectrum of a suspension of intact erythrocytes (final haematocrit 0.70) to which* α-L-*glutamyl*-L-*alanine was added (final concn. 26.8mmol/l of extracellular fluid) at time zero; (b)–(d), time series of $^1$H-n.m.r. HGSE spectra of the same suspension after addition of FeDx (1.0mmol/l of extracellular fluid) immediately following acquisition of spectrum (a)*

N.m.r.: 200 transients/spectrum for spectra (*a*) and (*b*) and 400 transients/spectrum for spectra (*c*) and (*d*), all in 8 kilobyte data locations. Spectral assignments: a, quaternary ammonium methyl protons of ergothioneine; b, dipeptide-glutamyl $H^{\gamma}$; c, glutamate $H^{\gamma}$; d, GSH-glutamyl $H^{\beta}$; e, dipeptide-glutamyl/glutamate $H^{\beta}$; f, alanine $H^{\beta}$; g, dipeptide-alanyl $H^{\beta}$; h, lactate methyl; i, DSS methyl.

peptide resonances results in an unacceptably large diminution in the intensity of intracellular resonances; thus it was more practical to work with small residual extracellular resonances in the spectra.

The subsequent time series of spectra obtained after the addition of FeDx (Figs. 6*b*–6*d*) shows two important changes: an increase in the free glutamate $H^{\gamma}$ (c) and free alanine $H^{\beta}$ (f) resonances. The increasing intensity of the (intracellular) ala-

nine $H^{\beta}$ resonance could be due to either a membrane transport process where alanine is produced from the dipeptide intracellularly or, since alanine is transported into human erythrocytes, from extracellular cleavage of the dipeptide by haemolytically-released peptidases with subsequent transmembrane flux of alanine. However, the concomitant increase in intensity of the (intracellular) glutamate $H^{\gamma}$ resonance can only result from a coupled peptide transport–intracellular hydrolysis process since glutamate is impermeant to human red cells and extracellular cleavage would thus not cause an increase in signal intensity due to the presence of FeDx in the extracellular milieu. This is unequivocal evidence that α-glutamyl peptides are transported into human erythrocytes; furthermore, comparison of the rate of hydrolysis measured in this experiment (using the glutamate $H^{\gamma}$ resonance) with that measured using a suspension of cells of exactly the same constitution but without FeDx (thus enabling extracellular and intracellular resonances to be measured *in toto*, giving the total rate of hydrolysis including any contribution from haemolysed cells if they had been present) showed no significant difference. This indicates that haemolysed cells make a negligible contribution to the observed rate of hydrolysis of α-glutamyl dipeptides in suspensions of intact erythrocytes; the measured rate is thus the dipeptide transmembrane flux rate.

*Assay of glutaminase activity*

Glutaminase activity can be readily monitored by using 400 MHz $^1$H-n.m.r. since the glutamine and glutamate $H^{\gamma}$ protons have significantly different chemical shifts (Middlehurst *et al.*, 1984). Incubation of rabbit or human erythrocyte lysates or whole cell suspensions (haematocrit 0.70–0.80) with glutamine (5 mmol/l) led to no detectable production of glutamate (i.e. less than 100 μmol/l) after 4 h of incubation at 37°C *in situ* (spectra not shown). We thus concluded that glutaminase activity in rabbit and human erythrocytes is negligible.

## Discussion

Erythrocytes require a continuous supply of glutamate to maintain the intracellular concentration of the multifunctional tripeptide glutathione. For human erythrocytes the required rate is approx. 11.5 μmol/h per l of packed cells based on a mean GSH concentration of 2.2 mmol/l of packed cells (Beutler, 1975) and a half-life of 4 days (Dimant *et al.*, 1955). Canine red cells obtain glutamate via a highly specific transport system for acidic amino acids (Ellory *et al.*, 1983). The impermeability of human erythrocytes to glutamate, however, has resulted in their source of this amino acid remaining obscure. It has recently been shown that human erythrocytes possess a $Na^+$-dependent, saturable transport system for the entry of glutamine (Ellory *et al.*, 1983). Considering the large plasma concentration of glutamine (approx. 500 μmol/l, the highest plasma concentration of any of the amino acids), these authors suggested that the influx and subsequent glutaminase-catalysed deamination of glutamine could provide the human erythrocyte with the necessary glutamate for GSH synthesis.

At the physiological plasma concentration of glutamine the rate of transport of glutamine into human red cells, given the mean steady-state kinetic parameters quoted by these authors ($K_t$ = 125 μmol/l and $V_{max.}$ = 190 μmol/min per l of packed cells), would be approx. 150 μmol/min per l of packed cells, which is well in excess of the required rate of glutamate supply. The feasibility of their proposal for the source of human red cell glutamate consequently hinges on the level of intracellular glutaminase activity. Rapoport (1961) reported a significant level of glutaminase activity in human erythrocyte lysates (250 μmol/min per l of packed cells); however, no protocol was given for these experiments and it is very likely that the preparations were contaminated with leucocytes. Later work (Sandring *et al.*, 1968) showed that both glutaminase I (EC 3.5.1.2) and glutaminase II (EC 2.6.1.15) activities were *absent* from haemolysates when leucocytes were carefully removed. This latter result is supported by the present $^1$H-n.m.r. studies in which we found no evidence of glutaminase activity in leucocyte-free rabbit or human erythrocyte lysates or whole cell suspensions during 4 h of incubation with glutamine. It must therefore be concluded that plasma glutamine cannot be the source of human erythrocyte glutamate despite the existence of a specific transport system for glutamine in the red cell membrane.

The results presented here indicate that glutamate supply to human erythrocytes may be via the assimilation of α-glutamyl-peptides. We have shown that the model (representative) peptides α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate enter the human erythrocyte through saturable membrane-transport systems describable by Michaelis–Menten kinetics (see Table 1). Furthermore, we have demonstrated that the cytosolic red cell peptidases have a vast capacity to hydrolyse both dipeptides, though especially α-L-glutamyl-L-alanine, with both processes also describable by simple Michaelis–Menten kinetics. For both dipeptides the transport process is the rate-determining step in the pathway leading to the production of intracellular glutamate from extracellular α-glutamyl-peptide.

The α-glutamyl-dipeptide transport systems described here are considerably more 'efficient' than the iminodipeptide transport system of human erythrocytes described previously (King & Kuchel, 1984). At micromolar substrate (S) concentrations (the expected range for most plasma peptides) the rate of peptide transport approximately equals $(V_{max.}/K_t)\cdot[S]$, since $K_t \gg [S]$. The $V_{max.}/K_t$ ratio is thus an 'efficiency index' for these transporters under normal physiological conditions. This ratio is equal to 1.39 for α-L-glutamyl-L-alanine, 1.76 for L-alanyl-α-L-glutamate and only 0.15 for glycyl-L-proline ($K_t = 4.7$ mmol/l and $V_{max.} = 0.698$ mmol/h per l of packed cells; King & Kuchel, 1984). Thus, at similar physiological concentrations, the rates of assimilation of α-glutamyl dipeptides are considerably faster than that of glycyl-L-proline.

The kinetic parameters presented here also show that transport of L-alanine in the form of α-glutamyl-dipeptides is more rapid than transport of the free amino acid over the entire physiological concentration range. L-Alanine uptake by human erythrocytes is separated into two components (Winter & Christensen, 1964): a $Na^+$-dependent saturable system describable by Michaelis–Menten kinetics ($K_t = 300\,\mu$mol/l and $V_{max.} = 4.8\,\mu$mol/min per l of packed cells) and a non-saturable route described by an apparent diffusion coefficient ($K_D = 0.007$/min). At very low concentrations the rate of L-alanine absorption via the saturable system can be described by an apparent first-order rate constant ($V_{max.}/K_t$) which equals 0.95/h. Adding the rate constant describing L-alanine flux through the non-saturable route ($K_D = 0.42$/h) gives a first-order rate constant of 1.37/h describing the total L-alanine flux into human erythrocytes at low extracellular L-alanine concentrations. Since this value is lower than the apparent first-order rate constants describing the influx of the α-glutamyl-dipeptides (i.e. the $V_{max.}/K_t$ ratios discussed previously) then it is clear that L-alanine always enters faster when bound to glutamate. This is unlike the lower rates of glycine and proline absorption by human erythrocytes when they occur together as glycyl-L-proline (King & Kuchel, 1984), but is similar to the enhanced absorption of amino acids by human intestine when they are in the form of small peptides (e.g. Adibi & Morse, 1977).

If we consider the absorption of micromolar concentrations of α-glutamyl-peptides by human erythrocytes to be a first-order process with a rate constant of 1.58/h (the mean of the $V_{max.}/K_t$ ratios reported here) then the plasma concentration of α-glutamyl-peptides would only need to exceed 7.3 μmol/l to provide enough glutamate to sustain GSH synthesis. This concentration, although not measured, is entirely feasible in view of substantial evidence that a large proportion of the peptide absorbed by the intestinal epithelium following a meal may pass intact into the portal blood (Gardner, 1975; Sleisenger *et al.*, 1977). In fact, one study showed that in some cases up to 40% of the absorbed amino-nitrogen from infused partial protein digests passed into the serosal fluid as peptides for uptake by the portal circulation (Gardner, 1978). Furthermore, the author showed that glutamate was one of the few amino acids to consistently enter the serosal fluid in significantly higher concentration peptide-bound than free (up to 13-fold higher peptide-bound).

Plasma peptide concentrations have rarely been measured but it is known that the plasma concentration of glycylglycine in the peripheral circulation may reach 50 μmol/l after a high protein meal (Adibi & Morse, 1971). As well as being derived from the diet, plasma α-glutamyl-peptides may also be derived from endogenous protein breakdown and clot dissolution. Consequently, we propose that the human erythrocyte glutamate pool for GSH biosynthesis is maintained via the absorption and subsequent hydrolysis of plasma α-glutamyl-peptides. It is interesting to note that γ-glutamyl-Xaa dipeptides can also be absorbed to some extent by human erythrocytes (York *et al.*, 1984); however, these do not provide a source of glutamate (unless Xaa = glutamate) since the *N*-terminal glutamate is always converted intracellularly to 5-oxoproline by γ-glutamyl-amino acid cyclotransferase.

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