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No evidence of high capacity α-glutamyl-dipeptide transport into human erythrocytes

In a recent letter Young *et al.* (1987) reported results which led them to contradict our claim (King & Kuchel, 1985) that human erythrocytes are highly permeable to α-L-glutamyl-L-alanine and L-alanyl-α-L-glutamate. They deduced, therefore, that these peptides cannot serve as a physiological source of glutamate for glutathione synthesis in human red cells. We agree with the new conclusions and confirm that the interpretation of our earlier experimental results was incorrect. The method we used to infer peptide transport was based on measuring the appearance of signals from free amino acids in the ^1H spin-echo n.m.r. spectra of red cells treated with peptide; the high concentration of intracellular peptidases catalyse the hydrolysis of the peptides, and the rate of formation of free amino acids was interpreted as being due to peptide transport across the cell membrane into the cytoplasm.

It is now realized that, especially in high-haematocrit suspensions (even in non-spinning n.m.r. tubes), invariably approx. 1% of the cells haemolyse over a 2 h period. This releases peptidases to the extracellular medium where they catalyse peptide hydrolysis. The phenomenon is illustrated in Fig. 1. Fresh, washed red cells (haematocrit 32%; this is less than half that which was used in our previous n.m.r. experiments) were incubated with peptide at 37 °C. N.m.r. spectra were recorded periodically at a probe temperature of 37 °C. Spectrum A was obtained 7 min after addition of L-alanyl-α-L-glutamate; the spectral features are similar to those reported previously (King & Kuchel, 1985). The small inverted peak a indicates the presence of free alanine, whilst the large (truncated) resonance, adjacent to it at higher frequency, is from the corresponding methyl group in the peptide. Spectrum B, which was obtained after 291 min of incubation, contains two obviously new features; the alanine resonance has increased in intensity as has that denoted g, namely, the glutamate-H^{γ}. Spectrum C is of red cells (haematocrit 80%) obtained after washing the sample immediately after the acquisition of spectrum B. The small glucose peaks (the main complex centred at 3.4 p.p.m.) and lactate (methyl resonance at 1 p.p.m.) in spectrum C, relative to the

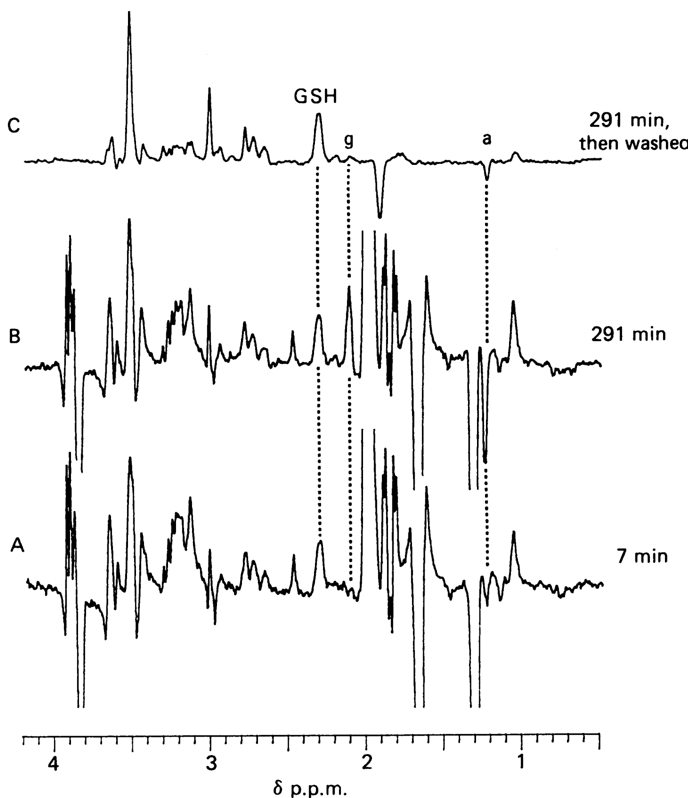

Fig. 1. ^1H spin-echo n.m.r. spectra of human erythrocytes

Sample preparation: venous blood was withdrawn (from B.E.C.) and immediately washed by two cycles of centrifugation (~ 3000 ***g*** for 5 min, 4 °C) in 0.154 M-NaCl/5 mM-glucose and three cycles (~2000 ***g*** for 5 min, 4 °C) in the same medium constituted with ^2H_2O. The buffy coat was aspirated along with the supernatant at each washing step. The final haematocrit was adjusted to 32% and 2.5 ml of the suspension was added to a 5 mm (o.d.) glass n.m.r. tube. At the start of the reaction time-course 70 μl of 500 mM-L-alanyl-α-L-glutamate was added to the cell suspension and immediately mixed by five times inverting and re-inverting the tube with movement of the trapped air bubble along the full extent of the tube.

N.m.r. conditions: we used a Varian XL-400 spectrometer operating in the pulse Fourier transform mode; the probe temperature was 37 °C; the residual $^1HO_2H$ resonance in the spectra was suppressed by selective irradiation prior to the spin-echo pulse sequence (e.g., King & Kuchel, 1985). Data was stored in 8192 memory locations. Each spectrum was derived from 128 free-induction decays and a line broadening factor of 1 Hz was used. The three spectra above were plotted so that the intensity of the glutathione-glutamyl-H^{γ} resonances (denoted by GSH) were of equal amplitude. A, Spectrum of cell suspension 7 min after addition of the peptide; B, the same sample after 291 min of incubation; C, cells from the sample in B obtained by washing four times in the ^2H_2O medium using the method of Young *et al.* (1987). Spectral assignments are not given in detail but 'a' denotes the free alanine-H^{β}, 'g' denotes free glutamate-H^{γ}. The chemical-shift scale was assigned with the ergothionine —N—$(CH_3)_3$ resonance set to 3.100 p.p.m.

previous two spectra, is a consequence of the large extracellular volume in the previous cases. The important points to notice in spectrum C are that very little free glutamate (peak g) is evident and the free alanine (peak a) is also small compared with Spectrum B. The intensity ratio (spectrum C/spectrum B) of free alanine is larger than that for free glutamate. The results indicate that considerably more alanine than glutamate had entered the cells; yet, if the peptide had entered and been subsequently hydrolysed, then, since alanine efflux is known to be faster than that of glutamate, the glutamate resonance should have been proportionately larger than that of the alanine. The converse is clearly evident in spectrum C. The results are consistent with the peptide having been hydrolysed outside, and glutamate and alanine having entered the cells as free glutamate and alanine.

Experiments similar to that mentioned above were carried out with α-L-glutamyl-L-alanine in the presence and absence of amphotericin B (at a concentration that we used previously; King *et al.*, 1983) yielding results very similar to those mentioned above. Very little glutamate was evident inside the cells after washing. Amphotericin B appeared not to influence, to any significant extent, the appearance of glutamate in the washed cell suspensions.

In conclusion, we agree with Young *et al.* (1987) that the rate of entry of α-glutamyl-peptides into human erythrocytes at 37 °C is very small. In our earlier work we were already concerned about the possibility of extracellular hydrolysis of peptides (see the discussion in King & Kuchel, 1985), so we carried out a control experiment that involved the suppression of extracellular resonances with a paramagnetic complex, ferrioxamine. The emergence of resonances in the ^{1}H n.m.r. spectrum assigned to free glutamate and alanine was interpreted as these species appearing inside the cells. However, full suppression of extracellular resonances was not achieved, and we now know that the emergence of the free alanine and glutamate resonances was a consequence of the failure to suppress these extracellular peaks fully. Furthermore, the differences in amplitude of the resonances from the two amino acids should also have indicated to us that the concentration of alanine inside the cell was greater than that of glutamate and was therefore inconsistent with a large amount of peptide transport. The implication of the present work on the interpretation of studies in which n.m.r. was used to monitor the transport of other peptides into erythrocytes has yet to be fully explored.

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