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The effect of histidine and cysteine on zinc influx into rat and human erythrocytes

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1. The effect of histidine and cysteine on the initial rate of zinc influx into rat and human erythrocytes in the presence of bovine serum albumin has been investigated.

2. The L-enantiomers of both amino acids promoted zinc influx into rat erythrocytes in a dose-dependent manner. L-Histidine, but not L-cysteine, also promoted zinc uptake into human erythrocytes. D-Histidine did not promote zinc uptake in either rat or human erythrocytes. In rat erythrocytes D-cysteine was significantly less effective than L-cysteine.

3. The stimulation of zinc influx into rat erythrocytes by 20 mM L-histidine was approximately 4·1 times that seen with human erythrocytes.

4. The influx of zinc in the presence of varying concentrations of L-histidine was linearly related to the calculated concentration of the zinc–bis-histidine complex but not to that of the zinc–mono-histidine complex or the free ionic zinc concentration.

5. These results are discussed in relation to the nature of the transport mechanisms involved.

The wide range of physiological roles ascribed to zinc makes it important to understand the mechanisms by which it may be taken up into cells. Aiken, Horn & Saunders (1992) reported that histidine promoted net uptake of $^{65}Zn^{2+}$ into rat erythrocytes *in vitro* in a dose-dependent and stereospecific manner. It was suggested that the zinc might be transported as a metal–amino acid complex on a transporter which normally carries amino acids, and that this could provide a mechanism for cellular accumulation of zinc under conditions where the anion exchange mechanism previously described by Kalfakakou & Simons (1990) and Alda & Garay (1989) would not make a significant contribution owing to the low ionic zinc concentration. This is likely to be the case in plasma, where the ionic zinc concentration is below 10^{-9} M (Magneson, Puvathingal & Ray, 1987).

We wished to extend these initial observations in several respects. Firstly, it was important to measure initial uptake rates, so that influx could be distinguished from net accumulation at 1 h, as previously measured. Secondly, it was decided to carry out all experiments in the presence of albumin so that the free ionic zinc concentration would be maintained at a low level. Thirdly, we investigated the effect on zinc uptake of cysteine, which is also known to be a major zinc-binding ligand in plasma (Giroux & Henkin, 1972).

Finally, we wished to investigate further the actual form in which zinc is transported and the carriers involved. We therefore adopted three separate approaches to the problem: by comparing the uptake rates in the presence of histidine in human and rat erythrocytes; by investigating the stereoselectivity of the stimulation by histidine and cysteine; and by correlating the measured influx rates with the calculated concentrations of free zinc, zinc–mono- and zinc–bis-histidine complexes at a range of histidine concentrations.

Previous experiments (Liou, 1993) suggested that the system L carrier was a likely candidate for the uptake of zinc–histidine complexes. This suggestion was based on the known properties of this system, in particular its relatively broad specificity, and the ability to show *trans* stimulation. This appeared to correlate with the finding that the L-histidine stimulation of zinc uptake in human erythrocytes was greater in erythrocytes preloaded with L-leucine. It has been shown that the system L and system y^+ carriers are capable of transporting histidine in human erythrocytes (Liou & Ellory, 1990).

It has also been reported that there is a considerable species difference in the expression of the system L carrier. The system L carrier in rat erythrocytes has a similar K_m to that in the human but, using phenylalanine-sensitive

leucine transport, the calculated V_{max} for the rat cells is approximately 450 times that seen in human cells (Yao, George & Young, 1993). This prompted us to investigate whether there was a quantitative difference between the histidine and cysteine stimulation of zinc uptake in human and rat erythrocytes.

METHODS

All blood samples were obtained on the day of the experiments. Rat (Wistar albino) blood was drawn by cardiac puncture under sodium pentobarbitone anaesthesia (80 mg kg^{-1}, I.P.). Coagulation was prevented by the addition of heparin (20 units ml^{-1}). Human blood was obtained, with local ethical committee approval, from healthy volunteers by venepuncture. Coagulation was prevented by the addition of EDTA (final concentration, 10 mM). Blood samples were centrifuged and the plasma and buffy coat were aspirated and discarded. The red cell pellets were then resuspended and washed three times in 50 ml of a solution containing 150 mM NaCl and 5 mM $CaCl_2$, then the final pellets were suspended in a buffer at pH 7·4, composed of 10 mM Hepes, 100 mM NaCl, 1% (w/v) bovine serum albumin (BSA; Sigma, Fraction V, initial fractionation by heat shock) and 125 mM sucrose ('suspension buffer') at a haematocrit of 10%. In those experiments involving the use of cysteine the suspension buffer also contained 2 mM mercaptoethanol to prevent oxidation to cystine. All amino acids were obtained from Sigma.

Incubation protocol

Polystyrene incubation flasks (Dilu Vials®, Elkay Products Inc., Shrewsbury, MA 01545, USA) were prepared containing $^{65}Zn^{2+}$ (New England Nuclear; specific activity 2·64 mCi mg^{-1}) and amino acids as appropriate dissolved in 5 ml of suspension buffer at twice the final required concentrations. Stock amino acid solutions (100 mM) were made each day and buffered to pH 7·4 before dilution in suspension buffer. The final concentration of zinc in the incubation solutions was 6·5 μM in the case of the histidine experiments and 4·5 μM in the case of the cysteine experiments. The flasks were incubated in a water-bath at 37 °C with gentle orbital agitation. The experiments were started by the addition of 5 ml of cell suspension to each incubation flask. The final haematocrit in the experiments was therefore 5%. Samples (0·5 ml) were taken in triplicate immediately and at 4, 8 and 12 min or 3, 6 and 12 min after the addition of the cells, depending on the experiment. The 0·5 ml samples were added to microfuge tubes (Eppendorf) containing 0·35 ml of silicone oil (550; Dow Corning) on top of which had been layered 0·65 ml of 10 mM EDTA in 150 mM NaCl ('stop solution'). The stop solution prevents further uptake of zinc and removes surface-bound zinc from the red cells. Samples were then spun at 13000 r.p.m. in a microfuge (Micro Centaur, MSE) for 1 min. The cells were pelleted through the silicone oil and the remaining incubation buffer and stop solution formed a layer above the oil. This layer was then aspirated and the inside of the tubes was wiped using cotton dental rolls. At the end of the experiment samples were taken from the incubation flasks for the determination of total radioactivity and for cell counts. The tubes containing the red cell pellets and the samples of incubation fluid were counted in a gamma counter. Cell counts were performed on samples from each incubation flask diluted in 150 mM NaCl using a Coulter counter with an aperture diameter of 70 μm for the rat cells and 100 μm for the human cells. The counts in the pellets taken at zero time consisted of background radiation, trapped incubation fluid and surface-bound zinc which the stop solution has failed to remove. Net uptake at subsequent times was therefore determined for each incubation by subtracting the average count at zero time from later measurements. All sample measurements were made in triplicate and each experiment was carried out at least four times using different human and animal donors each time. Results are expressed as means ± S.E.M., where n is the number of experiments. Significance of differences has been determined using Student's t test.

RESULTS

Figure 1 shows the net zinc uptake per 10^{13} cells against time for various concentrations of L-histidine in rat (*A*) and human red cells (*B*). There is very little uptake of $^{65}Zn^{2+}$ in the absence of histidine, which is to be expected since in the presence of albumin the concentration of free ionic zinc will be negligible (Fig. 4). The small amount of uptake which is present does not appear to increase with time and may represent trapped fluid or surface-bound zinc which has not been removed by the 'stop solution'. There is a concentration-dependent stimulation of zinc uptake by L-histidine such that the net uptake at 12 min is significantly greater than control values for histidine concentrations greater than 1·0 mM in both the rat and human experiments. Net uptake was linear up to 12 min in the human experiments but only up to 8 min in the rat experiments. It is likely that the greater rate of histidine uptake in the latter case results in a considerable increase in intracellular histidine concentrations with a resulting histidine-stimulated efflux in the case of the rat red cells. It is also possible that the external histidine concentration is being depleted. In the human red cells, D-histidine, over the same range of concentrations, produced a small but insignificant inhibition of $^{65}Zn^{2+}$ uptake compared with control incubations (Fig. 1*B*, only the 20 mM concentration data are presented for the sake of clarity). A similar result was obtained in experiments using rat red cells. This is to be expected if the D-histidine is chelating free, ionic zinc but the complex formed is not then a substrate for the transport process and, more importantly, suggests that the L-histidine stimulation is acting via a carrier-mediated transport system rather than simply rendering the zinc more diffusible.

Figure 2 shows the initial rate of zinc uptake per 10^{13} cells per minute, calculated over the first 8 min of the experiment, against the concentration of L-histidine for both the rat and human cells. The shape of the two curves is identical but the extent of the stimulation seen in the rat cells is considerably greater (4·7 times at 10 mM and 4·1 times at 20 mM) than that seen in the human. It should also be appreciated that the mean corpuscular volume of rat red cells is less than that of human red cells (61 fl compared with 80 fl; Albritton, 1952) and thus the uptake per unit

surface area is even greater in the rat compared with the human than the results shown in Fig. 2 would suggest.

Figure 3 shows the results of similar experiments carried out with L-cysteine. The difference between rat and human cells is even more pronounced (compare Figs 2 and 3) since cysteine did not stimulate uptake at all in human cells. L-Cysteine produces a significant stimulation of zinc uptake in rat cells at lower concentrations than is seen with L-histidine. Stimulation of uptake is significant at a concentration of 0·1 mM L-cysteine. At L-cysteine concentrations above 1 mM the stimulation of uptake begins to decline perhaps because the transport process is

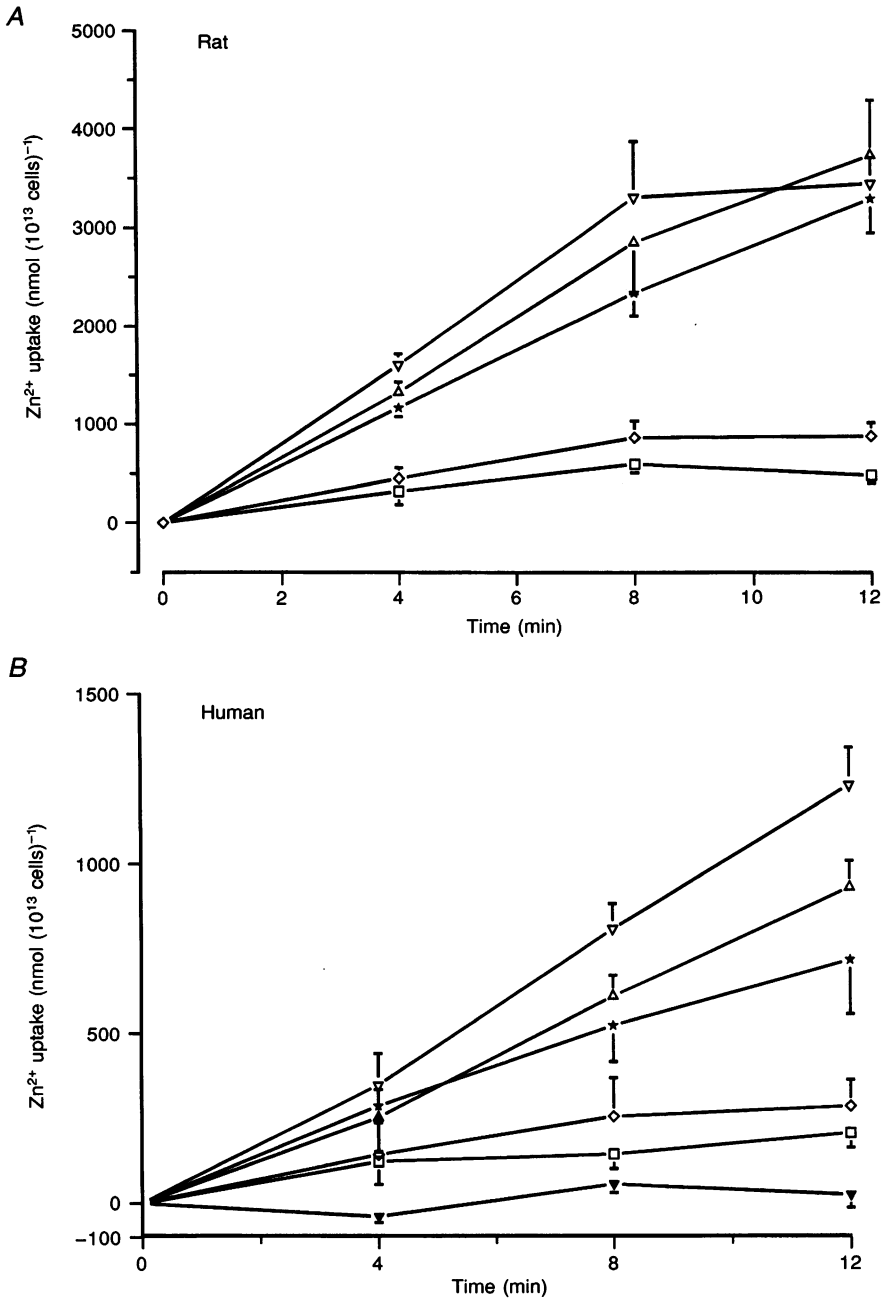

Figure 1. The effect of L-histidine on net zinc uptake

Net zinc uptake (nmol (10^{13} cells)$^{-1}$) over 12 min was determined at various L-histidine concentrations of 0 mM (□), 1 mM (◇), 5 mM (★), 10 mM (Δ) and 20 mM (▽) for rat (*A*) and human (*B*) red cells. The effect of 20 mM D-histidine (▼) is also illustrated for the human red cell experiments. Each point represents the mean of 5 (rat) or 4 (human) experiments with error bars equal to the S.E.M. where it is greater than the size of the symbol.

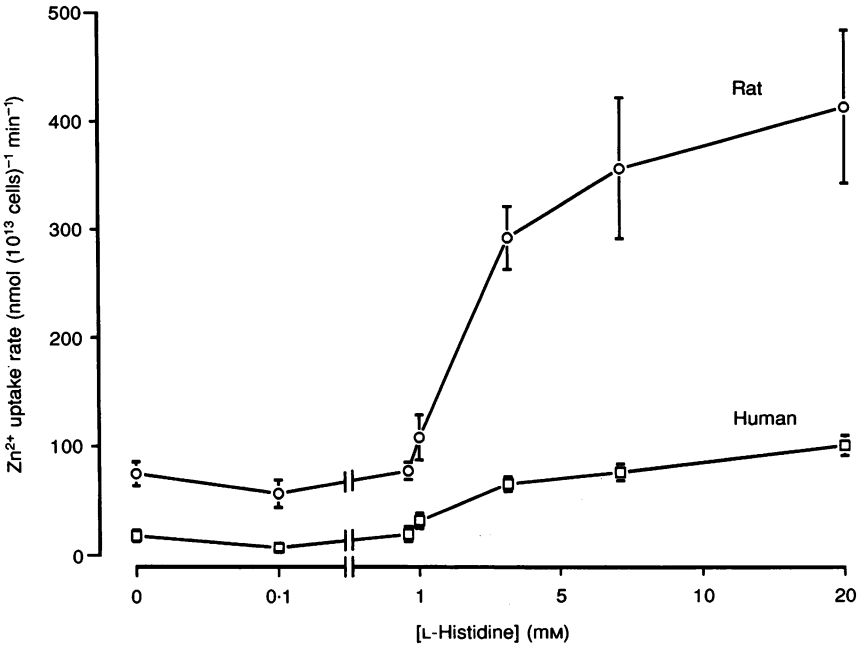

Figure 2. The effect of various L-histidine concentrations on the rate of zinc uptake
The rate of zinc uptake (nmol (10^{13} cells)$^{-1}$ min^{-1}) calculated over the first 8 min of the incubations at various histidine concentrations in rat (○) and human (□) red cells. Points represent the means of 5 (rat) or 4 (human) experiments with error bars equal to the S.E.M. where it is greater than the size of the symbol.

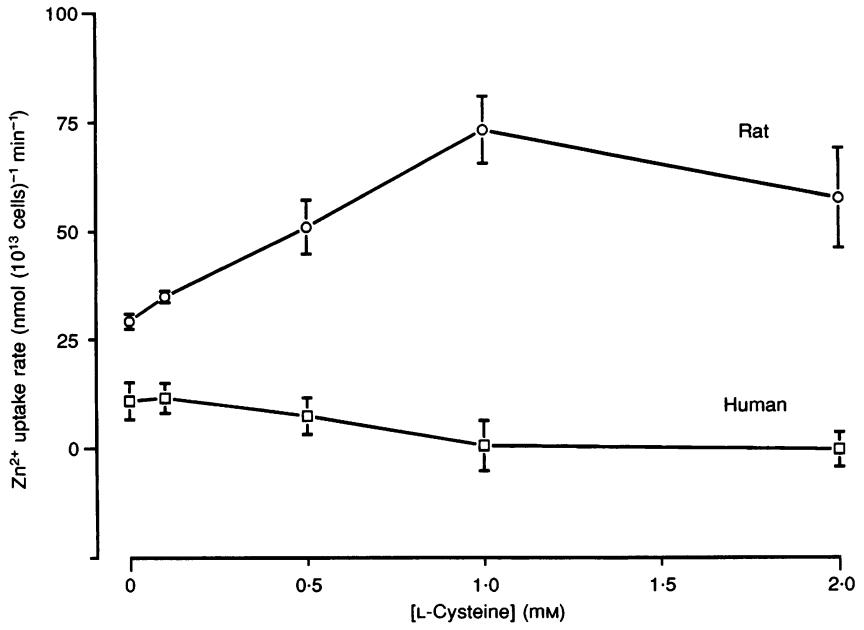

Figure 3. The effect of various cysteine concentrations on the rate of zinc uptake
The rate of zinc uptake (nmol (10^{13} cells)$^{-1}$ min^{-1}) calculated over the first 12 min of the incubations at various cysteine concentrations in rat (○) and human (□) red cells. Points represent the means of 6 (rat) or 5 (human) experiments with error bars equal to the S.E.M. where it is greater than the size of the symbol.

saturated or because L-cysteine or an L-cysteine–zinc complex is competing with the species being transported. There is some stimulation of uptake by D-cysteine (not shown here) but it is significantly ($P = 0{\cdot}039$) less than that seen with L-cysteine. In the experiments with human red cells there is no detectable stimulation of zinc uptake by L-cysteine (Fig. 3). All the experiments using L-cysteine were conducted with mercaptoethanol (2 mM) added to the stock amino acid solution and the suspension buffer. This was used to prevent the oxidation of cysteine to cystine. It is possible that the mercaptoethanol present in the cysteine experiments may have interfered in some way with the transport process in human cells and so a series of experiments was conducted to examine the effect of mercaptoethanol on the L-histidine stimulation of zinc uptake in human red cells. Using L-histidine concentrations of 0, 2, and 5 mM it was found that there was no significant difference between the histidine stimulation of zinc uptake in the presence or absence of 2 mM mercaptoethanol. (At 8 min in the presence of 5 mM L-histidine, the uptakes were 680 ± 140 and 648 ± 137 (means $\pm$ S.E.M.; $n = 4$) with and without 2 mM mercaptoethanol, respectively.) It therefore seems unlikely that the absence of a cysteine-stimulated zinc uptake in human cells is due to an interference with the transport process by mercaptoethanol.

The exact nature of the amino acid–zinc complex which is being transported remains to be determined. Zinc is able to combine with one or two histidine molecules producing a mono or bis complex. We have calculated the concentrations of the three forms of zinc at various concentrations of L-histidine in the presence of 1% BSA (Fig. 4) using log stability constants for histidine of $k_1 = 6{\cdot}7$, $\beta_2 = 11{\cdot}8$ (Dawson, Elliott, Elliott & Jones, 1986), for albumin of $k_1 = 6{\cdot}98$ (Giroux & Henkin, 1972) and pK (the $-\log$ of K_d) values for histidine of 9·17 and 6. This calculation was performed using a computer program (T. J. B. Simons, personal communication) which assumes that the interaction of zinc with albumin and with the amino acid are independent, and that no significant binding of amino acid to albumin occurs. This has recently been confirmed in the case of histidine by Masuoka & Saltman (1994). As the concentration of L-histidine is increased the free ionic zinc concentration falls steadily, the mono complex concentration initially rises slightly but then falls, whilst the concentration of the bis complex rises steadily. In Fig. 5 the uptake of zinc per minute over the first 8 min of the incubation in rat cells is plotted against the calculated concentrations of the various zinc moieties produced by different concentrations of L-histidine. There is an inverse relationship between zinc uptake and free ionic zinc concentration (not shown) and a linear relationship between uptake and the concentration of the zinc–bis-histidine complex. There is no clear relationship between zinc uptake and the concentration of the zinc–mono-histidine complex.

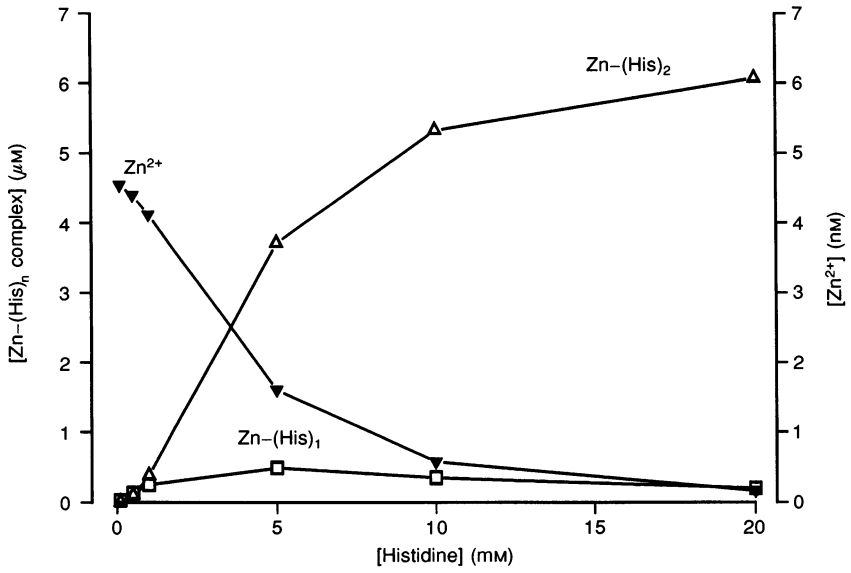

Figure 4. The effect of L-histidine on the concentrations of various zinc moieties

Concentrations of various zinc moieties: free ionic zinc (▼), the zinc–mono-histidine complex (Zn–(His)$_1$; □) and the zinc–bis-histidine complex (Zn–(His)$_2$; △) calculated for various concentrations of L-histidine in the presence of 6·5 μM zinc and 1% BSA.

DISCUSSION

The mechanism by which zinc is transported across the erythrocyte cell membrane under physiological conditions is still not clearly established. The experiments described in this paper were designed to obtain further information about the mechanism by which zinc-binding amino acids promoted uptake of zinc into erythrocytes *in vitro*, and to investigate further the suggestion that a zinc–amino acid complex might be transported on the system L carrier. The experiments were all carried out in a medium containing 150 μM BSA, to ensure that the free ionic zinc activity was kept at a low level, and would not vary, for example with

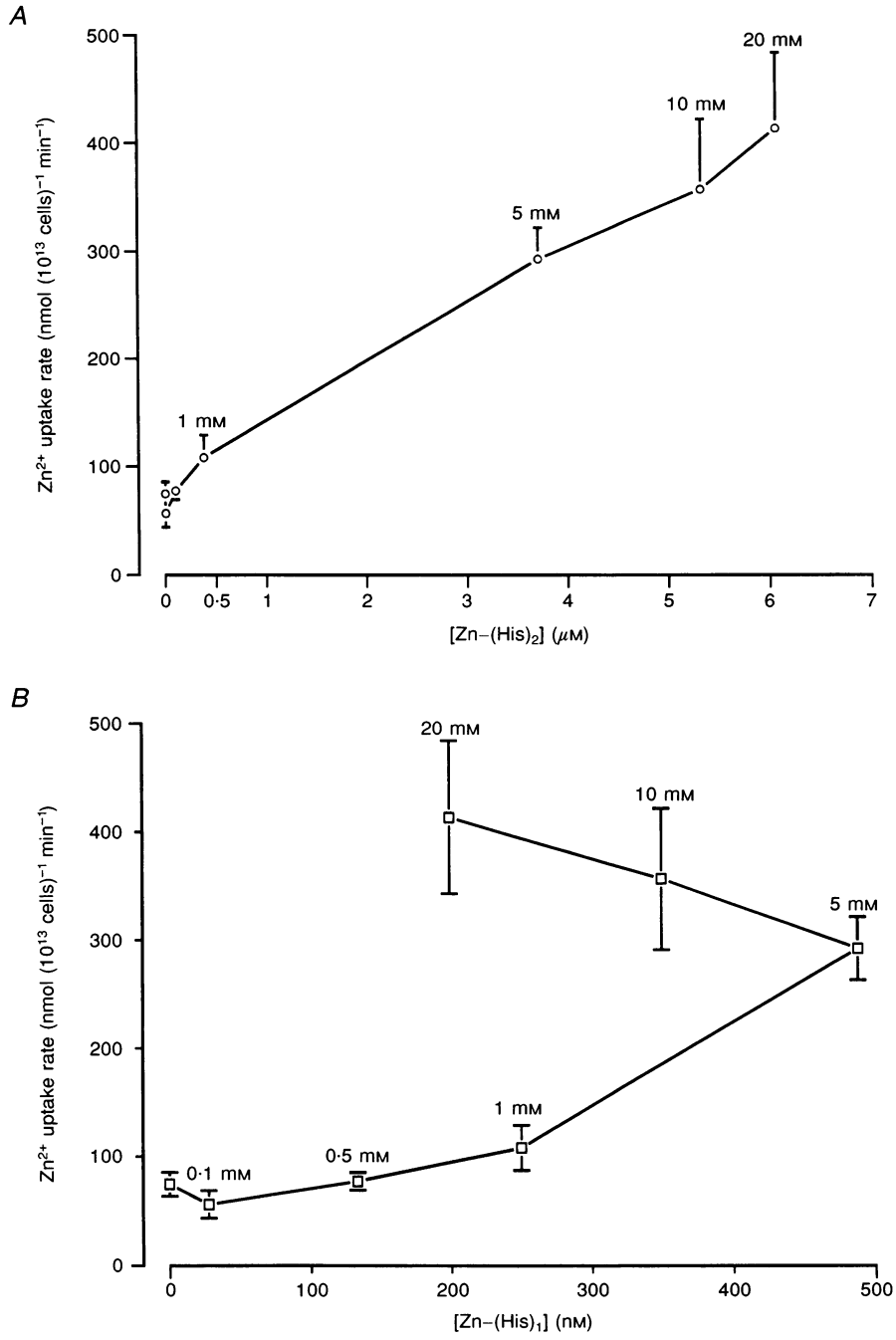

Figure 5. The relationship between the rate of zinc uptake by rat red cells and the concentrations of various forms of zinc complex

Using the data from the experiments depicted in Fig. 1*A*, the rate of zinc uptake is plotted against the calculated concentrations of the complex between zinc and 2 histidine molecules ($Zn–(His)_2$; *A*) and the complex between zinc and 1 histidine molecule ($Zn–(His)_1$; *B*). Points represent the mean of 5 experiments with error bars equal to the S.E.M. where it is greater than the size of the symbol. The numbers next to the points indicate the relevant L-histidine concentrations (in mM).

small changes in other zinc-binding macromolecules such as haemoglobin from damaged erythrocytes. Use of rapid centrifugation through silicone oil also enabled measurement of uptake at time intervals short enough to measure influx, so that meaningful comparisons of transport rate could be made.

The first finding to emerge clearly from these studies is that in rat and human erythrocytes histidine causes a dose-dependent stereoselective stimulation of zinc influx, which has not reached a maximum at 20 mM external concentration (Fig. 2). In the case of cysteine, the stimulation was maximal between 1 and 2 mM external concentration. The stimulation of influx by both amino acids showed stereospecificity. In the case of histidine, this was virtually absolute, with D-histidine causing only a non-significant inhibition compared with control in both human and rat erythrocytes. With cysteine in rat erythrocytes, there was a clear preference for the L-enantiomer although this was not as complete as with histidine. However precise estimates of any L-cysteine contamination of the D-cysteine preparation are not available. We have found no evidence in the literature for stereoselectivity in the formation of zinc–amino acid complexes except for the report by Morris & Martin (1970) that there was a small preference for the formation of the mixed D–L bis-histidine complex compared with the L–L or D–D complexes. This is of little direct relevance in experiments where only one enantiomer is present. It is clear that stereoselectivity is a property of the uptake mechanism, and strongly indicates the involvement of a carrier system. Earlier experiments (Liou, 1993) suggested that the system L carrier was a possible candidate. There are conflicting reports in the literature regarding the stereoselectivity of this transporter. Young, Jones & Ellory (1980) found that in human erythrocytes the apparent K_m for L-leucine uptake was approximately one-third of that for D-leucine, while the V_{max} was approximately half. Hider & McCormack (1980) state that the rate of uptake into human erythrocytes of both enantiomers of leucine was approximately the same, although their data appear to show that the initial influx was greater for L-leucine. Liou & Ellory (1990) reported that histidine transport into human erythrocytes was primarily by the system L and y^+ carriers. Information about the stereoselectivity of histidine and cysteine transport into erythrocytes by either of these carriers is not yet available, and the extreme stereospecificity of the histidine stimulation of zinc uptake cannot therefore be directly related to the properties of histidine or cysteine uptake by either carrier.

The second approach has been to compare the relative stimulation of zinc uptake by histidine and cysteine in rat and human erythrocytes. It was found that the stimulatory effect of both amino acids on the rate of zinc uptake was considerably greater in rat than in human erythrocytes, indeed cysteine had no stimulatory effect at all in human erythrocytes. This is compatible with the suggestion that the system L carrier may be involved, since it has been reported that this is expressed to a much greater degree in rat than human erythrocytes (Yao *et al.* 1993). These authors found that the V_{max} (measured at 1 °C) for phenylalanine-sensitive leucine transport in the rat erythrocyte was several hundred times that seen in human cells, although the K_m was similar. We have not measured V_{max} for the amino acid stimulation of zinc transport in the two species.

The third approach adopted has been to relate measured uptake rates to calculated concentrations of ionic zinc and zinc–histidine complexes. Ionic zinc concentration decreases as histidine concentration is raised (Fig. 4), and an uptake mechanism involving free ionic zinc is therefore unlikely. There is no clear relationship between the zinc uptake rate and the calculated concentration of the zinc–mono-histidine complex (Fig. 5*B*), whereas the zinc uptake rate appears to be clearly related to the zinc–bis-histidine complex concentration (Fig. 5*A*). It seems unlikely that the bis complex would itself be a substrate for an amino acid uptake system, even one with such broad specificity as the system L carrier, and evidence is lacking for uptake of histidine- or cysteine-containing dipeptides into erythrocytes. It is possible that the bis complex promotes uptake by enabling zinc to diffuse through an unstirred layer such as the extracellular glycocalyx, but this does not explain the marked stereoselectivity found. However, the lability of the zinc–amino acid complexes would mean that the bis complex could dissociate in the immediate vicinity of the active site of the carrier and thus promote the subsequent transport of the mono-histidine complex in a stereospecific manner. Owing to the higher stability constants for the formation of cysteine–zinc complexes compared with histidine, we calculate that the concentration of the zinc–bis-cysteine complex present at a total cysteine concentration of 2 mM would be comparable with that of the zinc–bis-histidine complex at a total amino acid concentration of 20 mM, and this is in agreement with the fact that cysteine stimulated uptake at lower concentrations than histidine.

It has not been possible to raise the concentration of the bis-histidine complex sufficiently to achieve saturation (Fig. 5*A*). However, the data so far obtained suggest that the apparent affinity of the complex for the uptake mechanism is high, possibly in the low micromolar range. This can be compared with the reported K_m for L-leucine uptake in the human erythrocyte of either 5·8 or 12·9 mM, depending on the method of curve fitting (Young *et al.* 1980). This would suggest that the system L and/or y^+ carriers would not be effective at the low bis complex concentrations present in our experiments. However, a novel variant, the y^+L carrier, has been recently described and partially characterized in human erythrocytes (Devés, Chávez & Boyd, 1992; Angelo & Devés, 1994). The K_m for

lysine was found to be 14 μM, and lysine transport was inhibited by leucine with a K_i (half-maximal inhibition constant) of 22 μM. When sodium in the medium was replaced by potassium, the apparent affinity for leucine was reduced approximately 30-fold. Lysine uptake showed *trans* stimulation by leucine, isoleucine and phenylalanine. It was also reported that lysine transport through this system was not inhibited by pretreatment with *N*-ethylmaleimide (NEM), which did block the y^+ carrier (Devés, Angelo & Chávez, 1993). This novel carrier is therefore a possible candidate for transport of zinc–histidine complexes, although information is needed about the ability to transport histidine and cysteine and especially about the selectivity for enantiomers of both amino acids in human erythrocytes. Preliminary experiments suggest that the stimulation of zinc uptake by histidine is resistant to pretreatment with NEM, which appears to exclude the participation of the y^+ carrier.

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