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EFFECTS OF AMINO ACIDS ON ZINC TRANSPORT IN RAT ERYTHROCYTES

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SUMMARY

1. A significant proportion of plasma zinc exists complexed with amino acids. The effect of amino acids on the accumulation of radioactive zinc by rat erythrocytes was studied *in vitro*, to investigate the hypothesis that zinc might be transported into cells as an amino acid–zinc complex.

2. L-Histidine (500 μM–10 mM) stimulated ^{65}Zn uptake; 50 mM-L-histidine gave a slight inhibition of uptake. D-Histidine (500 μM–10 mM) inhibited uptake in a dose-dependent manner. A non-zinc-binding amino acid, L-alanine, did not affect ^{65}Zn uptake.

3. The effect of L-histidine was sodium dependent and temperature dependent, but was DIDS insensitive. These properties suggest that zinc is being transported as a zinc–histidine complex, utilizing an amino acid carrier system. Uptake of zinc in the presence of L-histidine differed from the previously described ionic mechanism, and may represent a physiological route of uptake.

4. L-Histidine stimulated efflux of ^{65}Zn from pre-loaded cells.

5. The relevance of transport of a zinc–histidine complex is discussed with reference to histidinaemia, and as a significant zinc transport system in the presence of the very low ionic zinc concentrations found in plasma.

INTRODUCTION

The form of zinc that is transported into animal tissue *in vivo* has yet to be identified. Kalfakakou & Simons (1986) and Alda Torrubia & Garay (1989) have presented evidence for the uptake of an ionic form of zinc by human erythrocytes *in vitro*. Uptake was found to be inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), suggesting the involvement of an anion exchanger. However, both these studies entailed the use of ionic zinc concentrations in the micromolar range. Plasma zinc is 98% protein-bound, with the remainder mainly

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complexed with amino acids such as histidine and cysteine (Giroux & Henkin, 1972). Therefore the concentration of ionic zinc in plasma is likely to be very low, as has been shown by Magneson, Puvathingal & Ray (1987) by means of a highly sensitive enzyme-linked assay of Zn^{2+}. They reported a free Zn^{2+} concentration of $2{\cdot}1 \times 10^{-10}$ M in horse plasma.

Thus the physiological significance of ionic zinc uptake *in vivo* is not clear. An alternative mechanism has therefore been considered, namely, the possibility of coupling of zinc transport and amino acid transport. Since a significant fraction of plasma zinc exists as a zinc–histidine complex (Giroux & Henkin, 1972), and since avid uptake systems for this amino acid have been demonstrated in a variety of tissues (e.g. Oldendorf, 1971; Pardridge & Jefferson, 1975) including erythrocytes (Young & Ellory, 1977), we have examined the hypothesis that zinc may be transported across the erythrocyte membrane as a zinc–histidine complex.

Such a mechanism has previously been postulated by Sivarama Sastry, Viswanathan, Ramaiah & Sarma (1960). They found that histidine (1 mM) inhibited the uptake of ^{65}Zn by 90% in horse erythrocytes, and explained this finding in terms of saturation of the histidine carrier (only one concentration of histidine was used). In view of the high affinity of histidine for zinc (Giroux & Henkin, 1972), this inhibition of zinc uptake could equally well be explained in terms of chelation of ionic zinc. Indeed, concentration-dependent inhibition by histidine of zinc uptake has been reported by Wensink, Molenaar, Woroniecka & Van den Hamer (1988) in rat synaptosomes, and was presented as evidence for ionic zinc transport.

In order to distinguish the effects of chelation from possible effects on amino acid-linked transport, the non-biological stereoisomer D-histidine has been used in the present study. Histidine transport has been shown to be stereospecific at several sites; for example at the blood–brain barrier (Oldendorf, 1973) and the renal tubule (Crampton & Smyth, 1953). Furthermore, transport of glutamine by the type N carrier, which also transports histidine, shows stereospecificity (Kilberg, Handlogten & Christensen, 1980). The experiments described in this paper have been designed to compare the effects of L-histidine and D-histidine on the uptake of ^{65}Zn by rat erythrocytes *in vitro*.

A preliminary account of this work has been published (Aiken, Horn & Saunders, 1990).

METHODS

Animals

Male albino Wistar rats (300–700 g) were anaesthetized with sodium pentobarbitone (200 mg/kg, intraperitoneal injection), and were bled by cardiac puncture into heparinized syringes (final heparin concentration 100 units/ml). The blood was centrifuged, the plasma was removed, and the erythrocytes were washed three times in three times their volume of ice-cold isotonic (154 mM) saline solution. Erythrocytes from three to five rats were pooled for each experiment.

Suspension

A suspension medium containing sodium chloride (100 mM), sucrose (125 mM) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES; 10 mM) was used (Simons, 1987). ^{65}Zn was added as zinc chloride, and final zinc concentration was 3·0 μM in all experiments. All incubations were carried out at pH 7·4 and 37 °C, using a packed cell volume of 5%. Aliquots of the suspension (4·4 ml) were incubated in sealed 20 ml vials, and were gassed every 30 min with humidified oxygen. In experiments where sodium bicarbonate was included in the suspension

medium, tubes were gassed with 5% CO_2 in oxygen by diffusion from the gas phase. Direct measurement confirmed that the pH did not fall below 7·37.

Uptake was measured 1, 2, 3, 4 and 6 h after the start of incubation, by centrifuging aliquots and washing the pellet twice with 50 volumes of ice-cold isotonic saline. Residual radioactivity of the pellet was counted by a Beckman 'Biogamma' counter (17% efficiency), and was expressed as a percentage of the total radioactivity available for uptake.

Test substances

Uptake of ^{65}Zn was studied in the presence of L- or D-histidine, L-alanine, bovine serum albumin (BSA), sodium bicarbonate and DIDS. Histidine stock solutions were 120 mM, adjusted to pH 7·4 using 1 M-NaOH or KOH. These solutions were found to be isosmolal with the suspension medium.

Sodium dependence of uptake

To study the sodium dependence of ^{65}Zn uptake, NaCl was replaced by KCl in the incubation medium. For sodium-free incubations, 1 M-KOH was used to adjust the pH of histidine stock solutions.

Effect of temperature

The effect of L-histidine on ^{65}Zn uptake at low temperature was studied, by incubating aliquots in an ice–water slurry. The measured temperature of the suspension was 1 °C.

Membrane binding

To ascertain whether or not histidine affected the binding of ^{65}Zn to the erythrocyte membrane, experiments were performed using 1 mM-ethylenediaminetetraacetic acid (EDTA) in isotonic saline as the solution for washing the erythrocytes after incubation. Apparent uptakes were not altered by this procedure.

Zinc efflux

Efflux of ^{65}Zn from rat erythrocytes was studied by initially loading the erythrocytes with isotope during a 3 h incubation period (conditions as above). The erythrocytes were washed three times in ten times their volume of ice-cold buffer, and were resuspended without ^{65}Zn. Efflux was measured 1–2 h after the start of re-incubation, and was expressed in relation to the ^{65}Zn loading immediately after resuspension. No significant difference in haemolysis was found between incubations with or without histidine.

Source of chemicals

L-Histidine monohydrochloride was from Lancaster Synthesis. Other amino acids, DIDS and BSA (fraction V) were from Sigma. All other chemicals were from BDH, and were of analytical grade. ^{65}Zn ($0·5–2 \times 10^{12}$ Bq/mol Zn) was obtained from Amersham International plc.

RESULTS

Control uptake values

The control uptake of ^{65}Zn at 37 °C varied between approximately 4 and 15% of the available ^{65}Zn, being lowest when albumin was added to the incubation medium. Some variation still occurred in experiments where no extra protein was added. This was likely to have been due to small differences in the extracellular protein concentration (due to haemolysis or plasma protein carry-over) which would have affected the ionic zinc activity and hence the relative contribution made by the anion exchange mechanism (see below).

Effect of histidine

In all experiments, uptake of ^{65}Zn was found to be essentially linear between 1–6 h. At 6 h, the maximum uptake obtained (that with 2 mM-L-histidine) was $37·5 \pm 4$% of the available label. Data were not obtained at times less than 1 h, but back-extrapolation of the 1–6 h uptake line does not pass through the origin. The values

presented are the cell-associated ^{65}Zn at 2 h, expressed as a percentage of the total counts available in the incubation without any allowance for zero uptake.

Figure 1*A* and 1*B* shows the effects of L- and D-histidine on ^{65}Zn uptake by rat erythrocytes. D-Histidine (Fig. 1*B*) inhibited uptake at a concentration of 500 μM, with progressively greater inhibition at higher concentrations.

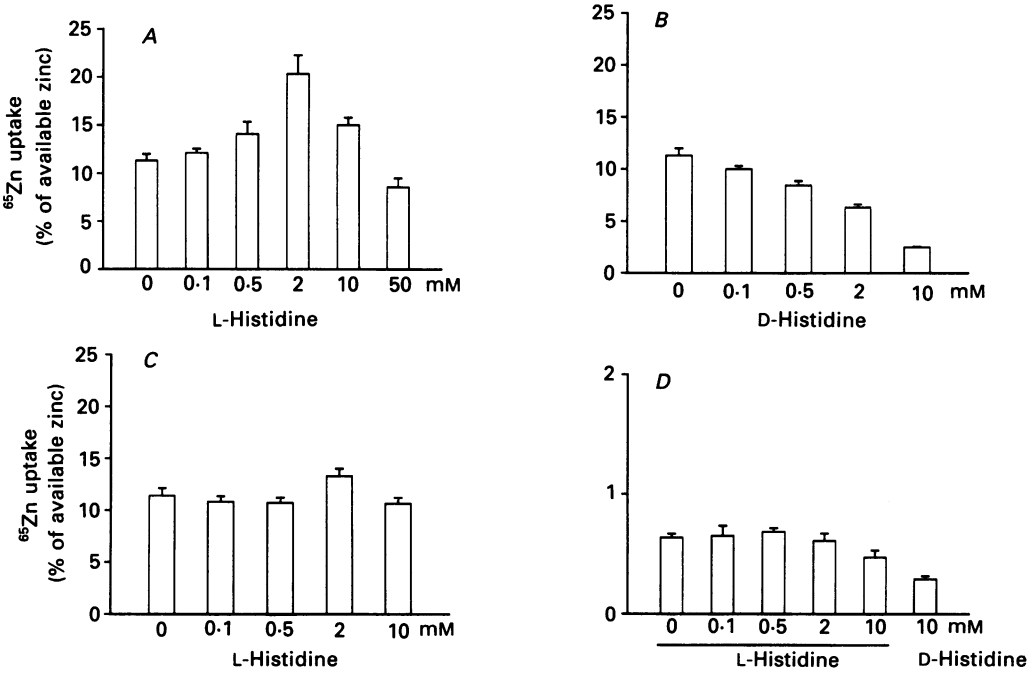

Fig. 1. *A*, effect of L-histidine on ^{65}Zn accumulation by rat erythrocytes. *B*, effect of D-histidine on ^{65}Zn accumulation by rat erythrocytes. *C*, effect of L-histidine on ^{65}Zn accumulation by rat erythrocytes in low-sodium buffer. *D*, effect of L- AND D-histidine on ^{65}Zn accumulation by rat erythrocytes at 1 °C. In all panels, net uptake at 2 h is shown; data from five experiments. Bars represent S.E.M.

The effect of L-histidine was biphasic (Fig. 1*A*). L-Histidine stimulated ^{65}Zn uptake at concentrations of 500 μM–10 mM. However, 50 mM-L-histidine inhibited uptake. Such concentrations did not cause haemolysis. L-Histidine concentrations of 100 μM (Fig. 1*A*) and 10 μM (not shown) had no effect on ^{65}Zn uptake.

Effect of bicarbonate

The presence of bicarbonate (5 mM) was found to promote uptake of ^{65}Zn (Fig. 2), as previously reported for human erythrocytes (Kalfakakou & Simons, 1986; Alda Torrubia & Garay, 1989). However, L-histidine caused further stimulation in the presence of bicarbonate, suggesting that separate mechanisms are involved.

Figure 2 shows the effect of DIDS (10 μM) on ^{65}Zn uptake, with and without L-histidine (10 mM). In these experiments, bicarbonate (5 mM) was included in all aliquots, since it was found that DIDS had little effect in the absence of bicarbonate. DIDS inhibited uptake of ^{65}Zn, as previously reported (Kalfakakou & Simons, 1986;

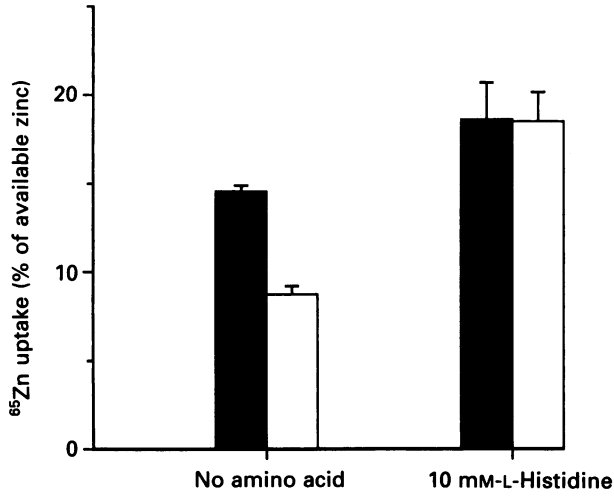

Fig. 2. Effect of DIDS (10 μM) on uptake of ^{65}Zn by rat erythrocytes with and without L-histidine (10 mM). Net uptake at 2 h is shown; data from five experiments. Bars represent S.E.M. Sodium bicarbonate (5 mM) was included in all incubations. ■, no DIDS; □, 10 μM-DIDS.

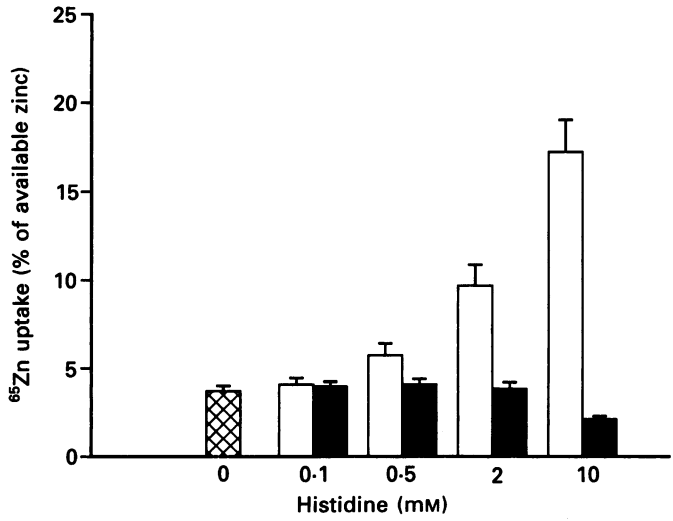

Fig. 3. Effect of histidine on uptake of ^{65}Zn by rat erythrocytes in the presence of bovine serum albumin (100 μM). Net uptake at 2 h is shown; data from five experiments. Bars represent S.E.M. □, L-histidine; ■, D-histidine.

Alda Torrubia & Garay, 1989). However, in the presence of 10 mM-L-histidine, DIDS had no effect.

Effect of albumin

In order to more closely simulate physiological conditions, the effect of histidine was determined in the presence of BSA (100 μM). Results are shown in Fig. 3. Uptake in the absence of histidine was inhibited by BSA (compare with Fig. 1*A*). L-Histidine

caused stimulation of uptake in the presence of BSA (Fig. 3), and this effect was proportionally greater than without BSA (Fig. 1*A*). D-Histidine caused inhibition only at the highest concentration used (10 mM).

Sodium dependence

Figure 1*C* shows the effect of L-histidine on ^{65}Zn uptake in normal and sodium-free media. Uptake in the absence of histidine was not sodium dependent, in agreement

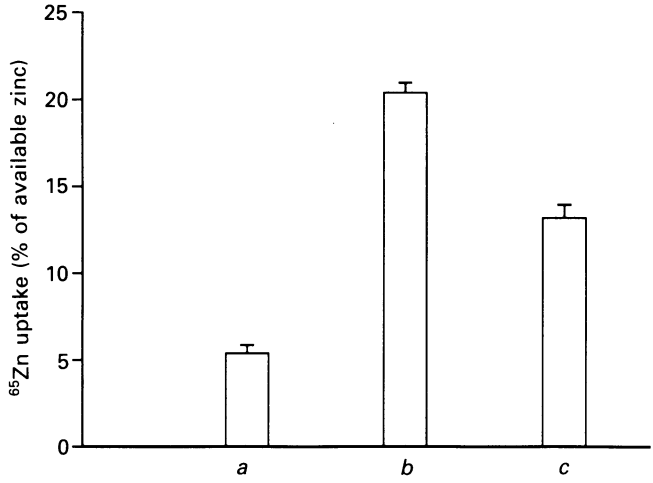

Fig. 4. Effect of pre-incubation with L-histidine (10 mM) on uptake of ^{65}Zn by rat erythrocytes. All erythrocytes were pre-incubated for 3 h before the addition of the ^{65}Zn. In *a*, no histidine was added at any time. In *b*, no histidine was present during the pre-incubation but 10 mM-histidine was added at the same time as the ^{65}Zn. In *c*, 10 mM-histidine was present during both the pre-incubation and the incubation with ^{65}Zn. Net uptake at 2 h is shown, data from five experiments. Bars represent S.E.M.

with the finding of Alda Torrubia & Garay (1989). However, virtually no stimulation of uptake by L-histidine was seen in the absence of sodium (Fig, 1*C*). This again suggests a separate mechanism of uptake in the presence of histidine. The significance of this sodium dependence will be discussed in relation to histidine transport.

Temperature dependence

^{65}Zn uptake by rat erythrocytes was strongly temperature dependent. Figure 1*D* shows that L-histidine did not promote ^{65}Zn uptake at 1 °C; indeed, at the highest concentration (10 mM), inhibition was seen. If L-histidine was promoting zinc influx simply by causing a non-specific increase in membrane permeability, then this effect ought to be observed at both 37 and 1 °C. The results (Fig. 1*D*) appear to discount this mechanism.

Effect of alanine

L-Alanine was chosen as a 'control' amino acid, because although it does not readily form complexes with zinc (Giroux & Henkin, 1972), it can be transported into erythrocytes (Young & Ellory, 1977). L-Alanine (100 μM–10 mM) did not affect ^{65}Zn

uptake by rat erythrocytes, uptake being $11{\cdot}39 \pm 0{\cdot}9\,\%$ in the absence of alanine and $11{\cdot}51 \pm 1.09\,\%$ with 10 mM-alanine, with uptake in none of the intermediate concentrations being significantly different from control.

Dependence of ^{65}Zn uptake on net histidine flux

As already stated, ^{65}Zn uptake was linear up to 6 h, even in the presence of high concentrations of L-histidine. Since incorporation into protein or other metabolism

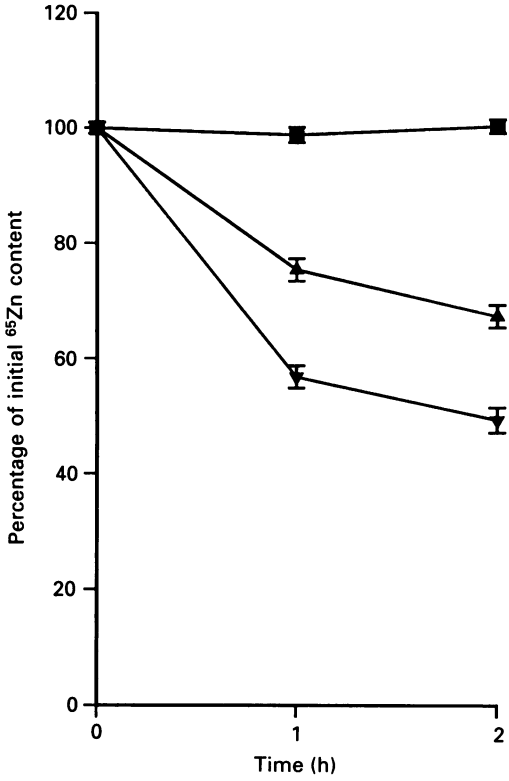

Fig. 5. Efflux of ^{65}Zn from rat erythrocytes following a 3 h loading period. Efflux is shown in the presence of 10 mM-histidine (▲,$n = 11$), in the presence of both 10 mM-histidine and 100 mM-BSA (▼,$n = 3$), and in control saline (■,$n = 10$). Bars represent S.E.M.

of histidine would not be expected in erythrocytes, these high concentrations (2–10 mM) should cause rapid saturation of the erythrocytes with L-histidine; that is, *net* histidine uptake would be greatest at the start of incubation, and would be negligible later on. Therefore, the steady net uptake of ^{65}Zn over 6 h does not seem to be directly related to net uptake of histidine.

The dependence of ^{65}Zn uptake on net histidine uptake was investigated by adding ^{65}Zn to rat erythrocytes after an initial 3 h incubation without ^{65}Zn. Uptake of ^{65}Zn was measured 2 h after this. L-Histidine (10 mM) caused a stimulation of uptake (Fig. 4) as previously. However, if L-histidine was present throughout the initial 3 h period (as well as during the 2 h uptake period,) ^{65}Zn uptake was less than for the

erythrocytes incubated without histidine (Fig. 4). This suggests that the reduced net influx of L-histidine during the 2 h following the addition of the ^{65}Zn in the pre-incubated erythrocytes results in less net ^{65}Zn uptake. However, these erythrocytes still took up more ^{65}Zn than did controls without histidine, indicating either that saturation of the erythrocytes with L-histidine is not complete, or that efflux of zinc linked with histidine transport does not occur as readily as uptake.

Efflux of ^{65}Zn from erythrocytes

Efflux of ^{65}Zn from rat erythrocytes following a 3 h loading period was negligible in the case of controls (Fig. 5). However, Fig. 5 shows that when erythrocytes were resuspended with L-histidine (10 mM) significant efflux of ^{65}Zn occurred over a period of 2 h. If 100 μM-BSA was also present after resuspension, even greater net efflux occurred (BSA alone did not affect net efflux; not shown). The differences between experimental groups could not be explained by differences in haemolysis.

These results will be discussed in relation to ^{65}Zn fluxes.

DISCUSSION

L-Histidine (500 μM–10 mM) stimulated the uptake of ^{65}Zn by rat erythrocytes *in vitro*. This effect did not appear to be due to a non-specific change in membrane permeability, since it was not seen at low temperature (Fig. 1*D*) and could not be induced by the non-zinc-binding amino acid L-alanine. Since a zinc-binding amino acid such as histidine would be expected to reduce uptake of ionic zinc, by lowering extracellular $[Zn^{2+}]$, the effect of L-histidine can be best explained by transport of a complex of zinc and histidine across the erythrocyte membrane. The observation that albumin reduced the control uptake suggests that transport of a zinc–protein complex was not a major factor, while the finding that L-histidine gave a proportionally greater stimulation of uptake over control in the presence of albumin may reflect competition for zinc between histidine and albumin. The finding that D-histidine did not have a significant inhibitory effect below 10 mM in the presence of albumin may have a similar explanation. Since the apparent uptake with L-histidine was not altered by EDTA washing, it does not seem that L-histidine is simply causing an increase in membrane-bound zinc.

The action of histidine was stereospecific (compare Fig. 1*A* with *B*), which again suggests the involvement of a transport system rather than passive permeability of a lipid-soluble complex.

The stimulation of uptake by L-histidine was sodium dependent (Fig. 1*C*), in contrast with ^{65}Zn uptake in the absence of histidine. The sodium-dependent amino acid transport system designated 'N', which is the most important specific system for histidine transport, has been shown to be present in erythrocytes from other species (see Ellory, 1987; Baker & Ellory, 1990, for reviews) as well as in hepatocytes (Kilberg *et al.* 1980). A system resembling type 'N' in its substrate preference, but having a different pattern of sodium dependence (suggestive of sodium–glutamine co-transport rather than sodium gradient dependence), has been described in rat muscle (Hundal, Rennie & Watt, 1987). Liou & Ellory (1990) have recently reported that histidine uptake in human erythrocytes may occur via several carriers, with

approximately 6% being sodium dependent. Since rat erythrocytes have not been widely used for transport studies, relatively few data on their histidine carriers are available. However, recent experiments on blood from rats of the same colony used for the experiments in this paper have indicated that histidine uptake in these rat erythrocytes is partially sodium dependent, and that it shows inhibition by leucine (J. C. Ellory & C. C. Liou, personal communication). This may indicate that the L-system carrier in these cells is capable of transporting histidine with the participation of sodium ions. This may be analogous to the reported sodium-dependent transport of dipolar amino acids by system Y^+, where the sodium–dipolar amino acid complex allows tripolar binding at the transport site (Christensen, 1990). The presence of a sodium-independent, non-concentrative, saturable leucine carrier with properties similar to the L-system carrier from human erythrocytes has recently been described in rat erythrocytes and reticulocytes by Felipe, Viñas & Remesar (1990). Further detailed study of the stoichiometry of the sodium dependence of the histidine stimulation of zinc uptake in these cells is obviously required.

The 'classical' inhibitor for uptake of histidine by the type 'N' carrier system is glutamine (Kilberg *et al.* 1980). Unfortunately, glutamine itself binds zinc (Giroux & Henkin, 1972), and experiments using glutamine to inhibit the histidine-induced stimulation of zinc uptake have not yielded clear results.

An alternative explanation of the effect of L-histidine (Fig. 1*A*) would be that the L-histidine molecule is capable of bringing a zinc atom into closer proximity with some sort of receptor site on the transport system. This action could be stereospecific. However, it is difficult to imagine how such an action could be sodium dependent (Fig. 1*C*).

Sivarama Sastry *et al.* (1960) did demonstrate inhibition of ^{65}Zn uptake by 1 mM-histidine. This is not in agreement with the present results, although the discrepancy may well be due to the different species used: horse as opposed to rat. Unfortunately, the report by Sivarama Sastry *et al.* (1960) does not mention the enantiomeric form of histidine that was used; the effect of a racemic mixture would be difficult to predict. Stimulation of ^{65}Zn uptake by L-histidine in human erythrocytes has been reported by Van Wouwe, Kos, de Goeij & Van den Hamer (1987). The concentration dependence of the histidine stimulation of zinc accumulation found in our experiments might give some indication of the molecular species involved. However, calculation of the concentrations of the candidate species (T. J. B. Simons, personal communication) gives the following values: at 1 mM-histidine, $[Zn^{2+}] = 1{\cdot}001 \times 10^{-8}$ M, $[Zn(hist)] = 0{\cdot}52$ μM and $[Zn(hist)_2] = 2{\cdot}47$ μM. At 10 mM-histidine, the values become $[Zn^{2+}] = 1{\cdot}18 \times 10^{-10}$ M, $[Zn(hist)] = 0{\cdot}06$ μM and $[Zn(hist)_2] = 2{\cdot}94$ μM (based on stability constants from Martell, 1974). If either Zn(hist) or ionic zinc were the transported species, marked inhibition would have been expected at 10 mM-histidine. This was not found; one possible explanation for this could be that $Zn(hist)_2$ is also a major transported species under these conditions, so that net uptake would not show the expected response to raised histidine. In any case, the kinetics would be difficult to predict in the presence of variations in protein concentration. Measurements of initial influx rate at defined albumin levels are required.

The sodium dependence of zinc uptake in the presence of L-histidine (Fig. 1*C*), and

the fact that this uptake is not DIDS sensitive (Fig. 2), imply a different mechanism of uptake from that previously suggested by Kalfakakou & Simons (1986) and Alda Torrubia & Garay (1989). This does not of course exclude the possibility of transport of an ionic form of zinc, although we have searched for alternative mechanisms of zinc uptake because of the very low $[Zn^{2+}]$ in plasma.

Carver (1965) quotes a mean plasma histidine concentration of 45 μM for the rat. This concentration is considerably lower than any concentration of L-histidine shown to have an effect on zinc uptake (see Fig. 1*A*). The physiological significance of histidine-linked zinc transport may therefore be questioned, although the histidine concentrations in hepatic portal blood after feeding would be expected to be considerably higher than the arterial concentration.

No qualitative change in these results occurred if BSA was included in the suspension medium (Fig. 3). However, it is still possible that lower concentrations of L-histidine would have an effect if physiological conditions were mimicked more adequately, or if zinc uptake could be studied over longer time periods. In this study, as in several previous ones, the total zinc concentration (3·0 μM) has been far higher than the physiological free ionic zinc concentration, although it is lower than the approximately 15 μM total zinc reported for human serum by Foote & Delves (1984).

The study of ^{65}Zn uptake in erythrocytes pre-incubated with L-histidine (Fig. 4) suggests that net influx of histidine is not required in order to stimulate ^{65}Zn uptake. The linear uptake of ^{65}Zn over 6 h in the presence of 10 mM-L-histidine could be explained in terms of a continuous shuttling of zinc into the cell, even in conditions where histidine influx would be expected to be balanced by efflux. This mechanism would require a reduced probability for a zinc–histidine complex to leave the cell; this would be reasonable in view of the high affinity of intracellular components (haemoglobin in particular) for zinc (Oelshlegel, Brewer, Knutsen, Prasad & Schoomaker, 1974). However, the pre-incubation experiment (Fig. 4) raises the possibility that efflux of a zinc–histidine complex could occur, and the study of ^{65}Zn efflux (Fig. 5) appears to confirm this. Histidine increased the rate of efflux, but the effect of histidine on efflux would be expected to be offset by the stimulation of uptake also caused by histidine. More efflux was seen when BSA and L-histidine were present, compared with L-histidine alone. The effect of BSA in this situation could be to inhibit re-uptake. However, it has recently been shown (Simons, 1991) that zinc efflux from red cells is stimulated by micromolar calcium concentrations, and any increase in calcium resulting from the addition of BSA would be thus expected to increase the zinc efflux. Measurement of calcium activity in similar incubation solutions with an Orion calcium-sensitive electrode suggests that the changes in ionic calcium due to addition of BSA and/or histidine are too small to explain the measured changes in efflux.

In the pathological condition of histidinaemia in humans, plasma histidine concentrations of up to 974 μM have been reported (Ishikawa, Saito, Morishita, Ito, Kato & Wada, 1987). The results presented here have clear implications for these patients. Henkin, Patten, Re & Bronzert (1975) have claimed that very high oral doses of L-histidine can alter zinc status in humans. Although enhancement of urinary zinc excretion was put forward as a mechanism, it is also possible that histidine could alter the uptake of zinc by tissues. Histidine could also stimulate

efflux of zinc from tissues, as suggested by these *in vitro* results (Fig. 5). Our recent preliminary report of the effect of raised plasma histidine concentrations on ^{65}Zn uptake by tissues of anaesthetized rats (Aiken, Horn & Saunders, 1989) showed that the effects of histidine *in vivo* were not stereospecific, a finding that is difficult to explain in terms of the transport mechanisms discussed here. Although it appears from the present work that histidine affects zinc transport *in vitro*, further studies are required in a variety of tissues, in order that the effects of histidine *in vivo* can be better understood.

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