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Effects of histidine on tissue zinc distribution in rats

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Histidine has been reported to affect body zinc status by increasing urinary zinc excretion. The effects of experimental histidinemia on distribution of ^{65}Zn in anesthetized rats were studied. Infusion of L-histidine at a rate sufficient to raise plasma concentrations to approximately 2 mM for 6 h starting 48 h after a single intraperitoneal ^{65}Zn injection did not alter ^{65}Zn activities in a variety of tissues when compared with anesthetized uninfused animals. However, plasma ^{65}Zn and erythrocyte ^{65}Zn were decreased, and liver ^{65}Zn was increased. If ^{65}Zn was injected intravenously during histidine infusion, net accumulation of zinc by some tissues was increased, but uptake by others was reduced relative to uninfused animals. In all cases, however, uptake expressed relative to plasma ^{65}Zn levels was increased when allowance was made for the more rapid fall in plasma ^{65}Zn during histidine infusion. Similar infusions of D-histidine produced quantitatively similar effects. Since enzymatic mechanisms and amino acid carriers would be expected to show stereoselectivity, such processes are unlikely to be involved in the zinc distribution changes described. The possibility of zinc transport by a hitherto unidentified carrier is discussed. These experiments confirm that histidinemia can affect zinc status, but any associated changes in urinary zinc excretion do not seem adequate to account for the tissue changes found.

Keywords: amino acids, histidine, zinc uptake

Introduction

The importance of dietary zinc is well-established and severe effects of zinc deficiency have been characterized (for instance, during fetal development: Hurley & Swenerton 1971); however, the mechanisms for uptake of zinc by tissues have received comparatively little attention, although the whole subject of zinc metabolism has been recently reviewed by several authors (e.g. Cousins 1985). The cellular uptake of zinc complexed with histidine, utilizing an amino acid transport system, has been proposed (Sivarama Sastry *et al.* 1960, Magneson *et al.* 1987). Recent experiments have provided *in vitro* evidence for such a transport mechanism in rat erythrocytes (Aiken *et al.* 1992) and in renal proximal cells (Gachot *et al.* 1992). The present paper deals with the effects of histidine on tissue zinc uptake in anesthetized rats.

It has been reported that very high doses of L-histidine administered orally to humans caused symptoms similar to those of zinc deficiency and that they could be reversed by administration of additional zinc (Henkin *et al.* 1975). It was suggested that histidinuria caused an increased loss of zinc in the urine, which in turn caused a depletion of body zinc. However, the effects of oral histidine on tissue zinc uptake and efflux, absorption from the gut and fecal zinc excretion were not considered. Any of these mechanisms might cause the changes in zinc status that were described and so we have endeavored to distinguish these different effects of histidine using an animal model of histidinemia. Part of this work has been published as an abstract (Aiken *et al.* 1989)

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Materials and methods

Animals and surgical preparation

Young adult females (180–225 g) from a randomly-bred closed colony of albino Wistar rats were transferred to individual cages on the morning of the experiment. A 14:10 h light:dark cycle was used (light 08.30–22.30), and experiments commenced between 13.00 and 16.00 h.

Rats were anesthetized by intraperitoneal (i.p.) injection of ethyl carbamate (2.0 g kg^{-1}). The right femoral artery and vein were cannulated, and a nylon tube was inserted into the bladder via the urethra. Blood pressure was monitored throughout the experiment from the femoral artery cannula, which was also used for withdrawal of serial blood samples for isotope counting, histidine assay and blood chemistry. Blood gases were analyzed before and after infusion of histidine (Radiometer BMS 3 Mk2 Blood Micro System).

Infusions

Solutions of histidine were infused intravenously (i.v.) using the following schedule: 1 ml min^{-1} for 2 min, then 4 ml h^{-1} for 28 min and thereafter 2 ml h^{-1}. Histidine solutions were prepared in water and the pH was adjusted to 7.4 with 5 M sodium hydroxide.

Either L-histidine (250 mM; 709 mOsmol kg^{-1}) or D-histidine (60 mM; 168 mOsmol kg^{-1}) was used. These concentrations were found to produce steady plasma histidine concentrations of approximately 2 mM for a period of at least 6 h when infused in the same total volume using the schedule described above, thus allowing for the different metabolism of the two enantiomers. In all experimental animals, plasma and urinary histidine was assayed by the method of Macpherson (1946).

Injection of ^{65}Zn

A tracer dose of the gamma-emitter ^{65}Zn was injected either i.v. or i.p. The injection solution consisted of high specific activity ^{65}Zn (approximately 3×10^{14} Bq g^{-1}) as zinc chloride (Amersham International plc) in approximately 400 μl of Tris (500 mM, pH 7.4). In all experiments, 123 000 d.p.m. per gram body weight was injected (equivalent to approximately 70 ng zinc per rat).

Effect of histidinemia on blood and tissue zinc equilibrium

In initial experiments, ^{65}Zn was administered i.p. 48 h before the start of infusion. The effects of L-histidine (6 h infusion) on efflux of zinc from tissues could therefore be studied. Uninfused rats were anesthetized and cannulated as above at 48 h, but not infused. Infused rates received a L-histidine infusion as described above and blood samples were removed for ^{65}Zn counting at intervals during the infusion. Both groups were killed by an i.p. overdose of ethyl carbamate 54 h after injection of ^{65}Zn.

Tissues were removed and γ activity was counted using a Beckman 'Biogamma' well-type scintillation counter (efficiency 17%). Tissue ^{65}Zn activity was expressed as d.p.m. per milligram of tissue (wet weight).

Uptake of ^{65}Zn

In a second series of experiments, ^{65}Zn was injected i.v. after 3 h of infusion, in order to compare directly the action of L- and D-histidine of the distribution of a tracer dose of zinc. Rats were killed 1 h later and tissues were sampled. As in the previous experiment, a group of rats were not infused, but were cannulated and kept anesthetized throughout the whole 4 h period.

Since it was not expected that plasma levels of ^{65}Zn would remain constant during the 1 h after injection, plasma was sampled throughout this period. Integrated mean plasma ^{65}Zn levels for each animal were calculated by a non-linear iterative least-squares curve fitting computer program.

Statistics

Data were analyzed by Student's paired and unpaired *t*-tests, and by one-way analysis of variance (ANOVA). Where *t*-tests were employed, corrections for multiple comparisons have not been made, although in cases where it is thought that significance levels could be unreasonably elevated by multiple comparisons, attention is drawn to the fact. Values are given as mean ± SEM.

Results

Rats pre-treated with ^{65}Zn

The mean plasma histidine concentration of six rats infused with L-histidine for 6 h commencing 48 h after zinc injection was 1.8 ± 0.16 mM, compared with the value of 40 μM reported for the plasma histidine concentration in untreated rats (Carver 1965). The plasma levels of ^{65}Zn in these rats are shown in Figure 1(a) during the 6 h of infusion. It can be seen that histidine infusion caused a large fall in the concentration of isotope in plasma. In uninfused animals, plasma ^{65}Zn levels remained steady over a 6 h period. The mean output of ^{65}Zn in the urine during the 6 h of infusion was equivalent to 1.2 ± 0.25% of the injected dose. Of the total L-histidine infused, 6.6 ± 1.55% was recovered in the urine.

^{65}Zn activity in erythrocytes during infusion of L-histidine is shown in Figure 1(b). The fall in activity within erythrocytes in the infused group was not proportionally so large as seen in plasma but was significantly greater than in the uninfused group with $P < 0.02$ (ANOVA). No significant changes in ^{65}Zn activities in other tissues were observed (unpaired *t*-tests, $P > 0.05$), with the exception of liver. Liver

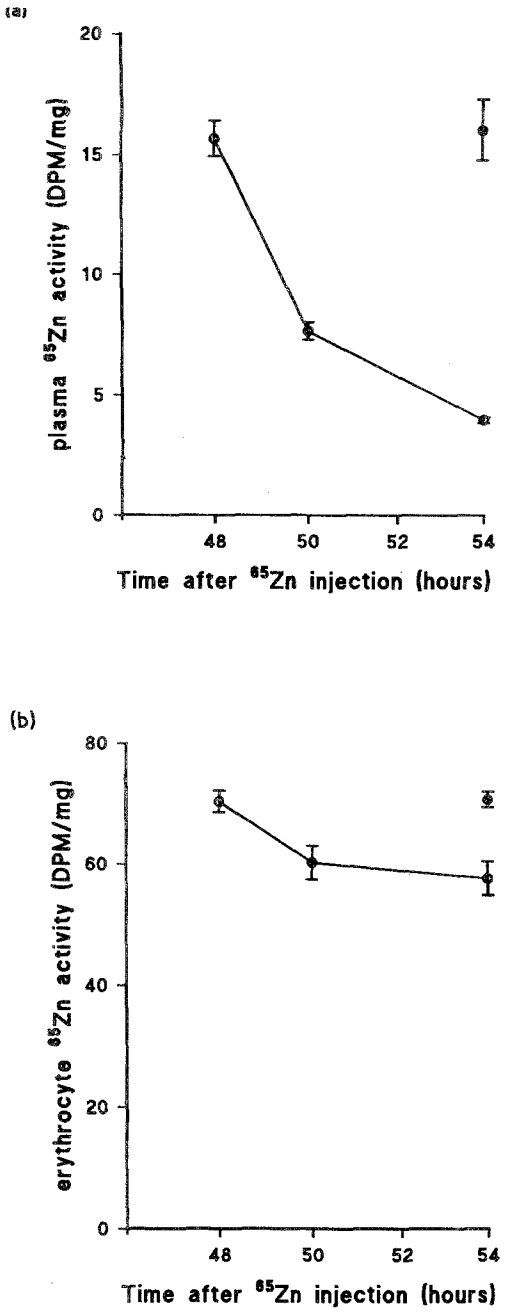

Figure 1. (**a**) Plasma ^{65}Zn activity in rats during infusion of L-histidine. ^{65}Zn was injected i.p. 48 h before commencing infusion. Rats were infused for 6 h (○) giving a mean plasma concentration of 1.8 ± 0.16 mM. Values for non-histidinemic plasma (●) were obtained from otherwise untreated rats anesthetized and bled by cardiac puncture 54 h after injection. Bars represent SE ($n = 6$). (**b**) Erythrocyte ^{65}Zn activity in rats during infusion of L-histidine. ^{65}Zn was injected i.p. 48 h before commencing infusion. Experimental rats (○) were infused for 6 h giving a mean plasma concentration of 1.8 ± 0.16 mM. Values for non-histidinemic rats (●) were obtained from otherwise untreated rats anesthetized and bled by cardiac puncture 54 h after injection. Bars represent SE ($n = 6$).

activity was 330 ± 15 d.p.m. mg^{-1} in uninfused and 468 ± 21 d.p.m. mg^{-1} in infused rats. This increase in liver activity is significant with $P < 0.0005$ (unpaired t-test), a difference which is valid even if an allowance is made for the number of tissues analyzed. Percentages of the isotope dose in the liver at the termination of the experiment were 12.2 ± 0.35% in uninfused and 14.8 ± 0.38% in infused animals. It should be noted that this 'shift' in the isotope distribution is quantitatively more important than the effect of L-histidine on urinary ^{65}Zn excretion.

Uptake of ^{65}Zn in rats

The tissue content of ^{65}Zn (d.p.m. mg^{-1}) 1 h after i.v. injection (and 4 h after the start of histidine infusion) is shown in Figure 2. In this series of experiments, another group of rats infused with D-histidine was also used. Mean plasma concentrations were 2.7 ± 0.20 mM for L-histidine and 2.7 ± 0.22 mM for D-histidine, respectively (six animals per group). There were no statistically significant differences in tissue ^{65}Zn content between the L- and D-histidine groups (unpaired t-tests). Although a t-test on the liver data yielded $0.02 < P < 0.05$, one result at this significance level might be expected due to chance.

Histidine altered the accumulation of ^{65}Zn when compared with the uninfused animals, in most tissues studied, although these effects were not stereospecific. Histidine reduced the ^{65}Zn content of erythrocytes, brain, liver and spleen, and perhaps kidney (Figure 2). However, in other tissues ^{65}Zn content was increased, most notably in skeletal muscle, ileum, bone and skin.

The plasma activity of ^{65}Zn during the 1 h following i.v. injection is shown in Figure 3. Results for the L- and D-histidine groups were very similar; in both cases ^{65}Zn levels in the period 5–60 min after injection were lower than in the plasma of uninfused rats (all rats received the same amount of zinc; plasma measurements before 5 min were not reliable due to inadequate mixing). The results for each group were fitted by an equation of the form: $y = a + \exp[b - c \times \log(x)]$ (using *P.Fit*; Biosoft, Cambridge, UK), and integrated mean plasma activites (d.p.m. mg^{-1}) were: 223 ± 6.5, 76 ± 6.5 and 78 ± 2.7 for uninfused, L-histidine and D-histidine groups, respectively. Histidine did not alter plasma clearance rates during the measured period (Figure 3); the lower plasma levels obtained in the infused animals are compatible with a larger volume of distribution.

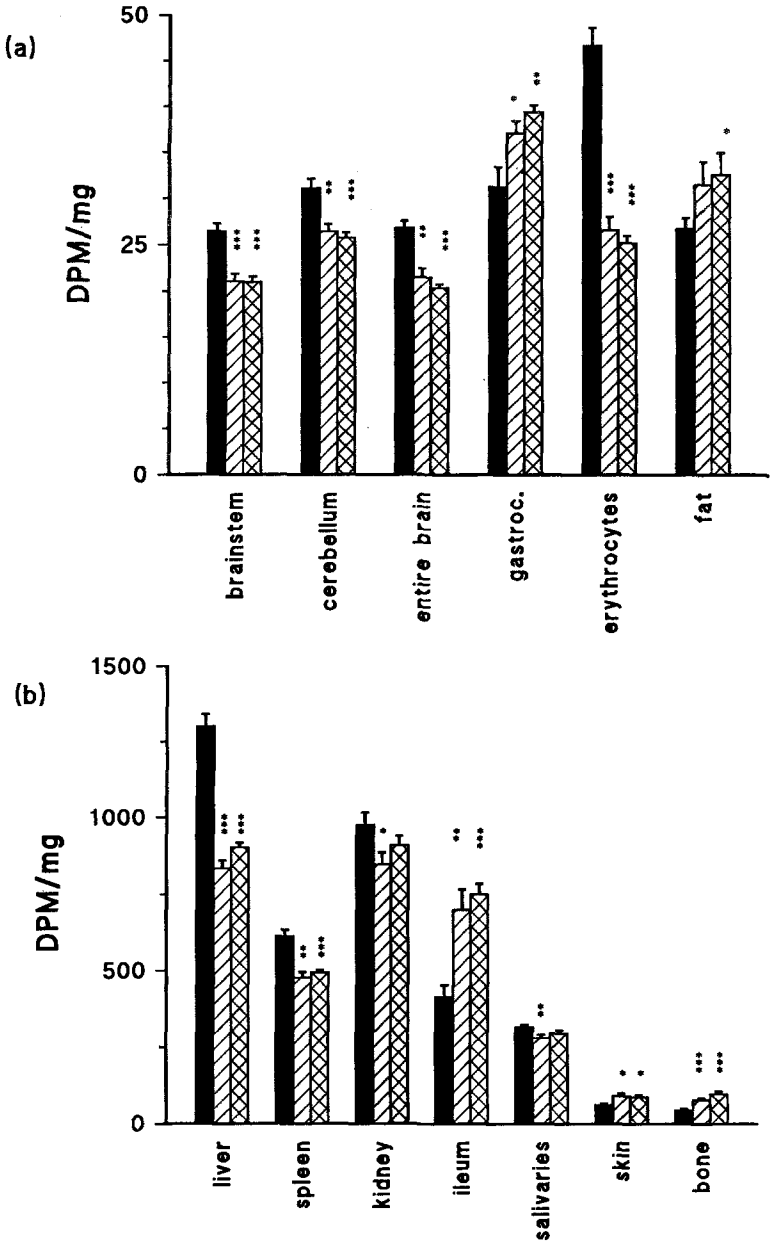

Figure 2. Tissue ^{65}Zn content 60 min after i.v. injection. Rats were infused with either L-histidine (▨) or D-histidine (▩) for 4 h, with an i.v. injection of ^{65}Zn (123 000 d.p.m. g^{-1}) at 3 h. Non-infused rats (■) were anesthetized for 3 h before injection, cannulated but not infused. Bars represent SE, with significance values as follows; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired t-test, comparisons with respect to uninfused value); $n = 6$ in all cases. Plasma histidine concentrations at the end of the experiment were 2.70 ± 0.2 mM for L-histidine and 2.66 ± 0.22 mM for D-histidine. To aid clarity, tissue uptakes are divided into (**a**) low uptake and (**b**) high uptake groups, and displayed with different y-axis scales.

In an attempt to correct the tissue uptake for changes in the plasma concentration of ^{65}Zn, tissue ^{65}Zn activities were expressed relative to the integrated mean plasma activities. These data are presented as ratios in Figure 4. Again, no significant differences were found between the L- and D-histidine groups. Figure 4 shows an increase in tissue-to-plasma ratio compared with non-histidine infused animals, in all tissues studied ($P < 0.001$, in all cases). Liver has not been included in this figure, because ^{65}Zn in the hepatic portal circulation was not determined and comparison with systemic arterial plasma would be meaningless.

The percentages of the ^{65}Zn dose excreted in the

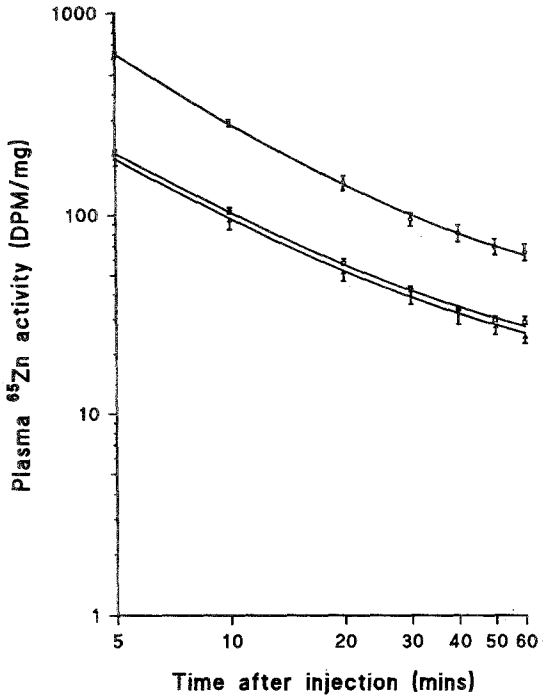

Figure 3. ^{65}Zn activity in plasma during the 1 h following i.v. injection. Experimental conditions were as described for Figure 2. Lines of best fit were obtained as described in the text: ○, non-infused; □, D-histidine; △, L-histidine.

urine during the 1 h were: 0.08 ± 0.04, 0.88 ± 0.22 and 1.3 ± 0.41 for uninfused, L-histidine and D-histidine groups, respectively. In these experiments, $5.8 \pm 1.63\%$ of the total infused L-histidine appeared in the urine during the 1 h period, compared with $37.9 \pm 9.22\%$ of the D-histidine. Urine flow was 720 ± 70 mg h^{-1} in the L-histidine group and 309 ± 65 mg h^{-1} in the D-histidine group. These results do not support a direct relationship between urinary histidine excretion and plasma zinc depletion. The uninfused rats produced very little urine, which may account for the low ^{65}Zn excretion. These results cannot account for the reduced accumulation seen in some tissues (Figure 2) or the more rapid plasma decay (Figure 3) in the infused animals.

Blood chemistry in infused rats

Infusion of L-histidine for 4 h caused marked metabolic acidosis. Blood pH and plasma bicarbonate concentrations for the three groups of rats used in the uptake study are shown in Table 1, both before and after infusion. The only significant change seen was that the L-histidine infused rats showed a pronounced fall in pH and $[HCO_3^-]$ at the end of the infusion period. Plasma osmotic pressure in the L-histidine groups rose from 344 ± 4.4 to 354 ± 5.5 mOsmol kg^{-1}, whereas the D-histidine group showed a non-significant fall. For the rats pretreated with ^{65}Zn and infused with L-histidine, values were similar to the L-histidine-infused rats shown in Table 1. The fall in bicarbonate concentration in the L-histidine group (Table 1) will be discussed in relation to the bicarbonate-dependent zinc uptake mechanism described by Kalfakakou & Simons (1986).

Discussion

The choice of appropriate controls for experiments of this type is not straightforward. Normally, separate controls are necessary to isolate the contributions made by such factors as osmotic pressure, acid–base status and plasma amino acid concentration. However, the findings that elevation of either L- or D-histidine concentration produced similar changes in zinc distribution when compared with uninfused animals despite producing different changes in blood chemistry (Table 1), osmotic pressure and renal function make it clear that such factors do not play a significant role in the observed effects. The D-histidine infusion provides a control for the chelating effects of histidine. Although stereoselectivity has been demonstrated in the preferential formation of the mixed D–L bis-histidine complex over the L–L or the D–D forms (Morris & Martin 1970), the quantity of the mixed bis complex formed in these experiments is insignificant, and it can be assumed that the L- and and D-histidine have the same zinc-binding characteristics but are not metabolized in a similar way. A conventional saline infusion would not provide an appropriate control group, as the effects on body fluid volumes and renal function would not be comparable with either the L- or D-histidine groups.

The high concentration of histidine in plasma (2–3 mM) in these experiments compared with the normal level of plasma histidine in the rat (40 μM; Carver 1965) together with the lack of stereospecificity observed suggests that the changes in tissue ^{65}Zn observed have little to do with normal mechanisms of zinc transfer in the body (although amino acid concentrations in hepatic portal blood are likely to be considerably higher than in arterial plasma and may be a factor in post-prandial hepatic zinc uptake). However, the results are important in confirming that marked changes in zinc distribution can occur in response to pathological changes in

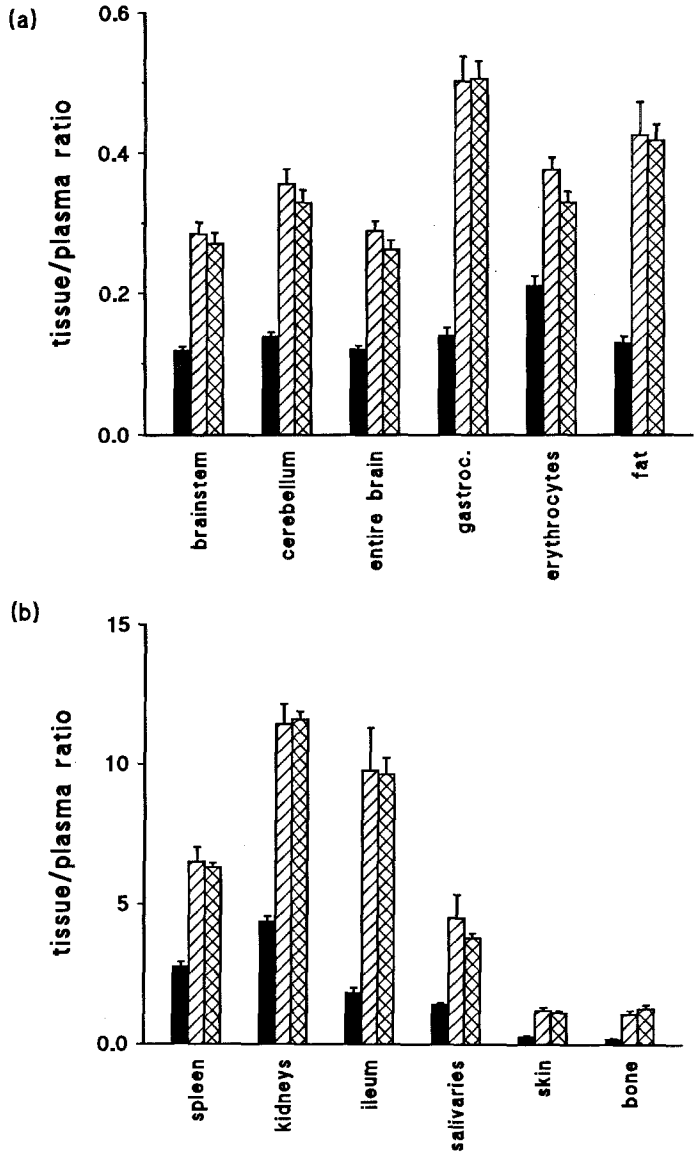

Figure 4. Tissue ^{65}Zn uptake expressed relative to mean plasma activity. Data are derived from the same experiments as Figure 3: (■) are from uninfused animals, (▨) from L-histidine infused animals and (▧) from D-histidine infused animals. Bars represent SE. All values from infused animals are significantly different from uninfused animals ($P < 0.001$). To aid clarity, tissue uptakes are divided into (**a**) low uptake and (**b**) high uptake groups, and displayed with different y-axis scales.

Table 1. Blood pH and bicarbonate concentrations in rats before and after infusion of histidine for 4 h

		Uninfused	L-histidine	D-histidine
pH	before	7.34 ± 0.02	7.32 ± 0.01	7.30 ± 0.01
	after	7.30 ± 0.01	7.18±0.02*	7.34 ± 0.01
[HCO_3] mM	before	16.1 ± 0.8	15.8 ± 0.6	15.6 ± 0.6
	after	16.1 ± 0.9	12.0 ± 0.5	15.8 ± 0.6

Mean values ± SEM for six rats are given. Values marked with an asterisk are significantly different from starting values ($P < 0.02$; paired Student's t-test).

plasma histidine concentration. The two experiments described here support the claim that histidinemia is able to modify tissue zinc levels (Henkin *et al.* 1975), but suggest that the actions of histidine on zinc distribution are much more complex than has hitherto been recognized.

In the first series of experiments in which rats were preloaded with ^{65}Zn 48 h before, infusion of L-histidine caused a decrease in plasma ^{65}Zn concentration (Figure 1a), and measurements carried out in collaboration with Professor M. W. M. Bradbury have shown that the effect on the total zinc

concentration (measured by atomic absorption flame photometry) in plasma is similar (unpublished data). This finding is comparable with observations in humans (Henkin *et al.* 1975, Geliebter *et al.* 1981) and in rats (Freeman & Taylor 1977, Rasmussen 1982, Wensink & Van der Hamer 1988).

The explanation of Henkin *et al.* (1975) that the effect of histidine on zinc status was due to increased urinary loss of histidine-bound zinc is not supported by the results of either of the present series of experiments. Only a minor loss of ^{65}Zn occurred in the urine. The results of the second series of experiments where ^{65}Zn was injected into histidinemic rats (Figures 2–4) suggest that the increased plasma levels of histidine result in a marked change in tissue uptake of ^{65}Zn with a reduction in brain, red cells, spleen and, especially, liver, but increases in skeletal muscle, gut, skin and bone. The tissues in which greater uptake of ^{65}Zn occurred constitute a high proportion of total body mass, thus even small increases in ^{65}Zn accumulation would be quantitatively important in producing a fall in plasma ^{65}Zn. These acute effects of histidine on ^{65}Zn distribution contrast with the lack of effect of histidine infusion on tissue zinc levels in rats preloaded with ^{65}Zn some 48 h earlier. This difference is probably due to most of the administered zinc becoming rapidly incorporated into slowly exchangeable pools. It is known that metallothionein-bound zinc does not readily exchange (Cousins 1985, Pattison & Cousins 1986). It can also be seen from Figure 1(b) that erythrocyte ^{65}Zn (which is mainly hemoglobin-bound) does not readily exchange in rats with a normal plasma histidine level.

The majority of plasma zinc not bound to macromolecules and therefore available for uptake into cells will be in the form of complexes with amino acids such as histidine and cysteine (Harris & Keen 1989). Since the real extracellular and intracellular concentrations of such complexes in these experiments is not known, it is not clear to what extent tissue ^{65}Zn was accumulated against a concentration gradient. Figure 4 suggests that in many tissues (including brain and skeletal muscle) it was not, while in others (such as kidneys and ileum) it probably was, suggesting that intracellular mechanisms exist for retaining the ^{65}Zn once it has penetrated the cells.

Figure 2 shows that there were no significant differences between the net uptake of ^{65}Zn at 60 min when L- or D-infused animals were compared. Some tissues showed greater uptake when infused were compared to uninfused animals, while others showed a decreased uptake. However, simple comparison of tissue levels in infused and uninfused animals is complicated by the difference in plasma levels in the hour following injection. Figure 4 therefore shows the uptakes expressed as a ratio of the integrated mean plasma ^{65}Zn activity to the tissue levels, and it can be seen that both D- and L-histidine groups showed a greater uptake of the available plasma zinc in all tissues measured when compared with the uninfused animals. However, the proportional increase varied considerably, from a 60% increase over uninfused values for erythrocytes and 130% for brain to about 280% for skeletal muscle and 400% for ileum. Such a comparison could not be made for liver, since hepatic portal blood was not sampled in these experiments.

Thus histidine, whether D or L, appears to increase the relative uptake of ^{65}Zn in all tissues studied. Due to the greater fall in plasma ^{65}Zn in histidinemic animals, there was less ^{65}Zn available in these animals for uptake into tissues. Only in tissues with the higher rate of uptake (e.g. ileum, skeletal muscle) was the uptake sufficient to produce a tissue level of ^{65}Zn which was actually greater than that found in the uninfused animals. These results suggest considerable differences in the effect of histidine on the accumulation of ^{65}Zn in different tissues. Professor M. W. B. Bradbury (personal communication) has suggested that one possible factor may be the level of *extracellular* ^{65}Zn–histidine complexes in different tissues, since the net tissue uptake measured would include extracellular and intracellular zinc. In the context of the present experiment erythrocytes did not have an extracellular space and brain is a special case, since histidine levels in brain extracellular fluid will be controlled by the combined effect of limited permeability across the blood–brain barrier and the sink effect of cerebrospinal fluid drainage. The observation that D- and L-histidine had similar effects on brain ^{65}Zn levels indicates that the stereospecific amino acid carrier normally involved in histidine exchange (Oldendorf 1973) did not make a significant contribution to brain ^{65}Zn uptake at the high histidine concentration used in these experiments. The measurements made on erythrocytes show clearly that both D- and L-histidine are capable of increasing intracellular levels of ^{65}Zn, but much of the tissue redistribution which followed histidine infusion in these experiments may be due to ^{65}Zn–histidine diffusion into tissue extracellular fluid.

In support of this it was observed that the ultrafiltrable (i.e. below 10000 molecular weight) zinc content of peritoneal fluid, which may be considered representative of extracellular fluid in

many tissues, did increase during histidine infusion. If such a shift in ^{65}Zn activity from plasma to extracellular fluid compartments occurred without an increase in cellular uptake the resulting reduction in plasma activity would cause an apparent (artifactual) increase in tissue to plasma ratio.

It is possible that the infusion schedules used change the concentration of another, so far unidentified, zinc-binding carrier, which may be involved in zinc uptake into tissues. For instance, a small peptide incorporating histidine might be produced from either L- or D-histidine and this may be capable of functioning as a 'carrier' molecule. The very slow uptake kinetics for zinc in some tissues previously reported (Sheline *et al.* 1943) are in keeping with such a hypothesis. Synthesis of such a carrier, possibly in the liver, may be the rate-determining step for tissue zinc uptake. Another possibility is that the rate of hepatic metallothionein synthesis is increased in response to raised histidine levels. The reported time-course of zinc-induced changes in metallothionein synthesis (Squibb *et al.* 1977) is compatible with the changes in zinc uptake seen in these experiments. However, it is not clear how the very different changes in blood chemistry produced by D- and L-histidine infusion might produce virtually identical changes in metallothionein-mediated uptake.

The uptake of Zn^{2+} ions by erythrocytes *in vitro* has been shown to be bicarbonate-dependent (Kalfakakou & Simons 1986, Alda Torrubia & Garay 1989). The decrease in plasma bicarbonate during L-histidine infusion (Table 1) might be expected to interfere with this uptake mechanism *in vivo*. However, the effects of histidine were similar when D-histidine was used. Since D-histidine did not lower plasma bicarbonate, it is not likely that the effects of histidine were due to interference with any mechanism dependent on plasma bicarbonate. In any case, the naturally occurring $[Zn^{2+}]$ in plasma is so low that the physiological importance of such a mechanism is debateable.

The effects of histidine on zinc distribution described here have obvious implications for the hereditary human disease of histidinemia. Plasma histidine concentrations of up to 947 μM have been reported in human patients (Ishikawa *et al.* 1987) and a mean plasma concentration of 2.2 mM has been produced in rats by feeding a diet supplemented with 8% histidine (Wensink & Van den Hamer 1988). The concentrations achieved by infusion in the present study are comparable to these. Further experiments are required in order to determine the effects of less severe histidinemia on zinc distribution. In particular, the variations in plasma histidine due to dietary intake of the amino acid need to be studied in order to determine whether zinc uptake may be affected.

The teratogenic effects of zinc deficiency have received much attention (Hurley *et al.* 1971). Since the present experiments show that histidine has a marked and reproducible effect on zinc distribution, even in a relatively short time, it would be of great interest to know how placental transfer of zinc and fetal zinc distribution are affected by alterations in maternal histidine levels. Figure 2 shows that in histidinemic conditions some organs, such as the brain, may take up less zinc. It has already been shown (Hurley & Swenerton 1971) that when pregnant rats are fed a low zinc diet, zinc cannot be mobilized from maternal stores and teratogenesis results. An experimental model using periods of induced histidinemia on pregnant rats may reveal how severely the fetus is affected by alterations in plasma histidine, and whether any such effects may be related to changes in zinc metabolism.

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