Biochemical Pharmacology, Vol. 39, No. 6, pp. 1005–1012, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

FACILITATED UPTAKE OF ZINC INTO HUMAN ERYTHROCYTES

RELEVANCE TO THE TREATMENT OF SICKLE-CELL ANAEMIA

ROBERT C. HIDER,*† LINDA EJIM,‡ PAUL D. TAYLOR,‡ ROSEMARY GALE,§ ERNST HUEHNS§ and JOHN B. PORTER§

*Department of Pharmacy, King's College London, ‡Department of Chemistry, Essex University, Colchester and §Department of Haematology, University College London, U.K.

(*Received* 11 *July* 1989; *accepted* 16 *October* 1989)

Abstract—The ability of a number of heterocyclic metal chelators to deliver zinc into red cells, to release the liganded zinc to haemoglobin and thereby cause a left shift in the oxygen dissociation curve of intact red cells has been investigated. Incubation of neutrally charged zinc–pyrone and zinc–pyridin-2-one complexes with red cells led to the rapid accumulation of zinc within cells, whereas unliganded zinc in the form of zinc acetate, zinc chloride or zinc sulphate accumulated only slowly. The rate at which zinc was delivered to red cells by pyrone and pyridin-2-one ligands increased with increasing lipid solubility of the ligands. The uptake of zinc into both normal adult and sickle red cells was associated with a dose-dependent increase in the oxygen affinity of haemoglobin. The degree of left shift in the oxygen dissociation curve following the incubation of red cells with zinc–pyrone and –pyridin-2-one complexes suggests that these complexes may find application as agents to increase the oxygen affinity of haemoglobin in sickle cell disease and thereby decrease the probability of intravascular sickling at low tissue oxygen tensions. Ethylmaltol appears to be a particularly useful agent due to its known low toxicity.

Although the pathology of sickle cell anaemia is well understood at a molecular and at a genetic level, there is still no effective treatment available to prevent or ameliorate intravascular sickling [1]. A variety of agents have been investigated previously for their ability to reduce the tendency for sickle haemoglobin (HbS) to polymerize in its deoxy form. Unfortunately, most agents have been shown to be either too toxic or ineffective in clinical trials [2]. One potential mode of action for an antisickling agent would be to interact with the haemoglobin molecule in such a way as to increase the affinity of HbS for oxygen, thereby decreasing the proportion of HbS in the deoxy state. Such "left shifting agents" can lead to reduced sickling and increased red cell survival as demonstrated by cyanate [3, 4] although this drug proved too toxic in clinical use.

Zinc is known to bind to isolated adult Hb with a stoichiometry of 2 Zn per tetramer, thereby causing a 3–4-fold increase in oxygen affinity [5, 6]. Unlike many agents which cause such a left shift in the oxygen dissociation curve, zinc does not alter the co-operativity of oxygen binding [7] or the Bohr effect [8]. Unfortunately unliganded zinc, such as zinc chloride, cannot pass into red cells freely, thereby limiting the degree of left shift that can be achieved [9]. A labile zinc–ligand complex which is capable of both crossing the erythrocyte membrane, and donating zinc to haemoglobin, might however find application as a left-shifting agent for the treatment of sickle cell disease. In this communication we have investigated the ability of a number of heterocyclic metal chelating agents, such as pyrones and pyridinones, to allow zinc to cross the red cell membrane and interact with haemoglobin so as to cause a left shift in the oxygen dissociation curve. Because the pyrones, maltol and ethylmaltol, are known to have a low degree of toxicity in animals [10] and are well established as food additives in man, their initial investigation has been more extensive than that of the pyridinones.

† To whom correspondence and reprint requests should be addressed.

MATERIALS AND METHODS

Maltol and ethylmaltol were purchased from Pfizer, (Sandwich, U.K.). The entire range of pyridinones were prepared as described by Hider and co-workers [11, 12]. Zinc solutions for absorption studies were prepared by mixing ^{65}Zn (Amersham International, Bucks, U.K.) with $ZnCl_2$, the appropriate ligand and buffered solution. All other chemicals were either purchased from the Sigma Chemical Co. (Poole, U.K.) or the Aldrich Chemical Co. (Poole, U.K.). Zinc diethylmaltol was prepared by adding a chloroform solution of ethylmaltol (0.02 moles) to an equal volume of an ethanol solution of zinc chloride (0.01 moles). After allowing the solution to stand for 5 min, a 10 M excess of solid Na_2CO_3 was added and the resulting mixture was stirred for 15 min. The filtrate of the mixture was rotary evaporated to yield a viscous oil. Trituration and subsequent recrystallization from ethanol gave zinc diethylmaltol as a white crystalline solid in essentially quantitative yield, mp. 271–272°; ν max (nujol) 1620, 1560, 1530 cm^{-1}; δ (d_6DMSO) 8.0 (d,2H), 6.4 (d,2H), 2.6 (q,4H), 1.0 (t,6H).

Speciation plots. The relative populations of Zn^{2+}, $[ZnL]^+$, $[ZnL_2]^\circ$ and $[ZnL_3]^-$ as a function of Zn : L

ratio, Zn concentration and pH of the solution were determined from affinity constants using MLGEN19 [13]. The affinity constants for the interaction between zinc and maltol are log K_1, 5.62; log K_2, 4.82; log K_3, 2.44 and pK_a, 8.73 [14]. The affinity constants for the interaction between zinc and 3-hydroxypyridin-2-one are log K_1, 5.57; log K_2, 4.55; log K_3, 2.18 and pK_a 8.66 [15]. The affinity constants for the interaction between zinc and 3-hydroxypyridin-4-one are log K_1, 7.35; log K_2, 6.38; log K_3, 5.38; and pK_a 9.74 [13, 16].

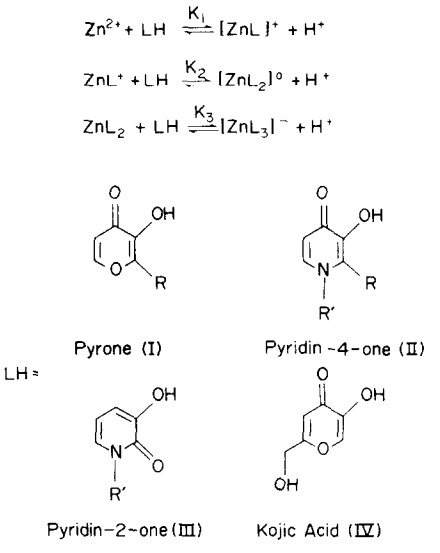
Determination of K_{part} *values.* The partition coefficients for free ligands and ^{65}Zn (in the presence of ligands) between *n*-octanol and aqueous Tris–HCl (20 mM, pH 7.4) was determined at 20° by the 'hand-shake' method. In each case the material to be measured was dissolved in the aqueous phase at a concentration of 10^{-4} M. After shaking for 1 min, the mixture was centrifuged at 1000 *g* for 30 sec. The two resulting phases were separated. Ligand concentration was determined spectrophotometrically over the 220 to 340 nm range, whereas ^{65}Zn was determined directly on a γ-counter. Values presented are the means of at least three independent determinations.

Erythrocyte uptake studies. Whole blood was centrifuged at 3000 rpm for 5 min at 4° on a Beckman model J-21 centrifuge. The plasma was removed by aspiration and discarded. The packed erythrocytes were then washed (2×) with approximately twice their volume of (Tris–HCl 20 mM, pH 7.4; NaCl 130 mM). Packed erythrocytes 0.5 mL were added to 2 mL of zinc complex in a 25-mL conical flask and incubated with shaking at 37° for the desired length of time. After incubation, 0.1-mL aliquots of the cell suspension were added to 0.4-mL MCC plastic tubes containing approximately 0.1 mL silicon fluid (ρ, 1.2) and 0.2 mL silicon fluid (ρ, 1.2) and 0.2 mL silicon fluid (ρ, 1.08). After centrifugation in a Beckman Microfuge B for 30 sec the erythrocytes separated from the supernatant. The centrifuge tubes were cut into two parts by the use of a hot scalpel and each part was placed into a vial insert. Thus, the ^{65}Zn content of both the incubation media and the centrifuged cells could be determined by γ-counting. The dry weight of the packed erythrocytes was determined by drying two 0.2-mL samples at 100° to constant weight.

Haemoglobin–zinc interaction. A haemoglobin solution was prepared by lysing packed erythrocytes with twice their volume of hypotonic buffer (20 mM, Tris–HCl, pH 7.4). The solution was centrifuged at 3000 rpm for 5 min at 4°. The supernatant contained haemoglobin at an approximate concentration of 4×10^{-3} M. A solution of zinc complex (zinc, 0.1 mM; ligand, 1 mM) was added to an equal volume of haemoglobin solution and incubated at 37° for 15 min. Two different procedures for separating the haemoglobin from the low molecular complexes were employed.

Gel permeation chromatography. A 0.5-mL aliquot was applied to a PD-10 (G25) column and eluted with (20 mM, Tris–HCl; 130 mM NaCl, pH 7.4). Thirty 0.5-mL fractions were collected and assayed for ^{65}Zn content on a γ-counter.

Ultrafiltration. A 2.0-mL aliquot of the solution was filtered through a 10,000 MW cut off membrane filter (Millipore) in a stirred ultrafiltration cell. The membrane, retaining the high molecular weight ligands (>10,000) and the filtrate containing the low molecular weight ligands (<10,000), were estimated for ^{65}Zn content on the γ-counter.



Scheme 1.

Haemoglobin–oxygen dissociation curves. Heparinized blood samples were obtained from normal adult volunteers and sickle cell disease patients. Washed packed red cells (0.5 mL) were incubated in 2 mL 20 mM bisTris buffer, pH 7.1 containing either 2.5 mM zinc (acetate, chloride or sulphate) or 25 mM ethylmaltol or both for 15 min at 37° in a shaking waterbath, then washed three times with the bisTris buffer. Oxygen dissociation curves on red cells suspensions in the same pH 7.1 buffer were carried out using the method of Bellingham and Huehns [17] and Hills coefficient (*n* value) calculated. For the dose–response curves, 2.5 mM $ZnSO_4$, 25 mM ethylmaltol was diluted in bisTris buffer, maintaining the zinc:ethylmaltol ratio at 1:10.

RESULTS

Speciation and K_{part} *studies*

Zinc forms three different complexes with monoprotonated bidentate ligands (L) such as hydroxypyrones, ZnL^+, ZnL_2 and ZnL_3^-, (Scheme 1). Only the non-charged ZnL_2 complex is likely to permeate membranes by simple diffusion and therefore conditions were selected in this study which optimize the formation of this neutral compound. Using speciation plots it is possible to determine the optimum conditions for a given zinc concentration and pH (Fig. 1). With maltol the percentage of $Zn(maltol)_2$ which is present at pH 7.4 ([Zn] total = 5×10^{-4} M) increases with increasing maltol/zinc ratio, reaching a maximum of 65% with a ratio in the region 20:1 (Fig. 1a). Above this ratio the proportion of $[Zn(maltol)_3]^-$ increases. This trend is matched with the increased partition of ^{65}Zn into the octanol phase

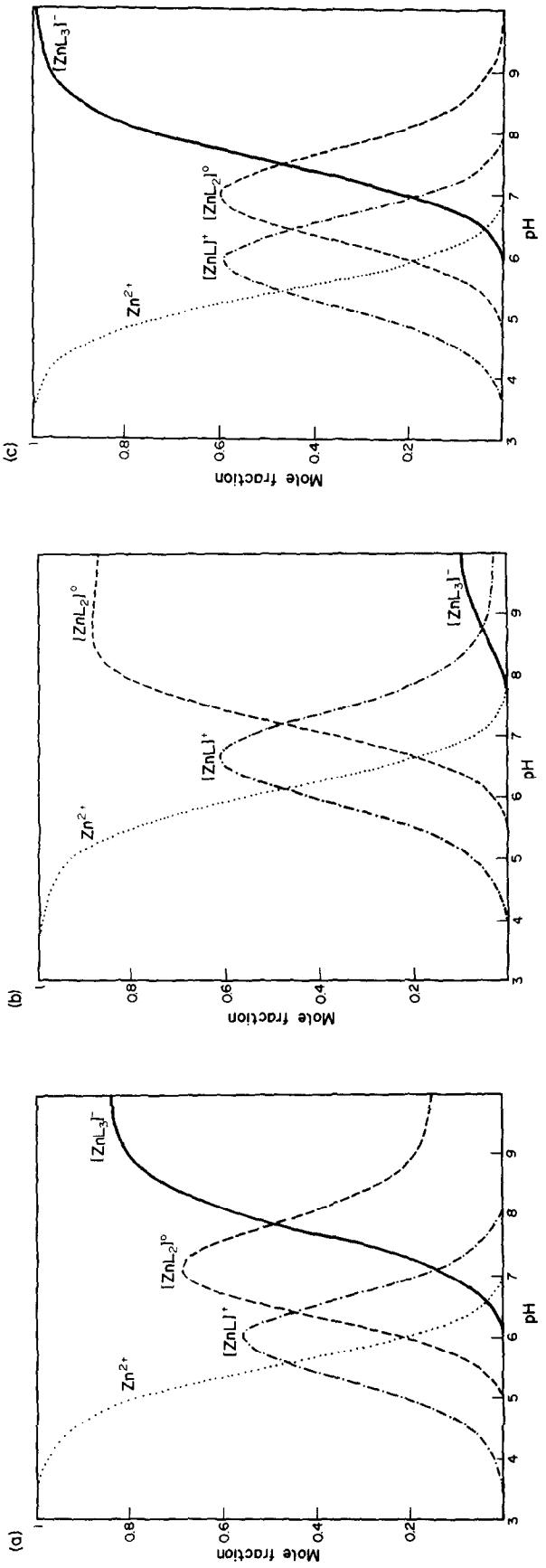


Fig. 1. The pH dependence of speciation plots for zinc-bidentate ligand systems. (a) zinc: ethylmaltol (1:20), $[Zn] = 5 \times 10^{-4}$ M; (b) zinc: 3-hydroxy-2-methylpyridin-2-one (1:10), $[Zn] = 5 \times 10^{-4}$ M; (c) zinc: 1,2-dimethyl-3-hydroxy pyridin-4-one (1:10), $[Zn] = 5 \times 10^{-4}$ M.

Table 1. The effect of the ligand:zinc ratio on partitioning of zinc ligand complexes between *n*-octanol and water

Ligand	K_{part} (ligand)	Ligand: zinc ratio	K_{part} (zinc complex)
Maltol	0.66	2:1	0.038
(I) R=Me		6:1	0.078
		10:1	0.100
		20:1	0.11
Ethylmaltol			
(I) R=Et	0.75	2:1	0.17
		6:1	0.65
		10:1	1.22
		20:1	1.24

with increasing maltol:zinc ratio (Table 1). From these values it is possible to estimate the K_{part} value of the neutral $Zn(maltol)_2$ complex. Thus for maltol the value is 0.17, whereas for the more hydrophobic ethylmaltol, it is 1.9. From these preliminary studies the ligand:zinc ratio in the region 10–20 appeared optimal for facilitating the partition of zinc into octanol. Parallel studies with 3-hydroxypyridin-2-ones (Fig. 1b) and 3-hydroxypyridin-4-ones (Fig. 1c) also identified optimal ratios of 10:1. This ratio was adopted for a wide range of ligands in a study designed to correlate the ^{65}Zn K_{part} value with the permeation of ^{65}Zn into erythrocytes.

^{65}Zn *uptake by erythrocytes*

A range of ligands was studied (Table 2). A large proportion of zinc (up to 99%) was absorbed by erythrocytes when the zinc was presented as a complex of either a hydroxypyrone (I) or a hydroxypyridin-2-one (III). In contrast when zinc was presented as a stable charged complex, for instance citric acid, EDTA, NTA or bipyridyl, no zinc (<1%) was taken up by the erythrocytes (Table 2). With lower affinity hydrophilic ligands, for instance glycine, lactate and catechol, zinc does not enter erythrocytes, but it is completely associated with the membrane and probably does not enter the cytoplasm (unpublished observations).

When zinc is incubated with hydroxypyrones (I) an extremely rapid uptake of the metal occurs (Fig. 2), the initial rate of entry being strongly dependent on the K_{part} value of the corresponding zinc dipyrone complex. A similar phenomenon occurs in the presence of hydroxypyridin-2-ones (III) (Fig. 3). The hydroxypyridin-4-ones have lower K_{part} values (Table 2) and behave like the relatively hydrophilic *N*-methyl-3-hydroxypyridin-2-one. Thus complexes with K_{part} values <0.2 only enter the erythrocyte slowly and donate the coordinated zinc to competing ligands on the outer cell surface. Under these circumstances equilibrium distribution is achieved within the first 2 min of incubation (data not shown).

In the presence of the hydroxypyrones and the hydroxypyridin-2-ones extremely large distribution ratios of zinc occur (>400). The zinc complexes enter the cells by simple diffusion as no saturation phenomena can be demonstrated (data not presented). Consequently, once inside the cell, zinc must dissociate from the complex and bind to a high

Table 2. Uptake of zinc by erythrocytes in the presence of different chelators

Class	Ligand	K_{part} (ligand)	K_{part} (Zn:L = 1:10)	% Zn in RBC after 60 min
Pyrones	Maltol	0.66	0.10	56
(I)	Ethyl maltol	0.75	1.22	96
	Isopropyl maltol	4.50	2.19	99
Pyrid-2-ones	3-Hydroxymethyl-pyrid-2-one	0.44	0.10	34
(III)	1-Ethyl-3-hydroxypyrid-2-one	0.50	0.66	78
	3-Hydroxy-1-propylpyrid-2-one	0.78	3.27	98
	3-Hydroxy-1-isopropylpyrid-2-one	3.10	2.70	99
Pyrid-4-ones	3-Hydroxy-1,2-dimethylpyrid-4-one	0.21	4×10^{-3}	17
(II)	1-Ethyl-3-hydroxy-2-methylpyrid-4-one	0.40	5×10^{-4}	27
	3-Hydroxy-2-methyl-1-propylpyrid-4-one	0.67	0.20	28
	3-Hydroxy-1-isopropylpyrid-4-one	0.95	0.11	22
Amino acids	Glycine		6×10^{-4}	13.9
	Glutamic acid		7×10^{-4}	12.8
	Picolinic acid		3×10^{-3}	0.6
Hydroxy acids	Citric acid		2×10^{-4}	0.5
	Malic acid		7×10^{-4}	8.4
	Lactic acid		1×10^{-3}	18.6
Ene diols	Ascorbic acid		3×10^{-3}	21.1
	Catechol		3×10^{-3}	27.5
General chelators	EDTA		3×10^{-4}	0.6
	NTA		2×10^{-4}	0.6
	Dipyridyl		3×10^{-3}	0.3

[Zn] = 10^{-4} M; [Ligand] = 10^{-3} M.

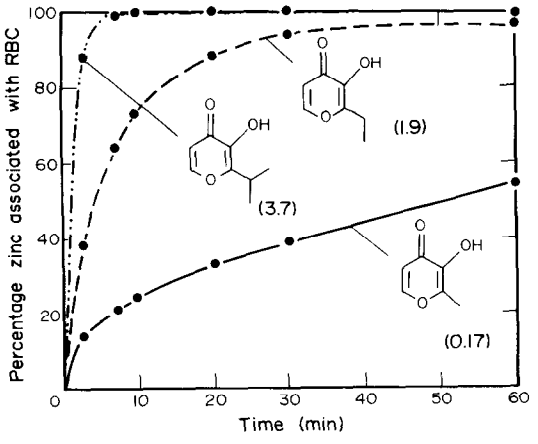


Fig. 2. Uptake of zinc by erythrocytes as a function of time in the presence of maltol, isopropylmaltol and ethylmaltol. Initial zinc concentration (5×10^{-4} M), initial ligand concentration (5×10^{-3} M). K_{part} values of zinc complexes are given in parentheses; N = 3.

affinity zinc site, which effectively behaves as a sink. In view of the findings of Brewer and co-workers [8, 9], haemoglobin was considered to be a likely candidate. In order to establish whether haemoglobin is capable of competing with hydroxypyrones and hydroxypyridinones for zinc, binding studies using ultrafiltration were undertaken (Table 3). It is clear that under the conditions of the experiment, haemoglobin is capable of removing zinc from both pyrones and pyridinones. Thus in view of its high intracellular concentration, haemoglobin is likely to provide the intracellular zinc binding site. Identical binding results were obtained when zinc haemoglobin complex formation was monitored by gel permeation chromatography.

Influence of zinc ethylmaltol on the haemoglobin–oxygen dissociation curve

When normal adult red cells were incubated with 2.5 mM $ZnSO_4$, 25 mM ethylmaltol, i.e. two zinc atoms per haemoglobin molecule, the oxygen dissociation curve P_{50} value at pH 7.1 was shifted from 27.5 mm Hg ($n = 2.5$) for untreated cells to 16.0 mm Hg ($n = 3.3$), $\Delta\log P_{50} = 0.235$ (Fig. 4a). Efficiency of intracellular uptake and binding to haemoglobin were not dependent on the zinc salt used, the same results being obtained with zinc acetate and chloride (Table 4). No significant shift was observed in the presence of either 2.5 mM zinc or 25 mM ethylmaltol alone (Table 4). Sickle cells also showed a left-shifted oxygen dissociation curve on incubation with zinc–ethylmaltol, P_{50} value for untreated cells 43.0 mm Hg ($n = 2.3$) and treated cells 20.5 mm Hg ($n = 2.4$), $\Delta\log P_{50} = 0.322$ (Fig. 4b). There was <1% formation of methaemoglobin after incubation. The increase in oxygen affinity was dependent on the dose of zinc present in both normal adult and sickle cells, with maximum shift in P_{50} occurring at an

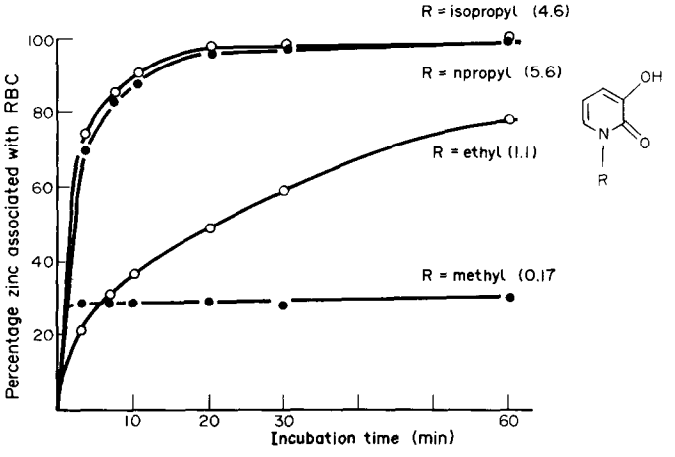


Fig. 3. Uptake of zinc by erythrocytes as a function of time in the presence of *N*-alkylpyrid-2-ones. Initial zinc concentration (5×10^{-4} M), initial ligand concentration (5×10^{-3} M). K_{part} values of zinc complexes are given in parentheses; N = 3.

Table 3. The binding of zinc to haemoglobin in the presence of a range of bidentate ligands

Ligand	Ligand: Zn ratio	Percentage ^{65}Zn bound to haemoglobin
Maltol	10	95 ± 3
Ethylmaltol	2	98 ± 2
1-Ethyl-3-hydroxypyridin-2-one	10	95 ± 4
1-Ethyl-3-hydroxy-2-methylpyridin-4-one	10	89 ± 2

Values are mean ± SD, N = 4.

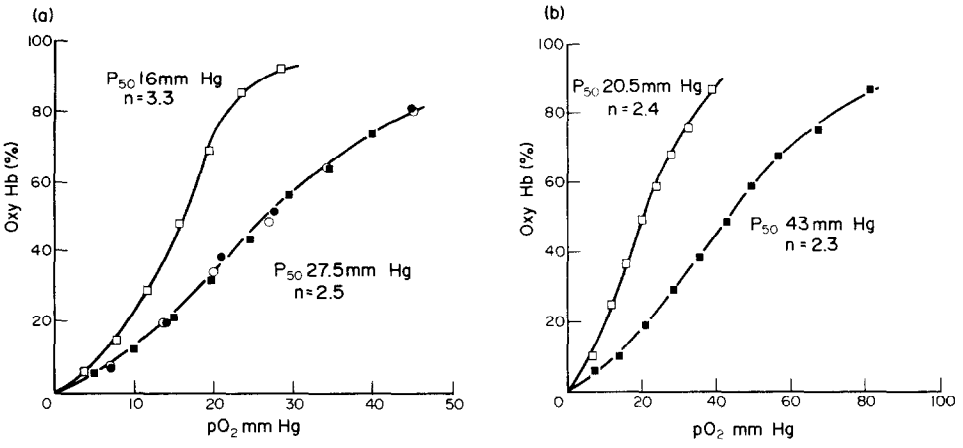


Fig. 4. Influence of zinc ethylmaltol on the oxygen dissociation curve of (a) normal human erythrocytes and (b) sickle cell erythrocytes incubated in bisTris buffer (20 mM, pH 7.1). (■) bisTris buffer; (●) 2.5 mM ethylmaltol; (○) 2.5 mM $ZnSO_4$; (□) 2.5 mM ethylmaltol and 2.5 mM $ZnSO_4$.

Table 4. Effect of zinc, ethylmaltol or both on oxygen affinity of normal adult erythrocytes

Incubation solution		P_{50} (mm Hg)	n value
BisTris pH 7.1		27.5	2.5
25 mM ethylmaltol		26.75	2.2
	2.5 mM zinc acetate	27.5	2.4
	2.5 mM zinc chloride	28.75	2.6
	2.5 mM zinc sulphate	27.25	2.3
25 mM ethylmaltol	2.5 mM zinc acetate	16.25	3.0
25 mM ethylmaltol	2.5 mM zinc chloride	16.0	3.3
25 mM ethylmaltol	2.5 mM zinc sulphate	16.0	3.3

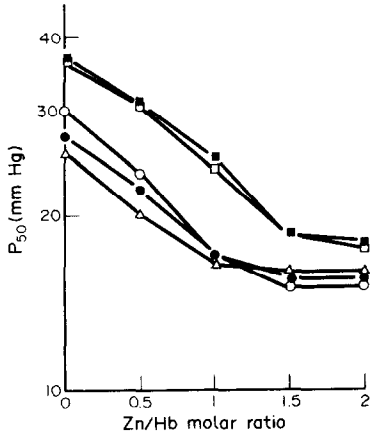


Fig. 5. Influence of zinc ethylmaltol concentration on the oxygen dissociation curve of normal adult erythrocytes (○, ●, △) and sickle cell erythrocytes (■, □).

approximate molar ratio of two zinc/haemoglobin molecule (Fig. 5).

DISCUSSION

The interaction of zinc with human haemoglobin leading to an increase in the oxygen affinity has previously been reported using solutions of haemoglobin, either unpurified [8] or stripped of all low molecular weight components [5]. The increase shown for the latter was 3.7-fold ($\Delta \log P_{50} = 0.57$) however, incubations of zinc chloride with whole blood showed only a 1.1-fold increase ($\Delta \log P_{50} = 0.04$) [9]. While clinical studies using oral zinc sulphate have shown a number of effects including improvement in wound healing [18], growth and weight of sickle cell disease patients [19], considerable relief from pain [20] and reduction in the number of irreversibly sickled cells [21], there has been no evidence to suggest that the zinc crosses the red cell membrane. In fact *in vitro* studies on the filterability of sickle cells showed that where improvement could be demonstrated, less than 0.05 zinc atoms were bound per haemoglobin molecule. This suggests that the effect was exerted on the sickle cell membrane and not on haemoglobin [22].

Very little is understood about the mechanism of zinc transport across the red cell membrane [23]. Picolinic acid has been reported to facilitate zinc absorption by intestinal cells [24], but in the studies presented here it did not increase the rate of entry into human erythrocytes (Table 2). Neither did hydroxycarboxylic acids, tricarboxylic acids, amino acids or dipyridyl. In contrast, both 3-hydroxy-

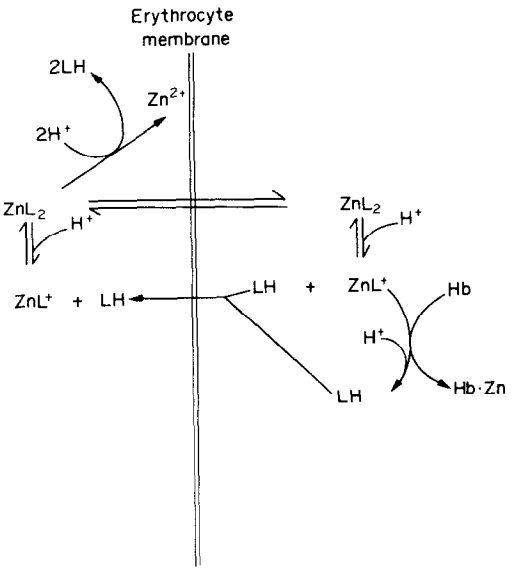


Fig. 6. Schematic representation of zinc accumulation by erythrocytes in the presence of hydroxypyrones or hydroxypyridin-2-ones (L). Hb, Haemoglobin.

pyrones (I) and 3-hydroxypyridin-2-ones (III) did facilitate the entry of zinc into red cells (Figs 2 and 3). The initial rate of entry was strongly influenced by the partition coefficient of the neutral zinc complex, with a partition coefficient greater than 0.5 leading to appreciable zinc uptake and accumulation by the red cell. The closely related pyridin-4-ones (II) were not as effective as a source of zinc transport under these conditions, despite being able to form neutral complexes with zinc (Table 2). This is probably due to the more extensive distribution of charge occurring with this class of molecule as compared with the hydroxypyridin-2-ones [25]. This necessitates a much larger side chain in order to increase membrane permeability to that observed with the hydroxypyridin-2-ones.

Both the pyrones and pyridin-2-ones facilitate the entry of zinc into red cells by a carrier mechanism, whereby the ligand simultaneously permeates the bilayer membrane with its co-ordinated zinc in a 2:1 ratio. Although 1:1 complexes and 3:1 complexes are possible (Fig. 1), the charged nature of these complexes limits uptake into the red cell. With the more hydrophobic ligands over 99% of the zinc originally present in the media had accumulated in the erythrocytes within 60 min and is bound to haemoglobin (Table 3). Clearly red cells would be unable to accumulate zinc against a concentration gradient by simple diffusion and yet enormous zinc distribution ratios were observed. This distribution ratio can be explained however by haemoglobin possessing a higher affinity for zinc than the bidentate ligand (pyrone or pyridin-2-one) and an overall uptake mechanism of the type indicated in Fig. 6 is proposed to explain the accumulation of zinc.

The binding of zinc to the intracellular haemoglobin resulted in the increase in oxygen affinity that had been reported in previous studies using haemoglobin solutions [5, 6] (Fig. 4). The increase was only observed in the presence of both zinc and ethylmaltol together (Fig. 4A), demonstrating that under normal conditions very little zinc crosses the red cell membrane. Ethylmaltol was equally effective at chelating zinc acetate, chloride and sulphate and releasing the zinc to haemoglobin (Table 4). The maximal shift in P_{50} value was observed when two zinc atoms were available to bind per haemoglobin molecule, which is consistent with the results of Gilman and Brewer [6]. It is not known precisely where the zinc binds to haemoglobin though at least three amino acid residues have been implicated, cysteine β93, histidine β143 and histidine β146 [5, 6]. Clearly however the β6 Glu to Val mutation of the sickle molecule does not interfere with binding of the zinc since the oxygen affinity increases were similar in both normal adult (Hb A) and sickle (Hb S) cells. It would also appear likely that although the binding of zinc to the β chain increases the oxygen affinity, it does not directly interfere with polymerization of the sickle molecule since the dose-dependent relationship between zinc bound and degree of left shift of the oxygen dissociation curve was almost the same for normal adult and sickle cells (Fig. 5). In contrast, sickle cells treated with agents such as urea, which disrupt the intermolecular bonds responsible for the aggregation process of the sickle polymer as well as increasing the oxygen affinity of the haemoglobin, show a far greater shift in P_{50} value than normal adult cells [26].

The results obtained with zinc–ethylmaltol are similar to those described for the anti-sickling agent cyanate. Carbamylation of the N-terminal amino groups of all four globin chains did not directly inhibit sickling, but the increased oxygen affinity of treated cells decreased the proportion of deoxyhaemoglobin present at any particular partial pressure of oxygen. This reduced the tendency to sickle at tissue oxygen tensions and therefore increased the red cell survival of cyanate-treated cells [3]. Experimental data suggest that maintenance of 25–30% Hb S in the oxy-conformation at venous oxygen tensions would help to prevent sickling [27]. Results with another agent BW12C, which stabilizes the oxy-conformation of haemoglobin and causes a left shift of the oxygen dissociation curve, have shown that up to 23% of the haemoglobin could be modified to the high affinity form without evidence of tissue hypoxia and with biochemical and rheological evidence for a transient decrease in the haemolytic rate [28]. Using zinc-ethylmaltol it should be possible therefore to modify the dose to achieve a clinical benefit by maintaining sufficient haemoglobin in the oxy-conformation, i.e. preventing the formation of sickle polymers, without compromising oxygen delivery to the tissues.

Clinical studies on many anti-stickling agents at effective doses have proved too toxic for use *in vivo* [2]. An advantage of using the hydroxypyrone ethylmaltol as an agent is that its toxicology and pharmacology have already undergone extensive investigation for use as a flavour-enhancing agent, e.g. in chocolate cakes and fruit drinks, with a safe level probably greater than 200 mg/kg/day [10]. Orally administered ethylmaltol is almost completely absorbed, rapidly metabolized and excreted in the

urine. However, under the conditions used in these experiments, once the ethylmaltol has released the zinc intracellularly it leaves the cell and would be removed by the washing procedure. Use of zinc–ethylmaltol with whole blood would not be expected to be as efficient at delivering zinc specifically to red cells due to competitive binding of plasma proteins such as albumin, which have a high binding affinity for zinc. Clinical use of zinc–ethylmaltol would probably therefore need to be extra-corporeal with red cells being treated *in vitro*, washed and then returned to the patient.

In conclusion, the results presented suggest that the treatment of red cells extra-corporeally with zinc–ethylmaltol would result in a left shift in the oxygen dissociation curve comparable in magnitude to that seen with other left shifting agents. This should reduce the tendency of treated cells to sickle *in vivo* at tissue oxygen tensions. Further investigation of the use of zinc–ethylmaltol for this purpose is indicated.

Acknowledgements—We wish to acknowledge financial support from the British Technology Group. Some of the data has been previously presented in the form of a patent (U.K. Patent GB 2118176).

REFERENCES

1. Luzzatto L and Goodfellow P, Sickle cell anaemia—a simple disease with no cure. *Nature* **337**: 17–18, 1989.
2. Serjeant GR, Therapeutic attempts to inhibit sickling. In: *Sickle Cell Disease*, pp. 359–370. Oxford University Press, Oxford, 1985.
3. May A, Bellingham AJ, Huehns ER and Beaven GH, Effect of cyanate on sickling. *Lancet* **i**: 658–661, 1972.
4. Gillette PN, Peterson CM, Lu YS and Cerami A, Sodium cyanate as a potential treatment for sickle disease. *N Engl J Med* **290**: 654–660, 1974.
5. Rifkind JM and Heim JM, Interaction of zinc with haemoglobin: binding of zinc and the oxygen affinity. *Biochemistry* **16**: 4438–4443, 1977.
6. Gilman JG and Brewer GJ, The oxygen-linked zinc binding site of human haemoglobin. *Biochem J* **169**: 625–632, 1978.
7. Gilman JG, Oelshlegel FJ and Brewer GJ, Hemoglobin-zinc interaction: functional and structural effects. In: *Erythrocyte Structure and Function* (Ed. Brewer GJ), pp. 85–101. Alan Liss Inc., New York, 1975.
8. Oelshlegel FJ, Brewer GJ, Knutsen C, Prasad AS and Shoomaker B, Studies on the interaction of zinc with human hemoglobin. *Arch Biochem Biophys* **163**: 742–748, 1974.
9. Oelshlegel FJ, Brewer GJ, Knutsen C, Prasad AS and Schoomaker B, Effect zinc on increasing oxygen affinity of sickle and normal red blood cells. *Biochem Biophys Res Commun* **53**: 560–566, 1973.
10. Gralla EF, Stebbins RB, Coleman GL and Delahunt CS, Toxicity studies with ethyl maltol. *Toxicol Appl Pharmacol* **15**: 604–613, 1969.
11. Hider RC, Kontoghiorghes G and Silver J, Pharmaceutical preparations, U.K. Patent GB 2118176, 1982.
12. Streater M, Taylor PD, Hider RC and Porter JB, Novel 3-hydroxy-2(IH)-pyridinones. Synthesis, Iron(III) chelating properties and biological activities. *J Med Chem*, in press.
13. Taylor PD, Morrison IEG and Hider RC, Microcomputer application of non-linear regression analysis to metal-ligand equilibria. *Talanta* **35**: 507–512, 1988.
14. Gerald C and Higel RP, Stabilité thermonamiques des complexes d'α-cetoenols le maltol et l'acide kojique avec les cations divalents: Mn, Co, Ni et Zn. *J Chem Res M* 4872–4886, 1978.
15. Stünzi K, Harris RLN, Perrin DD and Teitei T, Stability constants for metal complexation by isomers of mimosine and related compounds. *Aust J Chem* **33**: 2207–2220, 1980.
16. Stünzi H, Perrin DD, Teitei T and Harris RLN, Stability constants of some metal complexes formed by mimosine and related compounds. *Aust J Chem* **32**: 21–30, 1979.
17. Bellingham AJ and Huehns ER, Compensation in haemolytic anaemics caused by abnormal haemoglobins. *Nature* **218**: 924–926, 1968.
18. Serjeant GR, Galloway RE and Gueri MC, Oral zinc sulphate in sickle-cell ulcers. *Lancet* **ii**: 891–893, 1970.
19. Prasad AS and Cossack ZT, Zinc supplementation and growth in sickle cell disease. *Ann Intern Med* **100**: 367–371, 1984.
20. Brewer GJ, Oelshlegel FJ, Prasad AS, Knutsen CA and Meyers N, Paper presented at 1st National Symposium on Sickle Cell Disease, NIH, 1974.
21. Brewer GJ, Brewer LF and Prasad AS, Suppression of irreversibly sickled erythrocytes by zinc therapy in sickle cell anaemia. *J Lab Clin Med* **90**: 549–554, 1977.
22. Brewer GF and Oelshlegel FJ, Anti-sickling effects of zinc. *Biochem Biophys Res Commun* **58**: 854–861, 1974.
23. Rifkind JM, Interaction of zinc with erythrocytes. In: *Metal Ions in Biological Systems* (Ed. Sigel H), Vol. 15, pp. 275–317. Marcel Dekker, New York, 1983.
24. Evans GW, Picolinic acid and zinc absorption. In: *Biological Aspects of Metals and Metal-Related Diseases* (Ed. Sarkar B), pp. 81–88. Raven Press, New York, 1983.
25. Hider RC and Hall AD, Iron chelating agents in medicine: the application of bidentate hydroxypyridin-4-ones. In: *Perspectives of Bioinorganic Chemistry* (Eds. Hay RW, Dilworth JR and Nolan KB), JAI Press, London, in press.
26. May A and Huehns ER, The effect of urea on sickling. *Brit J Haematol* **30**: 21–29, 1975.
27. Franklin IM, Rosemeyer MA and Huehns ER, Sickle cell disease: the proportion of liganded haemoglobin needed to prevent crises. *Brit J Haematol* **54**: 579–587, 1983.
28. Keidan AJ, White RD, Huehns ER, Franklin IM, Joy M and Stuart J, Effect of BW12C on oxygen affinity of haemoglobin in sickle-cell disease. *Lancet* **i**: 831–834, 1986.