Zinc–Citrate Interactions in Blood Plasma. Quantitative Study of the Metal Ion Equilibria in the Zinc–Citrate–Histidinate, –Glutaminate and –Threoninate Systems and Computer Simulation of the Ability of Citrate to Mobilize the Low Molecular Weight Fraction of Zinc

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The present paper deals with the study of the influence of rising citrate concentrations in blood plasma on the extent of the low-molecular-weight fraction of zinc in the biofluid.

In addition to the results of an earlier work on the metal ion equilibria in the zinc-citrate-cysteinate system, the reliability of the computer simulations involved did require beforehand an investigation of the other potentially predominant mixed-ligand zinc–citrate complexes in plasma. The potentiometric study of the ternary complexation between zinc–citrate and –histidinate, –glutaminate, –threoninate are thus reported in turn.

The computer simulations carried out on the basis of these results show the tendency for zinc to be excreted urinarily under the influence of high concentrations of citrate in blood plasma, for example due to hyperparathyroidism, or to exchanged transfusions to infants. On this occasion the influence of high levels of citrate in plasma on the mobilization of other naturally–occurring metal ions into their low-molecular-weight fraction is also given.

Introduction

In many respects, citrate is a molecule of biological importance [1, 2]. Its presence in many naturally-occurring systems in which some of its functions interfere with the metabolism of metal ions [3–7] has drawn attention to the study of its potential interactions as a ligand in biological fluids [8–13], especially towards hard metal ions [13–17]. In particular, citrate has been shown to play a leading role in the complexation of Ca(II), Mg(II), Mn(II) and Fe(IIII) in blood plasma [18]. Zn(II) and Pb(II) had also been thought to be substantially complexed by citrate in the aforementioned study [18], but the initially high percentage of the zinc–citrate–cysteinate complex involved has more recently been proved to derive erroneously [19] from a miscalculated constant [11]. The latter study clearly established the need for the experimental determination of stability constants under the suitable conditions of ionic strength and temperature before they are used to simulate the metal ion equilibria in a given biofluid.

Part of our present research deals with the simulation of Zn(II) equilibria in biofluids [20–22] and particularly blood plasma [23, 24]. The possibly significant ternary complexation of citrate with zinc and the main amino-acids (other than cysteinate) in the zinc complex distribution prompted us to examine the corresponding systems. In addition, the excessive bone resorption due to hyperparathyroidism is known to be associated with the increase of the citrate plasma concentration [25]. As zinc is an essential cofactor of bone formation through collagen synthesis, we were also interested to know to which extent the rise of the citrate plasma concentration could result in a correlated increase of the low-molecular-weight fraction of zinc in plasma. Depending on the electrical charge of the potentially formed complexes, this increase would either promote zinc urinary excretion, or facilitate zinc diffusion into tissues, the latter effect possibly counteracting the excessive bone resorption.

The present study thus reports the experimental determination of the stability constants of the ternary complexes formed by zinc and citrate with histidinate, glutaminate and threoninate, these three amino-acids being the most predominant low-molecular-weight ligands in the zinc plasma distribution (after cysteinate). The effects of rising concentrations of citrate on this distribution are then briefly discussed, on the basis of the corresponding computer simulations.

Experimental

Formation Constant Determinations

Reagents

Citrate was purchased from Prolabo R.P. as a 'pro analysi' product and the amino-acids were purchased

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0020-1693/82/0000-0000/$02.75

from Merck as 'biochemical grade' substances. The titre of these ligands was systematically checked before use, by means of classical Gran plots. They were thus employed as supplied, stock solutions being kept under a nitrogen atmosphere.

The stock solution of zinc perchlorate (Pierce Inorganics B.V.) was prepared and titrated as specified in a previous paper [21]. The sodium hydroxide solutions were obtained by diluting BDH concentrated volumetric solutions in aqueous $NaClO_4$ 0.15 mol. dm^{-3} as described earlier [21], and were titrated against standard solutions of 'Normapur' Prolabo R.P. potassium hydrogenphthalate.

The titrations were performed by adding successive aliquots of sodium hydroxide to mixtures of zinc and ligand(s) previously acidified with 'Normatom grade' Prolabo R.P. perchloric acid, so that all the donor groups of the ligand(s) were initially protonated.

We used Merck reagent grade sodium perchlorate 0.15 mol. dm^{-3} as a background electrolyte to maintain the activity coefficients of the reactants constant and to ensure isotonicity of the solutions with blood plasma.

Apparatus and Technique

The potentiometric equipment was identical to that already described [21] and so was the electrode arrangement, opposing a glass electrode to a saturated sodium chloride calomel electrode. The system was calibrated in terms of concentrations, using readings from solutions of known-concentrations of hydrogen ions to determine the applicable formal potentials. The pH symbol used throughout this study should thus be understood in terms of $-\log [H^+]$.

The initial total concentrations of the reactants used in the titrations are given in Table I, along with the corresponding pH ranges. The temperature was fixed at 37 ± 0.02 °C throughout this study. Each experiment was stopped as soon as precipitate appeared in the solution, as firstly visualised by a steady drift in the mV-meter readings.

Calculation procedures

Estimations of the stability constants of the mixed-ligand complexes possibly formed in the three ternary systems investigated were refined by means of the MINIQUAD programme [26]. From a general point of view, the SCOGS programme [27] is able to accommodate estimations of constants more widely removed from the 'best' limit values than MINIQUAD [19]. It was thus also used for the calculations in the zinc-citrate-histidinate systems, in which the equilibrium constants of the various possible mixed-ligand species [19, 21] were difficult to assess on the usual statistical grounds [28]. In such cases the results obtained from SCOGS were finally refined by MINIQUAD.

TABLE I. Summary of the Titration Data Used for Calculating Stability Constants. Initial Total Concentrations of Zinc (C_M), First Ligand (C_L), Second Ligand (C_X), Strong Acid (C_H), and pH[a] Range.

System	C_M	C_L	C_X	C_H	pH range
	mmol. dm^{-3}				
Zinc–Citrate–Histidinate	10.15	10	10	25 79	1.9–8.9
	10 15	10	20	25.79	2.4–10.1
	10 15	20	10	25.79	1.9–9.6
	5.08	5	5	10.42	2 3–9 0
	5.08	10	10	25 27	1.9–10.6
	20.30	10	10	26 82	1 9–7.1
Zinc–Citrate–Glutaminate	10.15	9 97	9.90	25.79	2.8–8.9
	10.15	9 97	10.80	25.79	2.4–9 2
	10.15	19.94	9 90	25.79	3 2–9.1
	5.08	4 99	4.96	10.42	2 6–9.0
	5.08	9.97	9.90	25 27	2.8–9.4
	20.30	9.97	9.90	26.82	2.7–7 4
Zinc–Citrate–Threoninate	10.15	9 97	9 91	25.71	1.8–9.0
	10.15	9.97	19.82	25 71	1.9–9.1
	10 15	19.94	9.91	25.71	1.8–9 2
	5.08	4.99	4.96	10 39	2.2–8.9
	5.08	9 97	9.91	25.20	1.8–9.3
	20.30	9.97	9.91	26.74	1.7–7.3

[a]As defined in the text

An additional difficulty arose from the very structure of citrate. The protonation constants of its hydroxy group corresponds to the basic pH range in which the glass electrode measurements become less reliable [19]. To suppose that the number of dissociable protons (NDP) of citrate is equal to 4 would thus result in affecting the stability constants of the citrate complexes with the corresponding uncertainty. For this reason, the NDP = 3 hypothesis had already been considered by one of us as more satisfactory from a chemical point of view [19, 23]. For the MINIQUAD programme, the two alternatives are mathematically equivalent, although, as will be seen below, the calculations run in the NDP = 3 hypothesis do yield higher sums of squared residuals and R factors.

However, the NDP = 4 hypothesis had to be used beforehand for graphical reasons. In accordance with our customary approach [21], the search for the best model of ternary constants among the various possibilities investigated was carried out on both numerical and graphical grounds. The numerical fits were tested by comparing sums of squared residuals and R factors [26]. The graphical studies compared the experimental protonation curves of the pair of ligands investigated (including citrate and each of the three amino-acids in turn) with their simulations obtained by means of the PSEUDOPLOT programme [29, 21].

This kind of curve displays the average number of protons bound to the sum of the two ligands, as defined by the following expression

$$\bar{s} = \frac{C_H + NDP_L \times C_L + NDP_X \times C_X - C_{OH} - [H^+] + [OH^-]}{C_L + C_X} \quad (1),$$

as a function of pH. In expression (1), C_H and C_{OH} respectively represent the total concentrations of mineral acid and alkali in the solution, C_L and C_X standing for the total concentrations of citrate and amino-acid involved. As the PSEUDOPLOT programme can, by definition, simulate positive values of $\bar{s}$ only, citrate had therefore to be considered in the NDP = 4 hypothesis before the constants of the final 'best' model were transposed into their corresponding ones in the NDP = 3 hypothesis. Accordingly, both of these alternatives concerning citrate are met in Table II, where the constants of the parent binary complexes used in the present calculations are reported.

Results

The zinc–citrate–histidinate system had already been investigated by Daniele and Ostacoli [31], who had characterised the MLX and MLXH mixed-ligand species in the NDP = 3 hypothesis for citrate. We thus tried to refine constants for these complexes on the basis of our own data using the values given by the above-mentioned authors as input estimations, but the constant corresponding to MLXH was made negative by MINIQUAD. The research for the 'best' set of constants for this sytem thus had to be reconsidered.

Unlike the transition metal ions, the electronic configuration of zinc cannot be influenced by any ligand field stabilisation. It is thus difficult to estimate the configuration of zinc complexes from the properties of the ligand involved, which means that tetrahedral, squared pyramidal, distorted or regular octahedral structures are possible as well. Accordingly, we tried to assess the existence of each ternary species potentially formed between the parent ligands, using firstly SCOGS, then MINIQUAD. We reached the conclusion that in the NDP = 3 hypothesis for citrate, MLX was the main species formed, as its proportion reached about 40% in each experiment but the 2:1:1 ratio one (as expected). Two minor complexes were also characterised, the constants of which are given in Table III. Both of them improved the sum of squares of the models including MLX and each of them separately. Moreover, they were unequivocally proved to exist on the basis of graphical comparisons, an example of which is given by the 1:2:2 ratio experiment (Fig. 1), in which $ML_2X(OH)_2$ reached the percentage of 53%.

TABLE II. Stability Constants $\beta_{pqrs} = [M_rL_pX_qH_s]/[M]^r[L]^p[X]^q[H]^s$ of Parent Complexes of Citrate, Histidinate, Glutaminate and Threoninate at 37 °C and I = 0.15 mol. dm^{-3} $NaClO_4$, as Used in the Calculations (L = Citrate, X = Histidinate or Glutaminate or Threoninate).

System	p	q	r	s	log β	Ref.
Proton–citrate	1	0	0	1	12.050	[19]
(NDP = 4)	1	0	0	2	17.575	
	1	0	0	3	21.804	
	1	0	0	4	24.669	
Zinc–Citrate	1	0	1	1	16.754	[19]
(NDP = 4)	1	0	1	2	20.503	
	2	0	1	2	31.618	
	2	0	2	0	21.974	
Proton–Citrate	1	0	0	1	5.539	[19]
(NDP = 3)	1	0	0	2	9 775	
	1	0	0	3	12 644	
Zinc–Citrate	1	0	1	0	4.715	[19]
(NDP = 3)	1	0	1	1	8 441	
	2	0	1	0	7.361	
	2	0	2	−2	−2.214	
Proton–Histidinate	0	1	0	1	8.770	[21]
	0	1	0	2	14.643	
	0	1	0	3	16.400	
Zinc–Histidinate	0	1	1	0	6.336	[21]
	0	2	1	0	11.599	
	0	1	1	1	10.718	
	0	2	1	1	16.919	
Proton–Glutaminate	0	1	0	1	8.680	[30]
	0	1	0	2	10.864	
Zinc–Glutaminate	0	1	1	0	4 174	[23]
	0	2	1	0	7.664	
	0	2	1	−1	−2.137	
Proton–Threoninate	0	1	0	1	8.573	[30]
	0	1	0	2	10.721	
Zinc–Threoninate	0	1	1	0	4.467	[23]
	0	2	1	0	8.279	
	0	2	1	−1	−1.159	

No previous study could be found in the literature concerning the other two systems investigated. In both of them, the MLX and MLXH complexes in the citrate NDP = 3 hypothesis were successively made negative during MINIQUAD refinements. The only species that could be characterised in each of these systems was $M_2LX(OH)_2$, the existence of which was clearly demonstrated from the graphical comparisons between experimental and simulated protonation curves pertaining to the 2:1:1 experiments (Fig. 2).

Discussion

As far as the main ternary species in the zinc–citrate–histidinate system is concerned, our results

TABLE III. Stability Constants Obtained from These Studies. The Formula of the General Complex is the Same as in Table II. n = Number of Experimental Observations; S = sum of Squared Residuals; R = R Factor (see ref. 25); L = Citrate; X = Histidinate or Glutaminate or Threoninate.

System	p	q	r	s	log β	S	R	n
Zinc–Citrate–Histidinate	1	1	1	1	22.008 ± 0.030	0.8563 E–05	0.0047	294
(NDP_L = 4)	2	1	1	0	17.109 ± 0.062			
	1	1	2	−1	10 388 ± 0.220			
Zinc–Citrate–Glutaminate	1	1	2	−1	9.210 ± 0.039	0.1758 E–04	0 0079	299
(NDP_L = 4)								
Zinc–Citrate–Threoninate	1	1	2	−1	9.730 ± 0 043	0.2564 E–04	0.0090	293
(NDP_L = 4)								
Zinc–Citrate–Histidinate	1	1	1	0	9.973 ± 0 025	0.9840 E–05	0.0061	294
(NDP_L = 3)	2	1	1	−2	−6.985 ± 0.060			
	1	1	2	−2	−1.708 ± 0.231			
Zinc–Citrate–Glutaminate	1	1	2	−2	−2.815 ± 0 048	0.3356 E–04	0.013	299
(NDP_L = 3)								
Zinc–Citrate–Threoninate	1	1	2	−2	−2.299 ± 0.040	0.3002 E–04	0.013	293
(NDP_L = 3)								

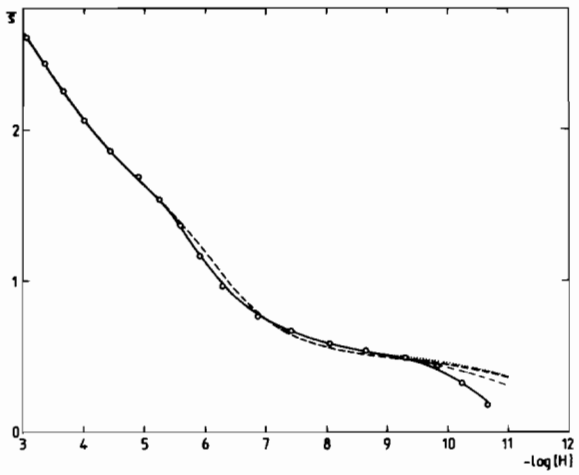


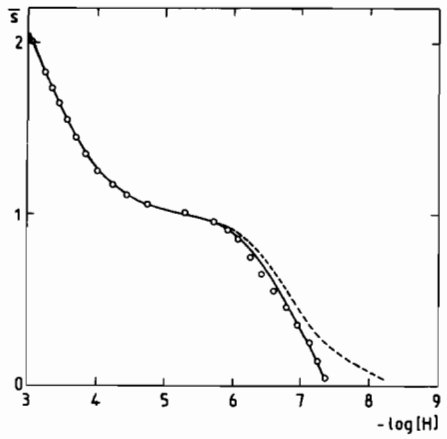
Fig. 1. Protonation curve of a mixture of citrate and histidinate in the presence of zinc. C_{Zn} = 5.08, C_{cta} = 10 00, C_{his} = 10.00 mmol. dm^{-3}. Every fifth experimental point is materialized. The broken line simulates the curve assuming no mixed-ligand species formation. The dotted line takes into account MLX only The dashed line (–·– –·) simulates the existence of both MLX and $M_2LX(OH)_2$. The solid line represents the final results in Table III (see text)

essentially confirm those obtained by Daniele and Ostacoli [31]. However, we did not characterize the same minor complexes as these authors. Their discovery of the MLXH species may arise from the absence of MX_2H in the constants they found on the zinc-histidinate system; it is noteworthy that their results on the zinc–citrate system also differ from ours in that they did not mention the existence of ML_2. Such discrepancies in the parent systems are likely to affect the mixed-ligand constant calculations [19], as already pointed for the zinc–histidinate system [31] in the study of the zinc–histamine–histidinate system [23].

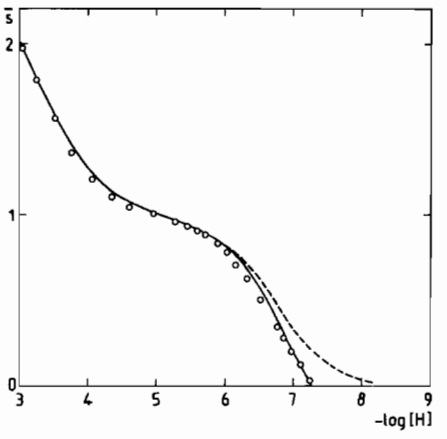
The $ML_2X(OH)_2$ complex in the citrate NDP = 3 hypothesis, which must be chemically understood in terms of ML_2X in the NDP = 4 hypothesis, was proved to be structurally possible from molecular model considerations, the dissociated hydroxy group of citrate being doubly involved in an octahedral configuration. As for $M_2LX(OH)_2$, its formation was logically expected since $M_2L_2(OH)_2$ is one of the main species in the basic range for the zinc–citrate system. This remark will also hold for the two other systems involving glutaminate and threoninate.

Let us now consider the ability of the systems investigated to give rise to the formation of mixed-ligand complexes. An empirical rule was expressed by Sovago and Gergely [32], according to which the stability of a mixed-ligand complex is higher when the difference between the $\log(K_1/K_2)$* values for the parent complexes is greater. This rule, which was first discovered for mixed-ligand complexes of diamines [32], was more recently extended to the case of amino-acids [33]. It does not hold in the present case, as the respective $\log(K_1/K_2)$ values are 2.069, 1.073, 0.684 and 0.655 for citrate, histidinate, glutaminate and threoninate, whereas the strongest association is observed between citrate and histidinate. In fact, the only mixed-ligand complex to be favoured is the zinc–citrate–histidinate one, presumably be-

*K_1 is defined as β_{ML} (or β_{MX}), K_2 as β_{ML_2}/β_{ML} or (β_{MX_2}/β_{MX}).



(a)



(b)

Fig 2. Protonation curve of a mixture of citrate and· a) glutaminate C_{Zn} = 20.30, C_{cta} = 9.97, C_{glu} = 9.90 mol. dm^{-3}; b) threoninate C_{Zn} = 20 30, C_{cta} = 9.97, C_{thr} = 9 91 mmol. dm^{-3}. Every third experimental point is materialized. The broken lines simulate the curves assuming no mixed-ligand species formation. The solid lines represent the final results in Table III.

cause of the particular affinity of the imidazole moiety of histidinate for the O donors of citrate. Indeed, this well-known phenomenon concerning the formation of copper mixed-ligand complexes is also valid for zinc [34].

The favourable formation of ternary complexes of citrate with histamine rather than with histidinate, already mentioned by Daniele and Ostacoli [31], is confirmed by the comparison of the present results in Table III with those of the zinc–histamine–citrate system [23]. This is probably due to the presence of the oxygen donor of histidinate as compared with the exclusive nitrogen atoms of histamine. Indeed, the formation of mixed-ligand complexes is generally favoured when the donor atoms of the parent ligands are dissimilar in the nature [35].

Computer Simulation of the Influence of Citrate on the Low-Molecular-Weight Fraction of Zinc in Blood Plasma

General Considerations

The total amount of a given metal ion present in a biofluid such as human blood plasma can be formally divided into four main fractions [18], namely:

(i) the metalloproteins in which the metal ion is embedded in a non-exchangeable form,

(ii) the labile metal–protein complexes,

(iii) the complexes arising from the low-molecular-weight (l.m.w.) ligands,

(iv) the free or hydrated ions.

The third of these fractions represents only a low percentage of the overall concentration of the metal ions, frequently less than 1%. Nevertheless, it is in large excess with regard to the concentration of the hydrated ions, and the size of its components makes it essential for a number of biological functions. Among those, the transfer of the metal ion through cell membrane is undoubtedly the most important, in that it determines the bioavailability of the latter.

The free concentration of the metal ions being buffered by the proteins present in the biofluid [36], any enhancement of the l.m.w. fraction due, for example, to the introduction of a powerful ligand into the milieu, will entail an immediate displacement of the metal from the fraction (ii) above. If the concentration of this ligand is maintained at a high level for a long period, then a slow consumption of fraction (i) will take place within the limits allowed by the lifetime of the metalloproteins, the initial mobilization of the metal into its l.m.w. fraction finally resulting in an alteration of the body reserves of the patient. Depending on the electrical charges of the main l.m.w. complexes formed, this alternation may induce either excessive urinary excretions, which is for example the case for zinc during total parenteral nutrition [20, 36], or excessive tissue deposition.

Simulation Studies

The ECCLES program [18] was used for this study. In accordance with the above observations, this program allows the quantitative evaluation of the mobilization of a given metal into its l.m.w. fraction, due to the influence of a complexing agent. This mobilization is usually materialised in drawing the plasma mobilizing index (P.M.I.), representing the ratio

$$\text{P.M.I.} = \frac{\text{total concentration of the l.m.w. metal ion fraction in presence of drug}}{\text{total concentration of the l.m.w. metal ion fraction in normal plasma}} \quad (2)$$

as a function of the plasma concentration of the exogenous substance under consideration [36–38].

In the present case the effects investigated are those of a naturally occurring substance in blood plasma. For this reason, the P.M.I. parameter has been plotted as a function of the ratio by which the average normal concentration of citrate [18] has been multiplied. Figure 3 shows the curve obtained for zinc, but also those corresponding to the other plasma naturally-occurring metal ions, given for the sake of comparison (except copper, which was almost not affected at all).

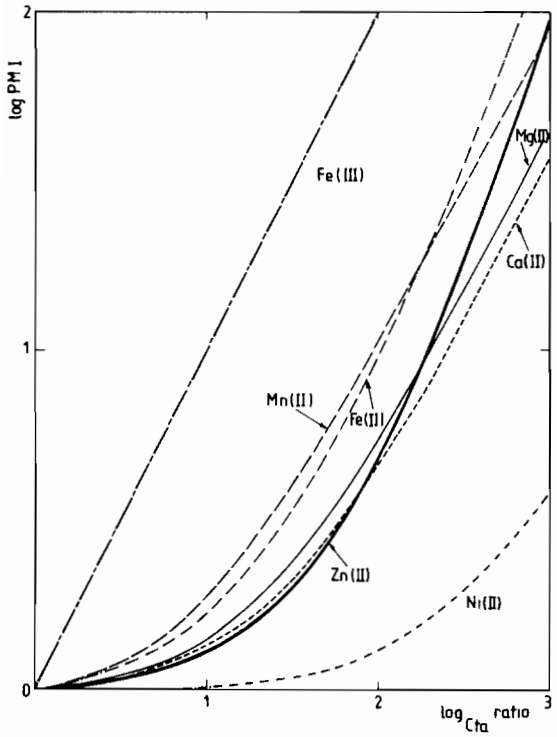


Fig. 3. Plasma Mobilizing Index of zinc, together with that of other naturally occurring metal ions, as a function of the ratio by which the normal plasma citrate concentration is raised.

Discussion

The reliability of such simulations clearly depends on that of the equilibrium constants on which the model is based. Concerning the distribution of the complexes involving both zinc and citrate, the concentration percentage of those experimentally investigated in the present study and in the previous one [19] represents more than 98% of their total amount.

Let us now consider the influence of citrate on the plasma zinc distribution. As far as hyperparathyroidism is concerned, the l.m.w. extra mobilization of zinc due to the high citrate concentration usually observed during this state is not expected to be of a determining importance. The order of magnitude of the plasma citrate concentration in this kind of affection is generally not more than twice the normal one [25], which results in an increase of about 5% of the l.m.w. zinc fraction. Nevertheless, it is noteworthy that the increase of the plasma concentration of citrate tends to favour the urinary excretion of the metal, since all of the predominant zinc complexes including citrate are electrically charged.

The latter remark could be of some importance towards other biological considerations. For example, it has been reported that the administration of citrate during exchange transfusions to infants may result in serious symptoms of calcium and magnesium deficiency [39]. It is interesting to note that the log P.M.I. curves of these metals due to rising plasma citrate concentrations (Fig. 3) have a shape very similar to that of zinc. It thus seems logical to expect that the hypocalcemia and hypomagnesemia associated with exchange transfusions to infants are paralleled by a correlative decrease of the plasma zinc concentrations, which might affect to some extent their growth conditions [40].

More generally, citrate is extensively used as taste additive to most of artificial beverages. More attention should probably be paid to the possibly resulting excretion of essential metal ions, first of all iron (see Fig. 3). Indeed, the l.m.w. fraction of Fe(III) is dramatically enhanced by any increase of the citrate plasma concentration, which is not surprising given the fact that citrate is the main l.m.w. ligand of Fe(III) in plasma [2, 18]. Moreover, all the complexes involving both Fe(III) and citrate are electrically charged, hence excretable *via* urinary route.

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