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Trace Metal Requirements in Total Parenteral Nutrition. Part 3. A Quantitative Study of the Metal Ligand Interactions in the Zinc-lysinate, Zinc-lysinate-histidinate, Zinc-lysinate-cysteinate and Zinc-threoninate-histidinate Systems in Artificial Nutritive Mixtures

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A theoretical approach has been recently put forward with a view to solving the problem of the supplementation of trace metals in the artificial feeding technique known as total parenteral nutrition. The most pressing need is the determination of the daily dose of zinc specific to each nutritive solution infused. This determination necessitates beforehand the checking, by computer simulation, of the distribution of the metal among the different complexes it forms in the solution. The reliability of the pertinent distribution depends to a crucial extent on the correlative reliability of the equilibrium constants on which the simulation model is based.

On our way towards a realistic level of confidence for this model, we have determined in the present work the stability constants of the complexes formed in the zinc-lysinate, zinc-lysinate-histidinate, zinc-lysinate-cysteinate and zinc-threoninate-histidinate systems. Implications of the results on the above considerations are briefly discussed.

Introduction

It is well established that artificial nutritive mixtures, especially when administered by intravenous route, can induce excessive metal ion excretion [1–4]. After physiological experiments carried out on animals [5–8] and man [9], this effect has been attributed to particular components of the mixtures, including the amino acids cysteine and histidine.

As far as zinc is concerned, its mobilization into low-molecular-weight complexes during prolonged total parenteral nutrition (TPN) has been recently investigated by computer simulation [10]. This study has confirmed the leading role of cysteine and histidine in the excessive urinary excretion of zinc observed for patients receiving TPN.

The TPN-induced extra losses commonly result in specific deficiencies within weeks [11]. Due to the outstanding role of zinc in nucleic acid and protein biosynthesis [12], deficiencies in this metal may affect the patients' recovery, especially after surgery [13]. For this reason, a theoretical approach has been proposed [14], with a view to allowing the determination of metal ion daily doses specifically adapted to the composition of the infused solutions. Such a determination depends on (i) the assessment of the free metal ion concentration in normal blood plasma, which can be deduced from the known protein binding of the metal in question [10, 15], (ii) the reliability of the equilibrium constants of the complexes representing the main portion of the metal ion in the nutritive mixture. For example, the reinvestigation of the zinc-cysteinate-histidinate system [16] did lower our initial estimation of the daily dose of zinc from 38 mg to 32 mg for the nutritive mixture under study [17].

As cysteine and histidine play a key role in the complexation of zinc during TPN [10], the ternary systems that they form respectively with the main amino acids in the nutritive mixture should also be studied under the required experimental conditions. The present paper thus reports the quantitative study of the zinc equilibria in the zinc-lysinate-histidinate, zinc-lysinate-cysteinate and zinc-threoninate-histidinate ternary systems; the investigation of the zinc-lysinate parent system is also described.

Experimental

Reagents

Lysine was supplied as free base by Sigma Chemical Co. and stored under dry nitrogen at low tem-

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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 range.

System	C_M	C_L	C_X	C_H	pH range
	mmol dm^{-3}				
Proton-lysinate		9.50		24.84	2.0–10.7
		4.75		14.90	2.1–10.8
		19.00		24.84	2.7–10.9
Zinc-lysinate	10.15	9.50		24.84	2.0–7.0
	5.07	9.50		19.87	2.2–7.4
	7.10	19.00		25.57	2.6–7.5
	5.07	19.00		25.36	2.7–8.2
	2.03	19.00		25.05	2.7–9.6
	1.01	19.00		24.95	2.7–10.0
Zinc-lysinate-histidinate	10.15	9.50	10.00	30.85	2.3–7.2
	10.15	19.00	10.00	30.85	3.3–8.7
	10.15	9.50	20.00	40.79	2.4–9.6
	5.07	4.75	5.00	15.42	2.5–7.8
	20.30	9.50	10.00	26.92	2.5–7.0
Zinc-lysinate-cysteinate	5.07	4.73	10.00	15.43	2.2–8.7
	5.07	9.47	5.00	15.42	2.6–8.5
	3.04	2.84	3.00	15.22	2.0–5.9
	3.04	5.68	3.00	15.22	2.2–8.8
	3.04	2.84	6.00	15.22	2.0–8.6
Zinc-threoninate-histidinate	10.15	10.00	10.00	30.43	1.9–8.1
	10.15	20.00	10.00	30.43	2.1–9.3
	10.15	10.00	20.00	40.23	2.0–9.6
	5.07	5.00	5.00	15.22	2.2–7.9
	20.30	10.00	10.00	29.40	1.9–6.6

perature. Histidine and cysteine were Biochemical Grade Merck products. All of the three ligands were potentiometrically checked using Gran titrations. They were thus employed without further purification. Zinc perchlorate solutions were prepared as described earlier [16], as well as sodium perchlorate, perchloric acid and hydroxide solutions.

Potentiometric Equipment

All the technical arrangements, including reaction cell system, mV meter, electrochemical cells and burette system were identical to those previously described [16]. The temperature was fixed at 37 ± 0.02 °C, the ionic strength being 0.15 mol dm^{-3} $NaClO_4$.

The reactant concentrations relative to the various titrations which were carried out are summarized in Table I, along with the corresponding pH* ranges.

*pH is given as $-\log [H]$ in the present case, all of the glass electrode measurements being made in terms of concentrations [18].

Computational Techniques

The MINIQUAD programme [19] was used for the formation constant calculations throughout this study. It was actually aimed at refining the preliminary formation constants guessed from (i) the features of the formation curves for protonation and binary complexation studies, (ii) statistical considerations in the case of the formation of mixed-ligand complexes.

Different sets of constants were produced by the combination of all the possible species, derived from the examination of the related formation curves. They were discriminated on the basis of (i) numerical fits compared in terms of sums of squares and R factors [19], (ii) graphical comparisons of PSEUDO-PLOT simulations [20].

The curves used for the protonation and ternary complexation studies expressed the variable $\bar{s}$, defined in expression [1], as a function of pH

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

TABLE II. Stability Constants $\beta_{pqrs} = [M_r L_p X_q H_s]/[M]^r [L]^p [X]^q [H]^s$ of Binary and Ternary Complexes as Obtained from this Study*, together with Those of the Parent Species Used in the Calculations, at 37 °C and I = 0.15 mol dm^{-3} $NaClO_4$. n = number of experimental observations; S = Sum of squares of residuals.

System	p	q	r	s	log β	S	n	Ref.
Zinc-hydroxide	0	0	1	−1	−9.03			[21]
Proton-histidinate	0	1	0	1	8.770			[16]
	0	1	0	2	14.643			
	0	1	0	3	16.400			
Zinc-histidinate	0	1	1	0	6.336			[16]
	0	2	1	0	11.599			
	0	1	1	1	10.718			
	0	2	1	1	16.919			
Proton-threoninate	1	0	0	1	8.573			[22]
	1	0	0	2	10.721			
Zinc-threoninate	1	0	1	0	4.467			[22]
	2	0	1	0	8.279			
	2	0	1	−1	−1.159			
Proton-lysinate	1	0	0	1	10.296 ±0.002	0.859E − 06	181	This study
	1	0	0	2	19.183 ±0.003			
	1	0	0	3	21.330 ±0.006			
Zinc-lysinate	1	0	1	1	14.386 ±0.014	0.135E − 05	240	This study
	2	0	1	1	19.844 ±0.028			
	2	0	1	2	28.507 ±0.010			
Zinc-lysinate-histidinate	1	1	1	0	11.075 ±0.034	0.631E − 06	204	This study
	1	1	1	1	20.328 ±0.018			
Zinc-threoninate-histidinate	1	1	1	0	9.863 ±0.046	0.449E − 05	238	This study

*No mixed-ligand species could be characterized in the zinc-lysinate-cysteinate system under the experimental conditions shown in Table I.

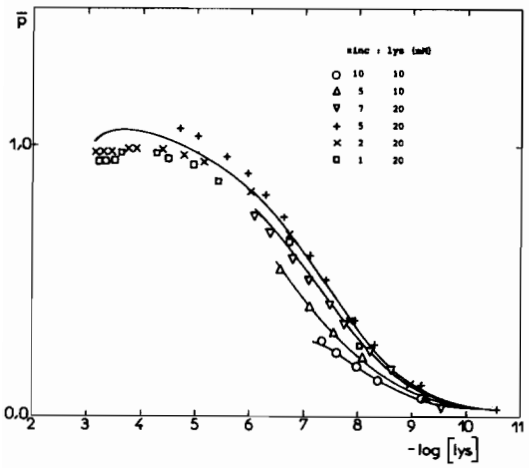

Fig.1. Formation curve of the zinc-lysinate system. About every fifth experimental point of each titration corresponding to the various metal to ligand ratios investigated has been materialized. The solid lines represent the simulations by the PSEUDOPLOT programme, as based on the "best" result shown in Table II.

In this equation, C_H, C_L, C_X and C_{OH} represent respectively the overall concentrations of strong acid, first ligand L, second ligand X and sodium hydroxide along the titration, NDP being the number of dissociable protons of the investigated ligand. For the zinc-lysinate system on the contrary, the variable

$$\bar{p} = \frac{C_L - [L] - [LH] - [LH_2] - [LH_3]}{C_M} \quad (2)$$

was plotted *versus* −log [L].

Besides the water dissociation constant pK_w = 13.38 used as previously found by one of us [18], some other formation constants, determined in earlier studies under the same experimental conditions, were used in the calculations. They referred to the zinc-hydroxide [21], proton-histidinate [16], zinc-histidinate [16], proton-cysteinate [18], zinc-cysteinate [18], proton-threoninate and zinc-threoninate [22] systems. The corresponding values are to be found in Table II when their use resulted in effective characterizations of new species.

Results

Lysinate Protonation

The equilibrium constants corresponding to the three protonation steps of the lysinate anion were shown in Table II.

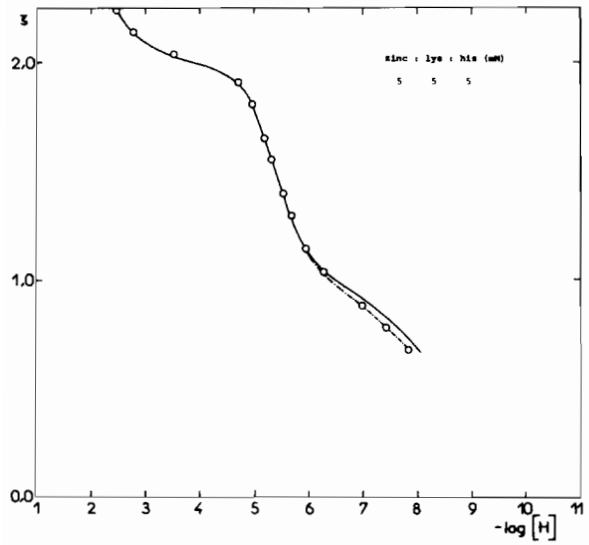

Fig. 2. Protonation curve of a mixture of lysinate and histidinate in the presence of zinc. About every fifth experimental point has been materialized. The solid line represents, the PSEUDOPLOT simulation assuming there is no ternary complex formation. The broken line simulates the existence of MLXH only. MLX was also unequivocally characterized in this system on grounds developed in the text.

Zinc-lysinate

In this system, the formation curve expressing $\bar{p}$ as a function of –log [lys] did not produce superimposable drawings for the various concentrations and metal to ligand ratios investigated (Fig. 1). The plots relative to the lowest of these ratios reached a maximum limit of about $\bar{p} = 1$, suggesting the existence of a ML complex, but the spreading observed between all the determinations seemed to indicate the formation of protonated species as well.

The protonation curves which were drawn [17] for the corresponding experiments in the presence of zinc showed shifts characteristic of the complexation. The inflexion point arising at $\bar{s} = 2$ in the absence of metal was observed at lower values, but never went below $\bar{s} = 1$. This suggested that lysinate would complex zinc in the LH form rather than through the dianion L.

Moreover, a recent study at 25 °C by Gergely and coll. [23] reports the existence of the four complexes ML, MLH, ML_2H and ML_2H_2. This tends to confirm the predominance of protonated species in this system, the more so as the above mentioned authors have postulated that the ML species was in fact the mixed hydroxo complex MLH(OH).

In order to identify unequivocally the stoichiometries of the possibly existing species, we used an additional graphical approach. As no $\bar{s}$ value lower than 1 had been determined in the presence of zinc, we decided to transpose LH into L*, by neglecting the first protonation step of lysinate. This implied that NDP_{L^*} was conventionally taken as zero and the following constants were derived from the original ones:

$$\log \beta_{L^*H} = \log \beta_{LH_2} - \log \beta_{LH}$$

$$\log \beta_{L^*H_2} = \log \beta_{LH_3} - \log \beta_{LH}.$$

The corresponding formation curves were less spread out than the classical ones in Fig. 1, and tended towards the apparent limit of $\bar{p}^* = 2$ [17]. Furthermore, after a slight inflexion at this particular value, the lowest metal to ligand ratio drawings went on rising up to near $\bar{p}^* = 3$. These features clearly suggested the formation of ML* (*i.e.* MLH), ML_2^* (*i.e.* ML_2H_2) and ML_2^*OH (*i.e.* ML_2H). As no "tail" characteristic of the existence of hydroxo species could be observed near $\bar{p}^* = 1$, no ML*OH (*i.e.* ML) complex was then logically expected.

Accordingly, among the various combinations of formation constants which were tried, involving each one of those corresponding to the four species above, the best numerical as well as graphical fits were obtained for MLH, ML_2H and ML_2H_2, whose values are shown in Table II. It is also noteworthy that the ML constant was made negative by MINIQUAD when refined together with the other three constants above.

Zinc-lysinate-histidinate

As appears in the above paragraph, no ML_2 was found in the zinc-lysinate system; the formation of the ternary species MLX was therefore not favourable. And yet the experimental formation curves of both the lysinate and histidinate ligands in the presence of zinc did not coincide with the corresponding PSEUDOPLOT simulations assuming there was no ternary complex formation (see Fig. 2 given as an example).

We thus tried and refined first the constant of a possible MLXH complex. When the resulting value was introduced in the PSEUDOPLOT data, the simulated protonation curves fitted pretty well with the experimental ones (Fig. 2). It is nevertheless of interest to note that the highest percentage reached by MLXH did not exceed 18% in the first experiment, 40% in the second and 24% in the fourth as listed in Table I.

In spite of the expectations above, we then additionally tried to refine the statistically estimated constant of a possible MLX species. It was not made negative during the MINIQUAD refinement which converged successfully, and was even calculated with a rather good accuracy, account being taken of its upper percentage of 16% only. Although MLX should undoubtedly be considered as a minor species,

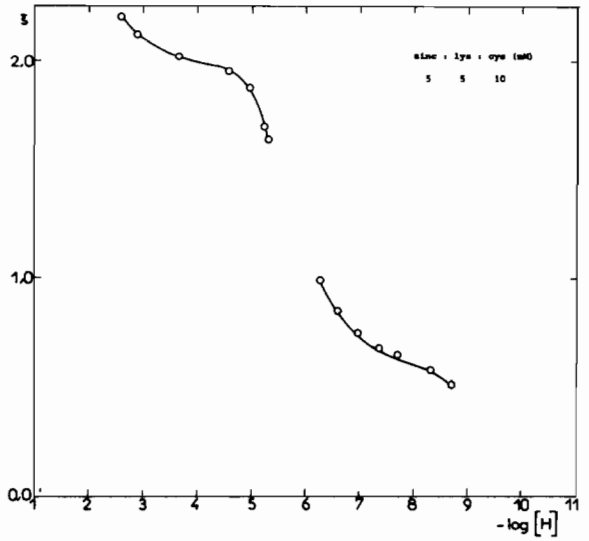

Fig. 3. Protonation curve of a mixture of lysinate and cysteinate in the presence of zinc. About every fifth experimental point has been materialized. The solid line represents the PSEUDOPLOT simulation assuming there is no ternary complex formation. As can be seen on the figure, a precipitate appeared in the solution around pH 5.5, which redissolved over pH 6.

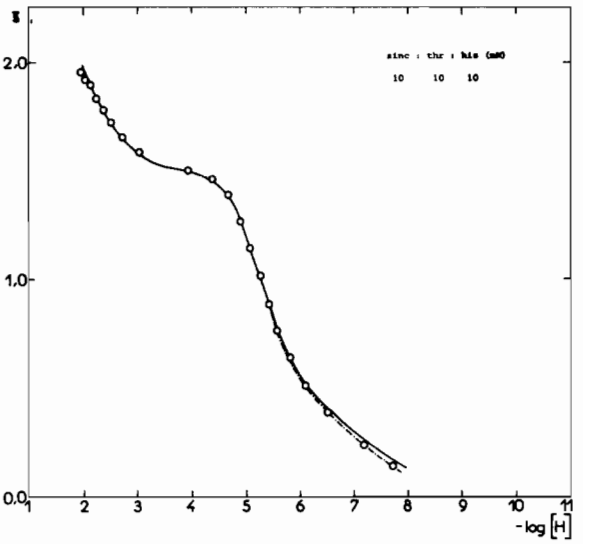

Fig. 4. Protonation curve of a mixture of threoninate and lysinate in the presence of zinc. About every fifth experimental point has been materialized. The solid line represents the PSEUDOPLOT simulation assuming there is no ternary complex formation. The broken line takes into account the MLX species as appears in Table II.

we chose to put it in our "best" set (Table II) for the following reasons:

(i) the standard error of the MLXH constant was significantly decreased when MLX was refined together with the former,

(ii) the sum of squares and R factor of the MINIQUAD programme were halved when MLX was introduced in the refinement of MLXH,

(iii) the existence of MLXH accounted almost totally for the difference observed between the experimental protonation curves and those which were simulated assuming there was no ternary species. Only the most basic range (pH > 9) of the curve corresponding to the third experiment in Table I was not properly interpreted: its interpretation was improved when MLX was introduced in the simulation data. Moreover, it is in this specific range that MLX reaches its maximum percentage; this can be taken as a logical confirmation of the preceding terms of our choice.

Zinc-lysinate-cysteinate

The most striking observation made on this system is the fairly good coincidence between the experimental protonation curves and those simulated on the basis of binary complexes only (Fig. 3). The formation of ternary species was thus not expected to be significant.

Accordingly, the attempt to refine the constants successively related to MLX, MLXH and even $MLXH_2$ – although less likely to exist from a chemical point of view – produced negative values.

Zinc-threoninate-histidinate

Although appearing non negligible, the ternary complexation was not expected to be very significant between zinc, threoninate and histidinate on the grounds of the usual graphical comparisons (Fig. 4). Indeed, the protonation curves of the sum of the two ligands in the presence of zinc which were simulated assuming there was no ternary complex formation were not very distant from the experimental points, especially for the solutions containing the higher concentrations of histidine.

The only species which could be found was MLX, with a rather poor accuracy. This is explained by the fact that its maximum percentage reached only 27% in the second experiment in Table I, for which the concentration of threonine is twice that of histidine. It is also noteworthy that even at this maximum percentage, the concentration of MLX was lower than the concentrations of the parent complexes ML_2 and particularly MX_2.

Discussion

Structure and Bonding Considerations

Although the formation constant of the $Cu(lys)_2$ complex was calculated by a number of authors [24–27], it has been well established [25, 27, 28] that this species is a minor one, and quite insignificant below $-\log[H] = 10$. Similarly, the zinc lysinate complex of the same stoichiometry could not be

characterized at all by Gergely *et al.* [23], and by ourselves in the present work either. As lysinate has been postulated to establish "glycine-like" bonds with copper [27] and zinc [23], its ω-amino group is not likely to play any role in the ternary coordination, and the mixed-ligand complexes generated by lysinate were thus expected to involve it in its monoprotonated form.

This has been confirmed by our observations in the zinc-lysinate-histidinate system, in which the MLXH species has been found predominant with respect to MLX. Moreover, the logarithm of the MLXH constant is of the same order of magnitude as the sum of those corresponding to the MLH and MX complexes: the respective values are 20.328 and 20.722, the latter being logically expected higher than the former for theoretical reasons [29].

As far as the zinc-lysinate-cysteinate system is concerned, we had already noticed in the previous part of this series [16] that glycinate and cysteinate formed negligible mixed-ligand species together with zinc. This is in line with the poor ability of glycinate to give rise to ternary coordination with other aliphatic acids [30]. As lysinate binds zinc in the "glycine-like" manner, it was logical to expect a very poor ability for the zinc-lysinate-cysteinate system to produce a significant ternary complexation. The fact that it proved impossible to find out any mixed-ligand complex in this system confirms this expectation.

Let us now consider the zinc-threoninate-histidinate system. The histidinate anion has been proved to be tridentate in the $Zn(his)_2$ complex [31]. This effectively accounts for the greater stability of the zinc-histidinate complexes [16] compared with those formed in the zinc-histamine system [32]. In addition, the structure of the above species is thought to be tetrahedral, with the carboxy-groups loosely bonded [31].

On the other hand, the binding of threoninate in the parent complex $Zn(thr)_2$ has been suggested to occur through the amino nitrogen and carboxyl oxygen [33], but more recent authors contend that there is an involvement of the hydroxy group in the coordination as well [34]. The ternary complexation of threoninate and histidinate with zinc, which would imply at least a loss of stability on one of the histidinate anions originally bound in the $Zn(his)_2$ species, should logically not be favoured. At most, the resulting stability ought to be similar to that of the Zn(gly)(his) complex [16].

If we compare now the experimental stability constant of the Zn(thr)(his) complex (9.86) with the statistical value (10.24) obtained from the following equation (35)

$$\log \beta_{MLX} = \tfrac{1}{2}(\log\beta_{ML_2} + \log\beta_{MX_2} + \log 4)$$

we can note a clear destabilization of this ternary species, which was not observed in the zinc-glycinate-histidinate system [16]. It is also noteworthy that such a phenomenon was not observed for the zinc-threoninate-histamine system [22] either. It thus seems to arise from antagonistic interactions of the carboxy-group of histidinate and the hydroxy-group of threoninate. This question would deserve a closer examination in the future.

Implications on zinc requirements in TPN

As developed earlier [10, 14] the approach developed by one of us for the supplementation of a given metal in TPN lies on the basic principle that the concentration of free metal in the nutritive solution should be the same as in normal blood plasma.

Once this concentration of free metal has been assessed, the problem remains as to determine the corresponding overall concentration to be introduced in the nutritive mixture. The exactness of this determination depends on the reliability of the parameters which express the concentration of each complex in terms of the total concentrations of its potential constituents, *i.e.* the stability constants. The total concentrations of the ligands involved are analytically measurable and are actually well known [10], but it is out of question to determine all the formation constants of the complexes to which they give birth, which are no fewer than 3400 for the 33 components in the nutritive solution under study [36].

The discrimination of the main complexes for each metal has thus been made on the basis of the simulated distribution of all the possible species [10], with the help of the ECCLES programme [15, 18] using three sorts of formation constants:

(i) reliable constants, available for the experimental conditions required,

(ii) interpolated constants, deduced from determinations carried out under different experimental conditions,

(iii) estimated constants, assessed from statistical considerations [35].

Our own feeling is that we can consider as a realistic level of confidence a proportion of 80% of the total metal concentration controlled by formation constants determined under the appropriate conditions. Our estimation of the optimal daily dose of zinc, which was temporarily found near 38 mg at the beginning of our study, has well decreased since then, as a result of our *in vitro* investigations of a number of systems [16].

After the present study, the dose of zinc comes down to only 20 mg per day, which is of the order of magnitude of the normal oral doses currently recommended [37]. Our final estimation of this dose for the nutritive mixture investigated will be reported in our next paper in this series, along with

the experimental determinations still necessary to reach the percentage limit stated above and the consecutive simulations.

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