Trace Metal Requirements in Total Parenteral Nutrition. Part 6. A Quantitative Study of the Copper(II)–Histidine Ternary Complexes with Leucine, Glutamic Acid, Methionine, Tryptophan and Alanine, and Final Evaluation of the Daily Doses of Copper and Zinc Specific to a Nutritive Mixture of a Given Composition

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Abstract

A computer-based approach was previously developed which made it possible to interpret the origin of the trace metal extra-losses induced by total parenteral nutrition (TPN) on a quantitative basis. The TPN-induced abnormal excretions of zinc and copper have been attributed to the enhanced mobilization of these metal ions into their plasma low molecular weight fraction by specific components of the nutritive solution, the mode of excretion (urinary for zinc/biliary for copper) being dictated by the electrical charge of the prevailing complexes. This implies that the trace metal needs during TPN must depend on the composition of the infusate.

It was thus proposed that every nutritive solution should contain total trace metal in such an amount that the corresponding free concentration be the same as that pertaining to normal blood plasma. In this way, the metal losses would not occur at the expense of the patient's body reserve.

Nevertheless, two conditions must be fulfilled before reliable doses can be calculated for a given nutritive mixture: (i) precise free metal ion concentrations in normal blood plasma must be assessed, (ii) a computer-based reliable distribution of each metal ion in the nutritive solution must be obtained. Now, the reliability of the involved simulation model directly depends on that of the equilibrium constants of the predominant complexes. A series of determinations was thus successively carried out for zinc and copper until a realistic degree of reliability (over 80%) was reached.

The present paper focuses on the determination of the formation constants of the mixed-ligand complexes formed by copper and histidine with five amino acids prevailing in the nutritive mixture under study, which brings the degree of reliability of the copper distribution up to 84%.

The final estimation of the zinc and copper absolute doses for the nutritive solution chosen as a reference is discussed with regard to the limitations of the present approach for which a number of applications are suggested.

Introduction

It is now well documented that long term total parenteral nutrition (TPN) eventually results in the appearance of overt trace metal deficiency symptoms, especially with regard to zinc and copper [1–4].

The solving of this problem is not straightforward since several factors may contribute to the occurrence of these disorders. Decreased intake arising from the lack of appropriate micronutrient allowances in the nutritive mixtures seems to be a major source of deficiency [5]. Increased utilization during anabolic phases [5], losses due to the gastrointestinal disease itself [3], or to stresses or trauma [6, 7], may also play a significant role. Nevertheless, for both zinc and copper, the depletion of the patient's body stores appears to stem mainly from the excessive losses specifically induced by the infusion of the TPN mixture [8], the excretion route depending on the nature of the metal [9, 10]. In that respect, the spectacular urinary excretion of zinc caused by TPN [1, 8, 9, 11] results in subnormal plasma levels of this metal from about 2 to 7 weeks after starting the treatment [12]. In contrast, copper urinary excretion is not significantly enhanced [8, 11], although the corresponding plasma levels also fall to a similar extent within the same period of time [5, 8].

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Many attempts were made to rationalize these observations, based on *in vitro* as well as *in vivo* experiments [13–15]. All of the derived conclusions focused on the fact that a small fraction of metal does exist in the form of low molecular weight (l.m.w.) complexes in blood plasma [13, 16–18], the extent of which would vary under the effect of the TPN infusion. However, no experimental approach is possible for investigating the distribution of this metal l.m.w. fraction in biofluids, if one takes into account (i) the infinitely small concentrations of the species involved, (ii) the complexity of the whole system of labile equilibria. It was thus not until computer simulation techniques were applied to metal speciation in blood plasma during TPN that these phenomena could be interpreted on a really quantitative basis [19, 20].

The dependence of the mobilization of a given metal into its l.m.w. fraction on the composition of the nutritive solution being infused was clearly established on this occasion. In particular, the clinical observations concerning the separate effects of the administration of individual amino acids on zinc urinary excretion in man [13, 21] and several animal species [13, 15, 22] were borne out by the calculations [19, 20]. Moreover, the mode of excretion of zinc and copper was shown to be directly related to the electrical charge of the main complexes formed by these metals in blood plasma during treatment. Clearly, the urinary excretion of zinc would arise from the formation of its electrically charged predominant complexes with cysteine and histidine, whereas the biliary excretion of copper would be due to the prevailing formation of its electrically neutral complexes with histidine and other monoprotic amino acids [23].

The dependence of the TPN-induced trace metal excretions on the composition of the nutritive solutions being administered is also especially important in that it clearly implies that *the metal doses to be incorporated into these solutions must be a function of their own composition.* No such conclusion had been reached before and the clinical assessments of the daily metal doses recommended for patients receiving TPN had so far been established regardless of the nutritive mixture composition [9, 10, 24].

It was therefore proposed that any nutritive solution should ideally contain such a total metal concentration that the corresponding free one be equivalent to that pertaining to normal blood plasma [19, 20]. In such a situation, the metal excretion induced by the infusion would not occur at the expense of the patient's body reserves, but entirely at the expense of the metal ion supplement.

In line with this principle, the calculation of the total trace metal concentrations that should be included in a given nutritive mixture will proceed directly from the computer-simulated distribution of each metal ion in this mixture. The reliability of the recommended metal doses will thus depend on (i) the exactness of the estimation of the free concentration of each metal ion in normal blood plasma, as deduced from the practical equilibrium constants available for its main protein complexes [19], (ii) the precision of the equilibrium constants which relate the free concentration of each metal ion to those of the various complexes it gives rise to with the potential ligands present in the solution. Concerning the latter point, it is realistic to consider that a satisfactory level of confidence has been reached when about 80% of the total metal concentration derived from the calculations is based on complex equilibrium constants checked under the appropriate experimental conditions. Accordingly, a series of studies had already been devoted to the determination of the stability constants relative to the main complexes of zinc in the nutritive mixture under consideration [25–27] before a daily dose could be proposed for this metal [27]. The same objective was pursued for copper, the preceding paper of this series showing the achievement of 56% reliability for the simulated distribution of this metal [23].

The present work completes this series and is aimed at assessing the optimum daily dose of copper specific to the nutritive mixture being considered. From the experimental point of view, it deals with the potentiometric investigation of the ternary complexation of copper and histidine, the main ligand of this metal in the nutritive mixture as well as in blood plasma [23], with leucine, glutamic acid, methionine, tryptophan and alanine, all these amino acids being involved in the temporary distribution of copper in decreasing order of importance [23]. The implications of the results on the evaluation of the recommended metal doses are discussed.

Experimental

Formation Constant Determinations

Reagents

All the amino acids were purchased from Merck as biochemical grade products. Their degree of purity was checked by the appropriate Gran titrations and they were proved to be reliably useable as delivered. Their stock solutions were stored under nitrogen atmosphere and were frequently renewed.

The stock solution of copper perchlorate was prepared and titrated for its metal content as previously described [28], its proton concentration being determined from direct potentiometric measurements.

TABLE I. Summary of the Titration Data Used in the Formation Constant Calculations. Initial Total Concentrations of Copper (C_M), Histidine (C_L), Second Amino Acid (C_X), Mineral Acid (C_H), and pH* Range Investigated. The Concentrations are expressed in mmol dm^{-3}.

System	C_M	C_L	C_X	C_H	pH range
Proton–leucine			5.00	10.44	2.13–11.26
			10.00	20.90	1 86–10.96
			10.00	26.02	1.73–10 78
			10.00	31 30	1.62–10.89
Copper–leucine	0.99		10.00	15.75	2.03–10 67
	1.99		10.00	15.83	2.02– 5 22
	0.99		5.00	10.53	2 12–11.00
	1.49		5.00	10.57	2.12– 8.75
	1.99		5.00	10.61	2.12– 5.72
	1 99		4 00	5.39	2.44– 6.37
	1.99		2.00	5.39	2.35– 6 24
Copper–histidine–leucine	4.96	5.00	5.00	16.09	2.14– 8.97
	4.96	5.00	10.00	26.53	1 89–10.32
	4.96	10.00	5.00	26.53	1 98–10.05
	4.96	10.00	10.00	31.75	1 93–10.33
	9 93	10.00	10.00	37.41	1.78– 6.78
	9.93	5.00	5.00	21.75	1.91– 5.61
Copper–glutamic acid	0.99		8.42	9.18	2.25–10.57
	1.99		8.42	13.81	2 03–10.25
	2 48		8.42	13 85	2.02– 9.97
	3.48		8.42	13.94	2.02– 9.67
	4.96		8.42	14 07	2.01– 6.60
	9.93		8.42	19.05	1.85– 5.42
	9.93		4.21	9.96	2.09– 5.23
Copper–histidine–glutamic acid	4.96	5.00	4 20	14.37	2 18– 7.24
	4.96	5.00	8.41	23.64	1.93–10 26
	4.96	10.00	4.20	19.01	2.24–10.05
	4.96	10 00	8.41	28.29	1.98–10.21
	9.93	10 00	8.41	28.72	1.95– 7 13
	9.93	5.00	4.20	14.80	2.14– 5.81
Proton–methionine			5.00	10.44	2.10–10.63
			10.00	20.88	1.84–10.67
			10.00	26.10	1.72–10.25
			10.00	31.20	1 61–10.80
Copper–methionine	0.99		10.00	14.01	2.07–11 05
	1.99		10.00	14.09	2.18– 5 21
	0.99		5.00	9 37	2 16– 5.58
	1.49		5.00	9.41	2.16– 5.41
	1.99		4.00	4.81	2.46– 5 82
	1.99		2.00	4.81	2.39– 6.42
Copper–histidine–methionine	4.96	5.00	5.00	14.35	2.20– 9.38
	4.96	5.00	10 00	23.63	1.96–10 37
	4.96	10 00	5 00	23.63	2 06–10.23
	4.96	10 00	10 00	28 27	2 00–10.30
	9.93	10.00	10 00	33 35	1.86– 8.61
	9.93	5.00	5.00	19.43	1.97– 5.85
Proton–tryptophan			4 92	10.44	2.12–10.64

(continued overleaf)

TABLE I. *(continued)*

System	C_M	C_L	C_X	C_H	pH range
			9.85	11.60	2.21–10.89
			9.85	20.88	1.86–10.83
			9.85	30.16	1.64–10.92
Copper–tryptophan	0.99		9.85	16.23	2.01–10.96
	1.99		9.85	16.32	2.00– 4.53
	0.99		4.92	10.43	2.12– 7.20
	1.49		4.92	10.48	2.12– 5 01
	0.99		2.46	5 71	2.34– 5 86
	0.99		1.97	4.22	2.46– 7.09
	1.99		1.97	4.31	2.44– 4.83
Copper–histidine–tryptophan	4.96	5.00	4.92	15.33	2.16– 7.99
	4.96	5 00	9.85	25.67	1.90– 9.97
	4.96	10.00	4.92	24.42	2.04– 9 82
	4.96	10.00	9.85	39.30	1.73–10.12
	9.93	10.00	9.85	35.19	1.81– 6.81
	9.93	5.00	4.92	15.76	2.10– 5.37
Proton–alanine			5.00	9.28	2.20–11.07
			10.00	18.56	1.94–11.43
			10.00	23.20	1.81–11.12
			10.00	27.84	1.70–11.26
Copper–alanine	0.99		10.00	14.01	2.12–11.11
	1.99		10.00	14.09	2.11–10.76
	2.48		10.00	18 77	1.93–10.54
	3.48		10.00	18.86	1.93–10.49
	4.96		10.00	18.99	1.92– 9.30
	9.93		10.00	24.07	1.77– 5.96
	9.93		5.00	14.79	1.94– 5.46
Copper–histidine–alanine	4.96	5.00	5.00	14.35	2.23– 9.52
	4.96	5.00	10.00	23.63	1.98–10.53
	4.96	10.00	5.00	23.63	2.09–10.38
	4.96	10.00	10.00	28 27	2.04–10.65
	9.93	10.00	10 00	33.35	1.87– 8.90
	9.93	5.00	5.00	19.43	1.99– 5.79

*See text.

Sodium perchlorate 0.15 mol dm^{-3} was used to maintain a constant ionic strength in the solutions investigated, this being isotonic with blood plasma. It was obtained from Merck as a reagent grade product and its stock solution was prepared as specified in a previous work [23].

Sodium hydroxide was purchased from BDH as concentrated volumetric solutions whereas perchloric acid was a Normatom grade Prolabo R.P. product. The stock solutions of both of these reagents were prepared and standardized as described earlier [25].

Apparatus and technique

The experimental determinations consisted of potentiometric titrations carried out with electrochemical cells using a Beckman glass electrode and a saturated sodium chloride Beckman calomel electrode fitted in an Ingold cell system.

The technical equipment was basically the same as that already used in the preceding part of this series [23]. For each titration, 20 cm^3 of solution were titrated against the standard solution of 0.1000 N sodium hydroxide delivered from an ABU 12 Radiometer Autoburette.

It is to be noted that a sufficient amount of perchloric acid was systematically included in the initial solutions to be titrated so that all the donor groups of the ligands were protonated at the outset of the experiment. The temperature was maintained at 37 °C inside the reaction cell, and a constant bubbling of purified and thermostatted nitrogen was set up

TABLE II. Stability Constants $\beta_{pqrs} = [M_rL_pX_qH_s]/[M]^r[L]^p[X]^q[H]^s$ of Parent Complexes at 37 °C and I = 0.15 mol dm^{-3} $NaClO_4$, as Used in the Calculations. L represents histidine and X stands for the second amino acid.

System	p	q	r	s	log β	Ref.
Proton–hydroxide	0	0	0	−1	−13.38	31
Proton–histidine	1	0	0	1	8.770	25
	1	0	0	2	14.643	
	1	0	0	3	16 400	
Proton–glutamic acid	0	1	0	1	9.176	34
	0	1	0	2	13 256	
	0	1	0	3	15.440	
Copper–histidine	1	0	1	0	9.893	35
	2	0	1	0	17.498	
	1	0	1	1	13.843	
	2	0	1	1	23.172	
	2	0	1	2	26.546	
	2	0	2	−2	9 204	
	2	0	1	−1	6.422	
Copper–hydroxide	0	0	1	−1	−7.60	36
	0	0	2	−2	−10.49	
	0	0	3	−4	−20.50	estimated from 37

throughout the titration. Each experiment was stopped as soon as a steady drift in the mV-meter readings revealed the appearance of a precipitate in the solution.

Full details concerning the data collected from the titrations are available elsewhere [29] along with those relevant to our previous work [23]. Table I thus reports the essentials of the corresponding experimental conditions, *i.e.* the initial total concentrations of the reactants and the pH ranges investigated. The electrode system being calibrated in the concentration scale, pH should be understood in terms of −log[H] throughout this study.

Calculation procedures

The MINIQUAD program [30] was used to refine the equilibrium constants. Their initial estimations entered as input data were derived from the formation curves of the protonation and binary complexation systems, and from statistical relationships for ternary system investigations.

In accordance with our usual combined approach [25, 28, 31, 32], the sets of constants that were the most likely to account for the experimental data of each system were preliminarily selected by comparing the goodness-of-fit parameters characteristic of the program [30]. Then, the final discrimination was

TABLE III. Formation Constants Obtained from these Studies. The Formula of the General Complex is the Same as in Table II. S = Sum of the Squared Residuals; R = R Factor as Defined in Ref. [30], n = Number of Experimental Observations; L = Histidine; X = Second Amino Acid.

System	p	q	r	s	log β	S	R	n
Proton–leucine	0	1	0	1	9.266 ± 0.001	0.235E − 06	0.0026	209
	0	1	0	2	11.590 ± 0.002			
Proton–methionine	0	1	0	1	8.779 ± 0.002	0.299E − 06	0.0028	227
	0	1	0	2	10.949 ± 0.002			
Proton–tryptophan	0	1	0	1	9.019 ± 0.002	0.491E − 06	0.0032	265
	0	1	0	2	11.341 ± 0.003			
Proton–alanine	0	1	0	1	9.399 ± 0.001	0.143E − 06	0.0017	207
	0	1	0	2	11.769 ± 0.002			
Copper–leucine	0	1	1	0	7.902 ± 0.004	0.163E − 06	0.0024	342
	0	2	1	0	14.533 ± 0 006			
	0	2	1	−1	2.324 ± 0.094			
Copper–glutamic acid	0	1	1	0	8.115 ± 0.005	0.689E − 06	0.0030	302
	0	2	1	0	14.504 ± 0.012			
	0	1	1	1	12.183 ± 0.006			
	0	2	1	1	18.682 ± 0.131			
	0	1	1	−1	1.079 ± 0.046			
Copper–methionine	0	1	1	0	7.490 ± 0.009	0.409E − 06	0 0045	211
	0	2	1	0	13.696 ± 0 018			

(continued overleaf)

TABLE III. *(continued)*

System	p	q	r	s	log β	S	R	n
Copper–tryptophan	0	1	1	0	7.957 ± 0.007	0.155E – 06	0.0024	270
	0	2	1	0	15.205 ± 0.009			
	0	1	1	1	10.430 ± 0.056			
Copper–alanine	0	1	1	0	7.947 ± 0.004	0.511E – 06	0.0029	292
	0	2	1	0	14.460 ± 0.009			
	0	1	1	1	10.401 ± 0.051			
	0	2	1	–1	2.378 ± 0.090			
Copper–histidine–leucine	1	1	1	0	17.183 ± 0.011	0.319E – 05	0.0047	373
Copper–histidine–glutamic acid	1	1	1	0	16.830 ± 0.019	0.296E – 05	0.0049	262
Copper–histidine–methionine	1	1	1	0	16.731 ± 0.016	0.477E – 05	0.0068	320
Copper–histidine–tryptophan	1	1	1	0	17.665 ± 0.015	0.394E – 05	0.0052	303
	1	1	1	1	21.468 ± 0.040			
Copper–histidine–alanine	1	1	1	0	17.000 ± 0.019	0.404E – 05	0.0059	295

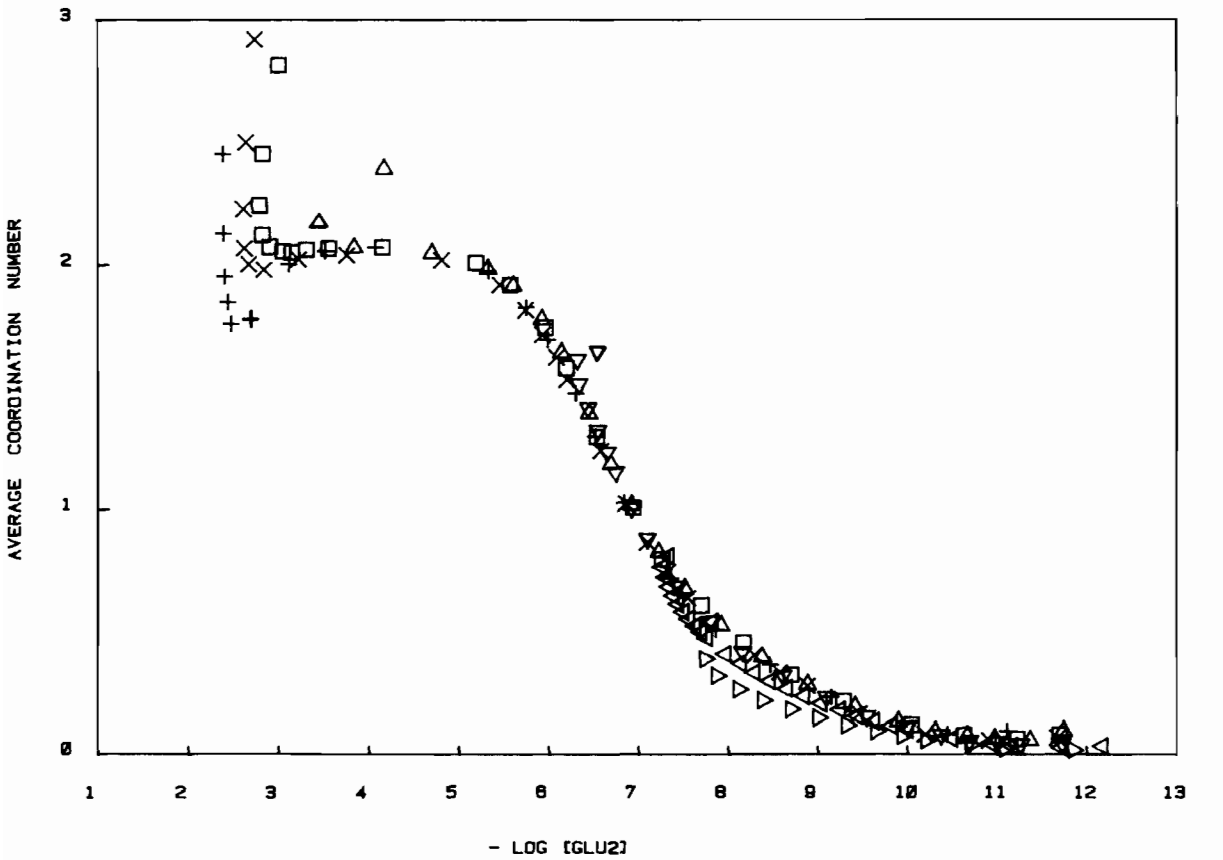

Fig. 1. Experimental formation curve for the copper–glutamic acid system. The following symbols +, ×, □, △, ▽, ◁, ▷ correspond to the respective order of the metal to ligand ratios shown in Table I.

derived from the graphical comparison of the experimental formation curves with their simulations, as obtained by means of our modified version [25] of the PSEUDOPLOT program [33] on the basis of the different sets of constants remaining in balance. Details on this procedure being available elsewhere [23, 25, 28, 31, 32], we will not develop it further in this paper.

Some stability constants relative to parent protonation and binary complexation equilibria were used in the present calculations as found in previous studies under the same experimental conditions. They are to be found in Table II.

Results

The formation constants for the systems investigated are to be found in Table III.

Discussion

Protonation equilibria

Account being taken of the very low total concentrations of trace metals with respect to those of the ligands present in biofluids such as blood plasma [23, 31] or artificial nutritive mixtures [19, 23], the metal-complexed fraction of these ligands often represents a nearly negligible part of their total amount. For this reason, the free concentration of each ligand depends to a crucial extent on its protonation degree, which is itself expressed as a function of the corresponding protonation constants. These constants are thus very important for simulation studies of metal complex distributions in biofluids.

As was the case in our various earlier works [23, 25–29, 31, 32, 35], the values determined in the present study were found to be a little lower than those previously obtained by Perrin *et al.* [18, 39]. These systematic discrepancies, which stem from the calibration of our electrode system in the concentration scale, have already been interpreted elsewhere [27, 40].

Binary systems

The copper–leucine system had already been investigated by two groups of authors: Hallman *et al.* [39] had characterized the MX, MX_2, MXH and MX_2H species, but later Brookes and Pettit [41] only mentioned the existence of MX and MX_2. We started our research by considering the latter two complexes only, then we added in turn MXH, MX_2H and MX_2H_2. The constants of all the protonated species

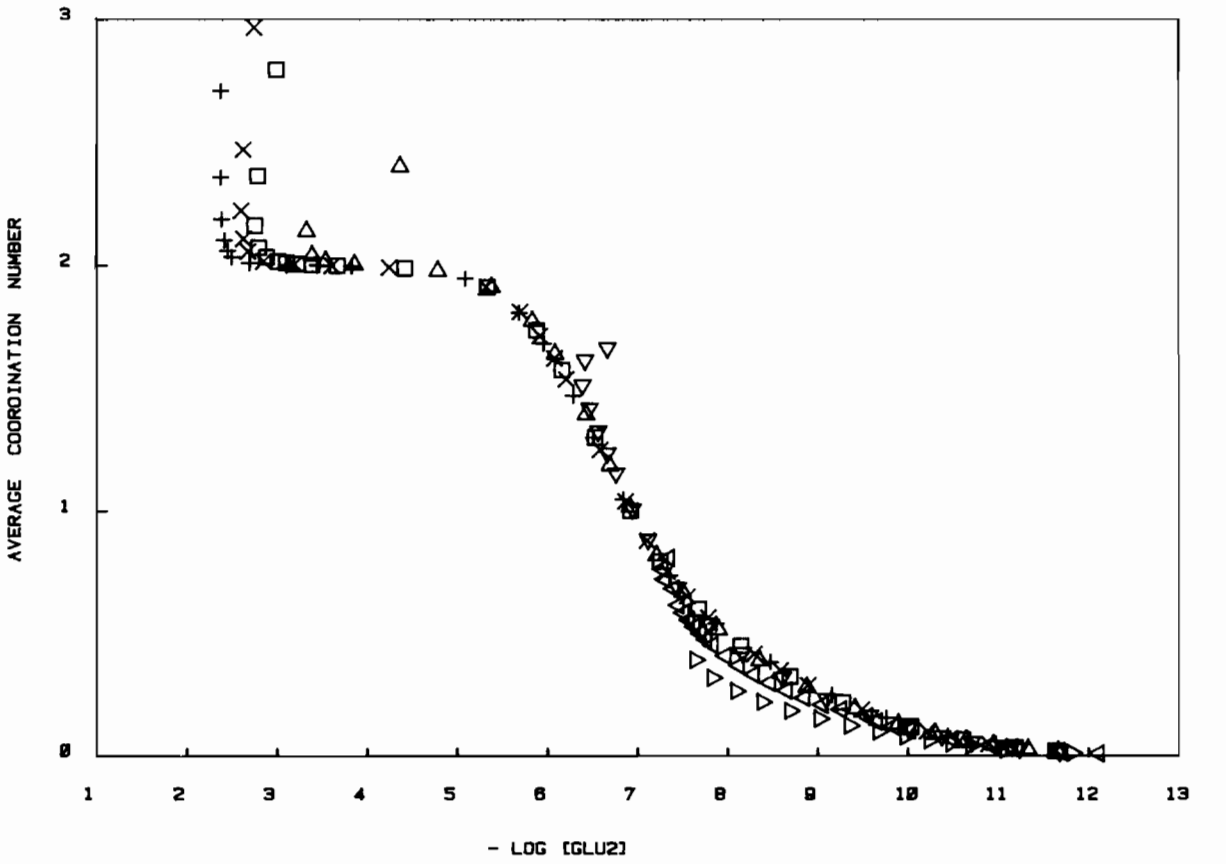

Fig. 2. Simulated formation curve as based on the results presented in Table III. The symbols are the same as in Fig. 1.

turned out to be negative during MINIQUAD refinements and the simulated formation curve based on MX and MX_2 did coincide satisfactorily with the experimental one up to $\bar{q} = 2$. Nevertheless, the 'tail' shape of the curve above this value revealed the existence of a hydroxo complex for higher free leucine concentrations at high pH. Accordingly, MX(OH), $MX_2(OH)$ and $MX_2(OH)_2$ were successfully tried. $MX_2(OH)$ was the only species to allow numerical as well as graphical satisfactory fits.

The copper–glutamic acid system had been investigated by several groups of authors who had characterized MX and MX_2 [39, 41], but also MXH [39, 42] and even MX_2H and MX_2H_2 [41]. Our results concerning the protonated complexes confirmed those obtained by Brookes and Pettit [41], but the upper part of the formation curve was characteristic of the existence of hydroxo species (Fig. 1). Among MX(OH), $MX_2(OH)$ and $MX_2(OH)_2$ successively taken into consideration, MX(OH) was preferred for graphical reasons, as clearly appears from the comparison of Figs. 1 and 2 given as an example of the PSEUDOPLOT approach described above.

The poor solubility of the copper–methionine complexes limited the pH range investigated for this system (see Table I). Nevertheless, values of $\bar{q}$ could be obtained up to about 1.8. The MX and MX_2 species were unequivocally characterized, but MXH, MX_2H and MX_2H_2 proved not to exist, which confirmed the results available in the literature [39, 41].

As far as the copper–tryptophan system was concerned, we confirmed the existence of the two MX and MX_2 simple complexes formerly mentioned by four groups of authors [39, 41, 43, 44], but we also characterized MXH as a minor species which significantly improved both numerical and graphical fits [29]. This finding is not surprising since complexes of this type may appear with copper and glycine-like amino acids [38, 55]. It is still noteworthy that even for free concentrations of tryptophan superior to 1.0×10^{-3} mol dm^{-3}, the $\bar{q}$ values remained constant around 2, the formation curve of this system showing no tendency to form hydroxo complexes [29].

Contrary to the above system, the formation for the copper–alanine equilibria displayed a 'tail' shape for free ligand concentrations above 1.0×10^{-3} mol dm^{-3} [29], this being characteristic of the formation of hydroxo species. MX(OH), $MX_2(OH)$ and $MX_2(OH)_2$ were thus researched in succession. The equilibrium constants for MX(OH) and $MX_2(OH)_2$ were made negative by MINIQUAD, but that of $MX_2(OH)$ was refined and allowed satisfactory PSEUDOPLOT graphical fits with the experimental curve above $\bar{q} = 2$. This does not confirm the results mentioned by Linder and Torrington [48], who had characterized MX(OH) together with $MX_2(OH)$. Regarding the possible protonated complexes, MXH [38, 48, 49] and even MX_2H and MX_2H_2 [50] had already been mentioned in addition to the 'classical' MX and MX_2 species [38, 45–50]. MXH was effectively characterized, but the stability constants for MX_2H and MX_2H_2 turned out to be negative during MINIQUAD refinements.

Ternary Systems

Three of the ternary systems investigated, *i.e* copper–histidine–leucine, copper–histidine–methionine and copper–histidine–tryptophan had already been studied by Brookes and Pettit [41], complexes of the MLX type had been characterized for all of them, MLXH being mentioned as a minor species in the copper–histidine–leucine system only. The MLX complex had also been proved to exist in the copper–histidine–alanine system [57].

Our results in Table III do not confirm all these findings in that we did not definitely establish the existence of a MLXH species in the copper–histidine–leucine system. Actually, the stability constant of the MLXH complex was not made negative by MINIQUAD, but its addition to MLX (i) did not improve the numerical fits to a significant extent, (ii) had no effect on the graphical fits, and (iii) slightly worsened the standard error on the MLX constant ($\log \beta_{MLX} = 17.209 \pm 0.012$; $\log \beta_{MLXH} = 20.951 \pm 0.084$ for $S = 0.303$ E – O5 and $R = 0.0046$). Moreover, the maximum percentage of MLXH reached only 7% within a short interval of pH for favorable concentration ratios. This species was thus finally discarded. This result is not really at variance with the conclusions given by Brookes and Pettit [41], who stated that species of this stoichiometry are not present in all such cases, and when present are always of minor importance.

For the copper–histidine–glutamic acid system, the stability constant of MLXH could also be refined together with that of MLX ($\log \beta_{MLX} = 16.848 \pm 0.019$, $\log \beta_{MLXH} = 21.003 \pm 0.124$ for $S = 0.278$ E – O5 and $R = 0.0048$) but the percentage of this species was always inferior to 5% and it was thus considered as negligible.

Similarly, MLXH was found to reach only 10% within a short pH range for the fourth experiment (Table I) of the copper–histidine–methionine system. Moreover, taking this species into account in the calculations worsened the numerical fits ($\log \beta_{MLX} = 16.756 \pm 0.018$; $\log \beta_{MLXH} = 20.648 \pm 0.088$ for $S = 0.494$ E – O5 and $R = 0.0069$) and the standard deviation of the MLX constant; it was thus also discarded.

Actually, the only system for which a MLXH species could be definitely characterized was the copper–histidine–tryptophan one (Table III), considering the existence of this complex improved

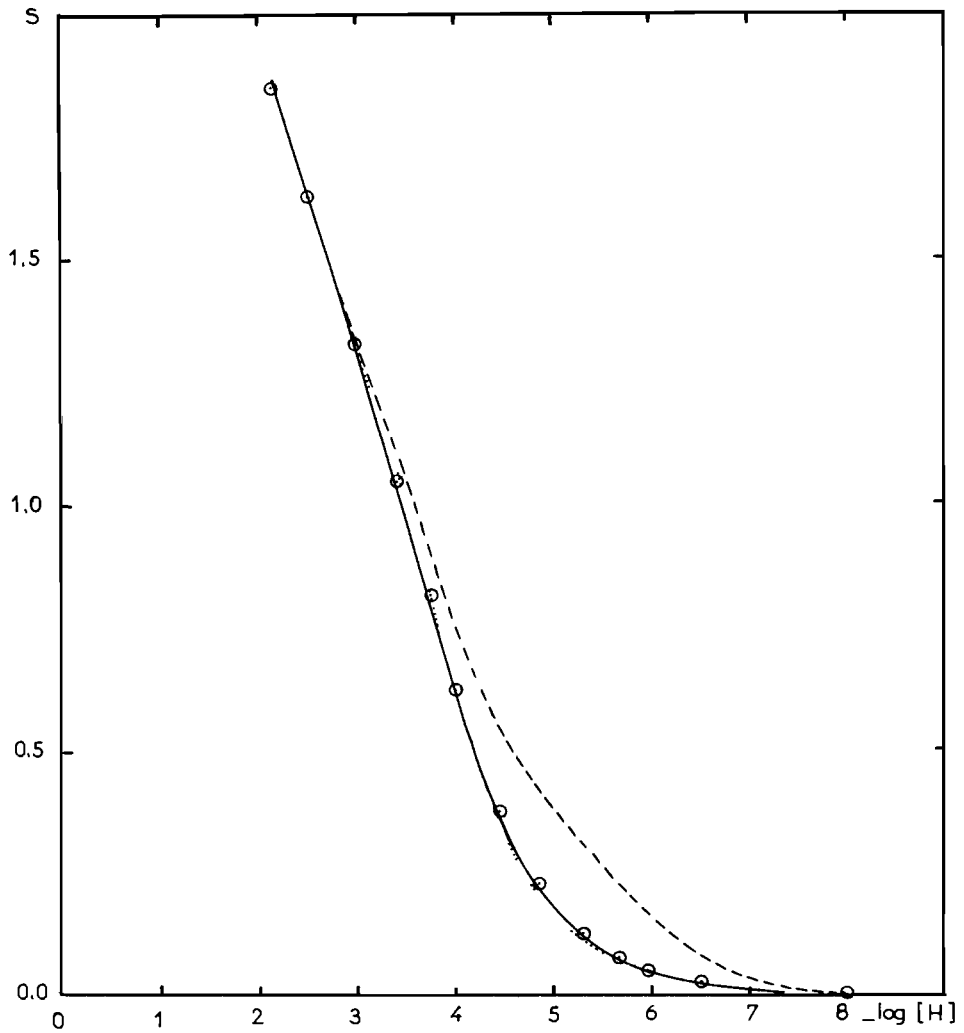

Fig. 3 Protonation curve of a mixture of histidine and tryptophan in the presence of copper: C_{Cu} = 4.96, C_{His} = 5.00, C_{Trp} = 4.20 mmol dm^{-3}. For the sake of clarity, about every fifth experimental point has been materialized. The broken line simulates the curve assuming no mixed-ligand species formation. The dotted line takes into account MLX only The solid line represents the final results as shown in Table III.

both numerical (log β_{MLX} = 17.643 ± 0.016 for S = 0.546 E – O5 and R = 0.0061) and graphical fits (see Fig. 3) to a significant extent. Moreover, its maximum percentages in the experiments summarized in Table I were 11%, 15%, 13%, 19%, 14% and 6% respectively, which cannot be considered as reflecting the influence of a negligible species.

The situation was quite different in the copper–histidine–alanine system, since the MLXH constant was made negative during MINIQUAD refinement.

From a fundamental point of view, it may be of interest to analyse the ability of each of these ternary systems to give rise to mixed-ligand coordination. As a number of similar systems involving both copper and histidine were recently investigated under the same experimental conditions [23], a comparison with the relevant results was desirable.

The theoretical background of the two classical relationships commonly used to estimate the propensity of a mixed-ligand complex to be formed from its 1:1 metal to ligand ratio parent ones

$$\Delta\log K = \log K_{MLX}{}^{ML} - \log K_{MX}{}^{M} = \log K_{MLX}{}^{MX} - \log K_{ML}{}^{M} \quad (1)$$

or from its 1:2 metal to ligand ratio binary analogues

$$\Delta\log \beta = \log \beta_{MLX} - \tfrac{1}{2}(\log \beta_{ML_2} + \log \beta_{MX_2}) - \log 2 \quad (2)$$

has been thoroughly discussed in the past [41, 51, 52] and is thus not worth detailing here. Recently, more sophisticated developments of relation (2) were published [53, 54] which take into account

TABLE IV Increments of Stability for the Formation of Mixed–Ligand Complexes of Copper and Histidine with Various Amino-Acids

System	Δ log K	Δ log β	Reference
Copper–histidine–threonine	−0.65	0.83	23
	−0.59	0.82	41
Copper–histidine–glycine	−0.95	0.52	23
Copper–histidine–valine	−0.90	0 58	23
	−0.55	0.81	41
Copper–histidine-phenylalanine	−0.84	0.47	23
	−0.54	0.75	41
Copper–histidine–leucine	−0.61	0.87	This work
	−0.69	0.77	41
Copper–histidine–glutamic acid	−1 18	0.53	This work
	−0.79	0.76	41
Copper–histidine–methionine	−0 65	0 83	This work
	−0 69	0.67	41
Copper–histidine–tryptophan	−0.19	1.01	This work
	−0 12	0.89	41
Copper–histidine–alanine	−0 84	0.72	This work

the ligand concentrations relative to the real medium being considered, but they actually apply to practical considerations which are implicitly encompassed by the computer simulation models used in the next paragraph. If we examine the increments of stability calculated on the basis of eqns. (1) and (2) which may be seen in Table IV, a number of remarks are in order.

The bonds of the MX copper complexes formed by the amino-acids involved in the copper–histidine mixed-ligand species mentioned in Table IV are all formed in the glycine-like manner. This was formerly established for threonine within the physiological pH range [55] and was also suggested later for glutamic acid in contrast with aspartic acid [41]. Their contribution to the calculations derived from relation (1) is thus expected to be of the same order of magnitude. Accordingly, the Δlog K values are found within the limits established by statistical considerations [52], but for glutamic acid and tryptophan.

The copper–histidine–glutamic acid complex appears indeed to be relatively destabilized. This is in line with the results obtained by Brookes and Pettit under different experimental conditions [41], also shown in Table IV for the sake of comparison. These authors proved that stereoselectivity was insignificant in the formation of this complex: such selective ligand–ligand interactions are thus unlikely to play a part in the effect observed, which seems to reflect the high ratio of the stepwise formation constants for the binary copper–glutamic acid system. As a matter of fact, log $K_{MX}{}^{M}$ − log $K_{MX_2}{}^{MX}$ is equal to 1.726, which is the highest difference observed for all the amino acids in Table IV.

The strong stabilization of the MLX copper–histidine–tryptophan species also corroborates the observations made by the above-mentioned authors [41]: the tendency of tryptophan to bind to the copper–histidine complex is almost equivalent to its affinity for the aquated copper ion. Contrary to glutamic acid, the high Δ log K reflects the lowest log $K_{MX}{}^{M}$ − log $K_{MX_2}{}^{MX}$ difference (0.709) for the amino acid series being considered. This result is confirmed by the corresponding Δ log β derived from relation (2): on this purely statistical basis, all the ternary complexes appear to be clearly favoured with respect to their parent binary analogues (Table IV), but tryptophan displays the most stabilizing effect. The same kind of observation [41] was formerly interpreted in terms of favorable intramolecular interactions involving aromatic ring stacking [56].

Computer Simulation Studies

The experimental determination of the stability constants of the above-mentioned copper complexes is likely to improve the reliability of the simulated distribution of this metal in any biofluid containing significant concentrations of the ligands involved. As the main amino acids composing the nutritive mixture under study are essentially the same as those prevailing in blood plasma, the influence of the present results on the distribution of copper in both of these biofluids will be successively examined.

All the corresponding simulations were carried out by means of the ECCLES program [58].

Distribution of copper into its l.m w. fraction in blood plasma

As has been quantitatively confirmed in our latest paper in this series [23], histidine is the major l.m.w. ligand of copper in blood plasma. The most signifi-

TABLE V. Predominant Low-Molecular-Weight (l.m.w.) Complexes Formed by Copper(II) in Blood Plasma, as Found by Computer Simulation at pH = 7.4, Using the Present Experimental Results together with Those Found in Ref. [23].

Complex species[a]	log β[b]	Percentage of total l.m.w. copper
Cu–$(his)_2$	17.50	19.3
Cu–his–thr	17.03*	18.5
Cu–his–ser	16.96	9.4
Cu–his–ala	17.00*	6.7
Cu–his–gly	16.94*	5.5
Cu–his–lys – H^+	27.05*	5.4
Cu–his–val	16.93*	4.8
Cu–his–gln	15.99	4.7
Cu–his–leu	17.18*	4.6
Cu–his–orn – H^+	26.74	2.3
Cu–his–pro	17.64	2.3
Cu–his–phe	16.69*	2.3
Cu–his–trp	17.66*	2.0

[a]The symbols are: his = histidine, thr = threonine, ser = serine, ala = alanine, gly = glycine, lys = lysine, val = valine, gln = glutamine, leu = leucine, orn = ornithine, pro = proline, phe = phenylalanine, trp = tryptophan. [b]The formation constants obtained from this study and ref. [23] are marked with an asterisk. The Cu–$(his)_2$ constant is taken from ref. [35].

cant finding of that study [23] was the demonstration of the negligibility of the copper–histidine–cystine and protonated copper–histidine–cystine complexes previously thought to be the predominant l.m.w. complexes of copper in this medium [39, 58–60]. The important implications of this finding as to the bioavailability of copper have already been discussed [23].

The incorporation of the present results into the blood plasma model does not induce major new changes in the copper simulated distribution, as may be seen from Table V. The only point still open to discussion concerns the comparison [57, 61] between this simulated distribution and the earlier physiological findings regarding copper [62, 63]. The solving of the problem will require further experimental determinations currently being carried on in our laboratories.

Distribution of copper in the nutritive mixture being considered and improved assessment of the corresponding daily dose of metal

The first aim of this study was to improve the reliability of the simulated distribution of copper in the nutritive solution taken as a reference [19]. This is indeed one of the two objectives to be met before the optimum daily dose of copper can be quantitatively assessed on the basis of the principles developed in the introduction.

TABLE VI. Percentage Distribution of the Predominant Copper(II) Complexes in the Nutritive Solution whose Composition is Given in Ref [19], as Found A) from the Preliminary simulation, B) from the Updated Simulation Using the Results obtained from this Study and from Ref. [23] at pH = 7.4.

Complex species[a]	log β[b]	Percentage of total metal[c]
A. Cu–$(his)_2$	17.50	14.0
Cu–his–thr	17.03	10 2
Cu–his–lys – H^+	27.24	8.8
Cu–his–gly	17.15	8.5
Cu–his–phe	16.85	7.7
Cu–his–val	17.13	7.0
Cu–his–cis^-	18.51	6 1
Cu–his–leu	17 13	5.5
Cu–his–cis – H	25.80	4.7
Cu–his–met	16.72	3.6
Cu–his–glu^-	17.45	3 4
Cu–his–trp	17.55	3.4
Cu–his–ala	17.04	3 3
B. Cu–$(his)_2$	17.50	17 2
Cu–his–thr	17.03*	12 4
Cu–his–leu	17.18*	9.5
Cu–his–lys–H^+	27.05*	7 0
Cu–his–phe	16.69*	6.5
Cu–his–gly	16.94*	6.5
Cu–his–trp	17.66*	6.1
Cu–his–met	16.73*	6.0
Cu–his–val	16.93*	5 5
Cu–his–ala	17 00*	4 7
Cu–his–ser	16.96	2.3
Cu–his–asp^-	17 15	2.3
Cu–his–ile	17.06	2.1

[a]The symbols are the same as in Table V, and cis = cystine, met = methionine, glu = glutamic acid, asp = aspartic acid, ile = isoleucine. [b]The stability constants obtained from the present studies and from ref. [23] are marked with an asterisk. The Cu–$(his)_2$ constant is taken from ref. [35]. [c]Above 2.0% for both parts A and B. All the species shown in Part A were investigated in ref. [23] or in the present work, but for Cu–$(his)_2$ which was investigated in ref. [35].

The inclusion of the present experimental results in the simulation model greatly improved its reliability percentage, since 84% of the copper to be added to the nutritive mixture is now represented by complexes whose formation constants have been checked under the proper experimental conditions, which is to be compared to the 56% resulting from our preceding work [23].

In order to allow a better appreciation of this improvement, Table VI shows the distribution of copper in its main complexes as obtained from the latest updated simulation, along with that corresponding to the preliminary simulation given for the sake of comparison.

TABLE VII. Free and Total Concentrations, and Predicted Dose for Calcium, Magnesium, Zinc and Copper in the Nutritive Mixture whose Composition is shown in Ref. [19], as Based on the Simulation in Table VIB. The volume of the daily infusate is taken as 2.58 dm^3.

Metal ion	Free concentration (mol dm^{-3})	Total concentration (mol dm^{-3})	Predicted dose (mg/day)
Ca^{2+}	1.13×10^{-3}	5.47×10^{-3}	566
Mg^{2+}	5.2×10^{-4}	$4\,63 \times 10^{-3}$	290
Zn^{2+}	1.0×10^{-9}	$1\,30 \times 10^{-4}$	21.9
Cu^{2+}	$1\,0 \times 10^{-16}$	$9\,96 \times 10^{-6}$	1.6

Based on the free plasma concentration of copper currently being considered, the calculation of the total concentration of this metal in accordance with the simulated distribution shown in Table VIA yields a daily dose of 1.63 mg for the 2.58 dm^3 of nutritive solution infused per day [19]. This result is reported in Table VII, together with the optimum doses of calcium, magnesium and zinc also derived from the updated simulation run for copper.

Regarding the data grouped in Table VII, a few remarks are in order:

Technical considerations on the assessment of the TPN metal doses

With respect to the evolution of the daily dose of copper throughout our studies, the ultimate improvement in reliability for the copper simulation model does not entail a significant change in the resulting amount. Indeed, the previous simulation had already yielded 1.61 mg [23], which is to be compared with the 2.05 mg found after the ascertaining of the zinc distribution [27] whereas 1.3 mg was obtained from the original distribution [19, 20].

The last two figures call for the following observation. As was pointed out in the discussion of the protonation equilibria, the protonation constants determined in all of our studies are systematically found to be lower than those previously obtained by Perrin's school, on which the original data files for the ECCLES program were essentially based [19, 20]. This means that as we are progressing towards a more precise knowledge of the metal complex equilibria, the free concentrations of the ligands involved are systematically increased, which results in the raising of the complex concentrations, and thus the parallel enhancement of the corresponding total metal concentrations since the calculations are based on constant free metal ones. The same kind of observation also holds for the other metals: after the final work devoted to zinc [27], the daily dose of this metal was calculated to be 21.05 mg but it has now been altered to 21.91 mg after the determinations intended for copper (see Table VII); as for calcium and magnesium, their doses have also respectively changed from 507 mg to 566 mg and from 257 to 290 mg during our zinc and copper analyses. This implies that *insofar as significant complexes of different metals involve common ligands, the determinations devoted to these metals should preferably be carried out simultaneously.*

Relevance of the daily dose of copper obtained from these studies

As regards the assessment of the daily dose of copper which was the prime objective of the present work, it is worth recalling that it compares favorably with the current estimate of the dietary requirement of this element [64]. Nevertheless, this dose cannot be considered as the ultimate result concerning copper in TPN, since account must be taken of the following limitations.

(i) The dose given in Table VII is specific to the composition of the nutritive mixture taken as a reference [19].

(ii) Although the reliability of the simulation model for this nutritive mixture is now quite satisfactory, the calculation of the dose of copper depends directly on the estimation of the free concentration of this metal in normal blood plasma, which still needs to be improved [23].

(iii) It remains unclear to what extent the binding of copper to amino acids is influenced by redox conditions, especially with regard to the cysteine/cystine couple. The Cu^+ species, even though probably not predominant as previously contended [65], is presumably not insignificant [66]. Nevertheless, in the present lack of reliable constants for the possible Cu^+ species, it seems realistic that our TPN calculations be based on the free concentration of Cu^{2+}, as derived from the total exchangeable concentration of copper and the conditional stability constant of the Cu^{2+} albumin complex [19, 20].

(iv) Concerning the matter of the free copper concentration in plasma, it is also worth recalling that it has deliberately been taken at a lower level than the value obtained by Agarwal and Perrin [67], on account of the risk of excess copper deposition in tissues, which could become harmful to the patients [10].

General observations on our computer-based approach to the assessment of TPN metal doses

The present approach has for the first time made it possible to quantitatively analyse the origin of the trace metal losses induced by TPN. It also enables one to establish the amounts of each metal which ideally need to be added to the TPN solution being investigated [19]. Nevertheless, its application is marked by two important practical limitations:

(i) its first lack stems from the fact that this approach is basically aimed at compensating for the metal excretions induced by the infusion of the TPN mixture, and does not allow for the specific losses due to the gastrointestinal disease itself (diarrhoea, fistulae, ...),

(ii) for calcium and magnesium whose free plasma concentrations can be directly deduced from ion selective electrode measurements [58], the limitations bound to the imprecision of the free copper concentration just pointed out above also hold true for zinc and other trace metals. More precise estimations of these free metal concentrations in normal blood plasma are thus needed before our approach can allow the calculation of reliable absolute metal doses.

However, this approach can be used to advantage for relative analyses of the metal requirements of nutritive solutions of different compositions. Indeed, its first important contribution was to allow the demonstration that metal doses must depend on the nutritive mixture composition. This point is of special interest when considering the following potential applications.

Depending on the status of the patients who often suffer from malnutrition at the outset of the treatment, TPN solutions of different amino acid compositions must be administered [68]. Once the metal requirements for a given nutritive solution can be assessed from clinical balance determinations (preferably based on our estimations) one could get a closer appreciation of the corresponding free metal concentrations pertinent to this solution, hence also to normal blood plasma. Our basic approach could then be generalized to apply to any nutritive mixture so long as a sufficient percentage of reliability can be reached for each relevant simulation model.

Such a generalization could be particularly interesting for the design of nutritive mixtures with a view to therapeutic applications. It is well documented that zinc is the necessary cofactor of a lot of enzymes involved in the biosynthesis of proteins and nucleic acids [69, 70], and so may be considered as a potential agent for tumor growth [71], whereas copper and some of its complexes exert an antineoplastic activity [72–74]. As has been pointed out by Askari *et al.* [68], it is highly desirable that a better knowledge of the 'normal' metal requirements can be obtained. It could indeed enable clinicians to administer TPN solutions specifically adapted to the evolution of the disease for patients suffering from cancers.

The same kind of application can also be contemplated in pediatrics. TPN-administered children are still more sensitive than adults to the composition of the nutritive solution and their growth rate is closely related to their zinc intake [70]. It is a common observation that TPN children in an apparently healthy state do not grow, presumably due to insufficient zinc supplementation [75]. Significant progress is thus to be expected from a better understanding of the relationship between the zinc dose to be administered and the composition of the infusate.

To conclude, the direct dependence of the metal needs on the composition of the nutritive mixture does substantiate the principle recently developed by Jeejeebhoy [12] according to which commercial solutions for TPN should preferably be prepared for each metal separately.

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