Copper–Histidine Ternary Complex Equilibria with Glutamine, Asparagine and Serine. The Implications for Computer-simulated Distributions of Copper(II) in Blood Plasma

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Abstract

The use of computer simulation techniques for assessing the percentage distribution of metal ions in biological fluids has recently been questioned on the grounds of observed discrepancies between such simulations and the results obtained experimentally by Neumann and Sass-Kortsak in their pioneering work on low-molecular-weight copper in reconstituted human serum. Recent work by our group has resolved the most serious of these discrepancies, but some worrying uncertainties remain regarding the ternary equilibria of copper(II) and histidine with glutamine, serine and asparagine.

This paper describes an experimental investigation of these equilibria and the effect that the newly-determined data have on the computer-simulated distribution of copper(II) in blood plasma. Simulations have also been performed using the actual low-molecular-weight compositions of the biofluids studied by Neumann and Sass-Kortsak. The results show good agreement between calculated and experimentally observed distributions and confirm the usefulness of the simulation techniques.

Introduction

It has been many years since the discovery that a small, but significant, fraction of the trace metal ions that normally occur in blood plasma consists of low-molecular-weight (l.m.w.) complexes, mainly involving amino acids [1, 2]. As a result of this discovery, it has become generally accepted that these l.m.w. complexes, which tend to occur as mixed-ligand ternary species [3], play an important role in the biological transport of the trace metal ions [1–4]. Indeed, given their small molecular size, such complexes seem to be the only forms capable of diffusing through biological membranes into tissues or to be filtered through the kidneys to be excreted in the urine.

Some years later, following the advent of high speed computers, programs were produced which made it possible to calculate the distribution of these complex species under simulated biological conditions [5, 6]. Further work, devoted particularly to the problem of metal–protein binding [7], led to the development of the ECCLES program [8]. This can simulate the distribution of metal ions amongst as many as 10 000 complex species and it has been widely used to investigate the l.m.w. equilibria established by both essential and toxic trace metals in blood plasma.

In a number of cases, applications of the ECCLES program have successfully rationalised various clinical observations, sometimes even on a quantitative basis [9–12]. In spite of this, the very principle of using such simulation models has been questioned [13, 14]. These criticisms have been based on the discrepancy between the simulated distribution of the l.m.w. copper(II) fraction and the experimental results of Neumann and Sass-Kortsak who performed the original *in vitro* experiments on reconstituted human serum referred to above [1].

It is a well-recognised principle [15] that the reliability of any simulation model depends on the corresponding certainty associated with the parameters it is given. For this reason, a number of equilibrium systems (most notably of zinc(II) [15–18])

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that were found to be significant in the initial plasma models have been experimentally re-investigated. The object has been to steadily improve the database of formation constants, many of which had had to be estimated for the early calculations. In some cases, even species which had been included on the grounds of some experimental evidence by Perrin and co-workers [5], were later found to be insignificant [16], a finding that has now been confirmed by further work from the original school [19].

As far as copper(II) is concerned, a series of investigations have recently been devoted to its distribution in solutions used routinely for the purpose of total parenteral nutrition [20, 21]. One outcome of these studies was the experimental determination of various formation constants that resulted in some significant changes to the simulated distribution of copper(II) in blood plasma [21]. In particular, ternary complexes with histidine and cystine, previously found to represent the main copper(II) species [22], were removed from the list of most predominant complexes [20]. These results, also independently confirmed [23], did much to lessen the differences between the simulated distribution of copper(II) and the data collected by Neumann and Sass-Kortsak. Nevertheless, it remains our declared intention to check experimentally the formation constants of all species with potential importance to the blood plasma model.

Accordingly, the present paper reports formation constant values determined for the copper–histidine ternary systems with serine, glutamine and asparagine. The effect of this new data on the simulated distribution of copper(II) in blood plasma has also been examined. Finally, some simulations have been performed for solutions having the particular concentrations applicable to the studies of Neumann and Sass-Kortsak. The overall objective of the work has been to clarify the present standing of the blood plasma models by comparing calculated results with some corresponding experimental observations.

Formation Constant Determinations

Materials

Glutamine, serine and asparagine hydrate were Merck biochemical grade products. Perchloric acid and sodium perchlorate were also purchased from Merck, as pro analysi reagents. The stock solutions of copper perchlorate, prepared by dissolving crystals supplied by G. Frederick and Smith Co. in dilute perchloric acid, were standardised for metal and proton concentrations as previously described [24].

Carbonate-free hydroxide solutions, prepared as before [24], were maintained constantly under nitrogen.

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

System	C_M	C_L	C_X	C_H	pH range
Proton–asparagine			5.00	9.80	2.14–10.50
			10.00	14.70	2.03–10.44
			20.00	24.50	1.92–10.27
			5.00	9.85	2.15–10.50
Copper–asparagine	1.02		10.00	9.89	2.24–9.73
	1.99		10.00	9.97	2.22–9.62
	3.10		10.00	10.06	2.22–9.29
	4.96		10.00	10.23	2.19–7.61
	9.93		20.00	25.37	1.85–7.10
	9.93		10.00	10.67	2.13–5.48
	9.93		5.00	10.67	2.06–4.69
Copper–histidine–asparagine	4.96	5.00	5.00	20.13	1.97–7.12
	4.96	5.00	10.00	25.06	1.91–9.63
	4.96	10.00	5.00	25.06	2.02–9.72
	4.96	10.00	10.00	34.91	1.84–9.95
	9.93	10.00	10.00	35.34	1.81–7.58
	9.93	5.00	5.00	25.49	1.80–5.72
Copper–histidine–glutamine	4.96	5.00	5.00	18.69	2.06–7.98
	4.96	5.00	10.00	23.26	2.02–9.85
	4.96	10.00	5.00	27.82	1.95–9.75
	4.96	10.00	10.00	34.73	1.83–9.91
	9.93	10.00	10.00	32.82	1.86–8.01
	9.93	5.00	5.00	20.47	1.94–5.64
Copper–histidine–serine	4.96	5.00	5.00	18.61	2.00–6.46
	4.96	5.00	10.00	23.16	1.96–9.45
	4.96	10.00	5.00	27.81	1.91–9.49
	4.96	10.00	10.00	32.38	1.88–9.81
	9.93	10.00	10.00	32.81	1.81–7.25
	9.93	5.00	5.00	19.12	1.97–5.58

[a]See text.

Technique

Potentiometric titrations were performed using Beckman glass and calomel electrodes fitted to an Ingold cell system. Measurements were made with a Beckman model 4500 potentiometer.

Titrated solutions were initially made sufficiently acidic to ensure that all ligand donor groups were fully protonated at the outset of each experiment. Titrations were stopped as soon as any precipitate appeared in the solutions, as evidenced by drifting potential readings. The temperature was maintained at 37 ± 0.02 °C inside the reaction cell. A nitrogen atmosphere was maintained in the vessel throughout all titrations by bubbling of the gas into the solution. Ligand–proton and metal–ligand ratios were varied significantly over the set of experiments performed for each system, as shown in the summary (Table I).

TABLE II. Formation Constants Determined in These Studies. The Formula of the General Complex is $M_rL_pX_qH_s$. S = Sum of Squared Residuals; R = R Factor as Defined in Ref. 25; n = Number of Experimental Observations; L = Histidine; X = Second Amino Acid

System	p	q	r	s	log β	S	R	n
Proton–asparagine	0	1	0	1	8.458 ± 0.001	1.96E – 07	0.0023	138
	0	1	0	2	10.609 ± 0.002			
Copper–asparagine	0	1	1	0	7.714 ± 0.005	6.65E – 07	0.0029	253
	0	2	1	0	14.210 ± 0.008			
	0	2	1	2	20.186 ± 0.023			
	0	2	1	1	17.417 ± 0.042			
	0	1	1	–1	0.675 ± 0.105			
	0	2	1	–1	3.941 ± 0.045			
Copper–histidine–asparagine	1	1	1	0	16.756 ± 0.011	2.54E – 07	0.0018	161
	1	1	1	1	20.057 ± 0.164			
	1	1	1	–1	5.702 ± 0.079			
Copper–histidine–glutamine	1	1	1	0	16.703 ± 0.006	4.28E – 07	0.0022	266
	1	1	1	1	20.108 ± 0.079			
	1	1	1	–1	5.844 ± 0.047			
Copper–histidine–serine	1	1	1	0	17.126 ± 0.007	6.78E – 07	0.0028	278
	1	1	1	1	21.003 ± 0.027			
	1	1	1	–1	6.786 ± 0.034			

The electrode system was calibrated in terms of hydrogen ion concentrations. Throughout this paper the symbol pH is used to represent $-\log[H^+]$.

Calculation Procedures

Initial estimates of formation constants and the stoichiometries of possible complexes were obtained, in the case of the binary systems, from the features of the protonation and formation curves and, for the ternary systems, from statistical considerations. Following our usual approach [15–18, 20, 21, 24], the MINIQUAD program [25] was employed to refine the formation constants and, on the basis of the usual numerical criteria, to select the initial sets of complexes. The final choice between combinations with similar MINIQUAD analyses was based on graphical comparisons between calculated curves and the corresponding simulated data obtained from the PSEUDOPLOT [26] and ESTA [27] computer programs.

A number of formation constants previously determined under the same experimental conditions as the present study were included in the calculations. In particular, these were for the systems proton–histidine [16], proton–glutamine, copper–glutamine and copper–histidine [28], proton–serine and copper–serine [29], the ionic product of water [15] and copper–hydroxide [21].

Results and Discussion

The formation constants obtained from the present work are shown in Table II.

(a) Proton–Asparagine

Surprisingly, the only protonation constants for asparagine under blood plasma conditions of temperature and ionic strength (37 °C, 150 mmol dm^{-3}) hitherto reported in the literature are values for KNO_3 cited by Perrin and Agarwal as unpublished results of McBryde [30]. Since asparagine may well play a leading role amongst the ligands complexing copper(II) in blood plasma [1], it is clearly important to ascertain reliable values for these constants. This is particularly so because in plasma the fractions of amino acids complexed to metal ions tend to be very small and, hence, the free amino acid concentrations are determined almost solely by the degree of protonation.

The asparagine protonation constants determined in this study (Table II) are significantly lower than those referred to by Perrin and Agarwal (*i.e.*, their values were 8.68 and 10.91, respectively). Although it is common [21] to find that protonation constants measured purely in terms of concentration are a little lower than those in which ligand concentrations and proton activities have been mixed (usually by 0.10–0.15 log units), the observed difference seems too large for this to be the cause in the present case. The implications of these differences for the computed distribution of copper(II) are significant: the likely consequence is a more important role for asparagine than has previously been assumed.

(b) Copper–Asparagine

Amongst the few quantitative studies of copper–asparagine interactions in solution [30, 32, 33], that

of Gergely *et al.* [32] has been the only one so far to detect the existence of hydroxo-species in addition to the stepwise MX and MX_2 complexes. The present data confirm the existence of the MX(OH) species. However, the $MX_2(OH)$ complex is preferred in our MINIQUAD refinement to the $MX_2(OH)_2$ complex reported by Gergely *et al.* In addition, this work reveals clear evidence for two acidic species, namely MX_2H_2 and MX_2H. Even a constant for MXH was refined by MINIQUAD (9.34 ± 0.26) but, if such a species exists, it occurred in too low a concentration to be considered significant. It is relatively common, with glycine-like amino acids, for complexes of the zwitterion (*i.e.*, the singly-protonated ligand) to be formed with copper [34] and other metal ions [35]. As these complexes are generally less stable than the corresponding acetates (due to the electron-withdrawing effect of the NH_3^+ group) and since they are only formed at low pH, they should not significantly affect the blood plasma simulations. They may, however, be relevant to other biological fluids, such as intestinal juice.

(c) Ternary Systems

The literature contains a few reports concerning the mixed-ligand complexes of copper and histidine with asparagine [13, 33], glutamine and serine [13, 36] but none of these deal with blood plasma conditions of temperature and ionic strength. Species of the type MLX and MLXH were characterised in all three systems by these previous investigations. Only Kruck and Sarkar [36] found evidence for the existence of MLX(OH) and this was only for copper–histidine–serine not copper–histidine–glutamine. The single study dealing with these three ternary studies under identical experimental conditions [13] found very similar values for each of the MLX formation constants. On this evidence, and from some solid-state investigations, it was concluded that the structure of the MLX complex in all three cases must involve copper(II) co-ordination by a terdentate histidine and a bidentate amino-acid partner in a '*cis*' configuration with an intramolecular hydrogen bond [13]. However, in a more recent solid-state structural study, Sasada *et al.* have contended that the histidine only behaves as a terdentate ligand in the asparagine ternary complex [37] and is bidentate in the copper–histidine–serine species [38]. Furthermore, they consider that their results substantiate the classification made by Neumann and Sass-Kortsak [1], according to which serine belongs to a group of ligands exhibiting moderate co-operativity with copper and histidine whereas asparagine, like threonine, shows pronounced co-operativity. Since the structure of the parent binary copper–asparagine complex is still a matter of debate [32, 33, 39], it is difficult to see how any firm conclusions about the structure of the ternary copper–histidine–asparagine species can be drawn.

As far as the present results, shown in Table II, are concerned, three types of complex appear common to each of the systems: MLX, MLXH and MLX(OH). Of these the MLXH species should be regarded as relatively minor, which is in accord with the observations of Brookes and Pettit [40] working on similar systems. The stabilities of the MLX complexes with asparagine and with glutamine are closely matched whereas the corresponding serine species is distinctly more stable. This conflicts with the uniform set of formation constants reported by other workers [13, 36]. Indeed, we find the copper–histidine–serine complex has a stability of the same order of magnitude as that of copper–histidine–threonine ($\log \beta = 17.03$) under the same experimental conditions [20]. This seems entirely reasonable given the structural similarity of serine and threonine and is in accord with independent results obtained at 25 °C [41]. Serine also resembles threonine more closely than asparagine in respect of the relationships which are sometimes used to quantify the increased stability of mixed-ligand complexes over the corresponding binary species [42, 43], as shown in Table III.

$$\Delta \log \beta = \log \beta_{MLX} - 0.5(\log \beta_{ML_2} + \log \beta_{MX_2}) - \log 2 \quad (1)$$

$$\Delta \log K = \log K_{MLX}^{ML} - \log K_{MX}^{M} = \log K_{MLX}^{MX} - \log K_{ML}^{M} \quad (2)$$

These provide a better method of assessing intrinsic co-operativity of ligands in mixed complexes than structural considerations [13, 38] or *in vitro* measurements at particular biological concentrations [1].

Simulation Studies

The simulation calculations were performed in two parts. First, the values determined in this work were introduced into our database of formation constants and the effect of this on the simulated distribution of copper(II) in normal blood plasma was determined. Secondly, a model was developed to simulate some of the *in vitro* data obtained by Neumann and Sass-Kortsak [1] for reconstituted serum. The ECCLES program [8] was used throughout.

Normal Blood Plasma

Table IV shows the latest simulated distribution of copper(II) in normal human blood plasma. Previous percentages [21] are shown for comparison.

TABLE III. Increments of Stability for the Formation of Mixed-ligand Complexes of Copper and Histidine with Amino Acids. References 20 and 21 refer to the same Experimental Conditions as this Work

	Δ log K	Δ log β	Reference
Copper–histidine–glycine	−0.95	0.52	20
	−0.86	0.55	13
Copper–histidine–valine	−0.90	0.58	20
	−0.55	0.81	40
	−0.81	0.56	13
Copper–histidine–phenylalanine	−0.84	0.47	20
	−0.54	0.75	40
Copper–histidine–threonine	−0.65	0.83	20
	−0.59	0.82	40
	−0.98	0.44	13
	−0.07	0.78	41
Copper–histidine–leucine	−0.61	0.87	21
	−0.69	0.77	40
Copper–histidine–alanine	−0.84	0.72	21
	−1.00	0.44	13
Copper–histidine–methionine	−0.65	0.83	21
	−0.69	0.67	40
Copper–histidine–tryptophan	−0.19	1.01	21
	−0.12	0.89	40
Copper–histidine–glutamic acid	−1.18	0.53	21
	−0.79	0.76	40
Copper–histidine–glutamine	−0.66	0.86	this work
	−0.80	0.61	13
Copper–histidine–asparagine	−0.85	0.60	this work
	−0.94	0.48	13
	−0.17	0.76	41
Copper–histidine–serine	−0.80	0.89	this work
	−0.88	0.54	13
	−0.77	0.65	40

TABLE IV. Distribution of Copper(II) in the l.m.w. Fraction of Normal Blood Plasma, as found by Computer Simulation at pH = 7.4, using (a) Stability Constants Available Before the Present Study and (b) Stability Constants Available After the Present Study. Species whose Percentage is less than 2.0 are Neglected

Complex species[a]	log β[b]	Percentage of total l.m.w. copper
(a) 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
(b) Cu–his–gln	16.70*	18.7
Cu–(his)$_2$	17.50*	15.1
Cu–his–thr	17.03*	14.4
Cu–his–ser	17.13*	10.8
Cu–his–ala	17.00*	5.2
Cu–his–gly	16.94*	4.3
Cu–his–lys–H$^+$	27.05*	4.2
Cu–his–val	16.93*	3.8
Cu–his–leu	17.18*	3.6
Cu–his–asn	16.76*	3.6

[a]The symbols are: his = histidine; thr = threonine; ser = serine; ala = alanine; gly = glycine; lys = lysine; val = valine; glu = glutamine; leu = leucine; orn = ornithine; pro = proline; phe = phenylalanine; trp = tryptophan; asn = asparagine. [b]Formation constants marked with an asterisk were determined by the same group of authors under the same experimental conditions (37 °C, $NaClO_4$ 150 mmol dm^{-3}).

The most striking result is the emergence of the copper–histidine–glutamine complex as the most predominant species. The percentage of copper bound to both the serine and asparagine complexes is also increased, albeit to a much lesser extent than with glutamine.

In some respects, the results are foreshadowed by the experimental data of Neuman and Sass-Kortsak. They found glutamine along with threonine and asparagine to be the major co-ordinating partners of copper and histidine. However, as discussed below, it should be noted that their experimental conditions did not closely correspond to those of normal blood plasma. This reasonably accounts for the fact that asparagine does not appear to be as important in the simulations as, at first sight, their observations may suggest.

It is pertinent to point out that some 89.3% of the l.m.w. copper(II) fraction is now attributed to complexes whose formation constants have been determined by the same group of workers under the same experimental conditions [20, 21, 28 and the present paper]. This permits some confidence that most, if not all, of the adverse effects caused by the inclusion of anomalous values have now been eliminated.

The continued dominance of the copper(II) distribution by histidine-containing complexes which are, almost without exception, of neutral charge is a most striking feature of the calculations. It provides an interesting rationale for the clinical observation that copper is excreted mainly through the bile rather than the urine [14].

Reconstituted Serum

The critics of computer simulations as a tool for investigating metal ion distributions in blood plasma

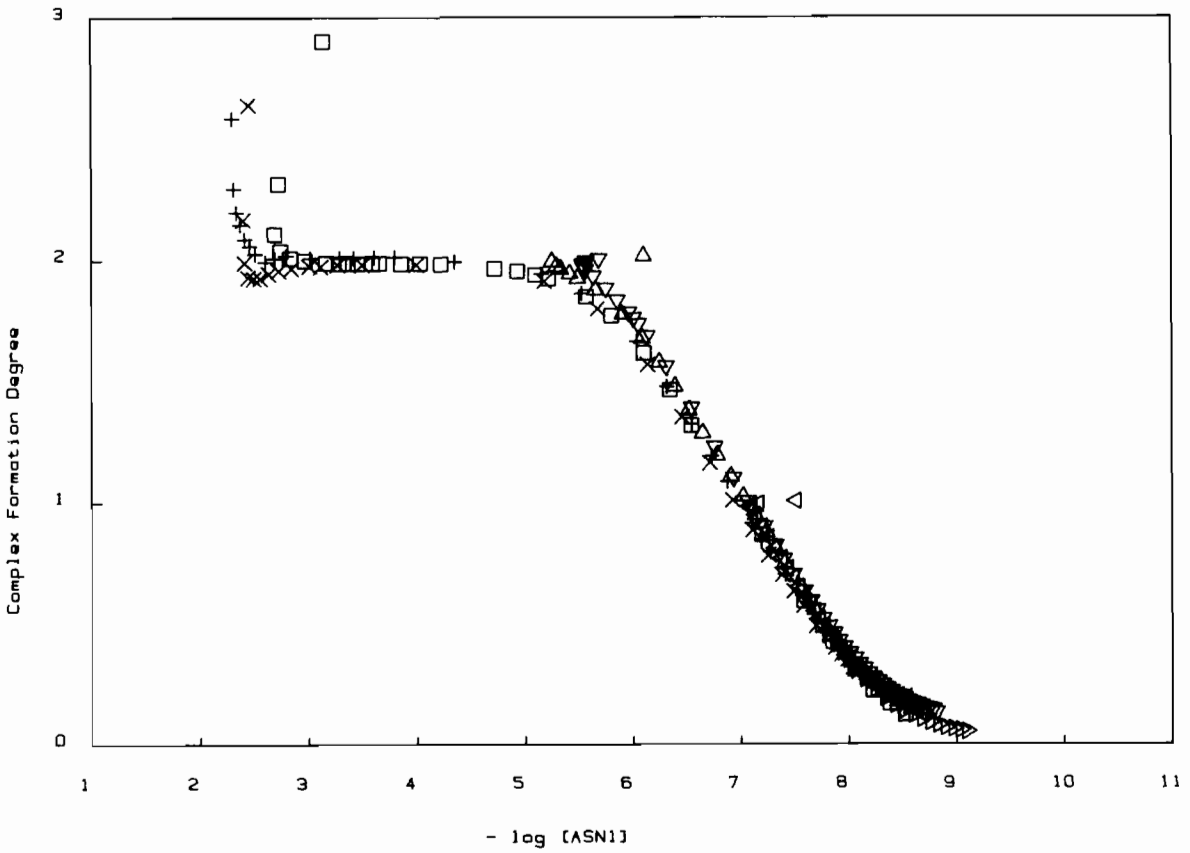

Fig. 1. Experimental formation curve for the copper(II)–asparagine system. The following symbols +, ×, □, △, ◁, ▷, correspond to the respective order of the metal to ligand concentration ratios shown in Table I.

(referred to in the introduction) have made much of the differences between Neumann and Sass-Korsak's results [1] and those calculated by the initial plasma model. For several reasons, these criticisms seem to us to be ill-founded. First, the original model had had to employ data that were acknowledged to be less than ideal; in many cases the formation constants had to be estimated or they remained to be confirmed by independent measurements. Secondly, Neumann and Sass-Kortsak employed experimental conditions which, for good reason, differed significantly from those of blood plasma; the concentrations of amino acids were not exactly the same and (potentially even more serious) copper ions were introduced in such high concentrations as to swamp the buffering capacity of the metal-specific site on the transport protein, albumin. Thirdly, the critics ignore the many and varied applications of the model which proved successful (e.g. see ref. 10). Finally, they demonstrate a lack of appreciation of the role which models play in the scientific method; in many respects, knowledge accrues only when there is agreement between hypotheses (as embodied in models) and experimental observations.

In spite of these reservations, it seemed worthwhile attempting to simulate the copper-mobilizing abilities of different amino acids in conjunction with histidine and to compare the results with the experimental data produced by Neumann and Sass-Kortsak (Table III in ref. 1). All the calculations referred to below were based on the concentration data provided in Table I of ref. 1, except for tryptophan whose concentration was reduced to 1×10^{-5} mol dm^{-3} so as to take albumin co-ordination into account [8].

Perhaps the most important matter which had to be considered before the simulations could be performed was the problem of metal–protein binding. This problem arises from uncertainties associated with all constants presently used to characterise the metal–protein equilibrium [8]. Traditionally, two kinds of approach have been followed. In the first, so-called 'conditional' protein-binding constants have been introduced into the simulations and the calculations have been carried out in the usual way based on the total metal ion concentration. Alternatively, it has been assumed that the protein binding can be implicitly taken into account by performing the

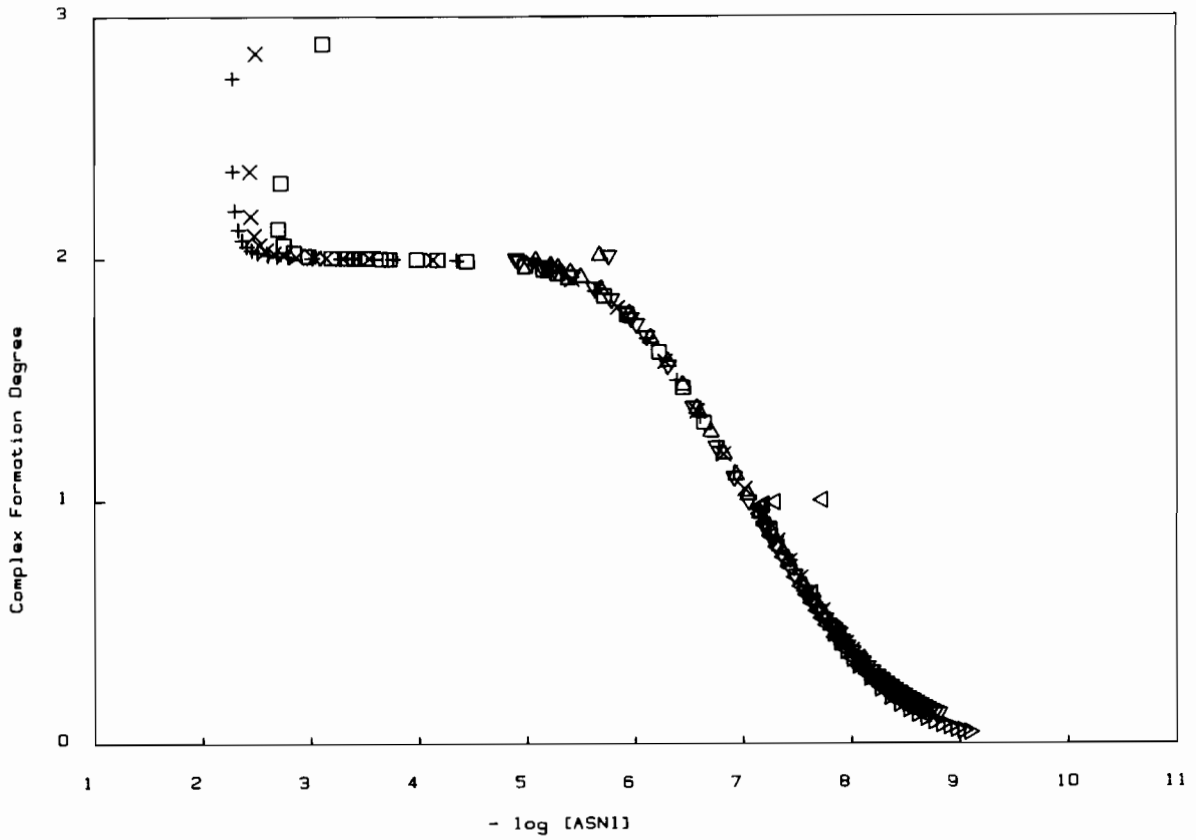

Fig. 2. Simulated formation curve for the copper(II)–asparagine system as obtained by means of the ESTA programme using results in Table II. The symbols are the same as in Fig. 1.

simulations with the free metal ion concentration held constant at some appropriate value. The latter approach assumes the metal–protein complex is very stable and effectively acts to buffer the level of the free metal ion concentration. Provided the level is sufficiently low, this has the advantage of permitting calculations of percentage distributions [7] and metal-mobilizing factors [9] in a manner that is independent of the exact nature and degree of the protein binding.

In the present studies, both kinds of approaches to metal–protein binding were investigated. Initially, it was felt that 'conditional' equilibrium constants for the copper–albumin interactions should be employed on account of the very high metal–protein ratios employed by Neumann and Sass-Kortsak. (At a copper:albumin ratio of 2:1, they were employing metal ion concentrations about one thousand times those prevailing in normal blood plasma). Of the 'conditional' constants which have been used by other workers [30, 44–46], that of Lau and Sarkar [45] from dialysis measurements ($\log K = 16.18$) seemed the most appropriate. On the grounds that (a) it was determined at 6 °C and (b) Perrin and Agarwal [30] have commented that it is probably too high, we judged a value of $\log \beta_1 = 15$ at 37 °C as being realistic. We also introduced a second constant for the Cu_2–albumin complex with a value of $\log \beta_2 = 27$ on the grounds that (a) binding by albumin of the first copper ion at the specific site is known to be considerably more avid than subsequent interactions [47] and (b) this gave a calculated proportion of copper bound to amino acids, as opposed to albumin, close to 10% – a value which is in good agreement with the results shown by Neumann and Sass-Kortsak in Fig. 1 of ref. 1 and which corresponds to a free copper ion concentration of 5.10^{-12} mol dm^{-3}.

Unfortunately, this first approach did not prove very satisfactory. On the one hand, using the above 'conditional' constants to simulate the distribution of copper in the presence of albumin alone (data corresponding to Fig. 6 of ref. 1) led to a calculated free copper concentration very much lower than that actually measured by Neumann and Sass-Kortsak. On the other hand, when these values were used in the various simulations of the experiments shown in Table III of ref. 1, the pairing of amino acids with histidine failed to mobilise more copper than the sum of the individual ligand contributions. This, it trans-

TABLE V. Computer Simulated Distribution of Copper(II) in the l.m.w. Fraction of Reconstituted Serum as Indicated in Table I of ref. 1. Species whose Percentage is Less than 2.0 are Neglected

Complex species[a]	Percentage of l.m.w. copper
Cu–his–thr	17.2
Cu–his–gln	16.3
Cu–$(his)_2$	15.9
Cu–his–ser	7.5
Cu–his–asn	4.7
Cu–his–ala	4.6
Cu–his–gly	4.5
Cu–his–leu	3.7
Cu–his–lys–H^+	3.6
Cu–his–val	3.3
Cu–his–glu	3.2
Cu–his–phe	2.2

[a]The symbols are the same as in Table IV, and glu = glutamic acid.

pired, was due to the sensitivity of the free copper concentration to changes in the concentrations of the ligands. In other words, the simulated decrease in free metal ion concentration caused by the addition of the amino acids was much larger than the effect of stronger complex formation attributable to the extra stability of mixed-ligand species. It may be concluded that, at present, models which depend on explicit inclusion of metal–protein binding relationships remain of uncertain value. Whenever the situation allows, calculations based on fixed free metal ion concentrations have much to recommend them.

Following this second approach, the distribution of copper in reconstituted serum was calculated using a free metal ion value of 10^{-13} mol dm^{-3}, which is one thousand times higher than its estimated level in normal blood plasma [21, 44]. This maintains the same ratio for total and free copper concentrations between reconstituted serum and normal blood plasma. The results are shown in Table V. It is noteworthy that differences exist with respect to the distribution in normal blood plasma shown in Table IV. These are due to the different amino acid concentrations used in the two sets of calculations, since the effect due to different free copper ion concentrations was shown to be insignificant. Note, in this respect, that the asparagine contribution is more important than in plasma.

Based on the degree of formation of the corresponding ternary complex shown in Table V, it is possible to classify the amino acids according to their association with copper and histidine in a manner that clearly corresponds to that of Neumann and Sass-Kortsak (Table III of ref. 1).

TABLE VI. Ratios of Copper(II) Mobilisation due to the Combined Addition of Histidine and One other Amino Acid with Respect to the Sum of Corresponding Individual Effects in Predialysed Serum: (i) as Obtained from Computer Simulation, (ii) Neumann and Sass-Kortsak's Results

Amino acid[a]	(i) Simulated ratios	(ii) Neumann and Sass-Kortsak's classification[b]
Threonine*	2.00	A
Glutamine*	1.94	A
Serine*	1.44	B
Asparagine*	1.28	A
Alanine*	1.27	B
Glycine*	1.27	C
Leucine*	1.21	C
Lysine*	1.21	C
Valine*	1.19	B
Glutamic acid*	1.19	B
Phenylalanine*	1.13	C
Isoleucine	1.10	C
Proline	1.09	C
Ornithine	1.08	C
Methionine*	1.07	C
Tryptophan*	1.06	C
Aminobutyric acid	1.01	C
Citrulline	1.01	C
Cystine*	1.00	C

[a]Amino acids marked with an asterisk correspond to those whose ternary systems with copper and histidine have been investigated by the same group of authors under the same experimental conditions [20, 21, 28 and present work]. [b]A, B and C classes, respectively, correspond to 2.1–2.4, 1.5–1.8, 1.3 or lesser values.

Finally, a further set of calculations were performed following the individual procedures carried out by Neumann and Sass-Kortsak. In particular, a classification of amino acids was derived by considering, in turn, each of the amino acids individually with copper and histidine. The results of these simulations are shown in Table VI, where they are compared directly with the classifications found by Neumann and Sass-Kortsak. The agreement is very satisfactory. Apart from the slightly weak computed binding by asparagine, the two orders are essentially the same.

Conclusion

In reply to those who criticise the concept of simulating physiological solutions, these authors cannot express a more succinct and cogent argument than that presented by Iversen, in a discussion of a model of homeostasis, many years ago [48].

'Many biologists of the old school react strongly against the use of mathematical models for inter-

preting biological phenomena, arguing that all biology is so fantastically complex that any model is bound to be an oversimplification of the grossest kind. On reflection, it will be evident, however, that a good simplification is a big advantage – as a matter of fact it is a precondition for a model being at all serviceable. If a map included all the details really present, it would be quite useless...'

'If we succeed in constructing mathematical models that include the main features of the structure of a biological system, such a simplification will not rule out the possibility of finding the biological laws. On the contrary, the model offers us the opportunity to understand the main points of what goes on in biology.'

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