Computer Simulation of Metal-ion Equilibria in Biofluids: Models for the Low-molecular-weight Complex Distribution of Calcium(II), Magnesium(II), Manganese(II), Iron(III), Copper(II), Zinc(II), and Lead(II) Ions in Human Blood Plasma

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An investigation by computer simulation into the nature of the metal-ion binding to low-molecular-weight ligands in human blood plasma is described. Although the absolute concentrations of the metal-complex species are controlled by protein binding, the percentage distribution of transition-metal ions amongst the low-molecular-weight ligands is not. Hence errors arising from the omission of protein–metal equilibria are successfully by-passed. The distribution of Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} amongst 5 000 complexes formed with 40 ligands has been computed. In order to cope with multicomponent systems of such a large size, a computer program has been developed. Ternary complexes account for the larger percentage of Cu^{II} and Fe^{II} species, all the former involving histidinate and all the latter, citrate. Binary complexes are favoured by Ca^{II}, Mg^{II}, and Mn^{II}. Zinc(II) and Pb^{II} form both binary and ternary complexes amongst the predominant species. In contrast with earlier work, ternary zinc citrate complexes are found to be important.

It has been well demonstrated that metal ions play a large number of important roles in biological systems.[1-3] Metal ions which are considered essential to human life include those of calcium, magnesium, manganese, iron, cobalt, copper, and zinc.[4] Furthermore man is constantly being challenged by pollutants including the toxic metal ions Pb^{2+}, Hg^{2+}, and Cd^{2+}.[5]

In human blood plasma, as well as in certain other biological fluids, the metal ions present may be classified into four distinct fractions: those which are incorporated rigidly into the metalloproteins and are non-exchangeable; those which are relatively loosely bound by other types of protein and are in labile equilibrium with similar ions in solutions; those which are complexed by the numerous low-molecular-weight ligands present, including amino-acid anions, carboxylates, carbonate, phosphate, salicylate, and ascorbate; and the free (or aquated) metal ions. The last three fractions can be regarded as a multicomponent system in which the individual complexing species are in competitive equilibrium with the free metal ions. Although biological systems never reach true equilibrium they often approach and sometimes attain a steady state. Moreover, 'to achieve high efficiencies of energy conversion, most biological systems operate near to reversible equilibria.'[4] Thus to assume that equilibrium exists is often justifiable. The low-molecular-weight fraction of metal ion complexes, although small by comparison with the protein–metal fraction, is of great significance owing to the important role of these complexes in many vital physiological and biochemical processes.[1,6] For example, the low-molecular-weight complexes are believed to be involved (*i*) as intermediates when metal ions are inserted into or removed from certain metalloenzymes or carrier proteins, (*ii*) in the transfer of certain metal ions across membranes, (*iii*) in keeping essential metals in solution, and (*iv*) in altering the potential of certain redox couples. It follows that the acquisition of a detailed knowledge of the equilibrium distribution of metal ions between low-molecular-weight ligands is highly desirable. This constitutes the objective of the work described in the present paper.

The total concentrations of Mn^{II}, Fe^{III}, Cu^{II}, Zn^{II},

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and Pb^{II} in normal human blood plasma are between 1×10^{-7} and 5×10^{-5} mol dm^{-3}.[1] The correspondingly minute concentrations of the respective free ions are far below the limits of detection of any analytical technique hitherto developed. In addition, the problems with experimental assessment of the equilibrium concentrations are heightened both by the large number of potential ligands that occur and by the fact that probes which interact chemically with the system are likely to perturb the very distribution that one is attempting to monitor. For these reasons, simulation of the metal ion–ligand equilibria using high-speed computers constitutes the only currently available method of estimating the equilibrium concentrations of the complexes involved.

Computer simulation of the complex-formation equilibria between the low-molecular-weight ligands and the metal ions of blood plasma has been pioneered by Perrin and his co-workers.[4,7-9] Perrin's earlier models were restricted to the metal ions Cu^{2+} and Zn^{2+} together with a selection of amino-acids (16 initially and 22 subsequently) as the ligands. In later developments the effects of inserting proteins (albumin and globulin) and the metal ions Ca^{2+} and Mg^{2+} were examined. In all the cases the major portion of the copper (80—98%) was found to be co-ordinated to histidine and cystine predominantly as the cystinatohistidinato-, protonated cystinatohistidinato-, and bishistidinato-complexes. By contrast the zinc formed complexes with a wider range of amino-acids. The results concerning the protein–metal complexes were not satisfactory, however. For example the concentration of the copper–albumin complex was negligible and the distribution of calcium between albumin and globulin differed distinctly from experimental results obtained by direct measurement.[10] Giroux and Henkin [11] reinvestigated the competition for zinc among amino-acids in the presence of albumin and obtained a distribution of the zinc amongst the low-molecular-weight complexes in essential agreement with Perrin's. Finally Brandegård and Österberg [12] simulated the calcium-ion reactions in blood plasma. They set up an eight-component model that included proteins, carbonate, phosphate, sulphate, amino-acids, and organic oxy-acids. Although their findings appear to be realistic, the omission of the calcium hydrogencarbonate complex may be expected to introduce distortion into their results owing to the relatively high concentration of this ligand.

All of the models referred to above are abbreviated examples of the conditions in blood since the number of complexes considered represents only a small fraction of the vast number of possibilities. Clearly there is a need for a more comprehensive study and this paper describes a model involving seven metal ions, 40 ligands, and *ca.* 5 000 complexes. In the light of present knowledge, it is considered impossible to incorporate satisfactorily the labile protein–metal equilibria into a model of the type under discussion. The reasons centre essentially around the difficulties (*i*) in determining the constants for the numerous individual binding sites on a given protein molecule, (*ii*) in taking into account interactive effects between the binding sites, and (*iii*) in defining the many individual complex species that can be produced with a given species of protein. Accordingly, an attempt is made here to circumvent these difficulties. The approach has been described in a preliminary report of the results.[13]

THE PLASMA MODELS

The choice of the low-molecular-weight ligands in the present study represents a selection of 40 from an initial list of *ca.* 100 compiled from various tabulations on the composition of blood plasma.[4,9,14-22] Criteria based on concentration and the availability and magnitude of formation-constant data for the ligand–metal complexes were used to make the selection. The chosen ligands together with their estimated total concentrations are listed in Table 1. Most of the concentrations are averages of

TABLE 1

Total-ligand concentrations in human blood plasma used in the computer simulations

Ligand	Concentration / mol dm^{-3}	Ligand	Concentration / mol dm^{-3}
Alanate	3.70×10^{-4}	Serinate	1.22×10^{-4}
Aminobutyrate	2.40×10^{-5}	Threoninate	1.50×10^{-4}
Arginate	9.50×10^{-5}	Tryptophanate	1.00×10^{-5}
Asparaginate	5.50×10^{-5}	Tyrosinate	5.80×10^{-5}
Aspartate	5.00×10^{-6}	Valinate	2.27×10^{-4}
Citrullinate	2.70×10^{-5}	Histamine	3.00×10^{-8}
Cysteinate	2.30×10^{-5}	Carbonate	2.45×10^{-2}
Cystinate	4.00×10^{-5}	Phosphate	3.81×10^{-4}
Glutaminate	5.21×10^{-4}	Silicate	1.38×10^{-4}
Glutamate	4.80×10^{-5}	Sulphate	2.11×10^{-4}
Glycinate	2.43×10^{-4}	Thiocyanate	1.40×10^{-5}
Histidinate	8.50×10^{-5}	Ammonia	2.40×10^{-5}
Hydroxyprolinate	7.00×10^{-6}	Citrate	1.13×10^{-4}
Isoleucinate	6.50×10^{-5}	Lactate	1.82×10^{-3}
Leucinate	1.24×10^{-4}	Malate	3.50×10^{-6}
Lysinate	1.78×10^{-4}	Oxalate	1.20×10^{-5}
Methionate	2.90×10^{-5}	Pyruvate	9.50×10^{-5}
Ornithinate	5.80×10^{-5}	Salicylate	5.00×10^{-6}
Phenylalanate	6.40×10^{-5}	Succinate	4.20×10^{-5}
Prolinate	2.11×10^{-4}	Ascorbate	4.30×10^{-5}

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several reported values. Some of the concentrations were reduced to correspond with the known protein binding of the ligand in question. These include salicylate and tryptophanate.[23-28] Urea, fatty acids, bilirubin, and folic acid were excluded from the model because they are almost totally bound by proteins.

The metal ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} were selected as being the most abundant in plasma or the best understood in terms of their biological roles. Owing to the problems involved with protein–metal binding it is not possible to arrive at an unequivocal set of concentrations except in the case of Ca^{2+}. Hence, in the computation of the distribution of the metal ions other than Ca^{2+} among the low-molecular-weight complexes (described below), it was decided to cover a range of plausible free concentrations for each, which bracketed an estimated average value. The estimated averages and ranges of concentrations for the free metal ions are listed in Table 2. These were obtained as follows.

TABLE 2

Metal-ion concentrations (mol dm^{-3}) used in the simulations

Metal ion	Free-concentration estimates used as average	Free-concentration range scanned
Ca^{2+}	1.14×10^{-3}	Fixed
Cu^{2+}	10^{-18}	10^{-19}—10^{-11}
Fe^{3+}	10^{-23}	10^{-24}—10^{-18}
Pb^{2+}	10^{-14}	10^{-16}—10^{-10}
Mg^{2+}	5.2×10^{-4}	5.10×10^{-4}—5.50×10^{-4}
Mn^{2+}	10^{-12}	10^{-15}—10^{-8}
Zn^{2+}	10^{-9}	10^{-11}—10^{-8}

Ca^{2+}.—The concentration of Ca^{2+}, being relatively high and directly determinable, for example by specific ion-sensitive electrodes, was fixed at a reported value of 1.14×10^{-3} mol dm^{-3}.[29] It should be noted that a degree of uncertainty arises from a fairly appreciable physiological variation.

Cu^{2+}.—Whereas the total concentration of copper in blood plasma is *ca.* 1.8×10^{-5} mol dm^{-3}, most is firmly bound to ceruloplasmin, the total exchangeable copper having a concentration of *ca.* 1×10^{-6} mol dm^{-3}.[4,30-33] The larger percentage of the latter is bound to albumin.[27] From a knowledge of the dissociation constant[34] of the copper–albumin complex, a lower limit for the concentration of free copper is estimated to be *ca.* 10^{-19} mol dm^{-3}. A conservative upper limit of 10^{-11} mol dm^{-3} is suggested by the fact that the free Cu^{2+} concentration is below the limit of detection by ion-selective electrodes[35] (*ca.* 10^{-9} mol dm^{-3}) and by the experiments of Neumann and Sass-Kortsak.[32] The latter workers found that at physiological ratios of copper to albumin *ca.* 0.4% of the metal was ultrafiltratable. It must be pointed out however that Neumann and Sass-Kortsak's investigations were made with total copper concentrations greatly in excess of the value in normal plasma and therefore estimates based on their results are bound to be strongly biased towards the upper concentration limit.

Fe^{3+}.—Almost all the iron is bound to transferrin. By using the value of the binding constant reported by Aasa *et al.*[36] one may infer the concentration of free Fe^{3+} to be 10^{-24} mol dm^{-3}. An upper limit to the free concentration of Fe^{3+} under physiological conditions of *ca.* 10^{-18} mol dm^{-3} is dictated by the solubility product of Fe^{III} hydroxide.[37]

Pb^{2+}.—The total concentration of lead in plasma varies quite considerably, but an average of the reported values is *ca.* 5×10^{-7} mol dm^{-3}.[1,14-16] When combined with the data of Gurd and Murray[38] this figure leads to an estimate of the maximum free concentration of $< 10^{-10}$ mol dm^{-3} in healthy human plasma. Since many other proteins compete for lead, the normal value is likely to be considerably lower than this. It is of interest that the symptoms of plumbism become apparent when the total lead concentration reaches *ca.* 3×10^{-6} mol dm^{-3}.

Mg^{2+}.—Using an exchange membrane, Heaton[39] concluded that ionized magnesium averaged 79% of the ultrafiltratable magnesium in serum. It may be inferred that the concentration of free Mg^{2+} lies in the range 5.1×10^{-4}—5.5×10^{-4} mol dm^{-3}.

Mn^{2+}.—From studies of the binding of Mn^{2+} to albumin,[40-43] the concentration of free Mn^{2+} is estimated to have a maximum value of 5×10^{-8} mol dm^{-3}. Conflicting assertions concerning the protein to which this metal is selectively bound have been reported.[42,44] Other reports[1,14-16] on the total concentration in plasma have been criticized[45] on the grounds of contamination. It therefore seemed reasonable to scan the concentration of free Mn^{2+} from an upper limit of 10^{-8} mol dm^{-3} downwards.

Zn^{2+}.—Although a value of 4.6×10^{-5} mol dm^{-3} for the total zinc concentration has been reported,[1,4,8,9,15,16] this seems to stem from an erroneous figure originally given by Vallee and Gibson.[46] The value of 1.6×10^{-5} mol dm^{-3} appears to be more reliable.[11,14,47-49] Since *ca.* 35% of the

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total zinc is rigidly bound to the metalloprotein α2-macroglobulin [14,50] the concentration of exchangeable zinc is estimated to be *ca.* 1×10^{-5} mol dm^{-3}. Albumin binds a large fraction of the exchangeable zinc in a labile equilibrium. From values of the zinc–albumin constant [11,51] the derived concentration of the free Zn^{2+} is *ca.* 10^{-9} mol dm^{-3}. Owing to the uncertainties in this figure, a range from 10^{-11} to 10^{-8} mol dm^{-3} seems reasonable.

TABLE 3

Trends [a] of stability [b] observed for amino-acid anion–metal complexes

Amino acid	Trend	CuL	CuL_2	MnL	MnL_2	ZnL	ZnL_2	ZnL_3
Simple amino acids [c]	0	8.0	14.7	2.5	4.4	4.6	8.5	10.9
Average	0	8.1	14.6	2.6	4.6	4.7	8.4	10.8
Alanine	0	8.0	14.6	2.4	4.3	4.6	8.6	10.7
Aminobutyric acid [c]	−	7.7	14.0			4.4	7.2	
Arginine	−	7.4	13.7			4.0	7.6	
Asparagine	−	7.7	13.7			4.5	7.8	10.0
Glutamic acid	+	8.7	14.9			4.8	8.5	
Glutamine	−	7.2	13.4			4.3	7.9	
Glycine	0	8.0	14.7	2.7	4.8	4.9	9.0	11.3
Histidine	+	9.8	17.5	3.2	6.2	6.3	11.7	
Isoleucine	0	8.0	14.7			4.4	8.1	
Leucine	0	8.0	14.7			4.5	8.6	
Lysine	0	9.3	14.6			3.5	7.0	
Methionine	−	7.7	14.1			4.2	6.9	
Ornithine	+	9.8	14.8			5.9		
Phenylalanine	−	7.7	14.4			4.5	8.4	
Proline	+	8.7	16.0	2.8	5.5	5.1	9.7	11.2
Serine	−	7.6	14.0	2.5	4.0	4.5	8.3	10.6
Threonine	−	7.6	14.0	2.5	3.9	4.4	8.1	10.1
Tryptophan	+	8.1	15.3			4.5	8.8	11.6
Tyrosine	+	9.1	15.1			6.1		
Valine	0	7.9	14.6	2.3	4.0	4.4	8.2	10.6

[a] 'Trend' means the strength of binding of the particular amino-acid relative to the average for the series of similar ligands. [b] Cumulative stability constants measured at 37 °C; $I = 0.15$ mol dm^{-3} $K[NO_3]$.[4,9] [c] Alanine, glycine, leucine, isoleucine, and valine.

The choice of complexes for the present simulation was based partly on the availability in the literature of reported experimental studies of the individual ligand–metal systems. Since mixed-ligand (ternary) complex formation occurs widely in systems containing metal ions and two or more different ligands,[9,52-54] as many as possible of these have been included. An extensive search of the literature up to March 1975 yielded pertinent formation-constant values for over 250 mononuclear binary, ligand–proton, and protonated ligand–metal complexes measured under physiological conditions, *ca.* 400 similar types of complex measured under conditions different from human blood plasma, and *ca.* 100 ternary mixed-ligand complexes. As is well known, ostensibly the same constants published by different authors often vary appreciably so all the values were critically evaluated. Some were omitted. Averages were taken whenever sufficient data were available. Extended Debye–Hückel-type ionic-strength corrections combined with extrapolations made by analogy with the experimental ionic-strength plots of Gergely *et al.*[55] and van't Hoff-type temperature corrections were applied where possible to those constants measured under non-physiological conditions. An additional 350 mononuclear binary or protonated ligand–metal complexes, for which no values had been reported, were deemed to be potentially important for the blood-plasma model. A variety of methods were used to estimate values for the missing constants. These were based on linear free-energy-relation principles including the extension proposed by Sigel,[56] the Irving–Williams rule, and educated guesses based on observed chemical analogies. The chemical analogies used are exemplified by the trends in stability observed for the amino-acid anion–metal complexes as shown in Table 3. The full list of 931 binary constants may be found in Supplementary Publication No. 21969 (71 pp., 1 microfiche).*

In view of the paucity of formation constants available for ternary complexes (see SUP 21969) and the expected dominant role of these in the low-molecular-weight fraction of blood plasma, estimations were made of *ca.* 4 000 additional ternary constants, considered to be necessary for inclusion in the model employing relevant binary complex constants, statistical factors, and stabilization factors.[57-59] Estimates of the stabilization factors were based where possible on experimentally determined stabilization factors of analogous complexes.[57-66] In order to correct the ternary formation constants which had been experimentally measured at other than physiological conditions, a scaling operation was performed on each

* For details see Notices to Authors No. 7, *J.C.S. Dalton*, 1976, Index issue.

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respective stabilization factor using expression (1).[57] Here, $\Delta\log\beta_{MAB}$(model) is the scaled stabilization factor applicable to the model (*i.e.* physiological) conditions, $\Delta\log\beta_{MAB}$(expt.)

$$\Delta\log\beta_{MAB}(\text{model}) = \Delta\log\beta_{MAB}(\text{expt.})\left[\frac{\beta_{MA_2}(\text{model}) \cdot \beta_{MB_2}(\text{model})}{\beta_{MA_2}(\text{expt.}) \cdot \beta_{MB_2}(\text{expt.})}\right] \quad (1)$$

is the observed scaling factor [58] obtained under non-physiological conditions = $\log\beta_{MAB}$(expt.) − ½[$\log\beta_{MA_2}$(expt.) + $\log\beta_{MB_2}$(expt.)] − log 2, β_{MAB} is the formation constant of the ternary complex, MAB, formed between a metal, M, and two ligands, A and B, β_{MA_2} and β_{MB_2} are the cumulative formation constants of the binary complexes, MA_2 and MB_2; the qualifier (expt.) refers to experimental constants determined under non-physiological conditions and (model) applies to formation constants measured at or corrected to model conditions. The scaled stabilization factor was then used together with the relevant binary complex constants (physiological conditions) and statistical factors [57–59] to obtain corrected ternary constants.

These choices of components and complexes comprise a model of *ca.* 5 000 species. Since the published programs COMICS [4,67] and HALTAFALL,[68] developed for computing distributions of species in multicomponent systems, are inadequate for handling models of the size proposed, a new program, ECCLES,* has been written. Details of the algorithm together with the symbolic coding may be found in SUP 21969. The program is based on a three-tier successive-approximations procedure, outlined in the Appendix, which gives rapid convergence whilst, at the same time, keeping the computer core-storage requirements considerably lower than would be possible with COMICS.

Using the average free metal-ion concentrations in Table 2 and the total concentrations in Table 1 for the ligands listed, the distribution of the metal ions amongst nearly 5 000 complexes formed was computed at three distinct pH values centring around the average value for plasma. Table 4 shows results for the predominant complexes found for each metal ion, expressed as the percentage of the total concentration of the relevant metal ion contained in the low-molecular-weight fraction of plasma. The computed total concentrations of the metal ions in the low-molecular-weight-fraction of plasma are listed in Table 5 together with the values estimated from experimental considerations as described above. Table 6 shows the computed free-ligand concentrations. For the sake of brevity, the model used to compute the information in Tables 4—6 is referred to, hereafter, as ' the primary model.' The effects of pH shown in Table 4 on the distribution of some of the metal ions is more marked than may have been anticipated. Both increases and decreases in the degree of complex formation can be observed for increases in pH. Ternary complexes account for the larger percentages of these formed by Cu^{II} and Fe^{III}. The more important Cu^{II} complexes contain histidinate whereas citrate features amongst the most predominant Fe^{III} complexes. Binary complexes are favoured by Ca^{II}, Mg^{II}, and Mn^{II}. Zinc(II) and Pb^{II} form both binary and ternary complexes amongst the respective sets of predominant complexes. Lead(II) appears to be bound mainly by cysteinate and cystinate. The other sulphur-containing amino-acid, methionine, does not appear to compete effectively for lead. This can probably be

TABLE 4

Percentage distribution of the metal ions Ca^{2+}, Cu^{2+}, Fe^{3+}, Pb^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} amongst low-molecular-weight ligands in human blood plasma as found by computer simulation

		Percentage of the total metal in in the low-molecular-weight fraction		
Complex	Charge	−log[H+] = 7.2	7.4	7.6
(*a*) Calcium(II)				
Protonated carbonate	+1	9	9	9
Citrate	−1	4	4	4
Lactate	+1	3	3	3
Phosphate	−1	2	3	4
Carbonate	0	1	2	3
(*b*) Copper(II)				
Cystinate histidinate	−1	16	21	28
Protonated cystinate histidinate	0	20	17	14
Bis(histidinate)	0	12	11	10
Histidinate threoninate	0	9	8	7
Histidinate valinate	0	5	5	5
Protonated histidinate lysinate	+1	5	5	4
Alanate histidinate	0	4	4	4
Histidinate serinate	0	4	4	4
Histidinate phenylalanate	0	3	3	3
Glycinate histidinate	0	3	3	3
Histidinate leucinate	0	2	2	2
Glutamate histidinate	−1	2	2	2
Glutaminate histidinate	0	2	2	2
Protonated histidinate ornithinate	+1	2	2	1
Histidinate prolinate	0	2	1	1
Histidinate isoleucinate	0	1	1	1
Histidinate tryptophanate	0	1	1	1
(*c*) Iron(III)				
Citrate hydroxide	−1	99	99	99
Citrate salicylate	−2	<1	<1	<1
Citrate glutamate	−2	<1	<1	<1
Citrate oxalate	−2	<1	<1	<1
(*d*) Lead(II)				
Cysteinate	0	76	80	82
Citrate cysteinate	−3	6	7	7
Protonated cystinate	+1	8	5	3
Protonated cysteinate phosphate	−2	2	3	3
Protonated carbonate	+1	5	2	1
Protonated bis(cysteinate)	−1	1	2	3
(*e*) Magnesium(II)				
Protonated carbonate	+1	5	6	6
Citrate	−1	5	5	4
Carbonate	0	1	2	4
Lactate	+1	2	2	2
Protonated phosphate	0	1	1	1
(*f*) Manganese(II)				
Protonated carbonate	+1	24	24	24
Citrate	−1	10	10	9
Carbonate	0	1	2	4
Oxalate	0	2	2	2
Protonated phosphate	0	1	1	1
(*g*) Zinc(II)				
Citrate cysteinate	−3	50	43	33
Bis(cysteinate)	−2	9	19	33
Cysteinate histidinate	−1	9	12	14
Cysteinate	0	4	3	2
Histidinate	+1	4	3	1
Protonated bis(cysteinate)	−1	1	1	2
Bis(histidinate)	0	2	1	1
Cysteinate glutaminate	−1	<1	1	1

* ECCLES = Evaluation of Constituent Concentrations in Large Equilibrium Systems.

[67] D. D. Perrin and I. G. Sayce, *Talanta*, 1967, **14**, 833.

[68] N. Ingri, W. Kakołowicz, L. G. Sillén, and B. Warnqvist, *Talanta*, 1967, **14**, 1261.

attributed to the relatively small formation constant of the Pb^{II}– methioninate complex and possibly to competition from Ca^{II} and Mg^{II}. The ternary cysteine–citrate–metal species is important for both Pb^{II} and Zn^{II}, suggesting that one of the aspects of toxicity caused by lead overload may be the displacement of zinc by lead from cysteine moieties of certain enzymes.

TABLE 5

Comparison of the computed total low-molecular-weight metal concentrations with total concentrations estimated from experimental considerations

Metal ion	Concentration of free metal ion mol dm^{-3}	Total low-molecular-weight metal concentrations obtained by computer simulation/mol dm^{-3}			Total metal concentrations estimated from experimental considerations/mol dm^{-3}		Approximate percentage of metal ion bound to protein
		$-\log[H^+] = 7.2$	7.4	7.6	*a*	*b*	
Ca^{2+}	1.14×10^{-3}	1.43×10^{-3}	1.46×10^{-3}	1.51×10^{-3}	3×10^{-4}	2.45×10^{-3}	45
Cu^{2+}	10^{-18}	6.01×10^{-12}	1.57×10^{-11}	4.14×10^{-11}	10^{-12}—10^{-9}	1×10^{-6}	>99
Fe^{3+}	10^{-23}	4.24×10^{-13}	6.68×10^{-13}	1.06×10^{-12}		2.2×10^{-5}	*ca.* 100
Pb^{2+}	10^{-14}	2.67×10^{-11}	5.88×10^{-11}	1.30×10^{-10}		5×10^{-7}	
Mg^{2+}	5.20×10^{-4}	6.48×10^{-4}	6.56×10^{-4}	6.67×10^{-4}	1.2×10^{-4}	9×10^{-4}	30
Mn^{2+}	10^{-12}	1.79×10^{-12}	1.83×10^{-12}	1.89×10^{-12}		10^{-8}—10^{-6}	
Zn^{2+}	10^{-9}	6.91×10^{-8}	1.84×10^{-7}	5.50×10^{-7}	10^{-7}—10^{-6}	1×10^{-5}	>95

[a] Low-molecular-weight complex concentration (excluding concentration of free metal ion). [b] Total exchangeable metal-ion concentration.

A comparison of the computed total low-molecular-weight metal-complex concentrations with those estimated from experimental considerations (Table 5) shows eminently satisfactory agreement between the corresponding figures. This provides some direct assurance that the model is not grossly in error. In view of the uncertainties in the estimates of the metal–ion concentrations, an investigation was made which involved varying the free concentration of each metal ion in turn through the ranges shown in Table 2, using the primary model at all three pH values as a reference. These free-metal-ion concentration scans yielded the striking observation that for each of the three pH values the percentage distribution was independent of the free metal-ion concentrations within the ranges chosen. This is valid to a precision of 1%. The explanation of this phenomenon lies in the fact that the formation of low-molecular-weight complexes in plasma is minimal. Indeed, the free concentrations of the low-molecular-weight ligands are usually several orders of magnitude larger than the complex concentrations. Thus the free-ligand concentrations are not significantly altered by changes in complex concentration, *i.e.* they are ' concentration buffered ' (*cf.* Tables 1, 5, and 6). The very low total concentrations of the transition-metal ions, the weak binding of Ca^{II} and Mg^{II}, and the lowering of the free metal-ion concentrations by protein binding are contributory factors. In consequence the concentration of each complex is almost directly proportional to the free metal-ion concentrations which are selected.

TABLE 6

Free-ligand concentrations in human blood plasma as obtained by computer simulation (for $-\log[H^+] = 7.4$)

Ligand	Concentration mol dm^{-3}	Ligand	Concentration mol dm^{-3}
Alanate	2.87×10^{-6}	Serinate	4.20×10^{-6}
Aminobutyrate	3.38×10^{-7}	Threoninate	6.87×10^{-6}
Arginate	3.40×10^{-6}	Tryptophanate	1.97×10^{-7}
Asparaginate	2.69×10^{-6}	Tyrosinate	3.49×10^{-9}
Aspartate	7.56×10^{-8}	Valinate	2.66×10^{-6}
Citrullinate	1.27×10^{-6}	Histamine	1.92×10^{-10}
Cysteinate	5.94×10^{-9}	Carbonate *	3.54×10^{-5}
Cystinate	4.61×10^{-7}	Phosphate *	3.40×10^{-8}
Glutamate	4.74×10^{-7}	Silicate	2.67×10^{-10}
Glutaminate	1.84×10^{-5}	Sulphate	2.04×10^{-4}
Glycinate	2.47×10^{-6}	Thiocyanate	1.40×10^{-5}
Histidinate	2.35×10^{-6}	Ammonia	6.55×10^{-7}
Hydroxyprolinate	8.59×10^{-8}	Citrate	2.67×10^{-5}
Isoleucinate	6.95×10^{-7}	Lactate	1.76×10^{-3}
Leucinate	1.32×10^{-6}	Malate	3.11×10^{-5}
Lysinate	4.88×10^{-9}	Oxalate	1.03×10^{-5}
Methionate	8.56×10^{-7}	Pyruvate	9.41×10^{-5}
Ornithinate	5.35×10^{-9}	Salicylate	1.20×10^{-11}
Phenylalanate	1.97×10^{-6}	Succinate	4.04×10^{-5}
Prolinate	2.60×10^{-7}	Ascorbate	5.36×10^{-8}

* These values may be too high. Current estimates of the appropriate solubility products suggest that they may represent supersaturation with respect to calcium ion. This is however, not impossible. Alternatively, it has been suggested that solid phases, dispersed by proteins into particles of colloidal sizes, may co-exist in plasma.[12] In any event the percentage distributions shown in Table 4 are unlikely to be significantly upset by this consideration.

Further support for the validity of the primary model was obtained from a simulation for each of the three individual pH values, 7.2, 7.4, and 7.6, using free metal-ion concentrations at the upper limits of the ranges in Table 2. At each respective pH this produced a computed distribution within 2% of the corresponding figures in Table 4.

DISCUSSION

One way in which models, such as are described in this paper, are significant lies in the background they provide to postulating metal-ion reactions that may take place in blood plasma or even within the cells supported by this medium. For example, certain of the predominant complex species may be suggested to participate in mechanisms whereby metal ions are incorporated into or removed from macromolecules. Thus according to the results in Table 4, the exchange of Fe^{3+} ions between transferrin molecules is postulated to take place *via* a mixed complex involving only one

citrate ligand rather than as the dicitrato-complex as previously suggested.[69,70] This is confirmed by the finding in the reported kinetic studies of Fe^{3+} exchange between chelates and transferrin [71] that the iron dicitrate is not the most reactive species. Similarly it may be concluded from Table 4 that exchange of Cu^{II} and possibly Pb^{II} between proteins in plasma involves mixed-ligand complexes whereas exchange of Mn^{II} involves binary complexes.

Another important application concerns the passive transport of transition-metal ions through biological membranes. It must be emphasized that considerably more is known about passive than active transport. In general, passive transport is restricted to electrically neutral species, the magnitude of the flux being dependent on the difference in concentration on either side of the membrane. In contrast, charged species cannot cross membranes at measurable rates [72] owing, in all likelihood, to the energy requirement being prohibitive for the transfer of a charge with small dimensions from an aqueous to a lipid phase. It appears, therefore, that because of their charges and their relatively low concentrations, the aqua-ions of transition metals in plasma are unlikely to diffuse across membranes. Hence it is suggested that only neutral complexes of transition metals are likely to be lipid soluble and therefore passively transported across membranes. In order to contribute to the understanding of both passive and active transport processes, computer models could, in principle, be used to determine the relative concentrations of the low-molecular-weight transition-metal complexes in the two biological fluids separated by a given membrane. The simulation could also be used to indicate how pH changes affect the distribution, especially with respect to the appearance or disappearance of charges on acidic or basic centres of the complexes. Thus the search for conditions which would either increase or decrease the flux would be facilitated. Examples of biological fluids which might be profitably simulated for the present purpose are intestinal fluid, cerebrospinal fluid, gastric juice, and urine. These models merely await the production of necessary experimental information on the metals and low-molecular-weight ligands that are present, together with estimates of their (total) concentrations.

Amongst the essential metals, iron is peculiar in possessing a unique form of homeostatic control in the human body. Although the mechanism of iron homeostasis is ill understood, it has been established that only about one tenth of the dietary intake of iron is absorbed and, moreover, the body possesses only a minimal ability to excrete this element. Thus, there appears to be a tendency for the body to preserve iron.[73-80] In contrast to the fate of the porphyrin moiety of haemoglobin, iron is removed in the protein degradation and stored for future use.[81] Table 4 shows that all the predominant low-molecular-weight Fe^{III} complexes of blood plasma are negatively charged. It is tempting to speculate that the impediment to transport across membranes resulting from the negative charges is associated with the body's inability to excrete iron.

Absorption of iron takes place throughout the intestine but mainly in the duodenum and the principle factor involved appears to be solubility. Any factor which tends to increase the solubility of iron, thereby preventing hydroxide precipitation as the pH rises on exit from the stomach, will promote iron absorption.[82-84] It is well established that normal iron-absorption paths can be by-passed by low-molecular-weight complexes.[80] So a knowledge of the manner in which various complexes are produced in biofluids will almost certainly be of use in combating both iron-deficiency anaemia and sideroses. Similarly, the present work is applicable to the other transition-metal ions included in the model.

Much remains which can be done to improve the detail of the blood-plasma model. The most pressing tasks are: (*i*) the accurate determination of all the as yet unmeasured formation constants for the important complexes (hydrogencarbonate complexes and magnesium and calcium amino-acid species, in particular); (*ii*) the experimental measurement of many more mixed-ligand complex-formation constants; and (*iii*) the improvement of estimates of the free metal-ion concentrations in plasma, especially that of magnesium. Since sodium chloride is the chief contributor to the ionic strength of blood plasma (0.15 mol dm^{-3}), there is considerable justification for using this substance as the supporting electrolyte in the formation-constant determinations. Also the temperature should preferably be that of the normal human body, namely 37 °C.

In spite of all the applications that can be envisaged, it is important to recognise that the negative evidence produced by the model may ultimately prove to be that of greatest value. The considerable stability of the

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computed distribution of metal ions amongst the low-molecular-weight complexes implies that neither metal-ion deficiency nor overload is likely to alter the gross distribution. Furthermore it is not difficult to envisage that the results may indicate that a given complex or even the entire low-molecular-weight metal-ion fraction may be eliminated as possible participants in various physiological processes under investigation.

APPENDIX

Outline of the ECCLES Algorithm.—It is required to find the free concentrations of the components in a multi-component metal–ligand system at equilibrium. The concentration of each complex species is then determined according to equation (A1). (The meanings of the symbols are listed at the end of the Appendix.) The solution is obtained by iterative improvement of the free-concentration estimates until they satisfy the mass-balance equations for each component as given by $T_i = X_i + \sum_j S_j k(i,j)$. Whilst a variety of optimization techniques are applicable to the generalized problem, a successive-approximation procedure has been selected as well suited to the very large systems which are envisaged.

$$S_j = \beta_j \prod_i X_i^{k(i,j)} \qquad \text{(A1)}$$

The first few iterations employ equation (A2) where

$$X_m{}^n = \frac{T_m{}^r X_m{}^0}{X_m{}^0 + \sum_j [G_j' S_j{}^0 k(m,j)]} \qquad \text{(A2)}$$

$G_j' = \prod_i (T_i{}^r/T_i{}^c)^{k(i,j)}/(T_m{}^r/T_m{}^c)$ and is an approximation for $G_j = \prod_i (X_i{}^r/X_i{}^c)^{k(i,j)}/(X_m{}^r/X_m{}^c)$. In SUP 21969 it is shown that (A2) may be expected to converge on $X_m{}^r$ provided that all the initial $X_i{}^c > X_i{}^r$. This requirement is automatically satisfied in the definition of G_j' in that the starting values for $X_m{}^c$ are taken as being equal to the real-concentration totals, $T_m{}^r$. These first few iterations rapidly produce values in the proximity of the solution. With the number of iterations as a criterion, this arrangement could be employed exclusively. In fact, however, the expression requires so much computation that it is only profitable to employ it in the early stages of the iteration procedure.

It is shown further in SUP 21969 that expression (A3) will

$$X_m{}^n = X_m{}^0(T_m{}^r/T_m{}^c) \qquad \text{(A3)}$$

always convert $X_m{}^0$ to a value $X_m{}^n$ which is smaller than the desired real concentration, *i.e.* it ' overshoots.' If the expression used by Perrin and Sayce [67] in COMICS is re-written as (A4) the denominator is seen to be intermediate

$$X_m{}^n = X_m{}^0[T_m{}^r/(T_m{}^r T_m{}^c)^{\frac{1}{2}}] \qquad \text{(A4)}$$

between $T_m{}^c$, which would overshoot, and $T_m{}^r$ which would yield no improvement.

Once the application of (A2) is completed the program moves into an intermediate phase in which the *iteration* formula of Perrin and Sayce, (A4), is used solely. This requires considerably less computation than (A2). As the solution is approached there is a fall off in convergence rate, as is usual with many successive-approximation techniques. Accordingly, when this stage is reached, expression (A3) is used on alternate iteration cycles. This improves the rate of convergence in the final stages.

Meanings of Symbols.—(*a*) *Concentrations.*

S = the concentration of a complex species
X = the free concentration of a component
T = the total concentration of a component

(*b*) *Indices.*

i = the general index for components
j = the general index for complex species
m = the specific component index
p = the index for a metal-ion component
q = the index for a ligand component
r = the index for a hydrogen-ion component

(*c*) *Superscripts.*

c denotes a ' calculated ' quantity
r denotes a ' real ' quantity
o denotes an ' old ' value in an iteration
n denotes a ' new ' value in an iteration

(*d*) *Parameters.*

β = the cumulative stability constant of a complex species as defined by the equation $\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$

k = the matrix which defines the components and their multiplicity in a complex species

(*e*) *Miscellaneous symbols.*

M = metal ion
L = ligand
H = hydrogen ion

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