Stability Constants of Complexes of Copper(II) Ions with some Histidine Peptides

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Values at 37 °C and 0·15M (KNO_3) for equilibrium constants of copper(II) complexes of glycylhistidine, glycylhistidylglycine, histidylglycine, and carnosine have been obtained from pH titration data by minimising the computed standard deviation in titre for a range of metal ion and ligand concentrations. Account was taken of the formation of protonated, hydrolysed, and polynuclear complexes as well as of ML_n-type complexes. A comparison is made of the copper-complexing abilities of these peptides in competition with α-amino-acids under physiological conditions.

HISTIDINE forms stable chelate complexes with copper(II) ion, involving co-ordination to an imidazole nitrogen atom. The enhanced stability of the complexes, relative to those of the simpler α-amino-acids, has been demonstrated in a computed equilibrium distribution of copper(II) and zinc(II) ions among a mixture of naturally occurring amino-acids at concentrations found in blood plasma.[1] A similar study of the relative metal-binding ability of histidine-containing peptides in competition with amino-acids and peptides of simple amino-acids might serve as a model for interactions between metal ions and proteins, but such a quantitative comparison has hitherto been precluded by the paucity of relevant stability-constant data.

Most of the equilibrium constants reported by earlier workers for copper(II) complexes of histidine-containing peptides[2-8] have been obtained from potentiometric titrations on a single solution or for a small range of metal ion and ligand concentrations with a simple postulation of the types of complexes formed.

Because of the multiplicity of possible equilibria in metal–peptide solutions, evaluation of constants from titration data requires either that simplifying assumptions, aimed at identifying the more important equilibria, be made or that suitable computer-based analysis is carried out with programmes such as LETAGROP[9] or

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[6] R. Osterberg and B. Sjöberg, *Acta Chem. Scand.*, 1968, **22**, 689.
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[8] G. F. Bryce, R. W. Roeske, and F. R. N. Gurd, *J. Biol. Chem.*, 1966, **241**, 1072.
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SCOGS.[10] We have used the latter approach, applying SCOGS to pH-titration data for a range of metal ion and ligand concentrations so as to refine equilibrium constants for all complexes which might be expected to be present. The procedure has been described elsewhere.[11] A species was considered to be negligible when the computed standard deviation in titre was unaffected by its inclusion and, on successive cycles of refinement, its estimated stability constant failed to converge and the computed concentration of the species progressively diminished.

EXPERIMENTAL

Materials.—Solutions of L-histidine (Fluka puriss.), glycyl-L-histidine hydrochloride (Mann Research Laboratories), glycyl-L-histidylglycine (Sigma Chemical Co.), L-histidylglycine (Sigma Chemical Co.), and carnosine (Fluka purum) were prepared immediately before use in boiled-out, glass distilled, water from material dried under vacuum over P_2O_5. Stock solutions (*ca.* 0·025M) of copper nitrate (B.D.H. AnalaR), acidified with known concentrations of nitric acid, were standardised against EDTA.[12] Potassium hydroxide solutions, containing less than 0·2% (mol/mol) of carbonate, were prepared under nitrogen from freshly washed pellets (Merck, p.a.) and standardised as described previously.[11]

Methods.—Details of the experimental technique used in the determination of ionisation constants of ligands and metal-complex stability constants are given elsewhere.[11] Practical constants, for 37 °C and I = 0·15 (KNO_3) mol l^{-1}, were refined from the titration data using the programme SCOGS [10] on a Univac 1108 computer. Copper ion concentrations were varied over a five-fold range and the ligand concentrations were varied ten-fold. (For copper with glycylhistidine and carnosine, concentrations were 1—5mM for the metal ion, 1—10mM for the ligand, and for copper with histidylglycine and glycylhistidylglycine half these concentrations.) The titration data have been deposited as a supplementary publication (SUP No. 21144, 19 pp.).* In the calculations the activity coefficient was assumed to be 0·80.[11]

To investigate the possible formation of mixed-ligand complexes between copper ions, amino-acid (glycine or histidine) and peptide, potentiometric titrations were also carried out on solutions having known and approximately equimolar concentrations of copper nitrate, amino-acid, and peptide. Titration curves were compared with those computed from the known pK values and stability constants on the assumption that mixed complexes were negligible. Significant differences between computation and experiment would be presumptive evidence of mixed complex formation and would permit the attempted refinement of stability constants for mixed complexes to account quantitatively for these differences.

To compare the metal-binding ability of the two ligands present in a system small computer programmes (trivially named as MINICOMICS) were used. They were written in the Basic-type language FOCAL and were designed to fit a small PDP-8/I computer (Digital Equipment Corporation, Maynard, Mass., U.S.A.) which also controlled a graphic plotter (model 7200A, Hewlett-Packard, Palo Alto, Calif., U.S.A.) to draw the pH-composition profiles for the components of specified systems. By computing and plotting in this way theoretical titration curves for comparison with the corresponding experimental curves it was also possible to obtain a visual check on the results obtained from SCOGS over the entire pH range (3—9) covered in the potentiometric titrations.

RESULTS

Data from all titrations for each system were treated simultaneously in refining the sets of constants given in Table 1. However, because of the number of species likely to be present, preliminary estimates of some of the stability constants were sought. Also, because SCOGS is programmed to refine constants for equilibria expressed in terms of metal ion, free ligand (L), and protons, it was sometimes preferred to express tabulated constants in terms of equilibria other than those used by SCOGS. (For example, Cu + HL $\rightleftharpoons$ CuHL, instead of Cu + H + L $\rightleftharpoons$ CuHL, and HL $\rightleftharpoons$ H + L.) Where this has been done in Table 1 the standard deviations given for the equilibrium constants have been obtained from the computed standard deviations in the overall constants, using a published procedure for calculating the propagation of mean square errors.[13]

TABLE 1

Equilibrium constants of copper(II) complexes at 37 °C in I = 0·15(KNO_3) mol l^{-1} solutions

Equilibrium	log K ± S.D.
(1) Glycylhistidine (pK_a values 2·66 ± 0·01, 6·61 ± 0·01, 7·97 ± 0·01)	
Cu + L $\rightleftharpoons$ CuL	8·68 ± 0·01
Cu + 2L $\rightleftharpoons$ CuL_2	15·41 ± 0·01
Cu + HL $\rightleftharpoons$ CuHL	4·28 ± 0·07
Cu(L − H) + H $\rightleftharpoons$ CuL	4·14 ± 0·01
Cu(L − 2H) + H $\rightleftharpoons$ Cu(L − H)	9·48 ± 0·01
Cu + L + HL $\rightleftharpoons$ $CuHL_2$	12·48 ± 0·08
CuL(L − H) + H $\rightleftharpoons$ CuL_2	7·73 ± 0·02
(2) Gly·His·Gly (pK_a values 3·03 ± 0·01, 6·36 ± 0·01, 7·68 ± 0·01)	
Cu + L $\rightleftharpoons$ CuL	8·52 ± 0·04
Cu + 2L $\rightleftharpoons$ CuL_2	15·78 ± 0·04
Cu(L − H) + H $\rightleftharpoons$ CuL	3·20 ± 0·04
Cu(L − 2H) + H $\rightleftharpoons$ Cu(L − H)	9·01 ± 0·01
CuL(L − H) + H $\rightleftharpoons$ CuL_2	7·37 ± 0·05
(3) His·Gly (pK_a values 2·32 ± 0·02, 5·39 ± 0·02, 7·15 ± 0·01)	
Cu + L $\rightleftharpoons$ CuL	8·02 ± 0·01
Cu + 2L $\rightleftharpoons$ CuL_2	14·15 ± 0·01
Cu(L − H) + H $\rightleftharpoons$ CuL	6·30 ± 0·06
CuL(L − H) + H $\rightleftharpoons$ CuL_2	8·49 ± 0·04
$Cu(L-H)_2$ + H $\rightleftharpoons$ CuL(L − H)	9·76 ± 0·05
Cu + L + HL $\rightleftharpoons$ $CuHL_2$	11·41 ± 0·05
$Cu_2(L-H)_2$ + 2H $\rightleftharpoons$ 2CuL	9·15 ± 0·07
(4) Carnosine (pK_a values 2·64 ± 0·01, 6·58 ± 0·01, 9·04 ± 0·01)	
Cu + L $\rightleftharpoons$ CuL	8·14 ± 0·01
Cu + 2L $\rightleftharpoons$ CuL_2	14·39 ± 0·02
Cu + HL $\rightleftharpoons$ CuHL	3·98 ± 0·02
Cu(L − H) + H $\rightleftharpoons$ CuL	6·24 ± 0·05
Cu + L + HL $\rightleftharpoons$ $CuHL_2$	11·21 ± 0·05
CuL(L − H) + H $\rightleftharpoons$ CuL_2	8·69 ± 0·03
2CuL $\rightleftharpoons$ Cu_2L_2	2·28 ± 0·05
$Cu_2(L-H)_2$ + 2H $\rightleftharpoons$ 2CuL	8·32 ± 0·03

* See Notices to Authors No. 7 in *J.C.S. Dalton*, 1973, Index issue.

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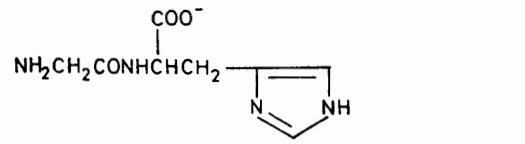
[13] W. E. Deming, 'Statistical Adjustment of Data,' Dover, New York, 1964, ch. 3.

Glycyl-L-histidine.—By analogy with other copper–peptide systems, the species CuHL, CuL, and Cu(L − H), where L is the anion (I), were considered likely to be the major complexes in the pH range 3·6—4·6 for copper(II) ion and glycylhistidine. (Also, pK values of 4·00, 4·50, and 9·25 have been reported [3,7] for proton loss from 1 : 1 copper–glycylhistidine complexes.) Analysis of the titration data in this pH range enabled approximate values of these stability constants to be obtained. These values were used with the data for pH 4·7—9·5 to seek estimates of constants for $CuHL_2$, CuL_2, CuL(L − H), and $Cu(L - H)_2$. Subsequent refinement of the set of seven constants led to values which reproduced the experimental titration curves to within a standard deviation in titre (SDT) of $2{\cdot}1 \times 10^{-3}$ ml in a maximum titration of 1 ml. There was no evidence that binuclear or higher complexes were formed. Computed and experimental titration data for copper–glycylhistidine are compared in the Figure.



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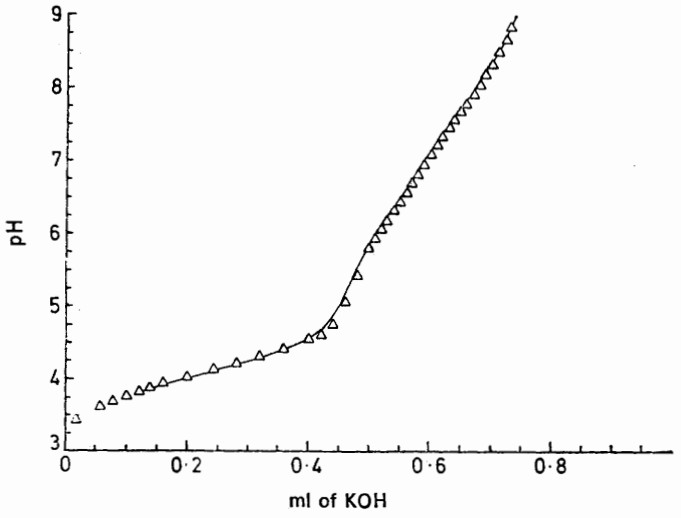


Computed curve and experimental points for titration of 0·0026M copper(II) and 0·0051M glycylhistidine

In the copper(II)–glycine or –histidine glycylhistidine systems (at concentrations around 0·005M) the titration curves, computed assuming no mixed-ligand complex formation, were almost identical with experiment, indicating that under these conditions mixed complex formation is not important. Similar results were obtained with the other peptides studied.

Other Peptides.—Except that equilibria involving CuHL and $CuHL_2$ were unimportant, quantitative analysis of the titration data for copper(II)–Gly·His·Gly closely paralleled the corresponding Gly·His results.

The greater part of the complex formation by L-histidylglycine is attributed to the five mono- and bis-complexes formed with copper(II) by the species L^- and $(L - H)^{2-}$. Formation of the binuclear complex $Cu_2(L - H)_2$ is also important.

Six of the equilibria in the carnosine system correspond to those for glycylhistidine. The other two are for binuclear complex formation.

DISCUSSION

The pK_a values of 2·65 and 7·97 for glycylhistidine are assigned to the carboxyl and protonated amino-groups, respectively. (Compare 3·23, 7·86 for glycylglycine.[14]) The pK_a of 6·61 is for the loss of a proton from the imidazolinium group. (Compare 7·08 at 25 °C and $I = 0{\cdot}16$ mol l^{-1} for acetylhistidine.[3]) The much greater value of log K_1 for copper(II)–glycylhistidine (8·68) than for copper(II)–glycylglycine (5·34 [14]) indicates differences in metal bonding. In the latter, the copper(II) ion is probably bonded through the terminal amino-group, the peptide nitrogen, and a carboxylate oxygen whereas in copper(II)–acetylhistidine (log $K_1 = 4{\cdot}35$ [3]) and monoprotonated copper(II)–glycylhistidine (CuHL, log $K = 4{\cdot}28$) metal bonding through the peptido- and imidazole-1-nitrogens is likely. Hence in 1 : 1 copper glycylhistidine the metal may be bonded through the amino-, peptido-, and imidazole-1-nitrogen atoms. X-Ray evidence on the crystalline copper glycylhistidine sesquihydrate is consistent with this: in the crystal these three nitrogen atoms are all approximately the same distance (2·0 Å) from the central copper ion while charge neutralisation is achieved by a carboxyl oxygen from another molecule of ligand.[15] Earlier potentiometric studies [3,7] and absorption spectra and o.r.d. measurements [16] also support this assignment. The high value of log β_2 (15·41) for bisglycylhistidinatocopper(II) would seem to imply six-co-ordination around the metal ion.

Earlier [7] results from the titration of a 1 : 1 mixture of copper(II) ion and glycylhistidine were interpreted as involving the loss of three protons from the 1 : 1 complex, with pK values of 4·00, 4·50, and 9·25 at 25 °C and $I = 0{\cdot}16$ mol l^{-1}. We find pK values of 4·14 and 9·48 (at 37 °C), and a third pK (of 7·73) for the loss of a proton from bisglycylhistidinatocopper(II) (CuL_2). The pK of 4·14 is probably for the ionisation of the peptide hydrogen atom and the pK of 9·48 may be for the loss of a proton from a water molecule co-ordinated to the copper ion in the complex. Martin and Edsall [3] attributed this pK to the ionisation of the pyrrole hydrogen of the imidazole ring. From the constants in Table 1 a pK of 3·57 ± 0·07 can also be calculated for $CuHL \longrightarrow CuL + H^+$.

The pK_a values of glycylhistidylglycine are assigned as for glycylhistidine, and similar metal binding is suggested to explain the high stability constants of the copper complexes CuL and CuL_2. The three pK values of the copper complexes are also similar to those for copper(II) with glycylhistidine but lower, as expected

[14] R. P. Agarwal and D. D. Perrin, *Trans. Royal Inst. Technol. Stockholm* (*Pure and Applied Chem.*), 1972, **34**, 387.

[15] J. F. Blount, K. A. Fraser, H. C. Freeman, J. T. Szymanski, and C. H. Wang, *Acta Cryst.*, 1967, **22**, 396.

[16] G. F. Bryce and F. R. N. Gurd, *J. Biol. Chem.*, 1966, **241**, 122.

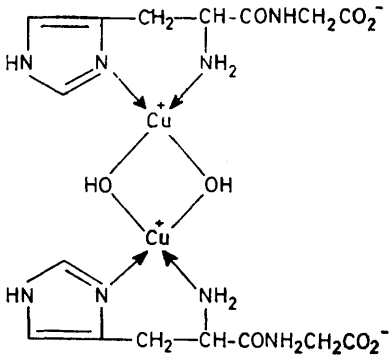
from the acid-strengthening effect of replacing carboxylate ion by an amido-group. The greater extent of formation of Cu(L — H) at low pH values would decrease the contributions made by species such as CuHL or $CuHL_2$. This probably explains why the latter were negligible over the pH titration range 3·4—9·4.

A study of atomic models suggests that the peptide, imidazole, and amine nitrogen atoms in histidylglycinatocopper(II) ion cannot all co-ordinate to the metal ion to form a square-planar complex. Agreement of values of log K_1 (8·02) and log β_2 (14·15) with those for the corresponding copper complexes with histidine methyl ester [8·14, 13·86 at 40 °C, I = 0·25 (KCl) mol l^{-1} [17]] suggests that only the imidazole and amine atoms are involved. Log K_1 is much greater than for the glycylglycinatocopper(II) complex (5·34 [14]), in which bonding is through the amino-nitrogen, the peptide nitrogen, and a carboxylate oxygen.

The weaker interaction of the peptide nitrogen in histidylglycinatocopper(II) is shown in its much higher pK values. The pK for CuL is 6·30 when HL is His·Gly: compare 4·14 for Gly·His, 3·20 for Gly·His·Gly.

By analogy with $[Cu_2(OH)_2A_2]^{2+}$, where A = histamine,[18] the blue histidylglycine complex $Cu_2(L - H)_2$ is believed to be the hydroxo-bridged species (II).

Log K_1 for copper(II)–carnosine is less than for glycylhistidine, although in both complexes co-ordination is probably through three nitrogen atoms. This could be due to the formation of two six-membered chelate rings in

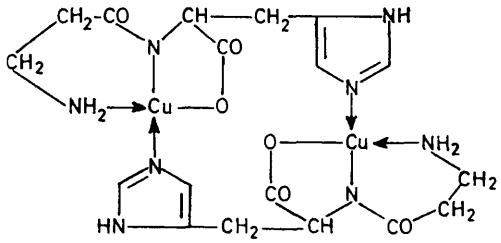


(II)

the former, as against a six- and a five-membered chelate ring in the latter. Alternatively, the difference may be due to the greater basicity of the amino-group, resulting from the interposition of the extra methylene group between the amino- and peptide portions of the molecule. By contrast, and as expected from their molecular environment, the pK values for the carboxyls and the imidazole portions of the two peptides are almost identical. The high values of log β_2 for the copper(II) complexes of carnosine and glycylhistidine suggest that the metal bonding is again similar. This resemblance is further supported by values of log K for CuHL (4·28 for glycylhistidine, 3·98 for carnosine).

The pK values of CuL and CuL_2 are similar for histidylglycine and carnosine. However, the different location of the free amino-group in carnosine precludes the formation of a copper(II)–carnosine complex analogous to (II). Different types of structure are postulated for the species Cu_2L_2 and $Cu_2(L - H)_2$ for carnosine, based on the observation of Freeman and Szymanski,[19] from crystallographic studies, that solid copper(II)–carnosine dihydrate is dimeric and bonded as shown in (III). Our species $Cu_2(L - H)_2$ may be identical with (III) with Cu_2L_2 as the corresponding complex in which the peptide hydrogen atoms are still present.

Using pH titration data for an 0·01M equimolar mixture of copper(II) ions and glycylhistidine, Martin



(III)

and Edsall [3] calculated the pK values for successive proton loss from the 1 : 1 copper complex on the assumption that all copper was present as the complex. Based on the equilibrium constants given in Table 1 we estimate that at pH values below 4·2 more than 30% of the total copper would have been present as copper(II) ion. Hence the reported pK values of 4·0 and 4·5 are probably composite and are due to the reactions

$$Cu^{2+} + H_2L^+ \longrightarrow CuL^+ + 2H^+$$
$$CuL^+ \longrightarrow Cu(L - H) + H^+$$

Similarly, for the 1 : 1 copper–carnosine system we believe that the pK values reported [3] as 5·00 and 5·55 are spurious and arise from the presence of uncomplexed ion and ligand and the formation of the binuclear complex $Cu_2(L - H)_2$. At pH 5·5 and total concentrations of 0·005M, computed composition ratios are $Cu_2(L - H)_2$, 33%; CuL, 26%; Cu^{2+}, 13%; Cu_2L_2, 13%; CuHL, 6%; Cu(L — H), 5%. Lenz and Martell [4] analysed the titration curve for a 1 : 1 approximately 0·005M copper–carnosine solution by assuming the formation only of CuHL, CuL, and Cu(L — H). However, calculations based on Table 1 show that for such a solution the major component over most of the pH range is the dimer $Cu_2(L - H)_2$. Above pH 6 it accounts for more than 70% of the total copper, exceeding 90% at pH 7·5. Even when the ligand is present in excess this species is still the major one. To a first approximation, the titration curves of copper–carnosine mixtures

[17] A. C. Andrews and D. M. Bebolsky, *J. Chem. Soc.*, 1965, 742.
[18] D. D. Perrin and V. S. Sharma, *J. Inorg. Nuclear Chem.*, 1966, **28**, 1271.
[19] H. C. Freeman and J. T. Szymanski, *Acta Cryst.*, 1967, **22**, 406.

TABLE 2

Computed distribution of Cu^{II} ions among 17 amino-acids and one peptide at 37 °C and pH 7·4

Constants and total concentrations were from ref. 1 and the present work. The concentration of each complex is expressed as a percentage of the total copper(II) present, and all complexes exceeding 0·2% of this total are shown.

	Distribution				
	No peptide	Gly·His	Gly·His·Gly	His·Gly	Carn
Complex	(%)	(%)	(%)	(%)	(%)
Cu·Cystine·His	45	16	2	42	45
Cu·H·Cystine·His	35	12	2	33	35
$Cu(His)_2$	19	7	1	18	19
$Cu(Gln)_2$	0·3			0·3	0·3
Cu·(OH)·His	0·2			0·2	0·2
$Cu·H(His)_2$	0·3			0·3	0·3
Cu·His	0·2			0·2	0·2
Cu(L − H)		61	88	0·9	(0·04)
CuL_2		2·5	2	4	
CuL(L − H)		1	3	0·3	
Cu(L − 2H)		0·5	2		

are fitted on the assumption that stoicheiometrically only one carnosine molecule is bound by each copper atom.[2]

The effects of the peptides on the computed distribution of copper(II) ions among amino-acids in blood plasma [1] were examined. In the absence of known values for average concentrations of individual peptides in human blood plasma the concentration of each peptide in turn was arbitrarily set equal to the average histidine content (74 μM). Results for pH 7·4 and 37 °C are given in Table 2. Under these conditions carnosine is unable to compete with histidine and cystine for copper(II), and histidylglycine is only marginally effective. The greater stabilities of the copper complexes of glycylhistidine and especially glycylhistidylglycine result in 65 and 95%, respectively, of the copper being displaced from the amino-acids by the peptides.

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