Studies on Transition-metal–Peptide Complexes. Part 7.† Copper(II) Complexes of Dipeptides containing L-Histidine

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The copper(II) complexes of three dipeptides (HA) containing L-histidine residues [L-carnosine, L-histidylglycine (HisGly), and glycyl-L-histidine (GlyHis)] have been studied by pH-metric, spectrophotometric, and, in part, ^{1}H n.m.r. and calorimetric methods. With L-carnosine and HisGly, the formation of a dimeric complex $[Cu_2A_2H_{-2}]$ was found, in which the co-ordination of copper(II) is glycylglycine-like, while the fourth co-ordination site is occupied by the imidazole N^3 nitrogen atom, forming a bridge between two copper(II) ions. An excess of HisGly suppresses the deprotonation of the peptide linkage and the complex $[CuA_2]$ is formed with histidine-like co-ordination. It is found that deprotonation of the peptide group is the easiest in the copper(II) complexes of GlyHis. The effect of co-ordination on deprotonation of the pyrrole-NH group is discussed.

Investigation of the complexes of β-alanine-containing dipeptides has recently received increasing attention;[1] β-alanine is a component of carnosine (β-alanyl-L-histidine), a naturally occurring dipeptide. Although its biochemical function has not yet been elucidated, Brown *et al.*[2] recently suggested the role of its copper(II) complex in Wilson's disease.

The copper(II)–carnosine dimeric complex was prepared and its crystal structure determined by Freeman and Szymanski.[3] The ligand was found to be co-ordinated to the metal *via* the N(amino), O(carboxylate), and N(amide) donor atoms, while the N^3 nitrogen atoms of the imidazole molecules bridge the copper(II) ions. At the same time, the possibility has also been suggested of binuclear complex formation in solutions of other copper(II)–small peptide systems.[3–5]

The importance of the histidyl residues of proteins in the binding of copper(II) has been emphasised by Sundberg and Martin[6] who, among others, drew attention to the necessity of clarifying the mode of co-ordination of the simplest histidine-containing dipeptides, such as glycyl-L-histidine (GlyHis), L-histidylglycine (HisGly), and carnosine (ligands HA), to copper(II). The same authors have also reviewed the results achieved in this field up to the early seventies. Reinterpreting the ^{1}H n.m.r. data of Ihnat and Bersohn[7] and also taking into account other spectral studies,[8] they confirmed that for the copper(II)–carnosine system in solution around neutral pH the dimeric complex exists, as was found in the solid.[3] As a result and also with consideration to other histidine dipeptides, some questions inevitably arose: (*i*) the co-ordination ability of the peptide linkage; this largely depends on the neighbouring donor groups, as for instance in the case of sulphydryl-containing dipeptides;[9] (*ii*) the role of the N^3 donor atom in the binding; and (*iii*) dimeric and bis-complex formation and their dependence on the metal to ligand ratio and pH.

The species existing in the equilibrium systems considered are mostly governed by the factors mentioned above. In fact, the recently elaborated computational methods make it possible to fit the potentiometric data with postulated species of different compositions. This may also explain why at times divergent results have recently been presented for the copper(II) interactions with GlyHis, HisGly, and carnosine.[10–12] Another reason might be that the various spectral studies are not always supplemented with wide-ranging equilibrium measurements[2,13–16] and *vice versa*.[10–12] Thus, for instance, the formation of $[CuAH_{-1}]$ is very probable in the copper(II)–GlyHis system,[6,17] but the processes taking place in the presence of excess ligand have not been satisfactorily clarified. In addition, evidence has been given[10,18] for dimeric complex formation between HisGly and copper(II) after the amide deprotonation between pH 5 and 8. On the other hand, on the basis of various spectral measurements at pH 5, in addition to the dimeric complex, the existence of various types of monomeric species has recently been supposed even in the copper(II)–carnosine system.[2,13,15] Moreover, both equilibrium studies[12] and e.s.r. investigations[14] allow the possibility of the formation of a complex with 1 : 2 composition.

Arising from the literature results, we aimed to contribute to the further clarification of the interactions of copper(II) with GlyHis, HisGly, and carnosine (ligands HA). The wide-ranging equilibrium measurements have been complemented with visible absorption spectra, and partly with ^{1}H n.m.r. relaxation and calorimetric measurements.

Experimental

L-Carnosine (BDH), glycyl-L-histidine hydrochloride monohydrate, and L-histidylglycine (Sigma) were used without purification. Other chemicals were obtained from Reanal. The concentration of the copper(II) chloride stock solution was checked gravimetrically.

The pH-metric titrations were performed in the concentration range 1×10^{-3}—6×10^{-3} mol dm^{-3} at metal to ligand ratios between 1 : 1 and 1 : 5. The pH-metric titrations and calculation of the stability constants were carried out as reported earlier.[5] The experiments were performed on a Radiometer PHM 64 pH-meter, using G202 B glass and K401 calomel electrodes. All titrations were carried out at $I = 0.2$ mol dm^{-3} (KCl) at 25 °C.

Visible spectrophotometric measurements were carried out on a Beckman ACTA MIV double-beam recording spectrophotometer. The use of flow cells made it possible to observe the pH and the absorbance of solutions simultaneously. Detailed experimental conditions of spectrophotometric measurements are listed in Table 1.

Hydrogen-1 n.m.r. relaxation studies were carried out on an N20 pulsed ^{1}H n.m.r. spectrometer. The variation of the transverse relaxation time of water protons was measured as a function of pH in the copper(II)–carnosine (1 : 1) system

† Part 6. A. Gergely and E. Farkas, *J. Chem. Soc., Dalton Trans.*, 1982, 381.

Table 1. Positions of the absorption bands ($\lambda_{max.}$/nm) of the copper(II) complexes

HA	$[Cu^{2+}]$/ mol dm^{-3}	[HA]/ mol dm^{-3}	[KOH]/[HA] 2.0	2.5	3.0	4.0	5.0
L-Carnosine	0.02	0.02	710		640	605	605
	0.04	0.04	710		635	605	605
	0.02	0.04	640	605	600		
HisGly	0.005	0.005			660	615	625
	0.005	0.01	665	645	625	625	
GlyHis	0.005	0.005	625		615	600	556
	0.005	0.01	620	605	575	558	

at different absolute concentrations (5, 10, or 20 × 10^{-3} mol dm^{-3}). The calculation of molar relaxation coefficients has been reported earlier.[19]

Samples containing copper(II) and carnosine in a mol ratio of 1 : 2 were studied calorimetrically on an LKB-8700 reaction and solution calorimeter. Carnosine was used in a concentration of 6 × 10^{-3} mol dm^{-3} during the experiments.

Results and Discussion

The pH-metric titration curves of carnosine, HisGly, and GlyHis are shown in Figure 1. It is evident from Figure 1 that up to pH 8 four equivalents of base are titrated in the copper(II)–carnosine (1 : 1) system, which can be explained either by the co-ordination of AH_{-1} with a deprotonated amide nitrogen or by the hydrolysis of the complex. The titration curve of the solution at a mol ratio of 1 : 2 reveals that 50% of the carnosine is titrated as free ligand. In accordance with the finding of Martin and co-workers,[20] this observation strongly suggests the formation of a species with 1 : 1 composition, in which the peptide amide is deprotonated.

As observed by Aiba *et al.*[10] the pH-metric curve for copper(II)–HisGly (1 : 1) is very similar to that for carnosine, but the titration of a fourth equivalent appears at higher pH in a separate process. When the metal to ligand ratio is 1 : 2, however, only three equivalents of base are titrated, without the appearance of free ligand. Thus, according to results found earlier,[11,12] if excess HisGly is present, bis-complex formation is probable in which deprotonation of the peptide linkage may be suppressed.

The titration curves for the copper(II)–GlyHis systems at any metal to ligand ratio can be regarded as similar to those for carnosine. Hence, the formation of a species $[CuAH_{-1}]$ takes place even in the presence of excess ligand. However, from the pH-metric data the formation of bis complexes between pH 6 and 9 cannot be excluded.

Spectrophotometric measurements were performed to identify the formation of different complexes in the equilibrium systems. The values thereby obtained serve as auxiliary data in the evaluation of the pH-metric results. The visible spectra of the samples at various metal to ligand ratios were recorded as a function of pH or equivalents of base added. The wavelengths of the absorption bands are listed in Table 1.

The interpretation of the spectral data strongly supports the conclusions obtained from the pH-metric titration curves. In particular, in the copper(II)–carnosine system at a 1 : 1 ratio, after the addition of four equivalents of base the absorption maximum is at 605 nm. The same band appears in a 1 : 2 solution when 2.5 equivalents of base are added, and there is no further change in the absorption up to pH 10. The results confirm those of the above-mentioned pH-metric measurements. Thus, the existence of a bis complex in the equilibrium system may be considered negligible, whereas the measurements might allow the interpretation that the species $[CuAH_{-1}]$ is formed. However, the intensity of the absorption obeys the Beer-Lambert law, and hence the process $[Cu_2A_2H_{-2}] \rightleftharpoons 2\,[CuAH_{-1}]$ does not seem to play a significant role in the equilibrium. Nevertheless, the spectral and pH-metric measurements alone do not provide sufficient evidence as to whether the formation of a deprotonated complex $[Cu_2A_2H_{-2}]$ or $[CuAH_{-1}]$ is favoured.

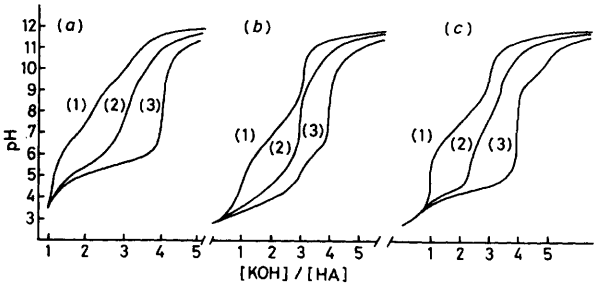


Figure 1. pH Titration curves of (*a*) copper(II)–L-carnosine, (*b*) copper(II)–HisGly, and (*c*) copper(II)–GlyHis. (1) [Cu] = 0; (2) [Cu] = 2 × 10^{-3}; (3) [Cu] = 4 × 10^{-3} mol dm^{-3}. [A] = 4 × 10^{-3} mol dm^{-3}

In Figure 1 there are two inflections in the titration curve of copper(II)–HisGly at a metal to ligand ratio of 1 : 1 at pH ~5 for three, and at pH ~8 for four equivalents. The first inflection point corresponds in the visible absorption spectrum to a maximum at 660 nm, while the second, with a significant blue shift, appears at 615 nm. In agreement with previous findings,[9,17] the former band also suggests participation of oxygen donor atoms in the co-ordination. The latter band is a consequence of the bonding of the deprotonated amide. Otherwise, this band does not occur when the metal to ligand ratio is 1 : 2 at any pH, which gives the possibility for conclusions as to bis-complex formation.

The spectral behaviour of the copper(II)–GlyHis system differs from those outlined above. At a metal to ligand ratio of 1 : 1, when two equivalents of base are added, the absorption band already appears at 625 nm. With two additional equivalents of base (*i.e.* a total of four), the absorption maximum shifts to 600 nm, suggesting co-ordination of nitrogen donor atoms to the copper(II).[10,17] If the ligand is in excess (1 : 2) this band occurs at 2.5 base equivalents, which is in good agreement with the process $[CuAH_{-1}] + HA$. In contrast with the copper(II)–carnosine system, with an increase of pH to *ca.* 9 the blue shift changes further to 575 nm. These findings support the suggestion of Agarwal and Perrin [11] and Brookes and Pettit [12] that bis complexes are formed with GlyHis above pH 6.

With regard to the results of both the pH-metric and the visible spectral experiments, conclusions were drawn on the species existing in the equilibrium systems. In accordance with this, computer analysis of the pH-titration data and calculation of the stability constants of the complexes formed between pH 3.5 and 8 were carried out. The values obtained are listed in Table 2; the compositions of the species require careful consideration.

(*i*) As demonstrated in Figure 2, the dimeric species $[Cu_2A_2H_{-2}]$, in which the amide participates in deprotonated form, dominates in the copper(II)–carnosine system. The pH-metric data, however, are well fitted with the assumption of the presence of the species $[CuAH_{-1}]$ as well. If this is true then there will be no obstacle to the formation of the monomeric complexes $[CuA_2H_{-1}]^-$ or $[CuA_2]$ either. The evaluation of the pH-metric data was therefore performed on these grounds. However, the assumption of these species was not confirmed by a better fitting of the experimental data.

The formation of bis complexes was recently suggested again

Table 2. Stability constants (log β_{pqr}) * of copper(II) complexes with L-carnosine, HisGly, and GlyHis: I = 0.2 mol dm^{-3} (KCl), 25 °C

	L-Carnosine	HisGly	GlyHis
HA	9.30	7.59	8.22
H_2A^+	16.14	13.53	14.99
H_3A^{2+}	18.67	16.49	17.50
$[CuAH]^{2+}$	13.26		12.45
$[CuA]^+$	8.25	8.85	9.06
$[CuA_2]$		15.06	15.96
$[CuAH_{-1}]$			4.91
$[CuA_2H_{-1}]^-$			8.02
$[Cu_2A_2H_{-2}]$	8.18	8.20	
$[CuAH_{-2}]^-$	−8.9		

* $\beta_{pqr} = [Cu_pA_qH_r]/[Cu]^p[A]^q[H]^r$.

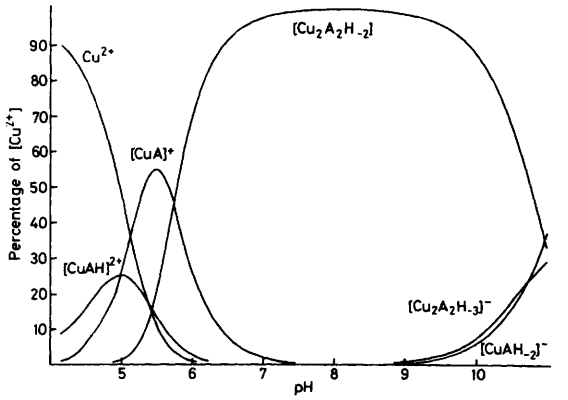


Figure 2. Concentration distribution of the complexes formed in the copper(II)–L-carnosine system as a function of pH; [Cu] = [A] = 2 × 10^{-3} mol dm^{-3}

on the basis of various spectral investigations in the case when the ligand is present in excess.[2,13,14] It was not questioned by Brown *et al.* and Antholine [2,13] that in an equimolar solution of copper(II) and carnosine the dimeric complex is formed exclusively. Our ^{1}H n.m.r. measurements confirmed this finding. In fact, for the molar relaxation coefficients of the complexes $[CuAH]^{2+}$, $[CuA]^+$, $[Cu_2A_2H_{-2}]$, and $[CuAH_{-2}]^-$, the following values were obtained: 2 000, 1 480, 330, and 2 400 mol^{-1} s^{-1}, respectively. The value for $[Cu_2A_2H_{-2}]$ is in good agreement with the data expected for complexes containing only axially bonded water molecules. (Previously [19] 400 mol^{-1} s^{-1} was found for the $[CuA_2]$-type complexes of several amino-acids.) In contrast, by assuming $[CuAH_{-1}]$ instead of $[Cu_2A_2H_{-2}]$, the molar relaxation coefficient is only 170 mol^{-1} s^{-1}; this value cannot be explained.

Calorimetric measurements support the formation of the dimeric complex $[Cu_2A_2H_{-2}]$ if excess ligand is present and the pH is between 6.5 and 9. For $\Delta H_{[Cu_2A_2H_{-2}]}$ a value of −50 ± 2 kJ mol^{-1} was obtained, which is far beyond that corresponding to $\Delta H_{[CuAH_{-1}]}$ (*ca.* −3 kJ mol^{-1}) from simple aliphatic dipeptides.[5] The large ΔH value may be best explained in terms of the structure determined by Freeman and Szymanski,[3] in which two N^3 (imidazole) nitrogens bridge the $[CuAH_{-1}]$ units. Indeed, the value of 50 kJ mol^{-1} may be considered as approximately referring to the heat of formation of the bridging donor atoms. At the same time, these thermodynamic data do not exclude the possibility that, when the ligand is present in larger excess, monomeric complexes are also formed.[2,13,14]

(*ii*) The concentration distribution of the species formed in

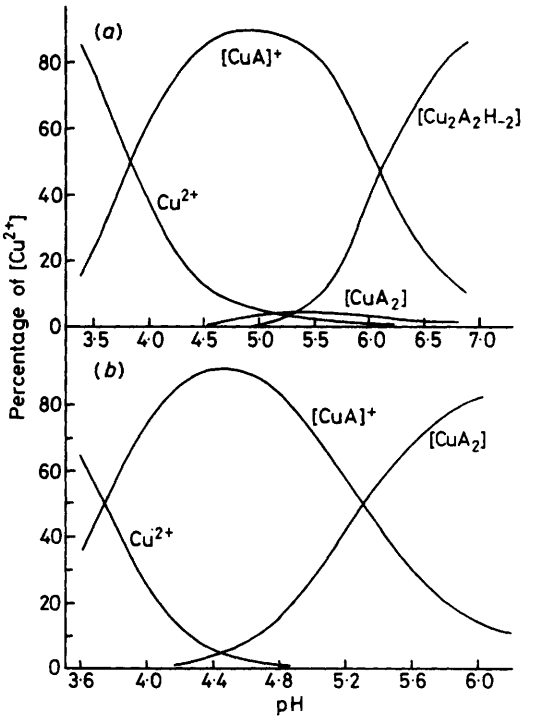


Figure 3. Concentration distribution of the complexes formed in the copper(II)–HisGly system as a function of pH; (*a*) [Cu] = [A] = 4 × 10^{-3} mol dm^{-3}, (*b*) [Cu] = 2 × 10^{-3}, [A] = 4 × 10^{-3} mol dm^{-3}

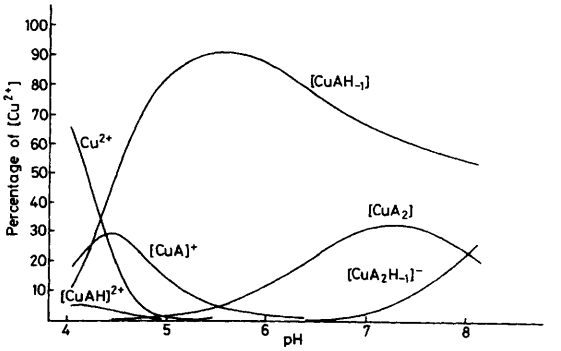


Figure 4. Concentration distribution of the complexes formed in the copper(II)–GlyHis system as a function of pH; [Cu] = 2 × 10^{-3}, [A] = 4 × 10^{-3} mol dm^{-3}

the copper(II)–HisGly system is plotted as a function of pH in Figure 3. It is clear that at a 1 : 1 mol ratio the dimeric complex $[Cu_2A_2H_{-2}]$ is formed exclusively around pH 7. However, in contrast with carnosine, an excess of HisGly suppresses [18] the deprotonation of the peptide-NH group, and the bis complex of $[CuA_2]$ dominates by pH 8 (not depicted in Figure 2). Otherwise, the value of log K_1/K_2 = 2.64 corresponds to the histamine-like co-ordination.[21] This type of bonding is well supported by the ^{13}C n.m.r. experiments carried out at pH 7.0 by Volter *et al.*[22]

(*iii*) In the case of GlyHis the most favourable is the formation of a monomeric complex $[CuAH_{-1}]$, as is clear from the concentration distribution curves in Figure 4.

In the presence of excess ligand the monomeric $[CuAH_{-1}]$ can transform to bis complexes with the stoicheiometric compositions $[CuA_2]$ and $[CuA_2H_{-1}]^-$. The concentration of $[CuA_2]$ can reach even 30%, which is very high in comparison to other copper(II)–dipeptide systems.[4,5]

An account of bis-complex formation can be given on the basis of the visible spectral measurements. In the pH range 6—8 the absorption bands shift to lower wavelengths; this cannot be explained *via* a decrease in the $[CuAH_{-1}]$ concentration by the substitution of the deprotonated AH_{-1} ligand with A. On the contrary, the AH_{-1} remains bonded to the copper(II), while a mixed type complex originates *via* the N^3 (imidazole) of the second GlyHis. The composition of this species is $[Cu(AH_{-1})(HA)]$ instead of $[CuA_2]$, which means that the amino-group of the second ligand is in protonated form. The deprotonation constant of the process $[CuA_2] \rightleftharpoons [CuA_2H_{-1}]^- + H^+$ is 7.94, a value reminiscent of the corresponding data for the free ligand ($pK_3 = 8.22$).

In connection with the complex composition, the histidine dipeptides above were regarded to involve the potential donor atoms N(amino), O(carboxylate), N(amide), and N^3 (imidazole). Nevertheless, the N^1H(imidazole) might be considered as a group, the deprotonation constant of which is strongly influenced [21] by both the metal and the presence of another ligand (in the case of the mixed complex). In agreement with this finding, differences between the behaviour of the three ligands at high pH have also been observed.

As Figure 1 shows, a fifth equivalent of base is consumed in the copper(II)–GlyHis system above pH 9. This process simultaneously results in a significant blue shift in the absorption band. In agreement with the finding of Morris and Martin,[17] the phenomenon can be interpreted by the formation of a tetrameric complex $[Cu_4A_4H_{-8}]^{4-}$, in which the fourth co-ordination site of the copper(II) is occupied by the deprotonated N^1 (imidazole) donor atom.

In an equimolar solution of copper(II) and HisGly there is a slight red shift in the absorption band above pH 11. This change can in all probability be explained by the formation of hydroxo-complexes. On the other hand, in the presence of excess ligand there is a new base-consuming process above pH 10, which strongly suggests that the histidine residue of HisGly in $[CuA_2]$ behaves similarly to simple histidine or histamine, *i.e.* deprotonation in parallel with the hydrolysis takes place at *ca.* pH 11.[21]

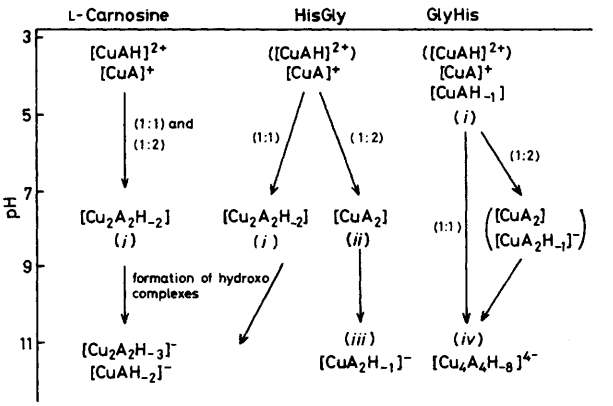
The features of carnosine in basic solution are totally or partly different from those of the other two ligands. Namely, up to about pH 12, deprotonation of the N^1H(imidazole) group cannot be observed at all. However, above pH 9, $[Cu_2A_2H_{-3}]^-$ and the hydroxo-complex $[CuAH_{-2}]^-$ are formed. The reason for the absence of deprotonation can easily be explained by the fact that the N^3(imidazole) nitrogens are not involved in the chelate ring formation in the dimeric complex.

Conclusions

The results in the previous section revealed that various complexes can be formed with the three histidine-containing dipeptides, depending on the available nitrogen donor atoms and on the metal to ligand ratio. The differences between the complex-forming abilities of the ligands are outlined in the Scheme, where the possible pathways of the interaction with copper(II) are shown. From the Scheme several results arise.

(*a*) The co-ordination of carnosine and HisGly is similar to that of glycylglycine in that each ligand is bonded *via* the amino, peptide, and carboxylate donor groups. In contrast with glycylglycine, however, dimers $[Cu_2A_2H_{-2}]$ are formed with carnosine and HisGly, in which the copper(II) atoms are joined *via* the N^3(imidazole) nitrogen donors.

(*b*) An excess of HisGly prevents deprotonation of the peptide linkage, and therefore in $[CuA_2]$ the ligand is bonded histamine-like. In the case of carnosine the bis complex is not present at all, while with GlyHis the bis-complex formation is partly connected with amide deprotonation.



Scheme. (*i*) deprotonated peptide-N; (*ii*) protonated peptide-NH; (*iii*) hydrolysis and deprotonation of N^1H(imidazole); (*iv*) deprotonation of N^1H(imidazole)

(*c*) The geometrical positions of the donor atoms in GlyHis makes the co-ordination of three nitrogens (amino, peptide, and imidazole) possible, which results in a very stable monomeric complex $[CuAH_{-1}]$. The monomer has one free co-ordination site where another GlyHis molecule can be bound *via* the N^3(imidazole) nitrogen with stoicheiometries of $[Cu(AH_{-1})(HA)]$ and $[CuA_2H_{-1}]^-$.

(*d*) the N^1H(imidazole) deprotonation is favoured only when the N^3(imidazole) nitrogen is involved in a chelate ring ($[CuAH_{-1}]$ for GlyHis, and $[CuA_2]$ for HisGly). The pyrrole-NH deprotonation of GlyHis is especially favoured because of the polynuclear complex formation *via* these donor atoms. (The deprotonation constant, pK, for the $[CuAH_{-1}]$ complex of GlyHis is ~10.5.)

(*e*) The imidazole residues of carnosine and HisGly do not take part in the chelate-ring formation. Therefore, the hydrolysis of $[Cu_2A_2H_{-2}]$ is favoured over the deprotonation of the N^1H(imidazole) group.

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Received 1*st April* 1982; *Paper* 2/554