Transition-metal Complexes of Carcinine, a Peptide-type Derivative of Histamine†

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The acid–base properties and copper(II), nickel(II) and cobalt(II) complexes of carcinine {3-amino-*N*-[2-(imidazol-4-yl)ethyl]propanamide} and of *N-tert*-butoxycarbonylcarcinine have been studied at 25 °C by pH-metric, spectrophotometric and, in part, ^{1}H NMR and EPR methods, and compared to those of histidine-containing dipeptides. Complexes of the type M(HL), ML and ML_2 (charges omitted) are found in the cobalt(II) and nickel(II) systems, with the additional presence of ML_2H_{-1} for the latter system. Copper(II) forms CuL_n ($n \leqslant 4$) complexes with *N-tert*-butoxycarbonylcarcinine molecules acting as monodentate ligands. The copper(II)–carcinine system in the range pH 3–9 shows the expected series of 1:1 complexes, more or less deprotonated depending on the pH, of type Cu(HL), CuL and $CuLH_{-1}$, and, for an excess of ligand, the unusual 1:4 complex CuL_4H_2 involving four N(3) inidazole nitrogens equatorially co-ordinated. At higher pH (>9) the monomeric $CuLH_{-2}$ and polynuclear $Cu_4L_4H_{-8}$ complexes are formed depending on the total concentration. The tetrameric species probably involves co-ordination of deprotonated N(1)-pyrrole nitrogens, with consequent formation of imidazolate bridges. The co-ordination ability of the peptide nitrogen in histidyl- and histamine-peptides is shown to depend on the size of the chelate rings formed and the existence of a neighbouring carboxyl group in the ligand.

Carcinine {β-alanylhistamine or 3-amino-*N*-[2-(imidazol-4-yl)-ethyl]propanamide} was discovered by Arnould and Frentz[1,2] in cardiac tissue of the Crustacean *Carcinus maenas* in 1973, and was then identified in several tissues of the rat, guinea-pig, mouse and human,[3] in levels as high as or higher than those reported for the related imidazole compounds carnosine (β-alanylhistidine), histidine and histamine (imidazole-4-ethanamine). The rapid incorporation (in a few minutes) of radioisotopic tracers (^{3}H-labelled histidine) into carcinine, carnosine and histamine in rat tissues showed that carcinine is metabolically linked to both histamine and carnosine.[3] The assumed relationship between these compounds seems to have special relevance in the physiologic response to stress.[3] It was also found that carcinine, unlike carnosine, exerted a dose-dependent positive inotropic effect, similar to that of histamine, in guinea-pig hearts.[4] Furthermore, carcinine and carnosine represent a large portion of mammalian non-protein intracellular nitrogen stores, and may serve as a reservoir of histamine. All these facts led to the conclusion that carcinine might have a role in the carnosine–histidine–histamine metabolic pathway and in both the mammalian cardiac physiology and cardiovascular response to stress.[4]

In all these compounds it is well known that the imidazole ring may play an important role as metal-ion binding site, as has been shown in the case of metalloproteins. The complexes formed between di- and tri-peptide derivatives of histidine and transition-metal ions, mostly copper(II), have been studied extensively.[5–12] Various types of complexes have been found with histidine-containing oligopeptides involving either the terminal amino or carboxylate ends as binding sites, the peptide nitrogen (after its deprotonation), or the N(3) nitrogen of the imidazole ring. The co-ordination ability of the peptide nitrogen, itself depending on the nature and the size of the resulting chelate rings, and the position of the imidazole ring in the peptide chain are indeed relevant factors in the structure of the complexes.

Thus two species appear to be important in the copper(II)–carnosine system at physiological pH, depending on the metal ion to ligand ratio. At high excess of ligand a monomeric complex CuL_4 is formed with four equatorial carnosine ligands each bound by the imidazole N(3) nitrogen[6,13,14] while a dimeric complex $Cu_2L_2H_{-2}$ is found at equimolar concentrations of ligand and Cu^{II}, in which the imidazole ring bridges the copper(II) ions *via* the N(3) nitrogen.[5] Tetrameric polynuclear complexes of the type $Cu_4L_4H_{-8}$, in which four copper(II) ions are bridged by imidazole rings *via* both N(3) and N(1) nitrogens, have also been proposed with both glycylhistidine[7] and glycylhistidylglycine[8] as ligands in the alkaline pH range (>9).

The biological interest of carcinine suggests the utility of similar investigations using this ligand, with the additional interest of allowing comparisons with the peptides mentioned above in order to understand their co-ordination properties. The present paper reports the protonation and metal-ion-co-ordination properties of carcinine, and further investigations are currently extending this field of research to a variety of histamine peptides.

Experimental

Materials.—*N-tert*-Butoxycarbonylcarcinine was synthesised in our laboratory from *N-tert*-butoxycarbonyl-β-alanine and histamine hydrochloride (Sigma products) according to a procedure to be described in a subsequent publication.[15] Carcinine was then obtained by release of the protecting *tert*-butoxycarbonyl group with hydrochloric acid. The structure of these compounds was confirmed by ^{1}H NMR spectroscopy, and their purity was checked by NMR spectroscopy, elemental analysis (C, H, N, Cl) and acid–base titration. Metal ions were obtained from the commercial perchlorates (Ni^{II} and Co^{II}; Alpha Ventron; Cu^{II}; Fluka). Stock solutions of metal perchlorates were standardized complexometrically.

pH-*Metric Measurements.*—The protonation and co-ordination equilibria were investigated by potentiometric titrations at 25.0 ± 0.1 °C under a nitrogen atmosphere at constant ionic strength (0.1 mol dm^{-3} $NaClO_4$). Changes in pH were followed

† *Non-SI unit employed:* G = 10^{-4} T.

Table 1 Stability constants of complexes of carcinine with H^+, Ni^{II}, Co^{II} and Cu^{II} (logarithmic values), $\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$, $I = 0.1$ mol dm^{-3} $NaClO_4$, $T = 298$ K, with estimated errors in parentheses (last two digits)

	Cation				
	H^+	Ni^{2+}	Co^{2+}	Cu^{2+}	λ_{max}^{d-d}/nm
(*a*) Carcinine					
β_{011} (HL)	9.23(01)				
β_{012} (H_2L)	16.07(01)				
β_{111} [M(HL)]		11.84(03)	11.06(10)	12.78(02)	760
β_{110} (ML)		4.21(03)	2.81(05)	6.91(05)	676
β_{120} (ML_2)		6.96(04)	5.31(01)		
β_{142} (ML_4H_2)				34.10(10)	592
β_{11-1} (MLH_{-1})				−0.36(06)	620
β_{11-2} (MLH_{-2})				−10.60(08)	592
β_{44-8} ($M_4L_4H_{-8}$)				−33.24(10)	562
β_{12-1} (ML_2H_{-1})		−2.93			
$pK_{ML}^{M(HL)}$		7.63	8.25	5.87	
$pK_{MLH_{-1}}^{ML}$				7.27	
$pK_{MLH_{-2}}^{MLH_{-1}}$				10.24	
$pK_{ML_2H_{-1}}^{ML_2}$		9.89			
(*b*) *N-tert*-Butoxycarbonylcarcinine					
β_{011} (HL)	7.04(01)				
β_{110} (ML)				3.850(05)	
β_{120} (ML_2)				7.25(01)	
β_{130} (ML_3)				9.93(02)	
β_{140} (ML_4)				12.41(04)	

by using an Orion (cat. no. 91-03) combined glass electrode and an Orion 901 pH-meter. For the quantitative evaluation of data[16] the correlation (1) was used between the experimental

$$E = E_0 + \frac{RT}{F} \log [H^+] + j_H[H^+] + j_{OH}[H^+]^{-1}K_w \quad (1)$$

electromotive force values (E) and the equilibrium hydrogen-ion concentrations $[H^+]$; j_H and j_{OH} are fitting parameters in acidic and alkaline media for the correction of experimental errors, mainly due to the liquid-junction potential and to the possible alkaline and acidic errors of the glass electrode, and K_w is the autoprotolysis constant of water ($10^{-13.75}$).[17] The protonation and complex formation constants were calculated as the average over six and six to ten independent titrations, respectively. The metal ion to ligand ratios were limited in the physiological pH range (6–7) towards lower contents of ligand on account of precipitation phenomena. Thus for the nickel(II)– and cobalt(II)–carcinine systems the metal ion to ligand ratios were varied from 1:2 to 1:10, and for the copper(II)–carcinine systems from 1:1.5 to 1:8, with metal-ion concentrations between 10^{-3} and 10^{-2} mol dm^{-3}. Each run used a 5 cm^3 sample titrated with a 0.03 mol dm^{-3} NaOH solution.

Spectroscopic Measurements.—The same concentration ranges were used as those for the potentiometric studies, except for some EPR spectra. The visible absorption spectra were recorded on a Varian DMS 100 UV/VIS spectrophotometer. The spectra obtained at different total concentrations and pH 3–12 were analysed as the sum of individual components relative to each complex, taking into account the values of the stability constants determined by potentiometry. The X-band EPR spectra were recorded on a Bruker ER-200 D spectrometer at liquid-nitrogen temperature and at 9.45 GHz. A FORTRAN program was used to analyse the spectra. In the case of anisotropic spectra, the g values, axially symmetrical hyperfine and quadrupolar tensors of copper(II) and nitrogen atoms, and linewidths (depending on the magnetic quantum number which characterizes each of them and on orientation) were fitted for both ^{63}Cu and ^{65}Cu isotopes. Proton NMR spectra were recorded on a Bruker AM-400 spectrometer, at 298 ± 2 K and 400 MHz, with acetone as internal standard.

Calculations.—The species formed in the investigated systems can be characterized by the general equilibrium process (2)

$$pM + qL + rH \rightleftharpoons M_pL_qH_r \quad (2)$$

(charges omitted) where M = Co^{2+}, Ni^{2+} or Cu^{2+} and L = carcinine or *N-tert*-butoxycarbonylcarcinine. The formation constants for this generalized reaction are denoted by β_{pqr}. The protonation and complex-formation constants defined by equation (2) were evaluated from the pH-metric titration data with the PSEQUAD computer program.[18]

Results and Discussion

Protonation Constants.—The pH-metrically determined protonation constants (β_{01r} with $r = 1$ or 2) of the free-ligand molecules are listed in Table 1. In the case of carcinine, log β_{011} (9.23) is assigned to protonation of the amino group, and log β_{012} (16.07) to the subsequent protonation of N(3) nitrogen in the imidazole ring ($pK_{N(3)} = 6.84$). In the case of *N-tert*-butoxycarbonylcarcinine, the terminal amino group is protected, thus log β_{011} (7.04) refers to protonation of the N(3) imidazole nitrogen. This value is slightly higher than the corresponding constant of carcinine, 7.04 against 6.84, classically due to an enhanced acidity of the diprotonated species in carcinine as compared to that of the analogous singly protonated species in *N-tert*-butoxycarbonylcarcinine. If neutral carcinine is denoted as L in the following, the species L, HL^+ and H_2L^{2+} are predominant at pH > 9, between 7 and 9, and below 6–7, respectively, in aqueous solutions of the free ligand.

Nickel(II)– and Cobalt(II)–Carcinine Complexes.—For both systems, precipitation occurs at pH > 10.3 and 8.7 for Ni^{II} and Co^{II}, respectively, even in the presence of an excess of ligand. The formation of the complexes can consequently be studied

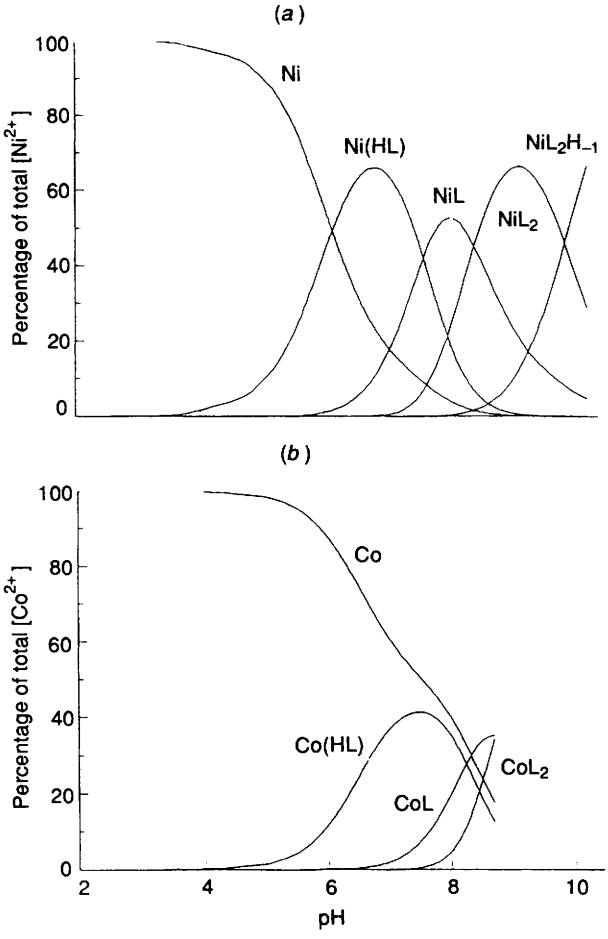
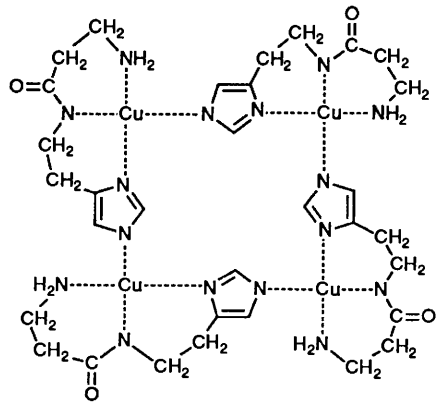


Fig. 1 Concentration distribution of complexes found in the nickel(II)–(*a*) and cobalt(II)–carcinine (*b*) systems as a function of pH; metal ion to ligand ratio 1:8, [metal ion] = 2×10^{-3} mol dm^{-3}



Scheme 1 Structure suggested for the tetrameric carcinine species $Cu_4L_4H_{-8}$

only for pH values lower than the upper limits mentioned above. Increasing the pH by addition of sodium hydroxide shows the formation of first M(HL) and then ML complexes in the range pH 5–6.5 and 6.5–8, respectively. The formation constants β_{111} and β_{110} of these complexes are listed in Table 1 and their concentration distributions as a function of pH are shown in Fig. 1. The values of β_{111} and β_{110} are quite close to those found for the corresponding complexes of carnosine, $\log \beta_{111}$ = 12.11 and 11.48 for Ni^{II} and Co^{II},[7,9] respectively, as compared to 11.84 and 11.0 for the carcinine complexes, and, in the same sequence, $\log \beta_{110}$ = 4.61 and 2.85[9] compared to 4.21 and 2.81. This shows that the carboxyl group which makes the difference between carcinine and carnosine has a subordinate role in the co-ordination. Additional information on the structure of these complexes was obtained from their ^1H NMR spectra. From pH 5 to 6.5 where only the (MHL) complex exists and the amino group of carcinine is protonated, there is a line broadening of the imidazole proton peaks, while the signals from the β-alanine residue remain sharp. This shows the existence of one binding site only, the N(3) nitrogen imidazole, the co-ordination shell of Co^{II} being presumably completed with water molecules. Increasing the pH to form the ML species is accompanied by further broadening of lines from the β-alanine residue, as expected for additional co-ordination by the amino group which is now deprotonated. The corresponding pK values for deprotonation pK_{ML}^{MHL} = 7.63 and 8.25 for the complexes of Ni^{II} and Co^{II}, respectively, are indeed substantially larger than that for N(3) of the free ligand (pK = 6.84), but smaller than that for the amino group (pK = 9.23), thus pointing to metal ion-promoted proton release from the terminal amino group (NH_3^+).

At higher pH, 8 for Co^{II} and 10 for Ni^{II}, the ML_2 complexes form for both metal ions with equatorial 4N co-ordination by amino and imidazole nitrogens. In the alkaline pH range, precipitation phenomena rule out the possibility of investigating the cobalt(II)–carcinine solutions. In the case of the nickel(II)–carcinine systems, however, it is still possible to detect an additional consumption of base leading to the ML_2H_{-1} complex. The high value of $pK_{ML_2H_{-1}}^{ML_2}$ (9.89, see Table 1) and the absence of a blue shift in the d–d band (see next paragraph) suggest the formation of hydroxo-complexes and not deprotonation of the peptide nitrogen or of the N(1) imidazole nitrogen. Further increase in the pH brings about precipitation of nickel(II) solutions too, but at about pH 11 the precipitate redissolves forming a yellow complex. The change of colour was also reported for nickel(II)–glycylhistidine[7,9,10] and assigned to the formation of a diamagnetic square-planar complex,[7,8] possibly a tetrameric polynuclear species $M_4L_4H_{-8}$ (*cf.* Scheme 1). Our ^1H NMR measurements confirm the formation of a diamagnetic complex because the proton lines become narrow at this pH.

Copper(II)–N-tert-*Butoxycarbonylcarcinine Complexes.*—Studies are limited to pH < 8 since precipitation occurs at higher pH. In this pH range there are two potential metal-binding sites which can be detected pH-metrically, namely the N(3) and peptide nitrogens. The consumption of a second base equivalent was not observed however on increasing the pH to 8, at any metal ion to ligand ratio (from 1:2 to 1:10). This rules out peptide deprotonation and co-ordination to copper(II), and consequently the presence of six-membered chelated ligand molecules. The ligand is likely to act in a monodentate manner towards copper(II), *via* the N(3) imidazole nitrogen, forming complexes of the type CuL_n, n = 1–4. The formation constants of these complexes determined pH-metrically (see Table 1), $\log \beta_{1n0}$ = 3.85, 7.25, 9.93 and 12.41 when n = 1–4, respectively, are quite reminiscent of those found for the copper(II)–*N*-acetylhistamine[11] (3.97, 7.05, 10.12 and 12.14, respectively) and –imidazole systems[14] (4.21, 7.55, 10.73 and 12.91). This confirms the assumption of imidazole rings co-ordinated to copper(II) *via* the N(3) nitrogens. The precipitate formed at pH > 8 does not redissolve in excess of base. Again the absence of a blue shift in the d–d band (see next paragraph) suggests the formation of uncomplexed copper(II) hydroxide $Cu(OH)_2$.

Copper(II)–*Carcinine Complexes.*—The pH-metric titration curves for the copper(II)–carcinine systems are shown in Fig. 2, as a plot of pH *vs.* the number of base equivalents per total ligand. These curves start on the acidic side with the neutralization of imidazolium protons and are drawn between pH 4 and 12. For all metal ion to ligand ratios there is an inflection point at pH 8 after consumption of two base equivalents per metal ion, and another weak inflection point at

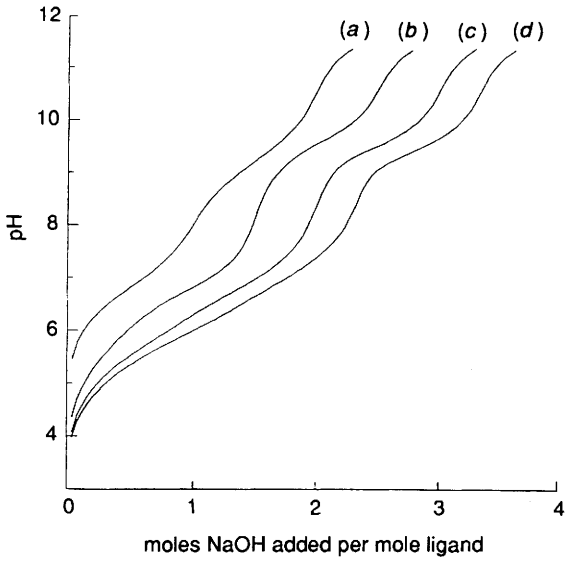


Fig. 2 pH Titration curves of copper(II)–carcinine at [L] = 1.5×10^{-2} and $[Cu^{II}] = 0$ (*a*), 3.75×10^{-3} (*b*), 7.5×10^{-3} (*c*), and 1.0×10^{-2} mol dm^{-3} (*d*)

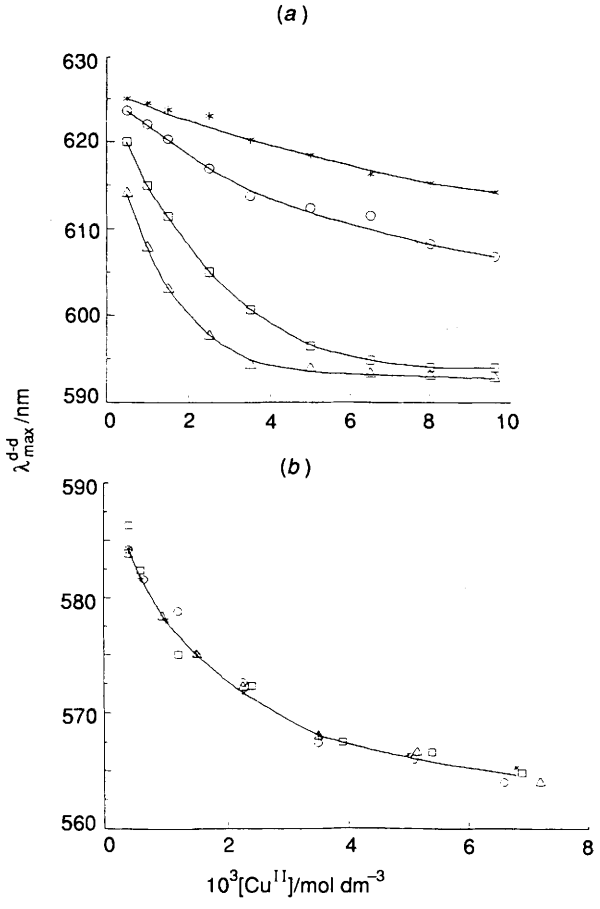


Fig. 3 Positions of the d–d band maximum for the copper(II)–carcinine system as a function of the total copper(II) concentration, at pH 8.54 (*a*) and 11.57 (*b*); Cu^{II}:L = 1:1.5 (*), 1:2 (○), 1.4 (□) and 1:8 (△)

pH 11 for a further addition of one base equivalent. Simulation of the titration curves shows the existence of the Cu(HL) and then CuL complex in the range pH 4–6 and 5–7, respectively, as in the case of the nickel(II)– and cobalt(II)–carcinine systems, where the monodentate, or bidentate, carcinine molecule is co-ordinated to the metal ion through the N(3) imidazole nitrogen [Cu(HL)], or through both N(3) and amino nitrogen (CuL) respectively.

Further deprotonation by one, and then two base equivalents, to reach pH 8 and then pH 11, strongly suggests the successive appearance of $CuLH_{-1}$ and $CuLH_{-2}$ species, respectively. The first complex should involve 3N co-ordination to copper(II) of one carcinine molecule through the N(3), amino and peptide nitrogens. Other possibilities however are the formation of monomeric multiliganded species, such as CuL_2, or of polymeric species such as the dimer $Cu_2L_2H_{-2}$, as in the case of the copper(II)–carnosine system.[5] Besides the criterion of the best fit to pH-metric titration curves, additional evidence from spectroscopic data was necessary to rule out these possibilities (see below). The formation of the $CuLH_{-2}$ complex requires further deprotonation of the above $CuLH_{-1}$ species, which may be achieved in either of two ways: deprotonation of a water molecule in the fourth equatorial position, or metal-ion promoted deprotonation of the N(1) pyrrolic nitrogen of the imidazole ring, probably accompanied by the formation of a tetrameric species as in the case of glycylhistidine[7,8] (at least in the absence of steric constraints[12]). Again, spectroscopic measurements were required to obtain additional information on the structure of these complexes.

Additional structural information was mainly obtained from UV/VIS spectroscopic measurements. It has long been recognized that peptide complexes with copper(II) exhibit colours changing from blue to purple as the number of co-ordinated nitrogen atoms increases; this is the familiar biuret reaction of proteins. The shift of the d–d band depends mainly on the nature of the four donor atoms in the equatorial plane of co-ordination. Blue shifts occur when nitrogen donors (peptide, amino, imidazole) are substituted by oxygen donors (carboxylate, carbonyl, H_2O, OH^-); the effects are approximately additive and a system of increments has been proposed to account for the observed wavelengths.[19] Increasing the number of co-ordinated nitrogens in carcinine complexes is thus expected to result in d–d bands more and more shifted to the blue. In fact, as the bands are broad, they cannot be detected individually, rather a progressive shift of the spectra and the maximum of their envelope, λ^{d-d}_{max}, as a function of the solution composition is observed.

Thus, for pH between 4 and 6, $\lambda^{d-d}_{max} = 760$ nm for the predominant species (*cf.* Table 1), as expected for one nitrogen donor only in the Cu(HL) complex. Other evidence for this complex is obtained from the same ^1H NMR measurements as those described above for the nickel(II)– and cobalt(II)–carcinine systems: relaxation data are consistent with monocoordination of carcinine *via* the N(3) nitrogen of imidazole. Increasing the pH to 7 shifts the d–d band with a component at $\lambda^{d-d}_{max} = 676$ nm; this shows 2N co-ordination in the CuL complex which is predominant at this pH.

A further shift to 620 nm is observed (at least in dilute solutions) when the pH is raised from 7 to 9.5, in line with an assumed 3N co-ordination in the $CuLH_{-1}$ complex. However using concentrated solutions at constant pH brings about an additional shift to 592 nm, showing the presence of a 4N co-ordinated species in equilibrium with $CuLH_{-1}$. Complexes formed from ligand HL after the consumption of two base equivalents are either polynuclear species or multiliganded monomers, of formulas $(CuLH_{-1})_n$ or CuL_nH_{n-2}, respectively, with $n = 1, 2 \ldots$, including the monomeric $CuLH_{-1}$ complex when $n = 1$. The blue shift of the d–d band increases with concentration. This point is made clear by plotting λ^{d-d}_{max} as a function of $[Cu^{2+}]$ in solutions of constant pH [8.54 in Fig. 3(*a*)]. Four curves are drawn on this graph, each one for a constant metal ion to ligand ratio, $R = 1:1.5$ to 1:8. All start from a common value $\lambda^{d-d}_{max} \approx 620$ nm (intercept at zero concentration), which corresponds to the monomer $CuLH_{-1}$. Each curve decreases to an asymptotic value of 592 nm, the faster the smaller is R. This observation eliminates the pos-

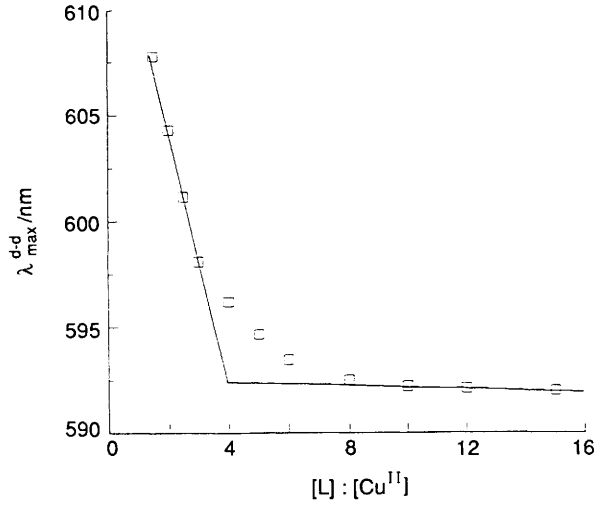


Fig. 4 Positions of the d–d band maximum in the copper(II)–carcinine systems as a function of the ligand to metal ratio at pH 8.54 and [L] = 2×10^{-2} mol dm^{-3}

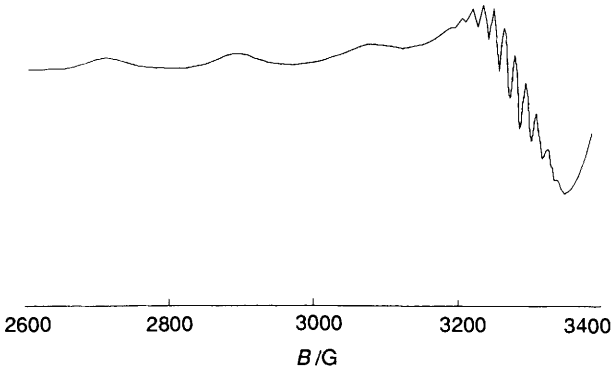


Fig. 5 Experimental (——) and computed (· · · ·) EPR spectra of copper(II)–carcinine solutions frozen at 77 K, pH 6.5, [Cu^{II}] = 4×10^{-3} mol dm^{-3}, and Cu^{II}:L = 1:150

sible existence of polynuclear species since the formation of $(CuLH_{-1})_n$ from monomeric $CuLH_{-1}$ according to the equilibrium (3) is independent of the quantity of carcinine in excess, if

$$n CuLH_{-1} \rightleftharpoons (CuLH_{-1})_n \quad (3)$$

we assume that copper(II) ions are totally engaged in the monomeric complex $CuLH_{-1}$ as a first step of complexation (see the distribution curves in Fig. 6). We are then left with the second possibility, in which the formation of CuL_nH_{n-2} according to equilibrium (4) is effectively promoted by an excess

$$CuLH_{-1} + (n-1)HL \rightleftharpoons CuL_nH_{n-2} \quad (4)$$

of free ligand HL. This accounts for increased proportions of CuL_nH_{n-2} when R is decreased from 1:1.5 to 1:8, and consequently for larger shifts of the d–d band and rapidly descending curves. The horizontal asymptote of these curves, corresponding to *ca.* 100% CuL_nH_{n-2}, can be reached only if the ratio R is smaller than 1:n. With the limited series of curves shown in Fig. 3(*a*) this seems to be achieved as soon as $R \leqslant 1:4$, suggesting that $n = 4$. This was confirmed by using an extended set of R values, obtained by simply adding variable amounts of copper(II) ion to an aqueous 0.02 mol dm^{-3} solution of carcinine maintained at a constant pH 8.54. Under these conditions a plot of λ^{d-d}_{max} *vs.* R shows a breakpoint when $R = 1:4$ (Fig. 4). This allowed us to conclude in favour of the presence of the species CuL_4H_2 in this pH range (7–10). Introducing this species, besides $CuLH_{-1}$, significantly improved the curve-fitting of the potentiometric data.

The co-ordination in the species CuL_4H_2 may involve the four N(3) imidazole nitrogens and probably two amino groups (the other two being protonated). Although there is presently no definite evidence as to the nature of the binding sites, several arguments seem to be in favour of equatorial co-ordination by four imidazole rings. First, in the analogous copper(II)–*N*-*tert*-butoxycarbonylcarcinine system described above, in which the protected amino group cannot participate in co-ordination, the Cu^{II} forms similar CuL_4 complexes. Secondly, in the analogous copper(II)–carnosine system[6,13,14] the formation of CuL_4 has also been reported in the presence of a large excess of ligand ($R = 1:200$), with the planar co-ordination of the four imidazole rings; the protonation conditions in this species are actually unknown since the pH-metric measurements are not conclusive. Thirdly, the similarity of λ^{d-d}_{max} between CuL_4H_2 (592 nm) and $Cu(Him)_4$ (Him = imidazole) (600 nm)[20] supports the assumption of planar co-ordination by four imidazole rings in both complexes.

The co-ordination shell about copper(II) in CuL_4H_2 could then be completed by two axial amino groups, thus increasing the stability of the complex. The number of co-ordinated nitrogens can in principle be deduced from the superhyperfine structure of the EPR spectra,[21,22] using frozen solutions at 77 K. However the superhyperfine structure of these spectra is not well resolved, probably because of the poor quality of the glassy solid obtained at 77 K. A somewhat better resolution was achieved (Fig. 5) with solutions containing a high excess of ligand (*e.g.* $R = 0.004:0.6$). We expect that the co-ordination sites remain unchanged in these solutions because of the same position of the d–d band and of the close similarity in the EPR parameters ($A_{\|} = 175$ G, $g_{\|} = 2.262$ and $g_{\perp} = 2.059$ to be compared to 174 G, 2.269 and 2.058, respectively, in solutions typically used for potentiometric measurements). The recorded EPR spectrum is shown in Fig. 5. The parameters [mentioned above, plus $A_{\perp} = 26$ G and $A(Cu\text{–}N_{\perp}) = 14.1$ G] agree well with those found for the copper(II)–carnosine system.[14] A simulation of the experimental spectrum is in accord with the co-ordination of four equatorial nitrogens to copper(II), at least under the present conditions of a high excess of ligand, but further investigations are needed to arrive at a definite conclusion.

At the alkaline end of the pH range investigated (10–12) there is again a significant blue shift of the d–d band at a pH where the above species $CuLH_{-1}$ and CuL_4H_2 have totally disappeared after consumption of one more base equivalent. This points again to the formation of new species. If we draw a graph [Fig. 3(*b*)] analogous to the one in Fig. 3(*a*), all four curves are superimposed upon each other, thus pointing to the presence of a polynuclear species $(CuLH_{-2})_n$ in equilibrium with the monomeric complex $CuLH_{-2}$. The value of λ^{d-d}_{max} for the monomeric complex is close to 592 nm in agreement with a value of 590 ± 9 nm computed using increments given in the literature[20] and with values previously determined for complexes of this type,[8] this suggests a 3N co-ordination as in the $CuLH_{-1}$ complex. The base-consuming process between pH 9 and 11.5 should then be assigned in dilute solution to the deprotonation of H_2O to OH^- in the fourth co-ordination site of copper(II). The blue shift in concentrated solution strongly suggests 4N co-ordination of copper(II) in the polymeric species $(CuLH_{-2})_n$. The fourth nitrogen donor in carcinine should be the N(1) pyrrolic nitrogen of imidazole. This requires first the deprotonation of this nitrogen atom. This a very weak acid ($pK > 14$) in the free ligand, but the chelated ligand (in $CuLH_{-1}$) is deprotonated in the range pH 9–11.[5,7,8] The N(1) and N(3) nitrogen donors of one imidazole ring however cannot be co-ordinated to the same copper(II) ion for steric reasons. The only way is to replace the hydroxide ion in $CuLH_{-2}$ by a N(1) pyrrolic nitrogen from another molecule; this results in polymeric structures involving closed loops of alternating copper(II) ions and bridging imidazole units. Following earlier suggestions[7,8] for the copper(II)–glycylhistidine system, a

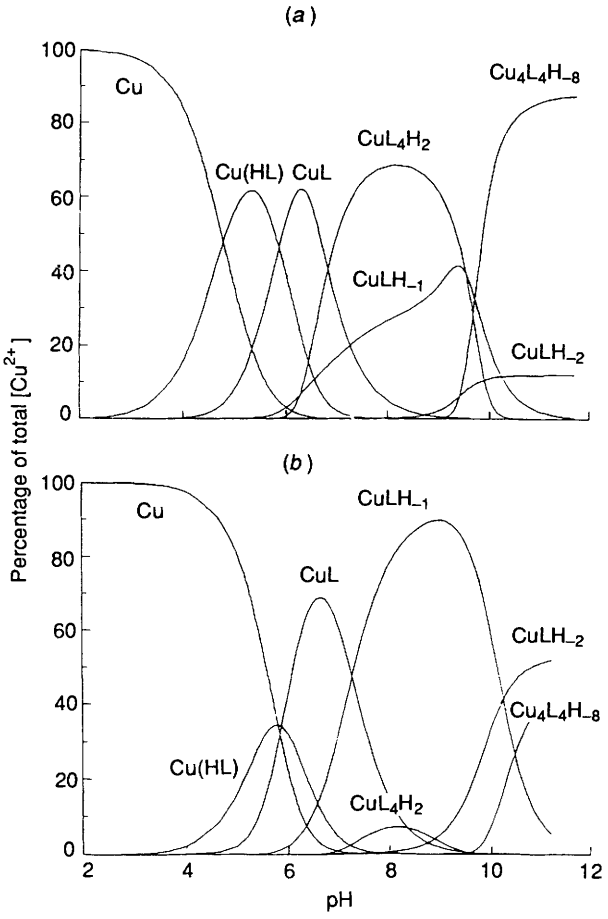


Fig. 6 Concentration distribution of complexes found in the copper(II)–carcinine system as a function of pH, at constant metal ion to ligand ratio $Cu^{II}:L = 1:4$ and $[Cu^{II}] = 9 \times 10^{-3}$ (*a*) or 1.0×10^{-3} mol dm^{-3} (*b*)

tetrameric species is preferred to any other on steric grounds, accounting for a planar equatorial co-ordination around the copper(II) ions without any bond distortion. The fitting of titration curves was consequently performed by assuming the presence of both species $CuLH_{-2}$ and $Cu_4L_4H_{-8}$ in this pH range. Again, the existence of an additional species besides $CuLH_{-2}$ improved the quality of fit significantly.

In conclusion, the whole set of pH-metric curves for the copper(II)–carcinine system between pH 4 and 12 was best fitted by considering six complexes, Cu(HL), CuL, $CuLH_{-1}$, CuL_4H_2, $CuLH_{-2}$, $Cu_4L_4H_{-8}$, and adjusting the set of six formation constants β_{pqr} listed in Table 1, together with the position of the d–d band for each complex. The distribution curves of these complexes as a function of pH are shown in Fig. 6, for two different concentrations of total Cu^{2+} and a constant metal ion to ligand ratio (1:4). These curves illustrate the predominance of multiliganded and polynuclear species, CuL_4H_2 and $Cu_4L_4H_{-8}$, over monomeric species, $CuLH_{-1}$ and $CuLH_{-2}$, respectively, in concentrated solution [Fig. 6(*a*)], with the opposite situation in dilute solution [Fig. 6(*b*)]. From these data we may derive constants for equilibria which may be useful to the discussion. The following points can be made.

(*a*) The formation constant of Cu(HL) from copper(II) ion and ligand HL [NH_3^+, NH, N(3)], calculated as $\log K_{Cu(HL)} = \log \beta_{111} - \log \beta_{011}$ has a value of 3.55 close to that found for the formation of CuL in the *N-tert*-butoxycarbonylcarcinine system. This similarity is additional evidence in support of co-ordination of copper(II) by the N(3) imidazole nitrogen in both complexes. Formation constants for the analogous complexes of nickel(II) and cobalt(II), $\log K_{M(HL)} = 2.61$ and 1.83, show the well known order of stability[23] of amino acids and peptide complexes: $Cu^{II} > Ni^{II} > Co^{II}$. Differences are still larger for the formation of the deprotonated complex of the type ML, where the stability constants $\log \beta_{110}$ are in the same sequence: 6.91, 4.21 and 2.81.

(*b*) Metal ion-promoted deprotonation of the amino group in Cu(HL) to yield CuL is classically observed with carcinine where pK goes from 9.23 for the free ligand to 5.87, 7.63 and 8.25 for the complexes of Cu^{II}, Ni^{II} and Co^{II}, respectively (again, in the expected sequence). The pK decrease on complexation, $\Delta pK = 3.36$ units for the copper(II)–carcinine system, is however somewhat lower than in the analogous Cu^{II}–Gly-His ($\Delta pK = 4.83$) and copper(II)–carnosine ($\Delta pK = 4.29$) systems,[10] a fact which may be tentatively assigned to further stabilization of the complex in the last two systems due to the presence of an additional co-ordinating carboxylate group.

(*c*) At higher pH, metal ion-promoted deprotonation of peptide nitrogens occurs. The pK for peptide deprotonation ($pK^{ML}_{MLH_{-1}} = 7.27$) is clearly greater than for other simple dipeptides for example (5.60 and 4.26 for carnosine and glycylhistidine, respectively[10]). The pK difference can be assigned to the presence of a carboxylate group in carnosine and to a further stabilization of the previously investigated copper(II)–glycylhistidine system compared to the copper(II)–carcinine system which is due to the formation of (5-6) instead of (6-6) chelates. This explanation is supported by investigations using analogous histamine dipeptides containing an α-amino acid residue in the place of β-alanine, *e.g.* glycyl- and sarcosyl-histamine, where much lower pK values are found for peptide deprotonation, 3.28 and 3.82, respectively.[24]

(*d*) The last deprotonation at the alkaline end of the pH range investigated converts $CuLH_{-1}$ species into $CuLH_{-2}$. The high pK value determined for this process, $pK^{MLH_{-1}}_{MLH_{-2}} = 10.24$, is consistent with deprotonation of a water molecule in the fourth equatorial co-ordination site of copper(II), forming a mixed ligand hydroxo-complex, in line with conclusions from UV/VIS spectroscopy (see above).

(*e*) The relative instability of $CuLH_{-1}$ complexes containing two six-membered chelates can also account for the formation of polynuclear multiliganded species (as in the case of carnosine, see Introduction). In the case of the copper(II)–carcinine system the dimer complex does not form, probably because there is no carboxylate group as a fourth binding site. However, in the presence of a small excess of ligand ([Cu]:[L] = 1:1.5), the same situation as found in the carnosine system at higher ligand excess, namely four monodentate N(3) imidazole nitrogens in the co-ordination plane, occurs with the subsequent formation of a tetraliganded complex CuL_4H_2, just as in the case of the *N-tert*-butoxycarbonylcarcinine system. These results clearly demonstrate that imidazole N(3) nitrogens can compete with amino and amido sites in peptides, significantly increasing the stabilities of the corresponding complexes. The model molecules presently investigated also help to understand the relatively infrequent occurrence of metal ion–peptide bonds in the case of proteins under biological conditions.

In strongly alkaline solutions a tetrameric 4N co-ordinated complex $Cu_4L_4H_{-8}$ occurs, in equilibrium with monomeric $CuLH_{-2}$, involving bidentate bridging imidazole units (Scheme 1). Although initially proposed for the Cu^{II}–Gly-His system,[5,7] no formation constant for this species seems presently available. A value has been determined for a derivative of glycylhistidine, the tripeptide Gly-His-Gly.[8] The value found for equilibrium (3) in this case, $\log K_{tet} = 7.60$, is clearly smaller than that presently found with carcinine, $\log K_{tet} = 9.16$. The increased stability of the tetramer in the latter system may be again assigned to the unfavourable presence in monomeric $CuLH_{-2}$ of two six-membered chelated rings with carcinine instead of (5-6) chelation with the above tripeptide. The structure of these polynuclear species again points to the exceptional role of imidazole N donors in histidine- or histamine-containing peptides.

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