Potentiometric and Spectroscopic Study of Ternary Complexes of Copper(II), 1,10-Phenanthroline and Oxidised Glutathione†

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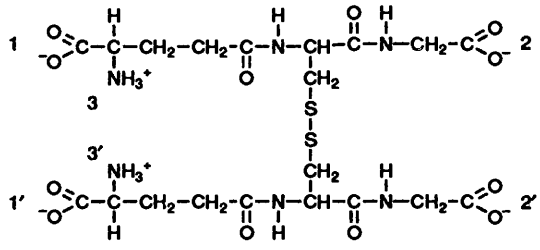
The ternary system consisting of oxidised glutathione (a S–S' bonded dimeric hexapeptide), 1,10-phenanthroline (phen) and copper(II) has been studied in aqueous solution in the range pH 3–8 by potentiometry, electronic spectroscopy and electron paramagnetic resonance. Potentiometric titrations have allowed the evaluation of stoichiometry and formation constants of the complexes in the system; EPR and electronic spectroscopy were used to define the chromophore of one particularly important adduct predominant at physiological pH, $[Cu(HL)(phen)]^-$ (H_6L^{2+} = completely protonated oxidised glutathione). The results obtained suggest that in the stable conformation of this complex only one glutamyl moiety is involved, the other two positions in the plane being occupied by two nitrogen atoms from phenanthroline.

The naturally occurring tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) is one of the most abundant non-proteic thiols. It is present in all living cells in a relatively high concentration and is involved in a number of intracellular processes, one of the most important being the protection of cellular membranes from oxidation of hydroperoxidase.[1] It is present in human erythrocytes at a 2–3 mmol dm^{-3} concentration,[2] and is readily oxidised to a S–S' bonded dimeric hexapeptide (H_2L^{2-}). Enzymes such as glutathione peroxidase and glutathione reductase mediate the relative concentration of glutathione and the dimer.[3,4] Glutathione also participates in redox reactions involving the Cu^{II}–Cu^{I} couple.[5] Usually, the rapid oxidation of its sulfhydryl group is catalysed by traces of metal ions such as copper(II)[6] Some complexes also show catalytic activity; for example, chelation of copper(II) by 1,10-phenanthroline (phen) strongly enhances the catalytic ability with respect to the oxidation of glutathione.[7,8] On the other hand, the dimeric hexapeptide can be reduced by some metal ions, such as copper(I).[9] Moreover, it has been suggested that the inhibitory activity of a copper(II)–D-penicillamine (3-sulfonyl-D-valine) complex in rheumatoid arthritis[10] is due to the formation of a copper(II) complex of the hexapeptide.[11]

Many examples could be given to stress the importance of copper and, in particular, of copper complexes with phenanthroline in biological environments. Such complexes play a role in the cleavage of DNA in neoplastic cells;[12,13] in the overall mechanism the metal centre cycles between +1 and +2 oxidation states to catalyse the reduction of oxygen to reactive radical species by the action of intracellular thiol reducing agents, such as glutathione itself. The radical species involved are suggested to be responsible for DNA fission. In this context, the study of the formation of the ternary complexes copper–biological target–phenanthroline can be important to suggest an involvement of a site-specific mechanism.

Despite the biological and chemical significance of the complexes of Cu with the hexapeptide, relatively few investigations on this system have been performed to date. This is probably due to the multiplicity of potential metal-binding sites and to the formation of complexes with many different stoichiometries, depending on pH of the solution. From polarographic measurements Li and co-workers[14] concluded that the hexapeptide only forms a 1:1 complex with a formation constant of 4×10^{14} dm^3 mol^{-1}. From EPR measurements on solutions of the binary system, it has been suggested that co-ordination takes place through the amino and carboxyl groups of the glutamyl residues; the existence of numerous protonated and various bis complexes was later proposed.[15–17] Moreover, Kroneck[18] reported that acid–base titration of a copper(II)–hexapeptide (2:1) solution up to pH 9.5 is accompanied by eight successive deprotonations of the hexapeptide. The proposed structure for the 2:1 complex involves a six-co-ordinate metal and a copper–copper interaction *via* a disulfide bridge; another particularly interesting feature is that Cu^{II} binds to all four peptidic nitrogens. A crystal structure of a dinuclear copper(II) complex of the hexapeptide has also been reported.[19]

Pursuing our interest in the study of ternary metal–phenanthroline complexes,[20,21] it seemed of particular importance to study the system consisting of oxidised glutathione, 1,10-phenanthroline and copper(II) in aqueous solution, together with the structural properties of one particularly important adduct, largely predominant at



H_2L^{2-}

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

physiological pH. On the basis of a definition of the chemical system obtained by potentiometric titrations in the range pH 3–8, electronic spectroscopy and electron paramagnetic resonance measurements were used. The EPR studies were particularly useful to define the chomophore in the ternary complex $[Cu(HL)(phen)]^-$, (H_6L^{2+} = completely protonated oxidised glutathione, see below) by using suitable computer programs[22] to simulate the spectrum of the complex without taking into account nitrogen nuclei, and then performing a convolution between the spectrum so obtained and the pattern of the nitrogens under the desired conditions.

Experimental

Materials and Methods.—Potentiometric titrations. High-purity (Milli-Q Millipore) water was used to prepare the solutions for potentiometric studies. Potassium nitrate (Fluka) was used in 0.1 mol dm^{-3} concentration as a buffer for ionic strength. The solutions of oxidised glutathione (Sigma, 99% purity) and of 1,10-phenanthroline (Aldrich) were standardised by acid–base titration with sodium hydroxide titrant solutions freshly prepared from Merck Titrisol ampoules, which were in turn standardised with potassium hydrogenphthalate (Fluka). They were also used in the potentiometric acid–base titrations of binary and ternary systems, as well as in the standardisation of HNO_3 solutions. The concentration of solutions of $Cu(NO_3)_2 \cdot 3H_2O$ (Fluka) was evaluated by following standard analytical procedures.[23]

Acid–base titrations were carried out at a temperature of 25.0 ± 0.1 °C at a fixed ionic strength (0.1 mol dm^{-3} KNO_3) using a Metrohm PHM 84 Research pH-meter. Addition of the titrant solution and recording of pH values were carried out under control of an Apple IIe personal computer with appropriate interfaces and software. Argon was bubbled through the samples to make sure that air was kept out of the solution, as well to mix it during the titrations with carbonate-free NaOH. Glass electrode calibration, in terms of hydrogen-ion concentration, was done according to ref. 24: the computer program MAGEC[25] was used to elaborate the results of a titration of 0.01 mol dm^{-3} HNO_3 with standard 0.1 mol dm^{-3} NaOH, at controlled ionic strength and temperature. The formation constants were computed on the basis of acid–base titrations on solutions containing a metal-to-ligand ratio which in the case of the binary copper–hexapeptide system varied from 2:1 to 1:4 and for the ternary system with phen was 1:1:1. The concentration used was 1×10^{-3} mol dm^{-3} for ligands and from 2×10^{-4} to 2×10^{-3} mol dm^{-3} for copper. The data obtained were elaborated by the program SUPERQUAD,[26] while routine programs were used to compute the concentration of the different species as a function of pH.

Spectroscopic measurements. The EPR measurements were carried out at room as well as at low temperature (frozen solution) under the same conditions as those of the potentiometric measurements, in the range pH 3–8. The Hamiltonian parameters were calculated from the spectra obtained for ligands and metal concentrations of 1×10^{-2} mol dm^{-3}, in order to get better resolution. X-Band EPR spectra were obtained on a Varian E-9 spectrometer equipped with a standard low-temperature apparatus, and on a Bruker 200D SRC spectrometer equipped with a high-sensitivity Bruker ER 4108 TMH cavity; they were calibrated against diphenylpicryl-hydrazyl powder (dpph, $g = 2.0036$). Microwave frequencies were measured with an XL Microwave model 3120 counter. The magnetic field was calibrated with a MJ-magnetometer by Jagmar (Poland). The temperature was controlled by using a Bruker ER 4111 VT variable-temperature unit. The spectrometer was interfaced with a PS/2 Technical Instruments Hardware computer and the data were acquired using the EPR data system Cs-EPR, produced by Stelar Inc. (Mede, Italy); suitable processing minimised baseline artefacts.[27] The spectra were simulated using COSMOS[22] (room temperature) and CUSIMNE[28] (rigid limit) computer programs. These programs take into account the simultaneous presence of the two copper isotopes (^{63}Cu and ^{65}Cu, isotopic abundances 69.17 and 30.83%, respectively; nuclear spin, $\frac{3}{2}$; ratio of nuclear moments of ^{65}Cu and ^{63}Cu, 1.07:1). All the programs were implemented on a Compaq 486/50L personal computer.

Electronic absorption spectra of the ternary system studied were recorded on a JASCO Uvidec 610 spectrophotometer, under conditions analogous to those of the pH-metric and EPR measurements, in the range pH 3–8.

Results and Discussion

Potentiometry.—The hexapeptide molecule can bear up to six acidic protons, two of which belong to ammonium groups and the other four to carboxylic groups. The predominant species of oxidised glutathione at neutral pH is H_2L^{2-}.

The disulfide bridge does not bind strongly to metal ions, so that the most important binding sites are the carboxylate and amino groups 1,1′, 2,2′ and 3,3′, respectively.[29] In view of the sometimes contradictory results reported in the literature, we reinvestigated the binary system copper–hexapeptide under our experimental conditions. First the stepwise acidity constants for H_6L^{2+} were determined. The resulting pK_a values ($pK_{a,1}$ = 2.34, $pK_{a,2}$ = 2.46, $pK_{a,3}$ = 3.43, $pK_{a,4}$ = 3.99, $pK_{a,5}$ = 9.06 and $pK_{a,6}$ = 9.59) can be referred to the acidity of sites 1,1′, 2,2′ and 3,3′, respectively. They are comparable with those previously reported,[11,29,30] within the limits of variability caused by the different temperature, ionic background and purity degree of the hexapeptide.

Titrations of solutions of the binary system copper–hexapeptide were performed for different metal-to-ligand ratios, the best fit being obtained for the stability constant values listed in Table 1. The concentration of the complex $[Cu_2L]$ is very low in solution where the metal-to-ligand ratio is 1:1; on the contrary, it is a significant species where the metal is present in excess, *i.e.* when the metal-to-ligand ratio is 2:1. Though using a metal-to-ligand ratio as low as 1:4, the data collected under our experimental conditions did not allow us to get evidence for the formation of $[CuL_2]$, as reported by Micheloni *et al.*[11] However, our results agree well with those more recently reported by Varnagy *et al.*[29]

In accord with these findings, an EPR reinvestigation of the binary system in 1:1 and 2:1 metal-to-ligand molar ratio at physiological pH suggests the formation of 1:1 complex. Although the disulfide can originate a 2:1 species, the characteristic signal at $g = 4$ due to the forbidden $\Delta m = 2$ transition in a spin-coupled dimetallic species[31] is not present in our EPR spectra. This is also in agreement with previous data reported by other authors,[18] as this species has been evidenced (for different molar ratios) only at very high pH (> 10).

As to the Cu–phen binary system, we used data reported in refs. 32–34, obtained by different methods, under experimental conditions similar to ours. The choice of not re-examining this

Table 1 Overall formation constants for the species present in the binary systems Cu–hexapeptide and Cu–phen. Standard deviations in parentheses

Species	log β	Species	log β
HL^{3-}	9.59 (0.01)	$[CuL]^{2-}$	14.34 (0.01)
H_2L^{2-}	18.65 (0.01)	$[Cu(HL)]^-$	18.72 (0.01)
H_3L^-	22.64 (0.03)	$[Cu(H_2L)]$	22.43 (0.01)
H_4L	26.07 (0.02)	$[Cu(H_3L)]^+$	25.51 (0.02)
H_5L^+	28.53 (0.04)	$[Cu_2L]$	17.39 (0.02)
H_6L^{2+}	30.87 (0.08)	$[Cu(phen)]^{2+}$	9.08*
$Hphen^+$	4.88*	$[Cu(phen)_2]^{2+}$	15.8*
		$[Cu(phen)_3]^{2+}$	21.00*

* Ref. 32.

system by potentiometric measurements was made on the basis of sound arguments discussed by Irving and Mellor.[35]

The study of the ternary system Cu–hexapeptide–phen, performed on solutions with 1 : 1 : 1 molar ratio, in the range pH 3–8, gave the results listed in Table 2. These data have been obtained by using the formation constants reported in Table 1 for the corresponding binary systems Cu–hexapeptide and Cu–phen.

On the basis of the values of the overall stability constants for the ternary protonated complexes $[Cu(H_3L)(phen)]^+$, $[Cu(H_2L)(phen)]$, $[Cu(HL)(phen)]^-$ and $[CuL(phen)]^{2-}$ the relevant acidity constants can be computed; $pK_{a,1}$ and $pK_{a,2}$ values of 3.23 and 4.17, respectively, indicate stepwise deprotonation of the 2,2′ carboxylic sites, while a $pK_{a,3}$ value of 9.13 suggests deprotonation of 3 (3′) amino group. From these values we can conclude that in the ternary complex $[Cu(HL)(phen)]^-$ the glutamylamino group is, at least in part, still protonated, suggesting a probable binding only with one side of the hexapeptide molecule *via* amino acid-like co-ordination.

The distribution diagram of the species in the ternary system Cu–heptapeptide–phen as a function of pH is reported in Fig. 1. Below pH 4 the predominant form is the binary $[Cu(phen)]^{2+}$ complex. With increasing pH, the ternary protonated $[Cu(H_2L)(phen)]$ species is also present, and then a marked stability of the ternary complex $[Cu(HL)(phen)]^-$ is evidenced, this being the most stable species in the system over a wide pH range, in particular at physiological pH values. This supports its importance in biological systems.

According to a criterion proposed by Sigel and co-workers[36,37] the stability of the ternary complex can be evaluated with respect to that of the 1:1 binary ones from the values of the equilibrium constant of the reaction [see equations (1) and (2)]. The value found is quite close

$$[Cu(phen)]^{2+} + [Cu(HL)]^- \rightleftharpoons [Cu(HL)(phen)]^- + Cu^{2+} \quad (1)$$

$$\log K_{eq} = \Delta \log K = \log K^{[Cu(phen)]^{2+}}_{[Cu(HL)(phen)]^-} - \log K^{Cu}_{[Cu(HL)]^-} \approx -0.76 \quad (2)$$

to the lower limit (*i.e.* −0.9) for ascertaining a significant shift of the equilibrium to the right-hand side. However, the distribution diagram clearly shows that copper is not present in the solution in free ionic form, revealing the marked influence of the experimental conditions, *i.e.* of the composition of the solution. On the other hand, we can compute the value of the constant for equilibrium (3) which accounts for the ability of

$$[Cu(phen)_2]^{2+} + [Cu(HL)]^- \rightleftharpoons [Cu(HL)(phen)]^- + [Cu(phen)]^{2+} \quad (3)$$

$$\log K_{eq} = \log \beta^{[Cu}_{[Cu(HL)(phen)]^-} - \log \beta^{Cu}_{[Cu(HL)]^-} - \log K^{[Cu(phen)]^{2+}}_{[Cu(phen)_2]^{2+}} \approx 1.6 \quad (4)$$

HL^{3-} to substitute one phen ligand to give a 1:1:1 ternary complex from a 1:2 binary one. Unfortunately, concomitant substitution of HL^{3-} by phen in corresponding 1:1 copper complexes is also involved, leading to an overall result consisting of two distinct energetic balances.

Spectroscopy.—The EPR spectra for the ternary system Cu–hexapeptide–phen were obtained at room temperature as well as in frozen solutions under the same conditions as those employed by potentiometric measurements, in the range pH 3–8. Also, in order to obtain good resolution, 1×10^{-2} mol dm^{-3} concentrations were used. The correlation between g_{iso} and g_m, calculated from the isotropic and from the anisotropic spectra, respectively, suggests that no change in the co-ordination around copper(II) occurs on passing from a room-temperature aqueous solution to a 5:1 water–glycol mixture in the frozen state. As we can see from the parallel region of the EPR spectrum of a frozen solution of the ternary system (Fig. 2) at pH 3, in agreement with potentiometric results, the EPR spectrum reveals the presence of the a binary $[Cu(phen)]^{2+}$ complex as the predominant species.[38] Some weak signals due to other species in the parallel region are also detectable. At pH > 4 the signal of a single species, different from that of binary $[Cu(phen)]^{2+}$ complex, progressively increases, while all the other signals progressively decrease. The species that becomes predominant from pH 4 upwards is thought to be the ternary complex $[Cu(HL)(phen)]^-$, in agreement with the potentiometric results. The concentration of this species reaches a maximum at pH 5–5.5, and is the only species evidenced in the EPR spectrum up to pH 8.

Table 2 Formation constants for the species identified in the Cu–hexapeptide–phen ternary system. Standard deviations in parentheses

Species	log β
$[Cu(H_3L)(phen)]^+$	34.44 (0.04)
$[Cu(H_2L)(phen)]$	31.21 (0.02)
$[Cu(HL)(phen)]^-$	27.04 (0.01)
$[CuL(phen)]^{2-}$	17.91 (0.04)

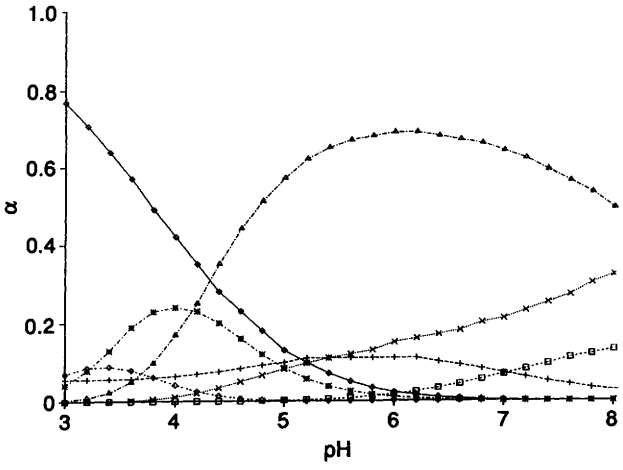


Fig. 1 Distribution diagram (molar fraction α *vs.* pH) of the species in the ternary system Cu–hexapeptide–phen. Species: ◇, $[Cu(phen)]^{2+}$; +, $[Cu(phen)_2]^{2+}$; □, $[Cu(phen)_3]^{2+}$; ×, $[CuL]^{2-}$; △, $[Cu(HL)(phen)]^-$; ✱, $[Cu(H_2L)(phen)]$; ◇, $[Cu(H_3L)(phen)]^+$

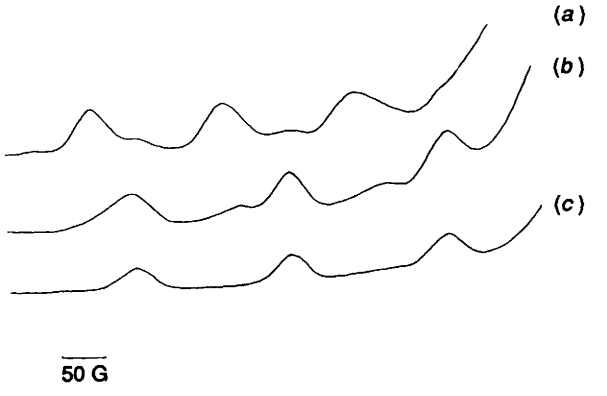


Fig. 2 The EPR spectra (parallel region) of the ternary system Cu–hexapeptide–phen (1 : 1 : 1) at different pH values: (*a*) pH 3, (*b*) 4 and (*c*) 5

From EPR data, a decrease in $g_{\|}$ and a corresponding increase in $A_{\|}$ is evidenced with increasing pH, suggesting that the planar ligand field becomes stronger, and also indicating an involvement in the planar co-ordination geometry of donor atoms from the hexapeptide. Also in the electronic spectra, on passing from acidic to physiological pH, a corresponding blue shift is evidenced. The transition at 612 nm in the visible spectrum recorded at physiological pH is typical of tetragonally distorted complexes with an in-plane CuN_2O_2 or CuN_3O chromophore.

The frozen-solution (110 K) EPR spectrum recorded on the ternary system at pH 6 is reported in Fig. 3(*a*), while in Fig. 3(*b*) the corresponding spectrum at room temperature (298 K) is shown. The simulated spectra are shown below. Within the range of experimental errors, a single solution is found for the set of the magnetic parameters leading to the best fit. The spectrum in Fig. 3(*a*) can be described by an axial spin Hamiltonian. The obtained values of $g_{zz} > g_{xx}, g_{yy} > 2.040$ suggest a $d_{x^2-y^2}$ ground state, characteristic of an octahedral stereochemistry for the complex, so confirming the results from visible spectra.[39,40] The values of $g_{\|}$ (2.24 ± 0.01) and of $A_{\|}$ [(182 ± 1) × 10^{-4} cm^{-1}] are in accord with chromophores in which copper is co-ordinated to three or four nitrogen atoms. Besides, in the perpendicular region of the EPR spectrum at pH 6, a superhyperfine structure is evidenced, which is attributable to the interaction between the unpaired electron of copper and nitrogen atoms of the ligands. Though the number of lines suggests the presence of more than two nitrogen atoms around the metal centre, direct evidence for the number of nitrogens co-ordinated cannot be gained and the use of Peisach–Blumberg plots[41] to deduce the co-ordination scheme is inadequate in the present case for discriminating between a 3N and 4N chromophore.

Since, as Fig. 3 shows, the superhyperfine patterns are not well resolved in the first-derivative experimental spectra, the second-derivative spectrum was computed and simulated [Fig. 4(*a*)]. The second-derivative display is helpful for analysing this superhyperfine pattern, as it emphasises sharp features with respect to broad ones, making discrimination easier. Shoulders in the spectrum become more evident signals with precisely defined turning points which are useful for accurate measurement of coupling constants. Distinguishing between three and four nitrogen atoms is however a difficult task, as the ratios of the intensities of the superhyperfine lines (Scheme 1) is similar. The intensity ratio 6:7 is hardly distinguishable from 16:19, so that the three central lines look in any case to have the same ratio. Of course, if the signal-to-noise ratio were high enough, the intensities of the outer lines for four nitrogens would be a definite proof. Long-time signal acquisition and signal averaging are highly desirable in this context. The best ratios which can be measured are, however, those between the intensities of the second and third lines and that of the central line, as outlined in previous work.[42,43]

In Fig. 4(*b*) (solid line) the second-derivative expansion (110 G) of the $M_I = \frac{3}{2}$ component is reported. The expanded portion of the EPR spectrum is compared with a simulation for two sets of 2N + 1N (· · ·) and one for 2N + 2N (— · —). Approximately the same superhyperfine coupling constants have been used. It is evident that, despite the fact that it is not simple to discriminate between the two cases, the previously described approach based on the intensity ratios of the second and the third lines in our case favours the 2N + 1N hypothesis. The simulation which gives the best fit allows a precise evaluation of the magnetic parameters: $g_{\|} = 2.23$, $g_{\perp} = 2.06$, $A_{\|,Cu} = 182 \times 10^{-4}$ cm^{-1}, $A_{\perp,Cu} = 7.67 \times 10^{-4}$ cm^{-1}, $A_{iso,NH_2} = 12.67 \times 10^{-4}$ cm^{-1}, $A_{iso,N(phen)} = 11.0 \times 10^{-4}$ cm^{-1}. A rotational correlation time (τ_C) of 164 ps was estimated.

Furthermore, the involvement of S atoms in the co-ordination can be excluded on the basis of the EPR results. Antiferromagnetic coupling between copper atoms in a dimeric structure can also be disregarded, as shown by the lack of any signal at a *g* value of around 4. In addition, stability data and EPR

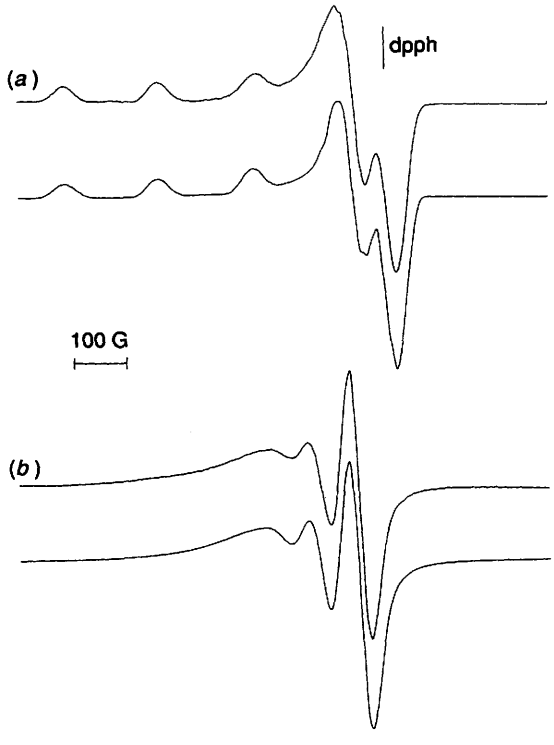


Fig. 3 X-Band experimental EPR spectrum of the $[Cu(HL)(phen)]^-$ ternary complex in (*a*) frozen solution (110 K, ν = 9.6251 GHz) and (*b*) a room temperature (298 K, ν = 9.6218 GHz). The simulated spectra leading to the best fit are shown below

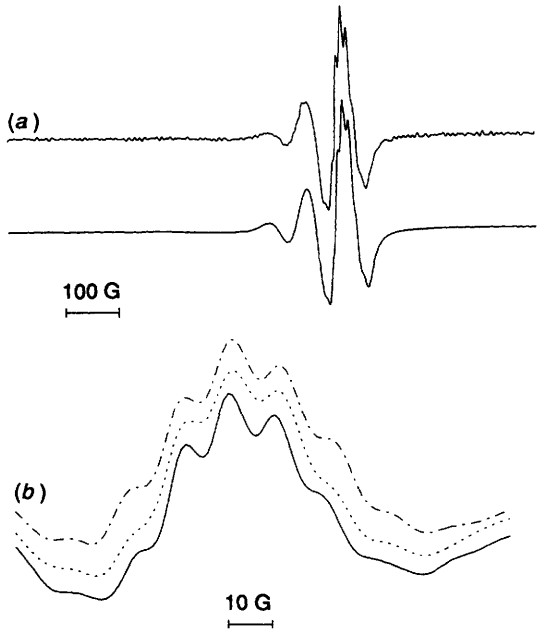
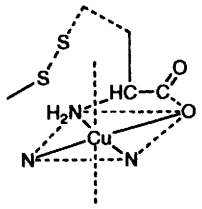


Fig. 4 (*a*) Second-derivative EPR spectrum of the $[Cu(HL)(phen)]^-$ ternary complex at room temperature and the simulated spectrum; (*b*) second-derivative expansion (110 G) of the experimental $M_I = \frac{3}{2}$ component (298 K, solid line) and simulations for 2N + 1N (· · ·) and for 2N + 2N (— · —)

Line number	4	3	2	1	0	1	2	3	4
3N		1	3	6	7	6	3	1	
4N	1	4	10	16	19	16	10	4	1

Scheme 1



Scheme 2

parameters obtained for the ternary complex are well comparable with those reported for similar complexes of copper(II) with 1,10-phenanthroline and different amino acids.[44-46]

In conclusion, the results suggest that the stable conformation of the ternary $[Cu(HL)(phen)]^-$ complex can involve only one glutamyl moiety. The carboxyl groups which are not involved in the equatorial binding scheme are located far from the metal, the apical position in the co-ordination sphere being possibly occupied by a solvent molecule at physiological pH. The carboxylic groups of ionised moieties of the hexapeptide do not chelate the copper atom. The co-ordination of the metal by a carboxylic group occurs in synergism with that with an amino group to give a five-membered stable ring, the other two positions in the plane being occupied by two nitrogen donor atoms from the phenanthroline-ligand, as shown in Scheme 2.

Acknowledgements

Financial support from Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Rome (40 and 60% funds) and from Consiglio Nazionale delle Ricerche, Rome, is gratefully acknowledged.

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Received 25*th October* 1994; *Paper* 4/06531J