Electronic Absorption and Electron Spin Resonance Studies on the Interaction between the Biologically Relevant Copper(II) Glycylglycine and L-Histidine Complexes with D-Penicillamine[1]

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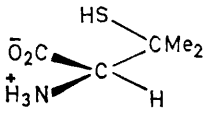
The ternary systems Cu^{II} + Gly-Gly + D-penicillamine, and Cu^{II} + L-His + D-penicillamine, were examined in neutral aqueous solutions by electronic absorption and e.s.r. spectroscopy. The Gly-Gly system was further examined by circular dichroism and electrophoresis measurements. The results are compared with those from the binary system Cu^{II} + D-penicillamine, and with the ternary systems where L-cysteine or dimercaptopropanol replace D-penicillamine. Evidence is presented for the formation of dimeric ternary complexes in the Gly-Gly and L-His systems at concentrations of Cu : Pen < 1 : 2. The relevance of the ternary complexes to the mode of mobilisation of the excess copper in Wilson's disease is discussed.

WILSON'S disease is a genetic defect resulting in an excessive accumulation of copper(II) ions in the liver, kidney, and brain, leading to neurological and other disorders and, ultimately, death if not detected and treated at an early stage. Several metal-binding reagents have been examined for their effectiveness in removing the excess copper from patients of Wilson's disease, *e.g.* cysteine, 2,3-dimercaptopropanol (BAL), EDTA, sodium diethyldithiocarbamate, sodium sulphide, and D-penicillamine (Pen) †, (I). It is the latter chelating agent which is currently used.[2] The mechanism by



(I) D-penicillamine

which penicillamine successfully removes copper, particularly the mode of interaction between Pen and copper(II) under biological conditions, is not known. However, a knowledge of the mechanism is vital in indicating the requirements for a more effective therapeutic chelating agent.

An excess of Pen is known to reduce copper(II) ions to copper(I) according to equation (1). There is some

$$4RSH + 2Cu^{II}_{aq} \longrightarrow 2[Cu^{I}SR] + RS{-}SR + 4H^{+} \quad (1)$$

evidence that the Cu^{I} complex formed is polymeric in nature.[3,4] Earlier workers [4] considered that the therapeutic action of Pen involved the reduction of the accumulated copper(II) to copper(I). However, other strong reducing agents, *e.g.* BAL, sulphide ion, and cysteine, are not as therapeutically effective as Pen. Others [5] consider that copper(II) will only chelate with the oxidised form of penicillamine, *i.e.* the disulphide $[H_2N{\cdot}CH(CO_2^-){\cdot}CMe_2{\cdot}S{\cdot}S{\cdot}CMe_2{\cdot}CH(CO_2^-){\cdot}NH_2]$, although oral administration of the disulphide form is known to be ineffective in promoting the removal of the excess of copper in Wilson's disease.[6] A third alternative advanced [7,8] is that of ' reductive chelation,' in which an initial chelation of the copper(II) by Pen is followed by reduction to mobile copper(I) species.

Although the views are diverse these studies have a common factor in that they were based on the interaction between Pen and aqueous copper(II) ions. In fact, it is now known through the work of Sass-Kortsak, Sarkar, and their co-workers,[9,10] that even in blood plasma there is a negligible amount of free copper(II) ion. It, therefore, seemed more appropriate to us to consider the interaction of Pen with chelated copper(II) ions rather than with free copper(II) ions; and, to determine (i) whether ternary species of the type $[L{-}Cu{-}(Pen)]$ can form under physiological conditions, where L is a biological ligand such as found in human serum, (ii) the oxidation state of the copper centre in such ternary species, and (iii) whether such species can act as transporters for copper ions through biological membranes.

EXPERIMENTAL

Earlier workers [11] commented on the lack of reproducibility of the Cu^{II}–Pen system. We traced this problem to two sources. First, the impurity of many commercial samples of penicillamine, particularly the hydrochloride salts of the DL and L forms of penicillamine which were severely contaminated with other thiols. We found both Koch–Light's ' puriss ' grade D-penicillamine and Ralph N.

† The abbreviations used throughout for the more common amino-acids and peptides are those recommended in the IUPAC–IUB tentative rules; see, ' Amino-acids, Peptides and Proteins,' ed. G. T. Young (Specialist Periodical Reports), The Chemical Society, London, 1970, vol. 2, p. 226.

[1] Presented in part at the XVI I.C.C.C., Dublin, 1974.
[2] J. M. Walshe, in ' The Biochemistry of Copper,' eds. J. Peisach, P. Aisen, and W. E. Blumberg, Academic Press, New York, 1966, p. 475.
[3] P. Kroneck, C. Naumann, and P. Hemmerich, *Inorg. Nuclear Chem. Letters*, 1971, **7**, 659.
[4] J. J. Vallon and A. Badinand, *Analyt. Chim. Acta*, 1968, **42**, 445.
[5] D. D. Perrin and R. P. Agarwal, in ' Metal Ions in Biological Systems,' ed. H. Sigel, Marcel Dekker Inc., New York, 1973, vol. 2, p. 167.
[6] J. M. Walshe, *Brit. Medicin. Bull.*, 1957, **13**, 132.
[7] J. Peisach and W. E. Blumberg, *Mol. Pharmacol.*, 1969, **5**, 200.
[8] Y. Sugiura and H. Tanaka, *Chem. Pharm. Bull.* (*Japan*), 1970, **18**, 368.
[9] A. Sass-Kortsak, *Adv. Clin. Chem.*, 1965, **8**, 1.
[10] B. Sarkar and Y. Wigfield, *Canad. J. Biochem.*, 1968, **46**, 601.
[11] E. W. Wilson and R. B. Martin, *Arch. Biochem. Biophys.*, 1971, **142**, 445.

Emmanuel's 'Gold Label' D-penicillamine to be satisfactory (>99% purity based on elemental analysis, optical rotation, and pH titration), and were used throughout this work. Secondly, the order of addition of reagents was important. If Pen was added to aqueous copper(II) solutions then non-reproducible results (particularly as a function of time) were generally obtained. This we attribute to the formation of polymeric copper(I) species making reaction (1) above not readily reversible and explains Wilson and Martin's [11] observations that the addition of chelating agents to aqueous Cu^{II}–Pen solutions did not shift the visible electronic-absorption maximum, contrary to the results presented here. The procedure adopted for the present work was to prepare the copper(II) chelate solutions and the Pen solutions separately at the required pH (7.2—7.5) and then to add the required amounts of the Pen solution to the Cu^{II} solution, adjusting the pH if necessary, and finally diluting to the required volume. All solutions and operations were de-oxygenated and handled under a nitrogen atmosphere. The solutions so obtained were found to be stable for at least a day. All other reagents were of AnalaR or comparable grade and were used without further purification. Copper(II) solutions were prepared from AnalaR $CuSO_4 \cdot 5H_2O$ and standardised with EDTA.

Electronic absorption spectra were recorded with a Unicam SP 800 spectrophotometer, thermostatted at 22—25 °C. Circular dichroism spectra were measured with a Roussel Jouan dichrographe B instrument calibrated with isoandrosterone. Electrophoresis experiments were carried out with an S.A.E. Shandon Electrophoresis model U77 apparatus. Separations were achieved using 0.1 mol dm^{-3} concentrations of reagents, Whatman No. 1 filter paper strips, 0.1 mol dm^{-3} $NaClO_4$ electrolyte, and a constant potential of 250 V. Glycylglycine was labelled with *ca.* 50 μCi of ^{14}C-Gly-Gly and the radioactivity distribution measured with a Panax radiochromatogram scanner. E.s.r. spectra were obtained on a Varian E-3 spectrometer, calibrated by comparison with a standard sample of Mn^{II} in MgO. Spectrum simulation was performed using a method similar to that described elsewhere [12] with final fitting done against a graph-plotter output.

RESULTS

Electronic Absorption Spectra.—Addition of D-penicillamine to aqueous copper(II) solutions gave the violet colour typical of many thiol–Cu^{II} complexes. For the ternary systems, Cu^{II} + Gly-Gly + Pen, Cu^{II} + L-His + Pen, and Cu^{II} + L-His + L-Cys, representative data are shown in Figures 1—3. The absorption spectra of the systems Cu^{II} + Gly + Pen and Cu^{II} + Gly-Gly-Gly + Pen (1 : 1 : *x* ratios) showed similar behaviour to the Gly-Gly system with increasing Pen concentration; the shifts in absorption maxima following the order Gly > Gly-Gly > Gly-Gly-Gly (at constant *x*).

Replacement of Pen by BAL resulted in the precipitation of Cu^{I} species even at relatively low BAL : Cu^{II} ratios. A similar effect was observed with L-Cys except in the Cu^{II} + L-His + L-Cys system.

Circular Dichroism and Electrophoretic Studies.—The Gly-Gly system was studied in more detail. Addition of Pen to solutions of Cu^{II} + Gly-Gly resulted in the appearance of a broad Cotton effect in the visible region (maximum at 580 nm) (Figure 1) which increased in magnitude up to the ratio 1 : 1 : 1 approximately and then decreased until at the 1 : 1 : 2 ratio the optical activity was <5% of the maximum observed. Over the whole range of Pen concentrations the maxima and minima of the Cotton effect

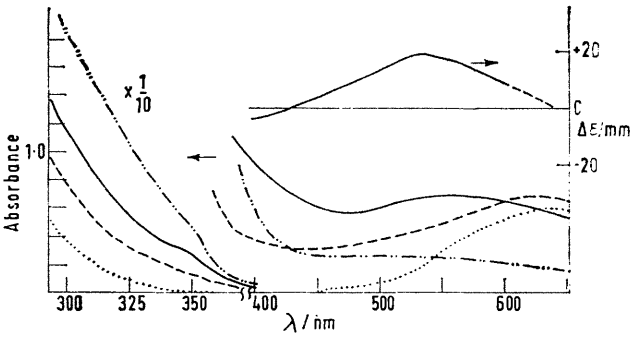


FIGURE 1 U.v.-vis. spectra and circular dichroism of the Cu^{II} + Gly-Gly + Pen system at 1 : 1 : *x* ratios: *x* = 0 (· · ·), *x* = 0.5 (— — —), *x* = 1.0 (——), and *x* = 2.0 (— · · — · ·). Cu^{II} 0.01 mol dm^{-3} in all cases

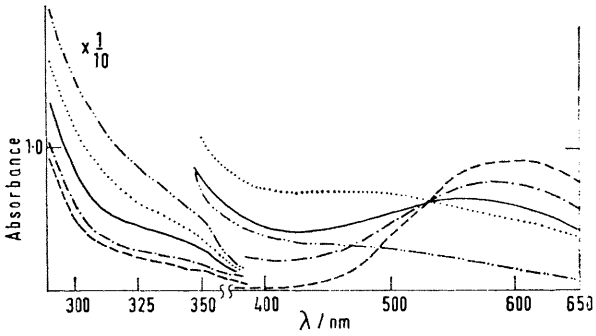


FIGURE 2 U.v.-vis. spectra of the Cu^{II} + L-His + Pen system at 3 : 1 : *x* ratios: *x* = 0 (— — —), *x* = 0.5 (— · — · —), *x* = 1 (——), *x* = 1.5 (· · ·), *x* = 2.0 (— · · — · ·). Cu^{II} 0.01 mol dm^{-3} in all cases

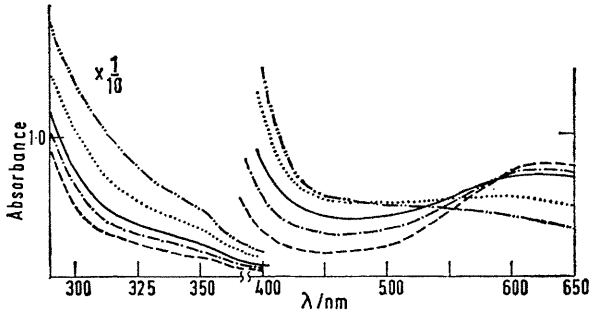


FIGURE 3 U.v.-vis. spectra of the Cu^{II} + L-His + L-Cys system at 3 : 1 : *x* ratios: *x* = 0.5 (— — —). *x* = 0.75 (— · — ·), *x* = 1.0 (——), *x* = 1.5 (· · · ·), *x* = 2.0 (— · · — · ·). Cu^{II} 0.01 mol dm^{-3} in all cases

was constant indicating the presence of a single optically active species. The maximum deflection corresponded to a value for $\varepsilon_L - \varepsilon_R$ of 0.3 based on the *total* copper present. Electrophoretic separations using ^{14}C-labelled glycylglycine revealed three major species: (*a*) a blue (reflectance maxima *ca.* 640 nm) electrically neutral radioactive species,

[12] J. F. Boas, R. H. Dunhill, J. R. Pilbrow, R. C. Srivastava, and T. D. Smith, *J. Chem. Soc.* (*A*), 1969, 94; T. Lund and W. E. Hatfield, *J. Chem. Phys.*, 1973, **59**, 885.

which was also the only fraction observed in the absence of Pen; (*b*) a violet (broad reflectance maxima at 500—530 nm) anionic and radioactive species; (*c*) a smaller amount of a pink non-radioactive species with a greater electrical mobility than species (*b*). Reasonable separations were achieved up to the ratio 1 : 1 : 1.

E.s.r. Spectra.—Frozen-solution spectra observed at 77 K were typical of Cu^{II} with the unpaired electron predominantly in the $d_{x^2-y^2}(b_{1g})$ ground-state. At room temperature only g_{av} and $A_{iso}(Cu)$ were measured. No superhyperfine coupling was detected. For each system examined the room temperature spectra were similar in form over the range of mixtures studied, so that peak heights could be taken as proportional to intensity and, hence, as an approximate measure of the amount of e.s.r.-detectable Cu^{II} in solution. Any corrections required for variation in spectrum width were found to be small except at the higher concentrations of Pen ($\geqslant$1 : 2 Cu : Pen ratio), but in this region the amount of Cu^{II} was only of the order of 5% of the total copper (Figure 4). There was an apparent small increase in Cu^{II} concentrations beyond the 1 : 2 ratio which can be attributed to reoxidation of copper(I) in the cavity. Only small changes in the g_{av} and A_{iso} values were observed with added Pen (or Cys) until the higher ratios were reached when more marked changes occurred.

For many of the spectra resolvable features were present which showed that, in the presence of Pen (or Cys), each spectrum was a mixture of two components; the first component diminishing in intensity with increased Pen to eventually allow the resolution of the second. The g_{av} and A_{iso} values appropriate to each species were then obtained by extrapolation. The e.s.r. results are summarised in the Table.

Features in the spectra of the frozen solutions at *ca.* $g = 2$ (only resolvable when Pen or Cys was present) allowed assignment of the $g_{\|}$, $g_{\perp}$, and A parameters. The g_{av} value from the frozen-solution spectrum was the same as that observed for the fluid-solution spectrum, indicating that rapid freezing had probably not significantly disturbed the equilibria present at room temperature. Again, the spectra of the frozen solutions could be resolved into two components. In the Cu : Pen 1 : 2 region the frozen-solution spectra were predominantly that of the second component (accounting for *ca.* 5% of the total Cu) and assignment of the spin Hamiltonian parameters for this component was confirmed by computer simulation of the spectrum (Figure 5).

The solutions in which water was the only ligand competing with Pen showed exceptions to the above behaviour. At room temperature only a single broad line was observed, and in the frozen solutions no structure was noted in the spectra until the Cu : Pen ratio was as high as 1 : 1.5. The spectrum then observed, arising from a species which we will call [Cu(Pen)]-1, was replaced by a different, but much weaker, one at the ratio 1 : 2. This latter spectrum, with lower $g_{\|}$ and $A_{\|}$ values, is attributed to a species called [Cu(Pen)]-2.

E.s.r. and molecular orbital parameters

		$g_{\|}$	$g_{\perp}$	$A_{\|}$/cm^{-1}	$A_{\perp}$/cm^{-1}	g_{av}	A_{iso}/cm^{-1}	P/cm^{-1}
Cu + Gly-Gly	Component 1	2.23	2.07	0.0177	0.0009 [a]	2.12	0.0067	0.026
+ Pen	Component 2 [b]	2.13	2.03	0.0184	0.0024	2.06	0.0087	0.022
Cu + His + Pen	Component 1	2.22	2.07	0.0197	0.0007 [a]	2.12	0.0070	0.029
	Component 2 [b]	2.13	2.03	0.0184	0.0024	2.06	0.0085	0.022
Cu + His + Cys	Component 1	2.22	2.06	0.0192	0.0009 [a]	2.12	0.0070	0.029
	Component 2 [b]	2.13	2.03	0.0184	0.0024	2.06	0.0075	0.022
Cu + Pen	[Cu(Pen)]-1	2.27	2.11	0.0149		2.19		0.024
	[Cu(Pen)]-2	2.14	2.03	0.0140				0.017

[a] Calculated from measured $A_{\|}$ and A_{iso}. Error $\simeq \pm 0.0005$ cm^{-1}. [b] Parameters obtained from simulated spectrum. Errors: $\delta g \simeq \pm 0.005$, $\delta A_{\|} \simeq 0.0002$ cm^{-1}, $\delta A_{\perp} \simeq 0.0002$ cm^{-1}. Errors in parameters obtained by direct measurement: $\delta g \simeq \pm 0.005$, $\delta A_{\|} \simeq 0.0005$ cm^{-1}, $\delta A_{iso} \simeq 0.0001$ cm^{-1} (component 1) $\simeq 0.0005$ cm^{-1} (component 2).

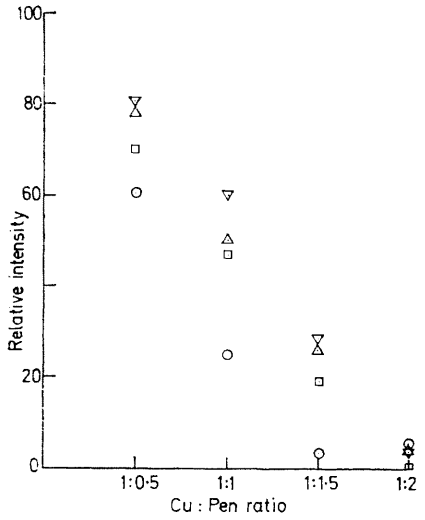


FIGURE 4 Intensity of the room-temperature e.s.r. signal *vs.* Cu : Pen ratio relative to that at Cu : Pen = 1.0. ⊙ Cu^{II} + Gly-Gly + Pen; △ Cu^{II} + His + Pen; ▽ Cu^{II} + His + Cys; ⊡ Cu^{II} + H_2O + Pen

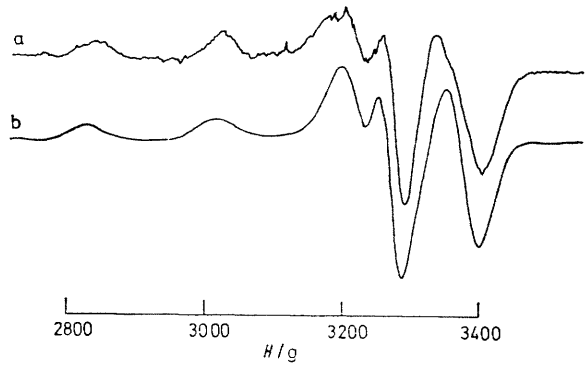
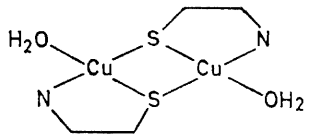


FIGURE 5 The e.s.r. spectrum from component 2 at 77 K; a, experimental $\nu = 9.26$ GHz; b, simulated using the parameters shown in the Table

DISCUSSION

Optical Spectra.—Under the conditions employed it may be assumed that the complexes initially present in the Gly-Gly and L-His systems are glyclyglycinato-copper(II), [Cu(Gly-$\bar{G}$ly-$\bar{O}$)],* and bis(L-histidinato)-copper(II), [Cu(L-His-$\bar{O}$)$_2$], respectively. The nature of the u.v.-vis. absorption changes and the isosbestic point at 580 nm for the L-His system are consistent with the formation of an intermediate ternary complex. The absence of an isosbestic point in the Gly-Gly system is not unexpected since the mode of formation of the ternary complexes [Cu(Gly-$\bar{G}$ly-O)($\bar{P}$en-$\bar{O}$)]$^{2-}$ and [Cu(L-His-$\bar{O}$)($\bar{P}$en-$\bar{O}$)]$^-$ is different; in the [Cu(Gly-$\bar{G}$ly-$\bar{O}$)] complex there is still one co-ordination site available (occupied by water). Apart from this, the nature of the u.v.-vis. changes are very similar in the two systems. The corresponding glycine and Gly-Gly-Gly systems show related changes. Strong support for the formation of a Cu^{II} ternary complex comes from the circular dichroism and electrophoresis measurements on the Gly-Gly system. In the former case the observation of a Cotton effect in the visible region of the spectrum can only be attributed to optically active *d–d* transitions arising from the co-ordination of D-Pen to Cu^{II} ions. The magnitude of the Cotton effect is similar to that observed in many copper(II) amino-acid complexes, suggesting that the optically active species is a major component. This species may be related to the violet anionic species observed in the electrophoresis experiments, which, from its radioactivity, must contain co-ordinated Gly-Gly, *i.e.*, [Cu^{II}(Gly-$\bar{G}$ly-$\bar{O}$)(D-$\bar{P}$en-$\bar{O}$)]$^{2-}$. The blue neutral species is the starting complex [Cu^{II}(Gly-$\bar{G}$ly-$\bar{O}$)]^0 and the pink, non-radioactive species is either some Cu^I species or [Cu^{II}(D-$\bar{P}$en-$\bar{O}$)$_2$]$^{2-}$. Further evidence for the co-ordination of Pen to Cu^{II} in the ternary systems comes from the strong u.v. absorptions noted on the addition of Pen (or Cys) to the copper(II) solutions. These intense absorptions, not found with other amino-acids but observed with other N,S donor ligands,[3,13] can be attributed to the formation of a Cu–S bond giving rise to a charge-transfer transition.

Of particular interest is the nature of the species present in the Cu^{II} + H_2O + Pen system at ratios of Cu : Pen < 1 : 2. Typical of these solutions is a major violet-coloured species ($\lambda_{max.}$ 520 nm).† This species has been described [7,11] as a mixed valence species following the earlier work of Klotz *et al.*[13] However, the evidence for a mixed valence species is weak and, more recently, Hemmerich and his co-workers [3] have presented evidence for a dimeric copper(II) species, (II), for the complex of the related ligand cysteamine. A prominent shoulder at 350 nm is observed for the ternary systems investigated here, which is much weaker in the Cu^{II} + His + Pen system. A similar band is found in some of the copper proteins (*e.g.* laccases) and has been shown [14] to arise from a diamagnetic Cu^{II}–Cu^{II} unit.



(II)

E.s.r. Spectra.—The e.s.r. intensity measurements (Figure 4) of the Cu^{II} + H_2O + Pen system show a decrease in Cu^{II} concentration following the stoicheiometry of equation (1). The intensity data further show that the presence of additional chelating agents appear to have no stabilising effect on the Cu^{II} state, *i.e.* there is no apparent increase in the amount of Cu^{II}. Indeed, with Gly-Gly present there is a significant decrease in the amount of e.s.r.-detectable Cu^{II} at comparable Pen levels, contradictory to the spectroscopic and electrophoretic measurements which suggest a major ternary Cu^{II} species is formed, particularly at the 1 : 1 : 1 ratio. This apparent contradiction can be explained in terms of an ' e.s.r. non-detectable ' Cu^{II} species, such as the sulphur-bridged dimeric species (II) proposed by Hemmerich.[3] No evidence was found from the e.s.r. spectra for any mixed-valence species. The problem is then to determine from the e.s.r. parameters the co-ordination environment of the copper(II) species of components 1 and 2 for each system.

The σ-bonding parameter β_1 may be obtained [15] from the expression

$$\beta_1^2 = -A_{iso}/P\kappa + (g_{av} - 2.0023)/\kappa \qquad (2)$$

where the symbols have their usual meaning.[16] However, because of the assumptions necessary in deriving values of P and κ we find that little reliance can be placed on the values obtained for β_1 other than in the most qualitative way.

Giordano and Bereman [17] have treated the parameter P as a variable to absorb the effects of electron delocalisation by use of formula (3). The authors suggest

$$P = (A_{\parallel} - A_{\perp})/[(g_{\parallel} - 2) - 5/14(g_{\perp} - 2) - 6/7] \qquad (3)$$

that identification of the bonding groups may be obtained from the value of P, *e.g.* for bonding to four sulphur atoms they find that [17] P lies in the range 0.020—0.016 cm^{-1} and for bonding to two nitrogens and two oxygens P = 0.026 cm^{-1}. We have calculated P

* The symbols Gly-$\bar{G}$ly-$\bar{O}$ and D-$\bar{P}$en-$\bar{O}$ indicate the di-anionic forms of these ligands.

† We find the extinction coefficient of this band to be approximately 50 and not 500 as earlier reported by Wilson and Martin.[11] B. Sarkar, personal communication, also reports that the extinction coefficient of this band is subject to counter-ion effects, although the sulphate ion was observed to have a negligible effect. This counter-ion effect will be a feature of our further studies.

[13] I. M. Klotz, G. H. Cerlinski, and H. A. Fiess, *J. Amer. Chem. Soc.*, 1958, **80**, 2920.
[14] R. Malkin, B. G. Malmstrom, and T. Vanngard, *European J. Biochem.*, 1969, **10**, 324.
[15] J. I. Zink and R. S. Drago, *J. Amer. Chem. Soc.*, 1972, **94**, 4550.
[16] C. M. Guzy, J. B. Raynor, and M. C. R. Symons, *J. Chem. Soc.* (*A*), 1969, 2299.
[17] R. S. Giordano and R. D. Bereman, *J. Amer. Chem. Soc.*, 1974, **96**, 1019.

by use of equation (3) for both our own results and for a selection of complexes whose co-ordination environment is fairly certain.[18] For component 1 of our Cu^{II} + Gly-Gly and Pen system $P = 0.026$, whereas for the corresponding His species $P = 0.029$. The second component in each case has $P = 0.022$. Where Pen is the only chelate $P = 0.023$ and 0.017 for [Cu(Pen)]-1 and [Cu(Pen)]-2 respectively. In all cases the change in P is consistent with a greater number of sulphur donor atoms bonding to copper(II) in components 2 than in components 1. In neither case can we definitely define the co-ordination chromophore since our calculations on other, well characterised, complexes show considerable overlap in P values, *e.g.* for a number of complexes involving nitrogen and/or oxygen donor atoms $P = 0.022$—0.029 cm^{-1}.

An alternative approach is that of Peisach and Blumberg,[7] in which $A_{\parallel}$ for a number of Cu^{II} complexes is plotted against their corresponding $g_{\parallel}$ values. In

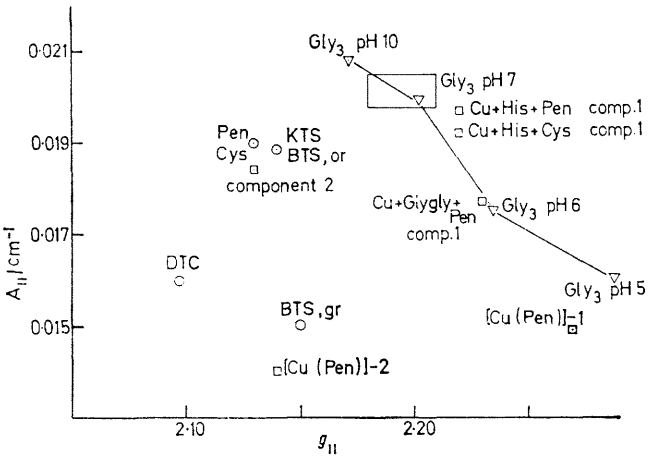


FIGURE 6 A plot of spin Hamiltonian parameters for Cu^{II} complexes. ⊡ This work; ⊙ from ref. 8, abbreviations as used by those authors; ▽ Cu^{II} + diglycylglycine at different pH values (K. E. Falk, H. C. Freeman, T. Jansson, B. G. Malmstrom, and T. Vanngard, *J. Amer. Chem. Soc.*, 1967, **89**, 6071); the Cu^{II} complexes with CuN_4 chromophores reported by G. F. Bryce, *J. Phys. Chem.*, 1966, **70**, 3549, lie within the rectangle outlined

such a plot it is found [7] that copper(II) complexes with the same co-ordination chromophoric group tend to cluster in the same region of the plot. This can be seen in Figure 6 which includes data for a number of well defined Cu^{II} complexes together with the parameters derived for components 1 and 2 of the systems reported in this work. This approach to the identification of the Cu^{II} environment in a complex can be rationalised since $A_{\parallel}$ is dominated by β_1^2, a measure of the σ-bonding; $g_{\parallel}$ involves both β_1^2 and β_2^2, the latter being a measure of the in-plane π-bonding, and also the magnitude of the $b_{1g} \longrightarrow b_{2g}$ splitting. Complexes with a CuN_4 chromophore are clustered at the top of the plot, successive replacement of N- by O-donor ligands results in $A_{\parallel}$ decreasing and $g_{\parallel}$ increasing, while replacement by S-donor ligands causes both $A_{\parallel}$ and $g_{\parallel}$ to decrease, consistent with greater covalency in such complexes.

It is obvious from the positions in the $A_{\parallel}$ *vs.* $g_{\parallel}$ plot of components 1 of the Gly-Gly and His systems and from the nature of the e.s.r. spectral changes, that these components correspond to the initial complexes present in solution, *i.e.* [Cu(Gly-$\bar{G}$ly-$\bar{O}$)] and [Cu(His-$\bar{O}$)$_2$] with CuN_2O_2 [19] and CuN_4 + CuN_3O [20] chromophoric groups respectively. Components 2 of these systems, which only predominate in solutions at the higher Pen : Cu ratios, are seen from Figure 6 to be in the CuN_2S_2 chromophore region, which is also consistent with the greater delocalisation found in components 2 from the calculated β_1^2 and P values. Delocalisation of electronic charge from the metal onto the ligands is required in the case of thiol ligands if the copper ion is to remain in the formal +2 oxidation state. The identical parameters for components 2 of the Gly-Gly and His systems suggest that they are the same species, most likely bis(penicillaminato)copper(II) with a CuN_2S_2 chromophore. In fact, the parameters noted here are very close to those noted by Peisach and Blumberg [7] for their alleged [Cu($\bar{P}$en-$\bar{O}$)$_2$]$^{2-}$ and [Cu($\bar{C}$ys-$\bar{O}$)$_2$]$^{2-}$ species measured under very different conditions. The similarity in e.s.r. parameters for the bispenicillaminato- and biscysteinato-complexes is also found in our work, *viz.* components 2 of the Cu^{II} + His + Pen and Cu^{II} + His + Cys systems. Interestingly, Peisach and Blumberg find that the copper(II) complex of 3-ethoxy-2-oxobutyraldehyde bisthiosemicarbazone of known CuN_2S_2 chromophore,[21] and the orange form of the butyraldehyde thiosemicarbazone (BTS) complex with a probable CuN_2S_2 chromophore, have similar parameters to the Pen and Cys complexes (Figure 6) and also exhibit ^{14}N-superhyperfine structure indicating extensive electron delocalisation.

The two components identified from the e.s.r. spectra for the Cu^{II} + H_2O + Pen system differ considerably from those obtained in the presence of the chelates Gly-Gly and His. These differences also show up in the calculated β_1^2 and P values. The $A_{\parallel}$ and $g_{\parallel}$ parameters derived for [Cu(Pen)]-1 are subject to a wide margin of error due to the poorly resolved e.s.r. spectrum, so that the species could have a CuN_2O_2, $CuNO_3$, or a $CuNSO_2$ chromophore, but certainly not CuO_4 (for the Cu^{II}_{aq} ion $A_{\parallel} \simeq 0.0127$ cm^{-1}, $g_{\parallel} \simeq 2.40$).[18] From the stoicheiometry, [Cu(Pen)]-1 in this case is almost certainly a 1 : 1 Cu : Pen complex, in which Pen co-ordinates most likely through the amino and sulphur groups, *i.e.* Cu(N,S)$(OH_2)_2$. The nature of component 2 is less certain, it is only observed at the 1 : 2 Cu : Pen ratio and accounts for only <5% of the total copper. It has $A_{\parallel}$ and $g_{\parallel}$ parameters similar to those of the green-form

[18] W. B. Lewis, M. Alei, and L. O. Morgan, *J. Chem. Phys.*, 1966, **45**, 4005.
[19] H. C. Freeman, *Adv. Protein Chem.*, 1967, **22**, 257.
[20] D. R. Williams, *J.C.S. Dalton*, 1972, 790.
[21] M. R. Taylor, E. J. Gabe, J. P. Glusker, J. A. Minkin, and A. L. Patterson, *J. Amer. Chem. Soc.*, 1966, **88**, 1845.

of $[Cu(BTS)_2]$ which reputedly [22] has a CuN_2S_2 chromophore, but the evidence for this is not strong.

Conclusions.—From the foregoing it is evident that, in the ternary systems, there is no e.s.r. detectable ternary complex, although there is evidence from the spectroscopic and electrophoretic measurements for the presence of copper(II). The two sets of data are consistent if the intermediate formation of a dimeric ternary complex occurs, as shown in (III) below, with a magnetic exchange (spin interaction) coupling of *ca.* 500 cm^{-1}. The molecular structure of the dimer poses

$$Cu^{II}L_n + D\text{-}\bar{P}en\text{-}\bar{O} \rightleftharpoons \tfrac{1}{2}\left[Cu^{II}L_2(D\text{-}\bar{P}en\text{-}\bar{O})_2\right] + L_{n'}$$

$$\tfrac{1}{2}\left[Cu^{II}{}_2L_2(D\text{-}\bar{P}en\text{-}\bar{O})_2\right] + D\text{-}\bar{P}en\text{-}\bar{O} \rightleftharpoons Cu^{II}(D\text{-}\bar{P}en\text{-}\bar{O})_2 + L_2$$

$$Cu^{II}(D\text{-}\bar{P}en\text{-}\bar{O})_2 \rightleftharpoons \tfrac{1}{x}\left[Cu^{I}(D\text{-}\bar{P}en\text{-}\bar{O})\right]_x + \overbrace{Pen\ \ Pen}$$

L—His; $n = 2, n' = 1$; Gly—Gly; $n = 1, n' = 0$

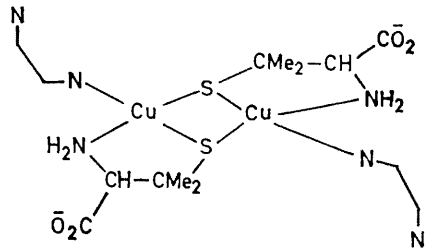
(III)

a problem unless axial co-ordination sites are used since there are insufficient co-ordination sites within the square plane of each Cu^{II} ion to satisfy the co-ordination requirements of each chelate. We believe the two most likely structures are (IV) and (V), with the possibility of a rapid equilibrium between the two forms.

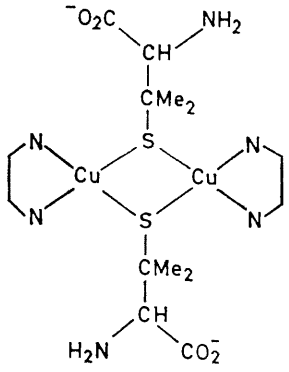
Probably the most significant result from the clinical viewpoint is the observed solubility of the [(Pen)-Cu^{I}] complexes as compared to the highly insoluble Cys and BAL analogues. In terms of mobilisation of the excess of copper, soluble blood-serum complexes are obviously important [9] and this factor may well reflect the greater therapeutic value of Pen as compared to other thiol reagents, *e.g.* Cys and BAL.* Furthermore, Harris and Sass-Kortsak [23] have presented evidence that low molecular-weight copper(II) complexes, *i.e.* bis(amino-acidato)copper(II) complexes, may function as transport species for copper(II) between blood and tissues. This could be an important role for the ternary copper(II) complexes involving penicillamine outlined in this work.

* More recently Walshe has found triethylenetetra-ammine to be effective in mobilising excess of copper from patients of Wilson's disease. This also forms highly stable and soluble copper(II) complexes (J. M. Walshe, *Lancet*, 1969, **2**, 1401).

Further evidence is being sought for the occurrence of the dimeric species and on the ability of these ternary



(IV)



(V)

(N N refers to either Gly-Gly or His co-ordinating through two N atoms.)

complexes to transport copper between the blood serum and tissue phases.

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[22] W. E. Blumberg and J. Peisach, *J. Chem. Phys.*, 1968, **49**, 1793.
[23] D. I. M. Harris and A. Sass-Kortsak, *J. Clin. Invest.*, 1967, **46**, 659.