An Electron Spin Resonance Study of Copper(II)–Peptide Complexes in the Liquid State*

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ABSTRACT: The complexes formed between Cu(II) and some peptides were investigated over a wide range of stoichiometry, pH, temperature, and physical state using electron spin resonance (esr) and optical rotatory dispersion (ORD). It was found, for the Cu(II)–glycylglycine system, that the low-temperature (−180°) solid-state esr results contain ambiguities which are not present in the liquid-state results; the latter can be interpreted in terms of covalent binding of Cu(II) by two magnetically equivalent nitrogen atoms. Isotopic substitution with ^{15}N in the glycylglycine yielded esr spectra indicating that Cu(II) is covalently bound by both the peptide and terminal amino nitrogen atoms and that the bonds are essentially equivalent. It is thus apparent that two structurally inequivalent nitrogen atoms can interact equivalently with the Cu(II).

The study was extended to more complicated Cu(II)–peptide complexes and to the *Pseudomonas* copper protein; an effect upon the esr spectra attributable to the rate of rotation of Cu(II)–peptide complexes was detected.

While the nature and disposition of at least four of the ligand atoms about iron in heme proteins is known, no system of ligands which binds copper in naturally occurring copper proteins has been identified. In the present study the problem of identification of these ligands has been approached using electron spin resonance (esr) spectroscopy. Some simple model chelates of Cu(II) with peptides of increasing complexity have been examined under a wide variety of conditions of stoichiometry, pH, temperature, and physical state. Esr studies of copper peptides and amino acids have also been made by Brill *et al.* (1964), Malmstrom and Vanngard (1960), Venable (1965), Warner and Weber (1953), Wiersema and Windle (1964), and Windle *et al.* (1963). These studies established the main esr characteristics of the complexes, but the existence of multiple forms under various conditions of pH and peptide:Cu(II) ratios has not been adequately considered, and in no case has an absolute identification of ligand atoms been made.

Materials and Methods

Peptides were obtained from Nutritional Biochemicals Corp. and from Cyclo Chemical Corp. Homogeneity was checked using chromatography with butanol–acetic acid–water and phenol–water solvents. ^{63}Cu, as CuO, was supplied by Oak Ridge National Laboratory and was of 99.9% purity. D_2O was supplied by Volk Radiochemical Co. and was of 99.64% purity. Glycine-^{15}N was supplied by Bio-Rad Laboratories and was of 96% purity in ^{15}N. It was recrystallized once from an ethanol–ether–water mixture and gave a single spot chromatographically. "Cu(II)-bisimidazole" [copper(4,4′-(5,5′)-bisimidazolylmethane)$_2^{2+}$] was kindly supplied by Professor J. Fruton.

Electron spin resonance spectroscopy was performed with a Varian Model V 4500 spectrometer using 100-kcycle field modulation and equipped with a Field dial VFR 2200 magnetic field regulator. Control of incident microwave power was achieved using a microwave bridge permitting continuous power attenuation, which utilizes a circulator. Temperature control for the esr experiments was achieved using the Varian liquid nitrogen accessory and a Varian EPR heater control unit. Temperature control was accurate to ±1°. For experiments above freezing temperatures, the samples were placed in a quartz capillary surrounded by hexane or benzene for heat transfer.

For the range of incident microwave power from 0.2 to 270 mw it was found that, in all cases, the relationship signal intensity $\sim\sqrt{\text{microwave power}}$ was followed, as expected if no saturation occurs. The esr spectra were run at modulation amplitudes such that modulation broadening was not a factor. Usually a modulation of 1.6 gauss was used.

Optical rotatory dispersion measurements were made with a Rudolph spectropolarimeter in the laboratory of Dr. J. Schellman at the University of Oregon

* From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon. *Received September 21, 1966.* Supported by grants from the U. S. Public Health Service, National Institute of Arthritis and Metabolic Diseases (AM 07180) and from the American Cancer Society (E225E). A portion of this work was presented at the International Conference on the Biochemistry of Copper, Harriman, N. Y., Sept 1965. Work done in partial fulfillment of the requirements for the Ph.D. degree at the University of Oregon Medical School. This work was supported by a predoctoral fellowship (1-FI-GM-25,525-01) from the U. S. Public Health Service.

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and with a Durrum-Jasco recording spectropolarimeter at the Durrum Instrument Co., to whom we are indebted.

A mixed anhydride procedure was used for the synthesis of the glycylglycine ethyl ester containing ^{15}N (Greenstein and Winitz, 1961). The peptides were checked for homogeneity using chromatography.

Glycylglycine ethyl ester forms, on the basis of the potentiometric titration curves, optical spectra, and esr spectra, the same Cu(II) complexes as does GG.[1] Where material was limiting, the syntheses were terminated at the ethyl ester form of the peptide.

Pseudomonas aeruginosa blue copper protein was kindly supplied by Dr. P. Ambler and was concentrated by lyophilization after removing excess $(NH_4)_2SO_4$ by passage through a G-25 Sephadex column. The pH of solutions of this protein was controlled by the use of buffers or, for pH 4.0 and 10.5 solutions, by direct addition of HCl or NaOH. An acetate buffer was used for pH 4.0, phosphate buffer was used for the pH 6.5 and 7.8 solutions, and glycine buffer was used for the pH 9.5 and 10.5 solutions. Cu(II) was determined by quantitative esr spectroscopy after acid digestion of the protein. The standards consisted of $CuCl_2$ dissolved in the same acid medium.

Results and Discussion

Esr Spectra (−180°) of Cu(II)–GG. Malmstrom and Vanngard (1960) determined the esr spectra at −180° of several Cu(II) complexes at a single pH, using excess ligand. Under these conditions the complexes gave the asymmetric esr absorption spectrum seen at low temperatures for a wide variety of Cu(II) complexes. As recently reported (Gould and Mason, 1966), the esr spectra of solutions of equimolar Cu(II) and GG at various pH values at −180° and an ionic strength of 0.16 in NaCl resemble the esr spectra of complexes of Cu(II) with the copper occupying an axially symmetric site (Sands, 1955) or a site with trigonal distortion (Venable, 1965). No obvious conclusions regarding possible differences among the complexes at the different pH values could be made from these spectra.

As reported previously (Gould and Mason, 1966), the −180° spectra of Cu(II)–GG frozen solutions show a strong dependence on ionic strength (NaCl concentration), while the esr spectra of liquid solutions show no ionic strength dependence. The spectrum for the Cu(II)–GG frozen solution with added NaCl is of the asymmetric type from which a $g_{||}$ and $g_{\perp}$ value can be determined, but the same frozen solution without added salt has a much more symmetrical spectrum and no value for $g_{||}$ can be measured.[2] The same type of behavior is observed for $CuCl_2$ with and without added salt.[3] The added salt may prevent regions of high Cu(II) complex concentration from separating out upon freezing where, in the absence of salt, Cu(II)–Cu(II) interactions could produce the symmetrical spectrum (Kozyrev, 1955; Vanngard, 1966). However, the line shape and width is independent of Cu(II) concentration over the range 0.05–50 mM.[3] These data can also be interpreted by assuming that the form of the complex is altered in the presence of salt at −180°. These salt effects are not seen in the esr spectra of the complexes in the liquid state so that the following discussion will be confined to these forms.

The Forms of Cu(II)–GG in Solution. Koltun *et al.* (1963) were able to fit a calculated titration curve for an equimolar Cu(II)–GG solution to one experimentally determined by postulating the following sequential reactions.

$$Cu^{2+} + GG^- \rightleftharpoons CuGG^+$$

$$CuGG^+ \rightleftharpoons CuGG + H^+$$

$$CuGG \rightleftharpoons CuGGOH^- + H^+$$

$$CuGG + CuGGOH^- \rightleftharpoons (CuGG)_2OH^-$$

$$CuGGOH^- \rightleftharpoons CuGG(OH)_2^{2-} + H^+$$

Their results agree with those of Dobbie and Kermack (1955) with the exception that Dobbie and Kermack have postulated the existence of a 1:2 Cu(II)–GG complex in a 1:2 molar mixture of Cu(II) and GG at pH values >10. Utilizing the experimentally determined equilibrium constants, Dobbie and Kermack (1955) calculated values for Cu(II):GG ratios and pH in which a maximum concentration of a given complex species will be present. The free form of Cu(II) (hydrated Cu^{2+}) should be present in greater than 90% concentration in an equimolar mixture of Cu(II) and GG with no added NaOH (pH 4); the complex species CuGG and $CuGGOH^-$ should be present in greater than 90% concentration in equimolar mixtures of Cu(II) and GG at 2 moles of NaOH/GG, pH 7, and at 3 moles of NaOH/GG, pH 10, respectively. Previously obtained esr results (Gould and Mason, 1966) confirm these observations in that only three distinct esr spectra are seen for mixtures of Cu(II) and GG at various pH values. These spectra can be

[1] Abbreviation used: GG, glycylglycine.

[2] The temperature dependence of the equimolar Cu(II)–GG pH 7 solution and of the equimolar Cu(II)–GG pH 10 solution with added NaCl is as predicted by Liehr and Ballhausen (1958) for an octahedral complex with tetragonal distortion. The method of Liehr and Ballhausen can be used to calculate $g_{||}$ and $g_{\perp}$ from the high-temperature spectra and compared to the experimentally determined $g_{||}$ and $g_{\perp}$ values at −180°. This gave, for the pH 7 solution, $g_{||}$ (calcd) = 2.40, $g_{||}$ (obsd) = 2.20, and $g_{\perp}$ (calcd) = 2.1, $g_{\perp}$ (obsd) = 2.05; for the pH 10 solution, $g_{||}$ (calcd) = 2.20, $g_{||}$ (obsd) = 2.21, and $g_{\perp}$ (calcd) = 2.05, $g_{\perp}$ (obsd) = 2.05. The agreement appears to be good at pH 10 but is not consistent with the results observed at pH 7. The errors inherent in estimating g values from experimentally determined curves (Blumberg, 1966) may underlie these results.

[3] D. C. Gould and H. S. Mason, unpublished results.

correlated with Cu(II) in the forms Cu^{2+}, CuGG, and $CuGGOH^-$. At intermediate pH values, the experimentally determined esr spectra can be approximated by a combination of these three spectra. Koltun *et al.* (1963) postulate the existence of a complex of the form $CuGG(OH)_2^{2-}$ which would be present in an equimolar mixture of Cu(II) and GG at 4 moles of NaOH/GG added. The esr spectrum under these conditions is the same as that seen at 3 moles of NaOH/GG. Either this species is not present or the presence of an additional OH^- in the coordination sphere of the Cu(II) has no effect on the esr spectrum. The species $CuGG^+$ cannot be obtained in high concentration (>10%) (Bryce *et al.*, 1965) and considerable amounts of Cu^{2+} and CuGG are present in addition. A careful analysis of the spectra in the pH range 4–7 would be needed to extract the spectrum of this species.

The data of Dobbie and Kermack (1955) indicate that in 1:2 molar mixtures of Cu(II) and GG, at pH values above 7, the Cu(II) should be distributed as $Cu(GG)_2^-$ and $Cu(GG)_2^{2-}$. Kim and Martell (1964) could fit their potentiometric data to proposed reactions by assuming the existence of only the $Cu(GG)_2^-$ complex. In the 1:2 Cu(II):GG mixtures, the esr spectra are, at all pH values, the result of the three spectra obtained for the 1:1 mixtures. The consumption of 4 equiv of NaOH in a 1:2 mixture of Cu(II) and GG can be explained, not by the formation of the complex $Cu(GG)_2^-$, but by the formation of the complex $CuGG(OH)^-$ and to the titration of one equivalent of the zwitterion form of GG (Doran *et al.*, 1964). There exist only two readily esr detectable forms of Cu(II)–GG complexes: that seen at pH 7 in an equimolar Cu(II)–GG solution, denoted by CuGG, and that seen at pH 10 in an equimolar Cu(II) and GG mixture, denoted by $CuGG(OH)^-$. The following discussion will be confined to these two complexes.

The Symmetry Problem for the Cu(II)–GG Complex. We have calculated possible configurations of the GG molecule about the Cu(II) using methods first proposed by Corey and Sneen (1955) and later applied to the calculation of ring geometries for the Co(II)–ethylenediamine complexes by Corey and Bailar (1959). This method assumes that the terminal amino and peptide nitrogen atoms, as well as the carbonyl oxygen, are ligands and that normal bond angles are retained in the GG molecule. Using these assumptions, neither a plane nor a center of symmetry was found to be possible at the Cu(II). Empirically, square-planar Cu(II) is the stable form of Cu(II) (Bowers and Owen, 1955), and some distortion of normal bond angles and lengths will occur to achieve this ground state.[4]

Optical Rotatory Dispersion of Cu(II)–GG-like Complexes. The experimental data will not be presented here as the results are essentially an abbreviated version of the work of Bryce *et al.* (1965). Assuming that the symmetry of the ligands about the Cu(II) is reflected in the d-orbital wave functions of the Cu(II) or those molecular orbitals which are involved in the "d–d" transition, then the lack of a plane or center of symmetry for these ligands will lead to a Cotton effect in the optical rotatory dispersion (ORD) curve in the region of the d–d transition (600 mμ). Bryce *et al.* (1965) found a Cotton effect in the 600-mμ band of the CuGG and $CuGGOH^-$ complexes when one or both of the glycines are replaced with optically active amino acids. However, the interpretation of this Cotton effect is uncertain. Piper and Karipides (1962) attribute the optical activity of the cobalt(ethylenediamine)$_3^{3+}$ complex to a distortion of the octahedral disposition of the nitrogen ligand atoms which abolishes the plane or center of symmetry. This would then be in agreement with the calculated model and the conclusions of Bryce *et al.* (1966). Mason and Norman (1965), on the other hand, have shown a deuterium effect on the circular dichroism of the cobalt(ethylenediamine)$_3^{3+}$ complex and have interpreted this in terms of a model in which the Co d-orbital wave functions extend to the carbon and hydrogen atoms as well as to the nitrogen atoms of the ethylenediamine molecules. According to this interpretation, the nitrogen ligand atoms could be symmetrically arranged about the Cu(II) with the asymmetry giving rise to the Cotton effect owing to the carbon and hydrogen atoms attached to the ligands. This interpretation could ac-

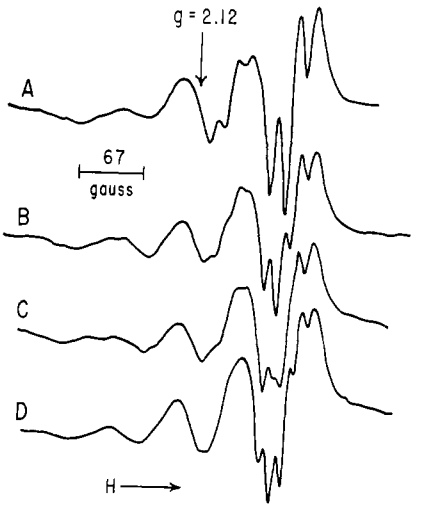

FIGURE 1: Esr spectra of the pH 7 Cu(II)–^{14}N,^{15}N-glycylglycine complexes; 10 mM $CuCl_2$ with 10 mM glycylglycine ethyl ester, 2 equiv of NaOH, pH 7; temperature 10°, liquid state. Curve A, μ-^{15}N-glycylglycine ethyl ester; curve B, ^{15}N(terminal amino),-^{14}N(peptide)-glycylglycine ethyl ester; curve C, ^{14}N-terminal amino),^{15}N(peptide)-glycylglycine ethyl ester; and curve D, μ-^{14}N-glycylglycine ethyl ester.

[4] These results are not in agreement with the crystallographic analysis of diaquoglycylglycinato copper(II) hydrate of Strandberg *et al.* (1961) as recently reviewed by Freeman (1966). They found that the three peptide ligand atoms plus one water molecule formed an approximate square with the Cu(II) slightly displaced from the center of the square along the normal to the plane of the square.

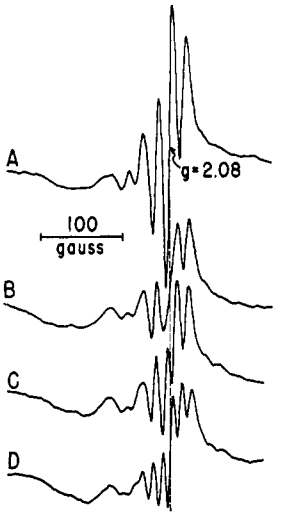

FIGURE 2: Esr spectra of the pH 10 Cu(II)–^{14}N,^{15}N-glycylglycine complexes; 10 mM $CuCl_2$ with 10 mM glycylglycine ethyl ester, 3 equiv of NaOH, pH 10; temperature 10°, liquid state. Curve A, μ-^{15}N-glycylglycine ethyl ester; curve B, ^{15}N(terminal amino),-^{14}N(peptide)-glycylglycine ethyl ester; curve C, ^{14}N-(terminal amino),^{15}N(peptide)-glycylglycine ethyl ester; and curve D, μ-^{14}N-glycylglycine ethyl ester.

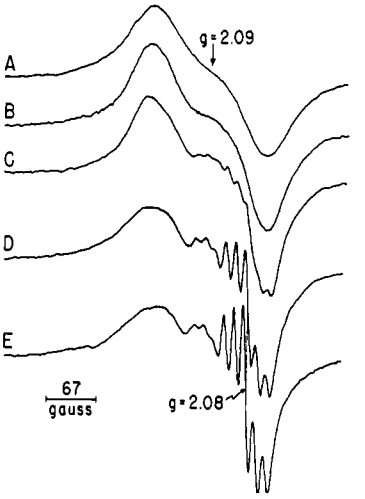

FIGURE 3: Temperature dependence of the esr spectra of the pH 10 Cu(II)–glycylglycine complex; 10 mM glycylglycine and 10 mM $CuCl_2$ with 3 equiv of NaOH added, pH 10, liquid state. Curve A, 85°; curve B, 60°; curve C, 30°; curve D, 18°; and curve E, −7°.

count for the Cotton effect seen in the 600-mμ band of the Cu(II)–glycyl-L-alanine complex at pH 10. Thus the problem of ligand symmetry about the Cu(II) remains unresolved for the Cu(II)–GG complexes in the liquid state.

Liquid-State Esr Results for the Cu(II)–GG Complexes. The esr spectrum of the complex CuGG consists of four main lines (Figure 1) with the high-field lines showing additional hyperfine structure. These four lines are consistent with the interaction of an unpaired electron with a copper nucleus of nuclear spin $I = {}^3/_2$ (Brill *et al.*, 1964). The model assumes binding of Cu(II) by a terminal amino nitrogen atom and a peptide nitrogen atom. The additional hyperfine structure on the $m = -{}^3/_2$ and $m = -{}^1/_2$ lines (Figure 1) consists of five hyperfine lines separated by 12.9 gauss. ^{14}N has a nuclear spin of $I = 1$, and unpaired electron interaction with two magnetically equivalent nitrogen nuclei would result in a five-line spectrum. Thus, the esr results are consistent with the interaction of an unpaired electron with two such nuclei. A further characteristic of importance is the relative intensities of the five hyperfine lines. If they are due to two equivalent nitrogen atoms then the ratios of the intensities should be 1:2:3:2:1. However, owing to the large degree of overlap in these lines and their asymmetry (Freed and Fraenkel, 1963), no definite assignment of intensities can be made from their spectra.

^{15}N has a nuclear spin of $^1/_2$ and unpaired electron interaction with two ^{15}N would result in a three-line hyperfine structure. As can be seen from Figure 2, curve A, the esr spectrum of the complex CuGG, formed from GG containing ^{15}N in both the terminal amino nitrogen and peptide nitrogen position, the five-line hyperfine structure seen for the CuGG complex formed from GG containing only ^{14}N has been reduced to a three-line hyperfine. The degree of separation of these lines is related to the Fermi contact term (Holton and Blum, 1962) and is proportional to μ/I, where μ is the nuclear magnetic moment and I is the nuclear spin quantum number. This predicts that the distance of separation of the ^{15}N hyperfine lines should be 1.4 times that of the ^{14}N hyperfine lines. The measured splitting of the five hyperfine lines (Figure 2, curve D) is 12.9 gauss; the splittings then observed for the ^{15}N peptide should be 18 gauss. The measured splittings (Figure 2, curve A) are 17.6 gauss.

The assumption of the equivalence of the nitrogen atoms implies that the ^{15}N(peptide),^{14}N(terminal amino)-GG–Cu(II) complex would give the same hyperfine structure as the Cu(II) complex of ^{14}N(peptide),-^{15}N(terminal amino)-GG. Qualitatively, the two spectra are the same in that both (Figure 2, curves B and C) show a four-line hyperfine structure. Quantitatively, the curves are not identical in that the distance between the four hyperfine lines is different for these two complexes. In addition, the assumption of equivalence is not consistent with the hyperfine structure which would be predicted by the Fermi contact term. This would predict that the hyperfine structure should consist of six lines with a separation between lines of 13, 10, 16, 10, and 13 gauss. Four lines with a separation (Figure 2, curve B) of 14.8, 13.4, and 14.8 are

observed experimentally.[5]

The esr spectra for the complex $CuGGOH^-$ is shown in Figure 3. The center of gravity of the lines has been shifted from $g = 2.12$ for CuGG to $g = 2.09$ (Figures 1 and 4, respectively). A four-line hyperfine structure that would indicate unpaired electron interaction with the copper nucleus is no longer apparent, although the curves suggest that the two low-field lines are broadened to the extent that only one line is apparent (Figure 4). No interpretation of these observations can be given at this time.

The complex $CuGGOH^-$ formed from GG containing only ^{14}N also shows a five-line hyperfine structure on the high-field line of the spectrum. The splittings are the same as those observed with the complex CuGG. Similarly, the complex $CuGGOH^-$ formed from GG containing only ^{15}N shows a three-line hyperfine structure in agreement with the observations on the complex CuGG. The $CuGGOH^-$ complex formed from GG with ^{14}N-terminal amino ^{15}N-peptide also shows a four-line hyperfine as did the complex formed at pH 7. However, the complex $CuGGOH^-$ with ^{15}N-terminal amino ^{14}N-peptide shows only a three-line hyperfine structure (Figure 3, curve B). The center line is asymmetric, and at low modulation amplitudes (0.16 gauss) there is a definite inflection in the center line indicating that the hyperfine is composed of four lines. The two center lines could not be completely resolved. As in the case of the complex CuGG, the two esr spectra for the complexes formed from mixed $^{14}N,^{15}N$-GG are in qualitative agreement, but the assumption of the equivalence of the two nitrogen atoms does not give exact agreement between the simple theoretical approach used here and the experimental observations. Either the assumption that the unpaired electron density is the same at both nitrogen nuclei is incorrect, or the errors due to the assumptions made in deriving the spin Hamiltonians have become apparent. Although the interpretation of the results is not complete, the data presented indicate that the hyperfine structure seen in the esr spectra of the Cu(II)–GG complexes is due to the nitrogen atoms and that both nitrogen atoms in a single GG molecule are interacting covalently with the Cu(II). The esr results provide unequivocal evidence that the peptide nitrogen atom is covalently bound to the Cu(II) in both the pH 7 and 10 complexes.

The esr results suggest the magnetic equivalence of the two nitrogen atoms in the GG molecule in terms of their bonds with Cu(II), but other experimental evidence indicates that these two nitrogen atoms are structurally not equivalent. The peptide nitrogen atom is adjacent to a carbonyl carbon atom, and infrared data indicate that it may form π bonds with the carbonyl carbon atom (Kim and Martell, 1964); the peptide nitrogen atom has ionized a proton and carries a partial negative charge (Freeman 1966); the Cu–N-(peptide) bond length is, from crystal structure analysis,

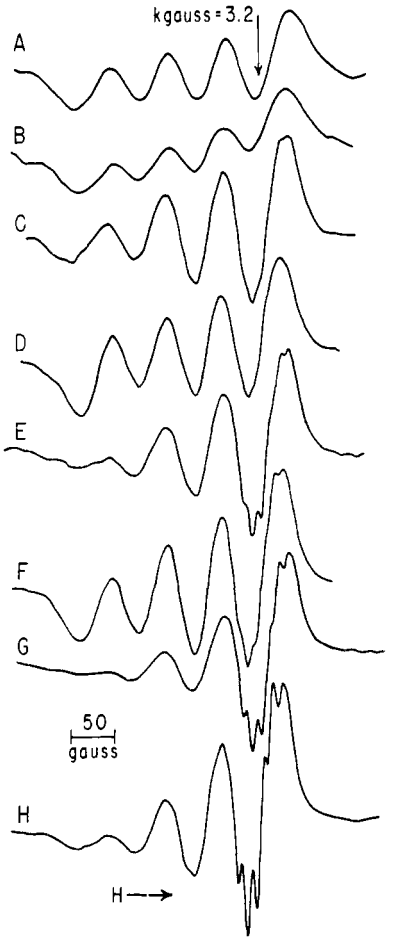

FIGURE 4: Esr spectra of 10 mM $CuCl_2$ with 10 mM glycylglycine (GG) and 10 mM glycyltryptophan (GT) with 2 equiv of NaOH added, pH 7, liquid state. Curve A, Cu(II)–GT, 60°; curve B, Cu(II)–GG, 60°; curve C, Cu(II)–GT, 30°; curve D, Cu(II)–GG, 30°; curve E, Cu(II)–GT, 20°; curve F, Cu(II)–GG, 20°; curve G, Cu(II)–GT, 10°; and curve H, Cu(II)–GG, 10°.

less than the Cu–N(terminal amino) bond length (Strandberg *et al.*, 1961). Yet, the esr evidence is somewhat consistent with the assumption that the unpaired electron density at the nitrogen nuclei is the same for the peptide nitrogen atom as it is for the terminal amino nitrogen atom. This implies that the covalent bonds between the Cu(II) and the nitrogen atoms are the same. We conclude that hyperfine structure which can be explained in terms of magnetically equivalent nitrogen nuclei does not imply their structural equivalence.

Maki and McGarvey (1958) calculated from the Fermi contact term, which has no angular dependence, a value of α'^2 of 0.25 for the Cu(II)–bis(salicylaldehyde) imine complex. The value of α'^2 is a measure of the unpaired electron density at the nitrogen nucleus; a value of α'^2 of 0.5 would indicate a totally covalent

[5] No explanation can be given for these results at this time.

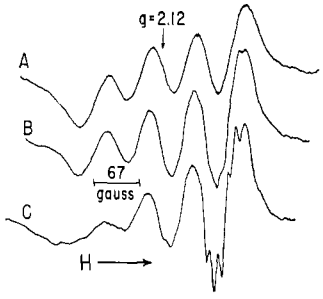

FIGURE 5: Temperature dependence of the esr spectra of the pH 7 Cu(II)–glycylglycine complex; 10 mM glycylglycine ethyl ester and 10 mM $CuCl_2$ with 2 equiv of NaOH, pH 7, liquid state. Curve A, 60°; curve B, 20°; and curve C, 0°.

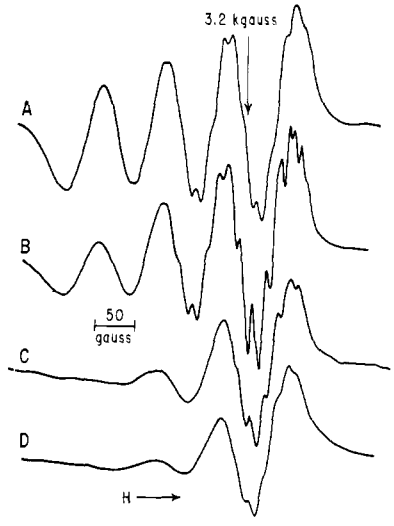

FIGURE 6: Esr spectrum of the Cu(II)–leucylglycylglycine complex; 10 mM leucylglycylglycine with 10 mM $CuCl_2$ at pH 10.7, 4 equiv of NaOH added, lqiuid state. Curve A, 64°; curve B, 24°; curve C, −8°; and curve D, −14°.

bond, and a value of 0.0 would indicate a totally ionic bond with the unpaired electron localized on the copper atom. The Cu(II)–bissalicylaldehyde complex has a nitrogen hyperfine splitting of 11.1 gauss, and the value of α'^2 is directly proportional to the degree of hyperfine splitting so that the Cu(II)–GG complexes with a nitrogen hyperfine splitting of 12.9 gauss have a value of $\alpha'^2 = 0.29$.

^{63}Cu and ^{65}Cu both give a four-line hyperfine structure, since both have $I = 3/2$. They occur naturally in the ratio 70 and 30%, respectively. However, ^{65}Cu has a nuclear magnetic moment greater than that of ^{63}Cu, and the separation of the four hyperfine lines for the ^{65}Cu spectrum will be slightly greater than that of the ^{63}Cu. As observed in the esr spectra of the Cu(II) complexes of 1,10-phenanthroline and 2,2′-dipyridine (Allen *et al.*, 1964), obtained at −180°, this overlapping can lead to anomalous hyperfine structure. The use of 99.9% ^{63}Cu gave no observable alterations in the esr spectra reported here.

Protons possess a nuclear magnetic moment and can give rise to hyperfine structure in esr spectra. Esr spectra of the pH 7 and 10 Cu(II)–GG complexes in D_2O were unaltered over those seen for H_2O solutions of these complexes. This would rule out hyperfine structure due to protons in the hydration sphere of the Cu(II) and also to the protons attached to the terminal amino nitrogen atom, since these protons would be exchanged with deuterium in D_2O solution (Scheraga, 1960; Klotz and Frank, 1965).

Temperature Dependence of the Cu(II)–GG Esr Spectra. The differences between the esr spectra for the Cu(II)–GG complexes with added NaCl at −180° (Figure 5) and those of the complexes in the liquid state (Figures 1 and 4) are that, while in both systems the complexes are randomly oriented, those in the liquid state are rotating while those in the frozen state are fixed. If the rotations in the liquid state are rapid compared to 9.5 kMcycles/sec, then the anisotropic (angular dependent) terms in the Hamiltonian will be averaged to zero, and the esr spectrum will be symmetrical. The presence of these anisotropic terms theoretically accounts for the asymmetric spectra observed at −180°, and the slow rotations of the copper proteins in the liquid state account for the similarity of their frozen- and liquid-state esr spectra (Morell *et al.*, 1964). The spectrum of the complex CuGG measured at 10° (Figure 6, curve H) resembles that obtained by Rivkind (1961) for Cu(II)–ethylenediamine under conditions where the rate of rotation of the complex was such that the anisotropy in the low-field lines was not averaged to zero. McConnell (1956) has shown that the relaxation time depends on M_I of the copper nucleus; the relaxation time increases as M_I increases. The line width of a hyperfine line is proportional to 1/(relaxation time) so that the $M_I = 3/2$ hyperfine line will be narrower than the $M_I = -3/2$ hyperfine line. Since the $M_I = 3/2$ hyperfine line is the high-field line (Lewis *et al.*, 1965), the results presented here are seen to be in agreement with the theory.

Rivkind altered the rotational rates of the Cu(II)–ethylenediamine complexes by changing the viscosity of the solvent. Glycerol, ethylene glycol, and propylene glycol form Cu(II) complexes at pH 7 and 10, so viscosity effects alone could not be ascertained in this way. Increasing the temperature would increase the rotational rates of these complexes. Murphy and Martell (1957) have measured the equilibrium constants for the reactions between Cu(II) and GG as a function of temperature and have found that, from 0 to 50°, the equilibrium constants show little variation so that proportions of the complexes CuGG and CuGG-OH^- alter very little over this temperature range. It seems justifiable to attribute the lack of symmetry in the esr spectrum for the complex CuGG at 0°,

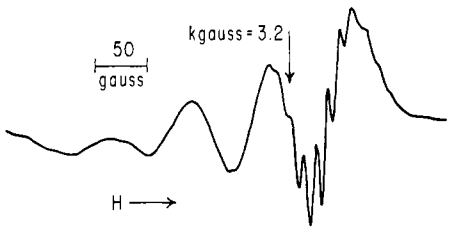

FIGURE 7: Esr spectrum of the Cu(II)–alaninamide complex; 10 mM $CuCl_2$ with 20 mM alaninamide, pH 10, 4 equiv of NaOH added/Cu(II); temperature −12°, liquid state.

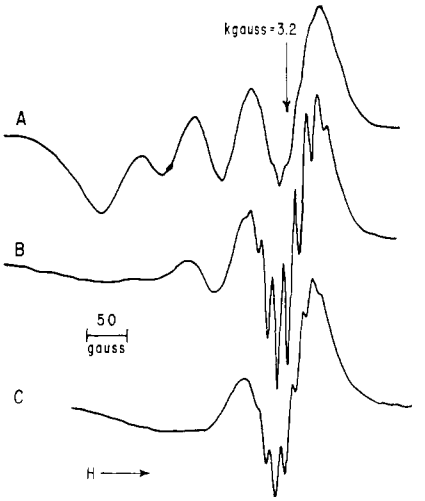

FIGURE 8: Esr spectra of the Cu(II)–bisimidazole complex; 10 mM $CuCl_2$ with 20 mM bisimidazole, at pH 7, liquid state. Curve A, 60°; curve B, 10°; and curve C, −5°.

and the approach to symmetry seen on increasing the temperature to the rotational motions of the complex. However, other mechanisms may be operative (Freed and Fraenkel, 1963).

Increasing the dimensions of the complexes without altering the binding groups of the Cu(II) would lead to decreased rotational rates. The peptide glycyltryptophan forms complexes with Cu(II) that are, potentiometrically, spectrophotometrically, and, on the basis of the hyperfine structure in the esr spectra, identical with the Cu(II)–GG complexes. Figure 6 compares the esr spectra of the pH 7 glycyltryptophan and glycylglycine–Cu(II) complexes at different temperatures. The esr spectrum of the Cu(II)–glycyltryptophan complex becomes symmetrical at a temperature 10° higher than that of the Cu(II)–GG complex; the asymmetry of the Cu(II)–GG spectrum is the same at a temperature T as the Cu(II)–glycyltryptophan spectrum at $T - 10°$. The low-field line of the Cu(II)–glycyltryptophan complex at 0° is broadened to the extent that it cannot be detected. Peptides that are larger than GG due to the presence of aliphatic groups (*e.g.*, glycylleucine and leucylleucine) show these same effects. These data are consistent with the proposal that the asymmetric curves seen at low (10°) temperatures and their conversion to symmetrical curves at higher (60°) temperatures is due to rotation of the complex ions. The situation is analogous to that discussed by Ingram (1958) for solutions of the biradical polymethylenebistriphenylmethyl. However, the asymmetric broadening of the lines shows that some anisotropy is present in these Cu(II)–esr spectra, contrary to what was assumed in using the Fermi contact term. The possibility that the rotations themselves are anisotropic must also be considered.

The temperature dependence of the esr spectrum for $CuGGOH^-$ (Figure 4) is much the same as that of CuGG. At no point is a four-line hyperfine present, and the exact interpretation of the temperature dependence is uncertain.

This provides a basis for correlating the results obtained with model compounds with those obtained with proteins. By increasing the size of the model complex without altering the binding groups of the Cu(II), the rotational rate of the complex can be decreased to the point where the asymmetric absorption consisting of $g_{||}$ and $g_{\perp}$ components is observed. Experiments are currently under way to enlarge upon these observations.

Other Cu(II)–Peptide Complexes. Cu(II)–glycine in the liquid state at pH values above 5 has a four-line esr spectrum resembling that of Cu(II)–GG at pH 7. Esr could not distinguish between the forms Cu(glycine)$_1$ and Cu(glycine)$_2$ (Dobbie *et al.*, 1955). Hyperfine structure, other than that attributable to unpaired electron interaction with the copper nucleus, was not observed for Cu(II):glycine ratios from 0.25 to 1 and pH values from 4 to 11. Sarcosine and glycylsarcosine form Cu(II) complexes which are esr spectrally equivalent to the Cu(II)–glycine system, in agreement with the work of Koltun *et al.* (1963).

Differences observed in the esr spectra of Cu(II)–dipeptide complexes in which the amino acids were aliphatic derivatives of glycine and contained no ligand atoms in the side chains can be attributed to the differences in the rotational rates among the complexes. Leucylleucine was the highest molecular weight studied. The peptides, leucyltyrosine and glycyltryptophan, form Cu(II) complexes which give the same esr spectra as the Cu(II) complexes of GG aside from the differences due to decreased rotational rates. The lack of Cu(II)–phenolic hydroxyl interaction was suggested by Dobbie and Kermack (1955) upon titrimetric evidence.

The Cu(II) complexes of diglycylglycine-like peptides have esr spectra with four hyperfine lines, characteristic of unpaired electron interaction with the copper nucleus, at all pH values between 5 and 11 (Figure 7). The spectra show the same type temperature dependence seen for the dipeptide–Cu(II) complexes. The additional hyperfine structure is most

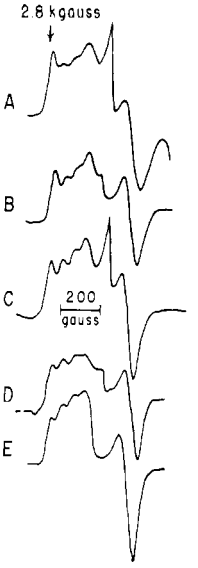

FIGURE 9: Solid-state esr spectra at −180° of a copper protein from *Pseudomonas aeruginosa*. Protein concentration is approximately 10^{-3} M (10^{-3} M in Cu(II)). Curve A, pH 10.5; curve B, pH 9.5; curve C, pH 7; curve D, pH 6.5; and curve E, pH 4. The magnetic field increases from left to right. The discontinuity in the curves at the high-field side of the 200-gauss marker represents a change in gain of $\times 10^{-1}$.

probably due to unpaired electron interaction with nitrogen nuclei. The number of lines (seven) is consistent with the interaction of an unpaired electron with three magnetically equivalent nitrogen nuclei; but the experimentally determined distance between the lines is not constant as it must be for this case. Peptides with ^{14}N replaced by ^{15}N will be needed for a complete analysis of these spectra.

The peptide cystinylbisglycine binds 2 atoms of Cu(II)/molecule of peptide. The absorption maximum (~610 mμ) and the esr spectra are much the same as those observed for the Cu(II)–GG complexes, indicating that the Cu(II) binding is much the same. It has recently been found that a copper–copper interaction occurs in the binuclear complex leading to a loss in esr signal intensity (Zuberbuhler and Mason, 1966). The exact form of the complex in which the interaction occurs has not been characterized.

Only at ligand:Cu(II) ratios $\geq$ 2:1 are the alaninamide– and bisimidazole– (Drey and Fruton, 1965) Cu(II) complexes stable above pH 6. The differences in the esr spectra seen for these complexes (Figures 8 and 9) can be correlated with the size of the complexes. The hyperfine structure seen on the high-field lines has a 12.5-gauss separation and consists of seven lines. These spectra are not the same as those observed for the leucylglycylglycine–Cu(II) complex (Figure 7). The seven-line hyperfine arising from three equivalent nitrogen atoms is difficult to rationalize on the basis of the stoichiometry. Isotopic substitution will be needed to interpret these spectra.

Esr of the P. aeruginosa Blue Copper Protein. The room-temperature esr spectra of the *Pseudomonas* blue copper protein were, within the limits of error due to the decreased signal to noise ratio, the same as the −180° esr spectra. Esr constants were $g_{max} = 2.056$, $g_{||} = 2.26$, and the hyperfine splitting constants were (low field to high) 0.006, 0.008, and 0.003 cm^{-1}, confirming Mason (1963). The extinction coefficient was 1.68×10^3/mole of Cu(II). The pH 4.0 and 10.5 solutions gave the same esr spectra in the presence of buffer or HCl–NaOH, indicating that the buffer anions were not involved. NaCl did not alter these spectra.

The equivalence of the solid-state (−180°) and liquid-state (20°) spectra of the copper protein is due to the slow rate of rotation of the protein in solution. The alterations in the $g_{||}$ region of the esr spectra (Figure 9) at the different pH values can only be correlated with pK's of known ligand groups if the binding constant of the Cu(II) to this group in the protein is known. While the changes observed in the esr spectra at the different pH values most probably reflect changes in the ligands of the Cu(II), no definite assignment of ligands can be made on this basis.

Thus, extension of these results to more complex and higher molecular weight peptides indicates that glycylglycine is perhaps not a good model for Cu(II)–protein interactions, and extrapolation of the results to the Cu(II)–protein systems will require a detailed analysis of the effect of the rotational rates of the complexes on the esr spectra.

Acknowledgment

We wish to thank Dr. T. Shiga, Dr. B. Allen, and Dr. W. Blumberg for helpful discussions and advice.

References

Allen, H. C., Kokoszka, G. F., and Inskeep, R. G. (1964), *J. Am. Chem. Soc. 86*, 1023.

Blumberg, W. E. (1966), *in* The Biochemistry of Copper, Aisen, P., Blumberg, W. E., and Peisach, J., Ed., New York, N. Y., Academic, p 399.

Bowers, K. D., and Owen, J. (1955), *Rept. Progr. Phys. 18*, 304.

Brill, A. S., Martin, R. B., and Williams, R. J. P. (1964), *in* Electronic Aspects of Biochemistry, Pullman, B., Ed., New York, N. Y., Academic, p 519.

Bryce, G. F., Pinkerton, J. M. H., Steinrauf, L. K., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 3829.

Bryce, G. F., Roeske, R. W., and Gurd, F. R. N. (1966), *J. Biol. Chem. 241*, 1072.

Corey, E. J., and Bailar, J. C. (1959), *J. Am. Chem. Soc. 81*, 2620.

Corey, E. J., and Sneen, R. A. (1955), *J. Am. Chem. Soc. 77*, 2505.

Dobbie, H., and Kermack, W. O. (1955), *Biochem. J. 59*, 246.

Dobbie, H., Kermack, W. O., and Lees, H. (1955), *Biochem. J. 59*, 240.

Doran, M. A., Chaberek, S., and Martell, A. E. (1964), *J. Am. Chem. Soc. 86*, 2129.

Drey, C. N. C., and Fruton, J. S. (1965), *Biochemistry 4*, 1258.

Freed, J. H., and Fraenkel, G. K. (1963), *J. Chem. Phys. 39*, 326.

Freeman, H. C. (1966), *in* The Biochemistry of Copper, Aisen, P., Blumberg, W. E., and Peisach, J., Ed., New York, N. Y., Academic, p 77.

Gould, D. C., and Mason, H. S. (1966), *in* The Biochemistry of Copper, Aisen, P. Blumberg, W. E., and Peisach, J., Ed., New York, N. Y., Academic, p 35.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. II, New York, N. Y., Wiley.

Holton, W. C., and Blum, H. (1962), *Phys. Rev. 125*, 89.

Ingram, D. J. E. (1958), Free Radicals as Studied by Electron Spin Resonance, London, Butterworths, p 231.

Kim, M. K., and Martell, A. E. (1964), *Biochemistry 3*, 1169.

Klotz, I. M., and Frank, B. H. (1965), *J. Am. Chem. Soc. 87*, 2721.

Koltun, W. L., Roth, R. H., and Gurd, F. R. N. (1963), *J. Biol. Chem. 238*, 124.

Kozyrev, B. M. (1955), Microwave and Radiofrequency Spectroscopy, The Faraday Society, Aberdeen, Aberdeen University, p 135.

Lewis, W. B., Alei, M., and Morgan, L. O. (1965), *J. Chem. Phys. 44*, 2409.

Liehr, A. D., and Ballhausen, C. J. (1958), *Ann. Phys. 3*, 304.

Maki, A. H., and McGarvey, B. R. (1958), *J. Chem. Phys. 29*, 35.

Malmstrom, B. G., and Vanngard, T. (1960), *J. Mol. Biol. 2*, 118.

Mason, H. S. (1963), *Biochem. Biophys. Res. Commun. 10*, 11.

Mason, S. F., and Norman, B. J. (1965), *Chem. Commun. 3*, 48.

McConnell, H. M. (1956), *J. Chem. Phys. 25*, 709.

Morell, A. G., Aisen, P., Blumberg, W. E., and Scheinberg, I. H. (1964), *J. Biol. Chem. 239*, 1042.

Murphy, C. B., and Martell, A. E. (1957), *J. Biol. Chem. 226*, 37.

Piper, T. S., and Karipides, A. (1962), *Mol. Phys. 5*, 475.

Rivkind, A. I. (1961), *Zh. Fiz. Khim. 35*, 2099.

Sands, R. H. (1955), *Phys. Rev. 99*, 1222.

Scheraga, H. A. (1960), *Brookhaven Symp. Biol. 13*, 71.

Strandberg, B., Lindquist, I., and Rosenstein, R. (1961), *Z. Krist. 116*, 266.

Vanngard, T. (1966), *in* Proceedings of the International Symposium on Electron Spin Resonance in Biological Systems, Ehrenberg, A., Malmstrom, B., and Vanngard, T., Ed., Stockholm, Wenner-Gren Symposium Publications (in press).

Venable, J. H. (1965), Ph.D. Dissertation, Yale University, New Haven, Conn.

Warner, R. C., and Weber, I. (1953), *J. Am. Chem. Soc. 75*, 5094.

Wiersema, A. K., and Windle, J. J. (1964), *J. Phys. Chem. 68*, 2316.

Windle, J. J., Wiersema, A. K., Clark, J. R., and Feeney, R. E. (1963), *Biochemistry 2*, 1341.

Zuberbuhler, A., and Mason, H. S. (1966), *in* Proceedings of the International Symposium on Electron Spin Resonance in Biological Systems, Ehrenberg, A., Malmstrom, B., and Vanngard, T., Ed., Stockholm, Wenner-Gren Symposium Publications (in press).