Multiple Forms of the Copper(II)–Carnosine Complex

By **Charles Eric Brown,*** Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226, U.S.A.
William E. Antholine and **Wojciech Froncisz,** Department of Radiology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226, U.S.A.

The effects of pH, temperature, and stoicheiometry on e.s.r. spectra of the copper(II)–carnosine complex have been measured at both *X*- and *S*-band. Four different complexes of Cu^{2+} and carnosine are found. At physiological pH a magnetically coupled copper(II) dimer is formed, but this is converted into a monomer in which the Cu^{2+} is co-ordinated to four equivalent carnosine molecules when the carnosine is present in excess. Anserine produces the same complexes as does carnosine, but homocarnosine and glycyl-L-histidine produce only the monomeric complex. The mechanism of crystallization of copper–carnosine and its possible importance in biological systems is reappraised.

L-CARNOSINE is a dipeptide (β-alanyl-L-histidine) present at high concentrations (*ca.* 1×10^{-3}—4×10^{-3} mol dm^{-3}) in skeletal muscle, nasal olfactory epithelium, and olfactory bulb of several species.[1-4] L-Anserine (β-alanyl-1-methylhistidine) and L-homocarnosine (γ-aminobutyryl-L-histidine), which are structural analogues of carnosine, are localized in skeletal muscle and the nervous system, respectively. It is accepted that carnosine and its analogues should play some physiological role in the specialized tissues where they are found,[5-12] but the observed distribution among organs of these peptides and of the enzymes that synthesize and degrade them [9,13] is not understood. It has been suggested that carnosine and anserine might be involved in regulation of anaerobic glycolysis of skeletal muscle by chelation of copper ions [5,6] and that the high activity of carnosinase in kidney is needed to avoid chelation of the cobalt in this organ by carnosine.[14] The latter suggestion is supported by the experimental observation that, although carnosine complexes cobalt(II) ion only weakly, mixed complexes of carnosine and cobalt(II) ion plus histidine or cysteine appear stable enough to exist *in vivo*.[14] Similar information concerning the stability of the copper(II)–carnosine and –anserine complexes is not available.

The literature contains several studies of the structure of copper(II)–carnosine, but each experimental technique appears to support a different structure. Experiments primarily with pH titration indicated structure (I) of Figure 1.[15-17] Ihnat and Bersohn [18] found with ^1H n.m.r. spectroscopy that the structure of the copper(II)–carnosine complex varies with pH. They stated that the carboxylate salt predominates at low values of pH and that structure (II) of Figure 1 (with $n = 1$) predominates at physiological values of pH. Structure (III) was stated to exist at pH >9, and it was suggested that two of these molecules would join to form the structure of crystalline copper(II)–carnosine [structure (IV) of Figure 1 [19]]. This study implied that structure (IV) should occur in solution only at high pH (*i.e. ca.* 12) where the primary amino-group of the β-alanyl residue is deprotonated. However, Boas *et al.*[20] made the somewhat contrary observation that frozen aqueous samples of copper(II)–carnosine or –anserine at pH 6.25 exhibit the e.s.r. spectrum of a copper(II) dimer, which would be predicted for the crystal. The study did not provide definitive information regarding the structure in aqueous solution at room temperature. Since the major

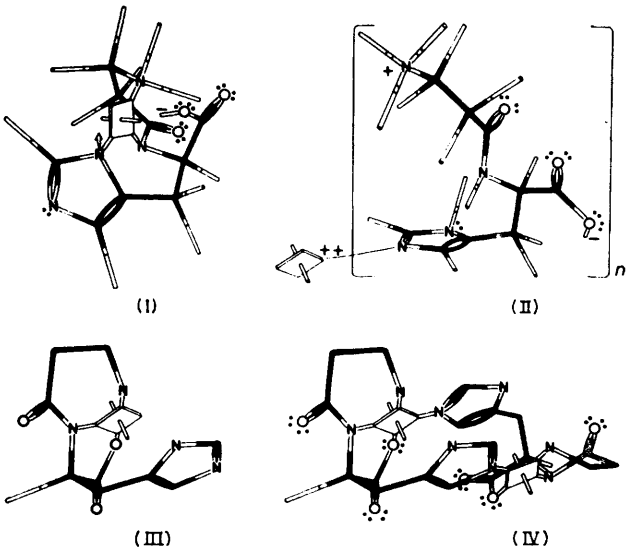

FIGURE 1 Structures that have been proposed for the copper(II)–carnosine complex. (I), The structure proposed to exist at or above neutrality on the basis of pH titration.[15-17] The arrow indicates the most likely position of the hydrogen atom on the imidazole ring. This nitrogen atom is methylated in anserine. (II), The predominant species in the physiological pH range when there is a 100—1 000-fold excess of carnosine (based on ^1H n.m.r. and e.s.r. data, $n = 4$). (III), The structure proposed by Ihnat and Bersohn [18] to exist in aqueous solution at pH >9. (IV), The structure of the crystal.[19] This is the same as or very similar to the structure of the dimer in solution at 77 K and ambient temperature. The hydrogen atoms have been omitted from structures (III) and (IV) for the sake of clarity

experimental differences between the ^1H n.m.r. and e.s.r. studies were the temperatures and the relative concentrations of copper(II) cation and carnosine, we have investigated the structure of the copper(II) complexes of carnosine and its analogues as a function of concentration of their components at various values of pH and temperature. This protocol makes possible a

more direct comparison of the results from the different techniques and provides explanations for the apparent contradictions in the earlier literature.

The results also provide an indication of which copper(II)–carnosine complexes might be expected to form in biological tissues. The *in vivo* concentration of copper is 20—60 μmol kg^{-1} of wet tissue,[21-23] of carnosine in skeletal muscle, nasal olfactory epithelium, and olfactory bulb is *ca.* 1×10^{-3}—4×10^{-3} mol dm^{-3},[1-4] and of anserine in skeletal muscle is *ca.* 1×10^{-3}—2×10^{-2} mol dm^{-3}.[1] One third of the total body copper resides in the skeletal muscle,[20] and the olfactory bulb, which receives axoplasmic flow from the primary olfactory neuron, has a higher concentration of copper than other regions of the central nervous system.[23] In addition it has been reported [5,6] that 5×10^{-2} mol dm^{-3} carnosine can reverse the inhibition of glycolysis by 47×10^{-6} mol dm^{-3} copper *in crude preparations of skeletal muscle*. Taken together, these data suggest that carnosine can chelate copper *in vivo* in spite of the general assumption that copper–carnosine complexes are weak and the knowledge that the concentration of free copper in biological tissues is much lower than the total concentration presented above.[24] The work presented here indicates that the structures of the various copper–carnosine complexes in aqueous solution are different from those generally accepted and that one of these complexes [structure (IV) of Figure 1] is more stable in solution at ambient temperature than expected. Although the copper complexes *in vivo* would be expected to be mixed complexes this work with the pure dipeptides provides the required insight regarding their chemistry to permit an understanding of the complexes that can be expected to form in the presence of a mixture of other ligands.

EXPERIMENTAL

Materials.—L-Carnosine, L-anserine nitrate, L-homocarnosine sulphate, and glycyl-L-histidine were purchased from Sigma Chemical Company. Sucrose was obtained from Schwarz/Mann. Sodium perchlorate, copper(II) sulphate, sodium hydroxide, and hydrochloric acid were purchased from Fisher. Deuterium oxide (99.8% isotopic purity), deuterium chloride, and sodium deuteroxide were from Merck, Sharp, and Dohme. The $^{63}Cu[SO_4]$ was prepared by dissolving ^{63}CuO from Oak Ridge Laboratory in a stoicheiometric amount of dilute H_2SO_4.

Methods.—All samples were prepared by mixing appropriately diluted stock solutions of $Cu[SO_4]$, peptide, and sucrose. All stock solutions were freshly prepared in distilled water, and spectra of the mixed samples were obtained within 24 h. Samples were stored at 4 °C. Replacement of exchangeable protons by deuterium was accomplished by repeatedly dissolving the sample in D_2O followed by flash evaporation to dryness. Solutions were adjusted to the appropriate pH at room temperature with dilute HCl, Na[OH], or their deuteriated analogues. No corrections were made for isotope effects. Corrections for the effect of temperature on the pH [25] also were not made because the samples did not visibly change colour during freezing as would be expected if the pH changed drastically, and the pH values at which the chemical structures appear to change are the same as determined by both e.s.r. and ^1H n.m.r. spectroscopy.

All e.s.r. spectra were obtained with spectrometers at the Biomedical ESR Center. The *X*-band spectra (9 GHz) were obtained with a Varian E-9 EPR spectrometer equipped with a dual-sample cavity. The *S*-band spectra (3.8 GHz) were obtained with an *S*-band bridge, cavity, and supporting equipment developed at the Center. Spectra of frozen samples were obtained by supporting the samples in the cavity with a finger Dewar filled with liquid nitrogen. Spectra of samples at temperatures above freezing were obtained with the Varian variable-temperature apparatus and a flat cell. The ^1H n.m.r. spectra were obtained at ambient temperature with a Nicolet NT-150 spectrometer and a 5-mm proton probe.

RESULTS

Characterization of Copper(II) Monomers.—The pH dependence of the structure of the copper(II)–carnosine complex in solutions with a 100—1 000-fold molar excess of carnosine to copper(II) ion demonstrated by ^1H n.m.r. spectral line broadening [18] is also found in the frozen solutions by e.s.r. spectroscopy (Figure 2). The e.s.r. spectra obtained at

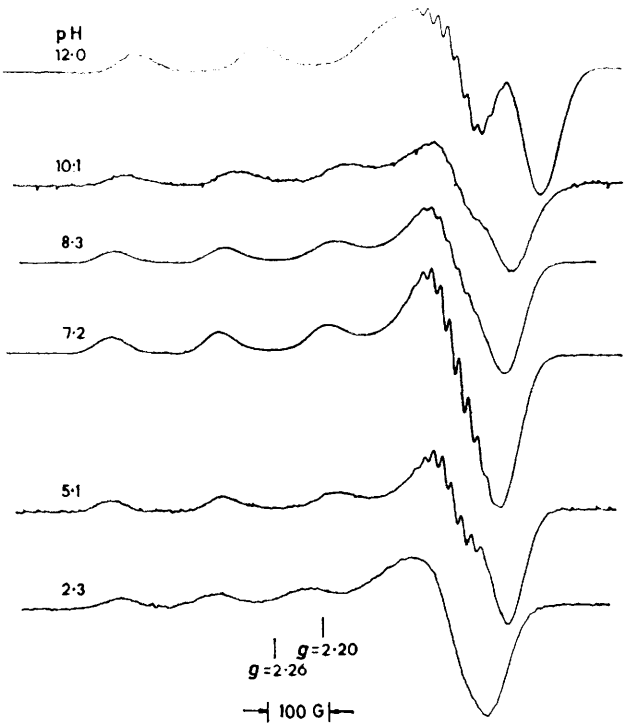

FIGURE 2 Effect of pH on the e.s.r. spectra (*X*-band) of aqueous solutions of copper(II)–carnosine complexes when the carnosine is present in excess. The solutions contain 9.10×10^{-4} mol dm^{-3} copper(II) ion, 0.910 mol dm^{-3} L-carnosine, and 0.27 mol dm^{-3} sucrose and are frozen at 77 K. All spectra indicate the presence of monomeric copper(II) complexes.

pH <5 do not exhibit nitrogen hyperfine splitting and are in agreement with formation of the copper(II)–carboxylate salt. No information regarding the stoicheiometry of this complex is available. Spectra obtained over the range pH *ca.* 5—12 indicate that two different complexes with nitrogen atoms co-ordinated to the copper(II) ion predominate at pH 7 and 12. The spectral resolution is lost at

pH *ca.* 9, probably as a result of overlapping of spectra from a mixture of complexes.

The e.s.r. spectra (*X*-band) of frozen (77 K) aqueous solutions containing a 100—1 000-fold excess of carnosine to Cu^{2+} at pH 7.2 (Figures 2 and 3) exhibit nitrogen hyperfine lines in the $g_\perp$ region that indicate the interaction of more than two nitrogen donor atoms with Cu^{2+}. Good simulations of both first- and second-derivative spectra (not shown) were made assuming four equivalent nitrogen donor atoms with A^N *ca.* 15 G and a coincident splitting of Cu^{2+} with A^N *ca.* 15 G,* but the experimental spectra for $g_\parallel$ were not sufficiently resolved to provide a definite measure of the stoicheiometry of the complex. However, one would expect the resolution of the e.s.r. spectrum to improve as the resonance frequency is decreased if the widths of the lines in the experimental spectra arise in part from the *g*-value

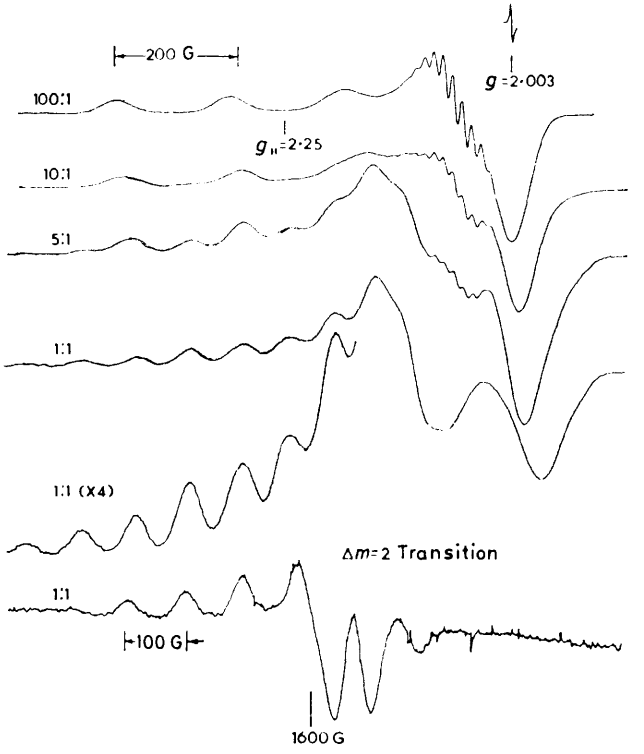

FIGURE 3 Effect of the ratio of molar concentrations of carnosine to Cu^{2+} (from 100 : 1 to 1 : 1) on the e.s.r. spectra (*X*-band) of frozen (77 K) aqueous solutions of 9.10×10^{-4} mol dm^{-3} copper(II) ion. All solutions contain 0.27 mol dm^{-3} sucrose and are adjusted to pH 7.2 ± 0.1. The half-field $\Delta m = 2$ transition of the copper(II) dimer is shown at the bottom of the Figure. Similar half-field transitions of lower intensity also were observed at concentration ratios of 5 : 1 and 10 : 1. Substitution of 0.82 mol dm^{-3} sodium perchlorate for the sucrose has no effect on the spectra

anisotropy. When the spectrum of this sample was recorded at *S*-band, the expected increase in spectral resolution was obtained (Figure 4). Nitrogen hyperfine splitting of the $g_\parallel$ low-field $I = +\frac{1}{2}$ transition with what appears to be a 1 : 4 : 10 : 16 : 19 : 16 : 10 : 4 : 1 pattern is observed at *S*- but not at *X*-band. This splitting positively confirms a structure in frozen aqueous solution at pH 7.2 with four equivalent in-plane nitrogen donor atoms in the equatorial co-ordination positions of the copper(II) ion.

* Throughout this paper: 1 G = 10^{-4} T

Similar experiments with the copper(II)–carnosine complex at pH 12 yielded spectra with lower resolution than those recorded at pH 7.2 (Figures 2 and 4). The nitrogen hyperfine lines in the $g_\perp$ region of the *X*-band spectrum indicate that more than two nitrogen donor atoms interact with the Cu^{2+}. The reduced resolution of the $g_\parallel$ low-field $I = +\frac{1}{2}$ transition of the *S*-band spectrum does not permit us to

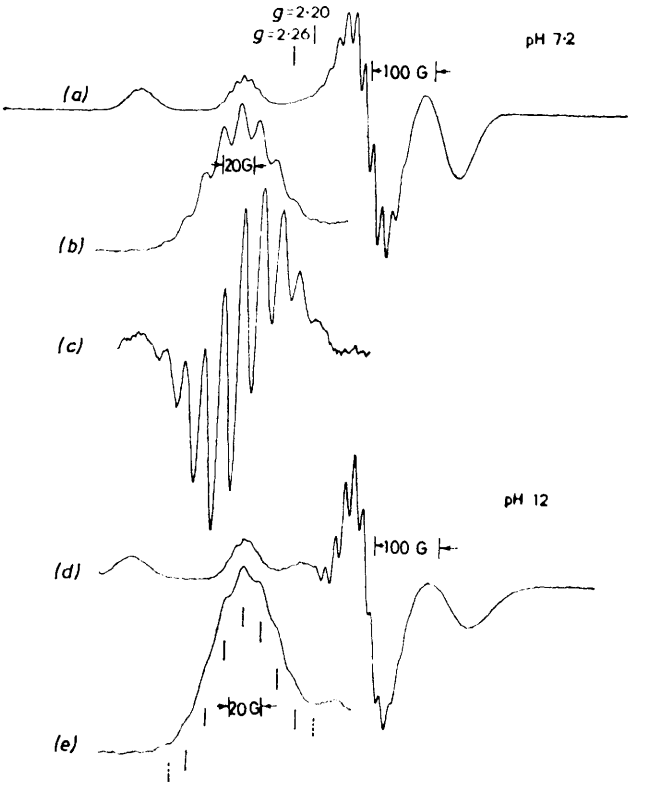

FIGURE 4 E.s.r. spectra (*S*-band) of frozen (77 K) solutions of 9.1×10^{-3} mol dm^{-3} $^{63}Cu^{2+}$ and 0.910 mol dm^{-3} carnosine in D_2O and 2.1 mol dm^{-3} ethylene glycol dimethyl ether. These spectra are in agreement with the copper(II) monomer having four equivalent nitrogen atoms co-ordinated to the equatorial positions at pH 7.2 and 3—4 nitrogen atoms at pH 12. Spectra (*b*) and (*e*) are expansions of the $I = +\frac{1}{2}$ transition in (*a*) and (*d*) respectively. Spectrum (*c*) is the second derivative of (*b*)

determine the exact stoicheiometry of the complex, but the presence of seven partially resolved peaks from nitrogen hyperfine splitting does indicate that either three or four nitrogen atoms are complexed to a single Cu^{2+} at pH 12. This conclusion is further substantiated by a *g* value of 2.20 and $A_\parallel$ for copper of 190 G (Figure 2), which is consistent with the co-ordination of three or four donor atoms.[26] Thus it can be concluded that the structure of the copper(II)–carnosine complex in frozen (77 K) aqueous solution at pH 7.2 *when there is a* 100—1 000*-fold excess of carnosine to copper*(II) *ion* is the same as that proposed by Ihnat and Bersohn [18] with the important difference that the stoicheiometry is four carnosine molecules per Cu^{2+} [structure (II) of Figure 1]. At pH *ca.* 12 there appears to be only one complex in frozen (77 K) aqueous solution, which has either three or four nitrogen atoms co-ordinated to the copper(II) ion when carnosine is present in a 100—1 000-fold excess (see Discussion).

When the e.s.r. spectrum of the above aqueous solution at pH 7.2 is recorded at ambient temperature no superhyper-

fine structure from nitrogen couplings can be observed. However, the *S*-band spectrum (Figure 5) is sufficiently well resolved to permit estimation of the isotropic g value (*ca.* 2.14) and of the isotropic copper hyperfine coupling constant (*ca.* 75 G). These estimates appear to agree with $g_{iso.} = 2.13$ and $A_{iso.}^{Cu} = 68$ G from the simulated spectrum at 77 K (*i.e.* $g_{\parallel} = 2.26$, $g_{\perp} = 2.06$, $A_{\parallel}^{Cu} = 175$ G, and $A_{\perp}^{Cu}$ *ca.* 15 G), which suggests that the complex may have

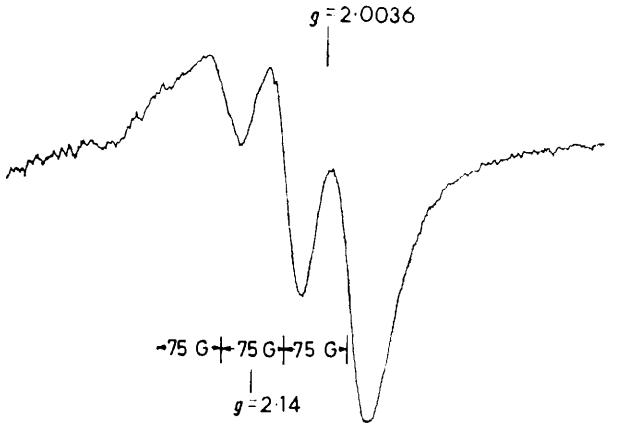

FIGURE 5 E.s.r. spectrum (*S*-band) of an aqueous solution of 9.1×10^{-3} mol dm^{-3} copper(II) ion and 0.910 mol dm^{-3} carnosine at pH 7.2 and ambient temperature

the same structure in both frozen and liquid solutions. According to the ^{1}H n.m.r. spectra of aqueous solutions of carnosine in the presence of copper(II) ion (Figure 6), the carnosine binds the copper(II) ion as in structure (II) in Figure 1. Since H^2 and H^4 of the imidazole ring (the resonances at *ca.* 8 and 7 p.p.m., respectively) experience paramagnetic relaxation enhancement to a much greater extent than the protons in the aliphatic region of the spectrum, the copper(II) ion must be co-ordinated to the imidazole ring at pH 7.2. This co-ordination is to N^3 of the ring [structure (II) of Figure 1] since the resonances of H^2 and H^4 are line-broadened equally. The amount of line broadening is proportional to the ratio of Cu^{2+} to carnosine, which indicates that the copper(II) ion in solution has a fixed number of binding sites and that these binding sites are saturated when there is a 100—1 000-fold excess of carnosine to Cu^{2+}. Thus the combined ^{1}H n.m.r. and e.s.r. data indicate that the copper(II)–carnosine complex has a fixed structure in solution when the carnosine is present in excess.

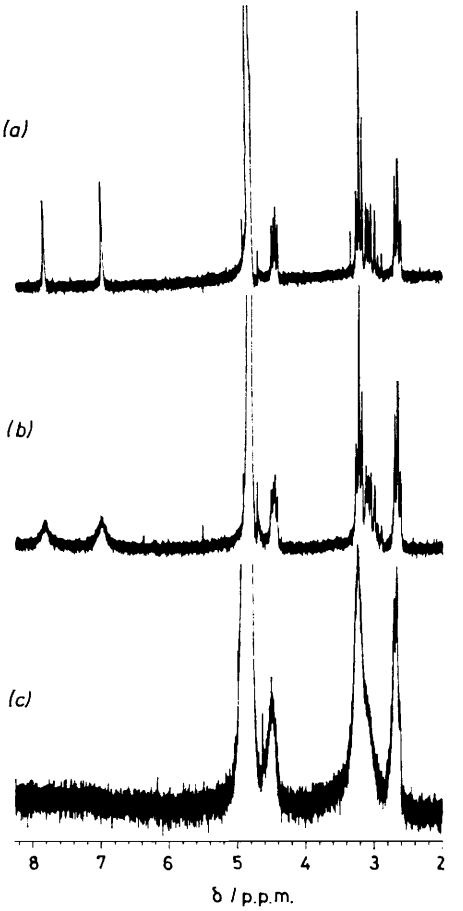

FIGURE 6 Proton n.m.r. spectra of 2×10^{-2} mol dm^{-3} carnosine in D_2O at pH 7.2. The concentrations of added Cu^{2+} are (*a*) 0, (*b*) 2×10^{-5}, and (*c*) 2×10^{-4} mol dm^{-3}. The large resonance at *ca.* 5 p.p.m. arises from HOD. Full assignment of the spectrum was reported by Ihnat and Bersohn [18]

This structure most likely has four carnosine molecules co-ordinated *via* N^3 of the imidazole ring to a single Cu^{2+}.* When the ratio of carnosine to Cu^{2+} approaches 1 : 1 the four co-ordination positions of the copper cannot be filled by co-ordination to a single functional group of the carnosine molecule, and formation of the copper(II) dimer is favoured.

Characterization of the Copper(II) *Dimer.*—The e.s.r. spectrum (*X*-band) of a frozen (77 K) aqueous solution containing equimolar concentrations of carnosine and Cu^{2+} at pH 7.2 exhibits both $\Delta m = 1$ transitions and a half-field $\Delta m = 2$ transition (Figure 3). The low-field $\Delta m = 1$ transition is well resolved into a seven-line pattern with the initial lines having apparent intensities of 1 : 2 : 3 : 4 : 3 : 2 : 1. This pattern, which was not observed by Boas *et al.*,[20] arises from coupling of the nuclei of the two copper(II) ions ($I = \frac{3}{2}$) of the dimer. The half-field,

* When a small molecule binds to a paramagnetic metal ion the observed spin–spin relaxation times represent an average of the relaxation times in the bound and free states if T_2 (bound state) $\gg \tau$ and $[\Pi\, \delta_{fb} T_2$ (bound state)$] \ll 1$ where τ is the lifetime of the bound state, δ_{fb} is the chemical-shift difference between the bound and free states, and T_2 (bound state) is the spin–spin relaxation time of the ligand proton when the ligand is on the paramagnetic metal ion. Since $1/T_2 = \pi(\Delta\nu)$ where $\Delta\nu$ is the linewidth at half-height of the peak in the ^{1}H n.m.r. spectrum, the spin–spin relaxation rates of the protons on a ligand molecule, and thus the orientation of that bound ligand relative to a given paramagnetic metal ion, can be measured from the linewidths of the peaks in the spectrum. When copper(II) is the paramagnetic metal, contributions to the line broadening other than just dipolar cannot be ruled out, but the linewidths can be used to gain insight into the general orientation of the ligand.[18] Since the copper(II) monomer and dimer complexes are in equilibrium in aqueous solution and the relative contributions of the various relaxation mechanisms to the ^{1}H n.m.r. spectra of these complexes are not known, we do not consider binding constants measured with this technique to be reliable. However, the linewidths of the ^{1}H n.m.r. spectra of the copper(II) monomer [structure (II) of Figure 1] do appear to be averages of the spectra of carnosine in the free and complexed state, and the line broadening is proportional to the ratio of copper(II) ion to carnosine. Thus the co-ordination of carnosine in this complex appears to be weak with rapid exchange of the ligand molecules. The copper(II) dimer appears to be more stable because of the much lower concentrations at which it can be detected.

$\Delta m = 2$, transitions confirm that this complex comprises magnetically coupled copper(II) ions in accordance with Boas *et al.*[20] Decreasing the line broadening from *g*-value anisotropy by recording the spectra at *S*-band does not provide any additional information. In fact, resolution is decreased because the $\Delta m = 1$ and 2 transitions overlap partially at the lower resonance frequency.

The e.s.r. spectra (*X*- and *S*-band) of a solution at ambient temperature containing equimolar concentrations of carnosine and Cu^{2+} at pH 7.2 (Figure 7) are sufficiently structure-

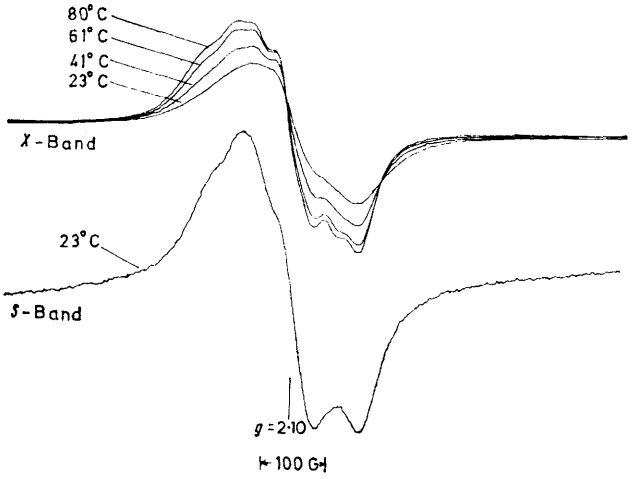

FIGURE 7 E.s.r. spectra (*X*- and *S*-band) of an aqueous solution of 9.1×10^{-3} mol dm^{-3} copper(II) and 9.1×10^{-3} mol dm^{-3} carnosine at pH 7.2. All spectra were obtained at temperatures above freezing and indicate that the copper(II) dimer is present in aqueous solution at ambient temperature. Note the increased resolution of the *S*-band compared to the *X*-band spectrum at the same temperature

less that one cannot delineate between monomer and dimer features. Therefore, the effect of varying the temperature of a 1×10^{-2} mol dm^{-3} solution of the copper(II) dimer from 5 to 80 °C on the signal intensity was investigated. Two isoclinic points (*i.e.* common points of intersection of a set of derivative spectra resulting in an isosbestic point for the absorbance [27]) indicate interconversion between two and only two distinct complexes. Double integration gives a 22% increase in intensity from 11 to 39 °C. A similar effect is observed with 1×10^{-3} mol dm^{-3} copper(II)–carnosine solution. This effect is completely reversible and demonstrates that the dimer structure is indeed present at ambient temperature in aqueous solution at pH 7.2 when the molar concentrations of Cu^{2+} and carnosine are equal. When the same experiment is performed with the monomer, very little variation of the integrated intensity is observed. It should be noted that other complexes exhibiting copper(II) dimer spectra at 77 K (analogous to that in Figure 3) have been observed to yield *X*-band spectra at ambient temperature analogous to that in Figure 7.[28,29] The *S*-band spectra of solutions of copper(II) dimer at ambient temperature are better resolved than those at *X*-band (Figure 7).

DISCUSSION

Perhaps the most important result of this work is the fact that one of the copper–carnosine complexes is much more stable than previously anticipated. Of the two possible structures at physiological pH, the dimer [structure (IV) of Figure 1] is the most stable. It can be detected with e.s.r. in frozen (77 K) solutions of a concentration as low as 1×10^{-5} mol dm^{-3}, at which the sensitivity of the spectrometer is limiting. The dimer structure (as measured by e.s.r.) is stable in aqueous solutions at pH 7.2 containing equimolar carnosine and Cu^{2+} to an upper concentration limit of *ca.* 3×10^{-2}—4×10^{-2} mol dm^{-3}, at which crystals begin to form. Since the structure of the dimer in frozen solution (77 K) appears to be the same as that of the crystal,[20] these results require a reappraisal of the mechanism of crystallization of copper–carnosine. These results also require further consideration of whether copper(II) complexes of carnosine and its analogues might be physiologically significant *in vivo*.

As the ratio of carnosine to Cu^{2+} in aqueous solution at pH 7.2 is reduced from 1 000 : 1 to 1 : 1 (Figure 3) there is a gradual transition of the e.s.r. spectrum from that of copper(II) monomer to that of the dimer. At ratios between 10 : 1 and 1 : 1 the spectra are composed of superimposed resonances from both complexes and thus this is the range of relative concentrations over which the monomer [structure (II) of Figure 1] is replaced by the dimer [structure (IV)]. An intermediate complex might be a monomer containing one carnosine molecule complexed *via* N^3 of the imidazole ring. This possibility is supported by ^1H n.m.r. line-broadening experiments performed at pH 7.2 in which the imidazole ring of carnosine is observed to experience greater paramagnetic relaxation enhancement as the ratio of carnosine to copper is decreased. When the ratio approaches 1 : 1, no ^1H resonances can be observed. This is in agreement with the result from e.s.r. that at ratios of from *ca.* 10 : 1 to 1 : 1 the copper(II) dimer [structure (IV) of Figure 1] begins to form.* These observations

* The accepted chemical structure of the copper(II) dimer [structure (IV) of Figure 1] is based on the following indirect evidence. First, this is the structure of copper–carnosine that was determined by *X*-ray crystallography.[19] Secondly, the distance between the two copper(II) ions that was measured by e.s.r. on a frozen (−196 °C) aqueous solution is in good agreement with that measured in the crystal.[20] We provide the additional evidence that the β-alanyl residue and the imidazole ring are involved in chelation of the copper(II) ions of the dimer. First, homocarnosine, which has the terminal amino-group extended by one methylene group, does not give the dimer. This molecule cannot make the six-membered ring upon chelation of the Cu^{2+} by both the terminal amino- and peptide nitrogen atoms that is necessary for stabilization of structure (IV). Secondly, ^1H n.m.r. spectral line-broadening indicates that the nitrogen atom of the terminal amino-group begins to interact with the copper(II) ion at the ratio of carnosine to copper that yields e.s.r. spectral evidence for the presence of the dimer in frozen solution. Over the range of 100—1 000 carnosine molecules in solution per copper(II) ion, the protons on the imidazole ring of the histidyl residue exhibit the greatest amount of paramagnetic line broadening, whereas the methylene protons of the β-alanyl residue exhibit little or no line broadening. Over the range of 1—10 carnosine molecules in solution per copper(II) ion the e.s.r. spectra exhibit an increasing proportion of the copper in the dimeric structure, and the ^1H n.m.r. spectra exhibit a parallel increase in the line broadening of the protons of the β-alanyl residue that is greater for the β- than for the α-methylene protons. Direct evidence for the functional groups that complex the copper(II) ion has not yet been obtained because the necessary ^{15}N-labelled intermediates are not readily available.

are not in agreement with structure (III) of Figure 1 being a precursor to the crystal [18] since this structure has no co-ordination to the imidazole ring. Ihnat and Bersohn [18] reported ^{1}H n.m.r. spectral line broadening at pH values around 12 that is basically the same as we report in Figure 6 at pH 7.2, which demonstrates the presence at pH 12 of co-ordination of Cu^{2+} to the imidazole ring. They argued that structure (III) of Figure 1 was the correct one at pH 12 because the solution also contained the dimeric complex [structure (IV)] in which the imidazole rings are co-ordinated to Cu^{2+}. However, the half-field $\Delta m = 2$ transition can be observed in frozen solution over a pH range of only *ca.* 5.8—8.1, but not at pH values around 12. This indicates that a structure corresponding to that of the crystal does exist in frozen solution at pH *ca.* 7 but does not exist at pH *ca.* 12. Therefore it is not possible for structure (III) of Figure 1 to be a predominant species at any of the pH values tested. The ^{1}H n.m.r. spectra of Ihnat and Bersohn [18] and our e.s.r. spectra (Figure 2) can best be explained by having a complex with structure (II) of Figure 1 over the pH range *ca.* 5—12. The change in the e.s.r. spectrum when the pH is raised above *ca.* 10 can be explained by the decrease in charge of the complex as a result of deprotonation of the unbound or very weakly bound primary amino-group of the carnosine molecules. The deprotonation may result in co-ordination of this nitrogen atom to the Cu^{2+} to produce structure (I) of Figure 1, but it does not result in the formation of a copper(II) dimer with a structure analogous to that of the crystal. The mechanism by which copper–carnosine crystallizes appears to involve the formation of stable copper(II) dimers in solution like structure (IV) of Figure 1. Crystallization of these dimers takes place when the solubility constant is exceeded. This mechanism is demonstrated to function in the physiological pH range but not at high values of pH.

Experiments with analogues of carnosine demonstrated that anserine produces both a copper(II) monomer and a dimer at pH 7.2 as does carnosine. The e.s.r. spectra of these copper(II)–anserine complexes were virtually identical to those of the analogous copper(II)–carnosine complexes (Figure 3). The e.s.r. spectra of copper(II) ion in the presence of a 100-fold excess of homocarnosine is qualitatively the same as those obtained with carnosine and anserine under the same conditions, which indicates that a copper(II) monomer complex [analogous to structure (II) of Figure 1] is formed by homocarnosine. However, there is no e.s.r. spectral evidence that the dimer is formed when the ratio of homocarnosine to Cu^{2+} approaches 1 : 1. The e.s.r. spectral evidence obtained with glycyl-L-histidine was virtually identical to that with homocarnosine.

It appears that the six-membered ring produced by the β-alanyl residue in the copper(II) chelate of carnosine and anserine is essential for the stability of the dimer. Increasing or decreasing the size of this ring by one methylene group (as with homocarnosine and glycyl-L-histidine, respectively) is incompatible with formation of the dimeric complex. The physiological significance of these specialized chelating capacities of carnosine, anserine, and homocarnosine in the tissues where they are found is not presently known. Homocarnosine, which occurs naturally in the brain, is not expected to form a stable complex with Cu^{2+} under normal physiological conditions because of the high concentrations of both metal and ligand that are necessary to detect the complex. However, the stability of the copper(II)–carnosine and –anserine dimers may explain the finding that patients with carnosinemia, carnosinuria, anserinuria, and a deficiency in serum carnosinase activity exhibit neurological damage that mimics that of Wilson's disease. These conditions are characterized by elevated concentrations of carnosine or anserine in serum and/or urine,[30-36] whereas Wilson's disease is characterized by elevated concentrations of copper in liver, kidney, and brain and an increased lifetime of the weak copper–serum albumin complex in the circulation.[37-40] The increased concentration of copper in the liver (and the resulting increased lifetime of the copper–serum albumin complex) is the result of an inability of patients with Wilson's disease to synthesize ceruloplasmin. We suggest that the copper is deposited in the kidney by formation of a copper–carnosine complex in the serum followed by hydrolysis of the carnosine in the kidney, which has the highest carnosinase activity in the body. Since formation of a copper–carnosine complex would be favoured by increasing the concentration of either the metal or the ligand in the serum, one would expect diseases involving the elevation of either carnosine, anserine, or copper in the serum to produce similar neurological damage if the copper were passing the blood–brain barrier in the chelated form.

We thank the Biomedical ESR Center and the Nicolet Technology Corporation for use of the e.s.r. and n.m.r. spectrometers, respectively, and Dr. John R. Pilbrow for his help in characterizing the e.s.r. spectra and preparing the manuscript. This investigation was supported by a Biomedical Research Support Grant from The Medical College of Wisconsin and by NIH grants.

[9/741 *Received*, 14*th May*, 1979]

REFERENCES

1 K. G. Crush, *Comp. Biochem. Physiol.*, 1970, **34**, 3.
2 F. L. Margolis, *Science*, 1974, **184**, 909.
3 D. Ferriero and F. L. Margolis, *Brain Res.*, 1975, **94**, 75.
4 A. Neidle and J. Kandera, *Brain Res.*, 1974, **80**, 359.
5 C. L. Davey, *Arch. Biochem. Biophys.*, 1960, **89**, 296.
6 C. L. Davey, *Arch. Biochem. Biophys.*, 1960, **89**, 303.
7 Y. Quereshi and T. Wood, *Biochim. Biophys. Acta*, 1960, **60**, 190.
8 S. E. Severin, *Int. Congress Biochem.*, 1964, 45.
9 J. Harding and F. L. Margolis, *Brain Res.*, 1976, **110**, 351.
10 C. E. Brown, F. L. Margolis, T. H. Williams, R. G. Pitcher, and G. Elgar, *Neurochemical Res.*, 1977, **2**, 555.
11 C. E. Brown, F. L. Margolis, T. H. Williams, R. G. Pitcher, and G. J. Elgar, *Arch. Biochem. Biophys.*, 1979, **193**, 529.
12 A. A. Boldreyev and V. B. Petukhov, *Gen. Pharmacol.*, 1978, **9**, 17.
13 J. F. Lenney, S. C. Kan, K. Siu, and G. H. Sugiyama, *Arch. Biochem. Biophys.*, 1977, **184**, 257.
14 C. E. Brown and W. E. Antholine, *Biochem. Biophys. Res. Comm.*, 1979, **88**, 529.

[15] H. Dobbie and W. O. Kermack, *Biochem. J.*, 1955, **59**, 246.
[16] R. B. Martin and J. T. Edsall, *J. Amer. Chem. Soc.*, 1960, **82**, 1107.
[17] G. R. Lenz and A. E. Martell, *Biochemistry*, 1964, **3**, 750.
[18] M. Ihnat and R. Bersohn, *Biochemistry*, 1970, **9**, 4555.
[19] H. C. Freeman and J. J. Szymanski, *Chem. Comm.*, 1965, 598.
[20] J. F. Boas, J. R. Pilbrow, C. R. Hartzell, and T. D. Smith, *J. Chem. Soc.* (*A*), 1969, 572.
[21] J. Donaldson, T. St. Pierre, J. L. Minnich, and A. Barbeau, *Canad. J. Biochem.*, 1973, **51**, 87.
[22] J. Smeyers-Verbeke, E. Defrise-Gussenhoven, G. Ebinger, A. Lowenthal, and D. L. Massart, *Clin. Chim. Acta*, 1974, **51**, 309.
[23] E. M. Widdowson and J. W. T. Dickerson, in 'Metal Metabolism: An Advanced Treatise,' vol. IIa, eds. C. L. Comar and F. Bronner, Academic Press, New York, 1964, p. 9.
[24] C. E. Brown and W. E. Antholine, *Neuroscience Abs.*, 1978, Abs. 1276.
[25] D. L. Williams-Smith, R. C. Bray, M. J. Barber, A. D. Tsopanakis, and S. P. Vincent, *Biochem. J.*, 1977, **167**, 593.
[26] J. Peisach and W. E. Blumberg, *Arch. Biochem. Biophys.*, 1974, **165**, 691.
[27] T. B. Marriott and O. H. Griffith, *J. Magnetic Resonance*, 1974, **13**, 45.
[28] T. D. Smith and A. E. Martell, *J. Amer. Chem. Soc.*, 1972, **94**, 3029.
[29] A. Gergely and T. Kiss, *Inorg. Chim. Acta*, 1976, **16**, 51.
[30] S. P. Bessman and R. Baldwin, *Science*, 1962, **135**, 789.
[31] P. M. Tocci and S. P. Bessman, in 'Amino Acid Metabolism and Genetic Variation,' ed. W. L. Nyhan, McGraw-Hill, New York, 1967, p. 161.
[32] T. L. Perry, S. Hansen, B. Tischler, R. Bunting, and K. Berry, *New England J. Med.*, 1967, **227**, 1219.
[33] T. L. Perry, S. Hansen, and D. L. Love, *Lancet*, 1968, June 8, 1229.
[34] P. J. Van Heeswijk, J. M. F. Trijbels, E. D. A. M. Schretlen, P. J. J. Van Munster, and L. A. H. Monnens, *Acta Paediat. Scand.*, 1969, **58**, 592.
[35] W. H. Murphey, L. Patchen, and D. G. Lindmark, *Clin. Chim. Acta*, 1972, **42**, 309.
[36] W. H. Murphey, D. G. Lindmark, L. I. Patchen, M. E. Housler, E. K. Harrod, and L. Mosovich, *Pediat. Res.*, 1973, **7**, 601.
[37] J. Scott, J. L. Gollan, S. Samourian, and S. Sherlock, *Gastroenterology*, 1978, **74**, 645.
[38] J. M. Vierling, R. Shrager, W. F. Rumble, R. Aamodt, M. D. Berman, and E. A. Jones, *Gastroenterology*, 1978, **74**, 652.
[39] G. E. Cartwright, *New England J. Med.*, 1978, **298**, 1347.
[40] J. M. Walshe, *Brain*, 1967, **90**, 149.