# Copper(II) Dimers in Solution: Evidence for Motional Averaging of Coupling Tensors without Chemical Dissociation

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The stability of the copper(II)–carnosine dimer in aqueous solutions at pH 7.2 has been investigated with Fourier transform–i.r. and e.s.r. spectroscopies. At subsaturated concentrations, the dimer dissociates into two identical monomers. However, the dimer does not dissociate in saturated solutions even at temperatures as high as 80 °C according to Fourier-transform i.r. spectra. Motional averaging of the dipolar coupling tensors occurs at temperatures at least as low as 15 °C. Thus at temperatures above the freezing point of the solvent the copper(II) dimer in saturated solution exhibits a spectrum with only four hyperfine lines. These are broader than the hyperfine lines of monomeric copper(II) complexes because of dipole–dipole interaction. This is the first spectroscopic confirmation to our knowledge that a copper(II) dimer need not dissociate in aqueous solution at temperatures above the freezing point of water.

L-Carnosine (β-alanyl-L-histidine) is present at concentrations of $1 \times 10^{-3}$—$4 \times 10^{-3}$ mol dm$^{-3}$ in skeletal muscle and nasal olfactory epithelium [1-4] where it has been suggested to play a role in regulating the transport and utilization of copper.[5] To understand how significant a role carnosine may play in the living body as a ligand for copper, it is necessary to determine whether copper–carnosine complexes are chemically capable of forming under physiological conditions of concentration, pH, and temperature.[6] With e.s.r. spectroscopy it is relatively easy to characterize copper(II) complexes at the temperature of liquid nitrogen (77 K), but characterization at ambient temperatures is not performed as readily. We have performed the following work to enable us to study copper(II)–carnosine complexes in the physiological temperature range and thereby to investigate their possible biological role(s).

Carnosine produces several chemically distinct complexes with copper(II) ion in aqueous solution.[6] Of these, two appear to be important in the physiological pH range. When the concentration of carnosine exceeds that of copper(II) ion by 100-fold or greater, a monomeric complex is produced with four equatorial carnosine molecules,[6] each bound by an imidazole nitrogen, and an axial water molecule in rapid exchange.[7] This complex has been described in detail previously.[6,7] At equimolar concentrations of carnosine and copper(II) ion a copper(II)–carnosine dimer with a structure as in Figure 1 (*a*) is produced,[6-9] and there is a gradual conversion from monomer to dimer as the ratio of carnosine to copper(II) is varied from 100 : 1 to 1 : 1.[6,7]

At 77 K and pH 7.2, frozen aqueous solutions of the copper(II)–carnosine (1 : 4) monomer exhibit e.s.r. spectra (*X*- and *S*-band) with four hyperfine lines in the $g_{\|}$ region of the spectrum from coupling to the nucleus of the single copper(II) nucleus ($I = \frac{3}{2}$).[6] At ambient temperature a four-line spectrum is also expected, but this is not observed experimentally [Figure 2(*a*)]. At 9.28 GHz only one line ($M_I = \frac{3}{2}$) is observed, whereas reducing the resonance frequency to 2.62 GHz yields three observable lines ($M_I = -\frac{1}{2}$, $\frac{1}{2}$, and $\frac{3}{2}$).[6] Electron spin resonance spectra that are calculated [10] with the assumption of isotropic tumbling with a correlation time of $4 \times 10^{-10}$ s (*i.e.* that expected for a complex with a molecular weight of 968 [10]) [Figure 2(*b*)] correspond very well to the experimental spectra [Figure 2(*a*)] over the entire range of resonance frequencies employed. Thus the increased resolution of the e.s.r. spectra of this copper(II)–carnosine (1 : 4) complex at lower resonance frequencies is determined mainly by the decreased contribution of anisotropy of the electron Zeeman interaction to the linewidth.[10] Although the spectrum is composed of a four-line pattern, not all the lines can be observed experimentally because they are too broad.

At 77 K, frozen aqueous solutions of equimolar carnosine and $Cu^{2+}$ at pH 7.2 yield e.s.r. spectra (*X*- and *S*-band) with both $\Delta M = 1$ transitions and a half-field $\Delta M = 2$ transition.[6] 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 arises from coupling of the nuclei of the two copper(II) ions ($I = \frac{3}{2}$) of the dimer.[8] The half-field, $\Delta M = 2$, transitions confirm that this complex comprises magnetically coupled copper(II) ions.[8] The splitting pattern was found not to be greatly affected by the concentration of the dimer in frozen solution.[6]

In sharp contrast, e.s.r. spectra (*X*- and *S*-band) of the same solutions when recorded at ambient temperature are sufficiently structureless that it is not possible to delineate between monomer and dimer features.[6] Furthermore, the intensities of spectra recorded with subsaturated concentrations of the dimer increase as the temperature of the sample is raised (Figure 3).[6] This change in intensity is completely reversible and is not observed with monomeric complexes under the same conditions. Proton n.m.r. spectra recorded at elevated temperatures and the concentration dependence of the effect of temperature on signal intensities were found to be consistent with conversion (in unsaturated solutions) of the dimer to a monomer with the structure shown in Figure 1(*b*).[11] Thus it appeared that dipolar coupling between the copper(II) ions of the dimer might be reduced in this case by chemical dissociation of the dimer into two identical monomers.[6,11] Such dissociation has been advocated by others.[7]

However, when the concentration of the dimer in solution is elevated until crystals of copper(II)–carnosine begin to form, the intensity of the e.s.r. spectrum of the saturated supernatant is found not to increase as the temperature is increased. At the same time, the spectrum was found to exhibit increased resolution at elevated temperatures. A four-line pattern was observed (unpublished observations). This suggested to us that a mechanism other than chemical dissociation might

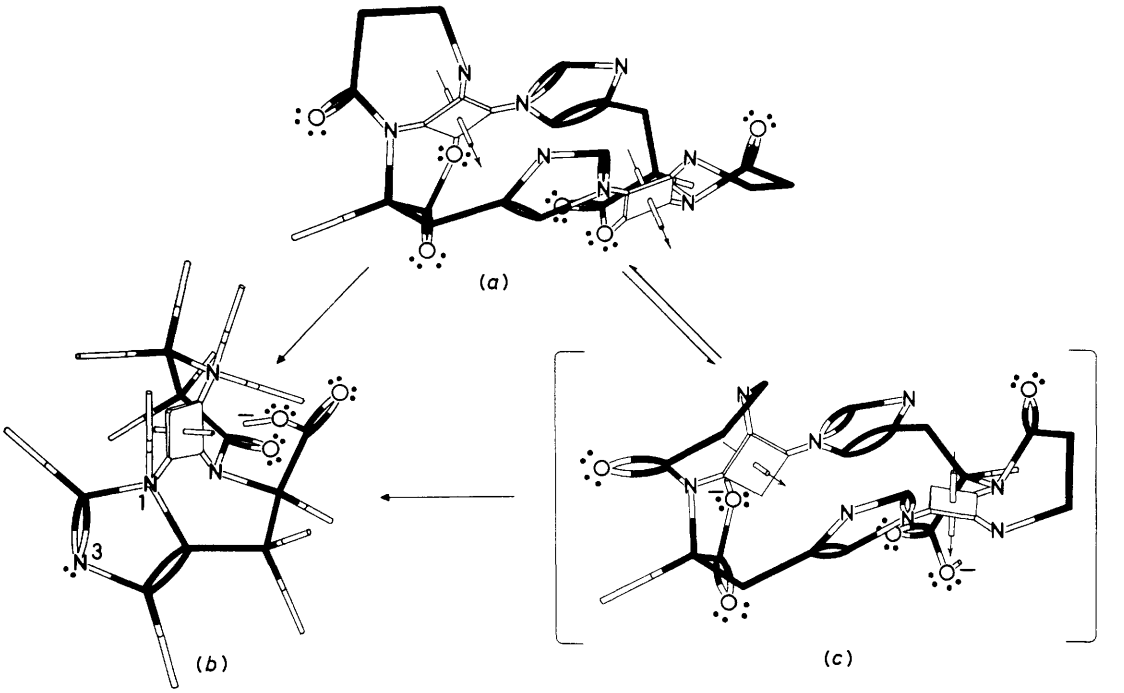


**Figure 1.** (*a*) Structure of the copper(II)–carnosine dimer in the crystal and in frozen aqueous solutions. (*b*) Structure of the copper(II)–carnosine monomer that is expected in dilute aqueous solutions at pH 7 and 11 when the concentrations of copper(II) ion and carnosine are equimolar. The remaining co-ordination positions should be occupied by $H_2O$ molecules at pH 7 and by hydroxyl ions at pH 11. (*c*) Possible intermediate in the dissociation of the dimer to the monomer

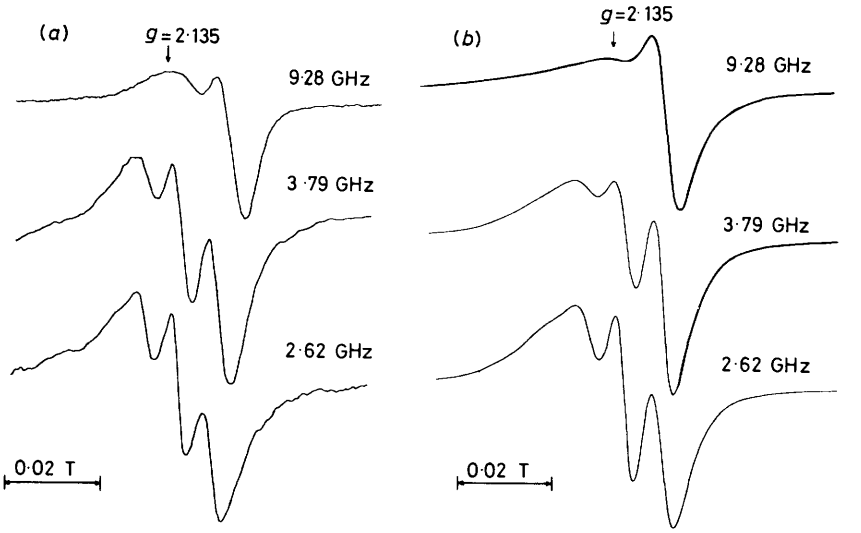


**Figure 2.** (*a*) E.s.r. spectra (*X*- and *S*-band) of an aqueous solution of $9.1 \times 10^{-3}$ mol $dm^{-3}$ copper(II) ion and $9.1 \times 10^{-1}$ mol $dm^{-3}$ carnosine at pH 7.2 and ambient temperature. The complex formed under these conditions is a copper(II)–carnosine (1 : 4) monomer. (*b*) Computer simulations of the spectra in (*a*). Conditions: $g_{\|} = 2.26$, $g_{\perp} = 2.048$, $A_{\|} = -553.9$ MHz, $A = -43$ MHz, $\tau = 4 \times 10^{-10}$ s. The apparent isotropic *g* value is indicated

average the dipole–dipole interaction between the copper(II) ions of the dimer.

The purpose of this paper is to provide evidence, based on Fourier-transform i.r. spectra of aqueous solutions, that the copper(II)–carnosine dimer is indeed in equilibrium with a monomeric complex in *dilute* solutions but does not dissociate chemically in *saturated* solutions at elevated temperatures. These results then are compared with e.s.r. spectra recorded under the same conditions to determine the physical parameters that give rise to the e.s.r. spectrum of the copper(II)–carnosine dimer in aqueous solution at physiological temperature. This is the first spectroscopic evidence to our knowledge that the copper(II)–carnosine dimer exists in aqueous solution at room temperature and above.

## Experimental

*Materials.*—L-Carnosine was purchased from Sigma Chemical Company. Copper(II) sulphate, sodium hydroxide, and hydrochloric acid were purchased from Fisher.

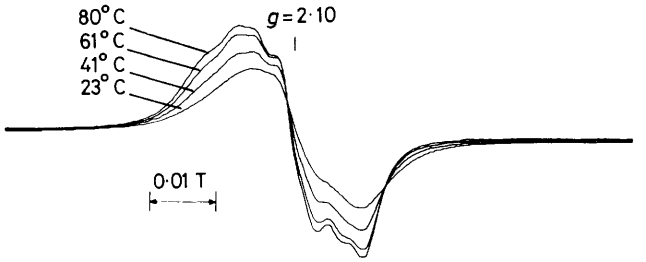


**Figure 3.** E.s.r. spectra[6] (*X*-band) of an aqueous solution of $9.1 \times 10^{-3}$ mol $dm^{-3}$ copper(II) ion and $9.1 \times 10^{-3}$ mol $dm^{-3}$ carnosine at pH 7.2. The apparent isotropic *g* value is indicated

*Methods.*—All samples were prepared by mixing appropriately diluted stock solutions of $CuSO_4$ and carnosine. After complete mixing the samples were adjusted to the appropriate pH at room temperature with dilute HCl or NaOH. All stock solutions were freshly prepared in demineralized water, and spectra of the mixed samples were obtained within 24 h. Samples were stored at 4 °C.

Fourier-transform (f.t.) i.r. spectra were recorded at the Nicolet Instrument Corporation with a Nicolet 7199 spectrometer. The aqueous samples at ambient temperature were held in a flow-through cell with $BaF_2$ faces and a pathlength of 50 μm. Spectra of frozen solutions were obtained by squeezing a thin film of solution between $BaF_2$ ' faces ' and then immersing the sample and faces in liquid nitrogen (77 K). After the sample and faces were completely cooled, they were removed from the liquid nitrogen, and the spectrum was recorded immediately. Spectra were recorded at elevated temperatures (*i.e.* up to 80 °C) with a thermostatted flow-through cell. In each case the i.r. spectrum of water at the same temperature was subtracted digitally from the spectrum of the sample solution.[12]

E.s.r. spectra were obtained with spectrometers at the National Biomedical ESR Center. The *X*-band spectra (9.09 GHz) were obtained with a Varian E-109 e.s.r. spectrometer, whereas the *S*-band spectra (3.78 GHz and 2.36 GHz) were obtained with an *S*-band bridge, cavity, and supporting equipment developed at the Center. The temperatures were regulated with a Varian variable-temperature apparatus.

## Results

*Fourier-transform Infrared Spectra of Copper*(II)–*Carnosine Complexes in Aqueous Solution.*—The f.t. i.r. spectrum of 400 mmol $dm^{-3}$ carnosine in $H_2O$ at pH 7.2 is presented in Figure 4(*a*). Addition of an equal volume of 400 mmol $dm^{-3}$ $CuSO_4$ and adjustment of the pH to 3.0 produces a copper–carboxylate salt[6,7,13] at a final concentration of 200 mmol $dm^{-3}$. One might expect formation of a simple carboxylate salt not to seriously affect the i.r. spectrum of the carnosine, and indeed this does appear to be the case [Figure 4(*c*)]. When the pH of this solution is raised above *ca.* 6, copper(II)–carnosine complexes precipitate. The saturated supernatant solution at pH 7 is expected to contain the copper(II)–carnosine dimer, but the i.r. spectrum of this solution is distinguished only by very weak peaks [Figure 4(*e*)]. The reason for the low intensity of these transitions is discussed below. Upon further raising the pH of such a solution to 11, the dimer is replaced by a copper(II)–carnosine monomer that is expected to have a structure like that shown in Figure 1(*b*) with hydroxyl groups occupying the unfilled co-ordination positions.[6] This solution exhibits an i.r. spectrum that is different from those obtained with unchelated carnosine and with copper(II)–carnosine at pH 3. Both the frequencies and relative intensities of two or three transitions are altered [Figure 4(*g*)].

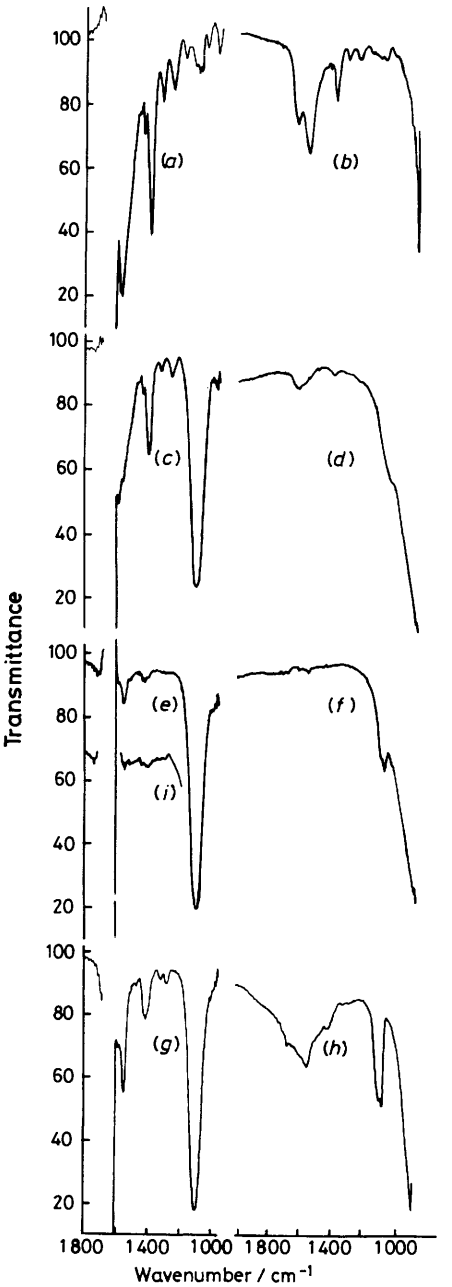


**Figure 4.** Fourier-transform i.r. spectra of aqueous solutions of $4 \times 10^{-1}$ mol $dm^{-3}$ carnosine, pH 7.2 [(*a*) and (*b*)]; $2 \times 10^{-1}$ mol $dm^{-3}$ copper(II) sulphate and $2 \times 10^{-1}$ mol $dm^{-3}$ carnosine, pH 3 [(*c*) and (*d*)]; saturated (*ca.* $5 \times 10^{-2}$ mol $dm^{-3}$) copper(II)–carnosine, pH 7.2 [(*e*) and (*f*)]; and saturated copper(II)–carnosine pH 11 [(*g*) and (*h*)]. The spectra on the left [(*a*), (*c*), (*e*), and(*g*)] were recorded at 20 °C, and the spectra at the right [(*b*), (*d*), (*f*), and (*h*)] were recorded at *ca.* −196 °C. The inset (*i*) is the f.t. i.r. spectrum of the saturated solution of copper(II)–carnosine, pH 7.2, at 80 °C. The intense absorption at *ca.* 1 100 $cm^{-1}$ in (*c*)—(*i*) arises from the sulphate counter ion

The dimer has a point of symmetry and thus would be expected to exhibit transitions of reduced intensity compared to the other monomeric carnosine complexes. That the low intensities of the peaks in Figure 4(*e*) are indeed the result

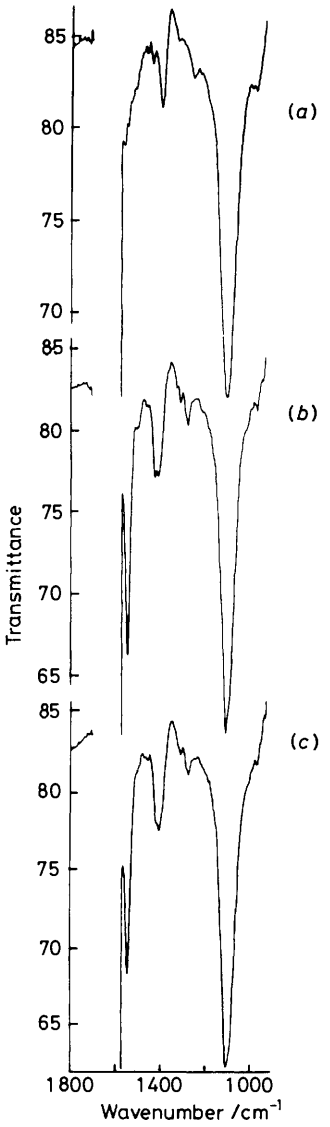


**Figure 5.** Fourier-transform i.r. spectra of aqueous solutions of $2 \times 10^{-2}$ mol $dm^{-3}$ copper(II) sulphate and $2 \times 10^{-2}$ mol $dm^{-3}$ carnosine at (*a*) pH 3, (*b*) pH 7.2, and (*c*) pH 11. All spectra were recorded at ambient temperature. The intense absorption at *ca.* 1 100 $cm^{-1}$ arises from the sulphate counter ion

of the dimer being the predominant species in saturated solution at pH 7 is supported by the following evidence. First, simple dilution of the saturated solution at pH 7 causes an apparent *increase* in the intensities of the peaks attributable to carnosine [*i.e.* compare Figure (4*e*) with Figure 5(*b*)], whereas dilution of the saturated solutions at pH 3 and 11 causes apparent *decreases* in spectral intensities [*i.e.* compare Figure 4(*c*) with Figure 5(*a*) and Figure 4(*g*) with Figure 5(*c*)]. This is exactly as would be expected, because dilution is known to favour disaggregation of the dimer to monomers with the structure shown by Figure 1(*b*), whereas the asymmetric monomer complexes do not change structure over this concentration range.[6,11] Second, the monomer complexes formed in dilute solution at both pH 7 and 11 are expected to have tridentate ligation of carnosine as shown in Figure 1(*b*). The primary difference between the complexes at these two pH values should be whether the remaining co-ordination positions of the copper(II) ion are occupied by water molecules or hydroxyl ions. In agreement with the conformations of the bound carnosine being so similar at both pH values, the i.r. spectra of dilute solutions at both pH 7 and 11 are very similar [Figure 5(*b*) and (*c*)]. Third, addition of histidine or cysteine to the saturated solution at pH 7, which is known to convert the symmetric dimer to asymmetric monomers with mixed ligands,[11] also causes the intensities of the peaks arising from carnosine to increase (data not shown).

*Effect of Temperature on the Fourier-transform Infrared Spectra of Saturated Solutions.*—We investigated the effect of temperature on f.t. i.r. spectra over a range of *ca.* −196 to +80 °C [*i.e.* the range over which e.s.r. and n.m.r. spectra of the copper(II)–carnosine dimer have been recorded [6]]. In general, freezing the sample has two effects on the i.r. spectra of aqueous solutions. First, the transition from water at 1 600 to 1 700 $cm^{-1}$, which causes serious interference in liquid samples, is not present in the frozen samples. Second, the i.r. spectra of the frozen samples are less resolved. Heating samples to temperatures above ambient, however, did not produce such effects on the i.r. spectra. The f.t. i.r. spectra of monomeric copper(II) complexes did not change between room temperature and 80 °C.

According to e.s.r. spectra [6] the symmetric dimer is expected to be the predominant species at −196 °C only in the pH range 5.8—8.1. At pH 3 and 11, only asymmetric monomeric species can be detected with e.s.r. spectroscopy.[6] Thus the i.r. spectra of frozen aqueous solutions of copper(II)–carnosine are expected to exhibit more intense peaks at pH 3 and 11 than at pH 7. This is indeed found [Figure 4(*d*), (*f*), and (*h*)].

Thus f.t. i.r. spectra can distinguish between the dimer and monomer species over the temperature range of at least −196 to +20 °C. At elevated temperatures i.r. spectra should also be able to distinguish between the copper(II)–carnosine dimer and monomer species. If the symmetrical dimer does not dissociate, then the i.r. spectrum recorded at elevated temperatures should exhibit only weak transitions just as is observed at ambient temperature [Figure 4(*e*)] and when frozen [Figure 4(*f*)]. In contrast, dissociation of the dimer at elevated temperatures, if it were to occur, would be expected to produce an i.r. spectrum with intense transitions between 1 200 and 1 600 $cm^{-1}$ just as is observed upon dilution [Figure 5(*b*)]. It was found in repeated experiments that at temperatures up to as high as 80 °C the spectrum continued to exhibit only very weak resonances in the range 1 200—1 600 $cm^{-1}$ [Figure 4(*i*)]. Thus, in saturated solutions, the copper(II)–carnosine dimer does not dissociate *chemically* as the temperature is raised to 80 °C.

*Electron Spin Resonance Spectra of the Copper(II)–Carnosine Dimer in Aqueous Solution.*—At a resonance frequency of 9.09 GHz and at 15 °C the e.s.r. spectrum of a saturated aqueous solution of the copper(II)–carnosine dimer is too featureless to ascertain the number of hyperfine lines [Figure 6(*a*)]. Reducing the resonance frequency to 2.36 GHz produces a small improvement in resolution but not enough to resolve the hyperfine lines from coupling to the copper(II) nucleus [Figure 6(*e*)]. However, raising the temperature to 80 °C produces a marked increase in spectral resolution to an apparent four-line pattern. The same four-line pattern is observed over the frequency range 9.09—2.36 GHz at this temperature [Figure 6(*b*), (*d*), and (*f*)].

Since the f.t. i.r. spectra indicate that the solution used for recording these e.s.r. spectra contains a dimer over the temperature range used, one must reconcile why the e.s.r. spectra exhibit only four lines, which would be expected for

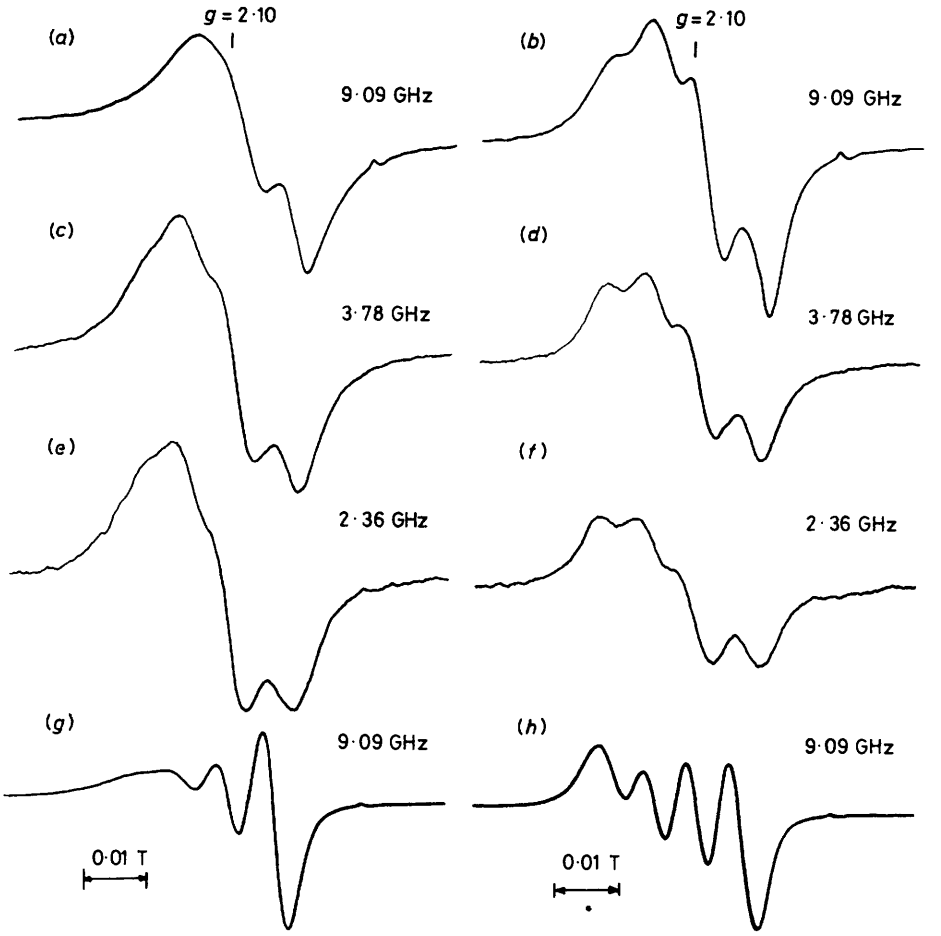


**Figure 6.** E.s.r. spectra (*X*- and *S*-band) of a saturated (*ca.* $5 \times 10^{-2}$ mol $dm^{-3}$) aqueous solution of copper(II)–carnosine at pH 7.2 [(*a*)—(*f*)] and the same solution after addition of excess histidine, pH 7.2 [(*g*) and (*h*)]. The spectra at the left (*a*), (*c*), (*e*), and (*g*) were recorded at *ca.* 15 °C, and the spectra at the right at *ca.* 70 °C. Simulation of the spectra of copper dimers in solution is hardly feasible because the mutual orientations of the two coppers are unknown and may depend on the experimental condition [*e.g.* Figure 1(*a*) and (*c*)]. The apparent isotropic *g* value is indicated

coupling to a single copper(II) nucleus ($I = \frac{3}{2}$). One must further ask what the predominant mechanism of line broadening in these spectra might be. The linewidths of the dimer spectra are broader than those of the copper(II)–carnosine (1 : 4) monomer, and the resolution of the dimer spectrum appears to be improved to a lesser extent upon reducing the resonance frequency from 9.08 to 2.62 GHz (*i.e.* compare Figures 2 and 6).

Addition of excess histidine to the saturated solution of the copper(II)–carnosine dimer that yielded the spectra in Figure 6(*a*), (*c*), and (*e*) gives rise to a much better resolved e.s.r. spectrum at ambient temperature [Figure 6(*g*)]. The added histidine converts the dimer primarily into a copper(II)–carnosine–histidine (1 : 1 : 3) monomer,[11] and the *X*-band e.s.r. spectrum exhibits only three of the expected four lines because the rate of molecular tumbling is not sufficiently rapid. [This is completely analogous to the situation with the copper(II)–carnosine (1 : 4) monomer in Figure 2.] Raising the temperature would be expected to improve the spectral resolution because the rate of molecular tumbling increases with temperature. In agreement with this expectation, the *X*-band e.s.r. spectrum of the copper(II)–carnosine–histidine (1 : 1 : 3) monomer exhibits a well resolved four-line pattern at 80 °C [Figure 6(*h*)]. This demonstrates two important points. First, the observed lack of resolution in Figure 6(*a*), (*c*), and (*e*) is not simply the result of the high concentration of copper(II) in the sample. The concentration of copper(II) in the solution which gave rise to Figure 6(*g*) is the same as that which gave rise to Figure 6(*a*), (*c*), and (*e*). Moreover, 10-fold dilution of these complexes does not change the widths of the hyperfine structure. Second, the spectral broadening in Figure 6(*a*), (*c*), and (*e*) indeed does not arise from slow molecular tumbling. The copper(II)–carnosine–histidine (1 : 1 : 3) monomer that is produced upon addition of excess histidine to the saturated solution of copper(II)–carnosine dimer has a molecular weight (*i.e.* $M = 755$) that is intermediate between the molecular weights of the dimer ($M = 579$) and the copper(II)–carnosine (1 : 4) monomer ($M = 968$). Thus, although the copper(II)–carnosine–histidine (1 : 1 : 3) monomer is tumbling less rapidly than the dimer, the e.s.r. spectrum of this monomer is better resolved. This brings into question the mechanism by which the copper(II)–carnosine dimer in liquid solution might produce four broadened hyperfine lines.

The spin Hamiltonian for a pair of copper(II) ions, labelled subscripts 1 and 2, has the form [14] shown in equation (1),

$$\mathcal{H} = \mathcal{H}_1 + \mathcal{H}_2 + \mathcal{H}_{ex} + \mathcal{H}_d \qquad (1)$$

where $\mathcal{H}_1$ and $\mathcal{H}_2$ represent the Zeeman and hyperfine terms,

$\mathcal{H}_{ex}$ represents the isotropic exchange coupling, and $\mathcal{H}_d$ is an anisotropic exchange term that includes pseudo-dipolar interactions and point-magnetic dipole–dipole interactions. These terms are given by equations (2)—(4) where β is the Bohr

$$\mathcal{H}_1 + \mathcal{H}_2 = \beta \boldsymbol{B}\cdot\boldsymbol{g}(\boldsymbol{S}_1 + \boldsymbol{S}_2) + \boldsymbol{S}_1\cdot\boldsymbol{A}\cdot\boldsymbol{I}_1 + \boldsymbol{S}_2\cdot\boldsymbol{A}\cdot\boldsymbol{I}_2 \quad (2)$$

$$\mathcal{H}_{ex} = J\boldsymbol{S}_1\cdot\boldsymbol{S}_2 \quad (3)$$

$$\mathcal{H}_d = \boldsymbol{S}_1\cdot\boldsymbol{D}\cdot\boldsymbol{S}_2 \quad (4)$$

magneton, $\boldsymbol{B}$ is the strength of the external magnetic field, $\boldsymbol{S}_1$ and $\boldsymbol{S}_2$ are operators for the electron spin angular momentum, $\boldsymbol{I}_1$ and $\boldsymbol{I}_2$ are nuclear spin angular momentum, $J$ is the isotropic electron exchange interaction constant, and $\boldsymbol{D}$ is a zero-field splitting parameter. Both $\boldsymbol{A}$ and $\boldsymbol{g}$ are tensor quantities that represent the hyperfine tensor and $g$ tensor, respectively. If the dimeric complex is tumbling rapidly in solution, the anisotropic effects will average to zero, and the spin Hamiltonian becomes equation (5), where $g$ and $A$ are

$$\mathcal{H} = g\beta B(S_{1z} + S_{2z}) + A(\boldsymbol{S}_1\cdot\boldsymbol{I}_1 + \boldsymbol{S}_2\cdot\boldsymbol{I}_2) - J\boldsymbol{S}_1\cdot\boldsymbol{S}_2 \quad (5)$$

the traces of the $g$- and $A$ tensors.

*According to equation* (5) *the freely tumbling copper*(II)*–carnosine dimer should exhibit only four hyperfine lines, similar to those observed with monomeric copper complexes, when* $|J| \approx 0$ (i.e. *when exchange interactions are negligible*). As the absolute value of $J$ increases, the number of lines increases, and the spectrum becomes more complicated.[15] Boas *et al.*[8] demonstrated that indeed the e.s.r. spectrum of the copper(II)–carnosine dimer in frozen aqueous solution can be explained by assuming only pure dipole–dipole interaction between the two copper ions and neglecting exchange interactions. Thus rapid tumbling of the copper(II)–carnosine dimer in aqueous solution should give rise to only four hyperfine lines as is observed experimentally.

Four possible contributions to the linewidths of the e.s.r. spectrum of the copper(II)–carnosine dimer can be identified. These are (*a*) unresolved superhyperfine structure, (*b*) spin-rotational mechanisms, (*c*) anisotropy of the nuclear hyperfine and Zeeman interactions, and (*d*) dipole–dipole interaction. Estimates of the relative contributions of these four mechanisms are as follows.

(*a*) *Unresolved superhyperfine structure.* The copper(II)–carnosine dimer is believed to have three nitrogen atoms ligated to each copper(II) ion [Figure 1(*a*)].[6] Since the usual superhyperfine splitting by nitrogen exhibits a coupling of *ca.* 0.0015 T, the contribution of unresolved superhyperfine structure to the linewidths in the e.s.r. spectrum of the dimer is expected to be 0.002—0.003 T.

(*b*) *Spin-rotational mechanisms.* The copper(II)–carnosine dimer has a molecular weight of 579 and thus a correlation time of $2 \times 10^{-10}$ s. Since for the dimer $g_{\parallel} = 2.20$ and $g_{\perp} = 2.05$,[8] a rather small contribution to the linewidth of *ca.* 0.00016 T is expected.[16]

(*c*) *Anisotropy of the nuclear hyperfine and Zeeman interactions.* Boas *et al.*[8] demonstrated that the copper(II)–carnosine dimer is described by the following spin Hamiltonian parameters: $g_{\parallel} = 2.20$, $g_{\perp} = 2.05$, $A_{\parallel} = 480$ MHz, and $A_{\perp} = 90$ MHz. These parameters give the anisotropy of hyperfine interaction, $\Delta A$, a value of 390 MHz and the anisotropy of Zeeman interaction, $B\beta(g_{\parallel} - g_{\perp})$, values of 650 and 180 MHz at resonance frequencies of 9.1 and 2.6 GHz, respectively. Assuming the correlation time, τ, to be $2 \times 10^{-10}$ s at 20 °C and $7 \times 10^{-11}$ s at 70 °C, the contributions of these anisotropies to the linewidths that are presented in the Table [17] are obtained.

**Table.** Contribution of anisotropy of the nuclear hyperfine and Zeeman interactions to the linewidths of the e.s.r. spectra of the copper(II)–carnosine dimer in aqueous solution

| $\theta_c$/°C | Microwave frequency/GHz | $10^4\Delta B$/T | | | |
|---|---|---|---|---|---|
| | | $M_I = -\frac{3}{2}$ | $M_I = -\frac{1}{2}$ | $M_I = \frac{1}{2}$ | $M_I = \frac{3}{2}$ |
| 20 | 9.1 | 68 | 39 | 18 | 4 |
| | 2.6 | 29 | 16 | 11 | 12 |
| 70 | 9.1 | 25 | 15 | 7 | 2 |
| | 2.6 | 13 | 9 | 6 | 6 |

(*d*) *Dipole–dipole interaction.* The point magnetic dipole–dipole interaction can be expressed by equation (6) where $r$

$$\mathcal{H}_d = \frac{1}{r^3}[\bar{\mu}_1\bar{\mu}_2 - 3(\mu_1\frac{\bar{r}}{r})(\mu_2\frac{\bar{r}}{r})] \quad (6)$$

is the distance between the magnetic dipoles $\mu_1$ and $\mu_2$ and $\bar{r}$ is the contribution of the vector between the two dipoles in the direction of the external magnetic field. This interaction is highly anisotropic. Its energy depends on the orientation of the dimer complex in the magnetic field and on the distance between the two magnetic dipoles. Tumbling of the dimer in solution will modulate the energy of the dipole–dipole interaction and thereby cause broadening of the lines. For a dimer with 5 Å between the copper(II) ions [*i.e.* the distance calculated by Boas *et al.*[8] for the copper(II)–carnosine dimer in frozen aqueous solution] the amplitude of the energy modulation is *ca.* 500 MHz. Thus the contribution of the anisotropy of dipole–dipole interaction to the linewidth is the same order of magnitude as that from anisotropy of the Zeeman and hyperfine interactions (Table).

The experimental width for the narrowest $M_I = \frac{3}{2}$ line is *ca.* 0.005 T, which is in good agreement with the above estimates [(*a*)—(*d*)]. The width of this line is not very sensitive to either the microwave frequency or the temperature. In contrast, the broadest $M_I = -\frac{3}{2}$ line is much more sensitive to both these parameters (Figure 6). The improved resolution at lower microwave frequency, when the Zeeman energy is diminished, is easily observed. This improvement in resolution is not as great as with the monomeric complexes because the intramolecular dipole–dipole interaction in the dimer does not depend on the microwave frequency or the magnetic field strength. Elevation of the temperature causes similar improvements in the spectra of both the dimer and the monomeric complexes. The close agreement between the predicted improvements in spectral resolution with reduced microwave frequency and elevated temperature (Table) and the experimental results (Figure 6) should not be overlooked.

## Discussion

The e.s.r. spectrum of an aqueous solution of the copper(II)–carnosine dimer recorded at the temperature of liquid nitrogen (77 K) differs greatly from those of the various monomeric copper(II)–carnosine complexes in aqueous solution at the same temperature.[6—8] The seven-line pattern of the dimer is easily distinguished from the four-line pattern of the monomer. However, when the temperature of a saturated solution with a ' dimer ' spectrum at 77 K is raised above the freezing point of the solvent water, a spectrum with only three or four broad hyperfine lines is observed. This raises the question of whether freezing might promote formation of the dimer form of the copper(II)–carnosine complex, whereas only a monomeric species might occur in the liquid state.

According to f.t. i.r. spectra, the dimer is stable in saturated aqueous solution at pH 7 over the temperature range of at least −196 to +80 °C (Figure 3). Thus the e.s.r. spectra of a saturated aqueous solution of equimolar copper(II) ion and carnosine at pH 7 recorded over the temperature range of ambient to 80 °C [Figure 6(*a*)—(*f*)] arise from a complex that chemically is a dimer. We have shown that the freely tumbling copper(II)–carnosine dimer should, indeed, exhibit only four hyperfine lines when exchange interactions are negligible [equation (5)]. However, less than the expected four lines may be observed experimentally if the contributions of anisotropy of nuclear hyperfine and Zeeman interactions and dipole–dipole interactions are too great. Elevating the temperature of the sample of either monomer or dimer to increase the rate of tumbling can reduce these contributions to the apparent linewidths. Decreasing the microwave frequency can have a similar effect in reducing the apparent linewidth.[18] Thus the theoretically expected four-line pattern may be observed by both raising the sample temperature and decreasing the microwave frequency. However, the resolution of the spectrum of dimer is worse than that of monomer because of the additional contribution of dipole–dipole interaction.

Other experimental procedures used to date have not permitted direct observation of the copper(II)–carnosine dimer in aqueous solution at ambient temperature and thus have led to the suspicion that the dimer does not exist under these conditions.[7,9,13,19] The results presented here demonstrate that the copper(II)–carnosine dimer does exist in aqueous solution at physiological pH and temperature. Although this dimer is readily disrupted by competing ligands and simple dilution, elevated temperatures do not appear to seriously affect its stability in saturated solution. These results provide the first direct evidence for our earlier assertion that crystalline copper(II)–carnosine, which is composed of dimers, is formed when the solubility constant of the dimer in aqueous solution is exceeded,[6] not when monomeric complexes react to form dimers.[7,9,13] At subsaturated concentrations, the solution contains a mixture of monomer and dimer, which gives rise to the apparent structure in the e.s.r. spectra shown in Figure 3. The dimer predominates in saturated solution. The copper(II)–carnosine dimer is demonstrated not to be able to exist at an appreciable level in the living body even though it is stable at physiological pH and temperature because the combination of subsaturated concentrations and competing ligands will yield monomeric complexes with mixed ligands.

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