

## Specific Interactions of the $\beta$ -Carboxylate Group of the Aspartic Acid Residue in Oligopeptides containing One, Two or Three Such Residues with Copper(II) Ions. A Potentiometric and Spectroscopic Study†

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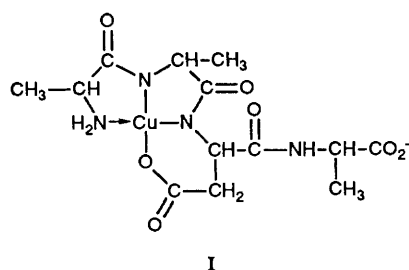
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Results are reported of a study of the influence of Asp and Glu residues in a peptide chain on the ability of the peptide to co-ordinate to copper(II) ions. The tetrapeptides Asp-Ala-Ala-Ala, Ala-Asp-Ala-Ala, Ala-Ala-Ala-Asp, Asp-Ala-Ala-Asp, Ala-Asp-Asp-Ala, Ala-Asp-Ala-Asp, Ala-Ala-Asp-Asp, Glu-Ala-Ala-Ala, Ala-Glu-Ala-Ala and Ala-Ala-Glu-Ala and the tripeptide Asp-Asp-Asp have been synthesised and their complexes with  $H^+$  and  $Cu^{2+}$  ions studied using potentiometry and spectroscopy (visible, CD and ESR). Results show that while the Glu residue has little influence on co-ordination equilibria, an N-terminal Asp residue stabilizes significantly the copper(II) complex with only one nitrogen atom co-ordinated (1N) as a result of chelation through the  $\beta$ -carboxylate group rather than the peptide C=O oxygen. This stabilization is much greater than with aspartic acid itself. Aspartic acid residues in the second or third positions of the peptide sequence stabilize 2N and 3N complexed species respectively, delaying significantly and, in some cases preventing completely, formation of 4N complexes. An Asp residue in the fourth position has a much smaller effect.

Recent studies of biologically active peptides like thymopoietin (Arg-Lys-Asp-Val-Tyr),<sup>1</sup> the N-terminal tetrapeptide fragment of fibrinopeptide A (Ala-Asp-Ser-Gly),<sup>2</sup> and angiotensin (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)<sup>3</sup> have shown that the  $\beta$ -carboxylate of the aspartic acid residue may influence dramatically the binding ability of oligopeptide ligands to metal ions. Copper(II) complexes with simple peptides are essentially planar. At approximately pH 5 co-ordination is through the terminal amino N and the carbonyl oxygen of the first peptide linkage to give a 1N complex. As the pH is increased the copper(II) ion promotes ionization of protons from successive peptide nitrogens, with the formation of  $Cu-N^-$  bonds, until the complex  $CuH_3L$  (a 4N co-ordinated species) is formed.<sup>4</sup> Although  $Cu^{II}$  favours co-ordination with nitrogen donors, the  $\beta$ -carboxylate of an Asp residue can co-ordinate effectively, and so interfere with this sequence, provided the complex is stabilized by chelation. For example, co-ordination in the complex plane to form a six-membered chelate ring is possible in the  $CuH_2L$  species with Ala-Ala-Asp-Ala (as shown in I).<sup>5</sup> As a result, the positions of Asp residues in a peptide chain have a significant influence on copper(II) interaction in solution with the peptides containing Asp residues, in particular they will influence the speciation as a function of pH. For example, the presence of an Asp residue in the third position in a peptide chain blocks involvement of the potentially very effective nitrogen donor of the third peptide bond (I) with the result that complexes involving four-nitrogen (4N) co-ordination could not be detected in such systems.<sup>1,5</sup>

Aspartic acid can behave as a tridentate ligand towards octahedral metal ions, bonding through the  $\beta$ -carboxylate



group to form two stable chelate rings (five and six membered). Glutamic acid, however, has a  $\gamma$ -carboxylate group in its side chain and this would require formation of a seven-membered chelate ring if it is to co-ordinate as a tridentate ligand. As a result co-ordination of glutamic acid is normally bidentate and comparable to that with alanine.<sup>6</sup> With  $Cu^{II}$ , tridentate co-ordination by aspartic acid would require apical co-ordination from one of the carboxylate oxygens, hence the stabilization found is significantly less than with octahedral ions such as  $Ni^{II}$ .<sup>6</sup> When Asp is the N-terminal residue of a peptide (as distinct from the free amino acid) more stabilization in the first complex to form (N,O co-ordination) is to be expected because bidentate N,O co-ordination involving a carboxylate  $O^-$  would be expected to be stronger than that through a peptide carbonyl C=O. This increased stability would be expected to be lost once co-ordination to the adjacent peptide  $N^-$  takes place. The influence of carboxylate co-ordination from Glu residues would be expected to be much smaller.

To examine more precisely the specificity in co-ordination to  $Cu^{II}$  of Asp and Glu residues in different positions in a peptide chain, and possible competition between two or more Asp

† Abbreviations used for the amino acid residues are those recommended by IUPAC-IUB in *Nomenclature and Symbolism for Amino Acids and Peptides*, *Pure Appl. Chem.*, 1984, **56**, 595.

**Table 1** Amino acid analyses

Peptide	Analysis	
	Asp	Ala
Asp-Ala-Ala-Ala	0.92	3.08
Ala-Asp-Ala-Ala	1.05	2.95
Ala-Ala-Asp-Ala	1.09	2.91
Ala-Ala-Ala-Asp	0.87	3.13
Asp-Ala-Ala-Asp	2.05	1.95
Ala-Asp-Ala-Asp	1.98	2.02
Ala-Asp-Asp-Ala	1.97	2.03
Ala-Ala-Asp-Asp	2.04	1.96
Asp-Asp-Asp	2.98	0
	Glu	Ala
Glu-Ala-Ala-Ala	1.03	2.97
Ala-Glu-Ala-Ala	1.05	2.95
Ala-Ala-Glu-Ala	1.03	2.97
FAB Mass spectroscopy (matrix = glycerol)		
Asp-Ala-Ala-Asp	390 + 1	
Ala-Asp-Ala-Asp	390 + 1	
Ala-Asp-Asp-Ala	390 + 1	
Ala-Ala-Asp-Asp	390 + 1	
Asp-Asp-Asp	363 + 1	
Ala-Asp-Ala-Ala	346 + 1*	
Ala-Ala-Glu-Ala	360 + 1*	

\* In *m*-nitrobenzyl alcohol.**Table 2** Protonation constants of peptides containing Asp or Glu residues at 25 °C and *I* = 0.10 mol dm<sup>-3</sup> (KNO<sub>3</sub>)

Peptide	log β				
	H <sub>L</sub>	H <sub>2</sub> L	H <sub>3</sub> L	H <sub>4</sub> L	H <sub>5</sub> L
Asp-Ala-Ala-Ala <sup>a</sup>	7.69(2)	11.29(3)	14.36(4)		
Ala-Asp-Ala-Ala <sup>a</sup>	7.96(1)	11.81(2)	15.03(2)		
Ala-Ala-Asp-Ala <sup>a,b</sup>	8.29	12.51	15.80		
Ala-Ala-Ala-Asp <sup>a</sup>	8.05(3)	12.06(3)	15.21(3)		
Glu-Ala-Ala-Ala <sup>a</sup>	7.77(1)	11.85(2)	14.98(2)		
Ala-Glu-Ala-Ala <sup>a</sup>	7.90(2)	11.97(2)	15.15(3)		
Ala-Ala-Glu-Ala <sup>a</sup>	8.10(2)	12.33(2)	15.58(2)		
Asp-Ala-Ala-Asp <sup>c</sup>	8.00(2)	12.64(2)	15.90(2)	19.02(4)	
Ala-Asp-Asp-Ala <sup>c</sup>	8.30(1)	12.65(1)	16.15(1)	19.08(2)	
Ala-Asp-Ala-Asp <sup>c</sup>	8.28(1)	12.91(1)	16.38(1)	19.52(2)	
Ala-Ala-Asp-Asp <sup>c</sup>	8.31(1)	12.98(1)	16.48(1)	19.91(3)	
Asp-Asp-Asp <sup>d</sup>	8.13(1)	12.91(1)	16.78(1)	19.82(1)	21.63(1)
Asp-Ala <sup>a</sup>	7.99(1)	11.60(1)	14.33(1)		
Glu-Ala <sup>a</sup>	7.98(1)	12.14(1)	15.30(1)		
Ala-Ala-Ala-Ala <sup>e,f</sup>	8.13	11.65			

<sup>a</sup> H<sub>2</sub>L. <sup>b</sup> Ref. 5. <sup>c</sup> H<sub>3</sub>L. <sup>d</sup> H<sub>4</sub>L. <sup>e</sup> HL. <sup>f</sup> Ref. 2.

residues, we have synthesised a range of oligopeptides and studied their interaction with Cu<sup>II</sup> in solution using potentiometry and spectroscopy (visible, CD and ESR). The ligands synthesised were (i) tetrapeptides containing an Asp residue at each position in the peptide sequence, (ii) tetrapeptides containing Glu residues in positions 1, 2 and 3 in the peptide sequence, (iii) tetrapeptides containing two Asp residues, Ala-Ala-Asp-Asp, Ala-Asp-Ala-Asp, Asp-Ala-Ala-Asp and Ala-Asp-Asp-Ala and (iv) the tripeptide Asp-Asp-Asp. For purposes of comparison we have also studied the dipeptides Asp-Ala and Glu-Ala.

## Experimental

**Peptide Syntheses.**—The tetrapeptide Ala-Asp-Ala-Ala was synthesised by standard liquid-phase methods identical to those used for the synthesis of Ala-Ala-Asp-Ala.<sup>5</sup> The coupling reagents were dicyclohexylcarbodiimide (dcci, Merck) and 1-hydroxybenzotriazole (Aldrich). The *tert*-butyloxycarbonyl groups were cleaved by 4 mol dm<sup>-3</sup> HCl–dioxane and the benzyl and benzyloxycarbonyl groups were removed by hydrogenolysis using 10% Pd/C in methanol–acetic acid (90:10).

The other peptides were synthesised by the solid-phase method using a Merrifield resin reticulated with 1% divinylbenzene, Bu<sup>t</sup>OCO-Ala, Bu<sup>t</sup>OCO-Asp-OC<sub>6</sub>H<sub>11</sub> and Bu<sup>t</sup>OCO-Glu-OC<sub>6</sub>H<sub>11</sub>. The cyclohexyl esters were chosen to avoid the formation of succinimide peptides during acid cleavage. The protecting groups were cleaved with HF in the presence of *p*-cresol. All peptides were purified by gel filtration [Sephadex G10 or G15, eluent acetic acid–water (5:100 v/v)]. Sample purity was checked using paper chromatography, HPLC (on Bondapack C<sub>18</sub> with an eluent gradient of water–methanol with 0.05% trifluoroacetic acid), 400 MHz proton NMR spectroscopy and potentiometry. Amino acid analyses were performed after peptide hydrolysis with 6 mol dm<sup>-3</sup> HCl using a Beckman 7300 analyser and, where samples were available, fast atom bombardment (FAB) mass spectra were recorded on a VG AutoSpec mass spectrometer. Results are given in Table 1.

**Spectroscopic Studies.**—Solutions of Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O (Fluka) and peptide with molar ratios of 1:1 were used with concentrations of 0.002–0.003 mol dm<sup>-3</sup>. Absorption spectra were measured on Cary 219 and Uvicon 810/P spectrometers and circular dichroism (CD) spectra on a Mark III Jobin-Yvon dichrograph in the 220–800 nm region. All CD results are expressed in terms of Δε = ε<sub>1</sub> – ε<sub>2</sub>. Electron spin resonance spectra were recorded on a Varian E102 spectrometer at liquid-nitrogen temperature at 9.13 GHz (X band). FAB Mass spectra were recorded on a VG AutoSpec mass spectrometer.

**Potentiometric Studies.**—Stability constants for H<sup>+</sup> and copper(II) complexes were calculated from titration curves carried out at 25 °C using total volumes of 1.5 cm<sup>3</sup>. Alkali was added from a 0.1 cm<sup>3</sup> micrometre syringe which had been calibrated by both weight titration and the titration of standardized materials. Experimental details were: Peptide concentration, 0.003 mol dm<sup>-3</sup>; copper concentration, 0.001–0.0028 mol dm<sup>-3</sup>; ionic strength, 0.10 mol dm<sup>-3</sup> (KNO<sub>3</sub>); pH range for complexation, 4–10.5; method, pH-metric titration using a micro combined glass-calomel electrode (Russel pH), calibrated in concentrations using HClO<sub>4</sub>;<sup>7</sup> number of titrations, three per ligand (average of 40 data points per titration); *T* = 25 °C; method of calculation SUPERQUAD.<sup>8</sup>

Standard deviations (σ values) quoted were computed by SUPERQUAD and refer to random errors only. They give, however, a good indication of the importance of the particular species in the equilibrium.

## Results and Discussion

**Hydrogen-ion Complexes.**—Protonation constants for the peptides studied are given in Table 2. The protonation represented by β<sub>HL</sub> is, in all cases, protonation of the amino N. The other protonations are of carboxyl groups with stepwise values (log *K*) always below 5 and generally below 4. For example Asp-Asp-Asp, with four carboxylate centres and one amino group able to co-ordinate protons, will have the formula H<sub>5</sub>L<sup>+</sup> at low pH but the protonation equilibria corresponding to protonation of the carboxylate groups will be macroconstants with contributions from all four protonation equilibria. The stepwise constants cannot, therefore, be assigned to protonation of any particular oxygen centre although assignment of β<sub>HL</sub> to protonation of the amino N is unambiguous. Carboxyl protonation equilibria will, in general, not be

**Table 3** Stability constants of complexes of Cu<sup>II</sup> with peptides containing Asp or Glu residues at 25 °C and *I* = 0.10 mol dm<sup>-3</sup> (KNO<sub>3</sub>)

Peptide	log β					CuH <sub>-1</sub> L <sub>2</sub>	CuH <sub>-2</sub> L <sub>2</sub>
	Cu(HL)	CuL	CuH <sub>-1</sub> L	CuH <sub>-2</sub> L	CuH <sub>-3</sub> L		
Asp-Ala-Ala-Ala (H <sub>2</sub> L)		6.04(2)	-0.34(2)	-7.82(2)	-16.88(3)		
Ala-Asp-Ala-Ala (H <sub>2</sub> L)		6.27(5)	1.97(1)	-7.77(3)	-17.65(2)		
Ala-Ala-Asp-Ala (H <sub>2</sub> L) <sup>a</sup>		5.36	0.57	-4.73	—		
Ala-Ala-Ala-Asp (H <sub>2</sub> L)		5.46(3)	-0.41(2)	-7.95(3)	-17.29(4)		
Glu-Ala-Ala-Ala (H <sub>2</sub> L)		5.37(3)	-0.22(1)	-7.71(2)	-17.03(3)		
Ala-Glu-Ala-Ala (H <sub>2</sub> L)		5.15(3)	-0.56(1)	-7.92(2)	-17.16(2)		
Ala-Ala-Glu-Ala (H <sub>2</sub> L)		5.59(2)	-0.13(1)	-7.28(1)	-16.73(2)		
Asp-Ala-Ala-Asp (H <sub>3</sub> L)	11.18(5)	6.83(2)	0.44(5)	-7.89(6)	-16.89(6)		
Ala-Asp-Asp-Ala (H <sub>3</sub> L)	10.97(8)	6.98(2)	2.61(1)	-4.57(3)	?		
Ala-Asp-Ala-Asp (H <sub>3</sub> L)	10.67(5)	7.11(1)	2.27(1)	-7.58(1)	-17.37(1)		
Ala-Ala-Asp-Asp (H <sub>3</sub> L)	11.20(2)	6.67(1)	0.86(2)	-4.58(1)	?		
Ala-Ala-Ala-Ala (HL) <sup>b</sup>	—	4.77	-0.45	-8.09	-17.33		
Asp-Asp-Asp <sup>c</sup> (H <sub>4</sub> L)	12.00(4)	7.83(2)	3.03(2)	-5.34(3)	?		
						CuH <sub>-1</sub> L <sub>2</sub>	CuH <sub>-2</sub> L <sub>2</sub>
Asp-Ala (H <sub>2</sub> L)		6.62(2)	1.92(1)	-7.90(6)		5.01(6)	-4.41(6)
Glu-Ala (H <sub>2</sub> L)		6.17(1)	1.86(1)	-7.62(1)		4.33(5)	-4.72(5)

<sup>a</sup> Ref. 5. <sup>b</sup> Ref. 2. <sup>c</sup> log β (CuH<sub>2</sub>L) = 15.78.

important when considering co-ordination of metal ions above pH 6. The value for log β<sub>HL</sub> is comparatively low when Asp is in the first position of the peptide chain but it increases as the Asp residue moves along the chain, approaching that for Ala-Ala-Ala-Ala (8.13<sup>2</sup>; cf. Ala-Ala-Ala-Asp, 8.05). In peptides containing Glu this effect is smaller because the carboxyl group is now an additional carbon atom from the nitrogen.

**Copper(II) complexes.**—Stability constants for complexes with Cu<sup>II</sup> are given in Table 3 and spectroscopic parameters in Table 4. Selected species distribution curves, calculated for 1:1 Cu:L mixtures (0.001 mol dm<sup>-3</sup>), are shown in Fig. 1. Species distribution curves for Ala-Ala-Ala-Ala are shown superimposed as dotted lines on the curves for tetrapeptides [Fig. 1(i)–(vi)].

**1N Co-ordination.** Complexes with peptides containing Asp as the N-terminal residue (Asp-Ala-Ala-Ala, Asp-Ala-Ala-Asp, Asp-Asp-Asp and Asp-Ala) all form 1N complexes in the pH range of 4.5–6.5 which are more stable than the corresponding complexes with Ala-Ala-Ala-Ala. With Asp-Ala-Ala-Ala [Fig. 1(i)] and Asp-Ala these are the CuL species but, with the peptides containing two or more Asp residues [Fig. 1(iii), (vii)], protonated complexes also form below pH 6 as a result of protonation of unco-ordinated carboxyl groups. Hence the 1N complexes will be the sum of Cu(H<sub>2</sub>L), Cu(HL) and CuL species. These 1N complexes were clearly identified from potentiometry and from spectroscopy (see Table 4). Complexes with 1N co-ordination normally have an absorption band in the 700–730 nm region but it is of low intensity and is normally obscured when the concentration is low or 2N complexes are also present. In the CD spectra it is also characterized by the absence of Cu–N<sup>-</sup> charge-transfer bands and has characteristic values for *A*<sub>||</sub> and *g*<sub>||</sub> in ESR spectra. These spectral characteristics were clearly identified in the pH region of 4–6 and with Ala-Ala-Asp-Asp and Asp-Asp-Asp over a smaller pH range. With Asp-Ala-Ala-Ala and Asp-Ala-Ala-Asp stabilization relative to Ala-Ala-Ala-Ala (Δ log value = 1.3 with Asp-Ala-Ala-Ala) is more marked than between aspartic acid and alanine (Δ log value = 0.45) supporting the suggestion that with Asp<sup>1</sup> peptides there is significant co-ordination to Cu<sup>II</sup> from the lateral carboxyl O in addition to the terminal N and possibly also the peptide C=O (if the carboxyl group can co-ordinate axially) as compared to the latter two centres alone with Ala-Ala-Ala-Ala. Similarly the CuL complex with Asp-Ala is about 1.1 log units more stable

than that with Ala-Ala.<sup>8</sup> The presence of a second aspartyl residue in the fourth position of the tetrapeptide sequence has only a minor effect, as expected. As Asp residue in the second position (Ala-Asp-) also has an effect on the stability constants of the 1N complexes, although this is less obvious from the species distribution curves because the 2N complexes are stabilized even more significantly. An Asp residue in the third or even the fourth positions of the tetrapeptide chain appears to promote a small stabilization in the CuL species. The origin of this stabilization is not immediately obvious but could be a result of the formation of large chelate ring involving the β-carboxylate group.

The presence of a Glu residue in the N-terminal position (Glu-Ala-Ala-Ala) showed a much smaller enhancement in the stability of the 1N complex, the CuL complex being only a minor species in the equilibrium. Hence co-ordination to Cu<sup>II</sup> of the lateral γ-carboxylate group of a Glu residue is, as expected, far less significant although it is more important in Glu<sup>1</sup> peptides than in glutamic acid itself.

**2N Co-ordination.** With simple peptides (e.g. Ala-Ala-Ala-Ala) and Cu<sup>II</sup>, 2N co-ordinated complexes (CuH<sub>-1</sub>L) are the major species in the pH range 6–7.5 and stabilization of 2N complexes would therefore result, in all probability, in a widening of this band of stability. Significant widening of this type is found with Ala-Asp-Ala-Ala [pH 5–9.5, Fig. 1(ii)], Ala-Asp-Ala-Asp [pH also 5–9.5, Fig. 1(v)], Ala-Asp-Asp-Ala [pH 5–7.3, Fig. 1(iv)] and with Asp-Asp-Asp [pH 5–8, Fig. 1(vii)]. The last two peptides also contain Asp residues in the third position, hence the ranges of existence of the 2N complexes are a little less than with peptides containing Asp<sup>2</sup> as the only Asp residue. Stabilization with Ala-Glu-Ala-Ala is negligible. A quantitative indication of the stabilizing effect of the Asp<sup>2</sup> residue may be obtained by comparing values for *K'* where log *K'* = log β(CuH<sub>-1</sub>L<sub>peptide</sub>) – log β(CuH<sub>-1</sub>L<sub>tetraalanine</sub>). With Ala-Asp-Ala-Ala log *K'* = 2.4 while with Asp-Ala-Ala-Ala it is 0.1 and the corresponding value between Asp-Ala and Ala-Ala is 0.2,<sup>8</sup> demonstrating that any stabilization in the 1N complexes as a result of β-carboxylate bonding from an Asp<sup>1</sup> residue is virtually destroyed on formation of a 2N complex. Other comparable log *K'* values are 2.7 for Ala-Asp-Ala-Asp, 3.0 for Ala-Asp-Asp-Ala and 3.5 for Asp-Asp-Asp. Binding of an Asp<sup>2</sup>-β-carboxylate group does not, however, prevent complete deprotonation and binding of the second and third peptide nitrogens to give 3N (CuH<sub>-2</sub>L) and possibly 4N (CuH<sub>-3</sub>L)

complexes at higher pH. This is particularly true with Ala-Asp-Asp-Ala [Fig. 1(iv)] and Asp-Asp-Asp which contain Asp residues in the third position. Competition from the  $\beta$ -carboxyl

group of these Asp<sup>3</sup> residues is sufficiently strong to promote breaking of the Cu-O<sub>2</sub>C(Asp<sup>2</sup>) bond to allow deprotonation of the Asp<sup>2</sup>-Asp<sup>3</sup> peptide N with subsequent Cu-N<sup>-</sup> co-

**Table 4** Spectroscopic data for the copper(II) complexes of peptides containing Asp or Glu residues

Peptide	Species	Spectral data		EPR	
		Absorption $\lambda_{\max}^a/\text{nm}$	CD $\lambda_{\max}^b/\text{nm}$	$A_{\parallel}$	$g_{\parallel}$
Asp-Ala-Ala-Ala	[CuL] 1N	730 (47)	764 (+0.08) <sup>c</sup> 600-630 (-0.02) <sup>d</sup> 270(sh) (+0.1) <sup>e</sup> 244(sh) (+0.29) <sup>f</sup> 277 (+0.5) <sup>g</sup>	150	2.33
	[CuH <sub>-1</sub> L] 2N	635 (82)	689 (-0.03) <sup>c</sup> 500(sh) (+0.02) <sup>d</sup> 306 (+0.2) <sup>h</sup> 260(sh) (-0.6) <sup>e</sup>	165	2.27
	[Cu <sub>-2</sub> L] 3N	570 (110)	570 (-0.29) <sup>i</sup> 308 (+0.3) <sup>h</sup> 274 (-0.2) <sup>e</sup> 236 (+0.45) <sup>g</sup>	190	2.20
	[CuH <sub>-3</sub> L] 4N	508 (127)	534 (-0.41) <sup>i</sup> 308 (+0.2) <sup>h</sup> 274 (-0.68) <sup>e</sup> 236 (+1.15) <sup>g</sup>	207	2.18
Ala-Asp-Ala-Ala	[CuH <sub>-1</sub> L] 2N	630 (80)	610 (+0.62) <sup>c</sup> 508 (-0.01) <sup>d</sup> 300 (-0.93) <sup>h</sup> 262 (+0.78) <sup>e</sup> 243(sh) (-0.1) <sup>f</sup> 229 (-1.8) <sup>g</sup>	165	2.27
	[CuH <sub>-2</sub> L] 3N	585 (80)	610 (+0.51) <sup>c</sup> 512 (-0.29) <sup>d</sup> 292 (-0.85) <sup>h</sup> 262 (+0.78) <sup>e</sup> 229 (-1.8) <sup>g</sup>	187	2.24
	[CuH <sub>-3</sub> L] 4N	520 (150)	536 (-1.01) <sup>c</sup> 304 (+0.63) <sup>h</sup> 272 (-1.46) <sup>e</sup> 240-220 (+3.4) <sup>g</sup>	205	2.18
Ala-Ala-Ala-Asp	[CuH <sub>-1</sub> L] 2N	648 (75)	692 (-0.12) <sup>i</sup> 300 (+0.26) <sup>h</sup> 260(sh) (+0.14) <sup>e</sup>	170	2.25
	[CuH <sub>-2</sub> L] 3N	564 (110)	552 (-0.32) <sup>i</sup> 306 (+0.29) <sup>h</sup> 272 (-0.14) <sup>e</sup> 234 (+0.50) <sup>g</sup>	185	2.21
	[CuH <sub>-3</sub> L] 4N	520 (140)	526 (-0.52) <sup>i</sup> 306 (+0.25) <sup>h</sup> 272 (-0.75) <sup>e</sup>	205	2.18
Glu-Ala-Ala-Ala	[CuH <sub>-1</sub> L] 2N	652 (82)	580-690 308 (+0.2) <sup>h</sup> 260(sh) weak <sup>e</sup> 234 (+0.17) <sup>g</sup>	170	2.25
	[CuH <sub>-2</sub> L] 3N	570 (121)	578 (-0.2) <sup>i</sup> 308 (+0.4) <sup>h</sup> 272 (-0.06) <sup>e</sup> 236 (+0.17) <sup>g</sup>	190	2.20
	[CuH <sub>-3</sub> L] 4N	520 (160)	536 (-0.45) <sup>i</sup> 308 (+0.45) <sup>h</sup> 274 (-0.64) <sup>e</sup> 232 (+1.34) <sup>g</sup>	210	2.18
Ala-Glu-Ala-Ala	[CuH <sub>-1</sub> L] 2N	640 (47)	600-670 (-0.1) <sup>i</sup> 308 (+0.19) <sup>h</sup> 265 (+0.25) <sup>e</sup>	172	2.248
	[CuH <sub>-2</sub> L] 3N	572 (95)	578 (-0.22) <sup>i</sup> 306 (+0.3) <sup>h</sup> 283 (-0.2) <sup>e</sup> (+1.5) <sup>g</sup>	188	2.204
	[CuH <sub>-3</sub> L] 4N	516 (120)	536 (-0.46) <sup>i</sup> 304 (+0.26) <sup>h</sup> 269 (-0.76) <sup>e</sup> 225 (+1.5) <sup>g</sup>	210	2.17

Table 4 (continued)

Peptide	Species	Spectral data		EPR	
		Absorption $\lambda_{\max}^a/\text{nm}$	CD $\lambda_{\max}^b/\text{nm}$	$A_{\parallel}$	$g_{\parallel}$
Ala-Ala-Glu-Ala	[CuH <sub>1</sub> L] 2N	650 (75)	600 (-0.15) <sup>i</sup>	170	2.25
			308 (+0.6) <sup>h</sup>		
	[CuH <sub>2</sub> L] 3N	572 (107)	260 (-0.55) <sup>e</sup>	188	2.21
		225 (-1.7) <sup>g</sup>			
Asp-Ala-Ala-Asp	[CuH <sub>3</sub> L] 4N	510 (150)	582 (-0.34) <sup>i</sup>	205	2.17
			312 (+1.04) <sup>h</sup>		
				274 (-1.24) <sup>e</sup>	
Ala-Asp-Asp-Ala	[CuL] 1N	705 (35)	534 (-1.1) <sup>i</sup>	150	2.310
			280 (-0.1) <sup>e</sup>		
	[CuH <sub>1</sub> L] 2N	650 (82)	700 (-0.25) <sup>c</sup>	170	2.240
		555 (-0.05) <sup>d</sup>			
Ala-Asp-Ala-Asp	[CuH <sub>2</sub> L] 3N	567 (131)	305 (+0.15) <sup>h</sup>	187	2.220
			270 (-0.20) <sup>e</sup>		
				570 (-0.35) <sup>i</sup>	
Ala-Asp-Ala-Asp	[Cu <sub>3</sub> L] 4N	518 (164)	310 (+0.50) <sup>h</sup>	207	2.172
			275 (-0.45) <sup>e</sup>		
				530 (-0.7) <sup>i</sup>	
Ala-Asp-Ala-Asp	[CuH <sub>1</sub> L] 2N	630 (85)	305 (+0.45) <sup>h</sup>	182	2.242
			270 (-1.5) <sup>e</sup>		
				650 (-0.06) <sup>i</sup>	
Ala-Asp-Ala-Asp	[CuH <sub>2</sub> L] 3N	555 (106)	295 (-0.3) <sup>h</sup>	203	2.204
			260 (+0.15) <sup>e</sup>		
				610 (-0.06) <sup>c</sup>	
Ala-Asp-Ala-Asp	[CuH <sub>1</sub> L] 2N	631 (74)	580 (-0.06) <sup>d</sup>	182	2.240
			480 (-0.01) <sup>d</sup>		
				305 (+0.35) <sup>h</sup>	
Ala-Ala-Asp-Asp	[CuH <sub>3</sub> L] 4N	523 (121)	270 (-0.40) <sup>e</sup>	212	2.170
			610 (-0.02) <sup>c</sup>		
				580 (+0.02) <sup>d</sup>	
Ala-Ala-Asp-Asp	[CuL] 1N	710 (35)	300 (-0.6) <sup>h</sup>	125	2.396
			260 (+0.7) <sup>e</sup>		
				530 (-0.7) <sup>i</sup>	
Asp-Asp-Asp	[CuH <sub>1</sub> L] 2N	644 (58)	303 (+0.1) <sup>h</sup>	185	2.240
			270 (-1.5) <sup>e</sup>		
				680 (-0.05) <sup>i</sup>	
Asp-Asp-Asp	[CuH <sub>2</sub> L] 3N	547 (123)	255 (+0.5) <sup>e</sup>	200	2.196
			590 (-0.4) <sup>c</sup>		
				490 (+0.4) <sup>d</sup>	
Asp-Asp-Asp	[CuH <sub>1</sub> L] 2N	644 (58)	300 (+0.5) <sup>h</sup>	185	2.240
			270 (-1.3) <sup>e</sup>		
				625 (+0.08) <sup>i</sup>	
Asp-Asp-Asp	[CuH <sub>2</sub> L] 3N	549 (118)	305 (+0.25) <sup>h</sup>	210	2.198
			260 (-0.60) <sup>e</sup>		
				580 (-0.15) <sup>d</sup>	
			310 (+0.10) <sup>h</sup>		
			280 (-0.25) <sup>e</sup>		

<sup>a</sup> Approximate absorption coefficient ( $\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ) given in parentheses. <sup>b</sup>  $\Delta\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  in parentheses. <sup>c</sup> B-Type d-d transition. <sup>d</sup> E-Type d-d transition. <sup>e</sup>  $\text{NH}_2 \rightarrow \text{Cu}$  Charge-transfer transition. <sup>f</sup>  $\text{CO}_2^- \rightarrow \text{Cu}$  Charge-transfer transition. <sup>g</sup> Intra-ligand transition. <sup>h</sup>  $\text{N}^- \rightarrow \text{Cu}$  Charge-transfer transition. <sup>i</sup> Combination of B and E transitions.

ordination supported by  $\text{Cu}-\text{O}_2\text{C}(\text{Asp}^3)$  binding. As a result the species distribution curves for Ala-Asp-Asp-Ala and Asp-Asp-Asp show both the  $\text{CuH}_1\text{L}$  (2N) and  $\text{CuH}_2\text{L}$  (3N) complexes to have abnormally wide ranges of existence compared to those with Ala-Ala-Ala-Ala. Again, the presence of an  $\text{Asp}^4$  residue does not have a significant influence on the complex speciation, as demonstrated by a comparison of the curves for Ala-Asp-Ala-Ala [Fig. 1(ii)] and Ala-Asp-Ala-Asp [Fig. 1(v)].

The wide range of existence of the 2N complexes when Asp is in the second position of the peptide chain is confirmed by the spectroscopic results as shown in Table 4. From all techniques used the 2N species could be identified unambiguously and their pH ranges of existence were similar to those calculated from potentiometry.

**3N Co-ordination.** Earlier studies with an Asp residue in the third position in a peptide sequence have demonstrated that binding of the  $\beta$ -carboxylate stabilizes the  $\text{CuH}_2\text{L}$  (3N) complex to such extent that co-ordination of a fourth N donor could not be detected.<sup>1,4</sup> Co-ordination in this complex (see I) would involve planar binding of  $\text{NH}_2$ ,  $\text{N}^-$ ,  $\text{N}^-$  and  $\text{CO}_2^-$  and this stabilization may be represented quantitatively by  $\log K'' = \log\beta(\text{CuH}_2\text{L})_{\text{peptide}} - \log\beta(\text{CuH}_2\text{L})_{\text{tetraalanine}}$ . With Ala-Ala-Asp-Ala the value is 3.4 and similar stabilization is also found with Ala-Asp-Asp-Ala ( $\log K'' = 3.5$ ), Ala-Ala-Asp-Asp ( $\log K'' = 3.5$ ) and Ala-Ala-Ala ( $\log K'' = 2.8$ ). Again the presence of Asp in the fourth position [Ala-Ala-Asp-Asp, Fig. 1(vi)] does not affect significantly the co-ordination equilibria which are very similar to those found with Ala-Ala-Asp-Ala.<sup>4</sup>

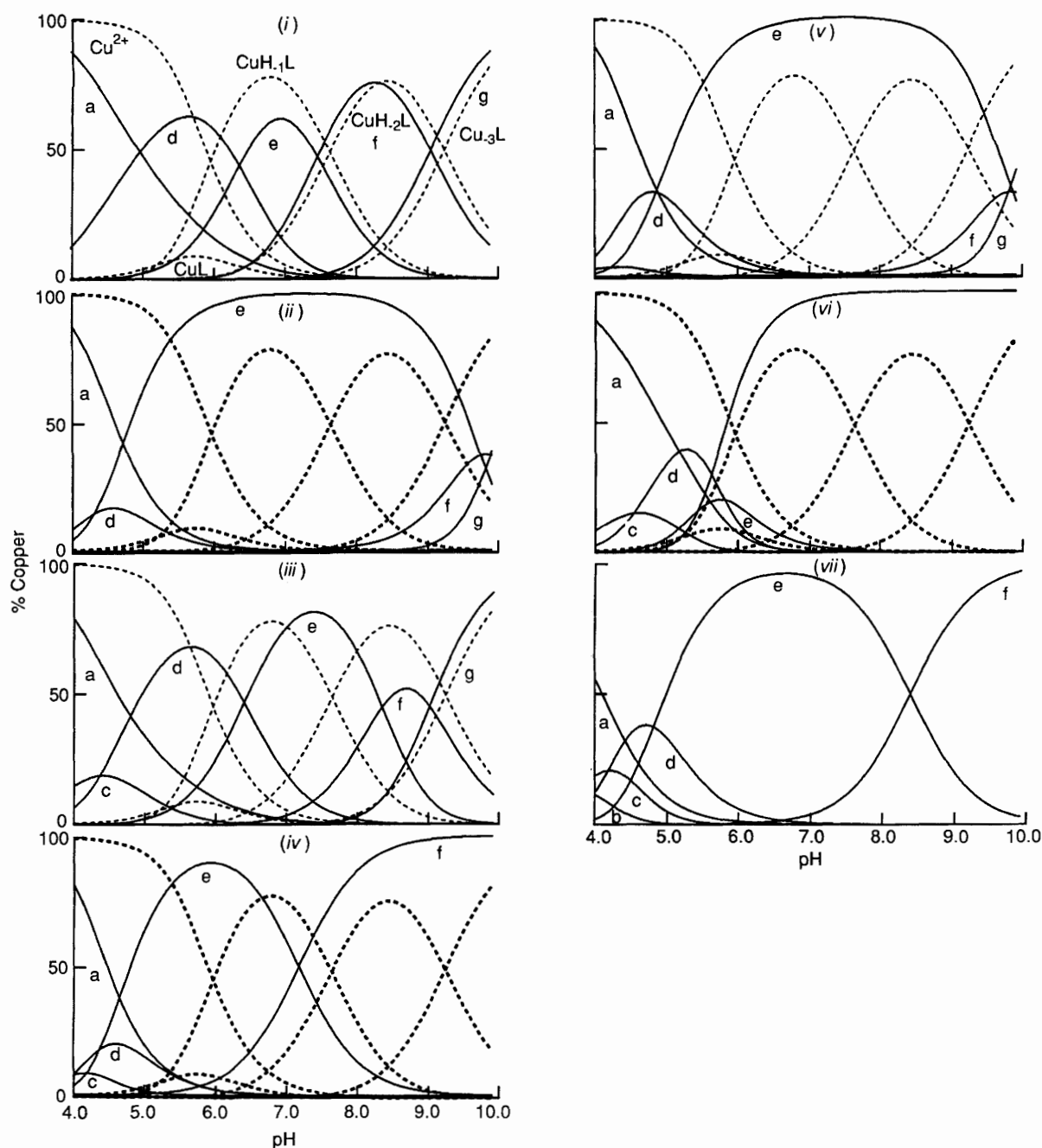


Fig. 1 Species distribution curves for 1:1 mixtures ( $0.001 \text{ mol dm}^{-3}$ ) of  $\text{Cu}^{\text{II}}$  with: (i) Asp-Ala-Ala-Ala, (ii) Ala-Asp-Ala-Ala, (iii) Asp-Ala-Ala-Asp, (iv) Ala-Asp-Asp-Ala, (v) Ala-Asp-Ala-Asp, (vi) Ala-Ala-Asp-Asp, and (vii) Asp-Asp-Asp. Dotted lines show the species distribution curves for complexes with Ala-Ala-Ala-Ala.<sup>2</sup> Species (a)  $\text{Cu}^{2+}$ , (b)  $\text{Cu}(\text{H}_2\text{L})$ , (c)  $\text{Cu}(\text{HL})$ , (d)  $\text{CuL}$ , (e)  $\text{CuH}_1\text{L}$ , (f)  $\text{CuH}_2\text{L}$  and (g)  $\text{CuH}_3\text{L}$ .

**4N-Co-ordination.** Complexes with 4N co-ordination ( $\text{CuH}_3\text{L}$ ) could be clearly identified by both potentiometry and spectroscopy (see Table 4) with Asp-Ala-Ala-Ala, Ala-Asp-Ala-Ala, Asp-Ala-Ala-Asp and Ala-Asp-Ala-Asp. When the peptide contained an  $\text{Asp}^3$  residue, however, these species could not be detected spectroscopically, a result identical to that reported earlier.<sup>1,4</sup> With Asp-Asp-Asp the potentiometric study suggested a  $\text{CuH}_3\text{L}$  complex above pH 10 [ $\log K(\text{stepwise}) \approx -11$ ] but omission of this species from the equilibria had only a minor effect on the other complexes. This could not be a 4N complex and the constant is compatible with hydrolysis of a co-ordinated water molecule. The data obtained for the  $\text{Cu}^{\text{II}}$ -Asp-Asp-Asp system indicate that all three Asp residues contribute towards the binding ability of the peptide. As a result the concentration of free  $\text{Cu}^{2+}$  ions at pH 5 is lower than with any other of the peptides studied (see Fig. 1).

### Conclusion

This study has demonstrated that, while the Glu residue has only a small influence on the ability of a peptide to co-ordinate to  $\text{Cu}^{\text{II}}$ , the Asp residue has a significant effect. While aspartic acid forms complexes with  $\text{Cu}^{\text{II}}$  which are only a little more stable than those with simple amino acids, peptides with Asp in the N-terminal position form 1N complexes which are significantly more stable as a result of co-ordination of the  $\beta$ -carboxylate group, rather than the peptide C=O oxygen with simple peptides. Aspartic acid residues in the second and third positions of the peptide sequence stabilize dramatically the  $\text{CuH}_1\text{L}$  (2N) and  $\text{CuH}_2\text{L}$  (3N) species respectively and delay or, in some cases, prevent co-ordination of a fourth nitrogen donor; residues in the fourth position have only a small effect on the stabilities of the complexes and do not affect the speciation significantly.

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