A Potentiometric and Spectroscopic Study of the Interaction of Angiotensin II and some of its Peptide Fragments with Copper(II) †

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The synthesis is reported of the peptide fragments of angiotensin II, Asp-Arg-Val-Tyr and MeCO-Tyr-Ile-His, together with the results of a potentiometric and spectroscopic (visible, circular dichroism, and e.s.r.) study of the interaction with Cu^{II} of angiotensin II itself and the peptides. Results show that, in the pH range 6—8, Cu^{II} appears to co-ordinate to the imidazole nitrogen of the histidyl side-chain together with neighbouring deprotonated peptide nitrogens. When the pH is raised above 8 the co-ordination centre moves to give a complex resembling that found in simple peptides, *i.e.* through the terminal amino nitrogen and its neighbouring peptide nitrogens.

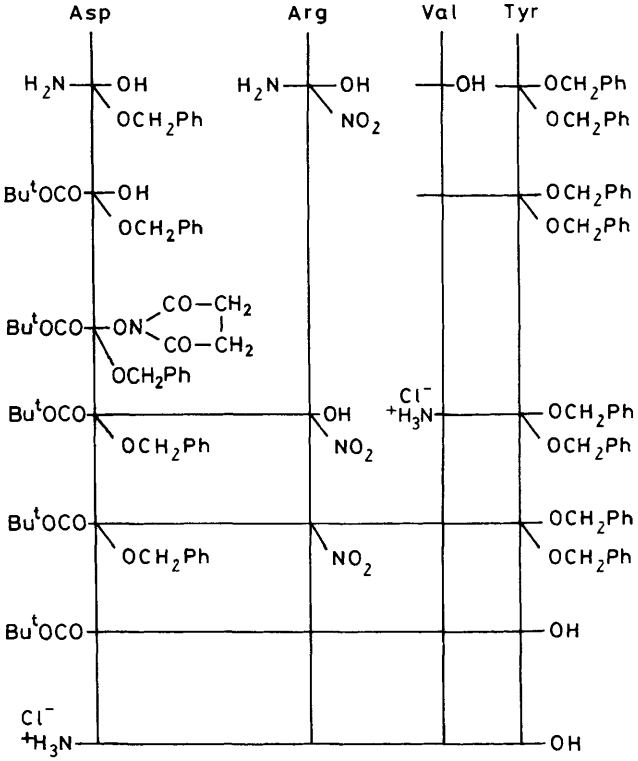
Angiotensin is a linear octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) which behaves as a hormone with a broad range of physiological activities. These are related mainly to the regulation of blood pressure, but it also influences several other processes, *e.g.* the regulation of salt and water excretion by the kidneys (the renin–angiotensin–aldosterone system).[1–5] The conformation and three-dimensional structure of the peptide have been studied extensively, but considerable controversy exists in the literature about its three-dimensional structure in solution.[6–10]

It has been shown that several biological activities of angiotensin are enhanced by certain cations, mainly Li^+, Na^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}.[11–14] It has been suggested that the cations induce conformational changes in the angiotensin molecule leading to a more physiologically active conformation.[11] However, more recently, it has been shown that the binding of metal ions results in no major change in the tertiary structure of the interior of the angiotensin II backbone.[15] Several studies on binding of metal probes to angiotensin II have been carried out with fluorescence and n.m.r. techniques.[15–19]

In this paper, we present the results of a study of the proton and Cu^{II} complexes of angiotensin II and three of its peptide fragments using potentiometric and spectroscopic (electronic absorption, circular dichroism, electron spin resonance) techniques. Since angiotensin II has two binding sites able to form initial co-ordinate bonds to Cu^{II}, *i.e.* the terminal amino group and from the imidazole nitrogen of the histidine residue,[20] we have also undertaken the synthesis and study of binding properties of two peptides which act as separate model compounds for these two binding sites. Peptides synthesised were Asp-Arg-Val-Tyr and MeCO-Tyr-Ile-His, which are respectively the N-terminal tetrapeptide fragment of angiotensin II and the intermediate tripeptide, blocked on its terminal amino group, containing the histidyl residue.

Experimental

Peptide Synthesis.—The tetrapeptide Asp-Arg-Val-Tyr and the tripeptide MeCO-Tyr-Ile-His were synthesised by standard liquid-phase methods as outlined in Schemes 1 and 2. The

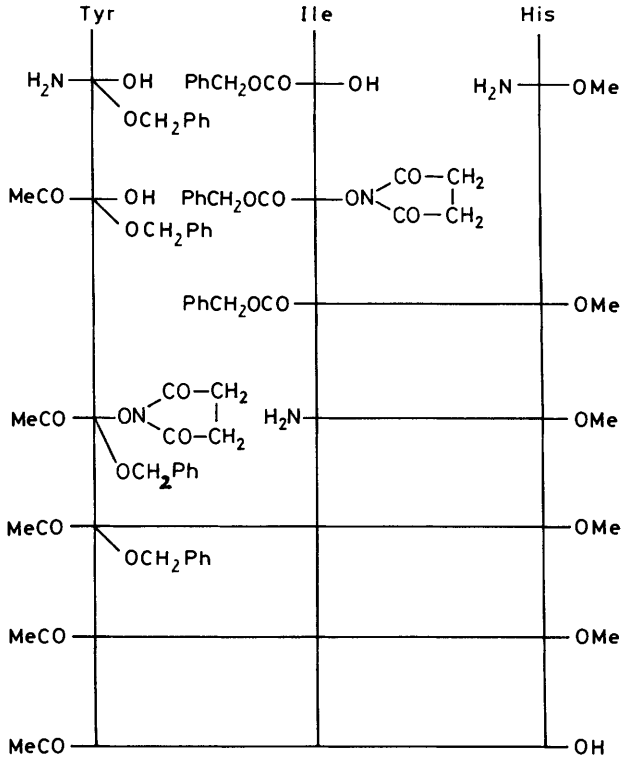


Scheme 1. Synthesis of Asp-Arg-Val-Tyr

coupling reagents were dicyclohexylcarbodi-imide (Merck) and 1-hydroxybenzotriazole (Aldrich). The Bu^tOCO groups were cleaved by 4 mol dm^{-3} HCl in 1,4-dioxane and the benzyloxycarbonyl and benzyl groups were removed by hydrogenolysis using 10% Pd/C in an acetic acid–water (90:10) mixture. The nitro group of guanidine was reduced using 'Pd black' as catalyst in the presence of cyclohexa-1,4-diene.[21] The methyl ester group was cleaved by saponification with sodium hydroxide. The peptides were purified by gel filtration (Sephadex G15, eluant water). Sample purities were checked by paper chromatography [Whatmann 1, eluant (% v/v): water (30), pyridine (35), and butanol (35)], and by h.p.l.c. on Bondapak C_{18}

† Abbreviations used for the amino acid residues are those recommended by I.U.P.A.C.–I.U.B. in 'Nomenclature and Symbolism for Amino Acids and Peptides,' *Pure Appl. Chem.*, 1987, **56**, 595.

with an eluant gradient of water (with 0.05% trifluoroacetic acid)–methanol. Amino acid analysis of Asp-Arg-Val-Tyr gave the following ratios: Asp, 1.02; Arg, 1.02; Val, 0.99; Tyr, 0.96. Angiotensin II and His-Pro-Phe were purchased from the Bachem Company.



Scheme 2. Synthesis of MeCO-Tyr-Ile-His

Spectroscopic Studies.—The metal ion source was $Cu(ClO_4)_2 \cdot 6H_2O$ (Fluka), and solutions with molar ratios of metal : peptide of 1 : 1 were used with concentrations of 0.001 and 0.002 mol dm^{-3}. Absorption spectra were measured on a Cary 219 spectrometer and circular dichroism (c.d.) spectra on a Mark III Jobin-Yvon dichrographe in the 200–800 nm region. All c.d. results are expressed in terms of $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$. Electron spin resonance spectra were recorded on a Varian E102 spectrometer at liquid nitrogen temperature, diphenylpicrylhydrazyl being used as a standard.

Potentiometric Studies.—Stability constants for H^+ and Cu^{2+} complexes were calculated from titration curves carried out at 25 °C using total volumes of 1.5—2 cm^3. Alkali was added from a 0.1-cm^3 micrometer syringe which had been calibrated by both weight titration and the titration of standardized materials. Changes in pH were followed using a glass electrode calibrated in H^+ concentrations with $HClO_4$.[22] All solutions were of ionic strength 0.10 mol dm^{-3} (KNO_3) and peptide concentrations of 0.002 mol dm^{-3}. Calculations were made with the aid of the SUPERQUAD computer program.[23] This allows for the refinement of total ligand concentrations and was able to confirm the purity of the peptides studied and in particular the absence of acetate, a frequent impurity in peptide samples, or other co-ordinating ions. In all cases duplicate or triplicate titrations were carried out at Cu : L ratios of 1 : 1 and 1 : 2. The standard deviations 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

Measured proton and Cu^{II} complex stability constants are given in Table 1, together with those of some related ligands. Spectroscopic data for copper complexes are given in Table 2.

Table 1. Formation constants of H^+ and Cu^{2+} complexes at 25 °C and I = 0.10 mol dm^{-3} (KNO_3), with standard deviations on the last figure given in parentheses

	log values				
H^+ complexes	β_{HL}	β_{H_2L}	β_{H_3L}	β_{H_4L}	β_{H_5L}
Asp-Arg-Val-Tyr (H_3L)	10.27(1)	18.04(2)	21.87(2)	25.01(4)	
Stepwise constants		7.77	3.83	3.14	
MeCO-Tyr-Ile-His (H_2L)	9.78(1)	16.80(2)	19.74(3)		
Stepwise constants		7.02	2.95		
MeCO-Gly-Gly-His (HL)[a]		7.18	3.08		
His-Pro-Phe (HL)	7.57(1)	13.11(1)	16.37(1)		
Stepwise constants		5.54	3.26		
Angiotensin II (H_3L)[b]	10.14(1)	17.89(1)	24.46(2)	29.13(2)	32.65(2)
Stepwise constants		7.75	6.57	4.67	3.52
Tetra-alanine (HL)[c]	8.13	11.65			
		3.52			
Cu^{2+} complexes	β_{CuHL}	β_{CuL}	$\beta_{CuH_{-1}L}$	$\beta_{CuH_{-2}L}$	$\beta_{CuH_{-3}L}$
Asp-Arg-Val-Tyr (H_3L)	16.34(1)	10.14(1)	2.34(2)	−7.13(2)	−17.90(3)
(stepwise)		6.20	7.80	9.46	10.77
MeCO-Tyr-Ile-His (H_2L)	14.26(2)	7.27(2)	−0.07(2)	−8.99(2)	−18.95(2)
(stepwise)		6.99	7.34	8.91	9.97
MeCO-Gly-Gly-His (HL)[a]		4.24	−2.26	−9.61	−18.86
His-Pro-Phe (HL)	12.21(5)	8.81(1)	1.81(1)	−9.09(3)	
		14.62(9)[d]	−2.8(1)[e]	12.90(1)[f]	
(stepwise)		3.40	7.00	10.90	
Angiotensin II (H_3L)		10.20(8)	1.48(2)	−7.65(1)	−16.95(1)
(stepwise)			8.72	9.13	9.30
Tetra-alanine (HL)[c]		4.77(5)	−0.45(1)	−8.09(1)	−17.33(2)
(stepwise constants)			5.22	7.64	9.24

[a] Ref. 38. [b] The empirical formula H_3L neglects the guanidinium proton. [c] Ref. 29. [d] $[CuL_2]$. [e] $[Cu(H_{-1}L)_2)]$. [f] $[Cu_2H_{-1}L_2]$.

Protonation constants were calculated first assuming ligand concentrations were as calculated from the titration curves and secondly, after allowing ligand and proton concentrations to 'float' in SUPERQUAD. It was found that the concentrations never changed by more than 1% and calculated constants remained constant within the calculated errors. These observations confirmed the purity of the peptides studied. The arginine residue contains a very basic terminal nitrogen atom as a result of resonance stabilisation of the protonated guanidine group (log $K \sim 12$).[24] Under the experimental conditions used, the pH never exceeded 10.5 and, at this value, the degree of deprotonation of the guanidine group is insignificant. Hence the proton was considered as unionisable under the conditions used and its protonation constant ignored. In all cases except His-Pro-Phe the first centre to protonate from basic solution of pH 10.5 was the tyrosyl oxygen (log $K \sim 10$), followed by the N-terminal nitrogen (log $K \sim 7$—8), the imidazole pyridine-like nitrogen (log $K \sim 6$), and finally the carboxyl groups (log $K \sim 3$—4). For all the ligands studied, the calculated constants were close to those expected, allowing for normal electronic effects within the molecules, suggesting no unexpected behaviour. Protonation constants of Asn-angiotensin (with the terminal Asp replaced by an aspartamine residue) have been studied previously by potentiometry and by n.m.r.[25,26]

Complexes formed by Cu^{II} were much more dependent on the particular peptide studied. The ligands are therefore best considered separately, combining the potentiometric and spectroscopic results.

Asp-Arg-Val-Tyr.—Potentiometric studies showed the existence of the series of complexes [CuHL], [CuL], [$CuH_{-1}L$], [$CuH_{-2}L$], and [$CuH_{-3}L$] (charges omitted throughout). Asp-Arg-Val-Tyr (H_3L) is a more complicated tetrapeptide than tetra-alanine (Ala-Ala-Ala-Ala, HL) since it possesses three additional co-ordination centres which may be able to bond to Cu^{II}; these are the side-chain carboxylate group (in Asp), the phenolic oxygen (in Tyr), and the guanidine nitrogen (in Arg). Studies of the interaction of arginine with Cu^{II} have shown it to behave in a glycine-like manner without any evidence of co-ordination through the guanidinium nitrogens[24] and all the experimental evidence shows that the co-ordination of Asp-Arg-Val-Tyr can equally well be explained without

Table 2. Spectroscopic data (G = 10^{-4} T) for Cu^{2+} complexes of angiotensin and related peptides

Ligand	Species		Visible λ/nm (ε)[a]	C.d. λ/nm (Δε)[a]	E.s.r. $g_{\|}$	E.s.r. $A_{\|}$/G
Asp-Arg-Val-Tyr	[CuHL]	1N	712 (36)	768 (+0.1)[b]	2.30	160
				604 (−0.06)[c]		
				256 (−1.13)[d,e]		
	[CuL]	2N	620 (70)	623 (−0.22)[f]	2.23	170
				314 (+0.30)[g]		
				272 (−0.56)[d]		
				246 (−0.88)[h]		
	[$CuH_{-1}L$]	3N	550 (100)	566 (−0.38)[f]	2.20	200
				314 (+0.38)[g]		
				278 (−1.28)[d]		
				248 (+0.20)[h]		
	[$CuH_{-2}L$]	4N	510 (140)	534 (−0.96)[f]	2.16	210
				314 (+0.56)[g]		
				276 (−1.79)[d]		
				246 (sh) (+2.5)[h]		
MeCO-Tyr-Ile-His	[CuHL]	1N	730 (34)		2.36	140
	[CuL]	2N	660 (43)		2.27	170
	[$CuH_{-1}L$]	3N	570 (77)	590 (−0.50)[f]	2.21	180
				320 (sh) (+0.50)[g,i]		
				240 (br) (+)[h,i]		
	[$CuH_{-2}L$]	4N	620 (sh) (60)	642 (+0.57)[b]	2.19	195
	[$CuH_{-3}L$]	4N	515 (110)	504 (−1.10)[c]		
				334 (+0.85)[g,i]		
				296 (−1.10)[e]		
				260 (+4)[h]		
His-Pro-Phe	[CuHL]	1N	656 (44)			
	[CuL]	2N	615 (98)	670 (+0.15)	2.22	183
				255 (−2)		
				237 (−4.2)		
				227 (−5.5)		
		3N?	600 (84)			
Angiotensin II	[CuL]	3N	610 (97)	581 (−0.54)[f]	2.22	160
				328 (+0.50)[g,i?]		
				284 (−0.24)[d]		
				260 (+0.52)[h]		
	[$CuH_{-2}L$]	4N	510 (140)	536 (−1.25)[f]	2.17	210
				324 (+0.50)[g]		
				284 (−1.70)[d]		
				255 (+2.5)[h]		
				232 (−4.78)[h]		

[a] Units of ε and Δε are $dm^3\ mol^{-1}\ cm^{-1}$. [b] *d–d* (*B*) transition. [c] *d–d* (*E*) transition. [d] NH_2–Cu c.t. transition. [e] Intra-ligand transition (^1L_b transition of the aromatic ring). [f] *d–d* transition. [g] N^-–Cu c.t. transition. [h] Intra-ligand transition. [i] N_{im}–Cu c.t. transition.

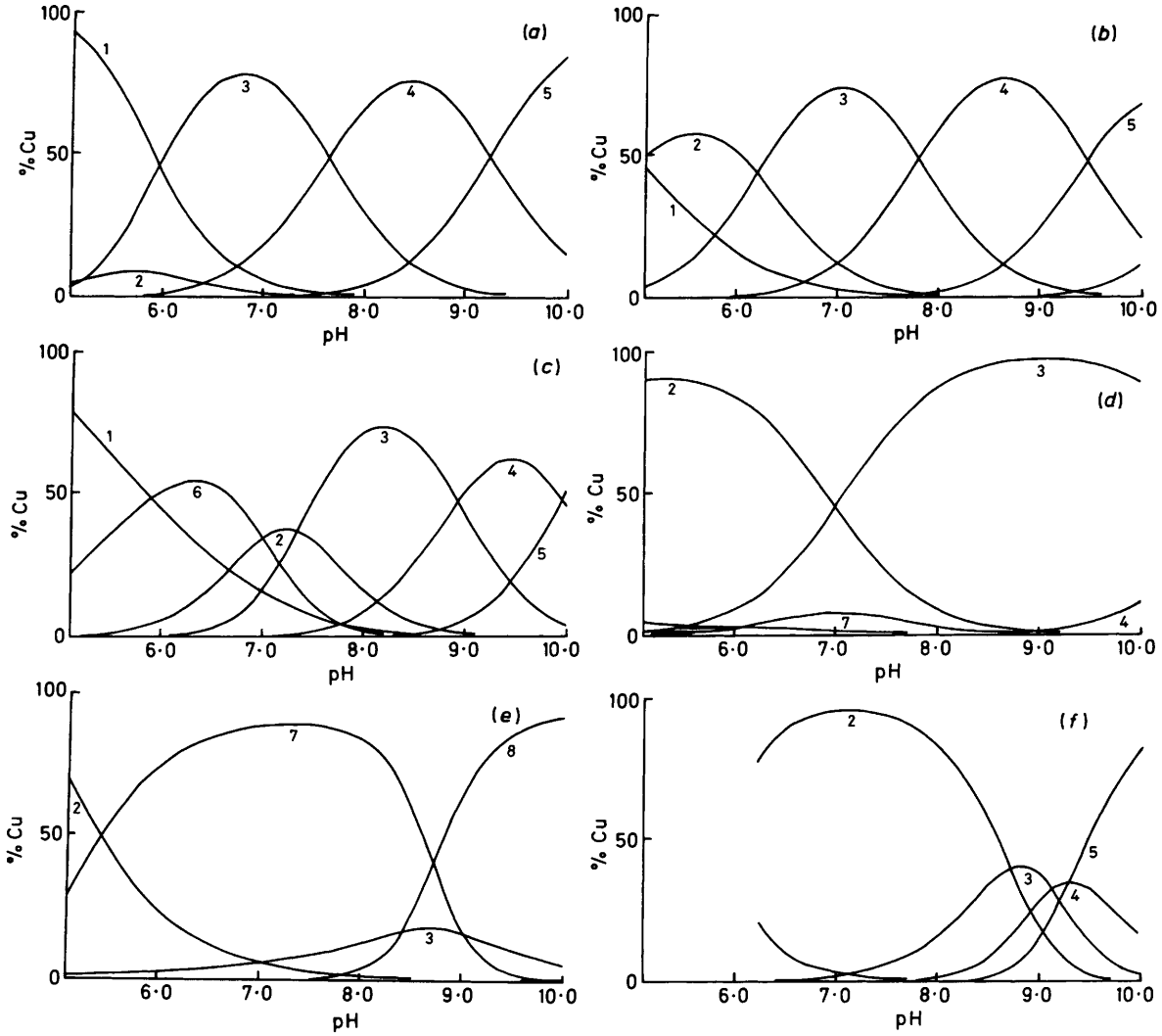


Figure 1. Species distribution curves for 1:1 mixtures (0.001 mol dm^{-3}) of Cu^{II} with (*a*) tetra-alanine, (*b*) Asp-Arg-Val-Tyr, (*c*) MeCO-Tyr-Ile-His, (*d*) His-Pro-Phe (1:1), (*e*) His-Pro-Phe (1:2), (*f*) angiotensin II. Species: 1, Cu^{2+}; 2, [CuL]; 3,[$CuH_{-1}L$]; 4, [$CuH_{-2}L$]; 5, [$CuH_{-3}L$]; 6, [CuHL]; 7, [CuL_2]; 8, [$CuH_{-2}L_2$]

direct involvement of the arginine side-chain. Interaction between a tyrosyl oxygen and Cu^{II} is well established in some peptides[27] but it depends strongly on the peptide conformation and, when present, can be detected unambiguously spectroscopically. Interaction between Cu^{II} and carboxyl groups of aspartyl residues is well established and is to be expected.[28–30]

Starting at low pH, the first complex to form would be expected to be the [CuHL] species, corresponding to the [CuL] species of tetra-alanine with the tyrosyl oxygen protonated. If the measured stability constant ($\log \beta_{CuHL} = 16.34$) is corrected for this protonation constant ($\log K = 10.27$) a value of $\log K = 6.07$ is obtained for the reaction Cu + L ⟶ CuL since both carboxylate protons can be assumed to be ionized. This is significantly higher than the corresponding value for tetra-alanine (4.77) and suggests significant bonding through the carboxylate group of the Asp residue to form a six-membered chelate ring, as is found in complexes of aspartic acid itself,[31] and peptides containing the Asp residue.[28,29] This complex would involve co-ordination through one nitrogen only and, although its presence could not be proved from the visible and c.d. spectra, it could be detected by e.s.r. spectroscopy (see Table 2).

As the pH is raised above 5, the normal mode of co-ordination of Cu^{II} is successively to ionize the neighbouring peptide protons to form Cu–N^- bonds giving, with simple peptides, the species [$CuH_{-1}L$], [$CuH_{-2}L$], and [$CuH_{-3}L$]. This also happens with Asp-Arg-Val-Tyr. The [CuL] species (corresponding to [$CuH_{-1}L$] with tetra-alanine) has a stability (after correction for protonation of the tyrosyl residue) close to that of tetra-alanine [log values of −0.13 (*i.e.* 10.14 − 10.27) and −0.45, respectively] suggesting similar modes of bonding, *i.e.* 2N co-ordination. Hence co-ordination through the β-carboxyl group of Asp must be broken during the reaction [CuHL] ⟶ [CuL]. This accounts for the very different values for this stepwise constant for Asp-Arg-Val-Tyr and tetra-alanine shown in Table 1 and is well illustrated in the comparison of the species distribution curves shown in Figure 1. As expected, values for the formation constants for the species [$CuH_{-1}L$] (3N) and [$CuH_{-2}L$] (4N), after correction for phenolate protonation (−7.93 and −17.40 respectively) are also close to corresponding values for tetra-alanine (−8.09 and −17.33 respectively). The final ionization, represented by [$CuH_{-2}L$] ⟶ [$CuH_{-3}L$], must be ionization of the phenolic proton of Tyr without subsequent co-ordination to Cu^{II}. This is confirmed by the similarity in the protonation constants of the

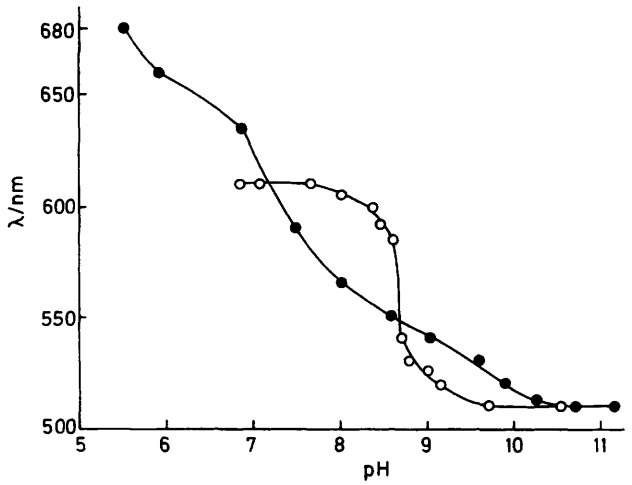


Figure 2. Variation with pH of the spectra of Cu^{II} complexes of Asp-Arg-Val-Tyr (●) and angiotensin II (○) in the visible region (*d–d* transitions)

metal complex and the free ligand (10.77 and 10.27 respectively) and the complete absence in the spectroscopic studies of bands around 400 nm characteristic of Cu^{II}←O^-(phenolic) charge transfer.[32]

The spectral studies confirm all the above deductions. The visible spectrum shows a regular decrease of the wavelength of the *d–d* band from 680 nm at pH 5.5 to 510 nm at pH 10.5, with plateaux at about 630, 560, and 510 nm (Figure 2). The c.d. spectra display negative Cotton effects for the *d–d* band (*B* and *E* transitions), at 630 nm (2N species), 570 nm (3N species), and 535 nm (4N species). In the charge-transfer (c.t.) region, there are two bands at 315 nm (N^-–Cu c.t. transition), and 275 nm (NH_2–Cu c.t. transition, see Table 2).[20] In this latter band, there is probably also an overlapping aromatic transition of tyrosine (^1L_b transition).[33] However, this band does not move to higher wavelength with deprotonation of the phenolic group, which implies that the NH_2–Cu transition has the major contribution.

In the e.s.r. spectra, the four species found by potentiometry could be detected (see Table 2). Values of $A_{\parallel}$ and $g_{\parallel}$ agreed well with 1N, 2N, 3N, and 4N species.[34] The 1N species is not always detectable by e.s.r. because it is usually formed in too small an amount, but here it is more stable due to the co-ordination of the β-COO^- of the Asp residue.

MeCO-Tyr-Ile-His.—In most peptides the pyridine-like nitrogen of an imidazole ring is the only donor centre (other than the terminal amino nitrogen) able to co-ordinate to Cu^{II} when the metal ion is not already held by other centres. This is because Cu^{II} cannot normally deprotonate peptide nitrogens if not already bound to a primary site. Once initially bound, Cu^{II} can then deprotonate neighbouring amides and bind to the nitrogen. In angiotensin this successive co-ordination would be towards the N-terminal end of the molecule since the first amino acid unit after His and going towards the carboxylate terminal is Pro, which contains a secondary nitrogen and so has no ionizable proton. Hence it acts as a 'break-point' to Cu^{II} co-ordination.[35] In addition, steric constraints make it difficult for the imidazole-N and the peptide nitrogens on each side of the His residue to co-ordinate simultaneously.[36] Hence Cu^{II} would be expected successively to deprotonate the His and Ile peptide nitrogens. Possible co-ordination in the His-Pro-Phe section of angiotensin was investigated by studying the complexes of His-Pro-Phe (see below). The tetrapeptide fragment MeCO-Tyr-Ile-His was synthesised to investigate this interaction, the terminal amino group being protected to prevent initial co-

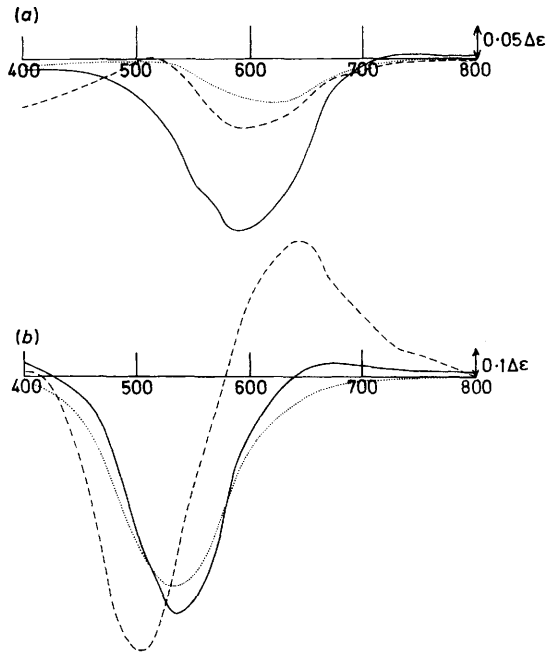


Figure 3. Circular dichroism spectra of Cu^{II} complexes of Asp-Arg-Val-Tyr (·····), MeCO-Tyr-Ile-His (------), and angiotensin II (——) at (*a*) pH 7, (*b*) pH 10.5

ordination to this donor centre. It should be noted that this peptide is not an ideal model for imidazole-N (N_{im}) co-ordination in angiotensin because it has the possibility of N_{im},COO^- chelate formation. However this is not a major contributory factor to complex stability.

The first complex expected would be the [CuHL] species, bonded through the N_{im} atom and also, probably, the carboxylate oxygen with the tyrosyl group protonated. The stability constant of this complex, after correction for tyrosyl protonation, is 14.26 − 9.78 = 4.48 log units. The corresponding value for imidazole itself is a little lower (4.2)[37] supporting this mode of co-ordination. Further evidence for the structure of this [CuHL] complex comes from the e.s.r. spectrum where the $g_{\parallel}$ and $A_{\parallel}$ values are typical of 1N co-ordination (see Table 2). The [CuL] species should be a 2N complex, the [$CuH_{-1}L$] would then be 3N, and [$CuH_{-2}L$] a 4N species. This is supported by the magnitudes of the stepwise deprotonation constants which are comparable to those of tetra-alanine (see Table 1). The proton ionized to form the [$CuH_{-2}L$] complex must be from the amide N of the N-acetyl group. The final ionization to form [$CuH_{-3}L$] would again be ionization of the phenolic proton without co-ordination to Cu^{II} since the protonation constant (log K = 9.97) is close to that of the free ligand (9.78), and no c.t. transitions around 400 nm could be detected in the spectra. The e.s.r. spectra (Table 2) confirm the existence of the 1N, 2N, 3N, and 4N species found in the potentiometric studies. Species distribution curves are shown in Figure 1 and c.d. spectra at intermediate and high pH in Figure 3.

The visible spectrum of the copper complexes of the peptide MeCO-Tyr-Ile-His is very different from that of the Cu complexes of Asp-Arg-Val-Tyr. From pH 6.5 to 8.5, the *d–d* band appears as a broad shoulder at *ca.* 590 nm. When the pH increases above 8.5, it appears as a band at 515 nm with a shoulder at 610 nm. The ratio of absorption coefficients of these two transitions (*ca.* 3:2) does not vary from pH 8.5 to 11, showing that the two transitions probably belong to the same species.

The above behaviour is better seen in c.d. spectra (Table 2). At pH 8, the spectrum displays a negative Cotton effect at 590 nm. On increasing the pH two well separated bands appear at 640 nm (positive) and 500 nm (negative) (Table 2). These two bands can be assigned to *B* and *E* transitions respectively. These transitions were also found to be well separated in Cu complexes of *N*-acetyl-glycylglycylhistidine, 490 nm (positive) and 590 nm (negative),[38] and in glycylglycylhistidine, 494 nm (positive) and 576 nm (negative).[39] The opposite signs of the Cotton effect between the Cu complexes of MeCO-Tyr-Ile-His and of Gly-Gly-His and its N-acetyl derivative might be due to the tyrosine residue which is known to modify the sign of the Cotton effect of the *d–d* band when it is in the first position of a peptide sequence.[40] The difference in energy between the *B* and *E* transitions in the present case is larger, probably as a result of a more distorted D_{4h} symmetry resulting from the steric hindrance of the side-chains.

The charge-transfer region is difficult to interpret due to the overlapping of several transitions. The band at 325 nm is probably due to the N^-–Cu c.t. transition and to one of the N_{im}–Cu c.t. transitions. The c.d. spectrum also displays a broad positive band between 230 and 270 nm, which could correspond to the second N_{im}–Cu c.t. transition, the second aromatic ^1L_b transition,[33] and some other intra-ligand transitions. At high pH, when deprotonation of phenolic oxygen occurs, there is a strong negative band at 295 nm which is due to the aromatic transition of the phenolate group. In this case, this transition is enhanced by complexation since the tyrosine residue is involved in a chelate ring around the metal ion.

His-Pro-Phe.—The peptide His-Pro-Phe was purchased to study the extent of interaction between Cu^{II} and the peptide nitrogen atoms between the His residue and the carboxylate terminal of angiotensin. Since Cu^{II} cannot bond to the peptide-N of the Pro residue[35] the only possible chelate ring is to the peptide-N of the terminal Phe residue, a ten-membered ring. Under normal circumstances such large rings are unlikely but a -Pro- residue in a peptide chain encourages the formation of a 'β-turn', so favouring chelate formation.[35] At low pH (<4) there was potentiometric evidence for a [CuHL] complex but this was only a minor species, probably bonded through the terminal amino-N or possibly the N_{im}.

Study of an N-protected tripeptide may have provided more useful results. The first major species to form was [CuL], a 2N complex bonded through the N-terminal nitrogen and the N_{im} in a histamine-like fashion. Hence the stability constant ($\log \beta$ = 8.81) is not greatly different from that of the Cu^{II} complex of histamine itself (9.55).[41] Such 2N co-ordination is supported by the c.d. spectra where the broad band around 255 nm (see Table 2) would contain components from at least three transitions, NH_2–Cu, N_{im}–Cu, and intra-ligand aromatic transitions. In the presence of excess tripeptide, Cu^{II} readily forms a bis-complex, [CuL_2], with $\log \beta$ = 14.62. The corresponding value with histamine is 16.0. In 2:1 tripeptide:metal mixtures this is the major species in the range pH 5.5—8.5 as shown in Figure 1(*e*). In 1:1 mixtures,a [$CuH_{-1}L$] complex forms above pH 6.5 and this must involve chelation through the Phe peptide nitrogen, forming a large chelate ring, to give a 3N complex. The N^-–Cu c.t. transition was not detected in spectroscopic studies using an excess of ligand because the [$CuH_{-1}L$] complex does not form to a significant extent under these conditions. Molecular models demonstrate that the 3N complex can form without undue strain but some steric hindrance is present, encouraging the formation of [CuL_2] when possible. The second deprotonation to give [$CuH_{-2}L$] only takes place above pH 10 (protonation constant 10.9 log units) and is presumably the result of hydrolysis of a co-ordinated water molecule.

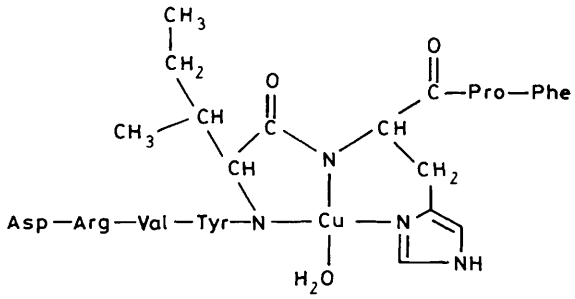


Figure 4. Suggested structure for the [CuL] complex of angiotensin II

Angiotensin II.—Between pH 5 and 7.5 angiotensin formed comparatively insoluble complexes with Cu^{II} making it necessary to use solutions with a maximum concentration of 0.0005 mol dm^{-3}. Above this pH range, however, the solubility increased significantly allowing precise potentiometric data to be obtained. Angiotensin has two potential donor centres which can initiate complexation (the NH_2 terminal and the N_{im} of the His residue). It is likely that the first centre to co-ordinate would be the N_{im} donor but it is also possible that the Cu^{II} binds to both centres initially leading to either a large chelate ring or a polymeric structure as has been suggested in the case of the copper(II)–Gly-Gly-Gly-His system.[42] Below pH 8 the major complexed species was [CuL] and between pH 8 and 9 the complex lost two protons almost simultaneously to give a [$CuH_{-2}L$] species. The intermediate [$CuH_{-1}L$] complex was detected as a comparatively minor species in titrations with a large excess of angiotensin but was insignificant in 1:1 mixtures. The [$CuH_{-2}L$] complex was further deprotonated above pH 9 to give [$CuH_{-3}L$]. This probably corresponds to ionization of the phenolic proton of the Tyr residue, without further co-ordination to Cu^{II} and the structure would then be effectively the same as that of the [$CuH_{-2}L$] complex.

In the pH range 6.5—8 both carboxyl groups would be deprotonated and, in all probability, the amino-terminal and the Tyr residue protonated. A [CuL] species is formed in this pH region and the spectroscopic evidence suggest that this is a 3N complex, comparable to that formed with MeCO-Tyr-Ile-His, *i.e.* bonded through the N_{im} donor rather than the terminal NH_2. Figure 2 shows that, around pH 7, angiotensin and Asp-Arg-Val-Tyr in the presence of Cu^{II} behave in very different ways. With Asp-Arg-Val-Tyr there is a regular decrease in $\lambda_{max.}$ as the pH increases while with angiotensin $\lambda_{max.}$ has a constant value of 610 nm from pH 6.5 to 8.5 and then decreases sharply to reach a value of 510 nm at pH 9.5. Hence the [CuL] species appears to have a wider pH range of existence with angiotensin than with Asp-Arg-Val-Tyr. In contrast, there is close similarity with MeCO-Tyr-Ile-His. The value of 610 nm for the *d–d* band energy is a little above that typical of 3N complexes of simple peptides (*ca.* 570 nm) but can be easily explained when ligand-field effects are taken into account since these are in the order $N_{im} < NH_2 < N^-$.[43] As a result a 3N species involving N_{im} and N^- co-ordination would be expected to have a higher value for $\lambda_{max.}$ than a 3N species involving NH_2–Cu and two N^-–Cu bonds.

If the Cu^{II} is bound first to the N_{im} of the His residue, [CuL] would probably involve deprotonation of amido-N of the His and the Ile residues to give a complex similar to the [$CuH_{-1}L$] species with MeCO-Tyr-Ile-His. The visible and c.d. spectroscopic results support this: *e.g.* $\lambda_{max.}$ (610 nm) with angiotensin is in agreement with results for the [$CuH_{-1}L$] species (a 3N complex) with MeCO-Tyr-Ile-His. If, on the other hand, the Cu^{II} is bound first to the terminal amino-N and does not involve the N_{im} donor centre, this nitrogen would be deprotonated

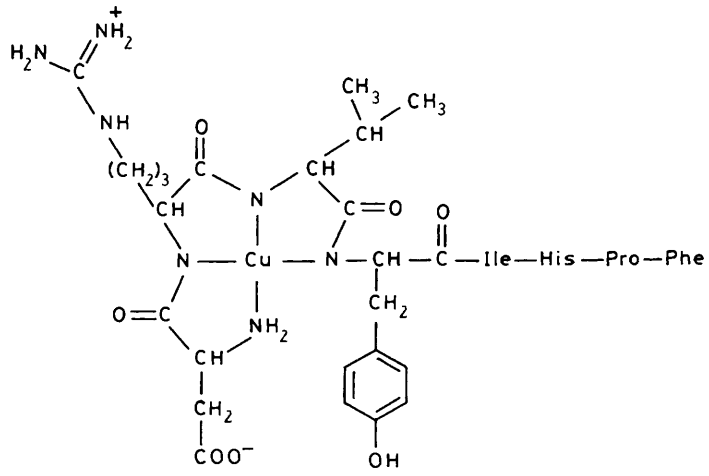


Figure 5. Suggested structure for the $[CuH_{-2}L]$ complex for angiotensin II

above pH 6.5 allowing only 2N co-ordination in a [CuL] species (presumably the terminal NH_2 and the deprotonated amido-nitrogen of the Arg residue making it comparable to the [CuL] complex with Asp-Arg-Val-Tyr). Although the e.s.r. results are compatible with this, the visible and c.d. spectroscopic results are not, as explained above. The low solubility found below pH 7.5 could be a result of the conformation of the 3N complex presenting four hydrophobic side-chains (Phe, Pro, Ile, and Tyr) to the polar solvent and protecting the metal ion inside a 'hydrophobic ball'. The alternative suggestion that the 3N [CuL] complex is bonded through the terminal NH_2, the N_{im}, and an amido-N^- forming either a large chelate ring or a polynuclear complex is less likely, although this could also account for the low solubility found. It is therefore suggested that the [CuL] species with angiotensin is a 3N complex involving the N_{im} and deprotonated amido-N donors of the His and Ile residues.

Above pH 8.5 there is a rapid double deprotonation to give the $[CuH_{-2}L]$ species. The value for $\lambda_{max.}$ of the *d–d* band for this species (510 nm) is identical to that found for the high pH complex of Asp-Arg-Val-Tyr (see Table 2) and is typical of 4N co-ordination through NH_2 and three N^- centres. In the case of the 4N species with MeCO-Tyr-Ile-His the value for $\lambda_{max.}$ is higher as a result of bonding through the N_{im} centre. What is more, at high pH the c.d. spectra of Cu^{II} complexes of angiotensin and of Asp-Arg-Val-Tyr are very similar, and different from that with MeCO-Tyr-Ile-His (Figure 3). At high pH we therefore suggest that Cu^{II} is bound to angiotensin in the same way that it is bound to Asp-Arg-Val-Tyr as shown in Figure 5. Comparison of Figures 4 and 5 shows the significant change in co-ordination which is thought to take place between pH 8 and 9.

The charge-transfer region of the c.d. spectrum is very complicated due to the overlapping of many transitions. Co-ordination of histidine normally gives c.t. transitions at *ca.* 330 [$\pi_1(N_{im}$–Cu] and 260 nm [$\pi_2(N_{im}$–Cu], the latter band being better seen in the crystalline state.[44] However the c.t. transition N^-–Cu is also located around 320 nm, making it impossible to use these bands to conclude whether or not the imidazole is involved in complexation.

The peptidic region of the c.d. spectrum is completely different in the copper complex compared to the free ligand[45] showing that major conformational changes have occurred on complexation, and between pH 8 and 11 the spectra, in this region, of solutions of the copper complexes also differ significantly.

Acknowledgements

We would like to thank Mrs. A. M. Caze for technical assistance in the spectroscopic studies.

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Received 21*st May* 1987; *Paper* 7/915