# The Unusual Co-ordination Ability of Vasopressin-like Peptides; Potentiometric and Spectroscopic Studies of some Copper(II) and Nickel(II) Complexes†

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The results are reported of a potentiometric and spectroscopic study of the $H^+$, $Cu^{2+}$, and $Ni^{2+}$ complexes of [Arg$^8$]vasopressin, [Arg$^8$]vasotocin and some synthetic analogues containing the D-valyl residue in place of the glutamine residue at 25 °C and an ionic strength 0.10 mol $dm^{-3}$ ($KNO_3$). The complexes of vasopressin and vasotocin with $Cu^{II}$ are the most stable Cu–peptide complexes with 4N co-ordination yet reported. This results from the favourable conformation of the binding site within the ring formed by the disulphide bridge of the peptide. The high stability is lost when a non-co-ordinating residue in the ring (Gln) is replaced by a residue of opposite chirality (D-Val) as a result of steric hindrance between the α-carbon atom of the Val side chain and the neighbouring carbonyl oxygen.

Arginine$^8$-vasopressin (AVP) and arginine$^8$-vasotocin (AVT) are naturally occurring neurophysical hormones with similar structural features, each possessing a 20-membered ring linked by a disulphide bridge with a tripeptide side chain.

AVP = Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

AVT = Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

Spectroscopic studies show that the similarities in their primary structure are followed by similar peptide backbone conformations, as well as the conformations about the disulphide bridge.[1-3] A recent study of the salt-dependent structural changes of this family of neurohormones has shown that some metal ions (*e.g.* lithium) may induce conformational rearrangements of the peptides with resulting changes in their binding domains in their targeted molecules, the neurophysins.[2] Conformational changes in oxytocin to give a vasopressin-like structure, induced by lithium ions, appear to be the result of two distinct effects: (*i*) an unspecific ionic strength effect which influences the C-terminal tripeptide tail of the hormone, and (*ii*) a specific effect of the cyclic backbone conformation of oxytocin generated by more selective interaction between metal ions and some amino acid residues of the peptide. The latter interactions are, however, uncharacterised.[2] The effect of other metal ions, *e.g.* $Mg^{II}$ and $Mn^{II}$, on the peptide–receptor interaction was also found to be profound, although the nature of the metal ion–hormone interaction is, as yet, not clear.

The 20-membered ring formed by the six-amino-acid backbone and two Cys side chains, and containing a S–S bridge, presents an unusual set of donor centres arranged around the potential metal ion binding site. The metal ions mentioned above ($Li^I$, $Mg^{II}$, and $Mn^{II}$) are unable to deprotonate the amide nitrogens in peptide bonds, and hence are unable to form direct bonds to the amide nitrogens; however, they could bond to the carbonyl oxygen or (less likely) the disulphide sulphur atoms. In contrast, copper, a biologically essential element, binds much more effectively to peptides since it can readily deprotonate the nitrogen of the peptide bond to form a stable Cu–$N^-$ bond. Hence vasopressin-like molecules should be very attractive ligands for $Cu^{II}$ ions, assuming that the steric arrangement of the amide nitrogens of the peptide bonds, dictated by the disulphide bridge, can accommodate the size of the metal ion. The interaction of $Cu^{II}$ ions with naturally occurring peptides may also be important in the understanding of the general behaviour of peptides in biological systems since several recent investigations strongly suggest the possibility of metal ion involvement in the regulation of the bioactivity of natural peptides.[4-6]

In this work we present the synthesis of five peptides and the results of potentiometric and spectroscopic studies of the complexes formed with $Cu^{II}$ and $Ni^{II}$ ions. The peptide ligands used in this study are given below. Hence D-VAVP is

[Arg$^8$]vasopressin, AVP =
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

[D-Val$^4$, Arg$^8$]vasopressin, D-VAVP =
Cys-Tyr-Phe-D-Val-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

Tyr(Me)[D-val$^4$, arg$^8$]vasopressin, TMeD-VAVP =
Cys-Tyr(Me)-Phe-D-Val-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

[Arg$^8$]vasotocin, AVT =
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

[D-val$^4$,arg$^8$]vasotocin, D-VAVT =
Cys-Tyr-Ile-D-Val-Asn-Cys-Pro-Arg-Gly-$NH_2$
(S–S)

[Arg$^8$]vasopressin with a D-Val residue in place of –Gln– in position 4; TMeD-VAVP is similar but with the tyrosyl side chain protected by methylation, and hence no longer possessing

† 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.*, 1984, **56**, 595.

an ionizable proton. D-VAVT is similarly [Arg$^8$]vasotocin with a D-Val residue in place of -Gln-.

## Experimental

*Peptide Syntheses.*—The protected peptide intermediates required for the synthesis of the five peptides were prepared by solid-phase synthetic methods, entirely on a resin.[7–9] Chloromethylated resin (Bio-Rad-Bio-Beads 5 × 1) was first esterified with Boc-Gly to a load of 0.62 mmol g$^{-1}$ and then the nona-peptide-resins were prepared using solid phase methods as described earlier.[7–9] Protected peptides were cleaved from the resin by ammoniolysis,[7] deprotected by sodium in liquid ammonia,[8] and the resulting sulphydryl compounds were subjected to oxidative cyclization with $K_3[Fe(CN)_6]$.[10] The crude peptides were desalted on Sephadex G-15 and then purified by gel-filtration on Sephadex LH-20.

*Potentiometric Studies.*—Stability constants for $H^+$, $Cu^{2+}$, and $Ni^{2+}$ complexes were calculated from titrations carried out at 25 °C using total volumes of 1.5 cm$^3$. Alkali was added from a micrometer syringe (0.1 cm$^3$) 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 $HClO_4$.[11] All solutions were of ionic strength 0.10 mol dm$^{-3}$ ($KNO_3$) and peptide concentrations were 0.003 mol dm$^{-3}$. Calculations were made with the aid of the SUPERQUAD computer program.[12] This allows for the refinement of total ligand concentrations and was able to allow for the presence of acetate in the ligands. Since they contained a positively charged arginine residue they were all prepared as acetate salts, as is normal with biopeptides. Small-scale replacement of acetate ions in such polar peptides with a different anion using gel-filtration results in the loss of most of the product and was therefore impractical. However, complexes of the acetate ion with $Cu^{II}$ and $Ni^{II}$ are very weak in comparison to those of the ligands studied here and could be ignored provided the concentration of acetate ion was known so that allowance could be made for the presence of the additional weak acid. 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.

*Spectroscopic Studies.*—Solutions were of the same concentrations as those used in the potentiometric studies, spectra being recorded at 1:1 metal:ligand ratios. Absorption spectra were recorded on a Beckman UV5240 spectrophotometer and circular dichroism (c.d.) spectra were measured on an automatic recording spectropolarimeter, JASCO-J-20. All c.d. spectra are expressed in terms of $\Delta\varepsilon$ ($\varepsilon_l - \varepsilon_r$). Electron spin resonance (e.s.r.) spectra were obtained on a JEOL JES-ME-3X spectrometer at liquid nitrogen temperatures and at 9.13 GHz.

## Results and Discussion

Protonation constants (log β values) for the ligands studied are given in Table 1. Potentiometric titrations clearly demonstrated two protonation steps (*ca.* pH 6.2 and pH 9.5) with AVP, D-VAVP, AVT, and D-VAVT with only one step with TMED-VAVP (pH 6.35). The protonation above pH 9 must be that of the phenolic oxygen of the Tyr residue (protected in TMED-VAVP) while that around pH 6.2 would be protonation of the N-terminal Cys amino group. The values for both these protonations are relatively low (by over a log unit) when compared to other oligopeptides. This must be a result of the electronic effect of the disulphide sulphurs, supported by the conformation forced on the molecule by formation of the disulphide bridge. Sulphur-containing side chains generally depress protonation constants significantly, having high Taft

**Table 1.** Stability constants of complexes of $H^+$, $Cu^{2+}$, and $Ni^{2+}$ with vasopressin analogues at 25 °C and $I$ = 0.10 mol dm$^{-3}$ ($KNO_3$)

(*a*) Protonation constants (log values)

| | $\beta_{HL}$ | $\beta_{H_2L}$ | Stepwise |
|---|---|---|---|
| AVP | 9.58(1) | 15.84(1) | 6.26 |
| D-VAVP | 9.54(1) | 15.70(2) | 6.16 |
| TMED-VAVP | 6.35(1) | | |
| AVT | 9.51(1) | 15.70(1) | 6.19 |
| D-VAVT | 9.52(1) | 15.73(1) | 6.21 |
| $(Gly)_4$[a] | 7.97 | | 7.97 |

(*b*) Copper complex stability constants (log values)

| | $\beta_{111}$ | $\beta_{110}$ | $\beta_{11-1}$ | $\beta_{11-2}$ | $\beta_{11-3}$ |
|---|---|---|---|---|---|
| AVP | 13.36(3) | 7.58(2) | 1.48(3) | −4.67(1) | −14.71(2) |
| D-VAVP | 13.02(9) | 7.64(2) | 1.95(2) | −6.47(2) | −16.32(2) |
| TMED-VAVP | | | −1.81(2) | −7.74(1) | −16.43(2) |
| AVT | 13.45(5) | 7.67(2) | 1.49(2) | −5.45(2) | −15.14(3) |
| D-VAVT | 12.85(3) | 7.39(1) | 1.00(1) | −7.32(1) | −17.33(1) |
| $(Gly)_4$[a] | | 5.08 | −0.42 | −7.31 | −16.60 |

log $K'$[b]

| | 1N | 2N | 3N | 4N |
|---|---|---|---|---|
| AVP | −2.48 | −8.26 | −14.36 | −20.51 |
| D-VAVP | −2.63 | −8.06 | −13.75 | −22.17 |
| TMED-VAVT | | −8.16 | −14.09 | −22.78 |
| AVT | −2.25 | −8.03 | −14.21 | −21.15 |
| D-VAVT | −2.88 | −8.34 | −14.73 | −23.05 |
| $(Gly)_4$ | −2.89 | −8.39 | −15.28 | −24.57 |

(*c*) Nickel complex stability constants (log values)

| | $\beta_{111}$ | $\beta_{110}$ | $\beta_{11-1}$ | $\beta_{11-2}$ | $\beta_{11-3}$ |
|---|---|---|---|---|---|
| AVP | 12.42(3) | 5.41(2) | | −11.2(2) | −20.01(2) |
| D-VAVP | 12.6(1) | 4.7(1) | | −12.4(2) | −22.7(1) |
| TMED-VAVP | | 2.71(5) | −4.38(3) | | −21.11(2) |
| AVT | 12.29(4) | 5.27(2) | | −12.3(2) | −21.12(3) |
| D-VAVT | 12.1(1) | 4.47(5) | | −12.62(2) | −20.7(1) |
| $(Gly)_4$[a] | | 3.64 | −4.45 | | −20.75 |

[a] Ref. 23. [b] log $K'$ = log $\beta_{xN}$ − log $\beta(H_2L)$ [HL with $(Gly)_4$ and TMED-VAVP], where $x$N corresponds to the species with $x$ nitrogen atoms co-ordinated.

**Table 2.** Spectroscopic data for Cu and Ni complexes

| | Species | | C.d. λ/nm (Δε)[a] | Absorption λ/nm (ε)[a] | E.s.r. $g_{\|}$ | E.s.r. $A$/G[b] |
|---|---|---|---|---|---|---|
| Cu–AVP | [CuHL] | (1N) | 740 (−0.07)[c] | 735 (18) | Not resolved | |
| | [CuH$_{-2}$L] | (4N) | 545 (−1.8)[c] | 520 (140) | 2.172 | 216 |
| | | | 320 (+0.46)[d] | 290 (sh) | | |
| Cu–AVT | [CuHL] | (1N) | 730 (−1.10) | | | |
| | [CuH$_{-2}$L] | (4N) | 535 (−2.4) | | | |
| | | | 315 (+0.77) | | | |
| Ni–AVP | [NiH$_{-2}$L] | (4N) | 475 (−2.1)[e] | 400 (100) | | |
| Ni–AVT | [NiH$_{-2}$L] | (4N) | 475 (−1.5) | | | |

[a] Units of ε are dm$^3$ mol$^{-1}$ cm$^{-1}$. [b] G = 10$^4$ T. [c] ($B$ + $E$) $d$–$d$ transition. [d] Cu–N charge-transfer transition. [e] ($A$ + $E$) $d$–$d$ transition.

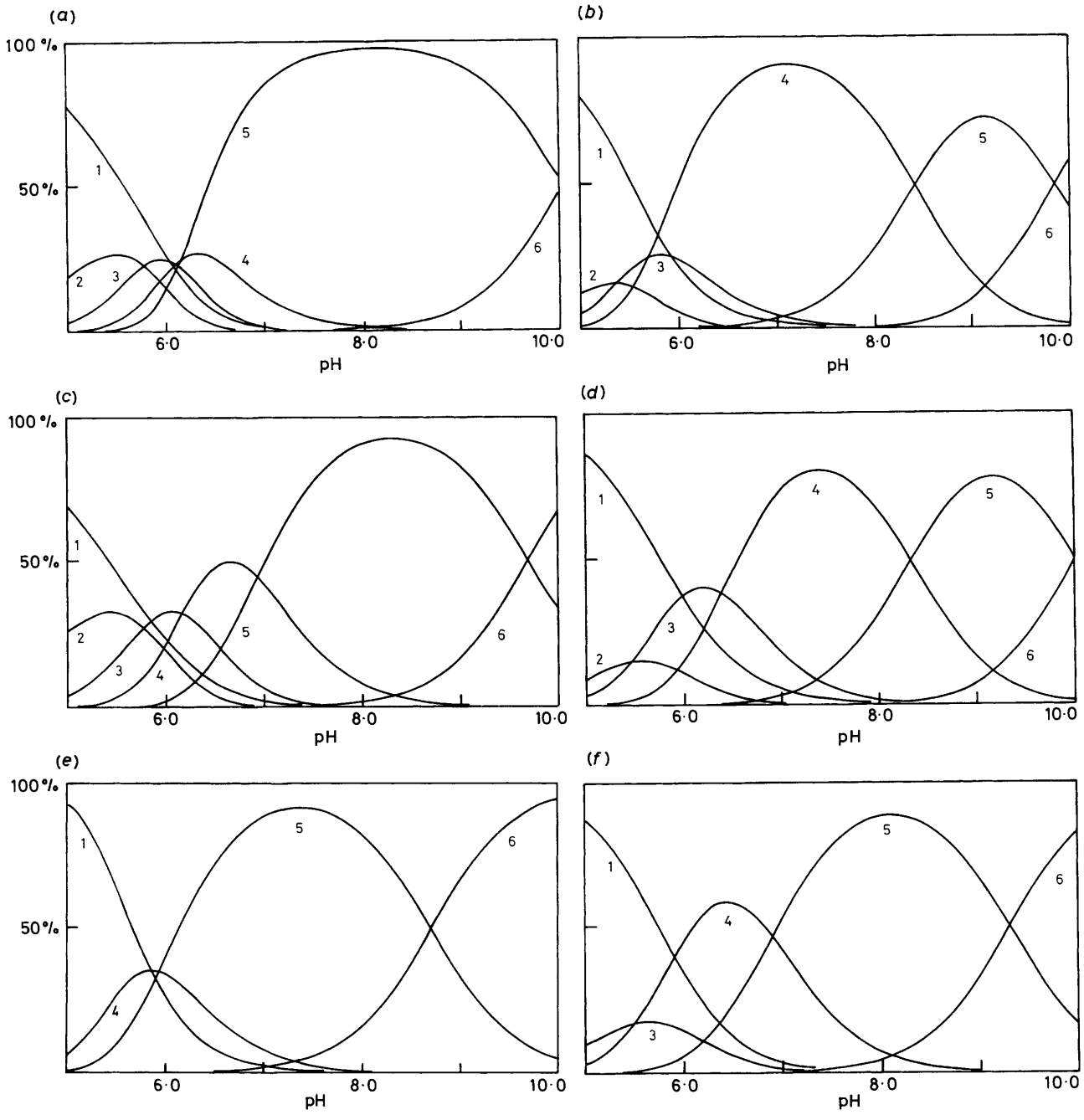


**Figure.** Species distribution curves for 1:1 complexes of $Cu^{2+}$ with: (*a*) AVP, (*b*) D-VAVP, (*c*) AVT, (*d*) D-VAVT, (*e*) TMeD-VAVP, and (*f*) $(Gly)_4$ (0.001 mol $dm^{-3}$): species 1, $Cu^{2+}$; 2, [CuHL]; 3, [CuL]; 4, $[CuH_{-1}L]$; 5, $[CuH_{-2}L]$; 6, $[CuH_{-3}L]$

parameters[13] (*e.g.* representative values for log $K_{HL}$ are for HL = alanine, 9.88, methionine, 9.20, and cystine, 8.80 and 8.03[14]). Protonation constants for cyclic peptides with disulphide bridges do not appear to have been reported.

Copper(II) and nickel(II) complex formation constants are also given in Table 1, and spectroscopic data for some of the complexes are given in Table 2 although the very small quantities of peptides available, particularly those containing D-Val substituents, prevented a full spectroscopic study of their complexes. All the peptides studied, apart from TMeD-VAVP, form five similar complexed species, [CuHL], [CuL], $[CuH_{-1}L]$, $[CuH_{-2}L]$, and $[CuH_{-3}L]$, although their stabilities and degrees of formation are, in some cases, profoundly different. Species distribution curves for 1:1 ligand:$Cu^{II}$ mixtures (0.001 mol $dm^{-3}$) are shown in the Figure and these demonstrate clearly the difference in behaviour between the peptides containing the D-Val residue and those containing the naturally occurring peptide backbone. Methylation of the tyrosine side chain in TMeD-VAVP removes an ionizable proton and hence reduces the number of species formed accordingly. It is clear from the stability constants given in Table 1 that the deprotonations represented by $[CuH_{-2}L] \longrightarrow [CuH_{-3}L] + H^+$ with ligands containing an unprotected Tyr side chain correspond to the ionization of this tyrosyl proton, since the protonation constants (log $K \sim 10$) are very close to those of the free peptides. In addition the spectra were not changed significantly when the pH was increased from 8 to 10.5.

With $Cu^{II}$, AVT and AVP behave similarly (see Figure). The

major complexed species is the $[CuH_{-2}L]$ complex, together with the corresponding species $[CuH_{-3}L]$ which differs only in ionization of the tyrosyl group. With AVP this complex is the major species above pH 6.3, and with AVT it is the major species above pH 6.8. Below these pH values individual complexes were much more difficult to identify although around pH 5 a complex bonded through one nitrogen (1N) could be identified spectroscopically (see Table 2). This was characterised by its absorption band at 735 nm, typical of 1N co-ordination, which was also observed in the c.d. spectra of both AVP and AVT with $Cu^{II}$. This complex must be bonded through the terminal amino nitrogen of the first Cys residue and would correspond to the [CuHL] species identified potentiometrically and shown in the Figure. Spectroscopic data (absorption, c.d., and e.s.r.) confirm the dominant species ($[CuH_{-2}L]$) to be 4N complexes. All the spectroscopic parameters given in Table 2 are characteristic of 4N co-ordination and correspond well with those found earlier for similar systems.[15–18] Hence the spectroscopic data confirm unambiguously the species distribution curves calculated from the potentiometric data, showing, as the pH is raised, the rapid adoption of 4N co-ordination. There was no spectroscopic evidence for $Tyr\text{-}O^{-}\text{–}Cu$ interaction since charge-transfer transitions in the region of 320 nm (typical of $Tyr\text{-}O^{-}\text{–}Cu$ bonding) were completely absent.

The most unusual feature of the species distribution curves is the dominance of the $[CuH_{-2}L]$ complex over such a wide pH range, accompanied by very rapid loss of two protons from [CuL]. The spectroscopic results show this $[CuH_{-2}L]$ species unambiguously to be a 4N complex, as would be expected for the empirical formula identified from potentiometry. The high stability of this 4N complex with AVP, making it the major species above pH 6.3, places it as one of the most stable 4N complexes formed between $Cu^{II}$ and peptides. Under normal circumstances [*e.g.* with tetraglycine, $(Gly)_4$, log $\beta_{11-3} = -16.6$] such a species is not formed below pH 9 (see Figure).The 4N Cu–AVP complex differs from the $(Gly)_4$ analogue in having a protonated Tyr-O (hence the differing empirical formulae) and in the presence of a macrocyclic ring. The crystal structure of the 4N $(Gly)_4$ complex shows the three peptide-N atoms and the $Cu^{II}$ to be almost coplanar, with the terminal N-donor somewhat out of plane.[19]

The unusual stability of this complex with both AVP and AVT can be attributed to the disulphide bridge which makes the peptides resemble other macrocyclic ligands, and presents the nitrogen donor atoms in the correct conformation and at the ideal separations for co-ordination to $Cu^{II}$ without requiring any significant conformational changes. Crystal structures of vasopressin and vasotocin have not been reported, the nearest molecules are deamino-oxytocin[20] (see below) which shows a

oxytocin = Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Gly-$NH_2$
(S–S bridge between the two Cys residues)

ring of the required size but, since it lacks the terminal $-NH_2$ group, it is not directly comparable to pressinoic acid, the cyclic moiety of vasopressin.[21] Attempts have also been made to calculate the ring conformation of vasopressin.[22] These last two studies show a ring of suitable shape but the conformations of the peptide N–H bonds are not ideal. Scale molecular models (Courtaulds models) allow the planar 4N co-ordination of $Cu^{II}$ to the terminal $NH_2$ nitrogen and the peptide nitrogen atoms of the first three peptide linkages without undue strain or molecular rearrangement, so supporting the mode of bonding suggested and high stability found.

Perturbation of the cyclic structure by the replacement of one of the constituent amino acid residues (Gln) by a residue of the opposite chirality (D-Val) has a profound effect on the stoicheiometry of the complexes formed. Species distribution curves for D-VAVT and D-VAVP are given in the Figure and these show that the major complex in the pH range 6—8 is now the $[CuH_{-1}L]$ species. {Although there was not sufficient material for a full spectroscopic study, results from a limited study of the absorption spectrum of a dilute solution show this to be a 3N complex (at pH 7, $\lambda_{max.} = 570$ nm, dropping to *ca.* 530 nm by pH 10) as required by the stoicheiometry, and confirm that the fourth nitrogen does not co-ordinate to give a $[CuH_{-2}L]$ complex until above pH 8.} With TMeD-VAVP similar behaviour is found (Figure); now the 3N complex is the $[CuH_{-2}L]$ species and this gives the 4N $[CuH_{-3}L]$ complex above pH 8. Hence the peptides containing D-Val behave similarly to $(Gly)_4$ itself, the 4N complex being a little more stable. The fact that results for the peptide with a protected Tyr side chain were otherwise identical to those with unprotected Tyr residues is confirmatory evidence for the absence of $Tyr\text{-}O^{-}\text{–}Cu$ bonding in the complexes with AVP and AVT. Co-ordination between the amido nitrogens of the Gln and Asn residues and $Cu^{II}$ would not be expected, and the experimental data gave no evidence for its presence. Similarly there was no evidence for participation of the disulphide sulphurs in the co-ordination schemes.

Examination of molecular models of the $[CuH_{-2}L]$ complexes demonstrates that, with a D-amino acid (D-Val) as the fourth residue, there is major steric hindrance between the carbonyl oxygen of the Phe-D-Val peptide bond and the first carbon atom of the valine side chain since these atoms are eclipsed in the preferred conformation. Steric hindrance is absent with an L–L peptide linkage. The resulting stereoselectivity, amounting to 1.80 log units with AVP and 1.84 with AVT, is one of the most dramatic examples with small molecules to be measured quantitatively.

Quantitative comparison of the stabilities of 4N $Cu^{II}$ complexes of AVP and AVT with those of $(Gly)_4$ is difficult as a result of the differing basicities of the ligands. Direct comparison of the pH at which 50% of the metal is held as a 4N complex is one approach. Values found [AVP, 6.45; AVT, 7.0; $(Gly)_4$, 9.3; D-AVP, 8.45; D-VAVT, 8.35] suggest that the AVP complex is more stable by a factor of *ca.* 700, while the D-VAVP analogue is more stable by a factor of only 7. With AVT the comparable figures are 200 and 9. This approach, however, makes no allowance for the significantly lower basicity of the vasopressin analogues resulting from the disulphide linkage which makes the terminal amino nitrogen a less able electron donor. The measured stability constants may be compensated for this lower basicity by using relationships such as: $\log \beta_{111} - \log \beta_{012}$ with 1N complexes of AVP and AVT and $\log \beta_{110} - \log \beta_{011}$ with $(Gly)_4$. Comparisons of complexes with the vasopressins with those for $(Gly)_4$ are complicated by the presence of the ionizable Tyr proton but values calculated are given in Table 3. These show the 'adjusted' stabilities for 1N and 2N complexes to be broadly comparable, demonstrating that the affinity for co-ordination to $Cu^{II}$ of the terminal amino-N and the neighbouring peptide-N are similar in vasopressins and in $(Gly)_4$. Values for the 3N species deviate somewhat more while values for the formation of 4N complexes suggest that the AVP species is more stable than that with $(Gly)_4$ by four orders of magnitude (4 log units) while the D-VAVP species only exceeds it by 2.4 log units.

Compared to $Cu^{II}$, interaction of $Ni^{II}$ with all the ligands studied was very slow indeed, resulting in lower confidence in the constants calculated. With D-VAVP the results can be regarded as tentative only, since they were obtained from titrations on an even smaller scale. Calculated stability constants are given in Table 1 and spectroscopic data for the complexes with AVT and AVP are given in Table 2. Spectroscopic studies above pH 9 showed the $Ni^{II}$ to be clearly square planar, diamagnetic and co-ordinated to four nitrogens,

forming yellow complexes. The paramagnetic–diamagnetic equilibrium, coupled with the steric constraints required for chelation within the cyclic peptide presumably account for the slow attainment of equilibrium. Results for TMeD-VAVP are comparable to the others when allowance is made for the absence of a tyrosyl proton. Hence [NiHL] ([NiL] with TMeD-VAVP) will be 1N complexes, [NiL] ([$NiH_{-1}L$] with TMeD-VAVP) will be 2N, and [$NiH_{-2}L$] ([$NiH_{-3}L$] with TMeD-VAVP) will be 4N. In none of the cases studied could a 3N-bonded complex be detected: a frequent occurrence with Ni–peptide complexes as a result of the paramagnetic–diamagnetic equilibrium. The final complexes to form will be 4N also, with the Tyr-O proton ionized. In practice the 4N complexes with protonated Tyr residues had only a small region of existence, hence the precision to which they could be measured was low. Compared to $Cu^{II}$ the most striking feature, however, is the much smaller difference in stability between the complexes with AVP and D-VAVP (and AVT and D-VAVT). What is more, their stabilities, after correction for the differing protonation constants, are more nearly comparable to those with $(Gly)_4$.[23] This is presumably a result of the larger size of the $Ni^{II}$ ion.

## Conclusions

The macrocyclic ring formed as a result of the disulphide bridge in peptides related to vasopressin forms an excellent macrocyclic chelate ring for $Cu^{II}$, the nitrogen donor centres being held spatially in ideal positions for co-ordination without the necessity for significant modification to the conformation of the ring. Significant strain is introduced when the chirality of the fourth amino acid residue is changed, resulting in a dramatic example of stereoselectivity.

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