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(35) M. C. Blok, E. C. M. Van der Neut-Kok, L. L. M. Van Deenen, and J. de Gier, *Biochim. Biophys. Acta*, **406**, 187 (1975).
(36) NOTE ADDED IN PROOF. Using low angle light scattering and photon correlation spectroscopy, we have recently determined the molecular weight and hydrodynamic radius of 12 min sonicated DODAC (setting 3 on a Branson B-12 sonifier) vesicles to be 30 million and 309 Å. These values were best accommodated in terms of the formation of single compartment prolate DODAC vesicles (U. Herrmann and J. H. Fendler, to be published in *Chem. Phys. Lett.*, 1979).

# Assignments of $^1H$ Nuclear Magnetic Resonances of the Cystyl, Asparaginyl, and Aromatic Residues of Arginine Vasopressin in $D_2O$. A Comparison with Lysine Vasopressin and Oxytocin in Terms of Solution Conformation

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**Abstract:** The resonances of the $C^\alpha$ and $C^\beta$ protons of the cystyl, asparaginyl, and aromatic residues of [8-arginine]vasopressin (AVP) in $D_2O$ at pD 3.8 and 20 °C were assigned in a rigorous manner by the use of isotopic isomers of AVP that contain specific replacements of protons by deuterons and by comparison of $^1H$ NMR characteristics of AVP to those of [8-lysine]vasopressin (LVP) and oxytocin (OT). Although there is extensive overlap of resonances of $C^\beta$ protons even at 360 MHz, all of the chemical shifts of these protons and most of the couplings between them and their vicinal $C^\alpha$ protons could be determined, at least to a first approximation. It was concluded that the cyclic moieties (residues 1–6) of AVP, LVP, and OT possess essentially the same overall backbone conformation, and that the side-chain conformation—or rotamer populations—about the $C^\alpha$–$C^\beta$ bonds of the cystyl residue (positions 1 and 6), the tyrosyl residue (position 2), and the asparaginyl residue (position 5) are similar. This study indicates that selective replacements of $C^\beta$ protons by deuterons are necessary to improve the accuracy of coupling constants extracted from 360-MHz spectra of AVP for use in conformational analysis.

## Introduction

The primary structures of some of the naturally occurring neurohypophyseal hormones are shown in Figure 1. The solution conformations of these peptide hormones and their analogues in a variety of solvents have been studied by NMR spectroscopy.[2]

The neurohypophyseal hormone arginine vasopressin (AVP)[3] has been studied by $^{13}C$ NMR in both $(CD_3)_2SO$ and $H_2O$[4,5] and by $^1H$ NMR in $(CD_3)_2SO$[6] and $D_2O$.[7] In a previous study we reported a comparison of the chemical shifts of the amide protons, the temperature dependencies of these shifts, the exchange rates of these protons for deuterons, and the coupling constants between vicinal amide and $C^\alpha$ protons of AVP, LVP, arginine vasotocin (AVT), and oxytocin (OT) in $(CD_3)_2SO$ by $^1H$ NMR spectroscopy and concluded that the backbone conformations of these peptides are, to a first approximation, similar.[6] A comparison of these characteristics for these peptides in aqueous solution by $^1H$ NMR spectroscopy has not been reported. Although few studies of AVP have appeared in the literature,[4-7] several studies of LVP by $^1H$ NMR in $(CD_3)_2SO$[8,9] and aqueous solution[9,10] and by $^{13}C$ NMR in both $(CD_3)_2SO$ and $H_2O$[4,11]—as well as several studies of desaminolysine vasopressin (dLVP) by $^1H$ NMR in $(CD_3)_2SO$[12] and aqueous solution[13]—have been reported.

Analysis of the $C^\beta$ proton region of OT in $D_2O$ to yield couplings between vicinal $C^\alpha$ and $C^\beta$ protons provides information on the conformations about the $C^\alpha$–$C^\beta$ bonds ($\chi^1$ values) of some of the amino acid residues.[14-17] A similar analysis of this region of AVP in $D_2O$ is complicated by a high degree of overlap of $^1H$ resonances in this region.[18] For example, the resonances of the $C^\beta$ protons of five of the eight residues that have side chains and those of the $C^\delta$ protons of $Arg^8$ overlap between the narrowly spaced limits of 2.8 and 3.5 ppm —i.e., 37 distinct lines are expected to be observed within a region of only 0.7 ppm. This region is of particular interest to us because it contains information on the conformation of the disulfide bridge.

The first step in an NMR analysis is the assignment of resonances. Here we report the use of isotopic isomers (isotopomers)[19] of AVP that contain specific $C^\alpha$ or $C^\beta$ protons replaced by deuterons to obtain assignments in $D_2O$ for resonances of $C^\beta$ protons in the region between 2.8 and 3.5 ppm and for those of the corresponding vicinal $C^\alpha$ protons. Assignment of resonances for half-$Cys^1$, $Tyr^2$, and $Phe^3$ are unequivocal, and those for $Asn^5$ and half-$Cys^6$ are rigorously justified. We also compare certain $^1H$ NMR characteristics of AVP, LVP, and OT and interpret these characteristics in conformational terms. We conclude that these three peptides have similar backbone conformations and that side-chain conformations—or rotamer populations—of residues in the cyclic moieties of AVP and OT are similar, with the exception of residue 3 (because of the difference in primary structure at this position) and possibly $Gln^4$ (which could not be analyzed in detail with the set of isotopomers used in this study).

## Materials and Methods

**Synthesis of Peptides.** Unenriched [8-arginine]vasopressin (AVP) was synthesized by the solid-phase method reported by Live et al.,[20]

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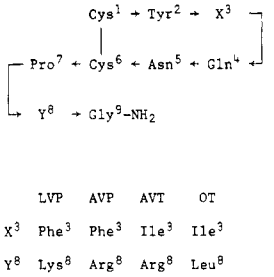


| | LVP | AVP | AVT | OT |
|---|---|---|---|---|
| $X^3$ | $Phe^3$ | $Phe^3$ | $Ile^3$ | $Ile^3$ |
| $Y^8$ | $Lys^8$ | $Arg^8$ | $Arg^8$ | $Leu^8$ |

**Figure 1.** The primary structures of the naturally occurring neurohypophyseal hormones lysine vasopressin (LVP), arginine vasopressin (AVP), arginine vasotocin (AVT), and oxytocin (OT). These peptides are comprised of a cyclic or ring moiety (residues 1-6) and a linear or tail moiety (residues 7-9).

and the isotopic isomers of AVP used in this study—viz., [1-hemi-[$\beta,\beta$-$^2H_2$]cystine,8-arginine]vasopressin (AVP I), [1-hemi[$\alpha$-$^2H_1$]-cystine,8-arginine]vasopressin (AVP II), [2-[$\alpha,\beta,\beta$-$^2H_3$]tyrosine,8-arginine]vasopressin (AVP III), and [3-[$\alpha$-$^2H_1$]phenylalanine,8-arginine]vasopressin (AVP IV)—were synthesized by the solid-phase method reported by Yamamoto et al.[21]

**Preparation of Samples.** A sample of AVP was prepared by dissolving 10 mg of its diacetate salt in ~10 mL of $D_2O$ to replace exchangeable protons by deuterons. After lyophilization, the peptide was again dissolved in ~10 mL of $D_2O$. After a second lyophilization, the peptide was dissolved in 0.7 mL of $D_2O$. After each of these three dissolutions, the pD was adjusted to 3.8 with $CD_3COOD$. The pD was determined by adding 0.4 to the reading from a pH electrode that had been calibrated with protic aqueous standards.[22] All $D_2O$ was 99.8% $^2H$ (Aldrich Chemical Co., Milwaukee, Wis.). Samples of the isotopomers of AVP, also initially present as their diacetate salts, were prepared at identical concentrations in a similar manner. Samples were placed in Wilmad no. 528-PP NMR tubes with an o.d. of 5 mm.

**$^1H$ NMR Spectra.** All $^1H$ NMR spectra were obtained at 360 MHz in the pulse and fast Fourier transform mode on a Bruker WH-360 spectrometer located in the Biology Department of Brookhaven National Laboratory, Upton, N.Y., and equipped with a Nicolet 1180 computer. A 9.0-$\mu$s pulse width was used for a pulse angle of 90°. One thousand scans of each sample were accumulated in 8192 20-bit words of computer memory. The spectrometer was operated in the quadrature mode,[23] with the carrier frequency set on the resonance of residual HDO and with a total spectral bandwidth of 4 kHz. No additional delay was imposed between scans, so the total time of accumulation for each scan was 1.024 s.

The discrete numerical representation of each free-induction decay (fid) was punched onto paper tape at Brookhaven National Laboratory, and this tape was read into the Nicolet 1080 computer located at the Physical Biochemistry Laboratory of the Rockefeller University, where all processing of data was performed. A spectrum generated by Fourier transformation occupied 4096 20-bit words of computer memory. Unenhanced spectra were obtained by transformation of an fid to which an exponential window equivalent to a line broadening of 0.3 Hz in the frequency domain had been applied. Resolution-enhanced spectra were obtained by use of optimal linear filtering;[24] the shape of a Fourier-transformed input line before filtering was assumed to be Lorentzian with a full width at half-height of 3 Hz; a value of $Q$ of 10 000 was used.

**Spectral Analysis.** To compensate partially for the limited number of computer memory locations, peak positions were determined by a three-point interpolation of a Lorentzian function.[25] Analysis and simulation were performed by an implementation of LAOCN3.[26] All positions of resonances and chemical shifts are reported downfield with respect to sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP), which was used as an internal standard in unenriched AVP.

**Spectral Assignments.** The resonances of the $C^\beta$ protons of the cystyl, asparaginyl, and aromatic residues were assumed to lie between 2.5 and 3.6 ppm.[2a,27,28] Vicinally coupled $C^\alpha$ and $C^\beta$ protons of these residues were identified by the technique of total spin decoupling, in which the perturbing frequency was applied to the resonances of the $C^\alpha$ protons. The resonances of the $C^\delta$ protons of the arginyl residue, which also fall in this region, were identified by decoupling of the vicinal $C^\gamma$ protons, which in turn were assigned on the basis of their

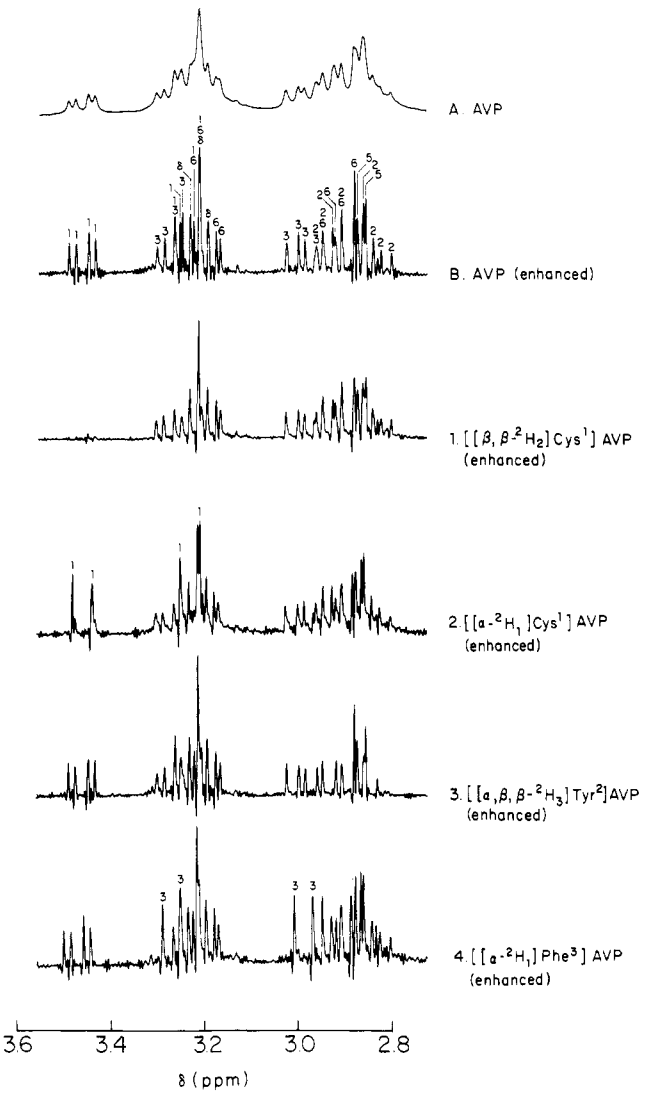


**Figure 2.** 360-MHz $^1H$ NMR spectra of the region between 2.7 and 3.6 ppm for AVP and selected isotopomers of AVP at pD 3.8 and 20 °C. Spectra A and B are the unenhanced and enhanced spectra, respectively, of unenriched AVP, and spectra 1-4 are the enhanced spectra of isotopomers AVP I-IV, respectively. All positions of resonances are downfield with respect to TSP, which was used as an internal standard in unenriched AVP. Numbers are used to label resonances of $C^\beta$ protons and correspond to the position of the residue in the peptide, and $\delta$ is used to label the resonances of the $C^\delta$ protons of $Arg^8$. See Materials and Methods for details relating to the acquisition and processing of data used to generate these spectra.

expected chemical shift[2a,27,28] and by further decoupling within this residue.

In the isotopomers AVP I and III the resonances of selected $C^\beta$ protons were eliminated by replacement of these protons by deuterons, and in AVP II and IV the multiplet pattern of the resonances of the selected pair of $C^\beta$ protons was changed from an octet to a quartet by replacement of the vicinal $C^\alpha$ proton by a deuteron. Unequivocal assignments of the resonances of these $C^\beta$ protons in AVP were made by comparison of the region between 2.8 and 3.5 ppm of the spectrum for AVP with that for each of the four isotopomers. This region was drawn for AVP and each of its isotopomers on separate pieces of translucent paper at identical scales of ~5 Hz/cm, and comparisons between these regions were made visually.

## Results and Discussion

**Assignments of $^1H$ Resonances in the Region between 2.8 and 3.5 ppm.** Figure 2 shows the region of the 360-MHz spectrum between 2.8 and 3.5 ppm for AVP and four isotopomers of AVP. The unequivocal assignments of the $C^\beta$ proton

**Table I.** Assignments of $^1H$ Resonances of Arginine Vasopressin in $D_2O$ at pD 3.8 and 20 °C Observed at 360 MHz in the Region between 2.8 and 3.5 ppm[a]

| resonance[b] | position, Hz[c] | residue | type of proton[d] |
|---|---|---|---|
| | | D | |
| 1 | 1259.5 | half-Cys$^1$ | $\beta$D |
| 2 | 1254.0 | half-Cys$^1$ | $\beta$D |
| 3 | 1244.4 | half-Cys$^1$ | $\beta$D |
| 4 | 1239.3 | half-Cys$^1$ | $\beta$D |
| | | M | |
| 5 | 1191.6 | Phe$^3$ | $\beta$D |
| 6 | 1186.0 | Phe$^3$ | $\beta$D |
| 7, 8 [e] | ~1177-1178 | [f] half-Cys$^1$ | $\beta$U |
| | | [f] Phe$^3$ | $\beta$D |
| 9, 10 [e] | ~1172-1173 | [f] half-Cys$^1$ | $\beta$U |
| | | [f] Phe$^3$ | $\beta$D |
| 11 | 1165.5 | Arg$^8$ | $\delta$D and $\delta$U[g] |
| 12, 13 [e] | ~1160-1163 | [f] half-Cys$^1$ | $\beta$U |
| | | [f] half-Cys$^6$ | $\beta$D |
| 14, 15, 16 [e] | ~1157-1159 | [f] half-Cys$^1$ | $\beta$U |
| | | [f] half-Cys$^6$ | $\beta$D |
| | | [f] Arg$^8$ | $\delta$D and $\delta$U[g] |
| 17 | 1151.9 | Arg$^8$ | $\delta$D and $\delta$U[g] |
| 18 | 1145.7 | half-Cys$^6$ | $\beta$D |
| 19 | 1142.7 | half-Cys$^6$ | $\beta$D |
| | | U | |
| 20 | 1091.8 | Phe$^3$ | $\beta$U |
| 21 | 1081.8 | Phe$^3$ | $\beta$U |
| 22 | 1077.2 | Phe$^3$ | $\beta$U |
| 23, 24 [e] | ~1067-1070 | [h] Tyr$^2$ | $\beta$D |
| | | [h] Phe$^3$ | $\beta$U |
| 25, 26 [e] | ~1062-1063 | [h] half-Cys$^6$ | $\beta$U |
| | | [h] Tyr$^2$ | $\beta$D |
| 27 | 1055.6 | Tyr$^2$ | $\beta$D |
| 28 | 1053.3 | half-Cys$^6$ | $\beta$U |
| 29, 30 [e] | ~1047-1049 | [h] half-Cys$^6$ | $\beta$U |
| | | [h] Tyr$^2$ | $\beta$D |
| 31 | 1039.1 | half-Cys$^6$ | $\beta$U |
| 32 | 1036.2 | Asn$^5$ | $\beta$D and $\beta$U[i] |
| 33 | 1032.6 | Tyr$^2$ | $\beta$U |
| 34 | 1029.4 | Asn$^5$ | $\beta$D and $\beta$U[i] |
| 35 | 1024.7 | Tyr$^2$ | $\beta$U |
| 36 | 1018.4 | Tyr$^2$ | $\beta$U |
| 37 | 1010.4 | Tyr$^2$ | $\beta$U |

[a] Subregions D, M, and U correspond to the separated downfield, middle-field, and upfield regions, respectively. [b] Resonances are numbered in order of spectral position from downfield to upfield. [c] Position is downfield relative to TSP as an internal standard; positions were determined from the enhanced spectrum of unenriched AVP. [d] The stereochemical assignments of the C$^\beta$ protons with relative downfield ($\beta$D) and upfield ($\beta$U) chemical shifts in the same residue are not known. [e] These resonances overlap and could not be separated by resolution enhancement. [f] Order of these overlapping resonances is not known. [g] There is no detectable difference in chemical shift of the two C$^\delta$ protons ($\delta$D and $\delta$U) of Arg$^8$; the deceptively simple triplet pattern of these resonances results from coupling to the two vicinal C$^\gamma$ protons. [h] Order of these overlapping resonances is probably as shown. [i] There is no detectable difference in chemical shift of the two C$^\beta$ protons ($\beta$D and $\beta$U) of Asn$^5$; the deceptively simple doublet pattern of these resonances results from coupling to the vicinal C$^\alpha$ proton.

resonances for half-Cys$^1$, Tyr$^2$, and Phe$^3$ were obtained by comparing the five enhanced spectra shown in this figure. The assignments of the C$^\beta$ protons for Asn$^5$ and half-Cys$^6$ were obtained by decoupling of these protons from their corresponding vicinal C$^\alpha$ protons and by comparison of the NMR characteristics for the C$^\alpha$ and C$^\beta$ protons of these residues with those reported for the corresponding residues in LVP and OT.[10b,14-16,31] The assignments of the resonances of the C$^\delta$ protons for Arg$^8$ were obtained by decoupling from their corresponding vicinal C$^\gamma$ protons at 1.67 ppm, the most upfield protons in AVP, which were assigned on the basis of their chemical shift.[27,28] Table I shows the assignments of the 37 resonances observed in the region between 2.8 and 3.5 ppm.

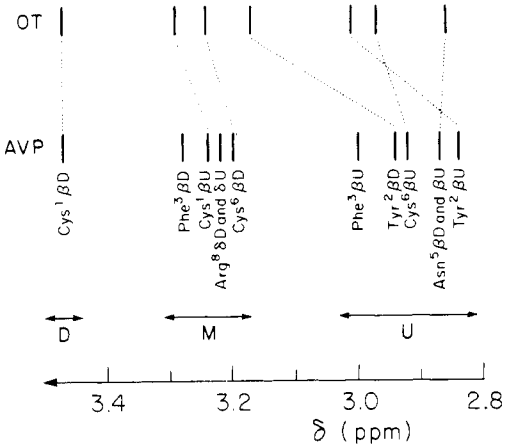


**Figure 3.** Positions of chemical shifts ($\delta$'s) of protons in AVP and OT in $D_2O$ at pD 3.8 and 20 °C between 2.8 and 3.5 ppm. All $\delta$'s are downfield with respect to TSP, which was used as an internal standard.

**Chemical Shifts and Coupling Constants for the Protons Whose Chemical Shifts Are between 2.8 and 3.5 ppm.** Tables II and III show the chemical shifts and coupling constants for the C$^\beta$ protons whose resonances are listed in Table I and some of these values for the corresponding protons of lysine vasopressin (LVP) and oxytocin (OT). The chemical shifts for the corresponding protons of AVP and LVP reported in Table II are quite similar in all cases, but there are several significant differences between the two vasopressins and OT—viz., the chemical shifts of C$^\alpha$ and C$^\beta$ protons of Tyr$^2$ of AVP and LVP are between 0.12 and 0.23 ppm upfield from those of the corresponding protons of OT. These differences are easily seen in Figure 3, which shows chemical shifts of protons in AVP and OT between 2.8 and 3.5 ppm. Also note in Table III that, within experimental error, corresponding coupling constants for C$^\beta$ protons in AVP and OT are identical.

In addition to the C$^\beta$ protons mentioned above, the only other protons whose resonances fall within the region between 2.8 and 3.5 ppm are the C$^\delta$ protons of Arg.[8] No difference in the chemical shifts of the two C$^\delta$ protons of this residue can be detected at 360 MHz, and the average value of this shift is 3.22 ppm. The average coupling constant between the two C$^\gamma$ and the two C$^\delta$ protons is 6.8 ± 0.5 Hz.

In comparing values of the chemical shifts of the C$^\beta$ protons of half-Cys$^1$ and Phe$^3$ that were obtained from analyses of the spectra of the isotopomers AVP II and AVP IV with the corresponding values that were obtained from an analysis of the spectrum for unenriched AVP, it was noted that replacement of a proton by a deuteron in the C$^\alpha$ position led to an upfield shift of approximately 0.01 ppm. This isotope shift is similar to values reported for other vicinal substitutions of $^2H$ for $^1H$.[29]

**Justification of Assignments.** Although the assignments of the resonances of the C$^\alpha$ and C$^\beta$ protons for half-Cys$^1$, Tyr$^2$, and Phe$^3$ are unequivocal, those for Asn$^5$ and half-Cys$^6$ are, in principle, interchangeable because the C$^\alpha$ and C$^\beta$ protons of both of these residues form isolated three-spin systems whose corresponding protons have chemical shifts in the same neighborhood. Tables II and III show that our assignments lead to good agreement between $^1H$ NMR characteristics for these residues in AVP and the corresponding ones in LVP and OT. First, the chemical shifts of the corresponding C$^\alpha$ and C$^\beta$ protons and the coupling constants between various pairs of these protons are quite similar in all three peptides for both

**Table II.** Comparison of Chemical Shifts ($\delta$'s) for Some $C^{\alpha}$ and $C^{\beta}$ Protons of Arginine Vasopressin (AVP), Lysine Vasopressin (LVP), and Oxytocin (OT) in Aqueous Solution[a]

| | $\delta$, ppm[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\alpha$ | | | $\beta$D[c] | | | $\beta$U[c] | | |
| residue | AVP | LVP | OT | AVP | LVP | OT | AVP | LVP | OT |
| half-Cys[1] | 4.30 | 4.27[d] | 4.27 | 3.47 | [e] | 3.47 | 3.24 | [e] | 3.29 |
| Tyr[2] | 4.65 | 4.64 | 4.77[f] | 2.94 | 2.92 | 3.17[f] | 2.84 | [e] | 3.01[f] |
| Phe[3] | 4.46 | 4.47 | [g] | 3.28 | 3.32 | [g] | 3.00 | 2.96 | [g] |
| Asn[5] | 4.79 | 4.79 | 4.74 | 2.87[h] | 2.88[h] | 2.86[h,i] | 2.87[h] | 2.88[h] | 2.86[h,i] |
| half-Cys[6] | 4.91 | 4.88 | 4.88 | 3.20 | 3.20 | 3.24 | 2.92 | 2.90 | 2.97 |

[a] Data for AVP were obtained in $D_2O$ at pD 3.8 and 20 °C. Values for LVP are taken from Table I of Von Dreele et al.;[10c] it is assumed that the solvent was $H_2O$, the pH was 4.6 (unless otherwise noted), and the temperature was 30 °C. Unless otherwise noted, values for OT at 20 °C are calculated from data obtained at pD 3.8 and 25 °C and reported in Table II of Wyssbrod et al.;[16] these values agree with those reported by Meraldi et al.[14] [b] Chemical shifts ($\delta$'s) for AVP and OT are downfield relative to TSP as an internal standard. Values for LVP were originally reported to be downfield relative to an external standard of $(CH_3)_4Si$ in $CCl_4$;[10c] an increment of 0.40 ppm has been applied to the originally reported values to minimize the average difference between chemical shifts of corresponding $C^{\alpha}$ protons in AVP and LVP. [c] See footnote *d*, Table I. [d] This value was not reported, but it was assumed to be the same as that for the $C^{\alpha}$ proton of Lys[8]. [e] This value was not reported. [f] A significant upfield shift is observed in going from OT to AVP and LVP. [g] Ile[3] replaces Phe[3] in OT. [h] There is no detectable difference in $\delta$ of the two $C^{\beta}$ protons of Asn[5] within compounds at 360 MHz. [i] An incorrect value of $\delta$ for the $C^{\beta}$ protons of Asn[5] was reported in Wyssbrod et al.;[16] the corrected value given here agrees with the one reported by Meraldi et al.[14] for OT in $D_2O$ at 28 °C.

**Table III.** Comparison of Geminal and Vicinal Coupling Constants ($^2J$'s and $^3J$'s) for Some $C^{\alpha}$ and $C^{\beta}$ Protons of Arginine Vasopressin (AVP) and Oxytocin (OT) in $D_2O$ at pD 3.8 and 20 °C[a]

| | $J$, Hz[b] | | | | | |
|---|---|---|---|---|---|---|
| | $\alpha$ to $\beta$D[c] | | $\alpha$ to $\beta$U[c] | | $\beta$D to $\beta$U[d] | |
| residue | AVP | OT | AVP | OT | AVP | OT |
| half-Cys[1] | 5.3 (5.3)[f] | 5.4 (5.8)[f] | ~4.6[e] (4.2)[f] | 4.9 (5.4)[f] | −15.0 | −15.0 |
| Tyr[2] | ~7.6[e,g] | 7.0 | 8.0 | 7.8 | −14.2 | −14.2 |
| Phe[3] | 5.5 | [h] | 10.0 | [h] | −14.0 | [h] |
| Asn[5] | [i] | [i] | [i] | [i] | [i] | [i] |
| half-Cys[6] | 3.0 (2.8)[f] | 3.3 (4.0)[f] | 9.8 (10.0)[f] | 9.7 (9.4)[f] | −14.2 | −14.3 |

[a] Values for AVP are calculated from positions of resonances reported in Table I, and, unless otherwise noted, those for OT, from data obtained at 25 °C and reported in Table II of Wyssbrod et al.;[16] the latter values agree well with those reported by Meraldi et al.[14] [b] Values of $^2J$ and $^3J$ are estimated to be within ±0.5 Hz of their true ones for AVP unless otherwise noted and within ±0.3 Hz for OT. [c] See footnote *d*, Table I; couplings are associated as indicated with downfield or upfield $C^{\beta}$ protons. [d] $^2J$ is assumed to be negative [H. J. Bernstein and N. Sheppard, *J. Chem. Phys.*, **37**, 3012 (1962)]. [e] Substantial overlap of resonances in the $C^{\beta}$ proton region prevented a precise analysis (see Table I). [f] These values were reported by Nicholls et al.[7] for AVP at pD 3.8 and at an unspecified temperature; no association of couplings with upfield or downfield $C^{\beta}$ protons was given. [g] This value was determined by subtracting the other value of $^3J$, obtained from the positions of the resonances of the upfield $C^{\beta}$ proton, from the sum of values of $^3J$, obtained from the spacing of the outer resonances of the $C^{\alpha}$ proton, and is estimated to be within ±0.7 Hz of its true one. [h] Ile[3] replaces Phe[3] in OT. [i] Because of the equivalence of the two $C^{\beta}$ protons of Asn[5], values of $^2J$ and individual values of $^3J$ could not be determined; the sum of values of $^3J$ could be determined, however, and is 13.6 ± 0.5 Hz for AVP and, as redetermined by us, 13.5 ± 0.5 Hz for OT.

residues. Second, the two geminal $C^{\beta}$ protons of Asn[5] appear to have the same chemical shift in each of the three peptides, thereby leading to the formation of a deceptively simple spectral pattern by the resonances of these protons.[30]

In some of the original reports of the assignments for the resonances of the $C^{\alpha}$ and $C^{\beta}$ protons of Asn[5] and half-Cys[6] in LVP[10b,c] in $D_2O$, the observed equivalence of two of the $C^{\beta}$ protons and the relatively upfield chemical shift were used as implied bases for assigning these resonances to Asn[5] rather than to half-Cys[6]. Although these arguments were not rigorous, they were highly reasonable and were not subject to serious challenge. In the case of OT, these assignments were rigorously made using the isotopomer [6-hemi[$\alpha,\beta,\beta$-$^2H_3$]cystine]oxytocin,[31] and other isotopomers enriched with $^2H$ and $^{15}N$ at appropriate positions in these residues also have been used to prove that the assignments are indeed unequivocal in OT.[17,31,32] Because of the close agreement in characteristics noted above for AVP, LVP, and OT, the assignments of both the $C^{\alpha}$ and $C^{\beta}$ protons for these residues in the former two peptides can be made with a high degreee of certainty.

**Conformational Interpretation.** $^1H$ NMR characteristics for five of the six amino acid residues that constitute the cyclic moiety of AVP, LVP, and OT are reported in Tables II and III.

The similarity of the chemical shifts ($\delta$'s) of the corresponding $C^{\alpha}$ protons of AVP and OT is compatible with a high degree of similarity in the basic backbone conformations of the cyclic moieties of these two peptides in $D_2O$. A small upfield shift, however, is observed for the $C^{\alpha}$ proton of Tyr[2] in going from OT to AVP (see below).

The similarity in coupling constants between corresponding $C^{\alpha}$ and $C^{\beta}$ protons and in most of the chemical shifts of corresponding $C^{\beta}$ protons of AVP and OT is compatible with similarity in the basic side-chain conformations—or rotamer populations—of residues in the cyclic moieties of these two peptides in $D_2O$, with the exception of residue 3 and possibly Gln[4].

**Cystyl Residue (Positions 1 and 6).** Slight upfield shifts of ~0.05 ppm are observed for three of the four $C^{\beta}$ protons of half-Cys[1] and half-Cys[6] in going from OT to AVP. Whether these slight changes reflect a difference in primary structure or a subtle difference in conformation remains to be determined. Within experimental error, there is no difference in corresponding couplings between vicinal $C^{\alpha}$ and $C^{\beta}$ protons in these two peptides. These data suggest that the conformation of the disulfide bridge is similar in OT and AVP.

**Tyrosyl Residue (Position 2).** The observed upfield shifts of 0.12 ppm for the $C^{\alpha}$ proton and 0.17 and 0.23 ppm for the two $C^{\beta}$ protons of Tyr[2] in going from OT to AVP are probably related to ring-current shifts from the next residue, since Phe[3] replaces Ile[3] in going from the former to the latter peptide. It should also be noted that Deslauriers and Smith[10a] reported that the aromatic $C^{\delta}$ and $C^{\epsilon}$ protons of Tyr[2] are shifted upfield by 0.19 and 0.12 ppm, respectively, in going from OT to LVP and offered these observations as evidence for stacking of the aromatic rings of Tyr[2] and Phe[3] in LVP in aqueous solution (also see Feeney et al.[10b] and Frič et al.[33]). These effects, however, may arise from other causes. For example, Walter

et al.[34] reported that the aromatic $C^\delta$ protons of $Tyr^2$ are shifted upfield by 0.11 ppm in going from OT to [3-D-alanine]oxytocin in $(CD_3)_2SO$. Inasmuch as neither of these peptides has an aromatic residue in the modified position (residue 3), the observed change cannot be ascribed to a ring-current shift.

Walter[35] suggested that the orientation of the side chain of $Tyr^2$ may depend upon the amino acid residue that occupies position 3 in the neurohypophyseal hormones (also see Walter et al.[36]). Our data on couplings between vicinal $C^\alpha$ and $C^\beta$ protons of $Tyr^2$ of AVP and OT in aqueous solution (Table II) indicate that the conformation—or rotamer populations—about the $C^\alpha$-$C^\beta$ bond, the first bond along the side chain, is probably similar in these two peptides. Nevertheless, this finding does not militate against the residue in position 3 affecting the orientation of the side chain of $Tyr^2$ when these peptides are bound to their respective receptors in the target tissues.

**Isoleucyl or Phenylalanyl Residue (Position 3).** Since residue 3 is one of the positions of change in going from OT to AVP and since the side chain of the isoleucyl residue with a single $C^\beta$ proton in OT and the phenylalanyl residue with two $C^\beta$ protons in AVP are quite different, one would not expect the same description of side-chain conformations for position 3 in these two peptides. Data are insufficient to determine whether the side chain of $Phe^3$ manifests rotational isomerism among the three classical staggered states about the $C^\alpha$-$C^\beta$ bond. If it does, then the observed couplings are compatible with relative populations of ~0.68 and ~0.26 for states I and II and of ~0.06 for state III—i.e., if this interpretation is correct, then state III, in which the $C^\gamma$ is gauche to both N′ and C′, is, for all practical purposes, frozen out, and $C^\gamma$ is restricted to spend most of its time in two positions relative to the backbone.[37] Even if isomerism among these three particular states is not manifest, the relatively large values for one of the two vicinal couplings and for the sum of these couplings probably indicate that the conformation about the $C^\alpha$-$C^\beta$ bond is relatively restricted in conformational space. If such a restriction results in the net stacking of the neighboring aromatic rings, then the upfield shifts observed for the $C^\alpha$, $C^\beta$, and aromatic protons of $Tyr^2$ in going from OT to AVP and LVP can be explained.[38]

**Glutaminyl Residue (Position 4).** Since no information was obtained on couplings between vicinal $C^\alpha$ and $C^\beta$ protons or on chemical shifts of the $C^\beta$ protons of $Gln^4$ in AVP, no comparison of the orientation of the side chain of this residue in AVP and OT can be made at this time. It should be noted, however, that Deslauriers et al.[11b] found evidence based on studies of $^{13}C$ spin-lattice relaxation ($T_1$) for the side chain of this residue being more restricted in its mobility in LVP than in OT. A detailed analysis of the $^1H$ resonances for this residue cannot be performed at this time because of the complexity of the five-spin system formed by the $C^\alpha$, $C^\beta$, and $C^\gamma$ protons and because of overlap from $^1H$ resonances from other residues. It would not be surprising, however, if a spectral analysis of the appropriate isotopomers of AVP and OT reveals a difference in the orientation of the side chain of $Gln^4$ in these two peptides.[39]

**Asparaginyl Residue (Position 5).** Individual couplings between vicinal $C^\alpha$ and $C^\beta$ protons of $Asn^5$ cannot be determined from the deceptively simple spectrum resulting from the equivalence of the two geminal $C^\beta$ protons in both AVP and OT. The similarity in the sum of these two couplings and in corresponding chemical shifts of the $C^\alpha$ and $C^\beta$ protons for $Asn^5$, however, suggests that the orientation of the side chain of this residue in these two peptides is similar.

**Linear Moiety (Positions 7–9).** Although this study is concerned primarily with the cyclic moiety (residues 1–6) of AVP, some information on $Arg^8$ in the linear moiety (residues 7–9) was obtained from the region between 2.8 and 3.5 ppm. The values of the chemical shifts of the two equivalent $C^\delta$ protons (3.22 ppm) and the average coupling constant between the two $C^\gamma$ and the two $C^\delta$ protons (6.8 ± 0.5 Hz) for $Arg^8$ of AVP are similar to the corresponding ones for arginine cation[40] and are compatible with a relatively unhindered conformational state of the side chain of this residue—i.e., there is probably free rotation about the $C^\gamma$-$C^\delta$ bond and perhaps about other side-chain bonds as well.

In a previous study Feeney et al.[10b] obtained the $^1H$ NMR spectra of LVP and OT in $H_2O$ and concluded that characteristics of the resonances of the amide protons indicated that these two peptides have similar backbone conformations. Their conclusion is similar to ours, which is based on $^1H$ NMR characteristics of $C^\alpha$ and $C^\beta$ protons.

Although we have emphasized the similarity in the conformations of AVP, LVP, and OT, subtle differences in these conformations should not be ignored. In addition to a mandatory difference in the side-chain conformations of residues 3 and 8 because of differences in primary structure, there may also be differences in the conformations of residues that were not included in this study—viz., $Gln^4$ in the cyclic moiety (as mentioned above) and $Pro^7$ and $Gly^9$-$NH_2$ in the linear moiety. Indeed, on the basis of studies of the spin-lattice relaxation times ($T_1$'s) of $^{13}C^\alpha$ nuclei in LVP and OT in $D_2O$, Deslauriers et al.[11b] concluded that the cyclic moieties of these peptides are of about equal flexibility, but that the linear moiety of LVP is significantly more mobile than that of OT. In addition, Nicholls et al.,[7] in their preliminary report on the comparative study of AVP and OT, emphasize the slight differences in corresponding couplings between $C^\alpha$ and $C^\beta$ protons for the half-cystyl residues in these peptides and argue that these differences in couplings reflect differences in the populations of conformations that contribute to the dynamic states of these peptides. We would agree that small differences in couplings might indicate slight differences in conformations—or populations of conformations—of AVP and OT, but we believe that more accurate determinations of couplings and more careful evaluation of possible conformations are needed before any such conclusion can be made. It should be noted that in a previous study of AVP and OT we reported that there were slight differences in $^1H$ NMR characteristics of these peptides in $(CD_3)_2SO$ and concluded that, although the backbone conformations are, to a first approximation, similar, there is probably a gradual shift in the average conformation in going from OT via AVT to AVP.[6]

**The Problem of Overlap of $^1H$ Resonances of AVP in the Range between 2.8 and 3.5 ppm at 360 MHz.** Figure 3 shows that the chemical shifts of eight $C^\beta$ protons of OT in $D_2O$ at pD 3.8 and 20 °C lie in the 0.7-ppm range between 2.8 and 3.5 ppm. It is not surprising that relatively accurate values of vicinal coupling constants needed for a conformational analysis have been readily extracted from $^1H$ resonances in this range by use of spectrometers operating from 270 to 300 MHz.[14–16] On the other hand, Figure 3 also shows that the chemical shifts of ten $C^\beta$ and two $C^\delta$ protons of AVP in $D_2O$ at pD 3.8 and 20 °C lie in the 0.7-ppm range between 2.8 and 3.5 ppm. Severe overlap of $^1H$ resonances in this range places a limitation on the accuracy of couplings that can be extracted, even when spectra are obtained at 360 MHz and resolution-enhancement techniques are used. Particularly severe at 360 MHz is overlap that obscures the resonances of the upfield $C^\beta$ proton of half-$Cys^1$, the downfield $C^\beta$ proton of $Tyr^2$, the two equivalent $C^\beta$ protons of $Asn^5$, and the upfield $C^\beta$ proton of half-$Cys^6$ (Table I and Figure 2). The first problem cannot be overcome by use of information from resonances of the $C^\alpha$ proton of half-$Cys^1$ because overlap of these resonances by those of the $C^\alpha$ proton of $Arg^8$ prevents their analysis. The second problem can be overcome by use of either resolution enhancement to extract

information from the $C^{\beta}$ proton region of $Asn^5$ or information from resonances of the $C^{\alpha}$ proton of this residue. In general, the third problem cannot be overcome except by use of information from the resonances of the $C^{\alpha}$ proton of half-$Cys^6$. We are, however, able to extract information about the upfield $C^{\beta}$ proton resonances of this residue from the spectrum of AVP III, in which the interfering resonances of the downfield $C^{\beta}$ proton of $Tyr^2$ had been removed.

Table I and Figure 3 show that part of the difficulty that results from overlap in the region between 2.8 and 3.5 ppm can be removed by replacement of the $C^{\beta}$ protons of $Asn^5$ and the $C^{\delta}$ protons of $Arg^8$ by deuterons. Note that resonances from the corresponding residues (positions 5 and 8) do not cause overlap in OT, and consequently, replacement of protons in these residues is not necessary. Whether resolution enhancement can be used to separate resonances in AVP once the above replacements in positions 5 and 8 are made remains to be seen. If not, then it will be necessary also to replace the $C^{\beta}$ protons of either $Phe^3$ or half-$Cys^6$ to solve the first problem, the most difficult of the three problems mentioned above, in which there is overlap of resonances of the upfield $C^{\beta}$ protons of half-$Cys^1$. Another approach to this problem is to replace the $C^{\alpha}$ proton of $Arg^8$ so that information from the resonances of the $C^{\alpha}$ proton of half-$Cys^1$ can be used.

## Conclusion

Overlap of $^1H$ resonances of AVP at pD 3.8 and 20 °C in the range between 2.8 and 3.5 ppm is extensive even at 360 MHz and when resolution-enhancement techniques are used. The extraction of accurate vicinal coupling constants from this region for use in a conformational analysis depends upon the use of selective replacement of protons—whose resonances overlap with resonances whose positions are needed for this analysis—by deuterons. We have used a number of specifically deuterated isotopomers of AVP to make unequivocal assignments of some of the resonances and to determine coupling constants. Information extracted from the 360-MHz $^1H$ NMR spectra of AVP and these isotopomers indicates that, to a first approximation, the backbone conformation of the cyclic moieties of AVP, LVP, and OT are essentially the same in $D_2O$. In addition, the side-chain conformations—or rotamer populations—about the $C^{\alpha}$-$C^{\beta}$ bonds of half-$Cys^1$, $Tyr^2$, $Asn^5$, and half-$Cys^6$ of AVP and OT also appear to be quite similar.

In this study we emphasized the basic similarities in the overall conformational features of AVP and OT in aqueous solution. Future studies will be aimed at detecting subtle differences between these conformations by NMR spectroscopy. Preliminary evidence that such differences exist has been provided by $^{13}C$ NMR studies,[11b,36] which have shown that there are small but detectable differences in chemical shifts and relaxation characteristics of certain corresponding nuclei in these two peptides, and by $^1H$ NMR studies of amide hydrogen exchange,[10d] which suggest that there might be different patterns—or, at least, characteristics—of intramolecular hydrogen bonding in LVP and OT.

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(39) Walter et al. (ref 36) discuss several possibilities for different orientations of the side chain of $Gln^4$ in the various neurohypophyseal hormones.
(40) See Table II of ref 27.

# Molecular Structures of the Dihydrodiols and Diol Epoxides of Carcinogenic Polycyclic Aromatic Hydrocarbons. X-ray Crystallographic and NMR Analysis

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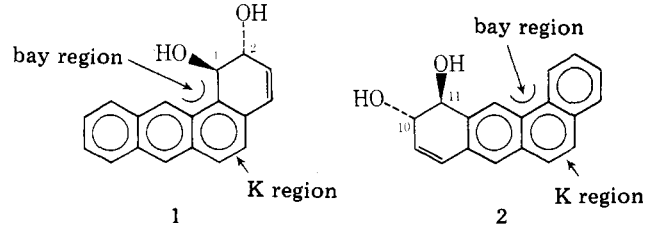
**Abstract:** The molecular dimensions and conformations of the *trans*-1,2- and 10,11-dihydrodiols of benz[*a*]anthracene have been determined from X-ray crystal structure analyses. The cell dimensions are $a = 14.399$ (3) Å, $b = 7.942$ (2) Å, $c = 11.579$ (3) Å, $\beta = 100.93$ (2)°, space group $P2_1/c$ for the 1,2-dihydrodiol, and $a = b = 18.905$ (3), $c = 7.529$ (1) Å, space group $P4_2/n$ for the 10,11-dihydrodiol. The trans hydroxyl groups are axial in the 1,2-dihydrodiol; the bulk of H(12) would be presumed to hinder the formation of the diequatorial conformer of the diol. This steric problem does not exist for the 10,11-dihydrodiol and the hydroxyl groups in the molecule in the crystalline state are diequatorial. NMR analyses in solutions of deuterated chloroform, acetone, or dimethyl sulfoxide give results which indicate that the diaxial conformation also predominates for the 1,2-dihydrodiol in solution. In the case of the less hindered 10,11-dihydrodiol there is an equilibrium of approximately 30% diaxial and 70% diequatorial conformers in solution. The experimentally determined dimensions for these two trans diols, together with previously determined dimensions of arene oxides, have given sufficient data for the calculation of approximate dimensions for the diol epoxides of benz[*a*]anthracene and of benzo[*a*]pyrene which have been implicated as the ultimate carcinogenic metabolites of these hydrocarbons. A characteristic of the covalent bond formed to a biological macromolecule from an atom in the bay region of an activated polycyclic aromatic hydrocarbon is that it is *axial* as a result of steric hindrance.

## Introduction

Trans dihydrodiols are among the principal metabolites of carcinogenic polycyclic arenes in mammalian cells.[2] While the majority undergo detoxification via conjugation and excretion, a significant proportion are metabolically transformed by the microsomal enzymes to diol epoxide derivatives. Many of the latter are highly mutagenic,[3-9] and the "bay region"[10] anti isomeric diol epoxides have been implicated as the principal ultimate active forms of benzo[*a*]pyrene[11] and other carcinogenic hydrocarbons.[6,7,12-15]

The factors which determine whether a particular dihydrodiol will undergo detoxification or activation are at present poorly understood. Since these pathways are likely to be dependent upon the molecular structures of the compounds involved, we have undertaken to investigate the structures of selected dihydrodiol metabolites by X-ray crystallographic analysis. We reported previously[16] the structure of (±)-*cis*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene, a representative "K-region"[10] dihydrodiol. We report here the crystal structures of (±)-*trans*-1,2-dihydro-1,2-dihydroxybenz[*a*]anthracene (**1**) and (±)-*trans*-10,11-dihydro-10,11-dihydroxybenz[*a*]anthracene (**2**), representative




non-K-region dihydrodiols in bay region and non-bay-region benzo rings, respectively. Although satisfactory crystals of the diol epoxides themselves could not be obtained, the analysis described herein provides three-dimensional coordinates from which it has proven possible to derive coordinates for the cor-

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