^{13}C Nuclear Magnetic Resonance Studies of the Peptide Hormones Oxytocin, Arginine Vasopressin, Isotocin, Mesotocin, Glumitocin, Aspartocin, Related Analogues, and Diastereoisomers. Use of Specifically Deuterated Hormone Derivatives for Assignments and Effects of Structural Changes on ^{13}C NMR Chemical Shifts in Peptides

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Abstract: The ^{13}C nuclear magnetic resonance (NMR) spectra of the naturally occurring peptide hormones oxytocin, arginine vasopressin (AVP), mesotocin, isotocin, aspartocin, and glumitocin were compared. Oxytocin derivatives specifically deuterated in the Half-Cys-1, Tyr-2, Ile-3, Half-Cys-6, Pro-7, or Gly-NH_2-9 positions were used to make unequivocal assignments of most of the α and β carbon atoms, and to sort out differences in assignments previously reported. Arginine vasopressin derivatives, specifically deuterated in the Half-Cys-1, Tyr-2, Phe-3, Half-Cys-6, or Gly-NH_2-9 positions, also were used for unequivocal assignments. The chemical shifts of invariant residues in these compounds were virtually unchanged from their positions in oxytocin despite the structural changes at positions 8 and/or 4, and differences in biological activities. Analogues with L-amino acid substitutions in the 1 position [(1-penicillamine]oxytocin), 3 position ([Phe^3]oxytocin), 4 position ([Leu^4]oxytocin), and 2 and 4 positions ([Leu^2,Leu^4]oxytocin, [Ile^2,Leu^4]oxytocin, and [Phe^2,Leu^4]oxytocin), also generally showed only minor ^{13}C NMR chemical shifts at invariant residues, though there were a few notable exceptions. An interesting observation was that except for the half-cystine residues, the Cα ^{13}C chemical shifts of L-amino acid residues were essentially the same whatever their sequence position in the 20-membered disulfide ring moiety of these peptides. However, there were large ^{13}C chemical shift differences (1.4–1.9 ppm) for the Cα of equivalent L-amino acids in the same molecule, when one residue was in the 20-membered ring moiety, and the other in the acyclic tripeptide moiety of the hormones. The α chemical shift of residues in the acyclic portion of the molecule were always upfield to those in the ring moiety. When D-half-cystine (positions 1 and 6) or D-tyrosine (position 2) were substituted into oxytocin or AVP, the ^{13}C NMR spectra of the diastereoisomeric peptide hormones often showed significant chemical shift perturbations even for residues quite remote from the substitution position. The similarities and differences of ^{13}C chemical shifts are briefly discussed in terms of the conformational and biological properties of these peptides.

The study of ^{13}C nuclear magnetic resonance (NMR) spectra of small (3–15 residues), biologically active polypeptides has grown steadily recently, and several studies have been directed to correlating chemical shift and relaxation (especially T_1) data to conformational and dynamic properties of these molecules in solution.[1-5] When natural abundance compounds are used, the proper assignment of NMR peaks to particular carbon atoms in specific amino acid residues is of critical importance in interpretation of data. For peptides with more than four residues, assignments are usually made by reference to chemical shifts found in smaller peptides. Summaries of such reference data such as is found in Figure 6 of ref 6 are very useful, and can be used in conjunction with the effects of pH on chemical shifts in terminal residues or in certain acidic and basic side-chain residues, etc., for NMR assignments. However, even in small peptides problems of unambiguous assignments can arise owing to the similarity of chemical shifts on carbons on different amino acid residues, specific effects due to sequence, structure, and conformation, etc.

The neurohypophyseal hormones oxytocin, H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2, and arginine vasopressin (AVP), H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2, and a few analogues have been examined in aqueous solution by ^{13}C NMR techniques by several groups,[7-10] and from these studies some tentative conclusions regarding the relationship of these data to biological function have been given.[7-9] In this paper a large number of specifically deuterated derivatives of oxytocin were used to unambiguously establish ^{13}C chemical shifts and to sort out the differences in assignments which have been reported for this hormone.[7a,8] Similar specifically deuterated arginine vasopressin derivatives have also been utilized to establish assignments for this compound. The assignments we obtain generally agree with those of Walter et al.[7a] The ^{13}C spectra of a number of other naturally occurring neurohypophyseal hormones[11] whose ^{13}C NMR spectra have not been reported were then examined. In general, their ^{13}C chemical shifts are nearly identical with those of oxytocin except for the specific residue changes. A number of oxytocin inhibitors and oxytocin and AVP diastereoisomers also were examined. The inhibitor analogues examined showed a few changes in the ^{13}C NMR chemical shifts (in addition to changes from residue replacements), and in some cases significant changes were observed. With respect to the oxytocin and AVP diastereoisomers, however, in many cases the chemical shift changes were extensive, both with respect to specifically modified residues as well as other parts of the molecule. These latter changes could not be accounted for by proximal structural changes, but appear to reflect specific conformational factors. Finally a few suggestions regarding the possible relationship of these findings to the biological activities of these compounds are made.

Experimental Section

Analytical Methods. Thin layer chromatography (TLC) was done on silica gel G plates using the following solvent systems: (A) 1-butanol–acetic acid–water (4:1:5, upper phase only); (B) 1-butanol–acetic acid–pyridine–water (15:3:10:12); (C) 1-pentanol–acetic acid–water (7:7:6); (D) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Amino acids and peptides were detected by ultraviolet light, iodine vapors, ninhydrin, and fluorescamine. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Amino acid analyses were obtained by the method of Spackman, Stein, and Moore[12] on a Beckman 120C amino acid

0002-7863/79/1501-0202$01.00/0

analyzer after hydrolysis in 6 N HCl for 22 h. Optical rotation values were measured at the mercury green line (547 nm) using a Zeiss Old 4 polarimeter. Elemental analyses were performed by Spang Microanalytical Laboratory or Chemalytics, Inc., and deuterium analyses were performed by Joseph Nemeth, Urbana, Ill.

Materials. In addition to the peptide and amino derivatives whose syntheses are reported here, the following peptide hormone derivatives and analogues were prepared and purified as follows: [9-[α,α-$^2H_2$]-glycinamide]oxytocin according to Glasel et al.;[13] [1-hemi[α,β,β-$^2H_3$]cystine]oxytocin by the method of Spatola et al.;[14] [6-hemi[α-^2H]cystine]oxytocin, [6-hemi[β,β-$^2H_2$]cystine]oxytocin, and [6-hemi-D-[α-^2H]cystine]oxytocin according to Upson and Hruby;[15] [1-hemi[α-^2H]cystine]oxytocin and [1-hemi-D-[α-^2H]cystine]oxytocin according to Hruby, Upson, and Agarwal;[16] [1-hemi[α-^2H]-cystine, 8-arginine]vasopressin, [1-hemi-D-[α-^2H]cystine, 8-arginine]vasopressin, [1-hemi[β,β-$^2H_2$]cystine, 8-arginine]vasopressin, [2-[α-^2H]tyrosine, 8-arginine]vasopressin, [2-D-[α-^2H]tyrosine, 8-arginine]vasopressin, [2-[α,β,β-$^2H_3$]tyrosine, 8-arginine]vasopressin, [3-[α-^2H]phenylalanine, 8-arginine]vasopressin, and [9-[α,α-$^2H_2$]glycinamide, 8-arginine]vasopressin by the method of Yamamoto et al.;[17] mesotocin and isotocin by the method of Hruby et al.;[18] aspartocin by Spatola et al. (unpublished); glumitocin by the method of Gitu;[19] oxypressin by published procedures;[20] [desglutamine^4]oxytocin by the method of Upson;[21] [4-leucine]oxytocin as previously reported;[22] [2,4-dileucine]oxytocin by the procedures of Hruby and Chan;[23] and [2-isoleucine, 4-leucine]oxytocin and [2-phenylalanine, 4-leucine]oxytocin as previously reported.[24]

Boc-DL-[α-^2H]Tyrosine and Boc-[β,β-$^2H_2$]tyrosine were prepared as previously reported.[17] Boc-*S*-3,4-dimethylbenzylcysteine was prepared according to the method of Smith.[25] Other Boc amino acid derivatives were purchased from Vega-Fox Biochemicals or Biosynthetica. The polystyrene resin 1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.07 mmol/g resin was purchased from Lab Systems, Inc., San Mateo, Calif. Solvents were purified for partition chromatography as previously described.[26]

Boc-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]Proline. A sample of 0.600 g (4.9 mmol) of [$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]proline provided by Professor A. T. Blomquist, Cornell University, was treated with 0.70 g (5.2 mmol) of *tert*-butyl azidoformate at pH 8.6 in 20 mL of dioxane–water (1:1) according to the method of Schnabel[27] using a Radiometer Autoburette. After the usual workup and recrystallization from ethyl acetate–hexane there was obtained 0.87 g (83%) of the title compound, mp 133–135 °C (lit. mp of unlabeled Boc-proline, 134–136 °C). On TLC in the solvent systems A, B, and C, a single spot was observed identical with authentic Boc-proline. Proton magnetic resonance indicated deuterium substitution in the labeled positions to be >95%.

[7-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]Proline]oxytocin. The solid-phase method[28] was used to synthesize the protected nonapeptide precursor, H-Cys(PMB)-Tyr(Bzl)-Ile-Gln-Asn-Cys(PMB)-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]-Pro-Leu-Gly-$NH_2$. The synthesis was performed on the Vega Model 95 synthesizer, an automated machine similar to that previously described.[29] The polymer support used was chloromethylated polystyrene, 1% cross-linked with divinylbenzene (Cl substitution = 1.07 mmol/g resin) which had been substituted with Boc-glycine as previously reported[14] to a glycine level of 0.38 mmol/g resin as measured by the modified[31] aldimine test.[32] Boc amino acids were used throughout. The sulfhydryl group of cysteine was *p*-methoxylbenzyl protected, and the tyrosine phenol group was benzyl protected. The procedures for deprotection of Boc protecting groups, neutralization, and coupling of amino acid residues to the growing peptide chain closely followed those previously used.[14,15] The exception was the coupling of Boc-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]proline to H-Leu-Gly-O-resin. In this case a single 3-h coupling using a 1.25 molar excess of the deuterated proline derivative and dicyclohexylcarbodimide (DCC) followed by a second coupling using 0.5 mol of each reagent was sufficient to obtain complete coupling as determined by the ninhydrin test.[32] From 3.0 g (1.1 mmol) of Boc-glycine-O-resin there was obtained 4.2 g of H-Cys(PMB)-Tyr(Bzl)-Ile-Gln-Asn-Cys(PMB)-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]Pro-Leu-Gly-O-resin. The peptide was cleaved from the resin by ammonolysis in anhydrous methanol saturated with anhydrous ammonia as previously reported.[14,15,33] There was obtained 0.900 g (64%) of Cys(PMB)-Tyr(Bzl)-Ile-Gln-Asn-Cys(PMB)-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]Pro-Leu-Gly-$NH_2$, mp 217–221 °C. A sample of 335 mg (0.24 mmol) of the protected nonapeptide in anhydrous ammonia was treated with Na to remove the protecting groups, and after removal of the ammonia the remaining peptide was converted to the disulfide form by oxidation under nitrogen with $K_3Fe(CN)_6$.[15,34] The product was purified by partition chromatography on Sephadex G-25[35] using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1). The product obtained (R_f 0.23) was further purified by gel filtration on Sephadex G-25 using 0.2 N aqueous acetic acid as eluent solvent. After lyophilization of the major peak there was obtained 84.2 mg (35%) of [7-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]-proline]oxytocin $[\alpha]^{24}_{547}$ −22.5° (*c* 0.5, in HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.01; Glu, 1.00; Pro, 1.02; Gly 0.94; Half-Cys, 1.92; Ile, 1.08; Leu, 1.00; Tyr, 0.91; NH_3, 3.20. TLC in solvent systems A, B, and C gave single uniform spots identical with unlabeled oxytocin. The 300-MHz ^{1}NMR spectrum of the title compound in an aqueous solution was identical with that of oxytocin except for the missing proline-7 residue protons as previously reported.[36] The milk-ejecting activity[37] was found to be identical with that of authentic oxytocin.

[2-DL-[α-^2H]Tyrosine]oxytocin and Separation of the Diastereomers by Partition Chromatography to Give [2-[α-^2H]Tyrosine]oxytocin and [2-D-[α-^2H]Tyrosine]oxytocin. The solid-phase synthesis of the precursor nonapeptide resin to the title compounds was accomplished using procedures similar to those used above. The quantity of Boc-glycine substituted resin used was 2.86 g with an amino acid content of 0.35 mmol/g resin (1.00 mmol). *S*-3,4-Dimethylbenzyl protection was used for cysteine protection, and Boc-Asn and Boc-Gln were coupled as their nitrophenyl esters using *N*-hydroxybenzotriazole catalysis.[15] The coupling procedures were as before. A three-fold excess of amino acid for DCC couplings and a four-fold excess for nitrophenyl ester couplings were used. Boc-DL-[α-^2H]tyrosine[17] was coupled using DCC (threefold excess of each reagent). There was obtained 3.85 g of Cys(DMB)-DL-[α-^2H]Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-O-resin. The peptide was cleaved from the resin using anhydrous ammonia in anhydrous methanol to give 0.879 g (70%) of Cys(DMB)-DL-[α-^2H]Try-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2, mp 218–222 °C. A 280-mg (0.22 mmol) portion of the nonapeptide was treated with sodium in liquid ammonia to remove the protecting groups and oxidized to the disulfide form with $K_3Fe(CN)_6$ to give a crude mixture of [2-DL-[α-^2H]tyrosine]oxytocin. The diastereomers were separated and purified by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1). The diastereoisomers were nicely separated with [2-[α-^2H]tyrosine]oxytocin eluting at R_f 0.24 and [2-D-[α-^2H]tyrosine]oxytocin eluting at R_f 0.39. The former compound was further purified by gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent solvent. There was obtained 45 mg (40%) of [2-[α-^2H]tyrosine]oxytocin, $[\alpha]^{22}_{547}$ −25.3° (*c* 0.5, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 0.96; Glu, 1.02; Pro, 1.04; Gly, 1.01; Half-Cys, 1.88; Ile, 1.00; Leu, 1.02; Tyr, 0.93; NH_3, 3.10. TLC in solvent systems A, B, and C gave single uniform spots identical with those of authentic oxytocin. Milk-ejecting activities[37] were identical with those of authentic oxytocin. The D diastereoisomer [2-D-[α-^2H]tyrosine]oxytocin was further purified by gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent solvent. There was obtained 47 mg (42%, 41% overall yield of both isomers from nonapeptide), $[\alpha]^{22}_{547}$ −83.5° (*c* 0.51, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 0.98; Glu, 1.02; Pro, 1.04; Gly, 0.98; Half-Cys, 1.85; Ile, 1.02; Leu, 1.00; D-Tyr, 0.91; NH_3, 3.20. TLC in solvent systems A, B, and C gave single uniform spots.

[2-DL-[β,β-$^2H_2$]Tyrosine]oxytocin and Separation of the Diastereomers by Partition Chromatography to Give [2-[β,β-$^2H_2$]Tyrosine]-oxytocin and [2-D-[β,β-$^2H_2$]Tyrosine]oxytocin. The solid-phase synthetic procedures used to prepare the protected nonapeptide resin to the title compound were essentially the same as those used to prepare [2-[α-$^2H_1$]tyrosine]oxytocin (vide supra). Boc-DL-[β,β-$^2H_2$]tyrosine[16] was added in a single coupling reaction using a twofold excess of the amino acid derivative and of DCC. After the synthesis there was obtained 3.96 g of Cys(DMB)-DL-[β,β-$^2H_2$]Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-O-resin. The peptide was cleaved from the resin with anhydrous ammonia in anhydrous methanol to give 0.880 g (70%) of Cys(DMB)-DL-[$\beta\beta$-$^2H_2$]Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2, mp 217–221 °C. A 316-mg sample (0.25 mmol) of the peptide was converted to crude [2-DL-[β,β-$^2H_2$]tyrosine]oxytocin as before. The diastereoisomers were separated by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1). The D diastereoisomer derivative eluted at R_f 0.41 and the all-L diastereoisomer

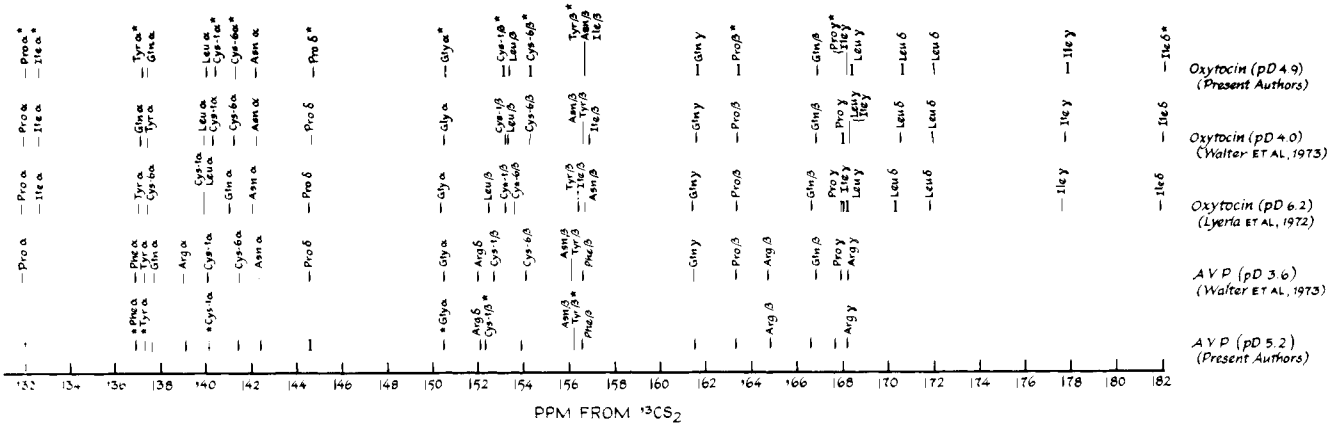

Figure 1. The ^{13}C chemical shift assignments for Cα, Cβ, and other aliphatic carbon atoms in oxytocin as determined by Lyerla and Freedman (ref 8), and in oxytocin and arginine vasopressin (AVP) by Walter et al. (ref 7a), and by this work using ^2H-labeled peptides (the asterisk denotes assignments checked in this work by specifically labeled hormone derivatives). Chemical shifts are given in parts per million upfield from $^{13}CS_2$, and were measured from internal 1,4-dioxane which is 126.1 ppm upfield from $^{13}CS_2$ (42).

derivative at R_f 0.23. After gel filtration on Sephadex G-25 there was obtained 47 mg (37.6%) of [2-[β,β-$^2H_2$]tyrosine]oxytocin, $[\alpha]^{23}_{547}$ −23.8° (*c* 0.51, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.03; Pro, 1.02; Gly, 1.00; Half-Cys, 1.92; Ile, 0.98; Leu, 0.97; Tyr, 1.00, NH_3, 3.10. TLC in solvent systems A, B, and C gave single uniform spots identical with those of authentic oxytocin. Milk-ejecting activities were identical with those of oxytocin. Gel filtration purification on Sephadex G-25 of [2-D-[β,β-$^2H_2$]tyrosine]oxytocin was also performed to give 53 mg (42.4%, 40% overall yield of both diastereoisomers from the nonapeptide), $[\alpha]^{23}_{547}$ −79° (*c* 0.54, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 0.98; Glu, 0.96; Pro, 1.08; Gly, 0.97; Half-Cys, 1.93; Ile, 1.00; Leu, 1.05; Tyr, 1.02; NH_3, 2.90. Thin layer chromatography in solvent systems A, B, and C gave single uniform spots.

^{13}C NMR Spectral Determinations. The ^{13}C NMR spectra were measured on peptide samples of 20–80 mg/mL in D_2O at 22.63 MHz using a Bruker WH-90 FT spectrometer interfaced with a BNC-12 computer, Diablo disk system, and Nicolet 293 controller system. The protons were decoupled with continuous proton noise decoupling at power levels sufficient to completely remove ^{13}C–^1H scalar coupling. The decoupler power and modulation frequency were maintained constant in recording individual spectra.

The FID was stored in 8K memory locations, and generally 5×10^4 scans or more were taken for each spectrum. The FID generally was Fourier transformed to a 6000-Hz sweep width. All measurements were made at a probe temperature of 26 ± 2 °C.

Sample tubes 10 mm in diameter were used and the pH was adjusted using CD_3CO_2D and NaOD. The pH values were determined before and after the ^{13}C NMR experiment with a calibrated Radiometer pH meter, and the values obtained (0.05 pH units) are given as pD (pD = pH + 0.4).[38]

Results

Previously two groups have made assignments of the ^{13}C NMR resonances of oxytocin[7a,8] in aqueous solution and assignments have also been made for arginine vasopressin in this medium.[7a] In the former case there are a number of differences. The correct assignments are critical to interpretation of chemical shift and relaxation data in terms of conformational and dynamic properties of the peptide. Therefore, for the purpose of establishing spectral assignments, specifically deuterium-labeled derivatives of oxytocin and of arginine vasopressin were prepared and used to make assignments. We have previously shown[4a] that deuterated peptides can be used to make unequivocal ^{13}C assignments. As a further aid the correlation table of Bovey[6] was used, and the ^{13}C NMR spectra of other naturally occurring neurohypophyseal hormone derivatives, mesotocin ([Ile[8]]oxytocin),[37] isotocin ([Ser[4], Ile[8]]-oxytocin),[40] glumitocin ([Ser[4], Gln[8]]oxytocin),[41] aspartocin ([Asn[4]]oxytocin), and also oxypressin ([Phe[3]]oxytocin),[20] were examined. The latter compounds have close sequence homology to oxytocin (and vasopressin). The results of these studies are shown in Figure 1, and the chemical shift data are tabulated in Table I. For further confirmation of the arginine vasopressin assignments, the previous ^{13}C NMR studies of Walter et al.[7a] of the naturally occurring neurohypophyseal peptides 8-arginine vasotocin ([Phe[3], Arg[8]]vasopressin) and 8-lysine vasopressin ([Lys[8]]vasopressin)] were valuable. As shown in Figure 1, all the asterisked carbon atoms were unambiguously determined by use of the deuterated analogues. Since we had oxytocin derivatives with deuterium substitutions at the Half-Cys-1 α and β, Tyr, α, β, 3′ and 5′, Ile α, Half-Cys-6 α and β, Pro α, β, γ, and δ, Leu α, and Gly α carbon atoms it was possible to settle all of the previous disagreements in assignment[7a,8] (Figure 1, Table I).

Previous differences in the assignments of the Cα resonances of Half-Cys-6 and Gln-4 had been noted[7a,8] (see Figure 1). Also some ambiguity remained for the Tyr-2 Cα resonance which was reported as upfield to Gln-4 Cα in Table I of Walter et al.,[7a] but indicated as downfield in Figure 2 of the same paper.[7a] Use of [2-[α-^2H]tyrosine]oxytocin clearly showed the Tyr-2 Cα downfield to the glutamine resonance in agreement with Figure 2 in Walter et al.[7a] A ^{13}C NMR spectrum of [6-hemi[α-^2H]cystine]oxytocin showed the Half-Cys-6 Cα to be at 141.4 ppm,[42] in agreement with Walter et al. In addition assignments previously made[7a,8] for the Cα of Half-Cys-1, Ile-3, Pro-7, and Gly-9 were corroborated using specific deuterated derivatives.

In the Cβ region of oxytocin previous assignment differences were noted for the Half-Cys-1 and Leu-8 resonances.[7a,8] Using [1-hemi[α,β,β-$^2H_3$]cystine]oxytocin, the assignments of Walter et al.[7a] were confirmed. In addition the previous assignments for the Tyr-2, Half-Cys-6, and Pro-7 β-carbon resonances were corroborated using deuterated analogues.

In residues with Cγ or further removed carbon atoms, the only difficulties in assignment are attributed to the γ carbons of the Pro-7, Ile-3, and Leu-8 residues all of which have resonances near 168 ppm. These resonances appear as a very closely spaced unysmmetrical doublet or triplet depending upon the pH.[7a,8] By use of [7-[α,β,β,γ,γ,δ,δ-$^2H_2$]proline]-oxytocin it could be shown that the most downfield absorption was due to the Pro-7 γ-carbon resonance. In oxypressin (Table I), which possesses Leu-8 and Pro-7 residues, but has a Phe residue in place of Ile in position 3, both of the γ carbons in Leu-8 and Pro-7 appear at 168.0 ppm, which is slightly downfield of the Cγ of Leu-8 and Ile-3 in oxytocin. On the other hand, in mesotocin, isotocin, and glumitocin (Table I), which possess no Leu-8 residue, but which all contain an Ile-3 residue, the resonance for the Cγ carbons in this region always

Table I. Chemical Shifts and Assignments of C_α, C_β, C_γ, C_δ, and Aromatic Carbon Resonances of Amino Acid Residues of Mesotocin, Isotocin, Glumitocin, Aspartocin, Oxypressin, Oxytocin, and Arginine Vasopressin in D_2O[a]

carbons pD	oxytocin 4.9	[Ile^8]oxytocin (mesotocin) 4.0	[Ser^4,Ile^8]- oxytocin (isotocin) 4.2	[Ser^3,Gln^8]- oxytocin (glumitocin) 4.0	[Asn^4]oxytocin (aspartocin) 3.0	[Phe^3]oxytocin (oxypressin) 3.0	[Arg^8]- vasopressin 5.2
Gly-9 αCH	150.6[b]	150.67	150.66	150.53	150.49	150.53	150.56[b]
Leu-8 αCH	140.07[b]				140.12	140.04	
βCH_2	153.40				153.43	153.13	
γCH	168.34				168.23	168.02	
δCH_3	170.55				170.57	170.48	
δCH_3	171.98				172.13	171.91	
Arg-8 αCH							139.06
βCH_2							164.73
γCH_2							168.28
δCH_2							152.13
ϵC							36.82
Ile-8 αCH		133.96	133.96				
βCH		156.55	156.84				
γCH_2		167.95	168.08				
γCH_3		177.68	178.02				
δCH_3		182.45	182.51				
Gln-8 αCH				139.42			
βCH_2				166.19			
γCH_2				161.58			
Pro-7 αCH	132.07[b]	132.21	132.21	131.82	132.21	132.01	131.98
βCH_2	163.47[b]	163.34	163.46	163.41	163.67	163.40	163.30
γCH_2	168.15[b]	167.95	168.02	167.96	168.23	168.02	167.64
δCH_2	144.75[b]	144.59	144.76	144.75	144.91	144.62	144.56
Half-Cys-6 αCH	141.37[b]	141.37	141.50	141.43	141.62	141.52	141.40
βCH_2	154.44[b]	154.26	154.50	154.50	154.43	153.96	153.94
Asn-5 αCH	142.34	142.35	142.41	142.34	142.46	142.21	142.38
βCH_2	156.71	156.55	156.84	156.90	157.08	156.32	156.32
Asn-4 αCH					142.46		
βCH_2					157.08		
Gln-4 αCH	137.54	137.50				137.27	137.63
βCH_2	166.91	166.65				166.91	166.54
γCH_2	161.65	161.58				161.58	161.65
Ser-4 αCH			135.39	135.40			
βCH_2			132.21	132.27			
Ile-3 αCH	132.72[b]	132.53	132.21	132.27	132.21		
βCH	156.71	156.55	156.84	156.90	157.08		
γCH_2	168.15	167.95	168.08	167.96	168.23		
γCH_3	177.83	177.68	178.02	177.96	178.00		
δCH_3	182.06	182.06	182.21	182.12	182.19		
Phe-3 αCH						137.27	136.92[b]
βCH_2						156.32	156.49
C_1						57.15	56.97
$C_{2,6}$						64.22	63.95
$C_{3,5}$						64.00	63.95
C_4						65.98	65.77
Tyr-2 αCH	137.28[b]	137.28	137.21	137.27	137.60	137.83	137.41[b]
βCH_2	156.71[b]	156.55	156.84	156.32	156.30	156.00	156.32[b]
C_1	65.10	65.20	65.32	65.72	65.60	65.55	65.55
$C_{2,6}$	62.89	62.70	62.67	62.67	62.70	62.45	62.64
$C_{3,5}$	77.26	77.48	77.26	77.40	77.40	77.48	77.21
C_4	39.20	38.80	38.58	39.03	39.10	39.03	38.81
Half-Cys-1 αCH	140.46[b]	140.40	140.33	140.39	140.90	140.04	140.10[b]
βCH_2	153.20[b]	152.97	152.76	153.04	153.13	153.13	152.29[b]

[a] Chemical shifts are reported in parts per million upfield from $^{13}CS_2$ measured from internal 1,4-dioxane (126.1 ppm). On this scale, Me_4Si (external) is 193.7 ppm upfield from $^{13}CS_2$. Conversion to the Me_4Si scale then is $\delta_C = 193.7$ ppm $- \delta_{CS_2, \text{scale}}$ (this table). The chemical shifts are accurate to 0.05 ppm. [b] Assignment check by use of specifically deuterated derivative (see text).

appears at 167.9–168.1 ppm. In all cases this is downfield to the resonances for Ile-3 or Leu-8 in oxytocin. On this basis we tentatively assign the more downfield resonance to the Ile-3 γ carbon and the more upfield resonance to the Leu-8 γ carbon. Owing to the complexity of the off-resonance ^1H decoupled spectrum of oxytocin in this region, it was not possible to use this method to distinguish between these carbons. All of the aromatic carbon assignments correspond to those of Walter et al. and are not repeated here.

The structure of 8-arginine vasopressin (AVP) differs from oxytocin in two respects: a Phe residue replaces Ile at position 3 and an Arg residue replaces Leu at position 8. Previously Walter et al. have assigned the ^{13}C NMR spectra of AVP based on its structural similarity to oxytocin and the close

similarities of their ^{13}C NMR spectra. In the Cα region, the Arg-8 Cα can be readily assigned at 139.1 ppm by reference to Figure 6 in ref 6. The only difficult assignments in this region arise for the Phe-3, Gln-4, and Tyr-2 Cα resonances which occur as a closely spaced triplet near 137 ppm. By utilizing the partially deuterated hormone derivatives, [2-[α-^{2}H]tyrosine]-8-arginine]vasopressin and [3-[α-^{2}H]phenylalanine]-8-arginine]vasopressin, we were able to show unambiguously that the downfield resonance at 136.9 ppm was due to the Phe-3 Cα and the middle resonance at 137.4 ppm was due to the Tyr-2 Cα. By elimination then, the upfield peak was due to the Gln-2 Cα (Table I). All of the other Cα resonances correspond closely to those found in oxytocin, though we did corroborate the Half-Cys-1 resonance by use of [1-hemi[α-^{2}H]cystine]-8-arginine]vasopressin and the Gly-9 resonance by use of [9-[α,α-^{2}H]glycinamide]-8-arginine]vasopressin. In the Cβ region two areas of ambiguity presented themselves. The first, which involved a closely spaced doublet at about 152 ppm was resolved by use of [1-hemi[β,β-$^2H_2$]-cystine]-8-arginine]vasopressin[17] and showed the upfield absorption at pD 5 to be due to that of the Half-Cys-1 Cβ. A more difficult problem was posed by a closely spaced unsymmetrical doublet at about 156.5 ppm (Figure 1) which contained the Tyr-2, Phe-3, and Asn-5 Cβ resonances. Use of the specifically deutered analogue [2-[α,β,β-$^2H_3$]tyrosine]-8-arginine]vasopressin[17] showed the Tyr-2 Cβ to be located at the downfield more intense portion of the doublet. Examination of the oxypressin proton decoupled ^{13}C NMR spectrum did not resolve the remaining assignment unambiguously, since in this case the upfield side of the doublet is more intense. We have tentatively placed Phe-3 Cβ in agreement with Walter et al.[7a] since in arginine vasotocin the Asn-5 and Tyr-2 Cβ chemical shifts are identical, but feel that further confirmatory work is needed. All other AVP assignments of γ and further removed carbon atoms including aromatic carbon atoms were readily made by reference to tables of Bovey[6] and to the spectrum of oxytocin, and are in agreement with those of Walter et al.[7a]

Having settled the assignments for oxytocin and AVP we next turned our attention to the naturally occurring and closely structurally related analogues of oxytocin: mesotocin, which differs from oxytocin only in the replacement of the Leu-8 residue by an Ile; isotocin, which in addition to the position 8 replacement in mesotocin also has a Ser-4 residue in place of a Gln-4 residue; glumitocin, which also has a Ser-4 in place of the Gln-4 residue, and a Gln-8 residue in place of the Leu-residue; and aspartocin, which differs only by an Asn-4 residue in place of the Gln-4 residue found in oxytocin. The ^{13}C NMR spectra of the aliphatic region of three of these compounds are shown in Figure 2. In addition we examined oxypressin ([Phe^3]oxytocin), which possesses the ring structure of vasopressin and the tripeptide side chain of oxytocin, and differs from oxytocin only at position 3 where Phe replaces the Ile residue. The assignments and chemical shifts for the Cα, aliphatic, and aromatic carbons of all of these compounds are given in Table I.

The ^{13}C NMR assignments for these compounds were generally very readily made owing to the remarkable constancy of the chemical shifts of the carbons in the invariant residues (Table I). Chemical shift changes greater than $\pm$0.30 ppm were very rare (Table I), and when changes did occur the shift could be readily observed and the assignment made by inspection. For example, the Ile-3 αCH_3 was found at 182.1 ppm in both oxytocin and mesotocin, and thus the Ile-8 αCH_3 in the latter compound could be assigned to the slightly upfield resonance at 182.5 ppm. In isotocin the Ile-3 moved 0.15 ppm upfield relative to its chemical shift in mesotocin (Table I), and the Ile-8 αCH_3 also moved slightly upfield (0.06 ppm; see Table I). Even residues adjacent to those where substitutions had been made showed little variation. A few exceptions, however, should be noted. The Ile-3 Cα moved downfield 0.51 ppm in isotocin and aspartocin and 0.45 ppm in glumitocin relative to its chemical shift in oxytocin, though none of the other Ile-3 aliphatic carbon resonances were similarly affected, nor were the adjacent Asn-5 Cα and Cβ chemical shifts. Interestingly the Tyr-2 Cβ chemical shift was shifted downfield about 0.41 ppm in glumitocin and aspartocin but no similar effect was seen in isotocin. It is perhaps not surprising that a change of the Leu-8 residue in oxytocin to the Ile-8 in mesotocin and isotocin should have only a minor effect in the chemical shifts of the adjacent Gly-9 and Pro-7 residues. However, even replacement with Gln-8 in glumitocin produced only minor changes with the largest being a 0.25-ppm downfield shift for the Pro-7 Cα. In oxypressin a few more significant chemical shifts were seen relative to oxytocin. For example, the Tyr-2 Cβ was shifted 0.7 ppm downfield and the Tyr-2 Cα 0.55 ppm upfield in oxypressin relative to its position in oxytocin, perhaps as a result of the proximity of the Phe-3 (which replaces Ile-3) and its associated aromatic ring current in the latter compound. On the other hand, the Gln-4 Cα and Cβ carbons are hardly affected with the largest shift being a 0.27-ppm downfield shift for the Cα of Gln-4. However, the Half-Cys-6 and Asn-5 β carbons are shifted downfield 0.5 and 0.4 ppm, respectively, relative to their chemical shifts in oxytocin. It is significant to note that in mesotocin and isotocin the Ile-8 and Ile-3 residues have identical βCH, γCH_2, and γCH_3 chemical shifts and only slightly different δCH_3 ^{13}C chemical shifts (Table I). However, the Cα chemical shifts of Ile-8 and Ile-3 are very different, differing by 1.4 ppm in mesotocin and 1.8 ppm in isotocin. In this regard it is interesting to note that the Gln-8 Cα in glumitocin has a 1.9-ppm chemical shift difference from the Gln-4 Cα in oxytocin and mesotocin; in all of these cases the residue 8 Cα is upfield relative to the residue 3 (or 4) Cα.

We next examined a number of analogues of oxytocin with single or double substitutions in the 2 and 4 positions which we had previously shown to be inhibitors of the neurohypophyseal hormones.[43] These included [Leu^4]oxytocin,[22,43] [Leu^2,Leu^4]oxytocin,[23,43] [Ile^2,Leu^4]oxytocin,[24,43] and [Phe^2,Leu^4]oxytocin.[24,43] In addition we examined [1-penicillamine]oxytocin ([Pen^1]oxytocin).[9] The assignments and chemical shift parameters for these compounds are shown in Table II. In most cases the assignments followed directly from these previously made for oxytocin. For [Leu^2,Leu^4]- and [Ile^2,Leu^4]oxytocin the chemical shifts for all carbons of constant residues were essentially the same ($\pm$0.4 ppm) as those found in oxytocin. The Ile-2 and Ile-3 residues ^{13}C chemical shifts were identical except for a small (0.25 ppm) difference for the Cβ. The Leu-4 Cα was considerably downfield (1.6 ppm) of the Leu-8 Cα in [Ile^2,Leu^4]oxytocin and this was also found to varying degrees in all the other Leu-4 substituted analogues (Table II). The Leu-2 Cα in [Leu^2,Leu^4]-oxytocin was somewhat less downfield (0.32 ppm) relative to the Leu-8 Cα. All other leucine carbon atoms showed either identical or only slightly shifted chemical shifts. In [Leu^4]-oxytocin a few small chemical shift perturbations (0.6–0.8 ppm) relative to the same carbon atoms in oxytocin were observed at Tyr-2 Cα, Asn-5 Cβ, Leu-8 δCH_3, and Ile-3 γCH_3. They were all relative upfield shifts. In [Phe^2,Leu^4]oxytocin, most chemical shifts were similar to those in oxytocin and the other analogues, but significant changes relative to oxytocin were observed, expecially at residue positions 5, 6, and 7. The Pro-7 Cβ and Asn-5 Cα were 1.4 and 1.8 ppm downfield, respectively, of their position in oxytocin, and the Half-Cys-6 Cα and Cβ were about 0.8 ppm downfield. In [Pen^1]oxytocin virtually all the chemical shifts are the same as found in oxytocin for the equivalent residues. The half penicillamine 1 Cα and Cβ resonances were readily assigned by their pH dependence[8a] and by reference to the spectrum of [Pen^1,Leu^2]oxy-

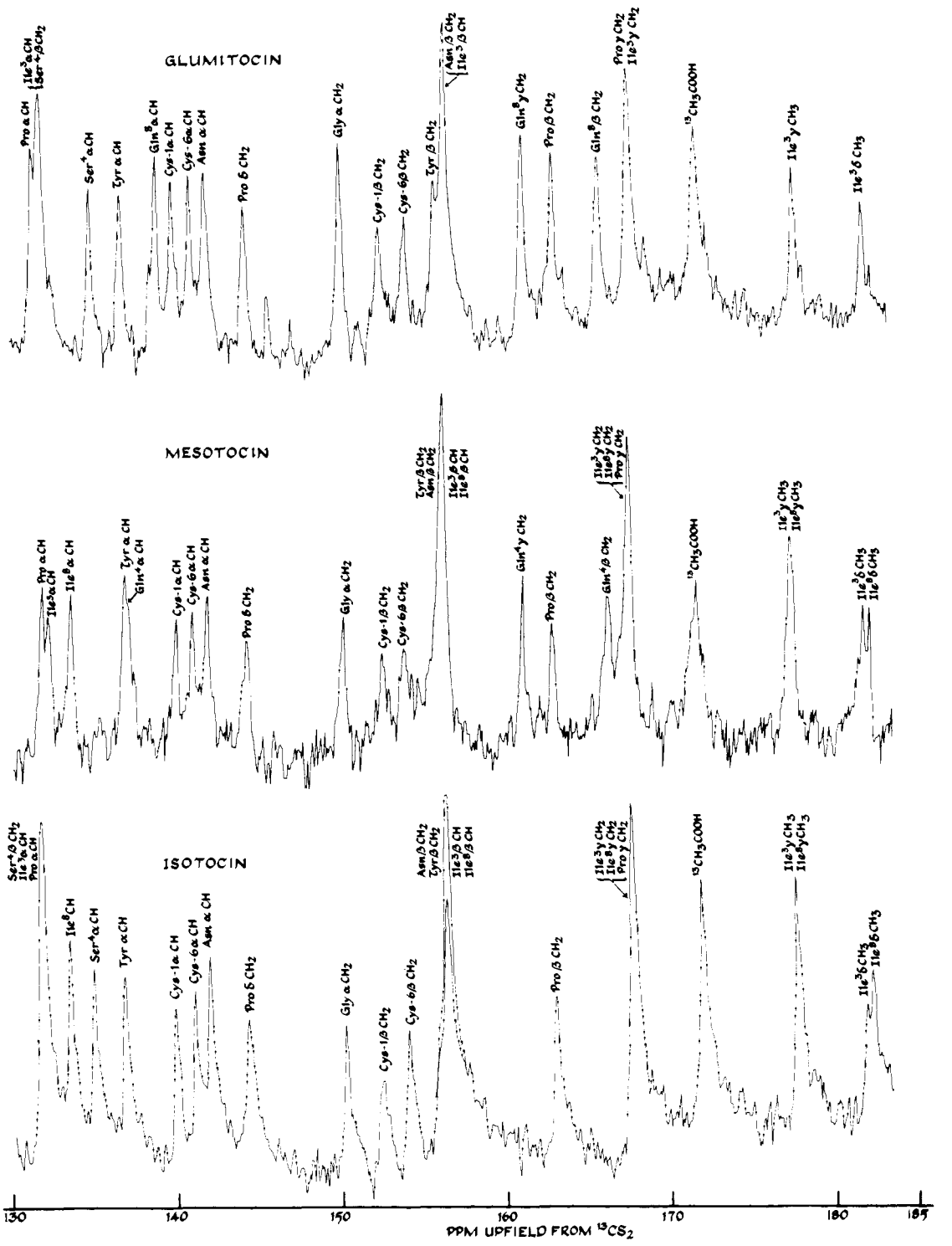

Figure 2. ^{13}C Nuclear magnetic resonance spectrum of the Cα, Cβ, and other aliphatic carbon atoms of the peptide hormones glumitocin, mesotocin, and isotocin. Chemical shifts are in parts per million upfield from $^{13}CS_2$, and were measured from internal 1,4-dioxane which is 126.1 ppm upfield from $^{13}CS_2$ (42).

tocin,[9b] and in both cases these resonances are considerably downfield relative to the comparable Cα and Cβ carbons in Half-Cys-1 in oxytocin (8.8 and 11.1 ppm, respectively). Significant chemical shift differences relative to oxytocin were noted only for the Half-Cys-6 Cα and the Asn-5 Cβ. The resonance at 155.8 ppm was previously assigned[8b] to the Half-Cys-6 Cβ and the downfield resonance at 153.0 ppm to the Asn-5 Cβ, primarily based on the NT_1 for the former resonance which was similar to that of the other aliphatic carbon atoms (mostly Cα) which make up the 20-membered disulfide-containing ring in [Pen^1]oxytocin. This places the Half-Cys-6 Cβ 1.4 ppm upfield from its position in oxyotcin and the Asn-5 Cβ at 3.7 ppm downfield from its position in oxytocin. If the assignments were switched the Half-Cys-5 Cβ would be 1.4 ppm downfield from its position in oxytocin and the Asn-5 Cβ 0.9 ppm downfield from its position in oxytocin. In either case they undoubtedly reflect the conformational differences between oxytocin and [Pen^1]oxytocin.[9]

As previously reported in our studies on the synthesis of specifically deuterated oxytocin[13-16] and arginine vasopressin[17] derivatives, we often prepared and separated diastereomeric analogues of the hormones. We report here the ^{13}C NMR of

Table II. Assignments of ^{13}C Resonances of α, β, γ, and δ and Aromatic Carbon Resonances of Oxytocin, [Pen^1]Oxytocin, [Leu^{2,4}]-Oxytocin, [Leu^4]Oxytocin, [Ile^2,Leu^4]Oxytocin, and [Phe^2,Leu^4]Oxytocin in D_2O[a]

carbons pD	oxytocin 4.9	[Pen^1]-oxytocin 4.3	[Leu^{2,4}]-oxytocin 4.4	[Leu^4]-oxytocin 5.2	[Ile^2,Leu^4]-oxytocin 5.1	[Phe^2,Leu^4]-oxytocin 5.3
Gly-9 αCH_2	150.60	150.50	150.53	150.78	150.60	150.41
Leu-8 αCH	140.07	140.15	139.94	140.14	140.01	140.53
βCH_2	153.40	153.22	153.26	153.54	153.33	153.65
γCH	168.34	167.61	168.26	168.51	168.40	168.21
δCH_3	170.55	171.0	170.85	171.14	170.94	170.49
δCH_3	171.98	172.0	171.98	172.42	172.10	171.92
Pro-7 αCH	132.07	131.65	131.94	132.03	132.08	132.33
βCH_2	163.47	163.35	163.34	163.95	162.93	162.1
γCH_2	168.15	167.6	168.00	168.10	168.12	168.21
δCH_2	144.75	144.7	144.82	145.02	144.81	144.46
Half-Cys-6 αCH	141.37	141.03	140.98	141.33	140.92	140.53
βCH_2	154.44	155.8	154.17	153.70	154.50	153.65
Asn-5 αCH	142.34	142.50	142.21	142.38	142.22	141.53
βCH_2	156.71	153.0	156.29	156.96	156.45	156.71
Gln-4 αCH	137.54	137.44				
βCH_2	166.91	166.72				
γCH_2	161.65	161.60				
Leu-4 αCH			138.44	139.10	138.44	138.30
βCH_2			153.26	153.54	153.02	153.27
γCH			168.00	168.10	168.12	168.21
δCH_3			170.85	171.14	170.94	170.49
δCH_3			171.98	172.40	172.10	171.92
Ile-3 αCH	132.72	133.1	133.44	132.24	133.31	132.60
βCH	156.71	157.4	156.97	157.35	157.23	156.71
γCH_2	168.15	168.0	168.28	168.51	168.40	168.21
γCH_3	177.83	178.24	177.63	178.49	177.63	177.83
δCH_3	182.06	181.66	182.12	182.21	182.25	182.06
Tyr-2 αCH	137.28	137.05		138.05		
βCH_2	156.71	157.0		156.96		
C_1	65.10	65.99		65.55		
$C_{2,6}$	62.89	62.89		62.45		
$C_{3,5}$	77.26	77.48		77.48		
C_4	39.20	39.20		39.2		
Leu-2 αCH			139.62			
βCH_2			152.71			
γCH			168.28			
δCH_3			170.55			
δCH_3			171.58			
Ile-2 αCH					133.31	
βCH					157.23	
γCH_2					168.40	
γCH_3					177.63	
δCH_3					182.25	
Phe-2 αCH						137.93
βCH_2						157.72
C_1						57.15
$C_{2,6}$						64.22
$C_{3,5}$						64.22
C_4						65.55
Half-Cys-1 αCH	140.46		139.94	140.14	140.40	140.53
βCH_2	153.40		153.26	153.54	153.33	153.27
Half-Pen-1 αCH		131.65				
βC		142.13				
$\gamma(CH_3)_2$		167.01				

[a] Chemical shifts are reported in parts per million upfield from $^{13}CS_2$ measured from internal 1,4-dioxane (126.1 ppm). On this scale, Me_4Si (external) is 193.7 ppm upfield from $^{13}CS_2$. Conversion to the Me_4Si scale then is $\delta_C = 193.7$ ppm $- \delta_{CS_2\ scale}$ (this table). The chemical shifts are accurate to 0.05 ppm.

five of these compounds (Table III). The nondeuterated oxytocin derivatives were known previously and their biological activities are reported in Table IV along with those of the other compounds whose ^{13}C NMR spectra are examined here. The vasopressin diastereoisomers are newer compounds and their biological activities are still under investigation.

The assignments of all of the ^{13}C resonances of the five diastereomeric derivatives of oxytocin and two diastereomeric derivatives of vasopressin (Table III) were made by comparison with our previous results with oxytocin, vasopressin, and their analogues. In addition we utilized the results from our previous work with ^{13}C-labeled derivatives of oxytocin and vasopressin[44-47] to establish some of the assignments. There were a number of significant changes in chemical shift, but except for

Table III. Chemical Shifts and Assignments of Carbon Resonances of Amino Acid Residues of Oxytocin, [1-Hemi-D-[α-^{2}H]Cys]oxytocin, [1-Hemi-D-[$\beta\beta$-$^2H_2$]Cys]oxytocin, [2-D-[α-^{2}H]Tyr]Oxytocin, [2-D-[$\beta\beta$-$^2H_2$]Tyr]Oxytocin, [6-Hemi-D-[α-^{2}H]Cys]oxytocin, AVP, [1-Hemi-D-[α-^{2}H]Cys]AVP, and [2-D-[α-^{2}H]Tyr]AVP in D_2O[a]

carbons pD	oxytocin 4.9	[1-hemi-D-[α-^{2}H]Cys]-oxytocin 6.2	[1-hemi-D-[$\beta\beta$-$^2H_2$]-Cys]oxytocin 6.0	2-D-[α-^{2}H]Try]-oxytocin 6.0	[2-D-[$\beta\beta$-$^2H_2$]-Tyr]oxytocin 6.1	[6-hemi-D-[α-^{2}H]Cys]-oxytocin 5.9	AVP 5.2	[1-hemi-D-[α-^{2}H]Cys]-AVP 6.2	2-D-[α-^{2}H]-Tyr]AVP 6.1
Gly-9 αCH	150.60	150.60	150.60	150.52	150.41	150.47	150.56	150.66	150.61
Leu-8 αCH	140.07	140.00	140.07	139.90	139.89	140.13			
βCH_2	153.20	152.74	152.84	153.16	153.15	153.59			
γCH	168.15	168.34	168.01	168.18	167.64	168.21			
δCH_3	170.55	170.55	170.55	170.45	170.12	170.48			
δCH_3	171.98	171.98	172.12	171.82	171.62	172.17			
Arg-8 αCH							139.06	139.16	139.11
βCH_2							164.73	164.83	164.73
γCH_2							168.28	168.28	168.24
δCH_2							152.13	152.16	152.10
ϵC							36.82	37.04	37.08
Pro-7 αCH	132.07	132.07	131.94	132.04	132.11	131.04	131.98	132.14	132.02
βCH_2	163.47	163.34	163.43	163.24	163.14	163.27	163.30	163.27	163.26
γCH_2	168.15	168.08	167.80	168.18	167.64	168.21	167.64	168.28	167.22
δCH_2	144.75	144.75	144.92	144.71	144.88	144.42	144.56	144.68	144.73
Half-Cys-6 αCH	141.37	141.25	141.17	141.61	141.79	140.53[b]	141.40	141.44	141.98
βCH_2	154.44	154.04	154.26	154.53	153.72	153.59	153.94	154.37	153.95
Asn-5 αCH	142.34	142.02	141.02	141.79	141.79	142.21	142.38	142.28	141.98
βCH_2	156.71	156.45	156.45	156.47	156.59	156.58	156.32	155.80	156.90
Gln-4 αCH	137.54	138.25	138.18	139.90	139.58	137.91	137.63	138.12	138.35
βCH_2	166.91	166.61	166.65	165.80	165.61	166.91	166.54	167.17	165.77
γCH_2	161.65	161.39	161.38	161.55	161.46	161.52	161.65	161.39	161.61
Ile-3 αCH	132.72	132.72	132.79	132.70	132.95	133.18			
βCH	156.71	157.36	157.16	157.52	157.48	156.58			
γCH_2	168.34	168.08	168.01	167.88	167.64	168.21			
γCH_3	177.83	177.37	177.65	177.92	177.63	177.76			
δCH_3	182.06	182.31	182.74	182.61	182.43	182.19			
Phe-3 αCH							136.92	136.75	137.63
βCH_2							156.49	157.30	156.90
C_1							56.97	56.53	57.15
$C_{2,6}$							63.95	64.22	64.66
$C_{3,5}$							63.95	64.22	64.22
C_4							65.77	66.21	65.98
Tyr-2 αCH	137.28	136.92	136.82		137.15	137.28	137.41	137.28	
βCH_2	156.71	156.66	156.60	157.00		156.58	156.32	157.30	155.70
C_1	65.10	65.38	65.10	65.67	65.77	65.55	65.55	65.55	65.55
$C_{2,6}$	62.89	62.96	62.45	62.67	62.45	62.68	62.64	62.45	62.89
$C_{3,5}$	77.26	77.48	77.13	77.48	77.48	77.48	77.21	77.70	77.70
C_4	39.20	39.25	38.58	39.24	38.94	39.03	38.81	39.03	39.03
Half-Cys-1 αCH	140.46		140.07	139.90	139.89	140.52	140.10		139.12
βCH_2	153.40	152.74		151.84		153.59	152.29	152.16	152.10

[a] Chemical shifts are reported in parts per million upfield from $^{13}CS_2$ measured from internal 1,4-dioxane at 126.1 ppm from the $^{13}CS_2$ resonance. On this scale, Me_4Si (external) is 193.7 ppm upfield from $^{13}CS_2$. Conversion to the Me_4Si scale then is $\delta_C = 193.7$ ppm $- \delta_{CS_2\ scale}$ (this table). The shifts are accurate to 0.05 ppm. [b] Obtained from ^{13}C NMR spectrum of [6-hemi-D-[β,β-$^2H_2$]Cys]oxytocin at pD 6.0. Otherwise spectra were virtually identical.

a 1-ppm downfield chemical shift for the Pro-7 Cα in [6-hemi-D-cystine]oxytocin, none of these were on the tripeptide side chain residues, which were essentially unchanged throughout. Changes, of course, were seen at the D residues. In the D-Half-Cys-1 derivatives both the α and β ^{13}C resonances are downfield from their positions in oxytocin, but both are quite pH dependent,[45-47] and since they move upfield with an increase in pH, the effects seen in Table III are not as great as seen at identical pH conditions. The other major chemical shift differences seen were at the residues adjacent to the D residues. For example, in [2-D-tyrosine]oxytocin the Half-Cys-1 Cα and Cβ were shifted downfield about 0.6 and 1.6 ppm, respectively, relative to their chemical shift positions in oxytocin. Interestingly the Ile-3 carbon resonances were hardly affected except for the Cβ. In [2-D-tyrosine, 8-arginine]vasopressin, however, the Half-Cys-1 Cα was shifted downfield about 1 ppm, but the Cβ was hardly changed relative to its positions in AVP. On the other hand, the Phe-3 Cα moved upfield about 1 ppm and the Cβ about 0.5 ppm upfield relative to their positions in AVP. Interestingly the largest chemical shift observed was for the Gln-4 Cα in [2-D-tyrosine]oxytocin, which was over 2 ppm *upfield* from its position in oxytocin. Even the Cβ of Gln-4 was about 0.8 ppm *downfield* from its position in oxytocin. In general, however, except for chemical shift changes due to stereochemical changes at adjacent residue, there were only minor shifts. It should be emphasized that for these compounds, however, some of the Cα and Cβ assignments must be viewed as tentative.

Discussion

Although assignment of the ^{13}C NMR spectra of peptides of the structural complexity of oxytocin, arginine vasopressin and its derivatives and analogues to specific carbon atoms of individual residues is often possible by reference to correlation

Table IV. Biological Activities of Oxytocin, Arginine Vasopressin, Mesotocin, Isotocin, Glumitocin, Aspartocin, and Analogues

compd	biological activities, units/mg				
	oxytocin	AVD	pressor	antidiuretic	milk ejecting
oxytocin	546 ± 18[a]	507 ± 23[b]	3.1 ± 0.1[a]	2.7 ± 0.2[a]	410 ± 16[a]
arginine vasopressin	12 ± 0.2[c]	100 ± 15[c]	487 ± 15[c]	501 ± 53[c]	116 ± 19[d] 30 − 120[c]
mesotocin	291 ± 21[e]	502 ± 37[e]	6.3 ± 0.8[e]	1.1 ± 0.1[e]	330 ± 21[e] 471 ± 70[d]
isotocin	145 ± 12[f]	310 ± 15[f]	0.06 ± 0.01[f]	0.18 ± 0.03[f]	219 ± 15[f]
glumitocin	~8[f]				53[f] 81 ± 6[d]
aspartocin	107 ± 29[g]	201 ± 12[g]	0.13 ± 0.03[g]	0.044 ± 0.0005[g]	298 ± 128[g]
oxypressin	20[h]	30[h]	3[h]	30[h]	65[h]
[1-hemi-D-cystine]oxytocin	~1.9 ± 0.1[i]	~0.02[i]	<0.01[i]	~0.02[i]	6.2 ± 0.2[i]
[6-hemi-D-cystine]oxytocin	~0.62[j]	<0.01[i]	anti[j]	<0.002[j]	<0.002[j]
[2-D-tyrosine]oxytocin	6.6 ± 1[k]	34 ± 3[k]	~0.02[k]	~0.02[k]	34 ± 9[k]
[4-leucine]oxytocin	13 ± 1[l]	44 ± 1[l]	weak depressor[l]	natriuretic, diuretic anti-ADH[l]	66 ± 3[l]
[1-penicillamine] oxytocin	inhibitor[m]	inhibitor[m]	nil[m]	nil[m]	1.6 ± 0.2[d] nil[m]
[2,4-dileucine]oxytocin	inhibitor[n]	n.d.[o]	<0.1[n]	diuretic, natriuretic[n,p]	n.d.[o]
[2-isoleucine, 4-leucine]oxytocin	agonist (Ca^{2+}, 1 mM), inhibitor (no Ca^{2+})[q]	n.d.[o]	~0.5[q]	diuretic natriuretic[p,q]	n.d.[o]
[2-phenylalanine, 4-leucine]oxytocin	inhibitor[q]	n.d.[o]	~0.5[q]	diuretic natriuretic[p,q]	n.d.[o]

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tables of the kind compiled by Bovey,[6] and by reference to other small model peptides, such information is often inadequate. This is especially the case for Cα and Cβ atoms of individual residues with similar or overlapping chemical shifts, for two (or more) identical amino acid residues which have different chemical shift values for one or more of their carbon atoms as a result of their different sequence positions, and for carbon atoms subject to specific anisotropic effects due to neighboring groups. In addition, carbonyl carbon atoms are generally very difficult to assign even in peptides of relative simplicity owing to their similar chemical shifts.[2,6] On the other hand, use of smaller peptide fragments of the compounds of interest can be most helpful for making assignments in peptides of this size.[3-7] The pH dependence of carbon atoms in the N-terminal and C-terminal residues are useful if the free amino and carboxyl groups are present (only the former is useful for oxytocin and related neurophypophyseal hormones). In addition the pH effect on chemical shift of other amino acid residues which possess titratable side-chain moieties, e.g., Asp, Glu, Lys, Arg, His, etc., can often be advantageously used for certain of the carbon atoms in these residues, but tyrosine-2 is the only such residue present in oxytocin and AVP. Therefore, in this paper extensive use of selectively deuterated derivatives of oxytocin and AVP were made to unambiguously assign a number of the chemical shift values of these peptide hormones to specific carbon atoms of specific residues. In general these were able to corroborate previous assignments, and in addition were able to make definitive assignments for several resonances where there had been differences in assignment or where some ambiguity in assignment remained. The use of deuteration, of course, is primarily applicable only to carbon atoms which possess a directly bonded proton. Thus for unambiguous assignment of carbonyl carbons, tertiary substituted carbons, and other nonprotonated carbons in peptides, ^{13}C enrichment will often be necessary, and of course can also be used for unambiguous assignment of other carbon atoms as well.

In any case we believe that the studies reported here, when interpreted in conjunction with previous studies,[7,8] have firmly established the ^{13}C NMR chemical shift assignments for essentially all the carbon atoms in oxytocin and AVP except for the carbonyl carbons.

Oxytocin and arginine vasopressin are the neurohypophyseal hormones present in man and several other higher mammalian species. However, at least seven other closely related structures are found in various vertebrates and these include lysine vasopressin, vasotocin, isotocin, mesotocin, glumitocin, aspartocin, and valitocin. As already noted, these compounds have many structural similarities but a rather large diversity of biological activities (see Table IV). These compounds therefore seemed an ideal case to see if ^{13}C chemical shift parameters might be related to bioactivities. The series was especially attractive because of the considerable previous effort which had been expanded to carefully determine and confirm ^{13}C NMR assignments for the parent structures, oxytocin and AVP. Thus one could have considerable confidence that any changes which might be observed would be real ones, and not artifacts based on incorrect assignments due to the complexity of these nonapeptide structures.

The ^{13}C NMR assignments for the naturally occurring neurohypophyseal hormone derivatives isotocin, mesotocin, aspartocin, and glumitocin, and the closely related oxytocin

analogue, oxypressin ([Phe^3]oxytocin), were all readily made owing to the close similarity in structure between these peptide hormones and oxytocin (and AVP), and the remarkable consistency of the chemical shift values for the invariant residues (Table I) including residues adjacent to the variable residues. Where significant chemical shift variations were seen, they were always readily assigned since the variations were generally quite small and did not involve "crossover" of ^{13}C NMR resonances. In these compounds, perhaps the most interesting chemical shift differences were noted for carbon atoms of amino acid residues located in the 20-membered disulfide-containing moiety of the neurohypophyseal hormone, relative to that same residue located in the acyclic tripeptide side chain of these hormones. The differences were seen primarily in the Cα chemical shifts, which were found 1.4–1.9 ppm upfield for the residues found in the side chain relative to those found in the ring. In contrast, the asparagine residues in the cyclic portion of aspartocin (Asn-4 and Asn-5) were virtually indistinguishable by ^{13}C chemical shifts. This general trend was also found in other substituted neurohypophyseal hormone analogues (vide supra) and may be of general application for peptides containing cyclic and acyclic moieties. Clearly further investigations along these lines are called for.

The ^{13}C NMR assignments for the analogues with modifications in the 4 and/or 2 position(s) of oxytocin could also generally be made with considerable confidence and created very few assignment problems. This was also true for [Pen^1]-oxytocin.

On the other hand, assignments for the diastereomeric analogues of oxytocin and arginine vasopressin, while generally quite straightforward, provided a number of difficulties. All of the diastereoisomers reported here contained D-amino acid substitutions in the "ring" portion of the neurophypophyseal peptide, and significant chemical shift changes were generally seen only for the carbon atoms of the residues in the ring moiety of the peptides. Chemical shift effects were noted for residues adjacent to the D-amino acid residue, but other residues were also significantly affected in some compounds. However, it appeared that most of the major chemical shift differences for these diastereomeric peptides relative to oxytocin and AVP could be primarily traced to the presence of a D-amino acid moiety and its effect on backbone structure, since most of the significant chemical shift differences were for the Cα and Cβ carbon on amino acid residues adjacent to the D-amino acid, though there were notable exceptions.

The question of the application of ^{13}C NMR chemical shift parameters to structure-function studies in peptide hormones and to studies of peptide hormone conformational differences is important. Previous studies have shown that when cis–trans isomerism obtains about X-Pro peptide bonds in biologically active peptides, ^{13}C NMR procedures[3c,4,7c,48] can be very useful in studying these conformational differences. In addition there is some evidence that in *rigid* peptides such as the ferrichromes[49] where some peptide strain is possible, there is some conformational dependence of the ^{13}C NMR chemical shift. The neurohypophyseal hormones oxytocin and arginine vasopressin appear to be quite flexible molecules in aqueous solution,[9,36,50,51] and the evidence presented in this paper and in previous studies[7,9] shows that the Half-Cys-6–Pro-7 peptide bond is trans. Thus one must look to other clues for possible conformational differences in the molecules examined. In the study of Deslauriers et al.[7b] on oxytocin, AVP, lysine vasopressin, and arginine vasotocin, a number of small chemical shift differences were noted to occur in oxytocin as a function of pH. These small chemical shift changes were suggested to be related to conformational changes in oxytocin as a function of pH. We observed similar effects for oxytocin, AVP, mesotocin, and the other naturally occurring neurohypophyseal hormones. Most of the chemical shift changes (including those removed from the titration site) appear to occur as the amino groups are titrated. We interpret these effects to indicate (in view of the conformational flexibility of these compounds) small changes in the relative populations of the conformations as a result of the state of ionization of the α-amino group. Other methods of conformational evaluation are needed to establish the precise nature of these changes.

It is interesting to *compare* the chemical shift differences (as a possible clue to conformational differences) in oxytocin and the related naturally occurring hormones isotocin, mesotocin, aspartocin, and glumitocin as well as the closely related analogue oxypressin at comparable pH values. The compounds have considerably different biological activities from oxytocin or each other (Table IV) and it might be suggested that these differences are due at least in part to conformational differences. However, as noted earlier, there were virtually no ^{13}C chemical shift differences of the invariant residues in these molecules, except for occasional changes on atoms in residues adjacent to residues where different amino acids had been substituted by nature. Thus it would appear that these latter effects are primarily due to the sequence changes, that these compounds have very similar conformations, and that ^{13}C NMR is not sufficiently sensitive to distinguish any small conformational differences which might exist for these compounds. An interesting observation, however, relates to the quite different chemical shifts (1.4–1.9 ppm) for the Cα carbon atoms of Ile (or Gln) when these residues are located in the 20-membered cyclic moiety of the hormones vs. when they are located in the acyclic tripeptide moiety of the peptides. A part of this might be due to the effects of adjacent residues on the chemical shift. However, as noted above and further observed in the 2- and/or 4-substituted oxytocin analogues, these latter sequence-dependent shifts generally appear to be quite small (less than 0.5 ppm). Thus it may be suggested that the chemical shift differences are related to the conformational restrictions placed on the residues in the cyclic moiety due to their presence in a 20-membered ring.

The 2- and/or 4-substituted oxytocin analogues [Leu^4]-oxytocin, [Leu^2,Leu^4]oxytocin, [Ile^2,Leu^4]oxytocin, and [Phe^2,Leu^4]oxytocin all have significantly different biological activity profiles compared with oxytocin (Table IV). However, except for the latter compound, they all had ^{13}C NMR spectra similar to that of oxytocin at the invariant residues. Thus it might be suggested that these compounds have conformations similar to each other and to oxytocin in aqueous solution. Once again in these compounds, significant differences are noted in most cases for the Cα of residues located in the ring moiety of the hormone analogues as compared to identical residues located in the acyclic portion of these peptides. The substantial chemical shift changes in [Phe^2,Leu^4]oxytocin suggest that perhaps this compound possesses a somewhat different average conformation than the other compounds, and thus it might be worthwhile to examine the conformational properties of this compound further.

We have previously examined the conformational and dynamic properties of [Pen^1]oxytocin and compared it to the conformational and dynamic properties of oxytocin.[9] These studies have shown that [Pen^1]oxytocin has a consderably different conformation in the ring moiety than oxytocin, while the tripeptide side chain moieties are not significantly different. It is thus not surprising that the carbon atoms of the side-chain residues have virtually identical ^{13}C NMR chemical shift parameters. However, except for significant chemical shifts of the Half-Cys-6 and Asn-5 Cβ, there also were no large (>0.5 ppm) differences in the chemical shifts for the invariant residues in the ring moiety of the two peptides. Further work will be necessary to establish what sorts of ^{13}C chemical shift parameters are conformationally sensitive and which are not.

Substituting D-amino acid residues in the peptide hormones

oxytocin and AVP did affect ^{13}C NMR parameters more than any of the other structural perturbation made here. This is expected since in general diastereomeric structures should have different chemical, physical, and biological properties. In some cases, most notably [2-D-tyrosine]oxytocin, fairly significant ^{13}C NMR chemical shift changes were seen throughout the molecule, especially the cyclic 20-membered ring moiety, relative to their positions in oxytocin, and this undoubtedly reflects conformational differences. It is interesting, however, that in other diasteroisomers, the chemical shift changes are often small at invariant residues. Generally, however, differences are seen in the vicinity of the D residue, and the D residue itself will often have significantly different chemical shift parameters than the L residue in an otherwise identical peptide. In this regard it is interesting to note that the pK_as of the α-amino group of [1-hemi-D-cystine]oxytocin and of [1-hemi-D-cystine, 8-arginine]vasopressin are about one pK unit less than in oxytocin and AVP, respectively.[45,46]

Care will be required to interpret changes in ^{13}C NMR chemical shift parameters with changes which may be related to the biological activities of these compounds, but conformational studies using other approaches should be useful for both of these purposes.

Acknowledgments. We thank Drs. David Wright and Nirankar Agarwal for the amino acid analyses. We thank Dr. Mac Hadley, Department of General Biology, University of Arizona, for the milk-ejecting assays, and Drs. J.-P. Meraldi and M. Blumenstein for some preliminary ^{13}C spectra.

References and Notes

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