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Evidence from Hydrogen-1 and Carbon-13 Nuclear Magnetic Resonance Studies That the Dissociation Rate of Oxytocin from Bovine Neurophysin at Neutral pH Is Slow†

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ABSTRACT: The interaction of the peptide hormone oxytocin with bovine neurophysins I and II (NPI and NPII) was studied using 270-MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy and observing the tyrosine-2 aromatic protons of the hormone. Using oxytocin and its diastereoisomer [1-hemi-D-cystine]oxytocin (which does not bind to the neurophysins), we investigated the T_2 (line width) values of these protons as a function of temperature, protein concentration at constant hormone concentration, and protein concentration at a constant hormone protein ratio and the T_1 values of these hormones in the presence and absence of the neurophysins. The results of these studies indicate that at neutral pH most of the effects observed can be accounted for by viscosity changes and suggest that the bound hormone is in slow exchange. This was corroborated by ^{13}C NMR studies. A total synthesis of specifically ^{13}C labeled (90% ^{13}C enrichment) [2-^{13}C]tyrosine (L and DL derivatives) was accomplished, and the enriched amino acid was incorporated into oxytocin and arginine vasopressin by total synthesis. The diastereoisomers of [2-DL-[2-^{13}C]tyrosine]oxytocin were separated and purified by partition chromatography. The interactions of these hormone derivatives with NPI and NPII were investigated at a variety of temperatures and hormone and protein concentrations but at constant pH (6.6). Under all conditions, [2-L-[2-^{13}C]tyrosine]oxytocin (**1**) and [2-L-[2-^{13}C]tyrosine, 8-arginine]vasopressin (**3**) interact strongly with NPI and NPII, and on binding, a 2.2-ppm downfield chemical shift of the labeled carbon atom is observed for both hormones at a 1:1 hormone to protein stoichiometry. These results indicate an equivalence of binding sites for both hormones to both NPI and NPII in the 1:1 complex. On the other hand, [2-D-[2-^{13}C]tyrosine]-oxytocin (**2**) does not significantly interact with the neurophysins. When an excess of labeled hormone **1** is added to NPI (or NPII) (e.g., a 1.4:1 hormone to protein mole ratio), free and bound peaks are observed. The spectra indicate that the hormone is in slow exchange under all conditions studied and that a 1:1 stoichiometry obtains for the oxytocin–neurophysin complex. An upper limit for the overall dissociation rate of about 2 s^{-1} can be estimated from NMR data leading to an upper limit of about 2×10^5 M^{-1} s^{-1} for the association rate. This small rate constant and the substantial chemical shifts associated with the binding process suggest that a substantial conformational change occurs at the tyrosine-2 position when the hormones bind to neurophysins.

Considerable information has been obtained in recent years on the interaction of the neurohypophyseal hormones, oxytocin and arginine (or lysine) vasopressin (AVP or LVP),[1] with a group of related proteins, called neurophysins, found in neurosecretory granules of the posterior lobe of the pituitary gland. From studies with both nonapeptide and tripeptide analogues and derivatives of oxytocin, Breslow and co-workers (Breslow, 1974; Breslow et al., 1971, 1973) found evidence that the three N-terminal residues of the hormones, ½-Cys-1, Tyr-2, and Ile-3 (Phe-3 in AVP and LVP), are critical for binding. Significant support for this hypothesis has come from various laboratories employing nuclear magnetic resonance (NMR) techniques (Glasel et al., 1973; Hruby et al., 1977b; Griffin et al., 1977; Alazard et al., 1974; Blumenstein et al., 1977; Balaram et al., 1973).

An area of disagreement about the oxytocin–NP interaction concerns the dissociation rate constant of the hormone–protein complex. The determination of this rate constant is important for several reasons. This rate is related to the strength of the protein–hormone complex. Also, a knowledge of this rate, as well as the equilibrium constant for interaction, allows a calculation of the overall association rate constant, which reflects the ease (or difficulty) of complex formation. In addition, the exchange rate is of great experimental significance in NMR studies, since a knowledge of this rate, or at least limits on its values, is often necessary for proper interpretation of NMR data. Our previous work (Blumenstein and Hruby, 1977) has

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[1] Standard abbreviations and nomenclature for amino acids, peptides, and peptide derivatives are used throughout. Amino acids, except glycine, are of the L configuration unless otherwise stated. Other abbreviations used include: AVP, arginine vasopressin; LVP, lysine vasopressin; NP, bovine neurophysin; NPI, bovine neurophysin I; NPII, bovine neurophysin II; NMR, nuclear magnetic resonance; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TLC, thin-layer chromatography.

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shown that near neutral pH the overall dissociation rate constant for oxytocin-NP binding is slow ($<10\ s^{-1}$) but that certain residues sense a much more rapid exchange ($>1000\ s^{-1}$).

Two proton NMR studies, both involving an observation of the aromatic protons of the tyrosine-2 residue of oxytocin (or LVP) and both done near neutral pH and under very similar conditions of protein concentration with large excesses of hormone, came to opposite conclusions about the exchange rate, with one study (Alazard et al., 1974) finding a fast-exchange condition with a rate $\gg 60\ s^{-1}$, and the other study (Balaram et al., 1973) finding slow exchange and a rate $\ll 125\ s^{-1}$. A deuterium NMR study conducted under quite different conditions at pH 2.5 found a rate $>1000\ s^{-1}$ (Glasel et al., 1973). A temperature-jump study at neutral pH found a rate of $10-20\ s^{-1}$ (Pearlmutter and McMains, 1977).

We have now repeated the ^1H NMR studies using suitable controls and can show that Alazard et al. (1974) misinterpreted their data and that the exchange rate *at neutral* pH is indeed very slow. We also present results of ^{13}C NMR experiments with [2-[2-^{13}C]tyrosine]oxytocin and similarly labeled AVP. The data from these latter studies confirm the slow exchange rate and the significance of the Tyr-2 residue in the binding process, show the similarity in the binding of oxytocin and AVP to the bovine neurophysins I and II at a 1:1 mole ratio, and give some evidence for a conformational change of the hormone on protein binding.

Experimental Section

Materials. The bovine neurophysins (NPI and NPII) were prepared as previously described (Blumenstein and Hruby, 1976) using a slight modification of the procedures of Breslow et al. (1971). NPII used in the proton NMR experiments was then rechromatographed on Sephadex G-50 using 0.1 N HCOOH as eluent solvent to remove salts and lyophilized.

N^{α}-Boc amino acids were purchased from Vega-Fox Biochemicals or Biosynthetica and were checked for purity just before use by thin-layer chromatography (TLC) in at least three solvent systems and by mixture melting point determination. N^{α}-*tert*-Butyloxycarbonyl-*S*-3,4-dimethylbenzylcysteine was prepared according to the method of Smith (1973; Upson and Hruby, 1976). Polystyrene resin, 1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.07 mmol/g of resin, was used. Solvents for partition chromatography were purified as previously described (Hruby and Groginsky, 1971). N^{α}-*tert*-Butyloxycarbonyl-*S*-benzyl-DL-[α,β,β-$^2H_3$]cysteine and [1-hemi-D-[α,β,β-$^2H_3$]cystine]oxytocin were prepared as previously described (Upson and Hruby, 1976).

Analytical Methods. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. TLC was performed 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–water (15:3:10:12), (c) 1-pentanol–pyridine–water (7:6:6), (d) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Amino acid and peptide spots were detected by ninhydrin, UV light, iodine, and fluorescamine. When an N^{α}-Boc group was present, the plate was first sprayed with 6 N HCl and heated at 110 °C for 15 min. Optical rotation values were measured at the mercury green line (547 nm) using a Zeiss Old 4 polarimeter. Amino acid analyses were obtained by the method of Spackman et al. (1958) on a Beckman 120C amino acid analyzer after hydrolysis in 6 N HCl for 22–24 h. The modified (Ehler, 1972) aldimine test (Esko et al., 1968) was used to quantitatively determine amino groups on the amino acid resin. The coupling steps in solid-phase synthesis were monitored for completion by the ninhydrin method (Kaiser et al., 1970). Partition chromatography was used for separation and purification of oxytocin diastereoisomers (Hruby et al., 1977a) and for purification of arginine vasopressin (Yamamoto et al., 1977) as previously reported.

Diethyl 2-Acetamido-2-p-methoxybenzyl[2-^{13}C]malonate. To a sodium ethoxide solution prepared from 0.35 g of Na and 45 mL of anhydrous ethanol was added 3.57 g (16.5 mmol) of diethyl acetamido[2-^{13}C]malonate (Koch Isotopes, Cambridge, Mass.) at 0 °C. To the stirred solution was slowly added 3.15 g (15.7 mmol) of *p*-methoxybenzyl bromide, and the mixture was stirred for 5 h. The solution was poured into 185 mL of water, the mixture cooled at 4 °C, and the precipitate filtered off to give 4.74 g (90%) of the title compound as white crystals: mp 95–96 °C [lit. mp (Yamamoto et al., 1977) 94.5–95.5 °C]; NMR ($CDCl_3$) δ 1.3 (t, 6 H), 2.0 (s, 3 H), 3.6 (s, 2 H), 3.8 (s, 3 H), 4.3 (q, 4 H), 6.8 (d of d, 4 H).

DL-[2-^{13}C]Tyrosine. A mixture of 4.7 g (14 mmol) of diethyl 2-acetamido-2-*p*-methoxybenzyl[α-^{13}C]malonate and 50 mL of 48% hydrobromic acid was heated for 1.25 h in a 110 °C oil bath. The solution was concentrated in vacuo, the residue redissolved in water, and the solution decolorized with Norite and filtered through Celite. The pH was adjusted to about 7 with concentrated ammonium hydroxide, the mixture was cooled to 4 °C, and the precipitate was filtered off, washed, and dried to give 2.18 g (86%) of DL-[2-^{13}C]tyrosine: NMR (CF_3CO_2H): δ 3.3 (d of d, 2 H), ~4.5 (complex, 90.5% ^{13}C, 1 H), 7.0 (d of d, 4 H). TLC in solvent systems a and b showed it to give single uniform spots, identical to authentic tyrosine.

Resolution of DL-[2-^{13}C]Tyrosine. A solution of 2.1 g of DL-[2-^{13}C]tyrosine in 15 mL of trifluoroacetic acid and 8 mL of anhydrous ether was cooled to 0 °C, and 1.75 mL of freshly distilled trifluoroacetic anhydride was added. The solution was stirred for 1 h, concentrated in vacuo, warmed on a steam bath with 5 mL of water, and concentrated in vacuo. The residue was slurried in ether, and the unreacted trifluoroacetate salt of DL-[2-^{13}C]tyrosine (660 mg) was filtered off (490 mg of DL-[2-^{13}C]tyrosine could be recovered). The filtrate was concentrated in vacuo, and crystallization from ether/toluene afforded 2.11 g (80%) of *N*-trifluoroacetyl-DL-[2-^{13}C]tyrosine, mp 189–192 °C [lit. mp for unlabeled compound (Shine and Niemann, 1952) 192.5–193.5 °C]. The product was dissolved in 50 mL of water and the pH adusted to 7.45 with 2 N LiOH. Carboxypeptidase A–DFP (0.5 mL, 25.8 mg/mL, 42.6 units/mg) was added, the solution was stirred at 37 °C for 22 h, the pH was adjusted to 5 with glacial acetic acid, and the mixture was cooled in the refrigerator. The precipitate was filtered off, dissolved in 1 N HCl, and decolorized with Norite as before. The pH was adjusted to 5 with NH_4OH, and the mixture was cooled, filtered, and dried to give 0.57 g (91%) of L-[2-^{13}C]tyrosine: $[\alpha]_{547}^{22}$ −11.5° (*c* 2.0, 1 N HCl) [lit. (Yamamoto et al., 1977) $[\alpha]_{547}^{21}$ −11.3 (*c* 2.4, 1 N HCl). TLC in solvents systems a, b, and c gave small uniform spots identical to L-tyrosine. The original filtrate containing the unreacted *N*-trifluoroacetylated D isomer was acidified to pH 3 with 1 N HCl, concentrated in vacuo, and treated with acetic anhydride (0.3 mL) in 15 mL of glacial acetic at reflux for 10 min. The mixture was concentrated in vacuo, and the residue was treated with 15 mL of 3 N HCl at reflux for 2.5 h. The mixture was concentrated in vacuo, and the residue was dissolved in water and decolorized with Norite. The mixture was filtered through Celite, neutralized with ammonium hydroxide, and cooled at 4 °C. The precipitate was filtered off and dried

in vacuo to give 0.50 g of DL-[2-^{13}C]tyrosine, identical to that obtained above.

N^{α}-tert-Butyloxycarbonyl-[2-^{13}C]tyrosine. A mixture of 0.57 g (3.1 mmol) of [2-^{13}C]tyrosine in 10 mL of peroxide-free dioxane and 10 mL of water at pH 10.0 was treated with 0.51 g (3.6 mmol) of *tert*-butyl azidoformate according to the method of Schnabel (1967). After the usual workup, there was obtained 0.71 g (81%) of N^{α}-Boc-[2-^{13}C]Tyr. The compound gave single uniform spots on TLC in solvent systems a, b, and c identical to authentic N^{α}-Boc-Tyr.

N^{α}-tert-Butyloxycarbonyl-DL-[2-^{13}C]tyrosine. The DL-[2-^{13}C]tyrosine(0.99 g) recovered from the trifluoroacetylation and the resolution followed by reracemization of the D isomer (see above) was treated with *tert*-butyl azidoformate as above. There was obtained 1.42 g (93%) of N^{α}-Boc-DL-[2-^{13}C]Tyr as a white powder, mp 127–129 °C [lit. mp (Yamamoto et al., 1977), 126–129 °C]. This compound gave single uniform spots on TLC in the solvent systems a, b, and c identical with authentic N^{α}-Boc-Tyr.

Solid-Phase Peptide Synthesis Methods. All solid-phase peptide syntheses were performed using either the automated instrument designed and built in our laboratory (Hruby et al., 1972) or a semiautomated instrument of our design. N^{α}-Boc-Gly was attached to the resin by the method of Gisin (1973) to a substitution level of 0.40 mmol of glycine/g of resin. The addition of each N^{α}-Boc amino acid derivative to the peptide chain was accomplished as in the synthesis of other ^{2}H-labeled arginine vasopressin (Yamamoto et al., 1977) and oxytocin derivatives (Hruby and Upson, 1976), except that 35% trifluoroacetic acid in 63% CH_2Cl_2 containing 2% anisole (v/v) was used for removing *tert*-butyloxycarbonyl protecting groups. *p*-Nitrophenyl ester derivatives of Boc-Gln and Boc-Asn were used, and 1-hydroxybenzotriazole was used as catalyst for coupling in solid-phase peptide synthesis (Upson and Hruby, 1976). N^{α}-Tosyl protection was used for arginine, and benzyl protection was used for cysteine sulfhydryl protection. Protected labeled amino acids were added at much reduced excesses (1.5–2.0 equiv) as previously reported (Yamamoto et al., 1977; Hruby et al., 1977a).

2-[2-^{13}C]Tyrosine,8-Arginine]vasopressin. The solid-phase synthesis was carried out using 2.5 g (1.0 mmol) of *tert*-butyloxycarbonylglycinate–resin. For washing and reaction steps, 30-mL portions of solvents or reagents were used. N^{α}-Boc[2-^{13}C]Tyr (0.51 g, 1.9 mmol) was added to the growing peptide chain in a single 90-min coupling using 1 equiv of dicyclohexylcarbodiimide (DCC). Following addition of the last amino acid residue to the peptide chain, the N-terminal Boc protecting group was removed to give 3.6 g of H-Cys(Bzl)-[2-^{13}C]Tyr-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin. The peptide resin was ammonylized in the usual manner (Yamamoto et al., 1977). There was obtained 0.997 g (71%) of H-Cys(Bzl)-[2-^{13}C]Tyr-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2. The protecting groups from a 350-mg (0.25 mmol) portion of the nonapeptide were removed by treatment with sodium in anhydrous liquid ammonia followed by oxidation with $K_3Fe(CN)_6$ in the usual manner. The product was purified by partition chromatography and gel filtration on Sephadex G-25 using 0.2 N HOAc as eluent solvent to give 83 mg of [2-[2-^{13}C]tyrosine, 8-arginine]vasopressin: $[\alpha]^{22}_{547}$ −23.8° (*c* 0.50, 1 N HOAc) [lit. (Yamamoto et al., 1977) for deuterated AVP $[\alpha]^{22}_{547}$ −23.4° (*c* 0.31, 1 N HOAc)]. The product gave a single uniform spot on TLC in the solvent systems a, b, and d, identical with authentic arginine vasopressin. The product had identical milk-ejecting activity (Hruby and Hadley, 1975) with authentic arginine vasopressin. Amino acid analysis gave the following molar ratios: Asp, 1.0; Arg, 1.0; Glu, 1.1; Gly, 1.0; ½-Cys, 1.9; Pro, 1.0; [2-^{13}C]Tyr, 0.90; Phe, 1.0.

Preparation of [2-DL-[2-^{13}C]Tyrosine]oxytocin and Separation and Purification of the Diastereoisomers. Solid-phase synthesis was accomplished using 2.5 g (1.0 mmol) of N^{α}-*tert*-butyloxycarbonylglycinate–resin following procedures generally used in the synthesis of oxytocin derivatives (Upson and Hruby, 1976; Hruby et al., 1977a). For washing and reaction steps, 25-mL portions of solvent or reagent solutions were used. N^{α}-Boc-DL-[2-^{13}C]Tyr was coupled to the growing peptide chain using 0.51 g (1.8 mmol) of the amino acid and 1 equiv of DCC with a 2-h coupling reaction. The ninhydrin test (Kaiser et al., 1972) indicated that incomplete coupling (~86%) had occurred. Therefore, a second overnight coupling was performed using 0.22 g (0.8 mmol) of N^{α}-Boc-DL-[2-^{13}C]Tyr and 1 equiv of DCC. Following addition of the last amino acid residue and removal of the N-terminal Boc group, there was obtained 3.5 g of H-Cys(Bzl)-DL-[2-^{13}C]Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-resin. The peptide was removed from the resin by ammonolysis to give 0.700 g (58%) of H-Cys-(Bzl)-DL-[2-^{13}C]Tyr-Ile-Gln-Asn-Cys-(Bzl)-Pro-Leu-Gly-NH_2. A 320-mg portion of the protected nonapeptide was treated with sodium in liquid ammonia to remove the *S*-benzyl protecting groups and then oxidized with $K_3Fe(CN)_6$ in the usual manner (Upson and Hruby, 1976). The diastereoisomers were separated from each other and from by-products by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1) (Hruby, Yamamoto, Upson, Smith, and Walter, manuscript in preparation). The [2-[2-^{13}C]tyrosine]oxytocin was eluted at R_f 0.23. After gel filtration on Sephadex G-25 there was obtained 44 mg, $[\alpha]^{22}_{547}$ −22.5° (*c* 0.5, 1 N HOAc). The product gave a single uniform spot on TLC in the solvent systems a, b, and c identical to authentic oxytocin. The compound had an identical milk-ejecting activity (Hruby and Hadley, 1975) with authentic oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Pro, 1.1; Gly, 1.0; ½-Cys, 1.9; Ile, 1.0; Leu, 1.0; [2-^{13}C]Tyr, 0.91. The [2-D-[2-^{13}C]tyrosine]oxytocin was eluted at R_f 0.39 (R_f 0.38, authentic [2-D-tyrosine]oxytocin independently synthesized). After gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent there was obtained 51 mg of [2-D-[2-^{13}C]tyrosine]-oxytocin as a white powder, $[\alpha]^{22}_{547}$ −79.1° (*c* 0.5, 1 N HOAc). The product gave single uniform spots on TLC in the solvent systems a, b, c identical with authentic [2-D-tyrosine]-oxytocin. The oxytocic activity on the rat uterus was found to be about 0.5 unit/mg, identical with authentic [2-D-tyrosine]oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Pro, 0.94; Gly, 1.0; ½-Cys, 1.9; Ile, 1.0; Leu, 1.0; D-[2-^{13}C]Tyr, 0.92.

NMR Studies. All ^{13}C and ^{1}H NMR spectra were run on a Bruker HX-270 spectrometer. For the ^{13}C work, sample preparation and acquisition of data were performed as in our earlier work (Blumenstein and Hruby, 1977). The ^{1}H studies were done as closely as possible to the conditions of Alazard et al. (1974). NP II was dissolved in D_2O, 0.1 N NaCl, "pD" (direct meter reading) 6.7 to a concentration of 60 mg/mL. The solution was lyophilized and the powder redissolved in 100% D_2O (Diaprep) to form a 60 mg/mL stock solution. Proton spectra were acquired using quadrature phase detection. For most spectra, a sweep width of ±1000 Hz and 16K memory was employed. The T_1 measurements were taken using a 180°–τ–90°–T sequence. Eleven τ values were used, and the value of T was five times the longest T_1 value. The temperature of all ^{1}H spectra was 18 °C unless otherwise noted.

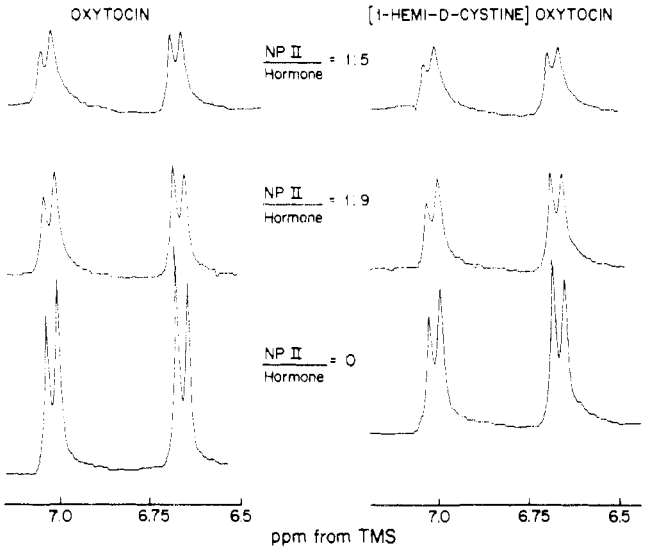


FIGURE 1: ^{1}H NMR spectra of the tyrosine-2 aromatic protons of oxytocin (1a) (left) and [1-hemi-D-[α,β,β-$^2H_3$]cystine]oxytocin (1b) (right) at 18 °C (pH 6.7) with no NPII present (bottom), with a NPII to hormone molar ratio of 1:9 (middle), and with a NPII to hormone ratio of 1:5. The hormone concentration was constant in all cases at 10 mg/mL. Spectra were obtained on an HX-270 NMR spectrometer equipped with quadrature phase detection.

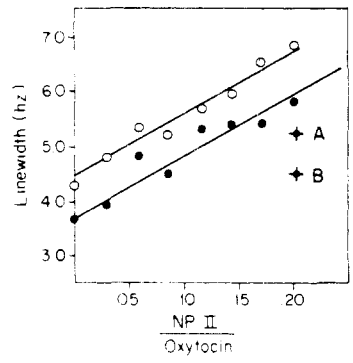


FIGURE 2: Plot of the ^{1}H line width (in Hz) of the aromatic protons in the tyrosine-2 of oxytocin (●) and of [1-hemi-D-[α,β,β-$^2H_3$]cystine]-oxytocin (○) as a function of NPII to hormone derivative ratio using an oxytocin concentration of 10 mg/mL. The conditions were the same as in Figure 1. For the two points marked +, the NPII oxytocin ratio was 1:5. For A the solution was diluted to an oxytocin concentration of 5 mg/mL (NPII concentration equal to 10 mg/mL), while for B the solution was further diluted threefold.

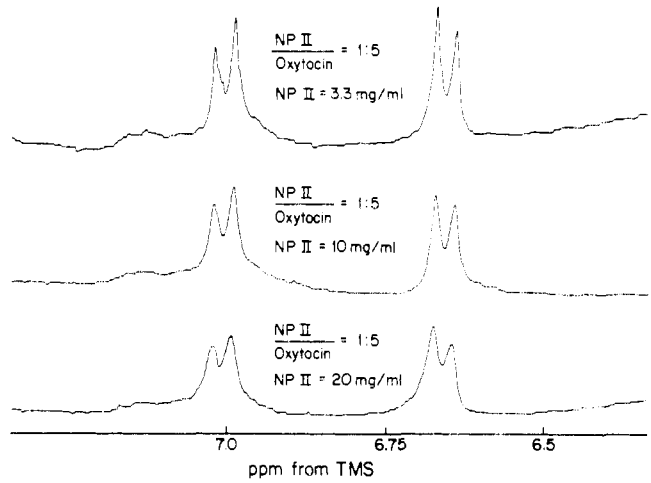


FIGURE 3: ^{1}H NMR spectra of the tyrosine-2 aromatic protons of oxytocin at a constant NPII to oxytocin molar ratio (1:5) but at different NPII concentrations of 3.3 (top), 10 (middle), and 20 mg/mL (bottom). All spectra were taken at 18 °C, pH 6.7.

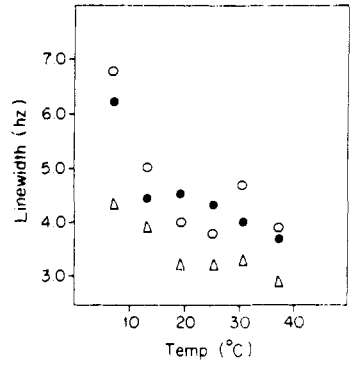


FIGURE 4: Plot of the ^{1}H line width (in Hz) of the aromatic protons of oxytocin (△), oxytocin + NPII (10:1 mole ratio) (●), and [1-hemi-D-[α,β,β-$^2H_3$]cystine]oxytocin + NPII (10:1 mole ratio) (○) as a function of temperature; pH = 6.7.

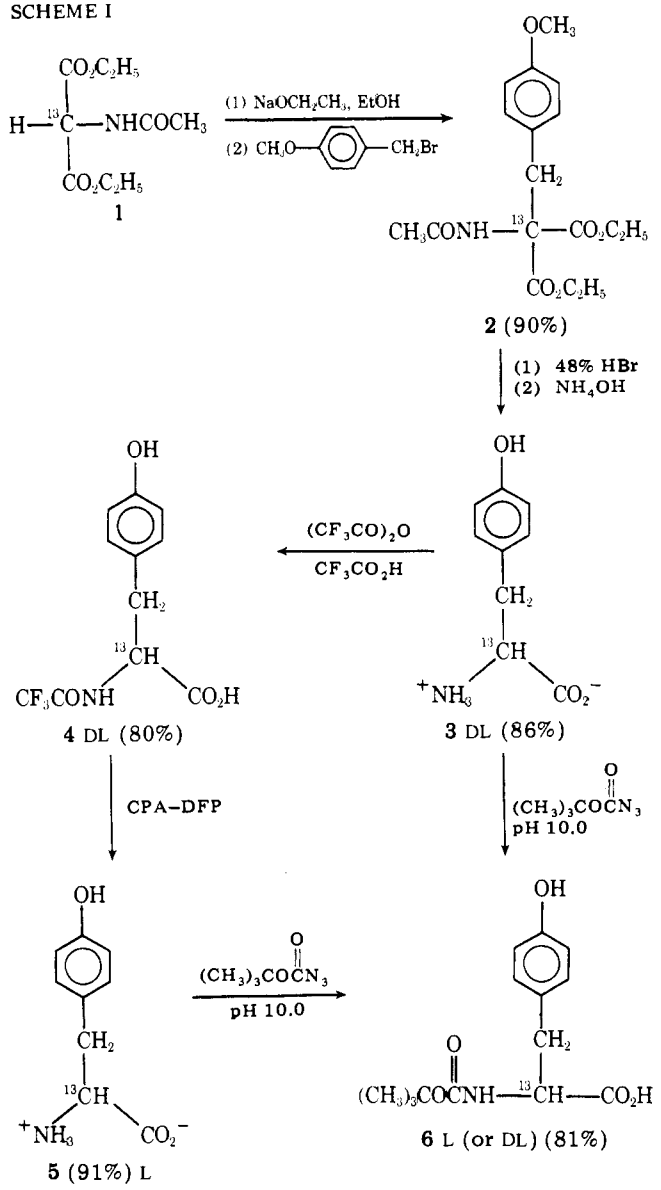
Results

^{1}H NMR Studies. The aromatic tyrosyl protons of oxytocin in the presence of varying amounts of NPII are shown in Figure 1a. There is an increase in the line width observed as more NPII is added, in agreement with the previous observation at Alazard et al. (1974). The ^{1}H NMR spectra of the tyrosyl aromatic protons of [1-hemi-D-[α,β,β-$^2H_3$]cystine]oxytocin in the presence of varying amounts of NPII are shown in Figure 1b. The results of a study of a more extensive series of protein concentrations are shown in Figure 2 and are very similar for oxytocin and the 1-hemi-D-cystine diastereoisomer. In Figure 3, spectra of the oxytocin–NPII mixture, mole ratio 5:1, are shown at different protein concentrations. The line width is dependent on protein concentration even when the protein–hormone ratio is kept fixed. The temperature dependence of the line width of the aromatic tyrosine protons of oxytocin in the absence of NPII, and of the same protons in oxytocin and [1-hemi-D-cystine]oxytocin in the presence of NPII, are shown in Figure 4. There is considerable scatter in the data, but in all three cases the line width decreases as the temperature is raised. Spin–lattice relaxation time (T_1) data for the tyrosine aromatic protons in oxytocin and [1-hemi-D-cystine]oxytocin in the presence and absence of NPII are given in Table I. There is a slight and equivalent decrease in T_1 values for *both* compounds in the presence of protein.

^{13}C Studies. Synthesis of ^{13}C (90%) Labeled Tyrosine and Peptide Hormones. The synthesis of DL-[2-^{13}C]tyrosine, resolution of enantiomers, and preparation of N^{α}-Boc derivatives suitable for use in peptide synthesis are outlined in Scheme I, and the experimental details can be found under Experimental Section. The synthesis of [2-[2-^{13}C]-tyrosine,8-arginine]vasopressin was accomplished by the solid-phase method using resolved [2-^{13}C]tyrosine. The preparation of [2-[2-^{13}C]tyrosine]oxytocin and its diastereoisomeric derivative [2-D-[2-^{13}C]tyrosine]oxytocin was accomplished by solid-phase peptide synthesis using Boc-DL-[2-^{13}C]Tyr and then separating the diastereoisomers by partition chromatography. The compounds were compared with authentic oxytocin and [2-D-tyrosine]oxytocin to establish identity (see Experimental Section).

^{13}C NMR Studies. ^{13}C NMR spectra of [2-[2-^{13}C]tyrosine]oxytocin alone and in the presence of NPI (1:1 stoichi-

SCHEME I



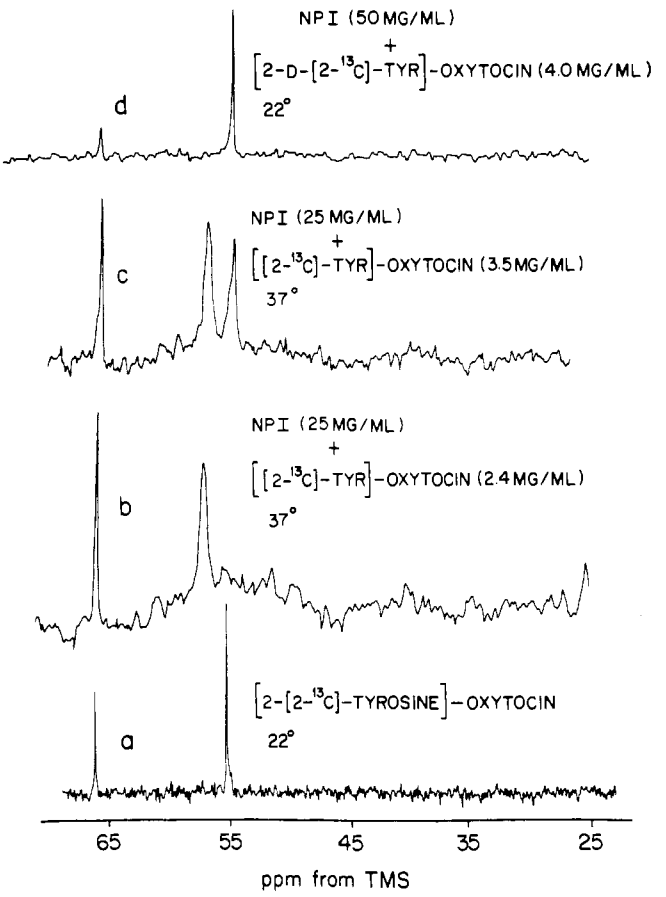


FIGURE 5: ^{13}C NMR spectra of (a) [2-[2-^{13}C]tyrosine]oxytocin (no neurophysin), (b) [2-[2-^{13}C]tyrosine]oxytocin + neurophysin I (NPI) in a hormone to protein molar ratio of 0.9:1.0, (c) [2-[2-^{13}C]tyrosine]-oxytocin + NPI in a hormone to protein molar ratio of 1.4:1.0, and (d) [2-D-[2-^{13}C]tyrosine]oxytocin + NPI in a hormone to protein ratio of 0.8:1.0. All spectra were run at pH 6.6, at 67.9 MHz, on an HX-270 NMR spectrometer. Repetition rates were 1 s and total accumulation times for a and d were about 15 min and for b and c about 3 h.

TABLE I: T_1 Values[a] of the Tyrosine-2 Aromatic Protons of Oxytocin and [1-Hemi-D-cystine]oxytocin in the Presence and Absence of Neurophysin II.

	ortho protons		meta protons	
	no NP	hormone/NP = 5:1	no NP	hormone/NP = 5:1
oxytocin	1.14 ± 0.03	0.92 ± 0.03	0.67 ± 0.02	0.61 ± 0.03
[1-hemi-D-cys]oxytocin	1.23 ± 0.04	0.94 ± 0.03	0.71 ± 0.02	0.63 ± 0.03

[a] T_1 values (in seconds) determined at 270 MHz, 18 °C, 0.1 M NaCl, pH 6.7.

ometry as well as excess hormone) and of [2-D-[2-^{13}C]tyrosine]oxytocin in the presence of NPI are shown in Figure 5. Upon hormone binding to the NPI, the resonance due to the enriched carbon is broadened and shifted downfield by 2.2 ppm. (The position of the bound resonance is 57.4 ppm from Me_4Si using as a standard internal dioxane at 66.5 ppm.) When excess labeled oxytocin is present, two resonances are observed for the enriched carbon, one due to bound hormone and one due to free hormone. Thus, the hormone–NP exchange rate is slow on the NMR time scale, and from the lack of exchange broadening we can set an upper limit on the exchange rate of about 10 s^{-1}. On the other hand, addition of NPI to [2-D-[2-^{13}C]tyrosine]oxytocin causes essentially no change in the chemical shift or line width of the resonance of the labeled carbon, an observation consistent with previous work (Breslow and Abrash, 1966) which showed that [2-D-tyrosine]oxytocin does not bind to bovine neurophysins.

The line widths of [2-[2-^{13}C]tyrosine]oxytocin at various protein concentrations (hormone/protein ratios are about 0.9:1.0 equivalents in all cases) and various temperatures are as follows (the concentration given is that of NPI): 11 mg/mL, 22 °C, 25 Hz; 25 mg/mL, 22 °C, 75 Hz; 25 mg/mL, 37 °C, 35 Hz; 65 mg/mL, 37 °C, 50 Hz (at 65 mg/mL, 22 °C, the enriched resonance could not be clearly observed).

Experiments with NPII and [2-[2-^{13}C]tyrosine]oxytocin were performed at 25 mg/mL protein and again a 2.2-ppm downfield chemical shift was observed; when excess hormone was added, slow exchange was observed as before. Similar experiments were performed using [2-[2-^{13}C]tyrosine,8-arginine]vasopressin, NPI, and NPII mixtures. Identical results were obtained as with the labeled oxytocin derivatives at a 1:1 protein hormone ratio, but as was true in our previous work (Blumenstein et al., 1977; Hruby et al., 1977), addition of

excess labeled AVP led to results which are as yet unclear and will not be discussed here.

Discussion

To complement our ^{13}C NMR studies of the interactions of oxytocin and AVP labeled on the α carbon of the Tyr-2 residue with neurophysins, we have reinvestigated the question of the observed exchange rates for the aromatic protons on this residue. This additional study was necessary, since one group of investigators (Alazard et al., 1974) found a condition of fast exchange for both oxytocin and LVP, while another group (Balaram et al., 1973) found slow exchange (only LVP was studied).

Addition of NPII to a solution of oxytocin results in a linear increase in the line width of the tyrosyl resonances of oxytocin (Figure 1). Alazard et al. (1974), who reported very similar data, used as a control for viscosity effects an oxytocin solution to which was added 15 mg of a high-molecular-weight crude protein isolated from pituitaries. The tyrosyl resonances of oxytocin showed no broadening upon addition of this protein. In our hands, this high-molecular-weight protein was insoluble under the conditions employed (0.1 M NaCl, pH 6.7), so we could not repeat this experiment. Since this protein is very heterogeneous and its exact composition may vary depending on previous steps in the purification, we recognize that the fraction used by Alazard et al. may well have been soluble. Of far greater importance is that their control experiment yielded no data on viscosity effects due to NPII. We have checked these effects in several ways. First, we found that addition of NPII to a solution of [1-hemi-D-cystine]oxytocin caused broadening of the tyrosyl protons very similar to that observed with oxytocin. Since our previous work (Blumenstein and Hruby, 1977) has shown that the 1-hemi-D-cystine diastereomer does not interact with NP, these changes could only be due to viscosity effects. Second, at a constant oxytocin/NP ratio, the line widths were dependent on protein concentration. Even the lowest protein concentration shown in Figure 3, 3.3×10^{-4} M (3.3 mg/mL), is well above the known oxytocin–NP dissociation constant of about 10^{-5} M (Breslow and Walter, 1972; Camier et al., 1973; Nicolas et al., 1976; Glasel et al., 1976). Thus, greater than 97% of the NP is complexed to oxytocin, and the percentage of oxytocin bound to NP does not change as the solution is diluted. Therefore, if the broadening were due primarily to either fast or slow exchange effects, it would be dependent only on the hormone/protein ratio and would be independent of concentration. Alazard et al. (1974) claimed that the increased oxytocin line widths at lower temperatures in the presence of NP were evidence for fast exchange. While our experimental data are similar to theirs, we also find similar temperature effects with [1-hemi-D-cystine]oxytocin in the presence of NPII, as well as with oxytocin in the absence of protein, indicating that the broadening is due to a viscosity increase. Our T_1 data also give no evidence for fast exchange. The changes in T_1 values (for both ortho and meta protons) upon the addition of NP are very similar for oxytocin and [1-hemi-D-cystine]oxytocin. These changes are again most likely due to a viscosity increase in the solution upon the addition of protein. It is conceivable that fast exchange between oxytocin and NP could lead to our T_1 results but this would only happen if the T_1 values for both the ortho and meta protons of oxytocin were the same in both the bound and free states. We regard this as a highly unlikely circumstance. The relative effect of viscosity on T_1 is much smaller than the effect on T_2, since, for the correlation times expected for free oxytocin ($2–5 \times 10^{-10}$ s), the T_1 vs. τ_c curve is near its minimum (Dwek, 1973) and T_1 is rather insensitive to τ_c.

From the above experiments, it is clear that when excess oxytocin is added to NPII the broadening observed in the ^1H resonance of the tyrosine aromatic ring in oxytocin is due to viscosity effects rather than fast exchange. Since no broadening due to fast exchange is observed, either fast exchange is present which does not lead to broadening or else the exchange rate is slow. The former could be true only if bound oxytocin gave rise to sharp tyrosyl peaks. This is not the case, since at a 1:1 oxytocin–NP ratio no sharp peaks are observed in the aromatic region of the spectrum. It can therefore be concluded that the exchange rate is slow. With excess hormone present, only resonances due to free hormone are observed, the bound ^1H resonances being broadened beyond detection.

Our ^1H results agree with those of Balaram et al. (1973) and explain completely the anomolous finding of rapid exchange by Alazard et al. (1974). The significance of the slow exchange rate will be discussed after mention of the ^{13}C results.

^{13}C Studies. The resonance due to the labeled carbon-2 of Tyr-2 in oxytocin exhibits a downfield shift of 2.2 ppm when the hormone binds to either NPI or NPII (Figure 5). None of the shift is due directly to the protonation of the hormone's N-terminal amino group upon binding (Blumenstein et al., 1977), since in free oxytocin the shift of this resonance is independent of pH (Walter et al., 1973). We have previously found an upfield shift upon binding of 2.7 ppm for the carbon-2 of the 1-hemicystine residue in oxytocin of which about 1.5 ppm could not be ascribed to protonation of this residue. The electronic environment of both of these atoms is thus severely perturbed as oxytocin binds to NP, a finding consistent with the important role which the side chains of residue 1 and 2 have in binding (Breslow, 1974). The exact cause of these substantial chemical shifts will be discussed in a further paper (Blumenstein, Hruby, and Viswanatha, manuscript in preparation).

The labeled ^{13}C line widths indicate correlation times for the bound α-tyrosyl carbon of about $1–5 \times 10^{-8}$ s, depending on temperature and concentration. These are similar values to those observed for the α carbon of the 1-hemicystine residue and indicate the tyrosine carbon in the hormones has little motion independent of NP, in contrast to carbons in the tripeptide tail of oxytocin (Glasel et al., 1973; Blumenstein and Hruby, 1976, 1977; Griffin et al., 1977).

Addition of excess oxytocin leads to a conclusion that slow exchange is present, in agreement with our previous ^{13}C studies, and in agreement with previous ^1H work, and the ^1H experiments presented above.

When [2-[2-^{13}C]tyrosine]AVP was added to NPI or NPII, the peak due to the enriched carbon is shifted downfield by 2.2 ppm, the same as observed for the labeled oxytocin derivative. This indicates that the environment of this carbon undergoes the same perturbation as was observed for oxytocin and is strong evidence that the NP–hormone interaction is very similar for oxytocin and AVP (up to a ratio of 1:1 hormone/protein), at least in the N-terminal portion of the molecule. Similarly, the environment of this carbon is identical whether either hormone is bound to NPI or NPII. The equivalence of the binding sites in NPI and NPII was also observed in hormones labeled in the α or carbonyl carbons of the 1-hemicystine residue (Blumenstein and Hruby, 1977; Blumenstein et al., 1977).

Rate Constants. From the data on the tyrosyl protons, as well as the ^{13}C NMR studies of [1-hemi[1-^{13}C]cystine]oxytocin (Blumenstein et al., 1977) and [6-hemi[1-^{13}C]cystine]-oxytocin (Blumenstein, Hruby, Viswanatha, and Yang, unpublished) with neurophysins, we can place an upper limit for the overall k_{off} of 2 s^{-1} at pH 6.6 and temperatures of 15–30

°C. Coupled with the value of the equilibrium constant for the oxytocin-NP interaction of about 10^5 M (Breslow and Walter, 1972; Camier et al., 1973; Nicolas et al., 1976; Glasel et al., 1976), this leads to an upper limit for the overall association rate constant of about 2×10^5 M^{-1} s^{-1}. This limit is considerably slower than that usually observed for interactions of small molecules with proteins and is likely indicative of a substantial conformational change in the protein and/or the hormone on interaction and/or a multistep binding process. Based on their conclusion that the hormone was in fast exchange with neurophysin at neutral pH, Alazard et al. (1974) deduced a much larger association rate constant and therefore postulated a ready accessibility of the binding site in neurophysin to residues 2 and 3 of oxytocin. However, in view of the findings of slow exchange presented here, this suggestion does not seem justified.

Previous spectroscopic studies have shown that significant conformational changes occur in neurophysin when hormone or hormone derivatives bind to them (Breslow and Weis, 1972). Furthermore, both the low association rate constant and the large ^{13}C chemical-shift changes which occur at several residues when the hormone binds to neurophysins (see above; Blumenstein and Hruby, 1977; Blumenstein, Hruby, and Viswanatha, manuscript in preparation) make it likely that the average conformation of oxytocin in the free state changes significantly when the hormone binds.

There is general agreement between the rate constants determined here and those determined by Pearlmutter and McMains (1977). However, our values are somewhat lower than those of the above workers. This discrepancy may be due to the different conditions (buffer, protein concentration) of the two studies. Also, Pearlmutter and McMains based their results on the rate of proton uptake which accompanies oxytocin-NP interaction, and it is possible that this rate may represent one microscopic step in the oxytocin-NP interaction rather than the overall reaction, which could be somewhat slower.

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