

# Biochemistry

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Volume 16, Number 24

November 29, 1977

## Interactions of Oxytocin with Bovine Neurophysins I and II. Use of $^{13}\text{C}$ Nuclear Magnetic Resonance and Hormones Specifically Enriched with $^{13}\text{C}$ in the Glycinamide-9 and Half-Cystine-1 Positions<sup>†</sup>

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**ABSTRACT:** The specifically  $^{13}\text{C}$ -labeled (90%  $^{13}\text{C}$ -enriched) neurohypophyseal hormone derivatives [9-[2- $^{13}\text{C}$ ]glycinamide]oxytocin (**1**), [1-hemi-L-[2- $^{13}\text{C}$ ]cystine]oxytocin (**2**), and [1-hemi-D-[2- $^{13}\text{C}$ ]cystine]oxytocin (**3**) have been synthesized by solid phase methods, and the interaction of these hormones with bovine neurophysin I and bovine neurophysin II studied using  $^{13}\text{C}$  nuclear magnetic resonance at 67.9 MHz and 22.6 MHz. Studies were made at a variety of hormone and protein concentrations and temperatures, but at constant pH (6.6). Under all the conditions derivatives **1** and **2** interact strongly with both neurophysins but the diastereomer **3** apparently does not. The line widths ( $1/\pi T_2$ ) of the labeled carbons of the hormones in a 0.9 to 1.0 hormone to protein mole ratio suggest considerable differences in the nature of the interaction(s) at the glycinamide-9 and half-cystine-1 residues of the hormones with the neurophysins, with the half-cystine-1 residue tightly bound with motional characteristics similar to those of the protein, while the glycinamide-9 residue of the hormones possesses additional motion. This extra motion of the glycinamide residue is equivalent to free rotation around one bond in the tripeptide tail of oxytocin. The half-cystine-1  $\alpha$ -carbon

atom of oxytocin in the free state and when bound to neurophysin had a chemical-shift difference of about 2.7 ppm under all conditions studied, while the chemical shift for the glycinamide-9  $\alpha$  carbon in **1** was the same or only slightly shifted in the free hormone as compared with the complex. Of the chemical-shift difference present with **2**, at most one-third can be due to a raising of the  $pK_a$  of the N-terminal half-cystine residue upon binding to neurophysin. Possible reasons for the additional shift include a conformation change of the oxytocin backbone upon binding to neurophysin, or a change in the dielectric constant of the surrounding medium. Most interestingly, the glycinamide-9  $\alpha$  carbon of **1** is in *fast exchange* ( $>1000 \text{ s}^{-1}$  at 37 °C) in the hormone-neurophysin I (or neurophysin II) complex, while the half-cystine-1  $\alpha$  carbon in **2** is in *slow exchange* ( $<15 \text{ s}^{-1}$  at 37 °C). A dynamic model of peptide hormone-neurophysin interactions is presented in which slow macroscopic exchange of the bound hormone with the bulk solution obtains, but with one or more much faster microscopic exchange rates contributing to the hormone-protein interaction.

**T**he neurohypophyseal hormones oxytocin and [8-arginine]-vasopressin (AVP)<sup>1</sup> are biologically important peptide

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<sup>1</sup> Standard abbreviation and nomenclature for amino acids, peptides, and peptide derivatives are used throughout. Amino acids are L unless otherwise stated. Other abbreviations used are: NP, bovine neurophysin; NPI, bovine neurophysin I; NPII, bovine neurophysin II; NMR, nuclear magnetic resonance; AVP, arginine vasopressin; DMB, 3,4-dimethylbenzyl; Boc, *tert*-butoxycarbonyl; TLC, thin-layer chromatography; UV, ultraviolet; DMF, dimethylformamide; Me<sub>3</sub>Si (or TMS), tetramethylsilane; EDTA, ethylenediaminetetraacetic acid.

hormones found in the posterior lobe of the pituitary gland of most mammals including man. They are found in neurosecretory granules (NSG) along with a class of proteins, the neurophysins, with which they can specifically associate in a noncovalent manner (for reviews, see Hope, 1975; Breslow, 1974). The neurophysins constitute a significant amount of the dry weight of mammalian posterior pituitaries and can be isolated in gram quantities (Breslow et al., 1971; Glasel et al., 1976) in pure form. Two major proteins, each of about 10 000 molecular weight, designated neurophysin I (NPI) and neurophysin II (NPII) can be obtained from bovine glands and their amino acid sequences have been determined (for NPII, see Chauvet et al., 1975; Wuu and Crumm, 1976) or are in progress (for NPI, see North et al., 1975). The hormones and many derivatives and analogues can be obtained in a highly purified form by contemporary organic synthesis (for recent examples, see for oxytocin, Upson and Hruby, 1976; for AVP, Yamamoto et al., 1977). The availability of the peptide hormones and the neurophysin proteins make these a unique and

important system for studying peptide hormone-macromolecule interactions and, furthermore, constitute an important model for related areas such as peptide hormone-receptor systems.

Previous studies of neurohypophyseal hormone-neurophysins, using a variety of physical methods and tripeptides related to the N-terminal region of hormones (Breslow et al., 1971, 1973; Balaran et al., 1973; for a review, see Breslow, 1975) or the hormones themselves and various derivatives and analogues (see, for example, Breslow and Abrash, 1966; Griffin et al., 1973; Alazard et al., 1974; Cohen et al., 1975; Glasel et al., 1973), have shown that the half-cystine-1 and tyrosine-2 residues of oxytocin and AVP are intimately involved in the hormone-protein interaction. In addition there is some evidence that the isoleucine-3 of oxytocin and the phenylalanine-3 residue of AVP are involved. On the other hand, the C-terminal residue of oxytocin and AVP, glycaminamide, appears not to be intimately involved in the association, but rather retains the same environment which it has in the unbound hormone. However, the precise nature of the interaction including the question of specificity and dynamics is still not established.

As one approach to obtaining a better understanding of this important system, we have been studying oxytocin and AVP interactions with the bovine neurophysins using  $^{13}\text{C}$  NMR spectroscopy. For this purpose we have prepared specifically labeled  $^{13}\text{C}$ -labeled amino acids and have incorporated them into the hormones by total synthesis. In a previous communication (Blumenstein and Hruby, 1976), we reported our initial studies on the interactions of [9-[2- $^{13}\text{C}$ ]glycaminamide]oxytocin (**1**) and [9-[2- $^{13}\text{C}$ ]glycaminamide, 8-arginine]vasopressin with NPII and showed that, at approximately equimolar protein-hormone ratios, the line width due to the bound hormone varied considerably with temperature and protein concentrations. Our interpretation of these data was that the glycaminamide residue of the bound hormone possessed considerable internal motional freedom at the lower concentrations (25 mg/mL and 11 mg/mL protein concentrations) and high temperatures ( $>25^\circ\text{C}$ ), but that raising the concentration and lowering the temperature significantly reduced this internal motion, probably as a result of aggregation of the protein-hormone complex.

In this communication, studies of the interactions of [1-hemi-[2- $^{13}\text{C}$ ]cystine]oxytocin (**2**) with NPI and NPII and further results of the interactions of **1** with NPII and NPI are reported. Studies at equimolar hormone-protein ratios and with excess hormone were made under a variety of conditions. The results from these studies allow us to draw more detailed conclusions concerning the motional characteristics of the bound hormone and, in addition, provide information on the ionization state of the N-terminal amino group of bound oxytocin, the environment of the  $\alpha$  carbon of the half-cystine-1 residue, and the exchange rate of oxytocin between the protein-hormone complex and the bulk solution.

## Experimental Section

**Materials.**  $[2-^{13}\text{C}]$ Glycine and *S*-benzyl-DL-[2- $^{13}\text{C}$ ]cysteine were obtained from Koch Isotopes.  $N^\alpha$ -Boc-amino acids were purchased from Vega-Fox Biochemicals or Biosynthetica and were checked for purity by thin-layer chromatography (TLC) in three solvent systems, and by mixed melting point determination.  $N^\alpha$ -Boc-*S*-3,4-dimethylbenzylcysteine was prepared according to the method of Smith (Smith, 1973; Upson and Hruby, 1976). 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. The bovine neurophysins were isolated and

purified as previously described (Blumenstein and Hruby, 1976; Glasel et al., 1976) using a slight modification of the procedures of Breslow et al. (1971). Solvents for partition chromatography were purified as previously described (Hruby and Groginsky, 1971).

**Analytical Methods.** Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are corrected. 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-pyridine-water (15:3:10:12); (c) 1-pentanol-pyridine-water (7:7:6); (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. 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 24 h. The modified (Ehler, 1972) aldimine test (Esko et al., 1968) was used to quantitatively measure amino groups on the amino acid-resin. The solid phase peptide synthesis coupling steps were monitored for completion by use of ninhydrin (Kaiser et al., 1970). Partition chromatography for purification and separation of diastereomers of oxytocin was performed as previously reported (Yamashiro et al., 1966; Spatola et al., 1974). Gel filtration on Sephadex G-25 was done by the method of Porath and Flodin (1959).

$N^\alpha$ -Boc-[2- $^{13}\text{C}$ ]glycine. The title compound was prepared as reported for  $N^\alpha$ -Boc-[ $\alpha,\alpha$ - $^2\text{H}$ ]glycine (Glasel et al., 1973). From 0.48 g of [2- $^{13}\text{C}$ ]glycine and 1.0 g of *tert*-butylazidoformate there was obtained 0.79 g (73%) of the title compound, mp 84–86 °C. Single spots were obtained on TLC using solvent systems A, B, and C identical with authentic  $N^\alpha$ -Boc-glycine.

$N^\alpha$ -Boc-*S*-benzyl-DL-[2- $^{13}\text{C}$ ]cysteine. The title compound was prepared as previously reported for  $N^\alpha$ -Boc-*S*-benzyl-DL-[ $\alpha$ - $^2\text{H}$ ]cysteine (Upson and Hruby, 1976). From 0.57 g of *S*-benzyl-DL-[2- $^{13}\text{C}$ ]cysteine and 0.5 g of *tert*-butylazidoformate there was obtained 0.65 g (75%) of the title compound, mp 108–110 °C. Single spots were obtained on TLC using solvent systems A, B, and C identical with authentic  $N^\alpha$ -Boc-*S*-benzyl-DL-cysteine. The NMR spectrum ( $\text{CDCl}_3$ ) showed:  $\delta$  1.45 (s, 9 H), 2.90 (s, 2 H), 4.4–4.6 (m, 0.1 H; multiplet doublet about 1 ppm upfield and downfield, 0.9 H), 5.35 (broad, 1 H), 7.30 (s, 5 H).

$N^\alpha$ -Boc-[2- $^{13}\text{C}$ ]glycinate-Resin.  $N^\alpha$ -Boc-[2- $^{13}\text{C}$ ]glycine (0.70 g, 4 mmol) was converted to the cesium salt according to the method of Gisin (1973), and the salt was added to 10 g of the chloromethylated polystyrene resin cross-linked with 1% divinylbenzene. The reaction was run in the usual manner and 10.5 g of the title compound was obtained. A small sample was treated with 50% trifluoroacetic acid in  $\text{CH}_2\text{Cl}_2$  and neutralized with 10% diisopropylethylamine in  $\text{CH}_2\text{Cl}_2$ . The results of a modified (Ehler, 1972) aldimine (Esko et al., 1968) test gave a glycine substitution of 0.25 mmol/g resin.

**Solid Phase Peptide Synthesis Methods.** All solid phase peptide syntheses were performed using a semi-automated instrument designed and built in our laboratory. The addition of each  $N^\alpha$ -Boc amino acid derivative to the peptide chain was accomplished using the procedures in Table I. *p*-Nitrophenyl ester derivatives of Boc-Gln and Boc-Asn were used, and 1-hydroxybenzotriazole was used as catalyst (Upson and Hruby, 1976). *S*-3,4-Dimethylbenzyl protection was used for cysteine sulphydryl protection (Smith, 1973; Yamashiro et al., 1973) except for the labeled cysteine in which *S*-benzyl protection was used.  $N^G$ -Tosyl protection was used for arginine. Labeled

TABLE I: Solid-Phase Peptide Synthesis Methods.

Step	Solvent or reagent	Time (min)	No. of times
Normal DCC Coupling			
1	CH <sub>2</sub> Cl <sub>2</sub>	1	4
2	Ninhydrin test		1
3	TFA-CH <sub>2</sub> Cl <sub>2</sub> -anisole (30:68:2)	2	1
4	TFA-CH <sub>2</sub> Cl <sub>2</sub> -anisole (30:68:2)	20	1
5	CH <sub>2</sub> Cl <sub>2</sub>	1	3
6	DIEA-CH <sub>2</sub> Cl <sub>2</sub> (10:90)	2	2
7	CH <sub>2</sub> Cl <sub>2</sub>	1	4
8	Amino acid <sup>a</sup> (1.5 equiv)-CH <sub>2</sub> Cl <sub>2</sub>		1
9	DCC (1.2 equiv)-CH <sub>2</sub> Cl <sub>2</sub>	30	1
10	CH <sub>2</sub> Cl <sub>2</sub>	1	2
11	100% EtOH	1	2
12	CH <sub>2</sub> Cl <sub>2</sub>	1	3
13	Amino acid (1.5 equiv)-CH <sub>2</sub> Cl <sub>2</sub>		1
14	DCC (1.2 equiv)-CH <sub>2</sub> Cl <sub>2</sub>	30	1
15	CH <sub>2</sub> Cl <sub>2</sub>	1	2
16	100% EtOH	1	2
Nitrophenyl Ester Coupling (for Asn and Gln)			
1	CH <sub>2</sub> Cl <sub>2</sub>	1	4
2	Ninhydrin test		1
3	TFA-CH <sub>2</sub> Cl <sub>2</sub> -anisole (30:68:2)	2	1
4	TFA-CH <sub>2</sub> Cl <sub>2</sub> -anisole (30:68:2)	20	1
5	CH <sub>2</sub> Cl <sub>2</sub>	1	3
6	DIEA-CH <sub>2</sub> Cl <sub>2</sub> (10:90)	2	2
7	CH <sub>2</sub> Cl <sub>2</sub>	1	4
8	DMF	1	5
9	Amino acid <sup>a</sup> (4 equiv)-DMF, 1-hydroxybenzotriazole (4 equiv)	210-300	1
10	DMF	1	3
11	CH <sub>2</sub> Cl <sub>2</sub>	1	2
12	100% EtOH	1	2

<sup>a</sup> All amino acids are *N*<sup>α</sup>-Boc protected.

*S*-benzyl-DL-[2-<sup>13</sup>C]cysteine was added at a much reduced excess (1.6 equiv) as previously reported (Upson and Hruby, 1976; Spatola et al., 1974).

[9-[2-<sup>13</sup>C]Glycinamide]oxytocin. Solid phase synthesis of the protected nonapeptide precursor to the title compound was accomplished using 3.5 g (0.91 mmol) of *N*<sup>α</sup>-Boc-[2-<sup>13</sup>C]glycinate-resin following the scheme outlined in Table I and using 30-mL portions of solution. Following addition of the last amino acid residue to the peptide chain, the *N*-terminal Boc protecting group was removed by performing the first six steps in Table I to give 4.42 g of H-Cys(DMB)-Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-[2-<sup>13</sup>C]Gly-NH<sub>2</sub>, mp 235 °C (dec). The protecting groups from a 285-mg (0.21 mmol) portion of the peptide were removed by treatment with sodium in anhydrous liquid ammonia (freshly distilled from sodium) and the sulfhydryl groups were oxidized under nitrogen (Walti and Hope, 1973) with 0.01 N K<sub>3</sub>Fe(CN)<sub>6</sub> solution (Hope et al., 1962). The product was purified by partition chromatography on Sephadex G-25 (block polymerizate) using the solvent system 1-butanol-3.5% aqueous acetic acid containing 1.5% pyridine (1:1). The fractions corresponding to the desired compound (*R*<sub>f</sub> 0.24) were pooled and the product was isolated to give about 100 mg. The product was purified further by gel filtration on Sephadex G-25 to give 90 mg (43%) of the title compound,  $[\alpha]^{22}_{547} -23.0^\circ$  (c 0.5, 1 N HOAc). The product gave a single uniform spot on TLC in the solvent systems A, B, and C, identical with authentic oxytocin. Amino acid analysis

following hydrolysis gave the following molar ratios: Asp, 1.00; Glu, 1.05; Pro, 1.10; Gly, 1.00; 1/2-Cys, 1.90; Ile, 0.95; Leu, 1.00; Tyr, 0.91. The milk ejecting activity was determined (Hruby and Hadley, 1975) and found to be 480 ± 60 units/mg, identical with authentic oxytocin.

*Preparation of [1-Hemi-DL-[2-<sup>13</sup>C]cystine]oxytocin and Separation of the Diastereomers.* Solid phase synthesis of the protected nonapeptide-resin precursor was done using 3.2 g of *N*<sup>α</sup>-Boc-glycinate-resin (0.32 mmol amino acid per g resin, 1.0 mmol) following the scheme outlined in Table I except for the addition of the final residue, *N*<sup>α</sup>-Boc-*S*-benzyl-DL-[2-<sup>13</sup>C]cysteine, where 0.25 g (0.8 mmol) and 0.19 g (0.6 mmol) of the amino acid and an equivalent amount of DCC were used in the first and second coupling steps, respectively. The Boc protecting group was removed as before and the peptide cleaved from the resin by ammonolysis to give 750 mg of H-DL-[2-<sup>13</sup>C]Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH<sub>2</sub>, mp 220-223 °C. A 300-mg (0.24 mmol) portion of the peptide was converted to crude [1-hemi-DL-[2-<sup>13</sup>C]cystine]oxytocin as before, and the two diastereomers 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) (Upson and Hruby, 1976; Yamashiro et al., 1966). The (1-hemi-L-[2-<sup>13</sup>C]cystine]oxytocin was eluted at *R*<sub>f</sub> 0.22. After gel filtration on Sephadex G-25 there was obtained 72.1 mg (58%),  $[\alpha]^{22}_{547} -22.8^\circ$  (c 0.5, 1 N HOAc). The product gave single uniform spots on TLC in the solvent systems A, B, and C identical with authentic oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.05; Glu, 1.08; Pro, 1.00; Gly, 1.00; 1/2-Cys, 2.00; Ile, 1.00; Leu, 1.06; Tyr, 0.98. The milk ejecting activity (Hruby and Hadley, 1975) was found to be identical with authentic oxytocin. In like manner gel filtration on Sephadex G-25 of [1-hemi-D-[2-<sup>13</sup>C]cystine]oxytocin gave 62.0 mg (49%, 54% overall yield of diastereomers)  $[\alpha]^{22}_{547} -68.7^\circ$  (c 0.5, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.05; Pro, 1.07; Gly, 1.00; 1/2-Cys, 1.95; Ile, 1.01; Leu, 1.00; Tyr, 0.92. The product gave single uniform spots on TLC in solvent systems A, B, and C identical with the authentic all protio derivative. The milk ejecting activity (Hruby and Hadley, 1975) was determined to be ca. 35 units/mg.

*NMR Studies.* Samples for NMR were prepared as described previously (Blumenstein and Hruby, 1976), and about 2  $\mu$ L/mL of dioxane was added for use as internal standard. pH values were determined on a Radiometer 26 pH meter. Values quoted are direct meter readings of the D<sub>2</sub>O solutions.

Spectra were run on one or more of the following instruments: Bruker HX-270 (MIT), Bruker WH-90 (Arizona), Bruker WP-60 (Tufts). We wish to thank Professor Leo Nuerger for generous use of the first instrument.

When samples of [1-hemi-[2-<sup>13</sup>C]cystine]oxytocin in the presence of neurophysin were run at 22.6 MHz (WH-90) or 15.1 MHz (WP-60), under several conditions it was difficult or impossible to detect the peak due to the enriched carbon above the natural abundance spectrum of the protein. Therefore all spectra of cysteine-enriched material reported here were run on a Bruker HX-270 spectrometer operating at 67.9 MHz.

Spectra were run with a pulse width of 20  $\mu$ s (about 90°), a repetition rate of 1 s, and 8K of memory. Most spectra were run using a sweep width of 15.151 kHz. Some spectra were acquired with a 10-kHz sweep width, and, for these spectra, controls were run to ensure that any "folding back" which occurred did not interfere with spectral interpretation. Due to the rather broad lines, exponential multiplication of the free

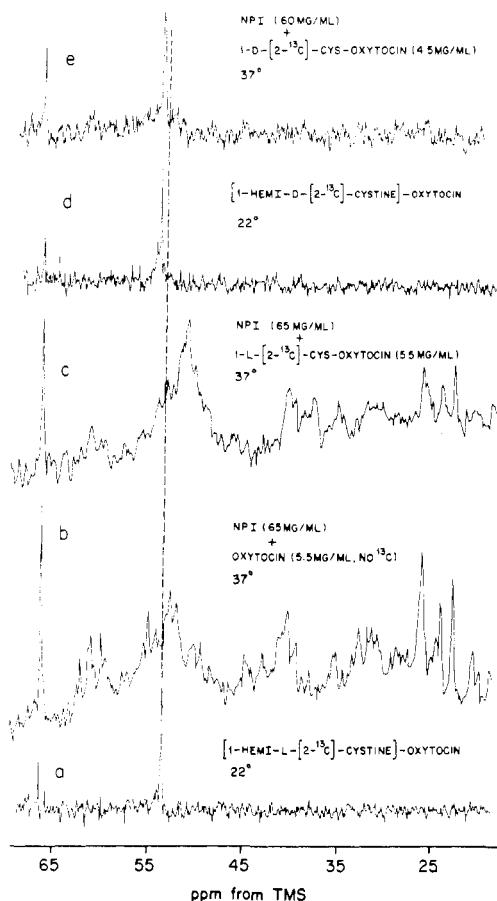


FIGURE 1:  $^{13}\text{C}$  NMR spectra of (a) [1-hemi-L-[2- $^{13}\text{C}$ ]cystine]oxytocin (no neurophysin); (b) neurophysin I (NPI) + oxytocin (no enrichment; hormone:protein ratio is 0.9:1.0); (c) NPI + [1-hemi-L-[2- $^{13}\text{C}$ ]cystine]-oxytocin (hormone:protein ratio as in b); (d) [1-hemi-D-[2- $^{13}\text{C}$ ]cystine]-oxytocin (no neurophysin); (e) NPI + [1-hemi-D-[2- $^{13}\text{C}$ ]cystine]oxytocin (hormone:protein ratio is 0.75:1.0). All spectra measured at 67.9 MHz on a Bruker HX-270 spectrometer, with a solution pH of  $6.6 \pm 0.1$  (direct meter reading in  $\text{D}_2\text{O}$ ). The total accumulation time, using a 1-s repetition rate and  $90^\circ$  pulses, for spectra a, d, and e was 10–20 min, while for b and c the accumulation time was 3–4 h. Spectrum b is displayed at about twice the amplitude of spectrum c. The sharp peak at 66.5 ppm is due to dioxane ( $2 \mu\text{L}/\text{mL}$ ).

induction decay corresponding to substantial line broadening (generally 9–18 Hz) was employed to improve the signal-to-noise ratio. Line widths of hormone resonances are the observed width less the width of the dioxane internal standard. Chemical shifts were measured from dioxane and are referenced to  $\text{Me}_4\text{Si}$  using a value of 66.5 ppm for the shift of dioxane. The number of transients accumulated was generally 10 000–15 000 (3–4 h).

Spectra of [9-[2- $^{13}\text{C}$ ]glycinamide]oxytocin with NPII and NPI were run on a Bruker WH-90 spectrometer as reported previously (Blumenstein and Hruby, 1976).

## Results

In Figure 1 are shown the  $^{13}\text{C}$  NMR spectra of the aliphatic region of [1-hemi-L-[2- $^{13}\text{C}$ ]cystine]oxytocin (**2**) in the presence and absence of NPI and of [1-hemi-D-[2- $^{13}\text{C}$ ]cystine]-oxytocin (**3**) in the presence and absence of NPI and of NPI in the presence of unlabeled natural oxytocin. A comparison of Figures 1b and 1c shows the peak at about 51 ppm to be due to the enriched carbon of the bound hormone. This peak shows a substantial line broadening as well as a chemical shift of about 2.7 ppm upfield relative to the peak observed in unbound oxytocin (compare Figures 1a and 1c; the position of the  $\alpha$

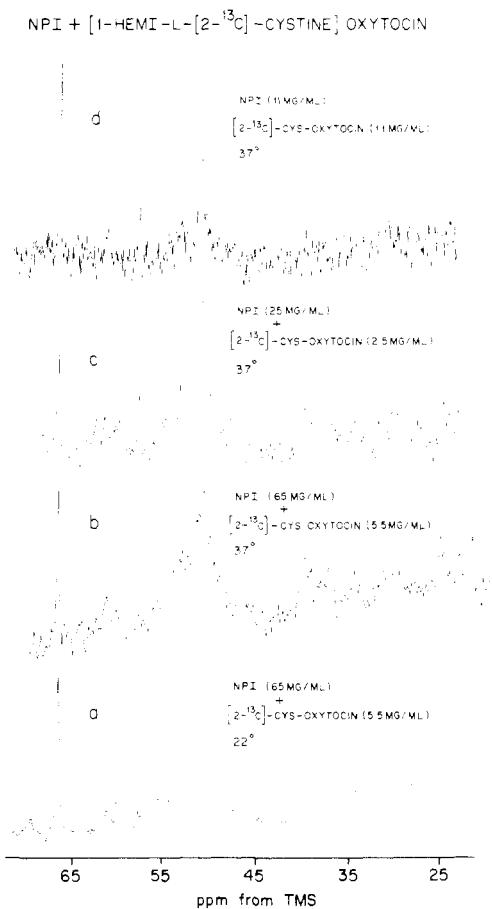


FIGURE 2: Spectra of NPI + [1-hemi-L-[2- $^{13}\text{C}$ ]cystine]oxytocin at hormone:protein ratios of about 0.9:1.0. Protein concentrations and temperatures are as follows: (a) 65 mg/mL, 22 °C; (b) 65 mg/mL, 37 °C; (c) 25 mg/mL, 37 °C; (d) 11 mg/mL, 37 °C. Spectral and solution conditions as in Figure 1. Accumulation times were 3–6 h. The peak in d at 57.5 ppm is due to EDTA (see text).

carbon of half-cystine-1 at pH 6.6 is in agreement with previous work (Walter et al., 1973)). Addition of NPI to a solution of the labeled 1-hemi-D-cystine analogue **3** caused essentially no change in the line width or chemical shift of the resonance signal due to the enriched hormone. The chemical shift of the  $\alpha$ -carbon atom of the half-cystine-1 residues of the free D diastereomer **3** is about 1 ppm downfield relative to the free L diastereomer **2**. The chemical-shift difference reflects both the different environment which this carbon senses in the two diastereomers as well as the lower  $\text{p}K_a$  of the N-terminal amino group of the D diastereomer **3** (Blumenstein and Hruby, unpublished) as compared with the natural hormone.

In Figures 2 and 3, the  $^{13}\text{C}$  NMR spectra of **2** in the presence of NPI and NPII are shown at various temperatures and protein concentrations. In the presence of NPI, the peak of the labeled carbon of the hormone is discernible under all conditions, although at low temperature (22 °C) and high protein concentrations it is severely broadened and not clearly resolved from the resonances due to the natural abundance  $^{13}\text{C}$  peaks of the protein. In the presence of NPII, the situation is worse since, under comparable conditions, the peak due to the labeled hormone is broader in the presence of NPII than in the presence of NPI. Furthermore, there is a rather prominent peak in the natural abundance spectrum of NPII (see Figure 3a) which has a chemical shift about the same as the labeled hormone. Because of the severe broadening of the bound hormone peak, often the largest peak in the  $\alpha$ -carbon region is due to the natural abundance peak(s) of the protein. However, at lower

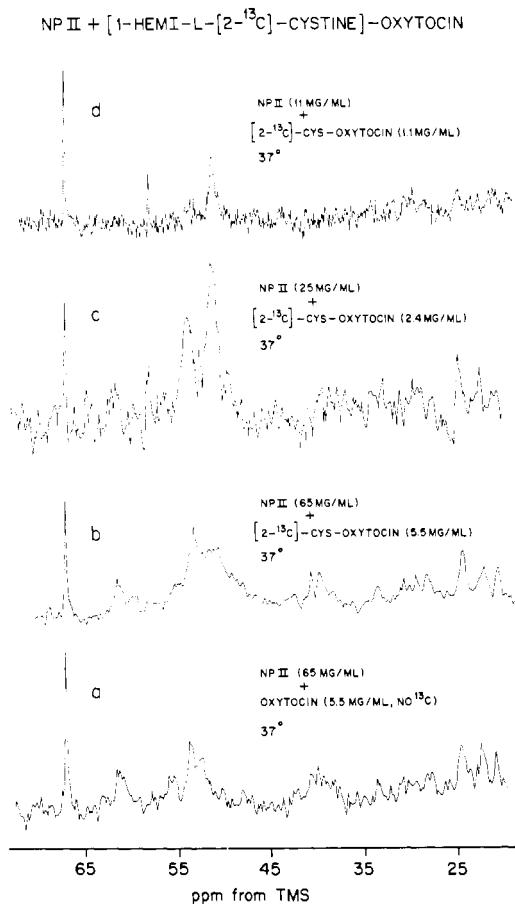


FIGURE 3: Spectra of NPII + oxytocin at hormone:protein ratios of about 0.9:1.0. (a) NPII (65 mg/mL) + oxytocin (no enrichment), 37 °C; (b-d) NPII + [1-hemi-L-[2-<sup>13</sup>C]cystine]oxytocin; (b) 65 mg/mL, 37 °C; (c) 25 mg/mL, 37 °C; (d) 11 mg/mL, 37 °C. Accumulation times were 3–6 h.

protein concentrations, it was found that the labeled peak in the bound oxytocin derivative **2** shows a chemical shift of about 3 ppm upfield relative to the peak in unbound oxytocin, just as was the case for the interaction of **2** with NPI.

None of the line-width or chemical-shift effects are due to precipitation since none was observed under any of the experimental conditions used (pH > 6). This is in agreement with Cohen et al. (1972) who found no precipitation above pH 6 for a 1:1 hormone-neurophysin complex even at a protein concentration of 60 mg/mL. The line widths obtained for the labeled carbon of **2** in the presence of NPI and NPII, and selected values for line widths of the labeled carbon of [2-[2-<sup>13</sup>C]glycinamide]oxytocin (**1**) in the presence of NPI and NPII are listed in Table II. To facilitate comparison with the data of **2**, the line widths listed for **1** are the actual line widths divided by two, since the  $\alpha$  carbon of the glycinamide-9 residue has two directly bonded protons, and the line widths are expected to be proportional to the number of directly bonded protons (Doddrell et al., 1972). In the *absence* of neurophysin, the line width for the labeled glycinamide-9  $\alpha$  carbon atom of **1** is less than 1 Hz per proton, and for the labeled half-cystine-1  $\alpha$ -carbon atom of **2** about 3 Hz per proton. The chemical shift for the labeled  $\alpha$ -carbon atom in **1** is 42.7 ppm under all conditions.

Some spectra in Figures 2 and 3 display a resonance at 57.5 ppm due to the four equivalent methylene carbon atoms of the carboxymethyl groups of EDTA. A peak of one-half this intensity due to the two methylene carbon atoms of the ethylene

TABLE II: Measured Line Widths in Hertz of [1-Hemi-[2-<sup>13</sup>C]cystine]oxytocin and [9-[2-<sup>13</sup>C]Glycinamide]oxytocin under Various Conditions of Neurophysin Concentration and Temperature.

[1-Hemi-[2- <sup>13</sup> C]cystine]oxytocin <sup>a</sup>			
	NPI (mg/mL)		
Temp (°C)	11	25	65
22	20	100	(>150) <sup>b</sup>
37	12	50	100
NPII (mg/mL)			
Temp (°C)	11	25	65
22	25–30	(~150) <sup>b</sup>	(>250) <sup>b</sup>
37	15	70	(~150) <sup>b</sup>
[9-[2- <sup>13</sup> C]Glycinamide]oxytocin <sup>a,c</sup>			
	NPI (mg/mL)		
Temp (°C)	25	65	
22	10	22	
37	3	12	
NPII (mg/mL)			
Temp (°C)	11	25	65
22	10	15	42
37	1.5	3	15

<sup>a</sup> Hormone to protein ratio is 0.9–1.0 in all cases. <sup>b</sup> Numbers in parentheses are estimates since labeled peaks could not be clearly resolved from natural abundance spectrum. <sup>c</sup> Values are linewidths divided by two (see text for explanation).

moiety in EDTA lies just downfield (about 0.1 ppm) of the peak due to the enriched  $\alpha$ -carbon atom in **2** and cannot be clearly distinguished.

In Figure 4 we show typical <sup>13</sup>C spectra obtained when an excess of **2** (about 1.4-fold) was added to NPI and NPII under a variety of conditions. The spectra are essentially a superposition of spectra obtained without an excess of hormone (Figures 2 and 3) plus the spectrum of the free hormone (Figure 1a). The observation of separate resonances due to free and bound hormones when excess hormone is present indicates that slow exchange is present for the hormone-protein complex. Both the free and bound resonances show negligible broadening due to exchange. (In the case of the bound resonance, the exchange broadening is the difference in line width observed between spectra obtained with hormone:protein ratios of 0.9 to 1.0, and 1.4 to 1.0, respectively. For the free hormone, the resonances compared are the free resonance observed with 1.4:1 stoichiometry, and the resonances seen with the diastereomeric derivative [1-hemi-D-[2-<sup>13</sup>C]cystine]oxytocin (**3**) in the presence of neurophysin, in which viscosity effects due to the protein occur, but where there is no peptide-neurophysin binding.) The exchange broadening of the bound peak is equal to  $1/\pi\tau$  ( $\tau$  is the residence time in the bound state and is equal to the reciprocal of the exchange rate), while for the free state the exchange broadening is  $[P_B/(1 - P_B)]1/\pi\tau$ , in which  $P_B$  is the mole fraction of the ligand in the bound state. If we set a conservative upper limit of 10 Hz for the exchange broadening of the free resonance, for which  $P_B = 1/1.4$ , then the upper limit for the exchange rate is about  $13 \text{ s}^{-1}$ .

In Figures 3 and 4, a slight temperature dependence of the position of the labeled resonance due to free oxytocin is observed. This temperature dependence is also seen in solutions containing no protein.

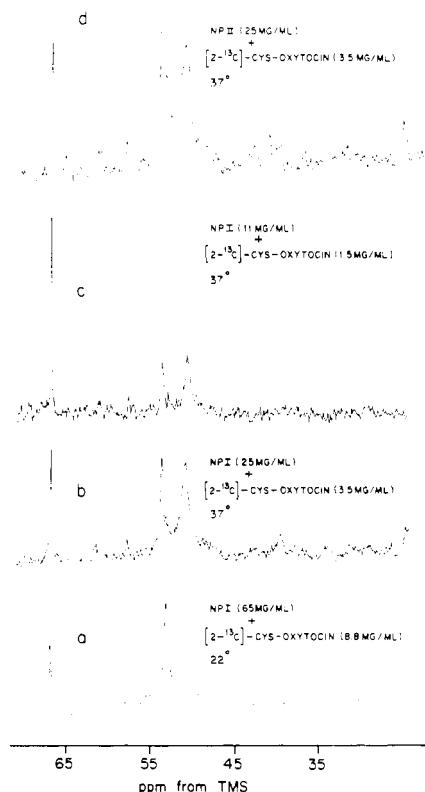
NPI, NPII + EXCESS [1-HEMI-L-[2-<sup>13</sup>C]-CYSTINE-OXYTOCIN

FIGURE 4: Spectra of NPI and NPII + [1-hemi-L-[2-<sup>13</sup>C]cystine]oxytocin at hormone:protein ratios of about 1.4:1.0. (a) NPI, 65 mg/mL, 22 °C; (b) NPI, 25 mg/mL, 37 °C; (c) NPI, 11 mg/mL, 37 °C; (d) NPII, 25 mg/mL, 37 °C.

Typical <sup>13</sup>C NMR spectra of [9-[2-<sup>13</sup>C]glycinamide]oxytocin (**1**) in a 1:1 and 2:1 molar ratio relative to NPII are shown in Figure 5. In these studies it is found that adding a twofold excess of hormone simply reduces the observed line width by a factor of two. The line width for the labeled carbon in the free hormone **1** is less than 1 Hz, but in the presence of NPII under the conditions shown in Figure 5 the line widths are about 95 Hz (0.85:1.0 mol ratio) and about 50 Hz (1.9:1.0 mol ratio), respectively. Thus, the glycinamide labeled hormone exhibits fast exchange (an average of free and bound resonances), and the exchange rate must be greater than the bound line width ( $\times\pi$ ). For example, the exchange rate for the [9-[2-<sup>13</sup>C]glycinamide]oxytocin-NPII complex at 16 °C (Blumenstein and Hruby, 1976) is  $>300\text{ s}^{-1}$ . If the labeled carbon atom in **1** were in slow exchange, a narrow intense peak (from the excess free hormone) superimposed on a broad peak (from the bound hormone) would have been observed under the conditions used in Figure 5. This seeming anomaly between the exchange rates deduced from the two labeled hormones will be discussed later.

In Figure 5 it appears that the position of the enriched resonance in the 1:1 complex is slightly upfield of its position in the 2:1 complex. We have found that in spectra where the height of the natural abundance background was significant relative to the height of the enriched peak, the natural abundance peak often experienced an upfield shift. We believe that this apparent shift was due to the spectral contribution of the background which was greater on the upfield side of the peak than on the downfield side.

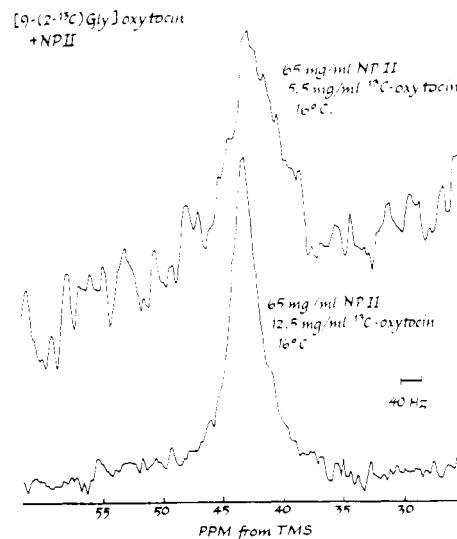


FIGURE 5: Spectra of NPII, 65 mg/mL, 16 °C, + [9-[2-<sup>13</sup>C]glycinamide]oxytocin. (Top) Hormone:protein ratio is 0.85:1.0; (bottom) hormone:protein ratio is 1.9:1.0. Spectra taken at 22.6 MHz on WH-90 spectrometer. Total accumulation time (0.2-s repetition rate, 90° pulses) for each spectrum was about 6 h.

## Discussion

*Observation of Labeled Resonances.* The experiments reported here on the interaction of the half-cystine-1 <sup>13</sup>C-labeled oxytocin derivative **2** presented certain technical problems. Our initial efforts to detect the <sup>13</sup>C-enriched peak of **2** in the presence of NPII were done at 22.6 MHz (Bruker WH-90 spectrometer) and were unsuccessful under most conditions. Despite the 90% <sup>13</sup>C label in **2**, the large line width of the resonance when the hormone is bound to NPI and NPII reduced the amplitude of the labeled resonance so that it became "buried" in the natural abundance spectrum. Though the resonance of the labeled carbon in **2** was detectable at 22.6 MHz in the presence of NPI, we decided to perform all experiments with **2** at 67.9 MHz (Bruker HX-270). At the higher frequency, the natural abundance background from the proteins is spread out over a threefold greater frequency range and it was expected that the width of the resonance due to the enriched carbon of the hormone would be frequency independent (Oldfield et al., 1975); the latter proved to be approximately true where line-width measurements could be made with reasonable accuracy at both frequencies. However, even at the higher fields there was often significant interference of the background with the labeled carbon in the hormone-neurophysin systems (Figure 3). Perhaps difference spectroscopy could have been profitably employed to obtain more accurate line widths for the labeled hormone. However, this would require large amounts of spectrometer time, and much larger amounts of protein and of unlabeled hormone. In any case, it is clear that, when working with globular proteins, specific enrichment of a particular carbon will not necessarily provide a clear spectrum. The resonance due to the enriched carbon in a globular protein or in a molecule firmly but noncovalently bound to a protein in stoichiometric quantities will generally be easily observable if one of the three following situations apply: (1) The labeled carbon possesses internal mobility (Blumenstein and Hruby, 1976; Nigen et al., 1973). (2) The labeled resonance occurs in a region of relatively little background, e.g., in the vicinity of the C-2 resonance in histidine (Browne et al., 1973; Hunkapiller et al., 1973). (3) The labeled carbon has no directly attached protons (Feeney et al., 1973;

Moon and Richards, 1974; Way et al., 1975; Pastore et al., 1976). In this regard we have recently prepared [1-hemi-[1-<sup>13</sup>C]cystine]oxytocin in which the carbonyl carbon of the half-cystine-1 residue is enriched (M. Blumenstein, V. J. Hruby, and Y. Yang, unpublished results). In preliminary studies we found the resonance of the labeled carbon to be sufficiently narrow even at high protein concentrations that it is easily observed at 15.1 MHz.

Despite the problems with **2**, we have obtained considerable information regarding its interaction with the neurophysins, and this will now be discussed.

*Line Width of Bound [1-Hemi-[2-<sup>13</sup>C]cystine]oxytocin.* From Figures 2 and 3 and Table II and from our previous studies (Blumenstein and Hruby, 1976), the following summary statements can be made regarding the line width of the resonances of the labeled half-cystine-1 in **2** when **2** is bound to NPI and NPII: (1) The line widths are dependent upon temperature and protein concentration. (2) The line widths of the labeled carbon in **2** are considerably greater than those of the labeled carbon of **1** (glycinamide-9  $\alpha$  carbon) under the same conditions. (3) The line widths of the labeled carbon in **2** in the presence of NPII are greater than those observed with NPI under the same conditions.

These results are consistent with a model in which the oxytocin-neurophysin complex undergoes reversible aggregation, and under all conditions there is additional motion at the glycinamide-9 residue of oxytocin. The aggregation appears to be somewhat more favored for NPII-hormone complex than for the NPI-hormone complex. By using the equation relating the value of  $T_2$  to the correlation time,  $\tau_c$ , (Doddrell et al., 1972) of the labeled carbon, calculated correlation times of about  $1 \times 10^{-8}$  s (11 mg/mL protein, 37 °C) to  $1 \times 10^{-7}$  s (65 mg/mL protein, 22 °C) are obtained for the half-cystine-1  $\alpha$ -carbon atom of oxytocin. Our measurements of viscosity indicate that changes in this parameter will contribute a maximum factor of 2.5 to the changes in correlation time. It is possible that larger microscopic viscosity changes may occur than those detected in the macroscopic measurements, but the large change in calculated correlation time is most likely indicative of a change in molecular weight and/or in internal mobility of the half-cystine-1 carbon.

At concentrations greater than a few mg/mL, the oxytocin-NPI complex has a molecular weight of about 20 000 (Nicolas et al., 1976). Our data at hormone-neurophysin complexes of 25 mg/mL and 65 mg/mL are consistent with a molecular weight range of from about 20 000 to 50 000 for the complex under various conditions.  $\tau_c$  of a protein is not only a function of its molecular weight, but also of its shape, charge distribution, hydrophobicity, etc., so that molecular weight estimations from  $\tau_c$  data are approximate. The data at 25 mg/mL and 65 mg/mL of protein-hormone complex are most reasonably interpreted by an equilibrium between dimeric (mol wt  $\approx$  20 000) and tetrameric (mol wt  $\approx$  40 000) forms of the hormone-protein complex. The narrower line widths obtained at 11 mg/mL for the complex of **2** with NPI and NPII might suggest that considerable amounts of the monomer are present, but previous results would suggest otherwise (Nicolas et al., 1976). Another possibility is that the  $\alpha$ -carbon atom of the half-cystine-1 residue in oxytocin possesses some mobility independent of the protein when it is bound. However, this seems unlikely since the amino group of the half-cystine-1 residue is thought to be a primary site of hormone-protein interaction (Ginsberg and Ireland, 1964; Breslow, 1975). Also, the large chemical shift of the half-cystine-1  $\alpha$  carbon is invariant with concentration. This would be unlikely if this position were to acquire significant motional freedom at the lower protein-

hormone concentrations only. A slight "breathing" of the hormone relative to the protein in the complex might decrease the line width while not affecting the chemical shift. Another possibility is that both the bound hormone and that part of neurophysin to which it is bound experience motion independent of the rest of the protein.

The narrower line width of the glycinamide-9  $\alpha$ -carbon atom in oxytocin and vasopressin relative to the half-cystine-1  $\alpha$  carbon suggests that independent motion of the "tail" of the bound hormone obtains under all conditions. The tail cannot possess free rotation around very many bonds, however, for this would lead to extremely narrow lines under all conditions, as observed, for example, for the  $\epsilon$ -methylene group of lysine in ribonuclease (Allerhand et al., 1971). Free rotation around one bond would lead to a narrowing by a factor of nine (Doddrell et al., 1972), and this is approximately what is observed under many of the conditions which were employed. Other models involving partial rotation about many bonds are also possible.  $T_1$  measurements obtained at different frequencies and specific labeling at other positions in the hormones will be used to help choose between different models.

*Chemical Shifts of Labeled Carbons.* The chemical shifts of the glycinamide-9 labeled  $\alpha$  carbon in oxytocin and AVP are very similar in free and bound hormone under all conditions. This is consistent with the above model, since the presence of motion in the tail of bound hormone limits the contact between the protein and the hormone. On the other hand, the half-cystine-1  $\alpha$ -carbon atom of oxytocin experiences an up-field shift of 2.7 ppm upon binding to NPII and to NPI. The  $\alpha$ -amino group of free oxytocin has a  $pK_a$  of 6.3 (Breslow et al., 1971), while studies have indicated that, when oxytocin is bound to neurophysin this group has a  $pK_a$  which is higher (Camier et al., 1973). Thus, at pH 6.6 (the pH of most of our studies), this group would go from a largely unprotonated to a protonated state on binding. In free oxytocin, this causes a chemical-shift change of about 1 ppm for the  $\alpha$  carbon (Walter et al., 1973). The large upfield shift for the half-cystine-1  $\alpha$  carbon on binding of **2** to NPI and NPII is consistent with this altered ionization state, but the reason for the additional chemical shift cannot be unambiguously determined. If as postulated (Ginsberg and Ireland, 1964), the  $\alpha$ -amino group is interacting with a negatively charged carboxyl group of neurophysin, the close proximity of the two groups could cause the shift of the  $\alpha$  carbon to differ from that observed in the unbound state. Alternatively, the oxytocin backbone could assume a different conformation when bound to neurophysin than when free in solution and this different conformation could affect the chemical shift. The shift is also consistent with the suggestion (Breslow et al., 1971) that the N-terminal region of oxytocin interacts with neurophysin to form a hydrophobic pocket, since a change in the dielectric constant of the medium surrounding the  $\alpha$  carbon would be expected to change its chemical shift.

*Stoichiometry of Binding.* Breslow et al. (1973) have determined that one oxytocin molecule is bound per neurophysin molecule (10 000 daltons) and Nicolas et al. (1976) have also found this (except at conditions of high salt). Glasel et al. (1976) found one strong site per 10 000 daltons and also found evidence for a much weaker site ( $K_1/K_2 > 100$ ). Our results also imply only one binding site per neurophysin. For both the cysteine and glycinamide enriched positions, addition of more than equimolar oxytocin results in a resonance which has shift and line-width characteristics identical with free oxytocin. With cysteine, the free resonance is directly observable, while with glycinamide the widths of free and bound forms are averaged, leading to a narrower line with excess hormone.

**Exchange Rates of Labeled Carbons.** Perhaps the most interesting finding of this study is that the exchange rate of oxytocin with neurophysin and the bulk solution appears to differ depending on which carbon atom is monitored. When the  $^{13}\text{C}$ -enriched  $\alpha$  carbon of glyciamide-9 in the hormones is monitored, fast exchange is observed under all the conditions examined and an exchange rate  $>300 \text{ s}^{-1}$  at  $15^\circ\text{C}$  is determined. The exchange rate at physiological temperature ( $37^\circ\text{C}$ ) appears to be  $>1000 \text{ s}^{-1}$ . On the other hand, when the  $\alpha$  carbon of the half-cystine-1 residue is observed, slow exchange is present, and an exchange rate  $<15 \text{ s}^{-1}$  at  $37^\circ\text{C}$  is calculated. We believe the slower exchange rate represents the macroscopic off rate for oxytocin from neurophysin, while the rate observed with **1** represents an additional microscopic process(es). A number of studies have suggested (for reviews, see Breslow, 1975; Cohen et al., 1975) that the major portion of the binding energy for the hormone in neurophysin-oxytocin (vasopressin) interaction is derived from the interaction of its first three residues (Cys-Tyr-Ile or Cys-Tyr-Phe). This part of the hormone is presumably held firmly to the neurophysin. However, as indicated by the studies reported here as well as others (Blumenstein and Hruby, 1976; Glasel et al., 1973), the glyciamide-9 residue is not so firmly held. Our data, therefore, suggest the following interpretation. Some of the time only the N-terminal region of oxytocin is interacting with neurophysin, and the glyciamide-9 residue does not sense being bound. However, for some of the time another part of the bound oxytocin has interaction with neurophysin, and during this time the motion of the glyciamide-9 residue is restricted and the residue is now in the "bound" state. This interaction is weaker than that involving the N-terminal residue of oxytocin and occurs with a much more rapid "off" rate. This off rate is greater than  $1000 \text{ s}^{-1}$  at physiological temperatures, and since this is the process which controls when the glyciamide-9 goes from the "bound" or broadened state to the "free" or unbroadened state, the glyciamide-9 residue gives the appearance of fast exchange. For the half-cystine-1 residue, whenever the hormone is bound, the  $^{13}\text{C}$  resonance is chemically shifted and broadened, and the exchange time is governed by the overall off rate of oxytocin from neurophysin, (less than  $15 \text{ s}^{-1}$  under our conditions). We have carried out preliminary experiments with oxytocin labeled in the carbonyl (C-1) carbon of the half-cystine-1 residue, and the  $\alpha$  carbon (C-2) of tyrosine-2 (Blumenstein, Hruby, Yamamoto, and Young, unpublished) and both of these positions also exhibit slow exchange.

Recently (Goetze and Richards, 1977) an NMR study of phosphorylcholine interaction with mouse myeloma protein M603 was done, and different exchange rates were deduced for the phosphate and the choline methyls. It was postulated that the phosphate group, which is not bound as deeply in the binding cleft as the choline moiety, experiences exchange with bound and free environments at a more rapid rate than the more tightly bound choline methyl groups.

Our finding of a slow exchange rate between oxytocin and neurophysin agrees with the data of Pearlmuter and McMains (1977), who determined off rates of  $10-25 \text{ s}^{-1}$  at pH 7.4. At pH 6.6 (our studies) where the binding is stronger, the exchange rate might be somewhat slower. Also Balaram et al. (1973) suggested that the exchange rate of lysine vasopressin with neurophysin was slow and determined an upper limit of  $125 \text{ s}^{-1}$ . There have been two studies suggesting a rapid rate of exchange between oxytocin and neurophysin. In one case, a proton NMR study was conducted at pH 6.75 (Alazard et al., 1974) under analogous conditions to those reported here. These authors suggested that the neurophysin-oxytocin system

exhibited fast exchange. In the latter study (as in the above study of Balaram et al. (1973)), a large excess (usually 10-fold) of hormone was present at all times. A broadening of the observed proton resonances from  $5.1 \text{ Hz}$  to  $5.8 \text{ Hz}$  on going from  $33$  to  $8^\circ\text{C}$  was seen, and from these and other data it was concluded, using the treatment of Sykes et al. (1974), that fast exchange was present. However, this increased line width at low temperature may have been due to an increase in the viscosity of the solution. Since the line width of the hormone resonances in the absence of protein is  $3.5 \text{ Hz}$  at  $18^\circ\text{C}$ , and the viscosity of water increases by over 80% in going from  $33$  to  $8^\circ\text{C}$ , the small increase in line width in the presence of protein does not provide clear evidence of fast exchange. If the line width in the absence of protein is negligible compared with the width in the presence of protein, viscosity effects will not be significant. However, in the study of Alazard et al. (1974), the line width of the free hormone is about 60% of the line width observed in the presence of protein, so neglect of viscosity effects is not justified.

In a second case where fast exchange has been suggested, a deuterium NMR study (Glasel et al., 1973) was made at pH 2.5 and an exchange rate of  $>300 \text{ s}^{-1}$  was suggested. However, at this pH, the binding constant is much lower (Camier et al., 1973) than at pH 6.6, and while part of this weaker binding is due to a slower on rate, a faster off rate is also likely.

In comparing our  $^{13}\text{C}$  NMR studies with the  $^1\text{H}$  NMR studies of Balaram et al. (1973) and Alazard et al. (1974), and the  $^2\text{H}$  NMR studies of Glasel et al. (1973), only in the  $^{13}\text{C}$  NMR work can the fully bound resonance be directly observed. In the  $^{13}\text{C}$  studies, as long as the bound resonance of the hormone has a chemical shift or line width which differs from that of the free resonance, the exchange rate which a given atom experiences can easily be determined by addition of a small excess (0.4-1.0 equiv) of hormone, since fast exchange leads to a single peak whose NMR parameters are a weighted average of free and bound forms, while slow exchange leads to two peaks, one due to bound hormone, and the other due to free hormone. The difficulty in obtaining the exchange rates when the observed resonance is due either totally or mostly to the free hormone is well illustrated by the studies of Balaram et al. and of Alazard et al., since in both studies the same resonance was monitored (the aromatic protons of the tyrosine-2 residue) and yet opposite conclusions were reached. Our study also illustrates the need to monitor resonances from atoms in different parts of the hormone, especially a flexible hormone such as oxytocin, since different atoms in the molecule "sense" different binding and dynamic processes.

The differing exchange rates of the glyciamide-9 and half-cystine-1  $\alpha$ -carbons are independent of temperature and concentration, and the 2.7 ppm chemical shift of the half-cysteine-1  $\alpha$ -carbon upon binding was observed under all conditions. Thus, while line width measurements indicate that the protein-hormone complex undergoes aggregation to sizes larger than dimers, this aggregation does not significantly affect the interaction between the hormones and the neurophysins in this part of the molecule. The aggregation may well be significant in the packaging of the protein-hormone complex in the neurosecretory granules.

#### Acknowledgments

We thank Dr. David Wright, Department of Chemistry, University of Arizona, for the amino acid analyses, and Dr. Mac E. Hadley, Department of Cell and Developmental Biology, University of Arizona, for the milk-ejecting activities.

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