slightly with heating rate, however. This relationship will hold for calcium ion concentrations which are sufficiently low so there is not much binding of calcium ion to the E′ form.

This analysis assumes that $\Delta H_{eff}$ and $\Delta S_{eff}$ are not strongly temperature dependent. This implies that there are not large heat capacity changes associated with the conformational change and ion binding steps.

For changes in melting temperature with ion concentration, the differentiated form of eq 5 can be used (eq 6). For small changes in melting temperature from an initial melting temperature, $T_m^0$, caused by a change in calcium ion concentration from $Ca^0$ to Ca, eq 7 applies. These arguments can be easily expanded to include the thermal stabilization induced by the binding of any other ions by replacing $x$ in these equations with the number of other ions which are coupled to the transition. Similarly $\Delta H_{eff}$ and $\Delta S_{eff}$ would become modified to include the $\Delta H$ and $\Delta S$ of binding of the ion.

$$-(dT_m/T_m^2) = (xR/\Delta H_{eff})d \ln [Ca] \quad (6)$$

$$T_m - T_m^0 = \Delta T_m = \frac{-xT_m^2R}{\Delta H_{eff}} \ln \frac{[Ca]}{[Ca^0]} \quad (7)$$

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# $^1$H Nuclear Magnetic Resonance Double Resonance Study of Oxytocin in Aqueous Solution†

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ABSTRACT: Peptide NH resonances in the 250 MHz $^1$H nuclear magnetic resonance (NMR) spectrum of oxytocin in $H_2O$ were assigned to specific amino acid residues by the "underwater decoupling" technique (i.e., decoupling from corresponding $C^\alpha H$ resonances, which are buried beneath the intense water peak). These experiments confirm previous assignments of A. I. Brewster and V. J. Hruby ((1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3806) and A. F. Bradbury et al. ((1974), *FEBS Lett. 42,* 179). Three methods of assigning NH resonances of peptides—solvent titration, underwater decoupling, and isotopic labeling—are compared. As the solvent composition is gradually changed from dimethyl sulfoxide to $H_2O$, oxytocin undergoes a conformational change at 70–90 mol % of $H_2O$. Exposure to solvent of specific hydrogens of oxytocin in $H_2O$ was studied by monitoring intensity changes of solute resonances when the solvent peak was saturated. Positive nuclear Overhauser effects (NOE's) of 14 ± 5 and 9 ± 5 were observed for the Tyr ortho CH and meta CH resonances, respectively. Comparative studies with deamino-oxytocin indicate that these effects result predominantly from intermolecular dipole–dipole interaction between aromatic side chain CH protons and protons of the solvent. The NOE's therefore indicate intimate contact between water and the aromatic CH hydrogens of the Tyr side chain. The extent of saturation transferred by proton exchange between water and NH groups varies with pH in a manner which appears to reflect the acid–base catalysis of the protolysis reaction. There is no indication that any NH protons are substantially shielded from the solvent.

Oxytocin [*cyclo*(Cys$^1$-Tyr$^2$-Ile$^3$-Gln$^4$-Asn$^5$-Cys$^6$)-Pro$^7$-Leu$^8$-Gly$(NH_2)^9$], a neurohypophyseal hormone, has, in addition to its physiological activity of stimulating the ejection of milk from mammary tissue, a broad range of well-defined biological properties (Sawyer and Knobil, 1974). Determination of the free solution conformation of oxytocin in water and in less polar solvents serves as a starting point for elucidation of the molecular mechanism of interaction of this hormone with its intracellular carrier protein neurophysin (for a recent symposium on this subject see *N.Y. Acad. Sci. 248,* 1975) and, eventually, also the mechanism of its interaction with oxytocin receptors, which are believed to be located on the outer surface of cell membranes (Schwartz and Walter, 1973). $^1$H nuclear magnetic resonance (NMR) studies of peptides are most readily performed in solvents such as $(CD_3)_2SO$, in which identifica-

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tion of the conformationally significant peptide NH resonances can be accomplished in a relatively straightforward manner by double resonance techniques (Stern et al., 1968; Urry and Ohnishi, 1970; Bovey et al., 1972). Most of the early $^1H$ NMR studies of neurohypophyseal hormones were performed with this solvent: oxytocin (Johnson et al., 1969; Urry et al., 1970; Walter et al., 1972; Brewster et al., 1973), lysine-vasopressin (Walter, 1971; Von Dreele et al., 1971a,b; Walter et al., 1972; Glickson et al., 1972a), arginine vasopressin, and arginine vasotocin (Walter et al., 1974a).

On the basis of $^1H$ NMR data, Urry and Walter (1971) have proposed a model for the preferred conformation of oxytocin in $(CD_3)_2SO$ solution, which has proven consistent with subsequent studies by $^1H$ NMR (Walter et al., 1972: Brewster et al., 1973), $^{13}C$ NMR (Walter et al., 1974b; Griffin et al., 1975), and potential energy calculations (Kotelchuck et al., 1972; Brewster et al., 1973). An important feature of this oxytocin model is a $\beta$ turn in the cyclic moiety involving the sequence Tyr-Ile-Gln-Asn, and a second hairpin turn involving the C-terminal sequence (Cys-Pro-Leu-Gly). Additional structures have been proposed on the basis of potential energy calculations and $^1H$ NMR studies (Brewster et al., 1973; Honig et al., 1973,), but these additional conformational states probably do not contribute significantly to the average conformation of oxytocin in $(CD_3)_2SO$ (Walter et al., 1974b; Glickson, 1975).

Extension of conformational studies of oxytocin to aqueous solution by $^1H$ NMR (Feeney et al., 1971; Glickson et al., 1972b; Brewster and Hruby, 1973; Bradbury et al., 1974) and by NMR of other nuclei (Glasel et al., 1973; Walter et al., 1973; Deslauriers et al., 1974a,b; Urry et al., 1974; Griffin et al., 1975) is the next objective. A major obstacle in $^1H$ NMR studies in $H_2O$ has been the difficulty of assigning peptide NH resonances. Three methods have been applied to oxytocin. (1) The "solvent transition" method correlates low field peptide NH resonances in $H_2O$ with previously assigned resonances in $(CD_3)_2SO$. Chemical shifts of oxytocin peaks are monitored while the solvent composition is changed from $(CH_3)_2SO$ to $H_2O$ in small increments (Von Dreele et al., 1972; Glickson et al., 1972b). (2) The "underwater decoupling" method (Dadok et al., 1972; Von Dreele et al., 1972) identifies peptide NH resonances coupled to specific $C^{\alpha}H$ hydrogens. Blind decoupling of $C^{\alpha}H$ resonances buried beneath the intense $H_2O$ peak is accomplished after the positions of these resonances have been determined from experiments in $D_2O$ solution. Peptide NH assignments of oxytocin obtained by Brewster and Hruby (1973) employing underwater decoupling differ substantially from those determined by Glickson et al. (1972b) using the solvent transition method. (3) The "isotope labeling" method involves detecting changes in spin coupling patterns resulting from selective isotopic replacement of peptide nitrogens ($^{14}N \rightarrow {}^{15}N$) and $C^{\alpha}H$ hydrogens ($^1H \rightarrow {}^2H$). Assignments of Tyr, Ile, and Leu NH resonances of oxytocin obtained by isotopic labeling (Bradbury et al., 1974) agree with the results of Brewster and Hruby (1973).

The initial objective of the present study is to assign all low field spectral resonances of oxytocin in $H_2O$, and to compare the ease, applicability, and reliability of the three aforementioned techniques for identifying peptide NH resonances with specific residues in $H_2O$. These assignments then serve as a basis for elucidating the aqueous conformation of oxytocin by $^1H$ NMR, and for comparing it with the structure of this hormone in $(CD_3)_2SO$. An important aspect of such a conformational analysis is determining which specific hydrogens of oxytocin are accessible to solvent and which are inaccessible as a result of intramolecular interactions. Four methods have been employed to measure solvent exposure of peptides: (1) rates of NH proton replacement by hydrogen isotopes (Hvidt and Nielsen, 1966; Molday et al., 1972; Stern et al., 1968), (2) temperature dependence of chemical shifts of NH resonances (Kopple et al., 1969; Ohnishi and Urry, 1969), (3) dependence of NH chemical shifts on the composition of a suitable solvent mixture (Pitner and Urry, 1972a,b; Kopple and Schamper, 1972a; Walter and Glickson, 1973), and (4) the degree of resonance broadening in the presence of a paramagnetic substance (Kopple and Schamper, 1972b). Each method has limitations. Deuterium exchange of oxytocin in $D_2O$ is very rapid and therefore difficult to monitor by NMR. Interpretation of temperature coefficients of chemical shifts in water is uncertain. The conformation of oxytocin may change with solvent composition. Paramagnetic ions often associate preferentially with water and may bind to specific sites on the hormone.

For these reasons we have been employing a technique, the solvent saturation method, which is applicable to peptides in aqueous solution and which reflects solvent exposure of NH and certain CH hydrogens[1] while not significantly altering molecular structure. The method, which involves monitoring changes in resonance intensity of specific solute peaks, has been applied to AII'[2] in $H_2O$ (Pitner et al., 1974) and to gramicidin S in a variety of organic solvents (Pitner et al., 1975). Rapidly exchanging (exposed) NH hydrogens experience transfer of saturation (decreased peak intensity) when labile hydrogens of the solvent are saturated (Von Dreele et al., 1972; Glickson et al., 1974; Waelder et al., 1975). NOE's (usually positive, i.e., increased intensity) are observed for solute CH resonances. It will be demonstrated that these NOE's result primarily from direct dipole–dipole interactions involving certain CH hydrogens in intimate contact with irradiated protons of the solvent. The solvent saturation experiment therefore provides a sensitive method for delineating the extent of solvation of NH protons and certain CH protons.

## Experimental Procedure

*Materials.* Oxytocin was prepared by the method of Hase and Walter (1973) using symmetrically substituted cystines as S-protecting groups and possessed an avian vasopressor activity of about 500 U/mg. Crystalline deamino-oxytocin (Ferrier et al., 1965) had an avian vasopressor activity of about 940 U/mg. $(CH_3)_2SO$ (Eastman Kodak Co., Rochester, N.Y.) was used without further purification. Water was deionized and glass redistilled. The pH was ad-

[1] Intermolecular NOE's will probably not be observed when the resonance originates from a hydrogen bonded to a carbon which has more than one proton directly attached to it. These hydrogens relax each other more efficiently than they would relax by dipolar interaction with solvent protons. In studies of peptides this effectively limits the solvent saturation technique primarily to aromatic CH protons, since with methods presently in use the $C^{\alpha}H$ hydrogens are obscured by the $H_2O$ peak.

[2] Abbreviations used are: deamino-oxytocin, [1-$\beta$-mercaptopropionic acid]oxytocin; AII', [Asn$^1$,Val$^5$]angiotensin; NOE, nuclear Overhauser effect; DSS, 2,2-dimethylsilapentane-5-sulfonate sodium salt; TSP, sodium $\beta$-trimethylsilyltetradeuteriopropionate. All other abbreviations are in accord with the IUPAC–IUB convention ((1972), *J. Biol. Chem. 247*, 977).

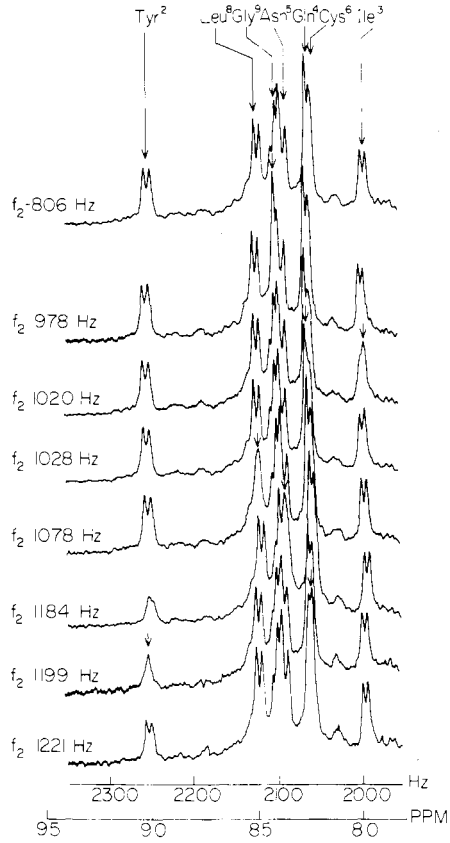


FIGURE 1: Underwater decoupling experiments to assign oxytocin (5% w/v, pH 3.8, 30 °C) peptide NH resonances in $H_2O$ (80 scans/spectrum in the correlation mode at 250 MHz). Decoupling rf power has been applied at the indicated frequencies ($f_2$) relative to internal DSS.

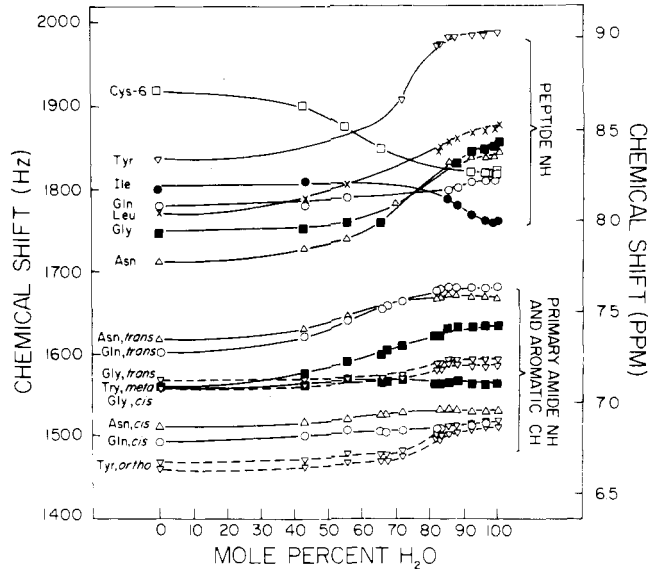


FIGURE 2: Chemical shifts of low field $^1H$ NMR resonances of oxytocin (220 MHz) as the solvent composition is progressively changed from $(CH_3)_2SO$ to $H_2O$ (24 °C). The data of Glickson et al. (1972) have been replotted in accord with corrected assignments of peptide NH resonances in $H_2O$ of Brewster and Hruby (1973).

justed with HCl and NaOH. Aqueous solutions of oxytocin and deamino-oxytocin were prepared by dissolving the lyophilized sample in 50 $\mu$l of glacial acetic acid, adding 300 $\mu$l of $H_2O$, and adjusting the pH.

*Methods.* Spectral measurements were performed at 250 MHz (Dadok et al., 1970) in the correlation mode (Dadok and Sprecher, 1974; Gupta et al., 1974; 1.6 sec/scan; frequency sweep) at ambient temperature, 30 ± 1 °C. The underwater decoupling experiments were performed as previously described (Dadok et al., 1972; Brewster and Hruby, 1973; Glickson et al., 1974). All chemical shifts in $H_2O$ are referred to the methyl resonance of the internal standard, DSS (Merck, Sharp and Dohme, Montreal, Canada). The study of the pH dependence of oxytocin spectra was carried out with the secondary internal standard TSP, whose methyl resonance is 0.015 ppm to high field of the DSS methyl peak (chemical shifts corrected to the DSS scale). Chemical shifts in dimethyl sulfoxide are referred to internal DSS ("solvent transition" study) or $Me_4Si$ (all other measurements in dimethyl sulfoxide).

## Results and Discussion

*Assignment of Resonances.* The peptide NH region of the 250-MHz spectrum of oxytocin in $H_2O$ solution (pH 3.8, 30 °C) appears in Figure 1. Decoupling rf power has been applied at specific frequencies ($f_2$) relative to internal DSS. The top spectrum in Figure 1 was obtained with off-resonance irradiation ($f_2$ = −806 Hz; i.e., 806 Hz to high field of the DSS methyl peak; this region of the spectrum contains no resonances). Each of the peptide NH resonances of oxytocin displays its characteristic doublet spin coupling pattern, except for the Gly resonance at 2100 Hz (8.40 ppm), which is a triplet as a consequence of coupling to two vicinal CH hydrogens. In the remaining spectra in Figure 1 the decoupling rf was applied successively at each of the $C^\alpha H$ resonance frequencies, which had previously been identified by Brewster and Hruby (1973) by decoupling experiments in $D_2O$ solution. Decoupling of each $C^\alpha H$ peak results in collapse of the corresponding vicinal peptide NH peak to a singlet. In this manner each of the NH peaks was identified with a particular residue of the hormone. These assignments agree with those previously reported by Brewster and Hruby (1973). The decoupling experiments are presented here because of the critical importance of these assignments and because these experiments illustrate the unequivocal manner in which peptide NH resonances can be identified by the underwater decoupling technique when the $C^\alpha H$ peaks are well resolved and unambiguously assigned.

The solvent transition data ($(CH_3)_2SO \rightarrow H_2O$) previously obtained (Glickson et al., 1972b) have been replotted using the corrected assignments of peptide NH resonances in water (Figure 2). In the previous study extensive overlap of peptide NH resonances of oxytocin near 80 mol % $H_2O$ led to erroneous assignments, but correct assignments were obtained with the more clearly resolved aromatic CH and primary amide NH peaks. As previously noted, simultaneous perturbation of peptide NH, primary amide NH, and aromatic CH resonances between 70 and 90 mol % $H_2O$ indicates a probable conformational change. Table I summarizes chemical shifts ($\delta$), coupling constants ($J_{N\alpha}$), and temperature coefficients ($d\delta/dT$) of oxytocin NH resonances; many of these parameters are altered in the course of the solvent transition. Spectral parameters of peptide NH resonances measured in different laboratories are in agreement; a small discrepancy exists in $d\delta/dT$ values of primary amide NH peaks in $(CD_3)_2SO$ as measured by Walter et al. (1972) and by Brewster et al. (1973). Most prominent is the change in $d\delta/dT$ of the Asn peptide NH, which is very small in $(CD_3)_2SO$ but quite large in $H_2O$.

Table I: Chemical Shifts ($\delta$) at 22 + 1 °C, Temperature Dependence ($d\delta/dT$) of Chemical Shifts, and $J_{N\alpha}$ of NH Resonances of Oxytocin.

| | $(CD_3)_2SO$ | | | $H_2O$ | | |
|---|---|---|---|---|---|---|
| Peptide NH | $\delta$ (ppm) | $-10^3 \times d\delta/dT$ (ppm/°C) | $J_{N\alpha}$ (Hz) | $\delta$ (ppm) | $-10^3 \times d\delta/dT$ (ppm/°C) | $J_{N\alpha}$ (Hz) |
| Tyr-2 | 8.35 ± 0.05[b] | 8.0 ± 4.5,[b] 6[d] | 7[d] | 8.99,[a] 9.03,[e] 9.04[c] | 5.5,[a] 6.5[e] | 7.5,[a] 7.2 ± 0.1,[c] 6.5[e] |
| Ile-3 | 8.21[b] | 9.8,[b] 9.5[d] | 4.4 ± 0.5,[b] 5[d] | 7.94,[a] 7.99,[e] 8.01[c] | 7.6,[a] 7.0[e] | 6.5,[a] 5.7 ± 0.3,[c] 6[e] |
| Gln-4 | 8.10[b] | 4.8,[b] 4[d] | 6.6 ± 0.5,[b] 6[d] | 8.20,[a] 8.23,[e] 8.26[c] | 4.3,[a] 5.5[e] | 4.0,[a] 3.7 ± 0.5,[c] 4[e] |
| Asn-5 | 7.79[b] | 0.23,[b] 0[d] | 6.1 ± 0.2,[b] 6[d] | 8.32,[a] 8.35,[e] 8.38[e] | 7.3,[a] 5.0[e] | 7.4,[a] 8.6 ± 0.3,[c] 8[e] |
| Cys-6 | 8.74[b] | 11.1,[b] 11.5[d] | 7.5 ± 0.1,[b] 7.5[d] | 8.19,[a] 8.23,[e] 8.24[c] | 5.0,[a] 5.5[e] | 7.0,[a] 7.0 ± 0.2,[c] 7[e] |
| Leu-8 | 8.06[b] | 6.8,[b] 7[d] | 7.3 ± 0.2,[b] 7.5[d] | 8.47,[a] 8.50,[e] 8.52[c] | 10.8,[a] 9.5[e] | 6.5,[a] 5.5 ± 0.1,[c] 6.5[e] |
| Gly($NH_2$)-9 | 7.96[b] | 5.9,[b] 6.5[d] | 5.6 ± 0.2,[b] 5[d] (av) | 8.37,[a] 8.39,[e] 8.42[c] | 8.6,[a] 7.0[e] | 5.4,[a] 6.1 ± 0.5,[c] 5.5[e] (av) |
| Primary amide NH | | | | | | |
| Asn, trans | 7.40[b] | 4.5,[b] 2.5[d] | | 7.60,[c] 7.64[e] | 6.5[e] | |
| Gln, trans | 7.29[b] | 5.0,[b] 2.5[d] | | 7.65,[c] 7.59[e] | 6[e] | |
| Gly($NH_2$), trans | 7.12 ± 0.02[b] | 5.2 ± 2.2,[b] 2.5[d] | | 7.44,[c] 7.39[e] | 4.5[e] | |
| Gly($NH_2$), cis | 7.09 ± 0.02[b] | 5.7 ± 2.2,[b] 3[d] | | 7.11,[c] 7.06[e] | 6[e] | |
| Asn, cis | 6.88[b] | 5.7,[b] 3[d] | | 6.96,[c] 6.90[e] | 6[e] | |
| Gln, cis | 6.80[b] | 5.7,[b] 3[d] | | 6.91,[c] 6.84[e] | 5[e] | |

[a] Data of Feeney et al. (1971), employing spectral assignments of Brewster and Hruby (1973). [b] Data of Walter et al. (1972) corrected to 22 °C. [c] Data of Glickson et al. (1972[b]) at 24 °C corrected to 22 °C employing temperature coefficients of Brewster and Hruby (1973). Peptide NH assignments modified according to Brewster and Hruby (1973). [d] Brewster et al. (1973). Assignments of peptide NH resonances of Ile[3] and Gln[4] by these authors have been interchanged for reasons discussed by Walter et al. (1974a). Otherwise the assignments of Brewster et al. (1973) agree with those of Johnson et al. (1969) and Walter et al. (1972). [e] Brewster and Hruby (1973). Chemical shifts at 28 °C corrected to 22 °C.

This suggests that the hydrogen bond involving the Asn peptide NH (Urry and Walter, 1971) is weakened or ruptured in the course of the transition from dimethyl sulfoxide to water. Changes in $J_{N\alpha}$ of Ile, Gln, and Asn further suggest some perturbation of the backbone orientation of the cyclic moiety of oxytocin.

These experiments illustrate the capabilities and limitations of the three techniques available for assigning NH resonances. Isotopic substitution is expected to be the most reliable procedure; however, this method is time consuming, limited to available isotopically labeled amino acids, and still can lead to incorrect assignments (e.g., Brewster et al., 1973; Griffin et al., 1975). Fortunately, double resonance techniques, even though more difficult to apply in water than in nonprotic solvents, yield equivalent results for peptide NH resonances. In view of the agreement of oxytocin peptide NH assignments determined by underwater decoupling with limited assignments obtained by isotopic enrichment, the decoupling procedure appears adequate, even for molecules of moderate structural complexity. The solvent transition method has been successfully applied to various molecules such as gramicidin S (Pitner and Urry, 1972a), stendomycin (Pitner and Urry, 1972b), various cyclic peptides (see Kopple and Schamper, 1972a, and references therein), various component peptides of luteinizing hormone-releasing hormone (Wessels et al., 1973), and lysine-vasopressin (Glickson et al., 1972b).[3]

Advantages of the solvent transition method over decoupling procedures include: (1) applicability to NH resonances which do not exhibit coupling to vicinal CH protons, (2) ability to indicate conformational changes which accompany solvent transitions, and (3) ability to distinguish between buried and exposed NH hydrogens when extensive conformational changes do not accompany the change in solvent (Pitner and Urry, 1972a; Kopple and Schamper, 1972a). Clearly the combined use of double resonance and solvent transition techniques is desirable whenever possible.

*Solvent Saturation.* The low-field region of the $^1H$ NMR spectrum of oxytocin at pH 3.80 is shown in Figure 3a. Saturating rf power has been applied far off resonance (4000 Hz to low field of the $H_2O$ peak) to correct for nonspecific changes in spectral intensity which may accompany double irradiation. Figure 3b shows the same region of the oxytocin spectrum when the water peak is saturated. Changes in spectral intensity are most readily detected and measured in the difference spectrum shown in Figure 3c. Positive peaks in the difference spectrum correspond to increased spectral intensity, whereas negative peaks indicate decreased intensity. The Tyr ortho CH and meta CH resonances exhibit positive NOE's of 14 ± 5 and 9 ± 5 %, respectively.[4] The intensity of the Tyr peptide NH is diminished by 57 ± 3 %. This decreased intensity results predominantly from transfer of saturation from water protons. Dipolar interactions and exchange modulation of scalar coupling with the $C^\alpha H$ protons (decreased intensity) may also modify the resonance intensity of this peak to a small extent (Noggle and Schirmer, 1971). The complex pattern near 2100 Hz results from decoupling of the Asn peptide NH, whose $C^\alpha H$ at 1078 Hz is only 12 Hz away from the irradiated $H_2O$ resonance at 1196 Hz. Gibbons et al. (1975) have observed similar effects in double resonance difference spectra involving $C^\alpha H$ and $C^\beta H$ protons of tyrocidine A. The Cys[6] $C^\alpha H$ at 1028 Hz is further removed from the $H_2O$ peak and may experience a small amount of rf power. Spin tickling may be responsible for the pattern of the Cys[6] peptide NH peak in the difference spectrum (Figure 3c). Definitive interpretation of the complex patterns associated with the Asn and Cys[6] peptide NH peaks would require additional experiments; these effects, however, are not of primary interest to this study.

Interpretation of the NOE's exhibited by the Tyr CH hy-

[3] The assignments of Glickson et al. (1972b) agree with those of Von Dreele et al. (1972) except for interchange of the Phe[3] and Cys[6] peptide NH resonances.

[4] Because of uncertainties in the measurement of NOE's, quantitative interpretation of these values should not be attempted.

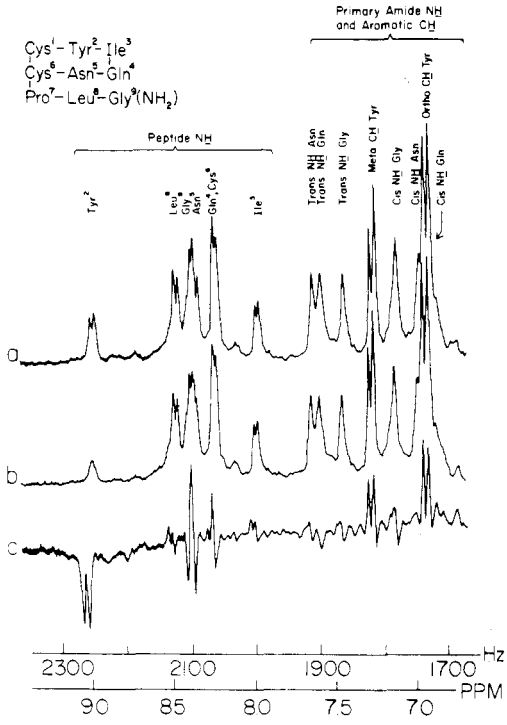


FIGURE 3: Solvent saturation study of oxytocin (5% w/v) in $H_2O$ (pH 3.8, 30 °C, 80 scans/spectrum accumulated in the correlation mode at 250 MHz): (a) spectrum accumulated with saturating rf power applied off-resonance 4000 Hz to low field of the $H_2O$ resonance (control experiment), (b) spectrum accumulated with $H_2O$ resonance (at 1196 Hz) saturated, and (c) difference spectrum (b − a amplified 2.68 times).

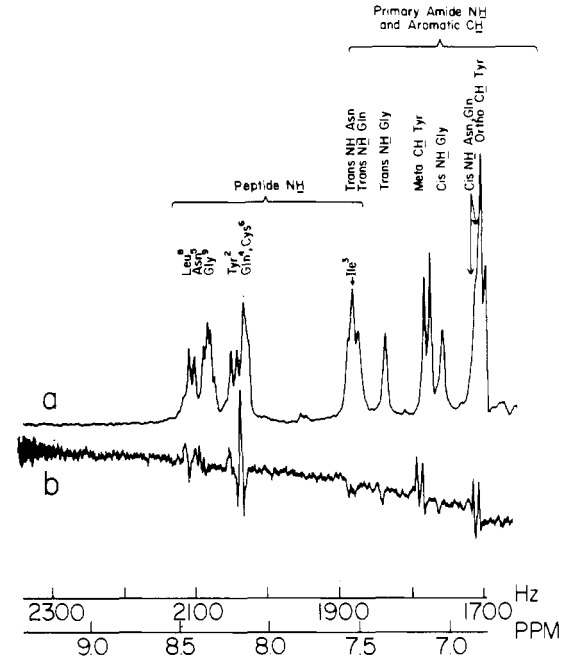


FIGURE 4: Solvent saturation study of deamino-oxytocin (1.7% w/v) in $H_2O$ (pH 3.8, 30 °C, 200 scans/spectrum accumulated in the correlation mode at 250 MHz): (a) off-resonance irradiated spectrum (4000 Hz to low field of the $H_2O$ peak), and (b) amplified difference spectrum (i.e., spectrum a substracted from spectrum accumulated while saturating the $H_2O$ peak).

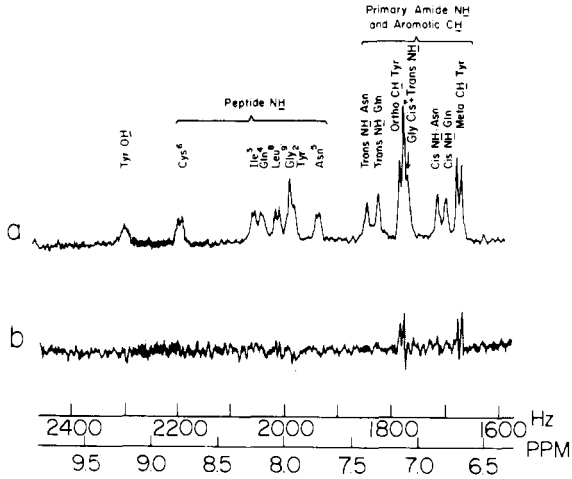


FIGURE 5: Solvent saturation study of deamino-oxytocin (1.7%) in $(CH_3)_2SO$ (30 °C, 200 scans/spectrum accumulated in the correlation mode at 250 MHz): (a) off-resonance irradiated spectrum (4000 Hz to low field of the $(CH_3)_2SO$ peak), and (b) amplified difference spectrum (i.e., spectrum a subtracted from spectrum accumulated with the solvent $CH_3$ peak saturated).

drogens depends on the detailed mechanism of relaxation of these protons (Noggle and Schirmer, 1971). In a recent study of AII′ (Pitner et al., 1974) a positive NOE of 12 ± 3 % was observed for the Tyr ortho CH resonance when the $H_2O$ peak was saturated; no significant change in spectral intensity was observed for the Tyr meta CH resonance of this peptide. It was noted that such NOE's result either from direct intermolecular dipole-dipole interaction between the CH and $H_2O$ protons or from intramolecular dipole-dipole interaction between the CH hydrogen and rapidly exchanging hydrogens of the hormone which have experienced substantial transfer of saturation. Distinction between an intermolecular and intramolecular mechanism is necessary in order to determine whether the NOE's serve as a direct index of exposure to solvent or as an index of proximity to rapidly exchanging solute hydrogens.

The observation of comparable NOE's for ortho CH and meta CH hydrogens of the Tyr residue of oxytocin (Figure 3) suggests that the intermolecular dipole-dipole interaction of the meta CH hydrogens with protons of the solvent is the dominant cause of the observed NOE's. Because of the inverse sixth power dependence of intramolecular dipole-dipole interactions on internuclear distance, dipolar interaction of phenolic OH hydrogens with the meta CH protons would be negligible in comparison with interactions involving more proximal ortho CH protons.

However, inspection of a space filling model of oxytocin suggests that intramolecular dipole-dipole interaction of the $Tyr^2$ meta CH hydrogens with two groups of at least partially saturated protons is possible—with the N-terminal $Cys^1$ amino hydrogens, which exchange too rapidly with the solvent to yield observable resonances, and with the Tyr peptide NH proton, which is partially saturated (Figure 3) as a result of rapid exchange with the solvent (its exchange rate is catalyzed by proximity to the $\alpha$-$NH_3^+$ group). Both of these intramolecular sites of saturation are absent in deamino-oxytocin. The spectral similarity between oxytocin and this analogue (compare Figures 3a and 4a) indicates that the conformations of these molecules in water are very similar. The only significant differences in their spectra are the higher field positions of the Tyr and Ile NH peaks in the spectrum of the deamino derivative. Both shifts can be ascribed predominantly to the deshielding effect of the positive charged amino group (Deslauriers et al., 1974a). That oxytocin and its deamino derivative have similar conformations in other solvents has previously been demonstrated

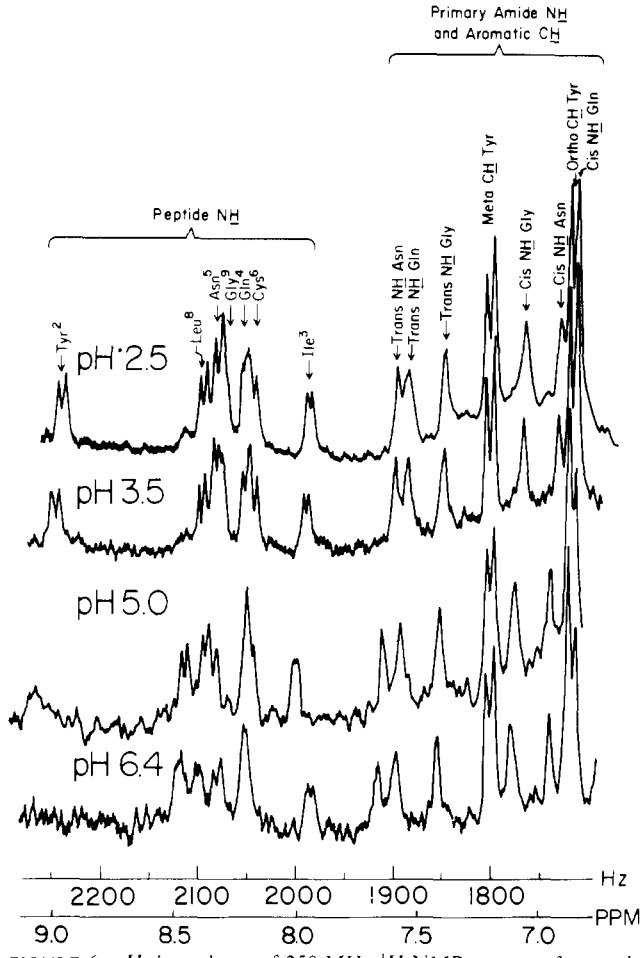


FIGURE 6: pH dependence of 250 MHz $^1$H NMR spectra of oxytocin (2% w/v in $H_2O$, 30 °C, 200 scans/spectrum accumulated in the correlation mode).

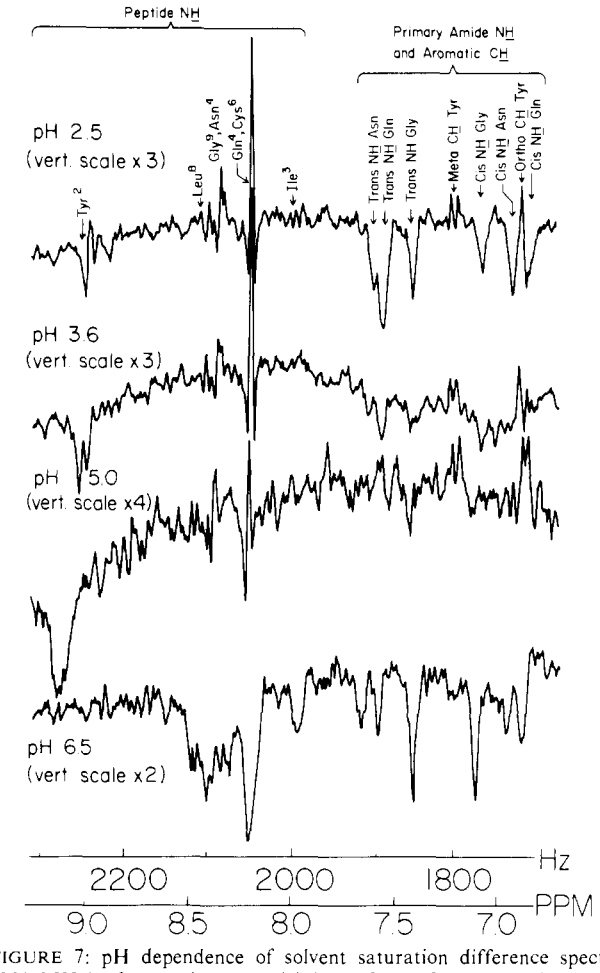


FIGURE 7: pH dependence of solvent saturation difference spectra (250 MHz) of oxytocin (3% w/v) in $H_2O$ (30 °C). At each pH the spectrum accumulated with $H_2O$ irradiated minus the off-resonance irradiated spectrum (rf irradiation 4000 Hz to low field of the $H_2O$ peak) is shown. The on-resonance and off-resonance irradiated spectra each consist of 800 scans accumulated in the correlation mode in four blocks of 200 scans. This was done to minimize field drift when the spectrometer was operated unlocked. A special program was employed to shift data points of the on-resonance and off-resonance irradiated spectra to assure optimum alignment between the two sets of spectra. This procedure also effectively removed any Bloch Segert shifts (which were very small).

(Johnson et al., 1969; Ohnishi et al., 1969; Brewster et al., 1973). Retention of comparable positive NOE's for the ortho CH and meta CH resonances of deamino-oxytocin accompanying saturation of the water resonance (Figure 4b) rules out the intramolecular effects. Further support for direct intermolecular dipole-dipole interactions producing the observed NOE's is obtained by performing the solvent saturation experiment in $(CH_3)_2SO$, a solvent which lacks exchangeable hydrogens and therefore cannot transfer magnetization by chemical exchange. In this medium, both the Tyr ortho CH and meta CH protons exhibit comparable positive NOE's (Figure 5). These experiments, as well as solvent saturation studies of gramicidin S in $(CH_3)_2SO$, methanol, and trifluoroethanol (Pitner et al., 1975), demonstrate that *positive NOE's resulting from saturation of solvent nuclei originate primarily from direct dipole–dipole interaction between solute protons and irradiated solvent nuclei in intimate contact with them.* The orientation of the Tyr side chain of oxytocin in water must permit contact of both its ortho CH and meta CH hydrogens with water.[5]

[5] By contrast, the Tyr meta CH hydrogens of AII′ must be inaccessible to water. Carbon-13 $T_1$ measurements indicate that internal rotation of the Tyr side chain of oxytocin in water is relatively free (Deslauriers et al., 1974b), whereas that of AII′ is hindered (Deslauriers et al., 1975). This suggests that some type of hydrophobic or steric interaction involving the Tyr meta CH but not the ortho CH hydrogens of AII′ may restrict rotation of the phenol ring. In this regard it is interesting to note that the primary structure of AII′ has a Val on either side of the Tyr. The Val methyl groups may perhaps interact with the Tyr meta protons.

*Effect of pH.* The low-field region of the $^1$H NMR spectrum of oxytocin in $H_2O$ at different pH's is displayed in Figure 6; chemical shifts are summarized in Table II. Rapid proton exchange with the solvent accounts for the absence of the Cys$^1$ $NH_3^+$ resonance and the disappearance of the Tyr NH peak, which broadens beyond detection between pH 5.0 and 6.4. In the latter pH range peptide NH resonances of Ile and Asn shift to high field by 20 and 10 Hz, respectively. This shift reflects titration of the N-terminal amino group, whose p$K_a$ is 6.3 (Breslow, 1961). Through-bond and through-space interactions associated with titration of the Cys$^1$ amino group also shift $^{13}$C resonances of oxytocin (Deslauriers et al., 1974a). Between pH 3.5 and 5.0 a general low-field shift of all peptide and primary amide NH resonances is observed. Since no ionizable groups in oxytocin are titrated in this pH range, the origin of this effect remains undetermined, but may involve association with acetic acid (p$K_a$ = 4.7), which was introduced during solution of the hormone.

Figure 7 displays difference spectra from solvent saturation experiments performed at various pH's as in Figure 3.

Table II: Chemical Shifts (Hz at 250 MHz) of Low-Field Peaks of Oxytocin (6% w/v, 30 °C) in $H_2O$.

| Peak | pH 2.5 | ($\Delta\nu$) | 3.5 | ($\Delta\nu$) | 5.0 | ($\Delta\nu$) | 6.4 |
|---|---|---|---|---|---|---|---|
| Peptide NH | | | | | | | |
| $Tyr^2$ | 2240 | (5) | 2245 | (28) | 2273 | | |
| $Leu^8$ | 2092 | (3) | 2095 | (23) | 2118 | (1) | 2119 |
| $Gly(NH_2)^9$ | 2073 | (9) | 2082 | (17) | 2099 | (1) | 2100 |
| $Asn^5$ | 2076 | (3) | 2079 | (11) | 2090 | (−10) | 2080 |
| $Gln^4$ | 2051 | (1) | 2052 | (3) | 2055 | (−4) | 2051 |
| $Cys^6$ | 2041 | (0) | 2041 | (11) | 2052 | (−1) | 2051 |
| $Ile^3$ | 1982 | (8) | 1990 | (15) | 2005 | (−20) | 1985 |
| Primary amide NH or aromatic CH | | | | | | | |
| Asn, trans | 1892 | (5) | 1897 | (19) | 1916 | (0) | 1916 |
| Gln, trans | 1882 | (3) | 1885 | (10) | 1895 | (2) | 1897 |
| $Gly(NH_2)$, trans | 1843 | (3) | 1846 | (11) | 1857 | (−2) | 1855 |
| Tyr, meta (1) | 1802 | (6) | 1808 | (0) | 1808 | (−4) | 1804 |
| (2) | 1794 | (4) | 1798 | (2) | 1800 | (−3) | 1797 |
| $Gly(NH_2)$, cis | 1761 | (6) | 1767 | (11) | 1778 | (2) | 1780 |
| Asn, cis | 1725 | (6) | 1731 | (11) | 1742 | (−1) | 1741 |
| Tyr, ortho (1) | 1715 | (3) | 1718 | (4) | 1722 | (−1) | 1721 |
| (2) | 1707 | (4) | 1711 | (4) | 1715 | (−2) | 1713 |
| Gln, cis | 1707 | (4) | 1711 | (11) | 1722 | (−1) | 1721 |

Table III: Percent Change in the Intensity of Amide NH Resonances of Oxytocin (3% w/v) at 30 ± 1 °C in $H_2O$.[a]

| | Peptide NH | | | | | | trans Primary Amide NH | | | cis Primary Amide NH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | Tyr | Leu | Gly | Asn | Gln Cys[6] | Ile | Asn | Gln | $Gly(NH_2)$ | $Gly(NH_2)$ | Asn | Gln |
| 2.5 | −13 | 0 | . . . | . . . | . . . | 0 | −17 | −39 | −19 | −12 | −20 | −19 |
| 2.9 | −25 | 0 | . . . | . . . | . . . | 0 | −14 | −30 | −13 | −6 | . . . | . . . |
| 3.6 | −37 | 0 | 0 | 0 | . . . | 0 | −5 | −13 | −4 | −8 | . . . | . . . |
| 5.0 | −100 | 0 | 0 | 0 | . . . | 0 | 0 | 0 | 0 | 0 | . . . | . . . |
| 6.5 | - - | −31 | −53 | −47 | −53 | −34 | −22 | −16 | −53 | −52 | −32 | −32 |

[a] Intensity changes of peaks which were too broad to observe are designated by (- -) and those which could not be measured accurately because of peak overlap or complexity of the pattern are designated by (. . .).

Table III summarizes the relative extent of saturation transfer from $H_2O$ at these pH's. When the water resonance is completely saturated, the fractional decrease in the intensity of the NH resonance is given by

$$\frac{I_0 - I}{I_0} = \frac{T_{1NH}}{T_{1NH} + \tau_{NH}} \quad (1)$$

where $I$ is the intensity of the NH peak when the $H_2O$ resonance is saturated, $I_0$ is the equilibrium intensity of this resonance, $T_{1NH}$ is the spin-lattice relaxation time of an NH proton (in the absence of exchange), and $\tau_{NH}$ is the mean exchange lifetime of the NH proton (Hoffman and Forsén, 1966; Noggle and Schirmer, 1971; Pitner et al., 1974).[6]

Protolysis of amides in aqueous solution is subject to acid-base catalysis in accordance with

$$1/\tau_{NH} = k_0 + k_A[H_3O^+] + k_B[OH^-] \quad (2)$$

where $k_0$, $k_A$, and $k_B$ are respectively rate constants associated with direct proton exchange with water, acid-catalyzed exchange, and base-catalyzed exchange (Berger et al., 1959; Hvidt and Nielsen, 1966; Molday et al., 1972). The pH at which a minimum exchange rate is observed ($pH_{min}$) is

$$pH_{min} = 1/2 \log (k_A/k_B K_w) \quad (3)$$

where $K_w$ is the ion product of water (Leichtling and Klotz, 1966; Glickson, 1969). Peptides exhibit minimum exchange rates at pH 2-3 (Hvidt and Nielsen, 1966; Molday et al., 1972). Consequently, between pH 2.5 and 6.5 only base catalysis is observed for peptides. Because of the inductive effect of the nearby $NH_3^+$ group, $k_B$ of the penultimate peptide NH (Tyr) is expected to be considerably greater than that of peptides more distant from the amino terminus (Molday et al., 1972; Sheinblatt, 1965, 1966). This explains the much greater extent of saturation transferred to the Tyr NH than to any other peptide NH proton between pH 2.5 and 5.0 (Figure 7, Table III). At pH 6.5 the Tyr NH does not contribute to the difference spectrum because it is broadened beyond detection by rapid chemical exchange. These characteristics—*low field resonance position and rapid exchange with the solvent as indicated by transfer of*

[6] Equation 1 was derived from the Bloch equations including contributions from chemical exchange and nitrogen intramolecular relaxation, but neglecting intermolecular relaxation by solvent protons. That these effects are negligible is indicated by calculations based on known bond angles and bond lengths of peptide groups and water and estimates of their respective correlation times (Deslauriers et al., 1974b). This assumption is also supported by the absence of measurable NOE's for oxytocin in $H_2O$ at pH's which yield low rates of amide proton exchange (Figure 7). Recently, Waelder et al. (1975) demonstrated that intermolecular relaxation of indole NH protons is negligible in water. Similar conclusions are expected to apply to amides. Equation 1 is valid only when water protons are completely saturated. A more general equation is given by Pitner et al. (1974). The intensity of the $H_2O$ resonance was monitored on the oscilloscope to confirm complete saturation. Complete transfer of saturation to the Tyr NH peak at pH 5.0 (Table III, Figure 7) further confirmed that the water peak was completely saturated.

*saturation and line broadening—may serve as general criteria for identifying the peptide NH resonance of penultimate residues of polypeptides in water.* The other peptide hydrogens experience significant transfer of saturation at pH 6.4.

For primary amides $pH_{min}$ is about 5 (Molday et al., 1972). Consequently these hydrogens exhibit both acid and base catalysis between pH 2.5 and 6.5 (Figure 7, Table III). In the acid-catalyzed pH range the Gln trans carboxamide NH hydrogens experience about twice the extent of saturation transfer as do the trans carboxamide NH hydrogens of Asn and Gly($NH_2$). In basic solution the C-terminal Gly($NH_2$) hydrogens experience the greatest extent of saturation. Interpretation of these results awaits the separate determination of $T_{1NH}$ (Bleich and Glasel, 1975) and $\tau_{NH}$ and the performance of comparative solvent saturation studies of suitable model compounds (e.g., comparison of such parameters of solvated Gln, Asn, and Gly($NH_2$) carboxamide protons) and other neurohypophyseal hormones (e.g., lysine-vasopressin). The pH dependence of the extent of saturation transferred to NH hydrogens of oxytocin is in qualitative agreement with known exchange characteristics of solvated peptide and primary amide NH hydrogens and suggests that changes of $\tau_{NH}$ with pH make the dominant contribution to the pH dependence of the extent of saturation transfer (eq 1). Thus the exchange rates of the peptide NH hydrogens of oxytocin appear to be at least qualitatively comparable to those of solvated peptide protons. There is no evidence of any NH hydrogens which are extensively shielded from the solvent. Walter et al. (1971) found that in 95% $(CD_3)_2SO$–5% $D_2O$ the exchange rates of the Asn, Leu, and Gly peptide NH protons were significantly slower than those of other peptide hydrogens of oxytocin. This suggests that the *peptide hydrogens of oxytocin are more accessible to water than they are to* $(CD_3)_2SO$, as has been noted previously (Feeney et al., 1971, Bradbury et al., 1974; Brewster and Hruby, 1973).

This conclusion is further supported by the temperature coefficients of peptide NH resonances in $H_2O$ (Table I). In water all the peptide hydrogens have comparable values of $d\delta/dT$, which are in the range expected for solvated peptides, whereas in $(CD_3)_2SO$ the small value of $d\delta/dT$ of the Asn peptide NH suggests internal hydrogen bonding of this proton. Values of $^{13}C$ $T_1$'s further suggest that oxytocin assumes a more mobile conformation in water than in $(CD_3)_2SO$ (Glickson, 1975). Protonated backbone carbons of the ring moiety of oxytocin have an average value of $NT_1$ of about 50 ms (Walter et al., 1974b) whereas the corresponding value in $D_2O$ solution is about 100 ms (Deslauriers et al., 1974b) ($N$ is the number of protons bonded to a given carbon atom). This difference can be largely attributed to the greater viscosity of $(CD_3)_2SO$ (about 2 cP at 30 °C; Crown Zellerbach technical bulletin) than $D_2O$ (1.04 cP at 32 °C; Prutton and Maron, 1951; Weast, 1968) which increases the correlation time for molecular rotation of the hormone in $(CD_3)_2SO$. The increase in $NT_1$ of backbone carbons of the acyclic tail of oxytocin relative to values of $NT_1$ of backbone carbons of the ring is significantly greater in $D_2O$ than in $(CD_3)_2SO$. This suggests that segmental motion of the acyclic tail of the molecule relative to the cyclic moiety is more rapid in aqueous solution.

These studies illustrate how solvent saturation experiments can be employed to elucidate the detailed interaction of the solvent with specific NH and CH hydrogens of complex biomolecules. Clearly much additional information can be obtained from more extensive intramolecular and intermolecular NOE experiments. Selective deuteration of biomolecules would greatly simplify quantitative interpretation of the data. These experiments would be interpreted within the framework of theoretical treatments of intramolecular (Noggle and Shirmer, 1971) and intermolecular NOE's (Alla and Lippmaa, 1971; Khazanovich and Zitserman, 1971; Sinevee, 1972; Krishna and Gordon, 1973). Such investigations as well as measurements of $T_{1NH}$, which will permit quantitative interpretation of transfer of saturation experiments in terms of proton exchange rates, are now in progress in our laboratories.

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