Conformational Studies on [Pro^3,Gly^4]-Oxytocin in Dimethyl Sulfoxide by ^{1}H Nuclear Magnetic Resonance Spectroscopy: Evidence for a Type II β Turn in the Cyclic Moiety†

Alberto Ballardin, Alan J. Fischman,‡ William A. Gibbons, J. Roy, I. L. Schwartz, Clark W. Smith, Roderich Walter,* and Herman R. Wyssbrod*,§

ABSTRACT: A model for oxytocin has been previously proposed in which residues 3 and 4 occupy the corner positions in a β turn (Urry, D. W., & Walter, R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 956). The analogue [Pro^3,Gly^4]-oxytocin (PGO) was used in proton magnetic resonance (^{1}H NMR) studies designed to probe the contribution of these corner positions in forming a β turn. Comparison of various ^{1}H NMR parameters obtained at 220 MHz for backbone amide protons of PGO with those for the corresponding protons of oxytocin suggests that, to a first approximation, these two peptides may have similar backbone conformations in $(CD_3)_2SO$. Theoretically, the L-Pro→Gly sequence in positions 3 and 4 of PGO would allow the formation of either a type I or type II β turn. The two coupling constants between vicinal amide and C$^\alpha$ protons for Gly^4 of PGO in $(CD_3)_2SO$ are compatible with a type II β turn in which ϕ_4, the dihedral angle about the bond between the backbone C$^\alpha$ and N′ atoms of Gly^4, is ~+92°, but not with a type I β turn. A survey of peptides studied in other laboratories by X-ray and/or ^{1}H NMR with reported type II β turns with L-Pro→Gly or Gly→Gly sequences in the corner positions revealed a close correlation between chemical shifts and vicinal coupling constants for the glycyl residue in the second corner position. It is suggested that this criterion can form an additional basis for the characterization of β turns. More studies are needed to determine the particular type of β turn manifest in the cyclic moiety of oxytocin per se, although a reasonable working hypothesis is that oxytocin, similar to PGO, has a type II β turn.

One of our major goals is to establish both NMR criteria for the determination of peptide conformation in solution and conformation-function relationships for peptides, especially the neurohypophyseal hormones and their analogues (e.g., see Walter, 1971; Walter et al., 1971b, 1974; Wyssbrod & Gibbons, 1973). On the basis of proton magnetic resonance (^{1}H NMR) studies, Urry & Walter (1971) proposed that oxytocin in $(CD_3)_2SO$ contains a β turn (Geddes et al., 1968; Venkatachalam, 1968; Urry & Ohnishi, 1970; Chandrasekaran et al., 1973) involving residues 2–5 in the 20-membered tocin ring (cyclic) moiety formed by residues 1–6 and that this β turn is stabilized by an intramolecular hydrogen bond between the backbone C=*O* of Tyr^2 and the backbone N*H* of Asn^5. Glickson (1975) has reviewed conformational studies of oxytocin from various laboratories and presented arguments in favor of this β turn being a major structural feature of the cyclic moiety.

Theory has shown that β turns in which both corner positions have an L configuration may be of either two types (Venkatachalam, 1968; Chandrasekaran et al., 1973), designated types I and II (Figure 1). Examples of both have been found in proteins (Chandrasekaran et al., 1973; Crawford et al., 1973; Chou & Fasman, 1977). The values of the coupling constants between the vicinal amide and C$^\alpha$ protons reported for the corner positions (Ile^3 and Gln^4) of oxytocin in $(CD_3)_2SO$ are compatible with both type I and type II β turns (see Table III of Walter et al., 1977).

It should be possible to synthesize oxytocin analogues with particular residues in the corner positions that create steric restrictions that energetically favor the formation of a specific type of β turn. Steric considerations (Venkatachalam, 1968; Chandrasekaran et al., 1973) indicate that a type II′ β turn[1] dissimilar to that proposed for oxytocin should be favored when a residue with a D configuration is placed in the first corner position (residue 3), while a type II β turn similar to that proposed for oxytocin is favored when such a residue is placed in the second corner position (residue 4). In a preceding paper, Walter et al. (1977) reported on their ^{1}H NMR study of [D-Ala^3]-oxytocin, a model for a type II′ β turn, and [D-Ala^4]-oxytocin, a model for a type II β turn, in $(CD_3)_2SO$; it was noted that certain ^{1}H NMR parameters of oxytocin more closely resembled those of the latter than those of the former analogue.

In a continuation of this work, we have synthesized

† From the Department of Physiology and Biophysics, Mount Sinai Medical and Graduate Schools of the City University of New York, New York, New York 10029 (A.B., J.R., I.L.S., and H.R.W.), the Rockefeller University, New York, New York 10021 (A.J.F.), the Medical Department, Brookhaven National Laboratory, Upton, New York 11973 (J.R., I.L.S., and R.W.), the Department of Physiology and Biophysics, University of Illinois Medical Center, Chicago, Illinois 60680 (C.W.S. and R.W.), and the Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706 (W.A.G.). *Received January 13, 1978; revised manuscript received June 22, 1978.* Supported, in part, by National Institutes of Health Grants AM-10080, and AM-18399, and, in part, by the Life Sciences Foundation, Inc. All 220-MHz studies were performed at the Rockefeller University on facilities operated by a consortium and supported, in part, by National Science Foundation Grant GB-12278. A preliminary report of this work was presented at the Fourth American Peptide Symposium, New York, New York (Wyssbrod et al., 1975).

‡ Fellow for the Camille and Henry Dreyfus Foundation.

§ Senior Investigator for the New York Heart Association.

[1] A type II′ β turn is a mirror image of a type II β turn and is similar to a type I β turn with respect to the orientation of the backbone carbonyl oxygen atom of the residue in the first corner position (Venkatachalam, 1968; Chandrasekaran et al., 1973).

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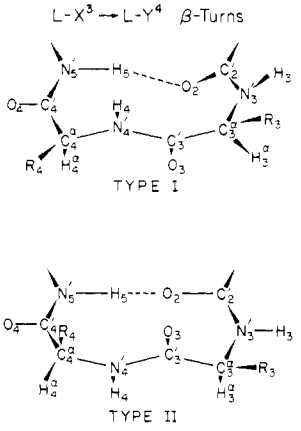

FIGURE 1: Types I and II β turns with amino acid residues of the L configuration in consecutive corner positions. Numbering of residues corresponds to that of the residues proposed to be involved in the cyclic (ring) moiety of oxytocin in $(CD_3)_2SO$, in which positions 3 and 4 are isoleucyl and glutaminyl residues, respectively. R denotes a side chain.

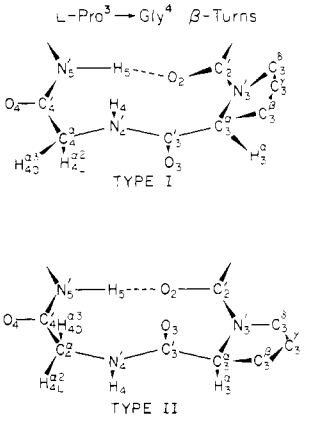

FIGURE 2: Types I and II β turns with prolyl and glycyl residues in the first and second corner positions, respectively. Numbering of these residues corresponds to that of the residues of [Pro³,Gly⁴]-oxytocin (PGO). The L C^{α} proton ($H_L^{\alpha 2}$) of the glycyl residue corresponds to the C^{α} proton of an optically active amino acid with an L configuration and, by the nomenclature proposed by Cahn et al. (1966), is designated the *pro-R* C^{α} proton, while the D C^{α} proton ($H_D^{\alpha 3}$) corresponds to the C^{α} proton of an amino acid with a D configuration and is designated the *pro-S* C^{α} proton.

[Pro³,Gly⁴]-oxytocin (PGO)[2] and studied some of its conformational features by ^{1}H NMR spectroscopy. Even though the conformation about the bond between the backbone C^{α} and N′ atoms is relatively constrained in prolyl residues (Leung & Marsh, 1958),[3] this conformation is compatible with that for the first corner position of both type I and type II β turns (Venkatachalam, 1968; Chandrasekaran et al., 1973). Moreover, a glycyl residue, having no side chain, may play the role of a residue with either an L or a D configuration, and, accordingly, all of the types I and II conformations accessible to corner residue sequences with L→L and L→D configurations should, at least in theory, also be accessible to the corner residue sequence L-Pro→Gly—see Figure 2 (Venkatachalam, 1968; Chandrasekaran et al., 1973). Indeed, L-Pro→Gly sequences as consecutive corner residues for both types I and II β turns have been found in proteins (Chandrasekaran et al., 1973; Crawford et al., 1973; Chou & Fasman, 1977).

In this paper, we utilize the ^{1}H NMR parameters of PGO to determine the type of β turn that might be manifest at corner positions 3 and 4 and compare the ^{1}H NMR parameters of PGO and oxytocin to determine if it is reasonable to assume that both molecules have similar backbone conformations.

Materials and Methods

Preparations of Samples for ^{1}H NMR Studies. [Pro³,Gly⁴]-oxytocin (PGO) was from the same source used previously (Wyssbrod et al., 1975). Except for INDOR studies, the sample was prepared by dissolving 22 mg (~22 μmol) of [Pro³,Gly⁴]-oxytocin acetate in 0.7 mL of $(CD_3)_2SO$ (99.9% D), thereby resulting in a concentration of approximately 32 mM or 3.1% (w/v). For the INDOR studies, 60 mg (~61 μmol) of peptide was dried for 96 h at 50 °C in vacuo to remove H_2O and then dissolved in 0.5 mL of solvent, thereby resulting in a concentration of approximately 122 mM or 12% (w/v). All chemical shifts and peak positions are reported downfield with respect to tetramethylsilane, $(CH_3)_4Si$, which was used as an internal standard in all samples.

^{1}H NMR Studies. All 220-MHz ^{1}H NMR studies were performed on a Varian Associates HR-220 field-sweep spectrometer equipped with a Nicolet 1080 computer and a TTI Fourier transform accessory located in the Spectroscopy Laboratory of the Rockefeller University. All studies were performed on this instrument unless noted otherwise. The temperature of the probe was controlled within ±2 °C. The double-resonance experiments involved total decoupling and were performed in either the field-sweep or the field-track mode, as previously described (Stern et al., 1968; Walter et al., 1972).

Two INDOR studies (Baker, 1962; Kowalewski, 1969; Gibbons et al., 1972a,b) were performed at 270 MHz on a Bruker HX-270 spectrometer located in the spectroscopy laboratory of the Department of Organic Chemistry of the Vrije Universiteit van Brussel in Brussels, Belgium. In the first study, designed to obtain *qualitative* information on the patterns and approximate positions of the various INDOR spectra, for each of the four fixed monitored (observed) frequencies f_1, the perturbing frequency f_2 was swept in a single scan over a spectral width of 1800 Hz over an interval of 2000 s (~1 Hz/s). In the second study, designed to obtain *quantitative* information on the positions of individual peaks in the four INDOR spectra, for each f_1, f_2 was swept alternatively upfield and downfield for a total of four pairs of sweeps over 90 Hz in 2000 s (~0.05 Hz/s); each of the eight scans was recorded separately on a width of 60 cm (0.15 Hz/mm); the extreme value for each upfield downfield sweep was eliminated, thereby resulting in six measurements for averaging; the average full width of each INDOR peak at half-maximal height was 5.6 Hz. Both INDOR studies were performed at 41 °C.

Spectral Analysis. The resonances of the isolated ABX spin systems formed by the single amide (X) and two C^{α} (AB) protons of Gly⁴ of PGO were analyzed by standard methods

[2] Abbreviations used are: Boc, *tert*-butyloxycarbonyl; 3D-Ala, [D-Ala³]-oxytocin; 4D-Ala, [D-Ala⁴]-oxytocin; δ_{NH}, chemical shift of the backbone amide proton; $\Delta\delta_{NH}/\Delta T$, temperature coefficient of δ_{NH}; f_1, monitoring (observing) frequency in an INDOR experiment; f_2, perturbing frequency in an INDOR experiment; HDX, hydrogen-deuterium exchange; I, intensity of a resonance signal; INDOR, internuclear double resonance; $^2J_{\alpha CH-\alpha CH}$, coupling constant between geminal C^{α} protons of a glycyl residue; $^3J_{NH-\alpha CH}$, coupling constant between vicinal amide and C^{α} protons; k_{HDX}, pseudo-first-order rate constant for HDX; OMe, methyl ester; PGO, [Pro³,Gly⁴]-oxytocin; $T_{1/2}$, half-time for HDX. Optically active amino acids are of the L configuration unless otherwise noted.

[3] Even though there is no free rotation about the bonds in the pyrrolidine ring of a prolyl residue, there is nevertheless substantial relative motion of vicinal atoms within the ring—i.e., there is considerable conformational averaging—because of rapid interconversion among various puckered forms of the ring (Deslauriers et al., 1974).

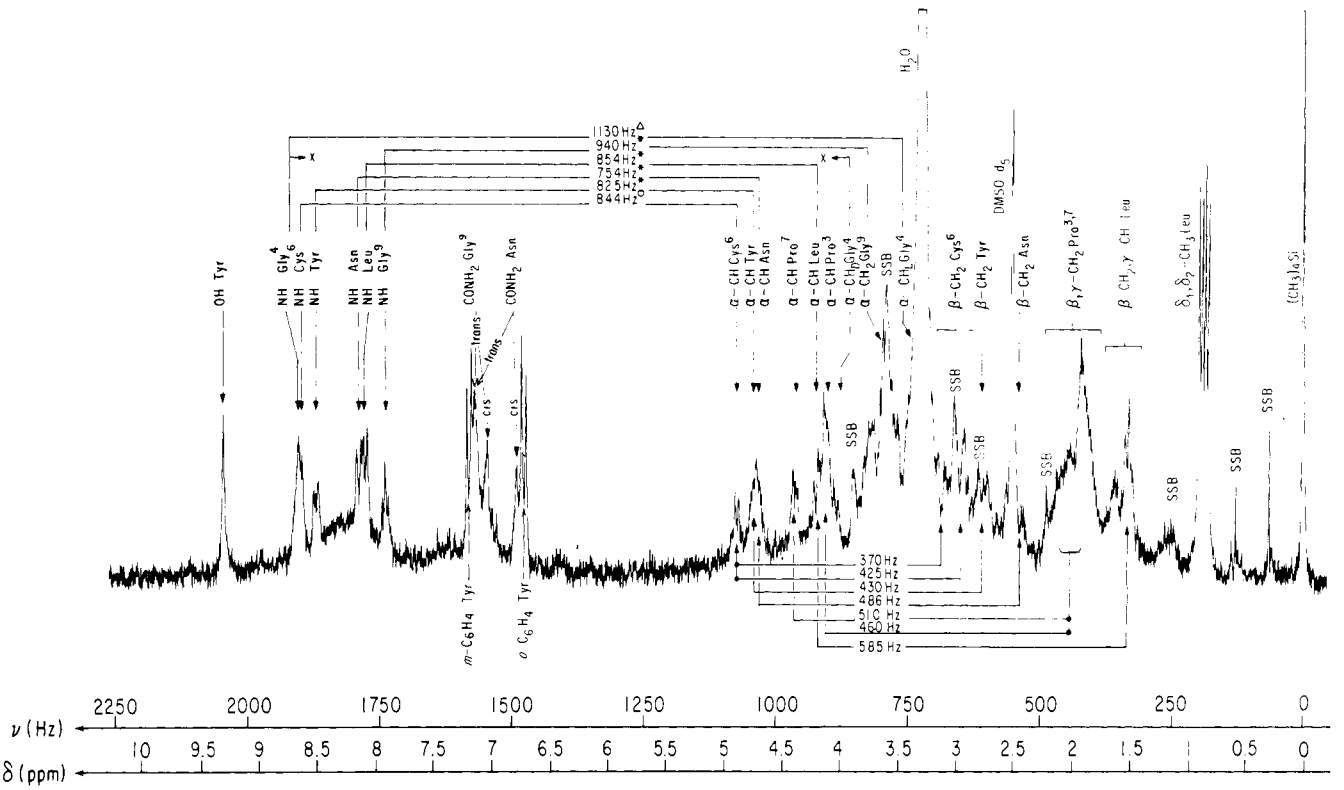

FIGURE 3: The assigned 220-MHz ^{1}H NMR spectrum of [Pro^3,Gly^4]-oxytocin (PGO) in $(CD_3)_2SO$ at 41 °C. The concentration is 3.1% (w/v). Me_2SO-d_5 indicates the resonance from [$^1H_1$,$^2H_5$]dimethyl sulfoxide. SSB denotes spinning side band. Couplings detected by double-resonance experiments performed at 28 (○), 41 (*), and 58 °C (△) are indicated by bridges, and separations of coupled resonances are given in Hz. The coupling between the amide and C$^\alpha$ protons of Gly^4 indicated by X was detected on a 270-MHz spectrometer. Backbone amide protons are designated by NH, side-chain and C-terminal carboxamide protons by $CONH_2$, and side-chain aromatic protons of the tyrosyl residue by *m*-C_6H_4 (meta) and *o*-C_6H_4 (ortho). The meta and ortho resonances correspond predominantly to the two δ and two ε protons, respectively. The downfield and upfield resonances of each pair of carboxamide protons correspond to the protons that are trans and cis, respectively, to the vicinal carbonyl oxygen atom (Anet & Bourn, 1965). The assignments of the prolyl C$^\alpha$ protons to residues 3 and 7 are tentative. The assignments of the L and D C$^\alpha$ protons of Gly^4 are unequivocal and are reversed from those reported earlier (Wyssbrod et al., 1975).

(Bernstein et al., 1957; Gutowsky et al., 1957) using the nomenclature of Pople et al. (1969). The C$^\alpha$ protons that resonate upfield and downfield, respectively, are arbitrarily designated A and B. In accordance with the nomenclature adopted, transitions 1-4, 5-8, and 9-12 arise predominantly from resonance absorption by the B, A, and X protons, respectively.

Conformational Analysis. Many Karplus relationships expressing $^3J_{NH\text{-}\alpha CH}$, the coupling constant between vicinal amide and C$^\alpha$ protons, as a function of ϕ, the conformation about the C$^\alpha$-N′ bond, have been reported in the literature (Barfield & Karplus, 1969; Bystrov et al., 1969a,b, 1973a,b; Thong et al., 1969; Ramachandran et al., 1971; Cung et al., 1972, 1973, 1974; Néel, 1972; Barfield & Gearhart, 1973; Ramachandran, 1973; Solkan & Bystrov, 1973; Bystrov, 1976; De Marco et al., 1978b). We have chosen to use the so-called *ferrichrome curve* proposed by De Marco et al. (1978b) in our analyses of the conformations of glycyl residues in PGO and other peptides because (1) this curve is calibrated in the pertinent region of ϕ and (2) glycyl residues are used in this calibration.

Values of ϕ are obtained from the following equations that approximately fit the upper and lower bounds for the Karplus relationship shown in Figure 5 of De Marco et al. (1978b):

$$^3J^{\text{upper bound}}_{NH\text{-}\alpha CH} = (5.20\cos^2\theta - 1.36\cos\theta + 2.70)\ \text{Hz}$$

and

$$^3J^{\text{lower bound}}_{NH\text{-}\alpha CH} = (5.79\cos^2\theta - 1.23\cos\theta + 1.53)\ \text{Hz}$$

where $\theta = \phi - 60°$ for amino acids of the L configuration and for the L C$^\alpha$ proton ($H^{\alpha 2}_L$) of a glycyl residue and $\theta = \phi + 60°$ for amino acids of the D configuration and for the D C$^\alpha$ proton ($H^{\alpha 3}_D$) of a glycyl residue.

Hydrogen-Deuterium Exchange (HDX) Study. Exchange was commenced by addition of 0.03 mL of D_2O to a 22-mg sample of [Pro^3,Gly^4]-oxytocin acetate dissolved in 0.7 mL of $(CD_3)_2SO$. The intensity, I, of the resonance of each backbone amide proton was determined as a function of time, t, following dissolution, the exchange rate, k_{HDX} (s^{-1}), determined for each exchanging proton was calculated as $-d(\ln I)/dt$, and the half-time for HDX, $T_{1/2}$ (h), was calculated as (1 h/3600 s) $\ln 2/k_{HDX}$.

Results

A preliminary report of this work has been presented (Wyssbrod et al., 1975).

Assignment of Proton Resonances. The 220-MHz ^{1}H NMR spectrum of ~3% (w/v) [Pro^3,Gly^4]-oxytocin (PGO) in $(CD_3)_2SO$ at 41 °C is shown in Figure 3. The resonances of side-chain protons of PGO were approximately assigned on the basis of their chemical shifts (Stern et al., 1968) and by comparison of resonances of similar protons in the previously assigned ^{1}H NMR spectrum of oxytocin (Johnson et al., 1969; Walter et al., 1972; Brewster et al., 1973), lysine vasopressin (Von Dreele et al., 1971; Walter et al., 1972), and desamino analogues of lysine vasopressin (Glickson et al., 1972). The C$^\alpha H$ resonances in PGO were then assigned by double-resonance experiments, which allow the detection of spin coupling between vicinal C$^\alpha$ and C$^\beta$ protons on the same residue. After the C$^\alpha$H resonance assignments were obtained, the NH reso-

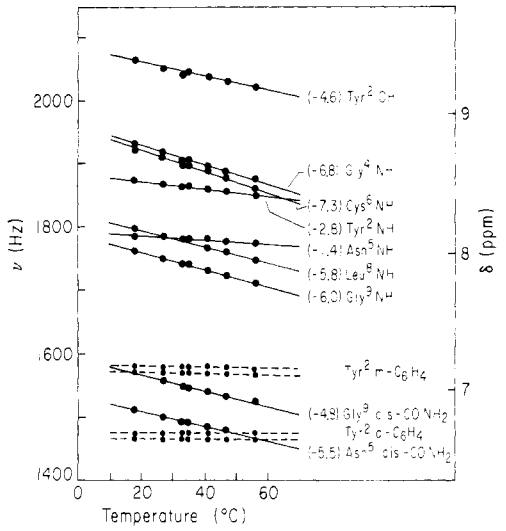

FIGURE 4: The temperature dependences of the chemical shifts of protons that resonate in the amide–aromatic region of the 220-MHz ^{1}H NMR spectrum of [Pro^3,Gly^4]-oxytocin (PGO) in $(CD_3)_2SO$ over the range 18–56 °C. Peak positions rather than chemical shifts are shown for the resonances of the aromatic protons. Values shown within parentheses are the dependences in ppb/°C; over the temperature range used, 0.1 ppb/°C corresponds to 0.8 Hz. See the caption of Figure 3 for a description of the designations *m*-C_6H_4, *o*-C_6H_4, and *cis*-$CONH_2$.

nances were assigned in a similar fashion by spin decoupling of vicinal amide and C$^\alpha$ protons on the same residue.

There are three potential sources of error in assigning the resonances in the ^{1}H NMR spectrum of PGO. The first is the overlapping C$^\alpha H$ resonances of Tyr^2 and Asn^5 around 4.69 ppm. This overlap would appear to make the assignments of the N*H* resonances of Tyr^2 and Asn^5 ambiguous. Fortunately, however, the N*H* resonances of Tyr^2 of PGO can be assigned with reasonable certainty on the basis of temperature-dependent line broadening—i.e., it has been observed that the N*H* resonances of the residue in position 2 of neurohypophyseal hormones—but not of their corresponding desamino analogues, in which the N-terminal amino group is replaced by a hydrogen atom—manifest a characteristic broadening as temperature is increased (Johnson et al., 1969; Walter et al., 1972; Glickson et al., 1972; Von Dreele et al., 1972; Brewster et al., 1973; Brewster & Hruby, 1973; Walter et al., 1974, 1977). In PGO, the N*H* resonances centered at 8.51 ppm at 41 °C (Figure 3) manifest temperature-dependent line broadening that is characteristic of residue 2 in other congeners and thus are assigned to Tyr^2; the N*H* resonances centered at 8.12 ppm at 41 °C are assigned to Asn^5 (Figure 3).

The second potential source of ambiguity in the assignments is the two multiplets of glycyl N*H* resonances, which are centered at 8.00 ppm and 8.76 ppm at 41 °C. Although the latter resonances are obscured by the N*H* resonances of Cys^6, the amide proton of Cys^6 exchanges for deuterium (N*H* → N*D*) much more rapidly than does the glycyl proton whose resonances overlap (see Table III below), and, thus, exchange can be used to unmask the glycyl N*H* resonances. Indeed, within 0.5 h after the addition of D_2O to PGO in $(CD_3)_2SO$ to a concentration of ~4% (v/v), the glycyl N*H* multiplet is completely isolated; these conditions are those used for the hydrogen–deuterium exchange (HDX) study (see Materials and Methods). When unmasked, a quartet—rather than the triplet characteristic of the N*H* resonances of Gly^9 in oxytocin in $(CD_3)_2SO$—is revealed. On the basis of this difference, the glycyl N*H* resonances centered at 8.76 ppm at 41 °C are tentatively assigned to Gly^4 (Figure 3). Furthermore, the remaining glycyl N*H* resonances centered at 8.0 ppm at 41 °C occur only 0.14 ppm from the N*H* resonances of Gly^9 in oxytocin (7.86 ppm), and these glycyl N*H* multiplets in both peptides are triplets (calculated from Table I of Walter et al., 1977). Thus, it is reasonable to assign the glycyl N*H* resonances centered at 8.00 ppm at 41 °C to Gly^9 (Figure 3).[4]

A third potential source of ambiguity in the assignments is the two prolyl residues (Pro^3 and Pro^7). The C$^\alpha H$ resonances of these residues are centered at 4.11 ppm and 4.31 ppm (Figure 3), thus indicating a difference in magnetic environments of the C$^\alpha$ protons of these two residues. On the basis of comparison with oxytocin, whose C$^\alpha H$ resonances of Pro^7 are centered at 4.39 ppm, the resonances centered at 4.31 ppm are assigned to Pro^7 of PGO, and, thus, those at 4.11 ppm, to Pro^3 (Walter et al., 1972). Inasmuch as the chemical shifts of the prolyl C$^\alpha H$ resonances are not interpreted by us in this paper, an error in these assignments would not be critical.

Temperature-Dependence Study. Figure 4 shows the temperature dependences of the chemical shifts of the amide protons ($\Delta\delta_{NH}/\Delta T$) and the peak positions of the aromatic protons of PGO in $(CD_3)_2SO$. Temperature coefficients have been employed to distinguish amide protons obstructed from contact with a solvent capable of forming hydrogen bonds (slightly positive, zero, or small negative values of $\Delta\delta_{NH}/\Delta T$) from those in contact with such a solvent (Stern et al., 1968; Ohnishi & Urry, 1969; Kopple & Ohnishi, 1969; Kopple et al., 1969a,b; Urry et al., 1970; Urry & Ohnishi, 1970; Llinás et al., 1970, 1972; Kopple, 1971; Bovey et al., 1972; Wyssbrod & Gibbons, 1973). Inaccessibility of an amide proton may result either from steric constraints or from involvement of that proton in intramolecular hydrogen bonding. It can readily be seen in Figure 4 that the values of $|\Delta\delta_{NH}/\Delta T|$ for the backbone amide protons of Tyr^2 and Asn^5 are significantly smaller than those for the other amide protons in PGO.

Hydrogen–Deuterium Exchange (HDX) Study. When D_2O is added to ~30 mM PGO in $(CD_3)_2SO$ to a final concentration of ~4% (v/v), all of the N-terminal amino, backbone amide, side-chain carboxamide and hydroxyl protons begin to exchange for deuterons. HDX is a pseudo-first-order rate process that can be characterized by a half-time ($T_{1/2}$), the time required for one-half of the protons to exchange for deuterons. Over 98% of the exchangeable protons in a particular group are replaced by deuterons after six $T_{1/2}$; for all practical purposes, HDX may be considered to be complete after this period of time. A slow rate of exchange (large value of $T_{1/2}$) may indicate that a proton is intramolecularly hydrogen bonded (Stern et al., 1968); on the other hand, it may reflect (a) steric constraints that limit the access of solvent to the site of exchange or (b) overall or local molecular rigidity[5] that hinders the molecule from assuming the transition state needed for HDX to occur. The values of $T_{1/2}$ for the six backbone amide protons of PGO are shown below in Table III;

[4] Methods for the synthesis of glycine stereospecifically labeled with deuterium in one of the α positions have been reported (e.g., Kainosho et al., 1975; Armarego et al., 1976). Recently, we synthesized (*R*)-[α-$^2H_1$]glycine by a new method (Fischman, 1978), which we shall report in detail in a later communication, and incorporated it into position 4 of PGO. Inspection of the ^{1}H NMR spectrum of this isotopic isomer confirms our assignments of glycyl proton resonances to residues 4 and 9 and reveals that the tentative assignments reported earlier by Wyssbrod et al. (1975) for the C$^\alpha$ protons of Gly^4 were reversed; the corrected assignments are shown in Figure 3.

[5] Unfortunately, terms such as *molecular flexibility* and *rigidity* are still rather vague. We hope that rigorous definitions of these terms will be developed in the future. In this paper, *rigidity* means *resistance to conformational averaging*. Thus, attractive intramolecular interactions would tend to impart rigidity to a molecule.

each of these protons in PGO exchanges at a slower rate than the corresponding proton in the naturally occurring neurohypophyseal hormones oxytocin, lysine vasopressin, arginine vasotocin, and arginine vasopressin and in the synthetic analogues desaminooxytocin, [Val^2]-oxytocin, [Gly^4]-oxytocin, and [D-Pro^7]-oxytocin (see Table III below and cf. Walter et al., 1971a, 1972, 1974; Brewster et al., 1973).

INDOR Studies. The value of the coupling constant between vicinal amide and C$^\alpha$ protons ($^3J_{NH-\alpha CH}$) reflects the dihedral angle ϕ, which defines the orientation about the C$^\alpha$–N′ bond (e.g., Stern et al., 1968; Bystrov et al., 1969a,b, 1973a,b; Ramachandran et al., 1971). One test of the hypothesis that residues 3 and 4 in neurohypophyseal hormones occupy the corner positions of a β turn is to measure the values of $^3J_{NH-\alpha CH}$ for these residues and to determine if the values of ϕ given by the Karplus relationship that relates ϕ and $^3J_{NH-\alpha CH}$ are compatible with conformations that have been proposed for β turns. In PGO, a prolyl residue, which has no backbone amide proton, occupies the proposed first corner position; the value of ϕ for this residue (ϕ_3) is constrained by the pyrrolidine ring to lie in the neighborhood of −60° (Leung & Marsh, 1958), a value compatible with both type I and type II β turns (Venkatachalam, 1968; Chandrasekaran et al., 1973).[6] In PGO, a glycyl residue occupies the proposed second corner position. Because a glycyl residue has two C$^\alpha$ protons, it is potentially possible to determine *two* values of $^3J_{NH-\alpha CH}$ and hence to gain additional information about ϕ_4. Unfortunately, ambiguity about ϕ_4 arises because of the uncertainty in assigning the chemical shifts of the two C$^\alpha$ protons (essentially, the problem is to determine which value of $^3J_{NH-\alpha CH}$ is related to which particular C$^\alpha$ proton).

The two C$^\alpha$ protons (A and B) and the single amide proton (X) of Gly^4 form an isolated ABX spin system. There are four X resonances in the N*H* region of the spectrum, eight AB resonances in the C$^\alpha$*H* region, and two combination resonances, which are of such low amplitude that they are buried in the noise of the spectrum; in some cases of ABX systems, information is lost as a consequence of degeneracy (viz., when the chemical shifts of A and B are almost identical—i.e., $\delta_A \approx \delta_B$). In principle, the sum of vicinal couplings ($\Sigma\, ^3J \equiv {}^3J_{AX} + {}^3J_{BX}$) can be obtained from a spectral analysis of the X region, while the individual couplings can be obtained only from an analysis of the AB region.[7] As mentioned above in the section on assignments, it is possible to unmask the four Gly^4 N*H* X resonances that overlap with the Cys^6 N*H* resonances; on the other hand, the eight Gly^4 C$^\alpha$*H* AB resonances either overlap with the Pro^3 C$^\alpha$*H* resonances or are buried under the large resonance peak of the H_2O that contaminates the sample (see Figure 1). Thus, it would be possible to obtain the sum of couplings, but not the individual couplings, from the 220-MHz ^{1}H NMR spectrum. We believed that it was important to gain the additional information inherent in the individual couplings, and so we decided to use INDOR spectroscopy (Baker, 1962; Kowalewski, 1969), which we had used previously to reveal hidden proton resonances in amino acids (Gibbons et al., 1972b) and peptides (Gibbons et al., 1972a). Furthermore, in the INDOR studies, we decided to be especially careful about exposure of our sample to air in order to minimize the uptake of H_2O and hence the amplitude of the H_2O peak.

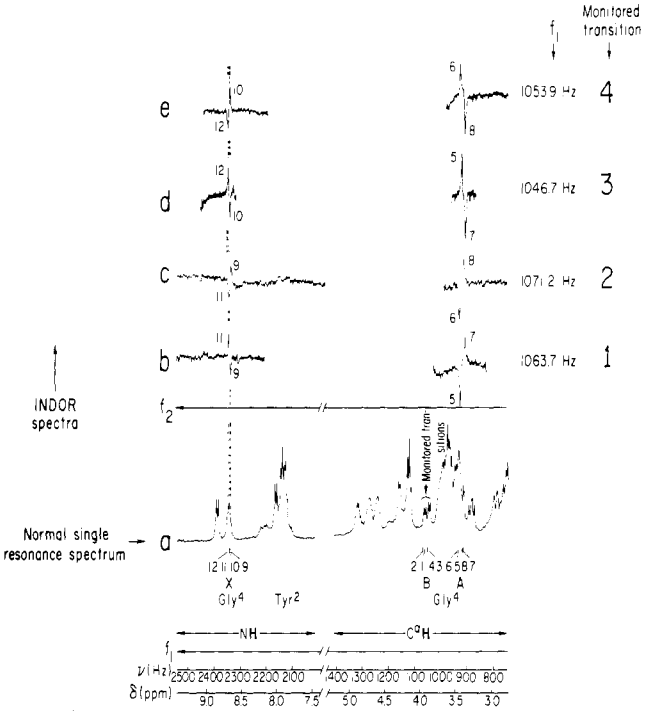

FIGURE 5: The normal single-resonance (a) and INDOR (b–e) ^{1}H NMR spectra of Gly^4 of [Pro^3,Gly^4]-oxytocin (PGO) in $(CD_3)_2SO$ at 41 °C. The concentration is 12% (w/v). The monitoring and perturbing frequencies are denoted by f_1 and f_2, respectively. See text for description of transitions and sweep rates for f_2.

Figure 5a shows part of the normal single-resonance 270-MHz proton spectrum of ~12% (w/v) PGO in $(CD_3)_2SO$ at 41 °C. Note that at 270 MHz the X resonances are separated from the Cys^6 N*H* resonances and the four downfield AB resonances (which originate primarily from the B proton) are separated from the Pro^3 C$^\alpha$*H* resonances, but that the four upfield AB resonances (which originate primarily from the A proton) still overlap with other resonances. The X subspectrum appears to be a triplet rather than a quartet probably because line broadening at the high concentration of PGO used in the INDOR studies causes the two inner resonances to overlap. The four A transitions (1–4) were monitored by the observing frequency f_1, and the corresponding INDOR spectra (Figure 5b–d) were generated by sweeping the perturbing frequency f_2 through the spectral regions containing the X and B resonances.

From the four INDOR spectra, it is possible to construct the topological spin-energy level diagram shown in Figure 6 (see Kowalewski, 1969, for details). For example, transition B_2 is connected only with transitions A_6, A_8, X_9, and X_{11} (see Figure 5c). Therefore, only lines representing these four transitions may be connected with the line representing transition B_2 in Figure 6. Because INDOR peaks 8 and 9 are positive (point upward), they represent *progressive* connections, in which the common energy level (spin state) lies between the two levels that are not in common. Thus, the diagram must be constructed so that spin state $\psi_{4'}$, which is common to transitions B_2 and A_8, lies vertically (on the energy or frequency axis) between states ψ_1 and ψ_7, which are not in common, and so that ψ_7 lies between $\psi_{4'}$ and ψ_8. Because INDOR peaks 6 and 11 are negative (point downward), they represent *regressive* connections, in which the common states lies either above or below the two states that are not in common. Thus, the diagram

[6] Recent convention (Kendrew et al., 1970a,b) is used throughout this paper to define ϕ.

[7] In principle, it is also possible to obtain individual couplings from an analysis of the X region and the value of the magnitude of the difference in chemical shift between A and B expressed in Hz ($|\Delta\nu_{AB}|$). The individual couplings cannot be obtained, however, if $|\Delta\nu_{AB}|$ is zero (in this case, the X resonances appear as a triplet, and the AB resonances, as a doublet; this pattern is termed *deceptively simple*).

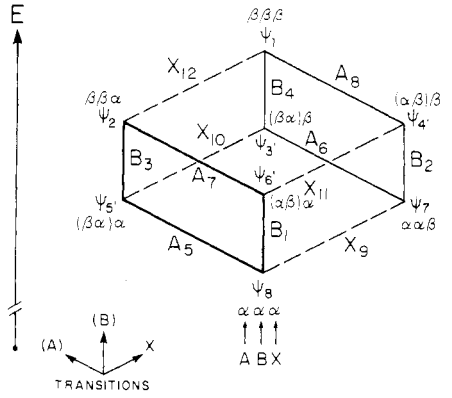

FIGURE 6: Topological spin-energy level diagram for an ABX spin system. Ordinate E denotes energy of the various spin states ψ. The spin-up state (the low-energy state for ^1H nuclei) is denoted by α, and the spin-down state (the high-energy state), by β. States in parentheses and primed states are mixed nuclear states. Spread of diagram along abscissa is only for the purpose of clarity. The AB subspectra appear as parallelograms (▬ and —), and the X subspectrum as four parallel lines (- - -) that separate the parallelograms. The transition numbers of this diagram are compatible with the INDOR spectra shown in Figure 5; see text for details.

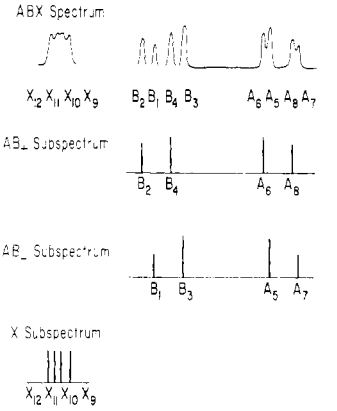

FIGURE 7: Diagrammatic representation of subspectra of the ABX spin system formed by the three protons of Gly^4 of [Pro^3,Gly^4]-oxytocin in $(CD_3)_2SO$. In this particular case, the AB_+ subspectrum corresponds to transitions of AB while X is in state β and the AB_- subspectrum, to those while X is in state α (see Figure 6).

must be constructed so that ψ_7 lies either above or below both $\psi_{3'}$ and $\psi_{4'}$ (in this case, below) and so that $\psi_{4'}$ lies either above or below both ψ_6 and $\psi_{7'}$ (in this case, above). Only four spin-energy level diagrams consistent with the four INDOR spectra can be constructed, and two of these can be eliminated on the basis of energetic considerations (e.g., to go from the lowest energy state ψ_8 to the highest energy state ψ_1 requires the same amount of energy regardless of the path taken; thus, for example, $\nu_5 + \nu_3 + \nu_{12}$ must equal $\nu_9 + \nu_2 + \nu_8$, where ν_i is the frequency of transition i).[8] Both of the remaining diagrams lead to the proper identification of the three subspectra (one X and two AB) needed for a spectral analysis. The diagram shown in Figure 6 is consistent with the assumption that the geminal coupling ($^2J_{AB}$) is negative (Bernstein & Sheppard, 1962).

From the topological spin-energy level diagram, it is possible to identify the three subspectra into which the resonances of an ABX spin system may be separated. The four transitions in the X subspectrum appear as approximately parallel lines (X_9-X_{12}). The two AB subspectra then appear as two isolated parallelograms separated by the lines that represent the X transitions. The AB subspectra whose center is more downfield (actually, at a higher frequency or energy) is designated the AB_+ subspectrum, and the other, the AB_- subspectrum. Figure 7 shows these subspectra in diagrammatic form. Once subspectra are identified, spectral analysis is unequivocal (Diehl et al., 1967). Table I shows the positions of the transitions in the subspectra,[9] and Table II, the chemical shifts and coupling constants obtained by analysis of these subspectra.

Comparison of Spectral and HDX Parameters. Table III summarizes the chemical shifts of the backbone amide protons (δ_{NH}), the temperature dependences of these shifts ($\Delta\delta_{NH}/\Delta T$), the half-time for HDX of these protons ($T_{1/2}$), and the coupling constants between vicinal amide and C^α protons ($^3J_{NH\text{-}\alpha CH}$) of PGO and oxytocin. The chemical shifts of amide protons and the peak positions of aromatic protons are shown as functions of temperature in Figure 4. Table IV summarizes the chemical shifts of the carboxamide protons of the side chain and the C-terminal group, the peak positions of the side-chain aromatic protons, and the temperature dependences of these parameters.

TABLE I: The 270-MHz Subspectra and Component Transitions for Gly^4 of [Pro^3,Gly^4]-Oxytocin (PGO) in $(CD_3)_2SO$ at 41 °C.[a]

proton	subspectrum[b]	transition[c] no.	change of spin state	nucleus of origin[d]	spectral position[e] (Hz)
$C^\alpha H_2$	AB_+	2	$\psi_7 \rightarrow \psi_{4'}$	B	1071.2[f]
		4	$\psi_{3'} \rightarrow \psi_1$	B	1053.9[f]
		6	$\psi_7 \rightarrow \psi_{3'}$	A	938.7[g]
		8	$\psi_{4'} \rightarrow \psi_1$	A	921.7[g]
	AB_-	1	$\psi_8 \rightarrow \psi_{6'}$	B	1063.7[f]
		3	$\psi_{5'} \rightarrow \psi_2$	B	1046.7[f]
		5	$\psi_8 \rightarrow \psi_{5'}$	A	934.0[g]
		7	$\psi_{6'} \rightarrow \psi_2$	A	917.0[g]
NH	X	9	$\psi_8 \rightarrow \psi_7$	X	2338.8[g]
		10	$\psi_{5'} \rightarrow \psi_{3'}$	X	2343.3[h]
		11	$\psi_{6'} \rightarrow \psi_{4'}$	X	2345.7[h]
		12	$\psi_2 \rightarrow \psi_1$	X	2350.2[g]

[a] Concentration of PGO is ~12% (w/v). [b] The AB subspectrum whose center is at a higher frequency (more downfield) is designated AB_+, and the other AB subspectrum, AB_-. [c] For nomenclature, see Pople et al. (1959), except that here the basic spin states α and β of each nucleus are taken to be the low and high energy states, respectively. [d] This is the predominant nucleus involved in resonance absorption; there is, however, some degree of mixing of the spin states of A and B, although it is quite low in this particular case. [e] All positions are downfield relative to $(CH_3)_4Si$ as an internal standard. [f] Position determined by INDOR monitoring frequency f_1. [g] Position determined by INDOR perturbing frequency f_2; sweep rate, ~0.05 Hz/s; average of six measurements; see ^1H NMR Studies in Materials and Methods for details. [h] Position estimated by ABX simulation using chemical shifts and coupling constants given in Table II.

Discussion

INDOR Studies. The normal single-resonance proton spectrum of the sample of [Pro^3,Gly^4]-oxytocin (PGO) used in the INDOR studies (Figure 5a) and that in the other studies (Figure 3) show the following differences even though acquired

[8] From Table I, $\nu_5 + \nu_3 + \nu_{12} = 4330.9$ Hz + $3\nu_{std}$, and $\nu_9 + \nu_2 + \nu_8 = 4331.7$ Hz + $3\nu_{std}$, where ν_{std} is the resonance frequency of the $(CH_3)_4Si$ standard. The difference in frequency between these two paths—viz., 0.8 Hz—is consistent with the estimated experimental error of ~0.3 Hz in the position of each transition.

[9] The positions reported in Table I are taken from INDOR spectra obtained at a *slow* rate of scan and not from those obtained at a *fast* rate shown in Figure 5b-e (see ^1H NMR Studies in Materials and Methods).

TABLE II: Chemical Shifts (δ) and Coupling Constants (J) for the Single Amide (X) and Two C$^\alpha$ (AB) Protons of Gly^4 of [Pro^3,Gly^4]-Oxytocin (PGO) in $(CD_3)_2SO$ at 41 °C.[a]

δ_A = 3.447 ppm[b]	$^2J_{AB}$ = −17.1 Hz[b,e]
δ_B = 3.911 ppm[b]	$^3J_{AX}$ = +4.7 Hz[b]
δ_X = 8.683 ppm[c,d]	$^3J_{BX}$ = +7.2 Hz[b]
	$\Sigma\,^3J$ = +11.9 Hz[b]; +11.4 Hz[c]

[a] Concentration of PGO is ~12% (w/v); ABX analysis of 270-MHz INDOR data shown in Table I; estimated maximum errors: ±0.001 ppm for δ and ±0.3 Hz for J. [b] From analysis of AB region. [c] From analysis of X region. [d] From data shown in Table III, δ_X is calculated to be 8.63 ppm at 41 °C for a concentration of PGO of ~3% (w/v). [e] The geminal coupling constant is assumed to be negative (Bernstein & Sheppard, 1962). [f] $\Sigma\,^3J \equiv {}^3J_{AX} + {}^3J_{BX}$.

at the same temperature (41 °C): (a) No Tyr^2 side-chain O*H* resonance is observed in the spectrum of the INDOR sample, while it is in that of the other sample, thereby suggesting that there is very rapid proton–proton exchange in the INDOR sample. (b) The Tyr^2 backbone N*H* resonances are shifted ~0.5 ppm upfield in the spectrum of the INDOR sample relative to that of the other sample, again suggesting rapid exchange in the INDOR sample (cf. Figures 3 and 5a). (c) The Asn^5, Leu^8, and Gly^9 backbone N*H* resonances are shifted ~0.1 ppm downfield in the INDOR spectrum. (d) The backbone N*H* resonances of Cys^6 are shifted downfield more than those of Gly^4 (~0.25 ppm) in the spectrum of the INDOR sample, thereby resulting in a separation of these resonances. (e) A large CH_3COO^- resonance is observed in the spectrum of the INDOR sample (not shown in Figure 5a), thereby suggesting that acetate is present in the form of acetic acid in addition to the amount needed as the counterion for PGO. (f) The Gly^4 N*H* resonances appear as a triplet in the spectrum of the INDOR sample, but as a quartet in that of the other sample.

Although great care was taken to minimize the amount of H_2O in the INDOR sample (see Materials and Methods), the presence of acetic acid in this sample may be responsible for an exchange-induced upfield shift in the Tyr^2 N*H* resonances, but not for the small downfield shifts observed for some of the other N*H* resonances. These downfield shifts may result, in part, from a systematic error, such as from computer read-out of peak positions or from a determination of the temperature, but the differential shifts (e.g., those that result in separation of the Gly^4 and Cys^6 N*H* resonances) cannot be explained in this manner. These differential shifts are most likely related to the aforementioned presence of acetic acid or to the high concentration used (~12% in the INDOR studies vs. ~3% in the other studies).

Meraldi et al. (1975) and Meraldi & Hruby (1976) warn that oxytocin—and presumably other neurohypophyseal hormones—may aggregate in dimethyl sulfoxide at temperatures and concentrations generally used for NMR studies. We have not systematically investigated the ^{1}H NMR spectrum of PGO as a function of concentration to determine if aggregation is responsible for the differences in backbone N*H* chemical shifts (δ_{NH}) observed for our different samples. Certainly the appearance of the Gly^4 N*H* resonances as a triplet rather than a quartet in the normal spectrum of the INDOR sample suggests line broadening related to increased aggregation, although such broadening may result merely from a general increase in viscosity of the solution caused by solute concentration per se. We feel confident, however, that the backbone conformations of the PGO in the two samples are quite similar regardless of the state of aggregation because there are no noticeable differences in corresponding coupling constants between vicinal amide and C$^\alpha$ protons ($^3J_{NH-\alpha CH}$) for any of the residues, except possibly for Gly^4: In a preliminary report (Wyssbrod et al., 1975), the individual values of $^3J_{NH-\alpha CH}$ for Gly^4 in PGO are stated to be 3.2 ± 0.6 Hz and 8.0 ± 0.4 Hz while in this report (Table II), 4.7 ± 0.3 Hz and 7.2 ± 0.3 Hz. A very low concentration of ~2 mg/0.7 mL (inadvertently not reported in Wyssbrod et al., 1975) was used in the preliminary INDOR studies, and it was difficult to ob-

TABLE III: ^{1}H NMR and Hydrogen–Deuterium Exchange (HDX) Parameters for Backbone Amide Protons of [Pro^3,Gly^4]-Oxytocin (PGO) and Oxytocin in $(CD_3)_2SO$.[a]

residue	δ_{NH}[b] (ppm)		$\Delta\delta_{NH}/\Delta T$[c] (ppb/°C)		$T_{1/2}$[d] (h)		$^3J_{NH-\alpha CH}$ (Hz)	
	PGO[e]	oxytocin[f]	PGO[e]	oxytocin[f]	PGO[e]	oxytocin[g]	PGO[h]	oxytocin[f]
Tyr^2	8.51[i]	8.27[i]	−2.76 ± 0.12	−6.4	<0.5	<0.02	7.5	*j*
Ile^3 (Pro^3)[k]		8.23		−11.6		<0.3		3.9 ± 0.5[l]
Gln^4 (Gly^4)[k]	8.76	8.11	−6.82 ± 0.34	−4.8	~6	≤0.8	L[m] 4.7[n]	6.6 ± 0.5
							D[m] 7.2[n]	
Asn^5	8.11	7.79	−1.40 ± 0.25	+0.2	~6	~0.7	8.1	6.1 ± 0.2
Cys^6	8.74	8.76	−7.30 ± 0.35	−11.1	<0.5	<0.02	7.3	7.5 ± 0.1
Leu^8	8.16	8.07	−5.82 ± 0.15	−6.8	~19	~0.8	7.3	7.3 ● 0.2
Gly^9	8.00	7.98	−6.01 ± 0.17	−5.9	~19	~1.4	5.5[o]	5.6 ± 0.2[o]

[a] Concentration of PGO and oxytocin is ~3% (w/v) unless otherwise noted. [b] Chemical shift at 20 °C downfield relative to $(CH_3)_4Si$ as an internal standard. [c] Temperature range is 18–56 °C for PGO and 18–44 °C for oxytocin. [d] Half-time for HDX in $(CD_3)_2SO$–D_2O (70:3 ≡ 140:6, v/v) at 22 °C. [e] Estimates of δ and $\Delta\delta/\Delta T$ are obtained from a linear regression of δ as a function of temperature, T; number of data points, n, is 7; standard error, s, of estimated δ < 0.01 ppm; s of estimated $\Delta\delta/\Delta T$ is shown following ± sign. [f] From Walter et al. (1972). [g] Concentration of oxytocin is ~3% (w/v); values of $T_{1/2}$ calculated from data presented in Walter et al. (1971a); solvent is $(CD)_3SO$–D_2O (20:1 ≡ 140:7, v/v); values reported here are 6/7 of the values calculated from the original data—i.e., a correction for concentration of D_2O is made in order to give estimated values of $T_{1/2}$ for oxytocin in $(CD)_3SO$–D_2O (70:3 ≡ 140:6, v/v). [h] Estimated maximum error is ±0.3 Hz. [i] The observed chemical shift of the backbone amide proton of Tyr^2 in oxytocin and oxytocin analogues can be highly variable from one preparation to another; this shift seems quite dependent upon such factors as the contaminating water or trace amounts of acid in the sample. [j] The coupling constant cannot be extracted because the signal is too broad. [k] Pro^3 replaces Ile^3 and Gly^4 replaces Gln^4 in PGO. [l] Corrected value reported here. [m] Unequivocal assignments: the L C$^\alpha$ proton of Gly^4, which corresponds to the C$^\alpha$ proton of an amino acid with an L configuration and to the *pro-R* C$^\alpha$ proton by the nomenclature proposed by Cahn et al. (1966), is assigned to A (see Table II), and the D C$^\alpha$ proton, which corresponds to the C$^\alpha$ proton of an amino acid with a D configuration and to the *pro-S* C$^\alpha$ proton, to B (see Table II). [n] Concentration of PGO is ~12% (w/v); values are taken from Table II. [o] Average vicinal coupling constant: $\{^3J_{NH-\alpha CH(L)} + {}^3J_{NH-\alpha CH(D)}\}/2$.

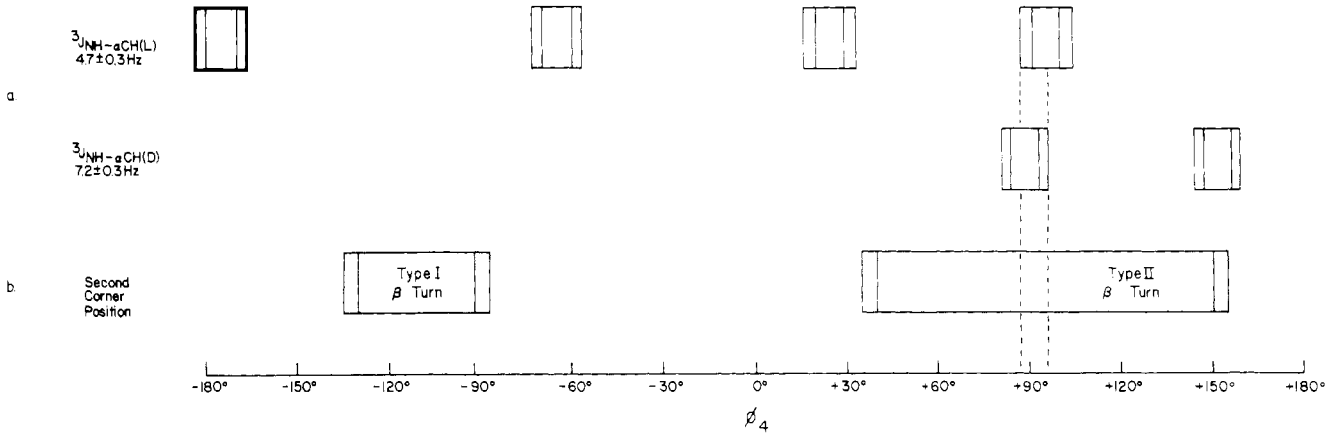

FIGURE 8: The observed individual values of the coupling constants between amide and C^α protons ($^3J_{NH-\alpha CH(L)}$ and $^3J_{NH-\alpha CH(D)}$) for Gly^4 of [Pro^3,Gly^4]-oxytocin in $(CD_3)_2SO$ at 41 °C and the ranges of values of ϕ that correspond to the observed values of $^3J_{NH-\alpha CH(L)}$ and $^3J_{NH-\alpha CH(D)}$ and to a glycyl residue in the second corner position of type I and type II β turns. (a) The value of ϕ for Gly^4 is assumed to be relatively fixed. The observed values of $^3J_{NH-\alpha CH}$ are used with the Karplus relationships given by Figure 5 of De Marco et al. (1978b) to determine compatible ranges of ϕ for each value; the inner ranges correspond to the estimated uncertainty in the relationship shown in this figure, while the outer ranges correspond to the additional uncertainty generated by the estimated limit in the error in the determination of $^3J_{NH-\alpha CH}$—viz., 0.3 Hz. Assignments of L and D C^α protons are unequivocal (see text). (b) The inner ranges for each type of β turn correspond to the permitted ranges reported in Table I of Chandrasekaran et al. (1973); region Ia of this table is used for the type I β turn, and regions Ib and III, for the type II β turn. The outer ranges expand the inner ranges by ±5° to account for the uncertainty generated by the 10° increments used by Chandrasekaran et al. (1973) in their calculations. Overlap between ranges compatible with individual couplings observed for Gly^4 of [Pro^3,Gly^4]-oxytocin occurs only in the neighborhood of +90°, which is compatible with a type II β turn; see text for additional details.

TABLE IV: ^1H NMR Parameters for Side-Chain Aromatic, Side-Chain Carboxamide, and C-Terminal Carboxamide Protons of [Pro^3,Gly^4]-Oxytocin (PGO) and Oxytocin in $(CD_3)_2SO$.[a]

proton		δ (ppm) PGO[b]	δ (ppm) oxytocin[c]	Δδ/ΔT (ppb/°C) PGO[b]	Δδ/ΔT (ppb/°C) oxytocin[c]
Tyr^2 meta	1[d]	7.18	7.12	−0.41 ± 0.07	−1.1
	2[d]	7.14	7.09	−0.40 ± 0.10	−1.4
Tyr^2 ortho	1[d]	6.70	6.67	0.16 ± 0.09	0
	2[d]	6.66	6.64	0.04 ± 0.08	0
Asn^5	cis	6.86	6.88	−5.55 ± 0.18	−5.7
	trans	*e*	7.35	*e*	−4.8
Gly^9-NH_2	cis	7.10	7.09	−4.84 ± 0.26	−5.7
	trans	*e*	7.12	*e*	−5.2

[a] Footnotes *a–c* of Table III also apply to this table. [b] See footnote *e* of Table III. [c] From Walter et al. (1972). [d] Peak positions rather than chemical shifts are reported. [e] At some temperatures these resonances are obscured by those from Tyr^2 meta protons (e.g., see Figure 3); linear regression analyses were not performed on these data.

tain spectra with a good single-to-noise ratio. Indeed, only the larger value of $^3J_{NH-\alpha CH}$ is deduced from these studies, and the smaller value of $^3J_{NH-\alpha CH}$ is obtained by subtraction of the larger value from the value of the sum of couplings ($^3J_{NH-\alpha CH}$) that is obtained from the difference in frequencies of the two outer Gly^4 N*H* resonances. Whether these differences in values of $^3J_{NH-\alpha CH}$ between samples are real remains to be determined.

Analysis of $^3J_{NH-\alpha CH}$ Values for Gly^4 of PGO. It is known that a Pro→Gly sequence can fill the role of consecutive corner positions in either a type I or type II β turn (see introductory section). Individual values of $^3J_{NH-\alpha CH}$ for Gly^4 of PGO are obtained in the INDOR studies. Panel a of Figure 8 shows the permitted ranges of ϕ_4, the conformation about the bond between the backbone C^α and N′ atoms of Gly^4, that are compatible with the pair of values 4.7 ± 0.3 Hz and 7.2 ± 0.3 Hz, which correspond to $^3J_{NH-\alpha CH(L)}$ and $^3J_{NH-\alpha CH(D)}$, respectively. Note that the observed value of $^3J_{NH-\alpha CH(L)}$ can correspond to four different ranges of fixed conformations, while that of $^3J_{NH-\alpha CH(D)}$, to only two different ranges. For a fixed conformation actually to be manifest, however, both individual couplings must correspond to the same conformation—i.e., the permitted ranges for the individual couplings must overlap. Overlap is observed only in the range from +88° to +96°. Thus, the only fixed value of ϕ_4 that is compatible with both couplings is +92 ± 4°. Panel b shows the permitted ranges of ϕ for a glycyl residue in the second corner position of both type I and type II β-turns (Chandrasekaran et al., 1973). Overlap is observed between the range permitted for a fixed value of ϕ_4—viz., +92° ± 4°—and that for a type II, but not a type I, β turn. In summary, individual values of $^3J_{NH-\alpha CH}$ for Gly^4 are compatible with Gly^4 occupying the second corner position of a type II β turn in PGO dissolved in $(CD_3)_2SO$.

Comparison of PGO with Peptides Reported to Have a Glycyl Residue in the Second Corner Position of a Type II β Turn. The solution conformation of gallichrome, the Ga^{3+} analogue of the cyclic hexapeptide ferrichrome, has been reported to be similar to the crystal conformation of the closely related analogue ferrichrome A (Llinás et al., 1972; Llinás & Neilands, 1976), and the solution and crystal conformations of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) have been reported to be quite similar to each other (Pease & Watson, 1978; Karle, 1978). Both of these peptides possess a type II β turn with a glycyl residue in the second corner position. The latter peptide, like PGO, possesses a prolyl residue in the first corner position. The solution conformations of other peptides with a Pro→Gly sequence have been studied by NMR spectroscopy, and it has been proposed that these residues also occupy consecutive corner positions of a type II β turn. Table V summarizes various ^1H NMR parameters for the glycyl residues of these model peptides and compares them to those of PGO.

In PGO the Gly^4 C^α protons have been unequivocally assigned with respect to chirality—i.e., the upfield C^α proton can be assigned to the *pro-R* proton, and the downfield proton, to the *pro-S* proton (Cahn et al., 1966).[4] In all cases reported in Table V, the glycyl C^α proton with the upfield chemical shift is associated with the smaller value of $^3J_{NH-\alpha CH}$, while the C^α proton with the downfield shift is associated with the larger

TABLE V: ^{1}H NMR Parameters for Glycyl Residues Occupying the Second Corner Position of Type II β Turns.[a,b]

peptide	residue in first corner position	T (°C)	$^2J_{\alpha CH(L)-\alpha CH(D)}$ (Hz)	$\delta_{\alpha CH(L)}$ (ppm)	$^3J_{NH-\alpha CH(L)}$ (Hz)	ϕ_L[c] (deg)
gallichrome	Gly	63	-16.9 ± 0.2	3.475	5.7 ± 0.2	71–89[e]
cyclo-(Gly-Pro-Gly)$_2$	Pro	27	-17.5 ± 0.5[f]	3.64	5.0 ± 0.5[f]	90–103
cyclo-(Ser-Pro-Gly)$_2$[g]	Pro	23	-15.5 ± 1.0[f,h]	*i*	3.5 ± 0.5	100–126
Boc-Val-Pro-Gly-Gly-OMe[k]	Pro	21	-17.0 ± 0.5[f]	3.712	4.0 ± 0.5[f]	94–117
cyclo-(Gly-Pro-Gly-D-Ala-Pro)	Pro	67	*m*	3.63	6.0 ± 0.5[n]	~60–89
[Pro^3,Gly^4]-oxytocin (PGO)	Pro	41	-17.1 ± 0.3	3.447	4.7 ± 0.3	87–104

$\delta_{\alpha CH(D)}$ (ppm)	$^3J_{NH-\alpha CH(D)}$ (Hz)	ϕ_D[c] (deg)	$\delta_{\alpha CH(L)} - \delta_{\alpha CH(D)}$ (ppm)	ϕ_{calcd}[d] (deg)	$\phi_{reported}$ (deg)	ref
3.777	6.4 ± 0.2	74–87	−0.302	80 ± 6	85 ± 6	De Marco et al. (1978b)
3.79	6.0 ± 0.5[f]	68–86	−0.15	83 ± 3	100	Schwyzer et al. (1972)
4.06	8.0 ± 0.5	87–115	~−0.5[i]	107 ± 8	60 ± 15[j]	Torchia et al. (1972)
4.159	7.0 ± 0.5[f]	77–96	−0.45	95 ± 1	55,[j] 80–90[l]	Khaled et al. (1976)
4.07	7.0 ± 0.5[n]	77–89	−0.44	83 ± 3	90,[j] 74[o]	Watson (1976); Pease & Watson
3.911	7.2 ± 0.3	81–96	−0.464	92 ± 4		

[a] Solvent is $(CD_3)_2SO$ unless otherwise noted. [b] Assignments of L and D C$^\alpha$ protons of the glycyl residue are unequivocal for PGO and assumed for all other peptides. [c] Limiting values of ϕ_L and ϕ_D are calculated from those of $^3J_{NH-\alpha CH(L)}$ and $^3J_{NH-\alpha CH(D)}$ by the upper and lower Karplus relationships shown in Figure 5 of De Marco et al. (1978b), which we describe in Conformational Analysis in Materials and Methods. [d] The value of ϕ_{calcd} is taken to be the mean of the upper and lower bounds of the overlapping range of ϕ_D; the ± values are taken as one-half the difference in these bounds of the overlapping range. [e] Ranges of values of ϕ_L and ϕ_D for the crystal conformation are 79° and 91°, respectively, as reported in Table III of De Marco et al. (1978b). [f] Our estimate of the error. [g] Only the conformation designated Ω_C is considered here; in this conformation, the peptide bond between the seryl and prolyl residues is assumed to be in a cis conformation ($\omega = 0°$). Nevertheless, the values of ϕ and ψ for the prolyl and glycyl residues are compatible with these residues occupying consecutive corner positions of a type II β turn. [h] Estimated from Figure 2 of Torchia et al. (1972). [i] From Figure 2 of Torchia et al. (1972), it is estimated that $\delta_{\alpha CH(L)}$ is approximately 0.5 ppm upfield from $\delta_{\alpha CH(D)}$. [j] Estimated from model building. [k] Solvent is $CDCl_3$–C_6D_6 (4:1, v/v). [l] Obtained from conformational energy calculations. [m] Not reported. [n] Solvent is $CDCl_3$, and temperature, 47 °C. Couplings in $(CD_3)_2SO$ remain within 0.5 Hz of those in $CDCl_3$. [o] Reported for the crystal conformation (Karle, 1978).

value. This criterion may form a strong basis for assigning *pro-R*(L) and *pro-S*(D) C$^\alpha$ protons of glycyl residues occupying the second corner position of type II β turns. We do not mean to imply, however, that this criterion applies only to this particular case—e.g., glycyl residues in other conformations might also manifest this correlation between chemical shifts and coupling constants.[10]

In summary, the ^{1}H NMR parameters listed in Table V for PGO and the close correlation of these parameters with those of other peptides support the assignment of a type II β-turn to the Tyr^2→Pro^3→Gly^4→Asn^5 sequence in PGO.

Analysis of the $^2J_{\alpha CH-\alpha CH}$ Value for Gly^4 of PGO. On the basis of molecular orbital calculations, Barfield et al. (1976) predict that the coupling between the geminal C$^\alpha$ protons ($^2J_{\alpha CH-\alpha CH}$) for glycyl residues is dependent upon the conformation on both sides of the C$^\alpha$ atom—i.e., upon ψ, the conformation about the C$^\alpha$–C′ bond, as well as ϕ, the conformation about the C$^\alpha$–N′ bond. For N^α-acetylglycinamide, a model for a glycyl residue, this dependency is given to within 0.3 Hz by the following equation:

$$^2J_{\alpha CH-\alpha CH}(\phi,\psi) = (-13.91 - 1.55\cos^2\psi - 2.80\cos^4\psi + 4.65\cos^2\phi)\ \text{Hz}$$

where the conventions for ϕ and ψ are given by Kendrew et al. (1970a,b). If the values of ϕ and $^2J_{\alpha CH-\alpha CH}$ are known, then the above equation can be solved for up to four values of ψ. Substitution of a value for ϕ of +92° and a range for $^2J_{\alpha CH-\alpha CH}$ of -17.1 ± 0.3 Hz from Table V for Gly^4 of PGO yields approximate ranges of $|\psi|$ from 28° to 21° and 152° to 159°. Chandrasekaran et al. (1973) list various combinations of values of ϕ and ψ in the first and second corner positions that are, on the basis of theoretical calculations, compatible with various types of β turns. Entries in which ϕ for the second corner position equals +90° may be found in region III of Table I of this theoretical paper. In this region, a glycyl residue in the second corner position serves as a residue with a D configuration, and possible values of ψ, to within ±5°, for this residue are listed as −40°, +20°, +30°, and +40°. Hence, a range of ψ of between +21° and +28°, as predicted from the above equation, is in excellent agreement with theoretical values for a type II β turn.

Possible Evidence for Intramolecular Hydrogen Bonding. Any β turn that might be formed by residues 2–5 could be stabilized by an intramolecular hydrogen bond between the backbone C=O of residue 2 and the backbone N*H* of residue 5. The relatively small magnitude of the temperature dependence of chemical shift for the backbone N*H* ($|\Delta\delta_{NH}/\Delta T|$) of Asn^5 in PGO suggests that this proton is obstructed from contact with solvent, possibly because of involvement in the formation of an intramolecular hydrogen bond (Table III). The intermediate value of $|\Delta\delta_{NH}/\Delta T|$ for Tyr^2 also suggests that this proton is somewhat inaccessible to the solvent, but to a lesser degree than the backbone N*H* of Asn^5. It would be of interest to see if the values of $\Delta\delta_{NH}/\Delta T$ for both Tyr^2 and Asn^5 in desamino-PGO approach zero, as in the case for these values in desamino-oxytocin (Urry et al., 1970).

The half-times for HDX ($T_{1/2}$) of the backbone amide protons in PGO appear to be significantly longer in PGO than

[10] It should be noted that the two C$^\alpha$ protons of glycyl residues in other relatively restricted conformations manifest different chemical shifts and values of $^3J_{NH-\alpha CH}$. For example, for the glycyl residue in the first corner position of the type II β turn in gallichrome, a value of ϕ in the neighborhood of −60° pertains and the C$^\alpha$ proton with the upfield chemical shift is associated with the larger rather than the smaller value of $^3J_{NH-\alpha CH}$ (De Marco et al., 1978a,b). Chemical shifts and coupling constants for glycyl residues in this position in other peptides have not been reported, and so a generalization cannot be made at this time about this particular case.

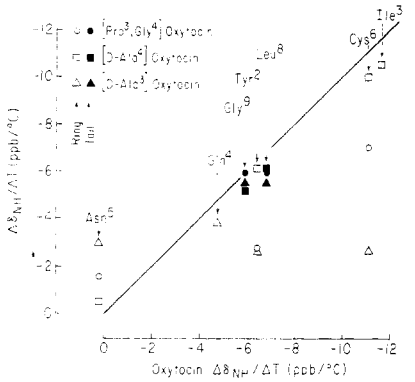

FIGURE 9: The comparison of the temperature dependences of the chemical shifts of the backbone amide protons ($\Delta\delta_{NH}/\Delta T$) for selected analogues of oxytocin with those of the corresponding protons for oxytocin per se. The data for [D-Ala^3]-oxytocin (3D-Ala) and [D-Ala^4]oxytocin (4D-Ala) are taken from Walter et al. (1977), and those for oxytocin, from Walter et al. (1972). The diagonal line corresponds to the locus along which corresponding protons have identical values of $\Delta\delta_{NH}/\Delta T$.

those of the corresponding protons in oxytocin (Table III). Inasmuch as the mechanism of exchange in $(CD_3)_2SO$–D_2O mixtures is not well understood, caution must be exercised in any direct comparison of corresponding values of $T_{1/2}$ in PGO and oxytocin, especially when the HDX studies are not performed under identical conditions—e.g., if different counterions are present in the two different samples or if the H^+ and OH^- activities of the two solutions are different. If, however, the differences in corresponding values of $T_{1/2}$ are not an experimental artifact, they may indicate that PGO is a more rigid molecule than oxytocin and that it is more difficult (energetically less favorable) for PGO than oxytocin to assume the requisite conformation—excited or transition state—for exchange of certain protons to occur (Walter et al., 1974). It should be noted that values of $T_{1/2}$ for backbone amide protons in the acyclic (tail) moiety (residues 7–9) as well as in the cyclic (ring) moiety (residues 1–6) are greater in PGO than in oxytocin, possible indicating cooperative interactions between the tail and ring, as has been suggested previously (Walter, 1971).

Similarities between PGO and Oxytocin. The similarity of corresponding ^1H NMR parameters of PGO and oxytocin suggests that the backbone conformations of these peptides in $(CD_3)_2SO$ may be quite similar (Table III). Nevertheless, except for Cys^6, there appear to be small, unexplained upfield shifts of corresponding backbone N*H* resonances in going from PGO to oxytocin (Table III). These upfield shifts are similar in appearance to those observed in going from arginine vasopressin to arginine vasotocin to oxytocin in $(CD_3)_2SO$ (Walter et al., 1974). The individual values of $^3J_{NH-\alpha CH}$ for Gly^4 of PGO seem to be compatible with a type II but not a type I β turn; the similarity of spectral parameters for PGO and oxytocin suggests that both of these peptides possess a type II β turn, but definitive proof of this conclusion for oxytocin requires additional data.

Comparison of PGO, [D-Ala^3]-oxytocin (3D-Ala), and [D-Ala^4]-oxytocin (4D-Ala) with Oxytocin. Figure 9 shows a comparison of the values of $\Delta\delta_{NH}/\Delta T$ for PGO with those of the corresponding protons for oxytocin. The data points for the linear peptide sequence (tail) (●) tend to lie on or near the diagonal line, while those for the ring moiety (○)—with the exception of the point for Asn^5—lie significantly below this line. One interpretation is that the amide protons of Tyr^2 and Cys^6 spend more time in a state obstructed from contact with solvent in PGO than in oxytocin. If so, then this interpretation is compatible with the suggestion based on HDX rates that PGO may be a more rigid molecule than oxytocin—i.e., conformational averaging is less manifest in PGO than in oxytocin. On the other hand, the data point for Asn^5 lies slightly above the line, suggesting that the N*H* of this residue spends *less* time in a solvent-obstructed state in PGO than in oxytocin; this difference may not be of much consequence, however, because the values of $|\Delta\delta_{NH}/\Delta T|$ for Asn^5 are quite small in both peptides.

Figure 9 also shows the comparison of the values of $\Delta\delta_{NH}/\Delta T$ for [D-Ala^3]-oxytocin (3D-Ala) and [D-Ala^4]-oxytocin (4D-Ala) with those for oxytocin. The data points for the tail moieties of both analogues (▲ and ■) lie near the diagonal line, while only those for the ring moiety of 4D-Ala (□) lie near this line. On the other hand, there are significant deviations from the diagonal line for data points for the ring moiety of 3D-Ala (△). One interpretation is that both oxytocin and 4D-Ala possess the same type of β turn in their ring moiety—viz., type II—and that these β turns help to endow both peptide backbones with similar conformational features, while oxytocin and 3D-Ala, on theoretical grounds, almost certainly possess different types of β turn and that differences in the backbone conformation for the rest of the ring moiety for these two peptides result from perturbations caused by the differences in the β turns (see Walter et al., 1977). It should be noted that values of $\Delta\delta_{NH}/\Delta T$ for Asn^5 and Cys^6 in PGO are midway between those for oxytocin and 3D-Ala, which—as mentioned above—almost certainly would have different types of β turn, while the value for Tyr^2 in PGO is practically equal to that in oxytocin. One possible explanation is that although oxytocin and PGO may possess similar types of β turn in the ring moiety—e.g., type II—the precise conformations of the residues that comprise the β turns are nevertheless slightly different (after all, in PGO, the conformation about the C^α–N' bond of residue 3 is relatively constrained and residue 4 has no side chain to cause steric restrictions) and these differences are reflected to some degree in the rest of the ring moiety.

Comparison of PGO in $(CD_3)_2SO$ and Aqueous Solution. Deslauriers et al. (1977, 1978) studied PGO in D_2O by ^{13}C NMR spectroscopy and found evidence for cis–trans isomerism about the peptide bond between Tyr^2 and Pro^3 and for an increased mobility of the C^α of Gly^4 relative to the C^α's of the other residues in the cyclic moiety. We have obtained the ^1H NMR spectrum of PGO in D_2O and also found evidence for two conformational forms, which we did not find for PGO in $(CD_3)_2SO$. Thus, the degree of conformational averaging in the cyclic moiety of PGO is greater in D_2O than in $(CD_3)_2SO$.

Conclusion

We believe that (a) [Pro^3,Gly^4]-oxytocin (PGO) and oxytocin have, to a first approximation, similar backbone conformations in $(CD_3)_2SO$, (b) conformational averaging is less prevalent in the backbone of PGO than in that of oxytocin, (c) residues 3 and 4 in the cyclic (ring) moiety of PGO occupy consecutive corner positions of a type II β turn, (d) a reasonable working hypothesis is that there is also a type II β turn in oxytocin, and (e) definitive proof for this hypothesis for oxytocin requires additional experimental data.

We also believe that the comparison of various corresponding physical parameters of PGO and oxytocin is only a small part of the larger problem of obtaining information on the proposed β turn in the cyclic moiety of oxytocin and that the real value of the study of oxytocin analogues with modifications at positions 3 and 4 will become evident only when a whole series of analogues representing all four types—viz., I,

II, I′, and II′—of β turn in the cyclic moiety has been prepared, studied, and compared with oxytocin.

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