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Proposed Conformations of Oxytocin and Selected Analogs in Dimethyl Sulfoxide as Deduced from Proton Magnetic Resonance Studies†

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ABSTRACT: The 220-MHz nmr spectra have been obtained and assigned for oxytocin, deamino-oxytocin, [4-glycine]oxytocin, [2-valine]oxytocin, [7-D-proline]oxytocin, and [1-β-mercaptopropionic acid,7-D-proline]oxytocin. Several deuterated derivatives were used for making unambiguous assignments. Conformational calculations based on measured values of the vicinal amide to α-proton coupling were used for proposing conformations for the molecules in dimethyl sulfoxide solution. Three energetically favorable conformations of oxytocin are found, one of which has a single intramolecular hydrogen bond involving the asparagine-5 backbone NH and the glutamine-4 carboxamide carbonyl. The tripeptide side chain is proposed to possess a trans–cis′ junction to the ring and is folded toward the ring. In dimethyl sulfoxide oxytocin appears to possess a nonrigid conformation. Deamino-oxytocin possess a conformation similar to oxytocin. The proposed conformation for [4-glycine]oxytocin has two transannular bonds: the asparagine-5 peptide NH and carbonyl to the tyrosine-2 carbonyl and peptide NH, respectively. The conformation of [2-valine]oxytocin appears to be quite different from that of any of the other peptide analogs examined, with no intramolecular hydrogen bond. Both D-proline-7 analogs differ from oxytocin and deamino-oxytocin in the orientation of the tripeptide side chain with respect to the ring. The influence of the amino acid substitutions on the conformation is discussed.

The nuclear magnetic resonance (nmr) spectra of the neurohypophysial hormone, oxytocin

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
1 2 3 4 5 6 7 8 9

and its analog, deamino-oxytocin, in dimethyl sulfoxide (Johnson *et al.*, 1969; Walter *et al.*, 1971; Deslauriers *et al.*, 1972; Brewster *et al.*, 1973), methanol–dimethyl sulfoxide (Urry *et al.*, 1970; Urry and Walter, 1971), and water (Feeney *et al.*, 1971; Glickson *et al.*, 1972; Brewster and Hruby, 1973) solutions have been studied. The spectra of the tripeptide side chain (Hruby *et al.*, 1971a) and of the ring without the side chain (Brewster *et al.*, 1972) of both oxytocin and deamino-oxytocin in dimethyl sulfoxide solution have also been reported. Conformations based on the results of these studies have been proposed (Urry *et al.*, 1970; Urry and Walter, 1971; Brewster *et al.*, 1972). Urry *et al.* (1970) predict a 1–4 turn (β turn) in the ring moiety stabilized by one transannular hydrogen bond in oxytocin and by two such bonds in deamino-oxytocin. They also predict a 1–4 turn in the side chain. In this paper, we show that energetically favorable alternative conformations may exist, including a particularly favorable conformation involving interaction of the glutamine-4 and asparagine-5 residues.

It is known from pharmacological studies on oxytocin analogs that tyrosine at position 2 is critical for the full display of the intrinsic activity of the hormone (see Rudinger *et al.*, 1972, for a recent review), and substitution at position 4 gives analogs with variable potencies and antihormonal activities (for recent summaries, see Rudinger, 1971, Flouret and du Vigneaud, 1969, and Walter, 1971). Accordingly, we have investigated [2-valine]oxytocin [Hruby and du Vigneaud, 1969) and [4-glycine]oxytocin (Drabarek, 1964), in which the residues at the 2 and 4 position in oxytocin, respectively, have

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been replaced. Both analogs possess low activities in the pharmacological effects usually associated with oxytocin (Table I).

It is also probable that the presence and orientation of the tripeptide side chain with respect to the ring is an important aspect of oxytocin structure and function. [7-D-Proline]-oxytocin (Ferraro and du Vigneaud, 1966) was studied as well as [1-β-mercaptopropionic acid,7-D-proline]oxytocin, hereafter referred to as deamino-7-D-proline-oxytocin (Ferraro and du Vigneaud, 1966). These analogs possess activities (Table I) very similar to those of the ring compounds of oxytocin and deamino-oxytocin, namely tocinamide and deamino-tocinamide (Hruby *et al.*, 1971b). It was hoped that these analogs would provide information regarding the orientation of the side chain with respect to the ring, and the effect of this relationship on overall conformation.

In this paper, we propose possible conformations for the five analogs of oxytocin mentioned, showing how the chemical substitutions made have resulted in apparent conformational changes.

TABLE I: Pharmacological Potencies of Oxytocin and Related Compounds.[a]

Compound	Oxytocic (Rat)	Vasodepressor (Fowl)	Milk Ejecting (Rabbit)
Oxytocin[b]	500 ± 10	507 ± 23	410 ± 16
Deamino-oxytocin (cryst)[c]	803 ± 36	975 ± 24	541 ± 13
Tocinamide[d]	3.2 ± 0.2	Nil	5.9[i]
Deaminotocinamide[d]	34 ± 3	Nil	
[7-D-Proline]-oxytocin[e]	13 ± 0.5	Nil	3.9 ± 0.1
[1-β-Mercaptopropionic acid,7-D-proline]-oxytocin[f]	~45	Nil	8
[4-Glycine]-oxytocin[g]	2.8 ± 0.1	5.5 ± 0.2	17 ± 1
[2-Valine]-oxytocin[h]	2.1 ± 0.1	6.8 ± 0.5	15.6 ± 0.6

[a] Potencies are expressed in units per milligram as mean potencies ± standard error. [b] Chan and du Vigneaud (1962). [c] Ferrier *et al.* (1965). [d] Hruby *et al.* (1971). [e] Ferraro and du Vigneaud (1966). [f] Drabarak (1964). [g] Hruby and du Vigneaud (1969). [h] Zaoral and Flegel (1972). [i] Ressler (1956).

Experimental Section

Preparation of Peptides. Oxytocin and related precursor peptides were prepared by standard solution methods of peptide synthesis (Bodanszky and du Vigneaud, 1958). Oxytocin was purified by partition chromatography on Sephadex G-25 (Yamashiro, 1964; Yamashiro *et al.*, 1966) followed by gel filtration on Sephadex G-25 (Porath and Flodin, 1959). Deamino-oxytocin was prepared by both solution (Hope *et al.*, 1962; Ferrier *et al.*, 1965) and solid-phase methods of peptide chemistry. Samples of oxytocin were also generously provided by Professor du Vigneaud. [1-[α,α,β,β-$^2H_4$]β-mercaptopropionic acid]oxytocin was supplied by Professor du Vigneaud. [1-Hemi[α,β,β-$^2H_3$]-L-cystine]oxytocin and [6-hemi[α,β,β-$^2H_3$]-L-cystine]oxytocin were prepared using solid-phase methods. Boc-*S*-benzyl[α,β,β-$^2H_3$]-DL-cysteine was used to introduce the deuterated amino acid into the growing peptide chain. Details of the preparation and purification of these peptides will be published elsewhere (Spatola *et al.*, in preparation). [7-L-[α,β,β,γ,γ,δ,δ]proline]oxytocin was prepared by solid-phase methods (Spatola and Hruby, unpublished results).

[4-Glycine]oxycotin was a generous gift from Dr. du Vigneaud or was made by published procedures (Drabarek, 1964). [2-Valine]oxytocin was also supplied by Dr. du Vigneaud or prepared by published procedures (Hruby and du Vigneaud, 1969). [7-D-Proline]oxytocin and deamino-7-D-proline-oxytocin were prepared by published procedures (Ferraro and du Vigneaud, 1966). The compounds had identical R_F values on partition chromatography in the solvent systems previously reported. The purified products gave single spots on thin-layer chromatography in at least two solvent systems and gave proper amino acid analyses after 24-hr hydrolysis in 6 N HCl using a Beckman 120C amino acid analyzer.

Nuclear Magnetic Resonance (Nmr) Methods. Samples were dissolved in dimethyl sulfoxide (99.5 and 100% D) to a concentration of about 7% w/v. Spectra were run on a Varian Associates 220-MHz nmr spectrometer, with variable-temperature accessory for the temperature studies. Deuterium exchange was carried out by addition of 6% v/v D_2O at ambient temperature. The decrease in area of the NH resonances was monitored as a function of time.

Conformational Calculations. The methods used to generate low-energy cyclic peptide conformations which are consistent with nmr data are detailed elsewhere (Tonelli, 1971, 1972; Tonelli and Brewster, 1972; Bovey *et al.*, 1972). The ring moiety in oxytocin and deamino-oxytocin is closed *via* formation of a S—S bond. Consequently, the conformation of the ring depends in part on the rotational states of the C—C, C—S, and S—S bonds in the cystine bridge, and upon the backbone conformations of the ring peptide residues. Thus, in the test for ring closure, the distance between the α-carbon atoms in half-cystine-1 and half-cystine-6 was calculated (Brant and Flory, 1965) as a function of the backbone conformations of the other residues in the ring.

To determine the range of distances which permit closure of the cyclic moiety, the distance between $C^{\alpha}_{Cys\text{-}1}$ and $C^{\alpha}_{Cys\text{-}6}$ was calculated as a function of the rotation angles about the C—C, C—S and S—S bonds in the cystine bridge. The C—C and C—S bonds were permitted (Flory, 1969) to adopt rotation angles of 0° and ±120°, while the S—S bond was fixed in the right-handed conformation with a dihedral angle slightly less than +90° (Linderberg and Michl, 1970), an angle similar to that suggested on the basis of circular dichroism studies (Urry *et al.*, 1968; Walter *et al.*, 1968). The C—C, C—S, and S—S bond lengths of 1.53, 1.81, and 2.08 Å were adopted (Semlyen, 1967), and the valence angles at each of the cystine bridge atoms was assumed (Semlyen, 1967) to be 110°.

Five different distances between $C^{\alpha}_{Cys\text{-}1}$ and $C^{\alpha}_{Cys\text{-}6}$ were found: 2.90, 4.50, 5.00, 5.75, and 6.80 Å. Of the conformations tested, those with distances within ±0.20 Å of any of the five possible $C^{\alpha}_{Cys\text{-}1}$ to $C^{\alpha}_{Cys\text{-}6}$ distances found above were considered to be cyclic structures. It should be noted that if oxytocin were a typical cyclic hexapeptide with six peptide residues closing the ring, then the distance between $C^{\alpha}_{Cys\text{-}1}$

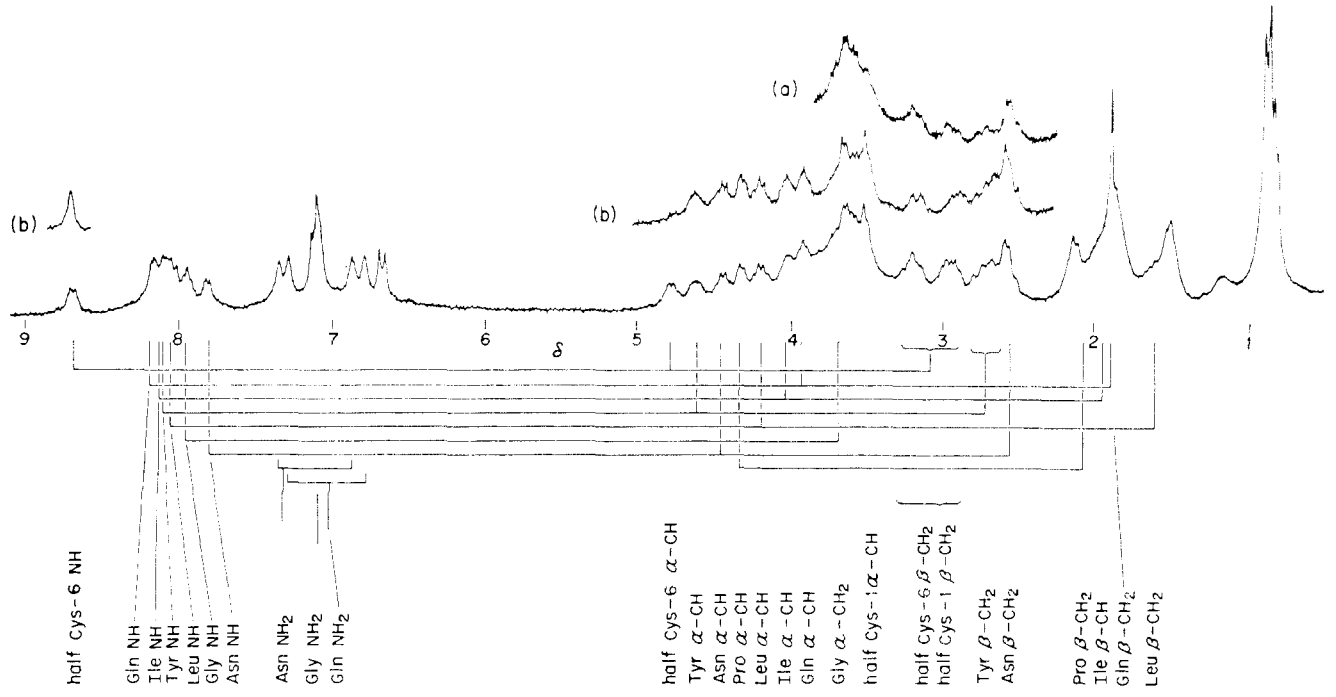

FIGURE 1: Spectrum of oxytocin. Insets are: (a) [1-hemi[α,β,β-$^2H_3$]-L-cystine]oxytocin and (b) [6-hemi[α,β,β-$^2H_3$]-L-cystine]oxytocin.

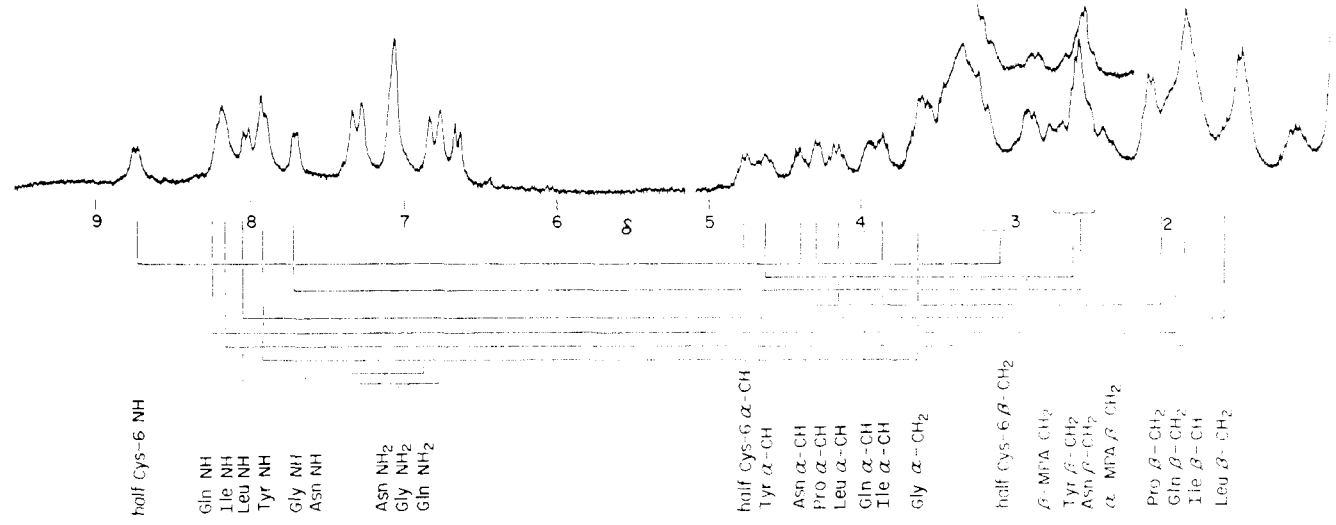

FIGURE 2: Spectrum of deamino-oxytocin. Inset is [1-$\alpha,\alpha,\beta,\beta$-$^2H_4$]$\beta$-mercaptopropionic acid]oxytocin.

and $C^{\alpha}_{Cys\text{-}6}$ would be (Brant and Flory, 1965) *ca.* 3.8 Å. Clearly the ring moiety in oxytocin and its various analogs is not a typical cyclic hexapeptide.

Results

Through homonuclear spin decoupling, it was possible to assign all the α, β, and peptide NH and some of the δ and γ proton resonances in oxytocin. Assignments of carboxamide resonances were confirmed from spectra of the precursor peptides, *e.g.*, Z-Asn-S-Bzl-Cys-Pro-Leu-Gly-NH_2 and Z-Gln-Asn-S-Bzl-Cys-Pro-Leu-Gly-NH_2, as well as from the spectrum of [4-glycine]oxytocin (see below). Spectra of [1-hemi-L-[α,β,β-$^2H_3$]cystine]oxytocin and [6-hemi-L-[α,β,β-$^2H_3$]cystine]-oxytocin permitted unequivocal assignments to be made for the α- and β-proton resonances of half-cystine-1 and half-cystine-6. Since the α-proton coupling to the peptide NH of half-cysteine-6 is absent in [6-hemi-L-[α,β,β-$^2H_3$]cystine]oxytocin, this compound also confirmed the assignment of this peptide NH peak (see Figure 1). Evaluation of the spectrum of [7-L-[$\alpha,\beta,\beta,\gamma,\gamma,\delta,\delta$-$^2H_7$]proline]oxytocin confirmed previous assignments for the α and β protons (Johnson *et al.*, 1969), but the δ protons were found at about 3.6 ppm, a position different than that previously suggested (Walter *et al.*, 1971). Comparison of the oxytocin spectrum with the spectrum of [2-valine]oxytocin (see below) together with the assignments of the half-cystine-1 and half-cystine-6 proton resonances allowed assignment of the tyrosine-2 resonances. Assignment of the asparagine-5 β-proton resonances agreed with those made for an analog in which the asparagine protons were replaced by deuterons (Brewster *et al.*, 1972).

The half-cystine-1 residue is replaced by a β-mercapto-propionic acid residue in deamino-oxytocin. The peaks belonging to this portion of the molecule were identified through their absence in the spectrum of 1-[$\alpha,\alpha,\beta,\beta$-$^2H_4$]$\beta$-mercaptopropionic acid]oxytocin (see Figure 2).

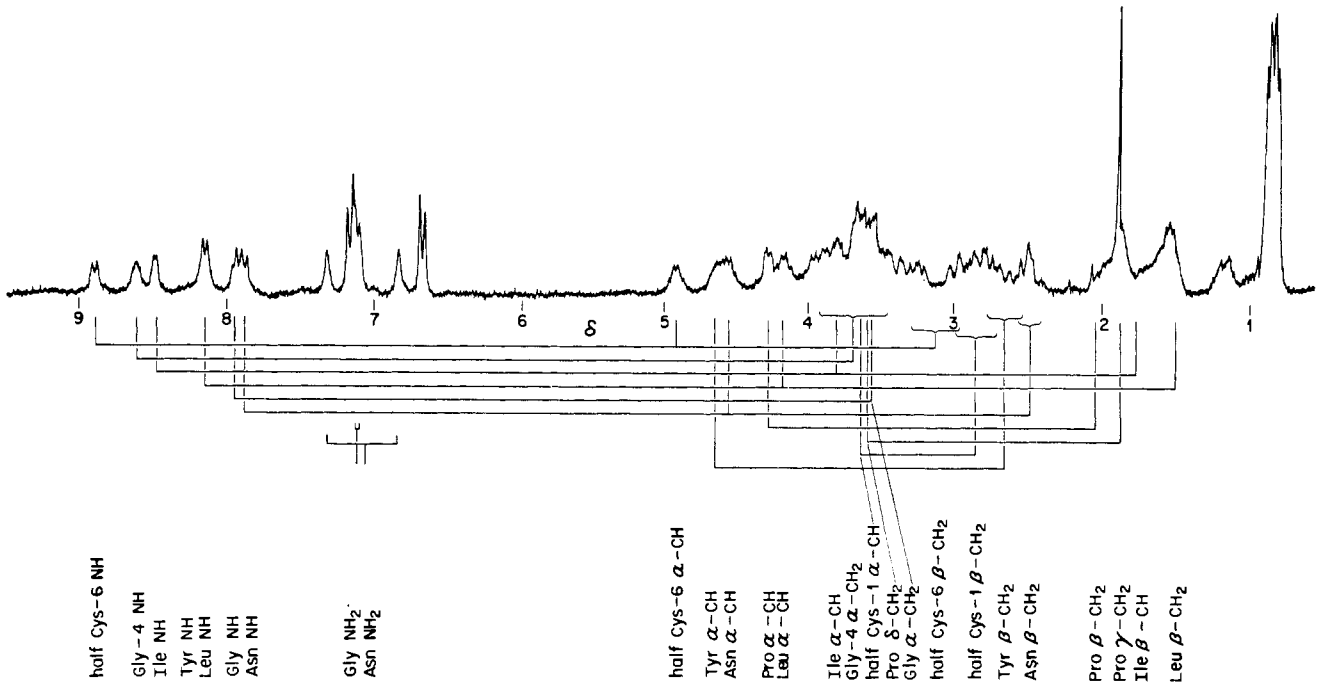

FIGURE 3: 220-MHz spectrum of [4-glycine]oxytocin.

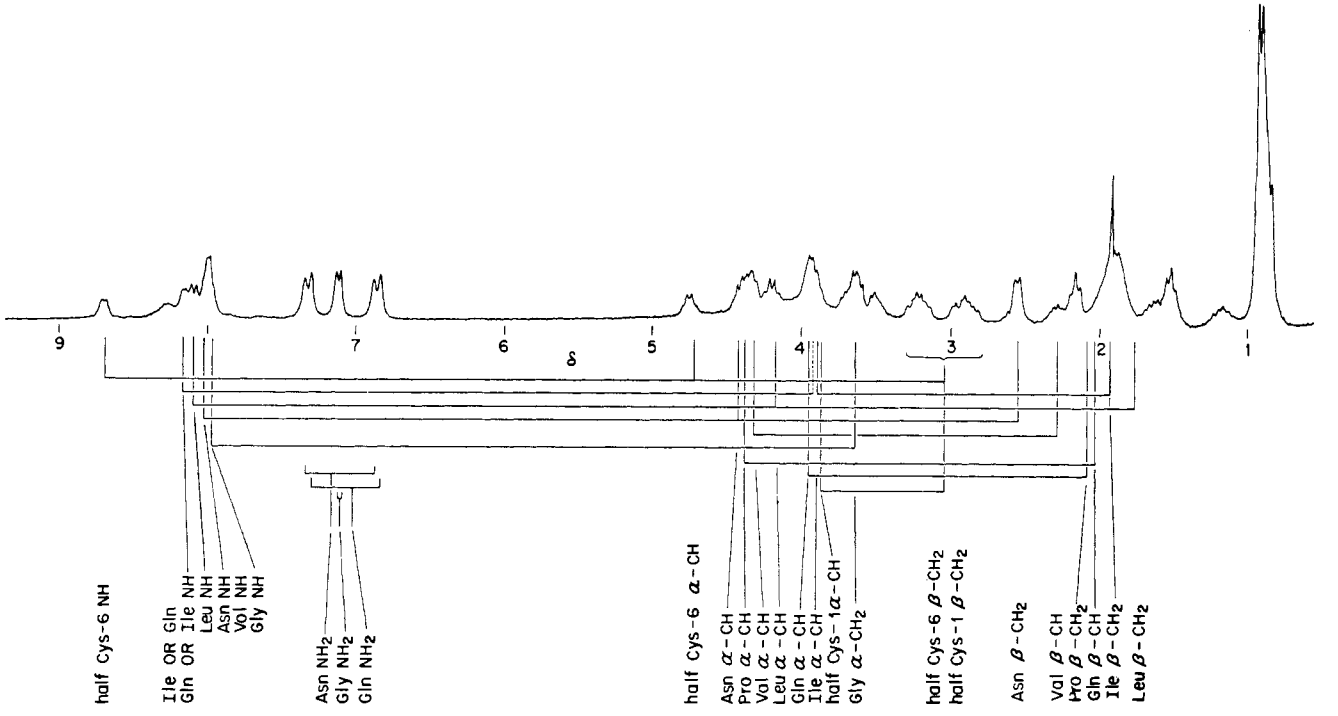

FIGURE 4: 220-MHz spectrum of [2-valine]oxytocin.

The spectra of [4-glycine]oxytocin, [2-valine]oxytocin, [7-D-proline]oxytocin, and deamino-7-D-proline-oxytocin are shown in Figures 3–6. Homonuclear spin decoupling and comparison with the spectrum and assignments for oxytocin and deamino-oxytocin permitted the assignment of most of the resonances in all of the spectra. Only in the case of [2-valine]oxytocin did any ambiguity remain. Here because of the spectral proximity of the isoleucine-3 β-CH and glutamine-4 β-CH_2 and the isoleucine-3 and valine-2 α-CH resonances, the peptide NH peaks of the former two residues could not be conclusively assigned. The two NH peaks had $J_{N\alpha}$ values sufficiently similar (5 and 6 Hz) so as not markedly to affect the conformational searches.

Deuterium exchange of the peptide NH and carboxamide protons was quite rapid in the six compounds, all of them exchanging to half-height within 1 hr. Though slight differences were observed, no clear differentiation between their exchange rates was found except that the half-cystine-6 peptide NH did exchange most rapidly—in 5 min. The carboxamide protons in both D-proline-7 analogs exchanged slightly more slowly than the peptide NH's. Since the concentration of water in the dimethyl sulfoxide, including the small amount

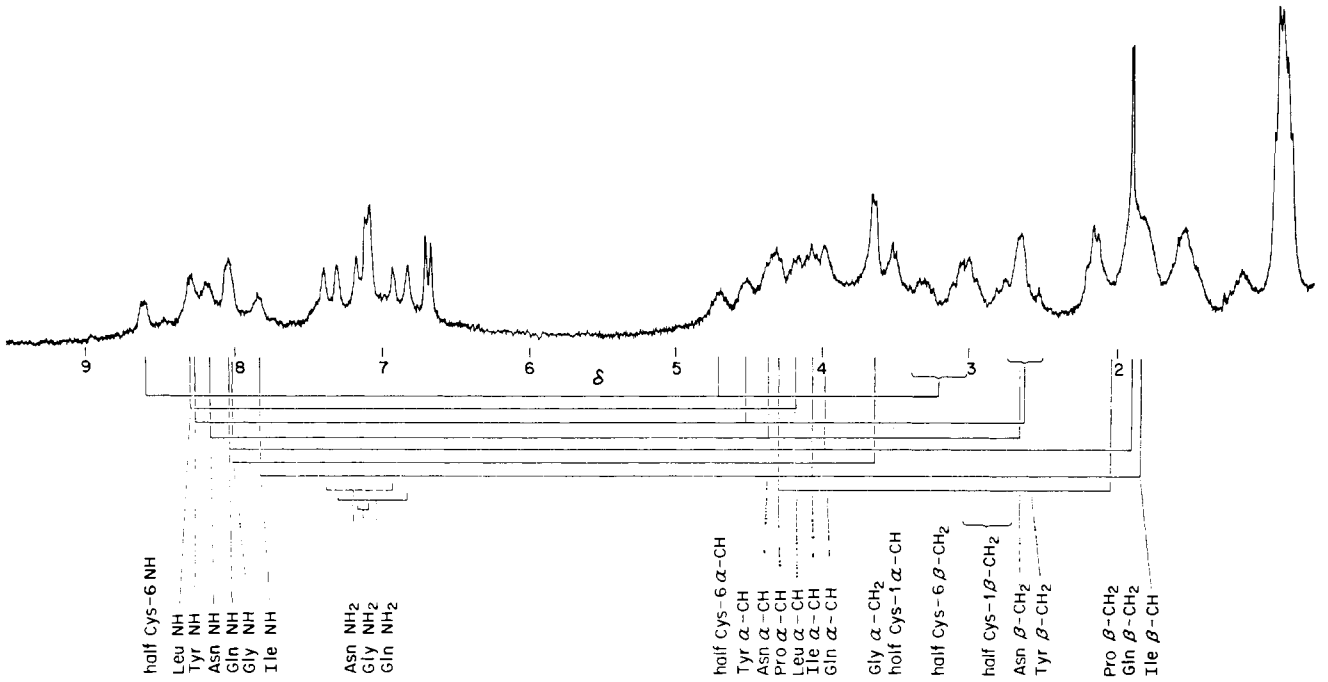

FIGURE 5: 220-MHz spectrum of [7-D-proline]oxytocin.

of D_2O added, is minimal, the acid–base catalysis of exchange can be assumed to be negligible. Therefore, the rate-determining factor on the deuterium exchange is likely the internal motion or "breathing" of the molecule.

It is expected that a proton which is either shielded from the solvent molecules or involved in an intramolecular hydrogen bond will show very little or no temperature dependence of its chemical shift, in contrast to those protons hydrogen bonded to a solvent such as dimethyl sulfoxide, which are expected to exhibit an upfield shift with increasing temperature.[1] The results of the temperature dependence studies for oxytocin and deamino-oxytocin are shown in Figure 7. For the peptide amide hydrogens of oxytocin there are some differences to those previously reported (Walter *et al.*, 1971). Whereas in tocinamide and deaminotocinamide (Brewster *et al.*, 1972) an internal hydrogen bond could be predicted to involve one of the carboxamide protons on the basis of its zero temperature dependence, the carboxamide protons in oxytocin and deamino-oxytocin show normal behavior. (The peptide NH chemical shift in *N*-methylacetamide exhibits a temperature coefficient of -6×10^{-3} ppm/°C in Me_2SO.) Results of the dependence of the chemical shifts of the peptide NH and carboxamide proton resonances on temperature for the four analogs are shown in Figure 8. The asparagine-5 peptide NH showed a zero or very small $d\delta/dT$ in three of the analogs, [4-glycine]oxytocin, [7-D-proline]oxytocin, and deamino-7-D-proline-oxytocin. In [2-valine]oxytocin, $d\delta/dT$ equals -3×10^{-3} ppm/°C for this peak, whereas the peak assigned to either isoleucine-3 or glutamine-4 had a zero temperature dependence. In [4-glycine]oxytocin, the tyrosine-2 peptide NH proton also exhibited a near zero temperature dependence. All of the carboxamide proton peaks in these four analogs studied had significant chemical-shift dependence on temperature. As in the spectrum of oxytocin, the glycine-9 carboxamide proton resonances of [2-valine]oxytocin appeared as nearly a single peak at ambient temperature, then gradually moved apart with increasing temperature, becoming separated by 0.04 ppm at 45°. Those in [4-glycine]oxytocin appeared as two peaks separated by 0.03 ppm at ambient temperature and showed only a small difference in their temperature dependence. In the D-proline-7 analogs, they were clearly separated by 0.07–0.10 ppm at ambient temperature and again showed rather small differences in $d\delta/dT$.

Peptide NH to α-CH coupling constants ($J_{N\alpha}$) were measured and did not change with temperature. In the case of [4-glycine]oxytocin, the tyrosine-2 peptide NH resonance was very broad, making it impossible to measure its $J_{N\alpha}$ accurately; we therefore placed no restrictions, other than those of low intramolecular energy, on ϕ_{Tyr_2}. The values of $J_{N\alpha}$ and the dihedral angles (Edsall *et al.*, 1966a–c) calculated from them and used in the search for cyclic conformers are listed in Tables II and III.

Discussion

The conformational calculations suggest two possible structures for oxytocin with a transannular hydrogen bond. In one structure (Table II, oxytocin-II) the asparagine-5 peptide NH is hydrogen bonded to the tyrosine-2 carbonyl, and a 1–4 turn is adopted as suggested by Urry *et al.* (Urry *et al.*, 1970; Urry and Walter, 1971). In the other conformation (Table II, oxytocin-III) the half-cystine-1 carbonyl partici-

[1] In a strong hydrogen-bond-accepting solvent such as dimethyl sulfoxide, all accessible NH groups may be considered to be hydrogen bonded, either intramolecularly *or* to the solvent. As the temperature is raised, the solvent-to-NH bonds are increasingly broken and the NH resonances move upfield. An NH proton, solvent-shielded or involved in an intramolecular hydrogen bond, resides in a nearly constant local environment during a temperature change provided the conformation is insensitive to temperature. Consequently, the chemical shift of an intramolecular hydrogen-bonded NH should show a relatively small temperature dependence, if any. Agreement between results from temperature studies (near zero temperature effect) and from deuterium-exchange studies (slower exchange) for solvent-shielded or intramolecularly hydrogen-bonded structures generally will be observed only in cases where a rigid structure obtains, since the temperature dependence of chemical shift is an equilibrium property, while deuterium exchange may be controlled by the kinetics of any conformational interconversions which occur in flexible structures.

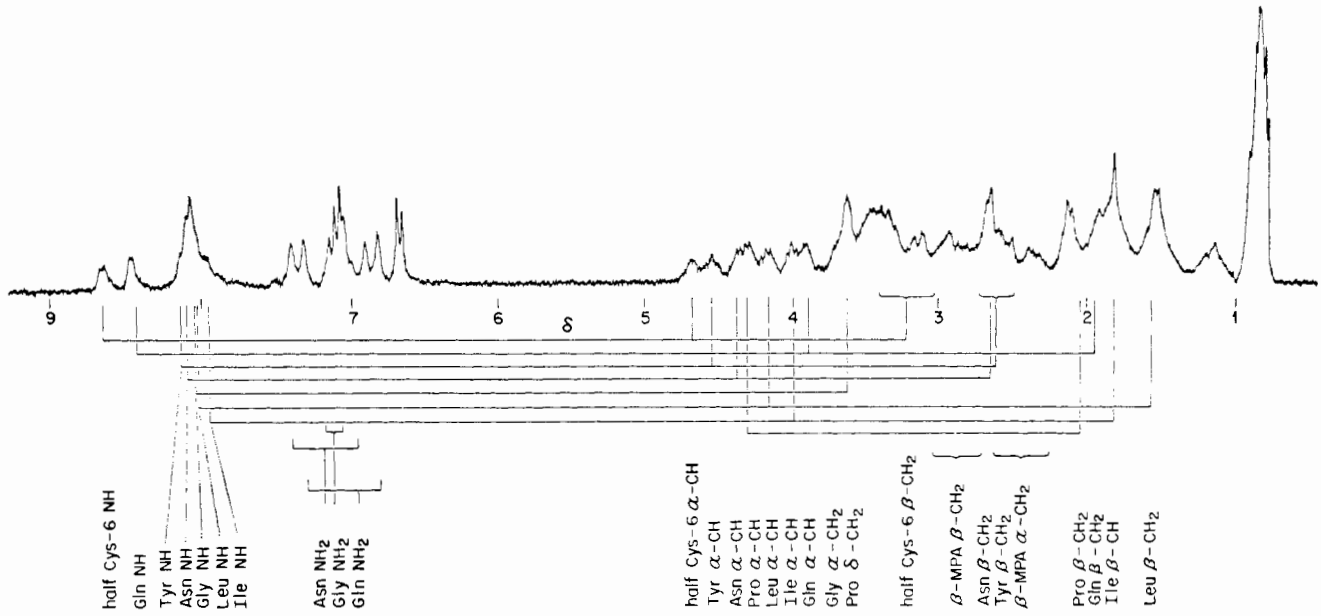

FIGURE 6: 220-MHz spectrum of deamino-7-D-proline-oxytocin.

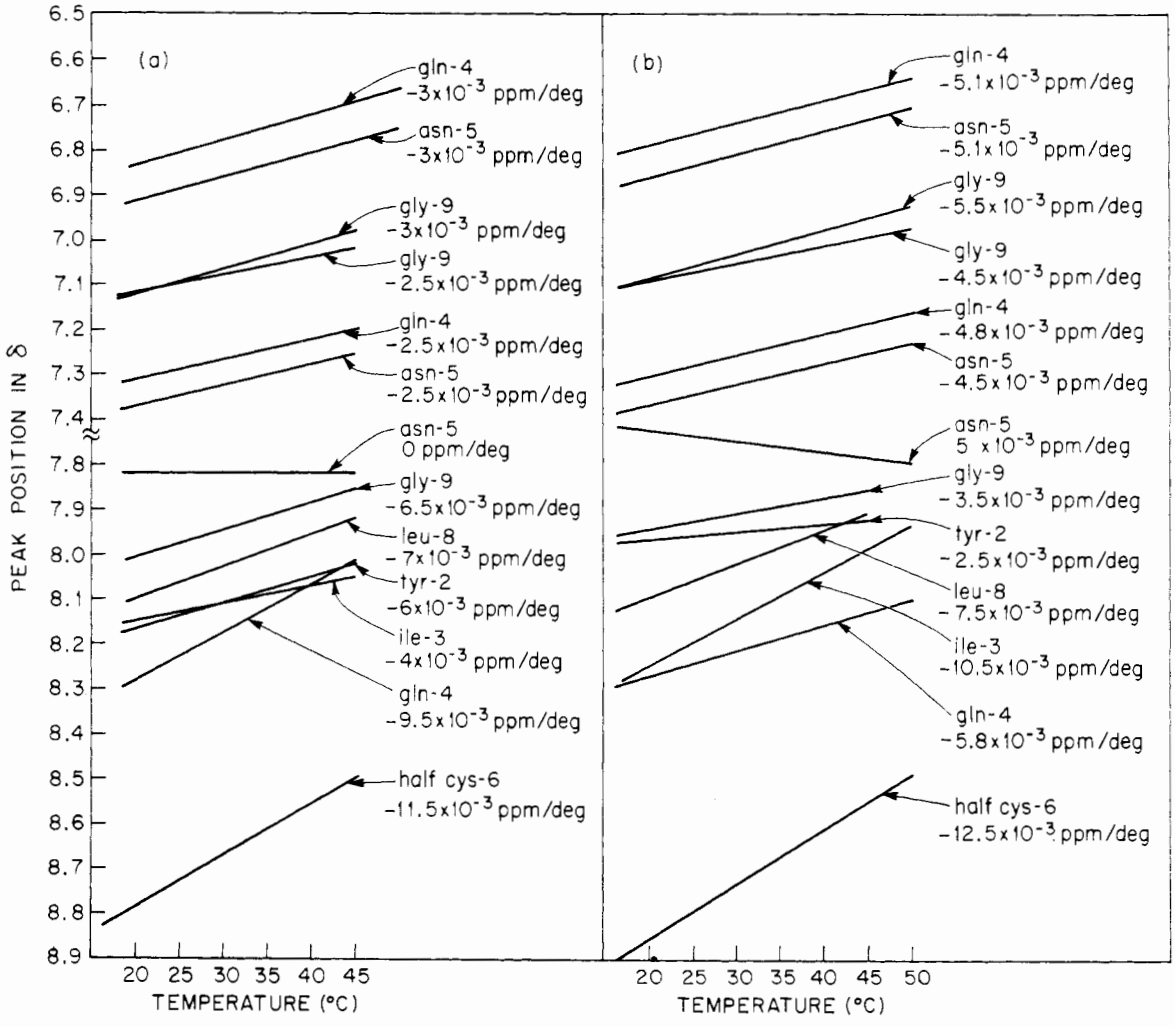

FIGURE 7: Temperature dependence of chemical shift of the peptide and carboxamide NH protons: (a) oxytocin and (b) deamino-oxytocin.

pates in a hydrogen bond with the same NH proton, producing a 1–5 turn. The latter structure has an intramolecular energy comparable to the 1–4 turn conformer. A 1–4 turn has been commonly found to be favored in cyclic hexapeptides (Stern *et al.*, 1968; Kopple *et al.*, 1972; Torchia *et al.*, 1972a,b), and the 1–5 turn has also been found in cyclic peptides (Duax *et al.*, 1972). As noted previously, however, the ring of oxytocin is closed by a disulfide bridge, rendering the analogy to

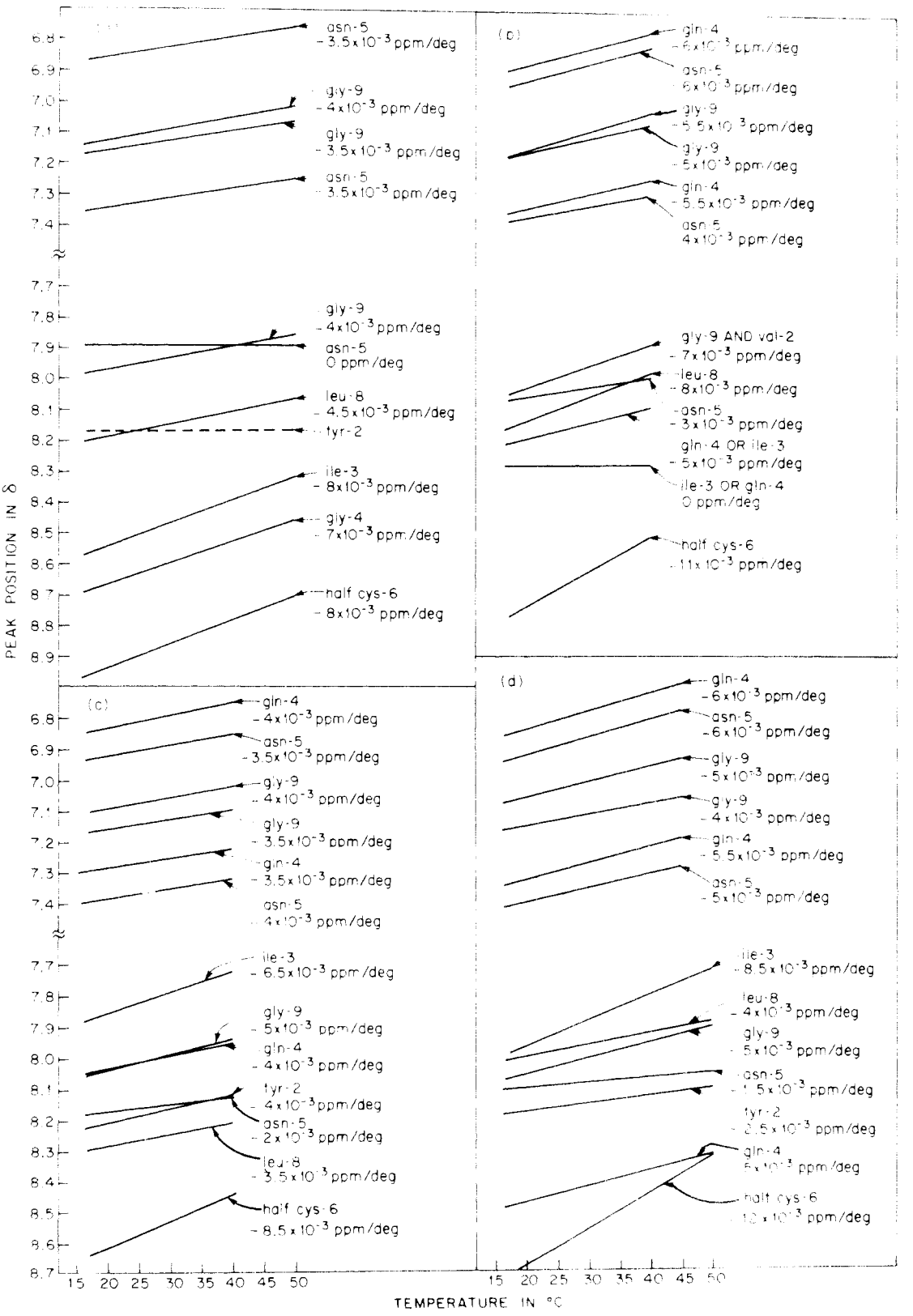

FIGURE 8: Temperature dependence of chemical shift of: (a) [4-glycine]oxytocin, (b) [2-valine]oxytocin, (c) [7-D-proline]oxytocin, and (d) deamino-7-D-proline-oxytocin.

ordinary cyclic hexapeptides less than complete. Our search for low-energy cyclic structures also yielded another conformation with an intramolecular energy comparable to the 1–4 and 1–5 turn structures. In this conformer, there is a hydrogen bond between the asparagine-5 peptide NH and the glutamine-4 carboxamide carbonyl. In addition to the favorable energy, this conformation (I, see Table II) not only emphasizes the importance of the asparagine residue in position 5 of oxytocin, as do conformations II and III (Table II), but also points up the significance of a residue such as glutamine,' having a carboxamide carbonyl, at position 4. The fairly rapid deuterium exchange of all the NH protons is

TABLE II: Experimental $J_{N\alpha}$ and Calculated ϕ and ψ (Edsall *et al.*, 1966) for Low-Energy Conformations of Oxytocin and Deamino-oxytocin.

	Oxytocin I			Deamino-oxytocin			Oxytocin II		Oxytocin III	
	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)	ϕ (deg)	ψ (deg)	ϕ (deg)	ψ (deg)
Half-cystine-1			270			0		280–300		120
Tyrosine-2	7	90	120	0–1	340	120	30	0	240	0
Isoleucine-3	5	105	120	4	110	120	105	270–300	105	120
Glutamine-4	6	100	330	5	105	330	210	240	20	240
Asparagine-5	6	100	330	6	100	300	20	240–270	20	240
Half-cystine-6	7.5	30	270–360	8	30	270–360	90	270–330	90	270–330
Proline-7		120	125		120	125	120	125	120	125
Leucine-8	7.5	30	120	7	30	120	30	120	30	120
Glycine-9	6 and 4	Flexible		5 and 5	Flexible		Flexible		Flexible	

consistent with a rather flexible molecule in rapid equilibrium with several conformers of equal energy. It would appear that the conformations above as well as others may all contribute to the conformation of oxytocin in Me_2SO.

It has been shown that the side-chain of oxytocin exerts considerable influence on the ring conformation since the conformation of the ring alone (Brewster *et al.*, 1972) is certainly different from that of the ring in oxytocin (different $J_{N\alpha}$ and chemical shifts, etc., are observed for corresponding residues). Carbon-13 nmr studies (Brewster *et al.*, 1973; Dorman and Bovey, 1973) indicate that a trans half-cystine-6 to proline-7 peptide bond (with a proline $\phi = 120°$) connects the side chain to the ring. Leucine-8 most probably assumes one of three orientations (Brant *et al.*, 1967): $\phi \simeq 30$ or 90°, $\psi = 120$ or 270–360°, or $\phi = 240°$, $\psi = 240°$ (Edsall *et al.*, 1966a–c). The glycine residue is probably flexible.

There are two possible values for ψ of proline-7 (Schimmel and Flory, 1968): the trans′ configuration with $\psi = 270$–360° and the *cis′* configuration with $\psi = 110$–150°. With a trans–trans′ orientation at the ring-to-tail junction only values of $\phi = 240°$, $\psi = 240°$ for leucine-8 allow the side chain to fold back toward the ring. With a trans–cis′ orientation, the side-chain folds back toward the ring when $\phi \approx 30°$ or 90° and $\psi = 120$ or 270–360° for leucine-8. In the spectrum of oxytocin and of deamino-oxytocin, the glycine-9 carboxamide protons appear as almost a single peak at 20–25°, gradually becoming two resonances separated by 0.04 ppm at 45°, and it is interesting to note that substitution of the glycine-9 proton by a methyl group as in [9-sarcosine]oxytocin gives an analog with greatly reduced activities (Cash *et al.*, 1962). We have chosen the structure (Table II) with a trans–cis′ ring-to-tail junction and a $\phi \simeq 30$ or 90°, $\psi = 120°$ for leucine-8 because, of the three possible side-chain orientations suggested here, only this one is not favored either energetically or sterically (Schimmel and Flory, 1968) with the substitution of a methyl group at the glycine-9 backbone NH. We do not find evidence for a 1–4 turn in the side chain as previously suggested (Urry and Walter, 1971). A space-filling model of conformation I is shown in Figure 9.

Our search for cyclic structures revealed that the lowest energy conformation found for deamino-oxytocin allows retention of the asparagine-5 peptide NH to glutamine-4 carboxamide carbonyl hydrogen bond, and also permits an across-the-ring hydrogen bond between the tyrosine-2 peptide NH and the glutamine-4 backbone carbonyl. By contrast, the NH and C=O groups of the tyrosine-2 and asparagine-5 residues are hydrogen bonded in the 1–4 turn structure (Urry *et al.*, 1970; Urry and Walter, 1971). The α-amino group cannot be easily accommodated in our proposed deamino-oxytocin conformer, due to steric overlaps with the glutamine-4 and asparagine-5 residues. Since the α-amino group is ionizable, it might be suggested that this property of the α-amino group also works to prevent the formation of the across-the-ring hydrogen bond in oxytocin.

For [4-glycine]oxytocin, temperature dependence studies suggest that the asparagine-5 peptide NH participates in a hydrogen bond, and since this analog possesses no carboxamide carbonyl at position 4, it cannot involve the hydrogen bond proposed in one of our suggested conformations of oxytocin (Table II, structure I). We find an energetically favorable conformation in which the asparagine-5 peptide NH is hydrogen bonded to the tyrosine-3 carbonyl. Also in agreement with a $d\delta/dT$ of 0 for the tyrosine-2 NH, we find this proton can be hydrogen bonded to the asparagine-5 carbonyl. This portion of the molecule, therefore, assumes a 1–4 turn which essentially corresponds to an energetically favorable LD (LG) bend (Venkatachalam, 1968; Ramachandran and Chandrasekaran, 1972). The tripeptide

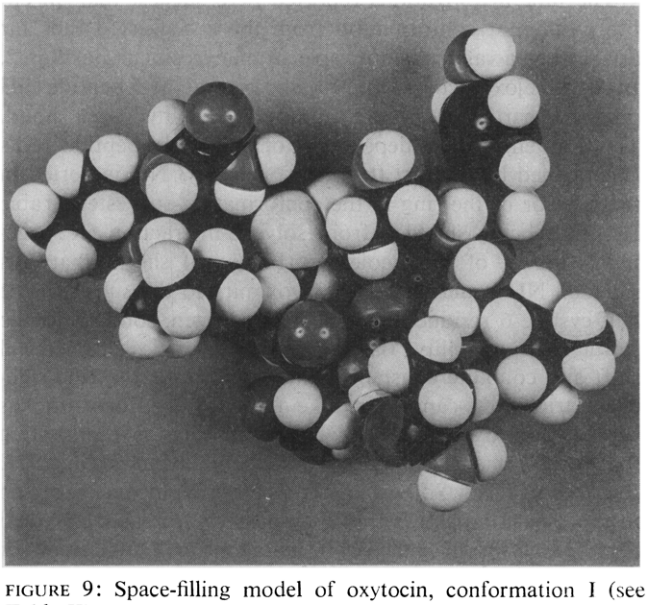

FIGURE 9: Space-filling model of oxytocin, conformation I (see Table II).

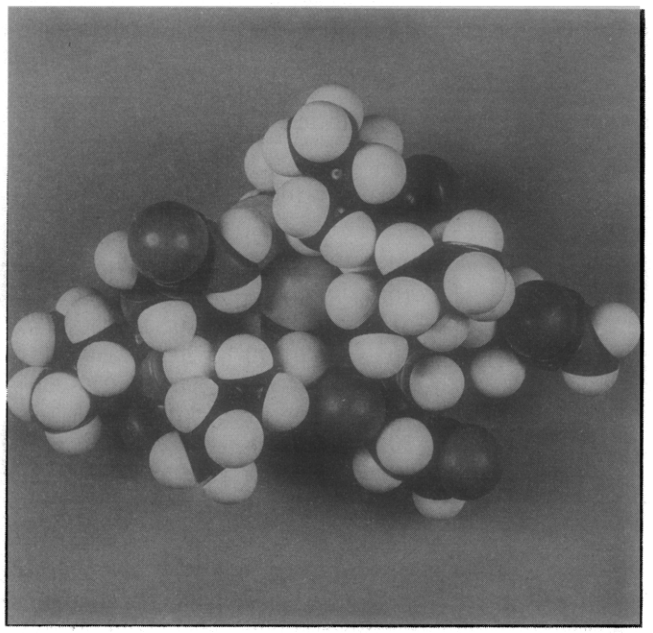

FIGURE 10: Space-filling model of [2-valine]oxytocin.

side chain can be assumed to possess the same conformational features as that suggested in oxytocin, in agreement with similarities in coupling constants and chemical shifts for the NH, α-CH, and β-CH and other protons in the two molecules. On steric grounds one would anticipate that a glycine substitution in the position 4 would be most favorable for a 1–4-like turn in the oxytocin ring (Crawford *et al.*, 1973). It is to be remarked that of many substitutions made in this position with hydrophobic amino acids (Rudinger, 1971; Fluoret and du Vigneaud, 1969), the glycine analog is the least potent in the pharmacological activities usually associated with oxytocin. It will be interesting to determine if the "loosening" of this apparent 1–4 turn occurs as one proceeds to more active compounds substituted in the 4 position (*e.g.*, [4-leucine]oxytocin, [4-valine]oxytocin, etc.), and a conformation more similar to others suggested for oxytocin, such as structure I, becomes more probable.

The nmr investigations reported here suggest that an entirely different conformation from those suggested thus far arises when a valine residue replaces the tyrosine at position 2. In [2-valine]oxytocin (Figure 10), the asparagine-5 peptide NH does not participate in an intramolecular hydrogen bond. The zero temperature dependence of one of the peptide NH's is explained by the fact that the isoleucine-3 NH is buried in the interior of the ring in the conformation suggested (Table III), thus being shielded from solvent interaction. A space-filling model of this molecule (Figure 9) shows that this shielded NH comes into close proximity to the sulfur of the half-cystine-6 residue and a weak hydrogen bonding to the sulfur is a possibility. Based on the similarities of chemical shift and coupling constant data, we conclude that the side-chain tripeptide can again take the same conformation as suggested for oxytocin. In view of our findings, it is interesting that careful pharmacological work (see Rudinger *et al.*, 1972, for a recent review) on various analogs of oxytocin substituted in the 2 position shows that they possess decreased maximal response ("partial agonism"), antagonism ("inhibitory or anti-hormonal"), markedly reduced potencies, etc., indicating that the presence of the hydroxyl group in particular is very

TABLE III: Experimental $J_{N\alpha}$ and Calculated ϕ and ψ (Edsall *et al.*, 1966) of Suggested Low-Energy Conformations.

	[4-Glycine]oxytocin			[2-Valine]oxytocin			[7-D-Proline]oxytocin			Deamino-7-D-proline-oxytocin		
	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)	$J_{N\alpha}$ (Hz)	ϕ (deg)	ψ (deg)
Half-cystine-1			300			300			270			300
Tyrosine-2	(6) br	30	330	0–1 Val-2	150 Val-2	120 Val-2	6	100	120	7	30	120
Isoleucine-3	4.5	120	270	5 or 6	105	330	6	100	120	6	100	120
Glutamine-4	5 and 6.5 Gly-4	260 Gly-4	210 Gly-4	5 or 6	100	120	6	100	330	4	110	330
Asparagine-5	7	30	330	6	100	240	6	100	330	5	105	300
Half-cystine-6	8	30	270–360	6.5	100	270–360	7	30	270–360	6.5	90	330
Proline-7		120	125		120	125		240	240		240	240
Leucine-8	7.5	30	120	7	30	120	4	0	120	5	20	120
Glycine-9	5.5 and 5		Flexible	(5 and 5)		Flexible	(5 and 5)		Flexible	(5 and 5)		Flexible

important, through not essential for the biological activities of oxytocin.

[7-D-Proline]oxytocin and deamino-7-D-proline-oxytocin are found to have conformations similar to those suggested for the ring portions of oxytocin and deamino-oxytocin, respectively. The critical feature in the D-proline-7 analogs which markedly differentiates their suggested conformations from those of oxytocin and deamino-oxytocin, respectively, is the relation in space of the ring and the side chain. We conclude from carbon-13 magnetic resonance studies (Brewster *et al.*, 1973; Dorman and Bovey, 1973) that the proline-7 to half-cystine-6 peptide bond remains trans. In oxytocin, a folding of the side chain toward the ring in Me_2SO solution has been suggested based on nmr data on the hormone itself and differences with the nmr of the ring and side-chain moieties of hormone when examined separately (Brewster *et al.*, 1972; Hruby *et al.*, 1971a; Urry and Walter, 1971; Walter, 1971). If we assume that the preferred orientation at the ring-to-side-chain junction is trans–cis′ ($\psi_{Pro_{7\text{-}D}} \approx 210$–$250$) and, following the same argument as in oxytocin, *i.e.*, assume that the probable values for ϕ and ψ of leucine-8 are approximately 0 and 120°, respectively, then when the proline at position 7 is of the D configuration the side chain is extended away from the ring and there is no possibility of interaction between the side chain and the ring. Further evidence of the absence of side-chain–ring interaction is provided by the observation that in the spectra of [7-D-proline]oxytocin and deamino-7-D-proline-oxytocin, the carboxamide NH protons of glycine are no longer nearly equivalent as they are in oxytocin. They are now separated by 0.07–0.10 ppm at 20° with little change in separation with rising temperature.

Considerable caution is necessary when discussing the "relationships of conformation to pharmacological activities" since it depends on which pharmacological response is referred to, and the solvent in which the studies are made (at the present time the nature of the environment at the hormone receptor and the nature of the hormone–receptor interactions are not known). On the basis of conformational studies in Me_2SO (Brewster *et al.*, 1972; this paper; Walter, 1971), it can be suggested that both the presence of the side chain and the occurence of some particular relationship(s) of the side chain to the ring are important and perhaps necessary for display of avian vasodepressor activity in oxytocin. In the case of oxytocic and milk-ejecting activities, however, a more subtle relationship apparently exists. First, the side-chain moiety is obviously not essential for the presence of oxytocic and milk-ejecting activities (tocinamide and deaminotocinamide are active). From the nmr studies, tocinamide (Brewster *et al.*, 1972) definitely possesses a different conformation in Me_2SO from that of the ring of oxytocin since chemical shifts, coupling constants, temperature dependence of chemical shifts, etc., are markedly different for equivalent residues. On the other hand, the ring moieties in [7-D-proline]oxytocin and deamino-7-D-proline-oxytocin probably have similar conformations in Me_2SO to those found in oxytocin and deamino-oxytocin, but possess oxytocic and milk-ejecting potencies similar to those of the corresponding ring compounds tocinamide and deaminotocinamide. Either the changed relationship of the side chain to the ring or some other structural feature(s) related to the receptor–hormone interaction or environment may be involved.

Conclusions

Oxytocin and deamino-oxytocin appear to have very similar conformations in dimethyl sulfoxide solution. For oxytocin, three conformations of equivalent energy are found which are consistent with the nmr and other available data. One of the preferred conformations of oxytocin contains a single hydrogen bond between the asparagine-5 backbone peptide NH and the glutamine-4 carboxamide carbonyl. The results suggest that oxytocin is conformationally labile in a rapid equilibrium with other conformations such as the two possessing across the ring hydrogen bonds. Nmr gives no evidence for hydrogen bonding in the tripeptide side chain, and we propose a trans–cis′ proline–half-cystine-6 junction, bringing the glycine-9 carboxamide protons close enough to the ring portion of the molecule to participate in some interaction(s) favoring the folding back of the side chain toward the ring and causing virtual equivalence of these protons at room temperature.

The presence of certain amino acid residues at particular positions is critical to the conformations of oxytocin and its derivatives in Me_2SO, and in some cases this appears to be related to the pharmacological activities displayed by these oxytocin analogs. Removal of the α-amino group on half-cystine-1 of oxytocin allows an additional hydrogen bond between the tyrosine-2 peptide NH and the glutamine-4 backbone carbonyl in deamino-oxytocin which would not be allowed in the presence of the α-amino group. Substitution of glycine for glutamine in [4-glycine]oxytocin removes the possibility of a hydrogen bond between the asparagine-5 peptide NH and the glutamine-4 carboxamide carbonyl but allows hydrogen bonds involving the asparagine-5 peptide NH and carbonyl with the tyrosine-2 carbonyl and peptide NH. [2-Valine]oxytocin, with the aromatic portion of the molecule absent, appears to assume an entirely different ring conformation from any of those suggested for oxytocin, with a buried isoleucine-3 peptide NH which is possibly weakly hydrogen bonded to a sulfur atom. When a D-amino acid residue links the tripeptide side chain to the ring in [7-D-proline]oxytocin, the side chain does not fold in toward the ring, but remains extended away from it.

These studies suggest that a number of conformations might exist for the neurohypophysial hormone oxytocin and its analogs in dimethyl sulfoxide. These molecules (especially the side-chain portions) appear to be quite flexible in dimethyl sulfoxide, and the conformations suggested here probably represent the most favorable ones in this solution.

Recently deuteron magnetic resonance relaxation studies on partially deuterated derivatives of oxytocin and related compounds (Glasel *et al.*, 1973a,b) showed that these molecules are quite flexible in aqueous solution. This is in agreement with an energy minimization study of oxytocin (Kotelchuck *et al.*, 1973) which also suggested that there may be considerable flexibility of the oxytocin molecule in aqueous solution. It would appear, therefore, that both in dimethyl sulfoxide and water, oxytocin is a relatively flexible molecule.

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