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Relation of the Conformation of Oxytocin to the Biology of Neurohypophyseal Hormones

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ABSTRACT **The conformation of oxytocin is related to the evolution and to some of the biological activities of neurohypophyseal hormonal peptides. On the basis of the three-dimensional structure, positions 3, 4, 7, and 8 are the only loci at which naturally occurring neurohypophyseal peptides may be expected to differ. The side chains of these amino-acid residues are the primary determinants of the differential specificity in interactions between neurohypophyseal hormones and their receptors.**

There are three general groups of structural modifications of neurohypophyseal hormones which can be correlated with specific changes in biological activity: (*a*) those affecting the stabilization of the backbone of the peptide, which would extensively perturb the spatial relationships among all the constituent amino acids and hence, affect both affinity and intrinsic activities uniformly; (*b*) those which, while retaining the stability of the backbone conformation, alter the steric environment and charge distribution of limited surface areas, and thereby can affect affinity and intrinsic activity differentially; and (*c*) those changing the steric and electronic requirements of moieties comprising the active surface of the neurohypophyseal peptide, without perturbing the peptide backbone of the hormone molecule and, hence, affecting intrinsic activity without altering affinity.

Neurohypophyseal hormones exhibit a variety of biological effects on contractile and membrane-transport phenomena. The initial event in the action of any hormone on its target cell, quite independent of any intermediary events and of the nature of the final response, requires a high degree of topological and chemical complementarity of the hormonal molecule and some macromolecular receptor. The elucidation of the peptide backbone of oxytocin as two β-turns (1) has afforded us a point of departure for a consideration of how the conformation of oxytocin can be related to the evolution of neurohypophyseal hormones and, in addition, of how the differential biological properties of naturally occurring neurohypophyseal peptides and their synthetic congeners can be envisioned.

The latter approach assumes that the "information" contained in the hormonal peptide finds full expression in the biological parameter measured. Expressed in terms of current receptor theory (2), a "stimulus", generated as a consequence of a hormone interacting with its specific target-tissue discriminator, depends upon three factors: (*a*) hormone concentration, (*b*) hormone affinity, i.e., the ability of the hormone to bind to its receptor, (*c*) hormone effectiveness, i.e., the ability to generate a stimulus at optimal receptor occupation (intrinsic activity). While the affinity parameter can usually be readily approximated experimentally, this cannot be said of intrinsic activity—neither for *in vivo* situations nor for *in vitro* situations. In fact, we postulate that all peptide hormones which initiate processes involving one or more coupling steps prior to a measurable response produce levels of stimuli in excess of that which can be expressed in the measured response. The existence of such saturation phenomena at any step preceding the final response has its biological significance in that it increases the sensitivity of the system (receptor saturation is not required for maximal response) and protects the system from potentially deleterious fluctuations of its components. Saturation phenomena may obscure the determination of the true intrinsic activity of the hormone. One approach by which this difficulty can be overcome is by supplementing the measurement of the final biological response with the measurement of a parameter that denotes an early step in the sequence of events—as close as possible to the initial hormone–receptor interaction.

For the "biologically active" conformation of oxytocin ("cooperative model"), in contrast to the structure observed in Me_2SO–MeOH (1) (or Me_2SO–D_2O) (3), we assume that the tyrosine side-chain folds back over the 20-membered ring component of the hormone. In placing the tyrosine side-chain in such a conformation, we are weighing its loss of rotational freedom against (*a*) the reduction of the hydrophobic surface area in contact with the hydrophilic environment, (*b*) the favorable proximity of the cross β-structure (comprised of the hydrogen-bonded peptide N—H and C=O groups of tyrosine and asparagine) to the aromatic moiety of tyrosine with the possibility of a pseudo π–π interaction between amide groups and aromatic ring, and (*c*) the possibility of intramolecular hydrogen bonding to the $CONH_2$ moieties of glycine, asparagine, and glutamine. Moreover, there is at present reason to believe that, to a first approximation, the peptide backbone conformation of all known naturally occurring neurohypophyseal peptides (including the vasopressins) should be the same as that of oxytocin, although the relative population of possible rotamers of individual amino-acid side chains probably differs among different neurohypophyseal peptides.

The cooperative model of oxytocin consists of a topological unit that is maximally intramolecularly stabilized. One side of the cross β-structure, with the exception of the protruding disulfide bond and C=O of isoleucine, is essentially featureless (hydrophobic surface), while the contours of the other side

Abbreviation: ArgVP, arginine vasopressin.

TABLE 1. *Neurohypophyseal peptides of vertebrates*

	1	2	3	4	5	6	7	8	9
1. Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly-NH_2
2. Mesotocin	—	—	—	—	—	—	—	Ile	—
3. Isotocin	—	—	—	Ser	—	—	—	Ile	—
4. Glumitocin	—	—	—	Ser	—	—	—	Gln	—
5. Arginine vasotocin	—	—	—	—	—	—	—	Arg	—
6. Arginine vasopressin	—	—	Phe	—	—	—	—	Arg	—
7. Lysine vasopressin	—	—	Phe	—	—	—	—	Lys	—
8. Elasmobranch Principle I	Unknown structure								
9. Elasmobranch Principle II	Unknown structure								

For phylogenetic considerations see Sawyer, W. H., in *Handbook of Experimental Pharmacology*, ed. B. Berde (Springer-Verlag, Berlin, 1968), Vol. 23, p. 717; and Vliegenthart, J. F. G., and D. H. S. Versteeg, *J. Endocrinol.*, **38**, 3 (1967).

of this structure are obscured by the overlying aromatic moiety of tyrosine, the C-terminal tripeptide, the side chain of asparagine, and possibly the side chain of glutamine (hydrophilic surface). This model utilizes each of the constituent amino-acid residues to its greatest effectiveness. The side chains of each of the component amino acids—with the exception of the isoleucine, glutamine, proline, and leucine residues in positions 3, 4, 7, and 8—play a critical role in the formation and intramolecular stabilization of the preferred backbone conformation. The side chains of residues 3, 4, 7, and 8 are free to engage in intermolecular interactions, while having a limited effect on the conformation of the peptide backbone (although bulky side chains help to stabilize the β-turn). On the basis of these structural considerations positions 3, 4, 7, and 8 are the only variable loci to be expected in naturally occurring neurohypophyseal hormones—and hormonal peptides that differ in positions 3, 4, and 8 have already been so identified (Table 1.). Alterations in positions other than 3, 4, 7, and 8 would destabilize the three-dimensional integrity of the molecule, and this would be inconsistent with the presumptions of evolutionary conservation.

The inherent limitation for naturally occurring alterations within these small polypeptides necessitates the existence of different receptors, i.e., receptors that must recognize different aspects of a molecule in order to mediate the different observed biological activities exhibited by a single hormone. The side chains of residues 3, 4, 7, and 8 are the distinguishing topological features of the molecule and, as such, are the primary determinants of the differential specificity in neurohypophyseal hormone–receptor interactions; however, this does not imply that all of these side chains are necessarily constituents of the "active sites" of the hormone in any particular "hormone–receptor" complex. The specific functional role of positions 3 and 8, for example, may be illustrated by a comparison of arginine vasopressin (ArgVP), arginine vasotocin, and oxytocin. This kind of comparison has led us to a unifying hypothesis for the interaction of these hormones with a number of receptors (Table 2)—unifying to the extent that the hormone is oriented so that its hydrophobic surface is in close contact with, or even in part enveloped by, a hydrophobic cleft on the receptor, while the opposite surface, containing the cluster of hydrophilic groups of the hormone, faces away from the cleft of the receptor towards the aqueous environment. Specifically, in the case of oxytocin, the hydrophobic surface will encompass the featureless surface of oxytocin extending from the isoleucine in position 3 to the leucine in position 8. In the case of ArgVP, the hydrophobic surface includes phenylalanine in position 3, but excludes arginine in position 8, inasmuch as the side chain of arginine will be part of the hydrophilic cluster. When ArgVP interacts with the antidiuretic receptor the arginine side chain appears to extend into the aqueous environment and hence there is no highly stringent requirement for specificity at this locus. Contrariwise, in view of the detrimental effect on pressor activity resulting from stereoisomeric aminoacid replacements in position 8, a highly specific complementary charge interaction is important (Table 3).

An amino acid of D-configuration in position 8 would, of course, also change the orientation of the end peptide moiety (-CONH-) of the second β-turn (4); nevertheless, the tripeptide tail could still be held close to the ring moiety by utilizing the carboxamide NH of asparagine. The fact that ArgVP has highly diminished uterotonic and milk-ejecting activities (as compared with oxytocin, see Table 2) can be explained by a combined effect of the unfavorable interaction of the charge-bearing side chain in position 8 of ArgVP with the hydrophobic receptor site of oxytocin as well as by the inability of the bulky duet of aromatic side chains in positions 2 and 3 (see below) to fit into the pocket of the receptor which is adapted to accommodate the isoleucine side chains of oxytocin. These combined effects reduce substantially the fit (and consequently the biological potency) of ArgVP for the oxytocin receptor. Conversely, the fact that

TABLE 2. *Biological actions of some neurohypophyseal hormones*[a]

Hormone	Uterotonic[b] (rat)	Milk-ejection (rabbit)	Antidiuretic (rat)	Pressor (rat)	Hydroosmotic[c] (toad)	Natriferic (frog)
Oxytocin	500	430	5	5	1.29×10^{-9} M	450
Arginine vasotocin	155	210	250	245	7.10×10^{-12} M	1140
Arginine vasopressin	12[d]	~70	500[d]	500	1.70×10^{-10} M	32.6

[a] Biological activities are expressed as USP units per mg, and taken from a review (Walter, R., J. Rudinger, and I. L. Schwartz, *Amer. J. Med.*, **42**, 653, 1967), unless otherwise noted.

[b] Isolated uterus in magnesium-free van Dyke–Hastings bathing medium (Munsick, R. A., *Endocrinology*, **66**, 451, 1960).

[c] Concentration for obtaining half-maximal responses in the isolated toad urinary bladder assay. Values are reported by Eggena, P., I. L. Schwartz, and R. Walter, *J. Gen. Physiol.*, **52**, 465 (1968).

[d] Meienhofer, J., A. Trzeciak, R. T. Havran, and R. Walter, *J. Amer. Chem. Soc.*, **92**, 7199 (1970).

TABLE 3. *Biological effects of optically isomeric amino acids in position 8 of neurohypophyseal peptides*

Amino acid in position 8	Vasopressor	Antidiuretic
Arginine[a]	500	500
Lysine[a]	250	245
Diaminobutyric acid[b]	150	120
D-Arginine[b]	4	114
D-Lysine[b]	0.75	10
D-Diaminobutyric acid[b]	4	120

[a] See Table 2.

[b] Values reported by Zaoral, M., J. Kolc, and F. Šorm, *Collect. Czech. Chem. Commun.*, **32**, 1250 (1967).

oxytocin has significantly diminished antidiuretic and pressor activities (as compared with ArgVP) is a result of the lack of matching hydrophobic sites at the antidiuretic and pressor receptors for position 8 of oxytocin, as well as the diminished potential for an energetically favorable interaction of the aliphatic side chain in position 3 of oxytocin with the vasopressin receptors.

Since oxytocin and ArgVP differ simultaneously in two loci, it is advantageous to consider arginine vasotocin, which differs from oxytocin and from ArgVP in only a single amino-acid substitution. It can be seen from Table 2 that arginine vasotocin exhibits an intermediary range of potency in its oxytocin- and vasopressin-like activities, consistent with the aforementioned conformational considerations. These activities are *pharmacologic* for arginine vasotocin, which serves as a molecular probe in these circumstances. However, when arginine vasotocin, the natural water-balance principle of lower vertebrates (5), is evaluated for its *physiological* activity by employing amphibian test systems, its potency is found to be strikingly higher than that of both oxytocin and ArgVP—a finding which is indicative of the precise molecular tailoring of the respective receptors for the particular requirements of the substituents in positions 3 and 8 of these hormones.

When our considerations of structural modifications are extended beyond positions 3, 4, and 8 to any other position in the molecule, we can differentiate in principle between three types of alteration: *A*, those which affect the stabilization of the backbone of the peptide, which would perturb extensively the relative spatial relationship of all the constituent amino acids; *B*, those which change the volume and charge distribution of limited surface areas, with retention of the stability of the basic backbone conformation; *C*, those which alter the steric and electronic requirements of moieties comprising the active site of the neurohypophyseal peptide, without otherwise perturbing the skeleton conformation. Each of these categories of structural alterations, here characterized in terms of conformational and topological changes, may be expected to have corollary effects on biological activity.

MODIFICATIONS OF TYPE A

Structural changes discussed in this category would alter the ability of the peptide to interact with all receptors, and uniformly affect both affinity and intrinsic activity. Such changes can be more or less drastic, as may be illustrated by the following three examples:

TABLE 4. *Comparison of the biological activities of oxytocin and oxytocin analogs with amino-acid substitutions in position 5*

	Uterotonic (rat)	Milk-ejecting (rabbit)	Antidiuretic (rat)	Pressor (rat)
Oxytocin[a]	500	430	5	5
[5-D-Asparagine]-oxytocin[b]	~0.2	—	—	—
[5-Alanine]-oxytocin	<0.05[c] 0.2–0.3[d]	<0.05[c] —	<0.01[c] 0.002[d]	<0.05[c] <0.01[d]
[5-Glutamine]-oxytocin[e]	1	7.3	—	0.01
[5-Serine]-oxytocin[c]	0.7	4.1	<0.01	<0.1
[5-Ornithine]-oxytocin[f]	~0.24	0.19	~0.002	<0.04
[5-Valine]-oxytocin[g]	~0.3	—	<0.001	~0.01
[5-Diaminobutyric acid]-oxytocin[h]	<0.1	—	<0.01	<0.01

[a] See Table 2.

[b] Dutta, A. S., N. Anand, and K. Kar, *J. Med. Chem.*, **9**, 497 (1966).

[c] Guttmann, St., and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1626 (1963).

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[f] Havran, R. T., I. L. Schwartz, and R. Walter, *J. Amer. Chem. Soc.*, **91**, 1836 (1969).

[g] Walter, R., and I. L. Schwartz, *J. Biol. Chem.*, **241**, 5500 (1966).

[h] Hase, S., and R. Walter, unpublished.

Replacements of the asparagine residue in neurohypophyseal hormones by amino acids which are incapable of stabilizing the backbone structure of both β-turns (1) increase significantly the conformational ambiguity of the resulting analogs and accordingly have a deleterious effect on all of their biological activities (see Table 4). Changes of the dihedral angle of the disulfide group (3, 6, 7) exercise a profound influence on the overall conformational integrity of the hormonal molecule. Even a minor modification such as the substitution of either sulfur moiety by selenium in oxytocin evokes a definite change in the biological activity spectrum. As shown earlier (7), crystalline deamino-1-seleno-oxytocin possesses a higher activity, while crystalline deamino-6-seleno-oxytocin has a lower activity than crystalline deamino-oxytocin. In accord with the idea that the type of structural change discussed in this section should affect all hormone-receptor interactions uniformly, the activity of deamino-1-seleno-oxytocin proved to be consistently higher than that of its 6-seleno isolog in all systems tested (Table 5).

In a third example, ArgVP and oxytocin are compared with [1,6-dialanine]-ArgVP (8) and [1,6-dialanine]-oxytocin; the latter has been particularly thoroughly studied for some of its biological activities (9). Although the conformational constraint usually imposed by the disulfide bridge is removed entirely in these acyclic analogs, they retain exceedingly low, but definite, biological activities, e.g. [1,6-dialanine]-oxytocin exhibited a rat uterotonic activity in the milliunit range

TABLE 5. *Biological effect*[a, b] *of the selective substitution of sulfur by selenium in neurohypophyseal peptides*

Compound	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Pressor (rat)	Antidiuretic (rat)
Deamino-1-seleno-oxytocin	1306 ± 46[c]	1217 ± 13[c]	479 ± 32	5.00 ± 0.18[c]	29.9 ± 1.5
Deamino-6-seleno-oxytocin	586 ± 36[c]	443 ± 7.0	282 ± 15	1.81 ± 0.08[c]	20.5 ± 2.1
Ratio:	2.23	2.75	1.70	2.76	1.46

[a] Expressed in USP units/mg.

[b] Ferrier, B. M., D. Jarvis, and V. du Vigneaud, *J. Biol. Chem.*, **240**, 4264 (1965) report for the disulfide-containing analog, deamino-oxytocin, the following values: fowl depressor 975 ± 24, oxytocic 803 ± 36, milk-ejection 541 ± 13, pressor 1.44 ± 0.06, and antidiuretic 19.0 ± 1.

[c] Values taken from ref. 7.

(about 0.95 mU/mg, as compared to oxytocin: 500 U/mg, see Table 2). From these results it was deduced (8, 9) that there exists a minute probability that these acyclic analogs will attain the active conformation of their cyclic congeners. This deduction is supported by the fact that the solution conformation of oxytocin (1) may be derived from its acyclic peptide in a stepwise fashion with minimal perturbations of the extended peptide chain.

MODIFICATIONS OF TYPE B

The structural modifications discussed in this section can affect both the affinity and the intrinsic activity of the resulting analogs despite retention of the peptide backbone conformation. Frequently a given analog of this category displays a gain of potency in one biological system, but a lesser gain or even a loss of potency in another. Such a nonparallel or opposite variation in activity reveals that the biological responses in question are mediated by different receptors. Obvious examples are the naturally occurring hormones already discussed, which clearly interact differently with "tocin" and "pressin" receptor groups (Table 2). Moreover, a further differentiation of receptors certainly exists within these groups, as revealed by many studies (10) which indicate that a neurohypophyseal hormone induces antidiuretic, natriuretic, and pressor responses in mammals via different receptors—and hydroosmotic and natriferic responses in amphibia also via different receptors.

From a study (11) of the uterotonic response to graded doses of a series of oxytocin analogs in which the isoleucine residue had been replaced by other aliphatic amino acids, it was concluded that the side chain in position 3 is important in determining the degree of affinity of the hormone for the uterine receptor, but not for its intrinsic activity. More recently, we found that the substitution of the aliphatic isoleucine residue by the aromatic phenylalanine results not only in a decreased affinity but also in an apparent decreased intrinsic activity (12, 13)—even with experimental conditions identical with those under which the analogs with aliphatic aminoacid residues in position 3 had been tested. We can now suggest a possible explanation for the differential action of oxytocin and vasopressin on the contractile elements of the uterine muscle in terms of differences in the conformation of these two hormones at the receptor. Unlike the isoleucine residue in position 3 of oxytocin, the phenylalanine in vasopressin competes effectively via a π–π interaction with the cross β-structure for the aromatic side chain of the tyrosine residue, resulting in a change of the predominant population of the rotamers of the tyrosine side chain (see above). In fact, on the basis of NMR studies, a stacking of the aromatic side chains has been proposed for lysine-vasopressin (14). A stacking of the aromatic side chains is also demonstrable by temperature and solvent effects in the circular dichroism spectra of lysine-vasopressin, effects not seen with oxytocin (15). In contrast to the isoleucine side chain, the combined aromatic unit of tyrosine and phenylalanine side chains would be unable to enter the appropriate hydrophobic pocket on the uterotonic receptor. Such a gross reduction of hormone–receptor complementarity may be responsible for the diminished affinity as well as the diminished intrinsic activity. It is anticipated that many other examples will be found in which structural modification of peptides will affect binding and thereby bring about a reduction in intrinsic activity.

MODIFICATIONS OF TYPE C

The peptides discussed in the previous section can exhibit differences in intrinsic activity as a consequence of changes in affinity for the receptor; in this section we consider structural modifications which result in changes of intrinsic activity without alteration of affinity. Inspection of the oxytocin model reveals that a progressive reduction in intrinsic activity—finally resulting in full antagonism—can be expected upon substitution of one or more groups which project into the hydrophilic area of oxytocin—while perturbing neither the conformation of the peptide backbone nor adding any additional volume at a region of the molecule where purely steric constraints are imposed by the receptor for binding. A relevant example is the series of oxytocin analogs in which the hydroxyl group of tyrosine is alkylated with groups of increasing bulkiness. Dose–response studies in the *in vitro* uterotonic assay and other tissues showed a progressive reduction of the intrinsic activity of these analogs as one goes from oxytocin to its *O*-methyl and its *O*-ethyl derivatives (for summary see ref. 16). In the rat uterotonic assay and particularly in the toad-bladder hydroosmotic assay, where it was possible to quantitate both the affinity and intrinsic activity parameters, it was found that [2-*O*-methyltyrosine]-oxytocin and [2-*O*-ethyltyrosine]-oxytocin possess the same affinity for the target tissue, but that [2-*O*-methyltyrosine]-oxytocin is a partial agonist whereas [2-*O*-ethyltyrosine]-oxytocin is an antagonist (17). This suggests that the occupation of part of the hydrophilic area of oxytocin by the increasingly bulky methyl and ethyl moieties progressively interferes with the interaction of catalytically important groups of the receptor with this hydrophilic area of the hormone, although binding of the hormone to the receptor at other loci is unimpaired.

The foregoing statement of relationships of a conformation of the peptide hormone, oxytocin, to various aspects of the biology of neurohypophyseal hormones constitutes a step forward in the development of our understanding of endocrine phenomena at the molecular level. Further progress in this direction will depend in large part on further refinements in the conformational analysis of neurohypophyseal hormones.

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Neurohypophyseal hormones are denoted in accordance with the IUPAC–IUB "Tentative Rules" (*Biochemistry*, **6**, 362, 1967) and standard abbreviations are used for amino-acid residues (*Biochemistry*, **5**, 2485, 1966). The amino acids (except glycine) are of the L configuration, unless otherwise noted.

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