# Active-Site Studies of Neurohypophyseal Hormones: Comparison of Oxytocin and Arginine-Vasopressin with Analogues Containing 4-D-Glutamine[1]

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*Received July 16, 1981*

**Abstract:** Previous studies have indicated that oxytocin at the uterine smooth-muscle receptor and vasopressin at the mammalian antidiuretic receptor utilize different structural and conformational properties to produce their biological effects. In both cases, however, the Gln[4] residue has been proposed to be of primary importance for receptor recognition (binding) but not critical for biological activity (transduction). On the basis of these considerations, it would be predicted that [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin would be weak but full agonists at the uterine and antidiuretic receptors, respectively. We have synthesized and purified the two D-Gln[4] analogues and examined their pharmacological activities in several assay systems for these hormones. In agreement with the predictions, [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin have greatly reduced potency at the in vitro uterine and in vivo antidiuretic assay systems, respectively, and both appear to be full agonists in these assays. However, the effects are quantitatively different, with [D-Gln[4]]oxytocin possessing 3.3 ± 0.2 units/mg of uterotonic activity ($^1/_{170}$ the potency of oxytocin) and [D-Gln[4],Arg[8]]vasopressin possessing only 0.45 ± 0.01 unit/mg ($^1/_{1100}$ the potency of arginine vasopressin) of antidiuretic activity. Based on carbon-13 nuclear magnetic resonance spectral data, both [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin have very similar conformations to oxytocin and arginine-vasopressin, respectively. Interestingly [D-Gln[4]]oxytocin has much more reduced biological activities relative to the native hormone in the avian vasodepressor (~0.04 unit/mg, ~$^1/_{12000}$ that of oxytocin) and milk-ejecting (0.09 ± 0.02 unit/mg, ~$^1/_{4500}$ that of oxytocin) assays and is a weak partial agonist in the pressor assay. [D-Gln[4],Arg[8]]vasopressin is a weak full agonist in the pressor assay (0.260 ± 0.004 unit/mg, $^1/_{1900}$ that of arginine-vasopressin) and also has weak uterotonic (~0.11 unit/mg) and avian vasodepressor (0.10 unit/mg) activities.

In aqueous solution the conformations of oxytocin, H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-$NH_2$, and arginine-vasopressin, H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-$NH_2$, appear to be quite similar to one another,[2,3] and similar observations have been made regarding their conformations in dimethyl sulfoxide.[4] Consideration of these and other conformational studies and structure–biological activity relationships of a wide variety of oxytocin and arginine-vasopressin analogues led Walter and co-workers to propose a "cooperative model" for the biologically active conformation of oxytocin at the uterine receptor[5] and of vasopressin at the antidiuretic receptor.[6] Utilizing conformationally restricted oxytocin analogues, oxytocin inhibitors, and structure–biological activity relationships, Hruby and co-workers have proposed a complementary "dynamic model" of oxytocin action at its biological receptors.[7,8] One approach we have utilized to further test the validity of these models is the use of stereoisomeric analogues. In favorable cases, a single configurational change will maintain the same overall conformation for the diastereoisomers as the native hormone. However, relationships between key side-chain groups which are important either for binding or for biological activity (transduction)[8] will be altered. In this regard, we recently reported the synthesis, biological activity, and some of the conformational properties of 2-D-tyrosine analogues of oxytocin and arginine-vasopressin.[9] These analogues were chosen because the "cooperative model" has proposed that in oxytocin the relationship of the tyrosine aromatic moiety to the 20-membered ring of the hormone was critical to the biologically active conformation of oxytocin at the uterine receptor[5] but was not critical to the biologically active conformation at the kidney receptor (antidiuretic receptor) of vasopressin. The high biological activity of the [D-Tyr[2],Arg[8]]vasopressin in the antidiuretic assay and the partial agonism of [D-Tyr[2]]oxytocin in the oxytocic assay[9] were supportive of these proposals, and the dynamic–conformational properties of conformationally restricted analogues[7,8,10,11] provided other supporting evidence for these proposals.

In this paper we utilize stereoisomeric analogues to examine the proposal that the 4-position residue in oxytocin and arginine-vasopressin (glutamine in both cases) is primarily important for receptor binding at the rat uterus and kidney antidiuretic receptors. We show by carbon-13 NMR that [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin have conformations similar to their respective hormones in aqueous solution and that both appear to have full activity, though at greatly reduced potency relative to the native hormones. We then examine whether these relationships hold at other neurohypophyseal hormone receptors (mammary gland, avian vascular system, and rat pressor system) and find that in some cases these other receptor systems appear to have somewhat different stereoisomeric requirements for biological activity.

(1) All optically active amino acids are of the L configuration unless otherwise indicated. Standard abbreviations for amino acids, protecting groups, and peptides as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [J. Biol. Chem., 247, 977 (1972)] are used. Other abbreviations used are: DMB, 3,4-dimethylbenzyl; TFA, trifluoroacetic acid; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide; HOAc, acetic acid; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance spectroscopy; AVP, arginine-vasopressin.

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Table I. Pharmacological Potencies of Oxytocin, Arginine-Vasopressin, and Their 4-D-Glutamine Analogues[a]

| compd | assay system | | | | |
|---|---|---|---|---|---|
| | uterotonic | avian vasodepressor | milk ejecting | antidiuretic | pressor |
| oxytocin | 546 ± 18[b] | 507 ± 23[c] | 410 ± 16[b]<br>438 ± 25[d] | 2.7 ± 0.2 | 3.1 ± 0.1 |
| [D-Gln[4]]oxytocin | 3.3 ± 0.2[e]<br>~0.1[f] | ~0.04[g,e]<br>~1.0[f] | 0.09 ± 0.02[e] | 0.05[e] | <0.1[e,h] |
| arginine-vasopressin | 12 ± 0.2[i] | 100 ± 15[i] | 30–120 | 503 ± 53[i] | 487 ± 15[i] |
| [D-Gln[4],Arg[8]]vasopressin | ~0.11[e] | 0.10[e] | | 0.45 ± 0.01[e] | 0.260 ± 0.004[e] |

[a] All activities are reported in units per milligram. [b] W. Y. Chan and V. du Vigneaud, *Endocrinology*, **71**, 977 (1972). [c] W. Y. Chan, M. O'Connell, and S. R. Pomeroy, *Endocrinology*, 72, 279 (1963). [d] Hruby and Hadley.[35] [e] This paper. [f] A. S. Dutta, N. Anand, and K. Kar, *J. Med. Chem.*, 9, 497 (1966). [g] Nonlinear dose-response curve. [h] This molecule was a partial agonist in this assay. A $1 \times 10^{-4}$ mmol dose gave no response, but a $1 \times 10^{-3}$ mmol dose or higher gave a maximum response for this compound which was much less (~30%) than the maximal response for vasopressin. [i] J. Meienhofer, A. Trzeciak, R. T. Havran, and R. Walter, *J. Am. Chem. Soc.*, **92**, 7199 (1970).

For the synthesis of the 4-D-glutamine analogues of oxytocin and vasopressin, we incorporated the D-glutamine residues into the growing peptide chain during the synthesis on a solid-phase resin. The solid-phase methodologies used were similar to those previously used for other oxytocin[9,12,13] and arginine-vasopressin[14] diastereoisomers in our laboratory. Both [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin were purified by partition chromatography on Sephadex G-25,[3,15] though somewhat different solvent systems were used for each diastereoisomer (see Experimental Section for detailed procedures). The purity of each diastereoisomer was assessed by amino acid analysis, optical rotation, thin-layer chromatography in several solvent systems, and by high-pressure liquid chromatography. The latter procedure (HPLC) was particularly useful for assessing the presence of oxytocin and arginine-vasopressin in the presence of [D-Gln[4]]-oxytocin[16] and [D-Gln[4],Arg[8]]vasopressin,[17] respectively. In each case, none of all L diastereoisomer was observed, indicating that there was no contamination with the native hormones (less than 1 part per 2000).

The pharmacological potencies of the 4-D-glutamine analogues and their natural congeners in five different assay systems specific for neurohypophyseal hormones are shown in Table I (see Experimental Section for details of assay methods). Both 4-D-glutamine analogues possess drastically reduced potencies relative to the native hormones in the in vitro rat uterotonic asssay, with [D-Gln[4]]oxytocin being about $^1/_{160}$ as potent as oxytocin and [D-Gln[4],Arg[8]] vasopressin being about $^1/_{100}$ as potent as arginine-vasopressin. In the case of oxytocin, this reduced potency is apparently only due to a decreased affinity for the smooth-muscle receptor, since the analogue can maximally stimulate tissue contraction relative to the native hormone, but requires a higher dose. These results can be contrasted with those for [D-Tyr[2]]-oxytocin,[9] which actually has a higher potency than [D-Gln[4]]-oxytocin (8.4 ± 0.3 units/mg[9] vs. 3.3 ± 0.2 units/mg) but does not maximally stimulate tissue contraction in this assay. Thus, the D-Tyr[2] analogue has reduced intrinsic activity,[18] while the D-Gln[4] analogue is a full agonist. These results are consistent with the suggestions of Walter[5] that the 2-tyrosine residue of oxytocin participates as an "active element" of the hormone for the uterine receptor, while the 4-glutamine residue is important primarily for the binding of the hormone at the uterine receptor. The question of whether [D-Gln[4]]oxytocin might have conformational properties drastically different from oxytocin was addressed in part by examination of the carbon-13 nuclear magnetic resonance (NMR) spectra. As shown in Table II, [D-Gln[4]]oxytocin has a carbon-13 NMR spectrum essentially identical with oxytocin, except for the expected differences at the 4 position due to the differences in chirality at this position in the two hormones and at the 3 position presumably due to its changed relationship to position 4. These results are consistent with the suggestions that [D-Gln[4]]oxytocin and oxytocin have approximately the same conformation. This is as expected, since Walter et al.[19] have shown that [D-Ala[4]]-oxytocin (which we have suggested[16] would be expected to have a similar conformation as [D-Gln[4]]oxytocin) has very similar conformational properties to oxytocin. These results are consistent with the "dynamic model" of oxytocin hormone action,[7,8,20] since displacement of the side-chain group of residue 4 relative to the other side-chain groups in the hormone in [D-Gln[4]]oxytocin appear to affect only binding but *not* attainment of full intrinsic activity.

Since arginine-vasopressin previously was shown to exhibit a reduced intrinsic activity, as well as low affinity in the in vitro uterotonic assay,[21] and a nonlinear dose–response curve was observed for [D-Gln[4],Arg[8]]vasopressin in this assay (Table I), a more complete profile of activity for this diastereoisomer in this assay was not examined.

In the avian vasodepressor and milk-ejecting assays, [D-Gln[4]]oxytocin also exhibited greatly reduced potencies (about $^1/_{10000}$ and $^1/_{4000}$ that of oxytocin, respectively). In the case of the milk-ejecting activity, full intrinsic activity was observed. However, the dose–response curve for this analogue in the avian vasodepressor assay was not linear with oxytocin, indicating differences in receptor specificity for configurational requirement in this assay system relative to the uterotonic or mammary gland systems. Interestingly, [D-Gln[4],Arg[8]]vasopressin was as potent or slightly more so than [D-Gln[4]]oxytocin in this assay system (Table I).

In the assay systems more specific for vasopressin, the rat antidiuretic and rat pressor assay, [D-Gln[4],Arg[8]]vasopressin shows drastically reduced potency relative to arginine-vasopressin (about $^1/_{1000}$ and $^1/_{2000}$, respectively. However, the diastereoisomer analogue appears to behave normally in these assay systems and as in the antidiuretic assay has a parallel dose-response pattern to arginine-vasopressin. Examination of the carbon-13 NMR spectrum of [D-Gln[4],Arg[8]]vasopressin relative to arginine-vasopressin shows that they have nearly identical spectra (Table II), except for the expected differences at the 4 position due to the different chiralities at this position in the two compounds. These results are consistent with the suggestion of Walter et al.[6] that the glutamine-4 residue in arginine-vasoprssin is important for receptor binding at the antidiuretic receptor and is not essential to the intrinsic activity of the hormone in its "biologically active" conformation at this receptor. However, the latter conclusion is somewhat speculative. The very low potency of the 4-D-glutamine analogues of vasopressin and oxytocin certainly indicates that the

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(13) V. J. Hruby, D. A. Upson, and N. Agarwal, *J. Org. Chem.*, **42**, 3552 (1977).
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(19) R. Walter, H. R. Wyssbrod, and J. D. Glickson, *J. Am. Chem. Soc.*, **99**, 7326 (1977).
(20) V. J. Hruby in "Topics in Molecular Pharmacology", A. S. V. Burgen and G. C. K. Roberts, Eds., Elsevier/North Holland, Amsterdam, 1981, in press.
(21) R. Walter, B. Dubois, and I. L. Schwartz, *Endocrinology*, **83**, 979 (1968); R. Walter and M. Wahrenberg, *Parmacol. Res. Commun.*, **8**, 81 (1976).

Table II. $^{13}$C Chemical Shifts of Amino Acid Residues of Oxytocin, Arginine-Vasopressin, [D-Gln[4]]Oxytocin, and [D-Gln[4],Arg[8]]Vasopressin[a]

| residue | C atom | oxytocin,[b] pD 4.9 | [D-Gln[4]]oxytocin, pD 4.4 | [Arg[8]]vasopressin,[b] pD 5.2 | [D-Gln[4],Arg[8]]-vasopressin, pD 4.4 |
|---|---|---|---|---|---|
| $^1/_2$-Cys[1] | α-CH | 53.24 | 53.69 | 53.60 | 53.40 |
| | β-$CH_2$ | 40.30 | 39.46 | 41.41 | 40.28 |
| Tyr[2] | α-CH | 56.42 | 56.60 | 56.29 | 56.28 |
| | β-$CH_2$ | 36.99 | 37.08 | 37.38 | 37.90 |
| | $C_1$ | 128.60 | 128.58 | 128.15 | 128.56 |
| | $C_{2,6}$ | 130.81 | 131.82 | 131.06 | 131.90 |
| | $C_{3,5}$ | 116.44 | 116.56 | 116.49 | 116.55 |
| | $C_4$ | 154.50 | 155.66 | 154.89 | 155.69 |
| Phe[3] | α-CH | | | 56.78 | 57.10 |
| | β-$CH_2$ | | | 37.21 | 37.11 |
| | $C_1$ | | | 136.73 | 136.79 |
| | $C_{2,6}$ | | | 129.75 | 130.35 |
| | $C_{3,5}$ | | | 129.75 | 130.00 |
| | $C_4$ | | | 127.93 | 128.56 |
| Ile[3] | α-CH | 60.98 | 59.81 | | |
| | β-CH | 36.99 | 37.78 | | |
| | γ-$CH_2$ | 25.55 | 25.64 | | |
| | γ-$CH_3$ | 15.87 | 15.58 | | |
| | δ-$CH_3$ | 11.64 | 11.44 | | |
| Gln[4] (D-Gln[4]) | α-CH | 56.16 | 53.69 | 56.07 | 53.75 |
| | β-$CH_2$ | 26.79 | 27.55 | 27.16 | 27.34 |
| | γ-$CH_2$ | 32.05 | 32.08 | 32.05 | 31.73 |
| Asn[5] | α-CH | 51.36 | 51.07 | 51.32 | 51.07 |
| | β-$CH_2$ | 36.99 | 37.43 | 37.38 | 37.90 |
| $^1/_2$-Cys[6] | α-CH | 52.33 | 51.81 | 52.30 | 51.75 |
| | β-$CH_2$ | 39.26 | 38.93 | 39.76 | 39.11 |
| Pro[7] | α-CH | 61.63 | 61.60 | 61.72 | 61.63 |
| | β-$CH_2$ | 30.23 | 30.31 | 30.40 | 30.37 |
| | γ-$CH_2$ | 25.55 | 25.96 | 26.06 | 25.70 |
| | δ-$CH_2$ | 48.95 | 48.93 | 49.14 | 48.96 |
| Arg[8] | α-CH | | | 54.64 | 54.60 |
| | β-$CH_2$ | | | 28.97 | 28.90 |
| | γ-$CH_2$ | | | 25.42 | 25.43 |
| | δ-$CH_2$ | | | 41.57 | 41.55 |
| Leu[8] | α-CH | 53.63 | 53.69 | | |
| | β-$CH_2$ | 40.30 | 40.40 | | |
| | γ-CH | 25.36 | 25.34 | | |
| | δ-$CH_3$ | 23.15 | 23.14 | | |
| | δ-$CH_3$ | 21.72 | 21.73 | | |
| Gly-$NH_2$[9] | α-$CH_2$ | 43.10 | 43.10 | 43.14 | 43.05 |

[a] Chemical shifts are measured in parts per million (ppm) from $Me_4Si$ (external). [b] Hruby et al. (1979).[3]

side-chain group of this residue is a major contributor to the binding interaction at the antidiuretic receptor. However, whether the glutamine-4 side chain is of little or no importance for transduction at the antidiuretic receptor is less certain. It is possible that though [D-Gln[4],Arg[8]]vasopressin gives a full biological response in the in vivo antidiuretic assay, it could be a partial agonist in the in vitro kidney medullary adenylate cyclase assay,[39] thus indicating an important, though not essential, role in transduction for this residue.

As expected, [D-Gln[4]]oxytocin also has greatly reduced antidiuretic and pressor activities (Table I). Particularly interesting was the weak partial agonist activity of [D-Gln[4]]oxytocin in the pressor assay system.

In summary, we have shown that substitution of an L-glutamine by a D-glutamine in the 4 position of oxytocin and arginine-vasopressin greatly reduced their potency in the rat uterotonic (and milk ejecting) and rat antidiuretic (and pressor) assay systems, respectively. Carbon-13 NMR studies suggest that the diastereoisomers have similar conformations in solution as the native hormones. These results are consistent with the suggestion[5] that in oxytocin the 4 position is primarily of importance for binding of the hormone to the uterotonic receptor and not for transduction and that the dynamic properties,[8,20] nonetheless, permit a full response at sufficiently high concentrations, perhaps as a result of the inherent flexibility of the hormone. The results also are consistent with the suggestion[6] that for vasopressin the 4 position is of importance for binding of the hormone to the antidiuretic receptor, but whether this residue has no importance for transduction is uncertain. Full in vivo antidiuretic activity was observed, but it is possible that [D-Gln[4],Arg[8]] vasopressin could be a partial agonist in the in vitro kidney medullary adenylate cyclase assay system. Finally, in all cases, it is unlikely that the activities reported here for the 4-D-glutamine analogues result from the presence of minor contamination with the native hormones, since in both cases the biological activity profiles of these diastereoisomers are much different from the native hormones in the five assay systems examined.

**Experimental Section**

Thin-layer chromatography (TLC) was done on silica gel G plates using the following solvent systems: (A) 1-butanol–acetic acid–water (4:1:5, upper phase only); (B) 1-butanol–acetic acid–pyridine–water (15:3:10:12); (C) 1-pentanol–pyridine–water (7:7:6); (D) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). The N-terminal free peptides were detected on the TLC plates using ultraviolet light, iodine vapors, ninhydrin, and fluorescamine. $N^{\alpha}$-Protected amino acids and peptides were first treated with aqueous 6 N hydrochloric acid, heated at 100 °C for 10 min, and then detected as before. Nuclear magnetic resonance (NMR) spectra were obtained using a Varian T-60 spectrometer, a Bruker WH-90 FT NMR spectrometer, or a Bruker WM-250 FT NMR spectrometer. Amino acid analyses were obtained by the method of Spackman, Stein, and Moore[22] on a Beckman 120 C amino acid analyser after hydrolysis in 6 N HCl for 22–24 h at 110 °C and are uncorrected for losses on hydrolysis. Partition chromatography[15] purification of oxytocin and arginine-vasopressin[14] derivatives was performed on Sephadex G-25 (block polymerizate). Final purification of the peptides was done by gel filtration on Sephadex G-25 using 0.2 N acetic acid for elution.

(22) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

Purification of solvents to remove metals and other contaminants was performed as previously described.[23] $N^{\alpha}$-Boc-protected amino acids and amino acid derivatives were purchased from Vega Biochemical Co. or from Biosynthetica or were prepared by published procedures. Before use, all amino acids were checked for purity by melting point, by thin-layer chromatography in solvent systems A, B, and C, and by the ninhydrin test of Kaiser et al.[24] Following partition chromatography, detection of peptides in eluents was made using UV spectroscopy (280 or 260 nm). The desired peptide fractions were isolated by addition of deionized water to the organic solvents, followed by rotary evaporation in vacuo at 25–30 °C and lyophilization of the aqueous solution. The high-pressure liquid chromatography was performed by the same methods as those used previously[16,17,25] for examining oxytocin and arginine-vasopressin diastereoisomers. Optical rotation values were measured at the sodium D line using a Perkin-Elmer 241 NM polarimeter.

**Solid-Phase Synthesis of [4-D-Glutamine]oxytocin.** The solid-phase synthesis of the nonapeptide-resin precursor to the title compound was accomplished using a semiautomated instrument of our design. A sample of 3.3 g of *N*-(*tert*-butyloxycarbonyl)glycine-benzhydrylamine resin (Gly substitution 0.30 mmol/g of resin) was prepared as previously described.[13] Removal of the *N*-Boc protecting groups, neutralization of the peptide resin salt, and addition of the next protected amino acid to the growing peptide chain followed our general scheme.[12-14] $N^{\alpha}$-Boc-Asn was added as its *p*-nitrophenyl ester. $N^{\alpha}$-Boc-D-Gln (3-fold excess) was added directly by dicycohexylcarbodiimide (2.4-fold excess), and *N*-hydroxybenzotriazole (4.8-fold excess) mediated coupling. Complete coupling required 24 h. The completion of coupling steps in the solid-phase syntheses was monitored by the ninhydrin test of Kaiser et al.[24] A negative test (>99.5% coupling) was indicated at each step in the synthesis except as noted above. The 3,4-dimethylbenzyl group[26,27] (DMB) was used to protect the sulfhydryl group of the cysteine residues. The total volume of solvent or solution used at each washing or reaction step was 35 mL. At the completion of the synthesis, the N-terminal Boc group was removed, the peptide resin was neutralized by repeating steps 1–7 of Table I,[12] and the peptide resin, H-Cys(DMB)-Tyr-Ile-D-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH-benzhydrylamine resin, was dried in vacuo: yield 4.3 g. A 1.3-g portion of the peptide resin was treated with 20 mL of anhydrous HF (freshly distilled from $CoF_3$) containing 2 mL of anisole for 60 min at 0 °C. The HF and anisole were removed in vacuo and the resin washed with four 25-mL portions of ethyl acetate. The peptide material was extracted into two 20-mL portions of 20% acetic acid and three 25-mL portions of 0.2 N aqueous acetic acid under nitrogen. The combined aqueous solution was diluted to 500 mL with deionized water, and the pH was adjusted to 8.5 with ammonium hydroxide. The sulfhydryl groups were oxidized using 60 mL of 0.01 N $K_3Fe(CN)_6$. After the solution was stirred for 30 min, the pH was adjusted to 5 with 20% aqueous acetic acid, and Rexyn 203 ($Cl^-$ form) was added to remove ferrocyanide and excess ferricyanide. The mixture was stirred for 20 min, and the resin was filtered off and washed with three 20-mL portions of 20% aqueous acetic acid. The combined aqueous solutions were combined with 50 mL of 1-butanol, the volume was reduced to about 200 mL by rotary evaporation at 20–30 °C in vacuo, and the solution was lyophilized. The solid was dissolved in 10 mL of 30% acetic acid, and the mixture was gel filtered on a Sephadex G-15 column (2.5 × 110 cm) using 30% acetic acid as the eluent solvent. The fractions corresponding to the peptide material were pooled and lyophilized to give about 300 mg of a pale cream powder. The powder was dissolved in 5 mL of the upper phase and 1 mL of the lower phase of the solvent system 1-butanol–3.5% aqueous acetic acid containing 1.5% pyridine (1:1) and placed on a 60 × 2.8 cm column of Sephadex G-25 (block polymerizate, 100–200 mesh) which had been equilibrated with the lower and upper phases for partition chromatography. The tubes corresponding to the major peak, $R_f$ 0.26, were pooled, 300 mL of water was added, the solvents were removed to 50 mL by rotary evaporation in vacuo at 20–30 °C, and the solution was lyophilized. The product was dissolved in 4 mL of 0.2 N acetic acid and then placed on a 2.8 × 60 cm column of Sephadex G-25 (200–270 mesh) for gel filtration using 0.2 N acetic acid as eluent solvent. The fractions from the major peak were pooled and lyophilized to give 103 mg (34% based on starting Boc-Gly-NH-resin) of [D-Gln[4]]oxytocin: $[\alpha]^{22}_{D}$ −36.1° (*c* 0.52, 1 N HOAc). TLC analysis in solvent systems A, B, and C gave single uniform spots. Reversed-phase HPLC on a $C_{18}$ column using identical techniques with those previously reported[16] showed the product to be of very high purity (>99.9%) and to contain no oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.06; Glu, 1.03; Pro, 0.97; Gly, 1.05; $^1/_2$-Cys, 1.90; Ile, 0.96; Leu, 1.04; Tyr, 0.89.

**Solid-Phase Synthesis of [4-D-Glutamine,8-arginine]vasopressin.** The solid-phase synthesis of the precursor peptide resin to the title compound was made using 2.5 g of Boc-glycinate-0-resin, which was prepared by the method of Gisin[12] using essentially the same procedures as used in the synthesis of [D-Gln[4]]oxytocin (Table I), except that the guanidyl group of arginine was tosyl protected and the $N^{\alpha}$–Boc groups of Asn and D-Gln were cleaved with 50% TFA in $CH_2Cl_2$. There was obtained 3.3 g of H-Cys(DMB)-Tyr-Phe-D-Gln-Asn-Cys(DMB)-Pro-Arg(Tos)-Gly-0-resin.

The protected peptide was cleaved from the resin in its C-terminal carboxamide form by stirring in 150 mL of freshly distilled anhydrous methanol saturated at −5 °C with anhydrous ammonia (freshly distilled from sodium). The flask was wired shut and stirred for 4 days. The solvents were removed by rotary evaporation in vacuo, and the peptide was extracted into DMF (2 × 50 mL) at 40 °C. The DMF solution was evaporated down to about 5 mL, and the product was precipitated by the addition of 50 mL of ether. The white powder obtained was reprecipitated from ethanol–acetic acid to give 0.50 g of H-Cys(DMB)-Tyr-Phe-D-Gln-Asn-Cys(DMB)-Pro-Arg(Tos)-Gly-$NH_2$, mp 144–148 °C. A 300 mg (0.20 mmol) portion of the protected nonapeptide in 200 mL of anhydrous ammonia (freshly distilled from sodium) was treated with sodium until a blue color persisted for about 1 min. The ammonia was evaporated to dryness under nitrogen,[28] and the powder was dissolved in 400 mL of 0.1% aqueous acetic acid under nitrogen. The pH was adjusted to 8.5 with 3 N ammonium hydroxide, and the cyclic compound was formed in the same manner as with [D-Gln[4]]oxytocin. The crude [D-Gln[4],Arg[8]]vasopressin was purified by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–ethanol–3.5% aqueous acetic acid containing 5% pyridine (4:1:8). The major peak ($R_f$ 0.10) was isolated as before and subjected to gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent solvent. The major peak was isolated and lyophilized to give 75 mg (35% yield based on starting Boc-Gly-0-resin) of a white powder: $[\alpha]^{22}_{D}$ −14° (*c* 0.52, 0.1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.00; D-Glu, 1.02; Pro, 0.97; Gly, 1.02; $^1/_2$-Cys, 1.98; Tyr, 0.92; Phe, 1.00; Arg, 1.00.

**$^{13}C$ NMR Spectra.** $^{13}C$ NMR spectra of [D-Gln[4]]oxytocin and [D-Gln[4],Arg[8]]vasopressin were measured at 62.9 MHz using a Bruker WM 250 spectrometer equipped with a 15-mm multifrequency probe. Peptide concentrations were 1.5 to 4 mg/mL in 5 mL of $D_2O$ containing a small amount of dioxane as a chemical-shift reference. The pH of each sample was adjusted to 4.0 (direct meter reading) using $CD_3COOD$.

The spectra were accumulated in quadrature mode using broad-band proton-noise decoupling. The 16K data point spectra were collected for 12–24 h using a 70° pulse width, a 15 150-Hz spectral width, and a 0.54-s acquisition time. The probe temperature was maintained at 22 ± 1 °C. Reported chemical shifts (Table II) are parts per million downfield from $Me_4Si$ (not present in the samples) calculated by setting the dioxane resonance to 67.6 ppm.

**Biological Assays.** For the rat uterotonic assay, mature, virgin Sprague–Dawley rats, weight 250–350 g, and in natural estrus were used. Uterine horns were isolated and mounted for bioassay in 10-mL baths according to the method of Holton.[29] For determination of specific activity (oxytocic potency), the bathing fluid was $Mg^{2+}$-free van Dyke–Hasting solution as modified by Munsick,[30] with the following composition (in millimolar concentrations): $NaHCO_3$, 30; $Na_2HPO_4$, 0.8; $NaH_2PO_4$, 0.2; NaCl, 115; KCl, 6.2; $CaCl_2$, 0.5; dextrose, 2.8. The reservoir and the baths were gassed with a mixture of 95% oxygen and 5% carbon dioxide. The pH of the bathing fluid was 7.4, and the bath temperature was 31 °C. Isotonic contractions were measured with a Grass polygraph used in conjunction with a Harvard smooth-muscle transducer (Model 386A) adapted for use with the polygraph. The four-point assay design[31] was used to evaluate the activity of the hormone analogues vs. the USP posterior pituitary reference standard.

**Avian vasodepressor assays** were run on conscious chickens using the method of Coon[32] as described in the U.S. Pharmacopeia,[33] as modified by Munsick et al.[34]

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**Milk-ejecting assays** were performed using mouse mammary tissue by the method of Hruby and Hadley.[35]

**Antidiuretic assays** were performed with anesthetized, male, Long-Evans rats (315-345 g) according to the method of Jeffers et al.[36] as modified by Sawyer.[37] Specific antidiuretic potencies were determined using the four-point design or matches against USP posterior pituitary reference standards.

Pressor assays were with urethane-anesthetized, male, Sprague-Dawley rats (250-350 g) as described in the U.S. Pharmacopeia.[38] [D-Gln[4]]oxytocin was found to be a partial agonist, showing only about 30% of the maximal response for arginine-vasopressin in this assay system at maximal stimulation. The response also was not parallel to arginine-vasopressin over the linear portion of the dose-response curve and, hence, no accurate determination of potency is possible (Table I).

**Acknowledgment.** The authors thank Dr. Clark Smith, University of Illinois at the Medical Center, Chicago, IL, for the oxytocic, avian vasodepressor, antidiuretic, and pressor assays, and Dr. Mac Hadley, University of Arizona, Tucson, AZ, for the milk-ejecting assays. We also thank Mr. G. Skala and Mrs. E. Skala for technical assistance and Mr. Jim Ormberg for the amino acid analyses results. This work was supported by Grant AM-17420 from the U.S. Public Health Service and by a grant from the National Science Foundation.

**Registry No.** Oxytocin, 680-77-9; arginine-vasopressin, 113-79-1; [D-Gln[4]]oxytocin, 3196-75-6; [D-Gln[4],Arg[8]]vasopressin, 76023-59-1; H-Cys(DMB)-Tyr-Ile-D-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH-benzhydrylamine, 80028-66-6; H-Cys(DMB)-Tyr-Phe-D-Gln-Asn-Cys-(DMB)-Pro-Arg(Tos)-Gly-$NH_2$, 80041-61-8.

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# Kinetic Analysis of Cytochrome $b_5$ Reduction by $Fe(EDTA)^{2-}$

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**Abstract:** The kinetics of cytochrome $b_5$ reduction by $Fe(EDTA)^{2-}$ have been studied as a function of temperature, pH, and ionic strength. The second-order rate constant for the reaction is 2.85(6) × $10^2$ $M^{-1}$ $s^{-1}$ [pH 7.0 (phosphate), $\mu$ = 0.5 M, 25 °C] with $\Delta H^{\ddagger}$ = 5.4(2) kcal/mol and $\Delta S^{\ddagger}$ = -29.2(8) eu. The ionic strength dependence between 0.05 and 0.5 M [pH 7.0 (phosphate), 25 °C] has been fitted to the Marcus ionic strength equation and yields a charge of -14.2 for cytochrome $b_5$ (oxidized). This charge has been used in calculation of the electrostatics-corrected self-exchange rate for cytochrome $b_5$ in this reaction ($k_{11}^{corr}$) to obtain a value of 4.3 $M^{-1}$ $s^{-1}$. Virtually identical ($k_{11}^{corr}$) values have been reported for cytochrome $c$ and cytochrome $c_{551}$. The pH dependence of the reaction has been analyzed in terms of reduction of a protonated ($k_a$) and an unprotonated ($k_b$) form of the protein to yield a p$K_a$ of 5.85 and values of 7.2($10^2$) $M^{-1}$ $s^{-1}$ for $k_a$ and 2.5($10^2$) $M^{-1}$ $s^{-1}$ for $k_b$ [25 °C (phosphate), $\mu$ = 0.5 M]. The activation parameters for this reaction have been studied as a function of pH and found to reflect an isokinetic relationship. This result suggests that the effect of pH on this reaction arises from the pH dependences of the driving force of the reaction and/or the electrostatic interaction between the protein and reagent.

## Introduction

Cytochrome $b_5$ is presently known to participate in at least three oxidation-reduction processes involving protein-protein electron transfer in vivo: (1) stearyl-CoA desaturation;[1] (2) cytochrome P 450 reduction;[2] and (3) methemoglobin reduction.[3] Despite this functional versatility, the electron-transer properties of cytochrome $b_5$ remain largely uncharacterized. As a first step toward a better understanding of this facet of cytochrome $b_5$ function, we have studied the reaction of the proteolytically solubilized form of the protein with $Fe(EDTA)^{2-}$. $Fe(EDTA)^{2-}$ was selected for this purpose because it has been shown to provide useful mechanistic information in studies involving a variety of other metalloproteins.[4]

## Experimental Section

Reagent grade chemicals were used throughout except where noted. Glass distilled water was used in initial measurements. In later studies this water was further purified by passage through a Barnstead NANOpure water purification system to produce water that routinely had a resistivity of 17-18 MΩ·cm. Residual oxidizing impurities present in Linde prepurified nitrogen were removed by passing the gas through two vanadous[5] and one photoreduced methylviologen[6] scrubbing towers. Measurements of pH were made with a Radiometer Model PHM 84 pH meter and combination electrode.

The hydrophilic fragment of cytochrome $b_5$ was prepared from fresh beef liver by a modified combination of the methods of Srittmatter[7] and Omura and Takesue[8] as follows. All operations were carried out at 4 °C.

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