Active Site Studies of Neurohypophyseal Hormones. Comparison of Oxytocin and Arginine Vasopressin Analogues Containing 2-D-Tyrosine[1]

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Received September 19, 1978

Abstract: On the basis of differences between the proposed "biologically active" model of oxytocin at the uterine smooth muscle receptor and the proposed "biologically active" conformation of vasopressin when bound to its mammalian antidiuretic receptor, it may be anticipated that [D-Tyr^2]oxytocin would exhibit markedly reduced uterotonic activity when compared to oxytocin, while [D-Tyr^2]vasopressin would retain most of the antidiuretic activity of its natural congener. To test this hypothesis [D-Tyr^2]oxytocin and [D-Tyr^2]arginine vasopressin were prepared by the solid-phase method of peptide synthesis and evaluated pharmacologically. DL-Tyrosine was introduced into the growing peptide chain and the diastereoisomers of each of the hormones were separated by partition chromatography. The biological results of the D-Tyr-2 analogues were in agreement with the prediction derived from the "biologically active" models. [D-Tyr^2]oxytocin possesses only 8.4 ± 0.3 units/mg in vitro rat uterotonic potency (cf. 546 ± 18 for oxytocin) and exhibited a reduced intrinsic activity. [D-Tyr^2]arginine vasopressin retained nearly 50% (207 ± 10 units/mg) of the antidiuretic potency of arginine vasopressin (cf. 503 ± 53 units/mg) and exhibited both the same affinity and intrinsic activity in the renal medullary adenylate cyclase assay of rat as the mammalian antidiuretic hormone.

The conformations of oxytocin and vasopressin qualitatively resemble one another.[4,10] One of the inherent differences between the structures of these molecules is the possibility for a ring-stacking interaction of the aromatic side chains of the neighboring Tyr-2 and Phe-3 residues in vasopressin,[5] which increases the time the tyrosine side chain spends pointing away from the 20-membered cyclic component of the antidiuretic hormone. The aliphatic Ile-3 residue in oxytocin does not offer this possible π–π interaction. In the proposed biologically active conformation of oxytocin at the uterine receptor (Figure 1A)[6,7] the aromatic ring of the tyrosine residue is oriented over the 20-membered ring of oxytocin where it is thought that the hydroxyl moiety participates as an "active element"[7] of the active site[8] of the hormone for the uterine receptor. Therefore, structural modifications which would eliminate or displace this orientation of the tyrosine side chain would be expected to reduce the ability of the resultant analogue to maximally stimulate the uterus in vitro. On the other hand, these same modifications should affect the antidiuretic activity to a lesser degree, since the orientation of the tyrosine side chain in the proposed biologically active conformation of vasopressin when bound to the antidiuretic receptor[9] is away from the 20-membered ring (Figure 1B).

One modification which can alter the preferred orientation of the tyrosine side chain is a change of configuration from L to D. Since there is evidence from ^{13}C nuclear magnetic resonance studies[10] that the overall conformational properties of the 20-membered covalent ring moiety of neurohypophyseal hormones is retained in the D-Tyr-2 analogues of oxytocin and vasopressin, the change from L- to D-tyrosine should result in analogues in which orientation of the tyrosine side chain over the 20-membered ring is unfavorable as a result of the specific change in configuration. In view of the differences between the biologically active model proposed for oxytocin when interacting with the uterine smooth muscle receptor[7] and the "biologically active" structure of vasopressin at the antidiuretic receptor,[9] [D-Tyr^2]oxytocin should exhibit markedly reduced uterotonic activity, while [D-Tyr^2]vasopressin should retain most of its antidiuretic activity. The purpose of this study was to test this contention by the synthesis and biological evaluation of 2-D-tyrosine analogues of oxytocin and arginine vasopressin.

For the synthesis of the 2-D-tyrosine analogues of oxytocin and vasopressin, appropriate DL-tyrosine derivatives were introduced into the growing peptide chain and the diastereoisomers of each of the hormones were separated. In order to maximize the usefulness of these analogues for independent biophysical and chemical studies, DL-tyrosine specifically labeled with deuterium was used in the syntheses (for examples of some applications see ref 11–15). In addition, the known analogue, [D-Tyr^2]oxytocin,[16] was also synthesized independently using D-tyrosine to verify the validity of the employed chromatographic separation techniques (Figure 2b) and to confirm the identity of products prepared in this study with those reported by Drabarek and du Vigneaud.[16] The solid-phase synthesis[17] of the nonapeptide precursor to [2-DL-[α,β,β-$^2H_3$]tyrosine]oxytocin was carried out by methods similar to those previously reported.[18,19] The all-L and 2-D-tyrosine diastereoisomers were separated by partition chromatography on Sephadex G-25[20,21] using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1). A typical separation is shown in Figure 2a. The solid-phase synthesis of [2-DL-[α-$^2H_1$]tyrosine,8-arginine]vasopressin and the separation of the diastereoisomers by partition chromatography were accomplished as previously reported.[22]

Pharmacological potencies of the 2-D-tyrosine analogues and their natural congeners are shown in Table I. Both D-tyrosine analogues possess drastically reduced potencies in the in vitro rat uterotonic assay. In the rat antidiuretic assay the dose response pattern of [D-Tyr^2]arginine vasopressin is similar to that of arginine vasopressin, and the 2-D-tyrosine analogue retains approximately 50% of the antidiuretic potency of vasopressin. On the other hand, [D-Tyr^2]oxytocin exhibits an unusual antidiuretic dose response pattern in this assay (Figure 3) and expression in terms of a specific activity value is not possible. At low doses it reproducibly increases urine flow, while at high doses it decreases urine flow.

Dose-response studies on the isolated rat uterus (Figure 4) show that the reduced uterotonic potency of [D-Tyr^2]oxytocin is due not only to an apparently decreased affinity for the

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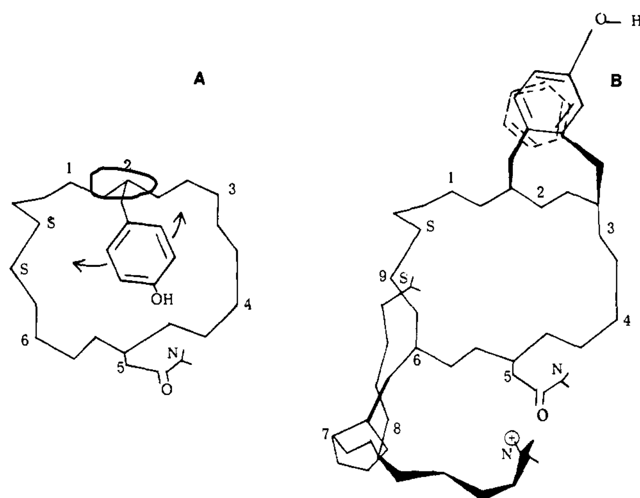

Figure 1. (A) Schematic representation (left) of the "active elements" of the biologically active conformation of oxytocin (i.e., the hydroxyl group of the tyrosine residue in position 2 and the side chain carboxamide group of asparagine in position 5) for the rat uterotonic activity. The tripeptide COOH terminus of oxytocin has been deleted for simplification. For further discussion, see ref 7. (B) The active elements (right) for lysine vasopressin (i.e., the side-chain carboxamide of Asn[5] and the side-chain amino group of Lys[8]) for the activation of the rat antidiuretic receptor. The stacking interaction of the aromatic side chains of Tyr[2] and Phe[3] is also depicted. Numbers indicate residue positions. For further discussion, see ref 9.

Table I. Pharmacological Potencies in Rat of Oxytocin, Vasopressin, and Their D-Tyr[2] Analogues

	uterotonic	antidiuretic	pressor
[D-Tyr[2]]oxytocin	8.4 ± 0.3[a]	nd[b]	nd[c]
	6.6 ± 1[d]	0.01-0.02[d]	0.01-0.02[d]
oxytocin	546 ± 18[e]	2.7 ± 0.2[f]	3.1 ± 0.1[f]
[D-Tyr[2]]arginine vasopressin	1.53 ± 0.09	207 ± 10	194 ± 11
arginine vasopressin[g]	12. ± 0.2	503 ± 53	487 ± 15

[a] This paper. [b] Not determined. See discussion in text and Figure 3. [c] Not determined. See Experimental Section. [d] Reference 16. [e] Reference 23. [f] Reference 24. [g] Reference 25.

smooth muscle receptor, but more importantly the analogue has a reduced ability to maximally stimulate tissue contraction, indicating that [D-Tyr[2]]oxytocin has a lower intrinsic activity[26] than oxytocin.[27] Since arginine vasopressin has previously been shown to exhibit a reduced intrinsic activity as well as lower affinity in the in vitro uterotonic assay,[28] the profile of uterotonic activity of [D-Tyr[2]]arginine vasopressin was not determined. The behavior of both 2-D-tyrosine analogues on the isolated rat uterus is consistent with the role proposed for the tyrosine side chain in the biologically active conformation of agonist neurohypophyseal hormones at the uterine receptor.

Dose-response studies of [D-Tyr[2]]arginine vasopressin in the in vitro rat medullary adenylate cyclase assay[29] reveal that this analogue not only retains the same affinity for the kidney receptor as arginine vasopressin, but also that both peptides possess the same intrinsic activity (Table II). On the other hand, oxytocin itself, as compared to vasopressin, generally displays a strongly diminished affinity and reduced ability to maximally stimulate kidney medullary adenylate cyclase preparations from several species,[30] and [D-Tyr[2]]oxytocin was therefore not tested in this system.

In summary, changing the configuration of Tyr-2 from L to D in either oxytocin or arginine vasopressin caused a dramatic loss of uterotonic potency and, as shown for [D-Tyr[2]]-oxytocin, a decrease in intrinsic activity. [D-Tyr[2]]arginine

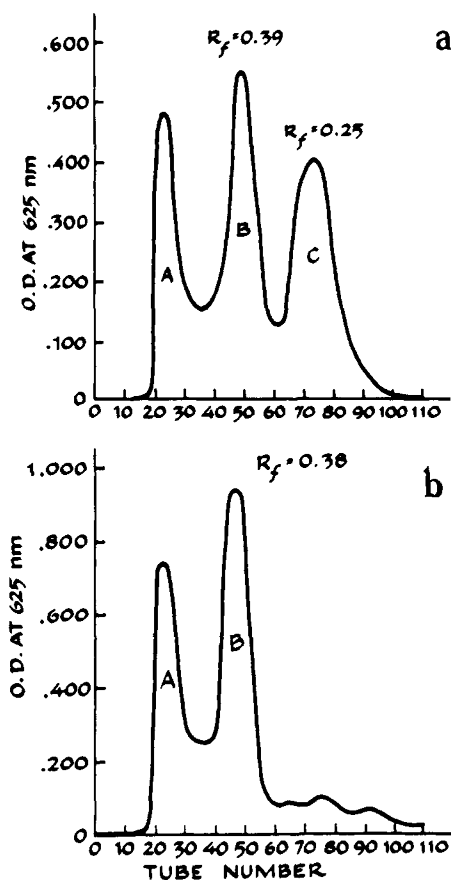

Figure 2. (a) Partition chromatography of [2-DL-[α,β,β-$^2H_3$]tyrosine]-oxytocin on Sephadex G-25 (upper) using the solvent system 1-butanol-3.5% aqueous acetic acid in 1.5% pyridine (1:1). Fractions 67-85 (C) gave [2-L-[α,β,β-$^2H_3$]tyrosine]oxytocin, fractions 43-53 gave [2-D-[α,β,β-$^2H_3$]tyrosine]oxytocin, and A is dimers and other byproducts of the synthesis. (b) Partition chromatography of [2-D-tyrosine]oxytocin on Sephadex G-25 (lower) using the same solvent system as in (a). Fractions 42-54 (B) gave [2-D-tyrosine]oxytocin; A is dimers and other byproducts of the synthesis.

vasopressin retains a high degree of antidiuretic activity as well as full affinity and intrinsic activity in the renal adenylate cyclase system, in line with the proposed vasopressin model, where the Tyr-2 side chain orientation is thought to point away from the ring in the biologically active conformation when the hormone interacts with the antidiuretic receptor.

Experimental Section

Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on silica gel G plates (E. M. Laboratories, Inc., or M. Woelm) 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 (5:5:1:13). Detection was made using iodine, ninhydrin, and fluorescamine. Optical rotation values were measured at the mercury green line (546 nm) using a Zeiss Old 4 polarimeter. Elemental analyses were preformed by Spang Microanalytical Laboratory or Heterocyclic Chemical Corp. Amino acid analyses were obtained by the method of Spackman, Stein, and Moore[31] on a Beckman 120C amino acid analyzer after hydrolysis in 6 N HCl for 22 h. Nuclear magnetic resonance (NMR) spectra were obtained using a Varian T-60 NMR spectrometer or a Bruker WH-90 NMR spectrometer. *N*-Boc-DL-[α-$^2H_1$]tyrosine and *N*-Boc-DL-[α,β,β-$^2H_3$]tyrosine were prepared as previously reported.[22] *N*-Boc protected amino acids were purchased from Vega-Fox Biochemical or from Biosynthetica, or were prepared by published procedures except as discussed below. Solvents for partition chromatography were purified as previously reported.[32]

Table II. Adenylate Cyclase Activity in Renal Medullary Homogenate of Rat in the Absence or Presence of Arginine Vasopressin and [2-D-Tyrosine]arginine Vasopressin[a]

	expt 1	expt 2	expt 3	expt 4	mean[c]
basal activity	24.3 ± 4.0	23.2 ± 1.0	25.2 ± 2.1	26.1 ± 2.1	24.7 ± 0.6 (4)
AVP (10^{-8} M)[b]	52.4 ± 2.5	61.7 ± 3.4	63.6 ± 7.8	80.1 ± 2.5	64.5 ± 5.8 (4)
AVP (10^{-6} M)	49.0 ± 1.3	78.7 ± 11.2	75.9 ± 4.6	77.5 ± 3.6	70.3 ± 7.1 (4)
[D-Tyr[2]]AVP (10^{-8} M)	52.8 ± 1.6	78.3 ± 2.4		70.3 ± 3.5	67.1 ± 7.5 (3)
[D-Tyr[2]]AVP (10^{-6} M)	52.5 ± 5.0	80.7 ± 8.4	75.0 ± 1.8	77.2 ± 3.0	71.3 ± 6.4 (4)

[a] Values are expressed in pmol cAMP min^{-1} mg^{-1} protein ± SEM. [b] AVP = arginine vasopressin. [c] *t*-Test for the difference between two means was performed for the following: basal activity vs. AVP (10^{-8} M), $P < 0.002$; basal activity vs. AVP (10^{-6} M), $P < 0.001$; AVP (10^{-8} M) vs. [D-Tyr[2]]AVP (10^{-8} M), $P \gg 0.5$; AVP (10^{-6} M) vs. [D-Tyr[2]]AVP (10^{-6} M), $P \gg 0.5$.

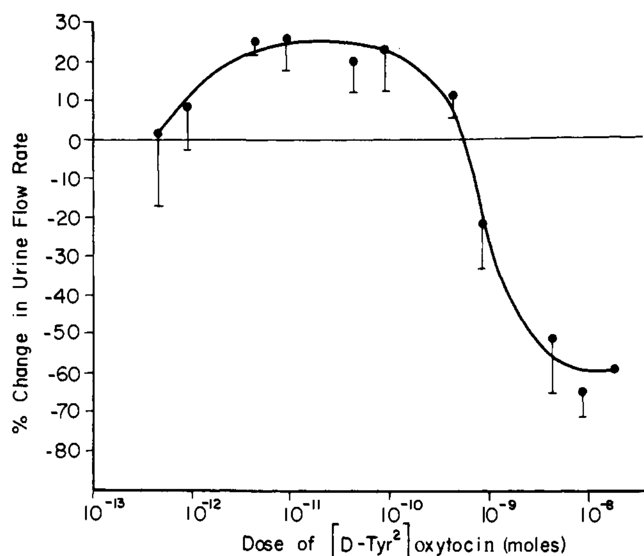

Figure 3. Antidiuretic response profile of [2-D-tyrosine]oxytocin as a function of dose determined in the hydrated rat.

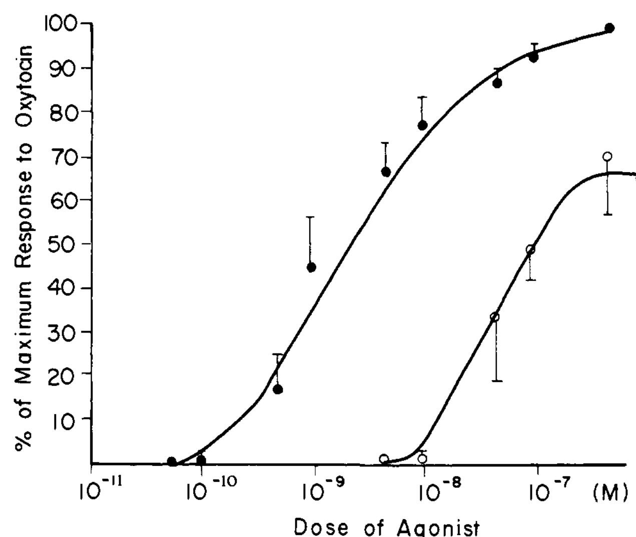

Figure 4. Dose-response relationship of oxytocin (●-●) and [2-D-tyrosine]oxytocin (O-O) on the in vitro rat uterus. Each point represents the average of ten experiments on six uterine horns. The difference in the maximal responses to the two peptides is significant ($p < 0.02$).

***N*-Boc-D-Tyrosine Dicyclohexylamine Salt.** A mixture of 22.4 g (124 mmol) of D-tyrosine (Vega-Fox Biochemicals) in 30 mL of deionized water and 50 mL of peroxide-free dioxane was treated with 28.6 g (200 mmol) of *tert*-butyl azidoformate at pH 10.6 according to the method of Schnabel.[33] When the reaction was complete the product was extracted with two 140-mL portions of ether and the pH adjusted to 3 with citric acid. The oil was extracted with three 200-mL portions of ethyl acetate and treated with Norite to remove colored material. The solvent was evaporated to a pale yellow oil (28.9 g). The oil was dissolved in 200 mL of anhydrous ether and 18.9 g (155 mmol) of dicyclohexylamine was added, and a white precipitate formed. The precipitate was filtered off, washed with two 50-mL portions of boiling ethyl ether, and dried in vacuo to give 31.8 g (71%) of Boc-D-tyrosine dicyclohexylamine salt: mp 213.5–214.5 °C; $[\alpha]^{23}{}_{546}$ −44.4° (*c* 2.1, DMF); NMR ($CDCl_3$) δ 1.37 (s, 9 H), 1.00–2.2 (bm, 22 H), 3.0 (m, 2 H), 3.8 (m, 1 H), 6.8 (q, 4 H). TLC in solvent systems A, B, and C gave single, uniform spots. Before use, the Boc-D-tyrosine was freed of dicyclohexylamine in the usual manner using an aqueous citric acid–ethyl acetate two-phase system to give free Boc-D-tyrosine, mp 93–95 °C (lit.[33] mp for L isomer 96–98 °C). The compound appeared as a single, uniform spot on TLC in solvent systems A, B, and C, identical with authentic Boc-L-tyrosine.

Solid-Phase Synthesis of [2-D-Tyrosine]Oxytocin. The solid-phase synthesis of the nonapeptide precursor to the title compound was accomplished on a semiautomated instrument.[34] A sample of 2.0 g of Boc-glycinate-*O*-resin (prepared from polystyrene resin) (1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.06 mmol/g, Lab Systems Inc., San Mateo, Calif.) was treated with Boc-glycine according to the method of Gisin[25] to give a substitution with the amino acid to an extent of 0.47 mmol/g resin. 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 the scheme outlined in Table III. The exception was the addition of Boc-D-tyrosine. After the initial coupling the ninhydrin test indicated that the coupling had only gone to about 84% completion. Therefore a subsequent overnight coupling was performed using 1.5 mmol of Boc-D-tyrosine and 1.2 mmol of DCC in dimethylformamide. After washing the coupling had gone to completion as judged by the ninhydrin test. The 3,4-dimethylbenzyl group[18,37,38] (DMB) was used to protect the sulfhydryl group of the cysteine residues. *tert*-Butyloxycarbonyl protected amino acid was used throughout. After each amino acid residue was coupled, the synthesis was monitored for completion of coupling by the use of the ninhydrin test.[36] A negative test (>99.5% reaction) was indicated at each step except as noted above. The total volume of solvent or solution used at each washing or reaction step was 25 mL. At the completion of the synthesis of Boc-Cys(DMB)-D-Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-*O*-resin the N-terminal Boc protecting group was removed, the peptide resin was neutralized by repeating steps 1–6 of Table III, and the peptide resin (3.02 g, 86% of theoretical) was dried in vacuo.

The sulfhydryl protected peptide was cleaved from the resin by stirring in 125 mL of freshly prepared anhydrous methanol saturated at −5 °C with anhydrous ammonia (freshly distilled from sodium). The flask was wired shut and stirred at room temperature for 3 days. The solvents were removed by rotary evaporation in vacuo, and the peptide was extracted from the resin with two 100-mL portions of DMF at 40 °C. The DMF solution was evaporated down to about 8 mL, 100 mL of deionized water was slowly added, the mixture was cooled at 4 °C overnight, and the precipitate was filtered off and washed with two 10-mL portions of water, ethanol, and ether. The white powder was dried in vacuo to give H-Cys(DMB)-D-Tyr-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2, 0.68 g (62%), as a white powder, mp 208–210 °C dec. The compound gave a single spot on TLC in solvent systems B (R_f 0.30) and C (R_f 0.18). Anal. Calcd for $C_{57}H_{80}N_{12}O_{12}S_2{\cdot}H_2O$: C, 56.69; H, 6.84; N, 13.92. Found: C, 56.43; H, 6.56; N, 14.08.

A solution of 295 mg (0.23 mmol) of the partially protected nonapeptide in 200 mL of anhydrous ammonia (freshly distilled from sodium) was treated with a sodium stick (to remove the DMB groups)

Table III. Solid-Phase Synthesis Procedures Used

	normal DCC coupling			nitrophenyl ester coupling (Asn and Gln)		
step	solvent or reagent	duration	no. of times	solvent or reagent	duration	no. of times
1	CH_2Cl_2	1 min	4	CH_2Cl_2	1 min	4
2	TFA/CH_2Cl_2/anisole 35/63/2	2 min	1	TFA/CH_2Cl_2/anisole	2 min	1
3	TFA/CH_2Cl_2/anisole 35/63/2	20 min	1	TFA/CH_2Cl_2/anisole 35/63/2	20 min	1
4	CH_2Cl	1 min	3	CH_2Cl_2	1 min	3
5	$DIEA/CH_2Cl_2$ (10/90)	2 min	2	$DIEA/CH_2Cl_2$ (10/90)	2 min	2
6	CH_2Cl_2	1 min	4	CH_2Cl_2	1 min	4
7	Boc-amino acid in CH_2Cl_2 (3.0 equiv)		1	DMF	1 min	5
8	DCC (2.7 equiv) in CH_2Cl_2	1-4 h	1	Boc-amino acid-ONP in DMF (4 equiv)	4-6 h	1
9	CH_2Cl_2	1 min	2	DMF	1 min	3
10	EtOH	1 min	3	EtOH	1 min	3

until the blue color persisted for about 1 min. The ammonia was removed by evaporation under nitrogen[39] followed by lyophilization. The white powder was dissolved in 500 mL of deaerated 0.1% aqueous acetic acid under nitrogen. The pH was adjusted to 8.5 with 3 N ammonium hydroxide, and the deprotected peptide was oxidized by stirring with 50 mL of 0.01 N $K_3Fe(CN)_6$.[40] The pH was adjusted to about 4 with 20% aqueous acetic acid, and the ferro- and excess ferricyanide ions were removed by Rexyn 203 (Cl^- form). The resin was removed by filtration and washed with three 20-mL portions of 20% aqueous HOAc. The solution was evaporated to about 200 mL on a rotary evaporator at 25 °C. The solution was lyophilized and the residue subjected to partition chromatography on Sephadex G-25 (block polymerizate, 100-200 mesh) which had been equilibrated with upper and lower phase using the solvent system 1-butanol-3.5% aqueous acetic acid in 1.5% pyridine (1:1, v/v) according to Yamashiro.[20,21] The fractions corresponding to the major product (R_f 0.38, Figure 2b) were pooled, and the peptide was isolated and then further purified by gel filtration on Sephadex G-25 to give 105.9 mg (45% from nonapeptide) of [2-D-tyrosine]oxytocin: $[\alpha]^{24}_{546}$ −82.2° (*c* 0.5, 1 N HOAc) [lit.[16] $[\alpha]^{22.5}D$ −62.6° (*c* 0.51, 1 N HOAc)]. TLC in solvent systems A (R_f 0.47), B (R_f 0.72), and C (R_f 0.57) gave single, uniform spots. Amino acid analysis gave the following molar ratios: Asp, 1.02; Glu, 1.04; Pro, 0.96; Gly, 1.04; half-Cys, 1.91; Ile, 0.98; Leu, 1.06; D-Tyr, 0.97.

Solid-Phase Synthesis of [2-DL-[α,β,β-$^2H_3$]Tyrosine]oxytocin and Separation of the Diastereoisomers by Partition Chromatography. The synthesis was carried out as above except for using a Vega Series 95 automated synthesizer, a machine similar to that described by Hruby et al.[34] using 3.75 g (1 mmol) of *N*-Boc-glycine-*O*-resin which was substituted with glycine to an extent of 0.27 mmol/g resin. The synthesis was run as previously reported[18,19] using a procedure slightly modified from that in Table III. All protecting groups were as before. The protected nonapeptide was cleaved from the resin as discussed above and precipitated from dimethylformamide-water. There was obtained 920 mg (74%) of H-Cys(DMB)-DL-[α,β,β-$^2H_3$]Tyr-Ile-Gln-Asn-Cys (DMB)-Pro-Leu-Gly-NH_2, mp 215-218 °C. TLC in solvent systems B and C gave single, uniform spots.

A 318-mg (0.25 mmol) portion of the partially protected nonapeptide was deprotected with sodium in liquid amminia and oxidized with $K_3Fe(CN)_6$ in the same manner as given above. The diastereomeric products were separated from one another and from byproducts of the synthesis by partition chromatography on Sephadex G-25 using the solvent system 1-butanol-3.5% acetic acid containing 1.5% pyridine (1:1). The separation of the diastereoisomers is shown in Figure 2. The fractions corresponding to [2-[α,β,β-$^2H_3$]tyrosine]oxytocin (R_f 0.25, fractions 67-85, Figure 2a) were pooled and isolated, and the product was further purified by gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent solvent. The fractions corresponding to the all-L isomer gave 57.8 mg of [2-[α,β,β-$^2H_3$]tyrosine]oxytocin as a white powder, $[\alpha]^{23.5}_{546}$ −24.9° (*c* 0.53, 1 N HOAc). TLC in solvent systems A (R_f 0.48), B (R_f 0.73), and C (R_f 0.57) gave single uniform spots identical with authentic oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.02; Pro, 1.05; Gly, 0.98; half-Cys, 1.90; Ile, 1.03; Leu, 1.00; Tyr, 0.90. The compound had identical milk-ejecting activities[41] with oxytocin within experimental error. The fractions corresponding to the D diastereoisomer (R_f 0.39, fractions 43-53, Figure 2a) were pooled and isolated and the product was subjected to gel filtration on Sephadex G-25 using 0.2 N acetic acid as eluent solvent. Isolation of the major peak gave 63.2 mg of [2-D-[α,β,β-$^2H_3$]tyrosine]oxytocin, $[\alpha]^{23.5}_{546}$ −78.4° (*c* 0.54, 1 N HOAc). TLC in solvent systems A (R_f 0.47), B (R_f 0.71), and C (R_f 0.57) gave single, uniform spots identical with authentic oxytocin. Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.04; Pro, 1.00; Gly, 1.00; half-Cys, 1.87; Ile, 0.96; Leu, 1.01; D-Tyr, 0.93. The biological activities are summarized in Table I.

Solid-Phase Synthesis of [2-DL-[α-$^2H_1$]Tyrosine,8-arginine]vasopressin and Separation of the Diastereoisomers by Partition Chromatography. The solid-phase synthesis was carried out by the same procedures as previously reported.[22] The diastereoisomers were separated by partition chromatography on Sephadex G-25 using the solvent system 1-butanol-ethanol-3.5% aqueous acetic acid containing 1.5% pyridine (4:1:5). The all-L hormone analogue, [2-[α-$^2H_1$]tyrosine,8-arginine]vasopressin, was isolated and further purified by gel filtration on Sephadex G-25 to give the product as a white powder, $[\alpha]^{21}_{546}$ −23.0° (*c* 0.51, 1 N HOAc). TLC in solvent systems B (R_f 0.46), C (R_f 0.12), and D (R_f 0.67) gave uniform spots identical with authentic arginine vasopressin. The compound also had identical milk-ejecting activities with authentic arginine vasopressin. The D diastereoisomer was isolated and further purified by gel filtration on Sephadex G-25. The [2-D-[α-$^2H_1$]tyrosine,8-arginine]vasopressin was isolated as a white powder, $[\alpha]^{23.5}_{546}$ −64.2° (*c* 0.52, 1 N HOAc) [lit.[22] $[\alpha]^{21}_{547}$ −64.3° (*c* 0.50, 1 N HOAc)]. TLC on solvent systems B (R_f 0.50), C (R_f 0.12), and D (R_f 0.68) gave single, uniform spots identical with the [2-D-[α-$^2H_1$]tyrosine,8-arginine]vasopressin previously prepared. Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 0.95; Pro, 1.03; Gly, 1.00; half-Cys, 1.83; Tyr, 0.98; Phe, 1.08; Arg, 0.95. The biological activities are summarized in Table I.

Biological Assays. For the *rat uterotonic assay* mature, virgin Sprague-Dawley rats in natural estrus weighing 250-350 g were used. Uterine horns were isolated and mounted for bioassay in 10-mL baths according to the method of Holton.[42] For the determination of specific activities (oxytocic potency) the bathing fluid was Mg^{2+}-free van Dyke-Hastings solution as modified by Munsick[43] 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 recorded with a Grass polygraph in connection with a Harvard smooth muscle transducer (Model 386A) adapted for use with the polygraph. The four-point assay design[44] was used to compare the hormone analogues with USP posterior pituitary reference standard. At least ten determinations on six uterine horns were performed for each compound. Results are the mean ± the standard error of the mean.

For *dose-response determinations on the rat uterus in vitro*, the millomolar concentration of $CaCl_2$ was 0.3 while the concentration of all other species was as above. Isometric contractions were recorded with a Grass polygraph in connection with a Grass force-displacement transducer (FT03C, springs removed). The tissue was subjected to a base-line tension of 1 g. The individual injection method[23] was used with doses being increased geometrically according to a $\frac{1}{2}$ log 10 procedure until a maximal response was reached. The tissue was rinsed

five times with bathing fluid between individual doses and 20 times after a complete determination until a smooth base line was reached. The order of testing the oxytocin standard (highly purified synthetic oxytocin possessing 500 units/mg oxytocic potency) and the analogue was randomized and the tissue was allowed to rest at least 30 min between determinations. The maximal response to oxytocin in the bath was determined for each tissue preparation and taken as 100%; this defined response was the standard reference point for comparison of all other responses. The values presented in Figure 4 are the average of ten determinations on six uterine horns and are given with the standard deviation at each point.

Antidiuretic assays were performed with anesthetized male, Long-Evans rats (315–345 g) according to the method of Jeffers et al.[45] as modified by Sawyer.[46] Specific antidiuretic potencies were determined using the four-point design or matches against USP posterior pituitary reference standard and values presented are the average ± standard error of the mean from eight determinations on four animals. The dose-response relationship of [D-Tyr^2]oxytocin shown in Figure 3 was determined using the identical assay procedure as above with doses increased geometrically according to a $\frac{1}{2}$ log 10 procedure. The results were obtained from three animals and each point is the mean ± standard deviation.

Adenylate cyclase activity was determined on vasopressin-sensitive enzyme isolated from rat renal medullopapillary (referred to as medullary) tissue which was dissected free of capsule and cortex as described by Harkcom et al.[29] Four enzyme preparations were used and triplicate determinations were performed with each preparation at each dose level including basal level (zero dose of peptide). Results are given in Table II and expressed as mean ± SEM.

Pressor assays were performed with urethane anesthetized, male, Sprague-Dawley rats (250–350 g) as described in the U.S. Pharmacopeia.[47] [D-Tyr^2]oxytocin possesses low pressor activity, the dose-response relationship of which was not parallel to standard and no estimation of its specific activity was possible.

Acknowledgments. The authors thank Drs. Thomas Dousa and Brian A. Jackson of the Mayo Clinic, Foundation and Medical School, Rochester, Minn., for adenylate cyclase assays, Mr. and Mrs. G. Skala for bioassays, and Dr. Nirankar Agarwal for the amino acid analyses. This work was supported by Grants AM-17420 (V.J.H.) and AM-18399 (R.W.) from the U.S. Public Health Service and by a Grant from the National Science Foundation (V.J.H.).

References and Notes

(1) All optically active amino acids are of the L configuration unless otherwise stated. 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: DMB, 3,4-dimethylbenzyl; TFA, trifluoroacetic acid; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide; HOAc, acetic acid.
(2) (a) University of Arizona. (b) University of Illinois at the Medical Center. (c) Taken in part from the Ph.D. Thesis of Donald A. Upson, University of Arizona, 1975. Recipient of a Lubrizol Foundation Scholarship.
(3) Recipient of a Smith, Kline, and French Postdoctoral Fellowship, 1973–1975.
(4) R. Walter in "Structure-Activity Relationships of Protein and Polypeptide Hormones", M. Margoulies and F. C. Greenwood, Eds., Excerpta Medica, Amsterdam, 1972, p 181.
(5) R. Deslauriers and I. C. P. Smith, *Biochem. Biophys. Res. Commun.*, **40**, 179 (1970).
(6) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1355 (1971).
(7) R. Walter, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **36**, 1872 (1977).
(8) K. Hoffman, *Brookhaven Symp. Biol.*, **13**, 184 (1960).
(9) R. Walter, C. W. Smith, P. K. Mehta, S. Boonjarern, J. A. L. Arruda, and N. A. Kurtzman in "Disturbance in Body Fluid Osmolality", T. E. Andreoli, J. Grantham, and F. C. Rector, Jr., Eds., American Physiological Society, Bethesda, Md., 1977, p. 1.
(10) V. J. Hruby, K. K. Deb, A. F. Spatola, D. A. Upson, and D. M. Yamamoto, *J. Am. Chem. Soc.*, **101**, 202 (1979).
(11) A. T. Blomquist, D. H. Rich, V. J. Hruby, L. L. Nangeroni, P. Glose, and V. du Vigneaud, *Proc. Natl. Acad. Sci. U.S.A.*, **61**, 688 (1968); B. W. Bycroft, C. M. Wels, K. Corbett, and D. A. Lowe, *J. Chem. Soc., Chem. Commun.*, 123 (1975); H. Kluender, F. C. Huang, A. Fritzberg, H. Schoes, C. J. Sih, P. Fawcett, and E. P. Abraham, *J. Am. Chem. Soc.*, **96**, 4054 (1974); F. H. Geisler, K. W. Jones, J. S. Fowler, H. W. Kraner, A. P. Wolff, E. P. Cronkite, and D. N. Slatin, *Science*, **186**, 361 (1974).
(12) V. J. Hruby in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins", Vol. 3, B. Weinstein, Ed., Marcel Dekker, New York, 1974, pp 1–188; A. I. R. Brewster, V. J. Hruby, J. A. Glasel, and A. E. Tonelli, *Biochemistry*; **12**, 5294 (1973); V. J. Hruby in ref 4, p 458; A. I. R. Brewster and V. J. Hruby, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3806 (1973); K. D. Kopple, A. Go, R. H. Logan, Jr., and S. Savrada, *J. Am. Chem. Soc.*, **94**, 973 (1972); R. Schwyzer, G. Grathwohl, J.-P. Meraldi, A. Tun-Kyi, R. Vogel, and K. Wüthrich, *Helv. Chim. Acta*, **55**, 2545 (1972); J. S. Cohen, M. Feil, and I. M. Chaiken, *Biochem. Biophys. Res. Commun.*, **236**, 468 (1971); A. F. Bradbury, A. S. V. Burgen, J. Feeney, G. C. K. Roberts, and D. S. Smyth, *FEBS Lett.*, **42**, 179 (1974); J.-P. Meraldi, V. J. Hruby, and A. I. R. Brewster, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1373 (1977), and references cited therein.
(13) J. A. Glasel, V. J. Hruby, J. F. McKelvy, and A. F. Spatola, *J. Mol. Biol.*, **79**, 555 (1973); D. T. Browne, G. L. Kenyon, E. L. Parker, H. Sternlicht, and D. M. Wilson, *J. Am. Chem. Soc.*, **95**, 1316 (1973); J. D. Cutnell, J. A. Glasel, and V. J. Hruby, *Org. Magn. Reson.*, **7**, 256 (1975).
(14) J. Feeney, A. S. V. Burgen, and E. Grell, *Eur. J. Biochem.*, **34**, 107 (1973); M. Blumenstein and V. J. Hruby, *Biochem. Biophys. Res. Commun.*, **68**, 1052 (1976); V. J. Hruby, D. Yamamoto, Y. C. S. Yang, and M. Blumenstein in "Peptides: Proceedings of the Fifth American Peptide Symposium", M. Goodman and J. Meienhofer, Eds., Academic Press, New York, 1977, p 179; M. Blumenstein and V. J. Hruby, *Biochemistry*, **16**, 5169 (1977).
(15) S. H. Koenig, R. D. Brown, T. E. Needham, and A. D. Matwiyoff, *Biochem. Biophys. Res. Commun.*, **52**, 624 (1973); J. A. Sogn, L. C. Craig, and W. A. Gibbons, *J. Am. Chem. Soc.*, **96**, 4694 (1974); W. Haar, S. Fermandjian, J. Vicar, K. Blaha, and P. Fromageot, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4948 (1975), and references cited therein; O. Convert, J. H. Griffin, C. Di Bello, P. Nicolas, and P. Cohen, *Biochemistry*, **16**, 5061 (1977), and references cited therein.
(16) S. Drabarek and V. du Vigneaud, *J. Am. Chem. Soc.*, **87**, 3974 (1965).
(17) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(18) D. A. Upson and V. J. Hruby, *J. Org. Chem.*, **41**, 1353 (1976).
(19) V. J. Hruby, D. A. Upson, and N. Agarwal, *J. Org. Chem.*, **42**, 3552 (1977).
(20) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).
(21) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Am. Chem. Soc.*, **88**, 1310 (1966).
(22) D. M. Yamamoto, D. A. Upson, D. K. Linn, and V. J. Hruby, *J. Am. Chem. Soc.*, **99**, 1564 (1977).
(23) W. Y. Chan and N. Kelley, *J. Pharmacol. Exp. Ther.*, **156**, 150 (1967).
(24) W. Y. Chan and V. du Vigneaud, *Endocrinology*, **71**, 977 (1962).
(25) J. Meienhofer, A. Trzeciak, R. T. Havran, and R. Walter, *J. Am. Chem. Soc.*, **92**, 7199 (1970).
(26) J. M. van Rossum, *Adv. Drug Res.*, **3**, 189 (1966).
(27) A reduced intrinsic uterotonic activity for [D-Tyr^2]oxytocin may explain the earlier observation of Drabarek and du Vigneaud[16] that the analogue, despite its agonist activity, also exhibited an inhibitory effect on the uterotonic activity of oxytocin.
(28) R. Walter, B. Dubois, and I. L. Schwartz, *Endocrinology*, **83**, 979 (1968); R. Walter and M. Wahrenburg, *Pharmacol. Res. Commun.*, **8**, 81 (1976).
(29) T. M. Harkcom, J. K. Kim, P. J. Palumbo, Y. S. F. Hui, and T. Dousa, *Endocrinology*, **102**, 1475 (1978), and references cited therein.
(30) T. Dousa, O. Hechter, I. L. Schwartz, and R. Walter, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1693 (1971); C. Roy, T. Barth, and S. Jard, *J. Biol. Chem.*, **250**, 3157 (1975).
(31) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(32) V. J. Hruby and C. M. Groginsky, *J. Chromatogr.*, **63**, 423 (1971).
(33) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).
(34) V. J. Hruby, L. E. Barstow, and T. Linhart, *Anal. Chem.*, **44**, 343 (1972).
(35) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(36) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(37) D. Yamashiro, R. L. Nobel, and C. H. Li in "Chemistry and Biology of Peptides", J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, pp 197–202.
(38) C. W. Smith, Ph.D. Thesis, University of Arizona, 1973, pp 57–60.
(39) M. Walti and D. B. Hope, *Experientia*, **29**, 389 (1973).
(40) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962).
(41) V. J. Hruby and M. E. Hadley in "Peptides: Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Eds., Ann Arbor Science Publishers, Ann Arbor, Mich., 1975, pp 729–736.
(42) P. Holton, *Br. J. Pharmacol. Chemother.*, **3**, 328 (1948).
(43) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(44) H. O. Schild, *J. Physiol. (London)*, **101**, 115 (1942).
(45) W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, **50**, 184 (1942).
(46) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(47) "The Pharmacopeia of the United States of America", 18th revision, Mack Publishing Co., Easton, Pa., 1970, p 771.