2-D-Tyrosine-oxytocin and 2-D-Tyrosine-deamino-oxytocin, Diastereoisomers of Oxytocin and Deamino-oxytocin[1]

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Diastereoisomers of oxytocin and deamino-oxytocin containing a D-*tyrosine residue in place of the* L-*tyrosine residue in the 2-position have been synthesized and tested for some of the pharmacological activities characteristic of oxytocin. The required intermediates, N-carbobenzoxy-S-benzyl-*L-*cysteinyl-*D-*tyrosyl-*L-*isoleucyl-*L-*glutaminyl-*L-*asparaginyl-S-benzyl-*L-*cysteinyl-*L-*prolyl-*L-*leucylglycinamide and S-benzyl-β-mercaptopropionyl-*D-*tyrosyl-*L-*isoleucyl-*L-*glutaminyl-*L-*asparaginyl-S-benzyl-*L-*cysteinyl-*L-*prolyl-*L-*leucylglycinamide, were prepared by the stepwise p-nitrophenyl ester method. In the case of the 2-*D-*tyrosine-oxytocin the nonapeptide intermediate was also obtained by the azide method. Removal of the protecting groups by reduction with sodium in liquid ammonia and ring closure through oxidation yielded 2-*D-*tyrosine-oxytocin and 2-*D-*tyrosine-deamino-oxytocin, which were isolated by countercurrent distribution. The 2-*D-*tyrosine-oxytocin possessed approximately 34 units of avian depressor activity, 6 units of oxytocic activity, and 34 units of milk-ejecting activity per milligram. The pressor and antidiuretic activities were negligible. The 2-*D-*tyrosine-deamino-oxytocin had about the same avian depressor activity as 2-*D-*tyrosine-oxytocin, about one-sixth of its oxytocic activity, one-half of its milk-ejecting activity, and very low pressor and antidiuretic activities.*

In the course of studies on the relationship between structure and biological activity in the posterior pituitary hormone oxytocin (Figure 1), attention has recently been paid to the effect of diastereoisomerism on the biological activities associated with the hormone. Substitution of a hemi-D-cystine residue for the hemi-L-cystine in the 1-position of the cyclic portion of oxytocin gave an analog (1-hemi-D-cystine-oxytocin) with an extremely low order of biological activity.[3,4] In contrast to this marked effect of diastereoisomerism in the 1-position, 8-D-leucine-oxytocin, in which the L-leucine residue in the penultimate position of the tripeptide side chain of the hormone is replaced by a D-leucine residue, was found to possess considerable avian depressor, oxytocic, and milk-ejecting activities.[5]

As a further contribution to these studies 2-D-tyrosine-oxytocin, a diastereoisomer which differs from oxytocin only in the configuration of the tyrosine residue in the 2-position of the cyclic moiety, has now been synthesized and tested for pharmacological activities characteristic of oxytocin. When we found that this compound possessed a substantial degree of these activities we decided also to synthesize the D-tyrosine analog of deamino-oxytocin, which lacks the free amino group on the hemicystine residue in the 1-position. It has already been demonstrated in this laboratory that the free amino group in oxytocin is not essential for manifestation of the biological activities of the hormone.[6,7] In fact, deamino-oxytocin appears to be more potent than oxytocin with respect to some activities. Also, deamino-deoxy-oxytocin has about the same potencies as deoxy-oxytocin.[8]

The synthesis of the D-tyrosine analogs of oxytocin and deamino-oxytocin was accomplished by use of the stepwise *p*-nitrophenyl ester method in a series of reactions similar to those used for the synthesis of oxytocin.[9] In the case of 2-D-tyrosine-oxytocin, the nonapeptide intermediate was also prepared by the azide method according to the procedure described by Honzl and Rudinger.[10] L-Isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[8] served as starting material. The crystalline heptapeptide was coupled with *p*-nitrophenyl N-carbobenzoxy-O-benzyl-D-tyrosinate in dimethylformamide to obtain the protected octapeptide. The carbobenzoxy group and the O-benzyl group were removed by means of hydrogen bromide in glacial acetic acid and the free octapeptide was obtained from the hydrobromide by treatment with triethylamine in dimethylformamide. The octapeptide then was allowed to react with *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate[11] to give the required protected nonapeptide intermediate for the synthesis of 2-D-tyrosine-oxytocin, namely, N-carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The same nonapeptide was also obtained by coupling the heptapeptide with N-carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosine azide. Coupling of the octapeptide with *p*-nitrophenyl S-benzyl-β-mercaptopropionate[6] yielded the corresponding protected intermediate for the synthesis of 2-D-tyrosine-deamino-oxytocin, namely, S-benzyl-β-mercaptopropionyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.

The protecting groups were removed from the

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(3) K. Jošt, J. Rudinger, and F. Šorm, *Collection Czech. Chem. Commun.*, **26**, 2496 (1961); **28**, 2021 (1963).

(4) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Am. Chem. Soc.*, **85**, 3686 (1963).

(5) C. H. Schneider and V. du Vigneaud, *ibid.*, **84**, 3005 (1962).

(6) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, Jr., *J. Biol. Chem.*, **235**, PC64 (1960); D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **237**, 1563 (1962).

(7) W. Y. Chan and V. du Vigneaud, *Endocrinology*, 71, 977 (1962).

(8) D. B. Hope and V. du Vigneaud, *J. Biol. Chem.*, **237**, 3146 (1962).

(9) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(10) J. Honzl and J. Rudinger, *Collection Czech. Chem. Commun.*, **26**, 2333 (1961).

(11) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).

intermediates by treatment with sodium in liquid ammonia by the method of Sifferd and du Vigneaud as used in the synthesis of oxytocin.[12] The resulting 2-D-tyrosine-oxytoceine and 2-D-tyrosine-deamino-oxytoceine were oxidized in aqueous solution at pH 6.8 by aeration followed by titration with potassium ferricyanide solution[6] to give 2-D-tyrosine-oxytocin and 2-D-tyrosine-deamino-oxytocin. The oxidation of the 2-D-tyrosine-deamino-oxytoceine proceeded more slowly than that of 2-D-tyrosine-oxytoceine. A similar observation was made with respect to the relative ease of oxidation of the sulfhydryl forms of deamino-oxytocin and oxytocin.[6]

The 2-D-tyrosine-oxytocin was purified by countercurrent distribution[13] in 1-butanol–1-propanol–0.05% acetic acid (2:1:3). Measurement of Folin–Lowry color values and avian depressor activities indicated the presence of one major component, which was isolated by concentration and lyophilization. The 2-D-tyrosine-oxytocin traveled faster than oxytocin, the partition coefficient (K) for 2-D-tyrosine-oxytocin being 0.57, whereas the partition coefficient for oxytocin under the same conditions was found to be 0.37. Upon partition chromatography of the D-tyrosine-oxytocin on Sephadex G-25 in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) according to the procedure used for chromatography of oxytocin,[14] the product appeared in a peak having an R_f of 0.45, whereas oxytocin under the same conditions moved much more slowly (R_f 0.24).[14] 2-D-Tyrosine-oxytocin also traveled faster than oxytocin when they were subjected to paper chromatography in 1-butanol–acetic acid–water (4:1:5), and chromatography of a mixture of the two peptides resulted in two distinct spots having R_f values of 0.65 (D-tyrosine-oxytocin) and 0.56 (oxytocin). On electrophoresis in acetate buffer at pH 5.6, 2-D-tyrosine-oxytocin moved to the cathode as a single spot with the same speed as oxytocin.

2-D-Tyrosine-deamino-oxytocin was purified by countercurrent distribution in two solvent systems. In 1-butanol–1-propanol–0.05% acetic acid (2:1:3) K was found to be 5.7. To increase the solubility of the peptide in the aqueous phase benzene was used in the second solvent system instead of propanol, and the ratio of the solvents was changed to 3:2:5. In this system K was approximately 1. In both solvent systems the material traveled as one peak. The homogeneity of the 2-D-tyrosine-deamino-oxytocin was also indicated by its behavior on paper chromatography and paper electrophoresis. The highly purified samples of 2-D-tyrosine-oxytocin and 2-D-tyrosine-deamino-oxytocin gave the expected amino acid and elemental analyses.

The pharmacological activities of these D-tyrosine analogs of oxytocin and deamino-oxytocin are shown in Table I along with the corresponding activities of oxytocin and deamino-oxytocin. The methods employed for the bioassays were the same as those already described for the latter two compounds.[7]

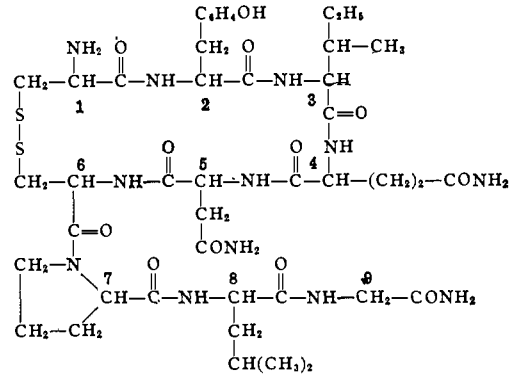

Figure 1. Structure of oxytocin with numbers indicating the position of the individual amino acid residues.

The differences in the partition coefficients and R_f values observed for oxytocin and its D-tyrosine isomer on countercurrent distribution and partition chromatography would be expected to result in the separation of any oxytocin that might conceivably have been present as a contaminant in the crude preparation of D-tyrosine-oxytocin as a result of some slight racemization of the D-tyrosine moiety during the course of the synthesis. Furthermore, the difference in the value of the ratio between the avian depressor and oxytocic activities of oxytocin and the ratio for the D-tyrosine isomer also indicates that the activities are not due to contamination with oxytocin. Thus, the biological potencies reported for 2-D-tyrosine-oxytocin would appear to be inherent activities of this diastereoisomer.

The results of this investigation show that the substitution of a D-tyrosine residue for the naturally occurring L-tyrosine residue in the peptide ring of oxytocin causes a pronounced loss of the biological activities of the hormone but does not destroy them completely. It is also of interest that in the case of D-tyrosine-oxytocin absence of the amino group from the peptide molecule does not appreciably change the avian depressor activity, but does cause a decrease in milk-ejecting and oxytocic potencies.

These two D-tyrosine analogs, which have a small degree of oxytocic activity, also exert an inhibitory effect on the oxytocic activity of oxytocin.[15]

Experimental[16]

Methyl D-Tyrosinate Hydrochloride. The esterification of the carboxyl group of D-tyrosine with methyl alcohol was carried out according to the method that Boissonnas, *et al.*,[17] applied to the esterification of L-tyrosine. The yield was 91%, m.p. 177–179°, $[\alpha]^{19}D$ −73.5° (*c* 3, pyridine). The corresponding L-isomer melted at 190°, $[\alpha]^{22.5}D$ +74.3° (*c* 3, pyridine).[17]

Methyl D-Tyrosinate. A solution of 10.5 g. of methyl D-tyrosinate hydrochloride in 50 ml. of water was cooled to 0° and treated with 11.8 ml. of an aqueous solution of potassium bicarbonate (1 g./ml. of water). After the mixture was stirred for 4 hr. at 0° the solid was filtered off and dried *in vacuo* over calcium chloride.

(12) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(13) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(14) D. Yamashiro, *Nature*, **201**, 76 (1964).

(15) This observation was made by Dr. W. Y. Chan of this laboratory.

(16) Capillary melting points were determined for all compounds and are corrected.

(17) R. A. Boissonnas, S. Guttman, P.-A. Jaquenoud, and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

Table I. Effect of Replacement of the L-Tyrosine by a D-Tyrosine Residue on the Pharmacological Activities of Oxytocin and Deamino-oxytocin

	Mean activities (units/mg.) and standard errors				
	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Pressor (rat)	Antidiuretic (rat)
2-D-Tyrosine-oxytocin	34 ± 3	6.6 ± 1	34 ± 9	0.01–0.02	0.01–0.02
2-D-Tyrosine-deamino-oxytocin	30 ± 1	1	16 ± 1	0.03–0.05	0.07
Oxytocin[a]	507 ± 15	486 ± 5	410 ± 16	3.1 ± 0.1	2.7 ± 0.2
Deamino-oxytocin[a]	733 ± 23	684 ± 32	400 ± 8	1.1 ± 0.1	15 ± 2

[a] See ref. 7.

This product (6.4 g.) was recrystallized from methanol and then from ethyl acetate, giving 4.7 g., m.p. 135–136°, $[\alpha]^{17.5}D$ −25.4° (*c* 1, methanol). The corresponding L-isomer melted at 135–136°, $[\alpha]^{20}D$ +25.75° (methanol).[18]

Methyl N-Carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosinate. N-Carbobenzoxy-S-benzyl-L-cysteine (8.2 g.)[19] was coupled with 4.7 g. of methyl D-tyrosinate in dimethylformamide with the use of dicyclohexylcarbodiimide according to the procedure that Bodanszky and du Vigneaud[11] used for the same coupling with methyl L-tyrosinate. The oil that separated upon addition of hexane was dried *in vacuo* over $CaCl_2$ and P_2O_5, crystallized from ethyl acetate–hexane, and recrystallized from 80% methanol, giving 8.5 g., m.p. 112–114°, $[\alpha]^{20}D$ −25.6° (*c* 1, methanol).

Anal. Calcd. for $C_{28}H_{30}N_2O_6S$: C, 64.4; H, 5.79; N, 5.36. Found: C, 64.3; H, 5.76; N, 5.41.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosine Hydrazide. A mixture of 4.3 g. of the dipeptide ester in 40 ml. of methanol and 1.5 ml. of hydrazine hydrate was allowed to stand at room temperaure for 48 hr., during which time the corresponding hydrazide crystallized out. After recrystallization from 300 ml. of ethanol the product weighed 3.7 g. and melted at 217–218°.

Anal. Calcd. for $C_{27}H_{30}N_4O_5S$: C, 62.1; H, 5.79; N, 10.7. Found: C, 62.3; H, 5.80; N, 10.2.

p-Nitrophenyl N-Carbobenzoxy-O-benzyl-D-tyrosinate. The carbobenzoxylation of the amino group and benzylation of the phenolic hydroxyl group of D-tyrosine was carried out according to the procedure used by Wünsch, *et al.*, for the preparation of the corresponding L-tyrosine compound.[20] The esterification of the carboxyl group was accomplished by the method of Bodanszky and du Vigneaud.[9] Recrystallization of the product from absolute ethanol yielded the protected ester, m.p. 148–149°, $[\alpha]^{20}D$ +9.3° (*c* 2, dimethylformamide). The corresponding L-isomer melted at 148–150°, $[\alpha]^{20}D$ −9° (*c* 2, dimethylformamide).[9]

Anal. Calcd. for $C_{30}H_{26}N_2O_7$: C, 68.4; H, 4.98; N, 5.32. Found: C, 68.6; H, 5.08; N, 5.37.

N-Carbobenzoxy-O-benzyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. L-Isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (1.73 g.) was dissolved in 20 ml. of dimethylformamide at 60°. The solution was cooled and 1.2 g. of *p*-nitrophenyl N-carbobenzoxy-O-benzyl-D-tyrosinate was added. The reaction was allowed to proceed for 20 hr. at room temperature. Solid began to separate after a few hours, and was found gradually to increase in amount. Ethyl acetate (50 ml.) was then added with shaking and the mixture was allowed to stand for another 2 hr. The product was collected, suspended in 25 ml. of ethyl acetate, filtered off, washed with 50 ml. of ethyl acetate, 50 ml. of ethanol, another 50 ml. of ethyl acetate, and dried *in vacuo* at 56° over P_2O_5, giving 2.48 g., m.p. 240–242°, $[\alpha]^{23}D$ −28.1° (*c* 0.7, dimethylformamide).

Anal. Calcd. for $C_{62}H_{81}N_{11}O_{13}S$: C, 61.0; H, 6.69; N, 12.6. Found: C, 61.0; H, 6.77; N, 12.6.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A. By p-Nitrophenyl Ester Method. Finely powdered protected octapeptide (2.3 g.) was suspended in 12.5 ml. of anhydrous acetic acid and treated with 19 ml. of hydrogen bromide in glacial acetic acid (31% w./w.). The mixture was allowed to stand at room temperature for 2 hr. and the solution was poured into 100 ml. of cold, dry ether. The supernatant liquor was decanted and the hydrobromide was washed three times by decantation with 100-ml. portions of ether. It was then filtered off and dried *in vacuo* for 2 hr. over potassium hydroxide and calcium chloride before being dissolved in 19 ml. of dimethylformamide. The solution was cooled to 0° and the pH was adjusted to 7 by the addition of triethylamine. The triethylamine hydrobromide was removed by filtration. *p*-Nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate (1.05 g.) was added to the filtrate and the reaction mixture was stirred for 3 days at room temperature. Ethyl acetate (100 ml.) was added and the product was collected, washed successively with 100 ml. of ethyl acetate, 100 ml. of ethanol, and 100 ml. of ethyl acetate, and dried *in vacuo* at 56° over P_2O_5, giving 1.65 g., m.p. 224–226°, $[\alpha]^{24}D$ −39.0° (*c* 1, dimethylformamide).

For analysis, a finely powdered sample (0.08 g.) was triturated with 12 ml. of 80% aqueous acetone, 50 ml. of hot ethanol, and 10 ml. of ethyl acetate, giving 0.05 g., m.p. 224–226°.

Anal. Calcd. for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 59.0; H, 6.55; N, 12.7. Found: C, 58.9; H, 6.72; N, 12.6.

B. By Azide Method. N-Carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosine hydrazide (0.79 g.) was dissolved in 9.6 ml. of 4.5 *N* hydrochloric acid in 95% tetrahydrofuran, cooled to −20°, and treated with a cold solution of 0.14 g. of sodium nitrite in 0.54 ml. of water. The mixture was stirred for 10 min. at −20°, diluted with 30 ml. of cold ethyl acetate, and washed

(18) E. Fischer and W. Schrauth, *Ann.*, **354**, 21 (1907); E. Fischer, *Ber.*, **41**, 850 (1908).
(19) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).
(20) E. Wünsch, G. Fries, and A. Zwick, *Ber.*, **91**, 542 (1958).

three times with 10-ml. portions of a saturated solution of sodium bicarbonate containing a small amount of sodium chloride. After being dried for a short time over sodium sulfate, the solution was added to a cold solution of 1.37 g. of L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in 50 ml. of dimethylformamide. The mixture was stirred for 12 hr. at 4° and then for 3 hr. at room temperature. Ethyl acetate (200 ml.) was added and the product was collected, dried, ground to a fine powder, and triturated successively with 50 ml. of ethanol and 50 ml. of methanol. It was then washed with ethyl acetate and ether, heated for 3 min. with 50 ml. of methanol, and dried *in vacuo* over P_2O_5 at 64°, giving 1.6 g., m.p. 232–235°, $[\alpha]^{20}D$ −39.1° (*c* 1, dimethylformamide).

*S-Benzyl-β-mercaptopropionyl-*D*-tyrosyl-*L*-isoleucyl-*L*-glutaminyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The procedure followed in general that described in the preceding section for preparation of the protected nonapeptide by the *p*-nitrophenyl ester procedure. The same protected octapeptide (1.46 g.) was suspended in 8 ml. of anhydrous acetic acid and treated with 8 ml. of hydrogen bromide in glacial acetic acid (25%, w./w.). The hydrobromide of the free base was precipitated with dry ether, separated, dried *in vacuo* over potassium hydroxide and calcium chloride, and dissolved in 10 ml. of dimethylformamide. The pH of the solution was adjusted to 7 by addition of triethylamine. The resulting triethylamine hydrobromide was filtered off and 0.46 g. of *p*-nitrophenyl S-benzyl-β-mercaptopropionate was added to the filtrate. After 4 days 200 ml. of ethyl acetate was added, and the mixture was allowed to stand at room temperature for 4 hr. The solid was collected and washed with 50 ml. of ethyl acetate and 50 ml. of ethanol. After being dried the product was triturated successively with 50 ml. of water, 30 ml. of methanol, and 30 ml. of ether, giving 0.94 g., m.p. 229–231°, $[\alpha]^{18.5}D$ −32.1° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{57}H_{79}N_{11}O_{12}S_2$: C, 58.3; H, 6.78; N, 13.1. Found: C, 58.1; H, 6.74; N, 13.0.

*2-*D*-Tyrosine-oxytocin.* N-Carbobenzoxy-S-benzyl-L-cysteinyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (200 mg.) was dissolved in 400 ml. of anhydrous liquid ammonia and treated with sodium until a faint blue color enveloped the solution and persisted for about 30 sec. One drop of glacial acetic acid was added and the ammonia was evaporated *in vacuo*, the last 50 ml. being removed by lyophilization. The residue was dissolved in 0.1% acetic acid and after adjustment of the pH to 6.8 the solution was aerated at a concentration of 1 mg./ml. for 2 hr., and then 0.8 ml. of 0.02 *N* potassium ferricyanide was added.[6] The ferrocyanide and ferricyanide ions were removed by passage through a column of ion-exchange resin AG 3X4 in the chloride form. Assay of the solution for avian depressor activity showed a total 3245 units. The solution was concentrated at a temperature below 20° to a volume of approximately 20 ml., placed in the first two tubes of a 200-tube Craig countercurrent distribution machine, and subjected to a total of 400 transfers in the solvent system 1-butanol–1-propanol–0.05% acetic acid (2:1:3). Determination of Folin–Lowry color values[21] after both 200 and 400 transfers indicated a main peak with a partition coefficient (*K*) of approximately 0.57, accompanied by two smaller, more slowly moving peaks. Avian depressor activity was present in tubes 130–165 and the peak occurred at tube 146. The curves obtained by plotting the Folin–Lowry color values and avian depressor values were in agreement with a calculated curve. The contents of tubes 135–155 were pooled, evaporated in a flash evaporator to a volume of approximately 40 ml., and lyophilized to give 46 mg. of fluffy, white product. Material isolated from countercurrent distribution was also submitted to partition chromatography on Sephadex. The D-tyrosine-oxytocin (53 mg.) was dissolved in 4 ml. of the upper phase of the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and applied to a Sephadex G-25 column (2.16 × 120 cm.) which had been equilibrated with both phases. Elution with the upper phase was performed and 9.6-ml. fractions were collected at a flow rate of 0.3 ml./min. The Folin–Lowry color values showed a main peak with an R_f of 0.45 consisting of fractions 20–30. The recovery of 2-D-tyrosine-oxytocin from this peak was 35 mg., $[\alpha]^{19.5}D$ −62.6° (*c* 0.51, 1 *N* acetic acid).

For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo*, and a loss in weight of 5.7% was observed.

Anal. Calcd. for $C_{43}H_{66}N_{12}O_{12}S_2$: C, 51.3; H, 6.60; N, 16.7. Found: C, 51.3; H, 6.72; N, 16.3.

The product was hydrolyzed in 6 *N* hydrochloric acid at 110° for 17 hr. and analyzed on the Beckman–Spinco amino acid analyzer according to the procedure of Spackman, Stein, and Moore.[22] The following molar ratios of amino acids and ammonia were obtained, with the value of leucine taken as 1.0: aspartic acid 1.1, glutamic acid 1.1, proline 1.1, glycine 1.0, cystine 1.1, isoleucine 1.0, leucine 1.0, tyrosine 0.9, and ammonia 2.9.

Samples of 2-D-tyrosine-oxytocin, oxytocin, and a mixture of equal amounts of each of the two peptides were dissolved in 50% ethanol, applied separately to a strip of Whatman No. 1 paper, and chromatographed at room temperature with the solvent system 1-butanol–acetic acid–water (4:1:5, descending). The chromatograms were developed with ninhydrin. The R_f values obtained were 0.56 for oxytocin and 0.65 for 2-D-tyrosine-oxytocin under these conditions. The mixture of the two peptides separated into two spots according to their R_f values. On electrophoresis at 4° in pyridine acetate buffer at pH 5.6, 300 v., no difference was observed in the speed with which the two diastereoisomers traveled to the cathode.

*2-*D*-Tyrosine-deamino-oxytocin.* The procedure employed for the conversion of S-benzyl-β-mercaptopropionyl-D-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide to 2-D-tyrosine-deamino-oxytocin followed closely that described for the preparation of 2-D-tyrosine-oxytocin from the corresponding protected nonapeptide. The oxidation of the reduced material was carried out by aeration of an aqueous solution at pH 6.8–7.0 at a

(21) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

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

concentration of 1 mg./ml. for 1.25 hr. followed by addition of 16 ml. of 0.02 *N* potassium ferricyanide. The ferricyanide and ferrocyanide ions were removed by passage through the resin AG 3X4 in the chloride form. The solution resulting from the reduction of 254 mg. of the protected intermediate and subsequent oxidation was found to contain a total of approximately 4180 units of avian depressor activity. This solution was concentrated below room temperature to a volume of approximately 20 ml., placed in the first two tubes of a 200-tube countercurrent machine, and subjected to a total of 350 transfers in 1-butanol–1-propanol–0.05% acetic acid (2:1:3). Determination of Folin–Lowry color values indicated one peak with a *K* of 5.7. The curve obtained by plotting these values and the one obtained from the avian depressor activity values were in agreement with a calculated curve. The contents of tubes 280–315 were concentrated to a volume of about 50 ml. and lyophilized to give 144 mg. of a fluffy, white product. This material (134 mg.) was redistributed in 1-butanol–benzene–0.05% acetic acid (3:2:5). After 300 transfers, a single peak was obtained with a *K* value of approximately 1. After concentration and lyophilization of contents of tubes 142–162, 108 mg. of 2-D-tyrosine-deamino-oxytocin was obtained, $[\alpha]^{19.5}D$ −60.4° (*c* 0.5, 1 *N* acetic acid).

For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* and a loss in weight of 5.1% was observed.

Anal. Calcd. for $C_{43}H_{65}N_{11}O_{12}S_2$: C, 52.0; H, 6.60; N, 15.5. Found: C, 52.0; H, 6.72; N, 15.4.

The 2-D-tyrosine-deamino-oxytocin was hydrolyzed in 6 *N* hydrochloric acid at 110° for 17 hr. and analyzed in the 30–50° system.[22] The following molar ratios of amino acids were obtained, with the value of glycine taken as 1.0: isoleucine 1.0, aspartic acid 1.0, glutamic acid 1.0, proline 1.0, glycine 1.0, leucine 1.0, tyrosine 1.0, cystine 0.25, mixed disulfide of cysteine and β-mercaptopropionic acid 0.53, and ammonia 3.0. The ratios for cystine and the mixed disulfide taken together fully account for the half-cystine residue in the 2-D-tyrosine-deamino-oxytocin.

Paper chromatography of the 2-D-tyrosine-deamino-oxytocin on Whatman No. 1 paper in butanol–acetic acid–water (4:1:5) gave one spot, R_f 0.7, when developed with either the platinum reagent of Toennies and Kolb[23] or the chlorine and starch–potassium iodide reagent of Rydon and Smith.[24] Under the same conditions deamino-oxytocin moves with an R_f of 0.68 and the mixture of the two diastereoisomers could therefore not be separated. On electrophoresis in pyridine buffer at pH 5.6, 300 v., and 4°, 2-D-tyrosine-deamino-oxytocin traveled as a single spot to the cathode with the same speed as deamino-oxytocin.

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(23) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
(24) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 923 (1952).

6-Hemi-D-cystine-oxytocin, a Diastereoisomer of the Posterior Pituitary Hormone Oxytocin[1]

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As part of the investigation of the importance of the stereostructure of the constituent amino acid residues of oxytocin to its biological manifestations, 6-hemi-D-cystine-oxytocin has been synthesized and tested for activities characteristic of this posterior pituitary hormone. In this diastereoisomer of oxytocin, the hemi-L-cystine residue in the 6-position of the ring, to which the tripeptide side chain is attached, has been replaced by a hemi-D-cystine residue. The required synthetic nonapeptide intermediate was synthesized, starting from L-prolyl-L-leucylglycinamide, by the p-nitrophenyl ester method. Reduction of the protected nonapeptide with sodium in liquid ammonia, followed by oxidation of the resulting disulfhydryl form with potassium ferricyanide, gave the 6-hemi-D-cystine-oxytocin, which was isolated by countercurrent distribution. This analog was found to be practically devoid of avian depressor, oxytocic, and milk-ejecting activities. 1-Hemi-D-cystine-oxytocin, in which the hemi-L-cystine residue in the 1-position of oxytocin is replaced by a hemi-D-cystine residue, shows only slight amounts of these activities. Thus, a change in the configuration of either half of the L-cystine residue in oxytocin practically destroys these pharmacological activities of the hormone.

One of the facets of the study of the relationship between chemical structure and biological activities of the posterior pituitary hormone oxytocin, has involved diastereoisomers of the hormone. The total synthesis[2] of this octapeptide amide (Figure 1), which contains all L-amino acid residues, provided an approach to the preparation of various diastereoisomeric forms

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(2) V. du Vigneaud, C. Ressler, J. M. Swan, P. G. Katsoyannis, C. W. Roberts, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).