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]

Maurice Manning and Vincent du Vigneaud

Contribution from the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received April 28, 1965*

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

(1) This work was supported in part by Grant HE-01675 from the National Heart Institute, U. S. Public Health Service. Dr. Manning wishes to acknowledge a Fulbright Travel Grant.

(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).

of the hormone by replacement of one of the naturally occurring L-amino acids with the corresponding D-amino acid in the intermediary synthetic steps. Comparison of the pharmacological properties of these diastereoisomers with those of oxytocin then enabled an assessment of the importance of the stereostructure of a particular constituent amino acid to the activities of the hormone.

The analog containing a D-leucine residue in place of the L-leucine in the penultimate position of the side chain of oxytocin (8-D-leucine-oxytocin) was found to possess oxytocic and avian depressor activities of approximately 20 units/mg. and a milk-ejecting activity of at least 50 units/mg.[3] These potencies represent about $^1/_{25}$ of the oxytocic and avian depressor potencies exhibited by oxytocin, and about $^1/_8$ of the milk-ejecting potency of the hormone. No appreciable rat pressor or antidiuretic activity was observed for the D-leucine analog.

Substitution of a hemi-D-cystine residue for the hemi-L-cystine residue bearing the free amino group in the 1-position of the ring of oxytocin[4-6] drastically lowered the activities characteristic of the hormone. The synthetic 1-hemi-D-cystine-oxytocin described by Hope, Murti, and du Vigneaud,[5] which had been subjected to extensive purification, possessed 2 units/mg. of avian depressor activity. However, they noted that the preparation might still contain a trace of oxytocin and hence that further investigation was required to determine whether this activity was an intrinsic activity of 1-hemi-D-cystine-oxytocin. It has recently been found in this laboratory[6] that this analog, purified by partition chromatography on Sephadex in 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9), possesses approximately 0.2 unit/mg. of avian depressor activity and 2 units/mg. of oxytocic activity. It was also found that, when countercurrent distribution in this solvent system was substituted for the partition chromatography, 1-hemi-D-cystine-oxytocin with the same oxytocic activity was obtained.

While the work in this laboratory on 1-hemi-D-cystine-oxytocin was underway, the preparation of this analog was described by Jošt, Rudinger, and Šorm.[4a] They suggested that the slight activity exhibited by their preparation may possibly have been due to contamination with a small amount of oxytocin, and subsequently reported[4b] that a highly purified preparation of 1-hemi-D-cystine-oxytocin exhibited approximately 0.1 unit/mg. of avian depressor activity, 0.25 unit/mg. of oxytocic activity, 0.38 unit/mg. of milk-ejecting activity, and 0.0033 unit/mg. of antidiuretic activity. They interpreted these data to indicate that the slight activities are intrinsic properties of the 1-hemi-D-cystine analog, since the ratios between the activities exhibited by the 1-hemi-D-cystine-oxytocin deviate from the corresponding ratios between these activities of oxytocin.

Nesvadba, Honzl, and Rudinger[7] have also prepared

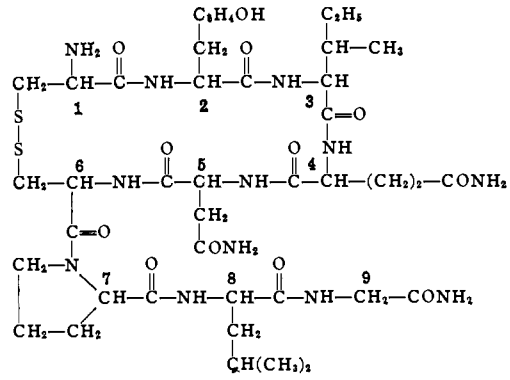

Figure 1. Oxytocin.

an analog of oxytocin using 3-L-alloisoleucine in place of L-isoleucine in the synthetic procedures. From their studies they tentatively concluded that 3-L-alloisoleucine-oxytocin has little or no avian depressor or oxytocic activity but that it may have appreciable milk-ejecting activity.

Replacement of the L-tyrosine residue in position 2 of the ring of oxytocin by a D-tyrosine residue was found by Drabarek and du Vigneaud[8] to reduce the avian depressor and milk-ejecting activities to approximately 34 units/mg. and the oxytocic activity to 6 units/mg. The pressor and antidiuretic activities of this analog were negligible.

When the potencies of the three diastereoisomers containing a D-amino acid residue in place of one of the naturally occurring L-amino acid residues are compared, it is evident that the most drastic loss of potency resulted from replacement of the hemi-L-cystine residue in the 1-position by a hemi-D-cystine residue. In view of this drastic loss of activity it was of interest to determine the effect of replacing the hemi-L-cystine residue in the 6-position by its enantiomer. It will be noted that the side chain is attached to the cyclic portion of the hormone at this position and hence it was considered likely that the configuration of the 6-hemi-L-cystine residue would also be of major importance to the manifestation of the activities characteristic of the hormone. 6-Hemi-D-cystine-oxytocin has now been synthesized and tested for the pharmacological activities characteristic of oxytocin.

The required protected nonapeptide intermediate, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-D-cysteinyl-L-prolyl-L-leucylglycinamide, was prepared from L-prolyl-L-leucylglycinamide by the stepwise *p*-nitrophenyl ester procedure used previously in this laboratory for the synthesis of oxytocin.[9] Synthesis of the protected tetrapeptide intermediate, S-benzyl-N-carbobenzoxy-D-cysteinyl-L-prolyl-L-leucylglycinamide,[10] was accomplished by using *p*-nitrophenyl S-benzyl-N-carbobenzoxy-D-cysteinate[5] in place of the S-benzyl-N-carbobenzoxy-L-cysteinate that was used in the step-

(3) C. H. Schneider and V. du Vigneaud, *J. Am. Chem. Soc.*, **84**, 3005 (1962).

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

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

(6) D. Yamashiro, D. Gillessen, and V. du Vigneaud, to be published.

(7) H. Nesvadba, J. Honzl, and J. Rudinger, *Collection Czech. Chem. Commun.*, **28**, 1691 (1963).

(8) S. Drabarek and V. du Vigneaud, Abstracts, Sixth European Peptide Symposium, Athens, Greece, Sept. 15–20, 1963, in press.

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

(10) The synthesis of this protected tetrapeptide and the corresponding decarbobenzoxylated tetrapeptide was first accomplished in this laboratory by Dr. Conrad H. Schneider. The authors wish to thank him for a supply of the former compound, which was helpful in early attempts to prepare 6-hemi-D-cystine-oxytocin by another route.

wise synthesis of oxytocin itself. This protected tetrapeptide, in contrast to the crystalline diastereoisomeric compound containing the protected L-cysteine residue, was obtained as a low-melting, amorphous solid. However, the N-carbobenzoxy-L-asparaginyl-S-benzyl-D-cysteinyl-L-prolyl-L-leucylglycinamide obtained from this tetrapeptide by reaction with *p*-nitrophenyl N-carbobenzoxy-L-asparaginate was crystalline. Chromatography of this protected pentapeptide on a thin-layer silica gel G plate gave no indication of contamination by the protected L pentapeptide. The higher protected peptide intermediates obtained in the subsequent synthetic steps were likewise found to be chromatographically homogeneous. In all cases the protected D-cysteine peptides traveled much faster than the corresponding protected L-cysteine diastereoisomers when they were subjected to chromatography under the same conditions in methanol–chloroform. It might also be mentioned that the protected D-cysteine peptides were more soluble than the corresponding protected L peptides, particularly in the case of the protected tetrapeptide. The protected nonapeptide was reduced with sodium in liquid ammonia to remove the benzyl and carbobenzoxy groups according to the method of Sifferd and du Vigneaud[11] as applied in the synthesis of oxytocin.[2] After removal of the ammonia the sulfhydryl nonapeptide was oxidized to the cyclic octapeptide at pH 7 by treatment with potassium ferricyanide.[12] The solution after removal of the ferricyanide and ferrocyanide ions showed no detectable avian depressor activity. Purification was effected by subjecting the crude product to countercurrent distribution[13] in the system butanol–propanol–0.05% acetic acid containing 0.1% pyridine (6:1:8) at 4°. After 1050 transfers the contents of the tubes representing the central portion of the symmetrical main peak ($K = 0.50$) were combined, concentrated, and lyophilized. The purified material appeared to be homogeneous upon subjection to paper and thin layer chromatography. Furthermore, a mixture of the 6-hemi-D-cystine-oxytocin and oxytocin could be separated by chromatography on thin-layer silica gel G in the system butanol–acetic acid–water (4:1:5). Elemental analysis and amino acid analysis[14] of the 6-hemi-D-cystine-oxytocin gave the expected values. It is of interest that the specific rotation of the 6-hemi-D-cystine-oxytocin in 1 *N* acetic acid is −81°, as compared with −23° for oxytocin[15] and −56° for 1-hemi-D-cystine-oxytocin.[6]

The 6-hemi-D-cystine-oxytocin was practically devoid of the pharmacological activities characteristic of oxytocin. It possessed no appreciable avian depressor activity, approximately 0.02 unit/mg. of oxytocic activity, <0.002 unit/mg. of milk-ejecting activity, and negligible pressor and antidiuretic activities. Thus it would appear that a change in the configuration of either half of the L-cystine residue occurring in the cyclic pentapeptide moiety of oxytocin practically destroys these biological manifestations of the hormone.

(11) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

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

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

(14) S. Moore, D. H. Spackman, and W. H. Stein, *ibid.*, **30**, 1185 (1958); D. H. Spackman, W. H. Stein, and S. Moore, *ibid.*, **30**, 1190 (1958).

(15) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957).

Experimental[16]

*N-Carbobenzoxy-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* L-Prolyl-L-leucylglycinamide[17] was prepared from the carbobenzoxy tripeptide, which had been synthesized by the method of Cash.[18] *p*-Nitrophenyl S-benzyl-N-carbobenzoxy-D-cysteinate[5] (9 g.) and the L-prolyl-L-leucylglycinamide (6 g.) were suspended in 20 ml. of chloroform and stirred at room temperature for 12 hr., during which time the reactants completely dissolved. The reaction mixture was then allowed to stand at room temperature for 2 days. The chloroform was removed *in vacuo* and the residual yellow oil was dissolved in 400 ml. of ethyl acetate. The resulting solution was extracted ten times with 60-ml. portions of 1 *N* hydrochloric acid, 15 times with 60-ml. portions of 1 *N* ammonium hydroxide, twice with 60-ml. portions of 1 *N* hydrochloric acid, and five times with 60-ml. portions of water. The solution was dried over anhydrous magnesium sulfate and filtered. Ethyl acetate was removed *in vacuo*, giving 11 g. of a glassy solid, $[\alpha]^{21}D$ −18.3° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{31}H_{41}N_5O_6S$: C, 60.8; H, 6.75; N, 11.4. Found: C, 60.8; H, 6.86; N, 11.3.

A portion of the protected tetrapeptide amide was hydrolyzed in 6 *N* hydrochloric acid at 110° for 17 hr. and then analyzed for amino acids and ammonia.[14] The following molar ratios were obtained (with the value of glycine taken as 1.0): S-benzylcysteine 1.0, proline 1.0, leucine 1.0, glycine 1.0, and ammonia 1.0.

*N-Carbobenzoxy-*L*-asparaginyl-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* Hydrogen bromide in acetic acid (32% w./w., 30 ml.) was added to a solution of 4.0 g. of the protected tetrapeptide amide in 20 ml. of dry acetic acid and the mixture was stirred at room temperature for 1 hr. The clear solution was poured into 400 ml. of cold, dry ether and the precipitated tetrapeptide hydrobromide was collected, washed with three 150-ml. portions of ether, and dried for 12 hr. in a vacuum desiccator over P_2O_5 and NaOH. The white solid was dissolved in 300 ml. of methanol and Amberlite IRA-410 resin (OH cycle) was added with stirring until the solution was free of bromide ion. The resin was filtered off and washed with methanol. The methanol was removed from the combined filtrate and washings *in vacuo*. The dry residue (3.0 g.) was dissolved in 5.5 ml. of dimethylformamide, and 3.5 g. of *p*-nitrophenyl N-carbobenzoxy-L-asparaginate was added. The resulting solution was stirred at room temperature and aliquots of the reaction mixture were subjected to chromatography on thin-layer silica gel G plates in chloroform–methanol (7:3) at intervals over a 24-hr. period, and it was found by following the disappearance of the free tetrapeptide that the reaction

(16) All melting points were determined in capillary tubes and are corrected.

(17) R. A. Boissonnas, S. Guttmann, P.-A. Jaquenoud, and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955); M. Zaoral and J. Rudinger, *Collection Czech. Chem. Commun.*, **20**, 1183 (1955); W. B. Lutz, C. Ressler, D. E. Nettleton, Jr., and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 167 (1959).

(18) W. D. Cash, *J. Org. Chem.*, **26**, 2136 (1961).

had reached completion after 24 hr. Spots were detected by spraying the plates with platinum reagent[19] and with modified ninhydrin reagent.[20] Dimethylformamide was removed from the reaction mixture by evaporation *in vacuo*. Ethyl acetate (40 ml.) was added to the residue and the solid that separated was filtered off, washed with two 100-ml. portions of cold ethyl acetate and three 100-ml. portions of hexane, and dried *in vacuo*, giving 4.4 g. of crystalline protected pentapeptide, m.p. 209–210°, $[\alpha]^{21}D$ −15° (*c* 1, dimethylformamide). A portion of this material was subjected to chromatography as already described, and only one spot was obtained on the chromatogram.

Anal. Calcd. for $C_{35}H_{47}N_7O_8S$: C, 57.9; H, 6.53; N, 13.5. Found: C, 57.9; H, 6.56; N, 13.4.

*N-Carbobenzoxy-*L*-glutaminyl-*L*-asparaginyl-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected pentapeptide (3.8 g.) was dissolved in 16 ml. of acetic acid, and hydrogen bromide in acetic acid (30% w./w., 14 ml.) was added. The solution was stirred at room temperature for 1 hr. and then poured into 400 ml. of cold, dry ether. The precipitated hydrobromide, after being collected, washed with dry ether, and dried *in vacuo* over NaOH and P_2O_5, was dissolved in 150 ml. of methanol and converted to the free base by treatment with Amberlite IRA-410 resin as described in the preceding section. The residue remaining after removal of the methanol was dissolved in 8.5 ml. of dimethylformamide, and 2.8 g. of *p*-nitrophenyl N-carbobenzoxy-L-glutaminate[9] was then added. The reaction mixture was stirred at room temperature for 20 hr. Subjection of aliquots of the reaction mixture to thin layer chromatography in the system methanol–chloroform (1:1) indicated that the reaction had reached completion. Dimethylformamide was removed *in vacuo* and 100 ml. of ethyl acetate was added to the residual oil. Solidification of the oil was induced by scratching with a glass rod. Hexane (150 ml.) was added and after 2 hr. the solid was collected, washed with hexane, and dried in a vacuum desiccator over P_2O_5, giving 4.5 g., m.p. 204–206°, $[\alpha]^{21}D$ −35.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{40}H_{55}N_9O_{10}S$: C, 56.2; H, 6.49; N, 14.8. Found: C, 55.9; H, 6.49; N, 14.5.

*N-Carbobenzoxy-*L*-isoleucyl-*L*-glutaminyl-*L*-asparaginyl-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected hexapeptide (3 g.) was dissolved in 10 ml. of acetic acid, and hydrogen bromide in acetic acid (30% w./w., 10 ml.) was added. After 1 hr. at room temperature, the solution was poured into 300 ml. of cold, dry ether. The precipitated hydrobromide was collected, washed with ether, dried in a vacuum desiccator over P_2O_5 and NaOH for 2 hr., and then dissolved in 100 ml. of methanol and converted to the free base by treatment with Amberlite IRA-410 resin. The amorphous residue remaining after removal of the methanol was dissolved in 10 ml. of dimethylformamide, 2 g. of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate[9] was added, and the mixture was stirred at room temperature. Subjection of aliquots to thin layer chromatography in the system methanol–chloroform (1:1) indicated that the reaction was complete after 22 hr. The dimethylformamide was removed *in vacuo* and the resulting solid was purified by trituration successively with three 50-ml. portions of ether, three 50-ml. portions of ethyl acetate, and one 50-ml. portion of ether–95% ethanol (1:1), giving 3 g. of protected heptapeptide, m.p. 205–207° (sinters 202°), $[\alpha]^{20.5}D$ −31.0° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{46}H_{60}N_{10}O_{11}S$: C, 57.1; H, 6.88; N, 14.5. Found: C, 56.5; H, 6.91; N, 14.4.

*O-Benzyl-N-carbobenzoxy-*L*-tyrosyl-*L*-isoleucyl-*L*-glutaminyl-*L*-asparaginyl-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* A solution of 2 g. of the protected heptapeptide in 15 ml. of acetic acid was treated with hydrogen bromide in acetic acid (33% w./w., 9.5 ml.), and the resulting solution was stirred at room temperature for 75 min. The clear solution was poured slowly into 300 ml. of cold, dry ether and the precipitated hydrobromide was collected, washed with dry ether, and dried in a vacuum desiccator over P_2O_5 and NaOH. The free heptapeptide was obtained from the hydrobromide according to the procedure already described for the pentapeptide. The amorphous solid remaining after removal of the methanol was dissolved in 8 ml. of dimethylformamide. *p*-Nitrophenyl O-benzyl-N-carbobenzoxy-L-tyrosinate[9] (1.3 g.) was then added and the mixture was allowed to stand at room temperature for 2 days. The dimethylformamide was removed *in vacuo* and the resulting solid was triturated successively with three 50-ml. portions of ethyl acetate and three 50-ml. portions of ether, giving 2.3 g. of protected octapeptide, m.p. 227–229°, $[\alpha]^{21}D$ −24.8° (*c* 1, dimethylformamide), which appeared to be homogenous when it was subjected to thin layer chromatography in methanol–chloroform (55:45).

Anal. Calcd. for $C_{62}H_{81}N_{11}O_{13}S$: C, 61.0; H, 6.69; N, 12.6. Found: C, 60.5; H, 6.70; N, 12.5.

*S-Benzyl-N-carbobenzoxy-*L*-cysteinyl-*L*-tyrosyl-*L*-isoleucyl-*L*-glutaminyl-*L*-asparaginyl-S-benzyl-*D*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected octapeptide (1 g.) was dissolved in 20 ml. of dry glacial acetic acid, and hydrogen bromide in acetic acid (33.7% w./w., 7 ml.) was added. The mixture was stirred at room temperature for 2.25 hr. and the clear solution was poured into 300 ml. of cold, dry ether. The precipitated hydrobromide was collected, washed with ether, and dried in a vacuum desiccator over P_2O_5 and NaOH. The free octapeptide was obtained from the hydrobromide by treatment with IRA-410 resin as already described for the other synthetic intermediates. It was important to use only enough resin for removal of bromide ions so as to avoid losses due to attachment of the free peptide to the resin, apparently through the phenolic group of the N-terminal tyrosine residue. The solid residue obtained on removal of the methanol was dried in a vacuum desiccator over P_2O_5 and dissolved in 8 ml. of dimethylformamide. *p*-Nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate[9] (0.7 g.) was added, and the mixture was stirred at room temperature for 3 days. Ethyl acetate (100 ml.) was added, and the resulting solid was collected and washed with 150 ml. of ethyl acetate and 100 ml. of ether. The product (950 mg.) was dissolved in 5 ml. of dimethylformamide and precipitated by the addition of 100 ml. of ethyl acetate, giving 0.75 g. of protected nonapeptide, m.p. 215–217°, $[\alpha]^{20}D$ −37.5° (*c* 1, dimethylformamide). When the protected nonapeptide was subjected to chromatog-

(19) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
(20) E. D. Moffat and R. I. Lytle, *ibid.*, **31**, 926 (1959).

raphy in methanol–chloroform (55:45), a single spot was detected which traveled much faster than the diastereoisomeric protected nonapeptide intermediate used in the synthesis of oxytocin.[2]

Anal. Calcd. for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 59.0; H, 6.55; N, 12.7. Found: C, 58.5; H, 6.46; N, 12.2.

6-Hemi-D-cystine-oxytocin. The protected nonapeptide (450 mg.) was dissolved in 300 ml. of anhydrous liquid ammonia at the boiling point and treated with sodium according to the procedure described for the synthesis of oxytocin.[2] The volume of ammonia was reduced to between 15 and 20 ml. by evaporation *in vacuo;* complete removal of the latter amount was effected by lyophilization. The residue was dissolved in 200 ml. of 0.25% acetic acid, the pH of the solution was adjusted to 7.0 with 2 *N* ammonium hydroxide, and the disulfhydryl nonapeptide was oxidized by treating the solution with potassium ferricyanide (24 ml., 0.011 *N*) by dropwise addition over a 30-min. period. The ferrocyanide and ferricyanide ions were removed by passing the solution through a column of AG3X4 (2.5 × 4 cm.) ion-exchange resin in the chloride form.[12]

The eluates and washings were combined and evaporated *in vacuo* below room temperature to a volume of 15 ml. This solution was devoid of avian depressor activity. It was placed in the first eight tubes of a 6-ml., 400-tube Craig countercurrent machine and subjected to a total of 1050 transfers in the solvent system butanol–propanol–0.05% acetic acid containing 0.1% pyridine (6:1:8) at 4°. After 800 transfers, a separation into four peaks, a faster-moving major peak with a *K* of 0.47 and three smaller ones with *K* values of 0.16, 0.20 and 0.30, had been accomplished, as determined by the Folin–Lowry color reaction.[21] After 1050 transfers, the *K* value of the major peak was 0.50. The curve was in agreement with the calculated curve for a substance with a partition coefficient of 0.50. The contents of tubes 335–365 from the central portion of the major peak were combined, concentrated to a small volume in a rotary evaporator, and lyophilized, giving approximately 59 mg. of a white fluffy powder, $[\alpha]^{20}D$ −81° (*c* 0.50, 1 *N* acetic acid). A second preparation, in which 250 mg. of the protected nonapeptide was used, gave a higher yield (60 mg.) of lyophilized product with properties that agreed with those of the first preparation. For elemental analysis a sample was dried at 100° over P_2O_5 *in vacuo* for 5 hr. with a loss of weight of 7%.

Anal. Calcd. for $C_{43}H_{66}N_{12}O_{12}S_2 \cdot C_2H_4O_2$: C, 50.6; H, 6.61; N, 15.8. Found: C, 51.0; H, 6.67; N, 16.1.

Amino acid analysis of an hydrolysate of the 6-hemi-D-cystine-oxytocin was performed on a Beckman–Spinco amino acid analyzer according to the procedure of Spackman, Stein and Moore[14] using the 30–50° system. The following molar ratios (with the ratio of glycine taken as 1) were obtained: aspartic acid 1.0, glutamic acid 1.0, proline 1.0, glycine 1.0, cystine 1.0, leucine 1.0, isoleucine 1.0, tyrosine 0.94, and ammonia 3.0. 6-Hemi-D-cystine-oxytocin traveled somewhat faster than oxytocin when they were subjected to thin layer chromatography. Oxytocin (100 μg.) and 6-hemi-D-cystine-oxytocin (100 μg.) as well as a mixture of 100 μg. of each of the two peptides were applied separately on a thin layer (250 μ) of silica gel G mounted on a glass plate (20 × 20 cm.) and chromatographed for 5.5 hr. at room temperature with the solvent system butanol–acetic acid–water (4:1:5, ascending). The R_f values of the spots obtained after development with the platinum reagent[19] were 0.30 for oxytocin and 0.33 for 6-hemi-D-cystine oxytocin. The mixture of the two peptides had been separated into two distinct spots that agreed with these R_f values. The two peptides were subjected to descending chromatography on Whatman No. 1 paper in butanol–acetic acid–water (4:1:5) over a 14-hr. period. The chromatogram was stained with the bromphenol blue–mercuric chloride reagent.[22] The sample of 6-hemi-D-cystine-oxytocin traveled as a single spot (R_f 0.69). Oxytocin (R_f 0.64) travels slightly slower under the same conditions. A mixture of the two peptides could not be separated by this means in contrast to thin layer chromatography. The 6-hemi-D-cystine-oxytocin was assayed against the U.S.P. posterior pituitary reference standard for certain pharmacological activities exhibited by oxytocin. This diastereoisomer of oxytocin showed no detectable avian depressor activity[23] and, in fact, the compound appeared to exert a weak inhibitory effect on the avian depressor response to the standard powder. The oxytocic activity[24] was extremely low, approximately 0.62 unit/mg. The milk-ejecting,[25] pressor[26] and antidiuretic[27] activities were practically nil, in each case less than 0.002 unit/mg. 6-Hemi-D-cystine-oxytocin was found to possess some degree of antipressor activity in the rat. When 0.1 mg. of the analog was injected simultaneously with 2–5 milliunits of U.S.P. reference standard the pressor response of the standard was greatly inhibited.

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