4-Deamido-oxytocin, an Analog of the Hormone Containing Glutamic Acid in Place of Glutamine[1]

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4-Deamido-oxytocin, an analog of oxytocin in which a glutamic acid residue replaces the glutamine residue in the 4-position, has been synthesized and tested for the pharmacological activities characteristic of the hormone. The last step in the synthesis of the analog, involving reduction of a protected nonapeptide intermediate with sodium in liquid ammonia followed by oxidation to the required octapeptide amide, was accomplished from both the carbobenzoxy nonapeptide VI and the tosyl nonapeptide V. Compound VI was prepared by coupling N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine azide with L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, and the corresponding tosyl nonapeptide V was obtained by coupling the appropriate protected tripeptide azide with the hexapeptide. The required hexapeptide and heptapeptide were prepared from the corresponding N-o-nitrophenylsulfenyl peptides III and VII. The 4-deamido-oxytocin (4-glutamic acid-oxytocin, I) was purified by countercurrent distribution and by partition chromatography on Sephadex. The analog possesses approximately 0.5 unit per mg. of avian depressor activity, 1.5 units per mg. of oxytocic activity, 11 units per mg. of milk-ejecting activity, and negligible pressor and antidiuretic activities. The characteristic pharmacological activities of oxytocin are thus drastically lowered by replacement of the carboxamide group in the 4-position by a carboxyl group. Furthermore, the ratio of oxytocic activity to avian depressor activity in this analog differs markedly from that in oxytocin.

As part of a systematic study in this laboratory of the significance of the chemical functional groups of oxytocin to its pharmacological properties, the role of the carboxamide groups of the glutamine and asparagine residues in positions 4 and 5 of the peptide ring of oxytocin has been investigated by the synthesis and study of various analogs differing structurally from the hormone in these positions. Substitution of the glutamine residue by an isoglutamine residue[3] or of the asparagine by an isoasparagine[4] resulted simultaneously in changes in ring size as well as in the proximity of the carboxamide group to the ring. It was found that these isoglutamine and isoasparagine analogs of oxytocin were practically devoid of avian depressor and oxytocic activities which are characteristic of the hormone.[3-5] Furthermore, the studies of Boissonnas and

co-workers showed that shortening of the side chain of the glutamine residue in position 4 of oxytocin by one methylene unit resulted in an analog (4-asparagine-oxytocin)[6] with a fairly high degree of these biological activities, whereas lengthening of the side chain of the asparagine residue in position 5 by one methylene unit gave an analog (5-glutamine-oxytocin) with extremely low biological activities.[6-8]

The effect of the absence of the carboxamide groups in the 4- and 5-positions of the hormone on its activities was assessed recently by synthesis of 4-decarboxamido-oxytocin (4-α-aminobutyric acid-oxytocin)[9] and 5-decarboxamido-oxytocin (5-alanine-oxytocin),[9,10] analogs in which the carboxamide groups of the glutamine and asparagine residues at the 4- and 5-positions, respectively, were replaced by hydrogen. The 5-decarboxamido-oxytocin was found to be practically inactive, but the 4-decarboxamido-oxytocin possessed a fairly high degree of the pharmacological activities characteristic of the hormone. We then became interested in determining whether an analog bearing a carboxyl group in place of the carboxamide group in the 4-position would also possess pharmacological activity.

The structure of this 4-deamido analog of oxytocin (4-L-glutamic acid-oxytocin, I) is shown in Figure 1. Synthesis of 4-deamido-oxytocin was accomplished according to the series of reactions indicated in the diagram in Figure 2. The protected pentapeptide N-carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II)[11,12] was employed as starting material. The carbobenzoxy group was removed by means of hydrogen bromide in glacial acetic acid and the free base was obtained from the resulting hydrobromide by treatment with Dowex IRA-410 (OH form) resin. Coupling of the pentapeptide with the p-nitrophenyl ester[13a] of γ-benzyl N-carbobenzoxy-L-glutamate,[13b] prepared by the dicyclohexylcarbodiimide method for making p-nitrophenyl

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esters,[11,14] led to N-carbobenzoxy-γ-O-benzyl-L-glu-tamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. However, difficulty was encountered in an attempt to prepare the free hexapeptide by use of the procedure already described for preparation of the pentapeptide, since some cleavage of the γ-benzyl ester group, as well as other side reactions, appeared to be occurring at the same time. The synthesis of the hexapeptide was accomplished by use of another N-terminal protecting group, the o-nitrophenylsulfenyl group recently introduced by Zervas, et al.[15,16] The removal of this group takes place under extremely mild conditions with 2–3 equiv. of hydrogen chloride in methanol or nonpolar solvents at room temperature within a few minutes.[15] Crystalline protected hexapeptide III was prepared by coupling L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide with γ-benzyl N-o-nitrophenylsulfenyl-L-glutamate[15] by the mixed anhydride[17] or the carbodiimide method[18] and also by coupling the pentapeptide with the p-nitrophenyl ester of γ-benzyl N-o-nitrophenylsulfenyl-L-glutamate. The protecting group was removed with hydrogen chloride in methanol and the resulting hexapeptide hydrochloride IV was converted to the free hexapeptide by treatment with IRA-410 resin (OH form).

The peptide chain was lengthened from 6 to 9 amino acid residues by coupling a protected tripeptide with the hexapeptide by the azide method which avoids racemization.[19] N-Tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine hydrazide[20] was converted to the corresponding azide[21] and coupled with the free hexapeptide to obtain the N-tosyl nonapeptide V. The corresponding N-carbobenzoxy nonapeptide VI was prepared by coupling N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine azide with L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, obtained from the hydrochloride by treatment with triethylamine. The preparation of the dipeptide azide from the corresponding hydrazide[22] was accomplished according to the modified procedure described by Honzl and Rudinger in a one-phase system.[23] The heptapeptide hydrochloride was obtained from N-o-nitrophenylsulfenyl-L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VII) by removal of the N-protecting group with hydrogen chloride in methanol. Compound VII was prepared by coupling N-o-nitrophenylsulfenyl-L-isoleucine[15] with the free hexapeptide

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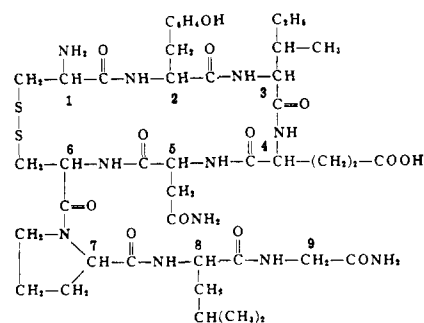

Figure 1. Structure of 4-deamido-oxytocin (I) with numbers indicating the position of the individual amino acid residues.

by the dicyclohexylcarbodiimide method. A somewhat less pure preparation of the N-carbobenzoxy nonapeptide VI was obtained by coupling N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine azide[24] with the hexapeptide.

Jaquenoud and Boissonnas have used the p-nitrophenyl ester of N-tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine in a coupling reaction with a hexapeptide.[25] The corresponding N-carbobenzoxy tripeptide p-nitrophenyl ester has been prepared in this laboratory by Ferraro according to the procedure used for the tosyl tripeptide. This nitrophenyl ester was coupled with the free hexapeptide in the hope of obtaining the protected nonapeptide VI. However, when the coupling product was subjected to amino acid analysis after hydrolysis with 6 N HCl it was found that 25% of the isoleucine was present as alloisoleucine. Hydrolysis of the starting carbobenzoxy tripeptide p-nitrophenyl ester led to only traces of alloisoleucine. It is assumed that during the coupling process racemization of the α-carbon atom of L-isoleucine led to formation of D-alloisoleucine. Recently Nesvadba, Honzl, and Rudinger[26] reported a similar case of racemization during the synthesis of 3-alloisoleucine-oxytocin. When they coupled N-tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-alloisoleucine with L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide by the carbodiimide method and converted the product to the oxytocin analog they found that 30% of the alloisoleucine was present as isoleucine.

4-Deamido-oxytocin (I) was obtained by removal of the protecting groups from the protected nonapeptides with sodium in liquid ammonia according to the method of Sifferd and du Vigneaud as used in the synthesis of oxytocin,[27] and by oxidation of the reduced material at pH 6.5–6.8 by aeration followed by addition of potassium ferricyanide solution (0.02 N).[28] The oxidized solutions were concentrated to a volume of approximately 50 ml. and subjected to countercurrent distribution.[29] The 4-deamido-oxytocin was fur-

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Z—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)
               |
               Bz

               II

               ↓ 1. HBr–HOAc, resin (OH)
                 2. NPS-Glu(OBz)-OC₆H₄NO₂

NPS—Glu(OBz)—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)
                        |
                        Bz

                        III

                        ↓ HCl–MeOH

——————————————HCl·Glu(OBz)—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)——
        | | | 1. resin (OH)
        | Bz | 2. NPS-Ileu + DCCI
        | IV
        | NPS—Ileu—Glu(OBz)—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)
   1. resin (OH) |
   2. Tos—CyS—Tyr—Ileu—N₃   Bz
        |
        Bz   VII

                                                                           ↓ 1. HCl–MeOH
                                                                             2. N(Et)₃ + Z—CyS—Tyr—N₃
                                                                                            |
                                                                                            Bz

Tos—CyS—Tyr—Ileu—Glu(OBz)—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)   Z—CyS—Tyr—Ileu—Glu(OBz)—
    | | |
    Bz   Bz   Bz

                                                                  Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)
                                                                           |
                                                                           Bz

        V   VI

   1. Na–liq. NH₃   1. Na–liq. NH₃
   2. oxidation   2. oxidation

                        ↓

Z = carbobenzoxy   CyS—Tyr—Ileu—Glu—Asp(NH₂)—CyS—Pro—Leu—Gly(NH₂)
Bz = benzyl
NPS = o-Nitrophenylsulfenyl   4-deamido-oxytocin (4-glutamic acid-oxytocin)
Tos = tosyl   I

Figure 2. Series of reactions for synthesis of 4-deamido-oxytocin.

ther purified by partition chromatography on Sephadex according to the procedure of Yamashiro.[30] Chromatograms of deamido-oxytocin derived from either the carbobenzoxy nonapeptide or the tosyl nonapeptide showed only one main peak containing the analog and very small amounts of impurities were indicated.

During the purification of the 4-deamido-oxytocin by both countercurrent distribution and partition chromatography on Sephadex, no peak in the position characteristic of oxytocin was observed. The purity and homogeneity of the analog were established by elemental analysis, amino acid analysis, paper chromatography, and paper electrophoresis. Furthermore, a titration curve revealed the presence of the expected one carboxyl, one amino, and one phenolic hydroxyl group.

4-Deamido-oxytocin possesses an avian depressor activity[31] of approximately 0.5 unit per mg. and an oxytocic activity[32] of approximately 1.5 units per mg. Two purified samples, one from the carbobenzoxy nonapeptide and the other from the tosyl nonapeptide, exhibited the same activities. The 4-deamido-oxytocin

also possesses a milk-ejecting activity[33] of approximately 11 units per mg. and negligible pressor[34] and antidiuretic[35] activities. The difference between the ratio of oxytocic to avian depressor activity in this analog (3:1) and that in oxytocin (1:1)[36] provides further evidence that the activities observed for the deamido analog are inherent activities and not due to contamination with oxytocin.

Experimental[37]

γ-*Benzyl N-Carbobenzoxy*-L-*glutamate p-Nitrophenyl Ester.* γ-Benzyl N-carbobenzoxy-L-glutam-

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ate[13b] (3.7 g.) was dissolved in 25 ml. of ethyl acetate. *p*-Nitrophenol (1.7 g.) was added and the solution was cooled to 0° before addition of 2.1 g. of dicyclohexylcarbodiimide. The mixture was allowed to stand for 30 min. at 0° and then for 2 hr. at room temperature. The N,N'-dicyclohexylurea was filtered off and washed with ethyl acetate, and the filtrate was concentrated to dryness *in vacuo*. The residue was crystallized from 20 ml. of 95% ethanol, giving 3.5 g., m.p. 111–111.5°, $[\alpha]^{19}$D −19.5° (*c* 3.2, ethyl acetate); lit.[13a] m.p. 111°, $[\alpha]^{25}$D −20.4° (*c* 3.2, ethyl acetate).

N-Carbobenzoxy-γ-O-benzyl-L-*glutamyl*-L-*asparaginyl-S-benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycinamide.* N-Carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II, 5.4 g.) was dissolved in 19 ml. of anhydrous acetic acid and treated with 19 ml. of hydrogen bromide in glacial acetic acid (33% w./w.). After 2 hr. at room temperature the solution was poured onto 140 ml. of cold, dry ether. The supernatant liquor was decanted and the hydrobromide was washed twice with 140-ml. portions of cold ether, each washing being followed by decantation. It was then filtered off, washed with ether, and dried *in vacuo* over sodium hydroxide and calcium chloride. The hydrobromide was dissolved in 100 ml. of methanol and passed through a column of Dowex IRA-410 (OH form) ion-exchange resin. The column was washed twice with 30-ml. portions of methanol. The eluate and washings were combined and evaporated *in vacuo* to a white solid which was dried for 2 hr. under high vacuum over phosphorus pentoxide. The free pentapeptide was dissolved in 19 ml. of dimethylformamide, γ-benzyl N-carbobenzoxy-L-glutamate *p*-nitrophenyl ester (4 g.) was added, and the solution was allowed to stand at room temperature overnight. Ethyl acetate (180 ml.) was added slowly under stirring and after 3 hr. at room temperature the precipitate was filtered off, washed with ethyl acetate and ethanol, and dried *in vacuo* over phosphorus pentoxide giving 6.3 g., m.p. 221–222° dec. After trituration with ethyl acetate, 6.2 g. of the protected heptapeptide was recovered, m.p. 222–223° dec., $[\alpha]^{19}$D −48.5° (*c* 1, dimethylformamide).

For analysis a sample (0.5 g.) was purified by precipitation from 80% ethanol, giving 0.42 g., m.p. 222–223° dec., $[\alpha]^{19}$D −49.1° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{47}H_{60}N_8O_{11}S$: C, 59.7; H, 6.39; N, 11.9. Found: C, 59.8; H, 6.51; N, 11.9.

N-o-Nitrophenylsulfenyl-γ-O-benzyl-L-*glutamyl*-L-*asparaginyl-S-benzyl*-L-*cysteinyl*-L-*prolyl* - L - *leucylglycinamide (III).* A. *By Mixed Anhydride Method.* γ-Benzyl N-*o*-nitrophenylsulfenyl-L-glutamate, prepared from 1.5 g. of its dicyclohexylammonium salt,[15] was dissolved in 20 ml. of dimethylformamide and cooled to −20°. Triethylamine (0.38 ml.), peroxide-free tetrahydrofuran (15 ml.), and isobutyl chloroformate (0.34 ml.) were added and the mixture was allowed to stand at −5° for 15 min. The solution was cooled to −20° and added to L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide prepared from the corresponding N-carbobenzoxy pentapeptide (II, 1.8 g.) as described in the preceding section. The solid was dissolved with stirring and the solution was allowed to warm to room temperature and stand for 2 hr. It was poured onto 100 ml. of cold water under stirring.

The crude product (2 g.) was recrystallized from 30 ml. of 80% methanol, giving 1.5 g., m.p. 182–184°. After a second recrystallization the melting point was 185–187°, $[\alpha]^{17}$D −50.8° (*c* 1, dimethylformamide). This substance was obtained in two crystalline forms (see C).

Anal. Calcd. for $C_{45}H_{57}N_9O_{11}S_2$: C, 56.1; H, 5.96; N, 13.1. Found: C, 56.1; H, 5.91; N, 12.9.

B. By Carbodiimide Method. γ-Benzyl N-*o*-nitrophenylsulfenyl-L-glutamate, prepared from 1.6 g. of its dicyclohexylammonium salt,[15] was dissolved in 5 ml. of dimethylformamide, cooled to −10°, and added to a precooled solution (5 ml.) of free pentapeptide prepared from 1.8 g. of II. Dicyclohexylcarbodiimide (0.55 g.) was added and the mixture was allowed to stand at −15° overnight and at room temperature for 1.5 hr. The N,N'-dicyclohexylurea was filtered off and washed with cold dimethylformamide. A few drops of acetic acid and 150 ml. of water were added to the filtrate slowly under cooling and stirring. The product was filtered off, dried, and triturated with 40 ml. of ethanol giving 1.6 g. After recrystallization from 36 ml. of 80% methanol, 1.5 g. of protected hexapeptide, m.p. 185–187°, $[\alpha]^{17}$D −50.0° (*c* 1, dimethylformamide), was obtained.

C. By Nitrophenyl Ester Method. L-Asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide obtained from 3.6 g. of II was dissolved in 15 ml. of dimethylformamide and γ-benzyl N-*o*-nitrophenylsulfenyl-L-glutamate *p*-nitrophenyl ester (2.7 g.) was added. The yellow solution was allowed to stand at room temperature overnight. Ethyl acetate (120 ml.) was added and after 4 hr. the precipitate was filtered off, washed with ethyl acetate, dried, triturated with ethanol (30 ml.), separated, and dried *in vacuo* giving 4.2 g., m.p. 189°. When this product was treated with boiling 80% methanol and the suspension was cooled, 3.9 g. of crystalline substance, m.p. 201–202°, was obtained. After recrystallization from methanol containing a few drops of water, the protected hexapeptide melted at 201–203°, $[\alpha]^{17}$D −50.9° (*c* 1, dimethylformamide).

Anal. Found: C, 55.8; H, 6.07; N, 13.0; S, 6.64 (Calcd. 6.65).

This higher melting form of the protected hexapeptide is less soluble in methanol than the form, m.p. 185–187°, described in the preceding sections. The two forms can be interconverted by crystallizing each form from 80% methanol and seeding with the other form. In a subsequent preparation of the protected hexapeptide by the *p*-nitrophenyl ester method, the lower melting form was obtained.

γ-O-Benzyl-L-*glutamyl*-L-*asparaginyl-S-benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycinamide Hydrochloride (IV).* The protected hexapeptide (III, 5.3 g.) was ground to a fine powder and suspended in 90 ml. of methanol before addition of 16 ml. of methanolic hydrogen chloride (approximately 1.2 *N*) with stirring. After 5 min. the solution was evaporated to half-volume *in vacuo* at 30–35° and peroxide-free ether was added. The ether was decanted and the residue was dissolved in 30 ml. of methanol and reprecipitated with ether. This procedure was repeated twice. After being allowed to stand in the refrigerator overnight, the hydrochloride was filtered off, washed with ether, and dried, giving 4.2 g. The hydrochloride was again dissolved in

methanol and reprecipitated with ether; $[\alpha]^{20}$D $-68.9°$ (*c* 1, methanol). On paper electrophoresis in pyridine acetate buffer at pH 4 the compound appeared as a single band.

Anal. Calcd. for $C_{39}H_{54}N_8O_9S \cdot HCl$: C, 55.3; H, 6.54; N, 13.2; Cl, 4.18. Found: C, 54.8; H, 6.65; N, 13.3; Cl, 4.38.

N-o-Nitrophenylsulfenyl-L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VII). Compound IV (2.3 g.) was dissolved in 30 ml. of methanol and passed through a column of Dowex IRA-410 (OH form) resin. The column was washed twice with 10 ml. of methanol. The eluate and washings were combined and evaporated to dryness *in vacuo* at 30–35° and the residue was dissolved in 10 ml. of dimethylformamide and precipitated by the slow addition of 100 ml. of ether. After being allowed to stand for several hours first at room temperature and then in the refrigerator the free hexapeptide was filtered off and washed with ether; wt. 2 g. For purification the compound was again precipitated from dimethylformamide with ether. Amino acid analysis of an hydrolysate of the hexapeptide by the method of Spackman, Stein, and Moore[38] showed the expected amino acids to be present in molar ratios to each other of approximately 1:1 and ammonia to occur in a molar ratio to any one amino acid of 1.8:1. N-o-Nitrophenylsulfenyl-L-isoleucine, prepared from 0.77 g. of its dicyclohexylammonium salt,[15] was dissolved in 5 ml. of dimethylformamide and added to 1.2 g. of the hexapeptide. The solution was cooled to $-20°$ and 0.33 g. of dicyclohexylcarbodiimide was added. The mixture was allowed to stand at room temperature overnight and the N,N'-dicyclohexylurea was separated by filtration. Cold water (100 ml.) was slowly added to the filtrate under stirring. The precipitate was filtered off, dried *in vacuo*, and triturated for 5 min. with 20 ml. of boiling methanol containing a few drops of water. After being cooled the product was filtered off and washed on the filter with methanol and then with ether. After repetition of the trituration, 0.9 g. of protected heptapeptide, m.p. 219–221°, $[\alpha]^{19}$D $-68.7°$ (*c* 1, dimethylformamide), was obtained.

Anal. Calcd. for $C_{51}H_{68}N_{10}O_{12}S_2$: C, 56.9; H, 6.36; N, 13.0. Found: C, 56.7; H, 6.48; N, 13.0.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI). The protected heptapeptide (VII, 0.8 g.) was finely ground and suspended in 8 ml. of methanol. Methanolic hydrogen chloride (approximately 1.2 *N*, 2 ml.) was added under stirring. The solid dissolved within 3 min. and the solution was immediately added to 70 ml. of dry, peroxide-free ether. The hydrochloride precipitated as a voluminous, gelatinous mass, which was immediately filtered off and washed with ether giving 0.48 g., m.p. 238° dec. On paper electrophoresis in pyridine acetate buffer, pH 4, the compound moved as a single band toward the cathode. Amino acid analysis[38] of an hydrolysate of the heptapeptide showed the expected amino acids to be present in molar ratios to each other of approximately 1:1

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

and ammonia to occur in a molar ratio to any one amino acid of 2.2:1.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine hydrazide (0.21 g.) was dissolved in 2.5 ml. of 4.5 *N* hydrochloric acid in 95% tetrahydrofuran and treated at $-20°$ under stirring with 0.25 ml. of a cold aqueous solution of sodium nitrite (18%). The mixture was stirred for 10 min. at $-20°$, diluted with cold ethyl acetate, washed three times with a saturated solution of sodium bicarbonate containing a small amount of sodium chloride and once with water, and dried over sodium sulfate. It was then added at $-20°$ to a mixture of 0.4 g. of the heptapeptide hydrochloride and 0.1 ml. of triethylamine in 12 ml. of dimethylformamide. The mixture was stirred at 4° for 20 hr. and then for 2 hr. at room temperature. Ethyl acetate (100 ml.) was added, and the mixture was cooled. The product was filtered off, washed on the filter with ethyl acetate, dried, and triturated briefly with boiling methanol, giving 0.35 g., m.p. 232–236°. It was then triturated with boiling ethyl acetate, filtered off, dried, dissolved in 3 ml. of warm dimethylformamide, and precipitated by addition of ether. The carbobenzoxy nonapeptide melted at 235–237°, $[\alpha]^{20}$D $-46.6°$ (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{72}H_{91}N_{11}O_{15}S_2$: C, 61.1; H, 6.48; N, 10.9. Found: C, 61.2; H, 6.80; N, 11.1.

N-Tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (V). N-Tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine azide[21] (0.4 g.) was added to a solution of 0.4 g. of γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in 5 ml. of dimethylformamide which had been cooled to 4°. The mixture was stirred for 16 hr. at 4° and then for 6 hr. at room temperature. Ethyl acetate (40 ml.) was added and the mixture was left at room temperature overnight. The product was then filtered off, washed with ethyl acetate, dried, and triturated with methanol giving 0.54 g., m.p. 228–232°. The tosyl nonapeptide was dissolved in 5 ml. of warm dimethylformamide and precipitated by addition of 30 ml. of ether giving 0.4 g., m.p. 228–232°, $[\alpha]^{28}$D $-27.9°$ (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{71}H_{91}N_{11}O_{15}S_3$: C, 59.4; H, 6.39; N, 10.7; S, 6.70. Found: C, 59.0; H, 6.34; N, 10.7; S, 6.63.

4-Deamido-oxytocin (4-L-Glutamic Acid-oxytocin) (I). A. From the Carbobenzoxy Nonapeptide. N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-γ-O-benzyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI, 300 mg.) was dissolved in 300 ml. of liquid ammonia (freshly distilled from sodium) and treated at the boiling point with sodium until a faint blue color enveloped the solution. One drop of glacial acetic acid was added and the ammonia was evaporated *in vacuo*, the last 50 ml. being removed from the frozen state. The fluffy residue was dissolved in 300 ml. of 0.1% acetic acid. After adjustment of the pH to approximately 6.8 the solution was aerated with carbon dioxide-free air for 1.5 hr. and then 2 ml. of 0.02 *N* potassium ferricyanide solution was added to ensure complete oxidation. Ferrocyanide and ferricyanide ions were removed by passing the solution down a column of AG

3X4 resin in the chloride form. The solution was concentrated in a flash evaporator to a volume of 50 ml., placed in the first five tubes of a Craig countercurrent distribution machine, and subjected to a total of 380 transfers in the solvent system 1-butanol-1-propanol-0.5% acetic acid containing 0.1% pyridine (6:1:8).[39] After 380 transfers determination of the Folin–Lowry color values[40] indicated a main peak between tubes 130 and 190 ($K = 0.7$). The contents of tubes 140–180 were concentrated and lyophilized to give 90 mg. of 4-deamido-oxytocin. In another experiment the distribution was carried out in the solvent system 1-butanol-1-propanol-0.05% acetic acid (2:1:3) for 500 transfers. The K of the main peak in this system was approximately 0.5.

The 4-deamido-oxytocin obtained after countercurrent distribution was also submitted to partition chromatography on Sephadex. In a typical experiment, 58 mg. of deamido-oxytocin was dissolved in 1 ml. of the upper phase of the solvent system 1-butanol-1-propanol-benzene-pyridine-0.1% acetic acid (6.5:1:0.5:1:9) and applied to a Sephadex G-25 column (2.15 × 118 cm.) which had been equilibrated with both phases. Elution with the upper phase was performed and eighty 8-ml. fractions were collected at a flow rate of approximately 20 ml. per hr. The Folin–Lowry color values showed a main peak having an R_f of 0.26, the R_f of oxytocin being 0.6 under the same conditions. The recovery of 4-deamido-oxytocin was 0.6 under the same conditions. The recovery of 4-deamido-oxytocin from the central portion of this peak was 35 mg., $[\alpha]^{19.5}D$ $-19.8°$ (c 0.05, 1 N acetic acid).

For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* and a loss in weight of 4.5% was observed.

Anal. Calcd. for $C_{43}H_{65}N_{11}O_{13}S_2$: C, 51.2; H, 6.50; N, 15.3. Found: C, 50.9; H, 6.56; N, 15.3.

A sample was hydrolyzed in 6 N hydrochloric acid at 110° and then analyzed for amino acids in the 30–50° system[38] on a Beckman Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid 1.0, proline 1.0, glycine 1.0, glutamic acid 1.0, cystine 1.05, isoleucine 0.9, leucine 1.0, tyrosine 0.9, and ammonia 2.1.

4-Deamido-oxytocin is easily separable from oxytocin both by paper chromatography in 1-butanol-acetic acid-water (4:1:5), where its R_f value is higher than that of oxytocin, and by paper electrophoresis in pyridine acetate buffers, pH 4 and 5.6, where it moves toward the cathode more slowly than oxytocin.

The titration curve of a sample of the 4-deamido-oxytocin shows three equal titrated regions corresponding to the γ-carboxy group (pK = 4.3-4.4), the α-amino group (pK = 6.5), and the phenolic hydroxyl group (pK = 9.7).

B. From the Tosyl Nonapeptide. The tosyl nonapeptide V (244 mg.) was reduced with sodium in liquid ammonia in the same manner as the carbobenzoxy nonapeptide, the only difference being that the 0.1% acetic acid solution of the reduced material was extracted four times with a total of 150 ml. of dry, peroxide-free ether before aeration. The solvent system 1-butanol-1-propanol-benzene-pyridine-0.1% acetic acid (6.5:1:0.5:1:9) was used for countercurrent distribution (600 transfers) and for partition chromatography on Sephadex. After 200 transfers one main peak ($K = 0.53$) was present as well as two smaller peaks, one having moved faster and the other slower than the main peak. After 600 transfers the contents of the main peak (tubes 186–230) was collected as two fractions. From tubes 186–210, 27.5 mg., and from tubes 211–230, 23 mg. of material, were obtained after concentration and lyophilization. Chromatograms of these fractions on Sephadex showed highly similar patterns containing one main peak. From the two fractions, 23.5 and 18 mg. of 4-deamido-oxytocin were recovered, respectively. On paper electrophoresis in pyridine acetate buffers, pH 4 and 5.6, this product showed patterns identical with those from the product described in section A.

(39) The use of solvent systems containing pyridine in the countercurrent distribution of oxytocin and related compounds was found by Dr. Derek Jarvis of this laboratory to be advantageous in avoiding emulsification and consequent time loss.
(40) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).