The Synthesis and Pharmacological Study of 4-Decarboxamidooxytocin (4-α-Aminobutyric Acid-oxytocin) and 5-Decarboxamidooxytocin (5-Alanine-oxytocin)∗

VINCENT DU VIGNEAUD, GEORGE S. DENNING, JR., STEFANIA DRABAREK,† AND W. Y. CHAN

From the Department of Biochemistry, Cornell University Medical College, New York 21, New York

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Oxytocin, the main oxytocin-milk-ejecting-avian depressor principle of the posterior pituitary gland, the structure of which is shown in Fig. 1, possesses a number of chemical functional groups: a primary amino group in position 1, a phenolic hydroxyl group in position 2, three carboxamide groups in positions 4, 5, and 9, and a disulfide linkage in the 20-membered cyclic moiety. The significance of the presence of these functional groups to the possession of biological activity of the hormone has been investigated in this and other laboratories through the synthesis and study of various analogues of oxytocin. The importance of the phenolic hydroxyl group and the primary amino group to the biological activity of oxytocin has already been examined by synthesis of analogues of the hormone lacking these groups, namely, deoxy-oxytocin (2-5), deamino-oxytocin (6, 7), and deaminooxytocin (8).1 These studies showed that the phenolic hydroxyl group in oxytocin is not essential for the production of the biological responses characteristic of the hormone, but its presence does contribute strongly to the avian depressor and oxytocic potencies of oxytocin. Deamino-oxytocin, in which the free amino group is replaced by hydrogen, has very high avian depressor and oxytocic activities in comparison with oxytocin, and exhibits an antidiuretic activity approximately 5 times that of oxytocin (6, 7). Thus the amino group of oxytocin is not required for the manifestation of biological activity. The activities of deamino-deoxy-oxytocin (8) are similar to those of deoxy oxytocin. When the disulfide linkage of oxytocin was replaced by hydrogens through the use of Raney nickel, the resulting dethiol-oxytocin was found to be devoid of avian depressor activity (9) and oxytocic activity (10).

In a continuing effort to probe the importance of the functional groups to the production of pharmacological properties of oxytocin by replacement of the functional groups by hydrogen, two analogues, 4-decarboxamidooxytocin and 5-decarboxamidooxytocin, have been synthesized in which the CONH group in position 4 or in position 5 of the ring of the hormone is substituted by a hydrogen atom. 4-Decarboxamidooxytocin (4-α-aminobutyric acid-oxytocin) was obtained by replacing the glutamine residue in the peptide ring by an L-α-aminobutyric acid residue. In 5-decarboxamidooxytocin (5-alanine-oxytocin) the asparagine residue was substituted by an L-alanine residue.

The syntheses of the two analogues were carried out by use of the stepwise p-nitrophenyl ester procedure used for the synthesis of oxytocin (11, 12). In the preparation of the 4-decarboxamidooxytocin, the protected pentapeptide, N-carbobenzyoxyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (12), served as starting material. After removal of the amino-protecting group, the free base was coupled with p-nitrophenyl N-carbonoxyl-L-α-aminobutyrate to give the protected hexapeptide. The chain was then lengthened by the stepwise p-nitrophenyl ester method to give the protected nonapeptide, N-carbobenzyoxyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-α-aminobutyryl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.

In the synthesis of 5-decarboxamidooxytocin, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide obtained from the corresponding protected tetrapeptide was allowed to react with p-nitrophenyl N-carbenzyloxyl-L-alanine to give the required protected pentapeptide. The successive attachment to this peptide of L-glutamine, L-isoleucine, L-tyrosine, and L-cysteine residues by use of the p-nitrophenyl ester method resulted in the protected nonapeptide, N-carbobenzyoxyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-alanyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.

In both syntheses from the tetrapeptide through the octapeptide stage, hydrobromic acid in glacial acetic acid was used for the removal of the carbenzyloxyl groups. The free bases were obtained from the hydrobromides by treatment with IRA-410 resin (in the OH cycle) and, in the case of the hydrobromides of the free octapeptides, by triethylamine. All the protecting groups of the protected nonapeptides were removed by treatment with sodium in liquid ammonia (13). The reduced materials so obtained were then oxidized at pH 6.5 to 7.0 by aeration followed by titration with a solution of potassium ferricyanide (6, 7). The solutions of the analogues from the oxidation steps were assayed for avian depressor activity. After concentration to about 20 ml, they were purified by countercurrent distribution in

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the solvent cyclohexanone propanol 0.05% acetic acid (2:1:3). The partition coefficient (K) for 4-decarboxamido-oxytocin was found to be approximately 0.68, and for 5-decarboxamido-oxytocin, approximately 0.53. The contents of the tubes containing the active materials were combined, concentrated, and lyophilized.

The two analogues so obtained gave the expected amino acid and elemental analyses. Amino acid analysis of acid hydrolysates of the hormone analogues was carried out according to the method of Spackman, Stein, and Moore (14) in a Beckman/Spinco amino acid analyzer. For 4-decarboxamido-oxytocin, the usual 50°C system (15) was initially employed. No separate peak appeared on the chromatogram representing the α-aminoibutyric acid under these conditions, but the cystine peak was abnormally high, representing a molar ratio of 1.89 instead of 1.0. Hence it was assumed that the peaks of cystine and α-aminoibutyric acid had coincided. When the analysis was run in the 30-50°C system, the separation of these two acids was satisfactory.

The 4-decarboxamido-oxytocin and 5-decarboxamido-oxytocin were tested for the biological activities characteristic of oxytocin. Four-point assay design was used in all the bioassays. Avian depressor assays were performed on conscious chickens according to the procedure employed by Munsiek, Sawyer, and van Dyke (16). Oxytocic assays were performed on natural estrous rat uteri according to the method of Holton (17) as modified by Munsiek (18), with the use of magnesium-free van Dyke-Hastings solution. Rat pressor assays were carried out on urethane-anesthetized male rats as described in the United States Pharmacopeia (19). Assays for antidiuretic activity were performed on male rats according to the method of Jeffers, Livezey, and Austin (20) as modified by Sawyer (21). All potency values were measured against the U.S.P. Posterior Pituitary Reference Standard.

The potencies of the 4-decarboxamido-oxytocin and 5-decarboxamido-oxytocin are presented in Table I along with those of oxytocin and the analogues so far prepared. The relative response of deamino-oxytocin to that of oxytocin has been determined at each stage of estrus (23).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Depressor (lowl)</th>
<th>Oxytocic (sat)</th>
<th>Pressor (sat)</th>
<th>Antidiuretic (sat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin</td>
<td>567 ± 15</td>
<td>486 ± 3</td>
<td>3.1 ± 0.1</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Deamino-oxytocin</td>
<td>733 ± 23</td>
<td>684 ± 32</td>
<td>1.1 ± 0.1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Deoxy-oxytocin</td>
<td>60 ± 30</td>
<td>30 ± 0.4</td>
<td>~0.6 ~0.5</td>
<td></td>
</tr>
<tr>
<td>Deamino-deoxy-oxytocin</td>
<td>68 ± 2</td>
<td>21 ± 1</td>
<td>~0.04</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>4-Decarboxamido-oxytocin</td>
<td>108 ± 5</td>
<td>72 ± 2</td>
<td>~0.1</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>5-Deoxy-oxytocin</td>
<td>None</td>
<td>0.2-0.3</td>
<td>None</td>
<td>0.002</td>
</tr>
</tbody>
</table>

All melting points are corrected, capillary melting points.

EXPERIMENTAL PROCEDURE

N-Carbobenzyox-L-α-aminoibutyric acid—Carbobenzylox chloride, 10.8 g, was added dropwise to a solution of 5 g of L-α-aminoibutyric acid in 4 N sodium hydroxide. The mixture was then extracted with ether, and the water layer after acidification with concentrated HCl to pH 2 was again extracted with ether. The ether solution was evaporated and the residual colorless oil was dissolved in benzene-acetone (1:1). After removal of the solvents, the oily residue crystallized on being allowed to stand. The product recrystallized from ether-hexane (1:1) weighed 2.2 g, m.p. 79-80°C, [α]D -11.1° (c, 1 in glacial acetic acid), [α]D 12.9 -10.5° (c, 1 in absolute ethanol), and [α]D 13.4 -9.8° (c, 1.2 in 95% ethanol); recorded value (24), [α]D 13.5 22° (c, 2.8 in ethanol).

That the difference between the values for optical rotation obtained by us and by Waley (24) was not due to racemization under the conditions used for the preparation was shown by removal of the protecting group from the N-carbobenzyox-L-α-aminoibutyric acid by catalytic hydrogenation. The resulting α-aminoibutyric acid had a rotation of [α]D 20 +8.7° (c, 2 in water) and [α]D 30 +20.6° (c, 1 in 5 N HCl); recorded values (35), [α]D 20 +21.2° (c, 1 in 5 N HCl).

\* All melting points are corrected, capillary melting points.

\* These values are those recently obtained in this laboratory for a highly purified sample of synthetic oxytocin (22).

\* These values are the mean values from assays on uteri from a large number of rats taken at random without regard to the stage of the estrus cycle. The relative response of deamino-oxytocin to that of oxytocin has been determined at each stage of estrus (23).

\* See (3-5).

\* See (10).

\p N-Nitrophenyl N-Carbobenzyox-L-α-aminoibutyrate—Three grams of N-carbobenzyox-L-α-aminoibutyric acid and 2.1 g of p-nitrophenol were dissolved in 45 ml of ethyl acetate, and the solution was cooled to 0°C and stirred while 2.61 g of dicyclohexylurea began to separate out immediately. The stirring was continued for 3½ hours at 0°C. Five drops of glacial acetic acid were added and the urea was filtered off and washed with 12.5 ml of ethyl acetate. The filtrate and washing were combined and concentrated. The oily residue with some crystals present was taken up in 10 ml of absolute ethanol, filtered to remove additional urea, and diluted with 50 ml of hexane.

\p N,N'-Dicyclohexylurea began to separate out immediately. The stirring was continued for 3½ hours at 0°C. Five drops of glacial acetic acid were added and the urea was filtered off and washed with 12.5 ml of ethyl acetate. The filtrate and washing were combined and concentrated. The oily residue with some crystals present was taken up in 10 ml of absolute ethanol, filtered to remove additional urea, and diluted with 50 ml of hexane. On being allowed to stand at -15°C, the product separated in crystalline form. Recrystallization from the same solvent system (33% ethanol-hexane) gave 2.3 g of a product with a melting point of 60.5-61.5°C and [α]D 20 -34.9° (c, 2 in dimethylformamide).
N-Carboxyanhydroy-L-α-amino butyryl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanyl-Leucylglycinamide—Finely powdered N-carboxyanhydroy-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanyl-Leucylglycinamide, 5.5 g, was dissolved in 22 ml of glacial acetic acid and treated with 22 ml of HBr in glacial acetic acid (32% weight for weight). After 2 hours at room temperature, the solution was poured into 400 ml of cold, dry ether. When the hydrobromide of the free pentapeptide had settled, the supernatant liquid was decanted and the precipitate was washed with three 150-ml portions of ether, each washing being followed by decantation.

After being dried in a vacuum over potassium hydroxide and calcium chloride for 3 hours, the hydrobromide was dissolved in 80 ml of dry methanol and passed through a column of ion exchange IRA-410 resin (in the OH cycle). The column was washed with 100 ml of ethanol, 100 ml of ethyl acetate, and again with 100 ml of ethyl acetate. The substance was dried to constant weight over P2O5 in a vacuum; weight, 5.33 g, m.p. 243-245°, [α]D -52.1° (c, 1 in dimethylformamide).

After being dried in a vacuum over P2O5, the product weighed 2.3 g and had a melting point of 238°-239°, [α]D 18° -56.1° (c, 1 in dimethylformamide).

\[
\text{C}_{13}\text{H}_{16}\text{O}_{4}\text{N}_8 \quad \text{Calculated: C 61.9, H 6.91, N 11.8} \\
\text{Found: C 61.9, H 6.91, N 11.8}
\]

4-Decarboxamido-oxytocin (4-L-α-aminobutyric acid-oxytocin)—A suspension of 1.75 g of the preceding protected octapeptide in 11.5 ml of acetic acid was treated with 11.5 ml of HBr in glacial acetic acid (32%). After 2 hours at room temperature, the hydrobromide of the free peptide was isolated in the usual manner and dried. Then it was dissolved in 15 ml of dimethylformamide. The solution was cooled to 0° and neutralized with triethylamine. The hydrobromide of triethylamine was removed by filtration and washed with dimethylformamide. P-Nitrophenyl N-carboxyanhydroy-S-benzyl-L-cysteinyl, 0.7 g, was added to the filtrate and the mixture was stirred for 4 days. No solid appeared after that time. Ethyl acetate was added and the resulting precipitate was collected and washed with 50 ml of ethyl acetate and 50 ml of ethyl acetate; weight, 1.8 g, m.p. 241-243°, [α]D 18° -52.8° (c, 1 in dimethylformamide).

C15H26O5N8S
Calculated: C 62.2, H 6.85, N 11.9
Found: C 61.9, H 6.91, N 11.8

4-Decarboxamido-oxytocin (4-L-α-aminobutyric acid-oxytocin) —A suspension of 1.75 g of the preceding protected octapeptide in 11.5 ml of acetic acid was treated with 11.5 ml of HBr in glacial acetic acid (32%). After 2 hours at room temperature, the hydrobromide of the free peptide was isolated in the usual manner and dried. Then it was dissolved in 15 ml of dimethylformamide. The solution was cooled to 0° and neutralized with triethylamine. The hydrobromide of triethylamine was removed by filtration and washed with dimethylformamide. P-Nitrophenyl N-carboxyanhydroy-S-benzyl-L-cysteinyl, 0.7 g, was added to the filtrate and the mixture was stirred for 4 days. No solid appeared after that time. Ethyl acetate was added and the resulting precipitate was collected and washed with 50 ml of ethyl acetate and 50 ml of ethyl acetate; weight, 1.8 g, m.p. 241-243°, [α]D 18° -52.8° (c, 1 in dimethylformamide).

C15H26O5N8S
Calculated: C 62.2, H 6.85, N 11.9
Found: C 61.9, H 6.91, N 11.8

Two hundred milligrams of protected nonapeptide were dissolved in 300 ml of anhydrous liquid ammonia. The solution was allowed to warm to its boiling point, and sodium was introduced by dipping a small bore glass tube containing sodium below the surface of the solution until a blue color appeared throughout (13). After 30 seconds, 3 drops of glacial acetic acid were added to remove the color and the ammonia was evaporated in a vacuum, the last 50 ml be removed from the frozen state. The fluffy residue was dissolved in 200 ml of 0.1% acetic acid. After adjustment of the pH to 6.8, the solution was aerated with CO2-free air for 12 hours. The oxidation was completed by the addition of potassium ferricyanide and ferricyanide ions were removed from the solution by means of AG3-X4 resin in the chloride form. This solution was found to contain a total of approximately 8800 units of avian depressor activity. It was concentrated in a flash evaporator to a volume of approximately 10 ml, placed in the first 2 tubes of a 10-ml 200-tube countercurrent machine, and subjected to a total of 350 transfers in the solvent system butanol-propanol-0.05% acetic acid (2:1:3). After 200 transfers, a separation into a main peak with a K value of 0.63 and two very small more slowly moving peaks had been accomplished, as detected by the Folin-Lowry color reaction (26). After 350 transfers, the distribution pattern remained the same, the main peak with K

\[
\text{C}_{15}\text{H}_{26}\text{O}_{5}\text{N}_8 \quad \text{Calculated: C 61.9, H 6.91, N 11.8} \\
\text{Found: C 61.9, H 6.91, N 11.8}
\]
p-Nitrophenyl N-Carbobenzyo-L-alanine—N-Carbobenzyo-L-alanine, 9.15 g, was dissolved in 140 ml of ethyl acetate and the solution was cooled. p-Nitrophenol, 6.85 g, and 8.45 g of dolycylohexylcarbodiimide were added and the mixture was stirred at 0°C for 30 minutes and then at room temperature for another 1.5 hours. After addition of 1 ml of glacial acetic acid, the N,N'-dicyclohexylurea was filtered off. On evaporation of the solvent a yellow oil remained. It was dissolved in hot ethanol, 70 ml, and allowed to crystallize. After recrystallization from the same solvent the product weighed 7.6 g, m.p. 78-79°C, [α]_D^28 = -42.0° (c, 2 in dimethylformamide). The values reported for the compound obtained by using tris-(p-nitrophenoxy)phosphine in pyridine (27) are: m.p. 79-79.5°C, [α]_D^28 = -88.1° (c, 1 in dimethylformamide).

C_{17}H_{20}O_4N_2

Calculated: C 58.5, H 7.09, N 13.6
Found: C 58.3, H 7.20, N 13.7

N-Carbobenzyo-L-alany-L-S-benzyl-L-cysteiny-L-proly-L-leu-cyclglycinamide—N-Carbobenzyo-S-benzyl-L-cysteiny-L-proly-L-leucylglycinamide, 6.11 g, was dissolved in 25 ml of warm acetic acid, cooled, and treated with 25 ml of HBr in glacial acetic acid (20% weight for weight) in the usual manner. The hydrobromide of the free base was precipitated with dry ether, dried, and dissolved in 75 ml of methanol. The solution was passed through a column of IRA-410 resin in the OH cycle. Removal of the solvent from the eluate left a crystalline residue which was taken up in 15 ml of dimethylformamide and coupled with 3.78 g of p-nitrophenyl N-carbobenzyo-L-alanine. The reaction was allowed to proceed for 18 hours and then 200 ml of ethyl acetate were added. No precipitate was formed, so the solution was evaporated and the residue was treated with 120 ml of ethyl acetate. On being allowed to stand, the product precipitated. After being washed twice with 20-ml portions of ethyl acetate, the protected pentapeptide was dried in a vacuum over P_2O_5; weight 6.08 g, m.p. 160-161.5°C.

For analysis, 0.5 g of the compound was recrystallized from 75 ml of boiling ethyl acetate to give 0.45 g of crystalline product, m.p. 161-163°C, [α]_D^28 = -59.3° (c, 1 in dimethylformamide).

C_{28}H_{40}O_8N_8S

Calculated: C 50.8, H 6.79, N 12.3
Found: C 50.8, H 6.80, N 12.4

N-Carbobenzyo-L-glutaminyl-L-alany-L-S-benzyl-L-cysteiny-L-proly-L-leucylglycinamide—The preceding protected pentapeptide, 5.49 g, was treated in 25 ml of dry acetic acid with 25 ml of HBr in glacial acetic acid (20% weight for weight), and the hydrobromide of the free base was isolated as already described. It was dissolved in 75 ml of methanol and the hydrobromic acid was removed by means of IRA-410 resin in the OH cycle. The residue which remained on evaporation of the solvent was dissolved in 15 ml of dimethylformamide followed by the addition of 3.54 g of p-nitrophenyl N-carbobenzyo-L-glutaminamide. The solution soon solidified into a hard cake. After 24 hours the mass was triturated with 175 ml of ethyl acetate; the solid was collected, washed with 100 ml of ethyl acetate and 100 ml of ethanol, and dried in a vacuum at 56°C; weight 6.0 g, m.p. 225-227°C, [α]_D^28 = -56.3° (c, 1 in dimethylformamide).

C_{39}H_{52}O_{12}N_8S

Calculated: C 57.8, H 6.71, N 13.8
Found: C 57.6, H 6.76, N 13.8

N-Carbobenzyo-L-isoleucyl-L-glutaminyl-L-alany-L-S-benzyl-L-cysteiny-L-proly-L-leucylglycinamide—The preceding protected hexapeptide, 5.40 g, was suspended in 60 ml of acetic acid, and 35 ml of HBr in acetic acid (30%) were added. After 23 hours at room temperature, 350 ml of dry ether were added. The precipitate was dissolved in 20 ml of acetic acid and treated with 20 ml of HBr in acetic acid (30%) and 2.84 g of p-nitrophenyl N-carbobenzyo-L-isoleucinate were added to the solution. After several hours at room temperature the semisolid mixture was dialyzed with 400 ml of ethyl acetate. The solid was collected, washed with 200 ml of absolute ethanol and 100 ml of ethyl acetate, and dried in a vacuum over P_2O_5 at 100°C; weight 5.45 g, m.p. 222-224°C, [α]_D^28 = 55.1° (c, 1 in dimethylformamide).

C_{26}H_{38}O_8N_8S

Calculated: C 55.5, H 7.09, N 13.6
Found: C 55.3, H 7.20, N 13.7

N-Carbobenzyo-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-alany-L-S-benzyl-L-cysteiny-L-proly-L-leucylglycinamide—Protected heptapeptide, 2.49 g, was suspended in 20 ml of acetic acid and treated with 20 ml of HBr in acetic acid (20%). After 2 hours at room temperature, dry ether was added and the precipitate was washed with ether. After being dried over CaCl_2 and KOH, the solid was dissolved in 50 ml of methanol and treated with IRA-410 resin in the OH cycle. Removal of the solvent left a residue, which was dissolved in 30 ml of dimethylformamide, and 1.56 g of p-nitrophenyl N-carbobenzyo-O-benzyl-L-tyrosinate were added. When the mixture was shaken, some solid soon started to separate. After 23 hours, 400 ml of ethyl acetate were added with thorough mixing. On the next day the solid was collected and washed with 50 ml of ethyl acetate, 200 ml of absolute ethanol, and again with 75 ml of ethyl acetate, and dried in a vacuum over CaCl_2 and then over P_2O_5.
at 100°C for 2 hours; weight 2.73 g, m.p. 238–239°C, [α]D 15.3°C (c, 1 in dimethylformamide).

C₄H₁₁O₂N₁₃S₃
Calculated: C 62.2, H 6.85, N 11.9
Found: C 62.1, H 6.86, N 11.7

5-Decarboxamido-oxytocin (5-Alanine-oxytocin)—The preceding octapeptide, 1.4 g, was suspended in 10 ml of acetic acid and treated with 5 ml of HBr in acetic acid (30%). After 2 hours at room temperature, 150 ml of dry ether were added and the precipitated hydrobromide was filtered off and washed with ether. After being dried in vacuum over KOH and CaCl₂, the hydrobromide was dissolved in 15 ml of dimethylformamide, and triethylamine was added to pH 7, followed by 0.61 g of p-nitrophenyl N-carbomethoxy-S-benzyl-L-lysinate. After 1 day at room temperature, the reaction mixture was mixed with 150 ml of ethyl acetate. The precipitate was washed on the filter with more ethyl acetate, 50 ml of ethanol, and 50 ml of ethyl acetate, dried in vacuum over CaCl₂, and then over P₂O₅ at 100°C for 2 hours; weight 1.28 g, m.p. 231–222°C, [α]D 53.2°C (c, 1 in dimethylformamide).

This protected nonapeptide, 277 mg, was dissolved in 250 ml of boiling ammonia, freshly distilled from sodium, and treated with sodium until a faint blue color enveloped the solution. Glacial acetic acid, 0.1 ml, was added, and most of the ammonia was removed in a vacuum, the last 50 ml being evaporated from the frozen state. The residue was dissolved in 200 ml of 0.1% acetic acid, and after adjustment of the pH to 6.5 to 7.0 the solution was aerated for 4 hours. The oxidation was completed by titration with 0.02 N potassium ferricyanide solution. The solution was deionized by passage through a column of ion exchange resin, AG3X4, in the chloride cycle. This solution was found to possess a total of 28 units of avian depressor activity. It was evaporated in a flash evaporator to a volume of 16 ml, and for further purification this solution was placed in the first 2 tubes of a 200-tube countercurrent machine and distributed in the solvent system butanol-propanol-0.05% acetic acid (2:1:3). Initially the crude 5-decarboxamido-oxytocin was purified in the solvent system butanol-ethanol-0.05% acetic acid (4:1:5). However, in this system the separation of analogue from by-products was very unsatisfactory, and with the increased amount of transfers the curve became more irregular. After 400 transfers, the partition coefficient for the main peak was 0.74, but after 900 transfers, the value changed to 0.88.

Determination of the Folin-Lowry (26) color values in the solvent system butanol-propanol-0.05% acetic acid after 2 transfers indicated two peaks with distribution coefficients of approximately 0.31 and 0.53. After 400 transfers, the pattern remained practically unchanged but a new peak appeared with a K value of 0.16. Avian depressor activity was present in tubes 125 to 160, which corresponded to the peak with a K value of 0.53. The contents of tubes 130 to 150 were pooled, evaporated in a flash evaporator to approximately 50 ml, and lyophilized; weight 49 mg, [α]D 13.3°C (c, 1.5 in n acetic acid). A sample was hydrolyzed in 0 N TCI at 100°C and analyzed in the Beckman/Spinco amino acid analyzer. The following molar ratios of amino acids were obtained with the values for glycine taken as 1: glutamic acid, 1.0; proline, 1.3; glycine, 1.0; alanine, 1.0; cystine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 1.0; and ammonia, 2.0.

DISCUSSION

The data presented in Table I summarize the biological effects observed thus far from the replacement of various chemical functional groups of oxytocin by hydrogen. The striking potencies of the deamino-oxytocin demonstrate that the amino group is not essential for the manifestation of the characteristic biological activities of oxytocin. Furthermore, the data show that the presence of the phenolic hydroxyl group is also not essential for the exhibition of activity, although this group does contribute strongly to the activities of the hormone. It is also of interest that the replacement of the amino group of deoxy-oxytocin with hydrogen does not lower the avian depressor and oxytocic potencies, since deamino-deoxy-oxytocin has approximately the same avian depressor and oxytocic activities as the deoxy analogue. On the other hand, there is a 10-fold decrease in pressor activity when the amino group of deoxy-oxytocin is replaced by hydrogen. The same effect on pressor activity is encountered on going from oxytocin to deamino-oxytocin, although in the latter case the change is less marked. From the standpoint of antidiuretic activity, there is little change in the potency of deamino-deoxy-oxytocin as compared with that of deoxy-oxytocin. However, it is noteworthy that the replacement of the amino group of oxytocin with hydrogen enhances the antidiuretic potency 5-fold.

The first studies in our laboratory involving the carboxamide groups of oxytocin were in connection with the synthesis of 4-isoglutamine-oxytocin (28, 29) and 5-isoaasparagine-oxytocin (30). These two analogues were originally synthesized to test the specificity of the glutamine and asparagine residues in the 4- and 5-positions for the activities of the hormone. Both of these analogues, in which the proximity and spatial relationship of the carboxamide groups to the disulfide ring as well as the size of the ring itself are changed from that found in oxytocin, were found to be biologically inactive. The carboxamide group in the 4-isoglutamine-oxytocin is linked directly to the ring instead of being separated from the ring by 2 methylene units as in oxytocin, and the size of the ring is increased from 20 to 22 atoms. In the case of the 5-isoaasparagine-oxytocin, the carboxamide group is attached directly to the ring and the ring size is increased from 20 to 21 atoms. Although the results with these two analogues demonstrated that neither the glutamine or asparagine residue in oxytocin could be replaced by its isomeric form without loss of biological activity, no assessment could be made as to whether this loss of activity was due to the change in ring size, to the change in the spatial relationship of the carboxamide groups to the rest of the molecule, or to both of these structural changes.

The current studies of the two decarboxamido analogues deal directly with the importance of the presence of the carboxamide groups at positions 4 and 5 in the hormone. The striking differences in the activities of the 4-decarboxamido-oxytocin (4-L-α-aminoobutyric acid-oxytocin) and 5-decarboxamido-oxytocin (5-L-alanine-oxytocin) clearly demonstrate that the presence of the carboxamide group of the asparagine residue at position 5 is vital for the exhibition of appreciable biological activity, in contrast to the carboxamide group of the glutamine residue at position 4. As shown by the data in Table I, the 4-decarbox-
amido-oxytocin possesses approximately 108 units per mg of avian depressor activity and 75 units per mg of oxytocic activity, whereas the 5-decarboxamido-oxytocin has an extremely low order of both these activities. With respect to pressor and antidiuretic activity, the absence of the carboxamide in position 4 results in a considerable decrease in both activities. The absence of the carboxamide in position 5 has an even more drastic effect; the 5-decarboxamido-oxytocin has practically no detectable pressor or antidiuretic activity.

In connection with these results on the biological activities of 4-decarboxamido-oxytocin, it is significant that the new hormone, isotoxin, isolated by Acher et al. (31) from the posterior pituitaries of teleost fishes, lacks a carboxamide group in position 4. The structure proposed by them, 4-serine-8-isoleucine-oxytocin, has been confirmed by synthesis (32, 33).

The three analogues of oxytocin prepared by Boissonnas et al. (34, 35) have afforded a series of analogues of particular significance to the question of the carboxamide groups. In this series, the glutaminyl-asparaginyl sequence was replaced by glutaminyl-glycyl (34), asparaginyl-glutaminyl, and asparaginyl-glutamyl (35). Of these three analogues, the only one to show a high degree of oxytocic or avian depressor activity was the one with the asparaginyl-asparaginyl sequence possessing 105 units of oxytocic activity and 202 units of avian depressor activity per mg (35). The other two analogues possessed 1 unit or less of these activities per mg. The results with these three analogues indicated that the substantial relationship of the 5-carboxamide group to the rest of the molecule is extremely important to activity.

Thus our studies involving the presence or absence of these carboxamide groups and those just discussed on the spatial relationship of these groups to the rest of the molecule point to the possibility that the carboxamide group of the asparagine residue is of special significance to the activity of oxytocin. The presence of this carboxamide group may be required for the attachment of the hormone to its receptor sites or for bringing about a particular conformation of the molecule which is a prerequisite for appreciable biological activity.

As shown in Table I, replacement of the disulfide moiety by hydrogen, giving rise to dethio-oxytocin, results in complete loss of activity. The remaining chemical functional groups of oxytocin by hydrogen. This aspect of the relation of structure to biological activity is being investigated.

**SUMMARY**

Two analogues of oxytocin in which the glutamine and asparagine residues have been replaced by L-α-amino butyryl acid and L-alanine residues, respectively, have been prepared by total synthesis according to the stepwise p-nitrophenyl ester method. Thus the carboxamide groups in positions 4 and 5 of the hormone have been replaced by hydrogen. The 4-decarboxamido-oxytocin (4-L-α-amino butyryl acid-oxytocin) was found to possess approximately one-fifth of the avian depressor activity and one-sixth of the oxytocic activity of oxytocin, whereas these activities in 5-decarboxamido-oxytocin (5-L-alanine-oxytocin) were of the order of 0.2 to 0.3 unit per mg.

These striking differences in the activities of the 4- and 5-decarboxamido analogues suggest an important role for the carboxamide group of asparagine in determining the biological effects of oxytocin.

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The Synthesis and Pharmacological Study of 4-Decarboxamido-oxytocin (4-\(\alpha\)-Aminobutyric Acid-oxytocin) and 5-Decarboxamido-oxytocin (5-Alanine-oxytocin)

Vincent Du Vigneaud, George S. Denning, Jr., Stefania Drabarek and W. Y. Chan