Synthesis and Some Biological Properties of 1-Deamino-4-Glu-Oxytocin (1-β-Mercaptopropionic Acid – 4-Glutamic Acid – Oxytocin) and its Use in Preparing a Hormone–Agarose Complex

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1-Deamino-4-Glu-oxytocin (1-β-mercaptopropionic acid – 4-glutamic acid – oxytocin) was synthesized by sequential reduction by sodium in liquid ammonia and oxidation by hydrogen peroxide of the octapeptide derivative, *S*-benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-γ-*O*-benzyl-glutamyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucyl-glycinamide. The oxidation analogue was isolated and purified by partition chromatography in two different solvent systems followed by exclusion chromatography on Sephadex G-25. It was found to possess approximately 13 I.U. of uterotonic activity, 34 I.U. of milk ejection activity, and 83 I.U. of milk ejection-like activity per milligram, measured on an isolated strip of lactating mouse mammary gland. 1-Deamino-4-Glu-oxytocin was coupled to AH-Sepharose 4B by way of the free γ-carboxyl group of its residue of glutamic acid. The water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride caused the coupling with approximately 70% effectiveness. The resultant peptide–agarose complex had low biological potency in the assay of milk ejection-like activity.

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La 1-désamino-4-Glu-ocytocine (1-β-acide mercaptopropionique – 4-acide glutamique – ocytocine) est synthétisée par réduction séquentielle à l'aide du sodium dans l'ammoniac liquide et par oxydation, avec le peroxyde d'hydrogène, du dérivé octapeptidique, le *S*-benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-γ-*O*-benzyl-glutamyl-asparaginyl-*S*-benzyl-cystéinyl-prolyl-leucyl-glycinamide. L'analogue obtenu par oxydation est isolé et purifié par chromatographie de partage dans deux systèmes de solvants différents et chromatographie d'exclusion sur Séphadex G-25. L'activité utérotonique de cet analogue est de 13 U.I., son activité d'éjection lactée de 34 U.I. et l'activité totale semblable à celle de l'éjection lactée, telle que mesurée sur une bande isolée de glande mammaire de souris en lactation, est de 83 U.I./mg. La 1-désamino-4-Glu-ocytocine est couplée à l'AH-sépharose 4B par l'intermédiaire du groupe γ-carboxyle libre de son résidu d'acide glutamique. L'hydrochlorure de 1-éthyl-3-(3-diméthylaminopropyl)-carbodiimide hydrosoluble entraîne le couplage avec une efficacité d'environ 70%. Le complexe peptide–agarose obtenu montre une faible action biologique dans l'essai de l'activité ressemblant à l'éjection du lait.

[Traduit par le journal]

Introduction

Hormones immobilized on resins are finding application in fractionation of proteins in the search for receptors. The synthesis of a deamino-oxytocin–agarose complex was undertaken with the aim of using it for isolation of oxytocin-binding fractions of plasma membranes from the hormone's target tissues. Deamino-oxytocin (1-β-mercaptopropionic acid – oxytocin) is a synthetic analogue of the neurohypophysial hormone oxytocin, in which the free amino group of the hormone is replaced by a hydrogen atom (Fig. 1). It is highly potent in milk-ejecting and uterotonic activities, which are characteristic of oxytocin, and binds strongly to uterus and mammary gland, the hormone's putative target tissues (1–3).

4-Glu-oxytocin (4-glutamic acid – oxytocin) is another synthetic analogue of oxytocin in which the residue of glutamine in oxytocin is replaced by one of glutamic acid (4). It thus has a free carboxyl group at position 4 (Fig. 1). It has very much reduced potency in uterotonic activity, but has retained considerable potency in milk-ejecting activity. This and other results obtained with analogues in which the glutamine residue

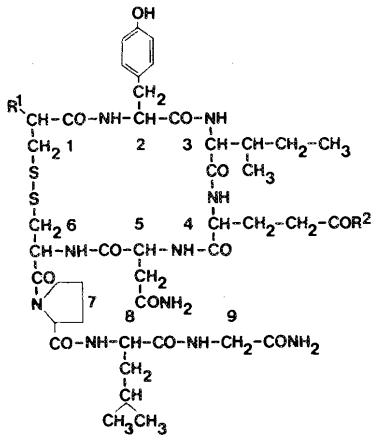

FIG. 1. Structure of oxytocin and analogues with structural modifications at positions 1 and 4. Oxytocin: $R^1 = NH_2$, $R^2 = NH_2$. 1-Deamino-oxytocin: $R^1 = H$, $R^2 = NH_2$. 4-Glu-oxytocin: $R^1 = NH_2$, $R^2 = OH$. 1-Deamino-4-Glu-oxytocin: $R^1 = H$, $R^2 = OH$.

of oxytocin was modified (5) show that the glutamine residue is not essential for the expression of biological activity. It was therefore decided to attempt to establish a bridge between agarose and the hormone at position 4. It was further decided to prepare deamino-oxytocin bound to agarose rather than oxytocin bound to agarose because of the synthetic advantages offered.

1-Deamino-4-Glu-oxytocin (I), if bound to agarose by coupling the free carboxyl group to the amino group of 1,6-diaminohexane bridge, will be transformed into a 4-substituted deamino-oxytocin (II) (Fig. 2). This bridge is available in the substituted agarose AH-Sepharose 4B (Pharmacia).

Materials and Methods

Biological Assays

Assays of uterotonic activity were made on isolated

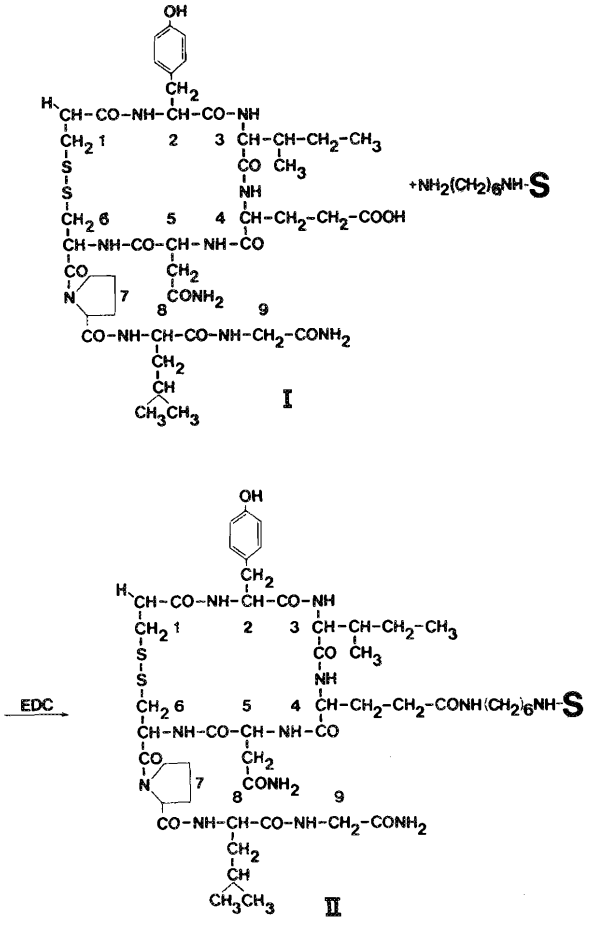

FIG. 2. Reaction of 1-deamino-4-Glu-oxytocin (I) with AH-Sepharose 4B to give a 4-substituted 1-deamino-oxytocin (II). S = Sepharose, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

uteri of rats according to the method of Holton (6) as modified by Munsick (7). Milk-ejection activity was measured in anesthetized lactating rabbits by the method of van Dyke *et al.* (8) modified by Chan (9). The milk ejection-like activity, which was the assay used routinely for following preparations and estimating degree of purification of products, was determined by measurement of contractions produced in an isolated strip of mammary gland of a lactating mouse, according to the method developed by Coch and coworkers (10). All assays used the United States Pharmacopeia posterior-pituitary reference standard, and all activities are expressed relative to this.

Synthesis of Peptide

S-Benzyl-β-mercaptopropionyl-tyrosine Dicyclohexylamine Salt

p-Nitrophenyl-*S*-benzyl-β-mercaptopropionate (I) (0.81 g, 2.5 mmol) and tyrosine methyl ester (0.4 g, 2.0 mmol) were dissolved in dimethylformamide (6 ml), and the solution was kept at room temperature for 3 days. It was poured into water, and an oil separated. The oil was dissolved in ethyl acetate and washed exhaustively with $NaHCO_3$ solution (5%), 1 *N* HCl, and water. The solution was dried over Na_2SO_4 and then evaporated to dryness to leave an oily residue. Thin-layer chromatography of the oil on silica gel in the solvent system $CHCl_3$–MeOH–AcOH (85:10:5) showed (iodine chamber) a single spot of *R* 0.77. The oil (0.6 g) was dissolved in acetone (3 ml), cooled to 0 °C, and treated, with stirring, by dropwise addition of 2 *N* NaOH solution (3 ml) over 15 min. Stirring was continued for 40 min at room temperature. Water (10 ml) was added, and the solution was acidified with 1 *N* HCl and extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4, and evaporated to give an oil. Thin-layer chromatography on silica gel in the system $CHCl_3$–MeOH–AcOH (85:10:5) showed a single spot (iodine chamber), R_f 0.67, with no contamination with the faster moving (R_f 0.77) methyl ester. The oil was dissolved in ethyl acetate (10 ml) and treated with dicyclohexylamine (0.6 ml). The precipitated salt was filtered and crystallized from methanol–ether in white plates, 0.6 g (54%), m.p. 185–186 °C, $[\alpha]_D^{20}$ +33° (*c* 2, methanol).

Mixed Disulfide of β-Mercaptopropionic Acid and Cysteine

The method of Hope *et al.* (1) was modified. Cysteine (0.7 g, 5.8 mmol) and β-mercaptopropionic acid (0.6 g, 5.7 mmol) were dissolved in water (15 ml) and the pH of the solution was adjusted to 7.5 with NH_4OH solution. The solution was stirred, with cooling in an ice bath, and H_2O_2 (30%, 0.5 ml) was added. Stirring and cooling were continued for 3 min. The pH was adjusted to 7 by the addition of concentrated HCl, and the insoluble cystine that had been formed was filtered off. The pH of the filtrate was adjusted to 3 with concentrated HCl, while cooling in an ice bath, and a solid separated. This solid was collected by filtration and dissolved in 1 *N* HCl. A small amount of insoluble material was filtered off and the pH of the filtrate was adjusted to 3 by the addition of NH_4OH solution. A white solid crystallized. It was collected by filtration, washed with water, and recrystallized by slow cooling of a hot aqueous solution to give 0.23 g (18%) of shining white plates, m.p. 200 °C (dec.), $[\alpha]_D^{20}$ −104.0° (*c* 1, 1 *N* HCl) (lit. (1): m.p. 202 °C, $[\alpha]_D^{20}$ −110.5° (*c* 1, 1 *N* HCl)). Thin-layer chromatography on silica gel in upper phase of the solvent system 1-BuOH – AcOH – H_2O (4:1:5) gave a single ninhydrin-positive spot of R_f 0.5. Cystine and cysteic acid have R_f values of <0.1.

S-Benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-γ-O-benzyl-glutamyl-asparaginyl-S-benzyl-cysteinyl-prolyl-leucyl-glycinamide (IV) (Fig. 3)

Method A. Azide Method—The hydrochloride of isoleucyl-γ-*O*-benzyl-glutamyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucyl-glycinamide (III) was prepared as described by Photaki and du Vigneaud (4). There was one observed difference in the preparation, in that the *o*-nitrophenylsulfenyl derivative of the heptapeptide crystallized from methanol containing a few drops of water, whereas in the reference quoted it was triturated with boiling methanol containing a few drops of water, and collected after cooling. *S*-Benzyl-β-mercaptopropionyl-tyrosine hydrazide (II) (0.21 g, 0.56 mmol) was transformed into its azide by treating a solution in 4.6 *N* HCl in tetrahydrofuran (2.5 ml) at −20 °C with 18% $NaNO_3$ solution (0.25 ml). This mixture was stirred at −20 °C for 10 min, diluted with ethyl acetate (15 ml), extracted three times with a saturated solution of $NaHCO_3$ containing NaCl, and then with water, and the organic phase was dried over Na_2SO_4. After evaporation of the solvent, the heptapeptide hydrochloride (270 mg, 0.28 mmol) was dissolved in dimethylformamide (8 ml) containing triethylamine (0.1 ml), and the solution was cooled to −20 °C. To this was added the azide solution, cooled to −20 °C, and the mixture was stirred over night at 2 °C and then at room temperature for 3 h. It was poured into ethyl acetate (75 ml). The gelatinous material that precipitated was collected by filtration, washed on the filter with ethyl acetate and methanol, and air dried to give 280 mg (79%) of white solid, m.p. 227–228 °C (dec.), $[\alpha]_D^{20}$ −32.8° (*c* 1, dimethylformamide). For analysis, the material was dried at 100 °C *in vacuo* over P_2O_5 for 24 h. *Anal.* Calcd. for $C_{64}H_{84}N_{10}O_{13}S_2$: C, 60.7; H, 6.70; N, 11.1. Found: C, 60.9; H, 6.70; N, 11.0.

Method B. DCC Method—The hydrochloride of the heptapeptide III (218 mg, 0.23 mmol), prepared as described under method A, and *S*-benzyl-β-mercaptopropionyl-tyrosine dicyclohexylamine salt (119 mg, 0.23 mmol) was stirred in dimethylformamide (5 ml) for 2 h until solution was complete. A solution of dicyclohexylcarbodiimide (DCC; 53 mg, 0.24 mmol) in dimethylformamide (0.2 ml) was added, and the mixture stirred at room temperature for 3 days. The insoluble material was filtered off and washed with dimethylformamide (1 ml). The filtrate and washings were added to water (50 ml) and the mixture was kept over night at 2 °C. The solid that separated was filtered, air dried, and triturated with methanol and ethyl acetate. It was dissolved in dimethylformamide (10 ml) and reprecipitated by the addition of ethyl acetate. It was filtered and air dried to give 78 mg (27%) of white solid, m.p. 224–226 °C (dec.), $[\alpha]_D^{20}$ −33.1° (*c* 1, dimethylformamide).

Thin-layer chromatography on silica gel of material prepared by both methods showed the products to be homogeneous in the solvent systems $CHCl_3$–MeOH–AcOH (85:10:5), R_f 0.77, and 1-BuOH–AcOH–pyridine–H_2O (15:3:10:12), R_f 0.84 (visualized in an iodine chamber).

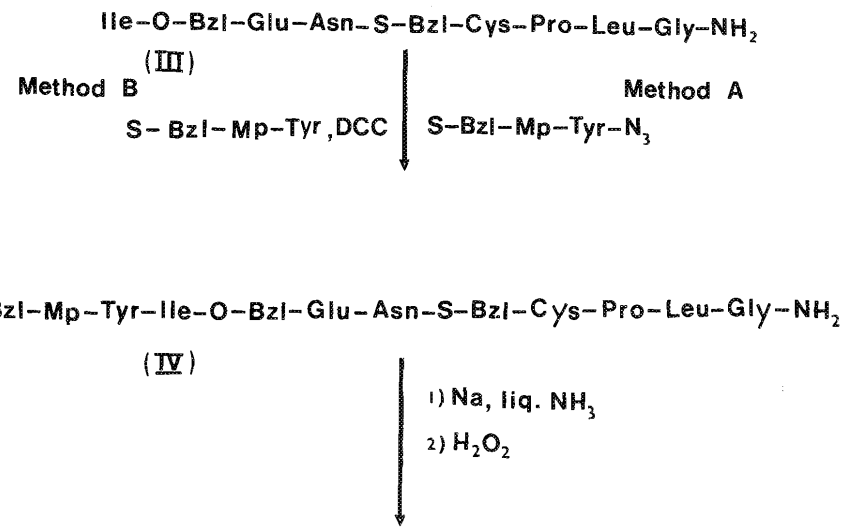

FIG. 3. Synthesis of 1-deamino-4-Glu-oxytocin (I). The two methods of preparation of the nonapeptide derivative (IV) are shown. Method A is preferred. Mp = β-mercaptopropionyl.

1-Deamino-4-Glu-oxytocin (1-β-Mercaptopropionic Acid – 4-Glutamic Acid – Oxytocin) (I)

The protected polypeptide derivative IV (200 mg, 0.16 mmol) was added to boiling NH_3 (250 ml) that had been freshly distilled from sodium. A sodium stick was held under the surface of the liquid until a blue color pervaded the solution and persisted for 1 min. The NH_3 was then removed by lyophilization (water pump). The residue was dissolved in water (200 ml), the pH of the solution adjusted to 8 with 1 *N* acetic acid, and H_2O_2 (30%, 0.5 ml) was added with stirring. Stirring was continued for 5 min. The pH of the solution was then lowered to 5 with acetic acid, and it was concentrated under reduced pressure at 20 °C to about 3 ml. It was filtered to remove a small amount of insoluble material, and put onto a column (1.5 × 75 cm) of Sephadex G-25 (fine) that had been equilibrated with lower phase of the solvent system 1-BuOH–1-PrOH–AcOH–H_2O (600:300:10.25:890) (12). Elution was carried out with the upper phase of the solvent system, and 1.1-ml fractions were collected. The peptide, as determined by Folin–Lowry colorimetric measurement (13), was eluted at the front. Fractions 35–48, which contained the salt-free peptide, were pooled, treated with water, and concentrated *in vacuo*. The addition of water and concentration were repeated until the organic phase was completely removed. The aqueous solution was lyophilized to give 53 mg (33%) of fluffy white powder.

The same Sephadex G-25 column was washed with a mixture of pyridine – 0.2 *N* acetic acid (1:4), then with 0.2 *N* acetic acid (12), and equilibrated with the lower phase and then the upper phase of the solvent system 1-BuOH–benzene–pyridine–AcOH–H_2O (500:500:15:35:950). The peptide obtained from the first column chromatography (20 mg) was dissolved in 1 ml of upper phase of the solvent system containing two drops of lower phase and was applied to the column. Elution was carried out with the upper phase, and 100 × 2.2-ml fractions were collected. Aliquots of 0.1 ml of each fraction were evaporated to dryness and used for Folin–Lowry colorimetric determination of their peptide content. A small amount of peptide was seen to be eluted near the front, and a larger amount was eluted as a symmetrical peak, with R_f 0.40 (R_f of deamino-oxytocin in this system is 0.19) (2). Fractions 35–54, corresponding to the major peak, were pooled, and concentrated after the addition of water. This was repeated until the organic phase was removed. The aqueous solution was then lyophilized to give 10 mg of a white powder. It was dissolved in dilute acetic acid (1 ml, 0.2 *N*) and submitted to exclusion chromatography on the same Sephadex column as used previously, after it had been washed with pyridine – 0.2 *N* acetic acid (5:4) and 0.2 *N* acetic acid. Elution of 1.75-ml fractions with 0.2 *N* acetic acid was carried out. Measurement of the optical density of these fractions at 280 nm showed that the peptide had been eluted as a single symmetrical peak (column hold-up volume 46 ml, elution volume of peak 108 ml) at the position at which a sample of synthetic oxytocin was eluted. The fractions corresponding to the leading and trailing halves of the peak were separately pooled, concentrated, and lyophilized to give 3 mg of fluffy white material from each half, $[\alpha]_D^{20}$ −81.8° (*c* 0.5, 1 *N* acetic acid).

For analysis, a sample was dried at 100 °C over P_2O_5

in vacuo with a weight loss of 7%. *Anal.* Calcd. for $C_{43}H_{64}N_{10}O_{13}S_2$: C, 52.0; H, 6.49; N, 14.1. Found: C, 52.6; H, 6.57; N, 14.0.

A sample was hydrolyzed in 6 *N* HCl at 120 °C for 20 h and analyzed for amino acids. The molar ratios of amino acids and NH_3 obtained were: Asp, 1.0; Pro, 1.0; Glu, 0.9; Gly, 1.0; Cys, 0.3; mixed disulfide of β-mercaptopropionic acid and cysteine, 0.3; Ile, 0.9; Leu, 1.0; Tyr, 0.9; NH_3, 2.2. The two disulfide compounds account for the residue of hemicystine in the analogue.

Similar results were obtained on repetition of the preparation and purification. Thin-layer electrophoresis on silica gel in pyridine – acetic acid buffer, pH 3.6, at 980 V showed the product to be homogeneous when visualized in an iodine chamber. In 3½ h it migrated 43 mm towards the anode, and 1-deamino-oxytocin migrated 7 mm towards the cathode.

Thin-layer chromatography on silica gel in the solvent systems (*a*) upper phase of 1-BuOH–AcOH–H_2O (4:1:5), (*b*) 1-BuOH–AcOH–pyridine–H_2O (15:3:10:12), (*c*) $CHCl_3$–MeOH–AcOH (85:10:5), and on cellulose in solvent system *a* showed a single iodine-positive spot in all cases. In system *a* on silica gel and cellulose, and system *b* on silica gel, the mobility was measurably different from that of 1-deamino-oxytocin. The R_f values of 1-deamino-4-Glu-oxytocin and deamino-oxytocin on silica gel were: *a*, 0.57 and 0.49; *b*, 0.67 and 0.70; *c*, 0.06 and 0.06 and on cellulose they were: *a*, 0.84 and 0.80.

1-Deamino-4-Sepharose 4B-oxytocin (II)

AH-Sepharose 4B (Pharmacia Fine Chemicals), 250 mg, with a capacity of approximately 6 to 10 μmol, was swollen in 0.5 *M* NaCl solution and washed with 0.5 *M* NaCl (100 ml) and with water (50 ml). To the stirred gel was added an aqueous solution of 1-deamino-4-Glu-oxytocin (3.3 mg, 3.4 μmol, in 0.5 ml water). With continued stirring, a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (40 mg, 208 μmol) in water (0.5 ml) was added in 0.1-ml aliquots at 10-min intervals. For the 1st hour, the pH of the suspension was kept at 5–6 by the addition of 0.1 *N* HCl. Stirring was continued for 24 h. The gel was filtered, and washed with water to give a volume of 15 ml. The gel was then washed with a solution of 1 *N* NaCl – 0.5 *M* $NaHCO_3$ (pH 7.95) until the volume of the washings was 50 ml. The second washing was repeated. The milk ejection-like activity and the uterotonic activity of the washings were determined.

1-Deamino-oxytocin was used to replace 1-deamino-4-Glu-oxytocin in a repetition of the experiment described in the preceding paragraph.

Results and Discussion

1-Deamino-4-Glu-oxytocin was synthesized (Fig. 3) by methods similar to those used by Photaki and du Vigneaud (4) to prepare 4-Glu-oxytocin. *S*-Benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-γ-*O*-benzyl-glutamyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucyl-glycinamide (IV) was prepared by coupling isoleucyl-γ-*O*-benzyl-glutamyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucyl-glycinamide (III) with *S*-benzyl-β-mercaptopropionyl-tyrosyl azide, or with *S*-β-benzyl-mercaptopropionyl-tyrosine by the dicyclohexylcarbodiimide method. The protected octapeptide derivative (IV) was treated with sodium in liquid ammonia to remove the protecting groups, and the resultant dithiol was oxidized to give 1-deamino-4-Glu-oxytocin (I), which was isolated by partition chromatography (12) in two different solvent systems and exclusion chromatography on Sephadex G-25.

The first partition chromatography in the system 1-butanol – 1-propanol – acetic acid – water (600:300:10.25:890) effected separation of peptide, which moved rapidly, from salts, which were held back. The second partition chromatography in the system 1-butanol – benzene – pyridine – acetic acid – water (500:500:15:35:950) resulted in a separation of the peptide into two fractions, of which the smaller faster-moving one is presumed to consist of polymeric material. The milk ejecting-like activity of the slower-moving material was measured and it was submitted to exclusion chromatography. The peptide was eluted in a single symmetrical peak with an elution volume virtually identical with that of oxytocin. Samples isolated from the leading and trailing halves of the peak were found to be of equal biological potency and of the same potency as the peptide before exclusion chromatography. These results suggest that a homogeneous peptide had been isolated.

The product was characterized by elemental and amino-acid analysis. Further evidence of its purity was obtained by thin-layer chromatography in several solvent systems and by thin-layer electrophoresis.

The milk-ejection activity of the 1-deamino-4-Glu-oxytocin was found to be 34.2 ± 5.0 U/mg, and the milk ejection-like activity, 82.8 ± 6.4 U/mg. The uterotonic activity was 13.3 ± 0.8 U/mg. The ratio of the milk-ejection:uterotonic activities of this analogue is thus 2.6, whereas the same ratio for deamino-oxytocin is 0.7 (2).

One of the possible contaminants of the product is 1-deamino-oxytocin. This could be formed during reduction of the protected precursor by sodium in liquid ammonia if the γ-*O*-benzyl group on the glutamic-acid residue was amidated rather than reductively cleaved. No amidation was detected in similar preparations of 4-Glu-oxytocin (4) and 9-Gly-oxytocin (14). The

TABLE 1. Effect of replacement of the amino and 4-carboxamido groups of oxytocin by a hydrogen atom and a carboxyl group, respectively, on the uterotonic and milk-ejection activities

Compound	Uterotonic activity (U/mg), rat uterus *in vitro*	Milk-ejection activity (U/mg), rabbit *in vivo*
Oxytocin	486 ± 5[a]	410 ±16[a]
1-Deamino-oxytocin	803 ±36[b]	541 ±13[b]
4-Glu-oxytocin	~1.5[c]	~11[c]
1-Deamino-4-Glu-oxytocin	13.3± 0.8	34.2± 5.0

[a]Chan and du Vigneaud (15).
[b]Ferrier *et al.* (2).
[c]Photaki and du Vigneaud (4).

characterization of 1-deamino-4-Glu-oxytocin did not indicate the presence of 1-deamino-oxytocin. Further, the two peptides have different R_f's in the second partition-chromatography solvent system, and different ratios of biological activities.

The replacement of the amino group on the hemicystine residue at position 1 of oxytocin by a hydrogen atom produces a hormone analogue (1-deamino-oxytocin) that is more potent than oxytocin itself in its milk-ejection and uterotonic effects (Table 1). 4-Glu-oxytocin, on the other hand, with a residue of glutamic acid in place of oxytocin's glutamine, has much reduced potencies in these actions, but there is considerably greater reduction of its uterotonic potency than of its milk-ejection potency. We have found that the presently described analogue, 1-deamino-4-Glu-oxytocin, is less potent than oxytocin and 1-deamino-oxytocin, more potent than 4-Glu-oxytocin, and more potent in milk-ejection than in uterine contraction. A consistent pattern appears when all four compounds are compared (Table 1). A similar pattern was reported for oxytocin and its analogues lacking one or both of the amino group and the 4-carboxamido group (5). The rather high level of milk-ejection activity of 1-deamino-4-Glu-oxytocin encouraged us to pursue its binding to agarose.

1-Deamino-4-Glu-oxytocin was coupled to AH-Sepharose 4B by the water-soluble coupling agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) according to the procedure used for the binding of folic acid to this Sepharose (16). Since folic acid is bound to the amino group of AH-Sepharose 4B by its glutamic-acid residue, it appeared to be a suitable model. The amount of peptide coupled was estimated by measurement of biological activity in the washings from the reaction. The first 15 ml of washings was found to contain 81 U of milk ejection-like activity. The next 50 ml of washings contained 0.5 U, and no biological activity could be detected in the subsequent 50 ml of washings (limit of detection was 120 μU/ml). The total activity washed out was therefore approximately 82 U, equivalent to 1 mg of 1-deamino-4-Glu-oxytocin. The amount of the analogue bound to 250 mg AH-Sepharose 4B is therefore estimated at 2.3 mg. This amount of binding (70%) appears to be similar to that found for folic acid (60%), which was similarly estimated by measurement of folic acid in the washings. 1-Deamino-oxytocin was fully recovered in the washings when it was treated with EDC in the presence of AH-Sepharose 4B, suggesting that the binding observed in the case of 1-deamino-4-Glu-oxytocin occurred exclusively at the 4-γ-carboxyl group. The fact that 1-deamino-oxytocin is recovered quantitatively and the observation that the ratio of the milk ejection-like and uterotonic activities in the washings from the coupling of 1-deamino-4-Glu-oxytocin to the agarose was the same as that of 1-deamino-4-Glu-oxytocin offer further evidence of lack of contamination by 1-deamino-oxytocin. The presence of deamino-oxytocin in significant quantity would have resulted in an altered ratio of biological activities in the washings.

The deamino-oxytocin – Sepharose 4B compound was found to have low potency in the assay of milk ejection-like activity. The assay was performed with a suspension of the compound, and the fluid bathing the mammary-gland strip was mixed by a flow of air bubbles. The peptide–Sepharose compound possessed 0.6 mU of milk ejection-like activity per milli-

gram. This, when expressed by weight of peptide present, is equivalent to approximately 60 mU/mg. It is probably not valid to compare the potency measured in this way with that of the free peptide. The gravitational and mixing forces may be enough to interfere with binding.

The 1-deamino-oxytocin – Sepharose 4B is presently being used in attempted fractionation of plasma-membrane receptors from mammary gland and from uterine smooth muscle.

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