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Solid-Phase Synthesis and Some Pharmacological Properties of [8-Phenylalanine]-Oxytocin*

J. W. M. Baxter, M. Manning,† and W. H. Sawyer

ABSTRACT: In an attempt to establish the identity of a principle designated as EOP I (elasmobranch oxytocin-like principle I), which has been isolated from the pituitary gland of the spiny dogfish (*Squalus acanthias*), [8-phenylalanine]-oxytocin has been synthesized and tested for some of the pharmacological activities characteristic of the neurohypophysial hormones. The synthesis was accomplished by means of the Merrifield solid-phase method as employed for the synthesis of oxytocin and glumitocin. After purification by gel filtration on Sephadex G-15, the product was obtained in 38% over-all yield.

Bioassays yielded the following activities with their 95% confidence limits: rat uterus (no Mg^{2+}), 108 (100–117) units/mg; rat uterus (+Mg^{2+}), 366 (332–406) units/mg; fowl vasodepressor, 191 (170–214) units/mg; rabbit milk ejection, 341 (316–368) units/mg; rat vasopressor, 0.97 (0.90–1.04) units/mg; rat antidiuretic, 1.16 (0.92–1.46) units/mg; and frog bladder activity, 1700 (1240–2360) units/mg. A comparison of these properties with those obtained for EOP I indicates that the latter is not [8-phenylalanine]-oxytocin.

The structures of seven naturally occurring neurohypophysial hormones are known (Table I). In these principles five different amino acids occupy position 8 of the common cyclic octapeptide structure, and two in each of positions 3 and 4.

A principle termed EOP I[1] has been isolated from the pituitary gland of the spiny dogfish (*Squalus ascanthias*) with biological properties similar to those of oxytocin analogs containing a neutral amino acid at position 8 (Sawyer, 1967), but differing in its pharmacological properties from any of the known neurohypophysial hormones or synthetic analogs with which it has been compared. So far, it has not been possible to obtain sufficient quantities of pure material to enable characterization studies to be carried out in the usual manner, *i.e.*, through amino acid analysis and subsequent sequence studies. In an attempt to elucidate the structure of EOP I, it was considered worthwhile to utilize knowledge of its physical and pharmacological properties with that of the evolutionary pattern of the neurohypophysial hormones, to predict what its structure might be and to test this prediction by the synthetic approach. Since evolutionary changes have occurred most frequently at position 8, it was thought possible that EOP I might be an analog of oxytocin containing yet another amino acid in this position. EOP I has been shown to have a slightly higher R_F value than oxytocin on a partition column (Sawyer, 1967). Thus it was reasoned that an amino acid possessing similar lipophylic properties to those of the leucine residue at position 8 in oxytocin might be present in position 8 of EOP I and it might indeed be phenylalanine. Because of this reasoning and also because of our general interest in structure–activity relationships of the neurohypophysial hormones we synthesized [8-phenylalanine]-oxytocin and compared its pharmacological properties with those of EOP I.

The key intermediate required for the synthesis of [8-phenylalanine]-oxytocin was the protected nonapeptide *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-phenylalanylglycinamide. This protected nonapeptide intermediate was synthesized by the solid-phase method (Merrifield, 1963, 1964) following essentially the procedure outlined in the synthesis of oxytocin (Manning, 1968) and of glumitocin (Manning *et al.*, 1968a). BOC-glycine was esterified to the chloromethylcopolystyrene–2% divinylbenzene resin and the stepwise synthesis was carried through eight cycles of deprotection, neutralization, and coupling with the appropriate BOC-amino acids to give the fully protected peptide-resin: *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-*O*-ben-

* From the Department of Biochemistry, McGill University, Montreal, Quebec, Canada, and the Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York 10032. *Received May 22, 1969.* This work was supported in part by a Studentship (to J. W. M. B.) and an Operating grant (to M. M.) from the Medical Research Council of Canada, a National Science Foundation Grant No. GB-4932X and a General Research Support Grant to Columbia University from the National Institutes of Health.

† To whom inquiries should be directed at the Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43614.

[1] Abbreviation used is: EOP I, elasmobranch oxytocin-like principle I.

TABLE I: Amino Acid Sequences of Known Natural Neurohypophysial Principles.

Known Neurohypophysial Principles	Common structure with variable amino acids in position 3, 4, and 8 denoted by X: 1 2 3 4 5 6 7 8 9 Cys-Tyr-(X)-(X)-Asn-Cys-Pro-(X)-Gly-NH_2 Amino Acids in Position 3	4	8
1. Vasotocin	Ile	Gln	Arg
2. Mesotocin	Ile	Gln	Ile
3. Isotocin	Ile	Ser	Ile
4. Glumitocin	Ile	Ser	Gln
5. Oxytocin	Ile	Gln	Leu
6. Arginine vasopressin	Phe	Gln	Arg
7. Lysine vasopressin	Phe	Gln	Lys

zyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-phenylalanylglycyl-resin. BOC-amino acids with protected side chains were *S*-benzyl-L-cysteine and *O*-benzyl-L-tyrosine. The final cysteine residue was added as the *N*-benzyloxycarbonyl-*S*-benzyl derivative. All coupling reactions to form peptide bonds were mediated by dicyclohexylcarbodiimide (Sheehan and Hess, 1955) in methylene chloride except those involving the carboxyl groups of asparagine and glutamine, which were allowed to react in dimethylformamide as their nitrophenyl esters (Bodanszky and du Vigneaud, 1959). Cleavage of the protected peptide chain from the resin was accomplished by ammonolysis (Bodanszky and Sheehan, 1964) following the procedure utilized in the solid-phase synthesis of oxytocin (Manning, 1968) to give the required protected nonapeptide amide intermediate. Conversion of this intermediate into [8-phenylalanine]-oxytocin was effected by reduction with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935; du Vigneaud *et al.*, 1953, 1954) followed by oxidation in aqueous solution at pH 6.5 with potassium ferricyanide (Hope *et al.*, 1962). The product was purified by gel filtration on Sephadex G-15 in a two-step procedure using 50% acetic acid and 0.2 N acetic acid, respectively, for elution in each step (Manning *et al.*, 1968b). The pharmacologically active material was obtained by lyophilization of the major component from the second elution step. Thin-layer chromatography and paper electrophoresis of the purified material indicated that it was homogeneous and that it did, in fact, possess chromatographic properties similar to those of oxytocin. Elemental analysis and analysis for amino acids and ammonia gave the expected results for [8-phenylalanine]-oxytocin. Assay of the [8-phenylalanine]-oxytocin against the USP Posterior Pituitary Standard by several methods gave the values indicated in Table II.

TABLE II: Some Pharmacological Activities[a] of 8-[Phenylalanine]-Oxytocin and a Comparison of Activity Ratios[b] with Those of EOP I.

	[8-Phe]-Oxytocin	Squalus EOP I
Rat uterus (no Mg^{2+})	108 (100–117)	
Rat uterus (+Mg^{2+})	366 (332–406)	
R_{Mg}[c]	3.4 ± 0.2	1.8–3.0
Rabbit milk ejection	341 (316–368)	
R_{ME}[d]	3.16 ± 0.17	1.5 ± 0.05
Fowl vasodepressor	191 (170–214)	
R_{FV}[e]	1.77 ± 0.11	1.55 ± 0.11
Bullfrog bladder	1700 (1240–2360)	
R_{FB}[f]	16 ± 3	2.4–4.2
Rat antidiuresis	1.16 (0.92–1.46)	
R_{AD}[g]	0.011 ± 0.0013	0.007
Rat vasopressor	0.97 (0.90–1.04)	
R_{VP}[h]	0.009 ± 0.0005	<0.02

[a] Activities expressed in units/mg with 95% confidence limits. [b] Calculated according to the method of Munsick and Jeronimus (1965). [c] Ratio of activity on the rat uterus in the presence of 0.5 mM Mg^{2+} to activity on the rat uterus in the absence of Mg^{2+}. [d] Ratio of rabbit milk ejection activity to activity on the rat uterus in the absence of Mg^{2+}. [e] Ratio of fowl vasodepressor activity to activity on the rat uterus in the absence of Mg^{2+}. [f] Ratio of activity on the frog bladder to activity on the rat uterus in the absence of Mg^{2+}. [g] Ratio of antidiuretic activity on the rat uterus in the absence of Mg^{2+}. [h] Ratio of rat vasopressor activity to activity on the rat uterus in the absence of Mg^{2+}.

Experimental Section

Materials and Methods. The *t*-butyloxycarbonyl- and benzyloxycarbonylamino acids were purchased from Schwartz BioResearch, Inc., and their purity was checked by thin-layer chromatography. The Merrifield resin (chloromethylated copolystyrene–divinylbenzene with 2% cross-linking, containing 1.40 mequiv of Cl/g) was purchased from Cyclo, Inc. The following procedure was used to effect the removal of fines. The resin (100 g) was suspended in 500 ml of methylene chloride in a 1-l. separatory funnel. As the resin settled out on top of the solvent the suspended fines were removed by draining off the solvent. The procedure was repeated and the resin was collected by filtration and dried. The glass synthesis vessel (Merrifield, 1963) was purchased from Scientific Glass-blowing Inc. The mechanical shaker used for rocking the synthesis vessel was purchased from Mann Research Laboratories. Dimethylformamide, trifluoroacetic acid, and triethylamine, all reagent grade, were each further purified by fractional distillation; the dimethylformamide under reduced pressure to prevent its decomposition. Dry methanol was prepared by standard methods (Vogel, 1956) and dry acetic acid was prepared by the method of Pictet and Geleznoff (1903). The 1 N HCl in glacial acetic acid solution was prepared by bubbling glacial acetic acid (500 ml) with hydrogen chloride under anhydrous conditions at room temperature for 30 min. The chloride content was estimated by the Volhard method (Stewart and Young, 1969) and adjusted to 1 N by dilution with glacial acetic acid. Peroxide-free dioxane was obtained by passing

dioxane through a column of activated alumina immediately before use. Amino acid analysis of the BOC-glycine-resin was carried out in the following manner. A 50-mg sample of substituted resin was treated with 1 N HCl in glacial acetic acid (30 ml) for 30 min to remove all the ion-exchange bound BOC-glycine. The resin was then filtered, washed with acetic acid and water, and dried. A 10-mg aliquot was hydrolyzed by refluxing in a 10-ml solution of a 1:1 mixture of 12 N HCl–dioxane (peroxide free) under an air condenser for 20 hr. The resin was removed by filtration and washed with 1 N HCl (three 5-ml portions). The combined filtrate and washings were evaporated to dryness under high vacuum, using a rotary evaporator. An aliquot of the residue was used for the estimation of glycine on the automatic analyzer (Spackman *et al.*, 1958). For the amino acid analysis of protected and free peptides, samples (1–2 mg) were weighed directly into a Pyrex hydrolysis tube to which was added 1 ml of constant-boiling 5.7 N HCl. After freezing the contents, the tube was sealed under vacuum and stored at 110° for 20 hr. The hydrochloric acid was removed under high vacuum on the rotary evaporator and an aliquot of the residue was used for the amino acid analysis. All analyses were carried out on a Technicon analyzer with a 4.5-hr run.

BOC-glycine-resin. The Merrifield resin (10 g) was suspended in 10 ml of ethanol. A solution of 1.0 g (0.57 mmole) of BOC-glycine and an equivalent amount of triethylamine (0.78 ml) in 3 ml of ethanol was added to the resin suspension. The mixture was stirred slowly at 80° under ahydrous conditions for 48 hr, using a magnetic stirrer. The esterified resin was washed successively with ethanol, water, and methanol, and dried *in vacuo* over P_2O_5. Amino acid analysis gave a value of 0.227 mmole of BOC-glycine/g of substituted resin.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanylglycyl-resin. The BOC-glycine-resin (3 g) containing 0.681 mmole of glycine was introduced into the reaction vessel, washed with methanol and methylene chloride, and allowed to swell in 30 ml of methylene chloride overnight.

The following general procedure was used for all washing steps in the synthesis. The resin was washed three times with 30-ml aliquots of each solvent over a 15-min period. When changing from one solvent to another an intermediate washing with a 1:1 mixture (30 ml) of each of the two solvents was employed.

The following cycle which incorporates minor variations of the original procedure as outlined by Merrifield (1964), was used to introduce each new residue into the growing peptide chain. (1) The resin was first washed successively with methylene chloride, ethanol, and glacial acetic acid. (2) The BOC group was removed by treatment with 1 N HCl in glacial acetic acid using the following procedure: 30 ml of 1 N HCl in acetic acid was added to the resin and allowed to equilibrate for 5 min. The resin was filtered and a second 30-ml aliquot of 1 N HCl in glacial acetic acid was added and allowed to react for a further 25 min. During this time the reaction vessel was occasionally removed from the rocker and shaken manually to prevent caking of the resin on the side of the vessel. For the deprotection of the BOC-glutaminyl residue the procedure outlined previously (Manning, 1968) utilizing trifluoroacetic acid (Takashima *et al.*, 1968) was followed. (3) The resin was washed with acetic acid, ethanol, and chloroform. (4) The free amino group was liberated from the hydrochloride or trifluoroacetate by treatment with triethylamine as follows. To a suspension of the resin in chloroform (10 ml) a solution of triethylamine (2 ml) in 20 ml of chloroform was added and allowed to react for 5 min with intermittent manual shaking. The resin was filtered, washed once with chloroform, and the step was repeated. (5) The resin was washed with chloroform and methylene chloride. (6) The resin was suspended in methylene chloride (10 ml) and a threefold excess (2.04 mmoles) of the appropriate BOC-amino acid, dissolved in 3 ml of methylene chloride, was added and allowed to equilibrate with the resin for 20 min. (7) An equivalent amount of dicyclohexylcarbodiimide (2.04 mmoles) in a 50% (w/v) solution of methylene chloride (0.8 ml) was added and allowed to react for 4 hr. The reaction vessel was agitated manually at intermittent intervals during this period. Eight cycles of deprotection, neutralization, and coupling were carried out in this manner on successive days with the following amino acid derivatives: BOC-L-phenylalanine, BOC-L-proline, BOC-*S*-benzyl-L-cysteine, BOC-L-asparagine-*p*-nitrophenyl ester, BOC-L-glutamine-*p*-nitrophenyl ester, BOC-L-isoleucine, BOC-*O*-benzyl-L-tyrosine, and *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine. The incorporation of the BOC-glutamine and the BOC-asparagine residues as their nitrophenyl ester derivatives led to the elimination of step 7 and the substitution of dimethylformamide for methylene chloride in steps 5 and 6.

After the incorporation of the final residue, steps 1–5 were repeated to bring about removal of all the *N*-Z-*S*-Bzl-L-cysteine which had been bound by ion exchange during the final coupling step to the quaternary ammonium groups on the resin. Estimation of the chloride content of filtrates and washings following the neutralization step at this stage gave a chloride value of 0.08 mmole; this value represents the quaternary groups formed both during esterification and during the subsequent neutralization steps.

At the conclusion of the synthesis, the protected peptide-resin was washed out of the reaction vessel with ethanol, dimethylformamide, and methanol, collected on a filter, and dried *in vacuo*, weight 3.728 g. The weight gain of 728 mg (0.574 mmole) at this stage indicated an 84.3% incorporation of protected peptide based on the initial BOC-glycine content (0.681 mmole) on the resin.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanylglycinamide. The protected peptide-resin (2.0 g) was allowed to swell in 80 ml of dry methanol for 3 hr and cooled to −10° in a salt–ice bath. Dry ammonia, from a boiling solution saturated with sodium, was bubbled through the cooled stirred suspension for 2.5 hr. The methanolic suspension was shown to be 16.4 N with respect to ammonia and the volume had increased to 130 ml. The flask was stoppered and stirring was continued at +4° for 18 hr. The flask was recooled at −10° and connected to the water pump *via* two soda–lime U tubes and evacuated at room temperature for 3 hr, whereupon the volume had decreased to 80 ml. The complete removal of the methanolic ammonia was accomplished by continuing the evacuation on a high vacuum pump for an additional 3 hr. An all-glass lyophilizer, with the center flask containing a 100-ml solution of a 1:1 mixture of 12 N HCl and glacial acetic acid immersed in a Dry-Ice–acetone bath, was interposed between the flask and the pump to trap the

evacuated ammonia vapors. The cleaved peptide was extracted with dimethylformamide (three 30-ml portions) and methanol (two 10-ml portions). The resin was removed by filtration and the dimethylformamide and methanol were removed *in vacuo* on a rotary evaporator. The residue was washed with methanol (three 30-ml portions), which was removed each time by evaporation, and dried *in vacuo* overnight. The product was purified by trituration with 95% ethanol (30 ml) and the insoluble precipitate was collected on a filter, washed with ethanol (two 10-ml portions) and diethyl ether (three 10-ml portions), and dried *in vacuo* over P_2O_5 to give the required protected nonapeptide amide as a white amorphous powder: weight 268 mg, mp 248–250°, $[\alpha]_D^{22°}$ −50° (*c* 0.5, dimethylformamide). *Anal.* Calcd for $C_{75}H_{90}N_{12}O_{14}S_2$: C, 61.98; H, 6.26; N, 11.83. Found: C, 62.22; H, 6.27; N, 11.61.

The yield of the purified protected nonapeptide amide from the ammonolytic cleavage and trituration was 70% of the amount expected, based on the weight gain of the resin. The yield based on the amount of glycine originally esterified to the resin was 59%. Amino acid analysis gave: Asp, 1.01; Glu, 1.02; Pro, 0.97; Gly, 1.05; Ile, 1.00; Tyr, 0.90; Phe, 1.00; Bz-Cys, 1.86; and NH_3, 3.1.

The protected nonapeptide amide gave a single spot, which traveled slightly faster than the protected nonapeptide amide intermediate of oxytocin (Manning, 1968) when subjected to thin-layer chromatography. The protected nonapeptide amides, dissolved in dimethylformamide (5–20 μg), were applied separately on a thin layer (250 μ) of silica gel H mounted on a glass plate (20 × 20 cm) and chromatographed for 3 hr at room temperature with the upper phase of the solvent system butanol–acetic acid–water (4:1:5, v/v, ascending) (Partridge, 1948). The R_F values of the spots obtained on development with platinum reagent (Toennies and Kolb, 1951) were 0.72 for the protected nonapeptide amide intermediate of [8-phenylalanine]-oxytocin and 0.70 for the protected nonapeptide amide intermediate of oxytocin. No spots were detected with ninhydrin reagent.

[8-Phenylalanine]-Oxytocin. The protected nonapeptide (75 mg, 0.052 mmole) was dissolved in 350 ml of anhydrous liquid ammonia, which was at its boiling point. Sodium was added from a small-bore glass tube until a faint blue color enveloped the solution for a 15-sec period. Dry glacial acetic acid (3 drops) was added to discharge the color and the ammonia was evaporated *in vacuo* under anhydrous conditions at the water pump. The residue was dissolved in 400 ml of 0.2% acetic acid and after adjustment of the pH to 6.8 with 2 N ammonium hydroxide, an excess of 0.011 M potassium ferricyanide (5 ml) (Hope *et al.*, 1962) was added to the stirred solution. After 10 min, 5 g of AG3-X4 resin (chloride form) was added and stirring was continued for 5 min to remove ferrocyanide and excess ferricyanide ions. The suspension was filtered through a bed of AG3-X4 resin (chloride form) (20 g wet weight) and washed through with 100 ml of 0.2% acetic acid. The filtrate and washings were combined and the pH was adjusted to 3 with glacial acetic acid. The solution was lyophilized to give 700 mg of crude product consisting of the required peptide, dimer, and inorganic salts. Purification was effected by the method of Manning *et al.* (1968b). The lyophilisate was dissolved in 3.0 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40–120 μ, column size 110 × 1.2 cm) which had been preequilibrated with 500 ml of 50% acetic acid. The sample was washed into the column with an additional 2 ml of 50% acetic acid and eluted with 50% acetic acid at a rate of 7.6 ml/hr, 60 fractions of 2.3 ml each were collected. A plot of the Folin-Lowry color values (Lowry *et al.*, 1951) of the various fractions showed the presence of two peaks with maxima at tubes 24 and 31, respectively, clearly separated from salt by 15 tubes. The contents of the second peak (tubes 29–38) which contained most of the active peptide, as detected by oxytocic assay (Munsick, 1960), were pooled, diluted with two volumes of distilled water, and lyophilized. Meanwhile the Sephadex column was reequilibrated with 500 ml of 0.2 N acetic acid over a period of 20 hr. The lyophilized powder (34.5 mg) from the second peak was dissolved in 2.0 ml of 0.2 N acetic acid, applied to the column, washed with a further 1 ml of 0.2 N acetic acid, and eluted and collected as before, at a rate of 9.2 ml/hr. The required peptide emerged as a single, nearly symmetrical peak preceded by a small amount of dimer. The contents of this main peak (tubes 50–65) were pooled, diluted with two volumes of water, and lyophilized, to give the desired product as a white fluffy powder (30 mg); this represents a yield of 55% in the reduction–reoxidation step of the protected nonapeptide and an over-all yield of 38% based on the initial glycine incorporation on the resin, $[\alpha]_D^{22.5°}$ −9.0° (*c* 0.5, 1 N acetic acid). For elemental analysis a sample was dried at 25° over P_2O_5 *in vacuo* for 24 hr. *Anal.* Calcd for $C_{46}H_{64}N_{12}O_{12}S_2$: C, 53.07; N, 16.15; H, 6.15. Found: C, 52.9; N, 15.95; H, 6.20. Amino acid analysis gave: Asp, 1.07; Glu, 1.14; Pro, 0.89; Gly, 1.00; Cys, 1.98; Ile, 1.05; Phe, 0.95; Tyr, 0.90; and NH_3, 3.4.

Aliquots of [8-phenylalanine]-oxytocin (40–60 μg) and oxytocin (40–60 μg) dissolved in water were subjected to thin-layer chromatography and run under the same conditions mentioned above for their protected nonapeptide amide intermediates. Single spots were detected using both the platinum (Toennies and Kolb, 1951) and the ninhydrin reagents, with R_F values of 0.32 for [8-phenylalanine]-oxytocin and 0.30 for oxytocin. Likewise only one component in the direction of the cathode was observed when paper electrophoresis of a further aliquot (100 μg) in two pyridine acetate buffers of pH 3.5 and 6.5 was carried out using the same detecting reagents.

Pharmacological Evaluation. A sample of the lyophilized peptide was dissolved in 50 mM acetic acid containing 5 g/l. of chlorobutanol (USP). This solution was stored under refrigeration and samples were removed as needed for biological assays. Activities on the rat uterus *in vitro*, suspended in solutions without Mg^{2+} and with 0.5 mM Mg^{2+}, were performed as described by Munsick (1960). Rabbit milk ejection, fowl vasodepressor, and isolated bullfrog bladder (hydroosmotic) assays were performed by methods described in Munsick *et al.* (1960). Antidiuretic assays were performed by the method described by Sawyer (1958) in rats under ethanol anesthesia. Vasopressor assays were done on pithed rats (Sawyer, 1966). All activities are reported in terms of the USP Posterior Pituitary Reference Standard.

Discussion

The synthesis of [8-phenylalanine]-oxytocin by the solid-phase method (Merrifield, 1963, 1964) as utilized for the synthesis of oxytocin (Manning, 1968) and glumitocin (Manning *et al.*, 1968a) further demonstrates the usefulness of this approach for the rapid and efficient synthesis of analogs of the

TABLE III: Pharmacological Activities (in USP units per milligram) of 8-Substituted Analogs of Oxytocin.

Oxytocin and Analogs	Rat Uterus	Fowl Vaso-depressor	Rabbit Milk Ejection	Rat Vasopressor	Rat Antidiuretic	Frog Bladder[a]
[8-Leu]-Oxytocin[b]	450	450	450	5	5	450
[8-Ile]-Oxytocin[b]	289	498	328	6	1	1,070
[8-Ile]-Oxytocin[c]	388	724	264		0.53	
[8-Val]-Oxytocin[b]	200	280	310	9	0.8	
[8-Val]-Oxytocin[d]	265			17	5	1,676
[8-Cit]-Oxytocin[e,f]	202	*ca.* 250	238	17	4	800
[8-Ala]-Oxytocin[b]	141	135	208	12	0.08	357
[8-Ala]-Oxytocin[g]	166	240		13		
[8-Arg]-Oxytocin[b]	114	285	210	245	250	100,000
[8-Phe]-Oxytocin[h]	108	191	341	1	1	1,700
[8-Lys]-Oxytocin[b]	78	210	180	130	24	15,000
[8-Gln]-Oxytocin[i]	58	95	256	34	6	1,270
[8-Orn]-Oxytocin[b]	42	90	95	103	2.5	*ca.* 80
[8-Gly]-Oxytocin[b]	16	7	47	0.6	0.16	530

[a] All frog bladder assays reported were done by W. H. S. and except for those on [8-Val]- and [8-Phe]-oxytocin have been reported in Sawyer (1965) and Sawyer *et al.* (1969). [b] Values reported by Berde and Boissonnas (1968). [c] Values reported by Rudinger *et al.* (1969). [d] Values from M. Manning, J. W. M. Baxter, and W. H. Sawyer, unpublished data. [e] Values reported by Bodanszky and Birkhimer (1962). [f] Values reported by van Dyke *et al.* (1963). [g] Values reported by Walter and du Vigneaud (1966). [h] Present communication. [i] Values reported by Sawyer *et al.* (1969).

neurohypophysial hormones. Comparison of the pharmacological properties of [8-phenylalanine]-oxytocin with those of EOP I (Table II) indicates that their properties are, as predicted, quite similar. [8-Phenylalanine]-oxytocin does, however, have greater relative activities on rabbit milk ejection and bullfrog bladder assays. Thus it appears that these peptides are not identical.

It is of interest to compare the pharmacological properties of [8-phenylalanine]-oxytocin with those of the known naturally occurring oxytocic principles and with other eight substituted analogs of oxytocin (Table III).

It can be seen that substitution of the leucine residue in position 8 of the oxytocin molecule by a phenylalanine residue results in the retention of much of the biological potency of the natural hormone. Thus [8-phenylalanine]-oxytocin possesses 76% of the milk-ejecting potency, 42% of the avian depressor activity, 24% of the oxytocic and antidiuretic activities, and 19% of the pressor activity of oxytocin. This correlates well with the findings as shown in Table III that substitution of the leucine residue in position 8 of the oxytocin molecule by other amino acid residues results in analogs which possess a high degree of pharmacological potency. This is the first reported instance in which the leucine residue has been replaced by an amino acid possessing an aromatic side chain and it is of interest to note that the rabbit milk-ejecting potency of this analog is higher than that of any other 8-substituted analog of oxytocin which has been synthesized to date.

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Restricted Reactivity of the ε-Amino Groups of Tobacco Mosaic Virus Protein toward Trinitrobenzenesulfonic Acid*

Robert B. Scheele† and Max A. Lauffer

ABSTRACT: The reactivity of the ε-amino groups of tobacco mosaic virus protein was investigated with trinitrobenzenesulfonic acid, a reagent specific for primary amines. The kinetics of trinitrophenylation was followed spectrophotometrically at room temperature in the pH range from 7.5 to 10.4. The half-times of the trinitrobenzenesulfonic acid reaction with tobacco mosaic virus protein were compared with the half-times of the trinitrobenzenesulfonic acid reaction with bovine serum albumin, poly-L-lysine, and denatured (performic acid oxidized) tobacco mosaic virus protein, under identical conditions. Trinitrophenylation of tobacco mosaic virus protein was found to proceed many times more slowly than the reaction of trinitrobenzenesulfonic acid with the other materials. At pH 7.5 tobacco mosaic virus protein did not react at all with the trinitrobenzenesulfonic acid, while the other materials did react. The reaction proceeds to completion at the higher pH values but denatures the tobacco mosaic virus protein. Whole virus did not react with trinitrobenzenesulfonic acid at pH 8.6.

A model involving two proton-sharing associations between lysine and tyrosine side chains is proposed to explain these observations and several anomalies of the chemistry of the ε-amino groups of tobacco mosaic virus protein previously reported in the literature.

The chemistry of the two ε-amino groups of tobacco mosaic virus protein (TMVP) has been investigated by many workers and found to be very unusual. Fraenkel-Conrat and Singer (1954) investigated the reaction of TMVP with fluorodinitrobenzene and found that only one of the ε-amino groups becomes dinitrophenylated. Ramachandran (1959) tried to react *O*-methylisourea with TMVP and found no reaction except in the case of denatured TMVP in which only one of the two ε-amino groups appeared to react. On the other hand, Fraenkel-Conrat and Colloms (1967) found that these groups react readily with acetic anhydride. Fisher and Lauffer (1949), using electrophoresis, found an increase in the net negative charge of whole virus, TMV, upon reaction with formaldehyde which they interpreted as an abolishment of positive charges of the ε-amino groups. However, the present authors have found that these groups cannot be titrated and apparently remain uncharged over the pH range from neutrality to the alkaline limits of titration reversibility (pH >11) (Scheele and Lauffer, 1967). In apparent contradiction to

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† Present address: Department of Biology, State University of New York at Buffalo, Buffalo, N. Y. 14214.