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Solid-Phase Synthesis of [4-Threonine]-oxytocin. A More Potent and Specific Oxytocic Agent Than Oxytocin*

Maurice Manning,† Esther Coy, and Wilbur H. Sawyer

ABSTRACT: As part of a study concerned with identifying a postulated evolutionary intermediate between the 4-glutamine- and 4-serine-containing neurohypophysial hormones, [4-threonine]-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 [8-phenylalanine]-oxytocin. After purification by gel filtration on Sephadex G-15, the product was obtained in 33% overall yield. [4-Threonine]-oxytocin has an oxytocic potency on the rat uterus of approximately 900 units/mg, twice the potency of oxytocin. It has a fowl vasodepressor potency of approximately 1480 units/mg, over three times the potency of oxytocin and has a milk-ejection potency of approximately 540 units/mg, about 20% greater then that of oxytocin. On the other hand, it possesses a rat vasopressor potency of approximately 0.43 unit/mg, only one-tenth the potency of oxytocin and a rat antidiuretic potency of approximately 3 units/mg, about half the potency of oxytocin. It may thus have a clinical application as a highly selective oxytocic agent.

The naturally occurring neurohypophysial hormones which have been characterized to date contain either a glutamine residue or a serine residue in position 4 of the common cyclic octapeptide structure. A mutation from serine to glutamine requires two base changes in the parent mRNA codons for these amino acids and this would seem to indicate the presence in nature of a hitherto uncharacterized 4-substituted intermediate as the "missing link" between the 4-serine- and 4-glutamine-containing neurohypophysial hormones. We have been concerned with trying to identify such an intermediate analog *via* synthetic means and have previously reported the pharmacological characteristics of three 4-proline-substituted analogs of the neurohypophysial hormones (Sawyer *et al.*, 1969). These analogs were found to possess very low activities in the standard assay systems and thus would be extremely difficult to detect in posterior pituitary extracts by the techniques currently in use.

In an extension of these studies, we now wish to report the synthesis and some pharmacological properties of [4-threonine]-oxytocin, an analog of oxytocin in which the glutamine residue in position four is replaced by a threonine

* From the Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43614, and the Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York 10032. *Received May 28, 1970.* This work was supported in part by the Medical College of Ohio, a National Science Foundation Grant No. GB-4932X, Research Grant AM01940, and a General Research Support Grant to Columbia University from the National Institutes of Health.

† To whom to address correspondence.

TABLE I: Pharmacological Activities (in USP or International Units per Milligram ± Standard Errors) of Oxytocin, [4-Threonine]-oxytocin, Deamino-oxytocin, and Other 4-Substituted Analogs of Oxytocin.

Oxytocin and Analogs	Rat Uterus	Fowl Vaso-depressor	Rabbit Milk Ejection	Rat Vasopressor	Rat Antidiuretic
Oxytocin[a]	450 ± 30	450 ± 30	450 ± 30	5 ± 1	5 ± 1
[4-Thr]-oxytocin[b]	(A) 890 ± 150	1487 ± 39	531 ± 27 (rat)	0.45 ± 0.014	2.1 ± 0.6
	(B) 976 ± 25	1474 ± 41	542 ± 23 (rab)	0.41 ± 0.012	3.9 ± 0.6
1-Deamino-oxytocin[c]	803 ± 36	975 ± 24	541 ± 13	1.44 ± 0.06	~19
[4-Ser]-oxytocin[a]	195 ± 30	230 ± 20	255 ± 45	<0.1	0.06 ± 0.01
[4-Val]-oxytocin[c]	139 ± 5	230 ± 14	~419	<0.005	~0.5
[4-Asn]-oxytocin[a]	108 ± 29	202 ± 12	300 ± 128	0.13 ± 0.03	0.044 ± 0.005
[4-Abu]-oxytocin[c]	72 ± 2	108 ± 5	225 ± 7	~0.1	~0.25
[4-Nva]-oxytocin[c]	61 ± 3	99 ± 4	182 ± 3	<0.001	0.04
[4-Orn]-oxytocin[d]	58 ± 1.6	163 ± 5	125 ± 7	<0.1	0.029 ± 0.006
[4-Ala]-oxytocin[a]	36 ± 6	65 ± 3	240 ± 55	<0.01	<0.01
[4-Ile]-oxytocin[c]	~37	81 ± 2	~184	<0.05	~0.02
[4-NLe]-oxytocin[c]	20.5 ± 0.5	51 ± 3	87 ± 4	0.015	<0.04
[4-Leu]-oxytocin[c]	13 ± 1	44 ± 1	66 ± 3	~0.07[e]	Diuretic
[4-Gly]-oxytocin[c]	2.8 ± 0.1	5.5 ± 0.2	17 ± 1	<0.005	<0.002
[4-Glu]-oxytocin[a]	~1.5	~0.5	~11	<0.01	<0.01
[4-Pro]-oxytocin[f]	~0.007		~0.04 (rat)	<0.2	<0.00006

[a] Values reported in Berde and Boissonnas (1968). [b] Present Communication; values from two separate syntheses. [c] Values reported in Flouret and du Vigneaud (1969). [d] Values reported by Havran *et al.* (1969). [e] Value reported by Chiu and Sawyer (1970). [f] Values reported by Sawyer *et al.* (1969).

residue. This analog has been found to possess strikingly higher activities than oxytocin in the oxytocic, and avian depressor assay systems and to possess slightly greater milk-ejection potency than oxytocin. Furthermore, it is a much weaker antidiuretic and vasopressor agent than oxytocin. These unexpected findings may have an important bearing on (a) the phylogeny of the neurohypophysial hormones, (b) the structure activity relationships of the neurohypophysial hormones, and (c) the potential clinical usefulness of this new analog.

The key intermediate required for the synthesis of [4-threonine]-oxytocin was the protected nonapeptide *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-*O*-benzyl-L-threonyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. 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 [8-phenylalanine]-oxytocin (Baxter *et al.*, 1969), with the following slight modification: each coupling step was carried out twice to ensure that each Boc-amino acid addition went to completion. 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*-benzyl-L-tyrosyl-L-isoleucyl-*O*-benzyl-L-threonyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycyl-resin. Boc-amino acids with protected side chains were *S*-benzyl-L-cysteine, *O*-benzyl-L-threonine, 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 dicyclohexycarbodiimide (Sheehan and Hess, 1955) in methylene chloride except the one involving the carboxyl group of asparagine, which was allowed to react in dimethylformamide as its nitrophenyl ester (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 [4-threonine]-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.*, 1968). 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. Elemental analysis and analysis for amino acids and ammonia gave the expected results for [4-threonine]-oxytocin. The synthesis has been repeated and the results reported here have been confirmed. Assay of the two separate preparations of [4-threonine]-oxytocin (A and B) against the USP Posterior Pituitary Standard by several methods gave the values indicated in the Table I.

Experimental Section

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-O-benzyl-L-threonyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycyl-resin. The Boc-glycine-resin (5 g) (purchased from Schwartz BioResearch, Inc.) containing 1.12 mmoles 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 procedure outlined by Baxter *et al.* (1969) was followed to introduce each new residue into the growing peptide chain with one additional modification: each coupling was carried out using a two-step procedure as follows. After deprotection and neutralization, the first step was carried out in the normal manner for a period of 4 hr. The resin was then washed successively with three 30-ml aliquots of methylene chloride, chloroform, and methylene chloride to remove all traces of side-products and by-products of the reaction. The coupling step was then repeated and allowed to continue for a period of 8 hr. Thus, in this new procedure all the major steps of deprotection, neutralization, and coupling are carried out twice as a means of achieving complete reaction at each stage. Eight cycles of deprotection, neutralization and coupling were carried out on successive days with the following amino acid derivatives: Boc-L-leucine, Boc-L-proline, Boc-*S*-benzyl-L-cysteine, Boc-L-asparagine, Boc-*O*-benzyl-L-threonine, Boc-L-isoleucine, Boc-*O*-benzyl-L-tyrosine, and *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine. All coupling reactions to form peptide bonds were mediated by dicyclohexplcarbodiimide (Sheehan and Hess, 1955) in methylene chloride except in the case of Boc-L-asparagine which was allowed to react as the nitrophenyl ester derivative (Bodanszky and du Vigneaud, 1959) in redistilled dimethylformamide.

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 6.20 g. The weight gain of 1.20 g (0.925 mmole) at this stage indicated an 82.5% incorporation of protected peptide based on the initial Boc-glycine content (1.12 mmoles) in the resin.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-O-benzyl-L-threonyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protected peptide-resin (2.99 g) was allowed to swell in 90 ml of dry methanol for 3 hr and cooled to $-10°$ in a Dry Ice–acetone bath. Dry ammonia, from a boiling solution saturated with sodium, was bubbled through the cooled stirred suspension for 2.5 hr. The flask was stoppered and stirring was continued at $+22°$ for 18 hr. The flask was recooled to $-10°$ and connected to the water pump *via* two soda lime U tubes and the cloudy suspension was evacuated with stirring at room temperature for 3 hr. 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 (five 30-ml portions) and methanol (three 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 (50 ml) and the insoluble precipitate was collected on a filter, washed with ethanol (two 30-ml portions) and diethyl ether (three 20-ml portions), and dried *in vacuo* over P_2O_5 to give the required protected nonapeptide amide as a white amorphous powder: weight 654 mg, mp 240–242°, $[\alpha]_D^{24}$ $-27.5°$ (*c* 1.0, dimethylformamide). *Anal.* Calcd for $C_{78}H_{97}N_{11}O_{14}S_2$: C, 63.40; H, 6.61; N, 10.44. Found: C, 63.81; H, 6.49; N, 10.10.

The yield of the purified protected nonapeptide amide from the ammonolytic cleavage and trituration was 99.5% 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 82%. Amino acid analysis (Spackman *et al.*, 1958) gave: Asp, 1.01; Thr, 0.92; Pro, 1.05; Gly, 1.00; Ile, 0.95; Tyr, 0.80; Bzl-Cys, 1.90; Leu, 1.05; NH_3, 2.20.

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 G 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 0.75 for the protected nonapeptide amide intermediate of [4-threonine]-oxytocin and 0.73 for the protected nonapeptide amide intermediate of oxytocin. No spots were detected with ninhydrin reagent.

[4-Threonine]-oxytocin. (A) The protected nonapeptide (150 mg, 0.101 mmole) was dissolved in 250 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 (9.5 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 510 mg of crude product consisting of the required peptide, dimer, and inorganic salts. Purification was effected by the method of Manning *et al.* (1968). The lyophilisate was dissolved in 5.0 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 9.6 ml/hr, 60 fractions of 2.4 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 27 and 32, respectively, clearly separated from salt by 15 tubes. The contents of the second peak (tubes 30–36) 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 (62.5 mg) from the second peak was dissolved in 3.0 ml of 0.2 N acetic acid, and eluted and collected as before, at a rate of 7.0 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 36–45) were pooled, diluted with two volumes of water, and lyophilized, to give the desired product as a white fluffy powder (41.8 mg); this represents a yield of 39.6% in the reduction–reoxidation step of the protected nonapeptide and an over-all yield of 33% based on the initial glycine incorporation on the resin, $[\alpha]_D^{24}$ −10.4° (*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 $C_{42}H_{66}N_{11}O_{12}S_2 \cdot CH_3COOH \cdot 5H_2O$: C, 46.70; H, 7.03; N, 13.62. Found: C, 46.90; H, 6.81; N, 13.60. Amino acid analysis gave: Asp, 1.05; Thr, 0.95; Pro, 1.19; Gly, 1.10; Leu, 1.07; Cys, 1.85; Ile, 0.99; Tyr, 0.93; NH_3 2.00.

Aliquots of [4-threonine]-oxytocin (40–60 μg) and oxytocin (40–60 μg) as well as a mixture of 40 μg of each of the two peptides dissolved in water were subjected to thin-layer chromatography and run under the 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 0.35 for [4-threonine]-oxytocin and 0.29 for oxytocin. The mixture of the two peptides had been separated into two distinct spots that agreed with these R_F values. Likewise only one component in the direction of the cathode was observed when paper electrophoresis of a further aliquot (50 μg) in two pyridine acetate buffers of pH 3.5 and 6.5 was carried out using the same detecting reagents.

(B) A second synthesis starting from Boc-glycine-resin was carried out as described above. A product possessing identical physical and chemical properties to those described for 4-[threonine]-oxytocin (A) was obtained.

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+}, were performed as described by Munsick (1960). Rabbit milk ejection, fowl vasodepressor, and rat vasopressor assays were performed by methods described in Munsick *et al.* (1960). Rat milk ejection assays were performed by the method of Bisset *et al.* (1967). Antidiuretic assays were performed by the method described by Sawyer (1958) in rats under ethanol anesthesia. All activities are reported in units based on the USP Posterior Pituitary Reference Standard.

Discussion

[4-Threonine]-oxytocin, by virtue of its rather unique pharmacological characteristics, occupies an intriguing position in regard to the phylogeny of the neurohypophysial hormones; particularly with respect to a postulated intermediate between the [4-serine]-containing and [4-glutamine]-containing peptides. In possessing greater and more selective oxytocin-like properties and weaker vasopressin-like qualities than oxytocin, it would appear to have superior adaptive value than oxytocin. Thus, it might very well be an oxytocic hormone in some mammalian species. There are precedents which lend support to this possibility. Thus it may be recalled that both arginine vasotocin (Katsoyannis and du Vigneaud, 1958) and mesotocin (Berde and Konzett, 1960) were first synthesized in the laboratory before their existence in nature was demonstrated.

There are still uncertainties as to how the neurohypophysial peptides have evolved. It is assumed that two lines diverged early in vertebrate phylogeny. One line contains the 4-glutamine peptides. The other line contains the 4-serine peptides (Geschwind, 1969).

A mutation from serine to threonine requires only one base change in their respective codons (Brimacombe *et al.*, 1965; Soll *et al.*, 1965) thus suggesting the possibility of a serine–threonine interchange during the course of evolution. Serine-containing neurohypophysial peptides are only known to occur among cartilaginous and actinopterygian fishes, however, and may not have been present during any stage in the evolution of mammals.

For [4-threonine]-oxytocin to have arisen from oxytocin requires the mutation of glutamine to threonine. This would entail two base changes with either proline or lysine as the intermediate step. The 4-proline analogs (Sawyer *et al.*, 1969) and [4-lysine]-mesotocin (Sawyer *et al.*, unpublished data) have been shown to possess very low activities and would be ineffective oxytocic hormones. Thus, although [4-threonine]-oxytocin might have adaptive advantages as a mammalian oxytocic hormone, it may never have evolved because the required postulated intermediate would be unlikely to survive long enough to undergo further evolution.

It is of interest to compare the pharmacological properties of [4-threonine]-oxytocin with those of other synthetic four substituted analogs of oxytocin (Table I).

It can be seen that substitution of the glutamine residue in position 4 of the oxytocin molecule by a threonine residue results in a dramatic enhancement of the oxytocin-like potencies with a corresponding decrease in the vasopressin-like characteristics of the natural hormone. Thus, [4-threonine]-oxytocin possesses three times the avian depressor activity, almost twice the oxytocic activity, 20% greater milk-ejecting potency while retaining only about one-half of the antidiuretic activity, and one-tenth of the pressor activity of oxytocin. This is in striking contrast to the findings reported for all other known 4-substituted analogs of oxytocin (Table I). It can be seen that while there is in some cases a retention of much of the biological potency of oxytocin, none possesses greater activities than oxytocin. The presence of the hydroxyl and methyl groups on the β-carbon atom of the threonine side chain at position 4 must be the key factor involved in bringing about such marked differences.

It may be recalled that a number of other analogs of oxytocin have been found to possess potencies greater than those of oxytocin. Thus deamino-oxytocin (Hope *et al.*, 1962) an analog of oxytocin in which the amino group in position one was replaced by hydrogen was shown to exhibit greater activity than oxytocin in the rat uterus, fowl vasodepressor, rabbit milk ejection, and rat antidiuretic and weaker activity than oxytocin in the rat vasopressor assay systems (Table I). The isosteric replacement of the sulfur atoms at position 1 and at both positions 1 and 6 by selenium in deamino-oxytocin to give rise to 1-deamino-1-selenocystine-oxytocin (Walter and Schwarz, 1968) and 1-deamino-1,6-selenocystine-oxytocin (Walter and du Vigneaud, 1966) also brought about a mixed enhancement of activities. However, a marked distinction between these analogs and [4-threonine]-oxytocin is apparent in regard to the differences in antidiuretic activities. Whereas, in the deamino derivatives this activity is increased over the corresponding potencies for oxytocin, in [4-threonine]-oxytocin the reverse is true.

It is interesting to point out that a selective enhancement of antidiuretic activity coupled with a marked decrease in pressor activity has been demonstrated in two 4-substituted derivatives of both [8-lysine]-vasopressin and [8-arginine]-vasopressin. Thus the ratio between antidiuretic and pressor potencies is approximately 1:1 for lysine–vasopressin and arginine–vasopressin, 70:1 for [4-α-aminobutyric acid]-8-lysine–vasopressin, and 200:1 for [1-deamino-4-α-aminobutyric acid]-8-lysine–vasopressin (Gillessen and du Vigneaud, 1967), 20:1 for [4-α-aminobutyric acid]-8-arginine–vasopressin and 95:1 for [1-deamino-4-α-aminobutyric acid]-8-arginine–vasopressin (Gillessen and du Vigneaud, 1970).

In the light of these findings and the findings presented in this report the extension of these studies toward the synthesis and pharmacological evaluation of the 4-threonine analogs of the other naturally occurring neurohypophysial hormones and their respective deamino derivatives is indeed warranted.

With its high oxytocic and low antidiuretic activity, [4-threonine]-oxytocin offers an attractive possibility for clinical application as an oxytocic agent. Water retention and acute water intoxication may occur during prolonged intravenous infusions of oxytocin. [4-Threonine]-oxytocin is twice as active as oxytocin on the rat uterus. When the antidiuretic effects of oxytocin and [4-threonine]-oxytocin are compared directly in assays on rats, one finds that [4-threonine]-oxytocin has only 36 $\pm$ 6% of the antidiuretic activity of an equal weight of oxytocin (Sawyer *et al.*, unpublished data). If [4-threonine]-oxytocin exhibits similar relative oxytocic and antidiuretic activities in women, it could be appreciably safer than oxytocin for infusion to induce labor.

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Preparation and Properties of 3-Nitrotyrosine Insulins*

J. W. S. Morris,† Daniel A. Mercola,‡ and Edward R. Arquilla†

ABSTRACT: Bovine insulin at 2.5 mg/ml, pH 7.4, was nitrated by a 3- to 12-fold excess of tetranitromethane. The products were separated by ion-exchange chromatography (Sephadex A-25) at pH 8.4 into unreacted insulin, mono-3-nitrotyrosine insulin, and di-3-nitrotyrosine insulin. The positions of substitution were determined by oxidative sulfitolysis, followed by ion-exchange chromatography to separate the A and B chains. Substitution occurred almost entirely on the A chain, establishing the diconjugated derivative as di-(A14, A19)-3-nitrotyrosine insulin. Chymotryptic hydrolysis of the monoconjugated derivative established that it was predominantly mono-(A14)-3-nitrotyrosine insulin. The nitrated derivatives were used to confirm Tyr A14 as an anomolously ionizing residue. It is shown that the presence of a Tyr A14-carboxylate hydrogen bond explains the chemical, optical, and titration data on insulin. The results of bioassays and immunoassays are presented.

Many techniques have been developed to study the disposition of tyrosyl residues of proteins in solution. These techniques can be divided into two classes: spectroscopic (near-ultraviolet difference spectrophotometry, spectrophotometric titration, near-ultraviolet circular dichroism, and solvent perturbation spectrophotometry) and chemical modification with reagents that are more or less specific for tyrosyl residues. These different techniques give consistant results with many proteins. Ribonuclease, for example, has three of its six tyrosyl residues buried by the criterion of spectrophotometric titration (Shugar, 1952). Only the three exposed residues are reactive to iodination (Donovan, 1963) and *N*-acetylimidazole (Riordan *et al.*, 1965). These findings have been confirmed by X-ray crystallography (Wyckoff *et al.*, 1967). However, the identification of those tyrosyl residues that contribute to the near-ultraviolet circular dichroism has not yet been accomplished (Simmons and Glazer, 1967; Simpson and Vallee, 1966; Pflumm and Beychok, 1969; Beaven and Gratzer, 1968).

The situation with insulin is much more complex. One (Inada, 1961) or two (Shugar, 1952) of the four tyrosyl residues is assumed to be buried because of an abnormally high p*K*. However, reactivity toward iodination indicates that Tyr A14 and Tyr A19 are "exposed" (de Zoeten and Havinga, 1961). Reactivity toward cyanuric fluoride indicates that Tyr A19 and Tyr B16 are "exposed" (Aoyama *et al.*, 1965), while limited titration data of desoctapeptide insulin indicates that Tyr B26 ionizes normally (Aoyama *et al.*, 1965). Moreover, difference spectrophotometry indicates that Tyr B26 is strongly interacting (Laskowski *et al.*, 1960), while circular dichroism suggests that this residue may be involved in the monomer–dimer aggregation site (Morris *et al.*, 1968). These two techniques also suggest that the environment of another tyrosyl residue, likely A14, is dependent on an anomolous carboxyl group (Laskowski *et al.*, 1960; Morris *et al.*, 1968). However, all the data to date do not permit an unambiguous assignment of the four tyrosyl residues of insulin, nor allow identification of those residues that are "exposed" and "buried" by the criterion of solvent perturbation spectrophotometry (Weil *et al.*, 1965; Menendez *et al.*, 1969).

Recently, tetranitromethane has been added to the available tyrosine reagents (Riordan *et al.*, 1966). Because more data on the tyrosyl residues of insulin are clearly needed, and because the tyrosyl residues of insulin have been implicated in the expression of biological and immunological activity (Arquilla *et al.*, 1968), we have prepared and characterized the 3-nitrotyrosine derivatives of insulin.

Material and Methods

We used two bovine zinc insulin preparations (Lilly, lot 2842 and Novo, lot 016666). Tetranitromethane was obtained from Baker, sodium tetrathionate from K & K Labs, α-chymotrypsin from Calbiochem, and 3-nitrotyrosine and 3,5-dinitrotyrosine from Nutritional Biochemicals. All other chemicals were reagent grade.

Nitration. To limit the extent of reactions, nitration was performed for short times on highly aggregated insulin solutions. Zinc insulin at 2.5 mg/ml in 0.11 M phosphate buffer, pH 7.4, was treated at 23° for 15–50 min with a 3- to 12-fold excess (with respect to tyrosyl residues) of tetranitromethane.

* From the Department of Pathology and the Department of Biophysics, University of California, Los Angeles, California. *Received April 15, 1970.* This work was supported by U. S. Public Health Service Grants 5R01 AM 06001-05 and AM 13441-01. A preliminary report of this work has been published (Morris *et al.*, 1969).

† Present address: Department of Pathology, University of California, Irvine, California 92664. Please send inquiries to this address.

‡ Postdoctoral Fellow, Southern California Diabetes Association. Present Address: Laboratory of Molecular Biophysics, University of Oxford.