[2-(3-Nitrotyrosine)]Oxytocin, a Weakly Active Analogue of the Hormone Bound by Neurophysin

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An analogue of oxytocin containing a nitro group *ortho* to the phenolic hydroxyl group of the tyrosyl residue was prepared by nitration of the hormone with tetranitromethane. [2-(3-Nitro-L-tyrosine)]oxytocin was bound by neurophysin although its pharmacological activity was virtually abolished. The oxytocic activity of the analogue on the isolated rat uterus was 1.1 i.u./mg.

Oxytocin and vasopressin, the polypeptide hormones of the neural lobe of the pituitary gland, are bound not only by receptors in target organs but also by the neurophysins. A structural feature that the hormones and the neurophysins have in common is a single tyrosine residue in each polypeptide chain. The tyrosine residue lies at position 2 in the hormones, and at position 49 in those neurophysins whose sequences have so far been determined.

An aromatic group at position 2 appears to be important not only for the pharmacological activity of the hormones, since the following synthetic analogues of oxytocin are almost inactive, [2-glycine]-oxytocin (Drabarek, 1964), [2-serine]oxytocin (Guttmann & Boissonnas, 1960) and [2-leucine]-oxytocin (Jošt *et al.*, 1963), but also for their binding to neurophysin (Breslow & Abrash, 1966).

Nitrated neurophysin has proved useful in the study of the importance of the tyrosine residue for the binding of the hormones (Furth & Hope, 1970). Nitration of the tyrosine residue in bovine neurophysin II with tetranitromethane did not affect the binding of [8-arginine]vasopressin. The position of this tyrosine residue at or near the hormone-binding site on the protein was revealed by difference spectra in the visible region, and confirmation of this finding has come from changes in circular dichroism resulting from neurophysin–hormone interactions (Breslow & Weis, 1972). Furthermore, negative nuclear Overhauser effects indicate the proximity of the hormone and neurophysin tyrosine residues in the protein–hormone complex (Balaram *et al.*, 1972).

It was decided to establish whether or not neurophysin would bind hormone analogues in which the tyrosine residue was replaced by 3-nitrotyrosine. The advantage to be gained from the use of colorimetry for the study of complex-formation has led us to prepare [2-(3-nitro-L-tyrosine)]oxytocin, a coloured analogue of the hormone.

Some time ago the synthesis of a fully protected nonapeptide intermediate of oxytocin containing a residue of 3-nitro-L-tyrosine in place of tyrosine was reported (Kaurov *et al.*, 1968), and more recently the

hormone analogue itself was synthesized in the same laboratory and reported to be inactive on the rat uterus (Auna *et al.*, 1971).

We now report the preparation of [2-(3-nitro-L-tyrosine)]oxytocin:

from synthetic oxytocin by nitration with tetranitromethane.

Methods

Tetranitromethane was from R. N. Emanuel Ltd., Alpertons, Middx., U.K., and 3-nitro-L-tyrosine and 3,5-dinitro-L-tyrosine were from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Optical rotations were measured on a Bellingham and Stanley polarimeter (model A, type P3) with a 2 dm tube. Chromatographic data refer to t.l.c. on Kieselgel G with butanol–acetic acid–water–pyridine (15:3:12:10, by vol.). Sulphur-containing substances were located with a solution of PtCl_4 in acetone as described by Toennies & Kolb (1951), with one modification, namely that the concentration of PtCl_4 was increased tenfold. The gel-filtration columns were prepared from Sephadex G-25 (fine grade; bead type, 20–80 μm), from Pharmacia (G.B.) Ltd., London W.5, U.K. Elemental analyses were carried out by A. Bernhardt, Elbach, Germany. Amino acid analyses were performed with an automatic analyser (EEL-206; Evans Electroselenium Ltd.) by the method of Spackman *et al.* (1958). Samples were hydrolysed *in vacuo* in 6M-HCl for 18 h. 3-Nitrotyrosine was eluted on the long column after phenylalanine and gave a colour yield 1.39 times that of tyrosine. Countercurrent distribution was run with a fully automatic train of 200 tube-units (H. O. Post and Co., Maspeth, N.Y., U.S.A.). Binding of the nitro analogue by neurophysin was measured by

equilibrium dialysis, as previously described for oxytocin (Hollenberg & Hope, 1967). Oxytocic activity was assayed on the isolated rat uterus (Holton, 1948), with the Mg^{2+} -free van Dyke-Hastings solution suggested by Munsick (1960) against synthetic oxytocin, standardized against the IIIrd International Oxytocic Standard (Bangham & Mussett, 1958). Spectroscopic measurements were carried out with the Unicam SP.800 recording spectrophotometer and the Zeiss PMQII spectrophotometer with 1 cm silica cells. Dimer-free oxytocin was prepared by the stepwise *p*-nitrophenyl ester method (Bodanszky & du Vigneaud, 1959), with the modification of the oxidation procedure devised in this laboratory (Wälti & Hope, 1973).

Results

Tetranitromethane is a highly specific reagent at pH 8 for the nitration of tyrosine residues in proteins (Sokolovsky *et al.*, 1966). The reagent was used successfully to convert the tyrosine residue in bovine neurophysin II into 3-nitrotyrosine (Furth & Hope, 1970). On the other hand treatment of glycytyrosine or insulin with tetranitromethane led to intermolecular cross-linking and little of the desired material (Boesel & Carpenter, 1970). It has been suggested that, where the tyrosine residues of proteins have been satisfactorily nitrated by tetranitromethane, these residues occupy positions such that coupling to other groups is prevented (Williams & Lowe, 1971).

To establish the extent of the reaction of tetranitromethane with the tyrosine residue of oxytocin, a sample of the hormone (0.1 mg) was dissolved in 50 mM-Tris-HCl buffer, pH 8.0 (2.0 ml), and stirred at room temperature with a 100-fold molar excess of tetranitromethane. The tetranitromethane (1.96 mg) was added as a solution in ethanol (1%, w/v), and the rate of nitration was followed on samples of the reaction mixture by measuring the increase in E_{350} (nitroform) as a function of time. The reaction was complete within about 50 min.

In preparative experiments, nitration of oxytocin was done on 50–100 mg of hormone under the conditions described above. Acetic acid was added to the reaction mixture after 1 h to stop the reaction, and [2-(3-nitro-L-tyrosine)]oxytocin was isolated by a combination of gel filtration and countercurrent distribution. Chromatography on Sephadex G-25 in 0.1 M-acetic acid separated the peptide from the nitroform effectively. Two coloured bands were clearly visible on the column: a fast-moving bright-yellow band followed by a pale-yellow band. Spectrophotometric readings gave the typical nitrotyrosine absorption at 428 nm in the faster-moving fraction; the slower-moving fraction had an absorption maximum at 350 nm typical of nitroform, which appeared therefore to be adsorbed by Sephadex.

Although gel filtration did not separate nitro-oxytocin from the components of the Tris buffer, countercurrent distribution was effective.

Dimer-free oxytocin (100 mg) was dissolved in 50 mM-Tris-HCl buffer, pH 8.0 (100 ml), and a 100-fold molar excess of tetranitromethane (980 mg) in ethanol (98 ml; 98%) was added with stirring. After 1 h at room temperature the yellow solution was acidified with acetic acid and concentrated in a flash evaporator at 30°C to a volume of approx. 15 ml. This residue was placed on a column (2 cm × 150 cm) of Sephadex G-25 and eluted with 0.1 M-acetic acid (25 ml/h). The effluent was collected in 12.5 ml fractions every 30 min, and the Folin-Lowry colour values (Lowry *et al.*, 1951) showed that the first yellow peak (fractions 15–35) contained the peptide material. Spectrophotometric readings on these fractions gave the nitrotyrosine absorption at 428 nm. The second peak (fractions 39–65) was Folin-negative and absorbed maximally at 350 nm (nitroform). The peptide-containing fractions were pooled and concentrated in a flash evaporator at 30°C to a volume of approx. 40 ml. The volume was made up to 50 ml with the lower phase of the solvent system butan-1-ol-propan-1-ol-0.05% acetic acid (2:1:3, by vol.) and the solution was placed in the first five tubes of the countercurrent-distribution apparatus. The distribution was carried through a total of 400 transfers. A separation into two main peaks with *K* values of 0.12 (Tris buffer) and 0.51 had been accomplished, as was detected by the Folin-Lowry colour values. The contents of tubes 120–180, containing [2-(3-nitro-L-tyrosine)]oxytocin, were pooled and concentrated in a flash evaporator at 30°C to a volume of approx. 25 ml. The solution was filtered to remove mechanical impurities, diluted to 50 ml with water and freeze-dried. The yellow non-crystalline material (48 mg; 46% yield) had $[\alpha]_D^{21.5} -67.8 \pm 3.5^\circ$ ($n = 5$) (c 0.05 in 1 M-HCl) and R_F 0.73. Oxytocin as a reference had R_F 0.70. The moisture content of the freeze-dried powder was 4.7% ($n = 2$) [P_2O_5 , 100°C at 267 Pa (2 mmHg)] (Found: C, 48.8, H, 6.2; N, 17.5. $C_{43}H_{65}N_{13}O_{14}S_2$ requires C, 49.1; H, 6.2; N, 17.3%). Amino acid analysis of three acid hydrolysates gave the following average molar proportions: ammonia, 2.8; aspartic acid, 1.1; glutamic acid, 1.0; proline, 0.9; glycine, 1.1; half-cystine, 1.8; isoleucine, 0.9; leucine, 1.0; 3-nitrotyrosine, 0.8; tyrosine and the dinitro derivative were absent. [2-(3-Nitro-L-tyrosine)]oxytocin gave an oxytocic activity of 1.1 ± 0.1 ($n = 3$) i.u./mg. The ability of neurophysin to bind the analogue was assessed by thin-film dialysis. A solution (0.5 ml) containing approx. 4 mg of bovine neurophysin II was introduced on the inside of an 18/32 Visking membrane. This solution was dialysed against 0.1 M-sodium phosphate buffer, pH 5.8 (9.0 ml), containing the nitro analogue (approx. 0.7 mg) in the outer compartment. The system was

allowed to equilibrate for 20h; the volume of fluid in each compartment was measured and the concentrations of the nitro analogue in the solutions were measured colorimetrically. The nitro analogue became concentrated more than 25-fold on the inside of the membrane containing the neurophysin, each milligram of which bound 0.103 ± 0.002 mg ($n = 3$) of the nitro analogue [i.e. 1 (0.97) mol of nitrated oxytocin/mol of neurophysin of mol.wt. 10000]. Thus the introduction of a nitro group into the tyrosine residue of oxytocin does not affect the ability of the peptide to form a complex with neurophysin. In contrast the pharmacological activity was decreased to a fraction of a per cent of the activity of the hormone itself. The reverse of this situation has been reported by us for another analogue of oxytocin (Wälti & Hope, 1972): [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin, a highly potent oxytocin analogue approximately isosteric with oxytocin, was not bound by bovine neurophysin II.

These results draw attention to the fact that oxytocin must satisfy two different structural requirements. On the one hand oxytocin must bind with and stimulate pharmacological receptors, and on the other hand it must bind with a neurophysin within neurosecretory vesicles. It is perhaps not surprising therefore that it has been possible to synthesize analogues, such as deamino-oxytocin and the hydroxy isostere, that are more active pharmacologically than the hormone itself. The binding of oxytocin by neurophysin is vitally dependent on the presence of a charged amino group at position 1, and the lack of the positive charge explains the inability of these two highly active analogues to form complexes with neurophysins.

The present results show that the introduction of a nitro group at the tyrosine residue of oxytocin did not impair the ability of neurophysin to bind the peptide, although pharmacological activity was almost abolished.

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