Short Communications

[1-(L-2-Hydroxy-3-mercaptopropanoic acid)]-oxytocin, a Highly Potent Analogue of Oxytocin Not Bound by Neurophysin

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Studies on the binding of the neurohypophyseal hormones, oxytocin and the vasopressins, to neurophysin show that the first three amino acid residues at the *N*-terminus are important (Stouffer, Hope & du Vigneaud, 1963; Breslow & Abrash, 1966; Hollenberg & Hope, 1967). In particular the terminal amino group at position 1 is essential for binding oxytocin as well as [8-arginine]-vasopressin (Fig. 1). This conclusion comes from work with synthetic analogues of the hormones in which the primary amino group was replaced by a hydrogen atom. Neither deamino-oxytocin (Fig. 1) nor deamino-[8-arginine]-vasopressin (Fig. 1) formed complexes with neurophysins. In contrast, the hormonal activities, e.g. milk ejection and anti-diuresis, are enhanced in the deamino analogues (Hope, Murti & du Vigneaud, 1962; Ferrier, Jarvis & du Vigneaud, 1965; Huguenin & Boissonnas, 1966). The primary amino group appears to have a precise biological role in relation to the neurophysins. The neurosecretory granules of the mammalian pituitary gland store the neurophysins and the hormones together (Dean & Hope, 1968). Presumably the proteins retain the hormones within the granules for transport and storage within neurons.

The nature of the hormone-binding site on neurophysin has not yet been defined. The possibility that an electrostatic linkage might be responsible was suggested by Haselbach & Piguet (1952) on the basis of electro dialysis experiments. However, the pK_a of the amino group in oxytocin is reported to be close to 6.3 (Breslow, 1961). At physiological pH (7.2–7.4) only 10% of the molecules would be positively charged. Unless this value is effectively increased in the micro-environment within a storage granule, e.g. by a proton-donating group in the neighbourhood of the binding site of the protein (Furth & Hope, 1970), we must consider the possibility that the contribution made by the amino group to the binding is through hydrogen bonds. It was with this in mind that we have synthesized [1-(L-2-hydroxy-3-

mercaptopropanoic acid)]-oxytocin, an analogue of oxytocin in which the amino group is replaced by a hydroxyl group (Fig. 1, X = OH).

The analogue of oxytocin was synthesized by a dipeptide–heptapeptide coupling. The sequence 3–9 was prepared as a protected heptapeptide by the stepwise *p*-nitrophenyl ester procedure (Bodanszky & du Vigneaud, 1959).

The free heptapeptide was recrystallized from water to remove diastereoisomerides (Hope & du Vigneaud, 1962). Sequence 1–2 was incorporated as *p*-nitrophenyl L-2-acetoxy-3-benzylthiopropionyl-L-tyrosinate, synthesized by a dicyclohexylcarbodi-imide coupling of *p*-nitrophenyl L-tyrosinate hydrobromide with the *O*-acetyl derivative of L-2-hydroxy-3-benzylthiopropionic acid (Hope & Wälti, 1970). The fragment had m.p. 137–139°C, $[\alpha]_D^{25} -34.3 \pm 0.3^\circ$ (c 1 in dimethylformamide) (Found: C, 60.1; H, 4.7; N, 5.1; S, 6.1. $C_{27}H_{26}N_2O_8S$ requires C, 60.2; H, 4.9; N, 5.2; S, 5.9%).

The crystalline heptapeptide was coupled with *p*-nitrophenyl L-2-acetoxy-3-benzylthiopropionyl-L-tyrosinate in dimethylformamide to give L-2-acetoxy-3-benzylthiopropionyl-L-tyrosinyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine amide as a solid in a yield of 95%. The acetyl group was removed from this peptide by treatment with methanolic ammonia. Amino acid analysis of an acid hydrolysate of the material by an E.E.L. 194 automatic amino acid analyser by the method of Spackman, Stein & Moore (1958) gave the following molar proportions with the value for isoleucine taken as 1: *S*-benzylcysteine, 1.1; ammonia, 2.9; aspartic acid, 1.1; glutamic acid, 1.1; proline, 0.9; glycine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 1.0.

For the preparation of [1-(2-hydroxy-3-mercaptopropanoic acid)]-oxytocin the *SS'*-dibenzyl peptide was reduced with sodium in liquid ammonia. The resulting dithiol was oxidized to the disulphide, after evaporation of the ammonia, by potassium

Fig. 1. Formulae of oxytocin ($X = \text{NH}_2$), [8-arginine]-vasopressin ($X = \text{NH}_2$, 3 = Phe, 8 = Arg), deamino-oxytocin ($X = \text{H}$), deamino-[8-arginine]-vasopressin ($X = \text{H}$, 3 = Phe, 8 = Arg) and [1-(L-2-hydroxy-3-mercaptopropanoic acid)]-oxytocin ($X = \text{OH}$).

ferricyanide in aqueous solution at pH 7.0 (Hope & du Vigneaud, 1962). The biologically active disulphide was purified by countercurrent distribution in the system butan-1-ol-toluene-0.05% acetic acid (3:2:5, by vol.). After 600 transfers the active material was concentrated in a single peak having a partition coefficient, K , of 0.33. The curve obtained by plotting the Folin-Lowry colour values (Lowry, Rosebrough, Farr & Randall, 1951) was in excellent agreement with the curve obtained from the values of the oxytocic activity on the isolated rat uterus and with a theoretical curve.

The combined contents of the tubes containing the active analogue were concentrated and freeze-dried. This material, $[\alpha]_D^{18} -91.5 \pm 2.5^\circ$ (c 0.05 in 1M-acetic acid), was dried at 100°C *in vacuo* over phosphorus pentoxide to constant weight (Found: C, 51.0; H, 6.7; N, 15.0; loss at 100°C , 6.2. $\text{C}_{43}\text{H}_{65}\text{N}_{11}\text{O}_{13}\text{S}_2$ requires C, 51.2; H, 6.5; N, 15.3%). Paper chromatography of the [1-(L-2-hydroxy-3-mercaptopropanoic acid)]-oxytocin on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase) gave a single spot at R_F 0.70 with the platinum iodide reagent of Toennies & Kolb (1951). Oxytocin has R_F 0.57 in this system. Amino acid analysis of an acid hydrolysate of the material by the E.E.L. 194 automatic amino acid analyser showed the presence of a peak on the chromatogram identical with the position of that found for an authentic sample of the mixed disulphide of cysteine and 2-hydroxy-3-mercaptopropanoic acid, 3,3'-dithio-L-2-hydroxy-L-2'-amino-dipropionic acid (Wälti & Hope, 1971). The following molar proportions were obtained, with the value

for tyrosine taken as 1: ammonia, 2.8; aspartic acid, 1.1; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; isoleucine, 1.0; leucine, 1.1; tyrosine, 1.0; cystine, 0.3; the mixed disulphide, 0.5. The two sulphur compounds fully account for the half-cysteine residue in the analogue. The presence of cystine would be expected from an acid-catalysed disulphide interchange (Ryle & Sanger, 1955). The oxytocic activity was assayed on the isolated rat uterus by using the method of Holton (1948) with the Mg^{2+} -free van Dyke-Hastings solution suggested by Munsick (1960) against synthetic oxytocin standardized against the Third International Oxytocic Standard (Bangham & Mussett, 1958). [1-(2-Hydroxy-3-mercaptopropanoic acid)]-oxytocin gave an activity of 1607 ± 20 ($n = 7$) i.u./mg. The value for oxytocin itself, isolated under comparable conditions, was 523 ± 8 ($n = 3$) i.u./mg.

The ability of a neurophysin to bind the hydroxy analogue and of oxytocin itself was assessed by thin-film dialysis in the 'alternate' cell described by Craig & Konigsberg (1961). A solution (0.5ml) containing approx. 1mg of bovine neurophysin-II was introduced on the inside of the 18/32 Visking membrane. This solution was dialysed against sodium acetate buffer, pH 5.8 and 10.1 (8.0ml), containing either oxytocin or the hydroxy analogue (approx. 10 i.u./mg) in the outer compartment. The system was allowed to equilibrate at 4°C for 42h; the volume of fluid in each compartment was measured and the solutions were assayed for oxytocic activity. The oxytocin became concentrated more than 25-fold on the inside of the membrane

containing the neurophysin, each milligram of which bound 47 i.u. of oxytocic activity [i.e. 1 (0.98) mol of oxytocin/mol of neurophysin of mol.wt. 10 000]. In the experiments with the hydroxy analogue the concentrations of this substance on the inside and the outside of the membrane were essentially the same (ratio 0.96, mean of two results).

Thus the analogue of oxytocin containing a hydroxyl group in place of the primary amino group is not bound by neurophysin, demonstrating that a strongly hydrogen-bonding hydroxyl group cannot be substituted for a primary amino group in oxytocin and retain the affinity for neurophysin.

We conclude that the binding of oxytocin by neurophysin requires a basic group in position 1 of the hormone molecule and that an electrostatic force is of primary importance for the formation of a protein-hormone complex.

The observation that removal of the primary amino group and replacement by a hydrogen atom in oxytocin led to an increase in pharmacological activity suggested that the presence of an α -substituent at position 1 was detrimental (Hope *et al.* 1962). It was surprising therefore to find that the [1-(L-2-hydroxy-3-mercaptopropanoic acid)] analogue with a hydroxyl group at position 1 in oxytocin possessed twice the activity found in the deamino analogue (803 ± 36 i.u./mg; Ferrier *et al.* 1965).

The basic property of the amino group in oxytocin is unlikely to account for the threefold difference in oxytocic activity between oxytocin and its [1-(L-2-hydroxy-3-mercaptopropanoic acid)] analogue because at the pH used for bioassay (pH 7.4) only 10% of the amino groups will be ionized. It seems likely that further studies with similar hydroxy analogues of oxytocin and vasopressin will

lead to a better understanding of the hormone-receptor interaction.

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- Bangham, D. R. & Mussett, M. W. (1958). *Bull. World Hlth Org.* **19**, 325.
- Bodanszky, M. & du Vigneaud, V. (1959). *J. Am. chem. Soc.* **81**, 5688.
- Breslow, E. (1961). *Biochim. biophys. Acta*, **53**, 606.
- Breslow, E. & Abrash, L. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 640.
- Craig, L. C. & Konigsberg, W. (1961). *J. phys. Chem., Ithaca*, **65**, 166.
- Dean, C. R. & Hope, D. B. (1968). *Biochem. J.* **106**, 565.
- Ferrier, B. M., Jarvis, D. & du Vigneaud, V. (1965). *J. biol. Chem.* **240**, 4264.
- Furth, A. J. & Hope, D. B. (1970). *Biochem. J.* **116**, 545.
- Haselbach, H. & Piguët, A. R. (1952). *Helv. chim. Acta*, **35**, 2131.
- Hollenberg, M. D. & Hope, D. B. (1967). *Biochem. J.* **105**, 921.
- Holton, P. (1948). *Br. J. Pharmac. Chemother.* **3**, 328.
- Hope, D. B. & du Vigneaud, V. (1962). *J. biol. Chem.* **237**, 3146.
- Hope, D. B., Murti, V. V. S. & du Vigneaud, V. (1962). *J. biol. Chem.* **237**, 1563.
- Hope, D. B. & Wälti, M. (1970). *J. chem. Soc. C*, p. 2475.
- Huguenin, R. L. & Boissonnas, R. A. (1966). *Helv. chim. Acta*, **49**, 695.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Munsick, R. A. (1960). *Endocrinology*, **66**, 451.
- Ryle, A. P. & Sanger, F. (1955). *Biochem. J.* **60**, 535.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Stouffer, J. E., Hope, D. B. & du Vigneaud, V. (1963). In *Perspectives in Biology*, p. 75. Ed. by Cori, C. F., Voglia, V. G., Leloir, L. F. & Ochoa, S. Amsterdam: Elsevier Publishing Co.
- Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Wälti, M. & Hope, D. B. (1971). *J. chem. Soc. C*, p. 2326.