INACTIVATION OF DEAMINO-CARBA ANALOGUES OF OXYTOCIN IN THE RAT UTERUS, LIVER AND KIDNEY

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The deamino-carba analogues of oxytocin are inactivated at a lower rate than oxytocin in homogenates of uterine, liver and kidney tissue. The enzymes responsible for the inactivation of oxytocin analogues are located in the 105000 *g* supernatant. The mechanism of the degradation of deamino-carba oxytocin analogues is discussed.

The insight into the role of inactivation processes involved in the metabolism of natural peptide hormones in target tissues has stimulated the synthesis of hormone analogues resistant to enzymic attack. The first metabolic studies led to the conclusion that the loss of biological activity of neurohypophysial hormones is due to the hydrolysis of the peptide bond between the N-terminal amino acids of the chain[1]. In tissues, aminopeptidase cleavage is probably preceded by reversible reduction of the disulphide bridge[2]. In order to eliminate the possibility of aminopeptidase degradation and reduction, an attempt was made to modify the chemical structure of oxytocin and thus produce analogues which would be metabolically more stable than the hormone itself. In view of this requirement, cyclic peptides with carba or dicarba bridges in place of the disulphide bridge were synthetized[3,4]. Scheme 1 shows the changes in the molecular structure of the parent hormone.** All the analogues are derived from deamino-oxytocin[5].

Studies on the pharmacological properties of the analogues showed the physiological consequences of changes in the hormone structure[6,7]. It was established that neither the terminal amino group nor the disulphide bridge (which was thought to be indispensable for the disulphide interaction with the receptor[8]) are necessary for evoking the biological response. However, certain differences between the effects of the individual derivatives on various tissues were observed. For example, the uterotonic effect of DCOT-1 and DCOT-6 is several times higher than that of oxytocin, and even DDCOT which has no sulphur atom in its molecule retains 20% of the oxytocin uterotonic activity. The antidiuretic action of these analogues on the kidney follows a similar pattern. It was, therefore, necessary to perform detailed

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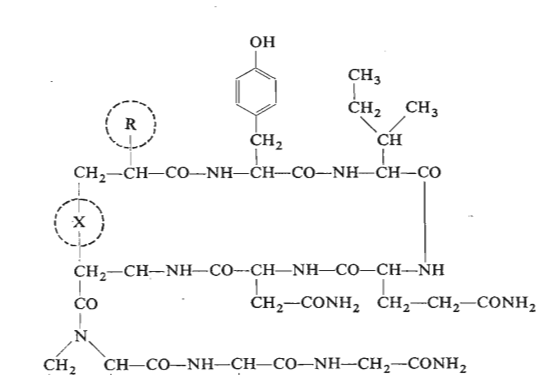
** Abbreviations: OT oxytocin, DCOT-1 α-deamino-cystathionine^{6,1}-oxytocin (deamino-carba^1-oxytocin), DCOT-6 β-deamino-cystathionine^{1,6}-oxytocin (deamino-carba^6-oxytocin), DDCOT α-aminosuberic acid^1-oxytocin (deamino-dicarba^{1,6}-oxytocin).

studies of the rate at which the individual analogues are inactivated in the uterus, liver, and kidney and investigate the pathways of their degradation. The solution of this problem could help to determine to what extent the rate of inactivation influences the character and degree of the biological response.



Oxytocin: X = —S—S—, R = NH_2 DCOT-6: X = —S—CH_2—, R = H

DCOT-1: X = —CH_2—S—, R = H DDCOT: X = —CH_2—CH_2—, R = H

SCHEME 1

EXPERIMENTAL

Material. Oxytocin was purchased from Spofa, Prague. DCOT-1, DCOT-6 and DDCOT were prepared at the Department of Organic Synthesis of this Institute[3,4]. Female rats of the Wistar-Konárovice strain, weighing 180—200 g, were used in the experiments.

Uterine homogenate and its subcellular fractions: The animals were killed by decapitation, the uterine horns were excised, freed of adipose tissue and endometrium, weighed, and placed in cold homogenization medium (1·5 mM-$MgCl_2$, 10 mM-KCl, 10 mM Tris-HCl; pH 7·2). The tissue was then cut into small pieces and ground in a mortar with sand. The homogenate (approximately 20% w/v) was filtered through a double layer of gauze and the sand was removed by repeated decantation. An aliquot of the homogenate was centrifuged at 105000 *g* for 60 minutes. The 105000 *g* supernatant and a suspension of subcellular particles were used for the determination of enzymic activity. All these steps were performed at 0—4°C.

Liver and kidney homogenates: Both organs were excised, weighed, and placed in cold homogenization medium. The tissues were cut into small pieces and homogenized in a Potter–Elvehjem homogenizer placed in an ice bath. The homogenates (approximately 20% w/v) were filtered through gauze and aliquots were centrifuged in the same way as the uterine homogenate.

Incubation: The individual analogues were incubated for 0—120 minutes with the uterine homogenate or its subcellular fractions, and 0—30 minutes with homogenates of liver and kidney tissue or their fractions. The incubation mixture had the following composition: 1 μM peptide, 50 mM sodium phosphate buffer — pH 7·4, and 0·3—0·8 mg of protein nitrogen/ml. The incubation was performed at 37°C and the reaction was stopped by placing the samples in a boiling water bath for 3 minutes. A 1 μM solution of the peptide tested was incubated without the enzymic preparation for 120 minutes and used as a standard.

Determination of oxytocin and its analogues: The remaining amount of oxytocin and its analogues in the samples at the end of the incubation was determined on the isolated rat uterus according to Munsick[9]. The results were evaluated by the four point test. The half-time of peptide decay was determined graphically from the time course of the decrease of biological activity. The degree of inactivation was calculated for the content of protein nitrogen in the incubation mixture.

RESULTS AND DISCUSSION

All the compounds studied were inactivated in the uterine homogenate. The following Table presents the mean values of the rate of inactivation expressed in terms of the half-time (min) of decay.

Homogenate	OT	DCOT-1	DCOT-6	DDCOT
Uterus	7·40	18·20	15·00	17·30
Kidney	0·44	2·55	2·76	1·89
Liver	0·74	8·30	5·30	4·80

On the average, DCOT-1, DCOT-6 and DDCOT were decomposed at approximately half the rate of oxytocin inactivation. As can be seen from Table I, the enzymes which degrade oxytocin analogues were located in the 105000 *g* supernatant and no inactivation occurred in the sediment. Slight inactivation of oxytocin was observed in the sediment. The rate of enzymic cleavage of oxytocin and its analogues was considerably higher in liver and kidney homogenates than in the uterus. The half-time of decay of the analogues was 5—10 times longer than that of oxytocin. As in the uterine tissue, the enzymes which inactivate oxytocin and its analogues were located in the 105000 *g* supernatant (Table I). Although the analogues were not inactivated in the sediment, oxytocin was degraded at a higher rate than in the uterine particulate fraction.

TABLE I

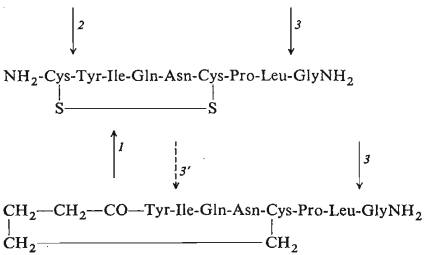
Inactivation of Oxytocin and its Analogues

Half-time of peptide decay (min).

Homogenate	Fraction[a]	OT	DCOT-1	DCOT-6	DDCOT
Uterus	P	18·80	[b]	[b]	[b]
	S	3·20	6·20	4·80	6·40
Kidney	P	0·61	[b]	[b]	[b]
	S	0·33	1·68	3·15	0·87
Liver	P	1·08	[b]	[b]	[b]
	S	0·85	2·40	3·85	2·80

[a]P 105 000 *g* sediment; S 105 000 *g* supernatant; [b]no inactivation.

The elimination of the primary amino group and the replacement of the disulphide bridge by a thioether group results in an enhancement of the uterotonic and antidiuretic activity of this type of oxytocin analogue[6]. One of the possible explanations of the increased activity is the limited enzymic cleavage. The present knowledge of the mechanisms by which oxytocin and the deamino-carba analogues are enzymically degraded is presented in Scheme 2. It is obvious that the three analogues studied cannot be inactivated by the reduction of the disulphide bond nor by aminopeptidase cleavage, *i.e.* by mechanisms which result in inactive oxytocin degradation products.



SCHEME 2

Enzymic Inactivation of Oxytocin and Deamino-dicarba Oxytocin

1 Reduction of the disulphide bridge, *2* aminopeptidase cleavage, *3* chymotryptic cleavage (carboxyamidase), *3'* chymotryptic cleavage (possible only in the case of deamino-oxytocin derivatives).

The inactivation of the analogues seems to be restricted to endopeptidase cleavage, most probably of chymotrypsin nature[10,11]. The oxytocin analogues lacking the terminal amino group are susceptible to chymotryptic cleavage not only between leucine and glycine amide but also between tyrosine and isoleucine[12].

Although the rate at which chymotrypsin attacks the individual bonds has not been determined yet, it may be expected that the bond involving an aromatic amino acid will be split at a higher rate. Recent findings, concerning the substrate specificity of the tissue enzyme which splits off glycine amide at the C-end of oxytocin[13,14], show that neither the uterine nor the kidney enzyme can attack the peptide bond between tyrosine and isoleucine in the peptide chain of DDCOT. These results imply that only the bond between leucine and glycine amide of the deamino-carba analogues can be split by carboxyamidase. The question of other possible inactivation mechanisms (*i.e.* the oxidation of the tyrosine side chain and/or the deamination of the individual carboxyamide groups) remains unsolved.*

The inactivation of oxytocin is more complicated; apart from non-specific tissue aminopeptidases which come into play after the reduction of the disulphide bridge, the hormone is also subject to endopeptidase cleavage. The fact that oxytocin rapidly loses its biological activity in homogenates under our experimental conditions supports the view that several enzymic processes participate in the degradation of the hormone. In our experiments with the uterine homogenate we found that DCOT-1, DCOT-6 and DDCOT are decomposed at half the rate of oxytocin inactivation. By contrast, the half-time of decay of the analogues is 5–10 times longer than that of oxytocin in kidney and liver homogenates. These findings seem to indicate that the kidney and liver homogenates inactivate oxytocin mainly by the reduction of its disulphide bridge, whereas the most important inactivation mechanism in the uterus is the enzymic liberation of the C-terminal glycine amide.

The time course of the loss of the biological activity of oxytocin and its deamino-carba analogues in the liver homogenate gives evidence of a more complicated reaction mechanism of inactivation. The occurrence of lag periods, or waves, in the time course of inactivation was observed by Pliška[15] in his studies in the inactivation of oxytocin and deamino-oxytocin in homogenates and minced liver tissue. The rapid decrease of the biological activity of carba analogues during the first minutes of incubation, followed by a temporary lag period in the decomposition of the hormone, was explained by the presence of a binding protein or a substrate which competes with oxytocin and deamino-oxytocin during the inactivation. Studies on the two-phase course of analogue inactivation are in progress.

Studies on the degradation of hormones in homogenates can serve only as a rough estimate of the physiological inactivation processes which take place in intact tissues. However, if the inactivation of oxytocin and its deamino-carba analogues is compared, it is obvious that the modification of the molecule of the parent hormone was successful in that it lowered the rate of peptide degradation in the target tissues.

* See note added in proof.

The modifications affect those groups which are not directly involved in evoking the biological response, and at the same time eliminate some possibilities of the enzymic decomposition of the analogues.

REFERENCES

1. Tuppy H., Nesvadba H.: Monatsh. *88*, 977 (1957).
2. Rychlík I.: *Oxytocin, Vasopressin and their Structural Analogues* (J. Rudinger, Ed.), p. 131. Published by Zdravotnické nakladatelství, Prague, and Pergamon Press, Oxford 1964.
3. Jošt K.: This Journal *36*, 218 (1971).
4. Jošt K., Šorm F.: This Journal *36*, 234 (1971).
5. du Vigneaud V., Winestock G., Murti V. V. S., Hope D. B., Kimbough R. D., jr: J. Biol. Chem. *235*, PC 64 (1960).
6. Krejčí I.: Unpublished results.
7. Barth T.: Unpublished results.
8. Schwartz I. L., Rasmussen H., Schoessler M. A., Silver L., Fong C. T. O.: Proc. Natl. Acad. Sci. US *45*, 1288 (1960).
9. Munsick R. A.: Endocrinology *66*, 451 (1960).
10. Folk J. E., Cole P. W.: J. Biol. Chem. *240*, 193 (1965).
11. Barth T., Pliška V., Rychlík I.: This Journal *32*, 1058 (1967).
12. Barth T.: Unpublished results.
13. Glass J. D., Dubois B. M., Schwartz I. L., Walter R.: Endocrinology *87*, 730 (1970).
14. Koida M., Glass J. D., Schwartz I. L., Walter R.: Endocrinology *88*, 633 (1971).
15. Pliška V.: Unpublished results.

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Note added in proof: The splitting of the prolyl-leucyl peptide bond of oxytocin by a human uterine enzyme was described (Walter R., Shlank H., Glass J. D., Schwartz I. L., Kerényi T. D.: Science *173*, 827 (1971)).