THE PREPARATION AND METABOLIC FATE OF TRITIATED N^{α}-ACETYL[2-O-METHYLTYROSINE]OXYTOCIN — AN INHIBITOR OF THE UTEROTONIC ACTION OF OXYTOCIN

Vera BOJANOVSKA*, Tomislav BARTH[a], Bohuslav ČERNÝ[b], Karel HAUZER[a] and Karel JOŠT[a]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 and*
[b] *Isotope Laboratory of Biological Institutes, Czechoslovak Academy of Sciences, 142 20 Prague 4*

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An inhibitor of the uterotonic action of oxytocin-[CH_3CO-^3H] [2-O-methyltyrosine]oxytocin — having biological activity and a specific radioactivity of 3—7 Ci/mmol was prepared by the reaction of [2-O-methyltyrosine]oxytocin with 2,2′-$^3H_2$-acetanhydride. The analogue was stable in human pregnancy serum. Chymotrypsin split the Tyr(Me)-Ile and Leu-GlyNH_2 peptide bonds. In the presence of subcellular fractions of homogenates of the rat uterus and mammary gland, several metabolic products were formed from the analogue.

The substitution of the primary amino group of cysteine by an acyl group, supplemented with the replacement of the hydroxyl of tyrosine in position 2 of the oxytocin peptide chain by a methoxyl group, is one of the possible ways of preparing antagonists of oxytocin[1,2]. N^{α}-Acetyl[2-O-methyltyrosine]oxytocin was found to be one of the most potent inhibitors in this series[3,4]. It is possible that the clinical application of this analogue may prove useful. In order to study its properties in more detail, we prepared the compound in labelled form. The metabolic stability of the analogue was investigated in experiments with subcellular fractions of homogenates of the target tissues for oxytocin in rats and in model interactions with chymotrypsin.

EXPERIMENTAL

Materials

2,2′-$^3H_2$-*Acetanhydride.* 20 mg of palladium oxide was reduced by hydrogen in 0·3 ml of dioxane. Dioxane was removed by lyophilization. 20·8 mg of sodium bromoacetate and 0·2 ml of dimethylformamide were added to the catalyst. Hydrogenation was performed with 40% tritium at room temperature and at a pressure of 85 kPa for 50 min; the amount of gas consumed

* Predoctoral Fellow from the Institute of Infectious and Parasitic Diseases, Bulgarian Medical Academy of Sciences, Sofia, Bulgaria.

was 4·4 ml. The 2-$^3H_1$-acetic acid was freeze-dried together with the solvent, dicyclohexylcarbodiimide (9·5 mg) was added and the mixture was left overnight in a refrigerator. The resultant mixture of 2,2'$^3H_2$-acetanhydride and solvent was freeze-dried.

[CH_3CO-^3H][2-O-*methyltyrosine*]*oxytocin* (^{3}H-AOT): 21·5 mg of [2-O-methyltyrosine]-oxytocin were added to the solution of 2,2'-$^3H_2$-acetanhydride in dimethylformamide and the mixture was kept for 60 h in a refrigerator. The product was precipitated with 10 ml of ether, isolated by centrifugation and purified on a column of CM-Sephadex C-25 in a system of water–pyridine–acetic acid (90 : 10 : 0·295), pH 6·5. The product was pure by chemical and radiochemical standards, its yield was 50% (with respect to [2-O-methyltyrosine]oxytocin) and its specific radioactivity was 3—7 Ci/mmol.

Storage of ^{3}H-AOT: 1·2 mg of ^{3}H-AOT with a specific radioactivity of 7 Ci/mmol was dissolved in H_2O so as to result in a concentration of 600 μCi/ml; the solution was placed in ampoules which were then sealed. The samples were stored at —18°C for 1 year without decomposition. After this period, other radioactive components began to appear. They were removed from the ^{3}H-AOT samples by gel filtration on a column of Bio-Gel P-2 (1 × 75 cm) in H_2O.

N^{α}-*Acetyl*[2-O-*methyltyrosine*]*oxytocin* (AOT) was prepared according to reference[3,5]. Dansyl-amino acids were purchased from Serva (West Germany).

Methods

Thin layer chromatography of ^{3}H-AOT and determination of the distribution of the radioactive compounds. ^{3}H-AOT chromatographed on Silufol UV 254 plates in a system of butanol–CH_3COOH–H_2O (4 : 1 : 1). The distribution of the radioactive compounds was measured by means of a Packard 7201 Chromatogram Scanner. The surfaces delimitated by the individual peaks were integrated in order to establish the percentage of the radioactive compounds present.

Determination of biological activity. The inhibitory potency of the labelled preparation was tested on the isolated rat uterus according to Holton[6] in the modification of Munsick[7]. The degree of inhibition was estimated from the shift in the dependence of the response (uterine contractions) on the log of oxytocin doses in the presence of the labelled inhibitor[8].

Cleavage of ^{3}H-AOT *by chymotrypsin*: To 50 μl of ^{3}H-AOT (6 μCi), 20 μl of 0·2M Tris-HCl buffer (pH 7·8) and 100 μl of chymotrypsin solution (1 mg/ml or 50 μg/ml) were added, the volume was made up to 200 μl with H_2O and the sample was incubated at 37°C. At regular intervals (0—120 min), 20 μl samples were withdrawn, boiled for 5 min and then analysed by TLC on Silufol sheets (Kavalier, Votice) in a solvent system of butanol–acetic acid–water (4 : 1 : 1).

Incubation of AOT *with chymotrypsin*: To 50 μl of AOT (2 mg/ml), 20 μl of 0·2M Tris-HCl buffer (pH 7·8) and 100 μl of chymotrypsin solution (1 mg/ml in experiment 1 or 50 μg/ml in experiment 2) were added, the volume was made up to 200 μl and the samples were incubated at 37°C. After 1 h of incubation, the enzymic reaction was stopped by 5 min boiling and the samples were treated as stated below. A sample containing chymotrypsin (1 mg/ml) was used as a blank solution (experiment 3).

Determination of N-*terminal amino acids* of the products of chymotryptic cleavage by means of the dansylation method: Samples from experiments 1—3 and a model mixture containing 100 nmol of Ile and 100 nmols of $GlyNH_2$ were treated according to the dansylation method of Hartley[9]. Afterwards, 5 μl samples were subjected to chromatography on Silufol sheets in a solvent system of toluene–ethylenechlorhydrin–acetic acid (150 : 80 : 6·7), using dansyl-isoleucine and dansyl-glycine as standards.

Cleavage of ^{3}H-AOT *by human pregnancy serum*: To 20 µl of ^{3}H-AOT (120 mCi/ml), 20 µl of 0·2M Tris-HCl buffer (pH 7·8) and 40 µl of human pregnancy serum were added, and the total volume was made up to 100 µl with water. The sample was incubated for 2 h at 37°C, then boiled for 5 min, centrifuged and a 10—20 µl aliquot was subjected to thin layer chromatography. Radioactivity was measured as stated above.

Incubation of ^{3}H-AOT *with subcellular fractions of rat uterine homogenate*: Three female rats of the Wistar-Konárovice strain, weighing 180—190 g, were estrogenized by a subcutaneous injection of Agofollin (Spofa) 24 h before the experiment. The rats were stunned, decapitated, the uterine horns were excised and placed in chilled buffer (0·2M Tris-HCl — 5 mM $MgCl_2$, pH 7·8; 1 g of tissue per 10 ml of buffer). The uterus was cut up and homogenized in a Kungel-Janke homogenizator at 5000 rpm, twice for 30 s. The homogenate was filtered through gauze and the filtrate was centrifuged at 4°C for 20 min at 600 *g*. The supernatant was saved, the sediment was washed and centrifuged repeatedly, and then resuspended in the buffer by means of a glass homogenizator. In this way we obtained two subcellular fractions — the 600 *g* fraction and the supernatant, both with 1·2—1·5 mg of protein/ml. The incubation of ^{3}H-AOT with the fractions was performed as follows: 50 µl of ^{3}H-AOT (120 mCi/ml) was diluted with 20 µl of 0·2M Tris-HCl buffer (pH 7·8), containing 5 mM $MgCl_2$, 50 µl of subcellular fraction were added, the volume was made up to 200 µl by adding H_2O and the sample incubated for 2 h at 37°C. The samples were then boiled for 5 min, centrifuged and 20 µl aliquots were analysed by thin layer chromatography. The distribution of radioactivity was detected in the above-mentioned way.

Incubation of ^{3}H-AOT *with subcellular fractions of the homogenate of lactating rat mammary gland:* A lactating rat (9—15 days post partum) was stunned, decapitated, the abdominal skin was cut open, and the mammary tissue was excised and placed in chilled 0·2M Tris-HCl buffer, pH 7·8, containing 5 mM $MgCl_2$ (1 mg of tissue per 10 ml). The homogenate of the mammary tissue and its subcellular fractions were prepared in the same way as those of uterine tissue. The same conditions were also chosen for the incubation of ^{3}H-AOT with the subcellular fractions and the analysis of the metabolic products.

RESULTS

Preparation of tritiated AOT: Chromatographic analysis proved the chemical purity of the compound. The preparation yielded approximately 50% of ^{3}H-AOT with a specific radioactivity of about 7 Ci/mmol. The compound was fully active biologically; in the same concentration as AOT it caused a shift in the dose-response curve for oxytocin. Its pA_2 was 7·58. Solutions of a concentration of 600—750 µCi/ml in H_2O were prepared for the experiments and stored for 12 months at −18°C without sign of the formation of other radioactive products. Storage for longer periods led to the appearance of a substance which could be removed by filtration on Bio-Gel P—2 in H_2O (ref. [18]). Judging by the mobility of the compound, it is possible that it is the dimer of ^{3}H—AOT.

Cleavage of ^{3}H—AOT *by chymotrypsin*: During 2 h of incubation with the lower concentration of chymotrypsin, ^{3}H—AOT (R_F = 0·37—0·42; chromatography in a solvent system of butanol–pyridin–H_2O (4 : 1 : 1)) was decomposed and two radioactive products were formed gradually. The appearance of the first product

with $R_F = 0{\cdot}27-0{\cdot}28$ was followed by the gradual formation of another product with $R_F = 0{\cdot}51-0{\cdot}54$. When the higher concentration of chymotrypsin was used, only the product with higher mobility was formed. ^{3}H-AOT by itself was not stable during 2 h of incubation; another product appeared with $R_F = 0{\cdot}08-0{\cdot}10$. The time course of the degradation of ^{3}H-AOT in the presence of the lower concentration of chymotrypsin is shown in Fig. 1.

The determination of the N*-terminal amino acids of chymotryptic cleavage products by the dansylation method*: The chromatography of dansylation products after hydrolysis proved the presence of dansyl-glycine and dansyl-isoleucine in experiments 1 and 2. These amino acids were not formed by the degradation of chymotrypsin itself; this was proved by incubating chymotrypsin alone and analyzing the products by the dansylation method.

Interaction of ^{3}H-AOT *with the subcellular fractions of rat uterine homogenate.* The appearance of certain radioactive products depended on the subcellular fraction used and on the duration of incubation. The incubation of ^{3}H-AOT with the supernatant fraction resulted in the formation of two products with higher R_F values ($0{\cdot}46-0{\cdot}48$ and $0{\cdot}56-0{\cdot}58$) than those of ^{3}H-AOT. After 2 h of incubation, the amount of the products represented 50% of the radioactivity taken into the experiment. The incubation with the 600 *g* fraction resulted mainly in a product characterized by $R_F = 0{\cdot}55-0{\cdot}60$. After 30 min of incubation, 20–25% of the total radioactivity was transformed into this product. When the incubation period was prolonged to 2 h, the amount of the product represented only 10% of the total radioactivity and another product appeared with $R_F = 0{\cdot}34-0{\cdot}36$.

Interaction of ^{3}H-AOT *with human pregnancy serum*: The labelled analogue was not degraded during the incubation with human pregnancy serum.

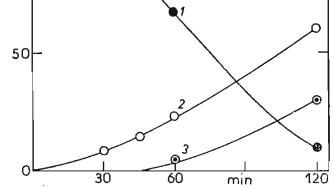

FIG. 1

Time Course of the Degradation of ^{3}H-AOT by Chymotrypsin (50 μg/ml)

Ordinate: % of total radioactivity; abscissa: duration of incubation in min. *1* ^{3}H-AOT, *2* compound with $R_F = 0{\cdot}27$ to $0{\cdot}28$, *3* compound with $R_F = 0{\cdot}51$—$0{\cdot}54$. For details, see Methods.

Interaction of ^{3}H-AOT *with subcellular fractions of homogenates of the lactating rat mammary gland*: We used the same experimental arrangement as when we analyzed the products of the hydrolysis of ^{3}H-AOT in the presence of fractions obtained from rat uterine homogenates. The incubation of the subcellular fractions with ^{3}H-AOT for 2 h gave a product with $R_F = 0{\cdot}71-0{\cdot}73$ in an amount representing 20–25% of the total radioactivity. Apart from this compound, another product with $R_F = 0{\cdot}28-0{\cdot}29$ appeared during the incubation with the 600 *g* fraction.

DISCUSSION

The preparation of a radioactively labelled antagonist of oxytocin makes it possible to investigate the stability of the compound under physiological conditions and discover some of the metabolic pathways in which the molecule is disintegrated. ^{3}H-AOT was prepared by the same method as the unlabelled analogue and the yield was approximately the same. When the compound was stored for more than 12 months at −18°C, another radioactive component was formed that could be detected by TLC and removed by gel filtration on Bio-Gel P−2. As previously mentioned, one of the motives for preparing the compound was the investigation of its metabolism. The data obtained so far on the metabolism of oxytocin and some of its analogues[10,11] allow us to anticipate the probable way in which the individual bonds are split. The substitution of the primary amino group of cysteine by an acetyl group protects the molecule from aminopeptidase (or oxytocinase) attack. Tritiated N-acetyl[2-O-methyltyrosine]oxytocin is stable in human pregnancy serum. This fact indicates that there are no other enzyme systems in pregnancy serum capable of degrading the compound.

Before investigating the disintegration of ^{3}H-AOT in subcellular fractions obtained from rat target tissues for oxytocin, a study was made of the splitting of ^{3}H-AOT by chymotrypsin. Chymotrypsin splits the bond between leucine and the terminal glycine amide of oxytocin. In deamino-oxytocin the bond between tyrosine and isoleucine is also available to chymotryptic cleavage[11]. The rate of hydrolysis of this bond is much higher than that of the bond between leucine and glycine amide, as found in the case of the deaminocarba analogue of oxytocin[11]. N-Acetyl[2-O-methyltyrosine]oxytocin has two bonds that could be split by chymotrypsin. The substitution of the primary amino group of cysteine by an acetyl group should have the same effect on chymotryptic cleavage as when the amino group is replaced by hydrogen, as in the deamino analogue. In order to detect all the products, two concentrations of chymotrypsin were chosen and the N-terminal amino acids of the individual cleavage products were detected by the dansylation method. The dansylation technique proved that both peptide bonds were split. However, it was not possible to estimate which one of them was split preferentially by chymotrypsin. On comparing

these results with those of the chromatographic analysis of the cleavage products of ^{3}H-AOT, one can see that radioactive material with $R_F = 0{\cdot}27 - 0{\cdot}28$ is probably a mixture of two products, each of which has one peptide bond split by chymotrypsin (Fig. 2).

After longer periods of incubation and in the presence of higher chymotrypsin concentrations, the final product has two peptide bonds split. The accessibility of the two bonds in the AOT molecule to chymotryptic cleavage differs from that of deaminooxytocin[11]. The fact that the peptide bond between O-methyltyrosine and isoleucine is less accessible to chymotryptic cleavage is apparently due to the methylation of tyrosine[11-13].

The cleavage of ^{3}H-AOT by subcellular fractions prepared from homogenates of the rat uterus and mammary gland was compared with the known inactivation pathways for oxytocin and its analogues. During the incubation of ^{3}H-AOT with the 600 *g* fraction obtained from the rat uterus, a product first appears which has the same R_F value as the final product of the chymotryptic cleavage of ^{3}H-AOT. After longer periods of incubation, the concentration of this compound decreases and another product appears which has a lower R_F value than ^{3}H-AOT. By contrast, the incubation of ^{3}H-AOT with the supernatant fraction of the rat uterus results in the formation of two radioactive fragments with higher mobility than ^{3}H-AOT.

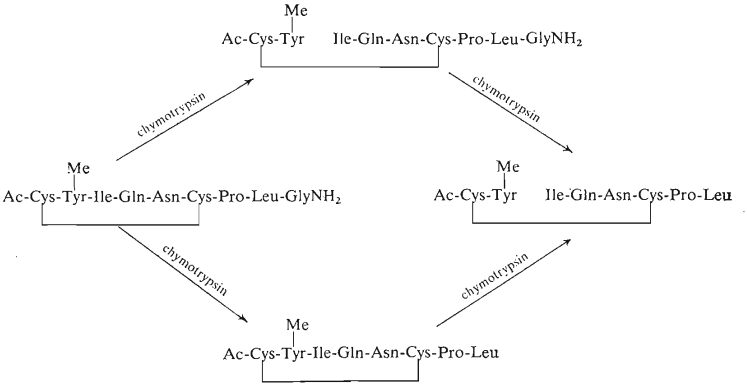

FIG. 2
Diagram of the Cleavage of AOT by Chymotrypsin

The product with the highest mobility corresponds to the final product of chymotryptic cleavage of ^{3}H-AOT, the other product could not be identified so far.

We could not find any model equivalent for the radioactive fragment with highest mobility formed during the incubation of ^{3}H-AOT with subcellular fractions prepared from the rat mammary gland. The compound with $R_F = 0{\cdot}27-0{\cdot}28$ detected after the incubation of ^{3}H-AOT with the 600 *g* fraction could represent a peptide in which one peptide bond had been split.

If we summarize the results we can see that several radioactive fragments were detected by analysing the products of the cleavage of ^{3}H-AOT by subcellular fractions obtained from the target tissues. The R_F value of one of the products indicates that it may be identical with the final product of chymotryptic cleavage, the other products could be intermediates in which only one peptide bond had been split. Apart from these, other radioactive fragments of unknown structure were detected. As mentioned above, aminopeptidase cleavage of the intact analogue can be excluded. However, after ^{3}H-AOT has been split by an endopeptidase it can be attacked by aminopeptidases. If, for example, the cyclic structure is disrupted between O-methyl tyrosine and isoleucine, the resulting derivative can be split by an aminopeptidase, isoleucine and other amino acids are liberated and radioactive fragments of the molecule appear. The so-called post-proline cleavage can also be mentioned in this connection; an enzyme catalysing this reaction was found in the human placenta and in lamb kidneys[14,15]. The fragment without leucyl-glycine amide is radioactive and it is probable that it is formed in the presence of the fractions of uterine homogenate. One can also expect the process of tyrosine demethylation to take place. This process is assumed to be responsible for protracted or two-phase biological effects of derivatives containing O-methyltyrosine[16,17].

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