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Enzymatic Inactivation of Oxytocin

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By chromatography on DEAE-cellulose, retroplacental serum is shown to contain at least two enzymes having cystine aminopeptidase properties. The main component has the ability to hydrolyze at least the first three N-terminally situated peptide bonds of oxytocin, as evidenced by the amino acid composition, Edman degradation and thin-layer chromatography of the cleavage products. By chromatography on Sephadex G-200, oxytocinase is eluted between the "7 S-" and "11 S-components".

Already in 1930, Fekete [1] observed that serum from pregnant women had the ability to inactivate extract from the posterior pituitary gland. About 10 years later, Werle and Effkemann [2] were able to show that the phenomenon involved an enzymatic break-down of the oxytocin present. The oxytocin-inactivating enzyme, oxytocinase, has consequently been studied by several workers, for instance by Page *et al.*,[3,4] Semm and Werle,[5,6] Šorm *et al.*,[7–10] and Tuppy *et al.*[11–14]

In the earlier investigations, the oxytocinase activity was estimated by allowing the enzyme to react with excess oxytocin, the residual substrate then being determined by conventional biological methods. The work in this field, however, was substantially facilitated when Tuppy and Nesvadba [11] in 1957 described L-cystine-di-β-naphthylamide as a new substrate for oxytocinase. The use of this reagent is based on the fact that oxytocinase is an aminopeptidase and releases from the substrate β-naphthylamine, which can be determined photometrically. Tuppy and Nesvadba [11] found that oxytocinase ruptures the bond between cystein and tyrosine in the oxytocin molecule, so that cysteic acid could be detected in the incubation mixture after inactivation of the oxytocin and oxidation with performic acid. Non-pregnant serum contains very little cystine aminopeptidase, but during pregnancy the cystine aminopeptidase activity increases concurrently with that of the oxytocinase.[12] The two activities likewise increase uniformly when the oxytocinase from retroplacental serum [12,15] is purified. Thus, L-cystine-di-β-naphthylamide seems to be a suitable substrate for oxytocinase determinations. However, certain enzymes are known to occur which are capable of hydrolyzing cystine-di-β-

naphthylamide but not oxytocin,[16] hence the former cannot always be used for the estimation of oxytocinase.

The purpose of the present paper is to report on the distribution of the cystine aminopeptidase activity of retroplacental serum by chromatography on Sephadex G-200 and on diethylaminoethyl (DEAE)-cellulose and to describe the course of the breakdown of oxytocin by oxytocinase preparations.

EXPERIMENTAL

Materials and apparatus

Retroplacental serum. Retroplacental blood, uncontaminated with amniotic fluid. was collected immediately after parturition and centrifuged as soon as possible (within 30 min after the bleeding). Red coloured serum, indicating haemolysis of red blood-corpuscles, was discarded. The serum used was yellow or only weakly reddish in colour.

Oxytocin. Synthetic oxytocin, "Syntocinon", 450 IU/ml, Sandoz, Basle.

Biodryex, a carboxymethyl cellulose derivative purchased from Lövdalens Industri AB. Centralpalatset, Stockholm C, who prepare it according to Palmstierna.[17]

Sephadex G-25 and *Sephadex G-200*, Pharmacia AB, Uppsala.

Cellulose powder, MN 300, Macherey, Nagel & Co., 516 Düren, West Germany.

Uvicord spectrophotometer, LKB-Produkter AB, Stockholm 12.

Water. Twice distilled, the second time in a glass apparatus.

All the other reagents used were of analytical grade.

Methods

All work with the retroplacental serum and the purified enzyme preparations was carried out at + 2°C, unless otherwise stated.

Chromatography of retroplacental serum. 25—50 ml of retroplacental serum was concentrated according to Palmstierna [17] to a volume of 10—30 ml with Biodryex, and then chromatographed on a column (3.5 × 45 cm) packed with 15 g of Sephadex G-200. The serum was then eluted with 0.1 M NH_4HCO_3 solution, 5 ml-fractions being collected. The transmission of the eluate was continuously recorded at 254 mμ by means of an Uvicord spectrophotometer, and the cystine aminopeptidase activity of the fractions determined according to Tuppy *et al.*[11] The fractions containing the highest specific activity were pooled and concentrated with Biodryex. The concentrated enzyme solution (5—10 ml) was adjusted to pH 7.5 with 0.1 M formic acid, dialyzed against 0.04 M phosphate buffer of pH 7.5 and chromatographed on a column (2.8 × 12.5 cm) packed with approx. 10 g of dry DEAE-cellulose. The column was equilibrated with 0.04 M phosphate-buffer and the elution started with 100 ml of the same buffer. Elution was then continued with the same buffer but of uniformly increasing ionic strength. This gradient elution was achieved as follows: 250 ml of 0.04 M buffer was introduced into a suitable flask equipped with a mechanical stirrer and having an outlet to the column. A second flask of similar dimensions, containing 250 ml 0.12 M buffer, was placed at the same level as the first flask and allowed to communicate with it. By this arrangement, an eluent of uniformly increasing ionic strength was obtained. The eluate was passed through the Uvicord scanner, collected in 5 ml-fractions, and the cystine aminopeptidase activity determined according to Tuppy.[11] The fractions showing peptidase activity were combined, concentrated with Biodryex and stored at −20°C.

The ionic strengths of the various fractions were estimated by measuring their specific resistance and comparing the latter with the resistances of solutions of the same buffer of known concentrations.

Inactivation of oxytocin with oxytocinase. 5 ml of Syntocinon containing about 5 mg of oxytocin was incubated at 37°C and pH 7.5 with 7 ml of an oxytocinase preparation, obtained from 50 ml of retroplacental serum. The activity of the preparation had been increased about 40 times based on the ratio cystine aminopeptidase activity : protein.

The oxytocinase was taken from the main activity peak after chromatography on DEAE-cellulose. The incubation was stopped after 2 h, after which time less then 20 % of the oxytocic activity remained.

Isolation of oxytocin and its cleavage products after incubation with oxytocinase. The proteins were separated from the peptides and amino acids by chromatography. The incubation mixture was run through a column (2.4 × 70 cm) packed with 60 g of Sephadex G-25, and eluted with 0.05 M acetic acid. Fractions of 5 ml were collected, and their optical densities measured at 280 mμ. The fractions containing peptides and amino acids were combined in three main fractions, designated I, II, and III. Each fraction was then run through a small column (0.5 × 8 cm) packed with Dowex 50 W-X2, 100 – 200 mesh. After being washed with 0.05 M acetic acid, the adsorbed material was eluted with two 1 ml-portions of 0.1 M pyridine-formic acid buffer of pH 5.0, and then with two 1 ml-portions of 1.0 M buffer, the eluates being collected in four fractions marked -A, -B, -C, and -D, respectively. The samples containing ninhydrin-positive substances were dried *in vacuo* over phosphorus pentoxide and potassium hydroxide before being analyzed further.

Two-dimensional thin-layer chromatography. 15 g of cellulose powder MN 300 was homogenized with 85 ml of water and spread in a thin layer on glass plates measuring 200 × 200 mm, using an apparatus from C. Desaga, Heidelberg, West Germany. The plates were dried at 105 – 110°C for 15 min. The chromatogram was developed in one direction with butanol:acetic acid:water (4:1:5), and in the second direction with pyridine:water (4:1). After the first run, the plates were heated at 70°C for 30 min and cooled to 25°C before the second run. The spots were detected by their UV-fluorescence and by the colour obtained at 100°C with a ninhydrin reagent (0.1 % ninhydrin + 1 % collidine in 95 % ethanol).

Hydrolysis. The peptides were hydrolyzed in evacuated glass tubes with constant-boiling hydrochloric acid at 105°C for 24 h.

Quantitative amino-acid analysis. Determination of amino acids in the hydrolysate was carried out in an automatic amino-acid analyzer according to Spackman, Moore, and Stein.[18]

Oxidation of peptides. The cystine in the peptides was oxidized according to Moore.[19]

Sequence determination of peptides. The determination of the amino acid sequence in the peptides was carried out with phenylisothiocyanate, according to Edman.[20,21] The peptide material was treated with phenylisothiocyanate in a buffer solution of pH 10 for 1 h at 40°C, giving the phenylthiocarbamyl derivatives. After drying *in vacuo* over phosphorus pentoxide, the N-terminal amino acids were released from the remaining peptides as the phenylthiazolinone derivatives by treatment with water-free trifluoroacetic acid at 40°C for 15 min. Dichloroethane and ether (0.75 + 0.90 ml) and water (0.25 ml) were added, after which the tubes were immediately shaken and centrifuged. The two phases were separated and the water phase was extracted twice with 0.25 ml of dichloroethane-ether. The combined organic extracts containing the thiazolinone derivatives were evaporated with a stream of air, whilst the water phase containing the residual peptide was evaporated *in vacuo* over P_2O_5. The thiazolinone derivatives of the N-terminal amino acids were converted to the corresponding phenylthiohydantoin (PTH) derivatives by warming for 75 min with 30 % ethanol, adjusted to pH 1 with hydrochloric acid. The sample was dried *in vacuo* over P_2O_5 and potassium hydroxide after which the PTH derivatives were extracted with a mixture of dichloroethane, ethyl acetate and heptane from a water phase, according to Wallén and Sjöholm.[22] The aqueous phase was transferred to the sample containing the residual peptide and dried over P_2O_5 *in vacuo*. The PTH-derivatives from the organic phase were identified and determined quantitatively by paper chromatography, according to Sjöquist.[23]

Determination of oxytocin. Oxytocin was determined according to Sjöholm and Rydén[24,25] on isolated strips from the mammary gland of lactating rats *in vitro*.

RESULTS

1. *Chromatographic fractionation of oxytocinase.* Fig. 1 shows the chromatography of 25 ml of retroplacental serum on Sephadex G-200, which had been

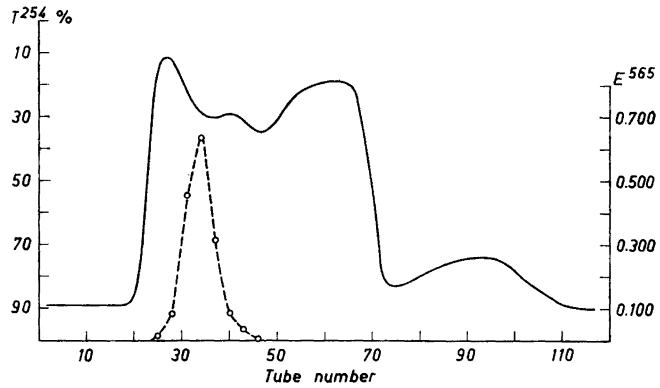

Fig. 1. Fractionation of retroplacental serum on Sephadex G-200 (3.5 × 45 cm) in 0.1 M NH_4HCO_3. ——— denotes the transmission at 254 mμ and — — O — — the distribution of the cystine aminopeptidase activity (E^{565}).

concentrated to 12 ml by Biodryex. In general, the cystine aminopeptidase activity could be increased 4—5 times by this procedure relative to the protein content. Fig. 2 depicts the chromatography on DEAE-cellulose of the concentrated enzyme preparation obtained after the Sephadex chromatography. Two peaks with cystine aminopeptidase activity were obtained. In the larger, which was eluted with about 0.06 M phosphate buffer, the cystine aminopeptidase activity could generally be further increased by a factor of 8—12. The specific activity could thus be increased about 35—60 times relative to that of the serum by the combination of these two chromatographic procedures.

2. *Analysis of the cleavage products from inactivated oxytocin.* The oxytocin was inactivated by an oxytocinase preparation, as described in the previous section. The amino acids and peptides obtained in the incubation mixture were separated from the protein components by gel filtration on Sephadex G-25 (Fig. 3). The fractions were analyzed for oxytocic activity and pooled as shown in the figure. After chromatographic separation on a Dowex 50 W × 2-column

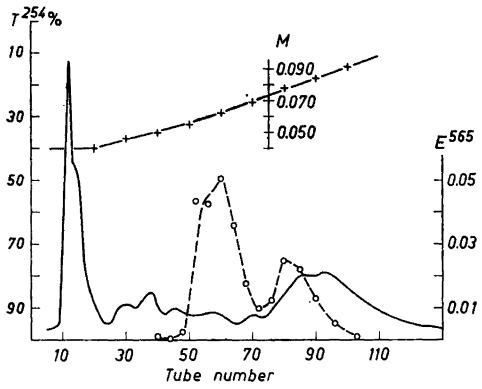

Fig. 2. Chromatography of gel-filtered retroplacental serum on DEAE-cellulose (2.8 × 12.5 cm) in phosphate buffer of pH 7.5 with increasing ion strength. ——— denotes the transmission at 254 mμ of the eluate and — — O — — the distribution of the cystine aminopeptidase activity (E^{565}). — — + — — indicates the buffer concentration of the eluate.

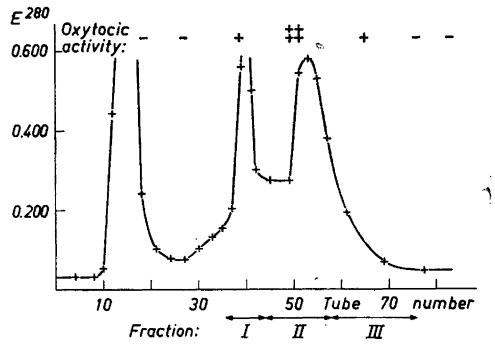

Fig. 3. Separation of the cleavage products of inactivated oxytocin from protein components on Sephadex G-25 (2.4 × 70 cm) in 0.05 M acetic acid.

as described earlier, the different fractions were submitted to two-dimensional thin-layer chromatography (see Fig. 4). As was evident from the ninhydrin reaction, the bulk of the products was present in sample I—C, where free isoleucin or leucin, as well as a considerable quantity of a peptide, which could be detected in lesser amounts also in other samples, were present. Isoleucine or leucine could also be detected in II—A. Large quantities of free tyrosine were present in III—C and III—D. Since the two-dimensional chromatography of the sample-pairs I—B and I—C, II—A and II—B, II—C and II—D, III—C and III—D, gave similar patterns, respectively, they were combined into four fractions. A portion of each of the combined samples was hydrolyzed and submitted to amino-acid analysis; the results are collected in Table 1. The amino-acid composition of I—B, C, and II—C, D was about the same, hence the two samples were combined.

All the samples were oxidized with performic acid, after which the peptides present were subjected to sequence determination. The results are collected in Table 1, where the PTH-derivatives obtained in the different steps are arranged in the same order as they occur in the intact oxytocin.

The sequence determination showed the presence of at least three different peptides. A peptide beginning with ileu-glu-asp-$CySO_3H$-pro-leu was obtained in largest amounts. This peptide is probably the oxidized heptapeptide that is obtained from oxytocin when the bonds between the N-terminal cystein and tyrosine and between tyrosine and isoleucin are broken. The second amino-acid sequence obtained was glu-asp-$CySO_3H$. These three amino acids seem to constitute the beginning of the peptide which remains when also the third bond in oxytocin — *i.e.* the one between isoleucine and glutamine — is broken. By characterisation of the first four amino acids of the third peptide, the latter was identified as intact oxytocin.

DISCUSSION

Flodin and Killander [26] found in the chromatography of blood serum on Sephadex G-200 that the blood proteins were separated into three main fractions, which were more or less homogeneous in the ultra-centrifuge. As can be seen from Fig. 1, the same main fractions can be discerned also in the chromato-

Table 1. Amino acid content and sequence determination of the cleavage products of inactivated oxytocin.

Fraction	Free amino acids detected by thin-layer chromatography	Amino acid content μmole amino acid		Sequence determination μmole PTH derivate, not corrected for losses Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
I—B,C	Ileu/leu	Asp 0.45 Ser 0.10 Glu 0.38 Pro 0.46 Gly 0.44	CySSCy + Ileu 0.49 Leu 0.42 Tyr 0.26	$CySO_3H$ 0.10 Tyr 0.03	Tyr + —	Ileu/leu + —	Glu 0.02 —	— —	— —
II—C,D		Asp 0.18 Glu 0.14 Pro 0.2 Gly 0.14	CySSCy ? Ileu 0.07 Leu 0.13 Tyr 0.07	Ileu/leu 0.23 Glu 0.03	Glu 0.11 Asp 0.06	Asp 0.06 $CySO_3H$ +	$CySO_3H$ + —	Pro + —	Ileu/leu + —
II—A,B	Ileu/leu	Asp 0.25 Thr 0.10 Ser 0.40 Glu 0.26 Pro 0.3 Gly 0.38	Ala 0.14 CySSCy ? Ileu 0.29 Leu 0.16 Tyr 0.11	Ileu/leu 0.13 Asp 0.07 Gly 0.15	Glu + —	Asp 0.02 —	— —	— —	— —
III—C,D	Tyr	Tyr 0.40		Tyr 0.15	—	—			

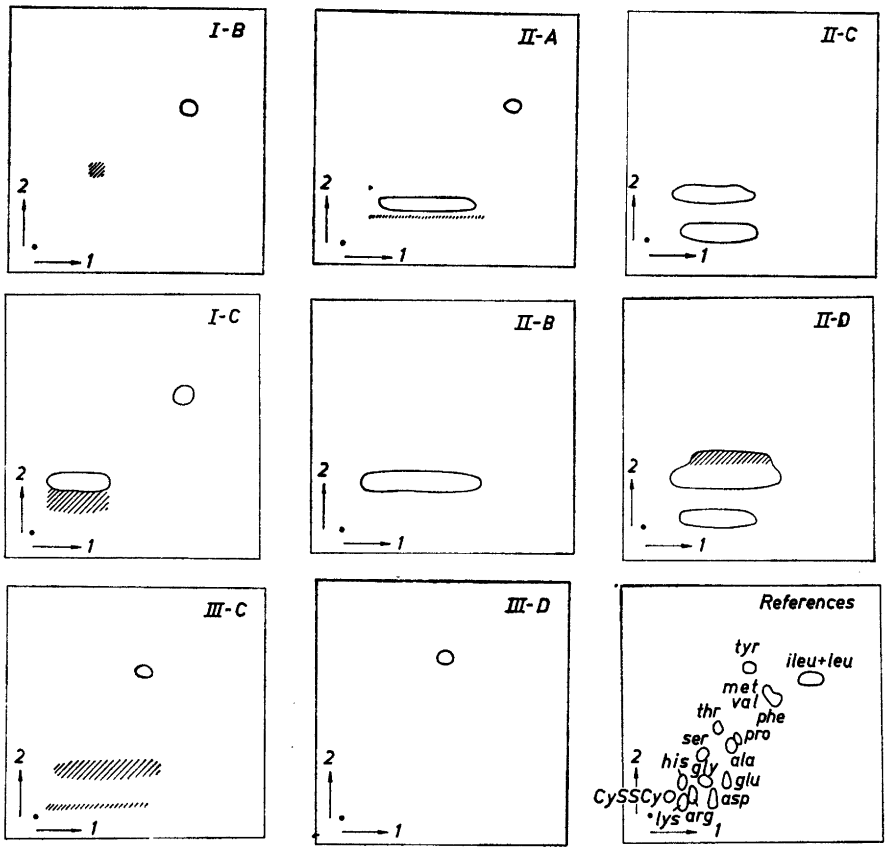

Fig. 4. Thin-layer chromatography on cellulose powder of the cleavage products of inactivated oxytocin. The different samples are obtained after chromatography on Sephadex G-25 and Dowex 50 W × 2. Solvent 1: butanol-acetic acid-water (4:1:5). Solvent 2: pyridine-water (4:1). Traces are indicated by shaded areas.

graphy of retroplacental serum. The cystine aminopeptidase activity could be located between the first and the second fractions. According to Flodin *et al.*,[26] components having a sedimentation coefficient of 19 S and 10—11 S migrate in the first fraction whilst those having a coefficient of 7 S migrate mainly in the second fraction. These observations indicate that oxytocinase should have a sedimentation coefficient of 7—11 S in the ultra-centrifuge.

At least two enzymes with the ability to hydrolyse L-cystine-di-β-naphthylamide were detected by the chromatography of gel-filtered retroplacental serum on DEAE-cellulose. This is in agreement with the findings of Page *et al.*,[3] who reported the occurrence of two cystine aminopeptidase fractions in retroplacental serum by starch gel electrophoresis. Wintersberger and Tuppy,[13] however, found only one cystine aminopeptidase band using the same technique. This fact, together with the author's own observation that

the ratio between the two activities found by the chromatography on DEAE-cellulose varies from one sample to another, indicate that large individual variations exist. Nothing is known at present, however, about differences in the specificity of the two activities, though several proteolytic enzymes are known to exist in different forms having the same enzymatic specificity. For instance, Bergström [27] has shown that bovine plasminogen can be present in two forms in some aninals, but only one fraction is present in other animals. However, since oxytocinase contains sialic acid [14] and possibly also other non-protein components, the different properties pointed out may be due to the different amounts of these accompanying components present in the two fractions, giving rise to discrepancies in their electrophoretic and chromatographic behaviour.

Oxytocinase has early been classified as an aminopeptidase.[7,8,11] Tuppy and Nesvadba have reported results indicating that the bond between cysteine and tyrosine in oxytocin is broken by serum from pregnant women. From the present work, however, it can be concluded that the break-down of the oxytocin molecule is more extensive. By hydrolysis and amino-acid analyses, sequence analyses and thin-layer chromatography of the cleavage products, it is shown that at least the first three peptide bonds of oxytocin are ruptured. Thus, by two-dimensional thin-layer chromatography, large amounts of both tyrosine and isoleucine have been identified, and these amino acids have also been found by the Edman degradation. Furthermore, the high yield of cysteic acid found in the first step of the sequence analysis might indicate that free cysteic acid is present in addition to the amount incorporated in the oxidized oxytocin. Unfortunately, a *complete* determination of the residual peptides of the oxytocin has not been possible to achieve, the technique of sequence analysis of small amounts of oligopeptides not yet being perfected. Edman's phenylisothiocyanate method seems at present to be the best one, but due to side reactions and losses of peptide material by the different extractions, the efficiency is limited. By model experiments with the method used, 67—75 % yields of glycine was obtained in the second and third steps from 0.6 μmole of leu-gly-gly, but the determination of smaller amounts of peptides, or of peptides containing glutamine, asparagine and tyrosine, would be expected to give still lower yields (Blombäck *et al.*[28]). In spite of these difficulties, however, the experimental procedures adopted gave adequate proof of the existence in the incubation mixture of, besides intact oxytocin, the two peptides beginning respectively with isoleucine and with glutamine mentioned above.

In addition, asparagine has been detected in the first step of a sequence-determination of fraction II—A,B. Though the succeeding amino acids have not been identified, it is not inconceivable that this asparagine constitutes the first member of a fourth peptide, indicating that even the fourth peptide-link in oxytocin is ruptured to a certain extent.

The amino-acid analysis of the hydrolyzed peptides revealed that all the amino acids constituting the oxytocin were present. However, only small amounts of cystine could be detected, in spite of the fact that cysteic acid was found in good yield in the sequence determination. Furthermore, serine and alanine as well as threonine, were found in the hydrolysate, although these amino acids were not detected in the sequence determination. As Yoritaka

and Ono [29] have shown, cystine can decompose when it is refluxed in 20 % hydrochloric acid, yielding mainly serine and some alanine. The presence of serine, alanine and threonine in the hydrolysates might be due to such a decomposition of cystine or might arise from small amounts of peptides formed by autodigestion of the oxytocinase preparation used.

The large amounts of free glycine found in step 1 in sample II—A,B is somewhat surprising. If not a contamination, this glycine must originate from the glycineamide in the C-terminal end of the oxytocin, thus suggesting that oxytocinase is still less specific than expected, having some carboxypeptidase activity as well. However, as long as the results have not been confirmed with more highly purified oxytocinase, definite conclusions cannot be drawn.

The sequence analyses also give some ideas about the course of the breakdown of oxytocin. Thus, since no peptide beginning with tyrosine has been detected, it seems that the bond between tyrosine and isoleucine is easily split and, because some oxytocin remains unaltered, is split easier than the bond between cysteine and tyrosine. The presence of large amounts of the peptide ileu-glu-asp proves that the bond isoleucine-glutamine is more resistent. These results are in very good agreement with the work of Tuppy *et al.*[14] on the specificity of oxytocinase. These authors investigated the ability of oxytocinase to hydrolyze the β-naphthylamide derivatives of different amino acids and found that tyrosine-β-naphthylamide is easily split, the rate of hydrolysis being about 4 times faster than in L-cystine-di-β-naphthylamide, while the L-isoleucine derivative is split more than 10 times slower than the tyrosine compound. Thus the oxytocinase here studied seems to have the same specificity as the purified enzyme of Tuppy *et al.*[12,14] It remains to determine if the other component of retroplacental serum with cystine aminopeptidase activity has the same specificity as the enzyme here studied, and also to establish the significance of these enzymes for the metabolism of oxytocin and other physiologically active peptides *in vivo*.

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