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Isolation and Identification of Vasopressin- and Oxytocin-immunoreactive Substances from Bovine Pineal Gland

PRESENCE OF N^{α}-ACETYLOXYTOCIN*

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Peptides with vasopressin- and oxytocin-immunoreactivity were purified from bovine pineal glands. Three immunoreactive peptides were purified by successive high performance liquid chromatography steps in sufficient quantities for identification by fast atom bombardment-mass spectrometry. Two peptides were characterized as authentic vasopressin and oxytocin. Their identities were in agreement with the observed immunoreactivities, high performance liquid chromatography behavior, and biological activity. The third peptide was identified as N^{α}-acetyloxytocin. The presence of the acetyl group was demonstrated by the molecular mass of the peptide, and the N^{α} position was shown to be the modified site by the presence of a blocked NH_2 terminus. N^{α}-Acetylation of oxytocin may be involved in altering the biological properties of the peptide.

The presence of neurohypophyseal hormones in the mammalian pineal gland has been extensively investigated by means of radioimmunoassays (RIA)[1] (1, 2) and bioassays (3–5). Accumulating data agree that the mammalian pineal gland contains substances having the same immunological and chromatographic properties as vasopressin and oxytocin (6, 7), and exclude the presence of authentic vasotocin (6–9). However, reports suggesting the existence of peptides different from authentic vasopressin and oxytocin continue to appear (10, 11). Since the proposed peptides detected in this organ have not been chemically defined, isolation of immunoreactive substances was undertaken. Large-scale extraction of bovine pineal glands combined with high resolution chromatography and specific radioimmunological detection allowed us to isolate three vasopressin- and oxytocin-immunoreactive peptides, which were identified by fast atom bombardment-mass spectrometry (FAB-MS). The results demonstrate the existence of authentic vasopressin and oxytocin and reveal novel forms of these peptides.

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[1] The abbreviations used are: RIA, radioimmunoassay; HFBA, heptafluorobutyric acid; PIC-B7, heptanesulfonic acid; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptides were prepared and provided by Drs. J. W. Van Nispen and H. M. Greven (Organon International B.V., Oss, The Netherlands). Sephadex G-50 (superfine) and CM-Sephadex C-25 were from Pharmacia LKB Biotechnology Inc. and charcoal from Merck (Darmstadt, Federal Republic of Germany). Sep-Pak C_{18} cartridges and 1-heptane sulfonic acid (PIC-B7 reagent) were purchased from Waters. Heptafluorobutyric acid (HFBA) was from Janssen Chimica Co. (Belgium). Glycerol and monothioglycerol were purchased from Aldrich and distilled under vacuum before use. Fluorescamine was from Pierce Chemical Co. All other chemicals were reagent grade. [^{3}H-Phe^3]Vasopressin and [^{3}H-Tyr^2]oxytocin were purchased from New England Nuclear with specific activity of 40.0 and 70.0 Ci/mmol, respectively.

Extraction of Bovine Pineal Glands—Bovine pineal glands from animals of different sex and age were collected in the slaughterhouse in different periods of the year and extracted according to Ref. 12. Briefly, fresh glands were desiccated in acetone (100 glands/10 ml) for at least 1 month and the powder was stored at 4 °C. Pooled acetone desiccated powder was precipitated at 50% ammonium sulfate, pH 3.9, followed by acetic acid extraction of the precipitate at 4 °C. The supernatant was dried and chromatographed on a CM-Sephadex C-25 column (2 × 50 cm) eluted by a linear gradient from 0.1 to 1.0 M ammonium acetate buffer at a flow rate of 10 ml/h. The major peak obtained was subjected to Sephadex G-10 filtration (1.5 × 40 cm) in 4% (w/v) sodium chloride. The biologically active fractions tested by the method of Munsick (13) were pooled and dried (E5 preparation). For these experiments approximately 50,000 bovine pineal glands were used. These yielded approximately 50 mg of E5 fraction.

Gel Filtration—The dried E5 fraction (50 mg) was dissolved in 4 ml of 10 mM ammonium acetate, pH 7.4, and fractionated on a pre-equilibrated Sep-Pak C_{18} cartridge by stepwise elution with 6-ml aliquots of 10% methanol, 55% methanol, and 100% methanol in 10 mM ammonium acetate, pH 4.15, respectively. More than 95% of vasopressin and oxytocin were recovered in the 55% methanol eluate as estimated from the elution of [^{3}H]vasopressin and [^{3}H]oxytocin under the same conditions. The 55% methanol fraction was dried *in vacuo* and dissolved in 4 ml of 0.1% (v/v) formic acid and then subjected to gel filtration on a Sephadex G-50 (Superfine) column (70 × 2.0 cm). Gel filtration was performed in 0.1% (v/v) formic acid at a flow rate of 4 ml/h and fractions of 4 ml were collected at 4 °C. Aliquots were taken and subjected to vasopressin- and oxytocin-specific radioimmunoassays. Pools were composed according to the immunoreactivity. The recovery in this step was about 90% as determined with [^{3}H]vasopressin and [^{3}H]oxytocin.

High Performance Liquid Chromatography (HPLC)—Pooled gel filtration fractions were dried *in vacuo* and the immunoreactive substances were further purified by successive HPLC steps. All HPLC effluents were monitored by UV absorbance at 210 nm (7). The first step involved reverse-phase HPLC on a μBondapak C_{18} column. A linear gradient running from 10 to 70% acidified methanol (0.15% (v/v) acetic acid) in 10 mM ammonium acetate, pH 4.15, over 40 min at a flow rate of 2 ml/min was used and 1 ml/fraction was collected. Four pools were made from these fractions according to the vasopressin- and oxytocin-immunoreactivities, dried *in vacuo*, and subjected to two different systems of paired-ion reverse-phase HPLC on μBond-

apak C_{18} columns. One paired-ion reverse-phase HPLC system employed a linear gradient running from 30 to 60% methanol containing 0.1% (v/v) HFBA in water containing 0.13% (w/v) HFBA over 30 min at a flow rate of 2 ml/min. The vasopressin immunoreactivity was finally purified by a second paired-ion reverse-phase HPLC system using a linear gradient running from 38 to 65% methanol containing 0.1% (v/v) PIC-B7 in 0.2% (v/v) PIC-B7 in water over 27 min at a flow rate of 1 ml/min. Selected immunoreactive fractions were pooled and analyzed by FAB-MS. The recovery in each HPLC step was about 80%.

Radioimmunoassay (RIA)—Radioiodination of vasopressin and oxytocin and RIAs were performed as described previously (14). The specificities of antisera were characterized by cross-reaction with synthetic peptides (7). Both vasopressin antiserum W1E and oxytocin antiserum O2 showed $CONH_2$-terminal specificities. The sensitivities of assays were 0.5 pg/tube for vasopressin and 4 pg/tube for oxytocin. Data were processed on an IBM Personal Computer.

NH_2-terminal Analyses—NH_2-terminal analyses of purified VP2 and OT2 were performed with fluorescamine (15). Purified VP2 (50 pmol) was mixed with 5000 dpm of [^{3}H]oxytocin in 50 μl of 0.5 M phosphate buffer (containing 10 mM EDTA), pH 7.0. The mixture was incubated with 50 μl of fluorescamine (200 μg/ml acetone) for 10 min at room temperature and then subjected to HPLC on a μBondapak C_{18} column using a linear gradient running from 20 to 80% acidified methanol in 50 min at a flow rate of 2 ml/min. Fractions were collected every 30 s. Vasopressin immunoreactivity was followed by vasopressin RIA. Aliquots from each fraction were counted in a β-counter to trace the derivatized form of [^{3}H]oxytocin. The purpose of the tracer was to determine the completeness of the fluorescamine reaction. The HPLC system was calibrated with synthetic vasopressin, oxytocin, and their N^{α}-acetylated forms. About 100 pmol of purified OT2 was mixed with 10,000 dpm of [^{3}H]vasopressin and analyzed in the same way.

Fast Atom Bombardment-Mass Spectrometry (FAB-MS)—All samples were dissolved in 5 μl of 1% acetic acid (v/v) and dried under vacuum directly onto the probe tip. Two μl of a mixture of glycerol:monothioglycerol (1:1, v/v) was then added, and mixed thoroughly with the sample before insertion into the ion source.

The FAB-mass spectra were recorded on a Kratos MS50 instrument equipped with a high field magnet, mass range 10,000 at 8 kV. The Ion Tech FAB gun was operated at 8 kV with a current of 30 μA with xenon as the bombarding gas. The scan speed was 100 s/decade over a mass range from *m/z* 2,000 to 300. All spectra were recorded in the positive-ion mode. The molecular masses of the constituent peptides were obtained by the subtraction of 1 Da (corresponding to one proton) from the mass of the protonated molecular ion (MH^+).

Bioassays—The antidiuretic and vasopressor activities of purified pineal vasopressin were estimated in *in vivo* bioassays performed as described by De Wied (16) and Dekanski (17), respectively.

RESULTS

Purification of Vasopressin- and Oxytocin-immunoreactive Substances from the Bovine Pineal Gland—Gel filtration of the bovine E5 preparation on a Sephadex G-50 column showed that vasopressin and oxytocin immunoreactivities were present in the small molecular weight fractions. These fractions were subjected to reverse-phase HPLC and separated into two main peaks of vasopressin immunoreactivity and two of oxytocin immunoreactivity (Fig. 1). The four components were coded VP1, OT1, VP2, and OT2 (Fig. 1). Two of them, VP1 and OT1, had the same retention time on HPLC as synthetic vasopressin and oxytocin. VP2 and OT2 eluted after vasopressin and oxytocin, respectively. No indication was obtained for the presence of vasotocin. Three pools of OT1:VP2, OT2, and VP1 were made and then subjected to paired-ion HPLC using an HFBA:methanol system. OT1 appeared as a single immunoreactive component detectable by UV absorbance (Fig. 2*A*). The oxytocin-immunoreactive peptide in fraction OT2 was resolved from several other substances (Fig. 2*B*). Paired-ion HPLC of VP1 resulted in a single vasopressin-immunoreactive peak (Fig. 3*A*), which appeared to have a heterogeneous composition as indicated by initial FAB-MS analyses. Peptide VP1 was obtained after

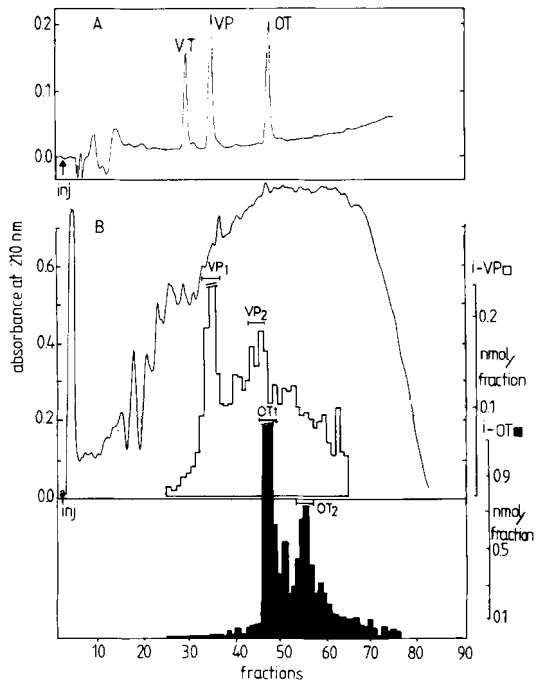

FIG. 1. **Reverse-phase HPLC fractionation of the gel filtration pool obtained from the E5 preparation of bovine pineal glands.** *A*, 10 μl of each of synthetic vasopressin (*VP*), oxytocin (*OT*), and vasotocin (*VT*) were separated on a μBondapak C_{18} column eluted by a linear gradient running from 10 to 70% of acidified methanol in 10 mM ammonium acetate, pH 4.15, over 40 min at 2 ml/min as described under "Experimental Procedures." *B*, UV profile and distribution of vasopressin immunoreactivity (*open bar*) and oxytocin immunoreactivity (*black bar*) obtained from the pineal fraction in the same HPLC fraction. Aliquots of each fraction were taken for vasopressin and oxytocin RIA. The *horizontal bars* indicate the fractions used to combine pools of OT1, OT2, and VP1.

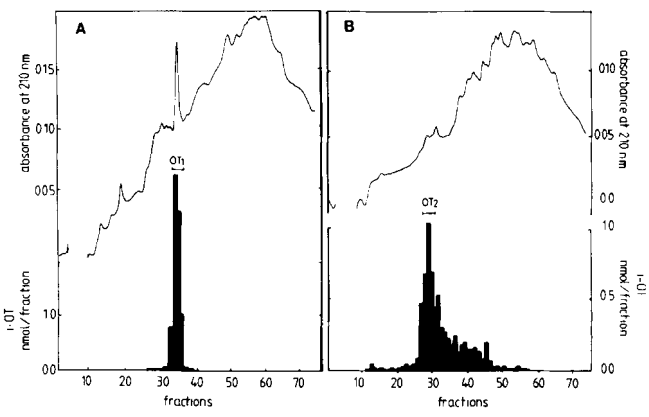

FIG. 2. **Purification of OT1 (*A*) and OT2 (*B*) by paired-ion reverse-phase HPLC.** Pools from the previous HPLC step were chromatographed on a μBondapak C_{18} column eluted by a linear gradient running from 30 to 60% of methanol containing the ion-pairing agent HFBA over 30 min at a flow rate of 2 ml/min. Fractions were collected every 30 s and aliquots were analyzed by oxytocin RIA.

further purification in a second HPLC system using PIC-B7 as the ion-pairing agent, but was not completely resolved from other substances (Fig. 3*B*).

FAB-MS Analyses of OT1, OT2, and VP1—The purified peptides OT1, OT2, and VP1 were analyzed by FAB-MS. Fig. 4*A* shows the MH^+ ions in the range of 942–1009 obtained from the FAB-mass spectra for OT1. Oxytocin (M_r = 1006) was seen as a protonated molecular ion (MH^+) at *m/z* 1007 in the molecular ion region recorded 1 min after insertion of

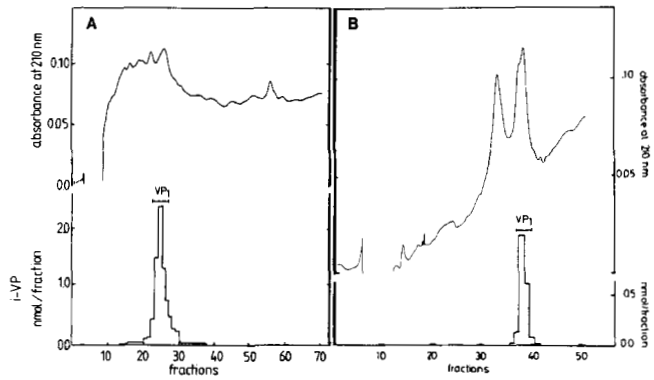

FIG. 3. **Purification of VP1 by two steps of paired-ion reverse-phase HPLC.** *A*, pool VP1 (Fig. 1) fractionated in the methanol:HFBA system as described under "Experimental Procedures" and the legend to Fig. 2. Fractions were collected every 30 s and analyzed by vasopressin RIA. *B*, top fractions of immunoreactivity were purified by a second paired-ion reverse-phase HPLC system using a linear gradient running from 38 to 65% of methanol containing PIC-B7 as ion-pairing agent over 27 min at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and aliquots from each fraction were used to measure the VP1 content.

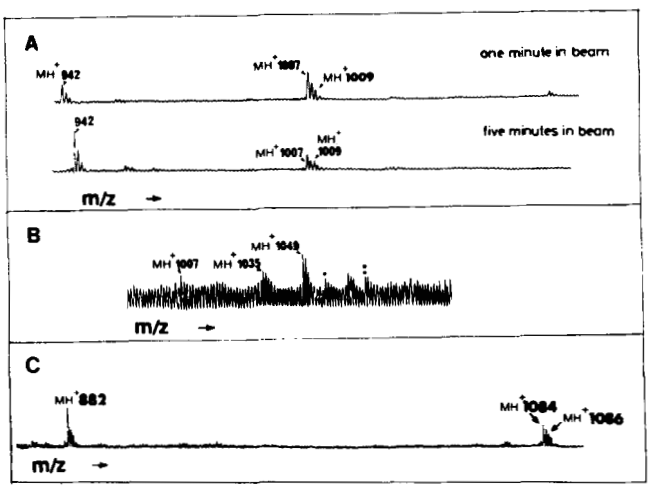

FIG. 4. **Partial positive ion FAB-MS spectra of the purified pineal OT1 (*A*), OT2 (*B*), and VP1 (*C*).** The protonated molecular ions (MH^+) for each peptide are indicated by *arrows*. *, sodiated ion (MNa^+) of formylated oxytocin; **, sodiated ion (MNa^+) of acetylated oxytocin.

the sample into the fast atom beam. After 5 min in the beam, a second molecular ion at *m/z* 1009 was observed (Fig. 4*A*). These observations are in agreement with the identity of OT1 as authentic oxytocin, which becomes reduced during the bombardment. The main molecular ion found in sample OT2 was at *m/z* 1049 (Fig. 4*B*), 42 daltons higher than that of native oxytocin. This increase is indicative of the addition of an acetyl group. Another molecular ion found at *m/z* 1035 indicated the presence of formylated oxytocin in sample OT2. In addition, the presence of sodiated ions (MNa^+) was also observed, which occur 22 daltons higher than the molecular ions of the formylated and acetylated species (Fig. 4*B*). [Arg⁸] Vasopressin was identified in sample VP1, based on the molecular ion at *m/z* 1084 together with the one at *m/z* 1086. This sample contained a second component with a molecular ion at *m/z* 882 (Fig. 4*C*). Aliquots of purified VP1 displayed the full antidiuretic and vasopressor activities of authentic vasopressin in the *in vivo* bioassays.

NH_2-terminal Analyses of OT2 and VP2—After the reaction with fluorescamine, OT2 still coeluted with synthetic N^{α}-

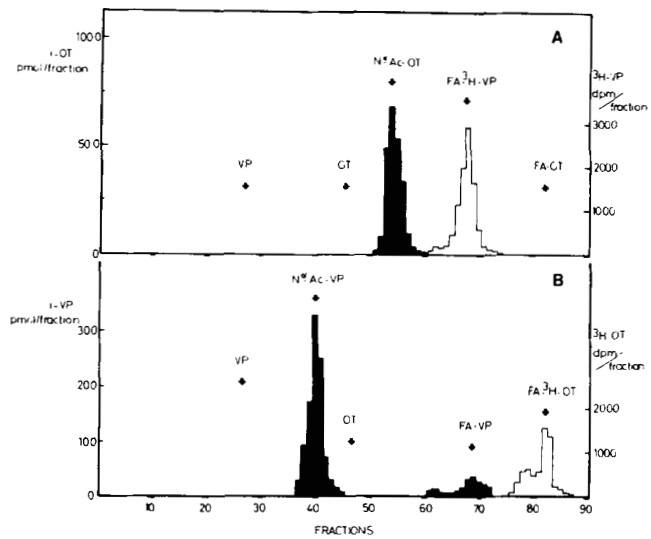

FIG. 5. **NH_2-terminal analyses of OT2 and VP2 by the reaction with fluorescamine (*FA*).** *A*, mixture of OT2 and [³H] vasopressin (*VP*) reacted with fluorescamine in a total volume of 100 μl and the products were analyzed by HPLC on a μBondapak C_{18} column as described under "Experimental Procedures." Oxytocin (*OT*) immunoreactivity in each fraction was measured by oxytocin RIA and aliquot from each fraction were counted by a β-counter to measure the radioactivity of [³H]vasopressin. *B*, mixture of VP2 and [³H]oxytocin reacted with fluorescamine and analyzed in the same way as OT2. *Black bars* indicate the immunoreactivity measured by vasopressin and oxytocin RIA. *Open bars* indicate the radioactivity measured by the β-counter.

acetyloxytocin on HPLC while [³H]vasopressin included in the sample was completely converted into the fluorescamine derivative, indicating the completeness of the reaction (Fig. 5*A*). The immunoreactivity of VP2 also remained at the elution position of synthetic N^{α}-acetylvasopressin after the reaction with fluorescamine, while [³H]oxytocin was completely derivatized. These observations showed that the NH_2-terminal groups of both OT2 and VP2 were blocked.

Extraction of [³H]Oxytocin and [³H]Vasopressin with Bovine and Rat Pineal Glands—Both [³H]oxytocin and [³H] vasopressin were co-extracted with bovine and rat pineal glands in 1 M acetic acid at 0 and 100 °C, respectively. The radioactivity was found only at the elution positions of synthetic oxytocin and vasopressin after separation by HPLC, indicating no artificial formation of the N^{α}-acetyloxytocin and N^{α}-acetylvasopressin.

DISCUSSION

Searching for vasopressin- and oxytocin-immunoreactive substances in the bovine pineal gland, we detected at least four such components and identified three of them. For the first time it was demonstrated by structural identification with FAB-MS that two of them have the structures of authentic vasopressin and oxytocin. No fragment ions could be detected in the FAB-MS analyses due to the low levels of samples available, but the MH^+ ions characteristic for the intact molecules of vasopressin and oxytocin were easily observed. In particular, when extending the time of the oxytocin sample in the fast atom beam, the MH^+ of the reduced form of oxytocin became clear. During all steps of purification of these vasopressin- and oxytocin-immunoreactive peptides, coelution with synthetic vasopressin and oxytocin occurred. Moreover, purified vasopressin showed antidiuretic and vasopressor activities in the *in vivo* bioassays with a potency similar to synthetic vasopressin. The results strongly indicate that the previously reported vasopressin and oxytocin im-

munoreactivities and bioactivities (18) are at least partially due to the presence of vasopressin and oxytocin.

A novel form of oxytocin was isolated and identified as N^{α}-acetyloxytocin. The identification of this peptide is based on two lines of evidence. First, the characterisitc MH^+ ion at m/z 1049, accompanied by traces of the reduced form of m/z 1051, is indicative of the presence of an acetyl group in the oxytocin molecule. Furthermore, the sodiated ion (MNa^+) of acetylated oxytocin was also observed. Second, the N^{α} position of the acetyl group is deduced from the blocked N^{α}-terminal group of the isolated peptide. This is shown by its failure to react with fluorescamine, a reagent specific for primary amino groups (15). In this experiment the completeness of fluorescamine derivatization was demonstrated by full reaction of [^{3}H]vasopressin included in the sample. These observations excluded the possibility of the acetyl group being esterified to the Tyr-OH group. Moreover, all chromatographic and immunological properties of the purified peptide were in agreement with its identity as N^{α}-acetyloxytocin.

Artifactual formation of N^{α}-acetyloxytocin due to the extraction procedure seems unlikely. First, [^{3}H]oxytocin and [^{3}H]vasopressin co-extracted with pineal glands in boiling or cold acetic acid were not detectably derivatized to acetylated forms. Second, N^{α}-acetyloxytocin was found to be present in the same quantity when pineal glands were extracted with 1 N HCl (containing 0.1% trifluoroacetic acid) (19) (data not shown). Therefore, N^{α}-acetyloxytocin may well be an endogenous peptide formed by post-translational modification of oxytocin.

It may be speculated that N^{α}-acetylvasopressin is also present in the bovine pineal gland, represented by the second peak of vasopressin immunoreactivity (VP2) in Fig. 1. This substance co-eluted with synthetic N^{α}-acetylvasopressin on HPLC, and appeared to cross-react to a lesser extent with a NH_2-terminal specific vasopressin antiserum (data not shown). It had a blocked terminal amino group. Unfortunately, the amount of this substance was too low to obtain structural information by FAB-MS.

The results described in this article do not explain the data of Noteborn *et al.* (20), who reported that pineal vasopressin and oxytocin, although eluting with comparable retention times to those of authentic vasopressin and oxytocin on HPLC, may have a derivatized Tyr residue of unknown identity. The Tyr derivative was resistant to hydrolysis in 6 N HCl for 18 h, and, therefore, could not represent an acetylated form of oxytocin and vasopressin. Our tentative data suggest that such a Tyr derivative may be formed during performic acid oxidation of the peptides in the presence of certain salts. Therefore, it remains to be investigated whether the reported Tyr derivative is artifactual or whether still other forms of vasopressin and oxytocin exist in the pineal gland. Furthermore, the previously detected vasopressin-like peptide in sheep and rat pineal glands which co-eluted with vasotocin (7) appeared to be absent in the bovine E5 fraction.

The finding of N^{α}-acetyloxytocin seems of biological importance. In the E5 fraction N^{α}-acetyloxytocin comprised approximately 20% of the oxytocin content. In preliminary experiments it was found that this peptide was also present in other parts of the central nervous system. Thus, N^{α}-acetyloxytocin may be a generally occurring form of oxytocin in the brain. It has been reported that synthetic N^{α}-acetyloxytocin has less than 1% of the uterotonic activity of oxytocin (21). Therefore, the finding of N^{α}-acetyloxytocin in the bovine pineal gland may point to a mechanism based on acetylation to alter or inactivate the biological activity of oxytocin. The biological importance of acetylation has previously also been demonstrated for other biologically active peptides, such as β-endorphin (22) and α-melanocyte-stimulating hormone (23).

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