ESTIMATION OF AN OXYTOCIN-LIKE SUBSTANCE IN HIGHLY PURIFIED EXTRACTS FROM THE BLOOD OF PUERPERAL WOMEN DURING SUCKLING

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SUMMARY

The presence of a substance in the jugular blood of lactating women with the chromatographic, electrophoretic, and pharmacological properties of oxytocin has been demonstrated.

The concentration of this substance in the jugular plasma, during suckling, was equivalent to 12–25 μ-u./ml. synthetic oxytocin.

These figures are in good agreement with those calculated from the rate of infusion of oxytocin necessary to elicit milk ejections similar to that produced during suckling.

INTRODUCTION

In previous work we have attempted to measure changes in oxytocin blood concentration during labour in man (Coch, Brovetto, Cabot, Fielitz & Caldeyro-Barcia, 1965). The procedure employed involved inactivation of oxytocinase by adding the plasma to boiling water. Proteins were precipitated by the use of HCl and small molecules eliminated by dialysis.

The oxytocic activity of extracts from jugular plasma measured in the milk-ejection assay increased throughout labour reaching maximum values in the second stage and decreasing during the puerperium. During the second stage of labour, the oxytocic activity of ‘jugular plasma’ was higher than that of peripheral plasma withdrawn simultaneously from the same patient. Subsequently we have tried to isolate oxytocin from extracts of jugular plasma by paper chromatography or paper electrophoresis in order to establish the identity of the hormone and to determine the amounts present in the extracts. Both techniques proved unsuitable because the oxytocic activity could not be separated.

In the procedure described here the use of trichloroacetic acid and the resin Amberlite IRC50-XE 64 to separate oxytocin from proteins and salts, as reported

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by several authors (Arimura & Dingman, 1960; Share, 1961; Yoshida, Motohashi, Ibayashi & Okinaka, 1965) allowed us to obtain extracts of blood that can be fractionated by chromatography or electrophoresis. With this method we were able to obtain oxytocin from the blood of puerperal women during intravenous infusions of synthetic oxytocin and also to separate an endogenous oxytocin-like substance from the jugular blood of puerperal women withdrawn during suckling.

MATERIALS AND METHODS

Extraction and recovery of oxytocin from plasma in vitro

Plasma of puerperal women was incubated for 1 hr. at 38° to inactivate any endogenous oxytocin and cooled to 0° to diminish the activity of plasma oxytocinase (Page, 1946; Méndez-Bauer, Carballo, Cabot, Negreiros de Paiva & González-Panizza, 1961; Garófalo, Brovetto, Fielitz, Cabot & Coch, 1967). Oxytocin (Syntocinon, Sandoz) was added in small amounts (0·5–1 m-u.) to 10 ml. of such plasma. The plasma was immediately poured into a 7·5 % (w/v) aqueous solution of trichloroacetic acid (3 vol. TCA to 1 vol. plasma) with continuous stirring. The resulting suspension was left to stand for 10–12 hr. at 4° and then centrifuged at this temperature for 10 min. at 2500 rev./min. The precipitate was washed twice with an equal volume of the 7·5 % solution of trichloroacetic acid. The supernatant and the washing liquids were neutralized with 2 n-NaOH to pH 7±0·1 and then acidified with glacial acetic acid to a final concentration of 5 % (v/v) (pH 2·5). The resultant solution was passed through a small chromatographic column (2 × 1·5 cm.) filled with Amberlite IRC50-XE64 resin in the hydrogen form. (To prepare the resin 10 g. were suspended in 100 ml. distilled water in a 100 ml. graduated cylinder of 3 cm. diameter; after 6 min. the supernatant was discarded and the procedure repeated six times. The resin was put in the column, washed with 30 ml. aqueous pyridine (30 %, v/v) to eliminate material extractable with this solvent, excess solvent was washed out with distilled water and the resin converted again to the hydrogen form by washing with 1 n-HCl, and later with H2O.) The column was then washed with 25 ml. 0·25 % (v/v) acetic acid and subsequently with 40 ml. distilled water. Oxytocin was eluted from the column by the passage of 30 ml. aqueous pyridine (30 %, v/v) for 1 hr. The solution thus obtained was freeze-dried. The small residue remaining after freeze-drying was suspended in 2 ml. of a mixture of acetone and water (2:1, v/v), centrifuged, and the supernatant was spotted as a band on a paper for electrophoresis or chromatography. Solvent was blown off by a stream of cool air. Spotting usually took 2 hr. Oxytocin was separated by paper electrophoresis in a 3 % acetic acid solution (pH 2·5) or by partition paper chromatography in n-butanol:acetic acid:water (4:1:5) as described elsewhere (Brovetto, Olhaberry, Gioia de Coch, Coda, Fielitz, Cabot, Fraga & Coch, 1967).

The position of oxytocin extracted from the blood in the chromatographic or electrophoretic strip was determined by a parallel run performed with a few μg. of synthetic oxytocin. The hormone was easily visualized by chlorination of the peptide bonds and liberation of iodine from potassium-iodide in acetic medium giving a dark blue colour (Reindel & Hoppe, 1954).

Elution was performed according to Dent (1947) with a 3 ml. Tyrode solution or
Oxytocin-like activity in puerperal blood

Oxytocin obtained from the blood of puerperal women during continuous infusions of synthetic oxytocin

Four experiments were performed: in all of them a control sample of jugular blood (60 ml.) was taken by catheterization before the infusion was started. These samples were treated in the same manner as those withdrawn during infusion.

Continuous intravenous infusions of synthetic oxytocin (Syntocinon, Sandoz) were administered at different rates to four patients during the early puerperium 1 or 2 hr. after suckling.

The rates of infusion were 128 m-u./min. in two patients; 240 and 480 m-u./min. in the other two. Thirty minutes after starting the infusion, 60 ml. of peripheral venous blood were withdrawn into a heparinized polyethylene coil which was immersed in an ice-water bath to diminish the oxytocinase activity of the plasma. The blood was centrifuged at 0° and 2500 rev./min. as soon as possible, and the supernatant was treated as described in the previous section.

Separation of an endogenous oxytocin-like substance from the blood of puerperal women during suckling

Since the experimental procedure for withdrawal of blood described below did not cause any emotional block of the milk-ejection reflex, as indicated by the increase of intramammary pressure, patients were not sedated. Samples of jugular blood were obtained from normal puerperal women during suckling and 1 or 2 hr. later, by catheterization, after an antecubital vein was punctured with a B.D. No. 16 TW needle (1-6 mm. o.d.). A polyethylene catheter No. PE 90 (1-27 mm. o.d.) was then introduced through the needle as far as the internal jugular vein. The position of the catheter in the innominate vein was controlled radiographically after injection of a contrast medium. Patency of the catheter was ensured by a continuous infusion of a 0-9 % NaCl solution. The effect of suckling was controlled in seven cases by recording the intramammary pressure by the method devised by Sica-Blanco, Méndez-Bauer, Sala, Cabot & Caldeyro-Barcia (1959). Figure 1 shows a record of the intramammary pressure changes in two lacteal ducts during withdrawal of a sample of jugular blood. Since sucking always produced strong contractions in the contralateral mammary gland we decided, in order to obtain samples in a more natural condition, to avoid mammary catheterization and measure the baby’s increase in weight over the period of blood sampling as a control of suckling. This weight gain was always positive, changing in accordance with the time of extraction of blood samples. Blood-pressure cuffs were applied to both arms of the patient at a pressure of 60 mm. Hg immediately before the baby was put to feed. Withdrawal of the blood samples was started at the moment when the baby started to suckle (Fig. 1). In order to ease and accelerate blood removal, the distal end of the coil was connected to a flask partially evacuated to a positive pressure of 100–150 mm. Hg.
The blood was collected in a heparinized polyethylene coil immersed in a bottle of ice-water. The time during which blood was collected ranged from 3 to 14 min. The samples were treated as described previously.

Seventeen samples processed to the freeze-drying step were combined into four large pools, and four samples were extracted individually.

Five jugular blood samples obtained before suckling were used for control experiments.

In one patient (No. 20) three blood samples each of 80 ml., were obtained: one during suckling, another 1 hr. later, and the third during a 30 min. intravenous infusion of synthetic oxytocin at the rate of 128 m-u./min. 2 hr. after suckling.

RESULTS

The recovery of oxytocin added in vitro to plasma of pregnant women near term, in nine experiments, was $33 \pm 12$ (s.d.) %. This figure includes results obtained either by paper chromatography or electrophoretic analysis.

In the corresponding nine control experiments no oxytocic activity could be found.

The most important losses connected with the method occurred during column chromatography (30%) and freeze-drying (37%).

These figures were calculated by measuring the loss of oxytocin in the freeze-drying stage (about 37%) and the loss of oxytocin when the hormone was passed through a chromatographic column, eluted with aqueous pyridine and freeze-dried (66%).

The difference between both figures was taken as the loss of chromatography plus elution.

The concentrations of oxytocin and oxytocin-like substance reported below were calculated assuming recoveries of 33% and neglecting the amount of oxytocin lost by the action of plasma oxytocinase under our experimental conditions (Garofalo et al. 1967).
Oxytocin-like activity in puerperal blood

Oxytocin was found in all the samples of blood collected during the infusions. Figure 2 shows a record of the assay of oxytocin obtained from peripheral plasma by paper electrophoresis. Blood concentrations of the hormone at different infusion rates are plotted in Fig. 4.

An endogenous oxytocin-like substance was obtained from three of the four pooled samples, two by electrophoresis and one by chromatography. The concentrations

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**Figure 2.** Record of the assay in the lactating rabbit of oxytocic activity in the blood of a puerperal woman during an i.v. infusion of synthetic oxytocin (see text). Injections of 0.5 ml. were given in 10 sec. by the retrograde arterial route. The oxytocic activity was separated by paper electrophoresis. X = doses of oxytocin-like activity. Oxytocin standard (μ-u./ml.).

**Figure 3.** Assay on the rat mammary gland strip (isometric contraction) of oxytocin-like substance from blood of puerperal woman during the act of suckling. Eluate (0.5 ml.) was added to the bath every 3 min. The rapid deflexion in the record is a 'washing-out effect'. The oxytocin-like substance (X) was separated by paper chromatography; S and Z correspond to eluates of the origin and the front of the chromatogram.
found in jugular plasma during suckling were equivalent to 19, 12 and 15 μ-u.
or oxytocin/ml. assayed by milk ejection in the rabbit.

The fourth plasma pool stored at 4° for more than 3 months had lost its activity
probably because of the actions of pyridine and of freeze-drying.

Using oxytocin assays on the isolated rat mammary gland strip, the oxytocin-like
substance was assayed in three cases, two extracts having been purified by paper

![Graph](https://via.placeholder.com/150)

**Fig. 4.** Concentrations of oxytocin found in plasma in four puerperal women
during intravenous infusions of synthetic oxytocin.

**Table 1. Values for the oxytocin-like activity in the jugular blood
of puerperal women during suckling**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Pooled samples</th>
<th>Individual samples</th>
<th>Control samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>3 2 3 4</td>
<td>10 11 20 21</td>
<td>20 25 31 36 37</td>
</tr>
<tr>
<td>Method of isolation of oxytocin-like material</td>
<td>E E C E</td>
<td>C C E E</td>
<td>E E E E E E</td>
</tr>
<tr>
<td>Assay method</td>
<td>ME ME IM IM</td>
<td>IM IM IM IM</td>
<td>IM IM IM IM IM</td>
</tr>
<tr>
<td>Oxytocin-like material (μ-u./ml. plasma)</td>
<td>19 15 12 6</td>
<td>18 23 25 11</td>
<td>&lt;10* &lt;10 &lt;14 &lt;12 &lt;6</td>
</tr>
</tbody>
</table>

* <10 indicates that no activity could be detected when the threshold of the method was 10 μ-u./ml.
plasma.

E = electrophoresis; C = chromatography; ME = milk ejection; IM = isolated strip of the rat
mammary gland.
chromatography and one by electrophoresis (see Fig. 3). The levels in jugular plasma were equivalent to 18, 23 and 25 \(\mu\)-u./ml.

Table 1 summarizes results obtained during suckling and in control experiments.

**DISCUSSION**

The substance obtained from the blood of puerperal women either during i.v. infusions of synthetic oxytocin or during normal suckling, had the same chromatographic, electrophoretic and pharmacological properties as synthetic oxytocin. Its chromatographic and electrophoretic behaviour excluded 5-hydroxytryptamine, lysine-vasopressin, adrenaline, noradrenaline acetylcholine, histamine and substance P as a source of, or a contribution to, the oxytocic activity (Heller & Lederis, 1958; Fitzpatrick & Walmsley, 1965; Brovetto et al. 1967). Bradykinin and hypertensin can also be separated from oxytocin by chromatography; they are excluded because they do not contract the isolated rat mammary gland in concentrations under 1 \(\mu\)-g./ml.

The oxytocin levels found were of an order which could be predicted from the following considerations:

1. The average oxytocin peripheral blood level found during i.v. infusions of 128 m-u. oxytocin/min. is about 30 \(\mu\)-u./ml, peripheral plasma. According to Sica-Blanco et al. (1959) the rate of infusion of synthetic oxytocin necessary to reproduce milk-ejection is around 4 m-u./min. (Sica-Blanco et al. 1959). If we assume a direct proportionality between the rate of infusion and plasma level, the concentration of oxytocin in peripheral plasma, during suckling, would come to 1 \(\mu\)-u./ml. Admitting that oxytocin concentration is five to ten times higher in jugular blood than in peripheral blood (Coch et al. 1965), the concentration of the hormone in jugular plasma would range from 5 to 10 \(\mu\)-u./ml.

2. According to a formula reported by Müller (1961), the total amount of a drug in blood, depends on the rate of infusion and on the half-life of the drug in the blood. Our samples of blood were taken usually on the third day of the puerperium. The half life of oxytocin in the plasma of such blood can be estimated as 9 min. (Garófalo et al. 1967). As the rate of infusion necessary to elicit milk ejection is about 4 m-u./min. according to Müller’s formula, the total amount of oxytocin in the blood would be 52 m-u. and its concentration about 10 \(\mu\)-u./ml. blood.

All the above facts strongly suggest that the oxytocin-like substance in the blood of puerperal women during suckling is oxytocin.

These results disagree with the values for oxytocin equivalent activity in the plasma of puerperal women reported previously by us (Coch et al. 1965), when an oxytoecic activity in jugular plasma of 200 to 300 \(\mu\)-u./ml. was found.

Still higher figures have been reported by Hawker, Walmsley, Roberts, Blackshaw & Downes (1961) under similar circumstances; they used a different method of extraction. The discrepancy may, at least in part, be attributed to the fact that the methods used by the previous authors were much less specific for oxytocin; hence, the activity of these extracts was probably the sum of the activities of oxytocin and other substances. However, the discrepancy could also be explained by assuming that the present method does not isolate the oxytocin quantitatively from blood.
Against this assumption is the agreement with the calculations reported above and the fact that a single i.v. injection of 2-4 m.u. oxytocin into an antecubital vein of a puerperal woman, roughly equivalent to a concentration of a few μ-u./ml. peripheral blood, produces milk ejection. The increase of oxytocic activity found during suckling needs further experimental confirmation.

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REFERENCES


