HORMONE AND ENZYME ASSAYS IN PREGNANCY

I. Studies on the placental and the tissue cystine-aminopeptidase activity in peripheral plasma from non-pregnant and pregnant women, and in plasma from the umbilical cord

By

Arne Christensen

ABSTRACT

The cystine-aminopeptidase (CAP) activity of placental origin (P-CAP) and of tissue origin (T-CAP) has been investigated in plasma from non-pregnant women, during normal pregnancy, in pregnancies complicated with severe pre-eclampsia and in plasma from the umbilical cord. In plasma from non-pregnant women the enzyme activity was significantly higher at pH 5.8 (T-CAP) than at pH 7.65 (P-CAP). During the pregnancy a gradual increase in both T-CAP and P-CAP took place. However, from about day 220 in the pregnancy the P-CAP became higher than the T-CAP. Between normal pregnancy and pregnancies complicated with severe pre-eclampsia there was only a low significant difference in the T-CAP activity, but highly significant difference in the P-CAP activity. The normal P-CAP pattern showed a gradual increase from about the 100th to the 270th day. A great spread in normal values was seen especially from about day 238 of gestation, due to variations in the enzyme activity of the women.

Abbreviations: P-CAP: Placental Cystine-Aminopeptidase ("plasma oxytocinase"). T-CAP: Tissue Cystine-Aminopeptidase ("tissue oxytocinase").

Presented in part at the 3rd European Congress of Perinatal Medicine, Lausanne 1972.
The coefficient of correlation between the P-CAP and the crude placental weight was calculated to +0.67.
The half-life of P-CAP was calculated to 8.3 days.
In plasma from the umbilical cord the T-CAP activity was significantly higher than the P-CAP, and was simultaneous that in plasma from non-pregnant women.

Oxytocinase is an aminopeptidase and since different substrates can be used for the estimation of the enzyme, the assay is rather unspecific (Tuppy & Nesvadba 1957; Sjöholm 1964; Yman 1970). When L-cystine-di-β-napthylamide is used as a substrate the enzyme measured is named cystine-aminopeptidase (CAP).

Semm (1960) discovered 2 aminopeptidases that were able to destroy oxytocin. He found one in tissues ("tissue" oxytocinase) and another in the peripheral plasma ("plasma" oxytocinase). Rydén (1966) reported optimal enzyme activity at pH 6.0–6.5 in some tissues while in others, such as the human placenta, the optimal activity was recorded at pH 7.3. Since 2 types of oxytocinase seem to be operating in the human body the question has been raised concerning the significance of measurement of plasma oxytocinase as an indicator of the placental function (Rydén 1966).

The CAP activity pattern in human pregnancy has been studied by several investigators (Babuna & Yenen 1966; Hensleigh & Krantz 1970; Chapman et al. 1971; Rydén 1971). The results, however, are conflicting probably due to different substrates and methods used.

The aim of the present study was to evaluate the interference of the 2 enzymes in the assay of CAP in plasma from non-pregnant women, during normal pregnancy, in plasma from the umbilical cord and in pregnancies complicated with severe pre-eclampsia. In addition, the intention was to study the CAP activity of placental origin during normal pregnancies and to determine the half-life of P-CAP.

MATERIAL AND METHODS

Subjects
Twenty healthy non-pregnant women (18 to 40 years old), 32 apparently normal pregnancies and 8 pregnancies complicated with severe pre-eclampsia were included in the present investigation. Severe pre-eclampsia was defined as a blood pressure systolic of 160 mmHg or higher and/or a diastolic blood pressure of 110 mmHg or higher associated with proteinuria of at least 20/00.

Collection and storage of the blood samples
Blood samples were drawn from the antecubital vein into heparinized test tubes, centrifuged and the plasma transferred to plastic tubes and deep frozen (~20°C).
In preliminary experiments it was demonstrated that the enzyme activity remained unchanged at room temperature for 6 h, at 4°C for 24 h and at -20°C for at least one year.

The method described by Babuna & Yenen (1966) has been used.

Reagents

Substrate solution. – 135 mg of 1-cystine-di-β-napthylamide (Sigma) was dissolved in 6.0 ml 0.1 N HCl by moderate heating and diluted to 50 ml with distilled water. Stored at 4°C.

Buffer solutions. – Veronal buffer pH 7.9: 104 ml 0.1 N HCl was mixed with 4.73 g sodium diethylbarbiturate and diluted to 500 ml with distilled water.

Phosphate buffer, pH 5.0 to 8.0, was made according to the Sörensen method.

Citrate-HCl buffer pH ≤ 5.0 was made according to the Sörensen method.

Trichloroacetic acid solution. – 10 % w/v.

HCl-acetone solution. – 2 volumes of 0.36 N HCl and 1 volume of acetone.

Sodium nitrite solution. – 0.1 % w/v. The reagent was stored at 4°C and freshly prepared every 3rd day.

Ammonium sulphamate solution. – 0.5 % w/v.

N-1 naphtylethylene diamine dihydrochlorid. – 0.05 % solution in 95 % ethanol.

The assay procedure

a) Initial mixture. – 0.6 ml plasma, 0.9 ml distilled water, 3.0 ml of the respective buffer.

b) Incubation mixture. – 75 ml initial mixture, 0.25 ml substrate solution.

One ml incubation mixture was pipetted into 3 corresponding test tubes. 1.0 ml of trichloroacetic acid was added to one of the test tubes which served as a blank. The 2 remaining tubes (duplicates) were placed in a water bath at 37°C for 120 min and the reaction was stopped by adding 1.0 ml of the trichloroacetic acid solution.

Determination of β-naphtylamine

After incubation the test tubes were centrifuged at 3000 r. p. m. for 20 min and 1.0 ml of the supernatant was pipetted into 3 corresponding tubes. The following steps were carried out in a semi-dark room: To the test tubes containing 1.0 ml of the supernatant, 2.5 ml HCl-acetone solution was added followed by 1.0 ml sodium nitrite solution. After 3 min 1.0 ml of ammonium sulphamate solution was added, and after another 2 min 2.0 ml naphtylethylene diamine solution. After each step in the procedure the test tubes were shaken well. The colour development took place at room temperature, but protected against light. After at least 60 min the samples were read in a spectrophotometer at 565 nm.

The enzyme unit

The enzyme activity was given as optical density (A O.D.) read in a spectrophotometer at room temperature after colour development for at least 60 min.

Reference plasma was produced as follows: Blood from 2 pregnant women was centrifuged and the plasma transferred to plastic tubes in aliquots of 1.0 ml and stored at -20°C, (reference plasma Nos. 1 and 2). The enzyme activity in the 2 plasma samples based on 20 measurements was O.D. 0.260 ± 0.005 (sd) and O.D.

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The T-CAP (pH 5.8) and the P-CAP (pH 7.65) activity in 20 non-pregnant women. The mean and variation are illustrated.

The enzyme activity at different pH in the incubation mixture during a single pregnancy. 1: After 125 days of pregnancy. 2: After 182 days of pregnancy. 3: After 223 days of pregnancy. 4: After 263 days of pregnancy. 5: After 282 days of pregnancy. 6: Six months after the delivery.
0.330 ± 0.005 (sd), respectively. The coefficient of variation was 1.8 per cent (No. 1) and 1.5 per cent (No. 2). The results of the measurements in each run of samples were accepted only if the activity in the corresponding reference plasma was within ± 2 sd of the original determination.

RESULTS

Non-pregnant women

The CAP activity found in plasma from 20 non-pregnant women is shown in Fig. 1. The enzyme activity was significantly higher at pH 5.8 (mean O. D. 0.072 ± 0.016 (sd)) than at pH 7.65 (mean O. D. 0.024 ± 0.004 (sd)).

The T-CAP and P-CAP curves during normal pregnancy

The enzyme activity curves (pH 3.5 to 8.0) were determined in 2 women

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Day in the gestation</th>
<th>pH 5.8 O. D.</th>
<th>pH 7.65 O. D.</th>
<th>Placental weight (g)</th>
<th>Infant weight (g)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>0.230</td>
<td>0.360</td>
<td>700</td>
<td>3850</td>
</tr>
<tr>
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<td>0.270</td>
<td>0.245</td>
<td>600</td>
<td>3750</td>
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<tr>
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<td>0.270</td>
<td>0.255</td>
<td>600</td>
<td>3380</td>
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<tr>
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<td>0.375</td>
<td>650</td>
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<tr>
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<td>0.225</td>
<td>0.280</td>
<td>600</td>
<td>3110</td>
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<td>0.255</td>
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</tr>
<tr>
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<tr>
<td>9</td>
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<td>0.270</td>
<td>0.350</td>
<td>650</td>
<td>3700</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
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<td>658</td>
<td>3566</td>
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<td>0.022</td>
<td>0.055</td>
<td>63.7</td>
<td>310.3</td>
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Pregnancies complicated with severe pre-eclampsia

<table>
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<tr>
<th>Case No.</th>
<th>Day in the gestation</th>
<th>pH 5.8 O. D.</th>
<th>pH 7.65 O. D.</th>
<th>Placental weight (g)</th>
<th>Infant weight (g)</th>
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</thead>
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<tr>
<td>1</td>
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<td>0.220</td>
<td>0.175</td>
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<tr>
<td>2</td>
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<td>0.200</td>
<td>450</td>
<td>2000</td>
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<td>3</td>
<td>271</td>
<td>0.235</td>
<td>0.150</td>
<td>550</td>
<td>2400</td>
</tr>
<tr>
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<td>0.200</td>
<td>650</td>
<td>2600</td>
</tr>
<tr>
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<td>0.180</td>
<td>450</td>
<td>1700</td>
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<td>0.225</td>
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<tr>
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<td>2106</td>
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<tr>
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<td>128.4</td>
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during pregnancy and in Fig. 2 the results from one of them are presented. As indicated in Fig. 2 a gradual increase in both T-CAP (pH 5.8 in the incubation mixture) and P-CAP (pH 7.65 in the incubation mixture) takes place. However, from about day 220 in the pregnancy the P-CAP became higher than the T-CAP.

The influence of T-CAP upon P-CAP in pregnancies complicated with severe pre-eclampsia

In order to determine whether there was any change in the T-CAP in pregnancies complicated with severe pre-eclampsia compared with normal pregnancies the T-CAP and P-CAP were measured in 9 normal pregnancies and 8 pregnancies complicated with severe pre-eclampsia. In Table 1 the results are presented. Between normal pregnancy and pregnancies complicated with severe pre-eclampsia there was only a low significant difference (0.025 < P < 0.05) in the T-CAP. However, there was a statistical highly significant difference in the P-CAP (P < 0.001). The duration of the pregnancies was between 268 and 286 days in both groups.

The P-CAP during normal pregnancy

P-CAP was determined during 32 normal pregnancies. The blood samples were collected from the 84th day in the pregnancy. The total number of assays

<table>
<thead>
<tr>
<th>Birth weight</th>
<th>No. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500–2999 g</td>
<td>3</td>
</tr>
<tr>
<td>3000–3499 g</td>
<td>12</td>
</tr>
<tr>
<td>3500–3999 g</td>
<td>13</td>
</tr>
<tr>
<td>Above 4000 g</td>
<td>4</td>
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</table>

<table>
<thead>
<tr>
<th>Sex of infants</th>
<th>No. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>15</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parity</th>
<th>No. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-para</td>
<td>17</td>
</tr>
<tr>
<td>B-para</td>
<td>10</td>
</tr>
<tr>
<td>C-para</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.
Distribution of birth weight, sex of infants and parity.
Fig. 3.
The normal P-CAP activity pattern in pregnancy based on 752 assays from 32 healthy women. (97.5 percentile, median. 2.5 percentile).

Fig. 4.
The P-CAP activity during 2 normal pregnancies.
Day to day variations in the P-CAP activity of normal pregnancies. 1: From 130 days of pregnancy. 2: From 190 days of pregnancy. 3: From 244 days of pregnancy. 4: From 263 days of pregnancy. 5: From 270 days of pregnancy.

The P-CAP activity after delivery in 3 cases.
was 752, mean 23.5 assays per woman, range 17 to 28. The distribution of birth weight, sex of the infants and the parity are presented in Table 2. Twenty-five of 32 infants had a birth weight between 3000 g and 3999 g. The distribution of the parity and sex of the infants in the material was fairly good.

The normal P-CAP pattern in 32 healthy women is presented in Fig. 3. The median, the 2.5 and 97.5 percentile are used in the statistical evaluation of the results. A gradual increase in the P-CAP was observed from about the 100th day of the pregnancy. The median showed an almost linear increase in the activity from day 100 to about day 270 in the pregnancy. There is a great spread in the normal values particularly from about 238 days of gestation.

In Fig. 4 the P-CAP during 2 normal pregnancies is presented. The variation in the enzyme activity from assay to assay showed only small deviations from the linear increase.

A better impression of the variations that might occur is given in Fig. 5 where day to day variations in 5 pregnancies are presented. Early in the pregnancy the variations are very small, some peaks are seen the last weeks before parturition. On the whole, the day to day variations are minor.

The enzyme activity in umbilical cord plasma in 15 cases of normal pregnancy at different pH in the incubation mixture. Mean and variation at pH 5.8 (T-CAP) and pH 7.65 (P-CAP). The drawn line: mean enzyme activity at different pH (abscissa).
The correlation between the P-CAP and the crude placental weight

Based upon the last assay carried out not later than 10 days before parturition the correlation between the enzyme activity and the crude placental weight was calculated to ± 0.67.

The half-life of P-CAP

The half-life of the enzyme was calculated from 10 subjects to be 8.3 days. Daily assays were performed after the expulsion of the placenta. In Fig. 6 3 cases are illustrated.

The CAP in plasma from the umbilical cord

The CAP found in plasma from the umbilical cord (15 cases) is shown in Fig. 7. The enzyme activity was significantly higher at pH 5.8 (T-CAP) (mean O. D. 0.130 ± 0.015 (sd)) than at pH 7.65 (P-CAP) (mean O. D. 0.0025 ± 0.005 (sd)).

DISCUSSION

In plasma from non-pregnant women the pH optimum was reached at pH 5.8 in the incubation mixture. It is reasonable to assume that this enzyme activity reflects the CAP activity in the tissues (“tissue oxytocinase”). The results are in good accordance with those previously reported by Semm (1960) and Rydén (1966). However, during the pregnancy there is a change in the pH optimum from pH 5.8 (T-CAP) to pH 7.65 (P-CAP). As both T-CAP and P-CAP increases during the pregnancy it could be argued that the measured rise in the P-CAP might partly be due to an increase in the T-CAP. This could be of practical value, since the liver is considered an important origin of the T-CAP (Müller-Hartburg et al. 1959; Oszacki & Grochowski 1964). Since the liver function can be affected in severe pre-eclampsia and cause an increased activity of T-CAP, false high P-CAP values could be obtained. The results, however, in the present work indicate that the T-CAP is almost unchanged in severe pre-eclampsia compared to normal pregnancies. Hence, the P-CAP in pregnancies complicated by severe pre-eclampsia reflects the metabolism of the placenta and only to a minor degree the metabolism of the liver (T-CAP).

At term in normal pregnancies the variation in the T-CAP was minor (O. D. 0.215–0.270) compared with the P-CAP (O. D. 0.245–0.405). Therefore the T-CAP seems to be rather constant from case to case compared with the P-CAP which is dependent on the functional state of the placenta. In addition, the present results indicate that measurement of T-CAP in peripheral plasma is of no value as a placental function test.

The wide range of variation in the P-CAP during normal pregnancies is
due to variation in the enzyme activity pattern of the women. The variation in the activity pattern in each woman did not show any differentiation that could explain the wide range particularly seen from about 238 days of pregnancy. The small variation in the day to day assays underline this fact.

It is difficult to compare the present results with those reported by other investigators (Babuna & Yenen 1966; Hensleigh & Krantz 1970; Chapman et al. 1971; Rydén 1971). Different methods have been used and the term “normal range” is not always defined. Rydén (1971) using the same substrate as in the present study, but a slightly different method, found an increase in the P-CAP up to about 38 weeks of pregnancy after which time a steep decrease occurred. This decrease could not be demonstrated in the present investigation.

The close relation between P-CAP and the crude placental weight indicates that the enzyme is produced in the placenta. The coefficient of correlation was higher than what has been reported by Rydén (1971).

The half-life of the P-CAP has been calculated to be from one week (Tovey 1969; Melander 1965) to 3 to 6 days (Rydén 1972). Based upon a study of 10 subjects in the present work, the half-life was calculated to 8.3 days (mean value). Hence, the P-CAP assay will reflect changes in the metabolism of the placenta rather slowly. The relatively long half-life may also partly explain the small day to day variations of the enzyme.

The P-CAP in cord blood was low and of the same order as in plasma from non-pregnant women. This is in good accordance with Riad (1962) and implies that the enzyme does not pass into the foetal circulation. Hence, the P-CAP merely reflects the metabolism of the placenta and not the foeto-placental unit as a whole. The high T-CAP in cord plasma (about twice the activity in plasma from non-pregnant women) reflects a high enzyme activity in the tissue of the newborn child.

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