Changes in Acid Soluble Nucleotides in the Rabbit Vagina in Relation to Duration of Oestrogen Treatment

By
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Abstract

1) By means of ion-exchange chromatography acid soluble nucleotides have been determined in the rabbit vagina in different hormonal conditions: i.e. normal mature rabbits, untreated castrated rabbits, and castrated rabbits treated with oestradiol for different periods of time (4–8 hours, 14–24 hours, 38–48 hours, 7–11 days, 18–28 days), as well as castrated rabbits treated with oestradiol + progesterone.

2) Castration results in a marked decrease in the NAD, ATP* and uridine nucleotide-fractions and inorganic phosphorus but an increase in the AMP-fraction.

3) As early as 4–8 hours after oestrogen treatment the nucleotides begin to return towards normal. Before 48 hours a normal nucleotide content (related to the amount of nitrogen) is obtained. The maximum oestrogen effect on acid soluble nucleotides is obtained after 7–11 days of oestrogen treatment.

4) After oestrogen stimulation a marked and early increase is observed of NAD- and uridine nucleotide-fractions, a somewhat smaller increase in ATP- and ADP-fractions and an insignificant increase in AMP-fraction.

5) The results indicate that castration induces dephosphorylation of ATP whereas oestrogen stimulation induces phosphorylation of AMP to ATP.

6) The significance of these results is discussed.

* The following abbreviations are used: TCA = trichloroacetic acid, AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, NAD = nicotinamide adenine dinucleotide (diphosphopyridine nucleotide).
Oestrogens have a dramatic effect on the target organs. There is practically no other hormonal effect in the body comparable to the rapid and marked increase in metabolism and synthesis produced by oestrogens in the uterus and the vagina.

The mechanisms of ovarian hormone action have been investigated mainly in two ways:

1) investigation of the effect of these hormones on substances which are important in the transport of energy, and
2) investigation of the time sequence of biochemical changes after administration of the hormones.

_Ad 1:_ The effect of ovarian hormones, mainly oestrogen, on high energy substances in the genital organs, e. g. adenosine nucleotides and different fractions of TCA-extracts has been studied by a number of research workers. In the rabbit uterus _Borell_ (1951 _a, b_) after 6 days of oestradiol treatment found a marked increase in easily as well as slowly hydrolysable acid soluble phosphates. He studied the metabolism of these phosphates by means of injected 32P-labelled phosphate and showed that oestrogen mainly influences easily hydrolysable phosphates while progesterone mainly affects slowly hydrolysable phosphates. Using a similar technique _Bengtsson_ (1953) found increases in the metabolism and amounts of acid soluble phosphates and in cell membrane permeability to phosphate in the rabbit vagina, following a single injection of oestrogen. _Walaas & Walaas_ (1950 _a, b_) found that oestradiol had no effect on the amount of labile phosphate in the rat myometrium and also that there was more labile phosphate in the uteri of pregnant than of castrated animals. They also demonstrated that oestrogen stimulates intracellular phosphate metabolism. _Menkes & Csapo_ (1952) found more ATP (2.40 μmol/g) in the oestrous rabbit uterus than in those of immature rabbits (1.98 μmol/g). Oestrogen stimulation also increased the ATP content of the uterus (cit. in _Corner & Csapo_ 1953).

The amount of adenosine phosphates in the human uterus under different hormonal conditions has been investigated by _Cretius_ (1957). In the uterus of menopausal women, non-pregnant women of fertile age and pregnant women he found that the amount of ATP (μmol/g) was 0.35, 0.61 and 1.25 respectively, that of ADP was 0.54, 0.85 and 1.64 and that of AMP was 0.90 and 1.41.

Thus, all these investigations have shown a marked effect of oestrogen on high energy phosphates in the genital organs.

Independently of the effect of hormones, the content of ATP in the rabbit uterus has been determined by _Lohmann_ (1928) who found 8 mg % adenosine polyphosphate; by _Csapo & Gergely_ (1950) who found 1.9 μmol ATP/g and by _Fleckenstein et al._ (1957) who found 0.40–0.75 μmol ATP/g. In the human uterus _Kinnunen & Pekkarinen_ (1952) found 5.4 mg % ATP.
Ad. 2: A second approach to the problem is to study the very first biochemical changes in the target organs after hormone stimulation. Behind this approach lies the assumption that such early changes may represent some of the most important starting mechanisms, which subsequently cause the increase in all metabolic and synthetic activities.

A number of facts are known about the sequence of biochemical changes after oestrogen stimulation. The following survey refers to the genital organs of different animals, mostly rats and rabbits. For complete references see Bengtsson (1953). The observation of a certain activity at a certain time after oestrogen stimulation does not imply that this activity did not start somewhat earlier.

Hours after oestrogen stimulation

1/2  Vasodilatation.
    Water uptake. Beginning of electrolytic changes.
1     Increase in O₂ consumption.
2     Increased permeability to phosphate.
2–6   Increased metabolism of acid soluble phosphates.
      Increased nucleotide metabolism (Mueller et al. 1958).
8–10  Increased mitotic activity.
      Increased muscular activity.
24–40 Cornified cells in the vaginal smear.

Thus, some of the earliest and presumably also some of the most important biochemical changes after oestrogen stimulation are found in the high energy phosphates.

The purpose of this investigation was to study more thoroughly the early changes in concentration of these important high energy substances after oestrogen stimulation and the evolution from a castrated to a strongly oestrogen stimulated rabbit vagina. This study is a continuation of previous work (Bengtsson 1953) and in addition is concerned with the effect of progesterone on acid soluble nucleotides in the rabbit vagina.

**MATERIAL AND METHODS**

The rabbits were of mixed breed, all fully mature, weighing between 2.5 and 4.2 kg. They were kept in separate cages and fed on the same diet: beets, dredge and hay, and fresh green plants when available. Two or three weeks after oophorectomy they received a single intramuscular injection of 0.5 mg oestradiol in oil («Dimenformon», Pharmacia). This more than physiological dose was chosen in order to guarantee
maximal stimulation of the vagina. When oestrogen stimulation was planned to last for more than two days, 20 µg oestradiol was given i. m. each day. Progesterone treated rabbits got 20 µg oestradiol i. m. daily for 7 days and 25 mg progesterone («Proluton» Schering A. G.) during the last two days. The animals were sacrificed by decapitation. By this means most of the blood was removed from the organs thus ensuring that the blood contributed but little to the content of water and orthophosphate in the vagina. The abdomen was then rapidly opened, and the intra-abdominal part of the vagina removed, cut open longitudinally, rapidly blotted on filterpaper and immediately placed on a special freezing table (Bengtsson 1953). When after some few seconds the vagina was frozen, thin chips were scraped off with a scalpel and, without being thawed, put into a test tube containing about 10 ml ice cold 5 % TCA. A thin layer of the vagina was left on the table in order to avoid contamination from the table. After shaking for several minutes, the fluid was filtered. The extraction was repeated twice and the vaginal tissue then carefully washed down on the filterpaper. The tissue and the filterpaper were submitted to wet combustion and nitrogen determined according to the Kjeldahl method. The extract was diluted to a known volume (50–100 ml), and an aliquot was taken for the determination of inorganic phosphorus according to the method of Martin & Doty (1949) with slight modification (Ernster et al. 1950; Borell 1951 b). The remainder of the extract was washed with chemically pure ethyl-ether until neutral reaction. The remaining ether was removed by aeration. The extract was then put on the column.

![Fig. 1.](image-url)

**Fig. 1.**

Ion-exchange chromatogram of test substances.
1 = AMP, 2 = UMP, 3 = ADP, 4–5 = UDP-derivates and 6 = ATP.
Ion-exchange chromatogram of acid soluble nucleotides from the vagina of a castrated rabbit. Peaks from left to right: NAD-fraction, AMP-fraction, ADP-fraction, U-fraction and ATP-fraction.

**Ion-exchange Chromatography**

The acid soluble nucleotides were separated according to the method described by Hurlbert et al. (1954). The column (Dovex 1, 200-400 mesh, 1 cm² × 10 cm) was prepared according to these authors. The mixing flask was filled with 200 ml distilled water. For the gradient elution the »formic acid system« was used in four concentrations (4 N formic acid, 0.2 M ammonium formate in 4 N formic acid, 0.4 M ammonium formate in 4 N formic acid and 0.8 M ammonium formate in 4 N formic acid). The eluate was collected by an automatic fraction collector (»Radi Rac«, type 3401 A and 3402 A, LKB Stockholm) in 4.5 ml fractions (0.5 ml/min). The optical density was measured in a Beckman spectrophotometer model DU at 260 mμ using distilled water as blank.

Nucleotides were expressed in μmol/g nitrogen, inorganic phosphorus in mg/g nitrogen. This was regarded as more accurate than referring to the wet or dry weight, which change considerably due to changes in water and electrolyte content. To get the amounts of nucleotides in μmol/g the OD of NAD was divided by 18, that of uridine by 10 and that of adenosine by 14.5.
Identification of the nucleotide fractions

In the test chromatograms, in which known amounts of AMP, ADP, ATP, UMP and UDP were chromatographed, the location of the peaks corresponded to the peaks obtained in the experiments (Figs. 1–3). The location of the peaks studied also corresponded to the distribution found by Hurlbert et al. (1954), using the same method. Furthermore, another method of determining acid soluble nucleotides in the rabbit vagina, i.e. gradient elution from Ecteola columns in the triethylammonium acetate system of Nilson & Sjunnesson (in press), which gives better separation of the nucleotides, has shown closely corresponding values (Bentsson et al., in press). It was also shown, however, that the peaks in the present study, obtained by the method of Hurlbert et al. (1954), are not completely pure and that the NAD- and ATP-fractions were somewhat more contaminated than other fractions. The results obtained by the two different methods are discussed in a forthcoming paper (Bengtsson et al.) The peaks of OD in this study are called "fractions", indicating that they are not absolutely pure.

All peaks, except that of uridine nucleotide-fractions in castrated animals, rose sufficiently above the background to allow of an accurate estimation of the OD.

The biological variation between animals in a certain group has been calculated for
the largest group, namely castrated animals. The standard error of mean was about 10% in all nucleotide fractions in this group.

The careful analysis of acid soluble nucleotides in the rabbit vagina performed by Bengtsson et al. (in press) is complicated, time consuming and expensive. The method used in the present investigation is on the whole without these disadvantages. Giving very similar results to those obtained by the more complicated method, this method, therefore, is very suitable for investigations in which a large number of experiments have to be done, as in this study.

RESULTS

The results have been grouped according to the hormonal condition of the animals. The hormonal states of castrated and hormone treated animals are clearly defined. A normal, mature female rabbit is in a more or less continuous oestrus. Thus, we may assume that all normal, mature, non-treated rabbits are in the same endocrine condition, and if minor variations occur, that these are irrelevant.

Castration produces a considerable decrease in all fractions of acid soluble nucleotides, with the exception of the AMP-fraction (Table 1, Fig. 4). The NAD-fraction decreases by about 30%, the ADP-fraction by about 8% and the ATP-fraction by 40%. The uridine fractions also show a marked decrease, but as these are very small in castrated animals, the values are somewhat difficult to determine accurately. The increase in the AMP-fraction is no less than 50%. The total decrease in acid soluble nucleotides/g nitrogen is about

![Table 1](https://example.com/table1.png)

Table 1.
Amount of acid soluble nucleotide fractions and inorganic phosphorus in the rabbit vagina under different hormonal conditions.
Nucleotides in μmol/g N.
Inorganic phosphorus in mg/g N.

<table>
<thead>
<tr>
<th>Hormonal condition</th>
<th>Number of experiments</th>
<th>NAD</th>
<th>AMP</th>
<th>ADP</th>
<th>U</th>
<th>ATP</th>
<th>Total nucl.</th>
<th>P₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>12.0</td>
<td>12.7</td>
<td>17.7</td>
<td>10.0</td>
<td>45.5</td>
<td>97.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Castrated</td>
<td>4</td>
<td>8.1</td>
<td>19.1</td>
<td>16.3</td>
<td>4.9</td>
<td>27.6</td>
<td>76.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Oestrogen 4–8 h</td>
<td>2</td>
<td>7.9</td>
<td>12.4</td>
<td>11.6</td>
<td>5.3</td>
<td>25.5</td>
<td>62.7</td>
<td>3.9</td>
</tr>
<tr>
<td>&quot; 14–18 h</td>
<td>2</td>
<td>12.7</td>
<td>10.5</td>
<td>16.1</td>
<td>8.1</td>
<td>35.8</td>
<td>83.2</td>
<td>4.7</td>
</tr>
<tr>
<td>&quot; 38–48 h</td>
<td>2</td>
<td>16.0</td>
<td>16.2</td>
<td>18.4</td>
<td>11.2</td>
<td>46.2</td>
<td>108.0</td>
<td>4.8</td>
</tr>
<tr>
<td>&quot; 7–11 d</td>
<td>3</td>
<td>24.7</td>
<td>20.2</td>
<td>36.2</td>
<td>19.7</td>
<td>69.6</td>
<td>170.4</td>
<td>6.7</td>
</tr>
<tr>
<td>&quot; 18–28 d</td>
<td>2</td>
<td>16.9</td>
<td>16.0</td>
<td>28.5</td>
<td>16.5</td>
<td>63.9</td>
<td>141.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Oestrogen + Progesterone</td>
<td>2</td>
<td>21.3</td>
<td>16.0</td>
<td>29.1</td>
<td>15.4</td>
<td>73.5</td>
<td>155.3</td>
<td>6.7</td>
</tr>
</tbody>
</table>

88
Changes of the amount of acid soluble nucleotides from the rabbit vagina in different hormonal conditions. 1 = ATP-fraction, 2 = ADP-fraction, 3 = NAD-fraction, 4 = AMP-fraction and 5 = U-fraction.

As the vagina atrofies after castration, which means a reduction of the nitrogen content, the absolute decrease of nucleotides per organ is much greater. The maximum oestrogen effect on acid soluble nucleotides is observed in animals treated for 7–11 days. Compared with castrated animals this group shows an enormous increase in all acid soluble nucleotides. It should be noted that the largest increase is found in the NAD-fraction (3 fold increase) and in the uridine nucleotide-fraction (3–4 fold; the values for castrated animals
are somewhat uncertain – see above). Next comes the ATP-fraction (2.5 fold increase) and the ADP-fraction (2.2 fold increase). The increase in the AMP-fraction is insignificant (from 19.1 to 20.2 \mu mol/g N). The total amount of acid soluble nucleotides increases 2.2 times.

If oestrogen treatment with the doses used (20 \mu g/d) is continued for a considerable time (18–28 days), the amount of acid soluble nucleotides/g nitrogen decreases (Table 1). It is not known why this decrease occurs. Prolonged treatment with large doses of oestrogen, however, produces severe degenerative changes in the rabbit uterus (Reynolds 1935; Zondek 1936). This has been confirmed in our laboratory. After 18 days treatment with 20 \mu g oestradiol i. m. daily, the uterus showed necrotic areas. This was more marked after 28 days of treatment. No such changes, however, could be found in the vagina. In spite of this negative finding it is quite possible that a depression of metabolic processes occurs without such gross changes in the histological structure of the vagina as in the uterus. Hence the 18–28 days oestrogen treated group is included in Table 1 only, but omitted from all other tables and figures. According to Bengtsson et al. (in press), uridine derivatives constitute the next largest group after adenosine derivatives in the rabbit vagina: up to 25 \mu mol/g tissue nitrogen for oestrogen treated animals. In the present investigation uridine nucleotides are found in not completely pure fractions as a »post-ADP-peak« and a »pre-ATP-peak«, which together represent 19.7 \mu mol/g nitrogen in 7–11 days oestrogen treated animals. In castrated animals the »pre-ATP-peak« disappears (Fig. 2), but reappears following oestrogen treatment.

Thus, the results from normal, castrated and 7–11 days oestrogen treated rabbits are in full agreement with the findings of Bengtsson et al. (in press).

A more thorough analysis of changes in nucleotide content of the vagina related to the duration of oestrogen stimulation (Fig. 4 and Table 1) shows in the 4–8 hours group a further decrease (as compared with castrated animals) in all acid soluble nucleotides, which now include the AMP-fraction. This unexpected decrease will be discussed later (p. 92).

In the 14–18 hours oestrogen treated group the results are different: the nucleotide content increases with the exception of the AMP-fraction which still decreases.

In the 38–48 hours and 7–11 days treated groups a further increase in all acid soluble nucleotides is observed.

As can be seen in Table 1, progesterone treatment for two days (combined with oestrogen pre-treatment) does not significantly change the nucleotide content as compared with 7–11 days treated animals, where the oestrogen stimulation is maximal.

The content of different acid soluble nucleotides in normal rabbits fits very well in between the 14–18 hours and the 38–48 hours oestrogen treated groups (Table 1). Hence, within as little as 48 hours, 0.5 mg oestradiol restores the
normal nucleotide content in the vagina (as related to the amount of nitrogen). Thus, oestrogen stimulation produces a marked increase in acid soluble nucleotides in the rabbit vagina which is closely related to the duration of stimulation. The distribution of different nucleotides calculated as a percentage of the total amount of acid soluble nucleotides also shows some interesting changes with different hormonal conditions (Table 2). The most obvious changes found after castration are: an increase in the AMP-fraction and a

Fig. 5.
Changes in distribution of AMP- and ATP-fractions (per cent of total amount of acid soluble nucleotides) in the rabbit vagina in different hormonal conditions.
Upper curve = ATP, lower curve = AMP.
Table 2.
The amount of different acid soluble nucleotide fractions expressed in per cent of total amount of acid soluble nucleotides in the rabbit vagina under different hormonal conditions.

<table>
<thead>
<tr>
<th>Hormonal condition</th>
<th>Number of experiments</th>
<th>NAD</th>
<th>AMP</th>
<th>ADP</th>
<th>U</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>12</td>
<td>13</td>
<td>18</td>
<td>10</td>
<td>47</td>
</tr>
<tr>
<td>Castrated</td>
<td>4</td>
<td>11</td>
<td>25</td>
<td>21</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>Oestrogen 4–8 h</td>
<td>2</td>
<td>13</td>
<td>20</td>
<td>18</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>&quot; 14–18 h</td>
<td>2</td>
<td>15</td>
<td>13</td>
<td>19</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>&quot; 38–48 h</td>
<td>2</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>&quot; 7–11 d</td>
<td>3</td>
<td>15</td>
<td>12</td>
<td>21</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>Oestrogen + Progesterone</td>
<td>2</td>
<td>14</td>
<td>10</td>
<td>19</td>
<td>10</td>
<td>47</td>
</tr>
</tbody>
</table>

decrease in uridine- and ATP-fractions. As early as 4–8 hours after oestrogen injection, the distribution of nucleotides tends to return towards the pattern observed in the normal vagina. After 14–18 hours the distribution differs only slightly from the normal. Neither more prolonged oestrogen stimulation nor progesterone treatment produces any further changes in this distribution. Thus, in the distribution of acid soluble nucleotides, a return towards a normal condition is found as early as 4–8 hours after oestrogen stimulation. The normalizing or stimulating effect of oestrogen on acid soluble nucleotides is first observed in the distribution and then in the amount of nucleotides.

Inorganic phosphate decreases after castration. Oestrogen stimulation induces a continuous increase (Table 1).

**DISCUSSION**

The decrease in high energy nucleotide phosphates in the vagina after castration and the increase after oestrogen stimulation show that the amount of energy available corresponds to the state of cellular activity. It is logical to assume that this increase in energy available is a prerequisite to the increase in activity. Thus, some of the first functions of oestrogen in the target organs seem to be to increase the permeability of the cell membrane to phosphates (Bengtsson 1953) and to stimulate the enzyme systems which build up the energy supply.

The decrease in the amount of acid soluble nucleotides after castration is understandable, but the further decrease after 4–8 hours oestrogen stimulation is quite unexpected. An apparent depression of some cellular activities shortly
after oestrogen stimulation is also indicated by other investigations: the data published by Brody (1958) and Brody & Westman (1960 a, b) show a decrease in the amount of nucleic acids of the rabbit uterus some few hours after oestrogen stimulation. Bengtsson (1953) found signs of decrease in labelling of acid soluble phosphates in the rabbit vagina shortly after injection of oestrogen and $^{32}$P.

It is difficult to explain the decrease shortly after oestrogen stimulation. The decrease in acid soluble nucleotides may be only relative, due to an increase in nitrogen content. Mueller et al. (1958) have shown that the increase in protein synthesis begins a few hours after oestrogen stimulation. However, it is doubtful whether this increase in protein synthesis is sufficient to change the ratio nucleotides/nitrogen to such an extent and so shortly after stimulation.

At about the same time after oestrogen stimulation as this decrease in nucleotides is observed, changes in mitochondrial structure are found. Mueller et al. (1958) demonstrated in the rat uterus that »in the period from 0–6 hours the cells become swollen, the mitochondria vesiculated, and the endoplasmic reticulum spreads apart«. In order to check if these mitochondrial changes also occur in the rabbit vagina, an electronmicroscopy study was made on the vaginas of castrated rabbits following 6 hours and 24 hours of oestrogen treatment (0.5 mg oestradiol i. m.). Six hours after oestrogen administration the same picture was found as that described by Mueller. Twentyfour hours after treatment, i.e. when the acid soluble nucleotides had reached the values of a normal rabbit, the mitochondria had returned to the normal stage. It is interesting to note that similar mitochondrial changes have been correlated with ATP, ADP and different ions (Lehninger 1959), but the exact significance of these changes is still unknown.

The absolute amount ($\mu$mol/g N) and the relative amount (% of total amount of acid soluble nucleotides) of AMP- and ATP-fractions show interesting changes (Figs. 4 and 5). After castration the ATP-fraction decreases and the AMP-fraction increases. After oestrogen treatment (up to 14–18 hours) the reverse is found: i.e. increase of the ATP-fraction and decrease of the AMP-fraction. The changes in the amount of the ATP-fraction in the vagina from the normal to the castrated and to the vagina of rabbits treated with oestrogen for 14–18 hours, reflect the changes in AMP-fraction (Fig. 5). This indicates that castration induces dephosphorylation of high energy phosphates whereas oestrogen stimulation induces phosphorylation of low energy phosphates. This phosphorylation of AMP to ATP seems to be one of the principal effects of oestrogen stimulation.

After castration the amount of NAD-fraction/g N decreases by 30 %. After only a few hours of oestrogen stimulation there is a relative increase in the NAD-fraction (Table 2) and from the castrated state to full oestrogen stimulation there is a 3 fold increase in the NAD-fraction (Table 1).
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