Abstract. The effect of oestradiol (Oe2) or ethinyl-oestradiol (EOe) on several enzymes, soluble protein and DNA in the endometrium was studied in ovariectomized adult rats. The most marked effect of Oe2 was the increase in the activity of lactate dehydrogenase (LDH). If the treatment was started on the day of ovariectomy, it took approximately 8 days to reach a constant elevation. If the administration of Oe2 was started one week after castration, the maximum response was seen after 4 days of treatment. Using the latter schedule a linear dose-response regression curve of LDH/DNA was obtained with λ = 0.086. This parameter is considered suitable for the comparison of the effects of different oestrogens.

Administration of Oe2 for 8 days beginning on the day of ovariectomy gave no linear dose-response curve of LDH/DNA.

Administration of EOe caused a very marked increase of the specific activity of pyruvate kinase, LDH, M-type LDH and some slight, but significant decreases in isocitrate dehydrogenase, glutamate dehydrogenase, acid phosphatase and alkaline phosphatase. The changes of β-glucuronidase were only slight. The content of DNA per wet weight of endometrium decreased after oestrogen treatment, the protein content remained reasonably constant. It is concluded that, after stimulation with oestrogen, the rat endometrium produces the energy needed for its own growth mainly via anaerobic glycolysis and that the Krebs cycle plays a relatively small role.

In the present paper we describe experiments performed to find an experimental design for the optimal response of endometrial enzymes to oestrogen administration, further to establish the type of response (increase or decrease) of the enzymes and to choose the best one for a quantitative comparison of various oestrogens.

Materials and Methods

In all experiments adult Cpb:ORGa rats, weighing 250–300 g and purchased from TNO, Zeist, The Netherlands, were used. All animals were kept at 22–24°C, relative humidity 45–55% with periods of 14 h light from 06.00–20.00 and 10 h darkness. They had free access to Hope Farm RMH-B chow (Woerden, Holland) and water. All rats were ovariectomized by the dorsal route under ether anaesthesia on day 1 of the experiment.

In order to establish the optimum period and duration of the treatment which would give the maximum response the investigation was started with two experiments. In the first one (Exp. 1) the administration of oestradiol was started on the day of ovariectomy and continued for various periods. In the second one (Exp. 2) oestradiol injections were started one week after ovariectomy and were again continued for various periods of time (Fig. 1). In these two experiments oestradiol 0.15 µg/300 g in arachis oil was injected sc twice daily.

In two following experiments the dose-response curve of oestradiol was established: in Exp. 3: 0.063-0.40 µg Oe2/300 g sc twice daily was administered for 8 days, starting on the day of castration. In Exp. 4: 0.025-0.16 µg Oe2/300 g sc twice daily was administered for 4 days, starting one week after castration (Fig. 2).
In the fifth experiment (Exp. 5) ethinyl oestradiol (4.0 and 16.0 mg/300 g po twice daily) was administered for 4 days, starting one week after castration (Table 3). Control animals were treated with arachis oil sc in experiments with oestradiol and with suspension vehicle po (0.2% methylcellulose and 0.9% NaCl in distilled water) in the experiment with ethinyl oestradiol.

At the end of each experiment the animals were decapitated 12 h after the last administration. The uteri were cleaned and the endometrium separated on a plate cooled by a mixture of water and ice. Endometrium was separated by careful scraping, collected in small preweighed cups and weighed to the nearest 0.1 mg. The samples were then immediately frozen on dry ice and kept in stoppered small plastic vials at −18°C until the moment of biochemical determination (usually not longer than one month). The endometria from 2–3 ovariectomized rats treated with placebo had to be pooled in order to obtain the required minimum amount of 20 mg tissue. In oestrogen-treated rats one animal provided enough tissue for one sample.

The tissue was homogenized in saline, pH 7.5 at 4°C to give a tissue concentration of 10 mg endometrium per ml homogenate. The homogenate was separated into supernatant and sediment by centrifugation at 20,000 × g for 10 min at 4°C. Enzymes and protein were determined in the clear supernatant and DNA in the sediment. In the alkaline hydrolysate of the sediment DNA was determined using the indole reaction (Bonting & Jones 1957). The determinations of extractable protein (Lowry et al. 1951), lactate dehydrogenase = LDH and its M-type = M-LDH (Bergmeyer 1970), β-glucuronidase = β-G (Talalay et al. 1946), acid phosphatase = ACP (Gutman & Gutman 1940) and alkaline phosphatase = AP (Bessey et al. 1946) were described in more detail in a previous paper (Jelinek & Jelinková 1977). The assays of pyruvate kinase = PK (E.C. 2.7.1.40), isocitrate dehydrogenase = ICDH (E.C. 1.1.1.42) and glutamate dehydrogenase = GLDH (E.C. 1.4.1.3) are based on the spectrophotometric determination of NAD or NADP at 346 nm and were performed at 25°C (Bergmeyer 1974). All the measurements of enzyme activities were made on the Beckman DB-G spectrophotometer fitted with a constant temperature cell housing. The rate of each enzyme reaction was linear during the time span of the assay. The two phosphatases (ACP and AP) were assayed using an Autolab (Linson Instruments A.B., Stockholm).

The enzyme activities are expressed in units (U) per mg protein or per mg DNA. One unit is defined as the amount of enzyme which converts 1 μmole of substrate per min under the prescribed conditions.

Statistical significance was calculated with the Wilcoxon test and is marked with an asterisk if P < 0.05. The λ of the dose-response curve is defined as the ratio

\[
\text{standard deviation of responses} \quad \lambda = \frac{\text{slope of regression line}}{.}
\]

In the figures and tables means ± sem are given.

Results

Selection of the optimum treatment period and of a suitable enzyme

We studied the LDH, β-glucuronidase, ACP and AP in the endometrium after administration of 0.15 μg oestradiol/300 g body-weight twice daily for various periods starting one week after ovariectomy (Exp. 2). Endometrial samples were taken 12 h after the last injection. Table 1 gives the activities of the 4 enzymes in U/mg protein. Of the enzymes studied the lactate dehydrogenase (LDH) displayed the greatest increase in activity with a maximum after 4 days of administration. AP decreased after oestrogen treatment as seen from comparisons with the corresponding placebo groups. The same is true for β-glucuronidase and ACP except for the longest period of administration (7 days). The activity of the two latter enzymes, however, decreases without treatment – with the time after castration, as seen from comparison of the 3 placebo groups. Judging from these experiments LDH promised to give the best dose-response curve to oestrogen treatment from the 4 enzymes studied.

To find the optimum beginning and duration of treatment we compared the results of the above mentioned experiment (Table 1) with another one in which oestradiol in the same dose was administered for various periods of time, but starting on the day of ovariectomy (Exp. 1). The result of this comparison is given in Fig. 1, this time expressed in U/mg DNA. If the treatment was started one week after ovariectomy, a maximum rise of LDH occurred after 4 days of administration, longer treatment resulted in smaller responses (Exp. 2). In Fig. 1 changes of LDH are presented which were observed when oestradiol was started on the day of ovariectomy. Although this also increased the LDH activity, there was obviously less influence on the duration of treatment and a lower maximum was reached (Exp. 1).

From these results a treatment period of 8 days was chosen when administration of the oestrogen was started on the day of ovariectomy, and a treatment period of 4 days when administration was started one week after ovariectomy. The dose-response curves of oestradiol were compared in these two treatment schedules.

Dose-response curves of oestradiol

The effects on LDH of increasing doses of oestradiol, administered in the two ways mentioned
Table 1.
Administration of oestradiol twice daily 0.15 µg/300 g body weight sc for various periods starting one week after ovariectomy (Exp. 2). Samples taken 12 h after the last injection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>mg DNA/g w. w.</th>
<th>Enzymatic activities in U per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LDH (U)</td>
</tr>
<tr>
<td>1) Placebo for 1 day</td>
<td>19.6 ± 0.6</td>
<td>0.97 ± 0.05</td>
</tr>
<tr>
<td>2) Oe2 for 1 day</td>
<td>12.5 ± 0.2*</td>
<td>1.28 ± 0.07*</td>
</tr>
<tr>
<td>3) Oe2 for 2 days</td>
<td>10.7 ± 0.4*</td>
<td>3.52 ± 0.13*</td>
</tr>
<tr>
<td>4) Oe2 for 4 days</td>
<td>9.2 ± 0.2*</td>
<td>4.40 ± 0.17*</td>
</tr>
<tr>
<td>1) Placebo for 3 days</td>
<td>19.1 ± 0.05</td>
<td>0.90 ± 0.04</td>
</tr>
<tr>
<td>2) Oe2 for 3 days</td>
<td>9.0 ± 0.06*</td>
<td>3.93 ± 0.24*</td>
</tr>
<tr>
<td>3) Oe2 for 5 days</td>
<td>10.4 ± 0.05*</td>
<td>2.94 ± 0.23*</td>
</tr>
<tr>
<td>4) Placebo for 7 days</td>
<td>21.8 ± 0.05*</td>
<td>0.76 ± 0.02*</td>
</tr>
<tr>
<td>5) Oe2 for 7 days</td>
<td>11.9 ± 0.03*</td>
<td>2.44 ± 0.12*</td>
</tr>
</tbody>
</table>

*= Significantly different from the first group in each of the two parts. N = no significance difference.
Eight samples per group in the first part; 7 samples per group in the second part. Mean ± sem.

![Graph](image)

Fig. 1.
Lactate dehydrogenase per DNA in rat endometrium: —— = administration started on the day of castration (Exp. 1); – – – = administration started 1 week after castration (Exp. 2); C = placebo-treated ovariectomized controls; ♀ = ovariectomy; E2 = oestradiol 2 x 0.15 µg/300 g/day sc given for the number of days indicated. Autopsy 12 h after the last injection.
Dose-response curve of LDH/DNA to oestradiol sc. The dose was given twice daily per 300 g body weight. 

--- = administration of Oe₂ for 8 days started on the day of castration (Exp. 3); --- = administration of Oe₂ for 4 days started 1 week after castration (Exp. 4); \( \lambda = \) standard deviation of assay 

The effect of the highest dose of oestradiol reached 1700% of that of the placebo. The effect was due to a dose-related increase in LDH and a dose-related decrease in DNA (Table 2). This linear regression can be considered suitable for the comparison of the effects of different oestrogens.

Table 2 shows also the changes of the other 3 enzymes in response to rising doses of oestradiol. There was a significant dose-related decrease in AP (down to 35%). ACP decreased to 60% of the control, the changes in β-GR were variable.

On the other hand, if the administration was started on the day of ovarietomy and continued for 8 days, a curved regression line not usable for testing of oestrogenic effect was found for LDH (Exp. 3) (Fig. 2).

Table 2.
Enzymatic activities after increasing doses of oestradiol (Oe₂) sc. Administration started 1 week after castration and was given for 4 days (Exp. 4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>mg DNA/g w. w.</th>
<th>Enzymatic activities in U per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LDH (U)</td>
</tr>
<tr>
<td>1) Placebo</td>
<td>21.4 ± 0.2</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>2) Oe₂ 2 × 0.025 μg</td>
<td>14.4 ± 0.5*</td>
<td>1.10 ± 0.04*</td>
</tr>
<tr>
<td>3) Oe₂ 2 × 0.040 μg</td>
<td>14.2 ± 0.5*</td>
<td>1.63 ± 0.12*</td>
</tr>
<tr>
<td>4) Oe₂ 2 × 0.063 μg</td>
<td>12.4 ± 0.5*</td>
<td>2.42 ± 0.12*</td>
</tr>
<tr>
<td>5) Oe₂ 2 × 0.100 μg</td>
<td>9.7 ± 0.3*</td>
<td>3.58 ± 0.20*</td>
</tr>
<tr>
<td>6) Oe₂ 2 × 0.160 μg</td>
<td>7.3 ± 0.6*</td>
<td>3.77 ± 0.17*</td>
</tr>
</tbody>
</table>

* = Significantly different from the first group.  N = no significant difference.
Seven samples per group. Mean ± SEM.
Table 3.
Comparison of enzymatic activities after 2 doses of ethinylestradiol (EOe) po. Administration started 1 week after castration and was given for 4 days (Exp. 5).

<table>
<thead>
<tr>
<th>Groups</th>
<th>mg protein/g w. w.</th>
<th>LDH (U)</th>
<th>M-LDH (U)</th>
<th>PK (mU)</th>
<th>ICDH (mU)</th>
<th>GLDH (mU)</th>
<th>β-GR (mU)</th>
<th>ACP (mU)</th>
<th>AP (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Placebo</td>
<td>56.7 ± 2.7</td>
<td>0.87 ± 0.03</td>
<td>0.66 ± 0.05</td>
<td>15.0 ± 2</td>
<td>44.3 ± 2.0</td>
<td>31.3 ± 3.0</td>
<td>6.7 ± 0.1</td>
<td>33.5 ± 1.2</td>
<td>43.2 ± 1.5</td>
</tr>
<tr>
<td>2) EOe 2 × 4µg</td>
<td>58.1 ± 1.5 N (7)</td>
<td>1.35 ± 0.04* (7)</td>
<td>1.07 ± 0.08* (7)</td>
<td>32.0 ± 4 (5)</td>
<td>33.3 ± 0.9* (7)</td>
<td>26.3 ± 2.8 N (7)</td>
<td>5.3 ± 0.5* (7)</td>
<td>28.6 ± 1.2* (7)</td>
<td>33.5 ± 1.4* (7)</td>
</tr>
<tr>
<td>3) EOe 2 × 16µg</td>
<td>58.8 ± 1.1 N (7)</td>
<td>3.42 ± 0.22* (7)</td>
<td>2.90 ± 0.25* (7)</td>
<td>107.5 ± 11* (7)</td>
<td>32.0 ± 1.1* (7)</td>
<td>12.5 ± 1.4* (7)</td>
<td>6.4 ± 0.4 N (7)</td>
<td>22.2 ± 1.2* (7)</td>
<td>14.9 ± 1.1* (7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>mg DNA/g w. w.</th>
<th>LDH (U)</th>
<th>M-LDH (U)</th>
<th>PK (mU)</th>
<th>ICDH (mU)</th>
<th>GLDH (mU)</th>
<th>β-GR (mU)</th>
<th>ACP (mU)</th>
<th>AP (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Placebo</td>
<td>19.5 ± 0.7 (7)</td>
<td>2.53 ± 0.16 (7)</td>
<td>1.92 ± 0.14 (7)</td>
<td>41.2 ± 7 (5)</td>
<td>129.5 ± 6.0 (6)</td>
<td>90.0 ± 8.4 (6)</td>
<td>19.5 ± 0.3 (7)</td>
<td>96.7 ± 2.2 (7)</td>
<td>126.3 ± 6.4 (7)</td>
</tr>
<tr>
<td>2) EOe 2 × 4µg</td>
<td>13.2 ± 0.6* (7)</td>
<td>6.01 ± 0.29* (7)</td>
<td>4.75 ± 0.37* (7)</td>
<td>141.0 ± 13* (5)</td>
<td>147.4 ± 6.7 N (7)</td>
<td>112.5 ± 9.2 N (7)</td>
<td>23.1 ± 1.9 N (7)</td>
<td>126.0 ± 3.7* (7)</td>
<td>147.9 ± 4.7* (7)</td>
</tr>
<tr>
<td>3) EOe 2 × 16µg</td>
<td>9.0 ± 0.3* (7)</td>
<td>22.53 ± 1.75* (7)</td>
<td>19.12 ± 2.00* (7)</td>
<td>705.0 ± 77* (7)</td>
<td>209.7 ± 8.8* (7)</td>
<td>84.4 ± 8.5 N (7)</td>
<td>42.0 ± 2.8* (7)</td>
<td>145.3 ± 8.9* (7)</td>
<td>97.6 ± 7.0* (7)</td>
</tr>
</tbody>
</table>

* = Significantly different from the first group. N = no significant difference.
Number of samples in parantheses. Mean ± SEM.
Effects of ethinyloestradiol

In a more extensive experiment we examined the effects of ethinyloestradiol on a broader spectrum of enzyme activities. EOe was administered in two doses (4 and 16 μg/300 g body weight) twice daily orally for 4 days starting one week after ovariectomy (Exp. 5).

Table 3 shows that the oestrogen induced a dose-related decrease in the concentration of DNA per wet weight of endometrium as well as in the activity of a number of enzymes when expressed in U/mg extractable protein (AP, ACP, GLDH, ICDH and partially β-GR).

On the other hand activities of LDH and PK rose, in particular the latter, but there was a large variation. During the increase of total LDH we observed a shift in the percentage of M-LDH from 76% in the controls to 85% after the higher dose of ethinyloestradiol.

When the enzyme activities were expressed in U/mg DNA, ICDH, β-GR and ACP slightly increased, GLDH and AP did not change significantly after EOe treatment, whereas the glycolytic enzymes PK, LDH and M-LDH increased enormously.

Discussion

Our previous study of endometrial enzymes in the rat oestrous cycle indicated that LDH responds to endogenous oestrogen by a marked increase. As far as β-glucuronidase, ACP and AP were concerned, it was difficult to draw conclusions on their response to endogenous oestrogen (Jelinek & Jelinková 1977). The mentioned study gave no indication as to the optimum duration of exposure to exogenous oestrogen which would cause the maximum changes in the enzyme activities.

The experiments reported here served dual purpose. The first was to find a biochemical parameter in the endometrium which would be suitable for testing the oestrogenic effect on this important target tissue and would allow to compare the oestrogenic potency of two or more compounds. The LDH activity increases very markedly after administration of oestradiol and it is possible to work out a treatment schedule in which this increase of LDH gives a linear response to the dose of oestrogen (Exp. 4). The optimum schedule in our experiments obviously was to begin the treatment one week after castration and continue for 4 days (Figs. 1 and 2).

The ratio LDH/DNA rather than LDH per protein or per dry or wet weight was chosen because of the steeper slope of the dose-response curve which is thus achieved. The dose-dependent decrease of DNA per g wet weight is probably due to the hyperplasia and oedema of the endometrium after oestrogen administration (Yochim & Pepe 1971 and Tables 1, 2 and 3). How this LDH/DNA parameter was used for comparing the relative oestrogenic potency of several compounds and how specific the test is will be reported in our next paper (Jelinková et al. 1981).

The second purpose was to obtain some information on those biochemical processes which are theoretically expected to play a role in the growth of endometrium induced by the administration of an oestrogen. We employed the schedule of starting the treatment one week after ovariectomy and administering the oestrogen for 4 days. We used oral administration of two doses of ethinyloestradiol (EOe) and a broad repertoire of enzymes (Table 3).

Although the activity of EOe administered sc does not differ very much from that of oestradiol, oral administration of EOe requires much higher doses.

We determined pyruvate kinase and lactate dehydrogenase (and its M-type) as representative of the glycolysis, isocitrate dehydrogenase as marker enzyme of the Krebs cycle and glutamate dehydrogenase was taken as a link between the pathways of transamination and the citric acid cycle. Finally we determined two lysosomal enzymes, namely acid phosphatase and β-glucuronidase and also the non-specific alkaline phosphatase.

The activity of several enzymes (AP, ACP, ICDH and GLDH per mg protein) decreases in response to EOe. The response of β-GR is only slight. The pyruvate kinase increases to an extent even greater than LDH; the variation in individual values, however, is too large to make it a fully reliable assay.

When we look at the lower part of Table 3 where the enzyme activities are expressed on the basis of DNA, we can see a very strong increase of pyruvate kinase and of lactate dehydrogenase and a shift of the LDH isoenzyme spectrum in favour of the M-type LDH (from 76%–85%).

The activity of isocitrate dehydrogenase increases only by about 60% following oestrogen administration. Another key enzyme of the Krebs
cycle – the malate dehydrogenase (which we determined in another series of experiments not mentioned here) – is influenced by oestrogens in a way similar to that of ICDH. The oestrogens do not influence the activity of GDH per DNA.

Thus, we can conclude that the energy production from glucose or amino acids via Krebs cycle does not play a major role in the growth of endometrium of castrated rats after the administration of oestrogens. The energy is formed mainly by anaerobic glycolysis which is also confirmed by the shift in LDH isoenzymes. This shift can be regarded as one of the compensatory mechanisms adapting quickly to tissue changes (such as hypoxia) to secure energy production even at decreased oxygen supply (Dawson et al. 1964; Goodfriend & Kaplan 1964).

Our findings are in good agreement with the literature: the partial pressure of oxygen in the lumen of the uterus of the rat decreases after administration of oestrogens to ovariectomized rats. The oxygen diffusion distance from the nearest capillary bed to the lumen increases (Mitchell & Yochim 1968). The nourishment of the upper layer of endometrium through the lumen is probably very poor (pO₂ is relatively low in comparison with arterial tension). However, the endometrium is able to derive its energy by other means than aerobic oxidation.

The oestrogen-stimulated glycolysis in rat endometrium is due to an induction of enzymes because actinomycin, cycloheximide and 5-fluorouracil prevented the oestradiol-induced enhancement of uterine pyruvate kinase (Singhal & Valadares 1970).

It is concluded that a period of one week's rest after ovariceotomy results in a more sensitive response of LDH to oestrogens and that administration of oestrogen for 4 days gives the maximum effect. This experimental design can be used for establishing a rather steep dose-response curve of LDH/DNA and this parameter is suitable for testing the relative potency of oestrogens. Further the energy for oestrogen-stimulated endometrial growth is apparently derived mainly from the anaerobic glycolysis.

Acknowledgments

The authors wish to thank Mr. T. S. van Helvoort, Mr. A. J. M. Degen and Mr. A. L. Kappen for their technical assistance.

References


Received on March 3rd, 1980.