THE EFFECTS OF PROGESTERONE AND OF PENTOBARBITONE ADMINISTERED AT THE DIOESTROUS STAGE ON THE OVARIANCYCLE OF THE RAT

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SUMMARY

Changes in the uterus:plasma and oviduct:plasma concentration ratios for radioactive iodide after the administration of progesterone at the dioestrous stage of the oestrous cycle have confirmed that significant oestrogen secretion begins on the afternoon of the day of dioestrous in a 4-day cycle. Progesterone injected at dioestrous delays ovulation and the change to a facilitatory effect on luteinizing hormone (LH) release does not occur until early on the day of pro-oestrus, about 12 hr. after the estimated time at which oestrogen secretion begins.

Pentobarbitone (Nembutal) injected at 13.30 hr. on the day of pro-oestrus blocks ovulation, but does not do so when injected 3 hr. later. When injected at either 13.30 or 16.30 hr. in dioestrous, it delays ovulation and may prevent it completely in that cycle. The injection of Nembutal at dioestrous does not appear to prevent the release of gonadotrophin responsible for oestrogen secretion and the delay of ovulation may be due to interference with some of the normal effects of oestrogen secreted at this stage of the cycle on the hypothalamo-hypophysial system. The delay of ovulation is not prevented by the injection of oestrogen with Nembutal at dioestrous but is prevented by the subsequent injection of progesterone at pro-oestrus.

The results are discussed in relation to other studies on the time of onset of oestrogen secretion in the oestrous cycle and to the change in the response to progesterone injection from inhibition to facilitation of LH release following the period of oestrogen secretion.

INTRODUCTION

Studies of the oestrous cycle of the rat have played an important part in the development of current views on the neural control of gonadotrophin secretion (Everett, 1964a). Much of this work has been concerned with the changes occurring during the ‘critical period’ on the afternoon of the day of pro-oestrus and leading to the discharge of an ovulatory surge of luteinizing hormone (LH). The detailed sequence of events at earlier stages of the cycle has received less attention. Recently,
however, evidence has been obtained from experiments involving ovariectomy and hypophysectomy at different stages of the cycle that the secretion of sufficient oestrogen to induce many of the changes seen later in the cycle may occur during a relatively short period late on the day before pro-oestrus and in response to a much briefer period of gonadotrophin secretion than was previously suspected (Schwartz, 1964; Schwartz & Talley, 1965; Lawton & Sawyer, 1968).

Progestosterone injected in the morning of the day of dioestrous delays ovulation but when injected on the morning of pro-oestrus advances ovulation by some hours (Brown-Grant, 1967a); the change in the response appears to follow the period during which oestrogen secretion is considered to begin. Experiments were therefore carried out to provide additional evidence as to the time of oestrogen secretion during the cycle and then to study in more detail the change from an inhibitory to a facilitatory effect on LH release in relation to the period of oestrogen secretion.

Oestrogen secretion was assessed indirectly by determining the increase in the uterus:plasma (U:P) and oviduct:plasma (O:P) concentration ratios for radioactive iodide after the administration of progesterone. This response is specific to steroids with pregnancy-maintaining properties and can be inhibited by oestrogen (Brown-Grant, 1967b). The time-course of the inhibition was first studied in ovariec-tomized rats. Sufficient oestrogen is secreted during the normal cycle to block the response to even large doses of progesterone (Brown-Grant, 1966b) and the early response to injections given between late metoestrus and early pro-oestrus was determined to establish the time at which effects from oestrogen secreted in the previous cycle disappeared and inhibition due to renewed secretion of oestrogen reappeared. The effects of progesterone on LH release when injected before and after the time of renewed oestrogen secretion were then determined. As the results obtained suggested that the gonadotrophin secretion responsible for oestrogen release occurred at about 14.00 hr. to 16.00 hr. on the day of dioestrous, the effect on ovulation of pentobarbitone (Nembutal) injected at this stage of the cycle either alone or in combination with steroids was investigated in rats with both 4- and 5-day cycles. In all these experiments the thyroid:plasma (T:P) concentration ratio for radioactive iodide was also measured to obtain information on the level of thyroid gland activity and as an indirect index of pituitary thyrotrophin (TSH) secretion.

MATERIALS AND METHODS

The animals were adult Wistar females from a closed colony, weighing 180–220 g. They were maintained from birth under the 14/10 hr. lighting conditions described by Everett (1948). By convention the midpoint of the dark period is taken as midnight and the pro-oestrous critical period is then from 14.00 to 16.00 hr. This convention was adopted in presenting new experimental results and in discussing results obtained in other studies although it was not used in earlier papers (Brown-Grant, 1962, 1963, 1966a, 1967a). Vaginal smears were taken daily between 06.00 and 07.00 hr.; no animal was used for an experiment until at least three consecutive cycles of constant length (either of 4 or 5 days duration) had been observed. The days and stages of the 4-day cycle are oestrus, metoestrus, dioestrous and pro-oestrus. The 5-day cycles, found in about 25% of the animals, differ from the 4-day cycle in that
the vaginal smears show 3 days with leucocytes. These days are metoestrus, dioestrus – 1 and dioestrus – 2.

On the day the animals were killed tissue: plasma radioactive iodide concentration ratios for the thyroid, uterus and oviduct were determined (T:P, U:P and O:P ratios). Organ weights, the state of the genital tract, occurrence of ovulation and egg numbers were also determined. Details of these methods are given in earlier papers (Brown-Grant, 1966b, 1969a).

The hormones used were progesterone (Organon Laboratories Ltd.) and oestradiol benzoate (British Drug Houses Ltd.). The steroids were injected in oil in a volume of 0·1 ml. s.c. or 0·05 ml. i.m. at the time and dose levels indicated. Human chorionic gonadotrophin (HCG) was obtained from Organon Laboratories Ltd.; the dose was 15 i.u. in 0·15 ml. 0·9 % NaCl solution, injected s.c. at 15.00 hr. Sodium pentobarbitone (Veterinary Nembutal, Abbot Laboratories) was diluted with saline so that 0·1 ml. contained 3·6 mg. and injected s.c. at a dose of 3·6 mg./100 g. body weight at the times stated.

Ovariectomy was carried out under Avertin (tribromoethanol plus amylene hydrate) anaesthesia and the animals were used 7–10 days later.

Values for groups of three or more animals are given as group means ± s.e. The statistical tests were the ‘t’ test, analysis of variance using the variance ratio, and Fisher’s exact probability test for a 2 × 2 contingency table, using Finney’s table of significance levels, as described by Siegel (1956).

The general design of the experiments was as follows: in the study of the time-course of oestrogen inhibition ovariectomized rats received 2·5 mg. progesterone s.c. at 08.00 hr. either alone or preceded or followed by oestrogen i.m. and were killed 24 hr. after the injection of progesterone. In the experiments on the effects of progesterone on U : P ratios in intact rats 1·25 mg. steroid was injected s.c. at various times between late metoestrus and early pro-oestrus in rats with 4-day cycles and the animals were killed the next morning except as noted under Results. When the effects on ovulation were studied 1·25 mg. progesterone was injected s.c. at different times during the day of dioestrus in rats with 4-day cycles and the animals were killed on the morning of the expected day of oestrus. The effects of pentobarbitone, injected at the times indicated under Results either alone or in combination with injections of steroids, were studied in rats with both 4- and 5-day cycles; the animals were killed 1–3 days after the injection of pentobarbitone.

RESULTS

The time-course of oestrogen inhibition in ovariectomized rats

Treatment of ovariectomized rats with progesterone resulted in U : P and O : P ratios 24 hr. later of 2·80 ± 0·28 and 3·36 ± 0·19 respectively in a group of six rats; when 1 µg. of oestradiol benzoate was injected 16 hr. before the injection of progesterone the values obtained were 0·90 ± 0·39 and 0·91 ± 0·25 for a group of six rats and were not significantly different from the values for six untreated ovariectomized rats (0·51 ± 0·02 and 0·84 ± 0·07).

In a second experiment ovariectomized rats received progesterone either alone or with 25 µg. oestradiol given 0, 2, 4, 8, or 12 hr. after the injection of progesterone.
The mean U:P ratios for groups of five rats were $4.82 \pm 0.73$ for rats which received progesterone only and $0.93 \pm 0.21$, $0.72 \pm 0.18$, $0.62 \pm 0.09$, $0.63 \pm 0.08$ and $1.56 \pm 0.40$ respectively when measured 24 hr. after the injection of progesterone. This suggests that a period of 8–12 hr. of unopposed action of progesterone is required for the effect on the U:P ratio.

**Table 1.** The effects of progesterone on thyroid:plasma (T:P), uterus:plasma (U:P), and oviduct:plasma (O:P) ratios for radioactive iodide, uterus and ovary weights

(Values in this and other tables are group means ± s.e. or values for individual rats. In these experiments the animals were killed at about 09.00 hr. on the day after injection except for the final group which was killed at about 14.00 hr. Values in bold are significantly different from those of controls killed at pro-oestrus. $P$ indicates injection of 1·25 mg. progesterone s.c. at the time stated. Organ weights are given in mg./100 g. body weight.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>T:P ratio</th>
<th>U:P ratio</th>
<th>O:P ratio</th>
<th>Uterus</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioestrus, control</td>
<td>5</td>
<td>$73 \pm 8$</td>
<td>$0.57 \pm 0.01$</td>
<td>$2.65 \pm 0.31$</td>
<td>$151 \pm 14$</td>
<td>$29.5 \pm 1.3$</td>
</tr>
<tr>
<td>Pro-oestrus, control</td>
<td>5</td>
<td>$95 \pm 11$</td>
<td>$0.62 \pm 0.01$</td>
<td>$0.86 \pm 0.16$</td>
<td>$200 \pm 17$</td>
<td>$27.3 \pm 1.2$</td>
</tr>
<tr>
<td>P, 15.30 metoestrus</td>
<td>3</td>
<td>$63 \pm 3$</td>
<td>$0.61 \pm 0.02$</td>
<td>$2.55 \pm 0.41$</td>
<td>$147 \pm 11$</td>
<td>$28.7 \pm 1.3$</td>
</tr>
<tr>
<td>P, 18.30 metoestrus</td>
<td>5</td>
<td>$111 \pm 37$</td>
<td>$0.58 \pm 0.03$</td>
<td>$3.27 \pm 0.24$</td>
<td>$155 \pm 10$</td>
<td>$27.1 \pm 2.1$</td>
</tr>
<tr>
<td>P, 06.30 dioestrus</td>
<td>5</td>
<td>$83 \pm 9$</td>
<td>$2.52 \pm 0.37$</td>
<td>$3.43 \pm 0.41$</td>
<td>$167 \pm 9$</td>
<td>$25.0 \pm 1.0$</td>
</tr>
<tr>
<td>P, 09.30 dioestrus</td>
<td>5</td>
<td>$75 \pm 9$</td>
<td>$1.09 \pm 0.14$</td>
<td>$1.84 \pm 0.15$</td>
<td>$172 \pm 6$</td>
<td>$25.1 \pm 2.1$</td>
</tr>
<tr>
<td>P, 12.30 dioestrus</td>
<td>5</td>
<td>$70 \pm 6$</td>
<td>$0.82 \pm 0.08$</td>
<td>$1.57 \pm 0.18$</td>
<td>$167 \pm 10$</td>
<td>$27.9 \pm 1.0$</td>
</tr>
<tr>
<td>P, 15.30 dioestrus</td>
<td>5</td>
<td>$76 \pm 10$</td>
<td>$0.69 \pm 0.04$</td>
<td>$1.15 \pm 0.10$</td>
<td>$169 \pm 11$</td>
<td>$26.4 \pm 1.2$</td>
</tr>
<tr>
<td>P, 15.30 dioestrus, killed p.m.</td>
<td>5</td>
<td>$80 \pm 10$</td>
<td>$0.61 \pm 0.01$</td>
<td>$1.14 \pm 0.14$</td>
<td>$162 \pm 9$</td>
<td>$27.2 \pm 1.1$</td>
</tr>
</tbody>
</table>

**Effects of progesterone at dioestrus on U:P and O:P ratios**

The results are shown in Table 1. Even when injected as late as 18.30 hr. on the day of metoestrus, neither U:P nor O:P ratios were increased as compared with controls killed at dioestrus. When injections were made at 06.30 hr. of dioestrus, U:P and O:P ratios were higher than in controls killed at pro-oestrus ($P < 0.01$). When injection was delayed until 09.30 hr. both U:P and O:P ratios were significantly below the values for rats injected at 06.30 hr. ($P < 0.02$, $P < 0.01$) and were still lower in rats injected at 12.30 hr. though these values were still significantly higher than those in controls killed at pro-oestrus ($P < 0.05$). There was no significant increase in rats injected at 15.30 hr. and killed the next morning. Although an increase in U:P ratio can be detected 8–12 hr. after progesterone injection (Brown-Grant, 1967b), it was possible that the failure to observe an increase in the U:P or O:P ratios of rats injected at 15.30 hr. was due to the shorter time-interval between injection and killing. A second group injected at 15.30 hr. was examined in the late afternoon of the next day but again no increase in U:P or O:P ratios was observed (Table 1).

**Other effects of progesterone**

Thyroid:plasma ratios, which normally rise between dioestrus and pro-oestrus, were lower in the progesterone-treated animals than in controls killed at pro-oestrus but the difference is not statistically significant for any one group though it is for the whole group of 20 animals ($P < 0.05$). Uterine weights were also lower than in controls; again the difference is not significant for any single group but for all rats injected at dioestrus the difference is highly significant ($P < 0.02$). The ballooning
of the uterus due to the accumulation of free fluid in the lumen that is normally seen at pro-oestrus was not observed in the progesterone-treated rats. Ovarian weights were not affected.

The vaginal smears of rats injected early on the day of dioestrus remained dioestrous in type; a mixed smear of an intermediate type was common in animals injected later in the day and in a few rats injected in the late afternoon the smear next morning was quite typical of pro-oestrus.

Table 2. Effects of progesterone (1-25 mg. s.c.) on $^{131}$I ratios, organ weights and ovulation in the rat

(Rats were killed on the day of expected oestrus. Progesterone (P) was injected at the times shown on the day of dioestrus in 4-day cycles or in the early hours of the day of pro-oestrus (the last group). N.O. = blockade of ovulation; O.V. = rats ovulated. Egg numbers are given per rat ovulating. Values in bold are significantly different from controls killed at oestrus. See Table 1 for abbreviations and other details.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>T:P ratio</th>
<th>U:P ratio</th>
<th>O:P ratio</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-oestrus control</td>
<td>5</td>
<td>95±11</td>
<td>0-62±0-01</td>
<td>0-86±0-16</td>
<td>200±17</td>
<td>27-3±1-2</td>
<td>—</td>
</tr>
<tr>
<td>Oestrus, control</td>
<td>OV 9</td>
<td>124±10</td>
<td>0-54±0-01</td>
<td>0-86±0-05</td>
<td>193±9</td>
<td>35-1±1-2</td>
<td>10-7±1-3</td>
</tr>
<tr>
<td>P, 06:30</td>
<td>N.O. 4</td>
<td>132±0-21</td>
<td>1-32±0-21</td>
<td>0-76±0-08</td>
<td>217±5</td>
<td>24-4±1-9</td>
<td>—</td>
</tr>
<tr>
<td>P, 09:30</td>
<td>N.O. 4</td>
<td>197±55</td>
<td>3-19±0-55</td>
<td>1-25±0-24</td>
<td>166±4</td>
<td>24-4±1-3</td>
<td>—</td>
</tr>
<tr>
<td>P, 12:30</td>
<td>N.O. 4</td>
<td>149±0-41</td>
<td>1-49±0-41</td>
<td>1-58±0-12</td>
<td>153±11</td>
<td>27-5±1-5</td>
<td>—</td>
</tr>
<tr>
<td>P, 15:30</td>
<td>N.O. 4</td>
<td>130±9</td>
<td>0-62±0-10</td>
<td>0-70±0-05</td>
<td>131±11</td>
<td>26-1±1-5</td>
<td>—</td>
</tr>
<tr>
<td>P, 18:30</td>
<td>N.O. 4</td>
<td>100±10</td>
<td>0-74±0-10</td>
<td>1-10±0-22</td>
<td>150±18</td>
<td>26-8±0-9</td>
<td>—</td>
</tr>
<tr>
<td>P, 01:30</td>
<td>OV 1</td>
<td>87±6</td>
<td>0-64</td>
<td>0-94</td>
<td>148±2</td>
<td>27-0</td>
<td>6</td>
</tr>
<tr>
<td>P, 04:30</td>
<td>N.O. 5</td>
<td>97±17</td>
<td>0-61±0-02</td>
<td>1-00±0-09</td>
<td>138±4</td>
<td>29-2±2-6</td>
<td>—</td>
</tr>
<tr>
<td>P, 08:30</td>
<td>OV 5</td>
<td>92±6</td>
<td>0-54±0-01</td>
<td>0-91±0-08</td>
<td>153±3</td>
<td>30-6±1-0</td>
<td>11-6±1-5</td>
</tr>
</tbody>
</table>

Incidence of ovulation

The results are shown in Table 2. Ovulation was blocked in 25 out of 26 animals injected between 06:30 hr. and 21:30 hr. of that day. When progesterone was injected at 03:30 of the day of pro-oestrus, ovulation was no longer blocked and may even have been advanced by some hours. The swollen segments of the oviducts were less obvious than in control rats killed at oestrus and in some cases loss of cumulus cells and dispersal of the ova had begun. Egg numbers were normal. The changeover from inhibition to facilitation (or at least to loss of the inhibitory effect) occurred some time in the early hours of the day of pro-oestrus.

Effects on U:P and O:P ratios

The changes in U:P and O:P ratios on the second day after the injection of progesterone at dioestrus may provide some indication as to the mechanism of this blockade. In nearly all cases (Table 2) these ratios were significantly higher than those in control animals killed at oestrus, the increases being most marked in the groups injected early in dioestrus at 09:30 or 12:30 hr. When ovulation was not blocked, no increase in U:P ratio was seen and O:P ratios were only slightly increased though the change was statistically significant. These findings are consistent with the idea that progesterone may be reducing or curtailing oestrogen secretion when given before about 14:30 hr. on the the day of dioestrus. The inhibitory action
of the small amount of oestrogen that may be secreted after progesterone injection may have affected the U:P and O:P ratios when these were measured the next day but 24 hr. later this inhibition was no longer effective and at this time high U:P and O:P ratios were observed.

Other effects

The changes in uterine weight also suggested that oestrogen secretion may have been reduced; the values were significantly below those found in control animals at oestrus for rats injected at 15.30, 18.30 and 21.30 hr. Some ballooning of the uterus was observed in all six rats injected at 06.30, one of those injected at 12.30 and one injected at 18.30, but not in any others. The amount of fluid was in all cases less than that normally observed at pro-oestrus. Ovarian weights of rats that failed to ovulate were also significantly lower ($P < 0.01$) than in controls killed at oestrus except for rats injected at 21.30 of dioestrus.

The changes in vaginal smears also support the view that oestrogen secretion has been affected. Except for the animals injected at 03.30 of pro-oestrus all of which had typical oestrous smears and all of which ovulated, the smears in the majority of the other animals were intermediate, containing both nucleated and cornified cells, on the day of expected oestrus. Of the 26 animals, two had pro-oestrous smears, six had fully cornified smears and 18 intermediate smears.

Finally the changes in T:P ratio in these experiments should be noted. The values were below those normally found on the day of oestrus, significantly so for rats injected at 06.30, 09.30, 12.30 and 15.30 hr. and not significantly different from the value observed in control animals killed at pro-oestrus. This is in agreement with an earlier study (Brown-Grant, 1967a). The rats that did ovulate on the day of expected oestrus, possibly prematurely in response to progesterone at 03.30 of pro-oestrus, also gave a T:P ratio below that for control rats at oestrus ($P < 0.05$).

Modification of the oestrous cycle by pentobarbitone injected at dioestrus

Preliminary experiments

Although the duration of the critical period during pro-oestrus had already been established for this colony (Brown-Grant, 1966a), preliminary experiments were carried out to confirm the earlier findings. Six rats with regular 4-day cycles received pentobarbitone at 13.30 hr. on the day of pro-oestrus and were killed the next day; ovulation had been blocked in all animals. Five rats were injected at 16.30 hr.; all had fresh tubal ova the next morning; egg numbers ($6.6 \pm 1.3$) were not significantly different from controls killed at oestrus (Table 2). The critical period of pro-oestrus was found to be about 14.00 to 16.00 hr. in confirmation of earlier studies. Fisher's exact probability test gives a $P$ value of $< 0.05$ for the difference in the incidence of ovulation between rats injected at 13.30 and 16.30 hr.

Effects of pentobarbitone injected at dioestrus

Rats were injected with pentobarbitone at 13.30 hr. on the day of dioestrus in a 4-day cycle and killed on the day of expected oestrus; ovulation was blocked in six out of eight rats. Four of these animals showed a dioestrous smear on the expected
day of pro-oestrus and all showed a pro-oestrous or mixed nucleated and cornified cell smear on the expected day of oestrum except for the two animals that ovulated, which showed a normal smear pattern. The other findings at autopsy did not differ from those of control animals killed at pro-oestrus (Table 3 and Table 1). It seemed possible that pentobarbitone at the critical period on the day of dioestrus was suppressing gonadotrophin and oestrogen secretion but this interpretation of the findings was not supported by further experiments.

Table 3. Effects of pentobarbitone (Nembutal = N) (3-6 mg./100 g., s.c.) at the times indicated on the day of dioestrus in a 4-day cycle on ovulation.

(All rats were killed on the day of expected oestrus. Oestradiol benzoate (OB) was injected at the same time as pentobarbitone, progesterone (P) at 11.30 hr. of pro-oestrus, and human chorionic gonadotrophin (HCG) (15 i.u.) at 15.00 hr. of pro-oestrus. Values in bold are significantly different from controls killed at oestrus (Table 2). N.O. = blockade of ovulation; OV = rats ovulated. See Table 1 for abbreviations and other details.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>T:P ratio</th>
<th>U:P ratio</th>
<th>O:P ratio</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>N, 10.30</td>
<td>OV 4</td>
<td>85 ± 13</td>
<td>0.53 ± 0.02</td>
<td>0.54 ± 0.05</td>
<td>226 ± 21</td>
<td>37.7 ± 2.4</td>
<td>15.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>N.O. 1</td>
<td>10</td>
<td>0.47</td>
<td>0.65</td>
<td>232</td>
<td>26</td>
<td>—</td>
</tr>
<tr>
<td>N, 13.30</td>
<td>OV 2</td>
<td>71, 111</td>
<td>0.56, 0.51</td>
<td>0.83, 0.61</td>
<td>172, 164</td>
<td>33.2, 29.1</td>
<td>4, 16</td>
</tr>
<tr>
<td></td>
<td>N.O. 6</td>
<td>112 ± 19</td>
<td>0.55 ± 0.01</td>
<td>0.51 ± 0.03</td>
<td>203 ± 9</td>
<td>28.3 ± 1.6</td>
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</tr>
<tr>
<td>N, 16.30</td>
<td>OV 2</td>
<td>105, 121</td>
<td>0.46, 0.48</td>
<td>0.63, 0.44</td>
<td>183, 232</td>
<td>39.5, 26.6</td>
<td>8, 8</td>
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<tr>
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<td>N.O. 7</td>
<td>70 ± 4</td>
<td>0.48 ± 0.02</td>
<td>0.53 ± 0.03</td>
<td>221 ± 14</td>
<td>28.2 ± 1.0</td>
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<tr>
<td>N, 18.30</td>
<td>OV 4</td>
<td>83 ± 3</td>
<td>0.48 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>195 ± 11</td>
<td>33.1 ± 1.7</td>
<td>13.9 ± 1.3</td>
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<tr>
<td></td>
<td>N.O. 1</td>
<td>71</td>
<td>0.51</td>
<td>0.40</td>
<td>179</td>
<td>27.2</td>
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<tr>
<td>N, 16.30 + 2.5 µg. OB</td>
<td>OV 2</td>
<td>55, 127</td>
<td>0.46, 0.49</td>
<td>0.48, 0.44</td>
<td>181, 159</td>
<td>34.1, 32.4</td>
<td>12, 12</td>
</tr>
<tr>
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<td>N.O. 8</td>
<td>109 ± 7</td>
<td>0.49 ± 0.01</td>
<td>0.47 ± 0.03</td>
<td>215 ± 16</td>
<td>30.9 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>N, 16.30 + 2.5 mg. P at pro-oestrus</td>
<td>OV 7</td>
<td>89 ± 7</td>
<td>0.53 ± 0.01</td>
<td>0.73 ± 0.05</td>
<td>185 ± 9</td>
<td>35.0 ± 1.9</td>
<td>8.6 ± 1.4</td>
</tr>
<tr>
<td>Saline, 16.30</td>
<td>OV 5</td>
<td>102 ± 11</td>
<td>0.51 ± 0.03</td>
<td>0.48 ± 0.03</td>
<td>179 ± 10</td>
<td>32.9 ± 2.3</td>
<td>11.2 ± 0.5</td>
</tr>
<tr>
<td>N, 16.30 + HCG at pro-oestrus</td>
<td>OV 5</td>
<td>72 ± 13</td>
<td>0.54 ± 0.02</td>
<td>0.87 ± 0.14</td>
<td>194 ± 8</td>
<td>40.9 ± 2.1</td>
<td>12.0 ± 1.3</td>
</tr>
</tbody>
</table>

First, the duration of the period during which pentobarbitone blocks ovulation when injected at dioestrus is not restricted—as it is at pro-oestrus—to the presumed period of gonadotrophin secretion. Rats were injected at 10.30, 16.30 and 18.30 hr. on the day of dioestrus. As shown in Table 3, ovulation was blocked in only one out of five animals injected at either 10.30 or 18.30 hr. but was blocked in seven out of nine rats injected at 16.30 hr. This is to be contrasted with the failure to block ovulation by the injection of pentobarbitone at 16.30 hr. in pro-oestrus noted above. Moreover, the sequence of vaginal smear changes was normal in these animals, even when ovulation was blocked. The findings at autopsy in the animals that failed to ovulate were again indistinguishable from those of controls killed at pro-oestrus. The animals that did ovulate gave values, including egg numbers, similar to those found in controls killed at oestrus except for the T:P ratios which were below the oestrus control values in all cases (P < 0.05 and < 0.02 respectively for rats injected at 10.30 and 18.30 hr.).

Secondly, if suppression of gonadotrophin release and hence of oestrogen secretion were the cause of the failure of ovulation, then exogenous oestrogen might be ex-
pected to reverse the effect. Rats were injected with pentobarbitone at 16.30 hr. of dioestrus and also received 2.5 µg. oestradiol benzoate i.m. at the same time; only two out of ten rats ovulated, about the same proportion as in rats receiving pentobarbitone only. Vaginal smear changes were normal in these animals and results obtained at autopsy did not differ significantly from those in controls killed at pro-oestrus (Table 3).

Thirdly, if oestrogen secretion were affected, then the usual changes occurring in the genital tract between dioestrus and pro-oestrus would not be observed. Rats were injected at 13.30 or 16.30 hr. of dioestrus and killed the next morning. The vaginal smear did remain dioestrous in six of these animals and the degree of ballooning of the uterus may have been less than normal for the pro-oestrous stage of the cycle. Objectively, the uterine weights had increased above dioestrus levels and did not differ from those of controls killed at pro-oestrus. The O:P ratios did not show the high values characteristic of dioestrus and did not differ significantly from pro-oestrous controls. The T:P ratio, however, was significantly below control values in both groups (P < 0.05, < 0.02, Table 4 and Table 5).

**Table 4. Effects of pentobarbitone (Nembutal = N) at different stages of the 4-day and 5-day cycle on ovulation, **$^{131}$**I ratios and organ weights**

(N.O. = blockade of ovulation; OV = rats ovulated. See Table 1 for abbreviations and other details.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>T:P ratio</th>
<th>U:P ratio</th>
<th>O:P ratio</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>N, 13.30 of dioestrus, killed at pro-oestrus</td>
<td>4</td>
<td>60±7</td>
<td>0.62±0.04</td>
<td>1.02±0.11</td>
<td>192±7</td>
<td>30.2±3.2</td>
<td>—</td>
</tr>
<tr>
<td>N, 16.30 of dioestrus, killed at pro-oestrus</td>
<td>6</td>
<td>60±4</td>
<td>0.58±0.02</td>
<td>0.85±0.14</td>
<td>188±8</td>
<td>31.0±1.4</td>
<td>—</td>
</tr>
<tr>
<td>N, 16.30 of dioestrus, killed 3 days later</td>
<td>OV 6</td>
<td>171±32</td>
<td>0.66±0.14</td>
<td>0.84±0.16</td>
<td>198±5</td>
<td>31.4±4.5</td>
<td>13.0±1.1</td>
</tr>
<tr>
<td>N, 16.30 of dioestrus, killed 3 days later</td>
<td>N.O. 7</td>
<td>96±12</td>
<td>0.53±0.02</td>
<td>0.75±0.08</td>
<td>145±12</td>
<td>28.5±1.3</td>
<td>—</td>
</tr>
<tr>
<td>N, 16.30 of dioestrus −1, killed 3 days later</td>
<td>OV 5</td>
<td>135±13</td>
<td>0.52±0.01</td>
<td>0.66±0.05</td>
<td>181±17</td>
<td>28.4±1.1</td>
<td>9.2±1.3</td>
</tr>
<tr>
<td>N, 16.30 of dioestrus −2, killed 2 days later</td>
<td>OV 3</td>
<td>83±7</td>
<td>0.53±0.01</td>
<td>0.79±0.17</td>
<td>201±5.6</td>
<td>33.2±2.4</td>
<td>8.7±0.7</td>
</tr>
<tr>
<td></td>
<td>N.O. 3</td>
<td>94±20</td>
<td>0.71±0.05</td>
<td>0.85±0.27</td>
<td>148±28</td>
<td>25.4±3.4</td>
<td>—</td>
</tr>
</tbody>
</table>

Finally, it was argued that if oestrogen secretion were suppressed or reduced, then the changeover from the inhibitory effect of progesterone injected at dioestrus to the facilitatory effect seen after injection at pro-oestrus in the normal cycle might not have occurred. Rats were injected with pentobarbitone at 16.30 hr. of dioestrus and with 2.5 mg. progesterone at 11.30 hr. the next day (expected day of pro-oestrus). When killed the next morning all seven animals had ovulated. Fisher's exact probability test showed the difference between this group and those that received pentobarbitone alone to be highly significant (P < 0.005). Egg numbers did not differ significantly from controls killed at oestrus (Table 3). The other results from these animals did not differ from control values except that the T:P ratio was lower than in control rats at oestrus (P < 0.02) and not significantly different from the pro-oestrous control value.
Additional experiments

Certain other experiments were carried out in connexion with the study of the blockade of ovulation by pentobarbitone injected in late dioestrus. Saline injected at 16.30 hr. did not affect ovulation ($P < 0.005$ in comparison with pentobarbitone-treated rats in Fisher's test). Values obtained at autopsy were not significantly different from oestrous controls (Table 3). The results of progesterone administration after the injection of pentobarbitone at 16.30 hr. of dioestrus suggested that the competence of the follicles to ovulate had not been adversely affected. This possibility was also tested by injecting 15 i.u. HCG at 15.00 hr. on the day of pro-oestrus into animals that had received pentobarbitone at 16.30 hr. of dioestrus. All five animals had ovulated when examined the next morning ($P < 0.005$ vs. animals receiving pentobarbitone only in Fisher's test) and egg numbers were slightly higher than in controls (Table 3). Ovarian weight was significantly increased ($P < 0.05$) and T:P values, as is usual when ovulation is induced by exogenous gonadotrophin (see Discussion in Brown-Grant, 1969b), were significantly below oestrous control values ($P < 0.01$) and did not differ from the pro-oestrous controls.

A further experiment extended the period of study after the injection of pentobarbitone at 16.30 hr. of dioestrus. If ovulation is blocked by the injection of pentobarbitone at pro-oestrus then it is postponed and occurs after a delay of 24 hr.; a second injection of pentobarbitone produces a further 24 hr. delay but subsequently atresia of the follicles occurs (Everett, 1964b; Everett & Sawyer, 1950).

Thirteen rats received pentobarbitone at 16.30 hr. of dioestrus and were killed about 3 days later, that is on the morning of the day after the expected day of oestrus. The usual smear pattern was observed initially, a pro-oestrous followed by an oestrous smear in all animals. On the day of killing, however, eight rats had a second fully cornified smear and five had metoestrous smears. Six of the animals had ovulated and all these had oestrous smears; the other seven animals did not have tubal ova but the ovaries contained several large follicles despite the metoestrous smear. It seems unlikely that these animals had ovulated the day before or would have ovulated the next day. Other findings in these animals are given in Table 4; T:P ratios in the animals that ovulated were high, significantly higher than in animals that did not ovulate ($P < 0.05$) but not different from those of controls killed at oestrus. Uterine weights were also significantly higher in animals that had ovulated ($P < 0.01$).

When ovulation is blocked by pentobarbitone injected at dioestrus, it is not only delayed but apparently rendered impossible in that cycle for about 50% of animals.

Experiments on rats with 5-day cycles

Finally a small number of animals with 5-day cycles were examined. The administration of pentobarbitone at the dioestrous - 1 stage of the 5-day cycle might affect ovulation and blockade at dioestrus - 2 (the day before pro-oestrus in a 5-day cycle) might be less consistent than in the 4-day cycle.

The first possibility was not supported by the results; five rats injected with pentobarbitone at 16.30 hr. on dioestrus - 1 had all ovulated when examined 72 hr. later on the morning of the expected day of oestrus; the values obtained (Table 4) do not
differ from those in controls killed at the oestrous stage of a 5-day cycle (see Table 3 in Brown-Grant, 1969b). Six rats were injected at 16.30 hr. on dioestrus −2 and killed 48 hr. later. Ovulation was blocked in only three animals. The values obtained do not differ significantly between animals that ovulated and those that did not, nor between ovulating animals and controls killed at oestrus though the T:P ratio was low as in 4-day rats that ovulate after pentobarbitone during dioestrus (Table 3). The incidence of blockade was lower but not significantly different from that produced by injection at 16.30 hr. of dioestrus in a 4-day cycle when examined by Fisher’s test.

**DISCUSSION**

The early (24 hr. or less) response of the U:P ratio to progesterone in intact rats was studied. Even late in metoestrus this response was still inhibited, probably by residual effects of oestrogen from the previous cycle and an increase was found only when injections were made in early dioestrus. If injection was delayed until 09.30 hr. a reduced response was obtained (Table 1). A period of 8–12 hr. of unopposed action is necessary for the response (Brown-Grant, 1967b), suggesting that oestrogen secretion was beginning during or shortly after the period from 14.00 to 16.00 hr. of dioestrus in this colony. When injected at 15.30 hr. no early response was seen, indicating that oestrogen levels may already have been high in the hours immediately after injection. This indirect estimate of the time of oestrogen secretion is in agreement with the findings of Schwartz (1964) and of Lawton & Sawyer (1967, 1968).

The effects of progesterone on the release of LH were then studied and related to the evidence that oestrogen secretion begins on the afternoon of dioestrus in a 4-day cycle. A dose of 1-25 mg. at 06.30 hr. of dioestrus delayed ovulation and the negative effect was also seen when injections were made later in the day, up to and beyond the time when oestrogen secretion was known to have begun. A change from the inhibitory to the facilitatory effects on LH release did not occur until between 21.30 hr. of dioestrus and 03.30 hr. of the day of pro-oestrus. This was considerably later than the time at which the early U:P response to progesterone was lost. The change to a facilitatory action may require a rather prolonged (up to 12 hr.) period of oestrogen action on the hypothalamo-hypophysial system.

Although reduced U:P responses were seen the next day when progesterone was given late in dioestrus (Table 1), blockade of ovulation was still produced by injections at these times. On the day of expected oestrus, despite the fact that measurements had been delayed for an additional 24 hr. after the injection of progesterone, U:P ratios were as high or higher than on the previous day (Table 2). Normally progesterone, even in large doses, acting throughout the period between pro-oestrus and oestrus does not produce an increase in U:P ratio (see results in rats injected at 03.30 of pro-oestrus in Table 2 and also results shown in Table 5 of a previous paper, Brown-Grant, 1967a). The implication of these findings may be that although minimal gonadotrophin (probably LH) release and subsequent oestrogen secretion occurs in rats injected shortly before the 14.00 to 16.00 hr. period on the day of dioestrus and this is sufficient to block or modify the early U:P response, full secretion of LH (and oestrogen) is prevented. Progesterone acting during pro-oestrus does not now have a
facilitatory effect on LH release and the uterus and oviduct can now respond with increase in $^{131}$I concentration ratios.

Continued daily injections of progesterone beginning early on the day of dioestrus are known to suppress ovulation indefinitely (Everett, 1964a; Hoffman & Schwartz, 1965). The mechanism involved could well be suppression of LH and oestrogen secretion during dioestrus. It is interesting to compare the effects of progesterone injection with the changes during pregnancy or pseudopregnancy. The U:P ratio is considerably increased on day 3 of pregnancy (the expected day of pro-oestrus in a 4-day cycle in relation to the ovulation at which fertilization occurred) implying progesterone secretion early in expected dioestrus, day 2 of pregnancy and, as in the case of the response to exogenous progesterone, indicating suppression of the usual LH release and oestrogen secretion between dioestrus and pro-oestrus (days 2 and 3 of pregnancy). To reproduce these high U:P values of pregnancy requires about 2-5 mg progesterone s.c. per day (Brown-Grant, 1965, 1966b, 1967a, b).

Clearly the rat is capable of producing more than enough progesterone early in pregnancy to suppress the early release of LH (and oestrogen) that normally occurs during the cycle. Blockade of ovulation during pregnancy or pseudopregnancy in the rat may be related to suppression of this first, oestrogen secretion-stimulating release of LH during the cycle rather than to direct suppression of the ovulatory surge of LH secretion that would be expected to occur between day 3 and day 4 (expected days of pro-oestrus and oestrus in a 4-day cycle). The suggestion that there is an abnormally early release of LH during pregnancy (Alloiteau, 1961) leading to an oestrogen "surge" that is necessary for blastocyst implantation (see DeFeo (1963, 1967) and Brown-Grant (1965) and (1966b) for references) may need to be re-examined in the light of what is now known about events during the cycle. The stage at which LH release was postulated, on good experimental grounds, is in fact the same in relation to the last ovulation as the oestrogen secretion-stimulating release now demonstrated to occur in the 4-day cycle, i.e. between day 2 and day 3 of pregnancy. It may be that rather than supplying an additional amount of oestrogen necessary for implantation, the true explanation of the sequence of events in pregnancy is that progesterone reduces LH and oestrogen secretion not to zero (for then implantation would fail as it does after early hypophysectomy or ovariectomy in pregnancy followed by treatment with progesterone alone) but to a much reduced level. Sufficient oestrogen is secreted to allow implantation and foetal development but not enough to prevent the action of progesterone in raising the U:P ratio to be inhibited or to allow the hypothalamo-hypophysial system to enter into a state in which progesterone exerts a facilitatory effect on LH release and leads to ovulation.

It is of interest to know whether this 'early' or 'oestrogen secretion-stimulating' release of LH during the cycle which can apparently be suppressed by progesterone, can also be suppressed by pentobarbitone as the later, ovulatory, surge can be (Everett, 1964a). Rats with 4-day cycles were injected with pentobarbitone at 13.30 hr. of dioestrus (the same dose at the same time of day 24 hr. later was shown to be effective in suppressing ovulation). Ovulation was blocked in the majority of animals injected at 13.30 hr. of dioestrus (Table 3) but was also in rats injected at 16.30 hr., although injection at this time in pro-oestrus did not block ovulation. However, the evidence that LH (and oestrogen) secretion is inhibited is not convincing.
The sequence of vaginal smear changes was not consistently affected; uterine ballooning on the expected day of pro-oestrus may have been reduced slightly but could not be assessed objectively. The increase in uterine weight was not significantly affected and O:P ratios did not remain high (Table 4). Moreover, exogenous oestrogen did not restore the normal sequence of events and lead to ovulation of the day of expected oestrus (Table 3). If the view is accepted that a facilitatory effect of progesterone on LH release is dependent on previous exposure to oestrogen, then the finding that progesterone injected at pro-oestrus would still induce ovulation in rats receiving pentobarbitone at dioestrus that would otherwise have failed to ovulate, is also evidence that oestrogen secretion had not been suppressed. After these studies had been completed, Lawton & Sawyer (1967, 1968) also reported that pentobarbitone given on the day of dioestrus in a 4-day cycle did not affect the ballooning of the uterus at pro-oestrus or vaginal cornification at oestrus. Similar studies have been carried out by Schwartz & Lawton (1968). They injected pentobarbitone at 14.00 and 16.00 hr. on the day before pro-oestrus in rats with either 4- or 5-day cycles and found little or no delay in uterine ballooning or vaginal cornification, and uterine weight changes were not affected. They also obtained similar results with a longer-acting barbiturate, barbitone, injected at 09.00, 13.00 or 16.00 hr. on the day before pro-oestrus. They concluded that the LH secretion of dioestrus and subsequent oestrogen production is not suppressed by these treatments, though they could not exclude some reduction of the level of secretion. With regard to the suppression of ovulation on the day of expected oestrus, their results at first sight appear to differ from those reported here. Pentobarbitone at 14.00 or 16.00 hr. blocked ovulation in only 18 out of 43 rats as opposed to 13 out of 17 in my experiments. The reason for the relative lack of success in blocking ovulation by pentobarbitone given in late dioestrus in their experiments is possibly related to their use of rats with 5-day cycles in the majority of their experiments. If their results are broken down to separate the results obtained in animals with 4- and 5-day cycles, the blockade rate is four out of four rats in the pentobarbitone experiments and six out of eight in all experiments for rats with 4-day cycles; for rats with 5-day cycles, the blockade rate with pentobarbitone is only 14 out of 35 and for all experiments 24 out of 50. These proportions are very close to those in 4-day rats shown in Table 3 and for the small number of 5-day rats shown in Table 4.

The mechanism of the blockade of ovulation in rats with 4-day cycles after injection with pentobarbitone late in dioestrus has not been established. Suppression of oestrogen secretion is a possible explanation, but the evidence is against this; a reduction in oestrogen secretion to a level at which the hypothalamo-hypophysial system does not spontaneously release LH on the evening of pro-oestrus is possible but the lack of effect of exogenous oestrogen is against this view as discussed above. Schwartz & Lawton (1968) suggested that progesterone, but not oestrogen, secretion may have been affected; the positive effects of progesterone in these circumstances (Table 3) are consistent with this view but there is at present no evidence that a facilitatory effect of progesterone is involved in the normal cycle, though such an effect has been postulated (Everett, 1944, 1961, 1964a; Rothchild, 1965; Brown-Grant, 1969b).

An alternative explanation, and one that is consistent with the facts at present
available, is that the barbiturates are interfering not with oestrogen secretion but with the action of oestrogen on the hypothalamo-hypophysial system that results in a state of this system such that on the next day (day of pro-oestrus) there is a release of an ovulatory quota of LH in response to some as yet unidentified neural triggering mechanism. This would be consistent with the equal, or even enhanced, effectiveness of pentobarbitone when given at the end of the critical period, when according to the results of Lawton & Sawyer (1967, 1968) and other results reviewed here, adequate gonadotrophin release to induce oestrogen secretion has already occurred. The failure to block the change to a facilitatory action of progesterone may indicate that this effect is less sensitive to barbiturate inhibition than the process responsible for establishing the conditions for 'spontaneous' LH release at pro-oestrus. Whatever the reason, the fact that a rat may be responsive to progesterone on the expected day of pro-oestrus although it would not have ovulated spontaneously, may be additional evidence against the view that a facilitatory action of progesterone is an essential feature of the normal cycle. Evidence that barbiturates may block the early action of oestrogen on the brain that later leads to LH release is provided by Presl (1961) who showed that phenobarbitone injected at the same time as oestrogen in pregnant rats prevented the release of LH but was much less effective when given 22 hr. after the injection of oestrogen. It would be of interest to know what effect pentobarbitone given in late dioestrus has on a third consequence of oestrogen action, the onset of behavioural oestrus.

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