THE PLASMA CONCENTRATIONS OF TESTOSTERONE AND LH DURING THE OVULATION CYCLE OF THE HEN (GALLUS DOMESTICUS)

By

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ABSTRACT

The plasma concentrations of testosterone and luteinizing hormone (LH) were measured by radioimmunoassay during the ovulation cycle of the hen. The baseline concentrations of testosterone were 100 to 250 pg/ml and the maximum concentration of 904 pg/ml was observed 8 h before ovulation. The maximum pre-ovulatory concentration of LH of 2.0 ng/ml, which was approximately twice the basal concentration, was observed 6 h before ovulation. The possibility that testosterone is the ovarian excitation hormone which initiates the ovulation-inducing surge of LH is discussed.

It has been recognized for some time that testosterone is a biologically active hormone in the laying hen and both Fraps (1955) and van Tienhoven (1961) reported that injections of testosterone induced ovulation prematurely in 41% of the hens. During the ovulation cycle the concentration of testosterone in plasma reaches a maximum 2 to 8 h before ovulation (Petersen et al. 1973; Etches 1974; Shahabi et al. 1975) and the concentration of LH in plasma increases 4 to 7 h prior to ovulation (Cunningham & Furr 1972; Furr et al. 1973; Wilson & Sharp 1973; Senior & Cunningham 1974; Shodono et al. 1975).

Hitherto, direct comparisons between the timing of the pre-ovulatory surges
of LH and testosterone have not been possible since the previous studies were conducted independently and with different sampling intervals. The objective of this investigation was to determine the temporal relationship between the plasma concentrations of LH and testosterone.

MATERIALS AND METHODS

Single comb White Leghorn hens aged approximately 500 days were maintained in individual cages and given continuous access to food and water. The lighting regime was either 14 h of light and 10 h of darkness or 18 h of light and 6 h of darkness. Blood (2.5 ml) was collected by heart puncture into heparinized syringes from 21 hens every two hours for 24 h during the interval between sequences and during the ovulation cycle. The blood was immediately cooled and the plasma separated by centrifugation was stored at -20°C for assay of testosterone and LH.

The times of ovulation were calculated from the times of oviposition which were recorded to the nearest minute. Ovulation of a mid-sequence egg was considered to occur 30 min after oviposition of the previous egg in the sequence (Warren & Scott 1935; Melek et al. 1973) and ovulation of the first egg of a sequence was considered to occur 27 h before its subsequent oviposition (Fraips 1955).

The procedure for the radioimmunoassay of testosterone was similar to that described for progesterone by Furr (1973). The antiserum (designated 550/3) was raised in goats against testosterone-3-[(α-carboxymethyl)-oxime-bovine serum albumin by Dr. B. J. A. Furr (I. C. I. Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire). Antiserum 550/3 cross-reacted with 5α-dihydrotestosterone, 5-androstene-3β,17β-diol and with the androstanediol series, but had negligible affinity for eight other androgens, cholesterol and representative oestrogens and progestagens (Table 1). The range of the standard curve was 1600 pg to 12.5 pg and the doses were arranged in sequential 1:1 dilutions.

Five hundred µl of plasma were extracted once with 5 ml of a mixture of light petroleum (b.p. 40 to 60°C) diethyl ether (4:1, v/v) and assayed without further purification. This mixture extracted 82.4% ± 0.4% of the [1,2,6,7-3H]testosterone, 77.7% ± 5.0% of the [1,2-3H]5α-dihydrotestosterone and 16.5% ± 0.1% of the [5,6-3H]5α-androstane-3β,17β-diol. The solvent “blank” was estimated by treating 500 µl of distilled water as a plasma sample. The inhibitory activity of this “blank” was consistently less than that of the lowest standard.

The specificity of the assay was further assessed by comparing the inhibitory activity of crude extracts of plasma with the inhibitory activity of the same sample after chromatographic purification. The 3,17-hydroxylated androgens and the plasma lipids were separated from testosterone and 5α-dihydrotestosterone by chromatography on a 2.5 cm x 0.5 cm column of Sephadex LH-20 with an elution solvent of cyclohexane:toluene:methanol (80:15:5, by vol.) (Carr et al. 1971). The first ml of eluate was discarded and the next 4 ml, which contained both testosterone and 5α-dihydrotestosterone were collected, evaporated to dryness and assayed according to the routine procedure. There were no significant differences between the potency estimates obtained with or without chromatography when they were compared by a paired t-test (t = 1.23, P > 0.05). The correlation between the potency of chromatographed and non-chromatographed samples was 0.94 (P < 0.01) and the regression equation was
Table 1.
The cross-reactions of antiserum 550/3 with various steroids. A value of 100 % was assigned to testosterone.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reaction (%/o)(^1)</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>0.01</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.01</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.04</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.01</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.44</td>
</tr>
<tr>
<td>Oestriol</td>
<td>0.01</td>
</tr>
<tr>
<td>4-Androstene-3,17-dione</td>
<td>0.4</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>0.8</td>
</tr>
<tr>
<td>5β-Androstane-3,17-dione</td>
<td>0.6</td>
</tr>
<tr>
<td>5-Androsten-3β-ol-17-one</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-Androstan-3β-ol-17-one</td>
<td>6.9</td>
</tr>
<tr>
<td>5α-Androstan-3α-ol-17-one</td>
<td>0.2</td>
</tr>
<tr>
<td>5β-Androstan-3β-ol-17-one</td>
<td>0.3</td>
</tr>
<tr>
<td>5β-Androstan-3α-ol-17-one</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>22.1 ± 4.8 (3)(^2)</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>27.4 ± 9.7 (3)(^2)</td>
</tr>
<tr>
<td>5β-Androstane-3α,17β-diol</td>
<td>13.7 ± 4.0 (3)(^2)</td>
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<tr>
<td>5-Androstone-3β,17β-diol</td>
<td>8.8 ± 1.5 (3)(^2)</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>66.4 ± 3.8 (5)(^2)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\(^1\) Cross-reaction = \(\frac{100 \text{ testosterone (pg)}}{\text{competing steroid (pg)}}\), at 50 % inhibition.

\(^2\) Cross-reaction (%/o) ± standard deviation (calculated on a between-assay basis).

The number of assays are indicated in brackets.

\(y = 1.09 x - 6.75\). The slope of the regression line was not significantly different from the expected value of one \((t = 0.65, P > 0.05)\).

To test the accuracy of the assay, testosterone was added to a pool of plasma from mature females. The regression equation between testosterone added \((x)\) and testosterone recovered \((y)\) was \(y = 0.94 x + 153\). The slope of the regression line was not significantly different from the expected value of one \((t = 0.58, P > 0.05)\).

The precision of the assay was estimated by calculating the within- and between-assay coefficients of variation according to the method of Rodbard (1971). The within-assay coefficients of variation of ten plasma samples taken from mature females in which the testosterone concentration ranged from 50 to 250 pg/ml and 500 to 2000 pg/ml were 7.8 % and 9.8 %, respectively. The between-assay coefficients of variation which were calculated from ten assays at standard concentrations of 50 pg and 400 pg were 8.9 % and 18.9 %, respectively.

LH was measured using the method described by Follett et al. (1975).
RESULTS

The concentrations of testosterone and LH prior to the last oviposition of a sequence are shown in Fig. 1. It is evident that the concentration of neither hormone varied with respect to oviposition and that baseline concentrations of approximately 100 to 250 pg of testosterone and 1.0 ng of LH per ml of plasma were observed during this period.

To establish when the pre-ovulatory surges of testosterone and LH in plasma occurred with respect to the first ovulation of a sequence, additional data were obtained so that the entire period between the last ovulation of a sequence and the first ovulation of the next sequence could be examined (Fig. 2). Fewer observations are included in these data because the first ovulation of the sequence was delayed by 24 or 48 h in several hens. Presumably, this was due to the combined effects of wakefulness and activity enforced upon the hens by the blood sampling procedure since similar effects were noted by Bastian & Zarrow (1955). It is apparent that the concentration of testosterone increased 10 h before ovulation and 2 h before the concentration of LH increased. Maximum concentrations of LH occurred 6 h before ovulation which was 2 h after the maximum concentration of testosterone was observed.

The concentrations of both LH and testosterone were measured prior to the ovulation of subsequent ova within a sequence. Since identical patterns were observed at all times in a sequence, the concentrations of testosterone and LH which preceded all ovulations were pooled and are shown in Fig. 3. The plasma concentration of testosterone rose 10 h before ovulation which was
The concentration of testosterone (●—●) and LH (○—○) in plasma collected at 2-hourly intervals during the 46 h period between the last ovulation of a sequence and the first ovulation of the next sequence. The vertical bars represent the standard error of the mean. The number of observations in each mean varied between 3 and 5.

The concentration of testosterone (●—●) and LH (○—○) in plasma collected at 2-hourly intervals between successive ovulations within a sequence. The vertical bars represent the standard error of the mean. The number of observations for each mean varied between 5 and 13.
**Fig. 4.**
The concentration of testosterone (●—●) and LH (○—○) in plasma collected at 2-hourly intervals from an individual hen during 16 h before ovulation.

4 h before a change in the plasma concentration of LH was detected. The maximum concentration of 904 pg of testosterone per ml of plasma occurred 8 h before ovulation and represented a three- to fivefold increase from the baseline value of 170 to 290 pg/ml. The maximum pre-ovulatory concentration of LH reached 2.0 ng/ml, which was approximately twice the basal concentration.

The large standard error of the mean concentration of testosterone 8 h before ovulation (Fig. 3) reflected the frequent occurrence of two peaks of testosterone in individual hens. The first peak invariably preceded the maximum concentration of LH by 4 h and the second coincided with the maximum concentration of LH. An example of this pattern is shown for an individual hen in Fig. 4.

**DISCUSSION**

*The radioimmunoassay for testosterone*

The radioimmunoassay was shown to be accurate by the quantitative measurement of unlabelled testosterone which had been added to plasma and by chromatographic purification of plasma extracts. The within- and between-assay coefficients of variation and the range of the standard curve were similar to those of previously published radioimmunoassay for testosterone (Collins et al. 1972; Cayotupa et al. 1972; Dufau et al. 1972; Ismail et al. 1972; Lucas & Abraham 1972; Nieschlag & Loriaux 1972; Barberia & Thorneycroft 1974).
The major deficiency of the assay is lack of specificity (Table 1). Since only 16% of the 5α-androstane-3β,17β-diol was extracted from the plasma by the mixture of light petroleum (b. p. 40 to 60°C):diethyl ether (4:1), it is likely that the combined influence of steroids belonging to the androstenediol and androstanediol series was negligible. However, the major cross-reacting steroid, which also possesses considerable biological activity (Wilson & Gloyna 1970; Wilson 1972) was 5α-dihydrotestosterone. The existence of this compound in chicken plasma has not been documented, although Gloyna & Wilson (1969) and Nakamura & Tanabe (1973) reported the potential of the comb, wattle, coccigeal gland and the epididymis of the cockerel to synthesize 5α-dihydrotestosterone in vitro. These observations are consistent with the idea postulated by Wilson & Gloyna (1970) that the formation and influence of 5α-dihydrotestosterone resides at the intra-cellular level in the target organ. Furthermore, it has been demonstrated that injections of 5α-dihydrotestosterone and testosterone have equal ovulation-inducing and ovulation-inhibiting actions (Etches 1975). Assuming that the ratio of the plasma concentrations of testosterone and 5α-dihydrotestosterone remain constant, this assay is suitable for measuring active testosterone equivalents during the ovulation cycle.

The plasma concentration of testosterone and LH

An increase in the concentration of testosterone always preceded an increase in the concentration of LH. It is conceivable, therefore, that testosterone is the ovarian excitation hormone which initiates the release of LH from the pituitary gland. This possibility is supported by several lines of evidence. For instance, if either the hypothalamus is lesioned or the pituitary gland ablated within 2 h of an injection of progesterone, ovulation will not be induced prematurely (Ralph & Fraps 1959; Rotchild & Fraps 1949). In view of this it could be argued that approximately 2 h elapse between excitation and LH release. This interval is similar to the lag observed in the present work between the increased plasma concentrations of testosterone and LH. In addition, Fraps (1955) and van Tienhoven (1961) found that the injection of testosterone induced ovulation prematurely and they suggested that this effect was mediated by the hypothalamus. Furthermore, the interval from the injection of testosterone to ovulation is greater than 9 h (Fraps 1955) although the interval from the injection of LH to ovulation is only 7 to 8 h (Fraps 1942).

If one of the functions of testosterone is to activate the LH release mechanism, then its concentration in plasma might be expected to fall before that of LH. This was not observed in the present experiments. In fact, in several hens, a second rise in the plasma concentration of testosterone occurred which coincided with the maximum concentration of LH (Fig. 4) and in all
hens the plasma concentrations of testosterone and LH decreased simultaneously. Thus, it would seem that the secretion of testosterone is potentiated by LH. It could also be suggested that testosterone plays a role in the maintenance of LH secretion and at maximum concentrations inhibits the ovulation-inducing release of LH. Alternatively, the prolonged secretion of testosterone may be necessary both to maintain accessory reproductive functions such as protein synthesis by the oviduct and to control patterns of sexual behaviour. As none of these possibilities were investigated, no conclusion can be made at the present time.

Although the temporal changes in the concentration of testosterone during the ovulation cycle were similar to those observed by Peterson et al. (1973) and Shahabi et al. (1975), the absolute concentrations varied considerably. Compared to the minimum concentration of 100 to 250 pg/ml and the maximum concentration of 904 pg/ml which were found in this investigation, the minimum and maximum observed by Peterson et al. (1973) were 320 pg/ml and 564 pg/ml, respectively, whereas the minimum and maximum values observed by Shahabi et al. (1975) were approximately 750 and 2500 pg/ml, respectively. The reasons for these differences are not clear but they could reflect differences in the assay procedures. Peterson et al. (1973) used a more polar solvent to extract the plasma and this may have increased the contribution of androgens which are more polar than testosterone to the inhibitory activity of the sample. Details of the assay procedure which was used by Shahabi et al. (1975) were not given.

Both the maximum and minimum concentrations of LH and the relative difference between them during the ovulation cycle were similar to those described by Cunningham & Furr (1972), Furr et al. (1973), Wilson & Sharp (1973), Senior & Cunningham (1974) and Shodono et al. (1975). Cunningham & Furr (1972) reported the existence of a minor increase in the concentration of LH 20 to 23 h before ovulation, although this increase was not found in the subsequent investigations conducted by Furr et al. (1973) and Wilson & Sharp (1973). However, in Figs. 2 and 3 it can be seen that the concentration of LH was raised during this time and similar results were reported by Shodono et al. (1975). This peak could be due to FSH as the assay might have a slight cross-reaction with this hormone. Since the peak occurs before the follicle matures its potential involvement in follicular maturation cannot be ignored.

Examination of the concentration of LH in plasma during the ovulation cycle using radioimmunoassay has consistently revealed a nadir 12 to 14 h before ovulation (Cunningham & Furr 1972; Furr et al. 1973; Shodono et al. 1975; Figs. 2 and 3). Nalbandov (1961) and Opel & Nalbandov (1961) showed that following hypophysectomy follicles became increasingly sensitive to the ovulation-inducing property of exogenous gonadotrophin and postulated that
the preparation of the follicle for ovulation required a withdrawal of gonado-
trophin. This interesting hypothesis is consistent with the observed pattern in
the plasma concentration of LH and deserves further investigation.

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