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Plasma Concentrations of Six Steroids and LH During the Ovulatory Cycle of the Hen, Gallus domesticus[1]

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ABSTRACT

Five laying hens were serially sampled via a brachial vein cannula at 2 h intervals for a period of 34 to 72 h. Plasma samples were grouped to the nearest 2 h interval relative to the estimated time of ovulation, and subsequently each sample was assayed for progesterone (P_4), estradiol-17β (E_2), estrone (E_1), testosterone (T), 5α-dihydrotestosterone (DHT), corticosterone (B), and LH. Mean concentrations of plasma LH, P_4, E_1, E_2, and DHT were highest 6 h prior to ovulation, while a peak of T preceded ovulation by 8 h. There was a surge of B preceding ovulation by 2 h; however, this is attributed to a peak related to oviposition and not to ovulation. There was a small daily surge of LH occurring at the onset of darkness regardless of whether an ovulation occurred on the subsequent morning. These hormone fluctuations are discussed with reference to the potential direct or indirect role of each hormone in the induction of ovulation.

INTRODUCTION

Several steroids have been implicated in the induction of ovulation in the domestic hen, including progesterone (P_4) (Wilson and Sharp, 1975; Etches and Cunningham, 1976), testosterone (T) (Wilson and Sharp, 1976; Croze and Etches, 1980), and corticosterone (B) (van Tienhoven, 1961; Etches, 1977). Quantification of hormone concentrations in serial blood samples taken throughout the ovulatory cycle have resulted in numerous reports of cycle-related rhythms of various combinations of two or perhaps three steroids and LH. Despite the finding that plasma concentrations of P_4 and LH begin to rise virtually simultaneously during a preovulatory surge (Laguë et al., 1975; Etches and Cunningham, 1976), Bonney and Cunningham (1977) have suggested that the preovulatory surge of LH is initiated by P_4, while Shahabi et al. (1974) have indicated that it is likely that hypophyseal trophic hormones trigger ovarian steroid secretion. There are also discrepancies concerning the timing of peak concentrations of various steroids and LH relative to ovulation. Senior and Cunningham (1974) have reported that in blood samples taken at 2 h intervals estradiol-17β (E_2) reaches peak concentrations 2 h prior to peak levels of LH, while Laguë et al. (1975), sampling at 20 min intervals, indicated that the peak of E_2 came slightly after peak concentrations of LH and coincident with peak concentrations of P_4. Williams and Sharp (1978) reported that concentrations of androgens generally peak with those of LH while Etches and Cunningham (1977) showed a T peak preceding that of LH by 2 h. Although Laguë et al. (1975) noted that in general plasma LH reached maximum concentrations before P_4, it was not possible to determine which hormone began to rise first.

Several workers have identified a diurnal rhythm of LH in the laying hen (Wilson and Sharp, 1973; Williams and Sharp, 1978) as well as in the immature pullet and cockerel (Scanes et al., 1978). This peak occurs just after the onset of the dark period. It has been suggested that in the laying hen this small increase in LH serves to stimulate P_4 secretion, which in turn initiates the preovulatory LH surge (Scanes et al., 1978).

Given the differences among these reports as to the mechanism by which the process of ovulation is initiated and as to the timing of peak concentrations of steroids and LH relative to ovulation, we felt it desirable to investigate the interrelationships of LH and as many

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steroids as possible throughout the ovulatory cycle in an attempt to better understand the interactions among hormones leading to ovulation. Therefore, the objective of the experiments described herein was to determine plasma concentrations of six steroids, (estrone [E_1], E_2, P_4, T, 5α-dihydrotestosterone [DHT], and B) and LH, assayed from the same plasma sample, throughout the ovulatory cycle in blood samples drawn at 2 h intervals.

MATERIALS AND METHODS

Animals

Cornell C strain white Leghorn hens in their first year of production, laying regular clutches of 2–5 eggs, were housed individually in cages 30 × 58 × 60 cm (w × d × ht). Feed (Cornell laying hen ration) and water were available ad libitum. Photoperiod consisted of 14L:10D with lights on between 0600 and 2000 h. Oviposition was recorded to the nearest 30 min interval throughout the sampling period. The time of ovulation was estimated from the time of oviposition and by subsequent digital palpation. The brachial vein of hens was cannulated with Silastic medical grade tubing (0.635 mm i.d. × 1.194 mm o.d.), and the hens were allowed to complete at least one normal clutch after surgery before sampling. Five hens were sampled at 2 h intervals (starting at 0200 h) for 34 to 72 h. A 2.4 ml sample of blood was drawn via the cannula at each interval. Blood samples were centrifuged at 1100 × g for 15 min, and plasma was stored at −20°C until assayed. Red blood cells were resuspended in sterile 1% saline and replaced at 4 h intervals. Blood samples drawn during the dark period were obtained by placing the hen near a dim green light, which allowed the sampler to obtain blood apparently without interferring with the hen's sleep.

Radioimmunoassays

The LH RIA has been modified from that originally described by Follett et al. (1972). Iodination was accomplished by the lactoperoxidase method (Bolton, 1977). The assay was accomplished by combining a 50 μl sample or standard (0.4 to 16.0 ng/ml) and rabbit anti-LH antibody on Day 1. On Day 2, 100 μl of [^{125}I]-LH (100 pg) were added, and 100 μl of anti-rabbit γ globulin (second antibody; obtained from Dr. W. R. Butler) were added 48 h later. After 48 h incubation, tubes were centrifuged, the supernatant decanted, and the pellet counted in a Beckman Gamma 4000 equipped with a DP 5000 data reduction unit. Sensitivity of the assay ranged between 300 and 400 pg/ml, while the mean within-assay coefficient of variation (CV) for low (1.1 ng/ml) and high (4.5 ng/ml) plasma pools was less than 6%, and the mean between-assay CV was less than 14% (n = 5).

Progesterone was extracted from plasma using petroleum ether, with an extraction efficiency of 85.8 ± 7.5 (SD)%. The P_4 antiserum (supplied by Dr. H. R. Behrman, Merck Institute for Therapeutic Research) crossreacts with 4-pregnen-11α-01-3, 20 dione (45%), 4-pregnen-11β-01-3, 20-dione (3%) and 5α-pregnen-3, 20-dione (17%). Further purification of the crude extract by Sephadex LH column chromatography (solvent system was isooctane:benzene:methanol, 62:20:18) did not significantly affect assay results ($t = 1.31$, $P>0.20$). Sensitivity of the assay was less than 25 pg/tube, while the mean within-assay CV for the four assays for low (0.445 ± 0.062 (SD) ng/ml) and high (3.682 ± 0.274 ng/ml) plasma pools was less than 7.6%, and the mean between-assay CV was less than 14.0%.

Corticosterone was extracted from the same aliquot of plasma, after the removal of P_4, with at least 10 volumes of benzene:toluene (2:1). Less than 4% of the B was extracted by petroleum ether, while less than 6% of the P_4 remaining after petroleum ether extraction was removed with benzene:toluene (2:1). Extraction efficiency for B was 85.5 ± 6.7%. The antibody was that described by Etches (1976). Further purification of the crude extract by Sephadex LH column chromatography (solvent system was toluene:methanol, 9:1), did not significantly affect assay results ($t = 1.21$, $P>0.20$). Sensitivity of the assay was less than 50 pg/tube, while the within- and between-assay CV was 9.8% (n = 14 samples) and 12.0% (n = 13 samples), respectively.

Testosterone, DHT, E_2, and E_1 were assayed at least in duplicate from the same aliquot of plasma after a single extraction with 10 times the volume of benzene:toluene (2:1). Estrone and E_2 were purified from the dried extract by Sephadex column chromatography using a 6.0 × 0.7 cm column with the solvent system isooctane:benzene:methanol (62:20:18). The androgens were eluted within the fraction 2.0 through 6.0 ml, and E_1 and E_2 were eluted within the fractions 6.0 through 8.0 ml and 9.0 through 12.5 ml, respectively. Estrone and E_2 were assayed separately using the same antiserum (S-310#5, obtained from Dr. G. E. Abraham, UCLA; this antiserum crossreacts 140% with E_1 and E_2 and 100% with estriol). The recovery of E_1 and E_2 was 76.3 ± 15.1% and 69.4 ± 14.5%, respectively. The within- and between-assay CV for E_1 was 6.6% and 10.8%, respectively, while the sensitivity of the assay was 17 pg/tube. Within- and between-assay CV for E_2 was 8.6% and 13.0%, respectively, and the sensitivity 26 pg/tube.

The androgen fraction collected from the above-mentioned column system was rerun on a Sephadex LH column (7.5 × 0.7 cm) using the solvent system isooctane:benzene:methanol (90:5:5). Dihydrotestosterone and T were eluted in the fractions 12 through 18 ml and 19 through 27 ml, respectively. The recovery of DHT and T was 74.9 ± 6.7% and 63.9 ± 6.5%, respectively. The T antiserum used was Abraham's S-741#2 antiserum (this crossreacts 70% with DHT and <0.10% with androstenedione), and the DHT antiserum was raised in sheep against 5α-DHT-17-hemisuccinate:BSA (this antiserum crossreacts 11.4% with androstenedione, 7.9% with T, and 6.0% with 20α-hydroxyprogesterone). The within-and between-assay CV for DHT was 10.4% and 14.8%, respectively, and the sensitivity of the RIA was 38 pg/tube. Within- and between-assay CV for T was 11.2% and 13.0%, respectively, while the sensitivity of the RIA was 22 pg/tube.

Statistical Analysis of Data

Concentrations of all hormones were grouped to the nearest 2 h interval relative to the estimated time

of ovulation. In addition, LH values were plotted relative to photoperiod.

Analysis of variance of our data is not permissible because within hens the data are not independent. We therefore used the following formula to calculate the probability that a peak concentration of a certain hormone would occur within the same interval prior to the time of ovulation: $P = T(X \div T)^n$, where P is the probability that all peak concentrations of a certain hormone fall within a specific interval, T = total number of 2 h intervals, X = the number of adjacent intervals in which a peak was observed, and n = number of ovulatory cycles. A peak of LH, P_4, T, DHT, and B in each hen was defined as the highest concentration found during an ovulatory cycle. Because previous workers have indicated that it is likely that two estrogen peaks (E_1 and E_2) occur within an ovulatory cycle (Peterson and Common, 1972; Laguë et al., 1975), the two highest values were designated as peaks.

We conducted paired t tests only for values at 2000 h vs those at 1800 h, and for values at 2000 h vs 2200 h, to test for a diurnal surge of LH occurring near the onset of darkness. For the 2200 h period, all values that preceded the preovulatory peak by 2 h were omitted (i.e., if peak concentrations of LH occurred at 2400 h, and 2000 h vs 2200 h comparison was omitted). An alpha value of less than 0.05 was considered significant.

RESULTS

Six ovulatory cycles from five hens were examined. Four of the six cycles represented the period preceding the first ovulation of the clutch (C_1 ovulation) while the remaining two preceded the C_2 ovulation. Progesterone, E_2, E_1, T, B, and LH were examined in all six cycles, whereas DHT was investigated in three of the six cycles.

Mean plasma concentrations of E_1 and E_2 are shown in Fig. 1. Highest concentrations of both estrogens were found 6 to 4 h prior to ovulation; however, the variability in the timing of the peak in individual birds precluded testing for statistical significance by the method outlined above. Five of six cycles showed a peak of E_2 between 6 and 2 h prior to ovulation (Fig. 2). In addition, three of six cycles exhibited a slightly smaller peak 22 to 18 h prior to ovulation. Five of 6 cycles had highest concentrations of E_1 8 to 4 h prior to ovulation, while no cycles showed an increase 22 to 18 h prior to ovulation.

Variability in the timing of peak concentrations of B relative to ovulation likewise precluded testing for statistical significance. Concentrations of B were highest in three of six cycles at 2 h prior to ovulation (Fig. 2). The highest values in two of the remaining three cycles were present 20 h prior to ovulation, and present 2 h after ovulation in the remaining cycle.

Concentrations of T were highest 10 to 6 h prior to ovulation (Figs. 2, 3; P = 0.0013), and highest concentrations of DHT were found 6 to 4 h prior to ovulation (Fig. 1; P = 0.041). Peak plasma concentrations of P_4 were consistently found 6 to 2 h prior to ovulation (Fig. 2; P = 0.0014), while highest concentrations of LH were always present 6 to 4 h prior to ovulation ($P<0.001$). In agreement with previous workers (Laguë et al., 1975; Etches and Cunningham, 1977), in no case was there a surge of T, P_4, or LH on the night preceding the last oviposition of the sequence.

Finally, when plasma concentrations of LH were plotted relative to photoperiod both for nights preceding an ovulation and for nights when ovulation did not occur the following morning (i.e., for the night preceding the terminal oviposition of the clutch), there was a small but statistically significant increase at 2000 h (the onset of darkness) compared with 2 h before darkness [on nights preceding an ovulation, $P<0.05$ for values at 2000 h vs 1800 h (n = 6), and $P<0.10$ for 2000 h vs 2200 h (n = 5); on nights not preceding an ovulation, $P<0.01$ for values at 2000 h vs 1800 h (n = 7), and $P<0.05$ for 2000 h vs 2200 h (n = 7); one-tail paired t test] (Fig. 4).

DISCUSSION

Plasma concentrations of E_1 and E_2 in the present study are in agreement with Peterson and Common (1972), Senior (1974), and Laguë et al. (1975). As noted by Laguë et al. (1975) a preovulatory peak of plasma P_4 was generally accompanied by an increase in E_1 and E_2 (Fig. 2). In contrast to results reported by Senior and Cunningham (1974), we do not find that the E_2 peak precedes that of LH by 2 h (Fig. 2). These findings, however, do not negate the possibility that an increase in plasma estrogen at this time may facilitate the release of the preovulatory LH surge at the level of the pituitary, as suggested by Bonney and Cunningham (1977).

It has been reported by some investigators that there is a smaller rise in estrogens 23 to 18 h prior to ovulation (Peterson and Common, 1972; Shodono et al., 1975). Results in the present study show no consistent elevation at this time for either estrogen.

Plasma B tended to be highest $\sim$2 h prior to ovulation (Figs. 1, 2). However, this peak

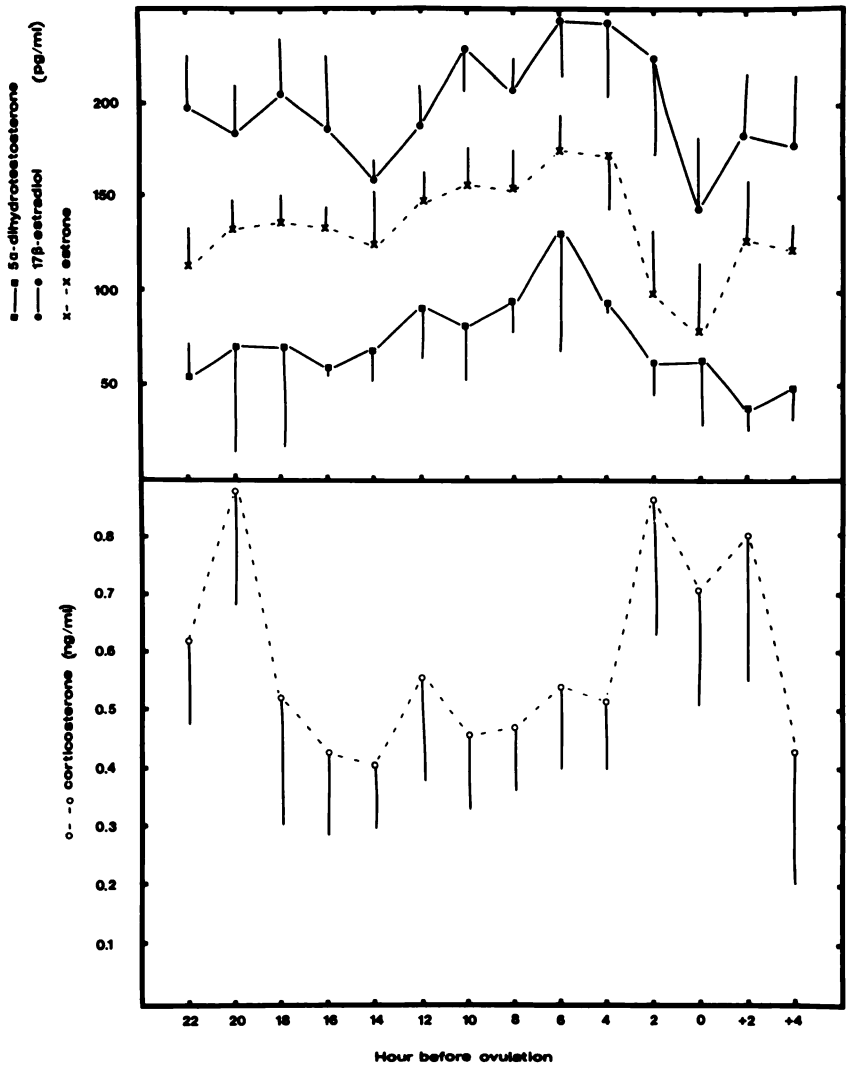

FIG. 1. Plasma concentrations of estradiol-17β (●———●), estrone (x———x), corticosterone (○———○), and 5α-dihydrotestosterone (■———■) relative to time of ovulation, irrespective of the stage of the ovulatory cycle. Mean ± SEM; n = 6 ovulatory cycles from 5 hens for estradiol, estrone, and corticosterone and n = 3 cycles from 3 hens for dihydrotestosterone.

occurred probably because of the coincident oviposition, as it has previously been shown that there is a significant surge of B coincident with oviposition (Beuving and Vonder, 1977; Johnson and van Tienhoven, 1980). There would appear to be no cycle-related fluctuation of B preceding ovulation by 4 to 10 h, as found for the remaining hormones (Fig. 1) (Johnson and van Tienhoven, 1980).

Plasma concentrations of T throughout the cycle are in close agreement with those reported by Etches and Cunningham (1977). Peak concentrations occur 10 to 6 h prior to ovulation, or in general 4 to 2 h before the peaks of P_4 and LH (Figs. 2, 3). The one exception found was hen 91, in which highest concentrations of LH, P_4, and T occurred simultaneously. This might simply be the result of our having missed the exact timing of peak levels as the result of the 2 h sampling interval.

Croze and Etches (1980) found that the median effective dose (ED_{50}) of T that would induce ovulation was 966 μg. They found that a dose of 1 mg T given s.c. routinely induces

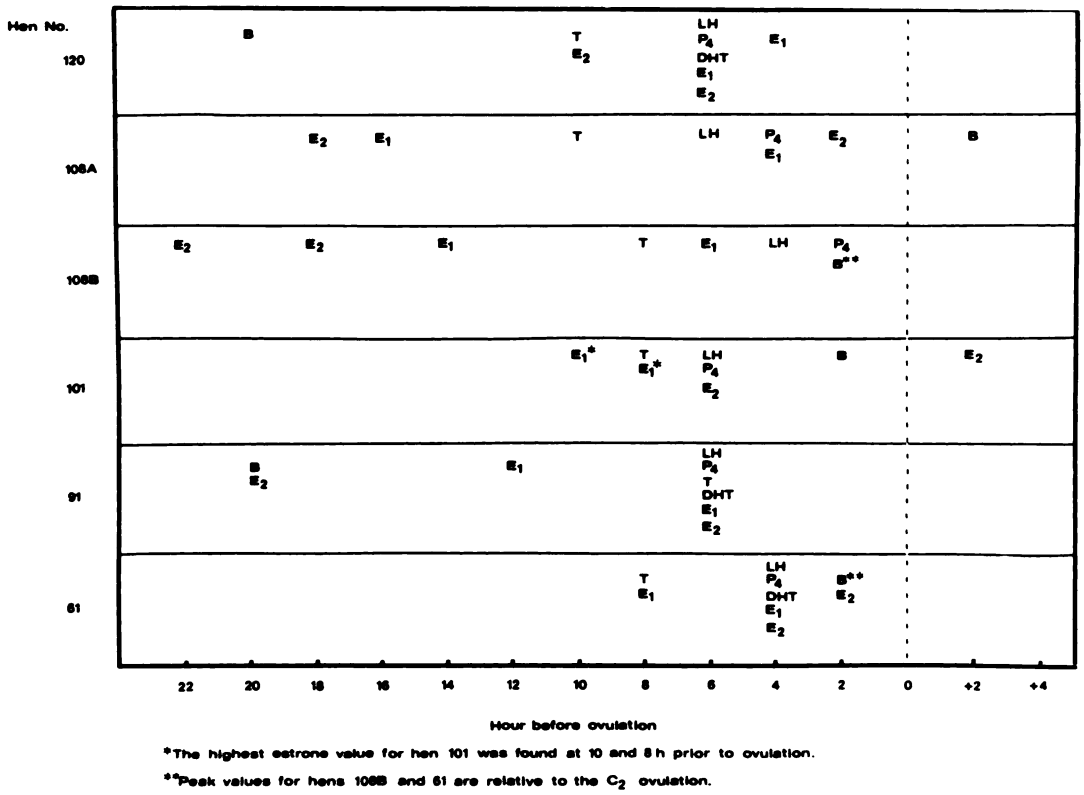

FIG. 2. Timing of peak concentrations of hormones in individual hens relative to ovulation. For estrone and estradiol-17β the two largest peaks are indicated. Ovulations for hen 108 have been designated as C_1 (108A) or C_2 (108B).

premature ovulation; however, in these hens plasma concentrations of T reach unphysiological levels (in excess of 10 ng/ml plasma). In addition, whereas 20 μg P_4 injected into the third ventricle of the brain is capable of inducing ovulation with an incidence of 72% (Johnson and van Tienhoven, unpublished), doses of 20, 40, and 80 μg T induce ovulation less consistently (less than 15% incidence; unpublished observations). These findings indicate that T is probably not directly involved in inducing ovulation, but do not rule out an indirect role of T in facilitating P_4 and/or LH release.

To our knowledge, there have been no previous reports of concentrations of DHT relative to the ovulatory cycle. Williams and Sharp (1978) have reported that basal concentrations of DHT and T are approximately 0.15 ± 0.04 and 0.44 ± 0.16 ng/ml, respectively, but do not indicate changes in concentrations of DHT relative to T throughout the ovulatory cycle. Our basal values of DHT and T are generally one-half of those reported by Williams and Sharp (1978). Though concentrations of DHT were investigated in only three hens, it would appear that there is an ovulatory cycle-related surge of DHT which peaks prior to or simultaneously with the peak of T (Fig. 2).

Croze and Etches (1980) have indicated that DHT injections can induce ovulation; however, the response is not dose-related. The preovulatory surge of DHT found in the present study is probably not directly involved in ovulation, as levels in the circulation are well below those needed for even T to induce ovulation. In all probability the increase in plasma DHT 6 to 4 h before ovulation is the result of peripheral metabolism of T released during the preovulatory surge.

Preovulatory plasma concentrations of LH appear to peak simultaneously or perhaps just prior to those of P_4 (Fig. 2). Consistent with reports by Laguë et al. (1975) and Etches and Cunningham (1976), our results from samples

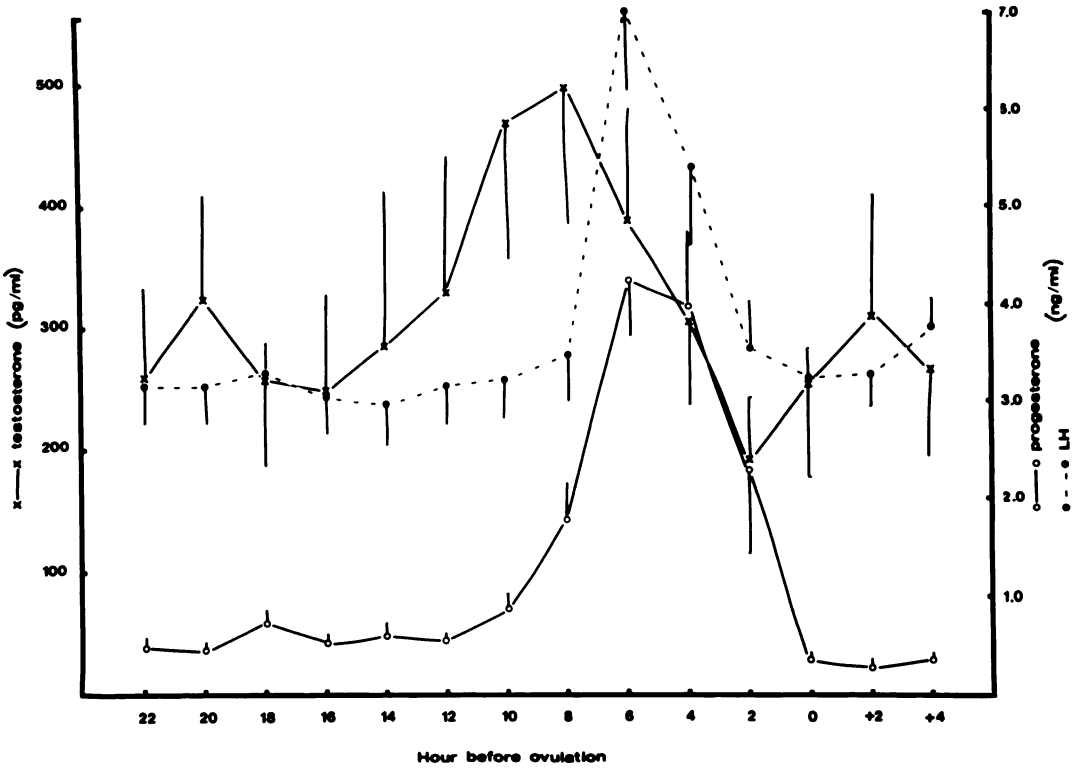

FIG. 3. Plasma concentrations of progesterone (o——o), testosterone (x——x), and LH (•——•) relative to time of ovulation, irrespective of the stage of the ovulatory cycle. Mean ± SEM; n = 6 ovulatory cycles from 5 hens.

drawn at 2 h intervals do not enable us to distinguish which of these hormones begins to rise first.

It has been demonstrated that injections of mammalian LH can induce ovulation (Gilbert, 1971), and, in addition, ovine LH has been shown to induce preovulatory-like surges of plasma P_4 and T, but not of estrogen (Shahabi et al., 1975). It has also been established that injection of P_4 stimulates LH secretion (Wilson and Sharp, 1975; Etches and Cunningham, 1976), and injection of LHRH stimulates secretion of P_4 (Etches and Cunningham, 1976). These data suggest the presence of a positive feedback mechanism between P_4 and LH that is involved in the induction of ovulation. We define a positive feedback mechanism as follows: The release of LH after an injection of progesterone would be similar in an ovariectomized and in an intact hen if progesterone has a stimulatory action only. If the positive feedback effect prevails, then a signal from the ovary is necessary to amplify the signal (LH release), and LH release is necessary to increase progesterone secretion until the peak concentration of one of the two is reached (e.g., because no more LH is available for release). In this case an injection of progesterone would result in a smaller release of LH in ovariectomized hens than in intact hens. However, Wilson and Sharp (1976) found in the ovariectomized, P_4- and E_2-primed hen that P_4 is capable of inducing a preovulatory-like surge of LH. This would indicate that only a stimulatory action, and not a positive feedback of P_4 on LH, is required to induce ovulation. Further investigations are needed to differentiate which of these two mechanisms exists in the hen.

The finding of a small daily surge of LH (Fig. 4) is in agreement with reports of Wilson and Sharp (1973) and Williams and Sharp (1978). However, their peak concentrations are found 1 h following the onset of darkness, whereas in the present study a rise in the concentration of LH is found in the sample taken at the time the lights went off (at 2000

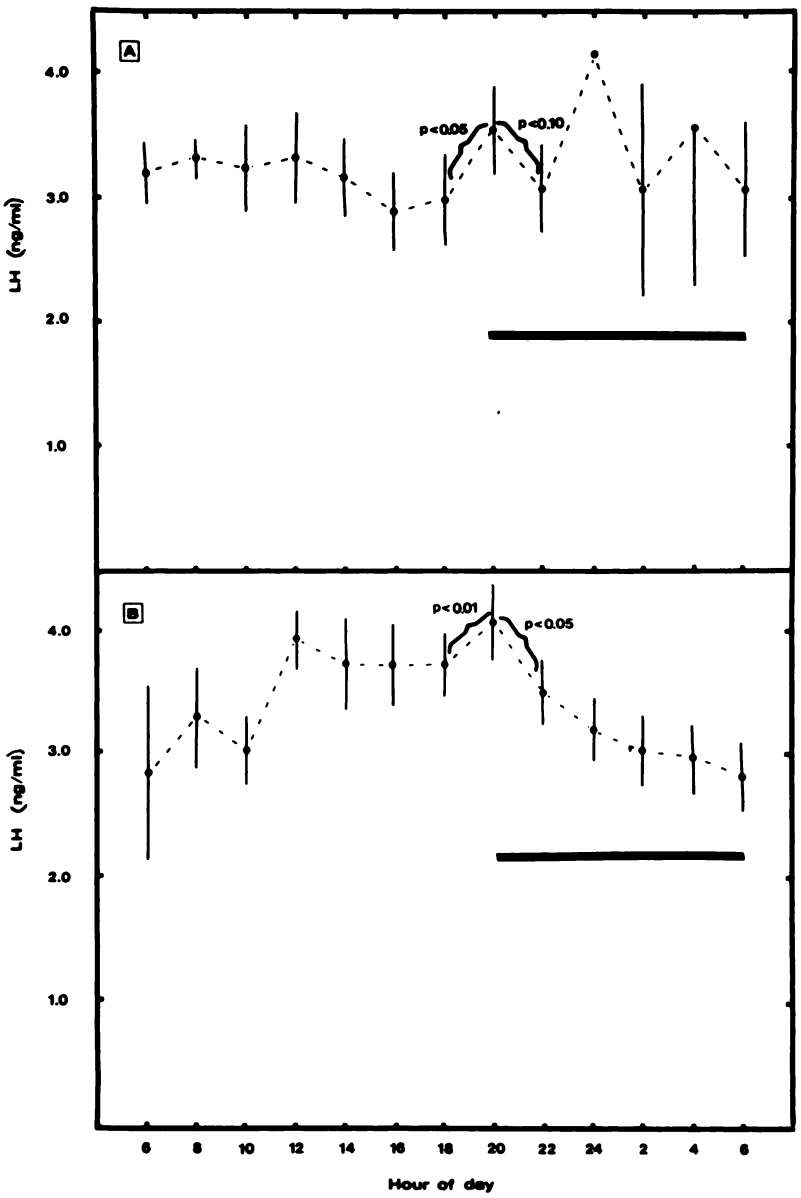

FIG. 4. Concentrations of LH relative to hour of day on nights (A) with a preovulatory LH surge (values 2 h prior to through 2 h after the preovulatory LH surge are excluded) and (B) with no preovulatory LH surge. Black bar denotes lights off.

h). The fact that this peak was found by Williams and Sharp (1978) to be of short duration, in combination with a possible confounding influence of the preovulatory surge of LH in samples drawn at 2200 h in the present study, may account for the lack of a significant difference between our values at 2000 h vs those at 2200 h in hens sampled on the night of a preovulatory LH surge (Fig. 4A). Shahabi et al. (1975) have shown that accumulation of P_4 occurs 6 h before ovulation within the C_1 follicle, but not within the C_2 or C_3 follicle. Therefore, this daily surge of LH may be involved in initiating P_4 release from only the most mature follicle. A daily surge is also found on nights when no preovulatory surge

occurs (Fig. 4B). This agrees with the findings of Wilson and Sharp (1973) and Scanes et al. (1978). Williams and Sharp (1978) have suggested that the lack of a P_4 surge and subsequent ovulation at this stage of the cycle is due to the lack of a mature follicle. Although the proposal that the daily LH surge initiates the preovulatory surges of P_4 and LH might explain the initiation of events leading to C_1 ovulation, it does not explain how, as the sequence progresses, the preovulatory surge occurs later and later in the dark period. Additional experiments are needed to determine whether this daily surge of LH is indeed necessary to initiate the preovulatory surges of P_4 and LH that lead to ovulation.

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