SUMMARY

Serum LH concentrations were less than 1 ng/ml immediately prior to castration of four 5-month-old Holstein bulls. A significant increase occurred 7 hr post-castration and serum LH remained elevated thereafter, interspersed with non-rhythmic episodic fluctuations of this gonadotropin. Single injections of testosterone or dihydrotestosterone, given iv or im, did not depress post-castration serum LH, whereas a single injection of estradiol (.5 or 2.0 μg/kg) caused a significant reduction in serum LH within 3 hr of administration. Repeated administration of testosterone propionate (100 mg TP at 12-hr intervals for 3 days) resulted in significantly reduced serum LH concentrations and episodic increases were eliminated. However, these concentrations of LH were still significantly higher than serum LH prior to castration. Administration of 5 μg gonadotropin releasing hormone (GnRH) at 4 hr and at 28 hr following a 4-day treatment with TP (119 mg at 12-hr intervals) resulted in a slight increase (P=.08) in total LH released as compared to the release of LH induced by injection of GnRH prior to TP treatment. We conclude that testosterone does not exert a negative feedback effect at the level of the pituitary. In fact, under the conditions of these experiments, testosterone slightly enhanced the ability of the anterior pituitary to respond to GnRH.

INTRODUCTION

Gonadectomy increases serum LH concentrations in male rats (Gay and Midgley, 1969), human males (Walsh et al., 1973), rams (Pelletier, 1968) and prepubertal bulls (Odell et al., 1970). Serum LH levels in castrate male rats were reduced with testosterone propionate (TP) or estradiol benzoate (EB) treatment (Gay and Dever, 1971), while elevated post-castration serum LH levels in rams were reduced with TP treatment (Crim and Geschwind, 1972; Pelletier, 1970; Pelletier and Ortavant, 1972). Implants of EB reduced post-castration serum LH in rams (Riggs and Malven, 1974).

Synthetic gonadotropin releasing hormone (GnRH) caused the release of LH in bulls in a dose response fashion when administered intramuscularly (im) at dosages of .3 and 3.0 μg/kg (Golter et al., 1973) or intravenously (iv) at dosages of 10 to 160 μg (Zolman et al., 1973). Castration altered pituitary responsiveness to 40 μg GnRH (iv) by causing an increased LH release (Mongkonpunya et al., 1974).

Bogdanove (1964) proposed that steroids may modulate the action of releasing hormones on the pituitary. Pretreatment of castrate male rats with EB, testosterone or dihydrotestosterone reduced the pituitary response to GnRH (Debeljuk et al., 1973). Pelletier (1974) showed a decreased pituitary response to GnRH at 6 hr, but an increased response at 36 hr after treatment with TP in castrate compared to intact rams. In contrast, Mongkonpunya et al. (1974) found no change in pituitary responsiveness to GnRH given to Holstein steers after 6 days of thrice daily treatment with 20 mg testosterone.

We conducted experiments using Holstein steers to determine the acute effects of castra-
tion on serum LH levels and the steroid dosage required to suppress post-castration serum LH levels. A final experiment was conducted to determine the site of negative feedback of testosterone on LH release by measuring pituitary responsiveness to GnRH before and after TP treatment.

METHODS AND MATERIALS

Experiment 1. Four prepubertal Holstein bulls weighing approximately 175 kg were castrated at 5 months of age. These same four steers were used in subsequent experiments. Blood samples (10 ml) were taken by jugular venipuncture before castration and at hourly intervals for 12 hr following castration. These blood samples, as well as all subsequent samples, were allowed to clot for 4 to 6 hr at room temperature and then were centrifuged at 1,110 x g for 10 minutes. The serum was decanted into storage vials and frozen until assayed for LH.

Experiment 2. In an attempt to reduce elevated post-castration serum LH concentrations, four steers, castrated 2 months previously, were treated im with TP (.5, 4.0 and 16.0 µg/kg), EB (.5 and 2.0 µg/kg), dihydrotestosterone benzoate (DHTB; .5, 4.0 and 16.0 µg/kg) or vehicle (2 ml corn oil). All dosages of steroid esters are expressed in terms of free steroid. Treatments were administered randomly every second day on 5 different days. Treatment with TP or DHTB failed to suppress elevated post-castration serum LH concentration as did four higher levels of TP (64, 256, 512 and 1,024 µg/kg) given iv in 50 ml ethanol-saline (2:1). Thus, for the last trial, 84 mg TP in 2 ml corn oil was injected into alternate sides of the rump (im) of two steers twice daily at 0800 and 2000 hr for 3 consecutive days. Blood samples were collected at 6 to 8 hr intervals during and for 1 day following the steroid injections.

Experiment 3. Gonadotropin releasing hormone, at dosages of 10, 40, 80 and 160 µg dissolved in 10 ml saline, was administered to the steers via indwelling jugular vein cannulae. Analysis of serum, collected at 10- to 30-min intervals prior to and following injection of GnRH, revealed a similar, maximal release of LH at all GnRH dosages. However, time to peak serum LH concentration tended to be positively dose related. To determine the threshold, lower dosages of 2.5, 5.0, 7.5 and 10 µg GnRH were similarly administered.

Experiment 4. The final experiment, using GnRH and TP treatment, encompassed a 7-day period. The GnRH, 5 µg in 5 ml saline, was injected into the jugular vein of four steers at 1200 hr on days 1, 5 and 6. Testosterone propionate, 100 mg in 2 ml corn oil, was injected into alternate sides of the rump (im) at 2000 hr on day 1, at 0800 and 2000 hr on days 2, 3, 4 and at 0800 hr on day 5. Two milliliters corn oil was injected on day 1 at 0800 hr, on day 5 at 2000 hr and on day 6 at 0800 and 2000 hr to maintain the constant presence of corn oil throughout the GnRH treatments. Ten milliliters blood samples were taken by jugular venipuncture at 0800 and 2000 hr on days 1 through 8, every 30 min from 1200 to 1400 hr on days 2, 3, 4 and 7 (days on which no GnRH was given) and every 30 min from 1000 to 1200 hr, every 10 min from 1200 to 1300 hr, and every 30 min from 1300 to 1500 hr on days 1, 5 and 6 (GnRH treatment days).

Data were analyzed using an analysis of variance with a randomized block or split plot design as appropriate. More detailed analyses were done with the aid of orthogonal contrasts (Steel and Torrie, 1960).

Radioimmunoassay. Serum LH was quantified by use of a double antibody radioimmunoassay for bovine LH similar to that described by Niswender et al. (1969). Bovine LH (BLH) standards were prepared from NIH-LH-B7 and the radioiodinations, similar to that described by Greenwood et al. (1963), utilized highly purified BLH (LER 1072-2) supplied by Dr. L. E. Reichert, Jr. 5.

Both antibodies, guinea pig anti-bovine LH (GPABLH) and sheep anti-guinea pig gamma globulin (SAGPGG), were prepared in our laboratory.

First Antibody. The GPABLH was prepared by intradermal injection of .1, .5 or 1.0 mg bovine LH (NIH-LH-B7), diluted in 1.0 ml saline and 1.2 ml adjuvant, into numerous sites in the back and footpads of guinea pigs. A total of seven injections were given at 3-week intervals. The first injection contained Freund's Complete Adjuvant (FCA) and all later injections contained Freund's Incomplete Adjuvant (FICA). Nine days following the third and all later injections, 2 ml blood was taken by heart

5 Dept. of Biochemistry, Emory University, Atlanta, Georgia.
puncture, centrifuged, and the serum subjected to a titration procedure to determine its binding affinity with BLH-\textsuperscript{125}I. The third and fourth bleedings from one guinea pig receiving .1 mg BLH were found to possess adequate binding ability when diluted 1:64,000. All serum samples were assayed for LH using the third bleeding.

Second Antibody. The SAGPGG was prepared by injection of guinea pig gamma globulin (.5 mg/kg) dissolved in 5 ml saline, 5 ml FCA (initial injection) or FICA (all subsequent injections) and .75 ml Combiotic (150,000 units penicillin, 187.5 mg dihydrostreptomycin). The emulsion was injected subcutaneously at numerous sites in the scapular area and back of a 50 kg ewe. The ewe received seven injections at 3-week intervals and a booster injection 4.5 months after the first injection. Approximately 500 ml of blood was withdrawn from the jugular vein 9 days following the third and all later injections and at monthly intervals for 4 months after the last injection. Titration of SAGPGG indicated its use in the assay at a dilution of 1:2.

RESULTS AND DISCUSSION

Validation of Double Antibody Radioimmunoassay. The LH radioimmunoassay cross-reacted negligibly with other pituitary hormones and the inhibition curves of NIH-LH-B7 standards were parallel with dilutions of serum and pituitary homogenates (figure 1). The parallel dose response with NIH-TSH is similar to that observed by Oxender et al., (1972) and indicates possible contamination with immunologically active LH even though the biologically active LH had been largely destroyed. Failure of serum LH to be altered following an iv dose of 50 \( \mu \)g thyrotropin-releasing hormone (a dose shown to significantly increase serum thyroxine in cows; Convey et al., 1973) lends further support to the conclusion that this assay does not cross-react with TSH. The interassay coefficient of variation was 12.8% and recovery of .61, 1.02, 2.05, 4.10 or 8.19 ng of BLH (NIH-LH-B7) added to 100 \( \mu \)l bovine serum was 108.7 \( \pm \) 2.65%. Inflated recoveries (\( >200\% \)) occurred in tubes to which 12.29 or 16.38 ng NIH-LH-B7 had been added per assay tube.

Consequently, all samples were assayed at a volume which would yield an estimated LH concentration of less than 10 ng per assay tube.

Two bovine serum pools were used to compare antibody developed at this laboratory (Lot I-3) with antibody supplied by G. D. Niswender\textsuperscript{6}. One pool of serum averaged 1.8 \( \pm \) .10 and 1.6 \( \pm \) .14 ng LH/ml for Lot I-3 and B225 antibodies, respectively (\( n = 5 \)). The other pool of serum averaged 14.2 \( \pm \) .35 and 10.5 \( \pm \) .11 ng LH/ml for Lot I-3 and B225 antibodies, respectively (\( n = 5 \)).

Physiological data from this assay are in good agreement with published results from other laboratories (Niswender et al., 1969; Oxender et al., 1972). In our assay, LH concentrations of cow serum obtained at mid cycle were 1.6 to 2.0 ng/ml and peak serum LH levels after injection of cows with 100 \( \mu \)g of GnRH (iv) averaged 22.2 ng/ml. In addition, pre-castration levels of .6 to 1.0 ng/ml and a post-castration range of 1.0 to 8.1 ng/ml in males compares favorably with published results (Mongkonpunya et al., 1974).

Experiment 1. The serum LH concentration (figure 2) was .8 \( \pm \) .04 ng/ml in four bulls immediately prior to castration, remained at .7 \( \pm \) .04 through 6 hr post-castration, then increased significantly to 2.0 \( \pm \) .39 at 7 to 8 hr following castration and to 3.7 \( \pm \) .75 ng/ml at 9 to 12 hr following castration. Episodic increases in serum LH concentration occurred in all four steers from 7 to 11 hr post-castration with a maximal mean serum LH concentration of 7.0 \( \pm \) 1.81 ng/ml. Similar increases in serum LH following castration have been documented in ewes (Reeves et al., 1972), rams (Pelletier, 1968), cows (Hobson and Hansel, 1972) and prepubertal heifers and bulls (Odell et al.,

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Mongkonpunya et al. (1974) found serum LH to be higher at 14 days (7.1 ± .6 ng/ml) than at 7 days (3.4 ± .4 ng/ml) after castration of Holstein bulls. Similarly, we observed higher serum LH levels 2 to 5 months after castration of the bulls used in the present experiment. The reduction in circulating testosterone levels by 2 hr post-castration, as reported for male rhesus monkeys (Resko and Phoenix, 1972), male rats (Coyotupa et al., 1973) and bulls (L. Swanson, unpublished data) suggests that the increased serum LH observed 7 to 11 hr after castration in this experiment could be due to reduced steroid levels.

Experiment 2. Steers treated with vehicle only had non-rhythmic fluctuations in serum LH (figure 3) which is in contrast to the rhythmic post-castration LH patterns observed in wethers (Riggs and Malven, 1974) and in ovariectomized ewes (Reeves et al., 1972). This discrepancy may be attributable to the longer sampling interval in our studies (30 min) as compared to 10-min and 15-min intervals in the other studies.

Intramuscular injection of EB (averaged over both dosages) resulted in a reduction (P = .02) of serum LH within 3 hr of administration (figure 3). Serum LH was further reduced (P < .001) from 3.7 ± .19 ng/ml during the first 6-hr period after EB to 2.6 ± .18 ng/ml during the subsequent 6-hr period.

The significant reduction in elevated post-castration serum LH concentrations after EB treatment in steers (figure 3) parallels findings by Bolt (1971) in rams and Riggs and Malven (1974) in wethers.

Repeated im injection of 84 mg TP reduced (P < .005) serum LH concentrations from 5.7 ± .70 prior to treatment to 2.1 ± .30 ng/ml during the 24-hr period following the last TP injection (figure 4). As determined by t-test, these levels (2.1 ± .30 ng/ml), though reduced, are significantly higher than serum LH prior to castration (.7 ± .008 ng/ml). Fluctuations in serum LH were also suppressed.

Pelletier (1974) observed a biphasic response following TP treatment (600 mg, im) of wethers; serum LH was depressed at 12 hr and 72 hr after TP. In a study of longer duration, Pelletier
(1970) observed depressed serum LH levels for 4 to 5 days following a single injection of TP (400 mg, im) to wethers. Pituitary LH was increased and hypothalamic LH releasing hormone (LRH) was reduced on day 2, suggesting that TP blocked LRH synthesis and LH release but not LH synthesis. Crim and Geschwind (1972) successfully reduced serum LH to precastrate levels in wethers given 10 mg TP/45.5 kg BW daily for 1 or 2 weeks. Thrice daily administration of 20 mg testosterone for 6 days reduced serum LH to levels not significantly higher than before castration in 9-month-old steers (Mongkonpunya et al., 1974). In contrast to androgens, only very low dosages of estrogens are required to suppress serum LH in steers. As shown in these experiments, as little as .5 μg/kg EB was effective (figure 3), suggesting a role for estrogens in regulation of gonadotropins in the male bovine. This extreme potency of estradiol in suppressing serum LH levels in males has also been noted in rats (Gay and Dever, 1971), in which EB was estimated to be 100 times more effective than androgens in preventing the post-castration increases in serum LH. In vitro conversion of androgens to estrogens by the rat brain led Naftolin et al. (1971) to suggest that estrogens could be the major active compound involved in feedback regulation of gonadotropins in males. But such aromatization by the brain has not been demonstrated in domestic animals. It should also be noted that while exogenous TP and estradiol did in fact reduce circulating levels of LH in the castrate steers in this experiment, neither TP nor estradiol were capable of reducing serum LH to precastrate levels.

Experiment 3. Various doses of GnRH (2.5, 5, 7.5 and 10 μg) were administered to determine a threshold level of GnRH that would elicit an LH release more susceptible to TP feedback. After deletion of data from one steer (which showed a 100% greater response to GnRH than steers given the same GnRH dosage), a significant dose response existed between GnRH dose and peak serum LH concentration and peak LH area (r = .99 and .99, respectively). Peak serum LH concentration increased from 15.1 (2.5 μg GnRH) to 36.1 ng/ml (10 μg GnRH). Using 10 min sampling intervals, the peak serum LH concentration occurred 20 to 30 min following GnRH administration. The 5 μg GnRH dosage was selected for Experiment 4 as it evoked an LH release (25.2 ng/ml) which could easily be differenti-ated from post-castration serum LH fluctuations.

An increase in peak serum LH concentration with increasing dosage of GnRH is characteristic of males as shown in rams (Hopkinson et al., 1974; Galloway et al., 1974), boars (Pomerantz et al., 1974) and bulls (Zolman et al., 1973). A dose response was not as obvious in this experiment, possibly because of the extreme sensitivity of these Holstein steers which produced near maximal LH release with 10 μg GnRH. The heightened sensitivity to GnRH in castrates has also been demonstrated in steers (Mongkonpunya et al., 1974) and in wethers (Reeves et al., 1970).

Experiment 4. The 4-day TP treatment reduced (P<.001) serum LH concentration from 5.9 ± .90 on the day before initiation of treatment to 3.6 ± .27 ng/ml during the 4-day TP treatment and to 2.4 ± .15 ng/ml during the 60-hr period following the TP treatment (table 1). Two days following the last TP treatment (day 7), mean serum LH concentrations began to increase due to elevated LH levels in one steer (R-3) on day 7. The average serum LH concentration for the remaining three animals (1.6 ± .06 ng/ml on day 7) suggests that steer R-3 was released from the TP-induced suppression.

Administration of 5 μg GnRH iv on the day before TP treatment (day 1) and at 4 hr (day 5) and 28 hr (day 6) after a 4 day TP treatment period failed to show any differences in pituitary response to GnRH as measured by maximum serum LH concentration (table 2). Analysis of the area under the response curve, as an indicator of total LH release, indicated an increased (P = .08) release of LH during and after TP administration.

These results indicate that TP had no effect on or may have enhanced pituitary sensitivity to GnRH; certainly it did not reduce it. In contrast, Debeljuk et al. (1973) observed that testosterone, dihydrotestosterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone and 5α-hydroxyprogesterone significantly decreased the release of LH after GnRH treatment in castrate male rats. They concluded that the suppressive action of sex steroids in rats is directed at least in part at the pituitary level. Galloway and Pelletier (1975) reported that wethers given testosterone (200 mg TP every second day for 2 weeks, im) had peak LH concentrations (in response to LRH) similar to untreated wethers but that the time interval
TABLE 1. SERUM LH CONCENTRATIONS (ng/ml) BEFORE, DURING AND AFTER TESTOSTERONE PROPIONATE TREATMENT (EXPERIMENT 4)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day^b</th>
<th>Y-1</th>
<th>B-2</th>
<th>Y-4</th>
<th>R-3</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-1</td>
<td>1</td>
<td>3.9 ± .12</td>
<td>11.1 ± 2.68</td>
<td>5.1 ± .47</td>
<td>3.4 ± .30</td>
<td>5.8 ± .90</td>
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<tr>
<td></td>
<td>2</td>
<td>2.6 ± .30</td>
<td>5.7 ± .80</td>
<td>4.9 ± .82</td>
<td>3.7 ± .27</td>
<td>4.2 ± .37</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.9 ± .40</td>
<td>8.1 ± 1.55</td>
<td>1.9 ± .09</td>
<td>3.6 ± .35</td>
<td>3.9 ± .62</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.3 ± .05</td>
<td>2.3 ± .13</td>
<td>1.9 ± .05</td>
<td>5.6 ± .51</td>
<td>2.8 ± .34</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.8 ± .14</td>
<td>2.0 ± .08</td>
<td>2.3 ± .13</td>
<td>3.7 ± .60</td>
<td>2.4 ± .21</td>
</tr>
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<td></td>
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<td>2.3 ± .14</td>
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<tr>
<td></td>
<td>7</td>
<td>1.2 ± .03</td>
<td>1.8 ± .02</td>
<td>1.9 ± .05</td>
<td>6.4 ± .05</td>
<td>2.8 ± .40</td>
</tr>
</tbody>
</table>

^aMean ± SE are presented for the 7 observations from each animal each day. Blood samples were collected at 0800, 1000, 1030, 1100, 1130, 1200, and 2000 hr on days 1, 5 and 6 and 0800, 1200, 1230, 1300, 1330, 1400 and 2000 hr on days 2, 3 and 4.

^bTP treatment (equivalent to 100 mg testosterone twice daily) was given on days 2, 3, 4 and 5.

between LRH injection (100 μg, iv) and the LH peak was lengthened-similar to intact rams. However, Mongkonpunya et al. (1974), in agreement with data in the present experiment, demonstrated that testosterone did not alter the increased pituitary sensitivity to exogenous GnRH in steers, suggesting a lack of feedback at the pituitary level. As discussed by Hooley et al. (1974) this apparent discrepancy may indicate that the steroid must be present in the animal for a definite length of time before it can modulate the action of the releasing hormone. Following a single 600 mg injection of TP, Pelletier (1974) observed a decreased response at 6 hr and an increased response to LRH given at 36 hr after TP. Mühlen and Köberling (1973) observed that treatment of normal men with testosterone every other day for 8 days had no effect on LH release induced by LRH, whereas the same dose of testosterone, spread over 4 weeks, depressed the LH-induced LH release. Perhaps 4 days of testosterone therapy, as used in the present experiment, was not long enough. Further work is needed to explore this possibility in cattle and the possibility that castrate animals may respond differently than intact animals to steroid feedback. And, as shown by Davidson et al. (1974), the time of castration with respect to the time of treatment has an important impact on the results observed. Galloway and Pelletier (1975) found that wethers castrated 3 hr prior to LRH treatment responded similarly to untreated or testosterone-treated long-term castrated wethers (castrated 6 months previously) in terms of total LH released but that the time to peak LH level was intermediate between intact or testosterone-treated long-term castrates and untreated long-term castrated wethers.

We are presently pursuing the possibility that factors other than testosterone may be

TABLE 2. SERUM LH CHARACTERISTICS AFTER TREATMENT WITH 5 μg GnRH (EXPERIMENT 4)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Day 1</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to peak (min)</td>
<td>15 ± 2.9</td>
<td>20 ± 4.1</td>
<td>18 ± 2.5</td>
</tr>
<tr>
<td>Peak concentration (ng/ml)</td>
<td>62.2 ± 14.27</td>
<td>69.2 ± 19.31</td>
<td>73.4 ± 10.74</td>
</tr>
<tr>
<td>Return to baseline (hr)</td>
<td>1.9 ± .12</td>
<td>2.5 ± .20</td>
<td>2.6 ± .12</td>
</tr>
<tr>
<td>Peak area (arbitrary units)</td>
<td>469.5 ± 88.28</td>
<td>842.3 ± 268.43</td>
<td>760.0 ± 139.43</td>
</tr>
</tbody>
</table>

^aGnRH was administered 8 hr before (Day 1), 4 hr (Day 5) and 28 hr (Day 6) after end of 4 days of testosterone propionate treatment (equivalent to 100 mg testosterone, twice daily, im).

^bDay 1 vs Days 5, 6; P=.08.
involved in regulating serum LH concentrations in the male.

LITERATURE CITED


Resko, J. A. and C. H. Phoenix. 1972. Sexual behavior and testosterone concentrations in the plasma of
the rhesus monkey before and after castration. Endocrinol. 91:499.


