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Feedback Effects of the Testis on Pituitary Responsiveness to Luteinizing Hormone-Releasing Hormone Infusions in the Ram*

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ABSTRACT. The present studies examined the effects of both short term and long term alterations in testicular hormone levels on pituitary gonadotropin responses during 4–6 h of constant infusions of LHRH (0.5 μg/min) in the ram. Short term changes in testicular hormone levels were produced either by testosterone (T) infusions [three animals (6.7 μg/min) and 3 animals (83 μg/min)] beginning 4 h before the LHRH infusion or by castration (n = 4) 2 h before the LHRH infusions. Long term alterations in testicular hormone levels were induced by T injections (T enanthate, 250 mg im twice weekly for 2 weeks) or castration (n = 4) performed 3–4 months before the LHRH infusion. Control LHRH infusions were performed in the same animals 2 weeks before the T studies. For the acute castrations, control studies were undertaken in four animals acutely sham castrated; for chronic castration normal animals were used as controls (n = 5).

Acute changes in testicular feedback induced either by castration 2 h before LHRH administration or by several hours of T infusion did not alter the normal patterns of LH and FSH increase and decrease during LHRH infusions. The normal biphasic pattern of increase in plasma LH levels and the monophasic pattern of FSH increase persisted, as did the development of pituitary refractoriness to LHRH stimulation in the last 2–3 h of LHRH administration. In contrast, more chronic changes in testicular feedback induced either by T injections for 2 weeks or by castration 3–4 months before LHRH administration produced definite alterations in pituitary responsiveness. LH secretion from the immediately releasable pool was markedly increased in the chronically castrated animals and decreased by 2 weeks of T administration. LH secretion from the second pool was less affected by 2 weeks of T therapy and was apparently unaffected by chronic castration. Chronic castration led to markedly increased basal FSH levels, but no further increase was seen during LHRH administration. These results demonstrate that in the ram 1) alterations in testicular hormone production require longer than 2–4 h to affect pituitary responsiveness to LHRH, 2) testicular feedback exerts a greater effect on the immediately releasable pool of pituitary LH than on the pool requiring longer LHRH stimulation for release, and 3) the pituitary refractoriness that develops after 2–3 h of LHRH stimulation in normal rams is not caused by the increasing plasma T levels but is an intrinsic pituitary phenomenon that could be due to an adverse effect of LHRH on its own receptors. (*Endocrinology* **106:** 329, 1980)

STUDIES of prolonged (4–6 h) constant iv infusions of LHRH into normal adult humans (1–4) and rams (5) have established that increases in serum LH levels occur in two phases. The first phase of increase begins within 5 min after commencement of LHRH administration, and serum LH levels phase of increase begins within 5 min after commencement of LHRH administration, and serum LH levels reach an early peak by 15–30 min into the infusion. This peak is followed by a plateau or slight decline in LH levels until approximately 45–60 min in rams and 90 min in humans when a second phase of LH increase begins, reaching a second peak at approximately 130 min in rams and 240 min in humans. The two phases of LH secretion have been interpreted as evidence for the existence of two pools of pituitary LH, one requiring longer stimulation for release than the other (1). It has been suggested that the first or early releasable pool may represent presynthesized stored hormone, possibly in secretory granules near the cell membrane, while the second or later pool may require new synthesis of LH or a protein necessary for the release of LH (1). Serum FSH levels in these studies have increased gradually throughout the LHRH infusions, with no discernible evidence of two phases of secretion.

Two apparent species differences between men and rams have emerged in the patterns of pituitary-testicular

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responses to constant LHRH infusions. 1) In the ram, the pituitary becomes relatively refractory after approximately 150 min of LHRH stimulation, and gonadotropin levels rapidly decrease in spite of continued LHRH administration (5). In men, gonadotropin levels remain at near-maximal levels for up to 19 h of LHRH infusion (6). 2) In rams, plasma testosterone (T) levels begin to increase within 15–30 min after LHRH infusions are begun, reaching maximal levels 300–400% above basal values within 2 h (5). In normal men, no change or only small increases in serum T levels occurs within 4 h of LHRH stimulation (2, 7, 8).

The present studies were undertaken to clarify the role the testis plays in controlling the pattern of serum gonadotropin increase during constant LHRH infusions in the adult male sheep. In particular, the main points under investigation were: 1) whether rising serum levels of testicular products such as T would exert a negative feedback effect during LHRH infusions, thereby causing the development of pituitary refractoriness to LHRH; 2) what effect removal of the testes either immediately before or several months before LHRH testing would have on gonadotropin responsiveness; and 3) what effect products of the testis would have on the two pools of pituitary LH.

Materials and Methods

Animals

Male, crossbred, sexually experienced, Corriedale sheep, aged 2–4 yr, were used. All studies were undertaken during the period of increasing day length (July to December). Animals were fed on irrigated pasture and brought into a closed shed 1–2 days before the studies.

Studies in normal rams

Sixteen normal rams each received two 4-h LHRH infusions, separated by 2 weeks. Synthetic LHRH (Hoechst Op 62) was dissolved in 0.9% saline and infused through an indwelling cannula in a jugular vein at 0.5 μg/min. The effect of short term iv T infusions upon gonadotropin responsiveness was assessed in six of these animals. T infusions were begun 4 h before the second LHRH infusion and continued until the end of the LHRH infusion (*i.e.* total length of the T infusion was 8 h). In three rams, the dosage of T infused was 6.7 μg/min, and in the other three, the dosage was 83 μg/min. The lower dosage was calculated to be slightly greater than the production rate of T from the normal ram testis (5 mg/day) (9). The higher dosage was designed to produce serum T levels approximating those found during LHRH infusions into normal rams (1500–2000 ng/100 ml) (5). T (Sigma Chemical Co., St. Louis, MO) was diluted in 30% ethanol-70% saline (0.9%) and infused through a jugular cannula.

Five rams received T enanthate in oil (250 mg im twice weekly) between their two LHRH infusions. This regimen was chosen to assess the effect of more chronic elevations of plasma T levels upon pituitary responsiveness to LHRH.

Five animals served as controls and received a 70% saline-30% ethanol infusion beginning 4 h before their second LHRH infusion and lasting until the end of that infusion (*i.e.* a total of 8 h).

Studies in castrated and sham-castrated rams

A single LHRH infusion was performed in 12 castrated or sham-castrated animals. Four animals were castrated 3–4 months before being studied; these were designated chronic castrates. Four animals were castrated 2 h before being studied and were called acute castrates. On the same day that the castrations were performed, 4 other animals received sham castrations and were studied 2 h later; these were called sham castrates. Sham castration consisted of incising the scrotum, handling the testes and spermatic cords, then suturing the scrotal incision. All operations took place at least 1 yr after the animals had reached sexual maturity. Anesthesia was obtained with sodium thiopental (Pentothal; 5 mg/kg iv). LHRH infusions in the acute castrates and sham castrates began 2 h after their operations and lasted 6 hr.

Blood sampling and hormone assays

Blood was obtained from an indwelling cannula in the jugular vein opposite the infusion line. Sampling was performed at 15-min intervals before and during the infusions and, in some cases, at 30-min intervals for 2 h after the infusions. Plasma was separated by centrifugation and stored at −20 C until hormone assay.

Plasma LH (10) and T (11) were measured by previously described RIAs. The LH standard was NIH-LH-S9 (biological potency, 1.07 × NIH-LH-S1). Plasma FSH was measured as described previously (12), except that a different anti-FSH antibody was used (59/7, prepared in this laboratory). This antibody was prepared in an adult male New Zealand White rabbit. Human FSH (LER 1536; 50 μg) in 0.5 ml saline was emulsified with 0.5 ml complete Freund's adjuvant, divided, and injected into 10–12 sites intradermally along the back. These injections were repeated 3 times at monthly intervals, and serum was obtained 3 weeks after the last injection. At a final concentration of 1:6000, this antiserum bound 15–28% of radioiodinated ovine FSH. Specificity of the antibody is detailed in Fig. 1, as is parallelism between serial dilutions of plasma samples and the standard curve. The assay standard was NIH ovine FSH S-6 (biological potency, 1.24 × NIH-FSH-S1). The FSH preparation used for iodination was Papkoff ovine FSH G4-150C. Assays were calculated using a previously described computer program (13). The sensitivity averaged 11 ng/ml. Within-assay variability was 5.7% and between-assay variability was 24% (mean of coefficients of variation of high, mid, and low range pools in 10 consecutive assays). All samples from each animal were run in a single assay.

Statistical analysis was performed using Student's *t* test for paired or unpaired observations (14).

Results

Normal rams

Control studies. After 15 min of LHRH infusion into normal animals (Figs. 2–4), plasma LH levels had in-

creased sharply, reaching a plateau which continued until 45 min when a second phase of increase began that lasted until approximately 2 h of the infusion had elapsed. In spite of continued constant LHRH administration, LH levels during the second 2 h decreased. Gradual increases occurred in plasma levels of FSH during LHRH administration (Figs. 2, 3, and 5), reaching peak levels at 2.5–3 h. No evidence for two phases of increase was discernable in the FSH levels.

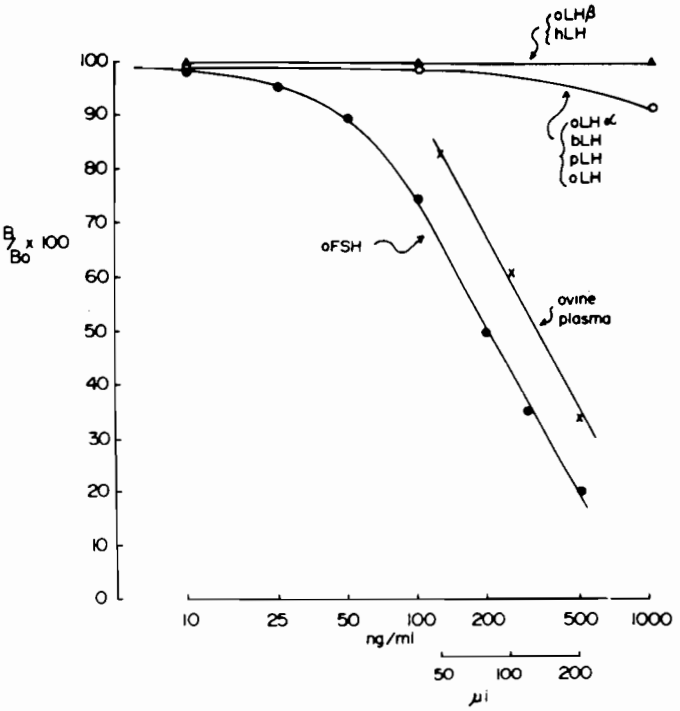

FIG. 1. Cross-reactivity and parallelism studies with the 59/7 anti-FSH antibody in the ovine FSH assay. Ovine LHβ and ovine LHα cross-reactivities were assessed with both Reichert and Papkoff preparations of each of these substances. hLH (LER 1533), bLH (NIH-B6), pLH (LER 786-3), and oLH (Papkoff G3222B and IV 28 BP and LER 1374A) represent partially purified preparations of human, bovine, porcine, and ovine LH, respectively.

When a second LHRH infusion was administered to five normal animals 2 weeks later with no intervening T treatment, LH and FSH responses were nearly identical to those during the first studies ($P > 0.2$ for areas under the curves for both LH and FSH, by paired t test; data not shown).

Short term T administration. The treatment of normal rams with T infusions of 6.7 or 83 μg/min, beginning 4 h before the LHRH infusions and terminating at the end of the LHRH infusions, did not significantly alter the LH or FSH responses to LHRH ($P > 0.2$, by paired t tests for areas under the curves for both LH and FSH). Only the results of the higher T dosage infusions are illustrated (Fig. 2), but those from the lower dosage infusions were essentially identical. The control infusions were ones performed in the same animals 2 weeks earlier with no T pretreatment.

Plasma levels of T during the T infusions were measured at 30-min intervals for 2 h immediately before the second LHRH infusions for each animal. At a dose of 6.7 μg/min, T levels were 657 ± 22 ng/100 ml (mean ± SE), significantly greater than those of normal untreated rams (295 ± 25; $P < 0.05$, by unpaired t test). During the 83 μg/min T infusion, plasma T levels were 1930 ± 232 ng/100 ml, significantly greater than those of normal untreated rams ($P < 0.01$) and of rams during the lower T dosage infusions ($P < 0.05$).

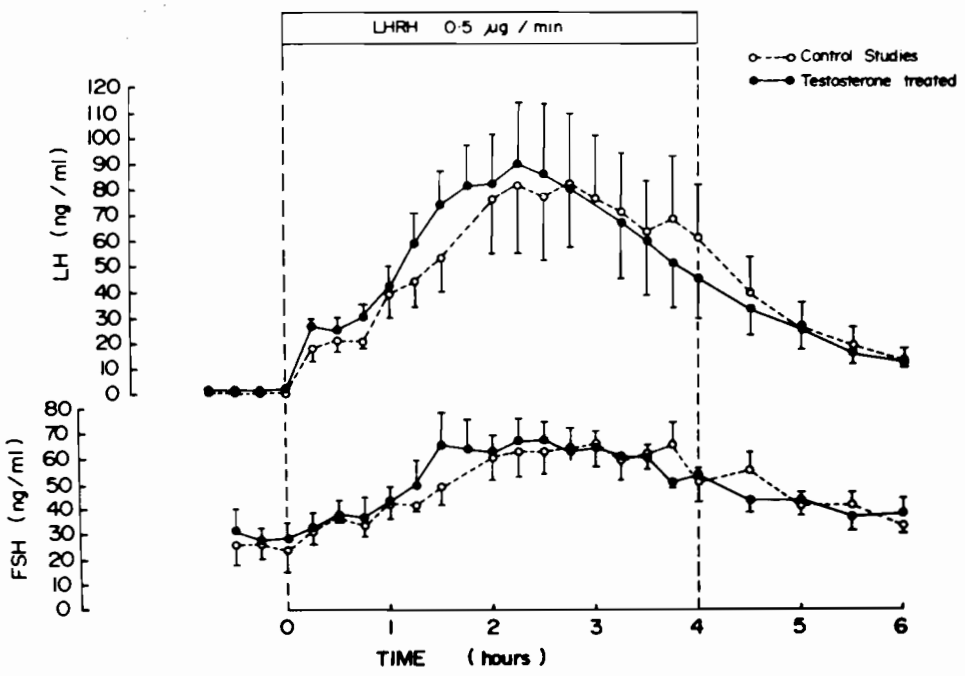

FIG. 2. Plasma LH and FSH levels (mean ± SE) in three normal rams before, during, and after two LHRH infusions (0.5 μg/min for 4 h) separated by 2 weeks. For 4 h before and during the second LHRH infusion, the animals received T (83 μg/min iv).

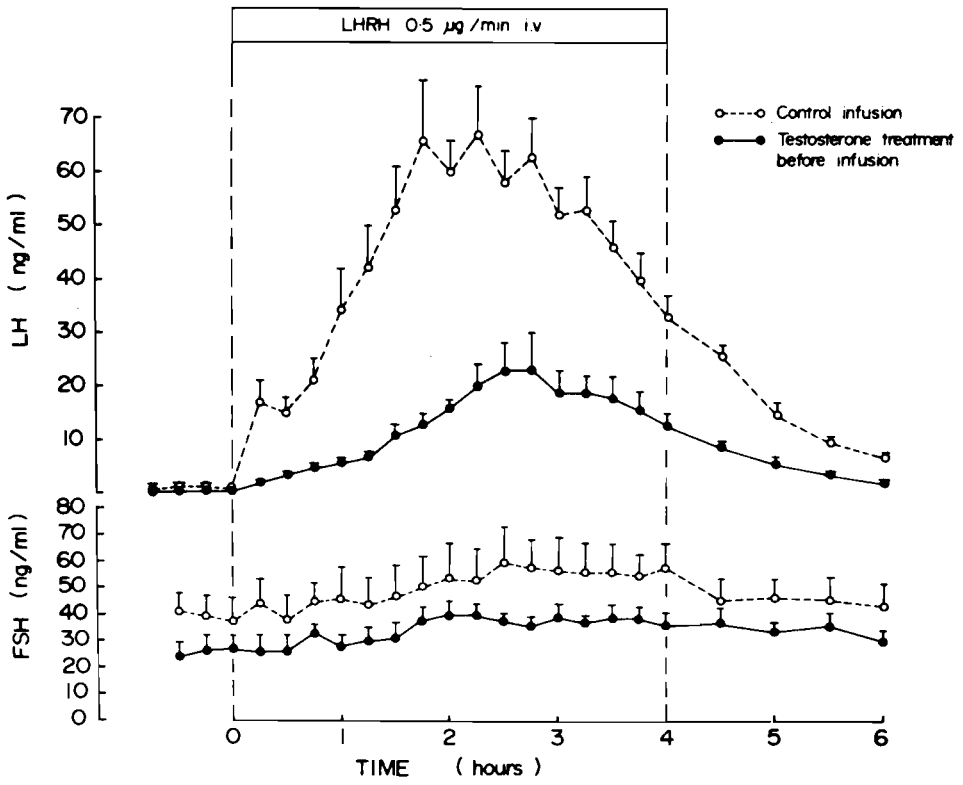

FIG. 3. Plasma LH and FSH levels (mean ± SE) in five normal rams before, during, and after two LHRH infusions (0.5 μg/min for 4 h) separated by 2 weeks. During the 2-week interval between the infusions, the rams received 250 mg T enanthate im twice weekly.

Long term T administration. After 2 weeks of T enanthate (250 mg im twice weekly), LH responsiveness to LHRH was markedly reduced (Fig. 3). Areas under the LH response curves were significantly lower after T treatment ($P < 0.01$, by paired t test). The difference was most marked during the first phase of LH secretion. Fifteen minutes after the commencement of the LHRH infusions, increases in serum LH levels in the untreated animals were 8-fold greater than the increases in these animals after receiving T. At 2.5 h (maximal levels), increases in the untreated animals were only 3-fold greater than in the same animals treated with T.

Although basal levels of FSH were moderately decreased after 2 weeks of T enanthate, the increase of FSH during LHRH infusion was not significantly different from that in the control studies ($P < 0.1$ for areas under response curves, by paired t test).

Plasma levels of T obtained during this form of T administration (measured at 30-min intervals for 2 h before the second LHRH infusion) were 1703 ± 171 ng/100 ml (mean ± SE). These values were significantly greater than those of normal rams ($P < 0.01$, by unpaired t test) and those in rams during T infusions of 6.7 μg/min ($P < 0.05$) but did not differ significantly from those of rams during T infusions of 83 μg/min ($P > 0.2$).

Castrated rams

Long term castration. In contrast to normal rams, no evidence for two phases of increase in plasma LH levels during LHRH infusions was found in the long term castrated animals (Fig. 4). Instead, LH values increased rapidly to reach stable high levels by 30 min. No further change occurred until approximately 2.5 h, when a decline began which lasted until the end of the infusions at 4 h. Although basal LH levels were higher in castrates than in normal rams ($P < 0.1$, by unpaired t test), the maximal levels of LH obtained during LHRH infusions were not significantly different ($P > 0.2$, by unpaired t test), and the patterns of decline after 2.5 h were nearly identical in the two groups of animals.

Basal FSH levels in chronic castrates (Fig. 5) were much higher than those in normal animals ($P < 0.01$, by unpaired t test), but no further significant increase occurred during LHRH stimulation ($P > 0.1$, by paired t test).

Short term castration and sham castration. There was no significant difference either in basal LH and FSH levels or in responses of those hormones to 6-h LHRH infusions between the groups of animals that were acutely castrated and those that were sham-castrated ($P > 0.2$, by unpaired t test; Fig. 6). In both groups, two phases of LH increase could be distinguished, and LH levels began to decline after approximately 2.5 h of LHRH stimulation.

In both groups, FSH levels revealed a gradual monophasic pattern of increase until approximately 2 h. After 3 h, FSH values began a decline in both groups; this decline continued through the last 3 h of the LHRH

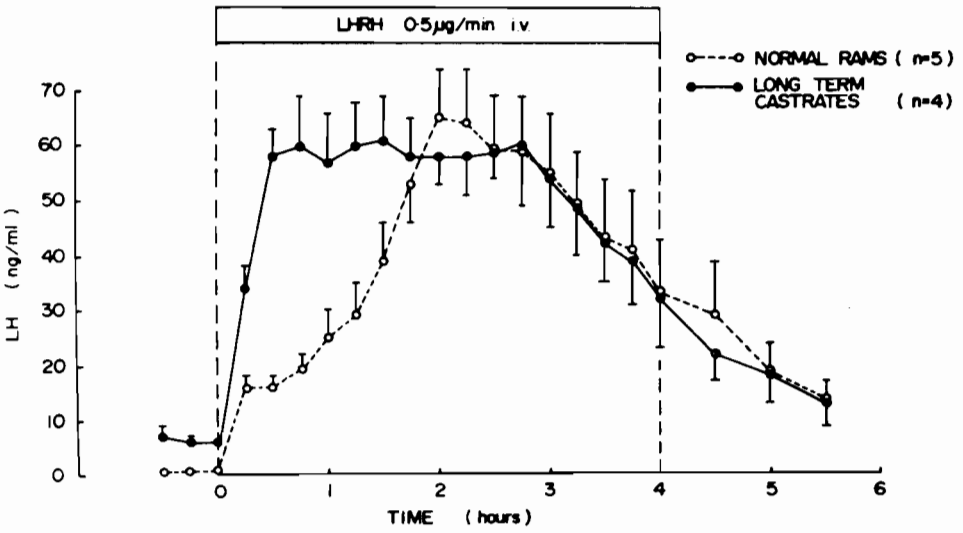

FIG. 4. Plasma LH levels (mean ± SE) before, during, and after LHRH infusions (0.5 µg/min for 4 h) in normal rams and long term castrates.

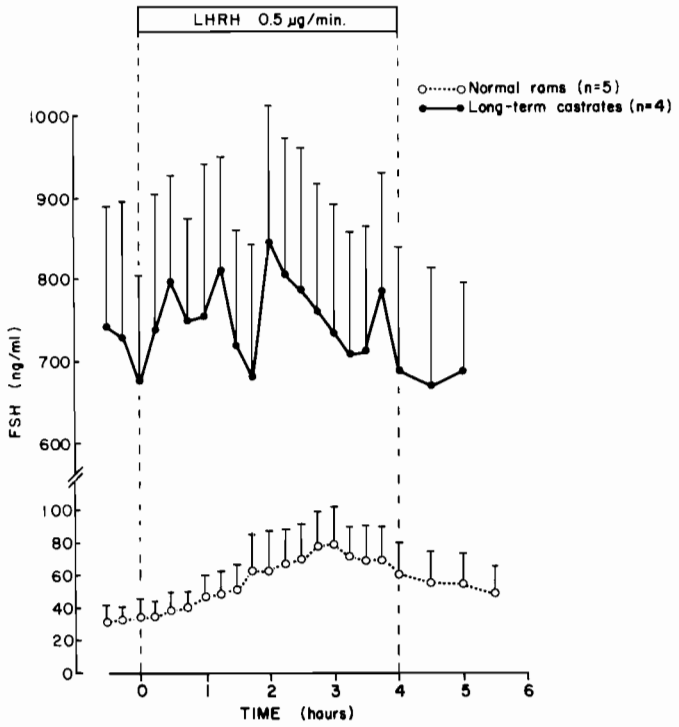

FIG. 5. Plasma FSH levels (mean ± SE) before, during, and after LHRH infusions (0.5 µg/min for 4 h) in normal rams and long term castrates.

infusions. No differences were apparent between the LH or FSH responses in either of these two groups of animals compared to those in normal animals (Figs. 2–5).

Plasma T levels revealed the expected marked increase during LHRH administration in the sham-operated animals, and this increase was eliminated in the castrated group (Fig. 6).

Discussion

The results of the present studies confirmed that in normal rams serum levels of LH increased in two phases and then declined during constant administration of LHRH over 4–6 h (5). Serum FSH levels increased gradually without evidence of two phases of release and declined toward the end of 6-h LHRH infusions (Fig. 6). The two phases of LH increase are consistent with the previous interpretation from studies in humans (1–4) and rams (5) that there are two pools of pituitary LH, one immediately releasable and the other requiring longer stimulation and possibly new protein synthesis for its release (1). The progressive decline in gonadotropin levels after 2–3 h in spite of continued constant LHRH administration implies the development of pituitary refractoriness to LHRH stimulation (5).

The present studies were designed to determine the acute and chronic feedback effects of the testis on the patterns of pituitary responses to LHRH infusion. Both acutely and chronically castrated animals and short and long term T administration were studied. In general, the results imply that neither acute increases nor decreases in testicular feedback affect pituitary responsiveness to LHRH in the ram. However, more chronic alterations in feedback have a marked effect, particularly upon the acutely releasable pool of pituitary LH. The results also demonstrate that feedback effects of the testis are not important in the development of pituitary refractoriness to LHRH that occurs during 4- to 6-h infusions of the releasing hormone.

Intravenous infusion of T, even at supraphysiological levels (83 µg/min), for 4 h before LHRH administration and continuing during the infusion of LHRH had no effect on either the LH or FSH response. However, with long term T exposure (T enanthate, 250 mg im twice weekly for 2 weeks), LH responsiveness was markedly decreased, particularly from the immediately releasable pool (Fig. 3). The plasma levels of T obtained by the im injections did not differ significantly from those obtained during the high dosage, short term infusion. These results

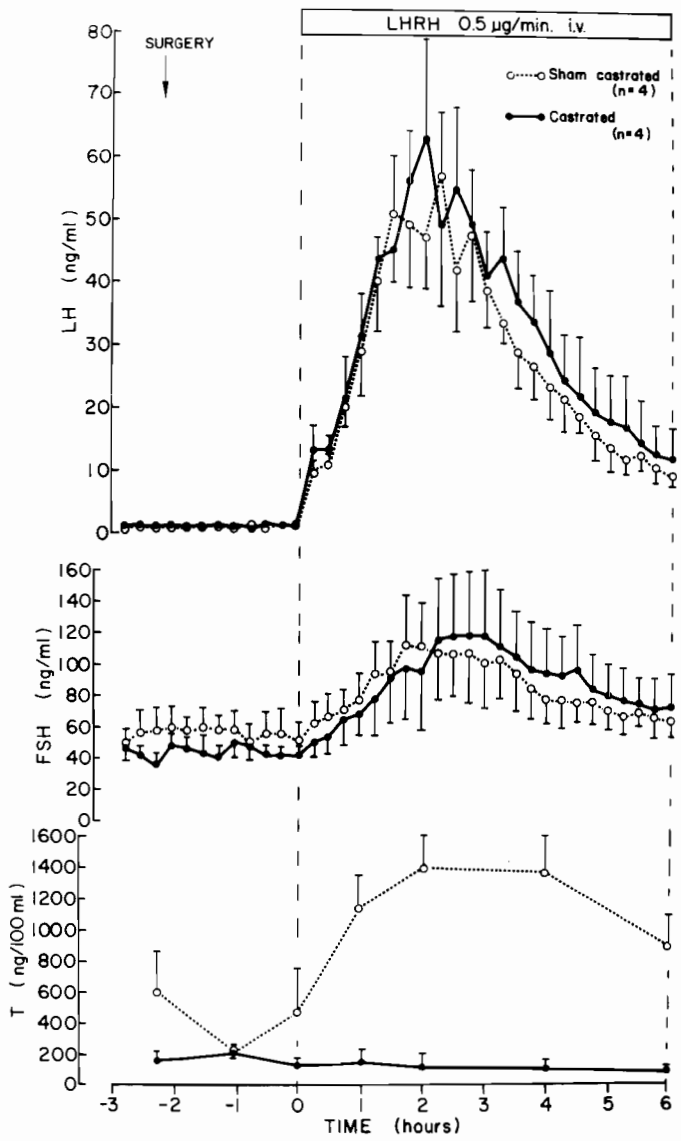

FIG. 6. Plasma LH, FSH, and T levels before and during LHRH infusions (0.5 µg/min for 6 h) into short term castrated and sham-castrated animals. Surgeries (*arrow*) were performed 2-2.5 h before commencement of the infusions.

demonstrate that it was the length of time the animal was exposed to high levels of T rather than the plasma level of T that was important in causing the suppressive effect. Whether this suppression was due to a direct effect of T on the pituitary or to inhibition by T of endogenous LHRH production with a secondary decrease in pituitary responsiveness cannot be determined from the results of this study.

In chronically castrated rams, LH secretion during the first hour of LHRH infusion was markedly greater than that in normal animals. LH values increased rapidly, reaching high stable levels by 30 min, and remained high until 2.5 h, when they began to decline. The maximal levels obtained from chronic castrates and normal animals did not differ. These results imply that the testis, under the conditions of this study, suppresses the first phase of LH secretion but is relatively unimportant in regulating the amount of LH produced during the second phase of secretion.

The testis also appears to be unimportant in causing the decrease in pituitary responsiveness that occurs late in 4-h LHRH infusions. Some product(s) of the testis appears to cause suppression of LH release from the early pool of pituitary LH during LHRH infusions as well as suppression of basal LH levels. T, possibly together with other gonadal steroids such as dihydrotestosterone and estradiol, is probably important in this regard. Preliminary studies of a few normal men (15) and data from patients with Klinefelter's syndrome (2) have suggested that administration of exogenous T for at least 2 weeks exerts a greater suppression of LH release from the first pool of LH than it does from the second.

Interestingly, serum FSH levels in chronically castrated rams, although elevated in the basal state compared to intact rams, did not increase significantly above basal values during LHRH infusion. These results are in contrast to the situation in humans; LHRH induces greater increases in serum FSH levels in patients with primary testicular deficiency than in normal subjects (2, 16). Presumably, the pituitary, freed from gonadal inhibition, secretes FSH at a maximal rate in the chronically castrated ram. An increase in basal FSH levels with a marked decrease in FSH responsiveness to LHRH after gonadectomy has also been reported recently in the prepubertal female (17).

The effects of acute castration on gonadotropin responsiveness to LHRH were examined to determine whether the responses of the testis (particularly steroid production) during LHRH infusions are important in determining the pattern of gonadotropin secretion. Acute castration was used to obtain animals with normal gonadal function until 2 h before the LHRH infusions and normal basal gonadotropin levels. Sham castrates were used as controls to rule out any possible effect of anesthesia or surgery on gonadotropin or T secretion. No evidence was obtained to indicate that the responses of the testes during 6-h constant LHRH infusions have any role in determining the patterns of gonadotropin secretion. Serum levels of LH and FSH obtained in the castrates were indistinguishable from those in the sham-operated group despite a marked increase in serum T levels in the latter group but not in the former. These results imply that the development of pituitary refractoriness during LHRH infusions is determined within the pituitary itself and is not dependent on gonadal feedback.

A possible explanation for the development of pituitary refractoriness is simple depletion of pituitary hormone stores during LHRH infusions. It is unlikely that this is

the sole or even the most important explanation. Pituitary refractoriness developed at approximately the same time during low dose LHRH infusions in which much less LH was secreted (5). In the present studies, FSH responsiveness also declined after 3 h of LHRH stimulation, although relatively small total amounts of FSH had been secreted. Rippell *et al.* (18) could not detect a decrease in pituitary LH content in ewes after an LHRH injection which induced a period of refractoriness lasting over 3 days.

It may be that refractoriness develops because exposure of the pituitary to high levels of LHRH over a relatively long period of time (*e.g.* 2 h) may adversely affect the function of pituitary receptors for LHRH. Although we are not aware of direct data to support this possibility in the case of LHRH, it has been demonstrated that the binding ability of cell membrane receptors for insulin (19) and hCG (20) is decreased by high ambient levels of these hormones. Nakai *et al.* (21) recently have reported the development of pituitary refractoriness to constant LHRH stimulation in arcuate nucleus-lesioned monkeys. This refractoriness could be avoided by intermittent administration of LHRH in low dosage (21). Chronic administration of LHRH or its potent analogs has also been shown to decrease pituitary responsiveness to LHRH in rodents (22, 23). Avoidance of this type of refractoriness could be an important reason for the pulsatile release of small amounts of LHRH and other hormones.

The concept of two pools of glandular hormone was postulated in earlier reports to explain the biphasic pattern of increase in insulin (24), glucagon (25), and LH (1) during constant stimulation by an appropriate agent. Recent studies in humans have shown that serum levels of TSH increase in two phases during constant iv infusions of TRH (26). In the case of LH, the concept of two pools has been supported by the finding that changes in the hormonal milieu may affect one pool of pituitary hormone differently from the other (3, 8). Evidence is accumulating from studies both *in vivo* (27) and *in vitro* (28, 29) that LH secretion from the second pool is dependent on synthesis of LH or of a protein necessary for its release. Similarly, the priming effect of LHRH (30), which probably depends upon mobilization of the second pool of pituitary LH, is eliminated by inhibitors of protein synthesis (31).

The results of the present study add further evidence for the concept of two pools of pituitary LH. Chronic castration in the ram appears to increase the responsiveness of LH secretion from the first pool but to have relatively little effect on responsiveness from the second pool; exogenous T administration for 2 weeks to normal rams may decrease LH responsiveness from the first pool more than it decreases responsiveness from the second. In addition, the present results demonstrate that the development of pituitary refractoriness during LHRH infusions is not due to testicular feedback but is an intrinsic pituitary phenomenon.

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