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The endocrine mechanisms involved in the initiation of sperm release from the vertebrate testis have not been elucidated. A seasonally breeding anuran amphibian, Hyla regilla, was selected as a model for testing the hypothesis that steroid action is involved in gonadotropin-stimulated sperm release. Experiments were carried out in vivo and in vitro utilizing three steroid inhibitors: 1) Flutamide, a nonsteroidal androgen receptor inhibitor, 2) CI-628, an estrogen receptor inhibitor, and 3) Cytadren, an aromatase inhibitor. The rationale for using these drugs was to test the effect of steroid inhibition on sperm release from LH-stimulated testes.

In vivo, Flutamide significantly inhibited sperm release compared to controls, by reducing the number of sperm released. CI-628 and Cytadren

tended to increase the number of sperm released.

In in vitro experiments excised testes were incubated in a Kreb's Ringers solution, prescreened for sperm, and then LH or LH and inhibitors were added. After incubation with the drugs, the number of sperm released into the medium were counted using a hemacytometer. The results of several experiments indicated that Flutamide treatment increased the incidence of total inhibition of sperm release.

Further insight into the effect of Flutamide was gained by comparing sperm release of contralateral testes. Again, Flutamide significantly inhibited sperm release primarily by total inhibition, but also by reducing the number of sperm released by testes that responded.

Preliminary results from the in vitro experiments using CI-628 and Cytadren tended to show a paradoxical response. In some cases these drugs inhibit the release of sperm while in other cases they stimulate sperm release.

In addition, experiments were carried out in vitro using contralateral controls to test the effects of various steroids on sperm release. In these experiments, E_2, estrone, estriol, progesterone, T, DHT and corticosterone had no sperm-releasing effect. A low dose of DHT when given in conjunction with LH tended to increase the number of sperm released. A high dose of DHT plus LH, however, significantly inhibited sperm release.

This study provides evidence for the hypothesis that steroids are involved in gonadotropin-stimulated spermiation. In particular, it is suggested that androgens may mediate the action of LH on the testis during the release of sperm. Estrogens also may be involved in

spermiation either directly or indirectly, by regulating androgen biosynthesis. The particular roles of estrogen and androgens in the regulation of testicular function need further study.

Endocrine Regulation of Sperm Release in an Anuran Amphibian

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INTRODUCTION

Comparative Morphology of the Tetrapod Testis and Spermatogenesis

The paired testis of anuran amphibians is characterized by dispersed seminiferous tubules consisting of follicle cells (Sertoli cells) and germinal epithelium surrounded by a matrix of connective tissue and interstitial cells (Leydig cells) (Houssay, 1954; Lofts, 1974). In seasonally breeding species these structures exhibit a definite cycle correlated with spermatogenesis. The early germ cells, primary spermatogonia, initially are surrounded and enclosed by a layer of undifferentiated follicular cells. These follicle cells later differentiate into Sertoli cells. The primary spermatogonia may divide and form additional nests of primary spermatogonia enclosed by proliferating follicular cells. The primary spermatogonia mitotically divide within the rest of follicle cells to form a germinal cyst of secondary spermatogonia. The germ cells contained within each cyst are all at the same stage of development. Although within a given region of the testis germinal cysts are at similar stages of development, some primary spermatogonia do not develop and thus provide germinal tissue for subsequent seasons (Lofts, 1974). In the anuran, the germinal cysts are all at the same stage of development throughout the testis, in contrast to the amniote testis which possesses the entire range of the spermatogenic cycle simultaneously (Lofts, 1974; Callard, I.P. et al., 1978).

During a multiplicative phase the secondary spermatogonia proliferate with up to eight cell divisions (Lofts, 1974). This multiplicative phase is followed by a maturation phase in which secondary spermatogonia mature into primary spermatocytes. Up to this time the follicular cells have developed only slightly and contain no cholesterol or lipids (Lofts, 1976; 1974; Doerr-Schott, 1964). Subsequently the primary spermatocytes undergo the first meiotic division forming smaller secondary spermatocytes within the germinal cyst. Sertoli cells and interstitial cells which have shown little accumulation of lipids begin to develop liposoidal droplets and become 3β-hydroxysteroid dehydrogenase positive (3β-HSD) (Callard, I.P. et al., 1978). This 3β-HSD activity is correlated with steroid biosynthesis in the testis (Lofts, 1974).

The second meiotic division of secondary spermatocytes yields cysts of spermatids. These germinal cysts break apart and the spermatids embed in the Sertoli cell cytoplasm with their tails projecting into the seminiferous tubule lumen. Concurrently, the steroidogenic properties of the interstitial cells are at a peak and plasma levels of androgens are highest (Lofts, 1974).

When spermatozoa are released, the Sertoli cells degenerate and form a cholesterol lipid matrix in the evacuated seminiferous tubules. The interstitial cells accumulate lipids, and there is a decrease in steroidogenesis and 3β-HSD activity (Callard, I.P. et al., 1978). This is followed by a depletion and regression of the cells (Lofts, 1974). The cholesterol lipid matrix in the seminiferous tubules gradually is depleted as the spermatogenic cycle is re-initiated (Lofts, 1964; 1974).

The urodele testis has a unique morphology. The testis consists of a series of lobes, and within each lobe the spermatogenic cycle is regionalized progressing from immature zones to mature zones from anterior to posterior (Tso and Lofts, 1977a, b). No seminiferous tubules are present; rather the germinal cysts are located in seminiferous lobules which drain into a collecting duct (Lofts and Bern, 1972; Lofts, 1974). A specialized region develops from the tissue associated with evacuated cysts, variously referred to as the yellow zone or glandular tissue. This region appears to be the major steroid-producing site in the urodele testis (Miller and Robbins, 1954; Lofts and Bern, 1972; Imai and Tanaka, 1978; Callard, I.P. et al., 1978, Moore et al., 1979).

In amniotes the testis consists of a permanent germinal epithelium surrounded by Sertoli cells, forming seminiferous tubules. These structures are interspersed in interstitial cell tissue and connective tissue (Roosen-Runge, 1977; Fawcett, 1978; Callard, I.P. et al., 1978). The Sertoli cells, via tight junctions, form two compartments within the testis that exclude plasma proteins and other particular blood-borne substances (Setchell and Main, 1975; Dym and Cavicchia, 1978). The two compartments formed include the basal compartment, containing spermatogonia and primary spermatocytes, and the adluminal compartment, containing secondary spermatocytes and spermatids, in communication with the fluid-filled tubular lumen (Setchell and Main, 1975; Dym and Cavicchia, 1978). The hypothesized functions of these compartments include: 1) providing separate microenvironments for germ cell differentiation and spermatid maturation and release, and 2)

preventing autoimmune responses against antigenic spermatids and spermatozoa (Dym and Cavicchia, 1978). The seminiferous tubules drain into a network of fluid-filled spaces, the rete testis, which empties into the epididymis, where sperm are stored prior to ejaculation (Roosen-Runge, 1977).

A spermatogenic wave occurs along the length of the seminiferous tubules so that the entire cycle is found in the testis at the same time (Fawcett, 1978). Regional groups of Sertoli cells may be synchronized, however (Dym and Cavicchia, 1978). Sperm release as a discrete process is thus difficult to isolate from spermatogenesis and experimental work in this area has been limited (Gravis, 1980).

Gonadotropin Regulation of Testicular Function: Mammals

In mammals, there is a clear dichotomy of function between the two endogenous gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Hall, 1970; Bartke et al., 1978; Dorrington and Armstrong, 1979). The Leydig cell is the target of LH; these cells contain specific LH receptors which, when bound increase cyclic AMP (cAMP) concentrations and protein kinase activity (Catt and Dufau, 1976; Bartke et al., 1978). The main effect of LH on Leydig cells is the increase in the production and release of androgens, probably mediated by increasing the activity of androgen biosynthetic enzymes and mobilizing cholesterol stored intracellularly (Bartke et al., 1978).

FSH receptors, on the other hand, are found only on Sertoli cells (Dorrington and Armstrong, 1979). The action of FSH in mediating

Sertoli cell responsiveness is also thought to be via a second-messenger cAMP-adenyl cyclase mechanism (Dorrington and Fritz, 1975). FSH stimulates the synthesis of androgen-binding protein (ABP). This may account for the spermatogenetic effect of FSH, for ABP localizes androgens near the proliferating germ cells (Bartke et al., 1975; Guerrero et al., 1975). FSH increases aromatase activity in immature rats; however, this may not be the case in the adult rat for aromatase activity in the Sertoli cell disappears after sexual maturation (Dorrington and Armstrong, 1979). FSH may indirectly affect Leydig cell function. FSH, as well as prolactin and growth hormone, increase LH-stimulated androgen release by Leydig cells by increasing the number of LH receptors (Bartke et al., 1978; Catt et al., 1980). However, the mechanism of this effect of FSH on Leydig cells is not known, but is presumed to involve a local messenger from the FSH-stimulated Sertoli cell.

Little information is available on the effects of gonadotropins on the release of sperm in mammals. It is difficult to separate gonadotropin effects on spermatogenesis from effects on spermiation. Sperm release disappears with hypophysectomy primarily because of the decrease in spermatogenesis (Hall, 1970). However, androgen-treated hypophysectomized males maintain spermatogenesis and sperm release, although at reduced levels, implying that gonadotropins are not required directly in these processes (Hall, 1970; Bartke et al., 1978; Dorrington and Armstrong, 1979). In summary all known effects of LH in the testis appear to be mediated by steroids, primarily androgens. Furthermore,

FSH involvement in testicular function also focuses on steroid-mediated or steroid-modulating effects.

Steroidogenesis and Regulation of Testicular Function: Mammals

The production of steroids by the testis has been studied extensively in mammals (Hall, 1970; van der Molen et al., 1975; Dym and Cavicchia, 1978; Moger, 1979). Steroidogenesis has been studied in male mammals primarily in relationship to observable changes during sexual maturation, and in the adult, to the spermatogenic process (van der Molen et al., 1975). Little reference has been given to a possible role of steroids in spermiation. However, it has been shown in several mammalian species that androgens, including testosterone (T) and dihydrotestosterone (DHT), can maintain fertility, and therefore sperm release, in hypophysectomized animals (reviewed by Hall, 1970; Bartke et al., 1978).

The Leydig cell is thought to be the major source of T (up to 98%) in the male rat, and production of T is LH-dependent (van der Molen et al., 1975; Moger, 1979; Lacroix et al., 1980). With maximum LH stimulation, precursor availability (see Figure 1a) is rate-limiting (Moger, 1979).

The ability of Sertoli cells to synthesize T is disputed. Sertoli cells do not have enzymes required for conversion of cholesterol to

Figure 1a. Abbreviated biosynthetic pathway of steroids in the testis. Cholesterol conversion to pregnenolone initiates steroidogenesis. 3β-Hydroxysteroid dehydrogenases (3β-HSD) convert pregnenolone and dihydroepiandrostane (DHEA) to progesterone and androstanedione (DIONE) respectively. DHEA can be converted to androstenediol (DIOL) or testosterone (T). DIONE can be reversibly converted to T. T may undergo aromatization to estradiol-17β (E_2) or 5α-reduction to dihydrotestosterone (DHT). E_2 may be converted to estriol or estrone. The enzymes responsible for steroidogenesis are unequally distributed among testicular tissues, so that products are produced in higher concentrations in localized areas.

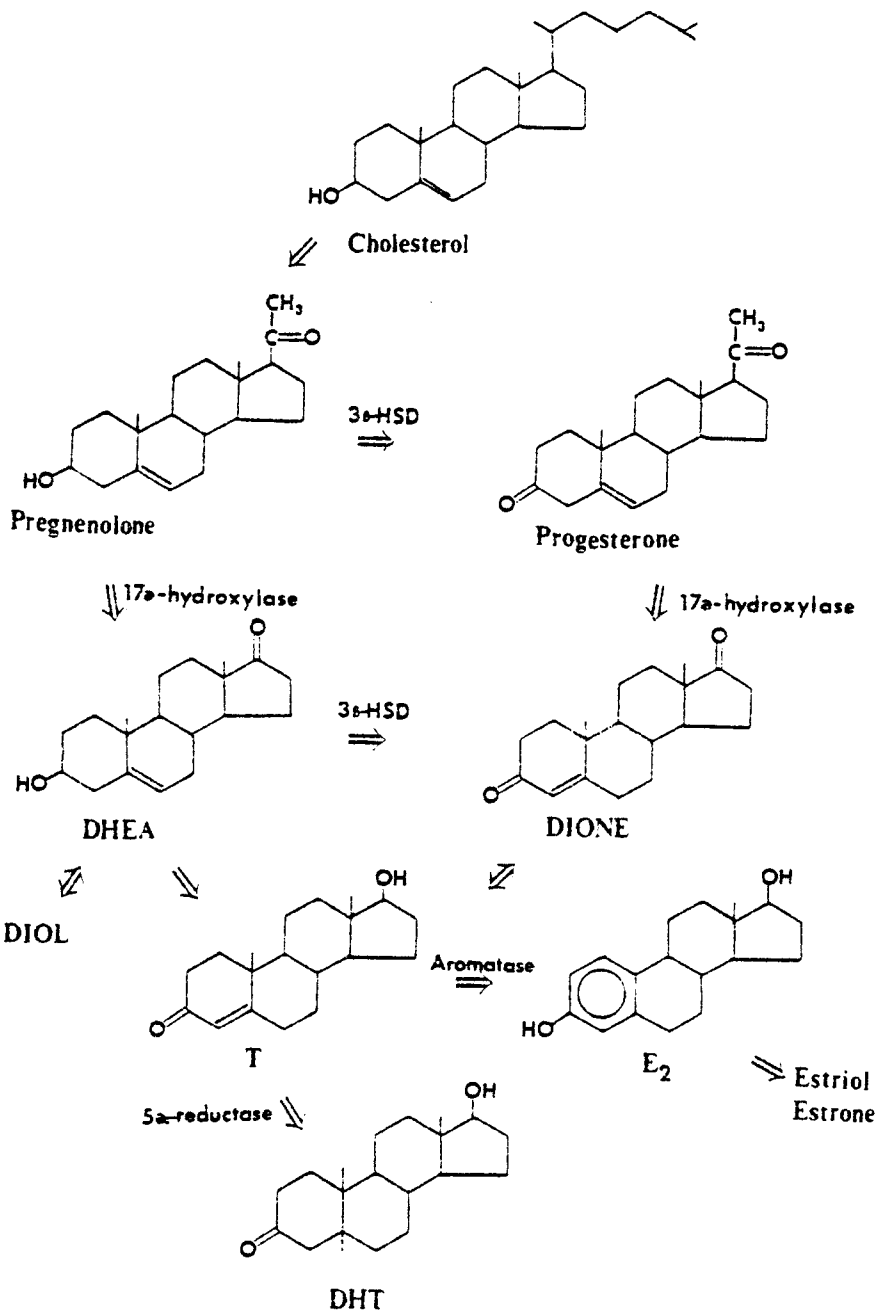

HO
Cholesterol
CH3
C=O
HO
3β-HSD
Pregnenolone
Progesterone
17α-hydroxylase
17α-hydroxylase
3β-HSD
DHEA
DIONE
DIOL
OH
Aromatase
T
E2
Estriol
Estrone
5α-reductase
DHT

Figure 1a.

pregnenolone (Dym and Cavicchia, 1978) and cholesterol cannot cross the blood-testis barrier (Setchell and Main, 1975). However, *in vitro*, isolated seminiferous tubules, containing Sertoli cells and germinal cells, can convert progesterone and pregenenolone to T (Drosdowsky *et al.*, 1975). These precursors readily cross the blood-testis barrier (Setchell and Main 1975). However, Purvis and Menard (1975) were unable to detect 17α-hydroxylase activity in isolated seminiferous tubules. Generally it is thought that the 17α-hydroxylase and 3β-HSD activity found in seminiferous tubules may be a result of contamination with interstitial tissue. Compared to the large quantities of T produced by the Leydig cells, the production of limited amounts of T by Sertoli cells may not be significant (Dorrington and Fritz, 1975; van der Molen, *et al.*, 1975; Setchell and Main, 1975). However, in seasonal breeders the local production of T by Sertoli cells could be important in maintaining the early stages of spermatogenesis when Leydig cell steroidogenic function is low (Purvis and Menard, 1975).

Testosterone (T) produced by the Leydig cells diffuses into the seminiferous tubules where it may 1) be converted to DHT or androstenediol (DIOL) by 5α-reductases, 2) be bound by androgen-binding protein (ABP) or, 3) be converted to estradiol-17β (E_2) by aromatase enzymes (Dorrington and Fritz, 1975). DHT is the main biologically active androgen in the target tissues (reviewed by van der Molen, *et al.* 1975). There is some controversy regarding the location of 5α-reductase activity. Van der Molen *et al.* (1975) and Nayfeh *et al.* (1975) found greater 5α-reductase activity in the interstitial tissue than in isolated seminiferous tubules. This 5α-reductase activity was LH-

dependent, but T independent. This contrasts with evidence that DHT and DIOL concentrations are greatest in the Sertoli cells. Rivarola et al. (1975) noted that DHT is the major metabolite in the seminiferous tubules and that DHT is absent in the interstitial cells. Several other studies have found 5α-reductase activity to be primarily localized in the seminiferous tubules (Payne and Kelch, 1975; Moger, 1979). Welsh and Wiebe (1976) observed that FSH increases metabolism of androgen by Sertoli cell. Dorrington and Fritz (1975), however, maintain that 5α-reductase activity is not dependent upon gonadotropin activity.

There is evidence that germ cells may be involved in androgen metabolism. Spermatogonia and spermatocytes, but not spermatids or spermatozoa, apparently convert T to DHT and DIOL (van der Molen et al., 1975; Dorrington and Fritz, 1975; Drosdowsky et al., 1975).

Androgen receptors are present in the seminiferous tubules, Leydig cells, spermatogonia, and spermatocytes (van der Molen et al., 1975). Sanborn et al. (1975), using a nuclear exchange assay, found that mature germ cells bound the greatest amount of androgen. Androgen binding by mature spermatids and epididymal sperm has also been noted by Galena et al. (1974) and Christensen et al. (1977). This is suggestive of a role of androgens in the release and/or maturation of sperm. However, others have not been able to find androgen binding by mature germ cells (Blaquier, 1971; Tindall et al., 1972).

A second major fate of T in relation to the Sertoli cell is to be bound by ABP. This protein serves to "protect" the T and DHT from further metabolism, thus competing with 5α-reductase and aromatase enzymes (Setchell and Main, 1975). ABP is thought to concentrate

androgens in specific regions of the Sertoli cell, i.e., near the germ cells, and promote spermatogenesis (Hansson et al., 1975). Another hypothesized role of ABP is that it carries androgens into the tubular lumen fluid, the rete testis and the epididymis, making androgens availabile to the developing and maturing spermatozoa (Bartke et al., 1975; Guerrero et al., 1975). ABP is produced in a dose-response manner to FSH. This response to FSH is potentiated by treatment with T or LH (Hansson, et al., 1975).

A third pathway for the metabolism of T in the Sertoli cell of the mammal is conversion to E_2. Following the discovery of estrogen production by the testis in mammals (Baggett et al., 1959) research has been aimed at defining the role of estrogen in the male. Initially, the role of estrogen was thought to be negative feedback on the pituitary (reviewed by Hseuh, 1978). However, a direct effect of estrogen on suppressing androgen biosynthesis has been indicated by decreased androgen production in gonadotropin-treated hypophysectomized males. Androgen biosynthesis is also depressed in gonadotropin-stimulated testicular fragments treated with estrogen in vitro (van Beurden et al., 1977; Chen et al., 1977; Hseuh et al., 1978; Moger, 1979; Sivelle, 1979). The site of action of E_2 in the testis is thought to be the Leydig cell, which has membrane-bound estrogen receptors (Brinkman et al., 1972; Mulder et al., 1973). It has been suggested that E_2 inhibits LH binding to LH receptors, accounting for the depression of androgen biosynthesis (Hseuh et al., 1978; Catt et al., 1980).

Association of estrogen receptors and LH receptors also has been indicated in studies of the phenomenon of hCG testicular

desensitization. Treatment with high doses of hCG results in a loss in the capacity of the testis to produce androgens in response to further challenge with this LH-like gonadotropin. Testicular desensitization can be blocked by an estrogen-receptor inhibitor, implicating estrogen in the hCG-induced loss of androgen biosynthetic capabilities (Catt et al., 1980). As hCG, like LH, primarily stimulates testosterone production, aromatization and resultant estrogen production would thus function in a localized negative feedback capacity.

In addition to negative feedback effects of estrogen on LH-stimulation of T production, estrogens directly interfere with androgen biosynthetic enzymes. E_2 directly inhibits 3β-HSD, 17α-hydroxylase, and to a lesser extent, 17-20 lyase and 17-ketosteroid reductase (reviewed by Hseuh et al., 1978).

Investigations regarding the site of production of estrogen in the testis have been confounded by factors such as age, method of measurement, and purity of cell cultures. E_2 is produced only by Sertoli cells and is thought to be dependent upon FSH stimulation in immature rats (Armstrong et al, 1975; Dorrington and Fritz, 1975). In the mature rat, however, isolated seminiferous tubules do not produce estrogens in response to treatment with FSH or T. This implies that the role of estrogens in the maturing rat may be different from that in adults (Dorrington and Armstrong, 1979; Dorrington et al., 1978; Pomerantz, 1980). In adult rats, de Jong (1974) measured highest basal E_2 levels in interstitial tissues but detected the greatest increase in tissue E_2 in seminiferous tubules. This reflects only tissue levels, for E_2 released into the incubation media was not assayed, and the

purity of the testicular fractions has been questioned (Payne and Kelch, 1975). Payne and Kelch, (1975) and Valladares and Payne (1979) maintain that aromatase activity in adult rats is restricted to interstitial cells and is responsive to LH treatment (but not FSH).

Regardless of the source of testicular estrogens, the presence of high affinity estrogen receptors on the Leydig cells points to a role of estrogen, possibly in local feedback control of androgens in testicular function (Mulder et al., 1973; Hseuh et al., 1978).

A much less understood level of regulation of testicular steroidogenesis is the effect of product inhibition. Examples of this include 1) progesterone inhibition of 3β-HSD and 17α-hydroxyprogesterone, 2) T inhibition of progesterone-17α-hydroxylase activity, and 3) androstenedione inhibition of 17α-hydroxypregnene-C-17 20-lyase activity (Nayfeh and Baggett, 1969; Oshima et al., 1970; Preslock and Steinberger, 1979). Furthermore, in addition to inhibition of biosynthetic enzymes, numerous steroids competitively interact with specific androgen or estrogen receptors, inhibiting steroid action (Muldoon, 1980).

In summary, the biosynthesis and regulation of steroids in the testis is a complex phenomenon. Two cell types, Leydig cells and Sertoli cells, respond to gonadotropins by producing steroids and proteins. Leydig cells produce androgens in response to LH, but the response can be modulated by FSH and steroids. Once Leydig cell LH-receptors are bound by LH and androgen biosynthesis is initiated, regulation at the biosynthetic enzyme level by steroids may occur.

Androgens synthesized by the Leydig cells enter peripheral circulation or may be locally metabolized in Sertoli cells. In the Sertoli cell, androgens may be reduced to DHT, aromatized to E_2, or bound by ABP. DHT binds to androgen receptors and thus mediates actions of androgens on germ cells directly. Estrogen may have a negative feedback function on Leydig cells, regulating androgen production. ABP "protects" androgens from further metabolism and may serve to localize or concentrate them in specific compartments of the testis.

Although much is known about synthesis and metabolism of steroids in the testis, there is still a great deal of controversy in some areas. Little is understood about the action of steroids in the testis once they are produced. Androgens are believed to affect spermatogenesis, possibly more in some stages than in others (Hansson et al., 1975). The effect of steroids on the release of sperm in mammals is not known. Russell (1978) and Gravis (1980) found that T and dibutyryl c-AMP (a potent c-AMP agonist) inhibited sperm release in hamsters. The action of these drugs was to disrupt "tubulobulbar complexes",the cytoplasmic extensions between Sertoli cells and maturing spermatids. These structures are thought to be important in chemical communication between the germ cell and Sertoli cell, responsible for initiation of a chemical trigger at the time of sperm release. Both Gravis (1980) and Russell (1978) tested only one dose of drug so that the inhibition of spermiation may not represent the response to a lower dose. In light of the fact that DHT and T can maintain spermatogenesis and sperm release in hypophysectomized animals, an inhibitory effect of T or dibutyryl c-

AMP on sperm release would not be expected. Certainly more work on the action of steroids in testicular function in mammals is needed.

Gonadotropin Regulation of Testicular Function: Amphibians

As in virtually all vertebrates, amphibian testicular function is regulated by the hypothalamal-pituitary axis (Callard, I.P. et al., 1980). Two gonadotropins have been isolated from each of three species of amphibians (Rana catesbeiana, Rana pipiens and Ambystoma tigrinum) (Licht and Papkoff, 1974; Licht et al., 1975; Farmer et al., 1977). In each of these species, one gonadotropin possessed LH-like activity, while the other was FSH-like. In the homologous species, the LH-like gonadotropin stimulated the production of androgen by the testis, while the FSH-like hormone affected spermatogonial proliferation (Muller and Licht, 1980). This pattern parallels the dichotomy of action of mammalian gonadotropins in these species and in mammals (Muller, personal communication; Muller and Licht, 1980).

The effects of mammalian LH and FSH on testicular function in other species do not support a consistent pattern of specificity of action of the two hormones (Licht, 1979; Muller and Licht, 1980). In Xenopus laevis, ovine LH and FSH are equipotent in stimulation of the production of androgen from the testis (Wiebe, 1970; Muller and Licht, 1980). Similarly, experiments using gonadotropins from more closely related species do not always conform to the pattern of LH and FSH specificity seen in mammals and in some amphibians. For example, Rana FSH is more potent than Rana LH in stimulating the production of androgens from

Taricha granulosa testes. Rana FSH also stimulates steroidogenesis in testes of Bufo marinus and Pseudoeurycea smithii, although Rana LH is more potent (Muller and Licht, 1980).

A similar overlap in function between LH and FSH has been found with respect to spermatogenetic effects. FSH primarily stimulates spermatogenesis in larval anurans and urodeles, but LH also increases spermatogenic activity (reviewed by Licht, 1979). Using homologous gonadotropins, Muller (1976) found that both Rana FSH and LH stimulated spermatogenesis in hypophysectomized adult frogs.

In summary, no consistent evolutionary pattern of gonadotropin specificity can be gleaned from the amphibian data. In general, it is difficult to predict how closely related species will respond to gonadotropins isolated from another genus or species.

Steroidogenesis and Regulation of Testicular Function: Amphibians

Less information is available regarding the production and regulation of steroids by the testis in nonmammalian vertebrates. The largest body of this nonmammalian work, however, has been carried out on amphibians (Callard, I.P. et al., 1978). Various steroids, including progesterone, estrone, E_2, estriol, T, androstenedione, DHT, and dihydroepiandrostenedione have been isolated from the plasma and testis of amphibians (reviewed by Ozon, 1972; Callard, I.P. et al., 1978). The anuran testis is thought to synthesize and metabolize steroids along the same pathways as in mammals (Ozon et al., 1972; Callard, I.P. et al., 1978). However, the urodele testis produces 11-ketotestosterone, a

steroid absent in mammals but common in fish (Callard, I.P. et al., 1978).

Interstitial cells in anurans and lobule boundary cells in urodeles are thought to be the major steroidogenic cells of the amphibian testis (Pesonen and Rapolai, 1962; Certain et al., 1964; Biwas, 1969; Vellano, 1969; Picheral et al., 1970; Tso and Lofts, 1977a, b; Imai and Tanaka, 1978; Callard, et al., 1980). Interstitial cell 3β-HSD activity in anurans is greatest during the breeding season and correlates with the development of secondary sexual characteristics (Lofts, 1974). However, in urodeles, the steroidogenic "glandular tissue" exhibits highest 3β-HSD activity following spermiation (Imai and Tanaka, 1978). In addition to steroidogenic glandular tissue, less differentiated lobule boundary cells possess some steroidogenic activity. The products of these lobule boundary cells may be important in local testicular function in seasons when glandular tissue is not present (Imai and Tanaka, 1978; Moore et al., 1979). The immature zone of the testis in the urodele shows little if any 3β-HSD activity and does not secrete significant quantities of androgens (Imai and Tanaka, 1978; Moore et al., 1979).

Sertoli cells, at least in anurans, may have some steroidogenic function (Callard, I.P. et al., 1978). Sertoli cell 3β-HSD activity in anurans correlates with the period of spermatogonial division, a process thought to be androgen stimulated (Lofts, 1974). The local steroidogenic function of Sertoli cells at this time may be important because interstitial cells are atrophic, and plasma androgens are low (Callard, I.P. et al., 1978, Specker and Moore, 1980). However,

androgens can be either stimulatory or inhibitory to spermatogonial proliferation in amphibians, depending upon previous hormone treatment (Moore, 1975). In addition to stimulating spermatogonial proliferation, steroids also may increase the rate of maturation of spermatids into spermatozoa (Rastogi et al., 1976; Callard, I.P. et al., 1978).

DHT is the major metabolite of T in anurans, including Rana esculenta, R. pipiens,. R. catesbeiana, Rana sylvaticus, B. marinus, and X. laevis (D'Istria et al., 1974; Muller, 1976; 1977a, b; Rastogi et al., 1976; Wada et al., 1976; Callard, I.P. et al., 1978). Plasma T is usually greater than DHT in urodeles, including Necturus maculosus, Pleurodeles waltii, T. granulosa and A. tigrinum, and in general the steroid levels are much higher than in anurans (Rivarola et al., 1968; Ozon, 1972; Moore and Muller, 1977; Callard, I.P. et al., 1978; Bolaffi and Callard, I.P., 1979; Bolaffi et al., 1979).

Testicular estrogen and aromatase activity have been found in (Bufo vulgaris and N. maculosus (Chieffi and Lupo, 1961; Callard, et al., 1980). Male R. catesbeiana possess aromatase activity only in neural tissue (Callard, G.V. et al., 1978). Plasma estrogen concentrations have been measured in male N. maculosus, R. catesbeiana, and R. temporaria (D'Istria et al., 1974; Callard, G.V. et al., 1978; Bolaffi and Callard, I.P., 1979). Short term incubations of Rana, Bufo, Taricha, or Necturus testicular slices (2 hr) with amphibian or mammalian gonadotropins contained no estrogen. (Muller and Licht, 1980). However, longer term incubation of Necturus glandular tissue (steroid secreting tissue of evacuated cysts) with FSH stimulated estrogen production (Callard, I.P. et al., 1978). The primary

estrogenic product from testes of Necturus estrone, derived from 17-hydroxyandrostenedione and androstenedione. Lesser amounts of E_2 were released, aromatized from T (Callard, et al., 1980). Aromatase activity has also been found in this region of Necturus testes (Callard, et al., 1980). The role of estrogen in the amphibian testis is not understood.

Although sketchy, information regarding testicular function in amphibians parallels the mammalian literature. Biosynthetic pathways of steroids as well as the enzymes for the metabolism of steroids appear to be the same as in mammals. The interstitial cell and Sertoli cell dichotomy of function in the synthesis of androgens and metabolism of androgens is also present in anurans. One major discrepancy, at least in some species of anurans, is the lack of specificity of function of the two gonadotropins, LH and FSH.

Much more information is needed in the area of regulation of testicular function in amphibians, particularly regulation at the intratesticular level. As in mammals, estrogen may have a central role in intratesticular regulation of androgen biosynthesis but at this time data are lacking.

Amphibians can be used as model animals for investigations into testicular function. The seasonal nature of the testicular cycle of male amphibians allows for manipulations not possible in intact, continuously breeding mature male mammals. Spermatogenesis, steroidogenesis, and spermiation are distinguishable events that can be "turned on" in amphibians and the endocrinological mechanisms can be studied.

Sperm Release in Amphibians

Currently there is no model accounting for the action of LH in the process of sperm release from the vertebrate testis. In mammals, sperm release from the Sertoli cells is continuous and is a difficult system to manipulate experimentally (Gravis, 1980). Male anuran amphibians have burst sperm release and thus represent a good experimental model to test the endocrine events associated with sperm release.

A summary of the spermiation responses noted in several anuran species is included in Tables 9 and 10 (Appendix 2). Houssay and Lascano Gonzalez (1929) first described the release of spermatozoa from male Bufo arenarum in response to implanted toad pituitaries. Release of sperm in response to anuran pituitary extracts was subsequently confirmed in B. arenarum (Houssay et al., 1929), and R. pipiens (Rugh, 1937; 1939). In 1946, van Oordt and Klomp coined the word "spermiation" in describing the response of Bufo bufo to injected gonadotropin.

Histological studies of spermiation (de Robertis et al., 1946; Burgos and Mancini, 1947a) established that Sertoli cells undergo hydration and vacuolization in response to gonadotropin. This results in the release of spermatozoa from the apical borders of the cells. Further evidence that water uptake is involved in sperm release is indicated by the fact that spermiation can be stimulated artificially by immersing the testis in hypotonic saline (van Oordt et al., 1954; Astrada and Taleisnik, 1957) or by injecting intact animals with distilled water (Giltz and Miller, 1950; van Oordt et al., 1954; Giltz, 1955). In addition, incubation of the testis in hypertonic saline can

inhibit gonadotropin stimulation of spermiation (van Oordt et al., 1954; Astrada and Taleisnik, 1957). However, the mechanism of the spermiation induced by hypotonic saline appears to be different from the response to gonadotropin (van Oordt et al., 1954).

Burgos and Vitale-Calpe (1967) using electron microscopy distinguished three stages in the response of Bufo testes to LH. The first stage was characterized by swelling of the endoplasmic reticulum, vacuoles and cisterns of the Sertoli cells, resulting in an increase in cell volume, pushing the embedded spermatozoa toward the apical border of the cell. The second stage showed a collapse of the previously swollen endoplasmic reticulum, concomitant with swelling of the apical cytoplasm, which further pushed the spermatozoa toward the lumen of the seminiferous tubule. In the third and final stage the apical cytoplasm ruptured and disintegrated so that the spermatozoa along with fluid and cellular debris were released into the lumen. This entire process of spermiation can be summarized by 1) water uptake by the endoplasmic reticulum, 2) transfer of fluid to the apical cytoplasm, and 3) rupture of the apical cytoplasm and release of sperm.

The mechanism of water uptake in response to sperm releasing agents, such as gonadotropins, is not clear. Mancini and Burgos (1948) described the disappearance of mucopolysaccharides from the Sertoli cell cytoplasm in response to treatment with gonadotropin, and postulated that this breakdown could increase the intracellular osmolarity and provide a gradient for water uptake. Further research on this problem is lacking.

Spermiation responses to gonadotropins have been observed in hypophysectomized animals, adrenalectomized animals, and in testes incubated *in vitro*. Further, the histological events of spermiation in these examples are similar to those *in vivo* (Burgos and Mancini, 1947b; Galli-Mainini, 1947a; Flores, 1948; Sulman, 1951; Houssay and Burgos, 1953; Crabbé, 1954a, b; Kissen, 1954; Phillips and Feldhaus, 1957; Ewy and Pigoniowa, 1957; van Dongen, 1958, 1962; van Dongen and de Kort, 1959; van Dongen *et al.*, 1959, 1960).

Galli-Mainini (1947a, b) introduced the use of anuran spermiation as an early pregnancy test, initially utilizing *B. arenarum*. The response of *Bufo* is quite specific to gonadotropins (hCG or pituitary extracts). The rate of spermiation to hCG was found to be dose dependent (Haskins and Sherman, 1949); most of the assays distinguished only between a positive or negative response with no quantification of relative magnitudes of sperm release.

When more species were utilized in pregnancy testing (see Galli-Mainini, 1948 for a review; Wiltberger and Miller, 1948, Houssay, 1954), it became apparent in such routine testing that discontinuous breeding animals exhibit a seasonal variation in responsiveness to gonadotropins (Samson, 1950; Wille, 1956a; Phillips and Feldhaus, 1957). Other species were not as specific as *Bufo* in the spermiation response, i.e., responses were noted to treatment with drugs other than gonadotropins.

Results of several investigations indicated that steroids may be involved in the release of sperm from the amphibian testis. Wille (1956a) observed a stimulatory effect of testosterone on sperm release in intact *R. temporaria* during the breeding season. Robbins and Parker

(1952) reported that spermiation occasionally occurred in R. pipiens following injections of bull testicular extracts. Both of these studies were in vivo and effects of steroids on the pituitary cannot be ruled out as the sperm-releasing mechanism.

Interestingly, epinephrine is the only other substance that consistently evokes a spermiation response in anurans. R. pipiens, R. esculenta, R. temporaria, Rana nigromaculata, Leptodactylus ocellatus and X. laevis spermiate in response to epinephrine and closely related compounds (Robbins and Parker, 1948, 1949; Galli-Mainini, 1948; Hinglais and Hinglais, 1949; Li and Chang, 1949; Cresseri and Bevacqua, 1950; Sulman, 1951; Clark et al., 1951; Houssay and Burgos, 1953; van Oordt et al., 1954). Spermiation in response to epinephrine appears to be similar cytologically to that in response to gonadotropins, except that in vivo no disappearance of mucopolysaccharides was noted (Cresseri and Bevacqua, 1950; van Oordt et al., 1954; van Dongen and deKort, 1959; van Dongen et al., 1959, 1960).

B. arenarum, Bufo fowleri, Bufo bufo asiaticus, Bufo viridus, and Hyla arborea do not spermiate in response to epinephrine (Galli-Mainini, 1948; Li and Chang, 1949; Sulman, 1951; Houssay and Burgos, 1953). The mechanism determining responsiveness to epinephrine or lack thereof is not known.

The action of epinephrine is thought to be on the testis, because spermiation has been shown to occur in hypophysectomized R. pipiens, R. esculenta, R. nigromaculata, L. ocellatus, and X. laevis (Robbins and Parker, 1948, 1949; Li and Chang, 1949; Sulman, 1951; Houssay and Burgos, 1953; Ewy and Pigoniowa, 1957). Wille (1957a) however, found

that epinephrine did not elicit spermiation in hypophysectomized R. temporaria. Furthermore, epinephrine evoked sperm release in vitro in testes from R. pipiens (Phillips and Feldhaus, 1957) and R. temporaria (van Dongen and deKort, 1959; van Dongen et al., 1959). Extirpation of the adrenals (a potential in vivo source of epinephrine) had no effect on hCG-initiated sperm release in R. pipiens or L. ocellatus (Sulman, 1951; Houssay and Burgos, 1953).

A possible neural mechanism of action of epinephrine was indicated by Clark et al. (1951) in that gonadotropin- or epinephrine-stimulated sperm release from R. pipiens could be blocked by adrenolytic drugs. However, adrenolytic drugs have been ineffective in blocking gonadotropin-stimulated sperm release in R. esculenta and epinephrine-induced spermiation in Leptodactylus (Houssay and Burgos, 1953; Ewy and Pigoniowa, 1957). Also, removal of sympathetic ganglia and sectioning of the spinal cord had no effect on epinephrine-induced sperm release in R. nigromaculata (Li and Chang, 1949).

Other information regarding the mechanism of action of epinephrine in the amphibian testis is lacking, but a few pertinent effects of epinephrine have been noted in the mammalian testis. Epinephrine reportedly increases cAMP and T levels in the rat testis (reviewed by Marsh, 1976) and increases aromatase activity in Sertoli cell-enriched immature rat testis cultures (Verhoeven et al., 1979). It is not known whether similar actions occur in the amphibian testis.

Thyroxine has been reported as inhibitory to testicular responsiveness to hCG in a sperm release assay, while propylthiouracil (PTU) increased responsiveness (Crabbé, 1954a, b; Wille, 1956b). However, in

epinephrine-treated testes, PTU decreased the sperm release response in terms of percent animals responding (Wille, 1957b). These results suggest a possible mechanism for hormonal regulation of testicular sensitivity to gonadotropins. Prolactin, which varies seasonally in anurans (Kuhn and Engelen, 1976) antagonizes thyroxine and thus could increase testicular responsiveness to gonadotropins.

More recently sperm release in anurans has been used as a bioassay for purification of gonadotropins in several taxa (Burzawa-Gérard, 1972; Licht et al., 1976). The specificity of spermiation in response to LH or FSH has been the object of much research, stemming from original interest in biological specificity of gonadotropins in vertebrates and from an interest in developing a highly specific bioassay for LH versus FSH (Licht et al., 1976). The results of such studies have been mixed, owing to both species differences in specificity and to early work with relatively impure hormones. In R. pipiens, mammalian LH was found to be more potent in evoking sperm release than FSH, using a percent responding criteria (Witschi and Chang, 1959; Burgos and Ladman, 1957). Robbins and Parker (1952), utilizing a relatively impure FSH-containing extract suggested that sperm release in R. pipiens was primarily initiated in response to FSH, but acknowledged the possibility of LH contamination. Lofts (1961) found that only LH stimulated sperm release in hypophysectomized R. temporaria. Licht (1973), using highly purified mammalian gonadotropins, concluded that spermiation is not specific for either hormone in R. pipiens, Hyla regilla and Eleuthrodactylus coqui. Less purified LH was more potent than FSH in R. pipiens, but the reverse was seen in Hyla and Eleuthrodactylus

spermiation responses. Furthermore, spermiation in Xenopus and Bufo is not specific for either highly purified mammalian hormone (Licht, 1979). Similarly, Rana and Hyla show no specificity for purified turtle gonadotropins or Rana gonadotropins in spermiation responses (Licht and Papkoff, 1974).

In summary, the biochemical mechanism of gonadotropin action in the release of sperm from the vertebrate testis is not known. In addition to gonadotropins, epinephrine and steroids sometimes can stimulate the release of sperm from amphibian testes. The relationship between epinephrine and gonadotropin-induced spermiation is not known, although steroids may mediate their common effect. In that the major known action of gonadotropins on the testis is steroidogenesis, the possibility that spermiation is mediated by steroids is a viable hypothesis.

MANUSCRIPT

The Role of Steroids in Gonadotropin-induced Spermiation of the Pacific treefrog, *Hyla regilla*

INTRODUCTION

The release of sperm from the amphibian testis in response to gonadotropins was studied extensively both as a method of clinical pregnancy testing (reviewed by Houssay, 1954) and, more recently, as a bioassay to define the specificity of nonmammalian gonadotropins (reviewed by Licht *et al.*, 1976). Gonadotropin-stimulated spermiation was noted in hypophysectomized animals and in testes incubated *in vitro*, indicating a direct effect of gonadotropin on the testes (reviewed by van Dongen and de Kort, 1959). However, very few recent studies have investigated spermiation, and the endocrine mechanisms of gonadotropin action on the vertebrate testis during sperm release have not been elucidated.

Results of several investigations indicate that steroids may be involved in the release of sperm from the amphibian testis. Wille (1956) observed a stimulatory effect of testosterone on sperm release in intact *Rana temporaria* during the breeding season. Robbins and Parker (1952) reported that spermiation occasionally occurred in *Rana pipiens* following injections of bull testicular extracts. Both of these studies were *in vivo*, and therefore the effects of steroids on the pituitary cannot be ruled out.

Even if the administration of testosterone to Rana stimulated the release of sperm by acting directly on the testis, the testosterone effect may not be mediated by an androgen receptor. Testosterone is an aromatizable androgen, capable of being converted to estrogen by aromatase enzymes. Aromatase activity has been found in the testis of Necturus maculosus (Callard, et al., 1980). Estrogen has been found in the testis of Bufo vulgaris (Chieffi and Lupo, 1961) and in the plasma of male Necturus, Rana catesbeiana, and R. temporaria (D'Istria et al., 1974; Callard, G.V. et al., 1978; Bolaffi and Callard, I.P., 1979). The physiological significance of testicular or systemic estrogen in the male amphibian is not known.

A number of studies, using toads, reported that neither androgens nor estrogens influenced spermiation (for a review see Galli-Mainini, 1948; Houssay, 1954). If LH stimulates spermiation by a mechanism independent of steroids, then this would be the only known action of LH not mediated by steroids. Perhaps the effect of LH on ovulation is analogous to the effect of LH on spermiation. It is well-documented that LH triggers ovulation in female anurans by stimulating endogenous steroid synthesis (Wright, 1961a, b).

The present study investigated the question of whether steroids are involved in the release of sperm from the vertebrate testis. The investigations used an in vitro quantitative assay of sperm release from testes of Hyla regilla, the Pacific treefrog. To determine the role of steroids, testes were incubated with two drugs that interfere with the action of steroids: Flutamide or CI-628. Flutamide is recognized primarily as an androgen-receptor inhibitor (Neri et al., 1972),

although it also may act as an aromatase inhibitor (McEwen, 1980). The main effect of Flutamide on the testis is the blocking of androgen-receptor mediated events. CI-628 (nitromiphene citrate) acts as an estrogen-receptor inhibitor by sequestering estrogen receptors in the nucleus, making them unavailable for further binding (McEwen, 1980). CI-628 thus inhibits estrogen-receptor mediated mechanisms. Inhibitory effects of these drugs on the release of sperm may then be interpreted as supporting the hypothesis that steroids are involved in the action of LH on the testis during the release of sperm.

MATERIALS AND METHODS

Adult male *H. regilla* (Pacific tree frogs) were collected during the breeding season (March and April, 1980, 1981) near Corvallis or Sisters, Oregon. They were housed at 10°C on a 12L:12D photoregime (lights on at 0600 hr). To obtain testes for the *in vitro* incubations, animals were anesthetized by immersion in MS-222 (ethyl m-aminobenzoate; 1% solution). Testes were removed immediately and washed in Kreb's isotonic medium (in mmoles/liter: 118.0 NaCl, 25.0 $NaHCO_3$, 4.8 KCL, 2.5 $CaCl_2$, 1.2 KH_2PO_4, and 1.17 $MgSO_4$). The medium was gassed with a mixture of 5% CO_2 and 95% air and a pH of 7.4 was carefully monitored. Incubations were carried out in 1.0 ml Kreb's medium at 27°C in clean 15 x 85 glass culture tubes. In experiments where pretreatment sperm counts were determined (all except for experiment IA) testes were incubated in Kreb's medium for 0.5 hr, at which time the number of sperm released into the medium was counted using a hemacytometer (Neubauer 1/400 sq. mm, 1/10 mm deep). Replicability of sperm counts were determined by repeated counts of a single sample (mean ± SD: $3.76 \times 10^6 \pm 0.21 \times 10^5$, n = 9). Following the pretreatment sperm count, experimental drugs or hormones dissolved in 0.1 ml amphibian Ringer's Solution were added to the medium containing the testes. The number of sperm released into the medium was counted using a hemacytometer at the end of a two hour incubation period. Three different experimental designs were used to test the spermiation responses of *Hyla* testes *in vitro* (Figure 1). An initial experiment (IA) utilized controls within the population of frogs. Testes were distributed among treatment

groups, avoiding the use of two testes from the same animal in a single treatment group. In this experimental design, however, nonresponding testes, either due to immaturity or previous evacuation of sperm, could not be distinguished from testes that did not respond as a result of experimental treatment. Furthermore, testes within a population that were capable of releasing sperm exhibited a broad range in the numbers of sperm released. The resultant variability tended to mask the effects of the experimental treatments.

More sensitive measures of sperm release were developed for the remaining experiments. Contralateral controls were used in experiments IB, IIA, IIB and IIC. In this design one testis from each animal was treated as an experimental while the contralateral was treated as a control. Right and left testes were assigned randomly to experimental and control treatment groups. Data analysis involved pair-wise comparisons of the experimental testes and their respective contralateral controls using a Student's Paired t-test.

Experiment IC used within-testis controls. Testes were sliced in half, one half was used for the experimental treatment while the other half served as a control. Anterior and posterior halves were distributed equally between experimental and control treatment groups. This design reduced experimental variability to that between regions of the same testis. Also comparisons between contralateral testes could be made.

In the latter two experimental designs, pretreatment sperm counts were taken following a 0.5 hr incubation period in Kreb's medium. Occasionally large numbers of sperm were present in the medium before

treatment, indicating that sperm release was already in progress. Usually this was found in both testes from the same animal. Final sperm counts from testes with high pretreatment sperm counts were not included in the analyses of the data.

Flutamide (SCH 13521, Batch 45978-43-II, Schering Corp.) CI-628 (nitromiphene citrate, CN-55, 945-27 Lot S, Warner Lambert Co.), testosterone (T = 17β-hydroxyandrost-4-en-3-one), dihydrotestosterone (DHT=17β-hydroxy-5α-androstan-3-one), progesterone (Δ^4-pregnen-3, 20-dione) estradiol-17β (E_2), estrone, estriol, and corticosterone (Δ^4-pregnene-11β, 21-diol-3, 20 dione) were suspended in amphibian Ringer's solution. Drugs were mixed with gum arabic, dissolved in one drop of 100% ethanol, and brought to volume with amphibian Ringer's. Ovine LH (NIA MMD-OLH-22, NIH) was dissolved in amphibian Ringer's solution. Preliminary experiments determined the dose-response and time-response of testes in vitro. Approximately 85% of the testes treated with 10 µg LH spermiated.

I. Spermiation in the presence of steroid inhibitors.

Three experiments were designed to test the effects of steroid inhibitors on gonadotropin-stimulated sperm release. In experiment IA, within population controls were used to test the effects of Flutamide (an androgen inhibitor) and CI-628 (an estrogen inhibitor) on LH-stimulated sperm release. Testes were incubated with 1) isotonic saline, 2) 10 µg LH, 3) 100 µg Flutamide plus 10 µg LH or 4) 100 µg CI-628 plus 10 µg LH. After two hours the number of sperm

released into the medium was determined. Data were analyzed, following a correction for heteroscedasticity with square root transformation (Sokal and Rohlf, 1969), using a one-way analysis of variance and a Student Newman Keul's test.

In experiment IB, contralateral controls were used to investigate the effect of Flutamide on gonadotropin-stimulated sperm release. Experimental testes were treated with 100 µg Flutamide plus 10 µg LH; contralateral controls were treated with 10 µg LH. After two hours of incubation, the number of sperm released into the medium was counted.

Within-testis controls were used in Experiment IC to test the effect of CI-628 on LH-stimulated sperm release. Because this population of frogs was less responsive to gonadotropin (reason unknown), the incubation medium volume was reduced to allow reliable sperm counts (volume = 0.2 ml). The dose of LH was increased slightly. Testis halves in experimental groups were treated with 1) 250 µg CI-628 plus 12.5 µg LH or 2) 25 µg CI-628 plus 12.5 µg LH. Control halves were treated with 12.5 µg LH. Data were analyzed using a Student's paired t-test.

II. Spermiation in the presence of exogenous steroids.

Two series of experiments were performed to test the effect of steroids on sperm release <u>in vitro</u>. One series used steroids plus LH, while the other tested the effect of steroids alone.

In Experiment IIA, contralateral controls were used to determine the effect of two doses of DHT on LH-induced sperm release. Preincubation sperm counts were determined, and then experimental testes were treated with 25 µg DHT plus 12.5 µg LH or 250 µg DHT plus 12.5 µg LH. Contralateral controls were treated with 12.5 µg LH. Following the two-hour incubation period, the number of sperm in the medium was counted.

Experiment IIB investigated the effect of estrogen on LH-stimulated sperm release. Experimental testes were treated with 1.0 µg E_2 plus 10 µg LH or 10 µg estrone plus 10 µg LH. Control testes were treated with 10 µg LH.

Several steroids at different doses were tested to observe any possible sperm-releasing effects. In experiment IIC, contralateral controls were used. Experimental testes were treated with 1) E_2 (100 µg, n=5; 10 µg, n=2; 1.0 µg, n=8; 0.1 µg, n=2), 2) estrone (10 µg, n=3), 3) estriol (10 µg, n=3), 4) progesterone (1 µg, n=2) 5) T (100 µg, n=8; 1 µg, n=2), 6) DHT (100 µg, n=5), or 7) corticosterone (25 µg, n=2). Contralateral testes were incubated in Kreb's medium plus isotonic saline to bring up to volume.

RESULTS

The antiandrogen Flutamide consistently and significantly inhibited release of sperm from LH-stimulated testes. In experiment IA (Table 1), testes treated with Flutamide plus LH released fewer sperm than LH-treated testes ($p < 0.05$). The release of sperm was not significantly different for testes treated with saline than treated with Flutamide plus LH. The analysis of the sperm counts from experiment IB (Figure 2) further demonstrates the inhibitory effect of Flutamide on gonadotropin-stimulated spermiation. In this experiment, Flutamide completely blocked spermiation in 11 out of 19 LH-treated testes. Furthermore, in the testes that did respond in the presence of the inhibitor, sperm release tended to be depressed ($p<0.1$), such that the mean number of sperm released from responsive testes was reduced by 54%.

The effects of the estrogen inhibitor CI-628, on LH-stimulated sperm release were less consistent. In experiment IA (Table 1) CI-628 enhanced spermiation when compared to the LH-treated group ($p < 0.05$). The effect of CI-628 was notable, for included in the mean number of sperm released was one testis that did not respond. Thus the stimulatory effect of CI-628 on the responsive testes more than compensated for this zero value.

In contrast to experiment IA, CI-628 treatment in experiment IC did not significantly enhance the effectiveness of LH in causing release of sperm. There was no significant difference between testis halves treated with either dose of CI-628 and their controls treated with LH ($p < 0.5$). However, the effect of CI-628 was consistent within each animal

(Table 2). The release of sperm from both halves of contralateral testes treated with CI-628 was affected in the same manner, either stimulated or inhibited, compared to control halves.

The exogenous steroids, including E_2, estrone, estriol, progesterone, T, DHT, or corticosterone in experiment IIC had no effect on sperm release. Similarly, treatment with E_2 plus LH or estrone plus LH (experiment IIB) had no effect on LH-stimulated sperm release *in vitro*.

A high dose of DHT plus LH was the only steroid treatment that notably affected sperm release (experiment IIA). This treatment resulted in a significant reduction in release of sperm when compared to contralateral LH-treated controls ($p < 0.1$) (Figure 3). The sperm counts from testes treated with a lower dose of DHT plus LH tended to to be higher but did not significantly differ, from LH-treated controls.

DISCUSSION

Currently there is no model accounting for the action of LH in the process of sperm release from the vertebrate testis. In mammals, sperm release from the Sertoli cells is continuous and experimental manipulation of the system has been limited (Gravis, 1980). Male anuran amphibians release sperm in bursts and thus represent a good experimental model to test the endocrine events associated with spermiation.

Other than a few suggestive studies in which androgens stimulated spermiation in amphibians *in vivo* (Robbins and Parker, 1952; Wille, 1956), there is no evidence for LH causing the release of sperm via steroids. However, if the LH action in spermiation is mediated without the involvement of steroids, this would be the only example of a non-steroid-mediated effect of the hormone.

The present experiments provide evidence for the involvement of steroids when LH stimulates the release of sperm from the testis of *H. regilla*. Flutamide, an androgen-receptor inhibitor, dramatically inhibited LH-stimulated sperm release. The effect of CI-628, an estrogen-receptor inhibitor, was paradoxical -- enhancing the release of sperm in some LH-treated testes while decreasing it in others. Although treatment of the testes with androgens, estrogens, progesterone or corticosterone did not evoke spermiation, the action of the inhibitors indicates that steroids are involved in spermiation.

These experiments suggest that androgens are involved in the release of sperm from *Hyla* testes. Based on what is known about the action of

Flutamide, the inhibitory effect of Flutamide on LH-stimulated spermiation is interpreted as evidence that androgens are involved. A stimulatory role of androgen in spermiation is further supported by the tendency of a low dose of DHT to augment release of sperm from LH-treated testes. The dose of androgen is critical, however, because a high dose of DHT significantly inhibited LH-stimulated spermiation.

The paradoxical effect of an anti-estrogen CI-628 provides evidence that the role of steroids during spermiation is influenced by the condition of the testis. When testes were sliced in half and contralateral pieces were treated with CI-628 (or saline), the anti-estrogen enhanced spermiation in testes from certain individuals and inhibited spermiation in testes from other individuals (Table 2). These results indicate that the condition of the testis influences the spermiation response. This may be a general phenomenon in studies of testicular function. Moore (1975), using a spermatogenic response in the testes of Ambystoma tigrinum, found that, depending on the condition of the testis, T can be either stimulatory or inhibitory.

CI-628, the anti-estrogen, may affect the release of sperm by directly inhibiting estrogen receptors. However, in mammals, estrogen receptors are typically found on interstitial cells, rather than the germ cell-associated Sertoli cells (Brinkman et al., 1972; Mulder et al., 1974). An alternative action of CI-628 on sperm release could involve an indirect effect of the inhibitor. Amphibians possess steroidogenic enzymes and biosynthetic pathways similar to mammals (Ozon, 1972; Lofts, 1974). In mammals, estrogen-receptors are thought to be associated with the LH-receptors of Leydig cells (Catt et al.,

1980). These estrogen receptors may exert a negative feedback function on Leydig cell androgen biosynthesis (Hseuh et al., 1978). The application of an estrogen-receptor inhibitor interferes with this intratesticular negative feedback by estrogen on Leydig cells (Catt et al., 1980). A possible indirect effect of an estrogen inhibitor, then, is elevated production of testicular androgen. Although estrogenic negative feedback regulation of T biosynthesis has not been investigated in amphibians, the dual effect of CI-628 on the testis during sperm release suggests that more than a simple, direct action of the inhibitor may be involved.

In summary, these experiments provide evidence that steroids are involved in LH action on the testis during the release of sperm. These experiments suggest that androgens may mediate spermiation, and that estrogens may also be involved. At this time more study is required to determine the particular roles that these two classes of steroids play in spermiation.

The sensitive method of controlling for the condition of the testis, using either contralateral controls or within-testis controls is recommended for future studies aimed at elucidating the endocrine mechanisms of the release of sperm from the vertebrate testis.

Table 1. Effect of inhibitors of steroids on the release of sperm from *Hyla regilla* testes *in vitro*.

Treatment	n	No. Testes Spermiating	Mean Sperm/ml x 10^5	95% Confidence Limits x 10^5
Saline	4	0	0^{∞}	--
10 µg LH	4	4	6.70*	$2.54 \leq \bar{x} \leq 12.8$
100 µg Flutamide + 10 µg LH	3	1	1.28^{∞}	$0.00 \leq \bar{x} \leq 5.60$
100 µg CI-628 + 10 µg LH	4	3	$12.7^{*\infty}$	$4.75 \leq \bar{x} \leq 23.2$

[π] Square root transformed sperm counts were analyzed using a one-way analysis of variance followed by Student Newman Keul's test. Mean values and 95% confidence limits represent back-transformed values.

* Treatment significantly different from saline-treatment group ($p < 0.05$).

$^{\infty}$ Treatment significantly different from LH-treatment group ($p < 0.05$).

Table 2. Effect of CI-628 on sperm release from LH-stimulated half testes of Hyla regilla: comparison of contralateral testes.

Treatment	Testis*	% Difference in spermiation of CI-628 plus LH-treated half from LH-treated control half**
250 µg CI-628 + 12.5 µg LH	1	+76
	1A	+43
	2	-69
	2A	-92
	3	-46
25 µg CI-628 + 12.5 µg LH	3A	-36
	4	-8
	4A	-41
	5	+93
	5A	+52

* Contralateral testes denoted by the same number, i.e., 1 and 1A.

** Mean difference in release of sperm (between CI-628 + LH-treated halves and LH-treated halves) are not significantly different using a Student's paired t-test at $p < 0.05$.

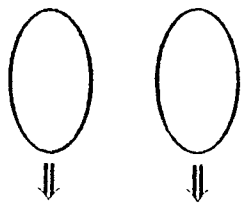
Figure 1. Three different experimental designs utilized in the experiments. In Experimental Design I (Experiment IA), testes were distributed randomly between treatment groups. For Experimental Design II (Experiments IB, IIA, B, C) one testis from each animal was treated with LH, as a control, while the contralateral testis was incubated with the experimental drugs. In Experimental Design III (Experiment IC) each testis was sliced in half. One half was treated as the control, while the other was treated as the experimental.

Experimental Design

I. Within Population Controls

A. LH vs. Saline
B. LH vs. LH & Inhibitors

II. Contralateral Controls



III. Within Testis Control

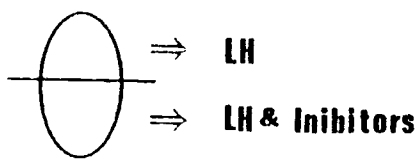


Figure 1

Figure 2. Effect of Flutamide, an androgen-receptor inhibitor, on gonadotropin-stimulated spermiation from testes of *Hyla regilla in vitro*. For simplicity of presentation, the mean and 95% confidence limits of both treatment groups are presented. Data were analyzed using a Student's paired t-test.

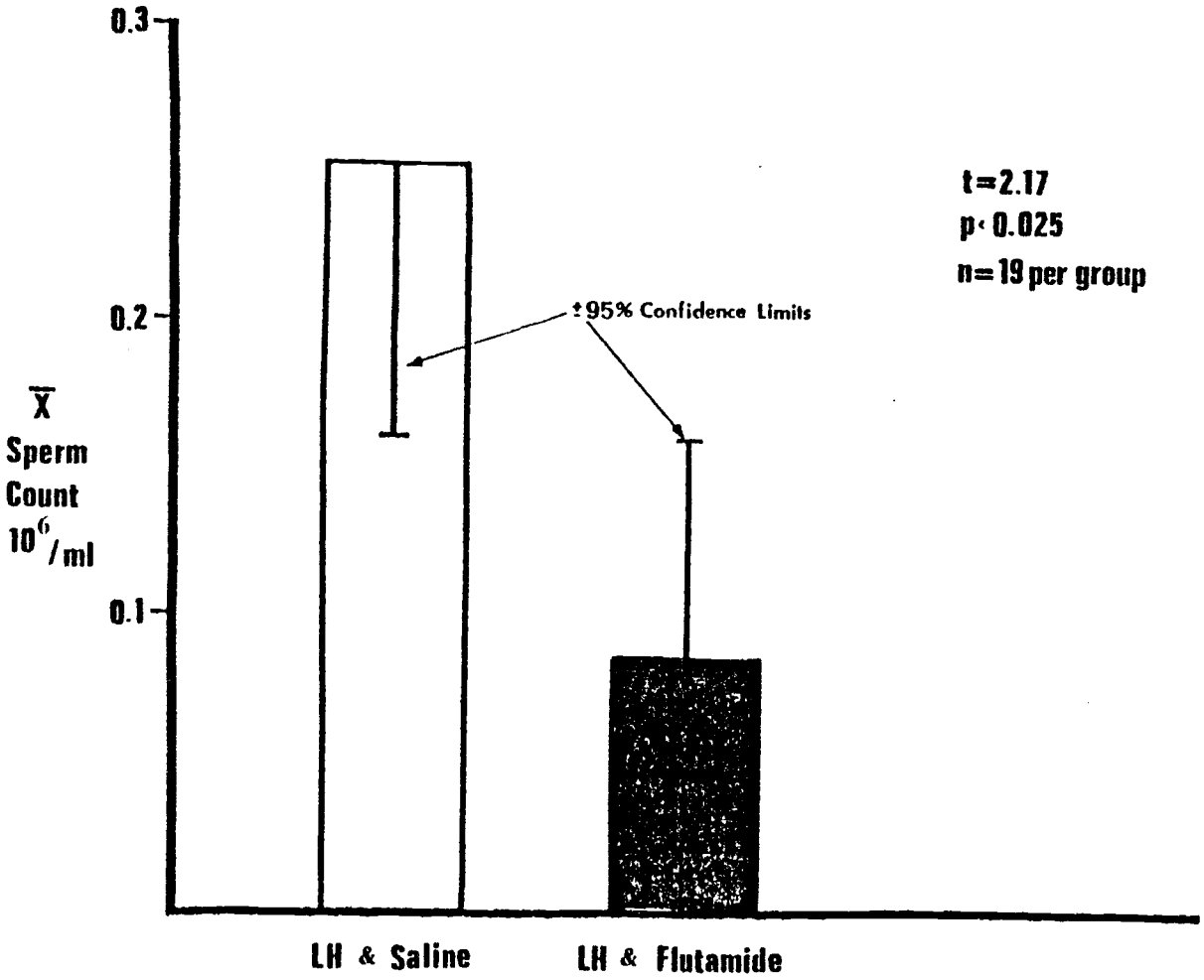

0.3
0.2
0.1
$\overline{X}$ Sperm Count 10^6/ml
±95% Confidence Limits
t=2.17
p< 0.025
n=19 per group
LH & Saline
LH & Flutamide

Figure 2.

Figure 3. Effect of two doses of DHT on gonadotropin-stimulated spermiation from testes of *Hyla regilla* *in vitro*. Mean percent of control sperm count represents the mean percent difference between the DHT plus LH-treated experimental testes and their respective LH-treated contralateral controls. Data were analyzed using a Student's paired t-test.

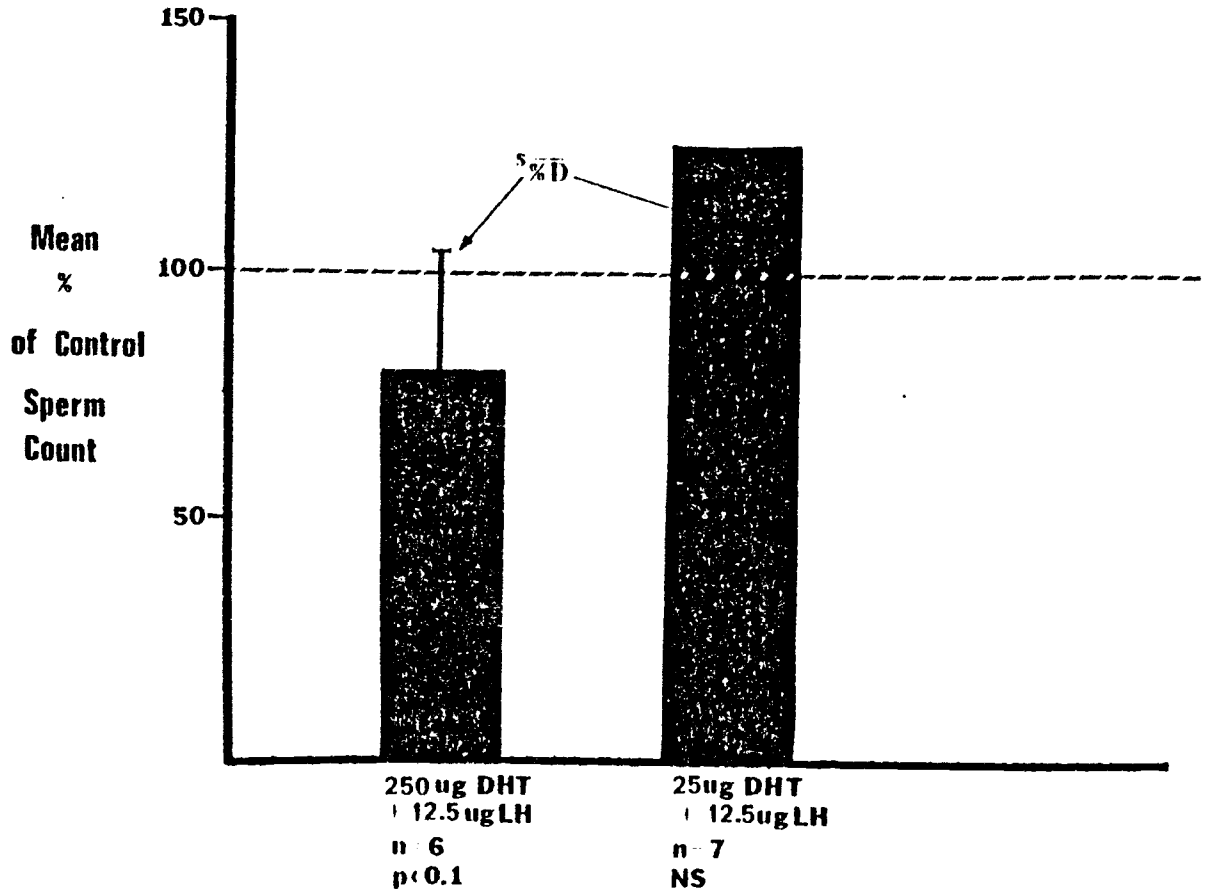

150
100
50
Mean
%
of Control
Sperm
Count
SEM
250ug DHT
+ 12.5ugLH
n = 6
p< 0.1
25ug DHT
+ 12.5ugLH
n = 7
NS

Figure 3.

SUMMARY AND CONCLUSION

The mechanism of action of gonadotropin in the regulation of male fertility is not known. This study provides evidence for the hypothesis that steroids are involved in gonadotropin-stimulated spermiation. In particular, it is suggested that androgens may mediate the action of LH on the testis during the release of sperm. Estrogens also may be involved in spermiation either directly or indirectly. The particular roles of estrogen and androgens in the regulation of testicular function need further study.

The seasonal, burst release of sperm from the male amphibian provides a manipulable model for experimental study. The method of controlling for the testis condition, using either contralateral controls or within testis controls, is recommended for further studies aimed at elucidating the endocrine mechanisms of the release of sperm from the vertebrate testis.

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APPENDIX 1

INTRODUCTION

In addition to the experiments presented in the manuscript portion of this text, several experiments are presented in this Appendix. Some of these experiments examined the LH-dose response of spermiation from intact animals and testes incubated *in vitro*. Other preliminary experiments tested the effects of steroid inhibitors on spermiation *in vivo*. In addition, several experiments that tested the effects of inhibitors on spermiation are presented.

MATERIALS AND METHODS

III. Dose response to ovine LH *in vivo*

Adult male *Hyla regilla* were collected during the breeding season, March-April, 1980 in Corvallis, OR and housed at 10°C on a 12L:12D photoregime (lights on at 0600). Animals were housed less than two weeks before use in experiments. All animals were verified as negative for sperm release on the day of injections by examining cloacal contents under a phase microscope. In Experiment III, five groups of animals were injected with 0.1 ml of 1) saline; 2) 1.0 µg LH/0.1 ml; 3) 2.5 µg LH; 4) 5.0 µg LH; or 5) 10.0 µg LH into the dorsal lymph sac. Care was taken to prevent leakage from the puncture in the skin. After injection the cloacal contents of the frogs were monitored every 15 minutes until a positive response was noted, up to a maximum time of two hours. Between testing, individuals were placed in a container with water.

IV. Dose response to ovine LH *in vitro*

Two different experimental designs were used to test for dose response of spermiation to ovine luteinizing hormone. In Experiment IVA testes were removed from anesthetized frogs (MS-222) and incubated in 1.0 ml of Kreb's Ringer's solution at 27°C. Testes were randomized according to treatment groups so as not to bias results of any one group with two testes from the same animal. Treatment groups included: 1) 0.1 µg LH, 2) 1.0 µg LH, 3) 5.0 µg LH, 4) 10 µg LH or 5) saline. After

two hours of incubation with the various doses of LH or saline, the medium was examined for the presence of sperm.

In the experiment IVB contralateral testes were treated with either 10 µg LH or saline, or 5 µg LH or saline. At the end of the incubation period, the numbers of sperm in the media were counted using a hemacytometer.

V. Implantation of inhibitors *in vivo*

In experiments VA and VB the effects of steroid inhibitors were tested *in vivo* using implants of Silastic capsules. The implants were slipped under the dorsal skin. Four days after the placement of the implants, animals were prescreened for the absence of sperm in the cloaca and then injected with 2.5 µg ovine LH (a dose previously yielding an 80% response). Injections were given into the dorsal lymph sac on the side opposite the implant, and care was taken to minimize leakage of the injectant out of the puncture hole. Following injection animals were checked for the presence of sperm in the cloaca every 15 minutes. Animals responding positively were rechecked for an hour following the initial response; those responding negatively were monitored at 15 min intervals for two hours. Positive responses were coded according to the approximate number of sperm contained in a drop under a 10X magnification field (see Table 6). The higher ranks were a subjective observation rather than an exact quantification, but the differences were of such a magnitude that approximate categorization was

not difficult. Data were analyzed using a Kruskall-Wallis or Mann-Whitney nonparametric statistic.

In Experiment VA, four groups of five male Hyla were implanted with 0.5 cm Silastic capsules filled with 1) Flutamide, a nonsteroidal androgen inhibitor; 2) CI-628 (nitromiphene citrate), an estrogen inhibitor; 3) Cyadren, an aromatase inhibitor; or 4) empty capsules. The animals were then treated as previously discussed. Eight days following the initial injections, (12 days after placement of implants) animals were reinjected with 2.5 µg LH and the sperm responses monitored as outlined above.

In Experiment VB, a dose response with Flutamide implants was undertaken. Four groups of ten animals each were implanted with 1) 1.0 cm empty Silastic capsule; 2) 1.0 cm capsule packed with Flutamide; 3) 0.50 cm capsule packed with Flutamide; 4) 0.25 cm capsule packed with Flutamide. Four days after placement of the implants the animals were injected with 2.5 µg of LH and the spermiation responses monitored as previously described.

VI. Pre-incubation with CI-628 in vitro.

Experiment number VI examined the effect of pre-incubation with CI-628 on gonadotropin-stimulated spermiation from testes which were divided into halves. Experiment VI utilized animals which had been held in captivity for 4 months (collected in Corvallis, OR). The testes were divided into halves, with one half serving as a control. Testis halves were washed with Kreb's Ringer's and then preincubated for 0.5 hr, at

which time the pretest sperm count was made. At this time 250 µg CI-628 or saline were added to the medium, and the testicular tissue was incubated for 1 hr. Then 25 µg LH was added to all samples, which were then incubated for 2 hr. After two hours of incubation, sperm counts were determined for all samples.

RESULTS

The results of the dose response of spermiation *in vivo* to ovine LH (Exp. III) are depicted in Table 3. From this it can be noted that over a narrow dose range, from 1 µg LH to 5 µg LH per animal, the percent animals responding went from 40% (2/5) to 100% (5/5). The control animals injected with saline did not spermiate. The rapidity of the spermiation response in this animal is indicated by the observation that in almost every case a positive response was noted in the first 15 min following injection. The only exception to this was one animal in the lowest dose-treatment group which responded in 30 min.

In experiments IVA and B spermiation exhibited a graded response to ovine LH. In the IVA (Table 4) the two lowest doses of LH (0.1 µg/ml and 1.0 µg/ml) were not significantly different from saline. An intermediate response in terms of numbers of animals responding was obtained at the 5 µg LH/ml dose. The highest dose, 10 µg LH/ml resulted in an 83% level of spermiation. The two higher doses, although significantly different from saline and the two lower doses ($p < 0.05$) were not significantly different from one another ($p < 0.2$).

Results obtained in the contralateral control dose response experiment IVB are presented in Table 5. Data were analyzed by a Student's t-test of mean differences (between LH or saline treatment) of the two doses of LH. Testes which did not respond to LH were not included in the analysis, as well as testes which released sperm into the medium but not elevated over the contralateral saline-treated control. Sperm counts of LH treated testes were significantly elevated

over saline treated controls ($p < 0.001$). Mean sperm counts of the two LH doses were not significantly different.

The effect of Flutamide in experiments VA and VB are presented in Table 6. Although none of the treatments are statistically different from controls within each experiment, several trends are noteworthy. First, Flutamide tended to inhibit the spermiation response *in vivo*. In fact, when the 0.25 cm implant data are combined from both experiments VA (4 days post-implant) and VB the Flutamide significantly inhibits sperm release ($0.02 < p < 0.01$) compared to controls from both experiments (Table 7). The control groups from experiments VA and VB, had 10/15 animals responding at the very high, high or medium level (other than an occasional negative 1/15). On the other hand, of all the Flutamide-treated animals, most of the animals (5/5 VA;6/8, 5/9, 6/9 VB) responded at the medium level or below. Reinjection after 12 days post placement of the implants in the Flutamide-treated animals (Experiment IVA) resulted in responses that were similar to the controls. In experiment VB, there appears to be no dose response to Flutamide.

The nature of the Experiment VA, VB and IA (see manuscript in this text) was such that although the experimental designs changed and were improved with time, the data in terms of numbers of animals spermiating in the presence of Flutamide could be analyzed using a single method. Examining the number of testes spermiating in the presence of 100 µg Flutamide plus 10 µg LH (Table 7), it can be seen that in experiments VA and VB this treatment did not significantly differ from controls. In the experiment IA, however, with a larger sample size, the total inhibition of sperm release in the Flutamide treated testes was highly

significant ($p < .007$). Combining probabilities from these three experiments, the total inhibition of gonadotropin stimulated sperm release by Flutamide is statistically significant ($p < 0.025$). This represents a 176% increase in the incidence of a totally inhibited spermiation response over that of LH-treated controls. In all three experiments, the level of response to LH alone remained stable, averaging 85% overall.

Results of CI-628 and Cytadren implants are less clear (Experiment VA). Neither of these treatments resulted in a significant difference in sperm release compared to controls. Some trends, however, can be noted. CI-628 tended to elevate the sperm release response in the 12-day post implant reinjection experiment (Table 6). In fact, two of the very high responses were noted as being extremely high, with the cloacal contents containing more sperm than had been noted in any other treatment group. Similarly, Cytadren tended to increase sperm release, although there was also an increase in the number of negative responses in this treatment group (Tables 6).

In experiment VI, CI-628 preincubated half-testes tended to release fewer sperm when stimulated by LH than LH treated controls, ($p<0.1$) (Table 8). In this experiment five out of seven testis halves treated with CI-628 showed a reduction in sperm release when compared to their respective controls. In one pair of treatments, CI-628 had no effect, and in the one treatment, CI-628 elevated gonadotropin-induced sperm release.

DISCUSSION

The spermiation dose response to mammalian LH of these male H. regilla closely parallels the doses noted by Licht (1973) using a highly purified LH (G3-206). He noted that 0.5 µg was the largest dose producing no spermiation, while 1.5 µg was the lowest dose resulting in greater than a 50% or median response. Similarly, it was noted that the Hyla responded generally within 45 minutes of injection. From this it is concluded that 2 hours is a sufficient amount of time to monitor sperm release; i.e., if an animal has not responded within 2 hours it is probably not responsive.

Spermiation from testes of male H. regilla in vitro is sensitive over a narrow range of doses of LH. Occasionally, sperm release is seen in saline treated testes, either due to spermiation already present in the animal or possible to damage to the testis during removal from the animal. The contralateral control experiments helped to eliminate some of the variability due to these influences and provided a sensitive test of treatments.

In addition to there being a difference between the number of testes responding to a given dose of LH, the numbers of sperm released into the medium appears to be dose dependent. The difference, although not statistically significant, between 5 µg LH/ml- and 10 µg/ml-induced spermiation in terms of sperm per ml represents a 50% increase with the higher dose. The biological meaning of this difference is difficult to assess, for data relating sperm counts to fertilizing ability in anurans is not available. In humans, an 83% reduction of sperm per ml

(an average value of 1.2×10^8 reduced to 0.2×10^8) results in clinical infertility (Guyton, 1976). As anurans rely on external fertilization, it is conceivable that a lower percent reduction in sperm count could affect fertility.

The median dose (dose at which 50% of the testes spermiated) of LH *in vitro* is approximately five times the median dose found *in vivo*. However, the meaning of this comparison is difficult to evaluate for factors such as blood volume or clearance alters the effective dose *in vivo*. Also, the testes incubated *in vitro* were not perfused and hormone delivery to the tissue may not be as effective as *in vivo*.

Several problems arise in the interpretation of inhibitor implant results and they should be considered preliminary. The method of estimating number of sperm is subjective and generally not acceptable for routine observations. Cloacal volume, and thus, apparent numbers of sperm per drop could be affected by water uptake by the frog. The effect of the inhibitors on water uptake is not known, but could easily bias results. Furthermore, handling stress, resulting in the emptying of the cloacae, could result in an apparent elevation of a spermiation response. The effect of the inhibitors on a stress response is also not known.

Another drawback of interpreting results of *in vivo* experiments is that the inhibitors may interact with the gonadal-hypothalamal-pituitary negative feedback system regulating gonadal function. By inhibiting negative feedback, elevated spermiation responses could be noted due to maximal activity of the gonadotropin releasing mechanism. The effects of CI-628 and Cytadren could be interpreted in this manner, particularly

since the response is more marked in the 12-day post implant experiments.

These experiments served as guides to further experimentation *in vitro*. In that the *in vivo* results of Flutamide implants concurred with *in vitro* experiments, the effects are worthy of consideration. The inhibitory effect of Flutamide on sperm release as suggested by these implant experiments supports the hypothesis that androgens are important in gonadotropin induced sperm release. The lack of a dose response to Flutamide in the *in vivo* experiments indicate that even the smallest dose was sufficiently high to inhibit the response.

The preincubation experiment (VI) using CI-628 *in vitro* points to an inhibitory role of estrogen in sperm release. Again, the complexity of interaction of estrogens and androgens in the testis preclude making any definitive statement regarding the particular role either group of steroids may play during the release of sperm.

Table 3. Experiment III. Effect of dose of LH on spermiation from intact *Hyla regilla*: percent animals spermiating.

Treatment	n	No. Animals Spermiating	% Animals Spermiating
Saline	5	0	0%
1 µg LH	5	2	40%
2.5 µg LH	19	18	95%
5 µg LH	5	5	100%
10 µg LH	5	5	100%

Table 4. Experiment IVA. Effect of dose of LH on spermiation from *Hyla regilla* testes *in vitro*: Percent testes spermiating.

Treatment	n	No. Testes Spermiating	% Testes Spermiating
Saline	7	1	14%
0.1 µg LH/ml	5	1	20%
1.0 µg LH/ml	5	1	20%
5.0 µg LH/ml	4	2	50%*
10.0 µg LH/ml	6	5	83%*

* Significantly different from saline ($p<0.05$)

Table 5. Experiment IV B. Effect of dose of LH on mean no. sperm released/ml from *Hyla regilla* testes *in vitro*.

Treatment	n	Mean sperm/ml x 10^6	SD sperm/ml x 10^6
Saline	4	0.30	0.60
10 µg LH/ml	4	2.28*	1.48
Saline	4	0	--
5.0 µg/LH ml	4	1.13*	1.05

* Significantly different from respective saline controls ($p < 0.05$) using a Student's paired t-test. The two doses of LH, however, are not significantly different using a Student's t-test on the mean difference (between the LH-treated and saline-treated contralateral testes) of the doses ($p < 0.2$).

Table 6. Spermiation response of male Hyla regilla implanted with steroid inhibitors when injected with 2.5 μg LH.

Treatment	n	Spermiation Response				
(4 Days Post-Implant)		Negative	Positive			
			Very low ~10 sperm	Low ~100	Med. ~500	High ~10^3	Very High ~10^4
Experiment VA							
Empty Capsules (1/2 cm)	5	-	-	-	2	1	2
CI-628 (1/2 cm)	5	-	-	-	3	-	2
Cyadren (1/2 cm)	5	3	-	-	-	2	-
Flutamide (1/2 cm)	5	1	-	2	2	-	-
Experiment VB							
(4 Days Post-Implant)							
Empty Capsules (1 cm)	10	1	0	0	2	4	3
Flutamide (1 cm)	8	1	-	1	4	1	1
Flutamide (1/2 cm)	9	2	-	2	1	3	1
Flutamide (1/4 cm)	92	2	-	2	3	-	

Table 6 continued

Experiment VA Treatment	n	Spermiation Response				
(12 Days Post-Implant)		Negative	Positive Very low ~10 sperm	Low ~100	Med. ~500	High $\sim 10^3$	Very High $\sim 10^4$
Empty Capsules (1/2 cm)	5	1	-	-	2	1	1
CI-628 (1/2 cm)	5	-	-	-	-	2	3
Cyadran (1/2 cm)	5	1	-	-	1	2	1
Flutamide (1/2 cm)	4	1	-	-	-	1	1

Table 7. Gonadotropin-stimulated spermiation from Hyla regilla in the presence of Flutamide, an androgen-receptor innibitor, in vitro: number of testes responding.

Experiment No.	Treatment*	n	No. Testes Spermiating	Exact Probability**
VA	Flutamide + LH	5	4	
				0.545
	LH	6	5	
VB	Flutamide + LH	3	1	
				0.143
	LH	4	4	
IA	Flutamide + LH	23	8	
				0.007
	LH	23	17	
			Combined	0.025

* Doses of LH: 10 µg LH/ml; Flutamide 100 µg Flutamide/ml

** Exact probabilities computed using a Fisher's Exact test. Combining probabilities from several experiments using different methods is in fact, a more stringent test.

Table 8. Effect of CI-628, an estrogen inhibitor, on LH-induced release of sperm from Hyla regilla testes in vitro.

Treatment	n	Mean sperm/ml x 10^5	SD sperm/ml
250 µg CI-628 plus 12.5 LH	7*	9.78	7.66
12.5 µg LH	8	18.8**	9.72

* One value was rejected due to an excessively high pretest count.
** Probability of mean difference using a Student's t test $p < 0.1$.

Table 9. Effects of gonadotropins on the spermiation response.

Species/ Gonadotropin	Treatment conditions: Intact Males	Hypox Males	Testis *in vitro*	Author	Notes
A. *Bufo arenarum*					
Toad pituitary	*			Houssay and Lascano Gonzalez, 1929	1st description
Toad pituitary	*			Houssay *et al.*, 1929	
Toad pituitary	*			deRobertis *et al.*, 1946	vacuolization of Sertoli cells
Toad pituitary	*			Houssay, 1947	
Toad pituitary hCG	*		*	Mancini and Burgos, 1947	
serum gonadotropin				Burgos and Mancini, 1947a, b	vacuolization of Sertoli cells
Gonadotropin	*			Mancini and Burgos, 1948	mucopolysaccharide
Pregnancy urine hCG	*			Galli-Mainini, 1947a, b; 1948	
Pregnancy urine hCG		*		Galli-Mainini and Pinto 1947	
Pregnancy urine	*			Flores, 1948	
Gonadotropin	*			Robbins and Parker, 1948	
Toad pituitary	*			Burgos, 1951	distension of seminiferous tubules
hCG	*			Burgos and Rubino, 1952, 1953	
Toad pituitary hCG	*			Houssay, 1954	
Gonadotropin	*			de Allende and Astrada, 1957	followed time course of spermiation

Table 9. continued

Species/ Gonadotropin	Treatment conditions: Intact Males	Hypox Males	Testis In vitro	Author	Notes
LH	*			Burgos and Vitale-Calpe, 1967	Increase H_2O, Na^+ content; histological description of sperm release.
8. *Rana pipiens*					
Frog pituitary	*			Rugh, 1937; 1939	
Pregnancy urine	*			Galli-Mainini, 1948	
Pregnancy urine	*			Wittberger and Miller, 1948	
Gonadotropin	*			Robbins and Parker, 1949	
hCG	*			Maskins and Sherman, 1949	time-dose response
hCG	*			Samson, 1950	seasonal sensitivity
Pituitary gonadotropin pregnancy urine/hCG		*		Clark et al., 1959	blocked by adrenolytic drugs
hCG	*			Sulman, 1951	adrenalectomy or adrenalectomy plus adrenal cortex extract had no effect
Gonadotropin, pregnancy urine		*		Kissen, 1954	
Gonadotropin			*	Phillips and Feldhaus, 1957	
hCG, LH, FSH,				Witschi and Chang, 1959	LH 1000X dose FSH

Table 9. continued

Species/ Gonadotropin	Treatment conditions: Intact Males	Hypox Males	Testis *in vitro*	Author	Notes
"Purified" LH, FSH, hCG	*			Burgos and Fadman, 1959	FSH stimulation attributed to LH contamination
C. *Rana temporaria*					
Frog pituitary extract	*			van Oordt *et al.*, 1951	
hCG	*			Wille, 1956a	seasonal sensitivity
hCG	*			Wille, 1956b	PTU increases spermiation T_4 decreases spermiation
Frog pituitary extract	*		*	van Dongen, 1958; 1962 van Dongen and deKort, 1959 van Dongen *et al.*, 1959, 1960	mucopolysaccharide content of Sertoli cell disappears with gonadotropin treatment
D. *Rana esculenta*					
Gonadotropin	*	*		Ewy and Pigonlowa, 1957	response not blocked by adrenolytic drugs
LH	*			Crăță, 1949	
hCG	*	*		Crabbé, 1954a, b	PTU increases responsiveness T_4 decreases responsiveness
E. *Bufo* species					
Gonadotropin	*			van Oordt and Klomp, 1946	coined word, "spermiation" *B. bufo*
Pregnancy urine	*			various, cited by Galli-Malnini 1948	various species used in pregnancy testing to date

Table 10. Effects of epinephrine on the spermiation response.

Species	Treatment conditions: Intact Males	Hypox Males	Testis In vitro	Author	Notes
Rana pipiens	+	+		Galli-Mainini, 1948	
R. pipiens	+	+		Robbins and Parker, 1949	
R. pipiens	+	+		Sulman, 1951	
R. pipiens	+			Clark et al., 1951	response blocked by adrenolytic drugs
R. pipiens			+	Phillips and Feldhaus, 1957	
Rana esculenta	+			Hinglais and Hinglais, 1949	
R. esculenta	+			Crosseri and Bovaqua, 1950	vacuolization of Sertoli cell
R. esculenta	+	+		Ewy and Pigonlowa, 1957	adrenolytic drugs did not block response
Rana temporaria	+			Hinglais and Hinglais, 1949	
R. temporaria	+			van Oordt et al., 1954; 1959	H_2O uptake by tubules
R. temporaria	+			Willo, 1957b	seasonal sensitivity
R. temporaria	+			Willo, 1957a	PTU decrease sensitivity to adrenalin

Table 10 continued

Species	Treatment conditions: Intact Males	Hypox Males	Testis *in vitro*	Author	Notes
R. temporaria	+			van Dongen, 1959 van Dongen *et al.*, 1959; 1960	Increase testicular weight and decrease H_2O uptake *in vitro* but not *in vivo*. No disappearance of mucopolysaccharide in Sertoli cells.
Rana nigromaculata	+	+		Li and Chang, 1949	sectioning of spinal cord and removal of sympathetic ganglia had no effect on response.
Leptodactylus ocellatus	+	+		Houssay and Burgos, 1953	response not affected by adrenalectomy, adrenolytic drugs did not block response
Xenopus laevis	+	+		Robbins and Parker, 1948	
Bufo arenarum	+			Houssay and Burgos, 1953; Galli-Mainini, 1948	
Bufo fowleri	+			Galli-Mainini, 1948	
Bufo bufo asiaticus	+			Li and Chang, 1949	
Bufo viridus	+			Sulman, 1951	
Hyla arborea	+			Sulman, 1951	