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Inhibin: From Concept to Reality*

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In numerous physiological and pathological conditions, the levels of two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), change independently (see review by Franchimont and Burger, 1975).

The secretion of LH is controlled by positive and negative feedback

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mechanisms induced by androgens and estrogens (see review by Setchell *et al.*, 1977).

In contrast, the secretion of FSH in both men and women, correlates inversely with gametogenesis.

I. Relationship between Gametogenesis and FSH Secretion: Definition of Inhibin

The concept of a gonadal hormone regulating FSH secretion and related directly or indirectly to gametogenesis originated many years ago. The hormone was called androhormone by Martins and Rocha (1931), inhibin by McCullagh (1932), and X hormone by Klinefelter *et al.* (1942). The concept has been strengthened by recent studies in both humans and animals that show an inverse relationship between gamatogenesis and the secretion of FSH.

Before puberty, the basal secretion of FSH and its response to exogenous LH-releasing hormone (LH-RH) are greater than those of LH. In contrast, as puberty develops, the levels of FSH, after a transitory rise, level off and may even fall. Thus, during pubertal development in the rat, the levels of LH increase in parallel with the development of spermatogenesis and testosterone secretion. In contrast, the level of FSH is high at the prepubertal stage when compared to adult levels. Serum FSH begins to fall coincidentally with the appearance of the first mature sperm and reaches adult levels at the time of complete spermatogenesis (Swerdloff *et al.*, 1971). Similarly, in boys and in girls the levels of FSH increase progressively from the first to the third stage of puberty and then level off from the third to the fifth stage. Moreover, if an LH-RH test is performed in males prior to the onset of puberty (stage 1), during puberty (stages 2 and 3), and after completion of sexual maturation (stage 5) it can be shown that the cumulative FSH response to 25 μg of LH-RH, reflecting the available pituitary reserve of FSH, diminishes significantly from stage 1 to stage 5 (adult) (Franchimont *et al.*, 1975a).

In women, the level of FSH increases at the beginning of the menstrual cycle, when the follicles are small. As the development of the follicle progresses, the level of FSH falls. Moreover, in women approaching the menopause, the level of FSH is greatly increased, sometimes up to five times the values observed at midcycle, whereas estradiol secretion remains normal and LH concentrations are unchanged (Korenman and Sherman, 1976). When the menopause

becomes established, the levels of FSH increase further to values that are clearly higher than those seen at the midcycle peak in menstruating women. LH, on the other hand, usually rises less dramatically and may not exceed midcycle peak levels. The changes observed at this stage of a woman's life are accompanied by a marked reduction of ovarian follicles and failure of their normal maturation.

In men suffering from germinal cell failure without damage of Leydig cells, due to various causes, the levels of FSH are often increased, whereas LH levels are frequently normal (Franchimont, 1972; Franchimont *et al.*, 1975b). In these patients, there is a linear relationship between the level of FSH and both qualitative (Franchimont *et al.*, 1972) and quantitative (De Kretser *et al.*, 1972) aspects of spermatogenesis observed in testicular biopsies. Thus, when spermatogenesis is arrested before the spermatid stage, the level of FSH is always elevated. When spermatids are present in the seminiferous tubules, the levels of FSH are usually normal (Franchimont *et al.*, 1972). This relationship does not occur with LH.

To interpret the consistently high FSH levels when spermatids are absent, one may postulate that spermatid maturation induces or permits the formation of a factor that controls FSH secretion. This interpretation would be consistent with the view that there is a specific stage in the process of spermatogenesis involved in testicular feedback on FSH secretion—at spermatid formation (Franchimont *et al.*, 1972) or at the point of maturation of spermatozoa (Johnsen, 1970). Alternatively, spermatids may have no specific function in FSH regulation. The depletion of these cells may merely be a reflection of a severe depression of the basal germinal cell population. The germinal cells could, under these conditions, produce less inhibitory factor, and FSH levels would increase as a result. The studies of De Kretser *et al.*, (1972) support this latter interpretation. These investigators have shown that there is an inverse correlation between FSH levels and the severity of reduction in germinal cells from spermatogonia to late spermatids. The most significant correlation was that found with the number of spermatogonia per tubular cross section.

Paulsen *et al.* (1972) suggested an additional explanation for the elevation in FSH observed in oligospermic men. The depletion of germinal cell elements may correlate with abnormalities of another process, such as Sertoli cell function, that actually controls FSH secretion. Since germinal cell depletion and Sertoli cell dysfunction are probably interrelated, the apparent correlation between FSH levels and germinal cell depletion may, in fact, reflect concomitant Sertoli cell abnormalities.

The regulating factor for FSH, related directly or indirectly to gametogenesis, in this review, will be called "inhibin" as proposed by McCullagh in 1932.

Definition of Inhibin

Inhibin can be defined as a peptidic factor of gonadal origin that specifically or selectively lowers the rate of secretion of FSH.

Other names have been given to substances isolated from a variety of biological fluids and possessing similar properties: folliculostatin (Schwartz and Channing, 1977), follitropin-suppressing principle (Sairam *et al.*, 1978), Sertoli cell factor (Steinberger and Steinberger, 1976).

We will nevertheless give the same name "inhibin" to all the various preparations described in the literature that meet the definition of this hormone, although we cannot exclude differences in their physiocochemical properties or physiological significance.

II. Sources of Inhibin and Techniques for Purification

Inhibin has been detected and partially purified from human seminal fluid (Franchimont, 1972; Franchimont *et al.*, 1975b), bovine seminal fluid (Franchimont *et al.*, 1975c; Sairam *et al.*, 1978; Chari *et al.*, 1978), ram rete testis fluid (RTF) (Setchell and Sirinathsinghji, 1972; Setchell and Jacks, 1974; Baker *et al.*, 1976; Davies *et al.*, 1978; Franchimont *et al.*, 1977, 1978; Cahoreau *et al.*, 1979), extracts of spermatozoa (Lugaro *et al.*, 1974; Setchell and Main, 1974), testicular extracts (Lee *et al.*, 1974; Keogh *et al.*, 1976; Baker *et al.*, 1976; Moodbidri *et al.*, 1976), ovarian extracts (Hopkinson *et al.*, 1975, 1977a; Chappel *et al.*, 1978), bovine follicular fluid (De Jong and Sharpe, 1976; Welschen *et al.*, 1977), porcine follicular fluid (Welschen *et al.*, 1977; Marder *et al.*, 1977; Lorenzen *et al.*, 1978), human follicular fluid (Chari *et al.*, 1979), as well as the culture medium of Sertoli cells (Steinberger and Steinberger, 1976) and granulosa cells (Erickson and Hsueh, 1978).

The extraction techniques used include homogenization in aqueous media (Lee *et al.*, 1974; Nandini *et al.*, 1976; Moodbidri *et al.*, 1976), precipitation with organic solvents (ethanol, acetone, ether) (Franchimont *et al.*, 1975b,c; Hopkinson *et al.*, 1977a; De Jong and Sharpe, 1976), and Deae-cellulose chromatography with elution by pH gradient (Franchimont *et al.*, 1977) or molarity gradient (Baker *et al.*, 1978), carboxymethyl chromatography (Baker *et al.*, 1978), precipitation with ammonium sulfate (Baker *et al.*, 1978; Chari *et al.*, 1979), and filtration using various molecular sieves.

In this review, experiments will be concerned with active material extracted from human seminal plasma and ram rete testis. Some details of the extraction will be given to provide the reader with information concerning the nature of the material used.

A. Extraction of Inhibin from Human Seminal Plasma (HSP) (Franchimont *et al.*, 1979a)

Human seminal plasma is centrifuged at 4°C for 10 minutes to remove spermatozoa. The supernatant is chromatographed in 3-ml fractions on a Sephadex G-100 column (2.6 × 92 cm), previously equilibrated with Sorensen phosphate buffer (0.05 *M*, pH 7.5). HSP elutes in three main peaks on Sephadex G-100. The first peak (HSP_1) elutes just after the void volume of the column. The second (HSP_3) and third (HSP_4) are markedly delayed. Fractions eluted between HSP_1 and HSP_3 are pooled to form fraction HSP_2. Fraction HSP_{3-4} consists of the tail of peak HSP_3 and the beginning of HSP_4 (Fig. 1). The fractions corresponding to these different zones are pooled and lyophilized. In the example, the protein concentrations estimated by Folin's method were 922 mg for HSP_1, 262 mg for HSP_2, 448 mg for HSP_3, 56.4 mg for HSP_{3-4}, and 13 mg for HSP_4 from 100 ml of HSP. Only HSP_3 and HSP_{3-4} fractions contained the inhibin-like substance capable of selectively inhibiting the secretion of FSH both *in vivo* (reduction of serum levels of FSH in rats 24 hours after castration) and *in vitro* (reduction of the FSH released in culture medium by LH-RH in rat pituitary cell culture).

B. Extraction of Inhibin from Ram Rete Testis Fluid (RTF) (Franchimont *et al.*, 1978, 1979a)

Ram rete testis fluid is centrifuged to eliminate spermatozoa and other cells and then lyophilized. A quantity of lyophilyzate corresponding to 300 ml of RTF is dissolved in 20 ml of eluting buffer and submitted to gel filtration on Sephadex G-100 (90 × 5 cm). The

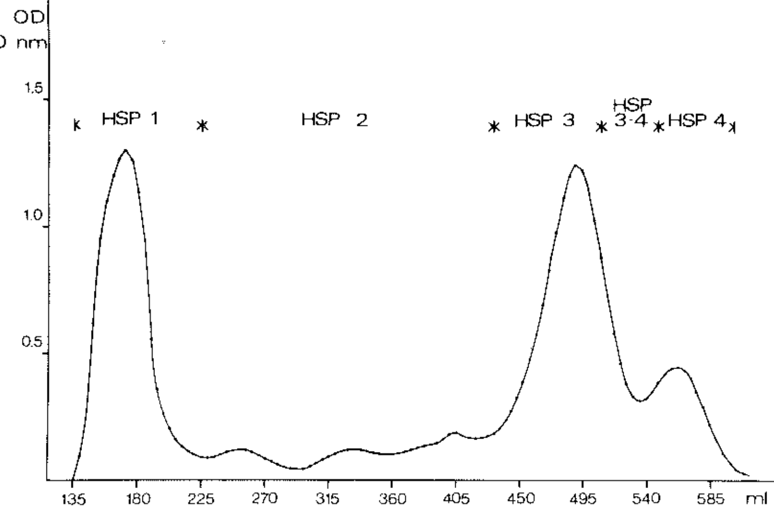

FIG. 1. Elution profile of human seminal plasma (HSP) filtered on Sephadex G-100. Abscissa: volume of elution; ordinate: optical density at 280 nm. External volume: 145 ml; salt peak (KI) is eluted from 566 ml. Reproduced from Franchimont *et al.* (1979a), with permission of the publisher.

Sephadex is equilibrated, and the elution is performed with ammonium bicarbonate buffer, 0.05 *M*, pH 7.5, at a constant flow of 60 ml/hour. In the *in vivo* and *in vitro* bioassays, RTF_{1a} and RTF_3 both possessed biological activity (Fig. 2).

Seventy milligrams of fraction 1a were submitted to preparative isotachophoresis Uniphor LKB 7900 on acrylamide–bisacrylamide

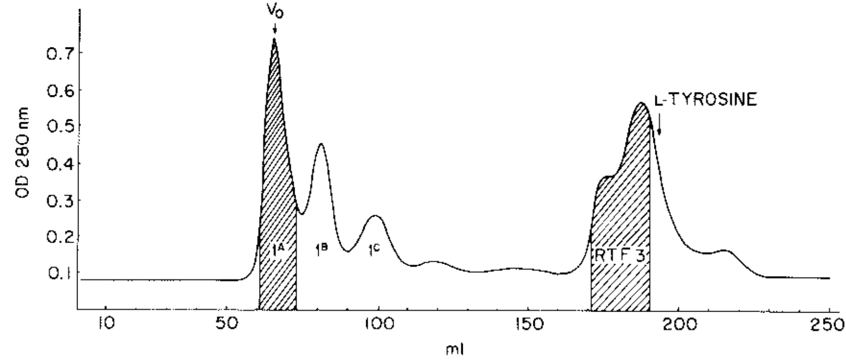

FIG. 2. Elution profile of lyophilized ram rete testis fluid (RTF) on Sephadex G-100 column (88 × 5 cm). Abscissa: milliliters of elution; ordinate: optical density at 280 nm. The hatched area represents biological activity.

(3.3%) gel containing riboflavin, 0.8 mg/dl; TEMED, 30 μl/dl; and ammonium persulfate, 10 mg/dl. The gel is polymerized in the leading buffer Tris-HCl, pH 7.5, with a final concentration of 0.01 *M*. The anodic and elution buffer is constituted by Tris 0.02 *M* added with HCl 1 *M* to reach pH 8. The terminating solution is constituted of Tris 0.02 *M* and valine 0.01 *M*, pH 9. The following discrete spacers, each 125 μmol, are used: ACES, MOPS, HEPES, Bicine, TAPS, Histidine. A constant current of 85 μA is applied, and voltage at the beginning of isotachophoresis is 0.350 kV. The elution rate is 10 ml/hour.

By optical density at 280 nm, seven peaks were obtained, one of which, RTF_{38}, was biologically active (Fig. 3).

RTF_{38} is submitted to semipreparative polyacrylamide gel electrophoresis (7.5%) in Tris-HCl buffer, pH 8.9, 0.2 *M*. Five components are isolated and recovered by this method, from the anode to the cathode: $RTF_{38a,b,c,d,e}$. RTF_{38c} is biologically active, migrates as a single band in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (R_f 0.51), is homogeneous in high-performance liquid chromatography and gives a simple band when submitted to immunoelectrophoresis with an anti-RTF_{38} serum.

III. Detection and Measurement of Inhibin

The study of inhibin was held up for a long time by the lack of biological tests suitable for its detection and measurement. Now, bioassays of inhibin activity are available *in vivo* and *in vitro*. Radioimmunoassays and radioreceptor assays are being developed.

Methods of measuring inhibin must obviously meet criteria of specificity, sensitivity, precision, and reproducibility. These criteria have been evaluated very little to judge from the literature, and to date most methods have been concerned with detection rather than quantitative measurement.

Furthermore, a reference preparation has been sadly lacking until quite recently, when Hudson *et al.* (1979) distributed a lyophilized preparation of ovine testicular lymph given an arbitrary biological potency of 1 U/mg.

In bioassays, both *in vivo* and *in vitro*, specificity is ascertained in three ways. First, inhibin activity is defined by its preferential action on FSH secretion; the threshold dose that affects the level of FSH is less than the one that affects LH secretion. The experimental condi-

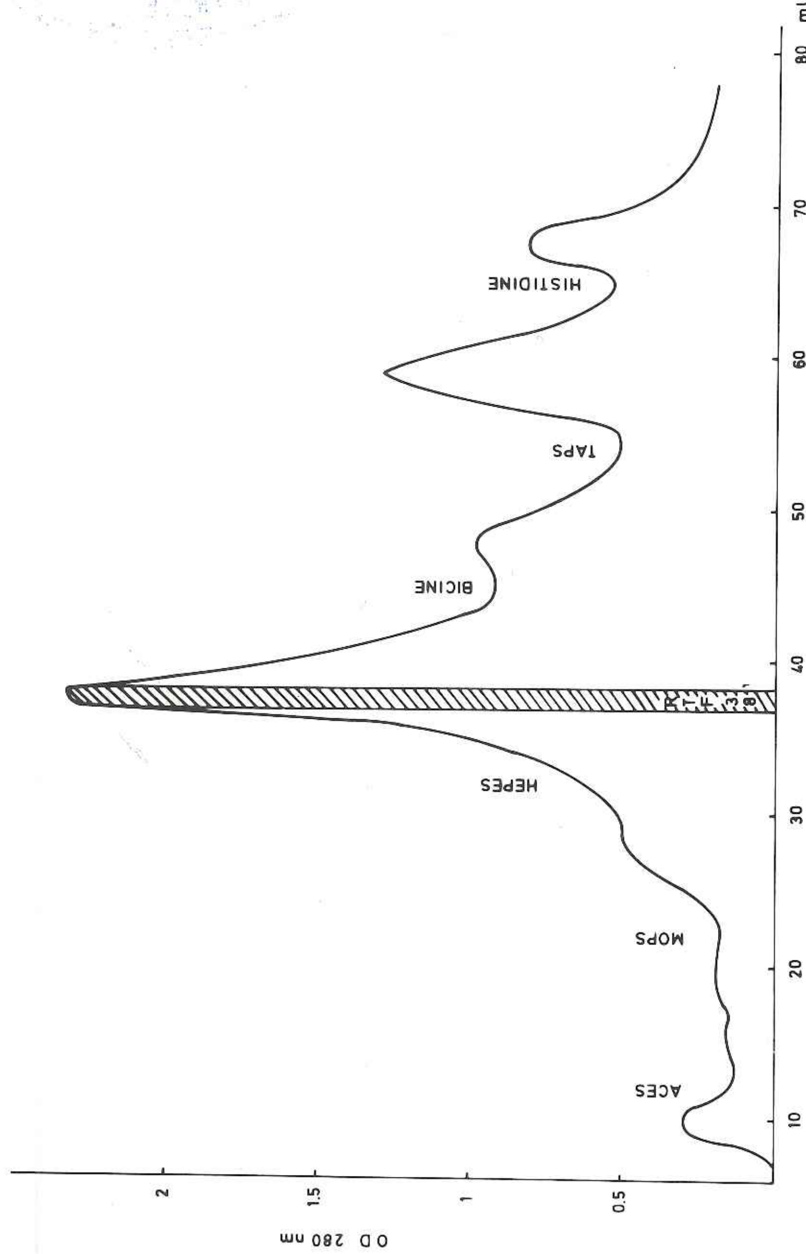

FIG. 3. Separation profile of ram rete testis fluid (RTF_{1a}) by preparative isotachophoresis. Abscissa: milliliters of elution; ordinate: optical density at 280 nm. The hatched area represents the biologically active fraction. Discrete spacers are indicated where they are eluted under experimental conditions described in the text.

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tions *in vivo* are often chosen to ensure this selective or specific response on FSH levels. Second, the secretion of other pituitary hormones, such as prolactin and TSH, should not be affected by inhibin. Last, the test preparation should not contain any substance known to be capable of interfering in the assay for example, sex steroids, fractions of gonadotropins, and enzymes.

A. *In Vivo* Tests

1. *Selective Reduction of Serum FSH Levels by Inhibin*

Reduction occurs in intact rats or rats castrated a few hours or several days previously (Fig. 4; Table I). The age and the sex of the rat affect this test (see review of Setchell *et al.*, 1977).

Many authors now use 35-day-old rats; at this age serum levels of FSH have risen sharply (Nandini *et al.*, 1976). Recently, castrated adult mice have also been used (Lee *et al.*, 1977). Other animals were used in earlier attempts to identify inhibin; these included castrated rams (Lee *et al.*, 1974; Baker *et al.*, 1976) and castrated rabbits (Franchimont *et al.*, 1977). The inhibin preparations were injected intravenously.

The duration, frequency, and routes of injection have varied from one study to another. One or several injections have been given over 24 hours or several days intravenously, subcutaneously, or intraperitoneally. It is worth stressing that the results are more consistent when injections are given twice daily for 72 hours, starting immediately after castration.

Finally, Hopkinson *et al.* (1977a) give dihydrotestosterone for 48 hours, in doses ranging from 20 to 120 μg per/100 gm body weight per day, at the same time as the test material to male and female rats (weighing 320–350 gm) that have just been castrated. This maintains LH at precastration levels whereas the rise in FSH after castration is unaffected.

These methods are useful for the detection of inhibin in biological fluids, but their lack of precision and reproducibility limit their application (Table I).

2. *Inhibition of Human Chorionic Gonadotropin (HCG)-Induced Ovarian and Uterine Weight Increase*

Another *in vivo* assay method measures the inhibition of the (HCG)-induced ovarian or uterine weight increase in immature rats or mice following the administration of preparations containing in-

TABLE I

SOME CHARACTERISTICS OF INHIBIN ASSAYS[a]

Parameters	*In vivo*: Inhibition of FSH levels 24 hours after castration[b]	*In vivo*: Inhibition of HCG-augmented uterine weight increase[c]	*In vitro*: Inhibition of LH-RH-induced FSH release	Radioimmunoassay: RTF homogeneous system, RTF_c as tracer and reference anti-RTF_{38} serum
Number of dose levels of any (standard or unknown) preparations	4	4	> 4	At least 10
Measured responses	Absolute FSH levels	Mouse uterus weight	Absolute FSH levels or % of FSH reduction	$B_x/B_o \times 100$
Slope (*a*)	−125.8	23.9	−46.5	−31.6
Index of precision (λ)	0.25–0.84	0.1	0.04–0.15	0.01–0.1
Coefficient of intraassay variation	12.3–21%	8.3%	3.2–6%	< 5%
Coefficient of interassay variation	32%	11.7%	17%	< 12%

[a] FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin; LH-RH, luteineizing hormone-releasing hormone; RTF, ram rete testis fluid.

[b] Franchimont *et al.* (1977).

[c] Ramasharma *et al.* (1979).

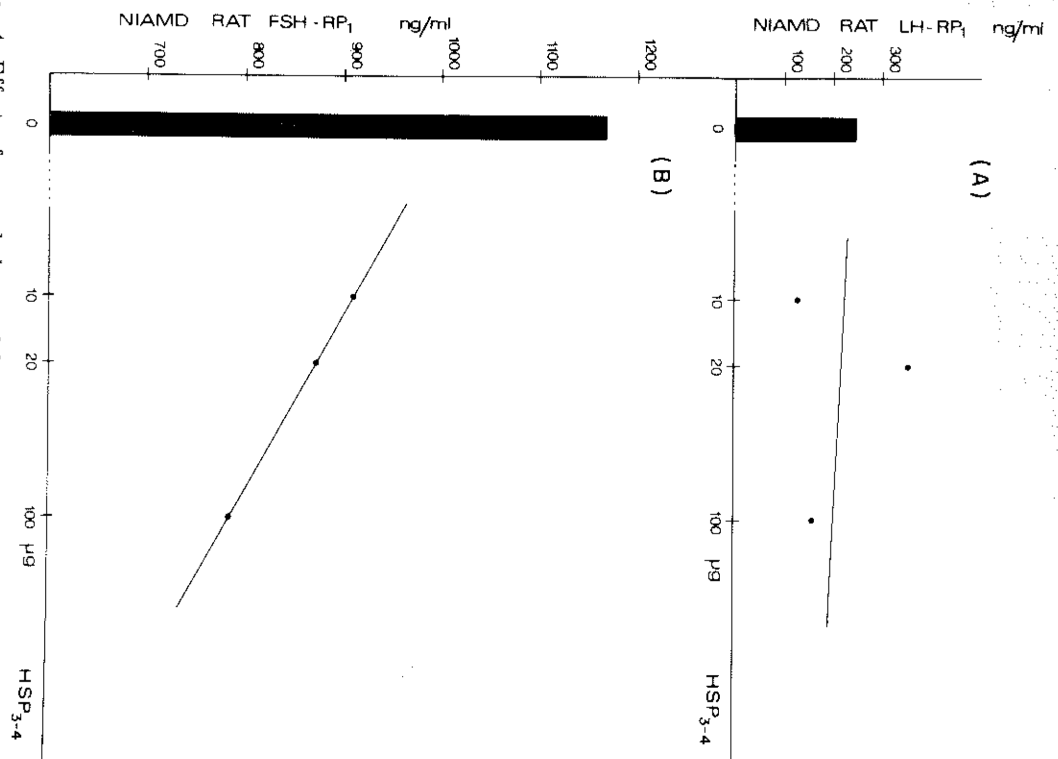

FIG. 4. Effect of several doses of human seminal plasma (HSP_{3-4}) on follicle-stimulating hormone (FSH) (panel B) and luteinizing hormone (LH) (panel A) levels in castrated rats. Male Wistar rats weighing 120 ± 10 gm were castrated at time 0. Twenty-two hours later, they received by tail vein either 0.5 ml of physiological saline (control group) or the material to be tested, diluted in 0.5 ml of 0.9% NaCl. Two hours later (24 hours after castration) the animals were anesthetized with ether and blood was removed by cardiac puncture for assay of FSH and LH. Each fraction was assayed in 6 rats, and the control group consisted of 10 animals. Black columns represent the control value of castrated rats treated with NaCl 0.9%. Formulas of regression lines and their significance are as follows. Panel A (LH): $Y = -22.2 \log X + 246.6$, $t = 0.094$ (NS), $r = 0.093$; panel B (FSH): $Y = -125.8 \log X + 1035.9$, $t = 55.1$, $r = 0.999$. There is a dose-response relationship for FSH levels only. Reproduced from Franchimont (1979a), with permission of the publisher.

hibin. Chari *et al.* (1976) have used ovarian weights in rats, and Setchell and Sirinathsinghji (1972) and Ramasharma *et al.* (1979) measured uterine weights in mice.

The explanation of this test rests on the fact that the increase in weight induced by HCG is possible only in the presence of endogenous FSH, which HCG liberates approximately 1 hour after administration (Ramasharma *et al.*, 1979) (Fig. 5). The inhibin preparations act by reducing endogenous FSH secretion. Although the results of individual experiments appear to be interesting, we agree with Setchell *et al.* (1977) that this type of test is often unreliable. The uterine and ovarian response, although simple to evaluate, is not reproducible from one assay to another presumably because of variability in response of the animals. It is also sensitive to slight variations in the dose of HCG. If too high a dose of HCG is given, the uterine and ovarian responses are reduced and the effect of inhibin is lost. Aspects of this method used by Ramasharma *et al.* (1979) are summarized in Table I.

B. *In Vitro* Assays

1. *Pituitary Cell Culture*

The monolayer pituitary cell culture to assay inhibin was first developed by Baker *et al.* (1976) in Melbourne. Dispersed anterior pituitary cells from mature rats in short-term culture are used according to the method of Hopkins and Farquhar (1973).

The cells are cultured for 2 days in the absence of test material, which is then added for the next 3 days. The culture medium is then removed for the assay of FSH and LH (Fig. 6). The cells are further cultured over a 6-hour period in semisynthetic medium, Dulbecco Modified Eagle's Minimum Essential Medium (DHEM), with or without the addition of a dose ($10^{-8}M$) of LH-RH. The culture medium is collected for the assay of FSH and LH under basal conditions or after stimulation by LH-RH.

At the end of this culture period, the cells are recovered, then destroyed by the addition of distilled water, and the gonadotropin content of the cells is determined.

There is a relationship between the logarithm of the dose of inhibin added to the culture, on the one hand, and, on the other, the secretion of FSH into the medium after 3 days of culture without LH-RH, after 6 hours of incubation with LH-RH, and the FSH content of the cells (Fig. 6.).

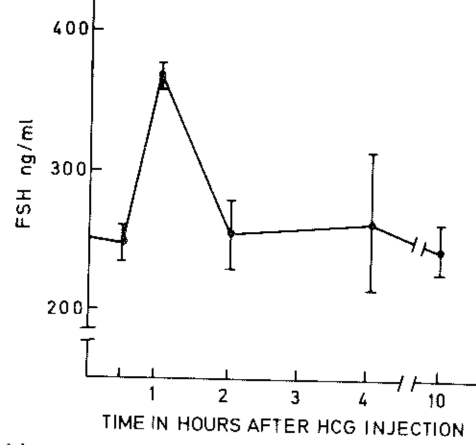

FIG. 5. Effect of human chorionic gonadotropin (HCG) injection on serum follicle-stimulating hormone (FSH) levels. HCG was administered at time 0, and serum samples were collected at different time intervals thereafter ($N = 5$). FSH in serum (ng/ml mean ± SD) was assayed using rat FSH radioimmunoassay provided by NIAMD. Reproduced from Ramasharma *et al.* (1979), with permission of the authors and publisher.

The selective effect of inhibin on FSH secretion is evident. In fact, the liberation of LH is affected only in response to LH-RH and by a much higher dose of inhibin than that required to impair FSH secretion. Furthermore, basal LH secretion after 3 days' incubation and intracellular LH content are not altered by increasing amounts of inhibin.

The slope of the reduction curve of FSH is steepest when FSH secretion is stimulated by LH-RH (Fig. 6). This condition forms the best *in vitro* bioassay for measuring inhibin (Table I).

In order to ensure the specificity of the observed response, we also measure the levels of prolactin and thyrotropin-stimulating hormone (TSH). Normally, these levels remain unchanged by the addition of LH-RH, irrespective of the presence or the absence of inhibin. The levels of these hormones fall if there is any nonspecific toxic effect on the pituitary cell culture.

2. *Incubated Pituitary Halves*

A second *in vitro* assay has been described by Setchell *et al.* (1977). Pituitary halves from adult rats, incubated in a suitable medium, retain a reasonable histological appearance for some hours and release FSH and LH into the medium. This release can be stimulated by LH-

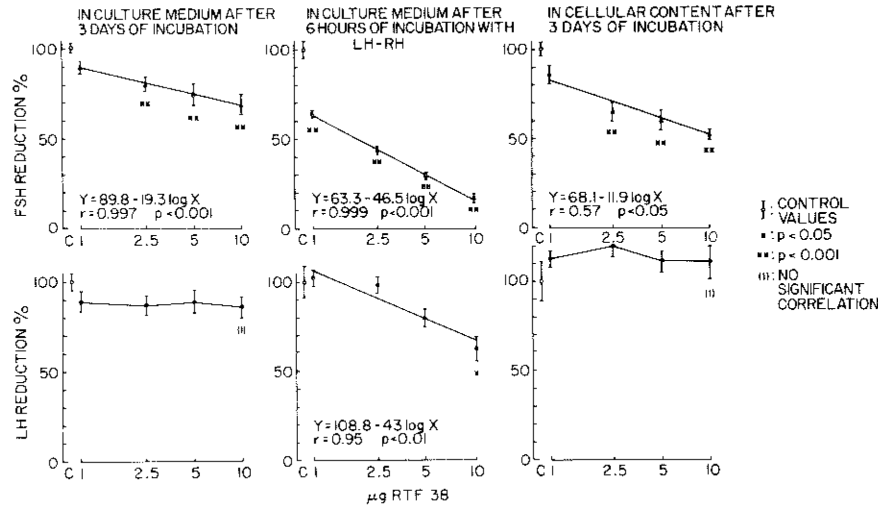

FIG. 6. Effect of increasing amounts of a preparation of inhibin (RTF_{38}) on follicle-stimulating hormone (FSH) (upper panels) and luteinizing hormone (LH) (lower panels) secretion by dispersed pituitary cells in culture medium after 3 days of incubation under basal condition (left) or after 6 hours of incubation with 10^{-8} *M* LH-releasing hormone (LH-RH) (center). The right-hand panels correspond to the intracellular FSH and LH contents in basal conditions after 3 days of incubation. Ordinate: reduction of FSH and LH secretion or intracellular content expressed as percentage of the control value (100%).

RH. Inhibin slightly reduces the basal production of FSH but has a marked effect on the LH-RH-stimulated release (Fig. 7). Release of LH is unaffected or may even be slightly increased. In our hands, this assay has been found to be less sensitive, precise, and reproducible, but much shorter and simpler, than the dispersed cell culture assay.

C. Radioligand Assays

Two preliminary methods for radioimmunoassay (Sheth *et al.*, 1978) and radioreceptor assay (Sairam *et al.*, 1978) have now been described. The published data are still too provisional to allow judging their quality or usefulness.

We have set up a radioimmunoassay for ovine inhibin with a molecular weight greater than 10,000.

The purified preparation of RTF_{38c} (see Fig. 3) was used as a tracer and reference preparation. This preparation was labeled according to the method of Greenwood *et al.* (1963). Labeled hormone was separated from radioactive salts by Sephadex G-25 filtration. One of the antisera obtained in rabbits with a semipurified inhibin preparation (RTF_{38}) was used as a final dilution of 1:200,000. A double antibody system allowed the separation of free labeled inhibin from labeled inhibin bound to antibody.

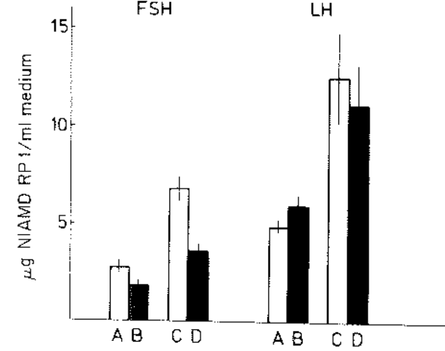

FIG. 7. Effect of Amicon-treated ram rete testis fluid (RTF) on the response to luteinizing hormone-releasing hormone (LH-RH) of incubated rat pituitary (RP) halves. Two pituitary halves were incubated for 4 hours at 37°C in an atmosphere of 95% O_2, 5% CO_2. LH-RH was added to a concentration of 4 ng/ml. All pituitary halves were preincubated for 30 minutes in medium alone. A, medium alone; B, medium + RTF; C, medium + LH-RH; D, medium + RTF + LH-RH. RTF produced a slightly lower basal follicle-stimulating hormone (FSH) release and markedly inhibited the effect of LH-RH on FSH with no significant effect on LH. Reproduced from Setchell *et al.* (1977), with permission of the authors and publisher.

Figure 8 illustrates inhibin curves obtained with the successive preparations of RTF obtained during the purification procedure. They are parallel. The ratios of the amount of RTF_{38c} to the quantities of crude RTF, RTF_{1a}, and RTF_{38} capable of displacing 50% of bound labeled RTF_{38c} were, respectively, 0.017, 0.1, and 0.32. A complete cross-reaction was observed between RTF preparations, on the one hand, and, on the other hand, HSP_{3-4}, bovine testicular extract, and bovine follicular fluid (BFF). In contrast, there was no cross-reaction with LH-RH, somatostatin, pituitary hormones, and RTF_3, the low molecular weight inhibin fraction of RTF. Table I describes some characteristics of this inhibin radioimmunoassay.

D. Present Position of Inhibin Assays and Outlook for the Future

There is no doubt that assays for inhibin have been improved since 1972, when the first work establishing the existence of this hormone was based on *in vivo* bioassays. These tests were poorly sensitive, not

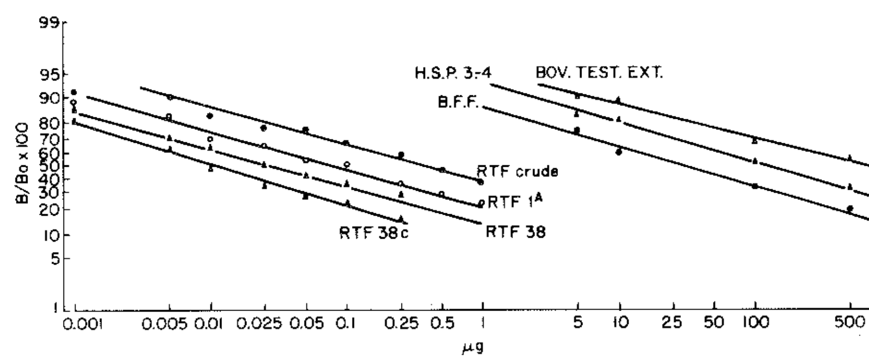

FIG. 8. Inhibition curves of several inhibin preparations expressed in logit-log in a radioimmunoassay for ram rete testis fluid (RTF) inhibin. Preparations of high molecular weight inhibin obtained at each step of purification from RTF gave parallel curves. Three other inhibin preparations from human seminal plasma (HSP_{3-4}), aqueous extract of bovine testis (Bov. Test Ext) and bovine follicular fluid (BFF) also displayed parallel inhibition curves, although with a much lower immunological potency.

precise, and barely adequate to detect inhibin in several biological fluids. The improvement has consisted in using an *in vitro* assay, the reduction of basal and/or LH-RH-induced FSH secretion by isolated pituitary cells. Its sensitivity and precision allow this technique to be used to follow the yield and specific activity of inhibin during the successive steps of purification. But this *in vitro* assay is still not adequate for measuring inhibin levels in biological fluids in order to establish the secretion of the hormone in normal and pathological conditions. Such physiopathological investigations require more sensitive assays, such as radioimmunoassay or radioreceptor assays. These ligand assays are still underdeveloped and not yet fully validated.

In future research on inhibin, the assays used should meet criteria for reliability, and the respective merits and limitations of the *in vitro* and *in vivo* assays need to be borne in mind. *In vivo* methods will still be of interest to confirm the inhibition of FSH secretion observed *in vitro*. Observation of an inhibitory effect *in vivo* also serves to demonstrate absorption of the active substance from the injection site and its transport to and action on the hypothalamohypophysial axis. Lack of biological effect *in vivo*, even though observed *in vitro*, could be explained by failure of absorption, by elevated metabolic clearance, by a homeostatic reaction masking the inhibitory effect of inhibin, or by inadequate experimental conditions.

The *in vitro*, assay is very attractive for several reasons already discussed—sensitivity, precision, broad linear log dose-effect relationship, and low requirement of active material. But some experimental

conditions are critical. First, pituitary cells, once attached to the dishes, must be incubated with and without inhibin preparations for the optimal time for a highly significant and dose-related effect; 3 days appears to be the optimal duration for incubation (Eddie *et al.*, 1978, 1979; de Jong *et al.*, 1979). Furthermore, the choice of sex and age of the pituitary donor animals may affect assay responsiveness. Many authors use adult male rats (Steinberger and Steinberger, 1976; Eddie *et al.*, 1978, 1979; de Jong *et al.*, 1979), although Lagace *et al.* (1979) use adult female rats. A preliminary experiment, however, has suggested to us that prepubertal male rats may be the best donors. Thus, 10^{-6} pituitary cells from immature male rats released identical amounts of FSH and LH under basal conditions and twice as much FSH as LH when gonadotropin secretion was stimulated by LH-RH. In contrast, the same number of cells from adult male rats secreted four times as much LH as FSH under basal conditions and two to six times as much LH as FSH when LH-RH was added to the culture medium. Under basal conditions and with LH-RH stimulation, the total amount of FSH secreted into the culture medium by 10^{-6} pituitary cells was three to four times higher when the donor animals were prepubertal than when they were adult. The specificity of *in vitro* assays must be assessed carefully. Absence of nonspecific factors capable of destroying cells, added LH-RH, and secreted gonadotropins are prerequisites. Furthermore, one should take into consideration the presence of substances, such as steroids (Labrie *et al.*, 1978) or possible unidentified factors (de Jong *et al.* 1979), capable of modifying the secretion of gonadotropins by the cells separately from the action of inhibin. These interferences may explain the lack of parallelism of inhibition curves and even stimulatory effects on gonadotropin secretion when crude extracts are used instead of purified preparations.

Finally, a model should be chosen that gives the widest range between the threshold doses acting on FSH and LH secretion. For that objective, the basal secretion of gonadotropins is the most appropriate condition, since basal LH levels are unaffected by doses of inhibin that produce significant suppression of the basal secretion of FSH (Fig. 6) (Steinberger and Steinberger, 1976; Labrie *et al.*, 1978; de Jong *et al.*, 1979). In contrast, inhibin reduces LH-RH-induced secretion of both gonadotropins, the secretion of FSH being affected more than that of LH (Fig. 6).

An internationally acceptable reference preparation should be provided that does not contain nonspecific factors that affect the assay or steroids and other substances that act on pituitary cells. It must give

an inhibition curve that is parallel to those obtained with inhibin preparations from several sources (see Fig. 25). Ovine testicular lymph, proposed by Baker *et al.* (1978), seems to be a good candidate. A control medium devoid of inhibin, such as the lymph collected from castrated rams (Eddie *et al.*, 1979), is also useful to serve as a "blank" in the evaluation of the assay data.

A radioimmunoassay (RIA) will permit investigators to follow purification procedures and to define inhibin secretion in health and disease. But to carry out a valid RIA, classic criteria must be met: purity of the tracer devoid of labeled contaminants and of damaged forms; specific antibody production; efficient technique for separating free labeled inhibin and labeled inhibin bound to antibody; absence of partial or complete cross-reaction with other substances and of nonspecific interferences by constituents of biological fluids (i.e., proteins, enzymes, and ions); complete immunological identity between the standard preparation and inhibin to be assayed; adequate sensitivity, precision, reproducibility, and accuracy. All these criteria should be assessed before applying the RIA to the measurement of immunoreactive inhibin in preparative samples and biological fluids.

IV. Physicochemical and Immunological Characteristics of Inhibin

1. *Inhibin Is Not a Steroid*

The sex steroids testosterone, 17β-estradiol, dihydrotestosterone, and progesterone have been measured in semipurified inhibin preparations after appropriate extractions. The preparations were found not to contain sufficient quantities of these sex steroids to explain the inhibiting effect on FSH secretion either *in vivo* or *in vitro* (Franchimont *et al.*, 1975b, 1979a; Hopkinson *et al.*, 1977a).

Furthermore, the biological fluids containing inhibin and used without solvent extraction, such as the culture medium of Sertoli cells (Steinberger and Steinberger, 1976), granulosa cells (Erickson and Hsueh, 1978), follicular fluid (Welschen *et al.*, 1977; de Jong *et al.*, 1978), and rete testis fluid (Setchell *et al.*, 1977), remained active even though they had been pretreated with charcoal alone or charcoal dextran for the purpose of removing most of the sex steroids.

It is also known that steroids such as 17β-estradiol and the androgens (testosterone and dihydrotestosterone) stimulate the secretion of FSH by pituitary cells in culture (Labrie *et al.*, 1978; Lagace *et al.*, 1979), and when an inhibitory effect appears, as is the case with estrogens, it is primarily more marked on the synthesis and on the release of LH both *in vivo* and *in vitro* than on FSH (see review by Setchell *et al.*, 1977; de Jong *et al.*, 1979).

2. *Inhibin Is Not the Androgen-Binding Protein*

French and Ritzen (1973) have shown that Sertoli cells under the influence of FSH secrete a protein that has the property of binding testosterone and 5α-dihydrotestosterone. Different preparations of inhibin extracted from human and bull seminal plasma and rete testis fluid have been tested for the presence of androgen-binding protein. None of them bind either testosterone, dihydrotestosterone, or tritiated 17β-estradiol (Franchimont *et al.*, 1975b,c, 1977, 1978).

3. *Inhibin Does Not Cross-react with Gonadotropins*

Active fractions of inhibin show no cross-reaction with FSH or LH of human, ovine, murine, or other origin. It is therefore not a modified gonadotropin (Franchimont *et al.*, 1975b, 1977).

4. *Molecular Weight and Amino Acid Composition*

There are inconsistencies in the estimates of the molecular weight of inhibin. These result from the fact that few of the studies have been performed with completely purified inhibin (Chari *et al.*, 1978; Sairam *et al.*, 1978). Inhibin also appears to be biochemically heterogeneous (Davies *et al.*, 1976; Franchimont *et al.*, 1978; Baker *et al.*, 1978).

Dialysis experiments using Amicon membranes and electrophoretic migration in sodium dodecyl sulfate (SDS) show that inhibin activity resides in fractions with molecular weights greater than 10,000 in most biological fluids studied: bull seminal plasma (BSP) (Chari *et al.*, 1978; Sairam *et al.*, 1978), ram rete testis fluid (Davies *et al.*, 1976; Franchimont *et al.*, 1977), porcine follicular fluid (Channing *et al.*, 1978), and the culture medium of Sertoli cells (Steinberger and Steinberger, 1976) and of granulosa cells (Anderson *et al.*, 1979).

The assigned molecular weight usually lies between 10,000 and 30,000. Inhibin from BSP has been obtained in a pure state by Chari *et al.* (1978), by acetone precipitation followed by filtration on Sephadex G-100, using 4 *M* urea–0.05 *M* sodium acetate buffer, pH 4, as eluent, then by straight elution analysis using carboxymethyl cellulose.

On the basis of gel filtration studies, SDS electrophoresis, and amino acid analysis, the molecular weight was estimated to be 19,000.

The amino acid composition was as follows: (Trp, Met, Arg)$_1$ His$_2$ Val$_4$ (Ile, Pro)$_5$ Ala$_6$ Thr$_7$ Phe$_8$ (Gly, Leu)$_9$ (Glu, Ser)$_{10}$ Tyr$_{11}$ Lys$_{13}$ Cys$_{14}$ Asp$_{21}$.

Sairam *et al.* (1978) have also managed to purify and characterize inhibin (which they call follitropin-suppressing principle) from bull ejaculate by ethanol precipitation, pH fractionation, then a series of chromatographic steps with Sephadex G-100, sulfopropyl Sephadex G-50 at pH 4.5, and DEAE-cellulose at pH 9. They obtained a protein with a molecular weight of approximately 15,000, active *in vivo* at doses of 5–10 μg in reducing the levels of FSH in castrated immature rats and inhibiting the uterine weight increase produced by 10 IU of HCG in 27-day-old female mice.

Some biological activity has also been found in protein fractions of much greater molecular weight, in the region of 90,000 (Davies *et al.* 1976, 1978; Baker *et al.*, 1976). Cahoreau *et al.* (1979) found the active fraction of RTF extracted by ethanol to have a molecular weight greater than 160,000 on the basis of Sephadex filtration and more than 100,000 on the basis of ultrafiltration on XM100 membranes.

On the other hand, inhibin-like biological activity has been found in peptide fractions of low molecular weight (< 5,000) extracted from ram testes by Moodbidri *et al.* (1976) and from human seminal fluid by Franchimont *et al.* (1978).

There are two fractions of RTF extract that show inhibin activity, one of high molecular weight (> 10,000), the other of low molecular weight (< 5,000). Only 3–6% of the total inhibin activity is located in the low molecular weight elution zone. The high molecular weight form gives rise to the low molecular weight form each time the former is subjected to chromatography on Sephadex G-200 (Fig. 9) (Franchimont *et al.*, 1978) or G-100 (Davies *et al.*, 1978). Moreover, when the large molecular weight fraction was submitted to gel filtration in 4 *M* urea, all the "inhibin" activity shifted to a fraction with a molecular weight less than 5000 (Davies *et al.*, 1978).

The high molecular weight form could be a polymer of low molecular weight forms, or a combination of natural inhibin with a carrier, or even a unique precursor that can liberate the active fragment under appropriate conditions. It could equally well be an agglomeration of two different substances in which the low molecular weight fragment might be absorbed on the larger protein. In our RIA, there is no cross-reaction between high and low molecular weight forms of inhibin.

As shown in Table II, there is no relationship between the several sources of inhibin and their molecular weights.

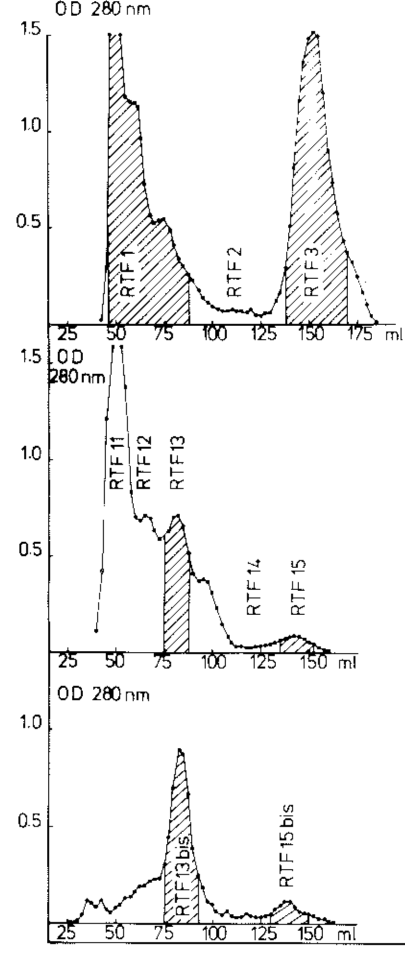

FIG. 9. Serial separation of ram rete testis fluid (RTF). *Upper panel:* After lyophilization, RTF (750 mg) was submitted to gel chromatography on Sephadex G-100 (88 × 1.6 cm column; PO_4 buffer 0.05 *M*, pH 7.5). Biological activity was located in RTF_1 and RTF_3 fractions. *Middle panel:* When RTF_1 (50 mg of protein) was further chromatographed on Sephadex G-200 (88 × 1.6 column; NH_4HCO_3 buffer, 0.05 *M*, pH 7), the resulting elution pattern consisted of five peaks: RTF_{11}, RTF_{12}, RTF_{13}, RTF_{14}, and RTF_{15}. RTF_{15} was eluted in the same position as RTF_3 when it was chromatographed on Sephadex G-200. *Lower panel:* When RTF_{13} (20 mg of protein) was submitted to further gel filtration on Sephadex G-200 (87 × 1.6 cm column; NH_4HCO_3 buffer 0.05 *M*, pH 7), the biological activity was again recovered in two peaks in the areas of high (RTF_{13} bis) and low (RTF_{15} bis) molecular weight. Hatched areas were biologically active.

TABLE II
MOLECULAR WEIGHT OF INHIBIN ACCORDING TO SOURCE

Sources of inhibin preparations	Approximative molecular weight	Method of determination	Authors
Rete testis fluid	10,000–20,000	Sephadex G-100[a]	Baker *et al.*, 1976
	15,000–25,000 and 80,000	Sephadex G-100	Davies *et al.*, 1976
	< 5,000, 20,000, 90,000	Sephadex G-100	Davies *et al.*, 1978
	1500	Sephadex G-75	Moodbidri *et al.*, 1976
	> 10,000 and < 5000	Sephadex G-100	Franchimont *et al.*, 1978
	≥ 160,000	Sephadex G-200	Cahoreau *et al.*, 1979
	> 100,000	Ultrafiltration	Cahoreau *et al.*, 1979
Human seminal plasma	19,000	SDS PAGE[b]	Sheth *et al.*, 1978
	< 5,000	Sephadex G-100	Franchimont *et al.*, 1978
Bovine seminal plasma	18,800	Sephadex G-100	Chari *et al.*, 1978
	21,500	SDS PAGE	
	15,000	Gel filtration	Sairam *et al.*, 1978
Ovine testicular extract	10,000–70,000	Sephadex G-100	Baker *et al.*, 1976
Human follicular fluid	23,000	SDS PAGE	Chari *et al.*, 1979
Bovine follicular fluid	> 10,000	Ultrafiltration	de Jong and Sharpe, 1976
Rat inhibin			
Sertoli cell factor	> 12,000	Dialysis	Steinberger and Steinberger, 1976
Seminiferous tubule culture	Not fractionated	Sephadex G-100	Eddie *et al.*, 1978

[a] According to their elution zone.
[b] SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

5. *Alteration of Biological Activity*

The active material with a molecular weight greater than 10,000 loses its activity when digested by trypsin, pepsin, or papain (Fig. 10). It is also thermolabile; boiling testicular extract (Nandini *et al.*, 1976) or culture medium from Sertoli cells (Steinberger and Steinberger, 1976) destroys the biological activity. Furthermore, heating RTF to 80°C for 30 minutes destroys its inhibin activity (Setchell *et al.*, 1977). We have also observed reduction of inhibin activity when the high molecular weight (> 10,000) extract of RTF is heated to 60°C for 30 minutes (Franchimont *et al.*, 1978).

6. *Lack of a Carbohydrate Moiety*

It is not clear whether inhibin possesses a carbohydrate moiety. Chari (1977) looked for possible carbohydrate components in extracts of inhibin from bull seminal plasma, using specific colorimetric techniques, but they found no evidence for carbohydrate. Furthermore, an extract of hamster ovaries obtained by Chappel *et al.* (1979) that

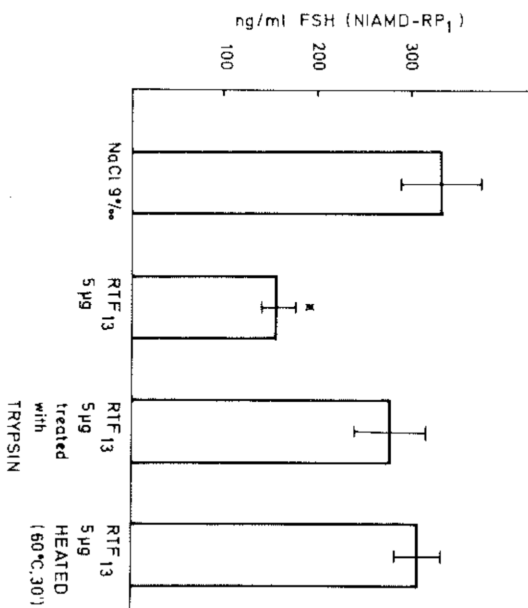

FIG. 10. Effect of an inhibin preparation (RTF_{13}) extracted from ram rete testis fluid (RTF) on luteinizing hormone-releasing hormone (LH-RH)-stimulated follicle-stimulating hormone (FSH) release in dispersed rat pituitary cell (RP) (10^5 cells) culture. Each column represents the FSH release (± 1 standard deviation) after 6 hours of incubation with LH-RH (10^{-8} *M*), when to the culture medium was added NaCl, 0.9% or 5 µg of RTF_{13} untreated, submitted to trypsin digestion, or heated at 60°C for 30 minutes. Only untreated RTF_{13} significantly (* = $p < 0.01$) reduced FSH release.

possessed inhibin-like activity did not contain any carbohydrate capable of being fixed on a column of concanavalin A.

7. *Immunochemical Behavior*

Beginning in 1975, we immunized rabbits with active fractions of HSP, BSP, and RTF. Antisera prepared in the rabbit and subsequently administered to rats led to an increase in endogenous FSH levels. We interpreted this to be the result of immunological neutralization of endogenous inhibin (Franchimont *et al.*, 1975c). The rabbits submitted to immunization under these conditions showed higher FSH levels than nonimmunized rabbits, whereas the levels of LH were identical to the control values (Table III).

Moreover, when 0.25 ml of antiserum raised against the active fraction (Ac_{II}) of BSP was injected daily into adult rats for 4 days a highly significant increase in endogenous FSH levels resulted, whereas the increase in levels of LH was barely significant. The concentration of testosterone in the rats treated with antiserum was no different from that in rats given normal rabbit serum (Fig. 11). This increase in endogenous FSH levels was significant because the nonspecific effect of rabbit immunoglobulins in the second antibody precipitation phase of the radioimmunoassays for FSH and LH had been carefully controlled.

By use of the RIA of ovine inhibin (RTF_{38c}) described in Fig. 8, cross-reactions between several preparations from different sources were established.

TABLE III
FSH and LH Levels in Rabbits Immunized with Inhibin Preparations[a]

Female Rabbits immunized with	FSH[b] (ng/ml)	LH[b] (ng/ml)
Control ($N = 4$)	468 ± 47SD	202 ± 101SD
RTF_{38}	1100	160
BSP Ac_{II}	1290	280
BFF	770	256

[a] FSH, follicle-stimulating hormone; LH, luteinizing hormone; RTF, ram rete testis fluid; BSP AC_{II}, active fraction of bull seminal plasma; BFF, bovine follicular fluid; SD, standard deviation.

[b] Assayed according to Dufy-Barbe *et al.* (1973) and expressed as nanograms of rabbit pituitary FSH and LH reference preparation.

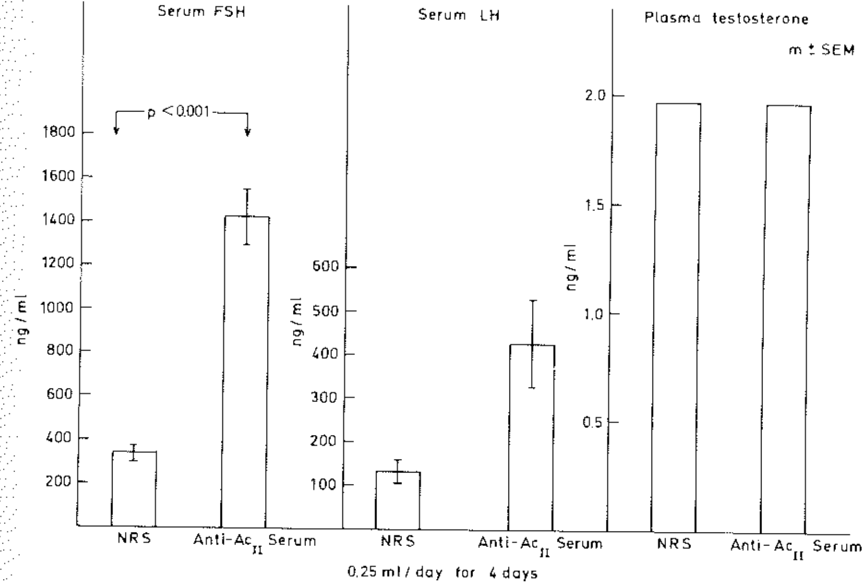

FIG. 11. Levels of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and of plasma testosterone in adult rats treated for 4 days with a normal rabbit serum (NRS) and with an anti-inhibin serum (anti-Ac_{II} serum). Anti-inhibin serum significantly increases FSH levels. The augmentation of LH was not significant. There was no change in plasma testosterone levels.

V. Biological Properties of Inhibin

Inhibin selectively impairs the secretion of FSH as much *in vivo* as *in vitro*. It also can cause a reduction in the secretion of LH, but much larger doses are required. This biological activity of inhibin occurs regardless of the species from which it originates. Inhibin from different species is active in monkeys, rats, mice, and rabbits.

A. Sites of Action of Inhibin

1. *Pituitary Action*

Inhibin certainly acts on the pituitary. No matter whether it is extracted from human seminal plasma or RTF (Setchell *et al.*, 1977; Baker *et al.*, 1976; Franchimont *et al.*, 1978), ram testicular lymph (Baker *et al.*, 1978), the culture medium of Sertoli cells (Steinberger

and Steinberger, 1976; Lagace *et al.*, 1979; de Jong *et al.*, 1978), or follicular fluid (de Jong *et al.*, 1978; Shander *et al.*, 1979), inhibin exerts an effect on basal FSH levels and on the FSH response to LH-RH in isolated cultured pituitary cells (Fig. 6). It is also true that an effect can be observed on the LH response to LH-RH but consistently much larger doses of the inhibin preparation are required and the inhibition curve is different from that for FSH. In Fig. 6, it is clear that a significant inhibition of LH-RH-induced FSH release was obtained with 1 μg of RTF_{38} per milliliter whereas 10 μg of RTF_{38} were needed to produce a significant reduction of LH levels under the same conditions.

The degree of inhibitory activity is quantitatively and temporally cumulative, since an increase both in the dose and length of exposure resulted in an increased degree of inhibition of pituitary release of FSH both in the presence and in the absence of LH-RH.

In vivo in male rats (Franchimont *et al.*, 1975b) and in female rats under precisely controlled conditions [preestrous rat in which preovulatory LH and FSH surges were blocked with phenobarbital (Wise *et al.*, 1979)], inhibin preparations inhibit the response of the gonadotroph to injection or perfusion of exogenous LH-RH (Fig. 12). With variation of experimental conditions (quantities of LH-RH and inhibin, route of injection of LH-RH, interval between pretreatment with inhibin and injection of LH-RH, etc.), the effect can be made more or less specific on FSH release (Wise *et al.*, 1979) or less discriminately on both FSH and LH secretion (Franchimont *et al.*, 1975b).

2. *Hypothalamic Action*

The data of Lugaro *et al.* (1974) suggested an action of inhibin at the level of the hypothalamus. These investigators injected their active extract (100 ng) prepared from bull spermatozoa into the third ventricle and observed a reduction in the levels of FSH. In contrast, no effect on LH was determined.

In vitro, we have shown that inhibin preparations extracted from HSP and RTF decrease the endogenous LH-RH content of isolated hypothalami of rats after short-term incubation with several concentrations of inhibin (Demoulin *et al.*, 1979b).

The entire hypothalami were dissected from the preoptic area to the mammillary bodies in adult male rats. The fragments were kept cold during the collection. The hypothalami were then incubated for 1 hour at 37°C in an atmosphere of air/CO_2 (95/5 v/V). Each tube contained an equivalent of 1 or 2 hypothalami in 1 ml of phosphate-buffered

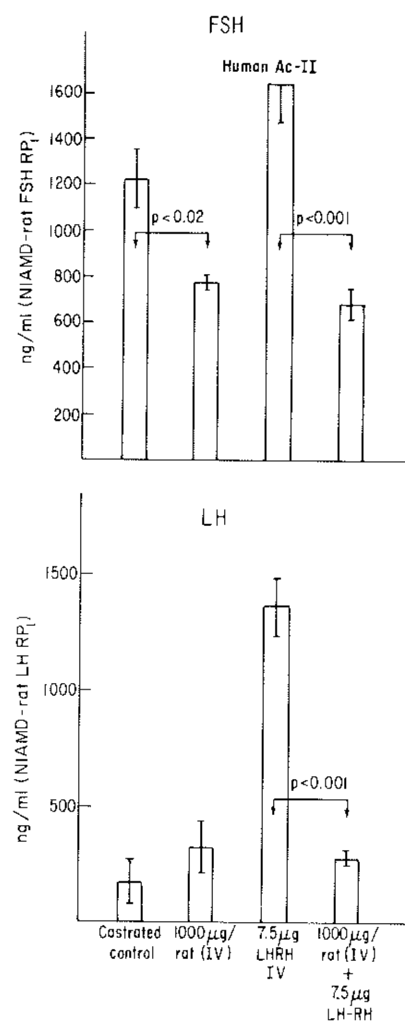

FIG. 12. Effect of a crude extract of human seminal plasma (HSP) (AC_{II}) on basal follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels and their response to LH-releasing hormone (LH-RH). Adult male rats (250 gm) were used 4 days after castration. They received intravenously either 0.5 ml of NaCl 0.9% alone or 1000 μg of the crude preparation of HSP dissolved in the same volume of diluent. Three hours later, some rats were injected intravenously (IV) with 7.5 μg of LH-RH. Twenty minutes later, they were killed and serum FSH and LH were measured. The columns represent the mean values (N = 6) ± SEM. Inhibin preparations significantly decreased basal FSH levels without affecting basal LH levels and also inhibited the FSH and LH responses to LH-RH.

saline supplemented with glucose (1.5×10^{-2} M) and bacitracin (2×10^{-5} M) (Rotsztejn *et al.*, 1976). The hypothalami were incubated in triplicate with several concentrations of inhibin fractions obtained from human seminal plasma (HSP_3) or ram rete testis fluid (RTF_{38}). After the incubation, the hypothalami were removed and homogenized by ultrasonication in 2 ml of 2 N acetic acid. After centrifugation at 3000 g for 10 minutes at 4°C, the supernatants were collected and stored at -20°C until assayed.

LH-RH (GnRH)-specific radioimmunoassays (Bourguignon *et al.*, 1979) were performed in aliquots of hypothalamic extracts previously neutralized with ammonium hydroxide.

The amounts of immunoreactive GnRH found in isolated hypothalami after incubation with increasing concentrations of inhibin are shown in Fig. 13. Using HSP_3, a small molecular weight fraction obtained from human seminal plasma, a dose-related decrease of intrahypothalamic GnRH was observed. This decrease was statistically significant when as much as 2.5 μg of HSP_3 were added per hypothalamus ($p < 0.001$). Using RTF_{38}, a high molecular weight fraction obtained from RTF, a dose-related decrease of GnRH content was also found. In the presence of 10 μg of RTF_{38} added per hypothalamus, the GnRH contained in the hypothalamus was significantly decreased ($p < 0.05$).

These observations demonstrate a dose-dependent effect of inhibin on hypothalamic structures synthesizing and storing GnRH. These data might be interpreted either as reduced synthesis or increased enzymatic degradation of GnRH. Measurements of GnRH peptidases, studies of GnRH contained in and released from nerve endings of mediobasal hypothalamus, and identification of GnRH metabolites are in progress in our laboratory.

3. *Direct Action on the Gonad*

As described in Section V,D,2, the incorporation of tritiated thymidine into testicular DNA was studied *in vivo* and *in vitro* in normal rats during puberty, aged 42 days, weighing 150 gm, and showing spermatozoa and/or spermatids from stage 12 in 40% of seminiferous tubules (Franchimont *et al.*, 1979b). *In vitro* fragments of the testis of these animals were incubated for 3 hours with different inhibin preparations in the presence of tritiated thymidine (1 μCi per milliliter of culture medium). Two inhibin preparations showed a powerful inhibitory effect on the incorporation of tritiated thymidine. These were

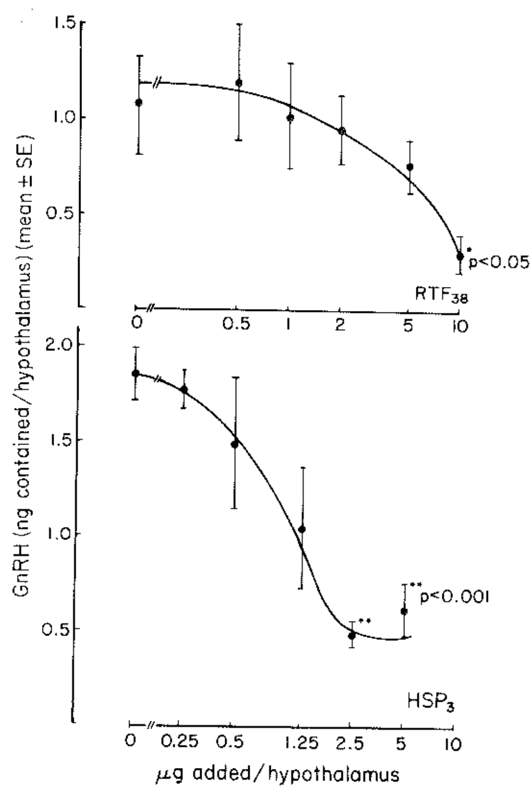

FIG. 13. Effect of increasing amounts of semipurified inhibin preparations extracted from ram rete testis fluid (RTF_{38}) and human seminal plasma (HSP_3) on hypothalamic luteinizing hormone-releasing hormone (LH-RH, GnRH) content. Ordinate: LH-RH (GnRH) content in nanograms per hypothalamus ± SEM. Abscissa: amount of inhibin preparations added per hypothalamus.

the active fractions from human seminal plasma (HSP_{3-4}) and RTF_3 that contained the substances of molecular weight less than 5000 (Table IV).

In contrast, under these experimental conditions the effect of the high molecular weight (> 10,000) fraction of RTF on the incorporation of tritiated thymidine into testis fragments maintained in organ culture was not statistically significant (Table IV). A comparison of RTF_3 (MW < 5000) and RTF_{38} (MW > 10,000) on an equimolar basis is needed before concluding that RTF_{38} has no gonadal action.

These preliminary findings suggest that certain inhibin preparations exert a direct inhibitory effect on the synthesis of DNA by actively dividing spermatogonia at the beginning of spermatogenesis.

TABLE IV

Tritiated Thymidine Incorporation into Testicular DNA *in Vitro*

Treatment[a]	Culture medium[b]	Dpm/mg DNA ($\times 10^{-3}$)	SD ($\times 10^{-3}$)	p
NaCL, 0.9%	5	63.4	11.6	
RTF_{38}, 20 μg	5	47.6	10	NS
RTF_3, 20 μg	5	34.6	6.8	< 0.005
HSP_{3-4}, 20 μg	5	38.7	9.4	< 0.002

[a] RTF, ram rete testis fluid; HSP, human seminal plasma.
[b] Each culture consisted of at least 20 testicular explants of 1 mm^3 volume.

B. Mechanism of Action of Inhibin at the Pituitary Level

1. *Specificity of Action on the Gonadotroph*

Inhibin, whether it be an extract of RTF or of bovine or human seminal fluid, specifically acts on gonadotropin secretion and has no effect whatsoever on TSH, prolactin, or growth hormone either *in vivo* or *in vitro* (Franchimont *et al.*, 1975b, 1978).

2. *Selective Action on FSH Secretion*

In vitro, inhibin exerts an effect not only on FSH, but also on LH release induced by LH-RH. Nevertheless, its action is more selective on FSH secretion. In fact, the minimum dose required to lower FSH levels significantly is much less than the dose that reduces LH levels. This selective action is even more apparent in experiments *in vitro,* in which basal levels of LH and LH cell contents are not lowered in the range of doses of inhibin used (Fig. 6).

In vivo the action of inhibin is the result of direct effects on the pituitary and on the hypothalamus, as will be shown.

3. *Action on the Synthesis and Release of the Gonadotropins*

In vitro, various preparations of inhibin (extract of RTF, follicular fluid, etc.) lead to a concomitant reduction of the quantity of FSH in the culture medium and within the cells after 72 hours of incubation in the absence of LH-RH (Fig. 14). This action is more marked on the cell content than on the quantities of FSH released into the culture medium. Under our chosen experimental conditions, no effect was

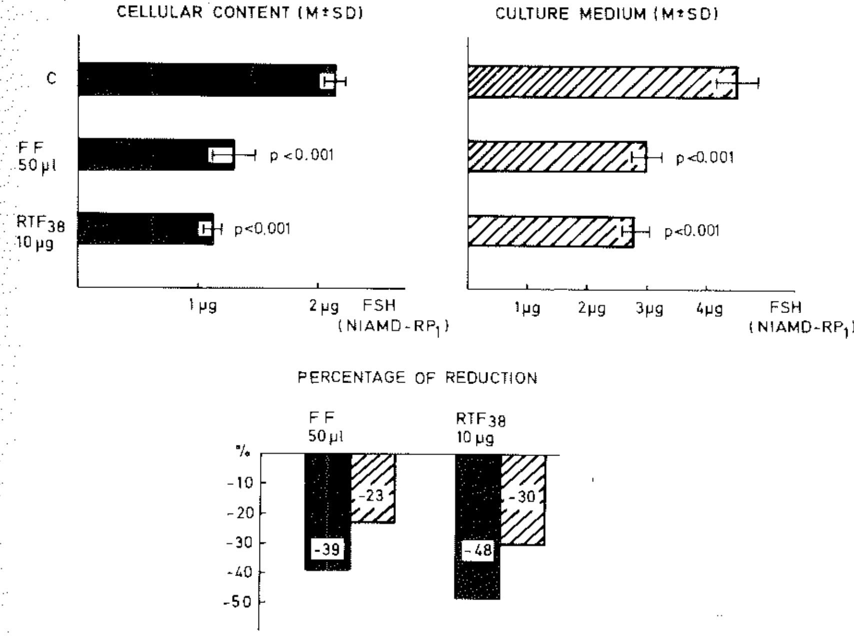

Fig. 14. Effect of 50 μl of steroid-free bovine follicular fluid (FF) and 10 μg of ram rete testis fluid (RTF_{38}) on amounts of follicle-stimulating hormone (FSH) cell content and in culture medium after 3 days of incubation. Bar C: Control values observed in the absence of inhibin preparations. The upper part of the graph indicates absolute FSH amounts ± SD, and the lower part represents the reduction of FSH amounts in cellular content (black columns) and in culture medium (hatched columns) expressed as percentage, the control values representing 0%. Under these experimental conditions, no effect was observed on luteinizing hormone intracellular and culture medium contents.

observed on the quantities of LH present in either the culture medium or the cells. These actions on the quantities of FSH in the two compartments show that the inhibin preparations tested have an effect on FSH synthesis under basal conditions. In fact, if the actions were limited to an inhibition of FSH release, the level of FSH would be reduced in the culture medium whereas the quantities of FSH in the cells would remain the same or be even greater than in the control cells (Franchimont *et al.*, 1978).

Convincing evidence of the action of inhibin on FSH synthesis has been provided by the experiments of Chowdhury *et al.* (1978). These authors studied the incorporation of [^{3}H]leucine into FSH and LH produced by organ cultures of rat anterior pituitaries cultured in a medium previously used for 2–5 days to culture Sertoli cells. The Sertoli cell factor present in the culture medium selectively reduced the

incorporation of labeled leucine into immunoprecipitable FSH without decreasing its incorporation into LH.

As both these experiments were performed *in vitro* in the absence of LH-RH, it is clear that the observed effect was caused by a direct inhibin-like action on FSH synthesis by the gonadotroph.

4. *Receptors to Inhibin in the Gonadotrophs*

Sairam *et al.* (1978) studied the binding of ^{125}I-labeled bovine inhibin by a crude membrane preparation from frozen ovine pituitary glands. Unlabeled preparations of inhibin from several species inhibited this binding, and LH-RH, ovine LH, bovine serum proteins, and rat liver extract failed to displace any of the isotope bound to the receptors.

It is evident from this experiment that the receptor for inhibin is different from that for LH-RH and that the action of the testicular hormone is not due to competition for LH-RH receptors.

C. Kinetics of the Action of Inhibin on Gonadotropin Secretion *in Vivo*

Experiments in which rats are given various inhibin preparations have been very useful for showing inhibin action, but the "one point" response to pulses of injected material does not allow the assessment of the effect of inhibin on the secretion of the gonadotrophs over a long period. The limited nature of these experiments is evident from the inconsistent results, often a specific inhibition of FSH, rarely on LH alone (Hodgen *et al.,* 1974), more frequently on both gonadotropins.

To overcome this difficulty, several studies were undertaken in animals in order to determine the kinetics of the action of different inhibin preparations. They all showed that a delay of several hours was necessary for inhibin to exert an inhibitory effect on FSH levels that would persist beyond the period of administration of the test material. Furthermore, these experiments revealed differences in the kinetics of the reduction of FSH and LH concentrations.

The first studies of the kinetics of inhibin action were made by Lee *et al.* (1974), Keogh *et al.* (1976), and Baker *et al.* (1976).

Crude testicular extracts equivalent to between 1.0 and 3.0 kg net weight of bovine testes were infused into five castrated rams over a period of 10–24 hours. Blood samples were collected before, during, and after the infusion either continuously (samples of 3–8 hours duration) or intermittently (three blood samples at 15-minute intervals every 3–8 hours). Under these conditions, extracts of bull testes infused into castrated sheep will lower plasma FSH levels after a lag period of about 12 hours. These decreases, which have ranged from 15 to 58% of preinfusion levels, persist for at least 24 hours after the infusion has been stopped. The levels of LH frequently showed a small decline within 3–5 hours of the beginning of the infusion but subsequently rose at 24 hours either with saline or testis extracts. Testosterone levels were unaffected.

More recently, Cahoreau *et al.* (1979) and Blanc *et al.* (1978) have clearly demonstrated that a nonsteroidal factor from RTF suppresses the secretion of both FSH and LH, but with different kinetics. Castrated or cryptorchid animals were bled every 15 or 30 minutes for 25 hours. Twenty milliliters of charcoal-treated RTF were injected at the fifth and at the sixth hour after the beginning of sampling. Human serum albumin or γ-globulins were injected similarly in controls. In all cases, the LH secretion pattern was altered first; plasma FSH levels were lowered much later, at a time when the LH secretion pattern had returned to normal. RTF injections resulted in the suppression of LH peaks for 3–5 hours starting in the first hours after treatment. FSH was progressively lowered after the first injection, and the maximum FSH inhibition was observed about 8 hours after the first injection and lasted for about 7 hours.

We have perfused rabbits, castrated at least 15 days earlier, with 0.9% saline solution followed 3 days later with an active fraction, P_{II}, of RTF obtained after precipitation with alcohol, washing with acetone, filtration on Sephadex G-200, and chromatography on DEAE-cellulose (Franchimont *et al.,* 1977). This is in the high molecular weight (> 10,000) category (Franchimont *et al.,* 1978). Blood samples were taken every 20 minutes for 8 hours, then at longer intervals. The test material was perfused for 6 hours from the end of hour 1 to the end of hour 7. As shown in Fig. 15, the level of FSH fluctuated widely during the control perfusion. When the active preparation was infused, one saw a marked reduction in FSH that appeared only 4–5 hours after the beginning of perfusion. From that time FSH values were stable, and they remained low for 24 hours after the start of the perfusion. The level of LH also fluctuated widely during the control perfusion and in the hour preceding the infusion of the active fraction. When the infusion was begun, the pulses of LH were lost and the levels of LH also fell. This effect was transitory, for at the end of the perfusion the LH values were similar to those seen during the control period (Fig. 16).

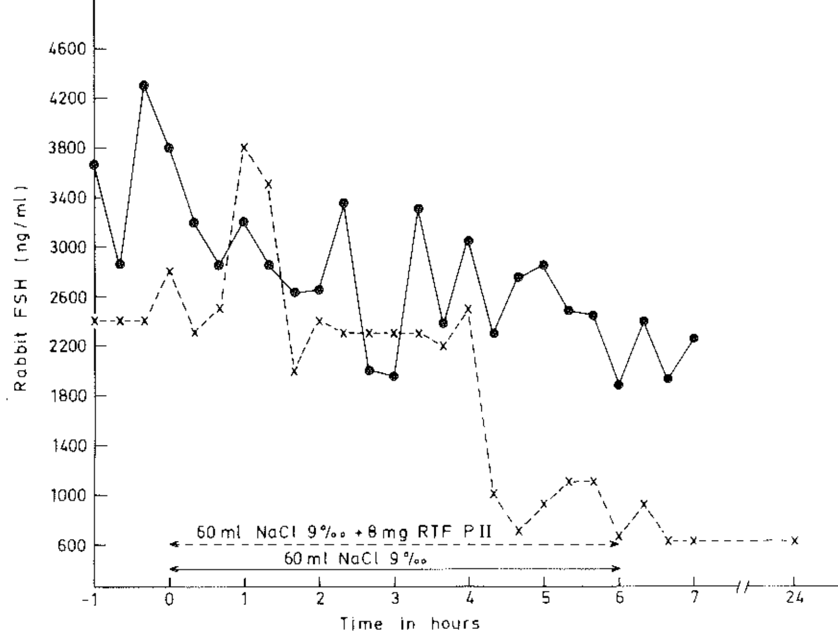

FIG. 15. Serum follicle-stimulating hormone (FSH) levels before, during, and after a 6-hour perfusion of 60 ml of NaCl, 0.9% (●—●) and 60 ml of NaCl, 0.9%, containing 8 mg of active fraction (PII) extracted from RTF in a castrated male rabbit (X---X). A significant decrease of FSH levels appeared after 4 hours of infusion and persisted for 24 hours after the beginning of the perfusion.

Kinetic studies are practically impossible to perform in rats because of the difficulty of taking frequent blood samples. It is interesting to know that the injection of inhibin preparations into rats castrated 2 or 3 weeks earlier leads to a simultaneous reduction of FSH and LH and, in some cases, to a reduction of LH without altering FSH levels (Hodgen *et al.*, 1974). Using intact adult rats injected every 8 hours for 24, 48, or 72 hours and with the blood sampled 4 hours after the last injection, Lee *et al.* (1977) found that at 28 hours LH levels were significantly suppressed but FSH levels were not. In contrast, at 52 hours and 76 hours LH secretion was unaltered and FSH was significantly suppressed.

In monkeys, the injection or perfusion of inhibin appears to have little effect on LH, but, characteristically, after a latent period there is a prolonged reduction of FSH levels.

Keogh (1978) showed that a preparation of ovine testicular lymph, when infused over a period of 24 hours, caused a 50% fall in the level

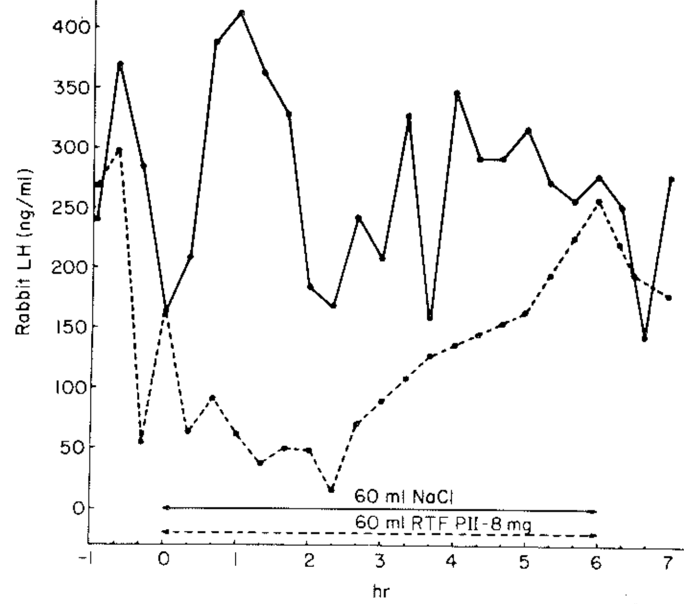

FIG. 16. Serum luteinizing hormone (LH) levels before, during, and after a perfusion of 60 ml of NaCl, 0.9% (●—●) and 60 ml of NaCl, 0.9%, containing 8 mg of active fraction (PII) extracted from ram rete testis fluid (RTF) in a castrated male rabbit (●---●). LH pulses disappeared with the beginning of the perfusion, and LH levels decreased for the first 3 hours. During the last 3 hours of perfusion, basal LH levels progressively increased to reach control values at the end of the perfusion.

of FSH that persisted for up to 48 hours. A control animal received an equivalent amount of protein prepared from the thoracic duct lymph of a castrated animal. No modification of LH levels was observed.

Channing *et al.* (1979) treated four long-term castrated adult female monkeys. The porcine follicular fluid (PFF) (15 or 5 ml) was administered subcutaneously in two doses 6 hours apart. Blood was sampled every 6 hours, 18 hours before and 3 days after the injections. The PFF administration led to a progressive decline of serum FSH that started within 18 hours and was maintained for about 3 days (Fig. 17).

These concordant kinetic studies show that the action of inhibin on pituitary secretion of gonadotropins *in vivo* is complex and clearly involves direct effects on the pituitary and the hypothalamus, as previously discussed. Species differences in the gonadotropin response are possible, in particular in monkeys, in which no action on LH was detected (Shashidhara-Murthy *et al.*, 1979; Keogh, 1978; Channing *et al.*, 1979).

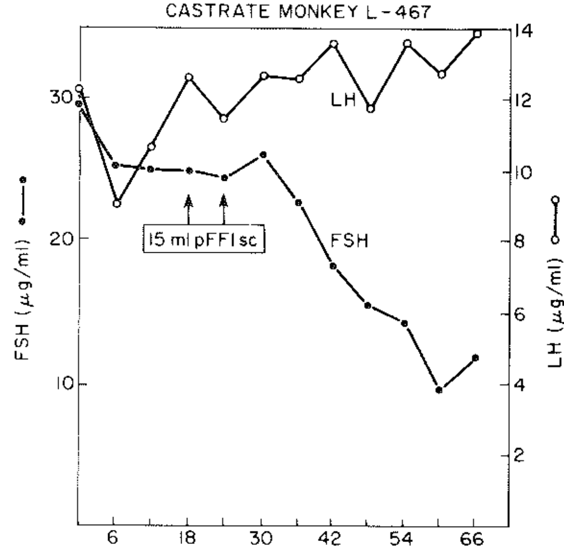

FIG. 17. Temporal course (abscissa, in hours) of inhibitory action of porcine follicular fluid (PFFl) upon serum luteinizing hormone (LH) (right ordinate) and follicle-stimulating hormone (FSH) (left ordinate) in a castrated monkey. The fluid was given subcutaneously in two 5-ml doses 6 hours apart followed by blood sampling every 6 hours thereafter for 3 days. A long-term castrated female monkey was used for this study. Serum LH and FSH levels were measured in each blood sample. Reproduced from Channing *et al.* (1979), with permission of the authors and publisher.

The brief reduction in LH secretion observed after administration of inhibin may be caused by one or both of the following mechanisms: reduced production and release of LH-RH by the hypothalamus (see Section V,A,2), or inhibition of pituitary LH release under LH-RH control (see Section V,A,1).

The existence of a latent phase before the inhibitory effect on FSH levels is observed, and the persistence of this effect well beyond the period of inhibin administration argues for a major action on pituitary synthesis of FSH.

The latent period and prolonged effect after administration of inhibin preparations cannot be explained by the half-life of FSH. The latent period is of the order of 4–12 hours in a variety of experiments whereas the half-life of FSH calculated from the slope of its disappearance curve in the absence of pituitary secretion is approximately 4.5 hours (Franchimont and Burger, 1975; Lincoln, 1978). Moreover, the inhibitory effect on FSH secretion lasts for 24 hours after the administration of inhibin. This time course favors an action of inhibin on FSH synthesis.

D. Interactions between Inhibin, Androgens, and Estrogens

Little work has been done on the problem of the interaction between the sex steroids and inhibin. Nevertheless, this problem is fundamental, as inhibin is not the sole factor regulating FSH secretion. Thus, 21 days after complete destruction of germ cells either by efferent duct ligation or by severe heating, the increase of FSH levels is only 30% of that observed 21 days after castration (Main *et al.*, 1978).

Some synergistic effects have been observed in *in vivo* experiments.

Serum FSH is only partially suppressible by estradiol in ovariectomized female mouse, even when estradiol is given in larger than physiological amounts. In these maximally estrogenized ovariectomized mice, ovarian transplantation, on the one hand, and administration of porcine follicular fluid, on the other hand, result in FSH blood levels that are within the range shown during the estrous cycle in intact females (Bronson and Channing, 1978).

Hopkinson *et al.* (1977b) studied the effects of testosterone alone; of an active inhibin preparation extracted from human seminal plasma, prepared by cold ethanol precipitation, acetone drying, and chromatography on Sephadex G-50; and of both substances together on the FSH and LH levels in adult male rats castrated 40 hours before sacrifice. Testosterone alone given subcutaneously in a dose of 50 μg/100 gm body weight had no effect on FSH levels whereas LH returned to precastration levels. The inhibin extract given alone had a limited effect on FSH levels. In contrast, testosterone and the extract given together led to a marked reduction of FSH to levels similar to those seen in intact adult animals. The levels of testosterone were the same in both groups of rats given testosterone either alone or with the extract of human seminal plasma. This experiment demonstrated a synergistic effect of testosterone and inhibin on the reduction of FSH levels and also a lack of any effect of inhibin on the absorption or clearance of testosterone.

In the same year, Marder *et al.* (1977) treated fluid from medium and large follicles of pigs with charcoal to remove steroids. The follicular fluid thus treated significantly reduced in a dose-dependent manner FSH levels in both ovariectomized and sham-ovariectomized rats. However, there was a lower threshold to porcine follicular fluid in

the sham-ovariectomized rats with higher estradiol levels than in ovariectomized animals, which suggests a synergistic effect of estradiol and ovarian inhibin on FSH negative feedback control.

In contrast, inhibin opposes the stimulating effects of androgens and estrogens on gonadotropin secretion induced by LH-RH *in vitro*. Lagace *et al.* (1979) studied the direct pituitary site for feedback action of sex steroids and inhibin and their interaction with LH-RH in the control of FSH and LH secretion in dispersed pituitary cell cultures. Estrogens stimulated both the LH and the FSH response to LH-RH, whereas androgens (testosterone and dihydrotestosterone) inhibited LH and stimulated FSH secretion. Porcine follicular fluid or granulosa cell culture medium as well as Sertoli cell culture medium, treated with dextran-coated charcoal to remove endogenous steroids, exerted an inhibitory effect on the LH-RH-induced release of both gonadotropins although the effect on FSH secretion occurred earlier and with lower concentrations than the effect on LH secretion. Furthermore, inhibin-containing media completely reversed the stimulatory effect of estrogens on LH-RH-induced LH and FSH secretion (Fig. 18) and of androgens on LH-RH-induced FSH secretion. An additive effect of dihydrotestosterone and inhibin present in bovine follicular fluid has been observed on LH release induced by LH-RH in dispersed pituitary cell culture (Fig. 18).

E. Effects of Inhibin on Gonadal Function

1. *Effect on Spermatogenesis*

Few experiments have been undertaken to study the effects of inhibin on spermatogenesis. De Jong *et al.* (1978) gave bovine follicular fluid, devoid of steroids, for 12 days to 21-day-old male rats and were able to show a delay in pubertal development of the testes compared with control animals, reduction of testicular weight, retardation of spermatogenesis, and decrease in the number of pachytene spermatocytes. These effects were produced even though the levels of FSH were reduced only during the first 4 days of treatment and the levels of LH were significantly increased.

We have given different inhibin preparations, extracts of human seminal fluid of low molecular weight (HSP_{3-4}) and of RTF with a molecular weight greater than 10,000 (RTF_{13}) and with a molecular weight less than 5000 (RTF_3), in doses totaling 160 μl/100 gm body weight administrated as four injections intraperitoneally over 36 hours to rats of different ages. We measured the incorporation of

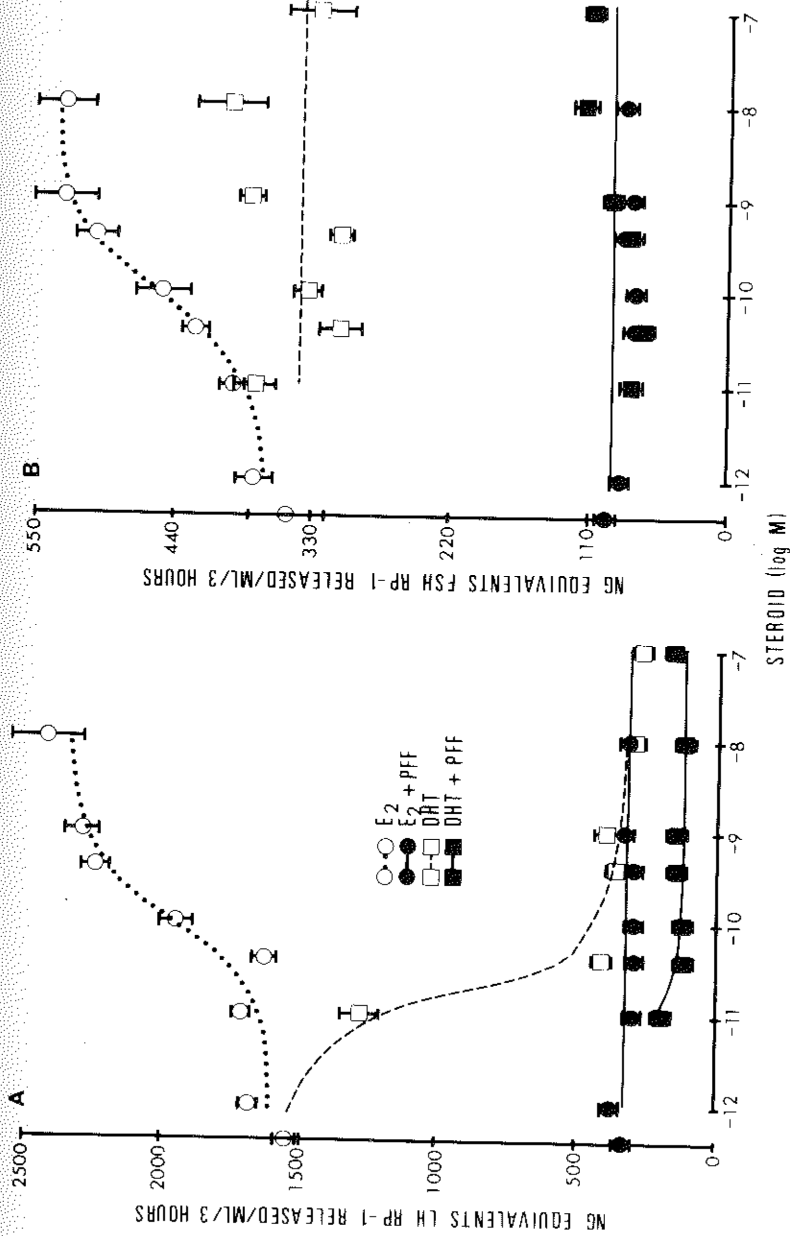

Fig. 18. Effect of increasing concentrations of estradiol (E_2) and dihydrotestosterone (DHT) in the presence (filled symbols) or the absence (open symbols) of porcine follicular fluid (PFF) on (A) the luteinizing hormone (LH) and (B) the follicle-stimulating hormone (FSH) responses to 0.1 nM LH-releasing hormone (LH-RH). LH-RH was present during a 3-hour incubation period after a 40-hour preincubation with the indicated steroids or porcine follicular fluid. Reproduced from Lagace *et al.* (1979) with permission of the authors and publisher.

tritiated thymidine injected 3 hours before sacrifice into testicular DNA and the labeling of germinal cells by autohistoradiography. The inhibin preparations markedly reduced the incorporation of tritiated thymidine into testicular DNA and the uptake by type B spermatogonia compared with the same preparation previously degraded by trypsin and heated to 60°C for 1 hour (Fig. 19). This effect was apparent in 42- to 49-day-old rats in which spermatogenesis commences and progresses at the same time as a significant incorporation of thymidine into testicular DNA is observed (Table V). In contrast, in adult rats, no effect was observed on the incorporation of [^{3}H] thymidine, which is ten times less than that seen in pubertal rats (Franchimont *et al.*, 1979b). Inhibin preparations specifically act on testicular DNA synthesis since they induce no modification of thymidine incorporation into hepatic DNA (Table V).

Inhibin thus appears to inhibit the synthesis of DNA implicated in the mitoses of germinal cells (particularly, spermatogonia type B) in pubertal animals. These cells, as they divide, signify the beginning of spermatogenesis. This effect is doubtless mediated by the reduction of the secretion of FSH. It is known, in fact, that after FSH withdrawal by injecting specific anti-FSH serum, there is also an inhibition of [^{3}H]thymidine incorporation into DNA (Murty *et al.*, 1979). But a direct effect of inhibin on the multiplication of germinal cells disclosed by the incorporation of tritiated thymidine in the testicular DNA has been observed *in vitro* with the low molecular weight preparations of inhibin: HSP_{3-4} and RTF_3 (see Table IV). The action of these inhibin preparations, as much *in vivo* as *in vitro*, resembles that of testicular chalones described by Clermont and Mauger (1974). These substances extracted from testes exert an inhibitory effect on germ cells, particularly on the multiplication of spermatogonia type A.

In adults, inhibin has no detectable effect either because the frequency of mitoses is insufficient for an effect to be observed, or because cell multiplication ceases to be dependent on FSH and secondarily on inhibin. Steinberger (1971) showed that in adult rats spermatogenesis can be maintained by testosterone alone.

2. *Effect on Gonadotropin Secretion during the Estrous Cycle and on Follicular Maturation*

Schwartz and Channing (1977) showed that the pattern of FSH secretion was modified during the estrous cycle of the rat by the injection of porcine follicular fluid previously treated with charcoal. Thus, the elevation of blood FSH levels that appears between proestrus and estrus in response to the natural preovulation peak of LH or an ar-

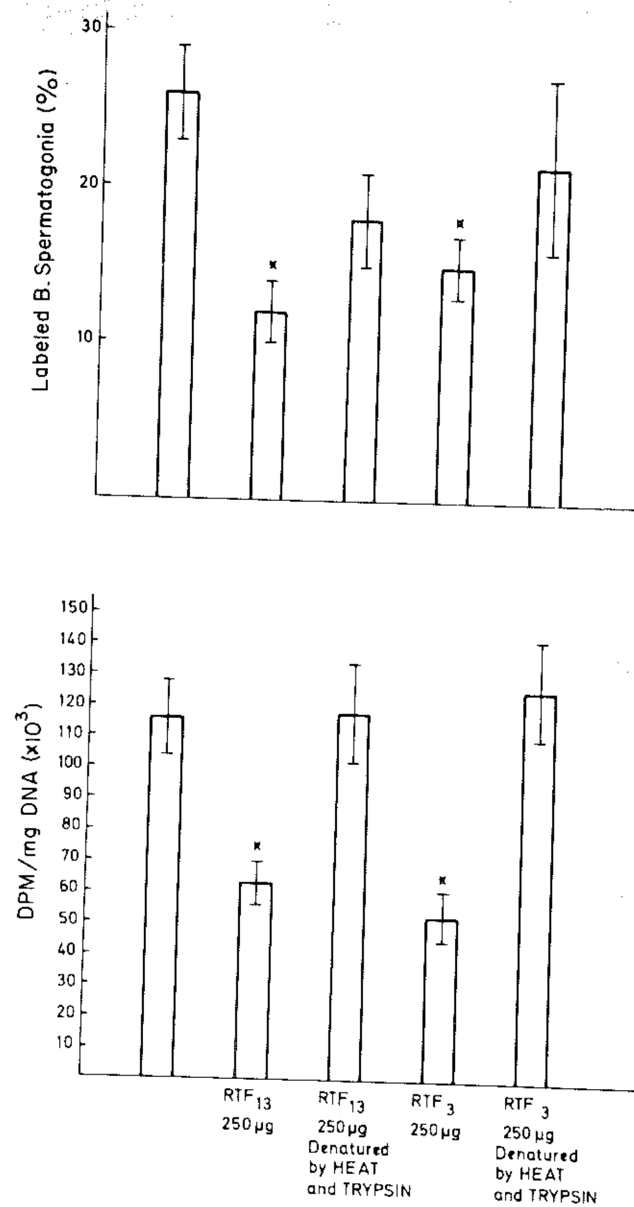

FIG. 19. Effect of NaCl, 0.9%, and two inhibin preparations from ram rete testis fluid (RTF_{13} and RTF_3) either untreated or denatured by heating and trypsin digestion on (upper part) the percentage of spermatogonia type B labeled with tritiated thymidine and detected by autohistoradiography; and on (lower part) incorporation of labeled thymidine into testicular DNA expressed as dpm/mg DNA ($\times 10^3$). RTF_{13} and RTF_3 significantly reduce (* = $p < 0.01$) the percentage of labeled spermatogonia and the thymidine incorporation into DNA compared with control animals treated with NaCl, 0.9% (first column) or with animals treated with denatured preparations.

TABLE V

INCORPORATION OF TRITIATED THYMIDINE INTO TESTICULAR AND HEPATIC DNA IN RATS AT DIFFERENT STAGES OF SPERMATOGENESIS MATURATION: INFLUENCE OF INHIBIN TREATMENT (160 μG/100 GM BODY WEIGHT)

Incorporation into	Age (days)	Percentage of seminiferous tubules containing spermatozoa and/or spermatids from stage 12[a]	N	Thymidine incorporation (dpm/mg DNA ($\times 10^{-3}$) $\pm$ SEM)			
				NaCl, 0.9%	RTF_{13}	RTF_3	HSP_{3-4}
Testicular DNA	42	40%	10	117 ± 5.0	64.3 ± 4.8[b]	53 ± 5.7[b]	67.1 ± 4.2[b]
	49	91%	6	43 ± 3.5	ND[c]	20 ± 1.7[b]	ND
	56	100%	6	23 ± 1.7	ND	23 ± 1.5	ND
Hepatic DNA	42	40%	5	184 ± 10.6	ND	196 ± 6.5	ND

[a] Seminiferous tubules (200–300) were examined by optical microscopy.
[b] $p < 0.01$ compared to animals of the same age treated with NaCl, 0.9%.
[c] ND, not determined.

tificial peak of LH (induced by the inhibition of the natural preovulation peak of LH by pentobarbital and replaced by exogenous LH) can be suppressed by the steroid-free fluid given intraperitoneally in two doses of 0.5 ml each (Fig. 20). This inhibitory factor does not alter the LH levels or modify estradiol and progesterone secretion rates and does not affect rupture of the follicle. Under these experimental conditions, treatment by steroid free porcine follicular fluid inhibited the second elevation of FSH, which may recruit follicles for the next cycle (Schwartz, 1969; Schwartz *et al.*, 1973).

De Jong *et al.* (1978) obtained somewhat different results in long-term experiments with much smaller doses of follicular fluid. These investigators injected adult female rats with charcoal-treated bovine follicular fluid daily over five estrous cycles. The dose was 0.25 ml/100 gm body weight for the first 17 days, subsequently 1 ml/100 gm. Under these conditions, the authors did not observe any changes in vaginal smears. Furthermore, the number of ova in the tubes from the second to the fifth estrous cycle was no different from controls treated

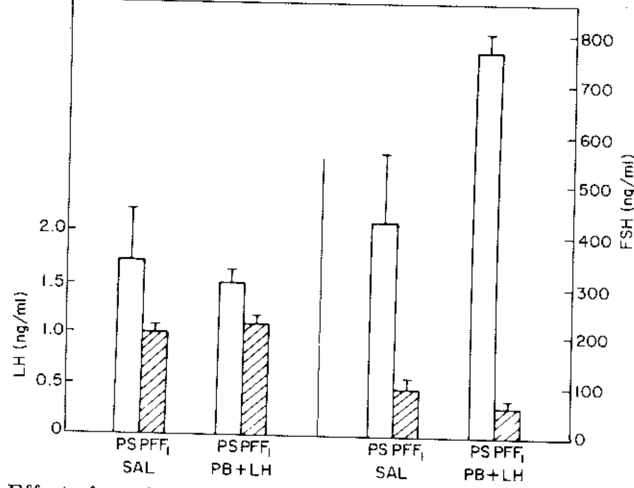

FIG. 20. Effect of porcine follicular fluid (PFF) and porcine serum (PS) on (LH) (left) and (FSH) (right) serum levels in rats at 4 A.M. on the day of estrus. Rats were treated with either saline (SAL) or pentobarbital (PB) at 1:30 P.M. and 3 P.M. on the day of proestrus; the pentobarbital-treated rats also received 8 μg of LH at 3:30 P.M. Porcine serum or porcine follicular fluid was injected at 3:45 P.M. (0.5 ml) and at 6:30 (0.5 ml). Rats exhibiting 4-day cycles were used. Standard error of the mean is indicated above each bar. The PFF led to a significant decrease of serum FSH levels in both experimental conditions, whereas serum LH was not significantly different as a result of the two pretreatments or in serum versus follicular fluid treatments. Reproduced from Schwartz and Channing (1977), with permission of the authors and publisher.

with bovine plasma. FSH levels fell 8 hours after the first injection, then there was an increase in FSH and LH levels as compared with control values. The blood levels are, nevertheless, difficult to interpret because they were not taken systematically during the five estrous cycles but at very wide intervals.

A most interesting experiment was performed by Channing *et al.* (1979), who demonstrated that inhibin inhibits follicular maturation and can modify the midcycle FSH peak in monkeys.

Porcine follicular fluid (PFF) from small and medium follicles was pooled, charcoal-treated, and injected intraperitoneally in 4-ml doses every 8 hours between days 1 and 4 of the menstrual cycle of 4 rhesus monkeys. Treatment was followed by laparotomy on day 12 to 14 of the cycle, with recovery of the preovulatory follicle. Serum FSH levels were measured in daily blood samples for one control menstrual cycle prior to treatment and throughout one treatment cycle. The PFF

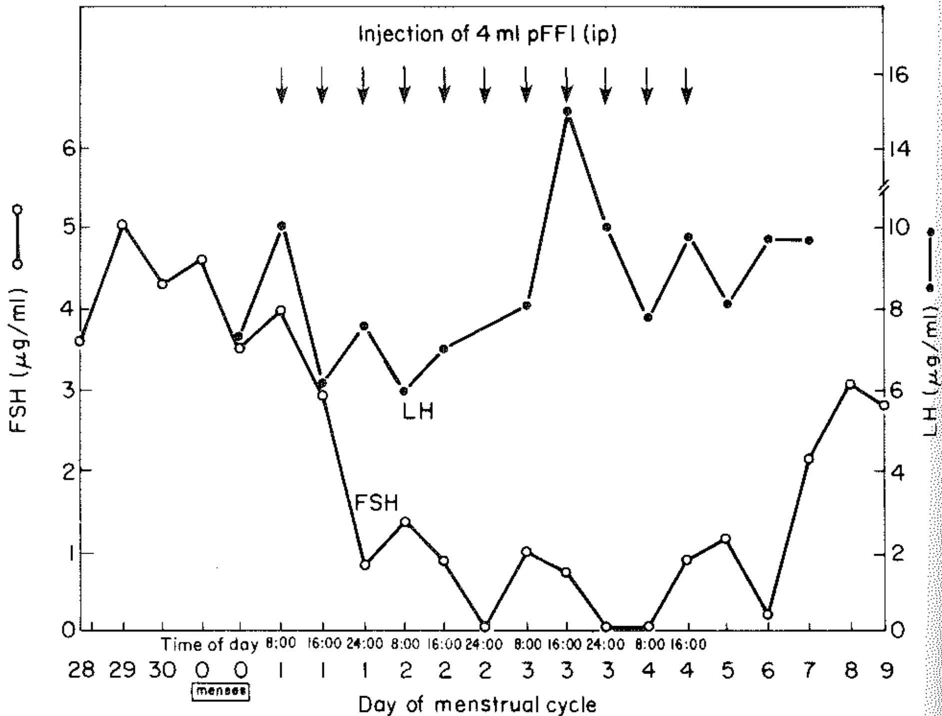

FIG. 21. Temporal course of inhibitory action of porcine follicular fluid (PFFI) upon serum (FSH) in a rhesus monkey given porcine follicular fluid every 8 hours for 4 days during the early follicular phase of the menstrual cycle. Reproduced from Channing *et al.* (1979), with permission of the authors and publisher.

caused a significant decrease in serum FSH levels within 24–36 hours of the start of the treatment in the 4 animals. FSH levels returned to preinjection control levels within 1–3 days after cessation of treatment (Fig. 21). There was a decline of less than 5% in serum LH levels. The follicle present on days 12–14 of the treatment cycle was smaller than normal and contained few granulosa cells (0.1×10^6 cells) compared to control preovulatory follicles, which contained 2 to 50×10^6 cells, i.e., less than 10% of the normal number of granulosa cells. The action of inhibin on follicular maturation is certainly mediated by FSH, but a direct effect of inhibin on the follicle is not excluded.

Two monkeys were given PFF for 4 days at midcycle. In one case in which the treatment was started 1 day prior to the expected midcycle surge of FSH, the midcycle FSH surge was delayed until after cessation of PFF treatment. In the other monkey, in which treatment was started 8 hours after the start of the FSH surge, the surge was shortened to about one-third of normal.

VI. Origin and Transport of Inhibin

A. Testicular Origin

The testis is the source of inhibin, as shown by its presence in testicular extracts. The site of production appears to be the seminiferous tubules because large quantities of the hormone are found in the rete testis, where the secretion of the seminiferous tubules accumulates. Eddie *et al.* (1978) have also identified a substance produced by cultures of rat seminiferous tubules that suppressed the LH-RH-induced secretion of FSH by pituitary cell cultures and inhibited the secretion of LH to a lesser extent.

The experiments of Steinberger and Steinberger (1976) have shown unequivocally that the Sertoli cell is directly involved in the synthesis and secretion of inhibin, called for this reason Sertoli cell factor. In fact, pituitary cells cocultured with isolated Sertoli cells consistently released significantly less FSH than pituitary cells grown alone or cultured with spleen or kidney cells. In contrast, the LH levels in the control and coculture were similar. Furthermore, the culture medium of viable Sertoli cells alone inhibited the spontaneous and LH-RH-induced FSH release in dispersed pituitary cell culture. Since only minimum or no inhibition of FSH release was caused by ruptured Sertoli cells, the inhibin appears to be synthesized by Sertoli cells *in vitro*.

Other evidence for the role of the Sertoli cell in the secretion of inhibin has been provided by Demoulin *et al.* (1979a). Mice testes were maintained in organ culture, and after 4 days the culture medium was removed and placed on dispersed rat pituitary cells. When the testes were cultured at 37°C, spermatogenesis was greatly altered by day 4, whereas the Sertoli cells maintained their normal light microscopic appearance. This medium depressed the LH-RH-induced FSH release without affecting LH release. After 8 days of culture, Sertoli cells were also affected and the inhibitory effect on FSH secretion disappeared (Figs. 22 and 23).

When testis was cultured at 31°C, spermatogenesis was altered histologically at day 8 whereas Sertoli cells remained in good condition for 20 days. The culture medium maintained its inhibin effect for the 20 days of the experiment. Thus, there was a relationship between the histological appearance of the Sertoli cells and inhibiting potency of the culture medium. In contrast, alteration of gametogenesis did not have any effect on the inhibin activity of the culture medium.

Although there is no doubt that the Sertoli cells are responsible for the secretion of inhibin, the possible role of spermatogenesis in the in-

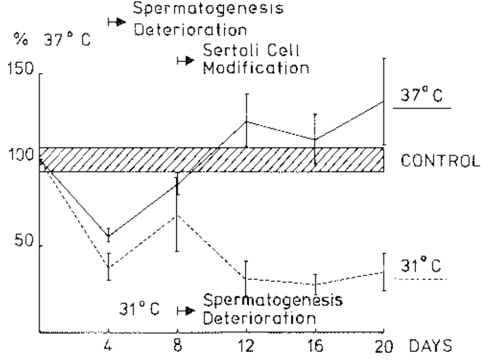

FIG. 22. Influence of testis organ culture medium on luteinizing hormone-releasing hormone (LH-RH)-induced follicle-stimulating hormone (FSH) release (expressed as percentage of the control release representing 100% ± SD = hatched area) by dispersed pituitary cells in culture. When the testis organ culture is performed at 37°C, spermatogenesis is deeply altered after 4 days and Sertoli cells are damaged after 8 days of culture. Release of FSH is significantly reduced by the testis organ culture medium collected on day 4 of culture, but not by the samples of culture medium collected later. When testis organ culture is performed at 31°C, spermatogenesis is altered after 8 days of culture, but no histological modification of Sertoli cells appears for the 20 days of culture. Testis organ culture medium collected on days 4, 8, 12, 16, and 20 of culture significantly depresses the LH-RH-induced FSH secretion, Reproduced from Demoulin *et al.* (1979a), with permission of the authors and publisher.

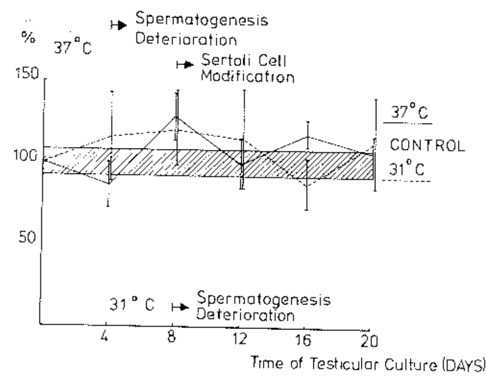

FIG. 23. Influence of testis organ culture medium on luteinizing hormone-releasing hormone (LH-RH)-induced LH release (expressed as percentage of the control release representing 100% ± SD = hatched area) by dispersed pituitary cells in culture. No significant modification of LH release was induced by any of the testis organ culture (at 31°C and 37°C) medium collected every 4 days.

duction of this secretion remains doubtful. Indeed, in some studies there was an inverse linear relationship between the level of FSH, and the qualitative (Franchimont *et al.*, 1972) and quantitative (De Kretser *et al.*, 1972) aspects of spermatogenesis observed in testicular biopsies. On the other hand, in many other studies, serum FSH levels were exceptionally high when late spermatid numbers were normal (Borsch *et al.*, 1973; De Kretser *et al.*,1974; Christiansen, 1975). Furthermore, inhibin activity was observed in extracts of human seminal plasma coming from oligospermic as well as normal subjects, but not from azoospermic patients (Franchimont, 1972). Moreover, it is known that Sertoli cells alone are not capable of reducing FSH levels in the absence of spermatogenesis. Such is the case in the Sertoli cell-only syndrome patients (De Kretser *et al.*, 1972). Finally, Hopkinson *et al.* (1978) studied the modification of germinal epithelium and the changes of gonadotropin levels in rats submitted to irradiation of the scrotum. In their experiments, gonadotropin levels were strongly negatively correlated with both tubular cytoplasm (mainly Sertoli cells) and the spermatid counts. Levels of FSH and LH were significantly higher on day 39 after irradiation as compared to day 26, and this increase coincided with an 85% decrease in the spermatid count. Over the same time interval, the spermatocyte and basal cell counts did not change significantly.

This possible relationship between spermatogenesis and the Sertoli cell resulting in the secretion of inhibin is not excluded by the experiments of Steinberger and Steinberger (1976). As a matter of fact,

cultures of Sertoli cells still contain germinal cells (less than 20%) and the rate of release of FSH-inhibiting substance into the medium declines after 7 days.

Cells of the germinal type might be able to release more or less specific factors that could modulate the secretion of inhibin. These germinal cell factors could persist for some time in spite of the disappearance or alteration of the gametogenic cells or exert a prolonged effect on the secretion of inhibin by Sertoli cells. Careful studies are needed to confirm or refute the hypothesis of this possible relationship between Sertoli cells producing inhibin and germinal cells (spermatids?) that provide the signal or the material for inhibin production.

B. Follicular Origin

In several animal species inhibin activity has been found in follicular fluid pretreated with charcoal to remove the sex steroids.

The models that are used to show this inhibin activity vary from one study to another. Thus, de Jong and Sharpe (1976) and Welschen *et al.* (1977) studied the reduction of FSH levels in adult male rats castrated less than 24 hours before sacrifice. Welschen *et al.* (1977) also studied the inhibition of FSH in unilaterally ovariectomized rats. Schwartz and Channing (1977) observed inhibition of the natural or induced FSH peak during preestrus in adult rats. Last, Marder *et al.* (1977) observed significant falls of FSH levels both in rats ovariectomized precisely in metestrus and in sham-operated animals. Under these various conditions, the levels of LH were little if at all modified. Data from the literature are contradictory concerning the levels of ovarian inhibin during follicular development. According to Lorenzen *et al.* (1978), the concentration of inhibin (which they call folliculostatin) diminishes with the growth of the follicle in the pig. In contrast, Welschen *et al.* (1977) found inhibin in small bovine follicles (5–10 mm in diameter), and maximum concentrations were reached in medium and large (11–20 mm in diameter) follicles. Very little inhibin was found in follicular cysts (diameter greater than 20 mm).

Inhibin, or folliculostatin, found in the follicular fluid of different animals appears to be produced by granulosa cells.

Erickson and Hsueh (1978) showed that the granulosa cells in culture secrete a substance that acts directly on pituitary cell cultures and preferentially suppresses FSH secretion. The inhibitory effect on FSH levels by the culture medium is greater the more numerous the granulosa cells in the culture. A slight reduction of LH release is

observed when the inhibitory effect of FSH reaches its maximum of approximately 60% (Fig. 24).

Granulosa cells acquire the ability to produce inhibin early in follicular development.

The inhibin-like substance secreted by porcine granulosa cells *in vitro* appears to have a molecular weight greater than 10,000 (Anderson *et al.*, 1979).

C. Similarity of Ovarian and Testicular Inhibin

Several arguments favor the identity of inhibin in extracts from biological fluids of males and females.

1. *Similarity of the Inhibitory Effects on Cultured Dispersed Pituitary Cells*

When different quantities of inhibin preparations extracted from human seminal fluid, RTF, and the follicular fluid of mares and women are incubated with pituitary cell cultures in the presence or the absence of LH-RH, the curves of reduction of FSH release into the culture medium, expressed as a percentage of the initial value, are parallel for the various preparations (Fig. 25).

Similar results were obtained by de Jong *et al.* (1978) and by Lagace

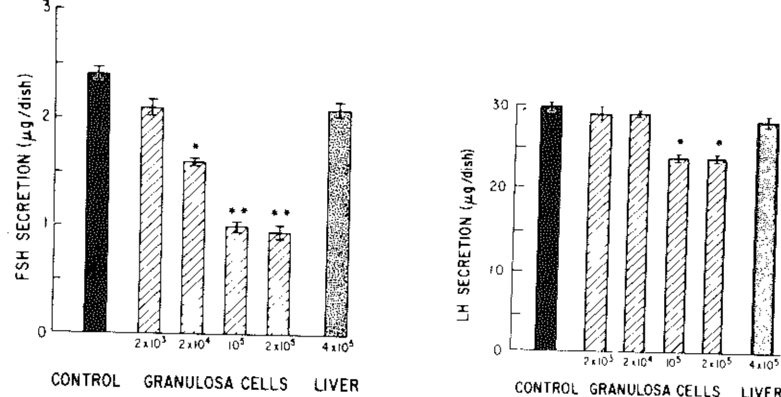

Fig. 24. Release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by pituitary cells incubated with spent media (800 μl) from increasing numbers of rat granulosa cells obtained from preovulatory follicles and maintained in culture for 3 days (mean ± SE of the mean of duplicate determinations of four separate dishes) (* = $p < 0.01$; ** = $p < 0.001$). Reproduced by Erickson and Hsueh (1978), with permission of the authors and publisher.

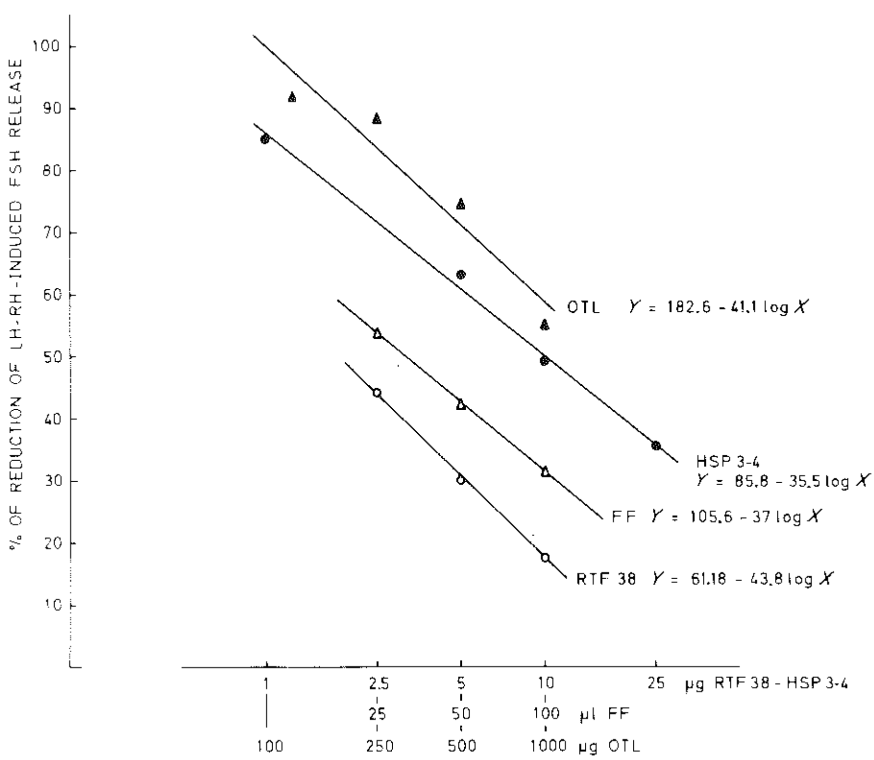

FIG. 25. Reduction of luteinizing hormone-releasing hormone (LH-RH)-induced follicle-stimulating hormone (FSH) release by progressive amounts of several preparations of inhibin. The curves are parallel. OTL, ovine testicular lymph, $a = 41.1$; HSP_{3-4}, fraction 3-4 of human seminal plasma (Fig. 1), $a = 35.5$; FF, mare follicular fluid, $a = 37$; RTF_{38}, semipurified fraction of ram rete testis fluid (Fig. 3), $a = 43.8$.

et al. (1979), who compared the biological activity of inhibin in porcine follicular fluid and Sertoli cell culture medium. In the experiments of both these groups of investigators, the effect of porcine follicular fluid on basal as well as LH-RH-induced LH and FSH release in anterior pituitary cells in culture was indistinguishable from the results obtained with Sertoli cell culture medium.

2. *Efficacy of Inhibin in Animals of Both Sexes*

The active material extracted from biological fluids from males. HSP, BSP, and RTF, reduces FSH levels in normal and castrated, adult and prepubertal, rats of both sexes.

Active preparations obtained from bovine or porcine extracts of

ovaries or follicular fluid reduce FSH levels in normal or castrated animals, as much in males as in females.

3. *Immune Reactions*

Anti-inhibin antisera have been obtained by immunizing rabbits with active preparations of BSP and RTF (Franchimont *et al.,* 1975c, 1977).

Injecting adult rats with these antisera (0.25 ml/100 gm body weight daily for 4 days) resulted in a rise of FSH levels toward the end of treatment in both male and female rats. This effect is attributed to the neutralization of endogenous inhibin. In contrast, LH levels did not increase significantly, and testosterone concentrations remained unchanged (Fig. 11).

Radioimmunological studies of the different preparations show complete cross-reaction between inhibin extracted from BFF and RTF. Inhibin extracts from BFF and RTF completely inhibited the binding of tracer to the antibody. The inhibition curves are parallel (Fig. 8).

D. Transport of Inhibin to the Hypothalamopituitary Axis

There are no data available that enable us to understand how inhibin leaves the ovary or testis and reaches the hypothalamus and pituitary.

In males, there are several possibilities. As the hormone appears to be produced within Sertoli cells, it could leave these cells from their basal surface directly into the lymph or the blood or from their luminal surfaces into the rete testis fluid or from both surfaces. It must not be forgotten that almost all the rete testis fluid is reabsorbed in the head of the epididymis (Setchell, 1970), and this results in a modification of the ionic and protein composition of seminal fluid.

VII. Possible Roles of Inhibin

Inhibin seems to be the feedback agent for quantitative information on rate of sperm production and on follicular maturation. Its first role is to modulate the hormones involved in the regulation of gametogenesis.

A. Negative Feedback on FSH Secretion

All inhibin preparations share the property of selective reduction of FSH secretion. This inhibition is rarely complete, and experiments to date do not permit an assessment of the intensity of FSH suppression under physiological conditions. The experiments are usually set up under circumstances in which FSH secretion is high (castrated animals), do not take account of the biological half-life of inhibin, which is still unknown, and use very impure preparations containing truly minute quantities of the active ingredient. Furthermore, inhibin is doubtless not the only factor that regulates FSH secretion (see Section V,D). Apart from a probable direct action of inhibin (discussed in Section VII,C), this gonadal hormone acts via the reduction of FSH secretion and its consequences.

1. *In Female Animals*

Follicle-stimulating hormone intervenes in the multiplication and functional differentiation of granulosa cells (secretion of FSH at the beginning of the cycle) and in the development of new follicles for the subsequent cycle (preovulatory peak of FSH). At present, one can say on the basis of studies in monkeys (Channing *et al.*, 1979) that early follicular deprivation of FSH induced by active fractions from follicular fluid is deleterious to the later growth of the follicle and of the granulosa cells. Furthermore, in rats (de Jong *et al.*, 1978) unilateral ovariectomy increases FSH levels without modifying the circulating estradiol concentration. Levels of FSH return to normal 28 hours after operation, when the remaining ovary contains twice as many large follicles as an ovary in an intact animal. Follicular fluid prevents this specific rise in FSH without affecting LH levels. One can guess that inhibin has a role in the short-term regulation of the number of developing follicles in the ovary.

In contrast, the fact that there can be ovulation in follicular fluid-treated rats who do not have a preovulatory FSH surge demonstrates the lack of requirement of preovulatory FSH for follicular rupture and oocyte maturation (Schwartz and Channing, 1977).

The role of FSH in the morphological changes of luteinization has been little studied. The effects of inhibin on this process remain unknown.

2. *In Male Animals*

The role of FSH in the initiation and maintenance of spermatogenesis remains in dispute.

During the pubertal period, FSH increases testicular weight and the diameter of the seminiferous tubules (Greep *et al.*, 1942; Simpson *et al.*, 1951). It further intervenes in the multiplication and differentiations of spermatogonia A_0 and A_1 and increases the number of pachytene spermatocytes. It restores the number of spermatogonia type A reduced by hypophysectomy in rats or lambs when testosterone is equally essential (Hochereau de Reviers and Courot, 1978). Treatment of immature male rats (25 days old) with specific anti-FSH serum for a period of 15 days brought about an arrest of spermatogenesis without having any adverse effects on accessory organs and their functions (Shivashanker *et al.*, 1977). Thus, germinal cells—but particularly type A spermatogonia, pachytene spermatocytes, and spermatids—are markedly reduced. All these data show that FSH is needed for the maintenance of different cells in seminiferous epithelium during the completion of the first wave of spermatogenesis.

Our experiments (see Section V,E,1) on the incorporation of tritiated thymidine into testicular DNA show that inhibin preparations reduce the synthesis of DNA *in vivo* and subsequently the mitotic activity of type B spermatogonias during puberty (in 42-day-old rats weighing 150 gm). In 53-day-old animals whose scrota were irradiated 11 days earlier, inhibin similarly reduced the incorporation of tritiated thymidine into DNA (Franchimont *et al.*, 1979b). This incorporation must occur in type A spermatogonia during regeneration (Clermont and Mauger, 1974).

In adult rats, rams, or bulls (Greep *et al.*, 1942; Simpson *et al.*, 1951), there is a positive correlation between the total number of Sertoli cells in the testis and of spermatogonia. Hypophysectomy does not affect the number of Sertoli cells, but it diminishes the stock of spermatogonia and the efficacy of spermatogenesis. In adult rats, FSH maintains the stock of spermatogonia and restores their divisions, whereas meiosis and spermiogenesis are better maintained by LH (Hochereau de Reviers and Courot, 1978). This action of FSH on the early phases of spermatogenesis confirms the earlier work of Means (1975). These authors show that FSH increases the mitotic rate and reduces degeneration among the spermatogonia. Furthermore, Orth and Christensen (1978) have shown by autoradiography that FSH binding sites occur over the surface of spermatogonia in concentrations similar to those of Sertoli cells in the basal compartment of the seminiferous tubules. Other authors believe that it is spermiogenesis that is dependent on FSH (Steinberger, 1971: Gemzell and Kjessler, 1964).

The work of Murty *et al.* (1979) similarly shows that FSH is needed for the maintenance of spermatogenesis in adults. Thus, chronic FSH

withdrawal in subhuman primates affects the fertility of the adult male. The administration of antisera specifically directed against FSH reduces the fertility rate to zero and reduces the number of spermatozoa to 44% of those found in control animals and the number of living spermatozoa is no more than 7.4%.

It seems logical to think that spermatogenesis is under the control of FSH as well as other hormones. FSH acts particularly on the multiplication of spermatogonia. Inhibin could depress this action. Conversely, a lack of inhibin when spermatogenesis fails would increase FSH with recruitment of spermatogonia in order to restore spermatogenesis.

B. Negative Feedback on LH Secretion

Inhibin also exerts an effect on LH secretion. This hormone is less sensitive to inhibin, probably because the mechanisms of inhibition of FSH and LH are different. Thus, inhibin has no inhibiting effect on the synthesis of LH by the gonadotroph *in vitro* unlike that of FSH (see Fig. 6). In contrast, the effect on secretion of endogenous LH-RH *in vitro* and on gonadotropin secretion by exogenous LH-RH, as much *in vitro* as *in vivo,* leads us to think that inhibin essentially acts on the release of LH (see Section V,B). It results in an inhibition of the secretion of LH that is more limited and much weaker than that on FSH, while tonic secretion of LH is maintained.

The physiological importance of the action of inhibin on the secretion of LH and its peripheral effects has not yet been seriously studied.

C. Is Inhibin a Cybernine?

Since 1976, various studies have shown that the ovary produces substances that modify its response to gonadotropins and prevent different stages of the reproductive cycle from developing. These substances may be included among the cybernines, which are products formed in a tissue that regulate locally the function of this parenchyma (Guillemin, 1978). Channing and co-workers showed that the granulosa cell produces a peptide factor, molecular weight approximating 2000, that inhibits the spontaneous maturation of isolated oocytes (Tsafriri *et al.,* 1976). This factor also opposes the production of progesterone by the cumulus surrounding the oocyte *in vitro* (Channing *et al.,* 1978).

Ledwitz-Rigby *et al.* (1977) have also demonstrated the presence of an inhibitor of luteinization in follicular fluid. It is present in large quantities in the fluid of small follicles and prevents the granulosa cells from luteinizing, i.e., from assuming the typical morphology and increasing progesterone production. It also reduces the formation of LH receptors and the production of cyclic AMP (cAMP) in response to exogenous LH (Channing *et al.,* 1978).

Other factors less well characterized biologically have also been found, on the one hand, in follicular fluid, an inhibitor of the effect of FSH that depresses cAMP production induced by FSH in granulosa cells of small follicles (Channing *et al.,* 1978) and, on the other hand, in the corpus luteum, LH receptor-binding inhibitor (LHRBI), which reduces binding of LH to the receptors on luteinized cells and on granulosa cells (Sakai *et al.,* 1977). LHRBI increases in the corpus luteum of the pig in proportion to its age and may play a role in the decline of luteal function. The secretion of progesterone under the influence of LH is also inhibited by this factor.

In aqueous testicular extracts, Reichert and Abou-Issa (1977) have provided evidence for a polypeptide factor, molecular weight approximately 1400, that inhibits the binding of FSH to freshly prepared testicular receptors.

In males, it nevertheless seems that some inhibin preparations exert a direct effect on spermatogenesis independent of FSH. In fact, one can observe an inhibition of the incorporation of tritiated thymidine into testicular DNA in the presence of inhibin compared with the values obtained in its absence. This action *in vitro* of inhibin makes it comparable to the testicular chalone described by Clermont and Mauger (1974, 1976) and defined as "an internal secretion produced by a tissue for the purpose of controlling, by inhibition, the mitotic activity of the same tissue" (Bullough, 1967). These authors think, though without definite proof, that chalones come from spermatogonia, and it is known that inhibin is secreted by Sertoli cells. Inhibin, produced by Sertoli cells and modifying the mitotic activity of germinal cells within the same seminiferous tubules, also meets the definition of a cybernine.

To say either that there is a common origin or structure of these ovarian and testicular factors and inhibin, or the opposite, would not be justified at this time. None of these factors have yet been tested to assess their effect on FSH secretion. Conversely, the possible action of inhibin on the maturation of the oocyte, on luteinization, and on LH binding to its receptors has yet to be studied.

As for the spermatogonial chalones described by Clermont and

Mauger (1974, 1976), it could be speculated that inhibin is implicated in the control of the numerical growth of spermatogonial stem cells. In adults, it would maintain the spermatogonial stem cell population in a steady state and, as a result, arrest the growth of seminiferous tubules. This action of inhibin could occur directly and/or via FSH secretion.

VIII. Summary and Conclusions

There is now convincing evidence for the existence of inhibin. This hormone is formed in the seminiferous tubules by Sertoli cells in males and by granulosa cells in the follicles of females.

Inhibin is not a steroid but is peptidic in nature. Its biological action is destroyed by digestion with trypsin or pepsin and by heat. It induces antibodies capable of neutralizing endogenous inhibin in adult male or female animals into which they are injected. The peptide is not identical to androgen-binding protein, nor is it a fragment of gonadotropins. Its molecular weight is not exactly defined, and it may exist in two forms. The relationships between these two forms have not yet been elucidated.

Inhibin exerts preferential inhibition on the synthesis and release of FSH by pituitary gonadotrophs maintained in culture. The dose required to exert an effect on LH is much higher. It reduces the amount of endogenous LH-RH in the hypothalamus maintained in culture. In the intact animals, the observed effect on gonadotropin levels is the result of the action of inhibin at both sites. Inhibin has no effect on the secretion of TSH, prolactin, or growth hormone either *in vivo* or *in vitro*. It undoubtedly has an effect on gonadal function in males and females via a reduction of FSH secretion, but a direct action on the gonad also is suspected.

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VITAMINS AND HORMONES, VOL. 37

Hormonal Control of Calcium Metabolism in Lactation

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I. Introduction

The increased need for calcium to satisfy the demands of milk production and at the same time to maintain the blood calcium concentration at an adequate level makes the normal physiological condition of lactation of special interest to endocrinologists concerned with the calcemic hormones and calcium homeostasis. The rat is an especially advantageous animal for study of lactation because the much higher rate of calcium excretion in milk in the rat than in the human, relative to body size, puts greater stress on homeostatic mechanisms in the rat and accentuates hormonal changes that may be only subtle in human lactation. Findings in the rat may help to focus attention on factors

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