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MINIREVIEW

Physiology and Biochemistry of Ovarian Inhibin[1] (42017)

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There are three recent reports of amino acid sequence studies on inhibin preparations. The preparation obtained in Bombay and partially sequenced by collaborators in Stockholm dealt with a preparation from human seminal plasma. This preparation had a molecular weight of approximately 14,000 (1). On the other hand, Seidah and colleagues in Montreal (2) reported a mixture of four peptides that they sequenced simultaneously to determine 31 of a possible 35 residues in a "complete" peptide. Three of the peptides sequenced as though they were derived from the larger complete peptide. The molecular weight of this peptide was estimated to be 4-5000. In a subsequent paper from this laboratory and two collaborators from the University of California in San Francisco (3), the principal peptide in the mixture was isolated and sequenced (31 residues) and a synthetic peptide with this sequence was shown to have inhibin activity equal to or slightly greater than the native peptide. We will comment further on these studies later in this review.

The landmark value of these studies lies in the fact that after many years investigators of inhibin are beginning to talk of exact biochemical structures that can be checked and verified in other laboratories. Most importantly, well-defined compounds can be used to compare results from laboratory to laboratory. Prior to now there was always wide discrepancy between reports (often these were little more than undocumented claims) from various laboratories. This led many to doubt the reality of an inhibin that suppressed the follicle stimulating hormone (FSH) secretion of the pituitary without affecting the luteinizing hormone (LH). Much still remains to establish the true physiological significance of inhibin and the exact nature of the native inhibin secreted by the Sertoli cells or granulosa cells of the gonads.

For these reasons and from a developing understanding of the nature of the inhibin problem in our own laboratory, particularly relating to the isolation of native inhibin, we feel this is a propitious time to attempt a review of the current status of inhibin research.

The designation of inhibin, a gonadal protein capable of specifically inhibiting follicle stimulating hormone secretion, originated with the early report of McCullagh (4) who observed that male rats made cryptorchid exhibited a castration-like appearance of the pituitary. McCullagh postulated that the cryptorchid gonad failed to secrete a protein material which acts by negative feedback on the pituitary gonadotrophs. In the past 10 years investigators examining the male gonad and its secretions have sought to isolate the active principle from testis extracts (5–7), rete testis fluid (8, 9), from seminal fluid (10–12), and from Sertoli cell conditioned medium (13). Because male inhibin appeared to be a physiologically active substance (see reviews 14–16) and because the Sertoli cell appeared to be a source, investigators in ovarian physiology sought for inhibin in ovarian extracts and follicular fluid. The first detection of inhibin in ovarian follicular fluid was reported by de Jong and Sharpe (17) (bovine) and Schwartz and Channing (18) (porcine). Since that time, the literature on

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ovarian inhibin has grown apace with that in the male inhibin arena. This present review will emphasize the physiology and the biochemistry of ovarian inhibin but with reference to other sources as appropriate. The physiology will be examined in terms of the effects of inhibin and the changes in inhibin secretion (blood, ovary) during physiological and pathological states. Some consideration will be given to the regulation of inhibin secretion by various hormones. The biochemistry will be focused on the purification and characterization of inhibin including a description of some available bioassay methods.

Finally we will consider the recent amino acid sequence reports for inhibin and attempt to project from these studies how these and other reports relate to a putative "native" inhibin molecule. To do this we will draw in part upon some unreported results from our own laboratory.

Inhibin Physiology. *A. Steroidal vs non-steroidal negative feedback action on pituitary: evidence for ovarian inhibin.* In the castrate female rat and mouse there are instances where administration of steroids, i.e., estrogen, progesterone, testosterone, fail to suppress serum FSH to baseline, intact animal levels, whereas administration of a crude steroid-free inhibin preparation (charcoal-treated porcine follicular fluid) does suppress serum FSH but not LH to baseline levels. These observations have been elegantly reviewed (19–22) and will only be briefly summarized here.

In the female rat there is a prompt postcastrational rise in FSH (3–8 hr), whereas the rise in LH is sluggish taking 1–2 days (23–26). Injections of massive doses of steroids including estrogen and progesterone failed to inhibit the postcastrational rise in serum FSH more than about 30%, whereas serum LH is suppressed (23, 27). The same situation exists in the castrate mouse (28). Furthermore, during the normal rat estrous cycle there is a postovulatory selective rise in serum FSH unaccompanied by a rise in serum LH (see reviews 21, 22, 27). This postovulatory surge in FSH is not suppressable with exogenous progesterone (29), estradiol (30), or testosterone (31). Sherman *et al.* (32) and Faiman and colleagues (33) observed that as women approach menopause they could still have normal menstrual cycles, normal estrogen, progesterone, and LH levels, but they had a selective rise in serum FSH.

In sharp contrast to the lack of inhibitory effects of steroids on serum FSH in castrate rodents, administration of steroid-free ovarian extracts or follicular fluid can selectively inhibit the postcastrational rise in serum FSH and not LH [rat: (31, 34); monkey: (35); horse: (36); hamster: (37)]. Unlike steroids, the steroid-free ovarian extracts can also inhibit the postovulatory rise in serum FSH in the rat (18, 38, 39). This activity present in the steroid-free extracts of ovarian homogenate or follicular fluid thus appears to have the characteristics of inhibin activity, i.e., it can selectively suppress serum FSH and not LH, and is not a steroid. Operationally it may be defined as inhibin. For inhibin to have a physiological role, changes in ovarian inhibin activity must correlate causally with changes in pituitary FSH secretion.

B. Effect of inhibin in the rodent. It was demonstrated that ovarian inhibin could suppress FSH secretion in a castrate rat comparable to serum FSH levels observed in intact rats. Porcine or bovine follicular fluid served as a suitable source of ovarian inhibin activity for physiological studies (38, 40) and had roughly the same inhibin activity measured in a pituitary cell culture assay (41, 42). Physiological effects of follicular fluid must be interpreted with caution since follicular fluid contains many other factors such as FSH binding inhibitor activity (see reviews 41, 43). Williams and Lipner (44) were able to mimic LH and FSH patterns of the intact rat with castrate rats by judicious timing of follicular fluid injections. They found that manipulating the steroid infusions alone was adequate to reproduce the pattern of LH, but not adequate to reproduce the pattern of FSH. They concluded that steroids regulate the timing of the gonadotropin secretion and follicular fluid inhibin regulates the intensity of the FSH surge. This conclusion is supported by observations on serum and ovarian vein inhibin activity in unilaterally castrate rats (see below, 45). When follicular fluid is administered to the castrate rat it produces a dose–response related suppression in serum FSH but not serum LH either administered intraperitoneally (34) or intravenously (46). If steroids (estrogen, progesterone) are ad-

ministered alone or with porcine follicular fluid, suppression of FSH to baseline levels is the result of additivity of the two agents (21, 27, 47, 48). The interaction between the follicular fluid actions and steroids varies with the type of administration; for example, in the ovariectomized mouse, estrogen can exert 20–30% suppression alone whereas administration of porcine follicular fluid can increase the suppression to greater than 50%.

These effects of porcine and bovine follicular fluid on serum FSH in the castrate rat could be attributed to effects on clearance of FSH. Schwartz recently carried out studies that partially rule out this possibility (21). She observed that after hypophysectomy the disappearance curve of serum FSH resembled that observed when porcine follicular fluid was administered.

Follicular fluid administration also suppresses serum FSH in intact rats if given at various times during the rat estrous cycle. It can selectively inhibit the primary preovulatory FSH surge without inhibiting the LH surge in the rat (49, 50) or hamster (51) or the secondary FSH surge occurring during estrus (18, 52). If the follicular fluid was given during late proestrous and estrus the secondary surge of serum FSH was blocked; in addition, follicular recruitment was also blocked (38). Hoak and Schwartz (53) went on to show that the inhibitory effects of the follicular recruitment could be reversed by administration of ovine FSH. This essential experiment was needed to prove that the effects of the follicular fluid were mediated by its inhibitory effects on serum FSH (i.e., due to its inhibin activity) and not due to some other adverse effects on the ovary. It must be cautioned here, and in the following rat studies, that all studies were carried out with crude follicular fluid which also contains FSH binding inhibitor(s) (54), as well as follicular protein (55) and a luteinization inhibitor (56) which also could bring about follicular demise. These studies rightfully should be repeated using pure inhibin which is free of these other inhibitors.

Phillips *et al.* (57) observed that porcine follicular fluid (PM-10 filtered) reduced the number of ova recovered from the oviducts in a dose-related manner on the afternoon of estrus following their treatment schedule. If the oviducts were examined for ova on the following morning (expected metestrus) the mean number of ova was not significantly different from the number observed in control animals. From the observation of ovulation rate and vaginal cytology it appeared that the treatment with porcine follicular fluid delayed ovulation. In another experiment it was found that pregnancy was decreased from 11.3 implants to 2.4 and 1.5 if the rats were treated two, three, and four times a day, respectively. If the rats were mated prior to porcine follicular fluid treatment the number of viable implants per rat (11.0) was not significantly different from the mean of 12.8 implants in serum-treated control rats. Since the pregnancy itself was not affected the inhibitory effect of the porcine follicular fluid was probably on the ovulation rate.

The site of inhibitory action of the porcine follicular fluid on ovulation and serum FSH levels is the anterior pituitary since the porcine and bovine follicular fluid can inhibit LHRH stimulated FSH and not LH secretion *in vivo* in the rat (45, 58) and in rat pituitary cell cultures (59–61). The additional inhibitory effects of crude inhibin preparations on hypothalamic LHRH content observed by Demoulin *et al.* (62); Lumpkin *et al.* (63), and Moodbidri *et al.* (64) could exist. However, it is difficult to envision how a specific inhibition of FSH secretion could be mediated solely by inhibition of LHRH content unless this inhibition is presumed to be very localized (23, 51).

C. Effect of inhibin in the monkey. As was the case in the rat, administration of charcoal-treated porcine follicular fluid to castrate female mature and infant monkeys as well as cycling monkeys, can depress serum FSH levels without altering serum LH except during the midcycle period and after GnRH administration (35, 65, 66). If the follicular fluid is injected during the first 3–4 days of the menstrual cycle the expected dominant follicle fails to mature properly and becomes atretic suggesting that deprivation of FSH early in the life of a follicle leads to adverse consequences in the later life of that follicle (65, 67).

D. Changes in inhibin secretion during various physiological states. For inhibin to qualify as a physiologically relevant hormone it should obey all of the classic roles of a hormone. It should be secreted into the blood

and be in a greater concentration in the blood draining the gland compared to blood entering the gland or in the general circulation. Removal of the gland should remove the effects of the hormone. The hormone should be secreted at physiologically appropriate times, (i.e., when FSH is elevated there should be generally less inhibin secreted and vice versa). As will be discussed below it appears that ovarian inhibin fulfills all of these qualifications and therefore can be called a hormone. The studies could only be made when an appropriate bioassay could be developed capable of measuring inhibin activity in ovarian vein blood. The rat anterior pituitary cell culture system originally described by Vale *et al.* (68), has proven satisfactory (see below for a discussion of inhibin bioassays). Rat ovarian venous blood has been assayed for inhibin activity and the activity is greater than that in the peripheral venous serum. Moreover, inhibin is secreted at appropriate times in the rat. There are low levels late on the afternoon of proestrus and on the morning of estrus for the secondary rise in serum FSH (52). We have recently measured the inhibin activity throughout the estrus cycle in rat follicular fluid and correlated it with ovarian vein levels (69). The follicular fluid levels of inhibin are about 1500 times greater than the ovarian vein levels. The follicular fluid levels of inhibin activity decreased about 2 hr later than the ovarian vein levels on the afternoon of proestrus. The observation of larger amounts of inhibin activity in follicular fluid compared to ovarian vein blood [pig: (67); rat: (38, 52, 69); monkey: (70); human: (71)], could reflect the fact that the interior of the follicles is avascular and inhibin made by the granulosa cell accumulates and is only slowly cleared from the follicular atrum. Alternately, since inhibin behaves as a high-molecular-weight complex (see below) its diffusion out of the follicle would be expected to be restricted.

In an elegant study comparing ovarian inhibin content in two strains of Merino ewes, Cummins *et al.* (72) observed that the Booroula ewes, which have a higher ovulation rate compared to the control strain have a lower ovarian content of inhibin and a higher serum FSH level. This suggests that the differences in ovulation rate could be due to differences in inhibin content.

Inhibin activity is also present in follicles and ovaries of infant monkeys and humans [Channing, Tanabe, Phillips, and Hahn, manuscript in preparation; reviewed by Hoover *et al.* (70)]. During puberty there is an increase in inhibin activity, perhaps reflecting a stimulatory action of FSH upon inhibin secretion. Follicles doomed for atresia as judged by their androgen/estrogen ratio (73) decrease their ability to secrete inhibin activity (74). The significance of their decrease in inhibin biosynthesis by atretic follicles may be that during the luteal phase of the menstrual cycle when there are more atretic follicles compared to the follicular phase (75) there may be a response with a rise in serum FSH at the end of the luteal phase which then can help to rescue follicular growth.

E. Hormonal control of secretion of inhibin by ovarian cell types. It is clear that the granulosa cell of the ovarian follicle is the principal source of ovarian inhibin since cultures of granulosa cells but not of thecal tissue or stroma secrete the bulk of inhibin activity [rat: (76, 77); monkey: (41, 70); human: (41, 70); cow: (79); pig: (80)]. Porcine, rat and bovine granulosa cells *in vitro* generally secreted more inhibin activity compared to human and monkey granulosa cells, as reflected in the inhibin content of the respective follicular fluids. Interestingly, as the porcine follicle matured, cultured granulosa cells increased their potential to secrete inhibin activity (80). This is probably due to prior exposure to some LH/FSH *in vivo* which is adequate to mature these cells, but not in quantities sufficient to fully luteinize them and thus decrease inhibin secretion. In contrast, levels of inhibin activity in follicular fluid from large porcine follicles were lower than levels in follicular fluid from small follicles recovered from pig. This seemingly anomalous observation could be due to increased blood flow to the large follicles which serves to clear the inhibin from the follicles in the blood. Two reports indicate that FSH stimulates inhibin production by granulosa cells *in vitro* [porcine: (80) and bovine: (79)]. The androgen enhancement of granulosa cell inhibin activity secretion may be responsible in part for the elevated follicular fluid levels of inhibin activity observed in women with polycystic ovarian disease (81).

F. Changes in follicular inhibin activity

associated with various clinical disorders. Clinical medicine has provided unique situations which can be explained by alterations in ovarian inhibin secretion (Table I). A possible example is polycystic ovarian disease (PCO) in which amenorrheic women secrete elevated amounts of androgens and have numerous small follicular cysts. These women have normal or low levels of serum FSH with normal or elevated levels of LH. If these women are given an LHRH challenge they secrete elevated levels of LH but secrete normal or subnormal amounts of FSH. We observed that the follicular fluid of these patients had a greater concentration of inhibin activity compared to similar sized pooled follicles obtained from normal women in the follicular phase of the menstrual cycle (81). Interestingly, the number of granulosa cells per follicle in the polycystic follicles was 10-fold less compared to similar sized normal follicles. This inhibin activity was detected in the ovarian vein of 3 out of 5 PCO patients whereas in normal women 2 out of 15 patients had detectable inhibin activity in ovarian vein blood (Tanabe and Channing, unpublished observations). It is tempting to conclude that the increased inhibin secretion in the PCO patients could be responsible for the altered FSH/LH ratio. However, steroid secretion in the appropriate ratio could also alter the FSH/LH secretion rate in PCO patients.

The selective rise of serum FSH prior to changes in serum levels of LH, estrogen, and progesterone during the year(s) immediately prior to menopause can best be explained by a decline in inhibin secretion as originally proposed by Sherman *et al.* (32) and Reyes *et al.* (82).

Bioassay of Inhibin. In the isolation and purification of a biologically active substance, it is essential to have a specific assay system by which the substance can be quantitatively monitored. However, in the case of inhibin, there is no concensus assay system nor reference standard. Much of the controversial data regarding the potency and the nature of this molecule (see following sections) probably results in part from the lack of such a standard assay and standard reference material for inhibin. Several excellent reviews have been published on the assays of inhibin (14, 19, 83–86). Baker and his colleagues have critically evaluated most of the available methods (83, 86). Here we intend only to review briefly the four basic types of assays which are generally acceptable and have a good dose–response relationship as well as index of precision. In addition, we will describe in detail the modified *in vitro* assay that we currently use in our laboratory.

A. In vivo assay. (1) Suppression of FSH-augmentation of hCG-induced uterine or ovarian weight increase (reversed Steelman-Pohley assay). In an attempt to measure the

TABLE I. CLINICAL SITUATIONS ASSOCIATED WITH ALTERED OVARIAN INHIBIN

Change in follicular inhibin activity	Clinical situation	Change in serum FSH/LH and other functions	References
Increase	Polycystic ovarian disease	Basal LH elevated basal FSH normal or low, LHRH stimulated FSH low, LHRH stimulated LH elevated	(81)
Increase	Human-menopausal gonadotropin (FSH/LH 1:1)-hCG-treated women	Usually no estrogen induced LH/FSH surge; no ovulation unless given exogenous hCG to induce ovulation	(41, 55, 70)
Decrease	Luteal phase of the menstrual cycle	Rise of FSH (selective) at the end of menstrual cycle due to presence of atretic follicles	(67, 75, 79, 127)
Decrease	Perimenopausal period	Elevated FSH, normal LH, estrogen, and progesterone levels	(Channing *et al.*, unpublished observations; 32, 82)

FSH content in the rete testis fluid, Setchell and Siranathsingh (87), found that there existed an antigonadotropic activity in the fluid, which could reduce the synergistic effect of endogenous FSH on the weight increase of the uterus and ovaries induced by hCG in immature female rodents. They proposed that this effect might be used as an assay for inhibin. Based on this proposal, Chari *et al.* (88) developed an inhibin assay measuring the suppression of ovarian weight induced by hCG treatment in immature rats. This assay is generally referred to as the reversed Steelman-Pohley assay. Using a similar protocol, Ramasharma *et al.* (89) developed another assay using immature mice and measured the inhibition of uterine weight instead of ovarian weight. The latter seemed to be more sensitive and specific than the former assay.

Although this assay is simple and convenient, it lacks the specificity required for the reliable estimation of inhibin activity. It might also measure material(s) that interfere with the action of gonadotropin on the target tissue in addition to, or instead of, inhibin that suppresses endogenous FSH secretions. It is well established that follicular fluid contains a variety of factors (16, 19), some of which might be expected to directly interfere with gonadotropin interaction with the gonads or other gonadal functions. These include binding inhibitors of LH (90) and FSH (54); luteinization inhibitor (56); and the follicular regulatory protein(s) (91). With the presence of such factors, it is not surprising that this assay provides unreliable estimates of inhibin activity.

(2) Suppression of serum levels of FSH. Measurement of the fall of the serum FSH level after single or multiple injections of inhibin preparations has been widely used as a direct *in vivo* assay of inhibin activity. Both rodents (7, 12, 34, 85, 92) and large animals such as rabbit (14), monkey (35), sheep (93), and mare (36) have been used for this type of assay. In rodents, response to inhibin treatment varied as to the age, sex, and the duration of castration (84, 92). In general, female rats are more responsive than male rats, and the most sensitive age is 75 days in female and 34 days in male. Furthermore, castrated animals are more sensitive to inhibin treatment compared to intact rats. The maximal suppression of FSH levels in these animals is often greater than 50% of the control. However, this sensitivity eventually decreases in chronically castrated animals. Lee *et al.* (92) therefore proposed that the acutely castrated animal would be preferable in this type of assay. Using the acutely ovariectomized female rats at metestrus, Schwartz and her colleagues (19) have developed a sensitive *in vivo* assay with good index of precision. This method is highly specific, but due to its requirement of large numbers of specially castrated animals on a specific day plus the narrow dose range of the tested samples, it is not practical for routine screening of inhibin activity in the large number of fractions obtained during a purification process.

B. In vitro assay. (1) Incubation with rat or mouse pituitary tissue. Inhibition of FSH release induced by GnRH in the short-term incubation of either the whole pituitary (94) or pituitary halves (83, 95, 96) has been used for detecting inhibin activity. This method in general is much less sensitive than the pituitary cell method (83). It was reported that the rete testis fluid caused the inhibition of LH release to a greater extent than that of FSH (83, 97) as measured by this method.

(2) Pituitary cell culture. Use of the enzyme-dispersed pituitary cell system for inhibin assay is more preferable than other methods. It has the highest sensitivity. It is 100 to 1000 times more sensitive than other methods (83) with one surprising exception: Franchimont *et al.* (98) claimed the reversed Steelman-Pohley assay was several times more sensitive than the pituitary cell culture.

The pituitary cell culture assay has the ability to handle a large number of samples with a wide range of test doses in one assay. Except for its labor-intensive aspect this method is ideal for screening fractions for inhibin activity during an isolation process. Furthermore, this method gives good dose–response curves and the potencies of the tested samples can be calculated by parallel line assay (99) method with a good index of precision. The main drawback in this method is its long turn-around time; it generally requires a 72-hr preculture period for the cells to form a monolayer and then up to 72

hr for the test material to show a significant effect in suppressing the FSH level in the cell culture. In addition, the cells are susceptible to nonspecific toxic agents which may influence their viability and metabolism (100, 101).

Several groups working in the inhibin field have adopted this method to assess inhibin activity; however, each group has a different protocol for their own assay. The differences are in age and sex of rats, the enzyme used for cell dispersion, concentration of cells used in each assay, culture medium, time of exposure to the test sample, and the measurement of either basal or LHRH-stimulated levels of FSH.

(a) Measurement of FSH under stimulation. Eddie *et al.* (59) developed an inhibin assay based upon the suppression of LHRH-stimulated secretion of FSH by the male rat monolayer pituitary primary cell cultures. The cultures were first exposed to test materials for 3 days, and then the levels of FSH in the media were measured by radioimmunoassay after the cultures were exposed for 6 hr to a maximally stimulating dose of LHRH. This method had good dose–response characteristics and FSH was suppressed to 10–20% of the control level. However, under LHRH stimulated conditions, the level of LH in the media was also reduced, but a higher dose of the test sample was required. They also found that androgens act also as an inhibin-like substance to suppress both the levels of FSH and LH. In the latter case, the dose–response curve was not parallel to that of inhibin, and the secretion of LH was more affected than that of FSH.

(b) Measurement of FSH secretion after stimulation by steroids. Using adult female rats, Shander *et al.* (61) developed another pituitary cell culture assay. They included both estradiol and progesterone to the cultures to stimulate FSH secretion. Either estradiol or progesterone alone had little effect on FSH secretion, but the addition of both steroids together produced about 60% increase in basal FSH secretion. Further addition of testosterone to the cultures would increase the FSH secretion to 200% of FSH basal level. The presence of steroids did not affect the basal LH level; neither did they cause a nonspecific effect in suppressing LH level by the tested material as was the case in LHRH-stimulated cultures. Study of the effect of various steroid concentrations affecting FSH secretion (Hoover, Anderson, unpublished observations) indicated the optimal concentration was $10^{-8}\,M$ estradiol plus $10^{-6}\,M$ progesterone. Exposure of cultures to the steroid-containing medium for 24 hr gave a three- to fourfold increase of FSH secretion as compared to the basal level. Although the steroids can augment the secretion of FSH several-fold from rat pituitary cell cultures, it is interesting that in ovine pituitary cell cultures, estradiol inhibits the basal secretion of FSH into the culture medium (102).

(c) Measurement of basal FSH secretion. Since in the LHRH-stimulated pituitary cell cultures inhibin becomes less specific and can inhibit both FSH and LH secretion, de Jong and his colleagues (84) used only the basal level of FSH to assess inhibin activity. This method is very specific, but the low level of basal FSH secretion from the cultures may reduce the sensitivity of the assay as compared to the LHRH-stimulated cultures.

Scott *et al.* (103) described another type of inhibin assay with male rat pituitary cell cultures. After the cultures were exposed to the test materials for 72 hr, instead of measuring FSH in the media, they removed the media and lysed the cells to measure the intracellular FSH content. In this assay, they found that inhibin could reduce the FSH level without affecting the LH level. Addition of androgens to the cultures would increase the cell content of FSH dramatically, while other steroids had very little effect. This method is also highly specific and has the advantage of requiring less cells per dose as compared to other pituitary cell cultures. However, in our experience the assay is less sensitive and the dose–response curve has a poorer slope than that method described below.

(d) Our modified pituitary cell assay. In our laboratory, we have developed a pituitary cell culture assay which is an adaptation from the method of Ying *et al.* (104) and that of Shander *et al.* (61). This assay can handle a large number of test samples at one time and allows us the flexibility to measure both medium (released) and cellular (content) FSH in one assay.

For preparation of pituitary cells, generally 40 pituitaries were collected from male Sprague–Dawley rats (275–300 g) in Hepes-buffered saline (pH 7.35), and were dispersed with two consecutive enzymatic solutions employing a concentration of one pituitary per milliliter. The first enzyme solution consisted of collagenase (CLS-II Worthington) 4 mg/ml and dispase (protease grade II, Boehringer Mannheim) 2 mg/ml in Hepes-buffered saline with 1% BSA. Digestion was carried out at 37°C for 70 min with constant shaking. The digested tissue was separated from the enzyme solution by gentle centrifugation (150*g* for 10 min) and the pellet was redigested in Hepes-buffered saline containing neuraminidase (Sigma N-2876), 8 μg/ml plus EDTA, 200 μg/ml for another 10 min at 37°C. After the second digestion, the cells were washed twice with culture medium, and plated into 19-mm 24-well culture clusters with a concentration of ca. 1.2 to 1.5×10^5 cells/well/0.5 ml. The culture medium consisted of F-12/DMEM/BGJB (6:3:1 v/v) with the following supplements: BSA 2 g/liter; Hepes, 2.38 g/liter; gentamycin, 50 mg/liter; glutamine, 2 m*M*, bovine fetal serum, 10%; and horse serum, 5%. Using this method of cell dispersion, we routinely get 1.5 to 2.0 million cells per pituitary for an average of ca. 2 pituitaries per one 24-well cluster for culture. With 40 pituitaries we could obtain ca. 20 clusters of cultures which can assay our standard reference material at six doses plus 38 samples at three doses each with triplicate replication of each dose.

Seventy-two hours were required for the cells to plate out into a uniform monolayer when incubated in a water-saturated incubator at 37°C in an atmosphere of 3% CO_2 in air. On the fourth day, the cultures were washed twice with culture media and new culture media (0.5 ml/well) containing 10^{-6} *M* progesterone plus 10^{-8} *M* estradiol. The samples to be tested were added to the cultures followed by another 3 days of incubation. At the end of this period, the media were removed for assay of FSH secreted by the cells. To the monolayer cells, 300 μl of 0.1% Triton X-100 in 0.05 *M* phosphate buffer was added to each well to lyse the cells. The cell lysates were then stored at −20°C for measurement of cell content of FSH. It has been our experience that the samples active in suppressing FSH secretion are always active in reducing the cell content of FSH, and that the FSH secretion into the media gives a more sensitive assay for inhibin activity. Thus we routinely assay the FSH levels in the culture media and use the cell lysates as a back-up in case problems develop with measurement of the FSH secretion.

In each assay our laboratory standard reference material was tested over the dose range from 0.0003 to 0.1 mg/ml. The dose–response curve usually covered the range of 0.001 to 0.01 mg/ml. The tested samples were assayed over a range from 0.0003 to 0.3 mg/ml. The most useful range was 0.001 to 0.1 mg/ml for screening inhibin activity in fractions obtained from the porcine follicular fluid. Samples that were not active at the dose of 0.3 mg/ml were considered inactive fractions. In our experience, sample concentrations higher than 1 mg/ml are usually toxic to the cells due to the osmotic shock. Osmotic shock to the cells will induce the release of both LH and FSH, resulting in a biphasic curve for the tested sample, i.e., inhibition of FSH at low doses followed by stimulation of FSH release at higher doses. Since some of our inhibin fractions are not readily soluble we routinely suspend them in 0.01 *M* phosphate-buffered saline at pH 9.0, 1 mg/ml overnight at 4°C. The samples are then diluted to adjust the pH at the appropriate concentrations with culture medium for assay.

In the preparation of our own laboratory standard reference material, we used the PM-10 retentate of porcine follicular fluid recovered from 1–5 mm follicles diluted with an equal volume of 0.2 *M* KCl and then precipitated with 90% ethanol overnight at 4°C. After centrifugation to remove the supernatant, the precipitate was dried *in vacuo*. The dried 90% ethanol precipitate was designated HSN-8-111.9P standard. This standard material is about 100 times more active than the ovine testicular lymph standard by Dr. Burger's assay and is about 8.5 times more active than a porcine follicular fluid standard used in Dr. Channing's laboratory (16). This preparation retained about 1% insoluble material in assay buffer and was less soluble in distilled water. For pituitary culture assays

we redissolved several grams of the material in water, centrifuged it to remove insoluble material, and lyophilized the soluble portion. This new preparation, HSN-8-111.9PS standard, is more convenient to dissolve and has about 10% greater specific activity than the old standard. It is about 2 to 3 times more active than raw porcine follicular fluid obtained from NIH based on solids present.

Our studies of the effects of progesterone and estradiol on the secretion of FSH from the cells, on the cell content of FSH, and on LH secretion are summarized in Figs. 1–3. In this series of HSN-8-111.9PS standard was assayed at seven doses for comparison.

The comparison of FSH secretion with and without steroid addition from the cell cultures is shown in Fig. 1. It is evident that the addition of both estradiol and progesterone increases the basal FSH secretion 40 to 50%. This is in good agreement with the results of Shander *et al.* (61) using female rats. This increment of basal FSH secretion makes the assay more sensitive than it would be in the absence of steroids. Dose–response to HSN-8-111.9PS in the cultures with steroids, was steeper (slope, −38.37; λ, 0.051) compared to cultures without steroids added

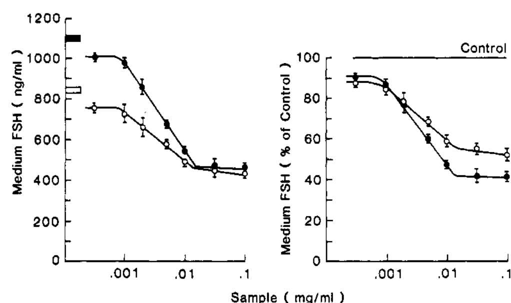


FIG. 1. Effects of an ovarian inhibin reference preparation (HSN-8-111.9PS) on cumulative FSH secretion into media after 3 days incubation with male rat anterior pituitary cell cultures with or without added estrogen and progesterone. FSH values are expressed as means ± SE (N = 3). Left panel, ng/ml; right panel, percentage of control. Control levels, left panel are indicated by the open block (without added steroid) or solid block (with added steroid) on the left vertical axis. Open circles, HSN-8-111.9PS added at the levels indicated to cultures without added estrogen and progesterone; solid circles, addition to cultures with added steroids (10^{-8} M estradiol and 10^{-6} M progesterone).

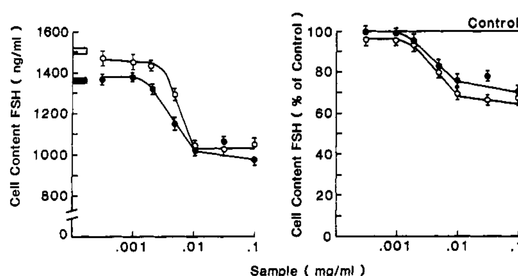


FIG. 2. Same as Fig. 1 except measurements are on the pituitary cell content after lysis as described in the text. These cells are from the same experiment as in Fig. 1.

(slope, −29.28; λ, 0.103). Their dose–response curves were not parallel.

The effect of steroid addition on cell content of FSH is shown in Fig. 2. The presence of steroids appeared to reduce the FSH level in the control wells from 85 to 90% of that observed without steroids; this in turn, made the dose–response curve of HSN-8-111.9PS standard in the cultures without steroids appear steeper than the other (slope, −34.0; λ, 0.12; as compared to slope, −31.18 and λ, 0.05). These curves are parallel. The maximal suppression of cellular FSH content obtained was about 60% of the control. This is much less than that reported by Scott *et al.* (103). Compared with the measuring of FSH secretion, measuring cell content of FSH is a less sensitive assay. Thus we measure the FSH secretion routinely and save the cell lysates as a back-up.

Figure 3 describes the effect of steroids on LH secretion. Addition of estradiol and progesterone increased basal LH secretion in control cultures, in the presence of steroids the inhibin standard failed to alter LH secretion. In the absence of steroids the inhibin standard produced an unexpected rise in LH secretion at the highest dose.

A typical assay of various fractions from a Fractogel column is shown in Fig. 4 (105). In this assay the dose–responses for both fractions B and C were parallel to that of the starting follicular fluid response, but fraction D was not. Using the parallel slope assay for potency calculation, it was found that fraction B had 8 times and fraction C had 12 times more activity than that of the starting mate-

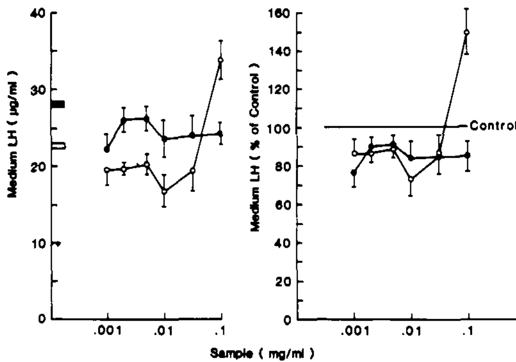


FIG. 3. LH patterns in the media collected in the experiment depicted in Fig. 1.

rial. The λ value in our assay is routinely between 0.05 and 0.22 and the coefficient of variation between assays was between 20 and 25%.

Biochemical Properties of Ovarian Inhibin. Although the renewed interest in inhibin dates back over a decade, we still cannot cite a satisfactory method for the purification of inhibin. There have been several reports of procedures for the purification of inhibin. However, these reports generally suffer from inadequate characterization of the end product, a lack of confirmation by other investigators, poor yields for the overall procedure (in many reports yield or recovery is not given), inadequate bioassay results, or combinations of several or all of the foregoing. The literature on the purification of inhibin is further complicated by the lack of a common reference preparation and the lack of a concensus assay for inhibin itself. Much of the earlier literature during the past decade has dealt with male inhibin derived from seminal plasma or testicular extracts. Such inhibin-containing materials represent starting material from 1 to 5% as potent as follicular fluid. Thus reports of purification approximating 500-fold (106) may, in fact, represent a preparation which is quite comparable in terms of specific activity to an ovarian inhibin preparation of, say 20- to 80-fold purification (107). Our comparisons are probably correct for the order of magnitude, but the lack of a common reference preparation against which the specific activities have been compared renders our assessment somewhat tenuous at best. The problem is exacerbated by the fact that rigorous comparisons have rarely been undertaken by a single laboratory.

Rete testis fluid is more troublesome to collect than seminal plasma or testicular extracts, but some investigators have used this material for fractionation. Rete testis fluid has been reported to have a ninefold greater potency (per milligram of protein) than follicular fluid (59). This report by Eddie *et al.* (59) is one of the rare instances where comparison of male and female inhibin sources has been made in a single lab and assayed against a common reference preparation.

Many of the early reviews of the inhibin literature dealt primarily with the physiological evidence for the existence of inhibin. Reviews that have appeared since 1977 have included more consideration of attempted purifications for the material. We will comment further on some of these reviews below.

In the present review our purpose is to address only the question of ovarian inhibin. It is inevitable, however, that we will eventually wish to make comparisons between the inhibin from male and female. (See Tables II and III for a summary of reported properties of male and female inhibin, respectively.) Indeed, Demoulin *et al.* (108) addressed this question specifically at a recent

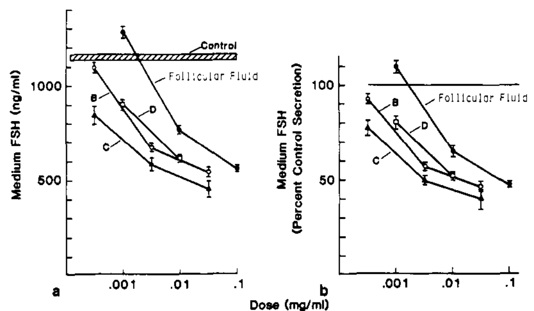


FIG. 4. Typical results from an inhibin assay of fractions from a Fractogel TSK-HW65 chromatogram of raw-follicular fluid. (Chromatography described in the report by Channing *et al.*, submitted) Fractions B and C are high-molecular-weight fractions that elute ahead of albumin and γ-globulin, Fraction D is from 60,000–160,000 mol wt range that includes albumin and γ-globulin. All lower-molecular-weight components were removed from the starting follicular fluid (right side curve). These late eluting fractions are inactive (data not shown).

TABLE II. SOME REPORTED PROPERTIES OF INHIBIN DERIVED FROM MALE SOURCES

Source	Species of origin	Molecular weight and method of estimation	Isoelectric point	Other properties	Yield/purification reported	References
Seminal plasma	Bovine	19K Gel exclusion	5–6.5		NR[c]	(10)
	Bovine	18K SDS electrophoresis gel			40%[a]/500-fold	(109)
	Human	4–5K[b]	Basic	Amino acid sequence determined	NR	(3)
	Human	>10K[b], <5K			Generally poor, yield varied from batch to batch of starting material	(118)
	Human	14K, Gel exclusion		N-terminal sequence given	NR	(1)
Rete testis fluid (RTF)	Ovine	>10K, <5K		Reduce testicular DNA synthesis	NR	(199)
	Ovine	90–240K (high pH), 60K, (low pH), 30K in SDS	5.2–5.8	Oxidation, reduction or cyanogen bromide all inactivate, proteases inactivate	70%/2- to 3-fold, 7%/30-fold	(120)
		>160K, Sephadex G-200				(121)
Testicular extracts	Ovine	1.45K, SDS–Urea			NR	(122)
Sertoli cell culture (SCC)	Rat	>10K		FSH stimulated the production of inhibin by SCC	NR	(123)

[a] Yield calculations are uncertain due to apparent activity "increases" over the starting activity (reverse Steelman-Pohley assay).
[b] SDS = Gel electrophoresis with SDS and sample reduction.
[c] Not reported.

TABLE III. SOME REPORTED PROPERTIES OF INHIBIN DERIVED FROM FOLLICULAR FLUID

Preprocessing of the follicular fluid	Species of origin	Molecular weight and method of estimation	Isoelectric point	Other properties	Yield/purification reported	References
None[a] (or steroid removal) Collection per follicle size	Pork	>10K (PM-10 membrane)		Heat (80°C, 1/2 hr) and trypsin sensitive	NA[b]	(124, 125)
Various	Pork	115K (Sephacryl S-300) 26K (in 8 *M* urea)	5.4–5.6		NR[c]	(126)
Various including Matrex Gel Red A	Bovine	60–70K	5.0–5.7	Dialysis produced about 30% loss of activity, heat sensitive	90%/20-fold 13.6%/81-fold	(107)
None	Equine	Not estimated		Heat denatured, 100°C/2 min	NA	(36)
Various	Human	23K (G-100, and CF25 membrane)	"Acidic"		"Good"	(127)
Various	Ovine	>80K (Gel exclusion)		Two components on HPLC	2.4%/20-fold	(117)
Granulosa cell culture	Bovine	100K (membrane XM 100A)		Heat inactivated (80°C/30 min)	NR	(79)
Granulosa cell culture	Rat	>10K				(77) (76)
Charcoal-treated ovarian extracts	Hamster	<30K		Inhibin activity present	NA	(128)

[a] For simplicity, "None" will indicate steroid may or may not have been removed by extraction or charcoal treatment. In most studies this step has not been found to alter the inhibin assay results significantly.

[b] NA = Not applicable. Some authors report slight loss of inhibin activity during steroid removal.

[c] Not reported.

conference and discussed the differences and similarities between male and female inhibin. In their conclusions they stated "at the present time, the existence of a protein hormone synthesized by the gonads and regulating FSH secretion is largely demonstrated in both sexes. Before complete purification and biochemical characterization of male and female inhibin [is available], the affirmation of their molecular identity will remain hypothetical." Sairam and Raj (109) took a more conservative approach in their 1981 report: "We shall use the term Inhibin-Like Activity (ILA) rather than inhibin in the following discussion for two important reasons. First, the active substance has not been isolated and completely characterized from any source, and secondly, the exact relationship between the activities found in various sources is ill-defined." We cannot yet state whether male or female inhibins are identical or dissimilar. We also cannot yet state whether inhibin preparations derived from various species are identical or dissimilar. Indeed, species differences would be less surprising. In fact we have found (Ward *et al.*, unpublished results) that porcine and bovine ovarian inhibin behave quite differently on Matrex gel Red A (i.e., porcine inhibin has a much lower affinity than has been described by Jansen *et al.* (107) for the bovine material). This we postulate may relate to species differences.

The inhibin literature has been reviewed rather regularly over the last several years. We will describe in this review how our own experimentation over the last 4½ years has evolved to our current concept of the inhibin problem and the attributes of the inhibin molecules obtained from porcine follicular fluid and indicate how our own conclusions relate to reports from other laboratories. Perhaps the most extensive and detailed review is that of Franchimont and colleagues (14). A very concise tabulation of inhibin research to 1980 is in the review by Hafez (110). The review by Chari (111), on the chemistry and physiology of inhibin presents a useful but rather uncritical review of studies largely from her own laboratory. The review by M. R. Blanc (112), presents a useful tabulation of the source and estimated molecular weights for several different inhibin preparations as well as historic information. The 1979 review by F. H. de Jong (113), entitled "Inhibin—Fact or Artifact" represents a brief but critical review of the literature on inhibin purification to that time. Ward *et al.* (114), reviewed the stability studies and molecular weight estimates for inhibin available to that date. In addition, that review contained new data on the radiation inactivation of inhibin that indicated a high molecular weight for inhibin in the range of 200,000. The landmark monograph on "Intragonadal Regulation of Reproduction," edited by Franchimont and Channing (115), contained a summary statement on the purification and characterization of ovarian inhibin by de Jong and colleagues (116) and a companion statement on the characterization of inhibin from bull seminal plasma by Sairam (106). In a 1983 review, Ward *et al.* (16) developed their arguments on the chemistry and physiology of inhibin as a high-molecular-weight complex and provided further evidence for this complex in terms of the sucrose density gradient centrifugation behavior of ovarian inhibin.

To design a purification strategy for a biological molecule the approach taken will most often depend on the molecular size of the molecule as a principal determining factor. In this respect the literature on inhibin is particularly perplexing. There are serious reports suggesting a molecular size ranging from 1500 to over 200,000 Da. Several laboratories reported molecular weight values clustering between 18,000 and 40,000 (see reviews cited above). Recent reports concerning ovarian inhibin molecular size have indicated values no lower than 60,000, but as high as 200,000 (16, 116, 117). In order to facilitate our own experimental design we attempted to provide evidence on the molecular weight of ovarian inhibin based on methodology other than molecular exclusion chromatography which formed the basis for virtually all of the previous reports. We studied ovarian inhibin by irradiation inactivation (114) and by sucrose density gradient centrifugation (16). By both of these methodologies we obtained evidence that ovarian inhibin was a high-molecular-weight complex. Indeed, in our hands the smallest molecular weight indicated for ovarian inhibin has been

65,000 [see unpublished data below and 16]. Since the high-molecular-weight forms of ovarian inhibin are readily demonstrated, we decided rather than pursue an ill-defined molecular form we would take advantage of the high-molecular-weight complex and this has been the basis for a recent report (105). In that report we defined conditions to obtain the high-molecular-weight inhibin complex, by Fractogel HW65 chromatography, well separated from the majority of the contaminating proteins, particularly γ-globulin and albumin. In current studies presented in preliminary form below, we have further demonstrated that this high-molecular-weight complex, upon treatment with chaotropic agents can dissociate into a lower-molecular-weight form at least as small as 65,000 Da. We are searching for further means to dissociate this complex to yet smaller-molecular-weight units with a high degree of efficiency and without proteolysis. At this time such experiments have been only partially successful. Nonetheless, we believe there must be still smaller forms of ovarian inhibin obtainable by well-defined procedures that will reconcile our findings with those of several other laboratories.

In our recent studies designed to isolate a high-molecular-weight inhibin complex we have successfully removed the albumin and γ-globulin (105), the two proteins in greatest abundance in follicular fluid. To illustrate our sequential studies still in progress, the partially purified complex (designated fraction C) has been applied to a high-performance liquid chromatograph on TSK 4000 column (Fig. 5A). This chromatography resolved two active fractions; TSK-C in Fig. 5A is in a molecular weight area of 200,000 or greater and fraction TSK-E in an area of approximately 65,000. (See assay results below Fig. 5. Note, the low-molecular-weight fraction TSK-I *stimulated* FSH release slightly.) In this experiment we demonstrated a rather gentle treatment (concentration, dialysis, ly-

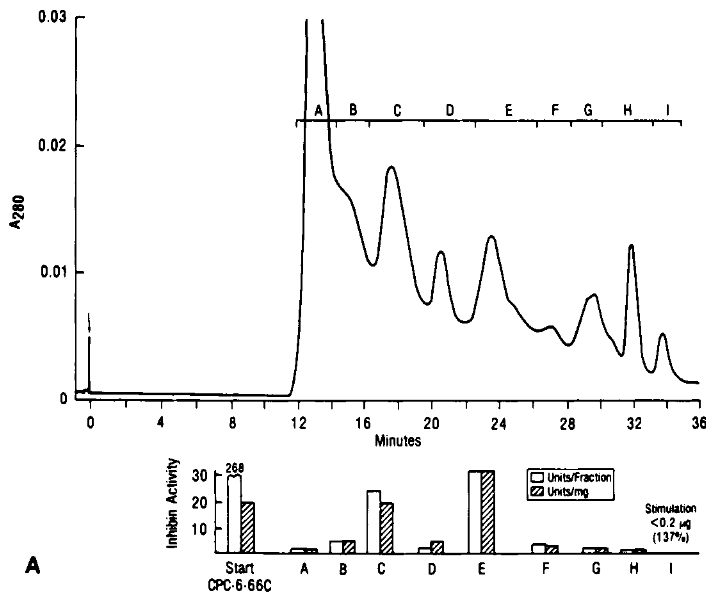


FIG. 5. (A) Elution pattern of the material in fractions C, Fig. 4, 14 mg 16 units/mg, submitted to HPLC chromatography on a TSK-4000 gel permeation column (0.7 × 60 cm) developed with 0.05 *M* phosphate buffer, pH 7.0, plus 0.14 *M* NaCl. Flow rate 0.5 ml/min. Fractions collected are indicated by the letters above the pattern; the inhibin activity in these fractions is summarized below the curve. Two active fractions were obtained, C and E. The latter indicates a 65,000 molecular weight inhibin has been generated with the handling of the higher-molecular-weight fraction from the Fractogel column. We believe protease activity is minimal or absent from this fraction and that the 65,000 molecular weight form of inhibin is a "native" form.

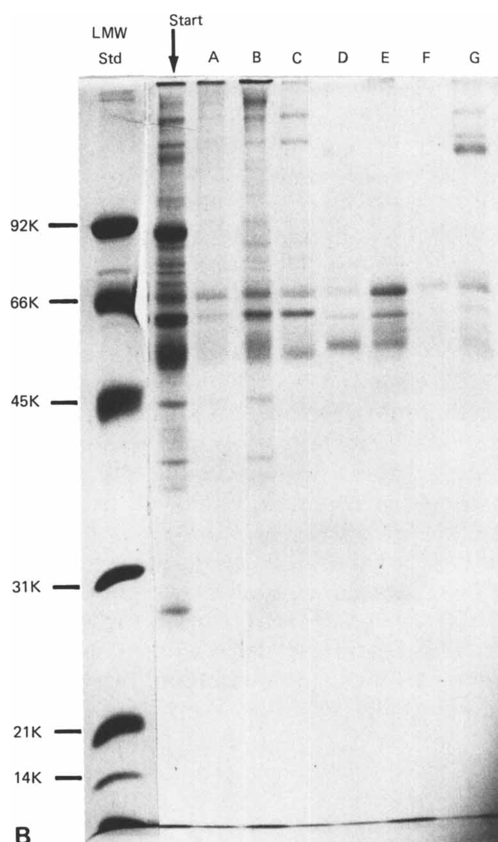


FIG. 5. (B) Polyacrylamide gel electrophoresis patterns of the fractions obtained in Fig. 5A. The samples were reduced and run under dissociating conditions with 0.1% sodium dodecyl sulfate in the gel. The active fractions C and E show significant heterogeneity. Presumably one or more of the bands common to these fractions represents inhibin.

ophilization, and molecular exclusion chromatography) led to the apparent dissociation of inhibin activity into two major fractions, although the activity recovery of the smaller form was only 25%. To extend these studies we are using dissociation with such chaotropic agents as urea or guanidine hydrochloride in attempts to induce this dissociation in very high yield. We have found, for example, that 2–4 *M* guanidine hydrochloride treatment converts the high-molecular-weight form (TSK-C) to the 65,000 mol wt form (TSK-E), with recovery of activity in variable yields. The conditions affecting yields are a concern of continuing investigation.

We have previously reported (114) that treatment of the 90% ethanol precipitate of porcine follicular fluid with guanidine hydrochloride greater than 0.5 *M* led to inactivation of inhibin activity. This result appears to be, in part, due to the type of handling necessary to prepare the samples for the *in vivo* assays that we were using at that time, as our current experiments indicate one can recover substantial inhibin activity after treatment with greater concentrations of guanidine hydrochloride.

We recognize that there are reports of ovarian inhibin preparations with molecular weights indicated as considerably less than 65,000 (Tables II and III). However, we believe it is important that in most instances the reported yields of activity have been very low (range from less than 1% to about 15%. References cited in Tables II and III.) None of the reported ovarian inhibin preparations have been characterized in detail. This is probably a consequence of the poor yields encountered, or, as in the case of our own preparations, the heterogeneity of the preparations so far achieved does not warrant a more detailed characterization.

Of the available reports (Tables II and III) it should be noted that in the case of a human seminal plasma inhibin preparation (118) a high-molecular-weight inhibin suddenly behaved as a low-molecular-weight (less than 5000) preparation. This followed a DEAE chromatography step. This characteristic is reminiscent of one of the molecular weight forms reported by Franchimont *et al.* (98), but in the latter case the treatment preceding the presumptive low molecular transition is less well documented. The material reported by Ramasharma and Sairam (118) has now been studied for amino acid sequence and the corresponding synthetic peptide shown to be active (see below).

As de Jong has discussed (113), the form of the inhibin assay may influence the size range in which one observes activity. This is in part a consequence of the fact that low-molecular-weight FSH binding inhibitors, LHRH inhibitors, etc. could influence some bioassays. It is at least a possibility that any given form of assay by its design may select for either a high-molecular-weight form or a lower-molecular-weight form. Ideally, of

course, an assay procedure should not be influenced by the physical form of the assayed sample. Moreover, we believe that the best interpretation for the 1500 mol wt inhibin products (64) would be that it represents analogues of LHRH that inhibit LH and FSH release. For the 5000 mol wt form of inhibin recent data from Seidah *et al.* (2) make it quite clear that the product is a mixture of closely related peptides in all probability generated by protease action in seminal plasma.

We have mentioned that in our own studies we can detect porcine follicular inhibin in physical forms that have been characterized as large as 200,000 mol wt or as small as 65,000. Franchimont *et al.* (98) have stated two forms of inhibin exist in rete testis fluid or human seminal plasma. Dubos *et al.* (117) have provided evidence for two forms of inhibin in ovine ovarian follicular fluid demonstrated by high-performance liquid chromatography. They cite the molecular weight as "approx. 80,000" for their higher-molecular-weight form, but from their data it would be more accurate to indicate 80,000 *or greater,* based on their Waters I-125 column data. Their two fractions from reverse-phase columns were not characterized as to size. The fraction with the greatest specific activity (23-fold purification) was obtained in only 2.3% yield. Thus the newer technology as yet has not provided a better purification.

We hypothesize (H-I) that the very low yields that are consistantly encountered during inhibin fractionation studies result from dissociation and/or proteolytic cleavage during processing such that activity is distributed throughout the several generated fractions. Inhibin in these fractions may not always be present in sufficient concentration to be detected, or proteolysis may destroy a significant part of the activity. Thus, only the fractions that happen to contain the most detectable levels are followed further. By this hypothesis the process could be repeated—the more handling the poorer the recovery. Nor would it necessarily lead to a homogeneous final fraction.

The foregoing hypothesis provides a rationale for believing that a procedure could be devised to isolate inhibin in a pure form for biochemical characterization. This hypothesis forms the basis for the current experimental approach in our own and other laboratories. However, other possibilities cannot be ignored until such time as a native inhibin is successfully isolated. For example, we might suggest an alternative hypothesis (H-II) in which inhibin activity results simply from a combined effect of several dissimilar factors that only in their concerted action lead to the activity designated as "inhibin." By this hypothesis the purification of inhibin as a single entity would be impossible, but the report by Seidah *et al.* (2) and their landmark paper that followed (3) effectively eliminates this second hypothesis.

There is yet a third hypothesis (H-III) to which we have alluded that could fit the data now available: namely, that inhibin exists as a discrete molecule, but during isolation it is degraded to an inactive form or a smaller active form as the report of Seidah *et al.* (2) indicates. The most likely degradation of this type would be proteolysis. Demonstration of proteolytic enzymes in follicular fluid is complicated. As we (114) and others (125) have noted proteases are not detected directly in follicular fluid. However, Beers (129) has shown that protease inhibitors present in bovine follicular fluid may be removed (by acid precipitation in his study) and thus enable the demonstration of proteolytic activity. Beers (129) was not considering inhibin in the study cited.

A recent report by K. J. Shaw, J. D. Compeau, P. C. Roche and G. S. diZerega (submitted) has demonstrated proteolytic activity in follicular fluid under conditions that are readily adapted to fractionation studies on inhibin, and on this basis we plan to test hypothesis H-III directly.

Proteases Present in Follicular Fluid and in Fractions Derived Therefrom. It has been well documented that many follicular fluid components are similar if not identical to those in serum (73, 130). These components include albumin, immunoglobulins, enzymes, salts, etc. Beers' studies (129) showed that proteases are also to be expected as components of follicular fluid. The activity of these proteases is inhibited in raw serum or follicular fluid either through protease inhibitors or through the presence of the proteases in their zymogen form (129). The proteases

become activated under certain conditions, as, for example, acid precipitation of inhibitor activity or conversion of the zymogen to its active form (e.g., plasminogen conversion to plasmin by a plasminogen activator) (129).

With the recent observations that porcine or bovine follicular fluid preparations contain plasminogen dependent and plasminogen independent proteases (129, K. J. Shaw, J. D. Campeau, P. C. Roche, G. S. diZerega, submitted) it was of interest for us to discern how protease activities were being distributed in our fractionation of inhibin from porcine follicular fluid.

Evaluation of protease activity is now done in our laboratory on a qualitative basis using polyacrylamide gels containing the protease substrate, gelatin (131). Samples migrate in 7.5 or 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) to inhibit protease activity (132). Following the removal of SDS with a Triton X-100 wash the gels are incubated in a glycine buffer at pH 8.3 overnight at 37°C. Following staining in 0.1% amido black and destaining, protease activities appear as clear zones against a blue background of stained gelatin. As is evident from the polyacrylamide gel shown in Fig. 6, it is clear there are protease activities in our fractions from the purification of inhibin.

When cold ethanol is used to precipitate inhibin activity from porcine follicular fluid, the bulk of inhibin activity is precipitated by 36% ethanol (14). Yet further fractionation of this precipitate by various methods often resulted in unexplained losses of inhibin biological activity. Figure 6 shows the protease activity in sequential ethanol precipitates of one batch of porcine follicular fluid. These results show that protease activity is present in the ethanol precipitates of porcine follicular fluid up to 36% ethanol (lanes 1–5), with relatively weak activity in the 37–50% (lane 6) and 51–90% (lane 7) ethanol precipitates. Interestingly, weak or no activity is detected in the 0–90% single-step ethanol precipitate (lane 8) at positions where clear-cut protease activity is present in the ethanol precipitates up to 36% (lanes 1–5), suggesting that inhibitors of protease activity are retained or the protease activity is eliminated in the 0–90% ethanol precipitate, but inhibitors are removed from the 0–36% ethanol precipitates.

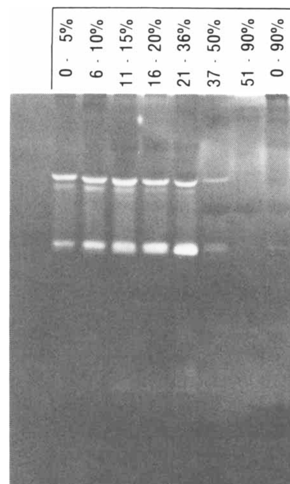


FIG. 6. Protease analysis of ethanol precipitates of porcine follicular fluid sampled by cuts or total precipitate. The protease analysis uses a 0.1% gelatin substrate in a 7.5% polyacrylamide gel electrophoresis slab. Samples were applied in 2% sodium dodecyl sulfate (SDS) to inhibit protease during electrophoresis. After electrophoresis the SDS was washed out with 2.5% Triton X-100 solution (1 hr) and the slab was incubated overnight in 0.1 *M* glycine buffer, pH 8.3, 37°C. Finally the gel was stained (amido black) and destained. The ethanol concentration to precipitate the cut from which the sample was obtained is indicated above the origin (lanes 1–7). Lane 8 contains a sample precipitated directly with 90% ethanol.

Additionally, these results suggest that one reason for variable recoveries of inhibin activity following the fractionation of the 36% ethanol precipitate of porcine follicular fluid may be due to the presence of activated protease and that hypothesis H-III is a strong consideration in inhibin purification.

In a separate study we wanted to determine if a seemingly gentle fractionation (column chromatography) of raw porcine follicular fluid would activate protease activity. Figure 7 shows that although detectable protease activity was found in the porcine follicular fluid, detectable protease activity is also seen in additional bands in several of the fractions obtained following chromatography on Fractogel HW-65F (lanes B, C, D, E). These results reinforce the idea that proteases can be activated and/or enriched in certain fractions following chromatography and may

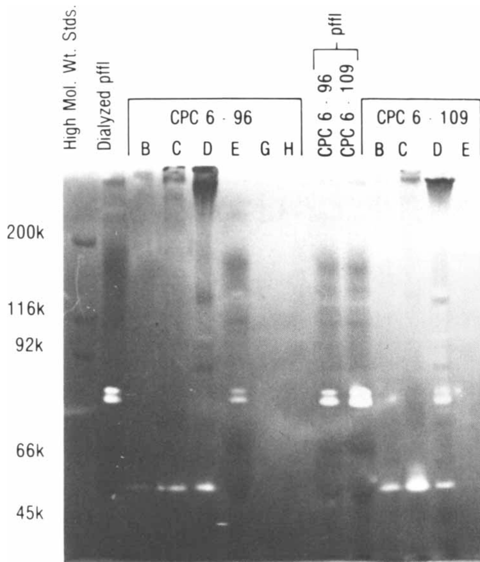
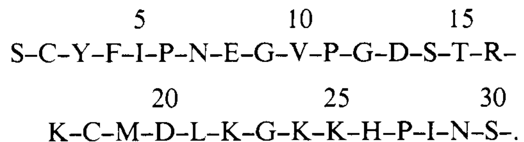


FIG. 7. Protease analysis of porcine follicular fluid fractions obtained from a Fractogel HW-65F chromatogram (Channing *et al.*, 1984). Fractions B, C, D are high molecular weight, greater than 160,000. Under these conditions the majority of the inhibin activity is in fractions C (Exp. CPC6-109) or B (Exp. CPC6-96). Protease activity is indicated by the unstained areas. An approximately 80,000 mol wt protease is observed in the porcine follicular fluid before or after dialysis. This fraction appears in fraction D or E, but another protease (released or generated in the processing) with a molecular weight about 50,000 can be seen with the inhibin fraction and those following (B, C, D).

play a role in the subsequent analysis of inhibin biological activity.

Questions which are now being addressed in our laboratory are: (1) Does the presence of protease in our fractions enriched with inhibin activity affect inhibin biological activity? (2) How can we inhibit or remove protease activity without significantly affecting the integrity and biological activity of the inhibin molecule or complex? (3) Does the presence of proteases contribute directly to the variety of molecular weight estimates made for inhibin? And finally (4) a corollary question to (1) and (3), can proteases "process" high-molecular-weight inhibin to specific, active smaller forms of inhibin? The report of Seidah *et al.* (2) has already provided an affirmative answer to question (4). We will close our review with a consideration of the three recent reports providing amino acid sequence data for inhibin-related molecules (1-3).

Sheth and colleagues (1) have reported the amino acid sequence for the first 30 residues on the N-terminus of a putative inhibin molecule isolated from human seminal plasma. This preparation had a molecular weight of approximately 14,000. It was isolated as a single peak from a reverse-phase high-performance liquid chromatogram. The activity, however, was not directly measured. The purified preparations applied to the chromatogram according to the authors "—already gave positive reactions in these assays at amounts corresponding to about 100 ng and 10 ng polypeptide, respectively. These values are several fold lower than those with other preparations reported—suggesting high biological potency and purity of the present inhibin-like material" (1). Obviously further characterization will be required. Assuming that it is subsequently demonstrated that the material Sheth and colleagues are sequencing is indeed a form of inhibin, their data indicate the following N-terminal sequence for this material (single-letter code):


```
        5          10          15
S-C-Y-F-I-P-N-E-G-V-P-G-D-S-T-R-
          20          25          30
   K-C-M-D-L-K-G-K-K-H-P-I-N-S-.
```

Residues 31 to approximately 130 have yet to be determined, 130 being the approximate total size.

Sairam and his colleagues in Montreal (2) reported amino acid sequence studies on a mixture of four structurally related peptides, the longest of which was about 35 residues. This preparation (mol wt 4-5000) was derived from human seminal plasma. A sequence was proposed for 31 of the possible 35 residues in the largest peptide. The other three peptides appear to be fragments of the larger peptide.

In a subsequent report (3), that we regard as a true landmark in inhibin research, this group isolated the major peptide from the above group of four peptides and showed that it was comprised of 31 amino acids. Its sequence was that deduced in the earlier report (2). Moreover, the Montreal group collaborated with Yamashiro and Li (University of California in San Francisco) to

synthesize the corresponding peptide and demonstrate that it was active to suppress FSH but not LH release by an *in vitro,* LHRH-stimulated mouse pituitary system. Indeed, the synthetic material was slightly more active than the natural peptide in its FSH suppressing activity. In a 10-hr incubation 25 ng gave approximately 74% suppression of FSH release. The amino acid sequence is:

```
    5         10
H-N-K-Q-E-G-R-D-H-D-K-
        15        20
   S-K-G-H-F-H-R-V-V-I-H-
              25        30
        H-K-G-G-K-A-H-R-G.
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This sequence bears little resemblance to the one proposed by Sheth *et al.* (1). Thus it appears that if the material studied by Ramasharma *et al.* (3) has homology with any part of the larger molecule studied by Sheth *et al.* (1) it is not near the N-terminus. Ramasharma *et al.* (3) reported a modest (32%) homology with a portion of yeast enolase, but essentially the inhibin active peptide sequence is a new peptide. A very remarkable characteristic is the high histidine content of the peptide (23%). With the lysine and arginine residues, the peptide is comprised of 45% basic amino acids.

We have referred to the report of Ramasharma *et al.* (3) as a landmark in inhibin research. We apply this description to their work since it now dispels the long-nagging question "Is there really a biochemical entity with inhibin activity, or is it possible the assay systems are responding to some beguiling artefacts?" Their work shows there is such an entity. Inhibin research can now move directly to answer the questions remaining; namely, what is the physiological role of inhibin (for which we have at least partial answers), and what is the biochemistry of the biosynthesis and secretion of inhibin (for which we can now devise a directed approach).

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