

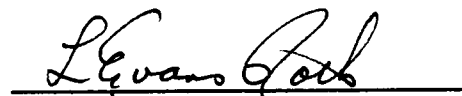
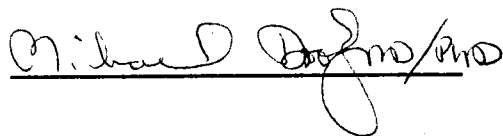
To the Graduate Council:

I am submitting herewith a thesis written by Holly Page Kelly entitled "Serum Gonadotropin Levels in Polycystic Ovarian Disease". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.



Thomas T. Chen, Major Professor

We have read this thesis
and recommend its acceptance:



Accepted for the Council:



Vice Provost
and Dean of The Graduate School

SERUM GONADOTROPIN LEVELS
IN
POLYCYSTIC OVARIAN DISEASE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Holly Page Kelly
August 1988

DEDICATION

To my parents, who always encouraged me to strive for a high standard of excellence in all endeavors I pursued. I could not have asked for better role models.

I wish to dedicate this in memory of Page Benson Kelly who passed away prematurely, but not before she taught me much about the importance of having ambition and perseverance. I love you, Page.

ACKNOWLEDGMENTS

Sincere appreciation goes to Dr. Thomas Chen who helped me endure through many trials and tribulations associated with this study. I am grateful for his unyielding insistence that I never compromise the quality of writing and research which went into this project. He, by example, has related the intensity and excitement with which one should approach teaching and research.

I would also like to express my gratitude to Dr. Michael Doody for his financial commitment, contagious positive attitude and scholarly insight. In addition, Dr. Evans Roth helped and encouraged me throughout the study and in the preparation of the text. Special thanks also go to Dr. M.L. Pan who graciously assisted me in the immunodiffusion and immunoelectrophoresis experiments.

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PREFACE

Numerous investigations have been conducted in the examination of reproductive disorders. Recently, many of these studies have focused upon symptoms related to polycystic ovarian disease (PCOD). PCOD is associated with a broad family of symptoms including hirsutism, anovulation, suboptimal gonadotropin secretion and infertility. These diverse symptoms make it impossible to draw broad conclusions from any single investigation. Instead, research is limited to examining only certain causative variables at one time. The resulting conclusions are then only valid within the limits of the specific experimental design.

Perhaps research has to be divided into subtopics in order to build an understanding of the symptoms and causes. Granted, the study and research completed so far have tremendous merit and have achieved much at defining reproductive disorders in general and PCOD in particular. We now know enough to focus on specific variables.

It is time for the diverse research groups to communicate with each other and allocate responsibilities, rather than engaging in duplication. I do believe that with today's state of research and technology, it is possible to organize a research project aimed at producing more valid, broad conclusions which can be applied with greater efficiency in the diagnosis and treatment of symptoms associated with reproductive disorders. In my opinion, I think it desirable for basic researcher, clinicians and biotechnologists to campaign for a cooperative effort in the search for answers and insights to the myriad of questions which plague reproductive biology.

ABSTRACT

The purpose of this study was to measure serum gonadotropin levels in two different groups of women. Radioimmunoassay (RIA) techniques were used to measure follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in women with normal menstrual cycles and in women with polycystic ovarian disease (PCOD).

Twenty-three women with no known reproductive disorders and 26-30 day menstrual cycles served as the control group. Fourteen patients of Dr. Michael Doody, a reproductive endocrinologist in the Department of Obstetrics and Gynecology at the University of Tennessee Hospital, with PCOD served as the patient group. Five blood samples were collected from each of the control subjects beginning on day 4 of the menstrual period and every third day thereafter through day 16. Only one blood sample was collected from the PCOD patients. Serum was separated from the blood and stored at -20 C until assaying was performed. All hormonal quantification was done in the same assay to eliminate inter-assay variations.

Results were analyzed using analysis of variance (ANOVA) and linear regression. No significant difference was found in the levels of FSH and LH, as a function of time (days), for the control subjects. The mean hormone levels for the control subjects were pooled to obtain one FSH and one LH group mean. A statistically significant difference resulted when the two groups' FSH and LH means were compared. The patients had lower FSH, but higher LH values. As a result, the LH/FSH ratios between the two groups were also significantly different, being

almost twice as large in the patients. A strong correlation between the two gonadotropins existed in the patient group, but only a moderate correlation was found in the control group.

The results show that patients with PCOD manifest low levels of FSH complete with high levels of LH. These findings support the working hypothesis that the women with PCOD would have lower FSH, but higher LH serum levels and that this would be reflected in a higher LH/FSH ratio. Knowledge about the gonadotropin profile helps the clinician diagnose and treat PCOD more effectively.

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CHAPTER I

INTRODUCTION

Several investigators agree that polycystic ovarian disease (PCOD) is often characterized by elevated levels of luteinizing hormone (LH) and normal to reduced levels of follicle stimulating hormone (FSH). It is not clear though whether abnormal gonadotropin secretion is a result of defects in the hypothalamo-pituitary axis, which governs reproductive functions, or is a manifestation of inappropriate ovarian steroid and/or inhibin feedback. Elevated levels of inhibin have been implicated in the discordant gonadotropin release by selectively suppressing FSH secretion. Other reports infer that inhibin levels are normal in PCOD and that other factors such as abnormal ovarian steroid production contribute to decreased levels of FSH in women with PCOD.

The purpose of this study was to determine if a relationship existed between gonadotropins (LH & FSH) and inhibin in women with no known reproductive disorders (control subjects) and in women diagnosed as having PCOD (patient subjects). However, the unexpected unavailability of anti-inhibin antibodies prevented an examination of inhibin levels in these two groups. The study was consequently limited to measuring serum FSH and LH levels.

Twenty-three women between the ages of 22-50 comprised the control group. All these women had regular menstrual cycle and were not using oral contraceptives throughout the duration of the study. Each consented to having blood drawn every third day during the follicular

phase of the reproductive cycle beginning on day 4 (day 0 = menses) and continuing through day 16. Five samples were collected.

Fourteen women with PCOD made up the patient group and were patients of Dr. Michael Doody, reproductive endocrinologist, Department of Obstetrics and Gynecology at the University of Tennessee Hospital. All the women were anovulatory, making it impossible to collect blood samples parallel to those of the control subjects. Only one blood sample from the patients was collected and analyzed in this study.

In light of these initial obstacles, the study's objective was modified to quantifying serum gonadotropins in both groups. The working hypothesis was that the patients would have higher serum LH but lower FSH levels and that this would be reflected in significantly higher LH/FSH ratios.

This project provides the basis for future studies in this important area. Blood samples have been preserved for such further study and baseline data have been obtained on these samples. In addition, established reproductive histories from the subjects based on the questionnaire which they completed at the study's outset can be utilized. The basis for proceeding with inhibin studies has been provided. Such work can commence as soon as an appropriate anti-inhibin antibody supply is available.

CHAPTER II

REVIEW OF LITERATURE

A. Physiology of Inhibin

Introduction

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropins secreted by the anterior pituitary. Both are essential regulators of male and female reproductive processes. The synthesis and release of both gonadotropins are regulated by a gonadotropin-releasing hormone (GnRH) (1), a decapeptide synthesized by neurons in the hypothalamus and released into the hypophyseal portal circulation. GnRH secretion is pulsatile in nature, a pattern which keeps the pituitary sensitive to GnRH stimulation (2) and contributes to the divergence seen in FSH and LH secretory patterns (3-5).

GnRH is not the sole regulator of gonadotropin secretion. Gonadal steroids play important roles in the negative and positive feedback control of LH and FSH, a topic extensively reviewed by Kalra and Kalra (6). Significant divergence in the circulatory pattern of FSH and LH secretion during the menstrual cycle (7-8), after ovariectomy (9-11) and during puberty (12-13) has been documented. To help explain this divergent pattern, the presence of a specific FSH-releasing factor (FSH-RF) has been repeatedly postulated (14-16). However, due to a lack of experimental evidence, this hypothesis is not widely accepted.

Paramount to the interactions between the hypothalamic GnRH, the pituitary gonadotropins and the gonadal steroids is the existence of a

feedback loop(s). The transition from prepuberty to puberty is thought to be associated with a decrease in the sensitivity of the hypothalamo-pituitary axis to gonadal steroids. Indeed, the "gonadostat hypothesis" put forth by several investigators (17-19) suggests that during sexual maturation the sensitivity of the hypothalamus to feedback inhibition by gonadal steroids is decreased. Victims with Turner's Syndrome, where the ovaries are non-functional, have elevated levels of FSH and LH long before the onset of puberty. Castration leads to a rise in gonadotropin secretion, presumably because of the removal of the negative feedback signal(s). These findings lend credence to the idea that gonadal steroids play some role in regulating pituitary gonadotropin secretion.

An alternative mechanism to explain human sexual maturation is the neuroendocrine control system which directs the pulsatile secretion of GnRH from the hypothalamus. Experiments on rhesus monkeys (20) showed that prepubertal monkeys receiving hourly infusions of GnRH developed menstrual cycles and that they returned to their immature sexual state upon discontinuation of the infusions. The evidence clearly demonstrates that the pulsatile pattern of GnRH secretion is required for the attainment of sexual maturation.

Steroid Effects on Gonadotropin Release

To appreciate the dynamics existing between circulating levels of gonadal steroids and the pituitary gonadotropins, two phenomena must be examined. The first is negative feedback, the processes involved in the suppression of gonadotropins as a result of elevating estrogen or

progesterone in the female or testosterone in the male. The second phenomenon is positive feedback which is unique to females and which concerns itself with a surge of, or increase in, gonadotropin secretion as a result of elevated estrogen or progesterone levels.

Estrogen and Feedback Control of FSH and LH

The complete removal of ovarian steroids, as in the case of menopause or ovariectomy, results in elevated levels of FSH and LH. Infusion of low levels of estrogen causes a decline of both gonadotropins to the basal levels seen in the early follicular phase of the menstrual cycle (21). Continued infusion of estrogen for 24-48 hours, however, produces a surge of both FSH and LH. In contrast to the negative feedback effects, higher levels of estrogen and a prolonged exposure to estrogen exert a positive feedback effect on gonadotropin secretion. The midcycle LH surge indeed coincides with peak estrogen concentrations. The surge of FSH is less pronounced than that of LH.

Progesterone and Feedback Control of FSH and LH

Progesterone also appears to exert both positive and negative signals on gonadotropin release. High levels of progesterone (4-8 ng/ml) hold FSH and LH secretion down to low levels as demonstrated during the luteal phase of the menstrual cycle. Progesterone presumably keeps FSH and LH low by enhancing the negative feedback effects of estrogen. In addition to the latter regulatory role, progesterone may act by blocking the positive feedback effect of estrogen on LH surge. This putative action is demonstrated by the absence of an LH surge in women who receive estrogen injections during the progesterone-dominant

phase (luteal phase) of the menstrual cycle.

At low concentrations, progesterone stimulates LH release and/or facilitates the positive feedback effects of estrogen. An LH surge can be induced, or its onset accelerated, when women are injected with low levels of progesterone during the estrogen-dominant phase (follicular phase) of the menstrual cycle. In summary, progesterone and estrogen elicit both positive and negative feedback effects on gonadotropin release. High and low levels of progesterone result in negative and positive feedback signals, respectively. The opposite results are obtained under estrogen feedback signals.

Inhibin and FSH

The regulation of FSH release from the pituitary cannot be explained completely or adequately by ovarian steroid secretion. The concept of a gonadal non-steroidal factor responsible for the regulation of FSH was first proposed by McCullagh in 1932 (22) who observed that cryptorchid rats exhibited hypertrophied cells in the pituitary. McCullagh postulated that, in the castrated-like condition, the secretion of a protein which acted on the pituitary via a negative feedback pathway was lost and called this proteinaceous substance "inhibin". It is well documented that inhibin preferentially regulates the secretion of FSH with little or no effect on LH regulation (23-26). Inhibin has been shown to suppress the synthesis and release of FSH in vivo (27), and in vitro (28-30).

Additional studies suggest that inhibin is involved with the selective suppression of FSH secretion. Several investigators (31-34)

concur that steroids alone are not completely effective in reducing the post-castrational rise of FSH to baseline levels of intact animals. However, steroid injections have been shown to suppress serum LH to basal levels (35-36). Marder et al. (37) reported that the post-castrational rise in FSH could be completely abated with injections of charcoal-treated porcine follicular fluid (ct-pFF), a presumed source of inhibin. In addition, Schwartz and Channing (38) demonstrated that porcine follicular fluid (pFF) suppressed the secondary surge of FSH seen during the morning of estrus in the rat. The normal pattern of LH was unaltered. These findings support the premise that simple steroid feedback mechanisms could not completely account for this secondary rise in serum FSH seen in the rat's cycle (34, 39). DePaolo et al. (40) have shown that the secondary surge of FSH coincides with the nadir levels of inhibin. This inverse relationship between inhibin levels and FSH secretion during estrus is thought to be essential for subsequent follicular recruitment (38).

In summary, it appears from these studies that inhibin is significantly involved, through a presumed feedback pathway, in 1) maintaining LH and FSH balance by preventing inappropriately high FSH secretion, 2) providing a signal for triggering secondary surges in FSH and 3) regulating folliculogenesis (41).

Source of Inhibin

Several studies report that the granulosa cells of the ovarian follicle are the principal source of inhibin (rat:42-43; primates:44-45; cow:46; pig:47) and that its secretion is stimulated by FSH (46-

48). Extensive work on the ovarian origin of inhibin has been carried out by Schwartz and Channing (49) and Rush et al. (50), while cyclic fluctuations of inhibin in ovarian tissue and ovarian venous blood have been reported by Chappel (51) and DePaolo et al. (52), respectively.

Further evidence suggests that inhibin is also secreted by the human corpus luteum (53-55) and that this form of inhibin is instrumental in the regulation of FSH and follicular growth during the luteal phase of the menstrual cycle. Immunoreactive inhibin was shown to circulate throughout the entire menstrual cycle (56), with its peak concentration seen during the mid-luteal phase. The direct correlation between serum progesterone and inhibin levels during the luteal phase strongly implicates the corpus luteum as a source of inhibin secretion.

The findings that inhibin was secreted by the human corpus luteum were initially met with surprise, because several investigators were unable to detect in vitro inhibin production by porcine and bovine luteal cells (57, 58). However, messenger RNA (mRNA) for the alpha subunit of inhibin has recently been detected in the rat corpus luteum (59). Furthermore, Woodruff et al. (60), using rat inhibin cDNA probes, identified the periods during follicular maturation in which the inhibin alpha and beta-A mRNAs are synthesized in the ovaries of cycling animals. Both types of mRNA were detected on all days of the estrous cycle, reached a peak on proestrus and dramatically declined following the primary surges of the gonadotropins. Woodruff and his colleagues concluded that the level of transcription is the major site of inhibin biosynthesis control and that the gonadotropin surges are one component of this control.

New findings suggest that the ovary is not the only source of inhibin. McLachlan et al. (61) recently reported that human placental tissues as well as umbilical cord blood contained inhibin immunoactivity. Messenger RNA encoding the alpha subunit of inhibin was detected in human placental tissues by Mayo et al. (62). Elevated inhibin levels were also detected during early gestation in women undergoing in vitro fertilization (IVF) (63), suggesting that trophoblast cells secrete inhibin.

The exact physiological role of placental inhibin is not known. McLachlan and his group (61) suggested that inhibin may regulate gonadotropin secretion during pregnancy. Suppressed maternal FSH levels are known to occur during gestation. In addition, Petraglia et al. (64) showed that incubation of placental cells with antiserum to inhibin resulted in a time- and dose-dependent increase in hCG concentration. This finding indicated that placental inhibin has an inhibitory effect on local gonadotropin secretion just as gonadal inhibin has an inhibitory effect on pituitary FSH release.

Placental inhibin may also play a paracrine role within the fetal-placental unit. There is striking homology between the inhibin-beta subunits and transforming growth factor-beta (TGF-B) such that they are thought to be of the same gene family (65). These two related proteins are important in regulating cell growth and/or differentiation. Padgett et al. (66) reported that significant homology existed between a decapentapeptide gene complex (DPP-C) known for its role in embryogenesis in *Drosophila* and proteins in the TGF-B gene family. It is

possible that inhibin may play a similar role in mammalian embryogenesis as DPP-C does in the *Drosophila*.

Regulation of Inhibin Secretion

Lee (67) found that pregnant mare serum gonadotropin (PMSG) primed female rats were a useful model in studying the secretion of inhibin. He reported a decrease in FSH levels concomitant with raised inhibin levels. This finding supports the accepted notion that a negative feedback pathway exists between inhibin and FSH. In another study, Lee et al. (68) showed that PMSG increased both ovarian and serum levels of inhibin in immature female rats. This gonadotropin is known to stimulate granulosa cell proliferation, to increase the mitotic activity of the antral follicle and to elevate the proportion of non-atretic follicles (69). These biological events can help explain why ovarian and serum inhibin production were increased in PMSG treated rats.

Welschen et al. (70) showed that estrogen implants in adult female rats did not produce a significant suppression in FSH secretion and consequently cannot be predominately responsible for the decrease in FSH levels. Therefore, Lee and his colleagues concluded that inhibin was the major factor responsible for suppressed FSH levels in the PMSG-primed rats.

The Role of Inhibin in Primates

A post-castrational rise of FSH and LH is known to occur in ovariectomized women and adult monkeys (71-73). Channing et al. (74) reported that serum FSH hypersecretion in primates was suppressed by charcoal-treated porcine follicular fluid (ct-pFF) without affecting

serum LH. Serum LH in monkeys was returned to baseline levels with estrogen treatment (73). These findings are consistent with similar studies done on rats (41, 43).

Channing and her colleagues (75) were the first to demonstrate that ct-pFF selectively suppressed FSH secretion in both intact and ovariectomized monkeys. DiZerega and Hodgen (76) proved that the same events occurred in women during the follicular phase of the menstrual cycle. However, inhibin in the ct-pFF was not selective for gonadotropin suppression during the midcycle FSH and LH surge. Shander et al. (77) reported that inhibin is capable of suppressing both GnRH-stimulated LH and FSH secretion. This may explain, in part, why inhibin affects both gonadotropins during ovulation.

The hypothalamo-pituitary axis is under functional restraint during childhood (gonadostat hypothesis). A negative feedback loop(s) involving gonadal steroids and inhibin contributes to, or accounts for, the reduced sensitivity of the axis which occurs at the onset of puberty. During this stage of development, the frequency and amplitude of GnRH are increased, thus enhancing gonadotropin secretion. In addition, the number of large ovarian antral follicles increases at puberty. Goodman et al. (78) proposed that FSH restraint is a function of the number of ovarian follicles. In turn, inhibin secretion from these follicles serves as a monitoring system, indicating to the hypothalamo-pituitary axis the follicle number remaining in the ovary.

An interesting hypothesis was proposed by Channing et al. (41) to explain the regulation of inhibin and FSH during adulthood. They

suggested that the axis monitors circulating inhibin levels as an indication of follicle number. In childhood, when follicular number is high, FSH levels are minimal. With age, follicles undergo atresia, therefore, inhibin concentration declines. Consequently, the feedback inhibitory restraint on FSH is removed concomitantly with increase development of antral follicles. Overexpression of folliculogenesis is prevented by steroidal and non-steroidal regulators acting at the intraovarian level. Cyclic changes during the menstrual cycle also act to regulate inhibin:FSH balance to maintain and facilitate the normal biological events of the menstrual cycle. Finally, as follicle number reaches its nadir around menopause, so too does inhibin. The stabilizing restraint which inhibin had on FSH secretion is lost, which attributes to the rise in serum FSH characteristically seen at menopause.

Summary

Complicated mechanisms including steroidal and non-steroidal feedback signals, pituitary gonadotropins and changes in the sensitivity of the hypothalamo-pituitary axis act in concert to contribute to the orderly maturation of the ovarian follicle and its oocyte. Inhibin selectively inhibits FSH secretion in a closed feedback loop. In turn, inhibin secretion is suppressed at ovulation, thus allowing for a secondary surge of FSH which is used, in part, for subsequent follicular recruitment.

Now that the inhibin protein has been isolated, sequenced and characterized all that appears unfinished is the development of a

reliable, standard radioimmunoassay. McLachlan et al. (79) reported an RIA for inhibin in follicular fluid and serum from both cow and human. The antiserum was developed against the 58kD species and ^{125}I 58kD and 31kD were both used as the radioligands, but only ^{125}I 31kD was effective when measuring serum inhibin.

With further development of an RIA for serum inhibin measurements, many more chapters on inhibin and its specific role in reproductive processes will surely be written.

B. Chemistry of Inhibin

Introduction

Although the existence of inhibin was postulated over 60 years ago, limited progress had been made in its isolation and purification until recently. The difficulties encountered in characterization were attributed to the lack of a reliable bioassay. Other factors included lack of inhibin standard preparation, differences in definition of "inhibin activity" and the apparent attachment of inhibin to large carrier proteins which consequently yielded multiple, heterogeneous species (80).

The introduction of the rat anterior pituitary monolayer cell culture (81-82) has partially overcome these problems. This in vitro system can specifically and reliably measure the inhibition of basal FSH secretion by inhibin.

Isolation and Purification of Inhibin

Several teams of investigators have reported within the last three years the isolation and purification of inhibin (83-88). Robertson et al. (83-84) used bovine follicular fluid for the isolation of inhibin. Ling et al. (85), Miyamoto et al. (86) and Rivier et al. (87) all isolated a 32 kiloDalton (kD) inhibin from porcine follicular fluid, while Leversha et al. (88) identified an ovine inhibin. In addition, the cDNA sequences are now available for pig (65), cow (89) and human (90, 91). The physiochemical properties as well as the amino acid sequences of these molecules will be discussed below..

Robertson et al. (83-84) reported the purification of inhibin from bovine follicular fluid (bFF), a 56kD protein that can be dissociated into two chains of 44kD and 14kD linked by disulfide bonds. In an attempt to improve the purification of bFF inhibin, they introduced a pH 4.75 precipitation step which produced two species of inhibin with molecular masses of 31kD and 58kD. Further lines of evidence gathered by this team suggested that the 31kD inhibin was a cleavage product of the 58kD form of inhibin.

Ling et al. (85), using porcine follicular fluid, reported the purification of two forms of inhibin, A and B. Furthermore, they found this molecule to be a heterodimer with an alpha-subunit molecular mass of 18kD and a beta-subunit of 14kD linked by disulfide bonds. Similar to that reported by Miyamoto et al. (86), the disulfide linkages are essential, because the bioactivity is destroyed when the bonds are reduced by dithiothreitol. Mason et al. (65) used the N-terminal amino acid sequence data available on these subunits and

reported that the two forms of inhibin are products of a gene family which also includes transforming growth factor-beta (TGF-B).

Miyamoto et al. (86), using porcine follicular fluid (pFF), reported the purification of a 32kD inhibin. This group identified the presence of four forms of inhibin in the pFF corresponding to molecular masses of 100kD, 80kD, 55kD and 32kD. The 32kD protein demonstrates 70% of the total inhibin activity and, when purified to homogeneity, was found to be composed of two subunits of 20kD and 13kD linked by disulfide bridges. However, irreversible loss of biological activity resulted from the cleavage of the bridges.

Using several reverse phase-HPLC steps and gel filtration, Rivier et al. (87) reported the existence of a highly potent 32kD inhibin in pFF. It consisted of two chains, A and B, with molecular masses of 18kD and 14kD, respectively. Amino acid composition of their purified protein revealed the presence of many cysteine residues, suggesting a globular structure for inhibin. In addition, their 32kD inhibin contained several proline residues, indicating a secondary structure with many turns.

Recently, two forms of inhibin from ovine follicular fluid (oFF) were identified (88). The 65kD form is similar in size to the high molecular weight form of the bovine and porcine inhibins reported by Robertson et al. (83-84) and Miyamoto et al. (86), respectively. The smaller ovine 30kD form is believed to be a proteolytic product of the 65kD species.

A comparison between the N-terminal amino acid sequences of the 31kD bovine and the 30kD ovine inhibins showed identical residues in

the first 13 positions. This is not surprising since the amino acid sequences of the beta-A chains in pig (65), cow (89) and human (90, 91) are identical. In addition, a high degree of homology was also observed among these species in the alpha chains.

In attempts to purify inhibin from porcine follicular fluid, both Vale et al. (92) and Ling et al. (93) noted fractions which stimulated FSH secretion. Vale's group reported the purification of an FSH releasing protein (FRP), which stimulated FSH but not LH biosynthesis and secretion. Using HPLC procedures, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Edman degradation they determined that the first 38 N-terminal residues were identical to those of the porcine inhibin beta-A chain. In total, 83 amino acids of porcine FRP were identical to the corresponding 83 of the 116 amino acid residues of porcine inhibin beta-A. These findings are consistent with the hypothesis that FRP is a homodimer of two inhibin beta-A chains linked by disulfide bonds.

Ling et al. (93) also noted fractions which could stimulate FSH release. They coined the term "activin" for their substance. The first 32 amino acid residues in the beta-subunit of both inhibin A and B were identical. Based on this finding, Ling and his colleagues proposed that their molecule was a heterodimer composed of two beta-subunits of inhibin A and B linked by disulfide bonds. It is also interesting to note that there is significant homology between the beta subunits of inhibin A and B and TGF-beta. This growth factor has been shown to be a homodimer with FSH-releasing activity (94).

The presence of the beta-dimers as well as inhibin in follicular fluid suggests a dual gonadal control of FSH secretion. Tsonis and Sharpe (95) demonstrated that when the two were added to pituitary cells, the biological activity of inhibin predominated over that of the beta-dimers, and hence, FSH secretion was decreased. However, it is not known yet whether the secretion of beta-dimers predominates under different physiological conditions. The proposed interaction between inhibin and the FRPs in the control of FSH secretion is presented in Figure 1 (96).

These recent data suggest the existence of a regulatory system involving the association of different subunits that give rise to biologically opposite effects. Two beta subunits of inhibin yield FRP, a potent stimulator of FSH release, while one beta subunit combined with a dissimilar alpha-subunit yields inhibin, a suppressor of FSH secretion.

Inhibin: Definition and Nomenclature

Investigators reporting on the purification of inhibin used slightly different terms to define and describe the properties of their inhibins. The need for a universally recognized terminology became apparent.

At a 1987 symposium entitled "Inhibin-Non-Steroidal Regulation of Follicle-Stimulating Hormone Secretion" (97), the following inhibin definitions and nomenclature were adopted.

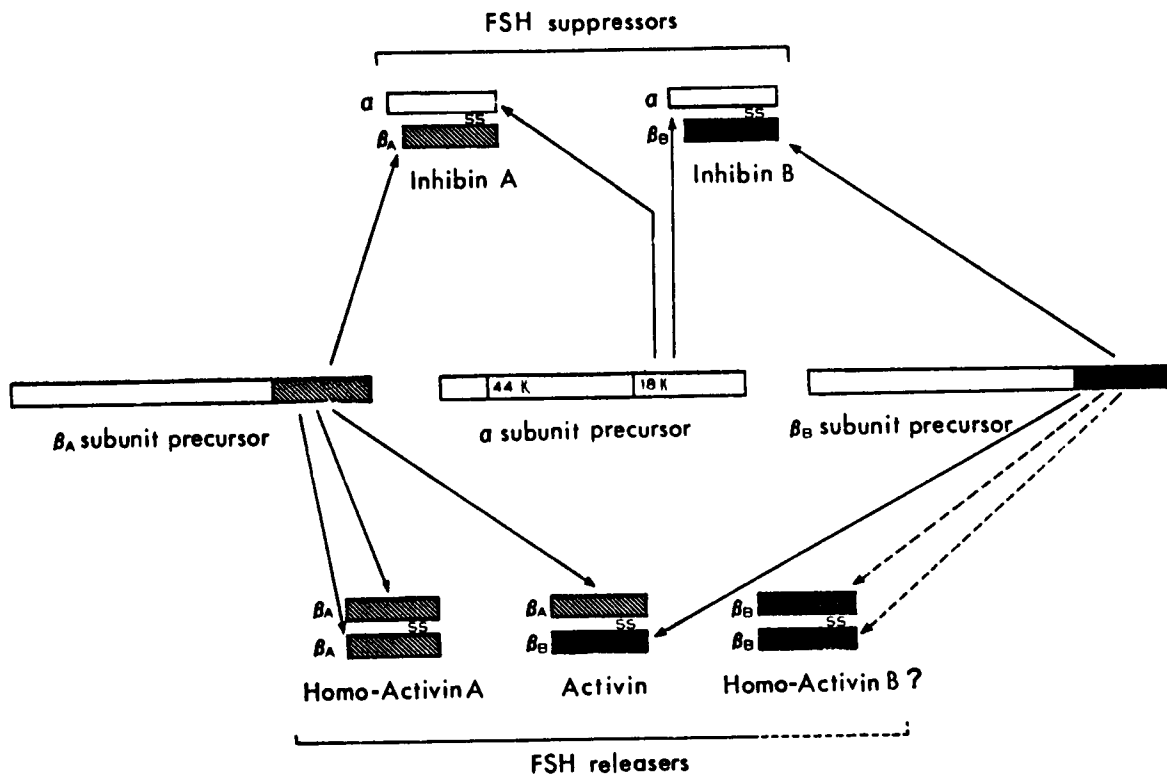


Figure 1. Inhibin and Activin Dimeric Proteins. Schematic representation of the formation of different dimeric proteins as products of gene rearrangement. Inhibin A and B are composed of a common alpha-subunit and a highly similar, but distinguishable beta-subunit (beta A beta B). Homodimers (beta A-beta A or beta B-beta B) or heterodimers (beta A-beta B) of beta-subunit form a new molecule, activins, with biological activities opposite to those of inhibins. (Reproduced from Ying SY. Inhibin, activins and follistatins:gonadal proteins modulating the secretion of follicle-stimulating hormone. Endocr Rev 9:283, 1988).

1. Inhibin is a glycoprotein hormone, which preferentially inhibits production and/or secretion of FSH.

2. The two subunits are to be called alpha and beta instead of A and B, respectively. The alpha subunit corresponds to the 20 kD subunit of the 31-32 kD inhibin.

3. The two different beta subunits will be called beta-A and beta-B. The beta-dimers will be called activin; activin A will be the homodimer of beta-A, while activin A-B will be the heterodimer beta-A and beta-B.

C. General Principles of Radioimmunoassay

Introduction

Radioimmunoassay (RIA) is a subclass of competitive protein binding assays in which radiolabeled hormones ("tracer") compete with unlabeled hormones for limited quantity of specific antibody. Before the advent of RIA, biological testing was limited to the measuring of substances in milligrams, and less often microgram amounts. RIA methodologies paved the road for measuring substances such as hormones, vitamins and drugs which are present in minute amounts in biological fluids. It is not uncommon now to accurately measure nanogram and picogram levels of substances via RIA techniques.

The development of RIA by Drs. Solomon A. Berson and Rosalyn S. Yalow in 1960 (98) is regarded by many as the single most important advancement in biological studies during the last three decades. This technique, together with other related procedures has significantly revolutionized biological studies in fields such as endocrinology, pharmacology, hematology and oncology. RIA has also exerted the same kind of influence on advancements in clinical research and biotechnology.

In the 1950's, Berson et al. (99) observed that diabetic patients injected with insulin developed antibodies which bound to ^{131}I -labeled insulin. Moreover, they demonstrated that this binding was competitively inhibited by unlabeled insulin (98). Recognition that binding of the tracer to the antibody was quantitatively a function of total insulin present and that unlabeled insulin can displace labeled insulin, formed the basis for the first RIA for insulin (99). Yalow was presented the Nobel Prize in 1977 for her pioneering efforts in the development of RIA.

Background and Theory

The basic concept of RIA involves a highly specific reaction between a hormone (antigen) and its antibody. Labeled and unlabeled hormones compete against each other for limited binding sites on the specific antibody. The general principle is illustrated in Figure 2. $^*\text{H}$ and H are the labeled and unlabeled hormone, respectively. Ab is the antibody to this hormone and the $^*\text{H}-\text{Ab}$ and $\text{H}-\text{Ab}$ are the labeled and unlabeled hormone-antibody complexes, respectively. The ability of a fixed amount of labeled hormone to bind to a limited amount of its specific antibody is competitively inhibited by unlabeled hormone in standard or unknown samples. As one can easily observe, the greater the concentration of unlabeled hormone, the less labeled hormone will be antibody-bound. A dose-response curve can be graphed. Although there are many methods for plotting a standard curve, one popular way is illustrated in Figure 3 where $\% \text{ B/B}_0$, the percent of tracer bound as a function of buffer control, is plotted against serial dilutions of the

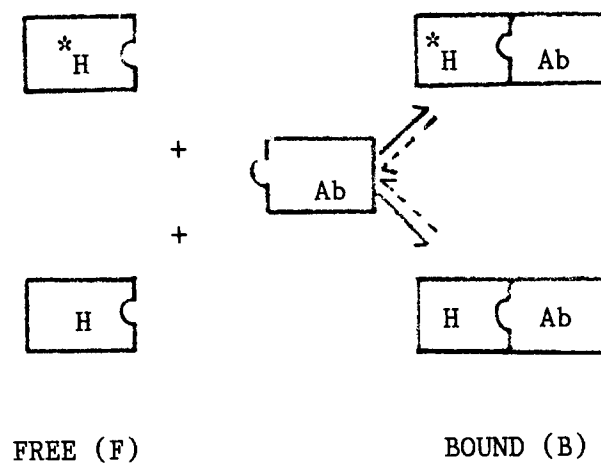


Figure 2. Antigen-Antibody Interaction in RIA. Competitive reaction that forms the basis of RIA.

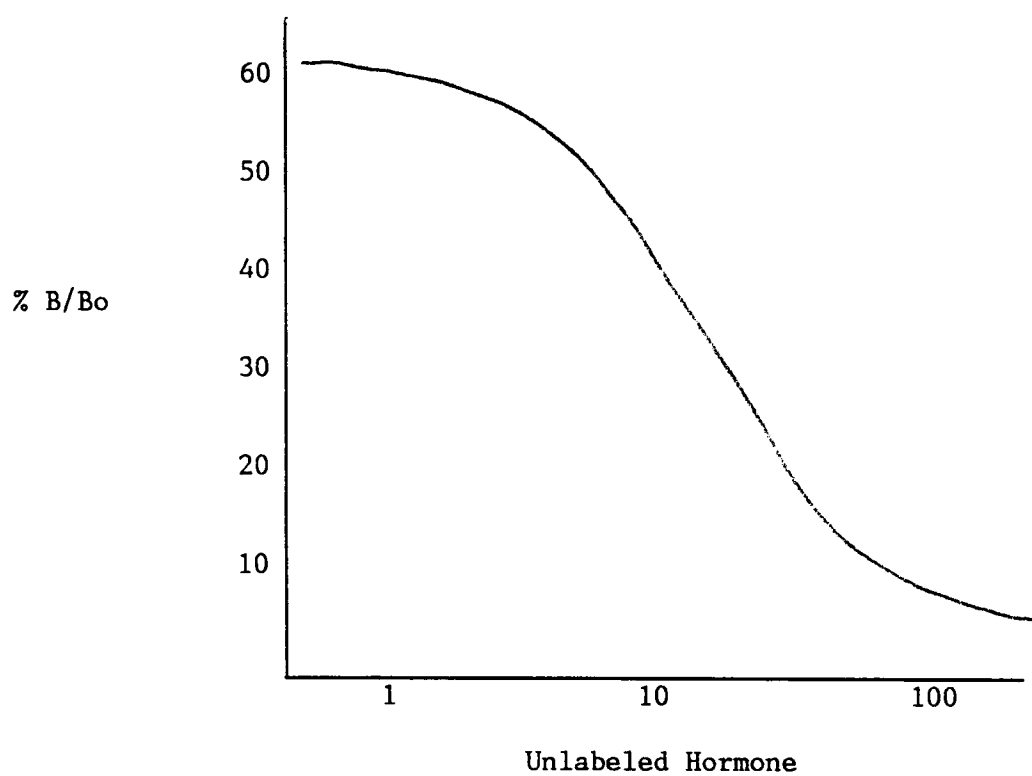


Figure 3. RIA Standard Curve

unlabeled hormone on a logarithmic scale (100). To determine the concentration of hormone present in unknown samples, the % B/B₀ is plotted and extrapolated from the standard curve. Linearization of the standard curve is made possible by the logit transformation introduced by Rodbard et al. (101). Two major advantages offered by this procedure are 1) standard curves from different assays can be compared and 2) parallelism between unknown samples and the standard curve can be measured.

Not only are standard curves used to assess the suitability of an antiserum for an assay but can also be used to determine the affinity constant (K) by means of a Scatchard plot (Figure 4) (102). By definition, K is the slope of the plot and represents the strength of binding between the hormone and its antibody. The maximum binding capacity (B_{max}) of the antibody is where the line crosses the abscissa.

A linear relationship will only exist when one species of antibody is present in the antiserum. However, most antisera contain a heterogeneous mixture of antibodies. Therefore, a Scatchard plot will show curvatures, and the curve with the steepest slope represents that antiserum which is most optimal for the assay. Scatchard analyses offer both qualitative and quantitative criteria to evaluate antibody properties (103).

An immune serum contains various species of antibodies, usually characterized on the basis of its antibody properties. Three properties must be assessed: sensitivity, specificity and titer. Sensitivity is defined as the least amount of unlabeled hormone which

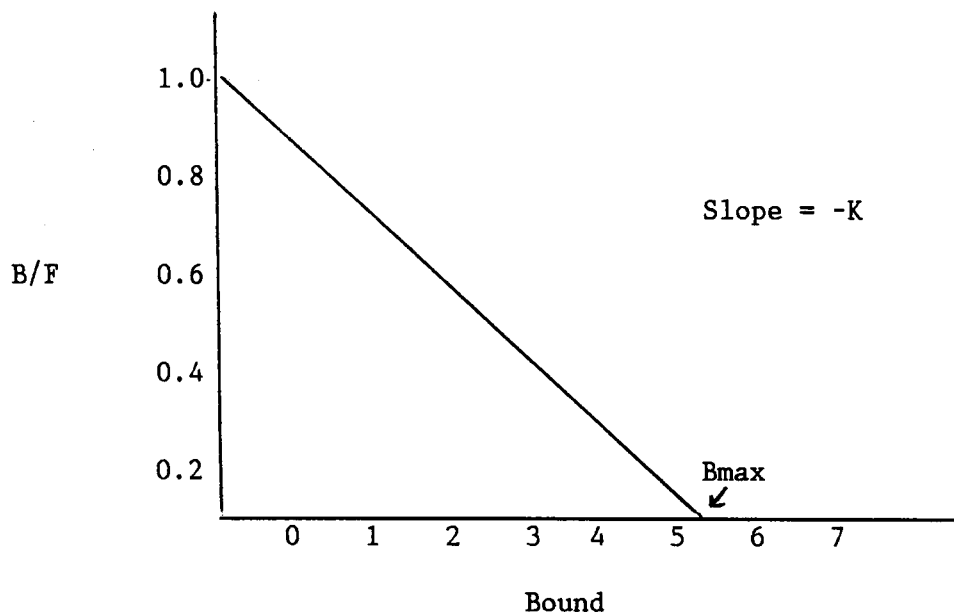


Figure 4. RIA Scatchard Plot

can be distinguished from a sample with no hormone (buffer control tube, Bo). In testing the sensitivity of an antiserum, it is helpful to know the immunoreactivity of the labeled hormone in relation to the particular antiserum. Figure 5 (104) illustrates the results of an experiment designed to assess the immunoreactivity of ^{125}I -hLH. Three antiserum dilution curves were used to examine the sensitivity of a given antiserum and to examine the extent to which this potential is lost in the labeled preparation. The antiserum was unable to distinguish between 0.1 ng/ml and 1.0 ng/ml of the tracer. However, when 0.1 ng/ml of ^{125}I -hLH was added to 0.9 ng/ml of unlabeled hLH, a third curve was generated which was to the left of the first two curves. The latter curve's position indicated a loss of immunoactivity due to labeling and consequently a loss of sensitivity.

It is well recognized that antisera vary in their ability to distinguish between different concentrations of labeled and unlabeled hormones. Consequently, a major limiting factor in assay sensitivity is the avidity of the antiserum. In other words, the sensitivity of an assay strongly depends on the energy of the reaction (avidity) between the hormone to be measured and its specific antibody. For practical purposes, sensitivity is reflected by the slope of the Scatchard plot, and therefore, is a function of the affinity constant, K (103).

Specificity is the "extent of freedom from interference by substances other than the one intended to be measured" (102). Measuring specificity is a prime requisite for the validation of an assay. It is well recognized that antibodies not only can bind to

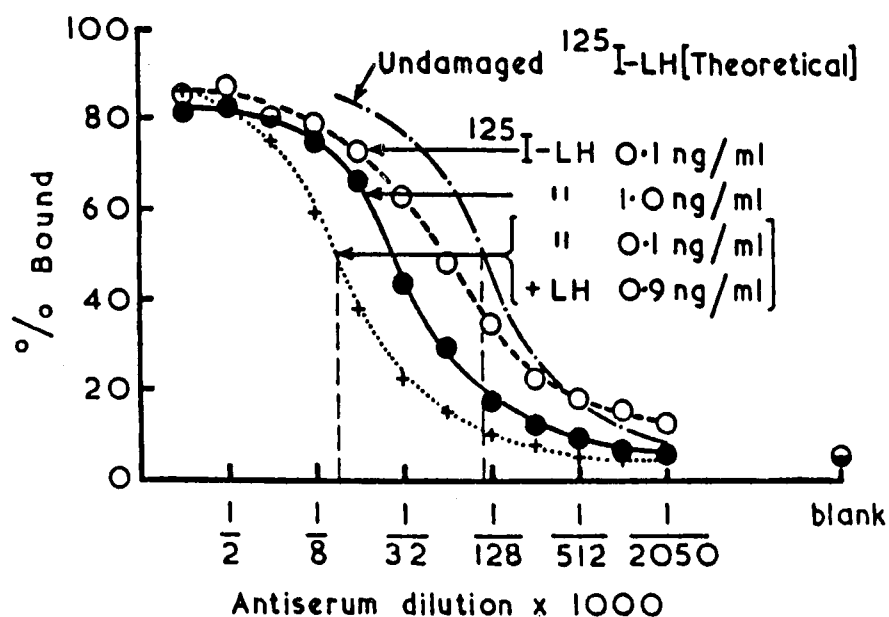


Figure 5. Immunoreactivity of ¹²⁵I-hLH. Antiserum titration curve to assess the immunoactivity of ¹²⁵I-hLH. The difference in the relative positions of the curve for 1.0 ng/ml ¹²⁵I-hLH (●—●—●) and 0.1 ng/ml ¹²⁵I-hLH + 0.9 ng/ml unlabeled hLH (*—*—*) indicates considerable loss of immunoreactivity due to labeling. The remaining curve (○—○—○) indicates the maximum possible separation to be expected for a ten-fold difference in antigen concentration. (Reproduced from Radioimmunoassay Methods. Kirkham KE, Hunter WM (eds.), Churchill Livingstone, London p 11)

their specific antigens, but also to closely related molecules. For example, cross-reactions between the glycoproteins FSH, LH and TSH as well as between hGH and hPl have been described. Such occurrences can clearly affect the reliability of radioimmunoassays.

It is essential that the standard hormone and the hormone in the unknown samples react in the same way with the antiserum, independent of the other constituents in the sample. To determine if the standard and unknowns are immunologically identical, serial dilutions of the unknown sample are prepared and assayed. The results are plotted on a standard curve and compared with the results using standard hormones. These two curves must be parallel to demonstrate that both substances are reacting in the same way with the antiserum.

Specificity is often tested by performing cross-reactivity studies using serial dilutions of closely related hormones. Essentially, one can graph the percent B/B₀ results on semi-log paper where the % B/B₀ is plotted on the Y-axis and the dose of the various competing hormones is plotted on the X-axis. Cross-reactivity is determined by dividing the dose of the standard by the dose of the competing hormone at the 50% point. The smaller this fraction is, the more specific is the antibody. An example of determining cross-reactivity is depicted in Figure 6 (105).

A titration curve is used to determine the optimal working dilution (titer) of a chosen antiserum. Normally, a dilution such that 40-60% of the labeled hormone is bound in the absence of unlabeled hormone is selected. Use of excess antibody results in loss of sensitivity,

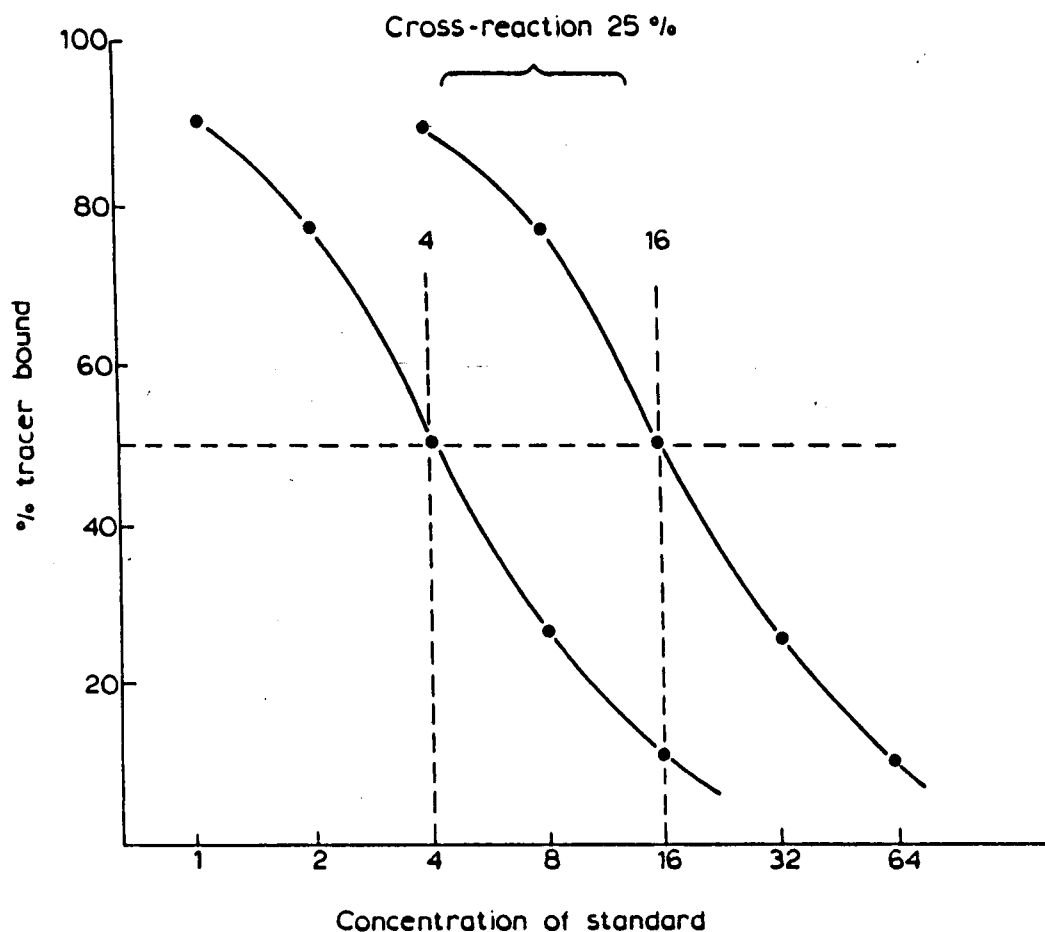


Figure 6. RIA Cross-reactivity Curve. Estimation of the percentage cross-reaction from the amount of material required to produce 50% inhibition in the assay. The curve on the left is for the standard, that on the right from the cross-reacting material. (Reproduced from Introduction to Radioimmunoassay and Related Techniques. Work TS, Work E (eds.), Elsevier Biomedical Press, New York p 188).

while the concentration of antibody which binds to significantly less than 40% of the labeled hormone results in loss of precision. This dilution generally will give an optimal ratio of sensitivity and precision in a radioimmunoassay. To determine this property, a fixed amount of labeled antigen (hormone) is incubated in serial dilutions of antiserum in the absence of unlabeled hormone. Figure 7 depicts a typical titration curve.

Two other criteria which need to be defined when validating an RIA are 1) precision and 2) accuracy. The objective of an RIA is to measure the concentration of a hormone present in a biological fluid. The actual concentration measured will deviate from the "true" amount present, because of experimental imprecision and inaccuracy. Accuracy is the extent to which the mean of an infinite number of measurements of a substance agrees with the exact amount of the substance which is actually present. Precision is the extent to which a given set of measurements of the same sample agrees with the mean. The intra-assay variance is determined by repeated measurements of the same sample in the same assay, and ideally should be below 10%. Inter-assay variance (reproducibility) is obtained when multiple measurements of the same sample are performed in different assays and/or by different people. Ideally, this parameter should be below 15%.

The standard index for precision is the coefficient of variation (C.V.) which is defined as the standard deviation of multiple measurements divided by the mean and multiplied by 100. Figure 8 demonstrates the conceptual difference between precision and accuracy (106).

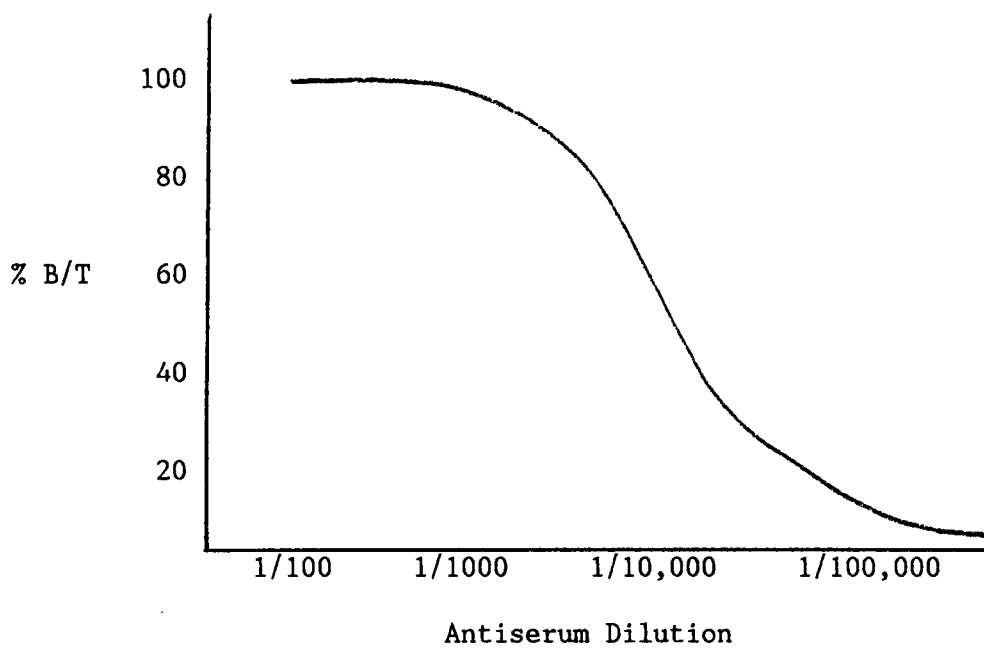


Figure 7. RIA Antiserum Titration Curve.

BINDING ASSAY PRECISION

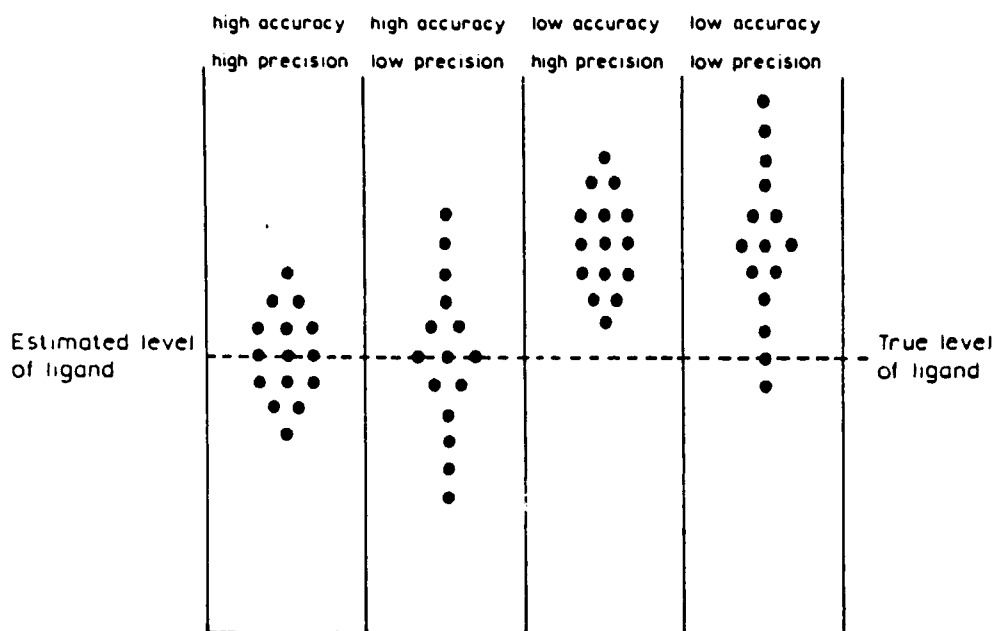


Figure 8. RIA Precision and Accuracy Diagram. The concept of precision (variation of repeated determinations) and accuracy (degree to which a measured concentration corresponds to the true concentration of a substance). (Reproduced from Introduction to Radioimmunoassay and Related Techniques. Work TS, Work E (eds.), Elsevier Biomedical Press, New York p 204).

D. Polycystic Ovarian Disease

Introduction

Polycystic ovaries were alluded to as early as 1845 by Chereau, but it was not until 1935 when Stein and Leventhal (107) reported a series of cases associating amenorrhea, hirsutism and obesity with polycystic ovaries. This "syndrome" is beset with such wide clinical and biological variability that within the past few years the eponym "Stein-Leventhal syndrome" has been replaced by "polycystic ovarian disease" (PCOD). This latter term is a generic description for a broad spectrum of pathophysiological manifestations.

It appears that several etiologies contribute to the development of this disease, but none is well understood. Generally associated features of PCOD include infertility, amenorrhea, anovulation, obesity and hirsutism; some or all of these features may be absent in individual patients.

Histopathology

The histopathology of PCOD is variable and often resembles many pathophysiological disorders of the adrenals, such as Cushing's syndrome and congenital adrenal hyperplasia. Gross anatomical features of polycystic ovaries include the presence of numerous fluid-filled cysts situated in a thickened cortex, a smooth pearly white tunica and elongated ovaries which may be twice the size of normal ovaries. Microscopic examination of polycystic ovaries reveals marked hyperplasia of the theca interna with groups of luteinized stromal-thecal cells ("androgenic cells").

At first, gross and microscopic descriptions of PCOD were believed to be consistent and predictable. However, results obtained from a large case study (108) forced reconsideration of this concept. Smith et al. (109) reported that 40% of the surgically removed polycystic ovaries were of normal size and that 46% had no tunica thickening. Since very little is known about the true frequency of the features associated with PCOD, strict adherence to a set of clinical criteria would deprive many women from the benefits of medical and/or surgical treatments. The possibility of PCOD should be kept in mind in all cases of anovulation and persistent menstrual disorders. Undoubtedly, the use of the laproscope will change our perception and knowledge of this disorder still further (110).

Symptomatology

Goldzieher and Green (108) published a report in 1962 detailing the symptomatology associated with polycystic ovaries (Table 1). These authors, as well as Netter et al. (111), suggested that the characteristic signs and symptoms are not found consistently enough to warrant calling this disorder a "syndrome". Netter and his colleagues have remarked that PCOD is a "fugitive syndrome with limits less well defined than those of the Sahara or Sudan".

Hormonal Profile of PCOD

Thecal cells of the stroma produce androgens, which are subsequently aromatized into estrogens by the ovarian granulosa cells. This conversion is activated by FSH. Estradiol, the most potent estrogen, directly regulates follicular maturation via its feedback actions

Table 1. Symptomatology Associated with Polycystic Ovarian Disease.

Symptom	Usable # cases	Incidence (%)	
		mean	range
Infertility	596	74	35-94
Hirustism	819	69	17-83
Amenorrhea	640	51	15-77
Obesity	600	41	16-49
Functional bleeding	547	29	6-65
Dysmenorrhea	75	23	----
Corpus luteum at surgery	391	22	0-71
Virilization	431	21	0-28
Biphasic basal body temperature	238	15	12-40
Cyclic menses	395	12	7-28

A summary of findings in 1079 cases compiled from 187 references (108). The usable number of cases refers to how many of the 1079 cases could be evaluated for the presence or absence of a particular symptom. (Adapted from Goldzieher JW, Green JA, The polycystic ovary. I. Clinical and histologic features. J Clin Endocrinol Metab 22:325, 1962).

on gonadotropin-releasing mechanism (112, 113). To assure proper interaction between androgens and estrogens, so as to modulate normal follicular function, it is imperative that FSH and LH act together under specific dynamics. In PCOD, it is well established that this normal sequence of events does not occur (114).

LH/FSH Ratios

Reports on the LH/FSH ratios in PCOD are conflicting. Gambell et al. (115), investigating serum gonadotropin levels in patients with PCOD, showed that LH levels may be low, normal or exaggerated with sporadic peaks. They, along with others (116-120), also reported that FSH, in general, appeared in the normal to subnormal range when compared to the follicular phase of the normal menstrual cycle. On the other hand, many investigators (121-124) concur that LH secretion in PCOD is a persistently high pulsatile pattern. Because patients with PCOD seem more sensitive to GnRH (118, 123-124), Rebar et al. (125) and Patton et al. (126) hypothesized that the elevated LH could be related to the heightened responsiveness of the pituitary to GnRH, resulting in the exaggerated pulsatile LH release. Figure 9 (127) illustrates the dynamics which may contribute to the aberrant LH/FSH secretion often associated with PCOD.

The variations reported in the LH/FSH ratios of PCOD may result from the fact that plasma samplings were measured once daily, or even less frequently. However, oscillations in gonadotropin and steroid release are well documented (128-129), and the importance of multiple

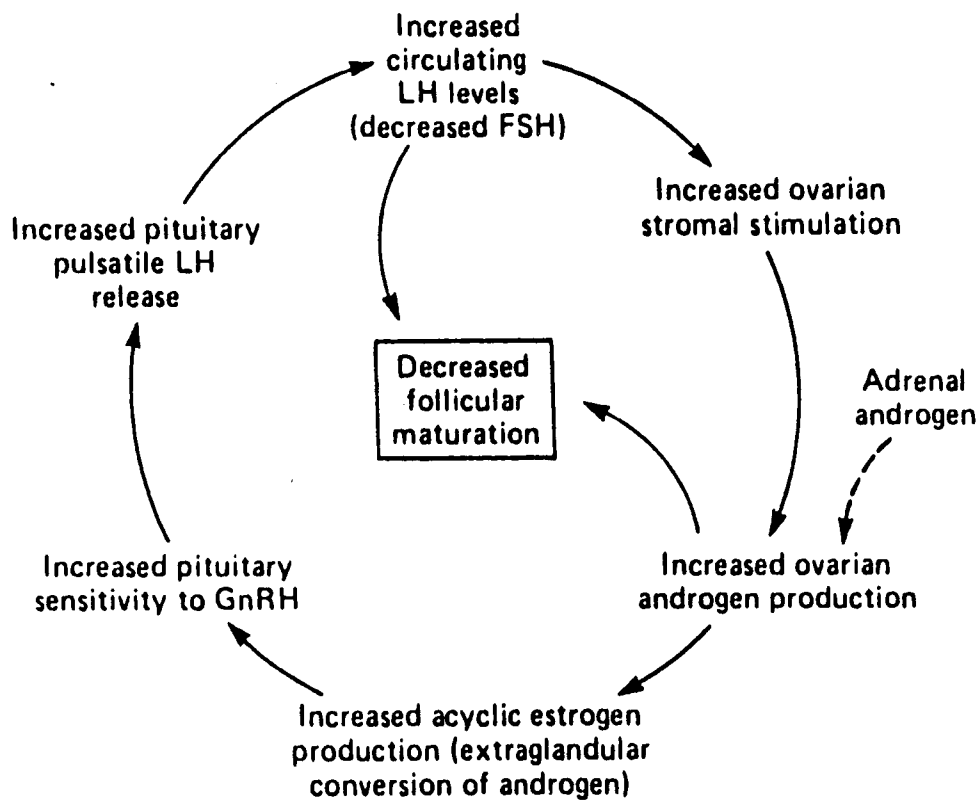


Figure 9. Pathophysiology of Chronic Anovulation. Diagram showing how anovulation can develop in women with polycystic ovary disease. (127). (Reproduced from Reproductive Endocrinology ed. 2, Yen SSC and Jaffe RB (eds.), Saunders, Philadelphia p 441)

sampling for obtainment of meaningful estimates cannot be overemphasized.

Steroids and LH/FSH Ratios

Data are conflicting in the attempts to explain the increase in LH/FSH ratios seen in PCOD. There have been reports correlating LH levels directly (130) or inversely (131) with testosterone levels. Other investigators reported that LH levels correlate with estradiol and estrone (119), or with estradiol (126, 132), or not with estrogen levels at all (133). It is obvious from these conflicting results that the prevailing steroidal environment in the ovary cannot alone adequately explain the status of the LH/FSH ratio.

Inhibin and Polycystic Ovary Disease

The involvement of an intraovarian factor(s) other than steroids is suspected. Yen (134) proposed that inhibin is at least one such factor. Chari et al. (135) demonstrated the presence of inhibin in human follicular fluid. Moreover, Channing et al. (136) and Tanabe et al. (137) found that inhibin levels correlate with steroid levels in follicular fluid. Tanabe et al. (138) recently conducted a study examining inhibin and steroid levels in follicular fluid from patients with polycystic ovaries. Their major objective was to evaluate the possibility that inhibin was linked with abnormal LH/FSH ratios found in PCOD. Inhibin content of all small normal follicles was considerably less than that found in polycystic ovaries (197 +/- 34 U/10ul compared to 332 +/- 13 U/10ul). In addition, androgen levels were greater in women with PCOD than in women with normally functioning

follicles. The study by Tanabe and his colleagues represent the first observation of inhibin activity in PCOD. They suggest that inhibin is indeed involved in the inappropriate gonadotropin secretion, which results in abnormal LH/FSH ratios.

A recent study undertaken by Buckler et al. (139) was unable to confirm the speculations put forth by Tanabe et al. (138), Franks et al. (140) and Yen (141) that serum levels of inhibin are higher in women with PCOD. Buckler's group reported that inhibin levels were no different during the early or mid-follicular phase of the menstrual cycle between PCO and control women. Moreover, they found that inhibin concentrations were actually higher in the control subjects during the late follicular, midcycle and mid-luteal phases. However, these authors acknowledged that one reason why they may not have found elevated levels of inhibin in their patients was because polycystic ovaries have many small follicles (2-6 mm), which have fewer granulosa cells. Since granulosa cells produce inhibin, more inhibin would be secreted from large follicles. However, if inhibin is also secreted by the corpus luteum, then it is not surprising that the PCO women would have lower levels of inhibin during the luteal phase, presumably because of a poorly developed, or absence of, a corpus luteum.

Buckler and his group also administered buserelin, an exogenous LHRH agonist, to both the PCO and infertile women in their study. These subjects responded with a parallel rise in both estradiol and inhibin, in much the same way as non-PCO women did in a previous report (48). Since the response to exogenous gonadotropin administration was

similar in normal and PCO women, these authors concluded that, "we found no evidence to indicate a primary defect in ovarian inhibin secretion in women with PCOS...".

The role of steroids must be taken into account when examining the factors involved in the disparity of LH to FSH secretion in patients with PCOD compared to that in normal women. Franchimont et al. (142) observed that the addition of androgens to bovine granulosa cell cultures resulted in an increased inhibin secretion, suggesting that androgens may stimulate inhibin secretion from granulosa cells. Channing et al. (41) stated a similar hypothesis that excess androgens stimulate inhibin release, which in turn suppresses FSH levels and triggers cyst formation. Inhibin may be in high concentrations in follicular fluid of polycystic ovaries (138, 143, 144), however, reports are not unanimous.

In view of these conflicting reports, Hurkadli et al. (145) investigated the possible role of inhibin in the etiology of PCOD. These investigators used rats and induced polycystic ovaries by thiouracil and human chorionic gonadotropin (hCG). The animals were divided into three treatment groups: 1) inhibin; 2) antibody to inhibin; 3) ovine FSH (oFSH). As expected, there was a decrease in ovarian weight (presumably due to reduced cyst formation) and a specific rise in FSH levels in rats treated with the anti-inhibin antibodies. Furthermore, testosterone levels were significantly decreased, while no changes in LH and prolactin were observed. A histological examination of the ovaries in this group indicated an arrest of cyst formation concomitant with growth of new follicles.

Treatment with inhibin resulted in a rise in serum testosterone, without affecting LH and prolactin levels. The decrease in serum FSH levels was accompanied by cyst formation. The study corroborates the hypothesis that inhibin does indeed play a causative role in the development of PCOD.

Summary

The cause(s) and pathophysiology of PCOD remain ambiguous and controversial. The most probable etiologies are a combination of altered gonadotropin secretion and a defect in gonadotropin action at the ovarian level. Figure 10 summarizes the possible causes leading to the development of PCOD.

If one presumes that the initial cause of PCOD is the elevation in pituitary LH, then a working hypothesis may suggest that this elevation causes the theca to produce excess androgens. High levels of androgens are believed to stimulate inhibin secretion from the granulosa cells, which in turn suppresses pituitary FSH secretion. In addition, down-regulation of LH receptors on granulosa cells may occur as a result of elevated LH levels (145). The latter, combined with suppressed FSH secretion may account for the aberrant follicular development. High levels of androgens are also capable of being peripherally converted to estrogens, which render the pituitary more sensitive to GnRH with a resultant release of more LH than FSH.

It is also possible that the initial cause of PCOD is an ovarian defect. The excess accumulation of inhibitor proteins such as ovarian maturation inhibitor (OMI) or luteinizing inhibitor (LI) can prevent

the ovarian follicles from properly developing, or can interfere with gonadotropin influence on follicular growth. Inhibin secreted by the granulosa cells contributes to the abnormal LH/FSH ratio, which further retards follicular maturation. (138, 145)

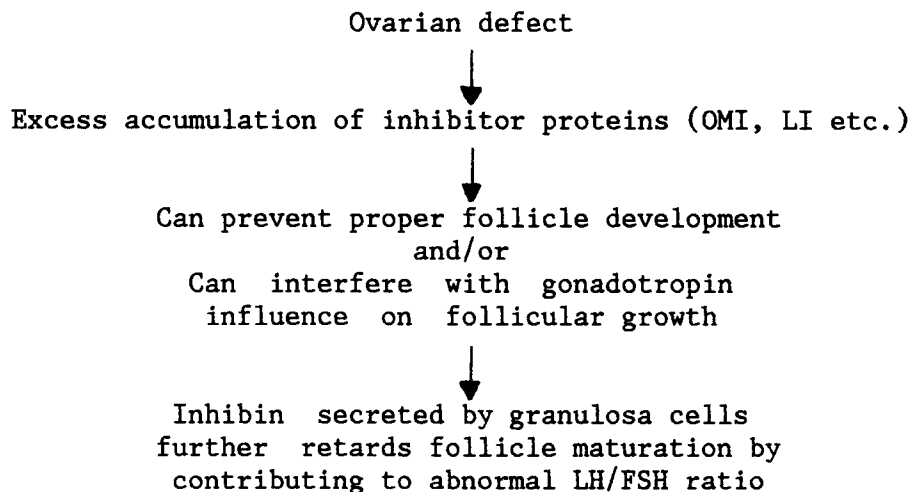
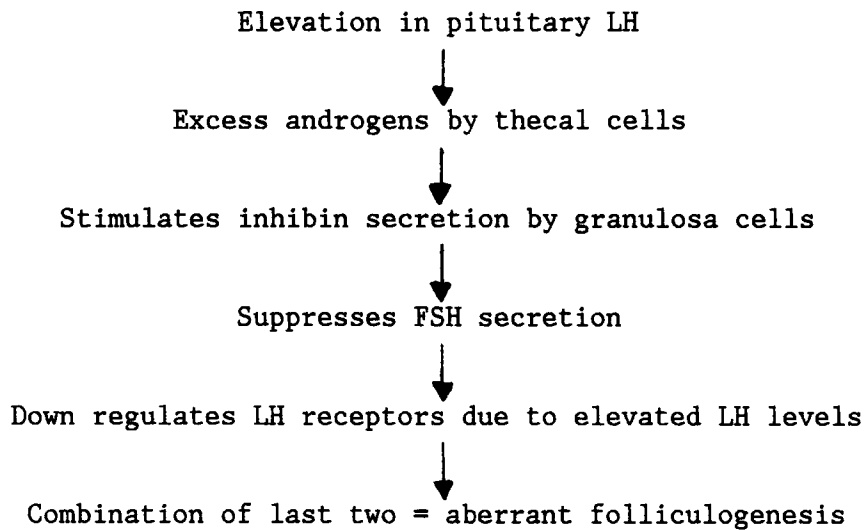


Figure 10. Probable Etiologies of PCOD. A combination of altered gonadotropin secretion and a defect in gonadotropin action at the ovarian level.

CHAPTER III

MATERIALS AND METHODS

A. Materials

Hormones and Reagents

The human follicle stimulating hormone (hFSH, NIADDK-hFSH-I-3, AFP-4822B) and human luteinizing hormone (hLH, NIDDK-hLH-I-3, AFP-8270B) used for radioiodination and the hFSH antiserum (NIADDK-anti-hFSH-6, rabbit) were prepared by Dr. A.F. Parlow (Torrance, CA). Dr. Leo R. Reichert (Albany, NY) made a dual hFSH-hLH reference preparation, LER 907. The potency of one mg of LER 907 was 53 IU FSH and 277 IU LH in terms of the second international reference preparation human menopausal gonadotropin (2nd IRP-hMG) as determined by RIA (Journal of Clinical Endocrinology and Metabolism (JCEM) 48:163,1979). Anti- hLH serum (NIDDK-hLH-I-2, rabbit) was produced by Dr. V.S. Fang (Chicago, IL).

A synthetic fragment of the alpha subunit of human inhibin which was suspected as having the most dissimilarity with rabbit inhibin was prepared by James Sparrow, a peptide chemist at Baylor College of Medicine, and was used for the immunization of rabbits; antisera collected from rabbits six months after initial injection were sent to Knoxville to be analyzed for the presence of anti-inhibin antibody. A partially purified form of the same fragment (2.08 mg/ml, 89% pure) was used for radioiodination.

Carrier-free Na ^{125}I in NaOH (Sp. Act. 17 Ci/mg I) was supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). Lactoperoxidase was obtained from Calbiochem-Behring (La Jolla, Ca), and Iodogen was supplied by Pierce Chemical Company (Rockford, IL). Chloramine T, sodium metabisulfite, human chorionic gonadotropin and minimum essential medium (Eagle's) with the nutrient mixture F-12 (HAM) were purchased from Sigma Chemical Company (St. Louis, Mo.).

Purity of hFSH and hLH

The biological potency of NIADDK-hFSH-I-3 is 3100 IU/mg by the hCG-Augmentation bioassay and its immunological potency (RIA) is 6887 IU/mg. Contamination with hLH is 400 IU/mg as determined by bioassay and 420 IU/mg as determined by RIA. Contamination with hTSH, hGH and hPr1 is 0.1, <0.01 and <0.01 IU/mg, respectively, as determined by RIA.

The biological potency of NIDDK-hLH-I-3 is 5900/mg and its RIA potency is 10,000 IU/mg. Contamination with hFSH and hTSH is 3 IU/mg and 0.04 IU/mg, respectively, as determined by RIA. Contamination with hGH and hPr1 is less than 0.05%, by weight, as determined by RIA.

The above information was provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK).

B. Methods

Preparation of Reagents

One-hundred micrograms of each of the hormones to be radioiodinated was solubilized in 1 ml of 0.5 M phosphate buffer, pH

7.5, equally divided into four aliquots (25ug/250ul) and stored in -20 C pending future use.

One milliliter of hFSH antiserum (1:25) preadsorbed with hCG was brought to 10 ml (1:250) in PBS containing 3% normal rabbit serum (NRS) and 0.05 M ethylenediamine tetraacetic acid (EDTA). Two hundred microliter aliquots were stored at -20 C.

One milliliter of hLH antiserum (1:10) was brought to 10 ml (1:100) in PBS containing 3% NRS and 0.05 M EDTA; 200 ul aliquots were stored at -20 C.

The reference preparation, LER 907, is a crude pituitary extract and served as the standard for both hFSH and hLH. Two hundred micrograms of LER 907 was solubilized in 2.5 ml PBS-BSA, and 8ug/100ul aliquots were prepared and stored at -20 C.

Radioiodination of hFSH and hLH

The chloramine T (C-T) method was used for labeling the hormones. One millicurie of carrier-free Na ^{125}I was added to 25 ug hormone in 250 ul of 0.5 M phosphate buffer, pH 7.5. Chloramine T (200 ug in 100 ul 0.05 M phosphate buffer, pH 7.5) was mixed with the hormone (8:1, C-T:hormone) and allowed to react for 60 seconds at room temperature. Sodium metabisulfite (400 ug in 200 ul of 0.05 M phosphate buffer pH 7.5) was then added to terminate the reaction. One-hundred microliters of transfer solution (16% sucrose) was mixed with the sample before loading it onto a Sephadex G-25 column. Ten 1-ml fractions were collected, and 10 ul from each fraction was counted using a Beckman Gamma 4000 counter to determine radioiodinated protein and free ^{125}I

peaks. The peak protein tube was aliquoted into ten-100 ul portions and stored at -20 C.

The lactoperoxidase procedure (LPO) was also employed to label the hLH. One millicurie of carrier-free Na ^{125}I was added to 12.5 ug hormone in 125 ul 0.1 M phosphate buffer, pH 7.1. Hydrogen peroxide (40 ng, 10 ul) and lactoperoxidase (1 ug, 10 ul) were allowed to react with the hormone solution for five minutes at 4 C. One-hundred microliters of transfer solution (16 % sucrose) was added to the sample before loading it onto a Sephadex G-25 column. Fractions were collected and aliquoted as described for the chloramine T method.

Human LH was also radiolabeled using the iodogen method. One-hundred micrograms of iodogen was solubilized in 2.5 ml methylene chloride, and 200 ul of this solution (40 ug/ul) was used to coat micro-centrifuge tubes. Tubes were placed in a multiport apparatus, air dried, capped and stored in -20 C until further use.

Carrier-free Na ^{125}I (0.5 mCi, 5 ul) was added to a pre-coated iodogen tube, followed by the addition of hLH-I-3 (12.5 ug in 125 ul 0.1 M sodium phosphate buffer, pH 7.1). Two reaction times were used: 1) 10 minutes and 2) 15 minutes. To terminate the reaction, the protein samples were each transferred to another polypropylene tube containing 200 ul 0.1 M protein-free sodium phosphate buffer, pH 7.1 for 10 minutes. This step allowed for any unincorporated iodide ions to return to iodine and to minimize the risk of iodinating the column proteins; i.e., albumin. The samples were each loaded onto Sephadex G-25 columns, and fractions were collected and aliquoted as described for the chloramine T method.

Radioiodination of Inhibin

One millicurie of carrier-free Na ^{125}I was added to 25 micrograms of human-inhibin alpha chain in 125 microliters of 0.5 M phosphate buffer, pH 7.5. Chloramine T (130 ug in 65 ul 0.05 M phosphate buffer, pH 7.5) was added at a ratio of 4:1 (C-T:hormone). The reaction proceeded for 30 seconds at room temperature and was terminated with sodium metabisulfite (260 ug in 130 ul 0.05 M phosphate buffer, pH 7.5). A final reaction mixture was loaded onto a Sephadex G-25 column to remove free ^{125}I . Ten 1-ml fractions were collected, and the peak protein tube was aliquoted into ten 100-ul portions and stored at -80 C.

Radioiodination was also performed using the lactoperoxidase procedure. Twenty-five microliters of a synthetic human-inhibin alpha chain (1 ug/ul 0.5 M phosphate buffer, pH 7.5) was resuspended in 100 ul of 0.1 M phosphate buffer, pH 7.1. One millicurie of carrier-free Na ^{125}I was added. Hydrogen peroxide (40 ng, 10 ul) and lactoperoxidase (1 ug, 10 ul) were allowed to react with the protein at 4 C for five minutes. One-hundred microliters of transfer solution (16 % sucrose) was added to the sample before loading it onto a Sephadex G-25 column to remove free ^{125}I . Ten 1-ml fractions were collected, and the peak tube was aliquoted into ten 100-ul portions and stored at -20 C.

Radioreceptor Assay

Fresh porcine corpora lutea (CL) were collected from Lay's Packing Plant (Knoxville, TN) and placed in ice cold minimum essential medium (MEM, Eagle's) supplemented with nutrient mixture F-12 (HAM), pH 7.4.

CL were decapsulated, and 5.4 grams of tissue was homogenized in 9 ml phosphate buffer containing 0.15 M NaCl (PBS) using a Dounce homogenizer. Serial dilutions of luteal tissue ranging from 120 mg/200 ul to 15 mg/200 ul were used, along with each of the six differently radiolabeled hLH (50 ul, 50,000 cpm). Unlabeled hCG (25 ng/ul, 100 ul) was used to compete with the tracer. PBS-BSA buffer was added for a final reaction volume of 1 ml. Assay tubes were placed in a shaking water bath and incubated for 24 hours at room temperature. All tubes, except totals, were diluted with 2.5 ml PBS-BSA and centrifuged at 3000 rpms for 20 minutes. The supernatant was aspirated, and the pellet was counted using a Beckman Gamma 4000 counter. Specific binding was determined by subtracting non-specific binding (NSB) from total binding. The assay tube receiving excess unlabeled hCG represented the NSB, because excess hCG far out competed the radiolabeled hCG for available receptor sites. Therefore, any radioactivity counted represented tracer which is non-specifically trapped in the tissue and/or bound to the glass.

Experimental Subjects

Normal women. Twenty-three women between the ages of 22-50; mean age of 30, served as control subjects. Each had a history of regular 26-30 day menstrual cycles and had no known reproductive/gynecological disorders. Hormone profiles of subjects were studied during the follicular phase of the menstrual cycle.

Polycystic ovarian disease (PCOD) women. Fourteen patients of Dr. Michael Doody, reproductive endocrinologist in the Department of

Obstetrics and Gynecology at University of Tennessee (UT) Hospital, served as the patient group. Five patients had not had a spontaneous menses for at least three months or longer, five had a menstrual period within four weeks of the blood collection and four had medically induced menses.

Experimental Protocol

This study was approved by the Human Subjects Review Boards from both the UT Knoxville Campus and the UT Hospital. All women completed a questionnaire (Appendix A) and gave informed consent (Appendix B) before undergoing any experimental procedures.

Normal women. Each woman had 10-ml blood samples drawn beginning on day 4 (day 0= menses) of the menstrual cycle and every third day thereafter for a total of five collections. Blood collection was performed between 10:00-11:00 a.m., except for four women whose blood was drawn between 6:00-8:00 p.m. Blood was allowed to clot and was then centrifuged for twenty minutes at 1500 g. Serum was collected and stored at -20 C until hormone assays.

PCOD women. All these women were anovulatory. Only one blood sample was collected upon an office visit. No consistency was apparent in the sampling of the blood.

RIA Protocol. See Appendix C for description.

CHAPTER IV

RESULTS

A. Validation of hFSH Assay

Characterization of hFSH Antiserum

Antibody titration. An antiserum titration curve involves the incubation of a fixed amount of tracer with different concentrations of the antiserum. Normally, a dilution such that 40-60 % of the labeled hormone is bound is selected. Using an antiserum dilution which is too concentrated or too dilute would result in decreased sensitivity of the assay. However, using a percent binding range of 40-60 % allows for a more equal opportunity for the radioligand and the unknown samples or standards to compete for antibody binding sites. An hFSH antiserum dilution of 1:5000 produced 41 % binding (B/T) and was selected as the working antiserum dilution. The percent binding over a range of anti-hFSH dilutions is presented in Figure 11.

Cross-reactivity assay. The cross-reactivity of the hFSH was comparable to data provided by Dr. Parlow. The results of the cross-reactivity determination are presented in Figure 12. The hFSH antiserum is species-specific in terms of recognizing rat FSH (rFSH). This explains why rFSH did not compete with hFSH for antibody binding sites. Likewise, inhibin also demonstrated no cross-reactivity with the hFSH antiserum as evidenced by a similar curve pattern as that produced when

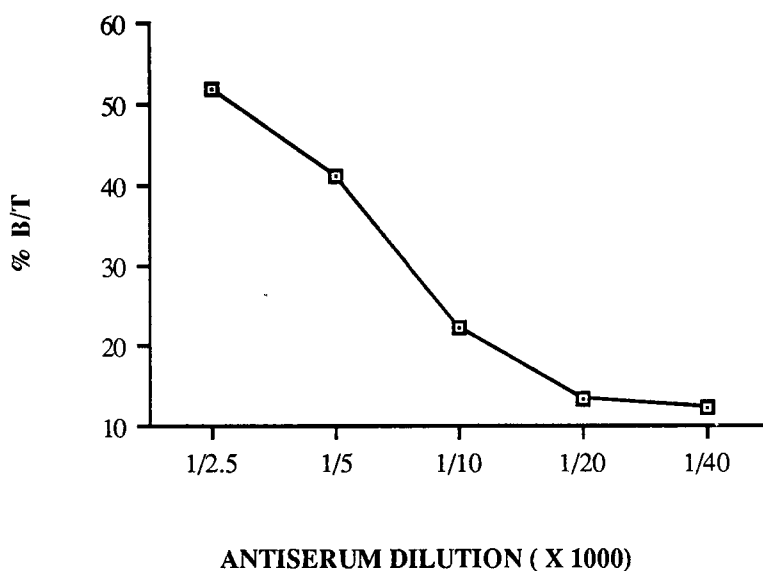


Figure 11. Human FSH Antiserum Titration Curve. An hFSH antiserum titration curve to determine the optimal hFSH antibody dilution to be used for subsequent radioimmunoassays. Radioiodinated hFSH (50,000 cpm) was incubated with serial dilutions of hFSH antibody for 24 hours at 4 C. Antiserum dilutions are ten times more dilute in the final assay.

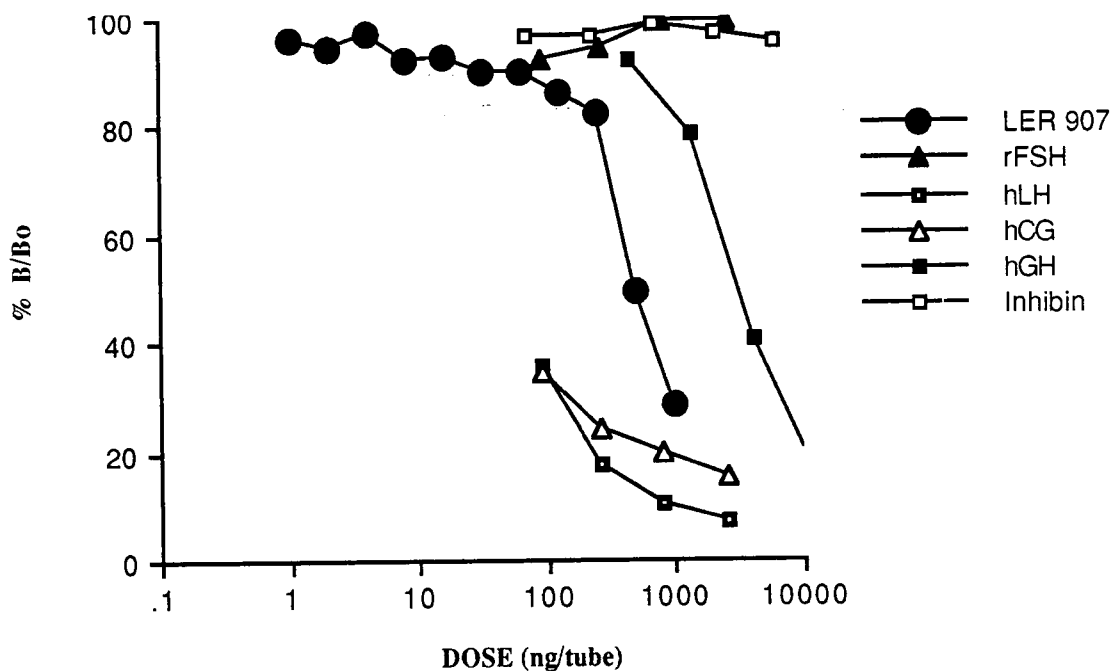


Figure 12. Human FSH Antiserum Cross-reactivity Curve. Determination of cross-reactivity between hFSH antiserum and various hormones. A 1:50,000 final dilution of hFSH antibody was incubated for 24 hours at 4 C with radioiodinated hFSH (50,000 cpm) and with each of the following unlabeled competitors: LER 907= hFSH standard reference; rFSH= rat follicle stimulating hormone; hLH= human luteinizing hormone (AFP 4226C); hCG= human chorionic gonadotropin (CR 125); hGH= human growth hormone; Inhibin= human alpha inhibin fragment.

using rFSH. The high degree of cross-reaction between hLH (AFP 4226C) and hCG (CR 125) can be explained by the fact that the antibody recognizes the alpha subunit which is shared by these two glycoproteins. The percentages of cross-reaction between the standard reference, LER 907, and hGH, hCG and hLH were 0.15%, 6.8% and 10.0%, respectively.

Sensitivity of hFSH assay. Construction of a standard curve involves the incubation of a fixed amount of tracer and antiserum with serial dilutions of an unlabeled hormone (standard). With decreasing concentrations of the unlabeled hormone, binding of the tracer increases. The sensitivity of the hFSH assay using the LER 907 as the reference standard was 70.5 ng based on an RIA data reduction program developed by P. Munson, D. Rodbard and M.L. Jaffe (NIH, Bethesda, MD). When substituting a more purified form of hFSH, hFSH I-2, for the reference standard, the sensitivity of the assay was markedly improved to 2.2 ng. These results are presented in Figure 13.

B. Validation of the hLH Assay

Characterization of the hLH Antiserum - batch #1

Antibody titration. The hLH antiserum-dilution assays indicated negligible binding to the hLH tracer. The most concentrated antiserum, 1:10,000 (final tube dilution), yielded only 1.8% of the total binding (B/T). The titration curve for hLH is presented in Figure 14.

The poor binding between the hLH antiserum and the hLH tracer prompted further examination of the radioiodination procedures. When problems with binding occur, the antiserum is seldom the cause since

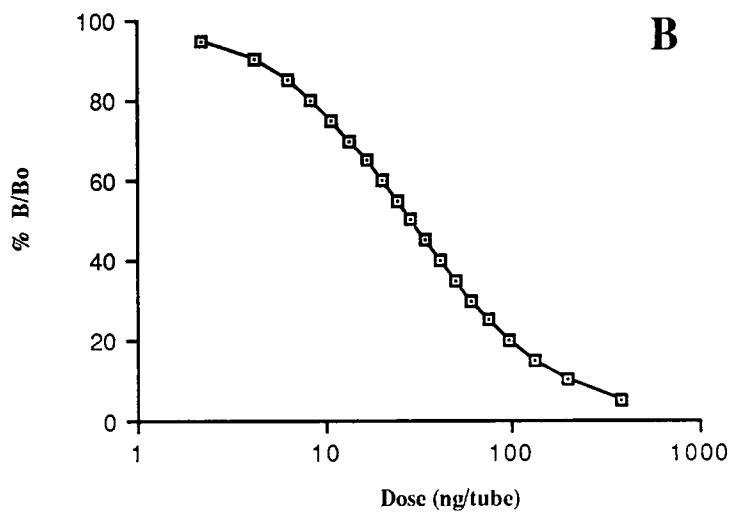
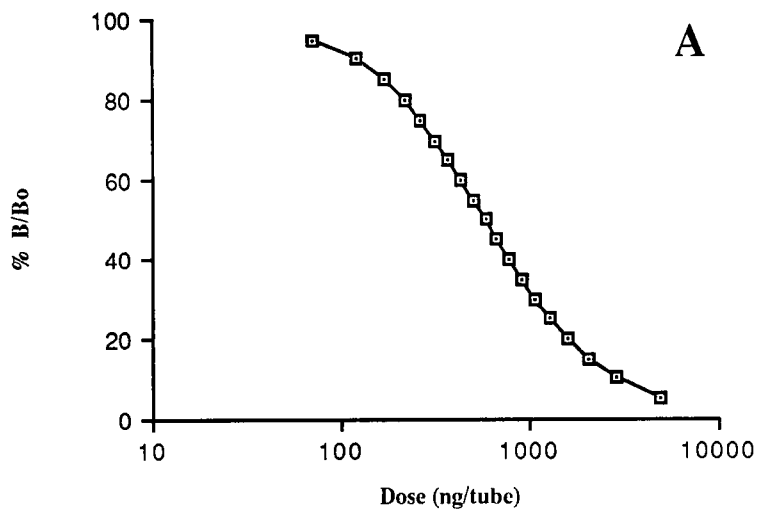


Figure 13. Human FSH Standard Curve. hFSH standard curves using an RIA data reduction program. A 1:50,000 final dilution of hFSH antiserum was incubated for 24 hours at 4 C with radioiodinated hFSH and serial dilutions of A) hFSH standard reference, LER 907 or B) a purified form of hFSH, hFSH I-2.

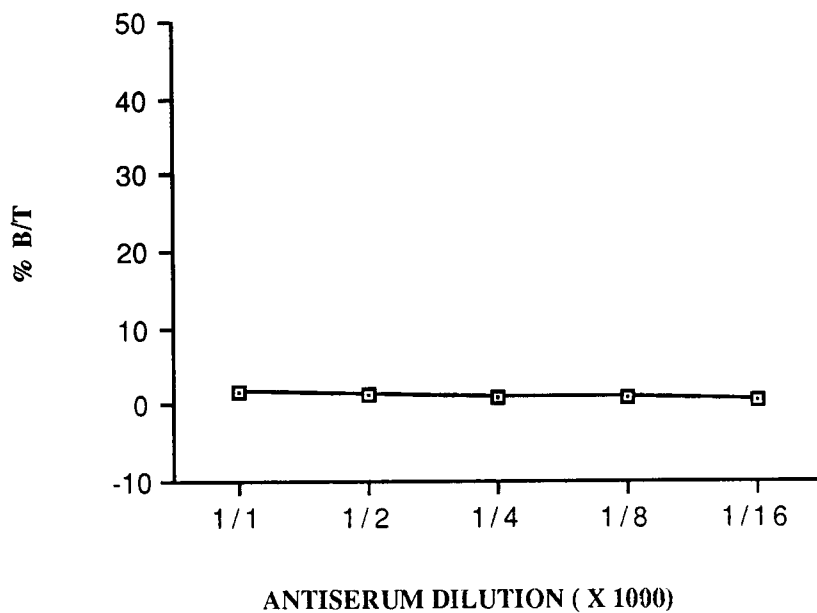


Figure 14. Human LH Antiserum Titration Curve (batch #1). An hLH antiserum titration curve to determine the optimal hLH antibody dilution to be used for subsequent radioimmunoassays. Radioiodinated hLH (50,000 cpm) was incubated with serial dilutions of hLH antibody for 24 hours at 4 C. Antiserum dilutions are ten times more dilute in the final assay.

antiserum deterioration is unlikely. To make sure the protein being used as the tracer was in fact labeled with ^{125}I , a fresh aliquot of the fraction containing the protein peak, collected from the original radioiodination procedure, was rechromatographed to further remove any free ^{125}I . A single major peak of radioactivity was eluted which represented the radiolabeled protein. Another antibody titration assay was performed with an extended incubation period of 48 hours. No improvement in binding occurred as the 1:10,000 final dilution resulted in 1.85% binding (B/T) (data not shown).

Modification of Radioiodination Procedures

Radioiodination of hLH was undertaken, employing modification of the chloramine T method. The lactoperoxidase (LPO) procedure and the iodogen method were also performed as described in Materials and Methods. Modifications of the chloramine T method included using different ratios of oxidant to protein: 4:1 and 8:1, and using different reaction times: 30 and 60 seconds. All six preparations resulted in less than 2% binding when incubated with the hLH antiserum (data not shown).

To further test the integrity of the hLH tracer, an assay was performed using the hFSH antiserum and the hLH tracer. A certain amount of binding would be expected because the antibody can recognize the identical alpha subunits which are shared by these two hormones. Twenty-four to twenty-eight percent binding (B/T) occurred between the hLH radiolabeled using the C-T method (8:1, 30'') and the two iodogen protocols and a 1:50,000 final antiserum dilution. In addition, dis-

placement by LER 907, hLH (AFP 4226) and hCG (CR 125) occurred, suggesting that the radioiodinated hLH was structurally intact because of its ability to compete with unlabeled hormones for hFSH binding sites (Figure 15).

A summary of the specific activities, percent incorporation of ^{125}I and the molar ratios of ^{125}I to protein for each of the six radioiodination protocols is found in Table 2.

As suggested by NIADDK, the goal of radioiodination is to achieve moderate specific activity (20-50 uCi/mg). The specific activity and the percent ^{125}I incorporation are similar for C-T 8:1 30'' and C-T 4:1 30'' (Table 2). However, since chloramine T is a strong chemical oxidant, the risk for protein damage is increased proportionally with increased concentrations of C-T. The specific activity and percent ^{125}I incorporation for C-T 4:1 60'' are higher than in the C-T 4:1 30'' protocol. However, the protein is subjected to structural damage by overradioiodination. Since moderate specific activity is recommended, the protocol using C-T 4:1 30'' was used in subsequent radioiodination procedures.

Radioreceptor Assay

To assess the structural integrity of the tracers obtained from the modified radioiodination procedures, a radioreceptor assay was performed using fresh porcine luteal homogenates. Structurally intact tracer should bind to LH receptors in the homogenates. All six newly labeled hLH bound to receptors in the luteal tissue as evidenced by a linear relationship between the amount of tissue and counts of radio-

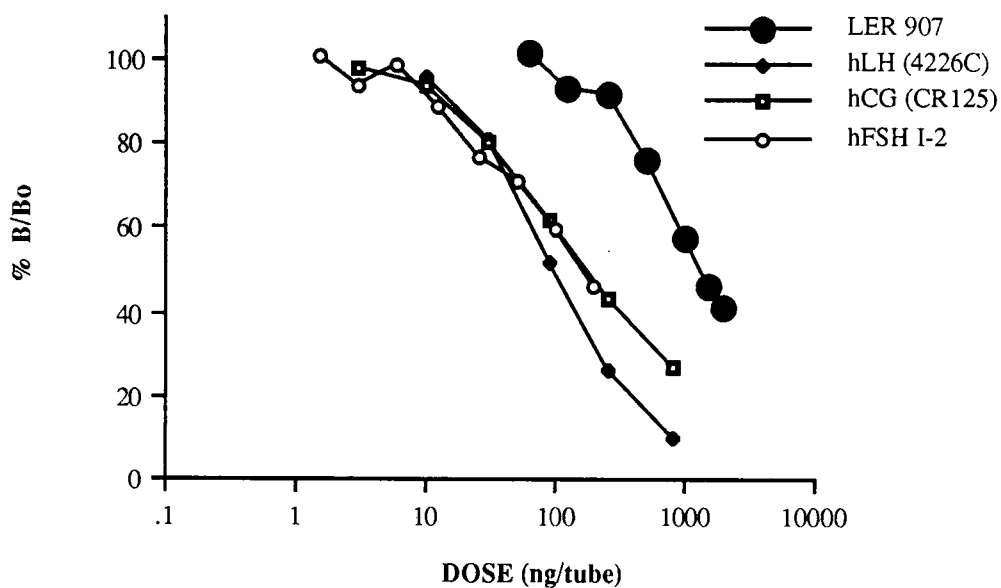


Figure 15. Human FSH Antiserum-Human LH Radioligand Cross-reactivity Curve. Determination of cross-reactivity in an hFSH assay using hLH as the radioligand to assess its ability to compete with various unlabeled hormones for hFSH antibody binding sites. A 1:50,000 final dilution of hFSH antibody was incubated for 24 hours at 4 C with radioiodinated hLH (50,000 cpm) and each of the following unlabeled competitors: LER 907= hFSH standard reference; hLH= human luteinizing hormone (AFP 4226C); hCG= human chorionic gonadotropin (CR 125); hFSH= human follicle stimulating hormone (I-2).

Table 2. Summary of Results from Modified Radioiodination of hLH and Bindability of hLH Tracer with hLH Antiserum and Luteal Tissues.

Radioiodination Protocol	Specific Activity uCi/ug	% ¹²⁵ I Incorporation	¹²⁵ I:hLH mol.ratio	%hLH-Ab binding (1:3000)	%tissue binding (120 mg)
C-T 8:1, 30''	28.6	29	0.45	NSB	5.9
C-T 4:1, 30''	30.0	31	0.45	1	2.9
C-T 4:1, 60''	77.9	80	1.22	1	2.8
LPO	19.5	20	0.31	1	3.1
Iodogen, 10'	25.3	52	0.40	NSB	2.2
Iodogen, 15'	23.0	47	0.37	NSB	1.3

* Note: Above figures based on 10 ug of protein in protein peak, which represents 80% recovery.

Abbreviations: C-T= chloramine T, LPO= lactoperoxidase, NSB= no specific binding.

activity specifically bound. In addition, the tracers can be competitively displaced by unlabeled hCG for binding sites. A summary of the tracers' bindabilities is presented in Table 2.

Characterization of the hLH Antiserum - batch #2

The data compiled from the aforementioned experiments strongly suggest that the antiserum was defective and not the radioiodinated hLH as originally thought. A second batch of hLH antiserum was received from NHPP and diluted ten-fold and aliquoted as described in Materials and Methods.

Antibody titration. The new antiserum was tested in an antibody titration assay, using hLH which had been radioiodinated by three procedures: 1) chloramine T, 2) lactoperoxidase and 3) iodogen. Using approximately 40,000 counts of tracer, each assay resulted in 10-20 times more binding over an antiserum range of 1:1000 to 1:128,000 (initial dilution) when compared to the binding results using the first antiserum. These data provided further support that the original antiserum was indeed faulty.

To further optimize the titer of the antiserum, a newly radioiodinated (C-T:hormone 8:1, 30') preparation of hLH was used. A working antiserum dilution of 1:16,000 resulted in 49% binding (B/T). The percent binding over a range of anti-hLH dilutions is presented in Figure 16.

Cross-reactivity assay. The results of the cross-reactivity determination are presented in Figure 17. Similar to the hFSH results,

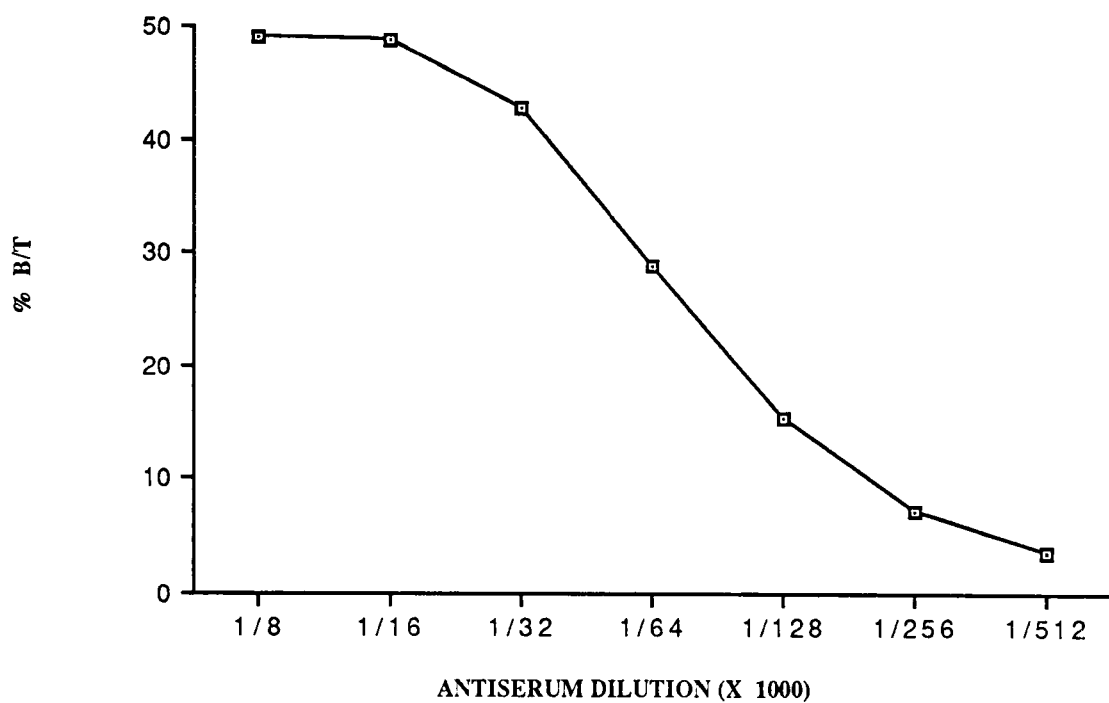


Figure 16. Human LH Antiserum Titration Curve (batch #2). An hLH antiserum titration curve, using a new batch of hLH antiserum from NHPP, to determine the optimal hLH antibody dilution to be used for subsequent radioimmunoassays. Radioiodinated hLH (40,000 cpm) was incubated with serial dilutions of hLH antibody for 24 hours at 4 C. Antiserum dilutions are ten times more dilute in the final assay.

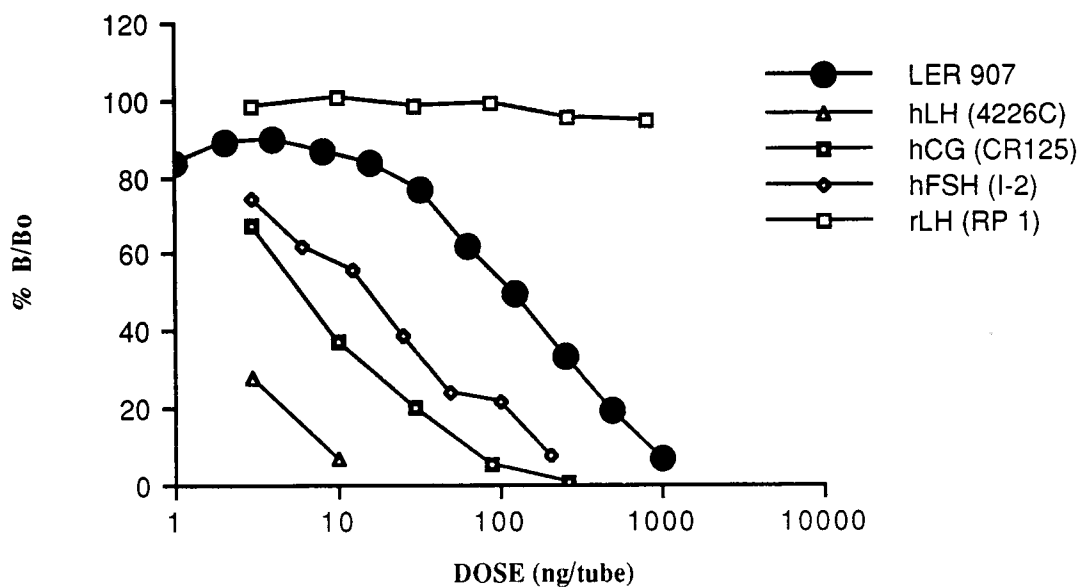


Figure 17. Human LH Antiserum Cross-reactivity Curve. Determination of cross-reactivity between hLH antiserum and various hormones. A 1:200,000 final dilution of hLH antibody was incubated for 24 hours at 4 C with radioiodinated hLH (20,000 cpm) and with each of the following competitors: LER 907= hLH standard reference; hLH= human luteinizing hormone (AFP 4226C); hCG= human chorionic gonadotropin (CR 125); hFSH= human follicle stimulating hormone (I-2); rLH= rat luteinizing hormone (RP 1).

rat LH (rLH) did not demonstrate any cross-reactivity, as indicated by a lack of binding over a wide range of dilutions. Purified forms of hLH (AFP 4226C), hFSH I-2 and hCG (CR 125) were all more specific than the LER 907 standard reference. These results were expected, because LER 907 is a crude pituitary extract with both LH and FSH activity. Cross-reaction between the standard reference, LER 907, and hFSH I-2 and hCG were 7.5% and 23.3%, respectively. Fifty percent binding was not obtained at the selected doses of hLH used in this cross-reactive assay.

Concerned that the LER 907 reference was not specific enough to provide a sensitive distinction between hLH and hFSH, the purified form of each was used in assaying gonadotropin levels in the collected sera.

Sensitivity of hLH assay. The sensitivity of the hLH assay when using the LER 907 reference was 11.3 ng based on an RIA data reduction program. This parameter was significantly improved to 584 pg when using hLH (AFP 4226C) as the standard reference (Figure 18).

C. Validation of Inhibin Assay

Characterization of Inhibin Antiserum

Antiserum titration. A series of RIA antibody titer assays was performed to establish optimal antiserum dilution which would bind 50% of the tracer normally used. Dilutions ranging from 1:3 to 1:59,049 did not result in precipitation of any radioactivity. Four elements which could have accounted for the lack of binding and/or precipitation were examined:

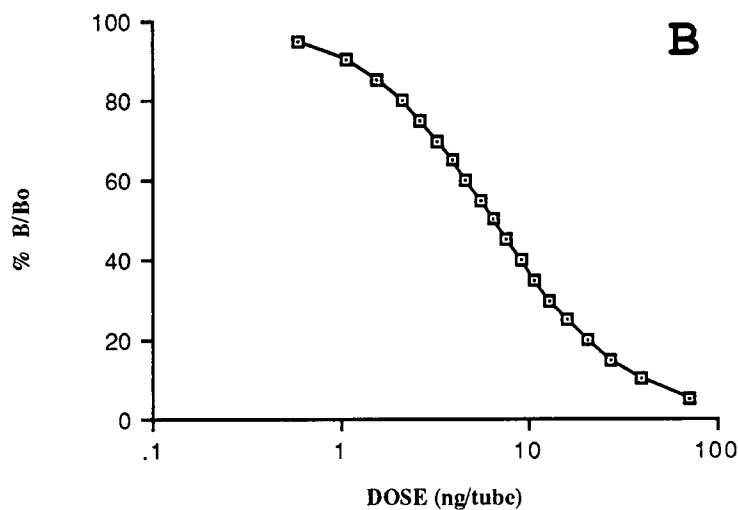
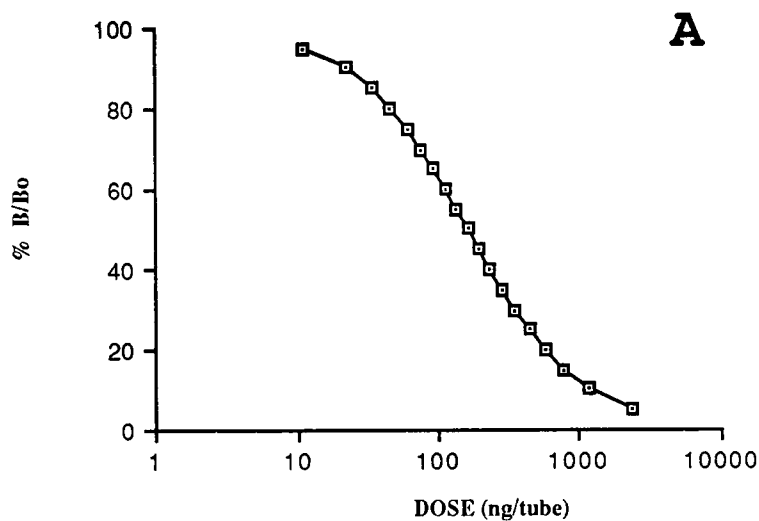


Figure 18. Human LH Standard Curve. hLH standard curves using an RIA data reduction program. An hLH antiserum was incubated for 24 hours at 4 C with A) radioiodinated hLH (20,000 cpm) and serial dilutions of LER 907, hLH standard reference or B) radioiodinated hLH (40,000 cpm) and serial dilutions of a purified form of hLH, hLH (AFP 4226C).

- 1) Protein A (Pansorbin),
- 2) the labeling of the antigen,
- 3) the antigen
- 4) the antiserum.

The first step in an RIA is the incubation of a radioligand and sample or standard hormone with a specific antibody. The second step is the separation of the antibody-antigen complex from free antigen. One rapid and reproducible method employs protein A-containing Staphylococcus aureus cells (Pansorbin). This immunoadsorbant technique relies on the binding of the Fc portion of IgG by protein A. Pansorbin precipitates primary immune complexes and serves as a solid support to which the primary antibody is fixed. In this study, the pellet obtained after centrifugation was firm and large, indicating Pansorbin's ability to precipitate the antibody-antigen complex. Therefore, the initial thought that Pansorbin was denatured proved to be unfounded.

Radiolabeled Antigen Characterization

To make sure that the radioligand used was actually labeled protein and not just free ^{125}I , each peak tube from the radioiodination procedure was rechromatographed on a Sephadex G-25 column. Two peaks were obtained in the same pattern as the original elution process. Therefore, the tracer solution appeared to be primarily labeled protein, and the possibility that the ligand was not successfully radioiodinated was eliminated.

Antiserum Characterization

The most likely element contributing to the lack of binding was the antiserum itself. Even though RIA is the most sensitive and specific technique to characterize antiserum, it employs radioisotopes which may alter the tertiary structure of proteins. Ouchterlony, immunoelectrophoresis, tube diffusion and dot blotting methods were all performed using the native antigen (inhibin) in attempts to screen for the presence of anti-inhibin antibody.

Ouchterlony Technique

Four milliliters of 1% agarose was layered on 50 x 75 mm slides using the sandwich technique. A center well and six surrounding wells were cut out and filled with 15 ul of inhibin and 15 ul of six different antisera, respectively. On separate plates, three different dilutions of the inhibin were used: 10 ug, 5 ug, 2.5 ug, while the antisera were used at full strength and at 1:5 dilutions. Slides were placed in a humid chamber and incubated at 4 C for 72 hours. The absence of precipitate lines suggested that no antibody-antigen binding occurred.

Immunoelectrophoresis Procedure

Pre-coated 50 x 75 mm slides were layered with 1% agarose. Four wells (troughs) were cut out and filled with different antisera mixed in agarose in a 1:5 ratio. After solidifying, one hole in each well was cut out and filled with 3-4 ul of the synthetic inhibin, and the slides were electrophoresed for one hour at 100V. No precipitation

line formed in any of the four wells, indicating an absence of an antibody-antigen complex.

Next, tube diffusion was performed in which antiserum mixed in 1% agarose was pipetted into Oudin tubes. The antigen (inhibin) was then layered on top, and the tubes were turned horizontally to minimize convection loss and incubated at room temperature. After a 24-hour incubation no zone of precipitation was observed, again indicating an absence of antibody-antigen complex.

Ammonium Sulfate Precipitation

Finally, the antiserum was concentrated by ammonium sulfate (AS) precipitation. This a non-specific way of enriching immunoglobulins. Gamma globulin fractions of serum proteins are insoluble in a 50% saturated AS solution. Therefore, mixing antiserum in saturated ammonium sulfate in a 1:1 proportion yields a globulin-containing precipitate. Removing the sulfate by dialysis or gel filtration produces a partially purified antibody fraction.

An 80% AS was prepared in water at 70 C. The saturated AS was added to a 1:2 antiserum-PBS mixture and the mixture allowed to stand at 4 C for two hours, after which it was centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS and centrifuged at 3000 rpm for another 20 minutes. The pellet was resuspended in 100 ul of PBS and dialyzed overnight at 4 C against PBS. This concentrated serum was used in the following dot blot procedure.

Dot Blotting

Nitrocellulose paper (NC) was cut into 5 x 5 mm squares and a 3-5 ul drop of inhibin was placed on each square. After several washings with 0.1% PBS-BSA and drying steps, the squares were incubated for two hours with the concentrated antiserum. Normal rabbit serum was used as a control. After PBS washing, the NC paper was then exposed to goat anti-rabbit IgG-horseradish peroxidase conjugate for two hours. After washing, diaminobenzidine (50 mg/100 ml PBS) and hydrogen peroxide (10 ul, 30%) were applied to the paper. After a two-hour incubation, no color change was observed, a result again suggesting that no antibody-antigen binding occurred under the experimental conditions.

D. Subjects' hFSH and hLH Serum Levels

The purpose of this study was to compare serum gonadotropin levels in women with PCOD and in women with normally functioning ovaries. Human FSH and LH serum levels were measured using RIA techniques. The LER 907 standard reference, commonly used for both FSH and LH assays, was substituted with more highly purified forms of hFSH (I-2) and hLH (AFP 4226C), respectively. This was done to render the assays more specific and sensitive in their measurements of the two hormones. The specific activities of the two purified forms were 5000 IU/mg and 5600 IU/mg, respectively.

The raw data obtained from the assays were transformed using a logit-transformation data reduction program. The transformed data were further converted to mIU/ml which is the clinically customary way to express gonadotropin levels.

While a number of possible statistical analyses existed, three were focused on for this study: 1) examination of day-to-day differences in LH and FSH levels within the control group, 2) examination of unpooled and pooled LH/FSH ratios in both control and patient groups and 3) examination of the correlation between LH and FSH levels in both the control and patient groups.

Figure 19 shows the controls' FSH and LH values over the five days. As a function of time, no significant differences resulted in the five different means for the two hormones. The LH/FSH ratios from day to day were also not significantly different (data not shown).

Patient FSH and LH means were compared to the corresponding means from day 4 of the control group. Significant differences were found ($P < 0.02$) for both hormones on all days (Figure 20) (only day 4 data is shown).

The LH/FSH ratio is a more realistic indicator of reproductive disorders than the individual FSH and LH levels alone. Since statistically significant differences were not observed in the controls' hormone levels and ratios as a function of time (days), the five means and ratios were pooled (Table 3). The hormone levels for all the control and patient subjects are presented in Table 4. A linear regression analysis was done to test for correlations between FSH and LH levels within individuals. The correlation was particularly evident within the patient subjects ($r = .9036$), but was only moderately present in the control subjects ($r = .4551$). (data not shown).

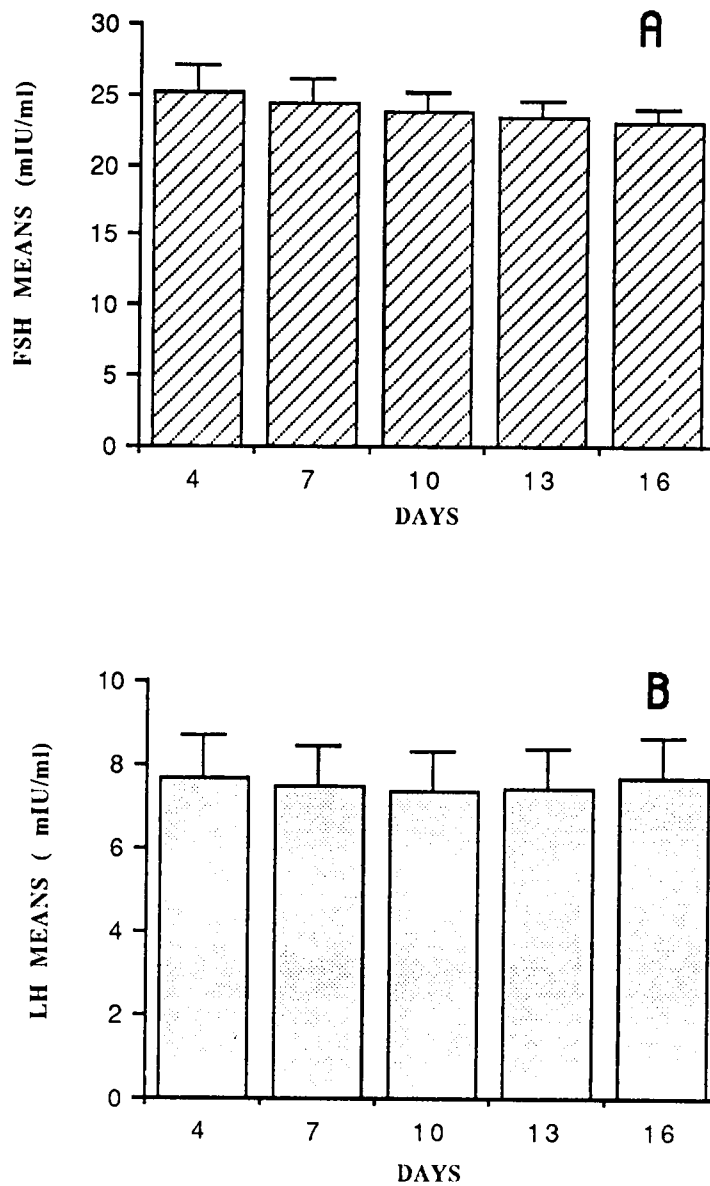


Figure 19. Control Subjects' Serum FSH and LH Levels. Mean ($\pm$ SEM) serum FSH and LH levels in the control subjects. Days are numbered from the start of menses. ($n=22$ on days 4 & 16; $n=23$ on days 7, 10 & 13).

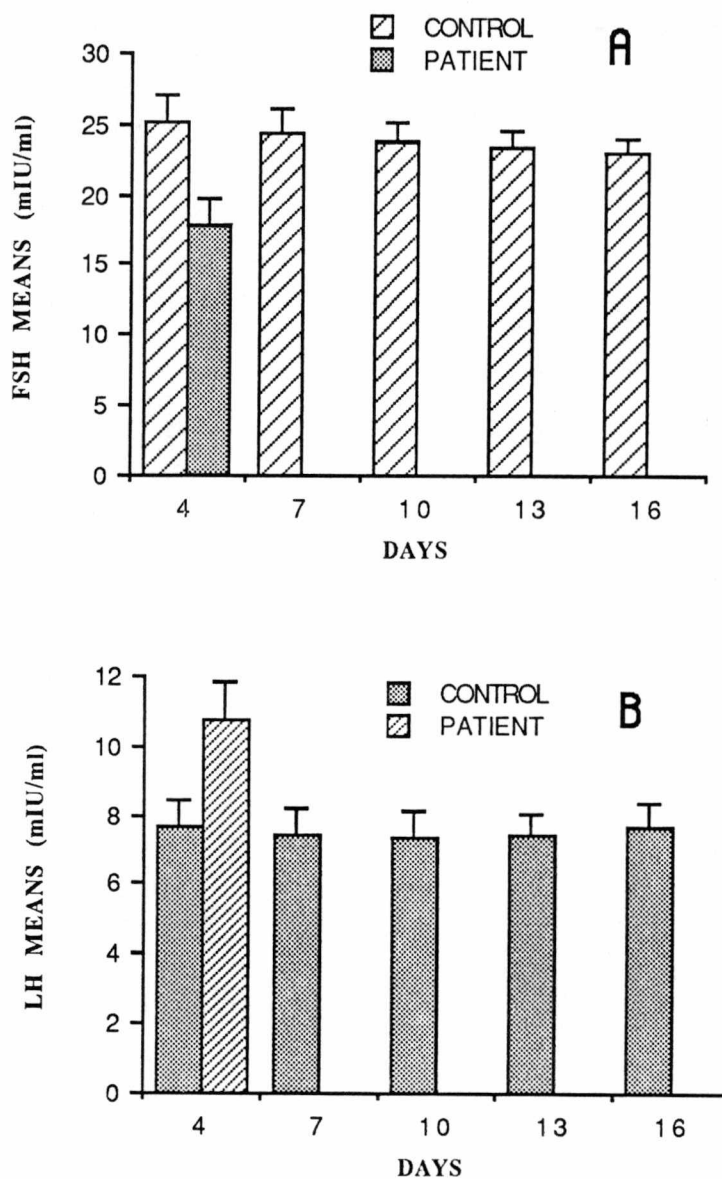


Figure 20. Comparison of Control and Patient Subjects' Serum FSH and LH Levels on Day 4. Mean ($\pm$ SEM) serum FSH and LH levels between control and patient subjects. The FSH mean is significantly lower ($P < 0.02$) in patient subjects, while the LH mean is significantly higher ($P < 0.02$) in patient subjects.

Table 3. A Comparison of Patient and Pooled Control Hormone Means and LH/FSH Ratios. (mIU/ml)

	Patient n=14	Control n=113	P Value
LH (+/- SEM)	10.76 +/- 1.05	7.65 +/- 0.36	0.0023
FSH (+/- SEM)	17.86 +/- 1.90	23.91 +/- 0.69	0.0020
LH/FSH Ratio (+/-SEM)	0.63 +/- 0.04	0.33 +/- 0.02	0.0001

Table 4. Serum FSH and LH Levels in Patient and Control Subjects.

PATIENT FSH and LH LEVELS (mIU/ml)

Subjects	FSH	LH
1.	8.7	9.3
2.	16.9	12.2
3.	32.3	19.9
4.	19.0	9.3
5.	14.8	8.4
6.	15.0	8.2
7.	25.4	12.9
8.	10.0	8.0
9.	16.1	9.6
10.	16.6	12.0
11.	15.8	9.8
12.	31.7	17.8
13.	12.6	6.0
14.	14.2	7.3
$\bar{X}$	17.9	10.8
SD +/-	7.1	4.0

Table 4 (continued)

Subjects	DAYS				
	4	7	10	13	16
Control LH Levels (mIU/ml)					
1.	17.2	13.3	14.0	11.3	13.6
2.	11.7	12.5	11.7	13.0	13.0
3.	13.2	13.9	15.0	15.7	15.7
4.	----	5.9	6.1	6.0	3.8
5.	7.3	5.7	4.7	5.7	5.5
6.	3.4	3.3	3.3	7.1	6.9
7.	13.4	10.5	10.5	8.0	6.8
8.	7.1	8.7	7.2	6.4	8.0
9.	12.7	13.8	5.7	7.5	6.1
10.	4.1	3.8	3.2	3.9	3.6
11.	6.2	6.7	6.8	8.4	6.8
12.	7.2	8.1	8.4	7.8	7.8
13.	5.1	2.7	2.6	2.7	4.7
14.	6.6	7.0	7.5	8.0	8.4
15.	5.3	4.6	5.9	3.7	3.0
16.	5.6	6.5	8.3	9.0	8.2
17.	3.9	3.1	1.0	3.3	3.8
18.	5.0	4.2	3.8	4.8	---
19.	5.3	2.5	5.5	5.1	6.5
20.	6.1	6.8	5.2	4.5	6.3
21.	6.4	9.9	12.2	11.9	12.7
22.	7.8	9.2	9.2	7.2	7.4
23.	8.8	8.9	11.3	9.3	9.8
$\bar{X}$	7.7	7.5	7.4	7.4	7.6
SD +/-	3.6	3.6	3.7	3.2	3.4

Table 4 (continued)

Subjects	DAYS				
	4	7	10	13	16
Control FSH Levels (mIU/ml)					
1.	26.4	27.8	25.8	25.0	28.7
2.	24.8	27.6	24.3	27.4	24.5
3.	28.4	30.3	36.9	33.4	29.4
4.	----	25.1	23.7	23.5	21.0
5.	23.4	22.5	23.2	26.7	22.9
6.	21.0	21.6	26.2	27.7	27.2
7.	49.7	33.0	37.6	35.9	28.5
8.	31.5	31.6	25.1	22.8	21.6
9.	47.6	47.7	23.5	23.7	26.7
10.	23.1	20.5	20.0	23.3	27.7
11.	28.5	29.2	36.7	30.8	23.9
12.	27.0	39.8	35.6	33.5	31.1
13.	27.7	24.3	20.2	17.9	21.4
14.	29.8	22.9	22.9	21.7	23.9
15.	16.7	15.9	16.0	15.7	14.4
16.	17.1	20.5	22.0	21.6	21.4
17.	14.7	12.5	12.8	13.5	12.5
18.	15.6	18.3	19.1	20.7	----
19.	20.1	18.2	19.6	22.3	24.2
20.	22.4	22.8	22.1	20.8	21.4
21.	23.3	22.7	24.3	22.3	23.1
22.	17.7	11.0	11.4	10.5	12.8
23.	15.3	15.5	17.0	16.5	17.2
$\bar{X}$	25.1	24.4	23.7	23.3	23.0
SD +/-	9.1	8.5	7.2	6.3	5.2

CHAPTER V

DISCUSSION

The aim of this study was to compare serum gonadotropin levels in women with PCOD to women with normally functioning ovaries. A significant difference was found in both the FSH and LH values between the two groups. The PCOD patients had lower FSH but higher LH concentrations than the controls. This difference was also reflected in a higher LH/FSH ratio in the PCOD patients. These findings confirm our hypothesis and are in accordance with results reported by other investigators (121, 125, 127).

In normal cycles, circulating levels of gonadotropins exhibit a cyclic pattern of secretion (146). The pattern is mainly influenced by two primary ovarian steroids, estradiol and progesterone. Most notably, estradiol acts mainly to suppress FSH while progesterone works mainly to suppress LH. The modulating effects of the ovarian steroids are believed to take place directly on the pituitary (147, 148). However, other studies suggest that ovarian steroids can regulate gonadotropin secretion by acting directly on the hypothalamus (149, 150).

During the follicular phase of the menstrual cycle, estradiol plays a major regulatory role in gonadotropin secretion and follicular maturation. In contrast, progesterone is thought to exert its full effects during the follicular phase only in the presence of estradiol (151). Chang and Jaffe (152) showed that progesterone administered

after estrogen pretreatment was able to trigger an acute LH surge and to initiate an FSH surge at midcycle.

As estradiol levels increase during folliculogenesis, FSH concentrations decline, a sequence illustrating the steroid's negative feedback effect on FSH secretion. On the other hand, estradiol has a positive feedback effect on LH secretion as demonstrated by the preovulatory surge of LH which coincides with peak estradiol concentrations.

An inspection of Figure 19 shows that FSH levels declined only slightly, with no significant difference over days. The LH levels likewise, did not differ significantly over time and did not reflect a characteristic midcycle peak (day 13-16) which should be associated with the normal menstrual cycle. Not only was the change in hormone levels from day to day not significantly different in the control subjects, but the LH/FSH ratios were not significantly different as a function of time, either.

These findings do seem perplexing, especially when the subjects are women with "normal menstrual cycles". Perhaps the overall sampling protocol is largely responsible for the absence of "normal" hormonal changes which are expected to occur in a "normal" menstrual cycle. For instance, blood samples, although taken at approximately the same time for each individual, were collected every third day. Given that gonadotropins are released in pulsatile fashion, the infrequent blood collection may not have accurately captured the true hormonal patterns taking place in vivo. To obtain more accurate data on the cyclic relationship of the gonadotropins, blood should be drawn every 10-15

minutes over a 12-24 hour period (153). Such a protocol would have been impractical for this particular study. In addition, the individual variations were large thus, preventing the elimination of obvious outliers. This factor may also be a result of the experimental design.

In spite of the inherent limitations of this study and the large variances, meaningful conclusions from the collected data can be made. A closer examination of Figure 19 indicates that the hormone levels correlate closely with patterns described in physiology and medical textbooks. The one notable exception is the absence of a peak near day 13. The infrequent blood sampling could explain this result. The levels may be slightly higher than those mentioned in textbooks, but this may be explained by the degree of cross-reactivity which the FSH and LH antisera had for hormones present in the serum. The antiserum used in the assays was polyclonal and was not specific for the distinct beta-subunits which differentiate the two gonadotropins. Therefore, any proteins possessing an alpha-subunit, which is identical among FSH, LH and thyroid stimulating hormone (TSH), would react with the antiserum. The potential of over-estimating the levels of gonadotropins is due to the cross-reaction between closely related hormones. This over-estimation could possibly have obscured any hormonal peak which might have occurred during the five blood samplings.

The length of the follicular phase is highly variable among individuals, whereas the luteal phase (time between ovulation and the start of the next menses) is highly predictable as far as hormonal patterns are concerned. The hormone profile is not the same for each woman. Not correcting for cycle length variability, i.e., setting day

0 as the day of ovulation, makes the data analyses less accurate. The deviations observed within the control group may reflect, in part, the inherent variabilities associated with the follicular phase of the reproductive cycle.

Measuring basal body temperature also would have helped with the interpretation of the hormone levels since it increases around ovulation. If the subjects were asked to measure their daily body temperatures throughout the study, a more accurate assessment of when ovulation occurred would have been possible.

Since parallel blood samples for both the control and PCOD patients were unavailable, the mean of the 14 patients' samples was compared with the corresponding control means from day 4, the day hormone levels during the anovulatory state are the most similar to the early part of the normal cycle.

There was statistically significant difference ($P < 0.02$) not only on day 4, but on all days examined (data not shown). FSH levels were lower in the patient group, while LH levels were higher compared to the control group. The finding of only a moderate correlation between FSH and LH levels in the control group is not surprising (data not shown, $r = .4551$). It is well recognized that divergence exists between FSH and LH release. LH is more sensitive to GnRH stimulation than is FSH, but FSH is more sensitive to estradiol regulation. In the normal cycle, all the components of the hypothalamo-pituitary-gonadal axis (H-P-G axis) are presumed functional. Therefore, a strong linear correlation would not be expected because FSH secretory patterns rarely parallel those of LH.

Within the patient group, a very strong correlation was found between FSH and LH ($r = .9036$). This finding suggests that a defect in one or more of the H-P-G axis components may be present. It is also possible that a defect in the steroid and/or inhibin feedback signals may exist as well. Thus, the typical divergent pattern of secretion which is known to exist between the gonadotropins would not be maintained.

The higher levels of LH found in the PCOD patients are consistent with findings reported by others. (125, 127, 154, 155). The cause of the increased LH in PCOD is not well understood, but many investigators speculate that estrogens mediate this action via their positive feedback signals on GnRH release (119, 125, 156, 157). Waldstreicher et al. (154) reported a strong correlation between LH pulse frequency and mean serum estradiol levels in women with PCOD.

The lower concentrations of FSH in the patients are in accord with other findings (121, 125, 127). Increased levels of inhibin have been correlated with lower FSH values found in women with PCOD (138). The aberrant FSH levels may also be a manifestation of elevated androgen secretion which is frequently associated with PCOD. Androgens also increase the concentration of estrogens, which in turn negatively feedback on FSH. It becomes apparent that assaying for ovarian steroids in addition to the gonadotropins would have added dimension to the study.

A great deal of significance was removed from the study when inhibin measurements were unavailable. However, this particular loss could have been compensated somewhat if steroids such as testosterone

and especially estradiol were measured so that the levels of FSH and LH could have been interpreted with more meaning when correlated with steroid levels. It would have been interesting to see if those individuals who had high LH levels also had high testosterone levels, especially in the patient group, since PCOD is closely associated with androgen excess. LH stimulates the secretion of androgens, which in turn negatively feedback on FSH, causing abnormal follicular development and follicular atresia. Androgen excess also results in greater peripheral conversion to estrogens, which in turn enhance GnRH release, causing a preferential LH release. A vicious cycle is perpetuated by the overall inappropriate steroid and gonadotropin secretions. Having combined data on both the gonadotropin and steroid profiles of the women in this study would definitely have provided a stronger framework from which to draw basic conclusions.

In addition, the study obviously would have been strengthened if there were parallel blood samples on which to make the comparisons. Using human subjects who were anovulatory understandably made it impossible to follow the same protocol for both groups. However, maybe if at least the same number of samples were collected more accurate comparisons could have been made.

Obtaining similar blood samples for two, instead of one cycle from the control groups might have added more insight to the interpretation of the data, however, suggesting this would probably have limited the number of subjects willing to partake in the study.

One additional piece of information that should have been obtained from the control subjects was for them to report when their next

menstrual period was, to calculate when ovulation occurred during the cycle in which they gave blood. Theoretically, ovulation offers a specific hormone profile which is reflected in gonadotropin levels.

Many reproductive disorders are beset with a varied combination of etiologies and often times these symptoms are not present in the same fashion in all patients with the same disorder. This study was only design to examine one component, i.e., gonadotropin levels during the follicular phase. In summary, the data confirm the hypothesis that FSH levels are lower, while LH levels are higher in women with PCOD. Consequently, the LH/FSH ratio is higher in the patient group than in the control group. The conclusions drawn from this study are valid and correspond well with other similar reports.

This project provides the basis for continuing studies of this important area. First, baseline data have been obtained on the blood samples, which have been preserved for additional analyses. The patients from which the samples were collected have established histories which can be utilized. Second, standard methods have been developed for assaying steroid and other hormone levels. Third, the basis for proceeding with inhibin studies has been provided. Such work can commence as soon as an appropriate anti-inhibin antibody source is available.

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APPENDIXES

APPENDIX A
THESIS QUESTIONNAIRE

THESIS QUESTIONNAIRE

DATE _____

1. NAME _____
2. AGE _____
3. YOUR HEIGHT, WEIGHT AND RACE ?
HEIGHT _____
WEIGHT _____
RACE _____
4. WHEN DID YOUR LAST MENSTRUAL PERIOD BEGIN ?
MONTH _____ DAY _____ YEAR _____
5. WHEN DID YOUR PERIOD START BEFORE THAT ?
MONTH _____ DAY _____ YEAR _____
6. HOW MANY PREGNANCIES, IF ANY, HAVE YOU HAD ? _____
7. ARE YOUR PERIODS REGULAR ?
YES _____ NO _____
8. HOW LONG DO YOUR PERIODS USUALLY LAST ?
DAYS _____
9. HOW MANY DAYS APART ARE YOUR PERIODS ?
DAYS _____
10. HOW OLD WERE YOU WHEN YOU HAD YOUR FIRST PERIOD ?
AGE _____
11. HOW MUCH TIME WENT BY BEFORE YOUR PERIODS BECAME
REGULAR ? (THAT IS, IF THEY EVER BECAME REGULAR)

12. PLEASE RATE THE AMOUNT OF CRAMPING, ON THE AVERAGE, YOU HAVE WITH YOUR PERIODS ON A SCALE OF 1 TO 10 (1 IS NO PAIN, 10 IS TRULY UNBEARABLE PAIN)
-

13. HAVE YOU EVER REQUIRED MEDICAL TREATMENT FOR VERY HEAVY MENSTRUAL BLEEDING ? FOR SEVERE CRAMPING ?

BLEEDING YES _____ NO _____

CRAMPING YES _____ NO _____

14. DO YOU MISS DAYS FROM SCHOOL OR WORK BECAUSE OF MENSTRUAL PROBLEMS ?

FREQUENTLY _____ OCCASIONALLY _____ NEVER _____

15. DO YOU HAVE MOOD CHANGES IMMEDIATELY BEFORE OR AT THE TIME OF THE ONSET OF YOUR PERIOD ?

FREQUENTLY _____ OCCASIONALLY _____ NEVER _____

16. BETWEEN YOUR PERIODS AND/OR TWO WEEKS AFTER STARTING YOUR LAST PERIOD, DO YOU HAVE:

ABDOMINAL PAINS YES _____ NO _____

SPOTTING YES _____ NO _____

CHANGES IN
VAGINAL DISCHARGE YES _____ NO _____

17. HOW WOULD YOU RATE THE AMOUNT OF STRESS YOU EXPERIENCE IN YOUR DAILY LIFE ON A SCALE OF 1 TO 10 ? (1 WOULD BE ALMOST NONE....10 WOULD BE UNBEARABLE STRESS)
-

18. DO YOU EXERCISE ON A REGULAR BASIS ? (MORE THAN TWICE A WEEK FOR AT LEAST TWENTY MINUTES)

YES _____ NO _____

19. HAVE YOU NOTICED ANY RELATIONSHIP BETWEEN DIETING OR EXERCISE AND THE REGULARITY OF YOUR MENSTRUAL PERIODS ?

DIETING YES _____ NO _____
EXERCISE YES _____ NO _____

AND THE SEVERITY OF YOUR MENSTRUAL PERIODS ?

DIETING	YES _____	NO _____
EXERCISE	YES _____	NO _____

20. HOW MANY MONTHS TOTAL (IF ANY) WOULD YOU ESTIMATE THAT YOU HAVE TAKEN ORAL CONTRACEPTIVES (BIRTH CONTROL PILLS) IN THE PAST ?

MONTHS _____

21. WHEN WAS THE LAST TIME YOU USED ORAL CONTRACEPTIVES ?

22. DO YOU HAVE WHAT YOU CONSIDER TO BE A GREATER THAN AVERAGE AMOUNT OF BODY OR FACIAL HAIR ? (IF SO, IS THIS A NEW PROBLEM OR HAS THIS BEEN PRESENT SINCE YOUR TEENS ?)

YES _____ NO _____

NEW PROBLEM ?

YES _____ NO _____

23. DO YOU HAVE GREATER THAN NORMAL PROBLEM WITH ACNE, IN YOUR OPINION ?

YES _____ NO _____

24. HAVE YOU NOTICED THAT YOUR BREASTS CONTAIN A FLUID WHICH CAN EASILY BE PRESSED OUT ?

YES _____ NO _____

25. DO YOU HAVE BREAST TENDERNESS OR ENGORGEMENT BEFORE OR ASSOCIATED WITH YOUR PERIODS ?

YES _____ NO _____

APPENDIX B

LETTER OF CONSENT

LETTER OF CONSENT

Your identity will be kept confidential. Only Dr. Doody and the Co-Director (Holly Kelly) will have access to the questionnaire responses, and data will be compiled and recorded according to a coding system designed by the Co-Director.

If you have any questions about this study, either now or later, please contact Holly Kelly, Walters Life Science Building, University of Tennessee, Knoxville 37996, or call 974-3790, or at home at 588-0919.

Your participation is voluntary; a choice made freely and without constraint or coercion. You are free to withdraw from participation at any time.

I have read and understand the explanation of this research study. I understand the risks of participation and my option to discontinue at any time without penalty. I agree to participate.

Name

Signature

Date

APPENDIX C
RIA PROTOCOL

RIA PROTOCOL FOR FSH AND LH

Caution: Set up assay on ice!

1. Set up labeled 12 X 75 mm borosilicate glass tubes in duplicate.
2. Add reagents as follows:
 - a. add appropriate volumes of buffer (PBS/BSA) so final reaction volume is 1 ml.
 - b. total count tubes- 100 ul of radioiodinated hormone.
 - c. nonspecific binding tubes- same as total tubes, except Pansorbin will be added on the second day.
 - d. buffer control tubes- same as total tubes, plus 100 ul of anti-FSH or anti-LH serum.
 - e. standard tubes- 100 ul of standard (.1 to 100 ng/ml), plus 100 ul of anti-FSH or anti-LH serum.
 - f. unknown samples- 40 ul of sample (1:10) from control subjects and 10 ul (full strength) from patient subjects, plus 100 ul of anti-FSH or anti-LH serum.
3. Add 100 ul of ^{125}I hFSH or ^{125}I hLH to all tubes.
4. Vortex gently and incubate at 4 C for 24 hours.
5. Add 100 ul of Pansorbin (1:15) to all tubes, except total count tubes.
6. Incubate at room temperature for 30 minutes.
7. Add 2.5 ml cold PBS-BSA to all tubes, except total count tubes.
8. Centrifuge at 3000 rpm for 20 minutes.
9. Aspirate the supernatants.
10. In a gamma counter, count the pellet of each tube including the total count and nonspecific tubes for one minute.

VITA

Holly Page Kelly was born in New York City on December 13, 1958. She graduated from Fox Lane High School in June 1977. The following August she enrolled at James Madison University, and in May 1981 she received her Bachelor of Science degree in Health Science. In July 1981 she accepted a teaching position at Martinsville City High School, Virginia, and remained there as a biology instructor/basketball and softball coach for three years.

In the Spring of 1985 she accepted a teaching assistantship at The University of Tennessee, Knoxville, and began study towards a Master's degree in Zoology. She received the Master of Science degree in August 1988.