To the Graduate Council:

I am submitting herewith a thesis written by Joan R. Hembree entitled "Plasma Cortisol and Progesterone Concentrations, Uterine Proteins and Corpora Lutea LH/hCG Binding Characteristics in Gilts Following Adrenocorticotropin or Hydrocortisone Acetate Administration During Diestrus." 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 Animal Science.

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H. G. Kattesh, Major Professor

We have read this thesis
and recommend its acceptance:

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Accepted for the Council:

The Graduate School

PLASMA CORTISOL AND PROGESTERONE CONCENTRATIONS, UTERINE PROTEINS AND CORPORA LUTEA LH/hCG BINDING CHARACTERISTICS IN GILTS FOLLOWING ADRENOCORTICOTROPIN OR HYDROCORTISONE ACETATE ADMINISTRATION DURING DIESTRUS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Joan R. Hembree

December 1983

To my parents,

Ralph and Shirley Hembree,

and to my brother Mitch,

with love and thanks

ACKNOWLEDGMENTS

The author expresses her sincere appreciation and respect to her major advisor, Dr. Henry G. Kattesh, for the opportunity to undertake this study and for his advice, friendship, and assistance in the course of this study.

Appreciation is expressed to the members of her graduate committee: Dr. T. T. chen, for his assistance in the laboratory, surgery, and for serving on the committee; Dr. R. L. Murphree, teacher and friend, for his direction and advice to the author during her undergraduate and graduate studies, and for serving on the committee; Dr. J. Koontz, for his valuable advice and counseling in reviewing this manuscript, and for serving on the committee.

The author thanks Terry Parrott and Mike Slowey for the many hours devoted to handling of animals and surgeries, Jack Underdown and the crew of the Blount Swine farm for their assistance in handling of animals, and Aaron Cole, Steve Banet, Ron Mann, Milton Orr, and the many other graduate students without whose assistance this study would not have been possible. Appreciation is also extended to Sue Grindstaff for her excellent typing job on this manuscript.

The author is grateful to her friends; Steve Banet, Lisa Bettison, Marco Fernandez, Aleta Gordon, and Chris Reynolds, for their invaluable support through the past two years, and to Dr. Heitmann and Berta Ward for their friendship and all of the Wednesday "conferences."

The author also extends her love and thanks to her parents, Ralph and Shirley Hembree, and to her brother Mitch, for their love and support during her academic career.

ABSTRACT

In a preliminary study four gilts fitted with jugular cannula were injected once or twice daily with either 2 mg hydrocortisone acetate (HA) in sesame oil per kg body weight, intramuscularly (IM) or 1 I.U. adrenocorticotropin (ACTH) in 14% gelatin per kg body weight, subcutaneously (SQ). Twice daily injection of ACTH significantly elevated ($P<.05$) plasma cortisol levels above that of the control animals (injected with 2 ml sesame oil, IM and 2 ml 14% gelatin, SQ, twice daily). Hydrocortisone acetate did not significantly elevate cortisol levels, although a second injection appeared to elicit a heightened cortisol response.

Twenty-three gilts were assigned to the following treatments administered twice daily at dosage levels established in the preliminary study; (1) nonbled control; (2) bled control, injected with the hormone carriers; (3) HA; and (4) ACTH. Treatments were administered days 2 through 7 (period one) or days 8 through 13 (period two) of the estrous cycle, with plasma samples collected daily during treatment and ovariectomies performed and uterine flushings obtained the day following the final day of treatment.

For both periods of treatment ACTH decreased and HA increased cortisol levels in a linear fashion over the days of treatment compared to the quadratic response of the bled controls. Within period two both HA and ACTH treatment resulted in a linear increase in progesterone levels compared to the quadratic pattern of the bled

controls; however, there was no significant effect of treatment upon progesterone profile compared to the controls in period one.

There were no significant differences between the nonbled and bled controls for any of the day 8 or 14 parameters measured, therefore the controls were pooled by period and all comparisons made relative to these pooled controls. There were no differences due to treatment detected within period one for either total uterine proteins or corpora lutea (CL's) luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor binding affinity or capacity. Within period two ACTH treatment reduced ($P<.05$) uterine proteins compared to the pooled controls, whereas HA had no effect. There was a trend ($P<.10$) for day 14 CL's LH/hCG receptor binding affinity to be reduced by HA and ACTH treatments although binding capacity was unaffected. Corpora lutea LH/hCG receptor binding affinity and capacity were negatively ($P<.05$) correlated. Based upon these data, mid- to late diestrus appears to be a physiological state sensitive to the HA and ACTG treatment used in this study. Total uterine proteins were reduced by ACTH treatment, which suggests that the uterine environment was altered. Since a proper uterine environment is essential for the survival of many embryos, alteration of uterine proteins by ACTH may effect embryonic survival.

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CHAPTER I

INTRODUCTION

An important aspect of swine production is the fecundity of gilts and sows as reflected in the number and viability of piglets born. Losses incurred due to impaired reproductive ability of gilts or sows, for whatever reason, reduces profits in the swine industry.

Several factors are associated with a reduction in the ability of gilts and sows to produce offspring. One factor which only recently has gained notoriety within the swine industry is stress. Stressful conditions related to intensified management practices (i.e., handling, overcrowding, and isolation) and environmental stressors, such as extremes in temperature and humidity, have been shown to adversely affect the fertility of gilts and sows, especially when they are evident during the early stages of gestation. Early embryonic losses approaching 35% occur normally in swine (Perry and Rowlands, 1962) but can approach 50% under severe stressful conditions (Tompkins et al., 1967).

The physiological mechanism behind stress-related early embryonic mortality in swine is unclear. One possible mechanism involved is the well documented antagonistic relationship of the hyperadrenal state and reproductive function. An abnormal elevation in peripheral plasma adrenal glucocorticoid levels either by direct (exogenously administered) or indirect (following acute stress or adrenocorticotropin (ACTH) administration) means has been associated

with impaired reproductive performance in several species (Fuquay, 1981; Andrews, 1977).

The established importance of the hypothalamic-pituitary-gonadal axis for the maintenance of normal reproductive function suggests that these hormones (ACTH, glucocorticoids) may exert their effects through involvement at some point in this axis. The actual nature of the interaction of adrenocorticotropins and glucocorticoids with this axis is still unclear.

The objectives of this study were: (1) to determine the appropriate levels of ACTH and hydrocortisone acetate (HA) needed to elevate circulating plasma cortisol levels; and (2) to examine specific endocrine (cortisol and progesterone levels) and physiological (luteal luteinizing hormone/human chorionic gonadotropin (LH/hCG) binding characteristics and total uterine proteins) responses in normal cycling gilts following ACTH and HA treatment during early to mid- or mid- to late-diestrus. Also, to insure accurate measurement and reliable techniques in determining LH/hCG receptor characteristics, extensive validation of the receptor assay was performed.

CHAPTER II

LITERATURE REVIEW

I. STRESS AND FEMALE REPRODUCTIVE PERFORMANCE

Low reproductive efficiency is a major concern in all farm animal industries. In particular, the swine industry, with its trend toward intensified confinement, is plagued with ever increasing losses of number of pigs born. A contributing factor is early embryonic loss (losses occurring within 25 days post-breeding), which is estimated normally to be around 35% (Perry and Rowlands, 1962) but can approach 50% under severe stressful conditions (Tompkins et al., 1967). Stressful conditions may be management related; examples being handling, overcrowding, and isolation. The environment itself may provide stressful conditions such as extremes in temperature and humidity, either alone or concomitantly with management related stress. These types of stress, which impose an event (physical, environmental, etc.) which significantly challenges the homeostasis of the animal, are implicated in increased losses due to lowered reproductive efficiency in livestock production (Moberg, 1976).

Stressful conditions which alter the normal reproductive function of female swine have been well documented. Movement of gilts from pasture lots into individual confinement housing systems increases the frequency of silent estrus and abnormally long estrous

cycles (Kraeling et al., 1982). Omtvedt et al. (1971) found that 8 to 16 days post-breeding, the time during which embryonic attachment is initiated, was the critical period when embryonic survival was most affected. These researchers also demonstrated that during mid-gestation (days 53 through 61) fetal survival was unaffected by thermal stress, whereas thermal stress applied during late gestation (day 102-110) decreased the number of live pigs farrowed. Chronic heat stress (24 hour duration) on day 1 through 5 of pregnancy has been demonstrated to increase embryonic mortality 30% above that of control animals (Tompkins et al., 1967). More recently, results of Wildt and coworkers (1975) showed 25% greater embryonic losses due to acute (2 hour duration) daily thermal stress from days 2 through 13 of pregnancy as compared to controls. Therefore, both chronic and acute heat stress has been shown to increase embryonic mortality in swine, particularly during the first two weeks of gestation. Impaired female reproductive efficiency due to thermal stress has also been reported in cattle (Stott and Williams, 1962; Labhstwar et al., 1963; Dunlap and Vincent, 1971; Ingrahm et al., 1974; Monty and Wolff, 1974; Moberg, 1976), sheep (Dutt et al., 1959; Dutt, 1963; Woody and Ulberg, 1964; Thwaites, 1969), rats (Macfarlane et al., 1957; Fernandez-Cano, 1958; Edwards, 1968; Fried et al., 1976), rabbits (Shah, 1956), and guinea pigs (Edwards, 1969). Early work by Christian and Lemunyan (1958) suggests that the decrease in reproductive efficiency encountered in stress may serve as an autoregulatory mechanism of

population control, thus being a common physiological mechanism of adaptation for many, if not all, animals.

Endocrine Mechanism of Stress-Impaired Female Reproduction

The precise endocrinological mechanism underlying decreased female reproductive capacities due to stress is unclear. However, one possible explanation is the well documented antagonistic relationship of the hyperadrenal state and reproductive function (Selye, 1946). Hyperadrenalism has been noted to occur in situations involving crowding and various other environmental stressors with an associated decrease in female (Selye, 1939; Christian and Lemunyan, 1958; Christian, 1971) and male reproductive capabilities (Christian, 1955; Skinner and Louw, 1966; Vincent, 1972; Fuquay, 1981; Young 1981). The increase in adrenal activity associated with stress, according to Selye (1939), is caused by increased adrenocorticotropin hormone (ACTH) production by the pituitary to compensate for abnormal metabolism and thus restore some degree of homeostasis, often at the expense of normal reproductive function.

Under normal conditions, the pituitary release of ACTH is dependent upon stimulation by corticotropin releasing factor (CRF) from hypothalamic neurosecretory cells. Corticotropin releasing factor is normally controlled by monoaminergic inhibiting (norepinephrine and gamma aminobutyric acid) and stimulating (acetylcholine) agents; however, other controlling agents may

exist (Jones and Hillhouse, 1977). Corticotropin releasing factor travels via hypophyseal portal vessels to hypophyseal corticotrophs and elicits the secretion of adrenocorticotropin. Adrenocorticotropin, in turn, travels via the general circulation to specific cells found in the cortical region of the adrenal gland where it binds to a high-affinity receptor and subsequently elicits increased production of corticosteroids. Corticosteroids inhibit the release (and/or production) of ACTH by negative feedback, probably acting at the hypothalamic and pituitary level (Tepperman, 1980). However, under stressful conditions the normal control systems may be overcome and ACTH elevated despite high circulating levels of corticosteroids. Elevated ACTH and corticosteroid levels are one aspect of the higher priority "General Adaptation Syndrome" (Selye, 1946) response to stress.

ACTH, Corticosteroids and Female Reproduction

Administration of ACTH has been documented to alter normal reproductive function in several species. Scholten and Liptrap (1978), mimicking the elevation in plasma glucocorticoid levels induced by heat stress with injections of long-acting ACTH, induced cystic ovarian follicles in gilts treated during the follicular phase of the estrous cycle. Adrenocorticotropin has also been demonstrated to inhibit ovulation in the sow (Liptrap, 1970; Liptrap, 1973), gilt (Barb et al., 1982), rat (Hagino et al. 1969),

and mouse (Christian, 1964; Pasley and Christian, 1972). Perhaps in a manner analagous to stress-induced embryonic mortality, researchers have increased embryonic losses up to 50% with the administration of ACTH during day 7 and days 11 through 15 of pregnancy (Velardo, 1957) and days 1 through 3 of pregnancy (Yang et al., 1969) in rats. Similar results were obtained by Kittinger and coworkers (1980) in mice by injecting ACTH days 1 through 8 of pregnancy. These results suggest that ACTH is, at least in part, a factor involved in stress-altered reproductive performance.

Corticosteroids have been directly implicated in altering normal female reproductive function. Elevation of plasma corticosteroid levels by administration of hydrocortisone acetate to sheep has been associated with up to 30% increase in early embryonic mortality (Howarth and Hawk, 1968). Corticosteroid administration has also been shown to inhibit ovulation in gilts (Barb et al., 1982) and immature (Smith et al., 1971) and mature rats (Baldwin and Sawyer, 1974), and increased the incidence of silent estrus in swine (Esbenshade and Day, 1980; Ford and Christenson, 1981).

II. ACTH AND CORTICOSTEROID ALTERATION OF NORMAL GONADAL FUNCTION

Although many researchers have observed changes in normal reproductive function after administration of ACTH and corticosteroids, the mechanism responsible for the alterations are still obscure.

Several studies on ovarian and testicular function after treatment with ACTH, corticosteroids or other stress-related hormones may provide insight into the mechanism of their action.

ACTH, Corticosteroids and the Ovulatory Follicle

Hagino and coworkers (1969) found that ACTH blocks pregnant mare's serum (PMS)-induced ovulation in immature rats only in the presence of the adrenal glands. They also found that dexamethasone, but not corticosterone, would block PMS-induced ovulation. The authors suggest the effects of ACTH is mediated by the adrenal rather than by direct action on the ovary. The different effects of corticosterone and dexamethasone may be due to their different biological potencies. There may be a threshold level of circulating corticosteroids, perhaps dependent on the biological potency (the ability of a compound to elicit a specific physiological response) of the corticosteroid used, before a physiological response can be elicited (Rousseau et al., 1972). In contrast, Liptrap (1970) demonstrated that ATCH, but not cortisol, inhibited ovulation in intact swine; however, adrenalectomy abolished ACTH inhibition of ovulation (Liptrap, 1973). In a later experiment, injection of 4 mg/kg bodyweight hydrocortisone acetate, double the dosage used in aforementioned study, blocked ovulation in swine (Scholten and Liptrap, 1978). These studies indicate that corticosteroids or other adrenal steroids may mediate ACTH effects by acting at the pituitary or hypothalamic level.

Corticosteroids have been demonstrated to inhibit the ovulatory luteinizing hormone (LH) spike in rats (Baldwin and Sawyer, 1974;

Baldwin, 1979), heifers (Stoebel and Moberg, 1979), and gilts (Barb et al., 1982). Several researchers have noted suppression of LH secretion in response to exogenous luteinizing hormone releasing hormone (LHRH) in ACTH and corticosteroid treated heifers (Matteri and Moberg, 1982) and rats (Baldwin and Sawyer, 1974) and with corticosteroid treated humans (Sakakora et al., 1975). In addition, Liptrap (1970) found that ACTH inhibition of ovulation in swine could be partially overcome by injection of human chorionic gonadotropin (hCG), which possesses LH activity. Padmanabhan and coworkers (1983) demonstrated that cortisol, but not ACTH, reduces LHRH induced secretion of LH by bovine pituitary cells in vitro. Baldwin (1979) noted that LH secretion was inhibited by chronically elevated plasma glucocorticoid levels in vivo although pituitary levels of LH were not affected. These studies suggest that corticosteroids may exert their inhibitory effect upon ovulation by acting directly on the pituitary to suppress LH secretion.

Other hormonal factors may be involved in ovulation inhibition. Adrenocorticotropin appears to stimulate the adrenal gland to produce progesterone as well as corticosteroids (Liptrap, 1970; Close and Liptrap, 1975; Benjaminson and Lunaas, 1980). Injection of progesterone has been associated with inhibition of ovulation and formation of cystic follicles in swine (Ulberg et al., 1951; Scholten and Liptrap, 1978); however, it appears to have a different mechanism of action than corticosteroids since it has been shown not to alter pituitary responsiveness to LHRH in vitro (Padmanabhan et al., 1983) or estrogen-dependent LH release (Baldwin, 1979).

ACTH, Corticosteroids and the Corpus Luteum

The endocrine mechanism behind ACTH and corticosteroid alteration of corpus luteum (CL) function is even more obscure than that behind inhibition of ovulation. Adrenocorticotropin and corticosteroids have been shown to interrupt pregnancy in rats, with ACTH affects abolished in adrenalectomized animals (Yang et al., 1969). Wagner et al. (1972) reported a decrease in midcycle peripheral plasma progesterone levels in heifers treated with ACTH. Carotid infusion of ACTH or hydrocortisone on days two through nine of the estrous cycle resulted in a decreased slope of progesterone increase, indicating either a slowed rate of CL development or progesterone synthesis. da Rosa and Wagner (1981) found that adrenalectomy blocked ACTH inhibition of progesterone synthesis. These studies suggest that ACTH exerts its inhibitory effect on CL function via the adrenal gland.

The possible effects of progesterone on LH release cannot be precluded. Recent studies in the ewe indicate that progesterone and estrogen synergistically act to alter pulsatile LH secretion (Goodman and Karsch, 1980; Goodman et al., 1981; Goodman et al., 1982). Apparently the ratio of these ovarian steroids is important in determining the frequency and amplitude of LH release. It may be suggested that administration of ACTH alters this ratio resulting in a decrease in the frequency or amplitude of LH secretion, thereby adversely affecting CL function. Immunization against LH or LHRH has been shown to terminate pregnancy in rabbits (Spies

and Quadri, 1967) and rats (Loewitt et al., 1969; Madwa and Maudagal, 1970; Nishi et al., 1976) in early gestation. Termination of pregnancy was counteracted by the administration of progesterone. These results indicate that some LH, even the low levels of early pregnancy, is important for CL maintenance and implies that termination of pregnancy by immunization against LH or LHRH is related to impaired CL production of progesterone.

Another possible mechanism of adrenal steroid termination of early pregnancy may be alteration of endometrial progesterone receptors. These receptors, through which progesterone exerts its essential role in regulating qualitative and quantitative aspects of porcine uterine protein secretions (Knight et al., 1973), probably play a vital role in the maintenance of a favorable uterine environment for embryos. The presence of endometrial progesterone receptors has been demonstrated in several species including rats (Walters and Clark, 1977), sheep (Kontula, 1975), swine (Diekmann and Anderson, 1979), and cattle (Atkins et al., 1980). Several researchers (Suthers et al., 1976; Jones et al., 1980; Svec et al., 1980) have reported that the glucocorticoid receptor has a second progesterone binding site and interactions with this site lead to an allosteric change in the receptor such that its affinity for the agonist is diminished. Svec and Rudis (1981) found that the progesterone binding site of cytosolic extracts of four separate glucocorticoid target tissues was similar. Perhaps the same phenomenon applies to endometrial progesterone

receptors since it has many characteristics similar to that of corticosteroid binding globulin (CBG) which has a high affinity for some adrenal steroids and is found both in tissue and plasma (Milgrom and Baulieu, 1970; Walters and Clark, 1977; Atkins et al., 1980).

These results indicate that high circulating levels of corticosteroids may directly compete with or reduce the affinity (via a second receptor site) of the binding of progesterone to its endometrial receptor. Progesterone binding would thus be reduced, which may impair the ability of the uterus to maintain embryos by altering quantitative and qualitative uterine protein secretion (Knight et al., 1974a; Bazer, 1975; Chen et al., 1975).

Adrenocorticotropin alteration of CL function may involve ACTH-induced hyperprolactinemia. Prolactin has been shown to be luteotropic during the early stages of pregnancy in the mouse (Bartke, 1973) and the formation of the CL of pseudo-pregnancy in the rat (Smith et al., 1975; Day et al., 1980); however, excessive production has been associated with impaired reproduction in both male and female humans (Bohnet et al., 1976; Carter et al., 1978). It is hypothesized that prolactin may act to stimulate adrenal steroid production (Carter et al., 1978), reducing pituitary gonadotropin secretion (Bohnet et al., 1976; Carter et al., 1977), and subsequent gonadal steroid output (Carter et al., 1977). Kittinger and coworkers (1980) found that ACTH treatment of both adrenalectomized and intact pregnant rats induced hyperprolactinemia

and reduced nidation sites, although the effect was less pronounced in adrenalectomized animals. These studies indicate that ACTH may stimulate prolactin releases, which affects the CL independently of adrenal steroids.

ACTH, Corticosteroids and Testicular Function

As in the female, elevation of corticosteroid levels, either by increase of pituitary ACTH secretion or by ACTH-secreting tumors, has been associated with impaired reproductive capabilities in males. Treatment with ACTH is reported to reduce testis weight and cause leydig cell atrophy in rats (Asling et al., 1951). Glucocorticoid excess has been associated with decreased libido and impotency in sexually mature male patients (McKenna et al., 1979).

Adrenal steroids, rather than ACTH, are implicated as the modulating agent of testicular function both by indirect and direct evidence. Adrenalectomy abolished the inhibitory effect of ACTH on testosterone production in rats, thus indirectly suggesting that the effect is mediated by adrenal steroids (Saez et al., 1977). Acute administration of corticosteroids reduced testosterone secretion in rats (Saez et al., 1977; Bambino and Hsueh, 1981), bulls (Welsh et al., 1979; Welsh et al., 1981), and men (Schaison et al., 1978), thus providing direct evidence of adrenal steroid involvement. In contrast, injection of a single dose of cortisol has been reported to stimulate, although chronic treatment reduced, testosterone secretion in boars (Liptrap and Raeside,

1975). These contradicting results may be due to species differences or, as pointed out earlier, be related to the biological activity of the corticosteroid injected and the length of time elevated plasma concentrations are maintained.

There are three potential mechanisms to explain corticosteroid associated decrease in testicular function. The first is that corticosteroids may decrease serum LH by acting at the hypothalamus or pituitary. Decrease in LH secretion due to elevated corticosteroid levels has been reported in bulls (Thibier and Rolland, 1976; Chantaraprateer and Thibier, 1978; Welsh and Johnson, 1981). Glucocorticoids are known to bind to cells of the hypothalamus in the pig (Stith and Weingarten, 1978) and could affect production of gonadotropin releasing hormone. A recent study by Liptrap and Raeside (1983) suggests that acute elevation of plasma cortisol may enhance the production of LH, and thus testosterone secretion, by increasing the responsiveness of the pituitary to gonadotropin releasing hormone. In addition to adrenal corticoids, progesterone secretion (presumably of adrenal origin) was increased by ACTH administration to bulls (Johnson et al., 1982). Progesterone may play a role analogous to corticosteroids in suppression of testicular function.

Another proposed mechanism is that adrenal secretions may decrease serum prolactin. In the leydig cell, prolactin has been shown to potentiate the actions of LH upon androgen biosynthesis and secretion (Hafiez et al., 1972; Catt et al., 1980). Several

researchers have found that elevated corticosteroids reduce prolactin secretion in male rats (Schwinn et al., 1976; Rossier et al., 1980). Adrenalectomy abolished ACTH-induced hypoprolactinemia in rats, which suggests that adrenal secretions mediate prolactin releases (Harms et al., 1975).

Lastly, adrenal secretions may directly inhibit testis function. Glucocorticoid treatment has been shown to decrease testicular testosterone production by hCG in vitro (Bambino and Hsueh, 1981) which is consistent with the evidence that glucocorticoid receptors exist in the testis (Ballard et al., 1974; Evain et al., 1976). Perhaps glucocorticoids act in some manner, via specific receptors, to alter steroidogenesis either by altering the cellular steroidogenic pathways or by altering receptors for other hormones, such as the LH receptor.

III. PROTEIN HORMONE REGULATIONS OF GONADAL STEROIDOGENESIS

Ultimately the regulation of ovarian function occurs at the cellular level. Gonadotropins have been well documented as the primary regulators of gonadal function which exert their effect by binding to specific, high-affinity receptors on the target cell surface.

A mathematical method commonly used to describe the interaction of a ligand with its receptor is Scatchard analysis (Dufau and Catt, 1978). The interaction of a hormone (H) with its

receptor (R) may be characterized by the following relationship:

$$[H] + [R] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [HR]$$

where [HR] represents the concentration of the hormone-receptor complex, K_a the association (or affinity) constant, and K_d the dissociation constant. At equilibrium the hormone-receptor interaction will follow the expression:

$$K_a = \frac{[H \cdot R]}{[H]\,[R]} = \frac{1}{K_d}$$

where K_a is the equilibrium association constant in liters per mole and K_d is the dissociation constant in moles per liter. Data for Scatchard analysis can be generated by incubating increasing concentrations of radioactive hormone or a fixed concentration of radioactive hormone with increasing concentrations of unlabelled hormone. After separation of bound and free hormone by one of several techniques (dialysis, filtration, centrifugation), the ratio of bound to free hormone (B/F) is plotted against bound hormone (B). A linear plot will result if the receptor binding sites are independent (non-cooperative) and homogenous. The point where the line intersects the abscissa is equal to the number of receptor sites and the affinity constant (K_a) can be determined from the slope of the line.

The major biological response elicited by gonadotropin binding to a receptor is steroidogenesis, although other processes are controlled such as growth, ovarian differentiation, and ovulation. Therefore, characteristics of hormone-receptor binding may provide some important clues about the possible interrelationship of ACTH and corticosteroid involvement on gonadal function.

Ovarian Cytodifferentiation and Receptor Formation

Studies in vivo on cytodifferentiation of rat ovarian cells have provided valuable information on the effects of the gonadotropins and their receptors on cellular differentiation and steroidogenic function. During the earliest stage of follicular development granulosa cells begin to proliferate in the primary follicle and "spontaneously" develop a variety of hormone receptors; follicle stimulating hormone (FSH) (Nimrod et al., 1976; Louvet and Vaitakaitis, 1976), estradiol (Richards, 1975), testosterone (Schrieber and Ross, 1976), progesterone (Schreiber et al., 1980a,b) glucocorticoids (Schreiber et al., 1982), and GnRH (Jones et al., 1980). Stimulation of the cells with FSH both in vivo and in vitro induces the granulosa cells to develop the capacity for estrogen and progesterone biosynthesis (Dorrington et al., 1975; Hillier et al., 1977; Erickson and Hsueh, 1978) and surface membrane receptors for LH (Zeleznik et al., 1974; Richards et al., 1976; Erickson et al., 1982), prolactin (Richards and Williams, 1976; Wang et al., 1979), and catecholamines (Coleman et al., 1979). In the rat, the preovulatory surge of LH and prolactin stimulates the

transformation of granulosa cells into granulosa-luteal cells which secrete primarily progesterone (Richards and Midgley, 1976). This transformation is characterized by LH down-regulation of its own receptors, but this loss is compensated for by the induction of new LH receptors by prolactin in the rat (Holt et al., 1976). Lastly, luteolysis occurs apparently by involving the rapid block of LH action by prostaglandin $F_{2\alpha}$ (Thomas et al., 1978). This model demonstrates that different controls may be expected at various stages of ovarian development.

LH Receptor Regulation and Ovarian Follicular Function

LH is the most important factor involved in the control of production of progesterone for the maintenance of pregnancy in all mammals (Niswender et al., 1980). The establishment of LH receptors during the early stages of follicular development may be a determinant of later ovulatory potential and thus the reproductive capabilities of the animal.

Porcine granulosa cells have been used extensively in cell culture studies. These cells specifically bind ^{125}I-hCG to a homogenous class of LH receptors in amounts which increase markedly during follicle maturation (Channing and Kammerman, 1973) without a change in binding affinity (Kammerman and Ross, 1975; Stouffer et al., 1976; Lee, 1976; May et al., 1980). Various media (such as serum-free, FSH and insulin-supplemented) have been used in the attempt to distinguish the hormonal factors responsible for the formation of LH receptors and subsequent steroidogenic capacity.

Recent evidence indicates that estradiol may serve as a biological amplifier of the positive action of FSH on follicular steroidogenesis, thus preparing the granulosa cells for high rates of steroidogenesis they ultimately express in the luteinized state (Veldhuis et al., 1982). However, conflicting results indicating that estrogens information which may be applicable to the more complex in vivo function of luteal cells.

LH Receptor in Regulation and Ovarian Luteal Function

In rats, prolactin appears to be necessary for adequate numbers of LH receptors in the developing CL (Richards and Williams, 1976). Prolactin receptors have been reported in porcline luteal tissue (Rolland et al., 1976) but the relationship to luteal function is still unclear. The number of LH receptors of the CL appears to be highly positively correlated with secretion of progesterone in women (Lee and Ryan., 1973), rats (Lee et al., 1975), cows, (Rao et al., 1976), and ewes Diekman et al., 1978). Less than 1% occupancy of receptors is required for maximal steroid secretion in the ovary (Diekman et al., 1978) and testes (Catt and Dufau, 1973). These "spare" receptors may serve as an area of homologous hormone regulation. Exposure of luteal tissue to high concentrations of LH or hCG results in the loss of as much as 90% of the receptors (Conti et al., 1976, 1977; Harwood et al., 1978; Dafau and Catt, 1978). However, Suter and coworkers (1980) found that losses induced by LH injection of ewes did not reduce progesterone production by the CL even though up to 85% of the receptors were lost. These

losses are thought to occur by the phenomenon of internalization (Goldstein et al., 1979; Willingham and Pastan, 1980) but the physiological significance of this phenomenon, as well as that of "spare" receptor, is unknown.

LH Receptor Regulation and Testicular Function

Extensive research has been performed on the LH receptors of the testes and their regulation. These studies may provide clues to possible regulatory mechanisms of ovarian LH receptors, particularly in regard to the possible alteration of the LH receptor by ACTH and corticosteroids.

LH is the primary tropic hormone controlling leydig cell function but other hormones are necessary for maintenance of their function. In hypophysectomized adult rats, Zipf and coworkers (1978) demonstrated that the combined effects of prolactin, growth hormone, and LH are necessary for the maintenance of LH receptors, with prolactin allowing LH to have positive effect on LH receptors, and that normal steroidogenic responsiveness is mainly dependent upon LH.

The leydig cell is of particular interest for it is the only system where the effect of ACTH and glucocorticoids on the LH receptor has been studied. Bambino and Hsueh (1981) treated hypophysectomized rats with dexamethasone, corticosterone or a synthetic progestin and observed alterations of LH receptor content and steroidogenesis. After five days of treatment both dexamethasone and corticosterone decreased the number of testicular

LH receptors, though the progestin had no effect. *In vitro* culture of testicular cells with glucocorticoids decreased testosterone production. These results demonstrate the direct inhibitory effect of glucocorticoids on testicular LH receptor content and steroidogenesis, suggesting that the adrenal glucocorticoids may play a role in regulating testis function.

CHAPTER III

MATERIALS AND METHODS

I. ANIMALS AND DATA COLLECTED

Experiment One

A preliminary study was conducted March and April 1982 to determine the appropriate dosage of adrenocorticotropin[1] (ACTH) and hydrocortisone acetate[2] (HA) to be used in subsequent studies. Six crossbred gilts (a combination of Landrace, Yorkshire, and Duroc breeds) approximately eight months of age were weighed, nose ringed, mixed, and moved to a half-acre pasture lot with open front shelters. Each gilt was fed approximately 2.2 kg of a 16% crude protein corn-soybean meal diet once a day and water was provided ad libitum. A mature boar was used twice daily (morning and evening) for estrus detection.

Four of the six gilts, demonstrating estrous cycles of 18 to 24 days, were surgically fitted with indwelling jugular cannula as described by Knight et al. (1982). Following surgery, the animals were placed in individual crates and bled twice daily 2 to 6 days prior to treatment to familiarize them with the bleeding procedure. Feed and water were provided as described earlier.

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[1]Adrenocorticotropin hormone, grade V porcine, Sigma Chemical Co., St. Louis, MO.

[2]Hydrocortisone 21-acetate, Sigma Chemical Co., St. Louis, MO.

Treatments administered were: (1) 2 ml sesame oil[3] injected intramuscularly (IM) and 2 ml of 14% gelatin[4] solution injected subcutaneously (SQ), (2) 2 mg HA in sesame oil per kg bodyweight (IM), and (3) 1 I.U. ACTH in 14% gelatin per kg bodyweight (SQ). The gelatin solution was prepared by dissolving 14 g of gelatin in 100 ml distilled water (w/v). The prepared ACTH-gelatin solution (100 I.U./ml 14% gelatin) was aliquoted into several sterile vials (5 ml solution/vial) and stored at 4C until used. The gelled solution was warmed at 37C in a water bath to be liquified prior to injection. The HA-sesame oil solution was aliquoted 15 ml per sterile vial at a concentration of 100 mg HA/ml sesame oil and stored at 4C.

Each treatment was administered to all four gilts in the aforementioned order with a 24 hour rest period provided between each treatment regime. Within treatments, animals were randomly allotted in pairs to two groups, one receiving only one injection (0800 hours) and the other a second injection 12 hours later (2000 hours). Blood samples (10 ml) were collected immediately prior to the first treatment and every 2 hours thereafter for 24 hours. Each sample was collected in a herparinized syringe, centrifuged at 750 x g for 10 minutes and plasma collected and stored at -20C until analyzed for cortisol concentrations.

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[3]Laboratory grade, Fisher Scientific Co., Pittsburgh, PA.

[4]Knox unflavored gelatin, Knox Gelatin, Inc., Eaglewood Cliffs, NJ.

Experiment Two

In a major study conducted in the spring and summer of 1982, 32 crossbred gilts of similar age, weight, and breeding to those used in the previous experiment were weighed, nose ringed, mixed, and moved in equal numbers to four pasture lots. Pasture conditions and management procedures were as described previously. Gilts were checked for estrus twice daily with the aid of a mature boar.

Twenty-three gilts demonstrating estrous cycles of 18 to 24 days were randomly assigned to the following treatment groups; (1) nonbled control, receiving no injections; (2) bled control, injected with 2 ml sesame oil (IM) and 2 ml 14% gelatin (SQ); (3) 2 mg HA in sesame oil per kg body weight (IM); and (4) 1 I.U. ACTH in 14% gelatin per kg bodyweight (SQ). Within each treatment group gilts were assigned to be treated on days 2 through 7 or days 8 through 13 of the estrous cycle (Table 1).

TABLE 1. Design of Experiment Two

	Day of treatment	
Treatment	2[a]-7	8-13
Nonbled control	3[b]	3
Bled control	2	3
Hydrocortisone acetate	3	3
Adrenocorticotropin	3	3

[a]Day 0 = first day of estrus.

[b]Represents number of gilts.

Injections were given twice daily at 0800 and 1700 hours during the respective treatment period. In addition, a 20 ml blood sample was collected via jugular puncture from each gilt immediately prior to the 1700 hour injection. Plasma was recovered and stored as described previously for later analysis of progesterone and cortisol concentrations.

Two to five days prior to the last day of treatment, gilts were transported to holding pens adjacent to surgery facilities and sorted into pens according to pasture assignments. On the day following the last day of treatment (day 8 or 14 of the estrous cycle) animals were anesthetized with halothane gas and the reproductive tract exposed by midventral laparotomy. Each uterine horn was flushed with 20 ml 0.33% hypotonic saline solution according to the procedure of Murray and coworkers (1972). The recovered fluid was stored at -20C until analyzed for total proteins. After the uterine flushings were collected, the ovaries were removed, weighed, number of corpora lutea (CL's) counted; and individual CL excized. The CL's were then frozen using dry ice and acetone and stored at -70C until analyzed for luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor characteristics.

II. HORMONE RADIOIMMUNOASSAYS

Plasma Cortisol

Plasma cortisol concentrations were determined by radioimmunoassay[5] according to the procedure of Barb et al. (1982) (Appendix A, page 111). Validation of the assay was performed by the extraction of .2 ml plasma containing the following amounts of added cortisol: 2.5, 5, 10, 25, and 50 ng. The actual amounts of cortisol recovered were: 3.65, 7.17, 13.77, 34.44, and 61.71 ng, respectively. The percent cross reactivity of various steroids and sterols tested with the cortisol antibody, as determined by Barb et al. (1982), were 31.8, 23.1, 6.5, and 5.1% for 17 α-hydroxyprogesterone, cortisone, corticosterone, and progesterone, respectively, and less than .01% by estrone, 17 β-estradiol, pregnenolone, cholesterol, 5 α-dihydroxy testosterone and testosterone. A pooled gilt plasma sample containing 13.0 ng cortisol per ml was used to determine the precision of the assay. The intra-assay coefficient of variation of eight duplicate determinations was 7.40% and the interassay coefficient of variation of four assays was 8.94%. Extraction efficiency was approximately 87%.

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[5]Cortisol antibody was kindly donated by Dr. R. R. Kraeling, Anim. Physiol. Res. Unit, USDA, ARS, Richard B. Russell Agr. Res. Center, Athens, GA 30613.

Plasma Progesterone

Plasma progesterone concentrations were determined by radioimmunoassay[6] according to the modified method of Niswender[6] (1973) (Appendix B, page 114). The cross-reactivity of the antibody, determined by Niswender (1973), was 34.5, 13.0, 8.0, 1.2, .7, .5, .2, and .03% for 11 α-hydroxyprogesterone, 5 α-pregnane-3,20-dione, 11 β-hydroxyprogesterone, 17 hydroxyprogesterone, deoxycorticosterone, 3 β-hydroxy-5 pregnen-20-one and cortisone. Precision of the assay was determined with a pooled gilt plasma sample containing 27.80 ng progesterone per ml. The intra-assay coefficient of variation of 12 duplicate determinations was 6.4% and the interassay coefficient of variation of 6 assays was 12.12%. Extraction efficiency averaged 64.36% (n = 36).

III. LH/hCG RECEPTOR ANALYSIS

Characterization of Radioiodinated hCG

In these experiments ^{125}I-hCG rather than ^{125}I-LH was used to assess LH binding capacity and affinity to porcine corpora lutea since hCG exhibits higher stability and binding activity after radioiodination (Dufua and Catt, 1978). Five µg hCG was radioiodinated with 2 mCi Na-^{125}I using a lactoperoxidase procedure (Morrison and Bayse, 1970) as outlined in Appendix C, page 117. To characterize the labelled hormone preparation, the specific

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[6]Progesterone antibody was obtained from Dr. G. D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, CO 80523.

activity and percentage of radioactive hormone that is capable of binding to excess LH/hCG receptors was determined. All experiments conducted to characterize the labelled hormone, as well as those conducted to validate the assay procedure, utilized surgically obtained porcine corpora lutea randomly pooled from several animals and prepared as outlined in Appendix D, page 121.

The specific activity of ^{125}I-hCG was determined by auto-competition curves (Ketelslegers et al., 1975) as shown in figure 1. An inhibition curve (curve A) was generated by adding nine concentrations of CR-121 hCG (.1, 2.5, .5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 ng/100 µl PBS-.1% BSA (buffer)) to tubes containing 80,000 cpm ^{125}I-hCG/100 µl buffer and 100 µl (containing 45 mg CL tissue) of membrane preparation in 700 µl buffer for a final reaction volume of 1 ml. A self-displacement curve (curve B) was generated by adding increasing quantities of ^{125}I-hCG (1.0, 2.5, 4.0, 5.0, 9.0, 20.0, 40.0, 50.0, 80.0, and 200.0 x 10^4 cpm/100 µl buffer) to 800 µl buffer in addition to 100 µl of the same membrane preparation used in curve A. Each curve in figure 1 is the mean of triplicates. Nonspecific binding was determined at each concentration of ^{125}I-hCG with 20 µg unlabelled hCG (Appendix D, page 121). After all tubes were incubated for 24 hours at 25C, the bound hormone was separated from free by centrifugation and the pellet formed was counted for bound radioactivity in a gamma spectrometer. The radioactivity specifically bound (total bound-nonspecific bound) in the absence of unlabelled hCG for curve A was designated as 100% binding

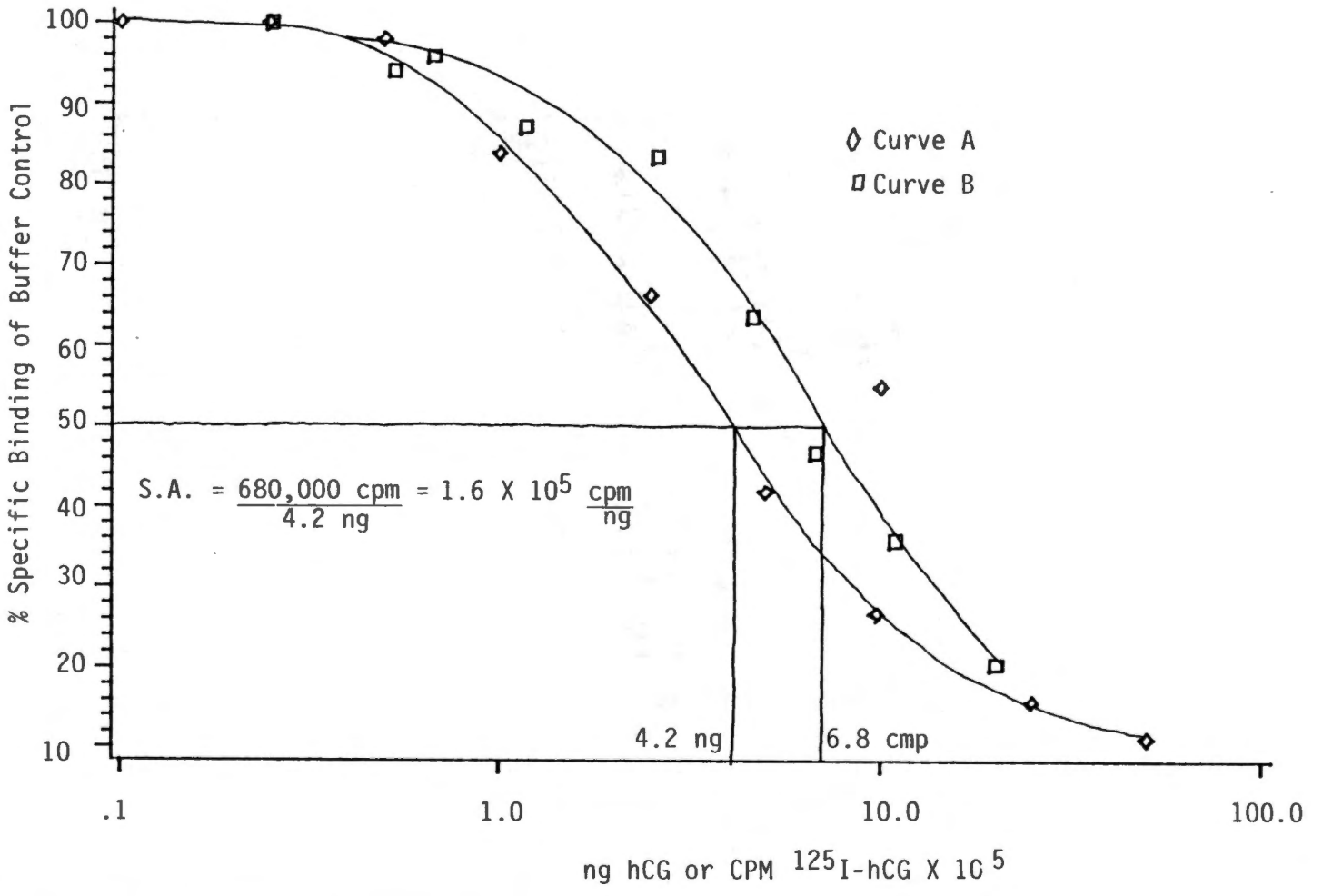


FIGURE 1. Determination of specific activity of ^{125}I-hCG binding to porcine luteal homogenates.

(buffer control). For curve B the bound/free ratio, corrected for nonspecific binding, was determined for each concentration of ^{125}I-hCG used. The concentration giving the highest bound/free ratio was designated as 100% binding (buffer control). Other points in curve A and B were expressed as a percentage of 100% binding.

Specific activity was calculated by dividing the cpm obtained at 50% binding (curve B) by the quantity of unlabelled hCG displacing 50% of ^{125}I-hCG (curve A). One ^{125}I-hCG preparation with a specific activity of 1.6×10^5 cpm/ng hCG was used (within four weeks of radioiodination) for all experiments.

Maximum binding of radioiodinated hCG to excess receptor was determined in triplicate by incubating 25,000 cpm/100 µl buffer ^{125}I-hCG with membrane homogenates containing .5, 1.0, 10, 20, 40, 60, 70, and 80 mg CL tissue with a final incubation volume of 1 ml. the maximum specific bindability of the preparation used in these experiments was 35% (Figure 2).

Validation of Hormone Binding Assay

The characteristics of ^{125}I-hCG binding to porcine luteal LH/hCG receptors was calculated using Scatchard analysis computer program by Faden and Rodbard (1976). To insure reliable estimates of binding affinity and capacity by Scatchard analysis, experiments were conducted to insure that nonspecific binding was carefully evaluated and corrected for, the concentration of receptors did

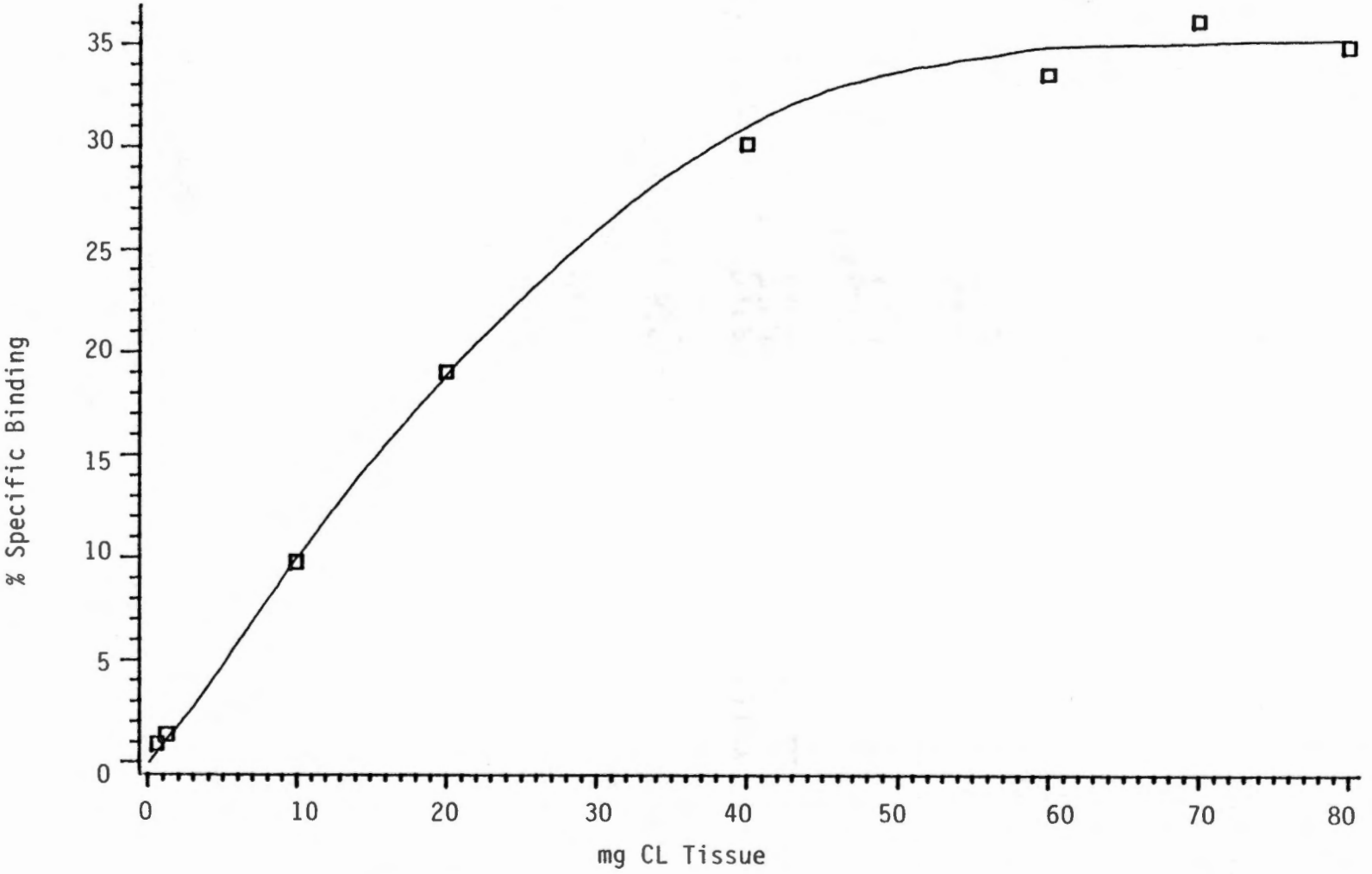


FIGURE 2. Binding of ^{125}I-hCG to varying concentrations of porcine luteal homogenates.

not alter the hormone-receptor interaction, and equilibrium was reached before bound and free hormone was separated.

Figure 3 shows the binding of ^{125}I-hCG as a function of the total amount added, representing the data generated in the determination of curve B (Figure 1). Specifically bound counts were determined as described earlier. The figure illustrates that ^{125}I-hCG binding to the porcine membrane preparation is a saturable phenomenon as has been demonstrated previously in bovine (Spicer et al., 1981) and rat (Lee and Ryan, 1973) luteal tissue homogenates.

To investigate the effect of varying concentration of receptors upon binding characteristics determined by Scatchard analysis, a binding assay (Appendix D, page 121) was performed on membranes equivalent to 29 and 34 mg of tissue weight (Figure 4). Comparable estimates of equilibrium association constants (1.10 and 1.11 x 10^{10} M^{-1}, respectively) and a similar number of binding sites (2.70 and 2.72 x 10^{-11} M, respectively) were obtained per mg of tissue, indicating that the binding of hCG is independent of receptor concentration.

Time- and temperature-dependence of the interaction of LH/hCG with its receptor was determined using three concentrations of hCG (CR121) (0.0, 0.5, and 5.0 ng/100 µl buffer), representing the range used in the binding assays, were added in triplicate to tubes containing 100 µl (containing 15 mg CL tissue) of membrane preparation, 100 µl (70,000 (cpm) ^{125}I-hCG and 700 µl buffer. Bound hormone was

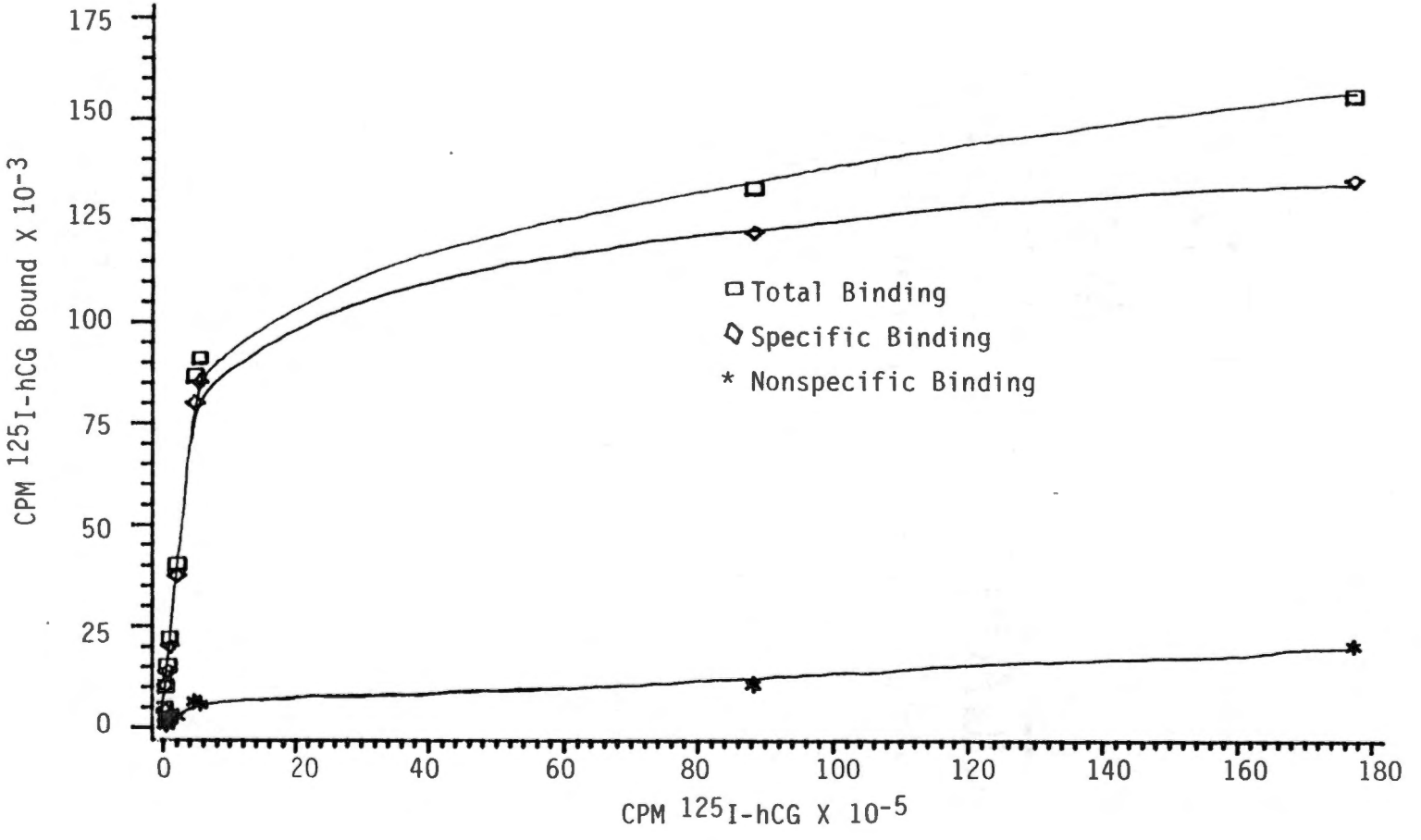


FIGURE 3. Specific binding of ^{125}I-hCG to porcine luteal homogenates as a function of ^{125}I-hCG concentration.

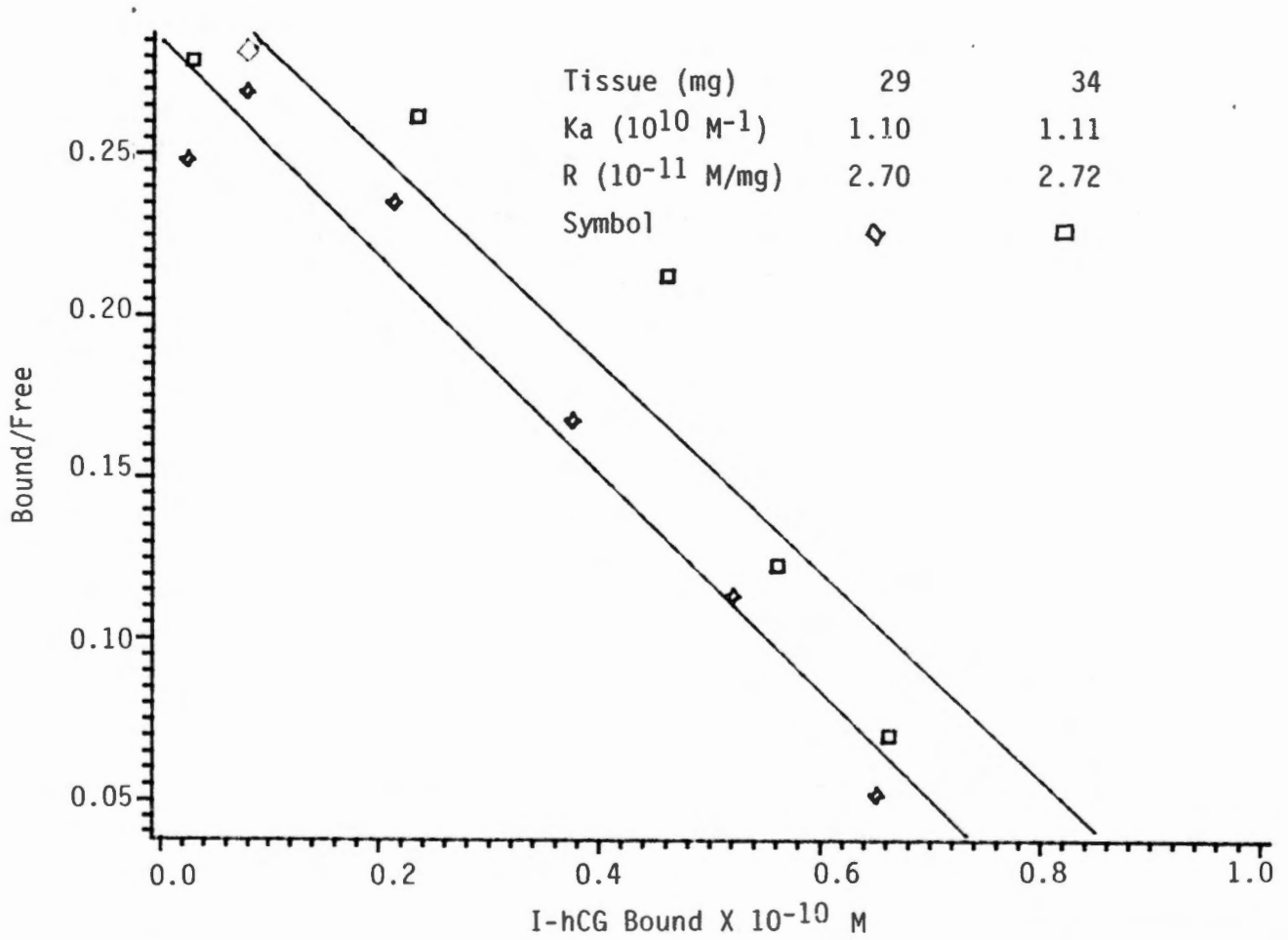


FIGURE 4. Effect of concentration of membrane homogenate on the estimation of LH/hCG receptor binding capacity and affinity.

separated from free after incubation at either 4C or 25C for .5, 2, 4, 8, 12, 16, 20, and 24 hours and counted as described previously. Nonspecific binding was determined, as described earlier, for each incubation time and hCG (CR121) concentration. The amount of radioactivity specifically bound by each concentration of hCG (CR121) after 24 hours of incubation was defined as 100% binding, with other points being expressed as a percentage of binding at 24 hours.

Figures 5 and 6 illustrate the results of time of incubation at 4C and 25C, respectively, on the binding of ^{125}I-hCG to membrane homogenates. At both temperatures equilibrium was reached by 20 hours of incubation regardless of hCG concentration. Tubes containing 0 and .5 ng hCG reached equilibrium later than tubes containing 5 ng hCG at both temperatures (20 hours vs 12 and 8 hours for 4C and 25C, respectively). The temperature dependence of the reaction is shown in figure 7, with the amount of ^{125}I-hCG specifically bound (0 ng hCG (CR121) added) plotted over time at 4C and 25C. Approximately 70% less ^{125}I-hCG was bound at 4C as compared to 25C following a 24 hour incubation. These results indicate that, under conditions used in the present experiment for performing the LH/hCG hormone receptor binding assay, equilibrium was reached by 20 hours (25C) of incubation. The reaction kinetics shown here agree closely with those determined for rat luteal homogenates (Lee and Ryan, 1973).

LH/hCG Binding Assay

Based upon the results obtained from the above experiments, an incubation period of 24 hours and a reaction volume of 1 ml

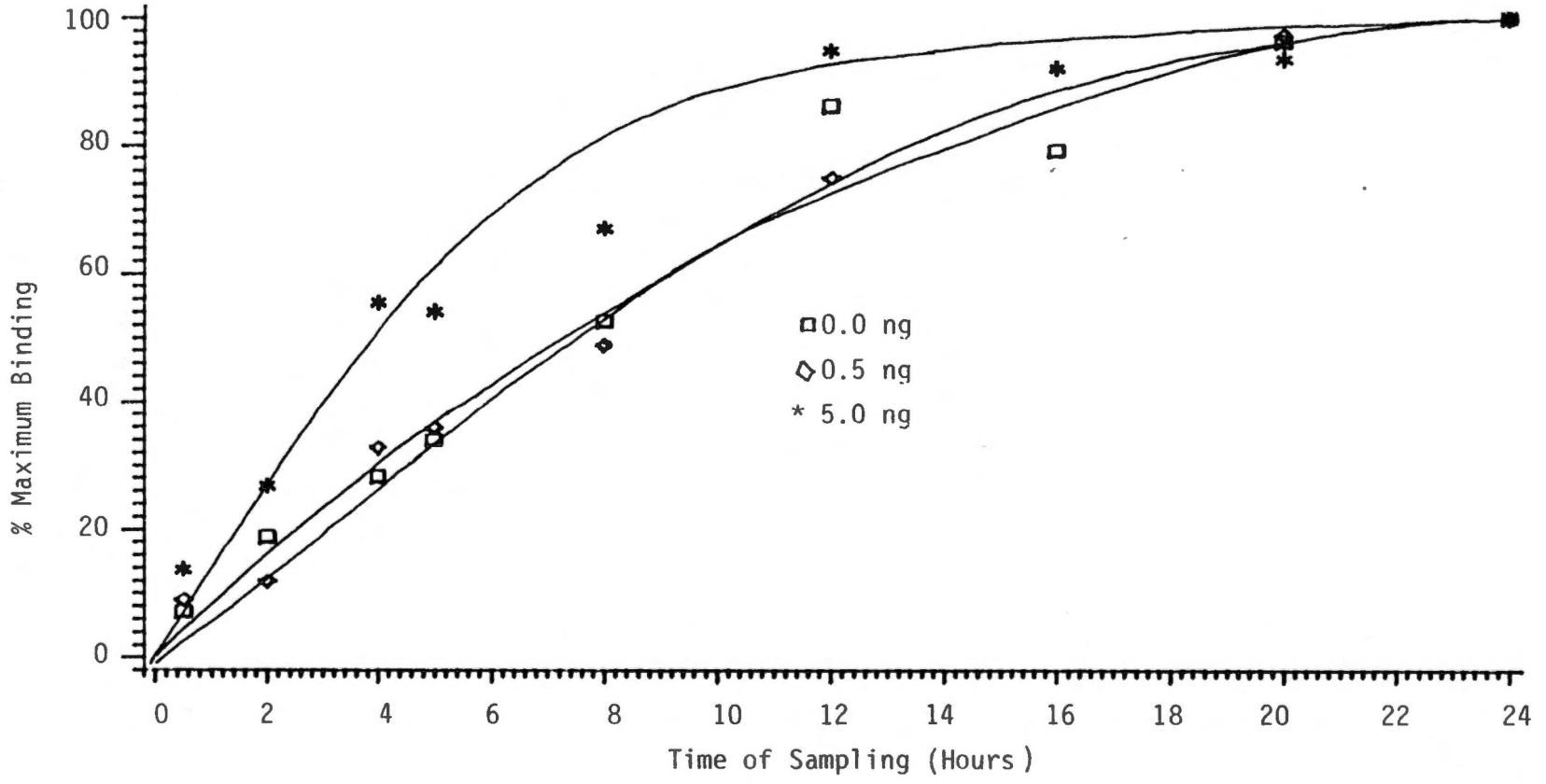


FIGURE 5. Binding of ^{125}I-hCG to porcine luteal homogenates at three concentrations of hCG (CR121) as a function of time at 4C.

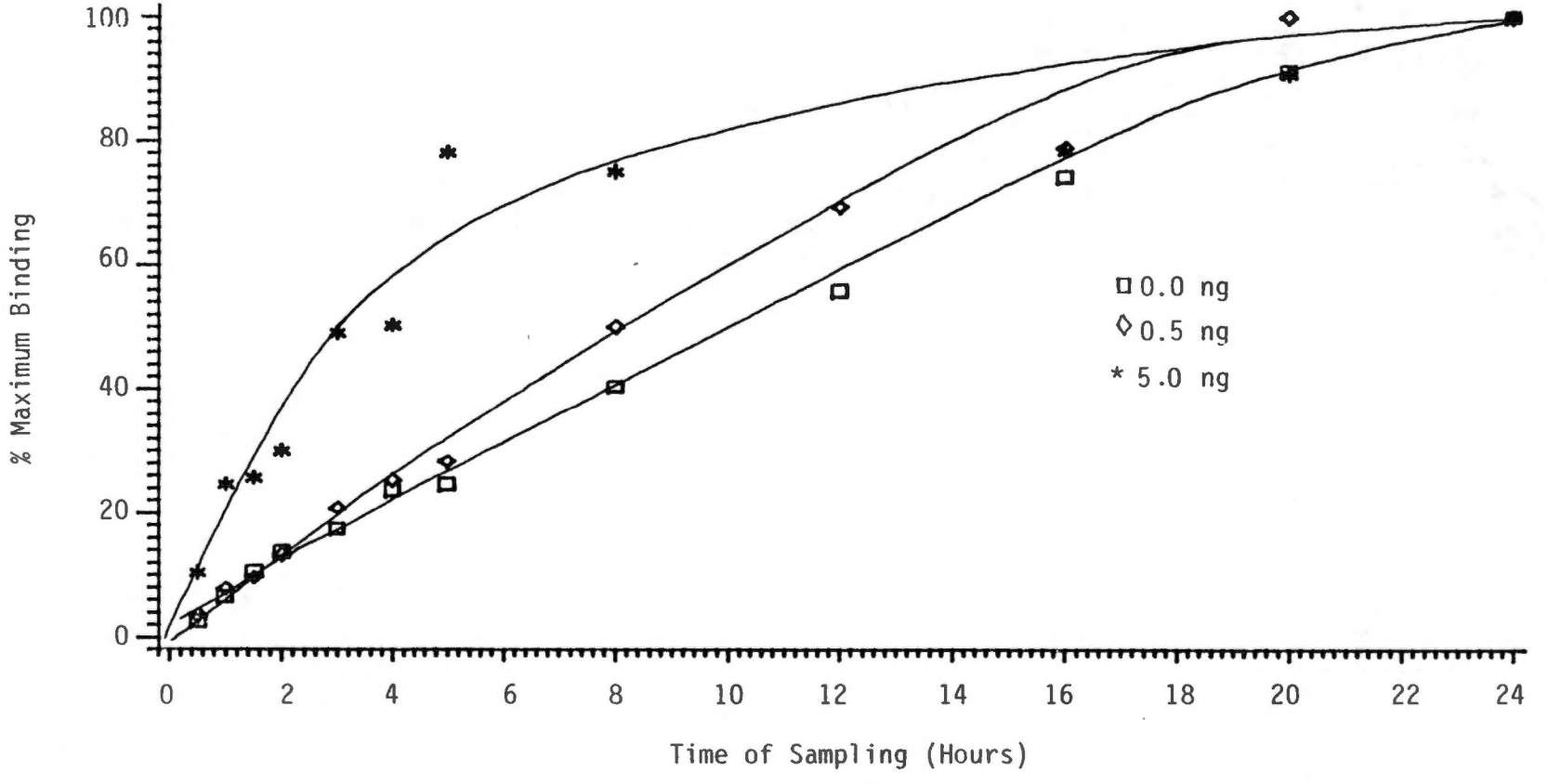


FIGURE 6. Binding of ^{125}I-hCG to porcine luteal homogenates at three concentrations of hCG (CR121) as a function of time at 25C.

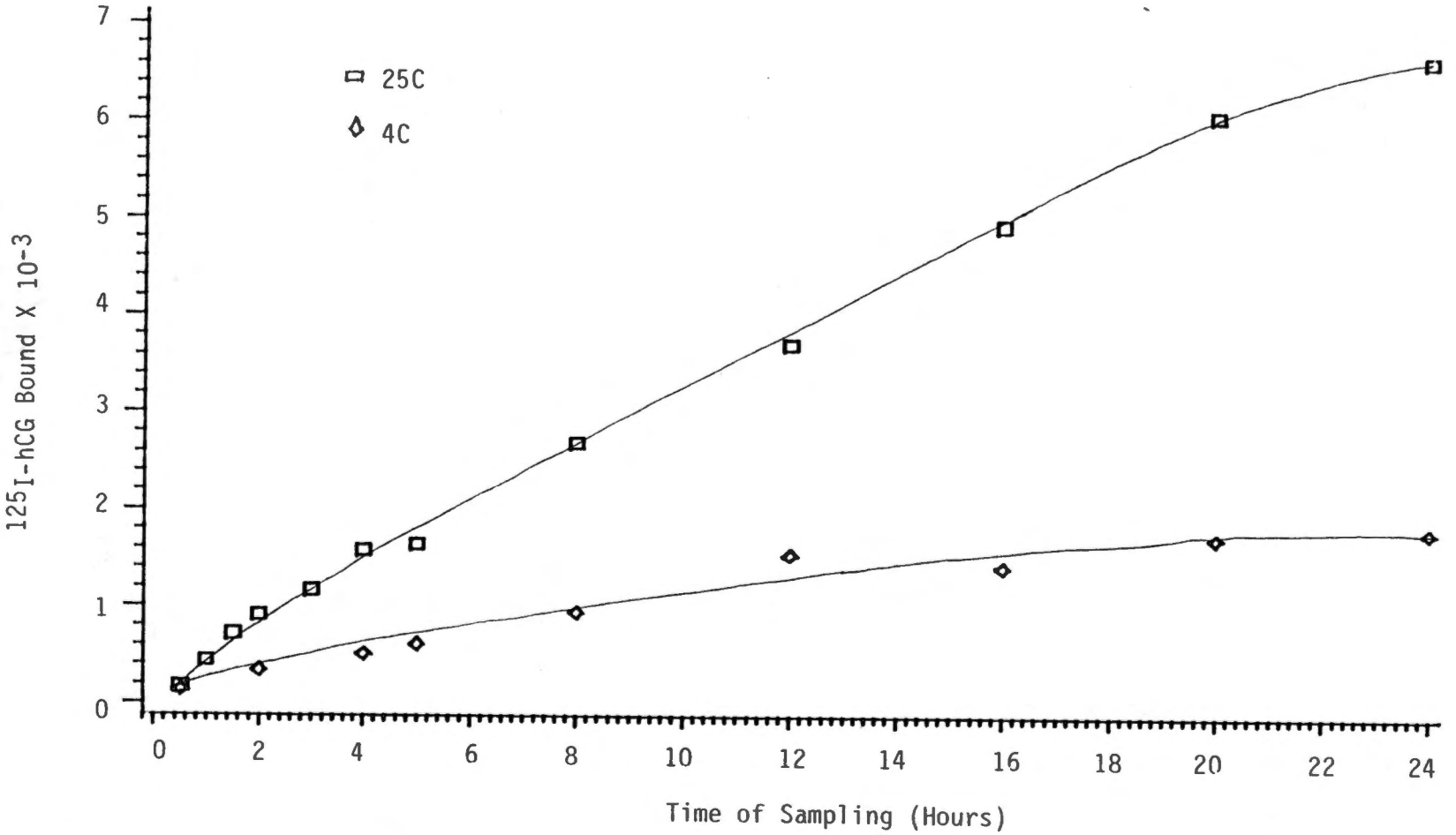


FIGURE 7. Binding of ^{125}I-hCG at 4C and 25C as a function of time.

was used for all analysis of porcine luteal LH/hCG receptors. Two (one per ovary) or six (three per ovary) CL from each pig were utilized to determine the LH/hCG receptor binding capacity and affinity. This was determined by generating an inhibition curve for each CL membrane preparation (Appendix D, page 121) and analyzing the results with a Scatchard analysis computer program (Faden and Rodbard, 1976).

A pooled membrane homogenate sample was used to estimate the precision of the assay. Several surgically obtained CLs were randomly selected and individually homogenized, centrifuged and resuspended in PBS - .1% BSA containing trypsin inhibitor as described in Appendix D, page 121. These membrane preparations were then pooled, mixed thoroughly, aliquoted (700 µl) into 12 x 75 borosilicate tubes and stored frozen at -70C. Each time an assay was performed the total and nonspecific binding of ^{125}I-hCG to a pooled sample aliquot was determined. The pooled sample specifically bound, on the average, 10,493 cpm. The inter-assay coefficient of variation of 9 assays was 13.38% and the intra-assay coefficient of 18 duplicated determinations was 6.0%. In addition, nonspecific binding was carefully monitored for each membrane preparation analyzed, averaging 4.02% of the ^{125}I-hCG bound.

V. UTERINE PROTEINS

Uterine proteins were prepared by a modified method of Knight et al. (1973). Modifications involved storage of the uterine

flushings at -20C prior to preparation and concentration by lyophilization rather than vacuum dialysis. After samples were lyophilized they were resuspended in 10 ml phosphate buffered saline and protein concentration determined by the method of Bradford (1976) (Appendix E, page 123). Total uterine protein recovered was calculated by multiplying protein concentration by the final volume of the sample (10 ml) and dividing by the percent recovery of the total 40 ml infused into the uterine horns.

V. STATISTICAL ANALYSIS

The response to treatment by pigs in experiment one was evaluated by determining the area under the curve for measured plasma cortisol concentrations determined over a 24 hour period by a Hewlett-Packard computer. The computed areas were statistically analyzed for treatment differences using a Student's T-test (Snedecor and Cochran, 1967).

Experiment two was arranged in a 4 x 2 factorial (Table 1, page 24) using a completely randomized design. The data were statistically analyzed by least square analysis of variance (Goodnight et al., 1982). When statistical analysis were calculated using plasma hormones (cortisol or progesterone) as dependent variables, the model used was as follows:

$$Y_{ijkl} = t_i + d_j + (dd)_{jj} + p_k + (td)_{ij} + (tdd)_{ijj} + (tp)_{ik} + (tdp)_{ijk} + (tddp)_{ijjk} + e_{ijkl}$$

where:

Y_{ijkl} = dependent variable

t_i = treatment

d_j = days of treatment (linear model)

$(dd)_{jj}$ = days^2 of treatment (quadratic model)

p_k = period of treatment

$(td)_{ij}$ = treatment by days interaction

$(tdd)_{ijj}$ = treatment by days^2 interaction

$(tp)_{ik}$ = treatment by period interaction

$(tdp)_{ijk}$ treatment by days by period interaction

$(tddp)_{ijjk}$ = treatment by day^2 by period interaction

e_{ijkl} = residual error

If differences due to treatment or any of the interactions were determined by F-test, analysis was performed to obtain biologically reasonable estimates for levels of discrete classes of effects (treatment, period) in interaction with a continuous variable (days). Means for each level of discrete effects were then obtained as linear functions of the generalized solution of the normal equation. Specific contrasts were performed using the generalized solution of the normal equations according to the method of Searle (1971).

All ovarian and uterine data were analyzed by least-squares analysis of variance using the following model:

$$I_{ijk} = t_i + p_j + (tp)_{ij} + e_{ijk}$$

where:

Y_{ijk} = dependent variable

t_i = treatment

p_j = period of sampling

e_{ijk} = residual error

The dependent variables analyzed were ovarian weight, number of CLs, CL LH/hCG binding affinity and capacity, and uterine proteins. Simple correlation estimates were determined among those variables to detect relationships.

CHAPTER IV

RESULTS

I. PLASMA CORTISOL AND PROGESTERONE LEVELS

Experiment One

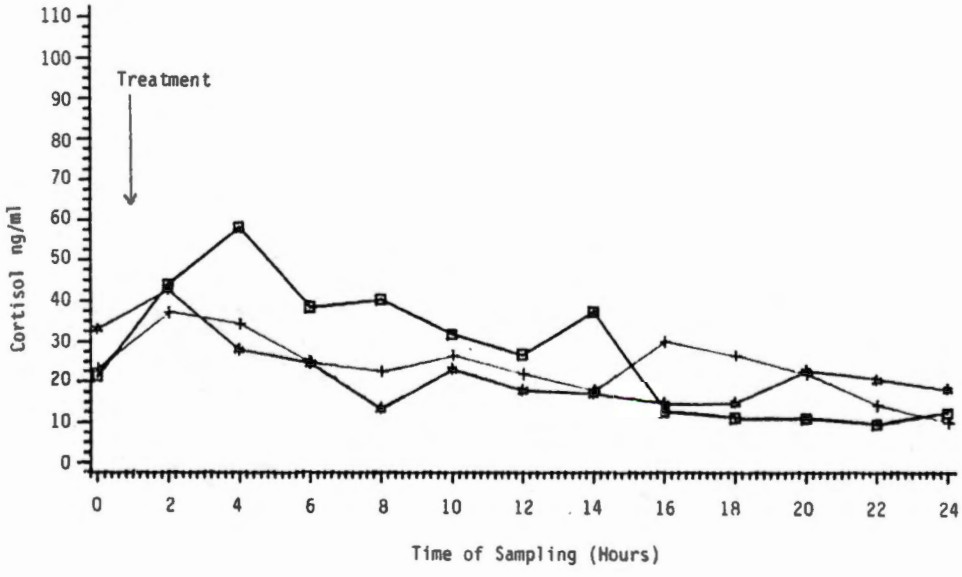
Plasma cortisol levels in control, hydrocortisone acetate (HA) and adrenocorticotropin (ACTH) treated gilts measured over a 24 hour period are depicted in Figure 8. No significant difference in mean total area ($\bar{X} \pm$ SEM) under the curve was noted in response to a single injection of HA (2871.6 $\pm$ 59.4 mm^2), ACTH (3598.1 $\pm$ 639.1 mm^2) or two injections of HA (2572.5 $\pm$ 932.6 mm^2) when compared to values determined for once injected (3157.2 $\pm$ 289.0 mm^2) or twice injected (2273.4 $\pm$ 250.2 mm^2) control animals. Two injections of ACTH significantly ($P<.05$) elevated the cortisol response curve (7854.4 $\pm$ 108.9 mm^2) above that of the twice injected controls.

Experiment Two

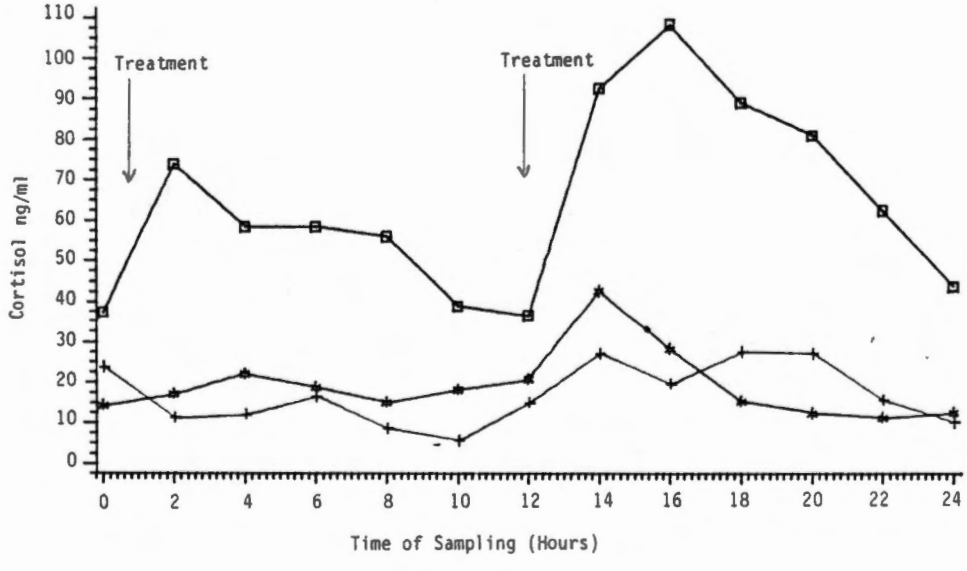
Daily plasma cortisol levels for bled control, HA and ACTH treated gilts during days 2 through 7 (period one) and days 8 through 13 (period two) are shown in Figures 9, 10, and 11, respectively. Cortisol levels for bled control animals (Figure 9) treated with sesame oil and 14% gelatin were randomly scattered over both six day bleeding periods with the points best illustrated ($P<.10$) in

FIGURE 8. Plasma cortisol concentrations of gilts treated with hydrocortisone acetate, adreoncorticotropin or 14% gelatin and sesame oil once (A) or twice (B) over a 24 hour period.

Treatment for both (A) and (B) is: □ = adrenocorticotropin; * = hydrocortisone acetate; + = control (14% gelatin and sesame oil).

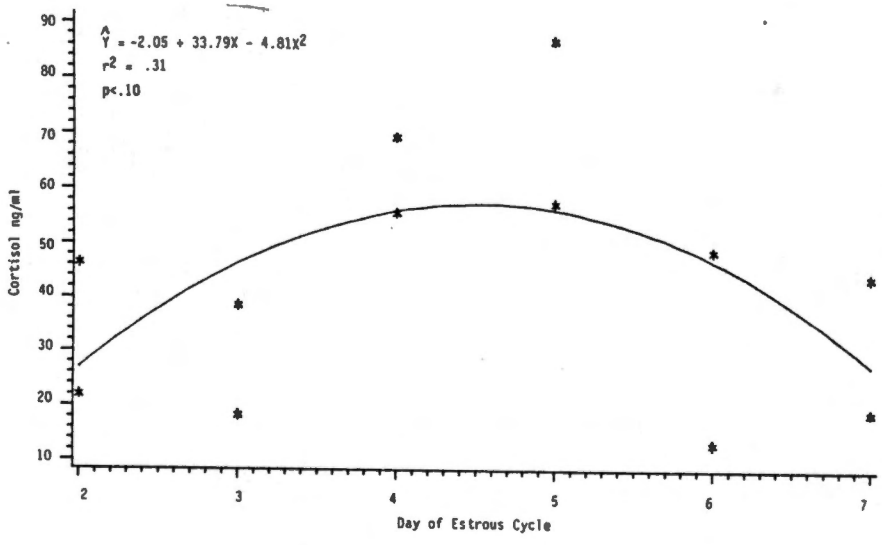


A. Single Injected

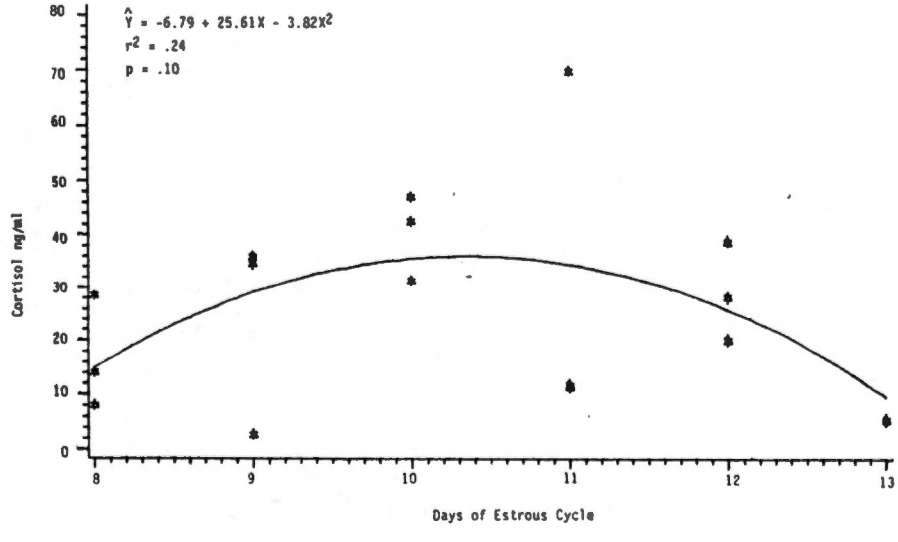


B. Twice Injected

FIGURE 9. Plasma cortisol levels of gilts treated twice daily with sesame oil and 14% gelatin during Period 1 (days 2 through 7) or period 2 (days 8 through 13) of the estrous cycle.

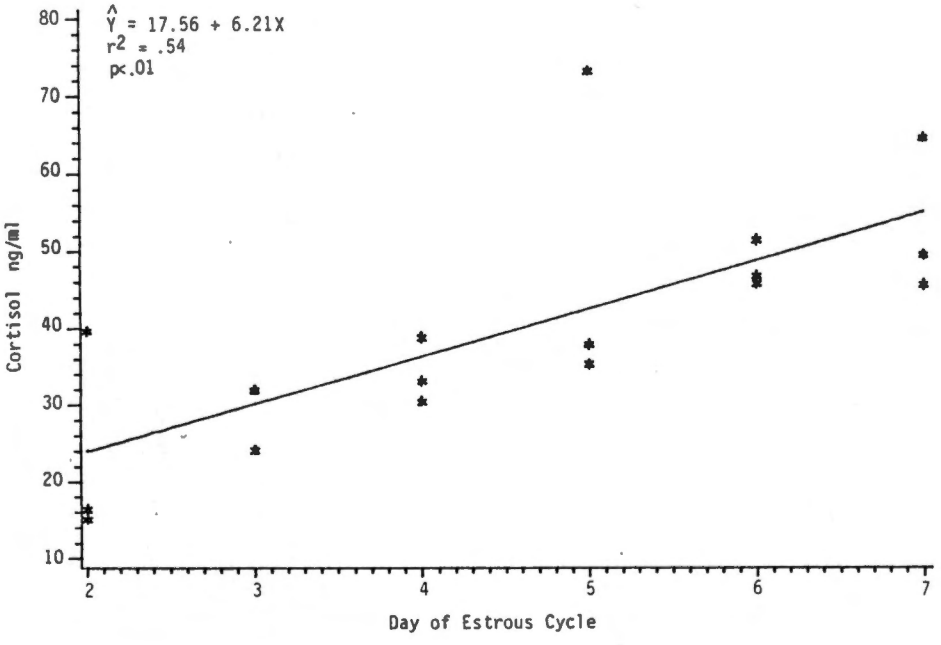


A. Period 1

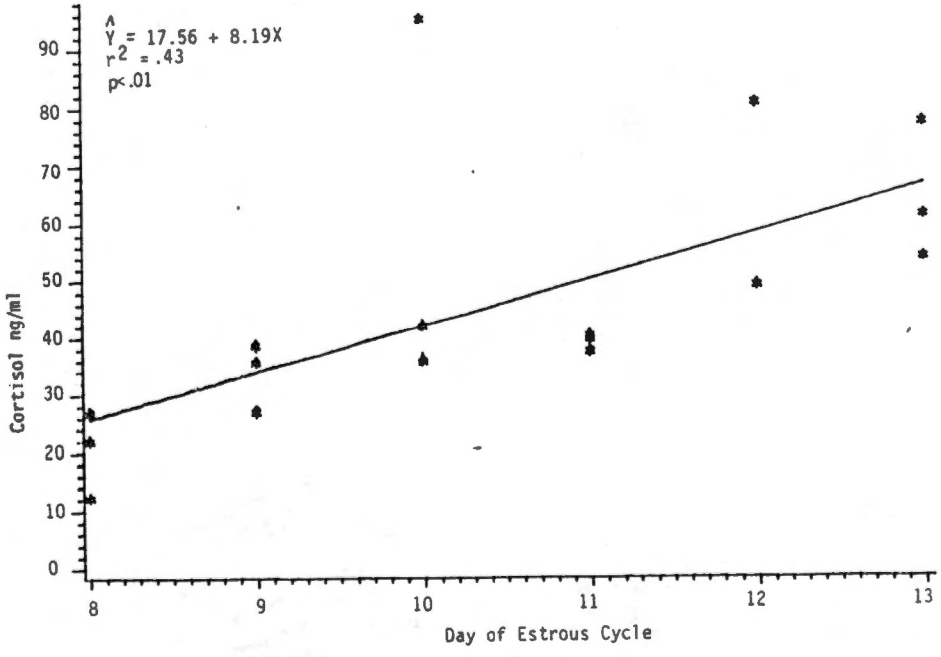


B. Period 2

FIGURE 10. Plasma cortisol levels of gilts treated twice daily with hydrocortisone acetate during Period 1 (days 2 through 7) or Period 2 (days 8 through 13) of the estrous cycle.

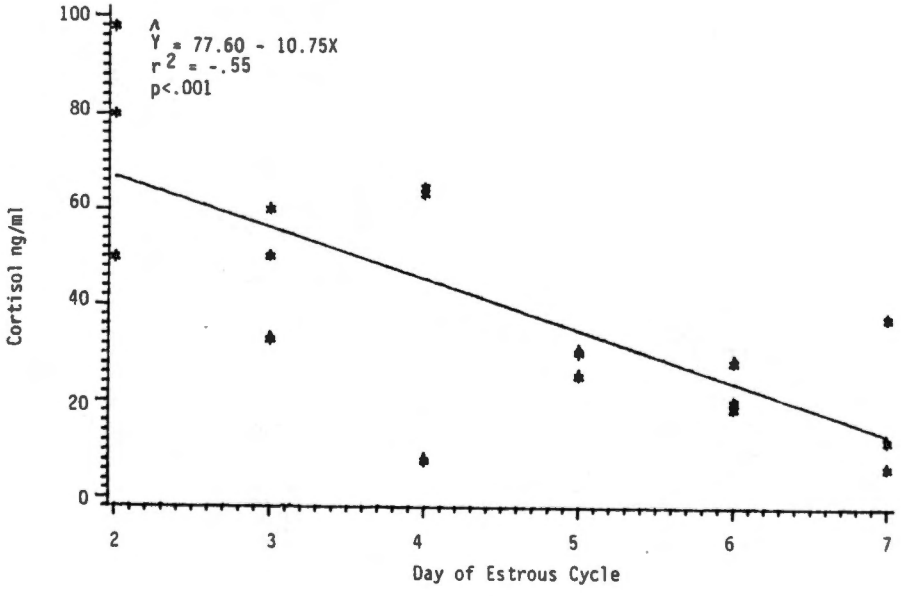


A. Period 1

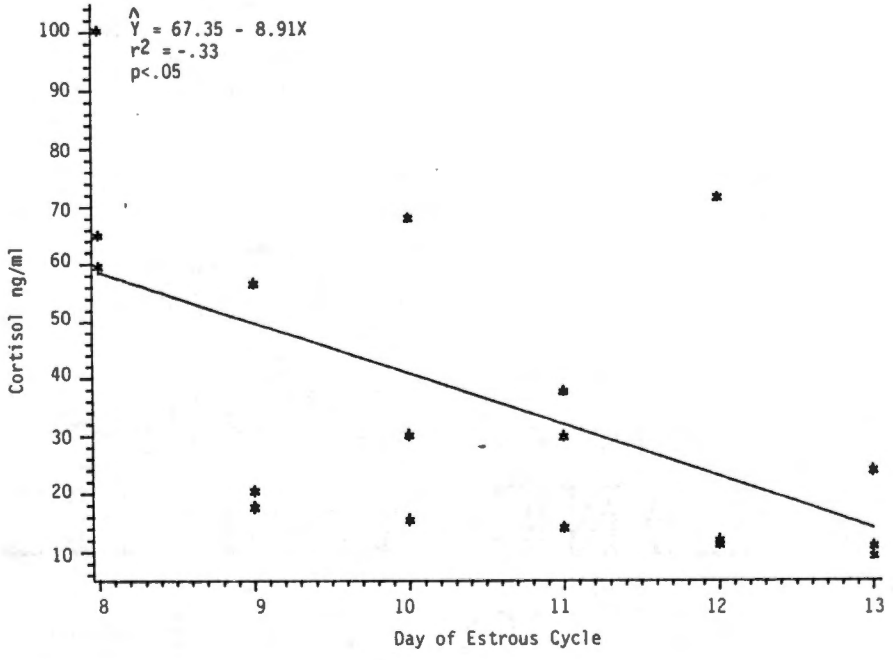


B. Period 2

FIGURE 11. Plasma cortisol levels of gilts treated twice daily with adrenocorticotropin during Period 1 (days 2 through 7) or Period 2 (days 8 through 13) of the estrous cycle.



A. Period 1



B. Period 2

a quadratic fashion. Only 24% (R^2 x 100) of the sample variation was explained by quadratic regression over the days of treatment with an overall mean ($\bar{X} \pm$ SEM) plasma cortisol concentration of 35.07 $\pm$ 18.77 ng/ml.

Plasma cortisol values of gilts treated with HA are depicted in Figure 10. Cortisol levels increased in a significantly linear fashion during both period one ($P<.001$) and period two ($P<.005$). For both periods of treatment cortisol levels increased from approximately 20 ng/ml to 55 ng/ml from the first to last day of treatment.

Adrenocorticotropin treatment resulted in a significant linear decline in plasma cortisol levels during period one ($P<.001$) and period two ($P<.05$) of treatment (Figure 11). Levels regressed from approximately 60 ng/ml on the first day of treatment to 20 ng/ml on the final day of treatment for both periods.

The result of analysis of variance of plasma cortisol values is shown in Table 2. Means were different ($P<.001$) between HA and ACTH treatments, although the sum of the hormone treatments did not vary significantly from the control. Treatment means were not different between periods and no interaction with period was apparent. Hydrocortisone acetate treatment resulted in different ($P<.001$) linear cortisol responses on the days of treatment when compared to those of ACTH. However, the sum of the cortisol responses of HA and ACTH treatment on days was not different from that of the control. These results indicate that both HA and ACTH treatment resulted in

TABLE 2. Analysis of Variance of Plasma Cortisol and Progesterone

Source	Plasma Cortisol (ng/ml) df	MS	Plasma Progesterone (ng/ml) df	MS
Treatment	2	10918.23***	2	271.52
Control vs HA, ACTH	1	497.55		
HA vs ACTH	1	10417.24***		
Period	1	426.86	1	1615.59***
Treatment x Period	2	252.22	2	117.28
Days				
Linear	1	147.41	1	1723.23***
Quadratic			1	755.11**
Treatment x Days				
Linear	2	15220.44***	2	207.66***
Control vs HA, ACTH	1	166.32	1	4.08
HA vs ACTH	1	15036.75***	1	201.00
Quadratic			2	240.03
Period x Days				
Linear	1	151.25	1	459.33
Quadratic			1	142.68
Treatment x Period x Days				
Linear	2	21.32	2	505.05†
Quadratic			2	726.83*
Control vs HA, ACTH			1	725.11***
HA vs ACTH			1	5.55
Residual	86	29741.85	73	6516.93
R^2		.39		.67

***p<.001.

**p<.01.

*p<.05.

†p<.10.

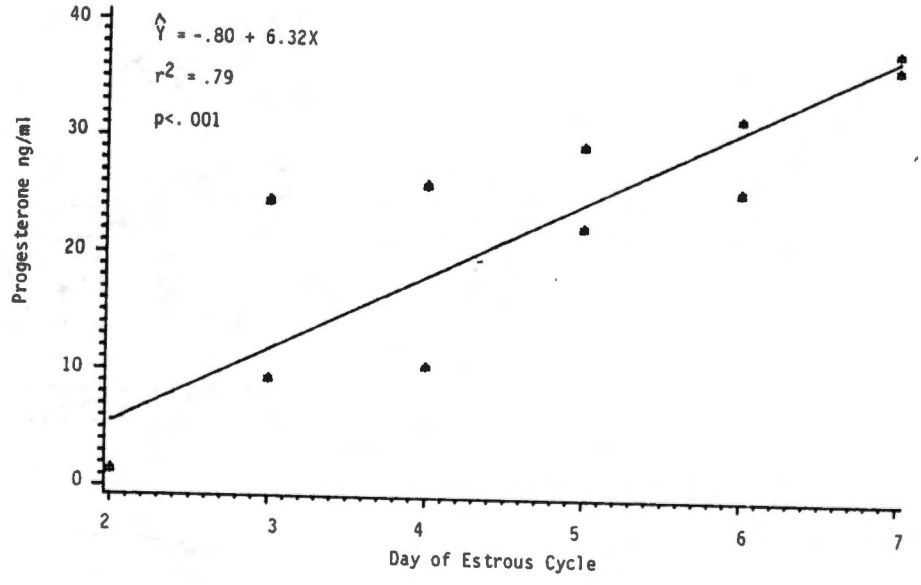
significantly different cortisol response curves over days of treatment, compared to each other and each treatment compared to the control.

Plasma progesterone levels over day of treatment for both period one and two are illustrated in Figures 12, 13, and 14. Progesterone values measured in control animals during period one increased ($P<.001$) in a linear fashion from initial levels of approximately 5 ng/ml (day 2) up to 40 ng/ml (day 7) (Figure 12). One of the original three control animals assigned to period two was eliminated from this study because of the presence of cystic ovaries. Progesterone values of the remaining two gilts over the second period of treatment were best represented ($P<.01$) in a quadratic fashion. Peripherally measured plasma progesterone peaked at approximately 45 ng/ml plasma on day 11, followed by a rapid decline to approximately 15 ng/ml on day 13.

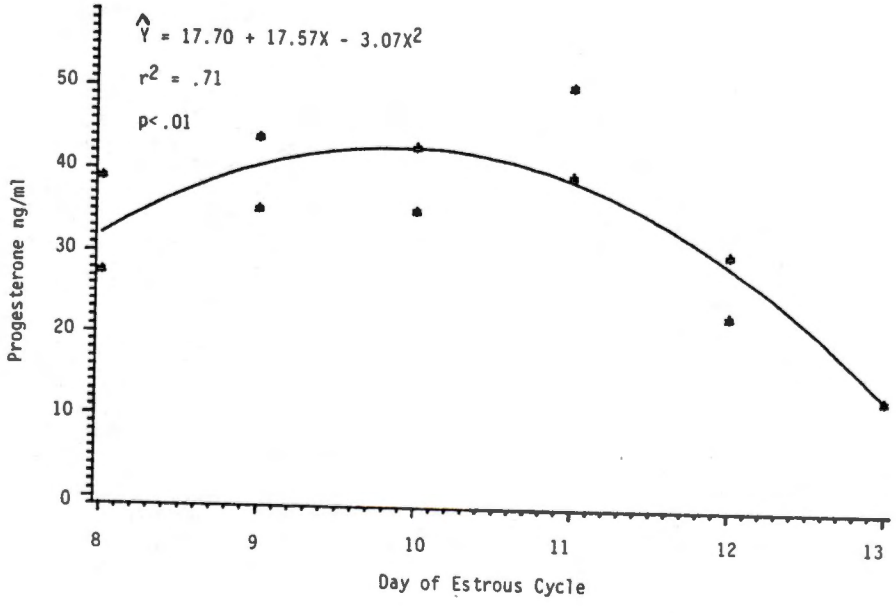
Progesterone levels of animals treated with HA during period one and two are shown in Figure 13. There was a significant ($P<.001$) linear increase in plasma progesterone levels over days of sampling for period one from an initial value of approximately 10 ng/ml (day 2) to 40 ng/ml (day 7). A similar linear increase ($P<.05$) in plasma progesterone was seen during the second treatment period, with values increasing from approximately 25 ng/ml (day 8) to 40 ng/ml (day 13).

Adrenocorticotropin treatment of gilts (Figure 14) during period one resulted in a significant ($P<.001$) linear increase in

FIGURE 12. Plasma progesterone levels of gilts treated twice daily with sesame oil and 14% gelatin during period 1 (days 2 through 7) or period 2 (days 8 through 13) of the estrous cycle.

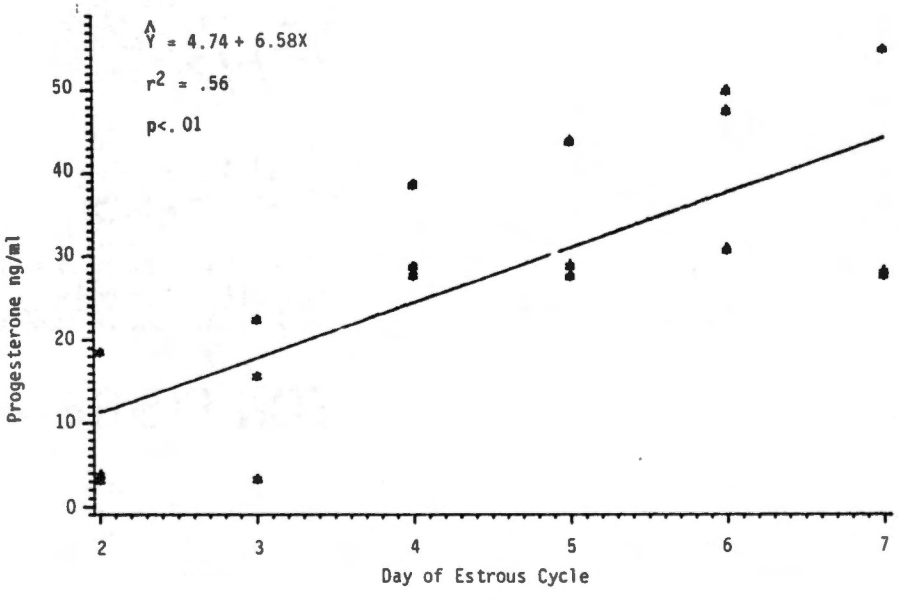


Period 1

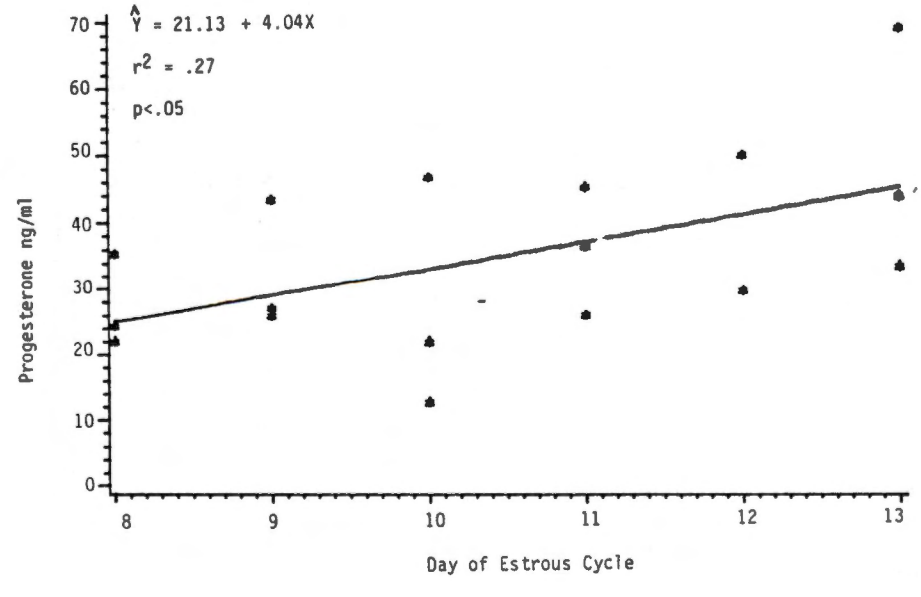


Period 2

FIGURE 13. Plasma progesterone levels of gilts treated twice daily with hydrocortisone acetate during period 1 (days 2 through 7) or period 2 (days 8 through 13) of the estrous cycle.

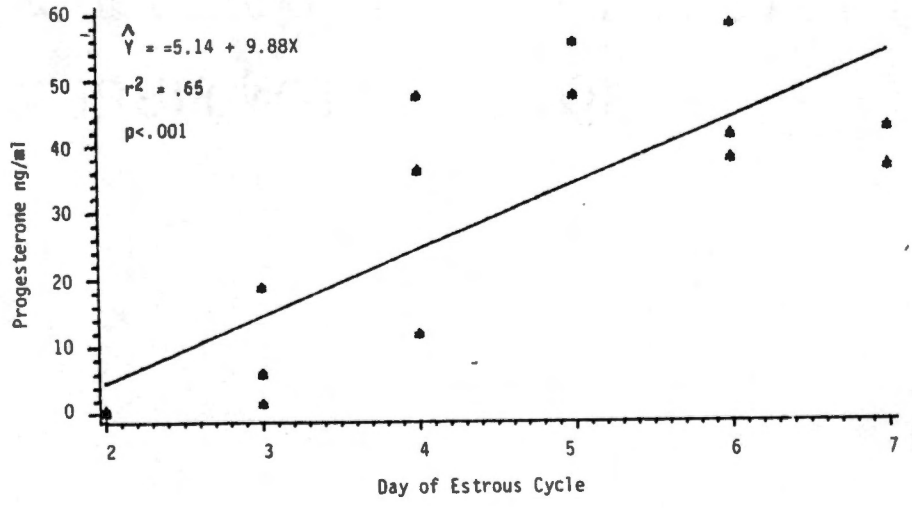


Period 1

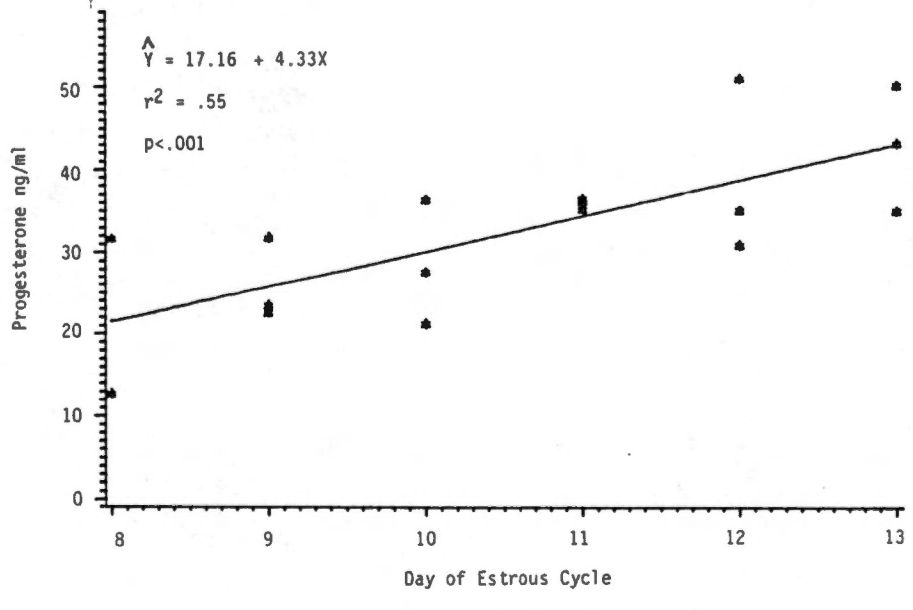


Period 2

FIGURE 14. Plasma progesterone levels of gilts treated twice daily with adrenocorticotropin during period 1 (days 2 through 7) or period 2 (days 8 through 13 of the estrous cycle.



Period 1



Period 2

progesterone levels from approximately 5 ng/ml (day 2) to 50 ng/ml (day 7). The progesterone response measured in ACTH treated gilts during period two also demonstrated a significant ($P<.001$) linear increase. Progesterone values increased from approximately 20 ng/ml (day 8) to 40 ng/ml (day 14).

Analysis of variance of plasma progesterone levels is shown in Table 2. The quadratic regression of progesterone levels over days and the interaction of that effect with treatment and period were significant ($P<.05$). Contrasts between HA and ACTH treatments within the treatment x period x quadratic regression on days indicate a significant ($P<.001$) difference.

Since periods were different ($P<.001$), least squares analysis of variance and contrasts were performed within each period (Table 3). Treatment differences were not significant in period one and they did not interact with either linear or quadratic regressions on days. Treatment was not significant in period two, but treatments did interact with quadratic regression on days, the sum of the progesterone response of HA and ACTH treated animals differing ($P<.05$) from that of the control. The progesterone response curve of HA compared to ACTH treatment did not differ with quadratic regression on days. These results indicate that the quadratic regression of progesterone levels on days for HA and ACTH treatments (within period two) were similar, with each treatment demonstrating a linear response different from the quadratic response of the control.

TABLE 3. Analysis of Variance of Progesterone by Period

Source	Period 1 df	Period 1 MS	Period 2 df	Period 2 MS
Treatment	2	318.79	2	90.74
Days				
Linear	1	2054.15***	1	194.66
Quadratic	1	809.68		
Treatment x Days				
Linear	2	371.11	2	340.23
Quadratic	2	266.55	2	658.00*
Control vs HA, ACTH			1	597.85*
HA vs ACTH			1	49.58
Residual	37	3410.82	36	3106.12
R^2		.74		.46

***p<.001.

*p<.05.

II. CORPORA LUTEA, TOTAL UTERINE PROTEINS AND LH/hCG RECEPTOR BINDING CHARACTERISTICS

There were no significant differences between the nonbled and bled control animals for any of the parameters (number and weight of corpora lutea (CL's), total uterine proteins, LH/hCG receptor binding affinity and capacity) measured within either period of sampling. Therefore, the two controls were pooled for each period and measurement, with all comparisons to the hormone treated animals based upon the pooled controls.

Corpora Lutea Number, Weight, and Total Uterine Proteins

Results of least squares analysis of variance of CL's number and weight are presented in Table 4. There were no significant differences due to treatment, period of sampling, or interactions of these independent variables for either CL's number or weight. Least square means of CL's data for HA or ACTH treated and pooled control animals are shown in Table 5. The overall mean ($\bar{X} \pm$ SEM) number of CL's observed per animal was 14.10 $\pm$ 1.08 (n = 22) and the average weight of the CL's analyzed was 910.35 $\pm$ 100.74 mg (N = 54).

Period of sampling ($P<.001$) and the interaction of period and treatment ($P<.05$) were different for measured total uterine protein (Table 4). Uterine proteins were greater ($P<.05$) for gilts in period two compared to period one for all treatments except ACTH treated gilts (Table 5). The overall mean ($\bar{X} \pm$ SEM) uterine protein

TABLE 4. Least Squares Analysis of Variance of Number of Corpora Lutea (CL), Weight of CL, Total Uterine Proteins, and Luteinizing Hormone/Human Chorionic Gonadotropin (LH/hCG) Receptor Binding Characteristics of Porcine Corpora Lutea

	Number of CL		Weight of CL		Total Uterine Proteins		LH/hCG Receptor Characteristics			
							Binding Affinity		Binding Capacity	
Source	df	MS	df	MS	df	MS	df	MS	df	MS
Treatment	3	30.25	3	.0030	3	65.04	3	1.408	3	.706
Period of Sampling	1	5.79	1	.0617	1	514.60***	1	6.774**	1	69.68**
Treatment x Period	3	32.17	3	.1386	3	79.27*	3	.830	3	8.808
Residual	14	172.50	14	1.486	14	106.10	14	7.254	14	64.184
R^2		.28		.11		.87		.58		.58

*** $p<.001$.

** $p<.01$.

* $p<.05$.

TABLE 5. Number of Corpora Lutea (CL), Weight of CL, Total Uterine Proteins, and Luteinizing Hormone/Human Chorionic Gonadotropin (LH/hCG) Binding to Porcine Luteal Receptors From Gilts on Day 8 or 14 of the Estrous Cycle

Treatment	Day of Estrous Cycle	Number of Gilts	Number of CL	Weight of CL (mg)	Total Uterine Proteins (mg)	LH/hCG Receptor Characteristics	
						Binding Affinity ($\times 10^{10} M^{-1}$)	Binding Capacity ($\times 10^{-10} M/CL$)
Controls:							
Nonbled	8	3	14 ± 2[a]	985 ± 188	11.07 ± 1.59[c]	3.11 ± .42	1.36 ± 1.28
	14	3	17 ± 2	836 ± 188	24.71 ± 1.59[d]	2.06 ± .42	5.85 ± 1.28
Bled	8	2	17 ± 3	769 ± 230	12.73 ± 1.95[c]	2.61 ± .51	2.83 ± 1.57
	14	3	12 ± 2	1027 ± 230(n=2)[b]	20.97 ± 1.95(n=2)[d]	1.98 ± .51(n=2)	4.54 ± 1.57(n=2)
Pooled	8	5	15 ± 2	899 ± 141	11.73 ± 1.26[c]	2.91 ± .31	1.95 ± .96
	14	6	15 ± 2	912 ± 141	23.21 ± 1.26[d]	2.03 ± .31	5.33 ± .96
Hormone injected:							
Hydrocortisone Acetate	8	3	15 ± 2	796 ± 188	13.70 ± 1.59[c]	2.98 ± .42	2.06 ± 1.28
	14	3	13 ± 2	1012 ± 188	26.54 ± 1.59[d]	1.23 ± .42	5.27 ± 1.28
Adrenocortico-tropin	8	3	13 ± 2	877 ± 188	13.30 ± 1.59[c,e]	2.48 ± .42	1.36 ± 1.57(n=2)
	14	3	12 ± 2	982 ± 188	17.87 ± 1.59[e]	1.39 ± .42	6.79 ± 1.28

[a]Least square mean ± standard error of the mean.

[b]n = number of gilts.

[c,d,e]Least square means within a column which have different letter superscripts are significantly different ($p<0.05$). Comparisons between days of estrous cycle occur only within treatments. Hormone treatments are compared only to the pooled control.

concentration for period one and two was 12.70 ± .84 mg and 22.52 ± .84 mg, respectively. Adrenocorticotropin treated gilts of period two had lower ($P<.05$) total uterine proteins (17.87 ± 1.59 mg) than the pooled control animals (23.21 ± 1.26 mg).

No difference ($P>.05$) in total uterine protein concentration was detected between treatments for animals measured in period one. Also there was no difference in total uterine protein concentration between the HA and pooled control animals within period two.

LH/hCG Receptor Characteristics

Initially six CL's, three per ovary, from three randomly selected animals were analyzed for LH/hCG receptor binding affinity and binding capacity. Comparisons of the binding affinity and capacity of CL's of each ovary demonstrated that there was no significant difference in these parameters between ovaries of each animal (Table 6). Based on these results, evaluation of binding capacity and affinity for all animals in experiment two were performed using two randomly selected CL's, one from each ovary. Corpora lutea weight ranged from .368 to 1.572 g and was found not to be correlated with either day 8 or 14 of the estrous cycle (Table 7). Therefore, binding capacity was expressed on a per corpus luteum (CL) basis.

Both LH/hCG receptor binding capacity and affinity of CL's differed significantly ($P<.01$) between periods of sampling. The overall mean ($\bar{X}$ ± SEM) binding capacity measured on day 8 (1.902 ± .717 x 10^{-10} M/CL) was significantly ($P<.005$) lower than that measured on day 14 (5.616 ± .680 x 10^{-10} M/CL). Conversely, the

TABLE 6. Luteinizing Hormone/Human Chorionic Gonadotropin Receptor Binding Affinity and Capacity of Homogenates of Six Corpora Lutea From Three Gilts

Gilt No.	Binding Capacity ($x10^{-11}M$)		Binding Affinity ($x10^{10}M^{-1}$)	
	Left Ovary	Right Ovary	Left Ovary	Right Ovary
89-3	1.8313	2.0359	2.2548	2.2446
	1.5432	1.3793	2.4063	2.7592
	2.0357	1.2003	2.1267	2.8587
5-2	3.7670	4.2624	1.1028	1.1061
	2.4557	2.6595	2.7233	2.5460
	4.2287	3.4690	1.9950	2.0162
20-6	1.1184	.9559	1.5223	1.3529
	1.2390	1.2727	2.3752	1.4754
	.9822	.8941	1.4837	2.0469

TABLE 7. Correlation Coefficients of the Dependent Variables Corpora Lutea (CL's) Number, CL Weight, Total Uterine Proteins, and Binding Capacity and Affinity of Porcine Luteal Luteinizing Hormone/Human Chorionic Gonadotropin (LH/hCG) Receptors on Day 8 and 14 of the Estrous Cycle

Parameter	CL Weight	Total Uterine Proteins	Binding Capacity	Binding Affinity
CL No.	-.5286†(8) -.5566†(14)	-.3232(8) .1632(14)	-1920(8) -.1602(14)	.5241†(8) .2470(14)
CL weight		-.4634(8) -.2587(14)	.0824(8) .1087(14)	.0581(8) -.2515(14)
Total uterine proteins			.5498(8) .1087(14)	-.6966*(8) -.2515(14)
Binding capacity				-.6606*(8) -.621688(14)

*p<.05.

†p<.10.

overall mean binding affinity was greater ($P<.005$) on day 8 ($2.794 \pm .220 \times 10^{10}$ M^{-1}) as compared to day 14 ($1.667 \pm .220 \times 10^{10}$ M^{-1}). There were no significant differences due to treatment for either CL LH/hCG receptor parameter as analyzed within either period. However, within period two there was a trend ($P<.10$) for binding affinity of the HA and ACTH treated animals (1.23 and 1.39 $\pm$ .42 $\times$ 10^{10} M^{-1}, respectively) to be lower than that of the pooled controls ($2.03 \pm .31 \times 10^{10}$ M^{-1}).

CHAPTER V

DISCUSSION

I. PLASMA CORTISOL AND PROGESTERONE RESPONSE

Experiment One

The results obtained in this experiment indicate that twice daily injection of adrenocorticotropin (ACTH) results in a more sustained elevation in plasma cortisol levels over 24 hours when compared to control treated animals. The cortisol levels obtained following ACTH administration are comparable to those seen in swine subjected to acute heat stress (Marple et al., 1972a).

Hydrocortisone acetate (HA) administration did not significantly elevate plasma cortisol levels over the 24 hour period. However, the approximate two-fold increase in cortisol following the second injection of HA (Figure 8, page 44) suggests that there was some response to the second injection. Barb et al. (1982) found that two 250 mg injections (10 hours apart) of HA in corn oil per gilt (no weight or age given) resulted in an initial two-fold increase followed by a gradual linear increase in plasma cortisol over 24 hours. In the present study 2 mg HA/kg body weight was administered to each gilt, with weights ranging from 111 to 133 kg (121.8 $\pm$ 22.9 kg; $\bar{X} \pm$ SEM). The carrier used in this study (sesame oil) and that used by Barb et al. (1982) (corn oil) may have influenced

the release rate of HA in a dissimilar fashion resulting in differences in the time of elevation of cortisol levels observed in these two studies.

Two of the four gilts used in this study were highly excitable and did not appear to become accustomed to the bleeding procedure. Cortisol levels may have been elevated by this behavior. The wide variation seen between individual animal 24 hour cortisol response curves in response to the control treatment (areas under the curve ranging from 2141 mm^2 to 3169 mm^2) may be indicative of these behavioral differences. Such elevations in cortisol levels may have masked any cortisol increase elicited by HA injection.

Based on the results of this experiment, twice daily injection of the hormone preparations was chosen as a means to elevate plasma cortisol concentrations in experiment two of this study.

Experiment Two

The overall mean ($\bar{X} \pm$ SEM) plasma cortisol concentration of the control animals (33.91 $\pm$ 4.03 ng/ml), with only 24% of sample variation being explained by quadratic regression over days of treatment (Figure 9, page 46), suggests that the sesame oil and 14% gelatin alone did not elicit a specific cortisol response. The wide range of plasma cortisol concentrations (2.7 to 86.7 ng/ml) was probably due to the unavoidable stress of handling and restraining of the animals during injection and bleeding. Cortisol increases of this nature are transitory, however, as can be seen by the

majority of samples collected having cortisol concentrations close to the overall mean.

The approximate three-fold linear ($P<.05$) increase in cortisol levels (20 ng/ml to 55 ng/ml) over the days of treatment in response to HA injections suggests that repetitive HA injection results in an accumulation of circulating cortisol. This linear increase over the days of treatment is similar to that observed by Barb et al. (1982) when HA in corn oil was administered to gilts twice daily for five days. The magnitude of change in cortisol concentrations in the present study over the days of treatment is similar to that reported during acute heat stress (32 C) of gilts (Scholten and Liptrap, 1978).

Adrenocorticotropin administration, on the other hand, increased cortisol levels to 60 ng/ml approximately nine hours after the first morning injection, but afterwards cortisol levels decreased in a linear ($P<.05$) fashion to approximately 20 ng/ml on the last day of treatment (Figure 11, page 50). A similar decrease in cortisol response was reported by Aberle et al. (1974). In their study, initial exposure of cycling gilts to elevated temperatures (35C) increased plasma cortisol levels; however, a second exposure several days later did not elicit a similar adrenal response. Marple and coworkers (1972a,b) also found that high temperature and humidity raised and maintained ACTH levels for several days, causing plasma corticosteroid levels to eventually fall below that of normal circulating levels.

Reduced plasma cortisol concentrations following repetitive ACTH treatment may result from desensitization of adrenal gland steroidogenic capacity due to over stimulation by ACTH. This is thought to be the case for patients with Addison's disease, who have very high circulating levels of ACTH but depressed cortisol levels (Besser et al., 1971). As stated by Collins and Weiner (1968), exposure to elevated temperatures leads to increased ACTH levels while adaptation involves reduced responsiveness by the adrenals and thus a reduction in glucocorticoid secretion. There is also the possibility that adrenal secretion rates in response to ACTH treatment are maintained but plasma clearance of cortisol is increased. Metabolic clearance rate of cortisol appears to be related to the level of circulating corticosteroid binding globulin (CBG); a high affinity, low capacity alpha globulin produced by the liver (Westphal and Devenuto, 1966; Westphal, 1967). Cortisol has been shown to be metabolized at a slower rate (Mills et al., 1960; Sandberg and Slaunwhite, 1963) and to be biologically inactive (Slaunwhite et al., 1962) when bound to CBG. Marple et al. (1972a,b) first suggested that prolonged heat stress may alter the metabolic clearance rate of corticosteroids. Kattesh and coworkers (1980) found that heat stressed ($\geq$28 C for 50 days during mid-gestation) pregnant gilts exhibited lower plasma glucocorticoid and CBG levels, suggesting that stress-acclimated swine maintain lower levels of glucocorticoids and metabolize the adrenal steroids at a faster rate. The decrease in plasma cortisol levels over six days of ACTH treatment seen in

this study may be related to a reduction in circulating CBG levels. Adrenocorticotropin treatment over long periods of time has been demonstrated to lower CBG levels (Acs et al., 1972) presumably by decreasing thyroid function via inhibition of thyrotropin-releasing hormone (TRH) release (Wilber and Utiger, 1969). CBG biosynthesis, at least in the rat, is under the control of thyroxin (Gala and Westphal, 1966; Burton and Westphal, 1972). Adrenocorticotropin treatment may possibly reduce thyroxin production which reduced CBG production, thus increasing clearance rate of cortisol from the circulation.

The cross-reactivity of the cortisol antibody used in this study with 17α-hydroxyprogesterone (31.8%) and progesterone (5.1%) may have resulted in erroneous estimation of plasma cortisol levels. There were no significant differences in cortisol levels due to period for individual treatments, despite the elevated progesterone levels of the second period. If significant cross-reactivity had occurred, the cortisol levels of the second period would have been elevated in accordance with heightened progesterone levels observed during this stage of the estrous cycle. Other progestins (such as 17α-hydroxy progesterone) appear to contribute little to total progestin levels in swine, with progesterone being the primary circulating form (Henricks et al., 1972). Since the cortisol antibody used in this study has only a 5.1% cross-reactivity with progesterone, over estimation of peripheral plasma cortisol levels relative to progesterone concentration appears unlikely.

Plasma progesterone levels of control treated gilts over period one (days 2 through 7) and period two (days 8 through 13) of this study were similar to those observed by Guthrie and coworkers (1972). Plasma values increased linearly from 5 ng/ml to 40 ng/ml within the first period. Progesterone concentrations of gilts measured over the second period remained high (approximately 45 ng/ml) from day 8 through day 11, rapidly declining to 15 ng/ml on day 13. The pattern of progesterone levels over the two periods is very similar to that found in swine by Masuda et al. (1967). The results of this study indicate that corpus luteum (CL) function as assessed by progesterone production was unaffected by injection of hormone carrier solutions or as a result of the handling procedure.

Treatment with HA or ACTH during period one did not alter circulating progesterone levels when compared to the controls. However, HA and ACTH treatments during period two significantly ($P<.05$) deviated the pattern of progesterone change compared to the control with progesterone levels increasing in a linear fashion over days 12 and 13. Progesterone levels measured on day 8 appeared to be depressed (25 ng/ml) compared to the controls (45 ng/ml) and peak progesterone levels were not reached until day 13 of the estrous cycle for HA treated gilts.

The increase in plasma progesterone of HA treated gilts during the second period suggests that the corpora lutea (CLs) are still functioning. Cortisol administration may be retarding the development of full steroidogenic capacity of the ovary, given the gradual rise to peak progesterone levels on day 13 rather than

day 11 of the estrous cycle. Liptrap (1970) demonstrated that corticosteroids administered daily to gilts from day 14 until the onset of estrus delayed the onset of estrus by one to three days and shortened its duration. Perhaps elevated progesterone levels, due to an elevation in corcicosteroid concentration, were responsible for this delay in estrus, since Coryn et al. (1979) demonstrated that ovulation in swine can be expected within four days after serum progesterone concentrations have fallen below 5 ng/ml.

Plasma progesterone levels may be increased by HA treatment as a result of a decrease in the rate of clearance from the circulation. Transcortin, or CBG, is known to have a greater binding affinity for progesterone than cortisol (Rosenthal et al., 1968; Migeon et al., 1968). Since cortisol exerts a negative feedback on ACTH release, ACTH levels may have been lowered in response to elevated cortisol levels seen in the HA treated gilts of the present study (Figure 10, page 48), in turn allowing increased TRH/thyroxin release with a resultant increase in CBG production by the liver (Gala and Westphal, 1966; Burton and Westphal, 1972). Thus, the gradual increase in plasma progesterone may be due to retention of progesterone in the circulation by binding to the CBG rather than by increased CL production.

The sum of the progesterone secretion pattern of gilts treated with ACTH or HA during period one did not differ significantly from that of the control animals of the same period. Adrenocorticotropin treated gilts demonstrated the same trend of linear increase in progesterone levels within period two as did HA treated gilts.

Scholten and Liptrap (1978) found that treatment of ovariectomized gilts with ACTH resulted in increased progesterone levels, presumably of adrenal origin. However, in this study ACTH elicited progesterone response different from the controls only during period two. If the adrenal was a significant source of progesterone, then an ACTH response should have been noted during both periods of treatment. Direct evidence of action of ACTH on ovarian function of swine has not been reported. However, direct perfusion of the ovary with ACTH does not appear to alter progesterone production in the bovine (Wagner et al., 1977).

Alternatively, ACTH may be acting indirectly, perhaps at the hypothalamic level, by altering the secretion of releasing factors in a manner analogous to that previously mentioned for TRH. Prolactin, which has been shown to be increased in the peripheral circulation of heat-stressed adrenalectomized rats (Harms et al., 1975), is known to induce luteinizing hormone (LH) receptors on rat luteal cells (Holt et al., 1976). In the cycling gilt prolactin and LH surge simultaneously at estrus, with at least the LH surge attributed to the formation of corpora lutea (CL's) (Brinkley, 1981; Van Der Weil et al., 1981). These researchers also observed an additional prolactin surge, independent of the LH rise, associated with elevated estrogen levels prior to estrus. The physiological significance of low levels of prolactin during all other times of the estrous cycle is unknown. However, in a recent study prolactin addition to porcine early luteal cells in vitro was found to stimulate progesterone secretion (Uregoraszczuk, 1983).

The similar progesterone response curves among the treatments in period one in contrast to that seen in period two suggests that the actions of ACTH and HA, whether directly or indirectly predicted, may depend on the physiological state of the ovary. Apparently ACTH and HA exert their effect on progesterone production during the time when the ovary is at peak steroidogenic production. The LH receptor is a likely site of modification by ACTH and HA given its importance in mediating progesterone production by the ovary (Niswender et al., 1980). Cortisol (and/or ACTH) may modify the binding capacity and/or affinity of the CL LH receptor directly or by indirect modification through another agent secondary to ACTH or HA, such as prolactin (Bambino and Hsueh, 1981).

This study is unique in that opposite extremes of cortisol response, as has been reported to occur during the stress adaptation syndrome (Selye, 1946) (high glucocorticoid levels following acute stress and lower levels after prolonged stress with adaptation), are examined as they may alter ovarian and uterine function. In the case of progesterone production, both ACTH and HA alter normal secretory patterns in a similar manner, although the mechanism behind this alteration is probably different for each hormone. Measurement of luteal luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor binding affinity and binding capacity and total uterine proteins may give further insights into the mechanisms involved.

II. UTERINE PROTEINS AND LH/hCG RECEPTOR CHARACTERISTICS

Uterine Proteins

In agreement with the observation of Murray et al. (1972), uterine proteins measured in the present study increased from day 8 to day 14 of the estrous cycle (Table 5, page 65). This period difference was significant ($P<.05$) for all except ACTH treated gilts, where day 14 protein levels were reduced. Period one (day 8) uterine protein levels were not affected by treatment, which coincides with the observation that progesterone levels were also unaffected by treatment within this period. Apparently CL function during period one, as reflected by plasma progesterone levels and total uterine proteins, are not sensitive to the treatments used in this study. Specific uterine proteins were not investigated in this study.

Within period two, total uterine protein levels of ACTH treated gilts was significantly ($P<.05$) lower (17.87 ± 1.59 mg; $\bar{X} \pm$ SEM) than that of the pooled controls (23.21 ± 1.26 mg) and HA treated (26.54 ± 1.59 mg) animals (Table 5, page 65). In contrast, HA treatment did not alter uterine protein concentration when compared to the pooled controls. This suggests that HA treatment had no effect on uterine protein secretion. It is interesting to note that the plasma cortisol increases induced by HA administration were gradual, not exceeding control plasma cortisol levels (33.91 ± 4.03 ng/ml) until approximately the fourth day of treatment in both periods (Figure 10, page 48). Adrenocorticotropin

administration, on the other hand, elevated plasma cortisol levels approximately twice that of the control levels on the first day of treatment, thereafter declining to levels similar to that of the controls (Figure 11, page 50). Perhaps the acute cortisol response following ACTH administration (versus the gradual response seen following HA) was, at least in part, responsible for the depression in total uterine proteins observed in the ACTH treated animals.

Progesterone response curves were similar for HA and ACTH treated animals during period two. However, total uterine protein levels measured in these animals were affected differently by treatment. Administration of progesterone to increase circulating levels above normal has been found to reduce the amount of uterine progesterone receptors during the luteal phase of the estrous cycle in the rat (Walters and Clark, 1980), guinae pig (Milgrom et al., 1973; Feiland Bardin, 1975), and hamster (Leavitt et al., 1974), a phenomenon known as down-regulation. Cortisol may alter the progesterone receptor itself by altering the affinity for its agonist, as has been demonstrated for the glucocorticoid receptor of rat heart, liver, kidney, and pancreas tissue (Svec and Rudis, 1981). The low levels of cortisol in the ACTH treated gilts may have allowed progesterone to better interact (by reducing indirect cortisol inhibition of the progesterone receptor) with the progesterone receptor of the uterus. This may have resulted in down-regulation of the receptor and decreased secretion of uterine proteins. The

higher cortisol levels in the HA treated gilts may have reduced the affinity of the uterine progesterone receptor for its agonist so that no down-regulation of the uterine progesterone receptors, and thus no decrease in uterine protein secretion, occurred.

There is conflicting information regarding the characteristics of uterine endometrial steroid receptors throughout the estrous cycle in swine. Diekman and Anderson (1979) found no differences in cytoplasmic and nuclear concentrations of progesterone or estrogen receptors as determined throughout the estrous cycle of the gilt. However, Deaver and Guthrie (1980) found that the number of uterine cytoplasmic estrogen receptors increased after the onset of estrous to a maximum on day 10, followed by a rapid decline to day 16. The conflicting results of these studies need to be resolved in future experiments investigating the characteristics of the uterine progesterone receptor of swine throughout the estrous cycle and pregnancy. Once uterine progesterone receptor of swine are fully characterized, in animals under normal conditions, experiments can then be conducted to investigate factors regulating these receptors.

The necessity of establishing a proper uterine environment for maintenance of embryos has been demonstrated in swine. Knight et al (1974a) found that progesterone supplementation in swine stimulated increased uterine secretory activity and that placental weight and length (directly related to placental surface area, degree of maternal attachment and consequent embryonic survival) were increased; conversely, immunization of gilts against purple acid

phosphatase, a specific uterine protein associated with the critical time of pregnancy recognition in swine (Murray et al., 1972), significantly reduced placental weight and length (Chen and Bazer, 1973). The demonstrated increase in embryonic losses due to ACTH or corticosteroid administration in rats (Velardo, 1957; Yang et al., 1969), mice (Kittinger et al., 1980), and the ewe (Howarth and Hawk, 1968) may be associated with an aberrant uterine environment. Improper uterine environment has been implicated in increased embryonic mortality due to heat stress in sheep by Woody and Ulberg (1964). These researchers found, by reciprocal embryo transfer, that ewes maintained at 90F did not provide as favorable of a uterine environment for embryo development as did ewes maintained at 70F. Arnold et al. (1982) found that daily administration of 80 U.S.P. units of ACTH over five day periods for the first 20 days of gestation did not alter fetal survival in pregnant gilts; however, the ACTH dosage level used in this study was not believed to be sufficient to elicit a significant adrenal glucocorticoid response (H.G. Kattesh, personal communication).

Estrogen may also play a role in regulating uterine protein secretions. Walters and Clark (1978) found that estrogen stimulates the synthesis of its own receptors in the rat endometrium. Knight et al. (1974b) noted that treatment of ovariectomized and intact gilts with increasing quantities of progesterone:estrogen at a ratio of 2000:1 was negatively correlated with total uterine proteins, in contrast to injections of increasing amounts of progesterone

which increased total uterine proteins in a dose-dependent fashion. These authors suggested that elevated estradiol levels may inhibit or delay uterine protein secretion of swine. The possible interplay of estrogen and progesterone in controlling uterine secretions in swine warrants further investigation.

The present study is one of the first to demonstrate a possible relationship between ACTH and HA administration on total uterine protein production in swine. Further studies need to be designed to examine total and specific uterine protein production during early pregnancy in response to ACTH and HA treatment.

Luteal LH/hCG Receptor Binding Affinity and Capacity

The overall binding affinity of the CL LH/hCG receptor was significantly ($P<.05$) higher on day 8 ($2.794 \pm .220 \times 10^{10}$ M^{-1}; $\bar{X} \pm$ SEM) than on day 14 ($1.667 \pm .220 \times 10^{10}$ M^{-1}) of the estrous cycle. Ziecik et al. (1980) found binding affinities of porcine CL's (using ^{125}I-LH) of $2.04 \pm .52 \times 10^{10}$ M^{-1}, $1.36 \pm .07 \times 10^{10}$ M^{-1}, and $1.96 \times .16 \times 10^{10}$ M^{-1} on days 8, 12, and 14 of the estrous cycle, respectively. The binding affinities determined in the present study are comparable to those described for luteal LH receptors in the cow (Gospodarowicz, 1973; Papaionannou and Gospodarowicz, 1975) and ewe (Diekman et al., 1978).

Treatment with HA or ACTH did not significantly alter CL LH/hCG binding affinity in the present study. However, within period two (day 14) HA and ACTH showed a trend ($P<.10$) toward lower binding affinity (HA, $1.23 \pm .42 \times 10^{10} M^{-1}$; ACTH, $1.39 \pm .42 \times 10^{10}$ M^{-1})

compared to that of the pooled control ($2.03 \pm .31 \times 10^{10}$ L/M). Ziecik et al. (1978) found that CL LH binding affinity in gilts was high on day 8 and 16 of the estrous cycle (when progesterone levels are low) but were low on day 10 and 12 (when plasma progesterone concentration is at a maximum). It is interesting to note that in the present study progesterone levels were elevated for gilts treated with HA and ACTH during period two and that the corresponding binding affinity of these animals appeared to be lower than that of the controls. The relationship between binding affinity and progesterone production warrants further investigation.

Binding capacity also differed significantly ($P<.05$) between periods of sampling. As expected, the overall mean ($\bar{X} \pm$ SEM) binding capacity on day 8 ($1.902 \pm .717 \times 10^{-10}$ M/CL) was lower than that measured on day 14 ($5.616 \pm .680 \times 10^{-10}$ M/CL). The binding capacity determined in this study is actually a measurement of the number of unoccupied sites. Less than 1% of the porcine LH receptors have been reported to be occupied by LH throughout the estrous cycle and up to 30 days of pregnancy (Ziecik et al., 1980). Also, the number of unoccupied binding sites has been shown to be directly correlated with serum progesterone levels in the ewe (Diekman et al., 1978) and the heifer (Spicer et al., 1981), thus suggesting that the assessment of the number of unoccupied binding sites is a physiologically appropriate measure of CL function.

The number of unoccupied receptor sites determined in this study are similar to that reported in the ewe (Diekman et al., 1978)

and the heifer (Spicer et al., 1981) when results are expressed on a per CL basis. In the ewe there is an approximate five-fold increase in luteal LH receptor sites from day 6 to day 14 of the estrous cycle (Diekman et al., 1978), an increase similar to that observed in the present study from day 8 to 14 of the estrous cycle of swine. The porcine CL binding capacity for LH determined by Ziecik and coworkers (1980) was reported on a per mg protein basis; therefore, a comparison of the findings of these researchers and that of the present study cannot be made.

No significant ($P>.05$) differences in CL LH/hCG binding capacity, as was the case for binding affinity, were detected in response to the treatments imposed in this study. The high standard error for CL LH/hCG binding capacity may have contributed to the inability to detect significant differences between treatments. Further studies utilizing a larger number of animals needs to be conducted to account for this individual variation and thus resolve whether HA and ACTH alter CL LH/hCG binding characteristics.

An inverse relationship between CL LH/hCG receptor binding affinity and capacity was evident on day 8 ($r = -.6606$) and day 12 ($r = -.6210$) of sampling. A similar negative correlation ($r = -.6216$) between porcine CL LH receptor binding capacity and affinity was reported by Ziecik et al. (1980).

Although CL LH/hCG receptor binding affinity and capacity were not significantly altered by the treatments imposed in this study, values for binding affinity were reduced following ACTH or

HA treatment during period two as compared to the pooled controls. Glucocorticoid treatment of male rats has been demonstrated to decrease the LH/hCG binding capacity of testicular homogenates and also to decrease testicular steroidogenesis (Bambino and Hsueh, 1981). These authors did not investigate the LH/hCG receptor binding affinity of the testicular homogenates. The possible effects of ACTH or HA on porcine CL LH/hCG receptor characteristics deserve further investigation.

Although LH levels were not measured in this study, there is strong evidence suggesting a relationship of the hyperadrenal state to circulating LH levels. Li and Wagner (1983a) found that continuous infusion of ACTH (day 2 of the estrous cycle to the next extrus) decreased plasma LH concentrations during the early phase (days 3 through 5) of the estrous cycle in intact, but not adrenalectomized, heifers. Hydrocortisone succinate (HS) infusion did not appear to change mean LH concentrations, although both treatments decreased the rate of progesterone increase during mid-cycle (da Rosa and Wagner, 1981). These authors conclude that ACTH, and perhaps HS, treatment depressed plasma progesterone levels probably due to insufficient circulating LH which resulted in an incomplete development and/or function of the corpus lutum. Elevated glucocorticoid levels appear to decrease pituitary responsiveness to GnRH challenge in the intact and adrenalectomized heifer (Li and Wagner, 1983b), rat (Baldwin and Sawyer, 1974), human (Luton et al., 1977; Sowers et al., 1980), ram (Fuquay and Moberg, 1980),

and cattle (Chantaraprateep and Thibier, 1978; Matteri and Moberg, 1982). These studies suggest that LH and/or synthesis secretion may be suppressed by elevated glucocorticoid levels acting at the hypothalamic and/or pituitary level, thus altering CL function. Bambino and Hsueh (1981) found that glucocorticoid treatment of male rats decreased LH/hCG binding capacity of testicular homogenates, so sites other than the hypothalamus and pituitary may be involved in the glucocorticoid alteration of gonadal function.

There is strong evidence indicating that LH is important for embryonic survival. Kato et al. (1983) found that administration of deglycosylated hCG, a potent antagonist of hCG in vivo, to pregnant rats during days 1 through 5 or 8 through 11 inhibited implantation or terminated pregnancy. Earlier studies have demonstrated that immunizing animals against LH or LHRH during early gestation terminates pregnancy in rabbits (Spies and Quadri, 1967) and rats (Loewit et al., 1969; Madwa and Maudagal, 1970; Nishi et al., 1976). These studies indicate that some LH, even the low levels of early pregnancy, is important for CL maintenance and thus the survival of many embryos. Pherhaps elevated ACTH or glucocorticoid levels may reduce embryonic survival by reducing LH levels or by changing the binding affinity or capacity of the CL LH/hCG receptor. These possibilities deserve further investigation.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A preliminary experiment, using four six month old normally cycling gilts with surgically implanted jugular cannula, was conducted to determine the dosage of adrenocorticotropin (ACTH) and hydrocortisone acetate (HA) needed to elevate plasma cortisol above that of normal levels. Twice daily injections of ACTH (1 I.U./kg body weight in 14% gelatin, subcutaneously (SQ) resulted in a significant ($P<.05$) elevation in plasma cortisol levels compared to the control treatment (injected with carrier alone: 2 ml sesame oil, intramuscularly (IM), and 2 ml 14% gelatin,(SQ). No differences were found between HA (2 mg/kg body weight in sesame oil, IM) and control treated animals, although a second HA injection appeared to elicit a heightened cortisol response.

Based on the treatments established in the preliminary study, a second study was conducted to investigate the effects of repeated daily injection of ACTH or HA during days 2 through 7 or 8 days through 13 of the estrous cycle. Twenty-three normal cycling gilts were randomly assigned to the following treatments: (1) nonbled control, receiving no injections; (2) bled control, injected with the hormone carriers; (3) HA; and (4) ACTH, injected twice daily using dosage levels determined in the preliminary study. Plasma samples were collected daily during treatment to be analyzed for plasma cortisol and progesterone concentrations. On the day following the last

day of treatment (day 8 or 14) animals were ovariectomized and uterine flushings obtained.

Injection of ACTH over six days decreased and HA treatment increased plasma cortisol levels in a linear fashion over the days of treatment compared to the quadratic response of bled controls during both treatment periods. There were no differences within period one in the progesterone profile of the HA or ACTH treated animals compared to the controls. Within period two both ACTH and HA treated gilts demonstrated a linear progesterone response compared to the quadratic response of the bled control animals.

There were no significant differences between the nonbled and bled controls for any of the parameters measured on day 8 or 14, therefore the controls were pooled by period and all comparisons to treated animals were made relative to those pooled controls. No significant differences due to treatment were detected within period one for either total uterine proteins or corpora lutea (CL's) luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor binding affinity or capacity. Within period two ACTH treatment significantly reduced ($P<.05$) uterine proteins compared to the pooled controls, whereas HA had no effect. Overall total uterine proteins were greater ($P<.05$) in period two ($22.52 \pm .84$ mg; $\bar{X} \pm$ SEM) compared to period one ($12.70 \pm .84$ mg). Treatment with HA or ACTH did not significantly alter corpus luteum (CL) LH/hCG binding affinity or capacity within either period, but within period

two (day 14) the hormone treatments tended ($P<.10$) to reduce binding affinity (HA, $1.23 \pm .42 \times 10^{10}$ M^{-1}; ACTH, $1.39 \pm .42 \times 10^{10}$ M^{-1}) compared to that of the pooled controls ($2.03 \pm .31 \times 10^{10}$ M^{-1}). The overall mean CL LH/hCG binding affinity was greater ($P<.01$) on day 8 ($2.794 \pm .220 \times 10^{10}$ M^{-1}) than on day 14 ($1.667 \pm .220 \times 10^{10}$ M^{-1}); conversely, binding capacity on day 8 ($1.902 \pm .717 \times 10^{-10}$ M/CL) was lower ($P<.01$) than that determined on day 14 ($5.616 \pm .680 \times 10^{-10}$M/CL) of the estrous cycle. Binding affinity and capacity was inversely ($P<.05$) correlated ($R = -.748$).

These results indicate that mid- to late-diestrus is a physiological state sensitive to the HA and ACTH treatment used in this study. Total uterine proteins were reduced by ACTH treatment, which suggests that the uterine environment is altered. Since a proper uterine environment is essential for embryonic survival alteration of uterine protein production by ACTH may effect embryonic survival. In future studies the effect of ACTH and HA administration to pregnant gilts might be investigated to explore the possible relationship of uterine protein secretions and embryonic survival. Utilization of larger number of animals may clarify the effect of ACTH and HA on CL LH/hCG receptor characteristics.

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APPENDICES

APPENDIX A

PROCEDURE USED FOR CORTISOL EXTRACTION AND QUANTIFICATIONS

Preparation of Phosphate Buffered Saline (PBS-ga) Solution

1. Solution A (0.02 M)

$NaH_2PO_4 \cdot H_2O$	2.78 g
Distilled H_2O	100 ml

2. Solution B (0.02 M)

$Na_2HPO_4 \cdot 12\ H_2O$	7.16 g
or $Na_2HPO_4 \cdot\ 7\ H_2O$	5.36 g
or Na_2HPO_4 (anhydrous)	2.84 g
Distilled H_2O	1000 ml

3. Phosphate buffer (PBS-a), pH = 7.4 (0.01 M)

Sodium Azide	1.0 g
Sodium Chloride	9.0 g (1.5 M)
Solution A	65 ml
Solution B	435 mg
Distilled H_2O	500 ml

4. 0.1% gelatin in phosphate buffer (PBS-ga)

Gelatin (Knox unflavored)	100 mg (0.1 g)
PBS-a	100 ml

Preparation of Dextran-Coated Charcoal (DCC)

Charcoal (Norit A)	.625 mg (add last)
Dextran (T70 or T80)	.0625 mg
PBS-ga	100 ml

Must be chilled and stirred continuously while in use. Store at 4C.

Extraction and Preparation for Assay

1. Add .10 ul (approximately 2750 cpm or .1 ng) of 1, 2, 6, 7 - 3H(N)-hydrocortisone (New England Nuclear, Boston, MS) in ethanol to each 15 x 85 glass extraction tube and to three miniscintillation vials.

2. Dry in vacuum oven (37C). Add 4.5 ml of scintillation cocktail (Aquasol-2, New England Nuclear) to the three scintillation vials, cap and vortex.
3. Add 200 µl of unknown plasma sample and 300 µl .01 M PBS-ga (pH = 7.4) to the extraction tubes. Vortex and equilibrate 30 minutes at room temperature before extracting.
4. Add 2 ml freshly distilled ethanol to all tubes, vortex and centrifuge (4C) at 3000 x g for 10 minutes.
5. Pipette 200 µl of the supernatant into scintillation vials for recovery and 100 and 200 µl aliquots into 12 x 75 borosilicate tubes for assay.
6. Dry recoveries and unknowns in vacuum oven (37C). Add 4.5 ml of scintillation cocktail to recovery vials, cap and vortex.

Preparation of Standard Curve

1. Prepare a stock solution of cortisol containing 100 ng/ml in ethanol. Prepare one standard curve for each set of 38 assay tubes by pipetting the appropriate volume of working stock solution (100 ng/ml) into 12 x 75 borosilicate tubes to give the following concentrations: .05, .1, .25, .5, .75, 1.0, and 1.5 ng. Place two blank tubes before the standards and one following the last assay tube for each set. Dry down standards in a vacuum oven (37C).

Cortisol Assay

1. Add 500 µl of cortisol antibody diluted 1:4600 in PBS-ga to all tubes.
2. Add 100 µl of 1, 2, 6, 7, $^{-3}$H(N)-hydrocortisone (New England Nuclear), containing 10,000 cpm (.36 ng), to all tubes.
3. Vortex and incubate overnight at 4C (at least six hours).
4. Following incubation, add 500 µl dextran-coated charcoal (DCC) to all tubes except the first blank tube of each standard curve, which receives 500 µl PBS-ga. Incubate 15 minutes at 4C.

5. Centrifuge (4C at 2000 x g for 15 minutes.
6. Pipette 500 µl of supernatant into miniscintillation vial and add 4.5 ml of scintillation cocktail. Cap and shake and transfer vials to liquid scintillation counter for measurement of radioactivity.

APPENDIX B

PROCEDURE USED FOR PLASMA PROGESTERONE EXTRACTION AND QUANTIFICATION

Preparation of phosphate buffered saline -.1% bovine serum albumin (PBS-.1% BSA) buffered (10 mM, pH = 7.4)

1. To 950 ml double distilled H_2O add

K_2HPO_4	1.74 g
NaCl	8.775 g

   Stir until dissolved, then add enough double distilled H_2O to bring volume up to 1,000 ml. Adjust to pH 7.4 with potassium phosphate monobasic (KH_2PO_4).

2. Add 1 g bovine serum album (BSA fraction 5 powder:Sigma Chemical Co., St. Louis, MO) to 1000 ml of phosphate buffered saline for a .1% BSA in PBS solution. Store at 4C; use within one week.

Preparation of dextran-coated charcoal (DCC)

1. Stock solution (10x)

Charcoal (Norit A)	5 g
Dextran (T70)	0.5 g
PBS-.1% BSA	200 ml

   Store at 4C.

2. Working solution (1x)
   Stir stock solution 10 minutes on ice prior to diluting. Then dilute 10x charcoal 1:10 by pipetting 7 ml into 63 ml PBS-.1% BSA. This is enough for a 140 tube assay. Let the diluted DCC solution stir for 10 minutes on ice prior to use. Must be stirred and chilled continuously while in use.

Scintillation cocktail preparation

Scintanalyzed toluene	4 l
2.5-diphenyloxazde (PPO)	16 g
p-bis-o-methylstyryl benzene (bis-MSB)	0.32 g

A mixture of 2.5 ml toluene based counting fluor and 0.5 ml Scintanalyzed 1,4-dioxane was used as a scintillation cocktail

Extraction procedure

1. Pippette 500 µl of plasma into 16 x 150 borosilicate tubes, then add 50 µl of [1,2,6,7-^{3}H] Progesterone (Amersham Co., Arlington Heights, ILL) containing approximately 1000 cpm in PBS-.1% BSA to each tube and into three miniscintillation vials. Add scintillation cocktail to the miniscintillation vials, cap, shake, and store at 4C until counted to determine recovery.
2. Vortex tubes containing plasma and recovery solution. Add 5 ml freshly distilled hexane, vortex thoroughly and leave overnight at 25C.
3. Freeze the bottom aqueous phase in dry ice and acetone and pour off the organic phase into corresponding 12 x 75 borosilicate tubes. Air dry at 25C. Add 5 ml hexane to the thawed plasma samples vortex, freeze, and pour off organic phase into corresponding 12 x 75 tubes containing progesterone from the first extraction. Air dry and repeat the extraction with 5 ml hexane.
4. After the organic phase from the final extraction is dried, add 2 ml PBS - .1% BSA to each tube, vortel and store overnight at 4C.

Preparation of standard curve

1. Prepare a stock solution of progesterone (Δ^{4}-pregnene-3,20-dione: Sigma Chemical Co., St. Louis, MO) containing

16 ng/ml in PBS-.1% BSA. Prepare one standard curve for each set of 40 assay tubes by serial dilution of the stock solution in 12 x 75 borosilicate tubes to give the following concentrations: 2, 1, .5, .25, .125, .0625, .0312, .0158, and .0078 ng/125 µl buffer. Place three blank tubes before the standard curve to determine total counts added, nonspecific binding and total binding. Prepare standard curve in triplicate.

Progesterone assay

1. Pippette 500 µl of extracted progesterone samples into scintillation vials for recovery, add scintillation cocktail, cap, and vortex. Pipette 50 and 500 µl in duplicate into 12 x 75 borosilicate tubes for assay.
2. Add 50 µl of progesterone antibody diluted 1:500 in buffer to all tubes except the first two (total count and nonspecific tubes).
3. Add 50 µl of [1,2,6,7-^{3}H] progesterone containing 10,000 cpm to all tubes.
4. Vortex and incubate overnight at 4C
5. Following incubation, add 500 µl DCC to all tubes except the first three which get .5 ml buffer only. Mix tubes thoroughly and let sit for 5 to 10 minutes.
6. Centrifuge (4C) at 300 x g for 15 minutes.
7. Pour off supernatant into miniscintillation vials and add scintillation cocktail, cap, shake, and transfer vials to liquid scintillation counter for measurement of radioactivity.

APPENDIX C

LACTOPEROXIDASE RADIOIODINATION OF HUMAN CHORIONIC GONADOTROPIN (hCG)

Reagents for lactoperoxidase radioiodination of hCG

1. 0.1 M sodium phosphate buffer, pH 7.1
   A. Mix 0.1 M sodium phosphate, monobasic (1.42 g/100 ml double distilled (dd) H_2O with 0.1 M sodium phosphate, dibasic (1.38 g/100 ml dd H_2O) to a final pH of 7.1.
      1. Approximately 70 ml dibasic phosphate to 30 ml monobasic phosphate.
      2. Store frozen (-20C) in small aliquots (100 µl).
2. Hydrogen peroxide stock solution
   A. Prepare in a container devoid of trace metals (Nalgene or similar nonglass material), diluting 30% hydrogen peroxide 1 to 400 with dd H_2O (add 250 µl hydrogen peroxide to 100 ml).
   B. Check the dilution with an ultraviolet spectrophotometer at 230 nm.
      1. This step is essential
      2. Absorbance should be 1.6 O.D. (molar absorptivity = 72.4).
   C. This solution can be stored indefinitely at 4C.
3. Hydrogen peroxide working solution.
   A. Add 20 µl of the stock solution to 4 ml dd H_2O, yielding a 40 ng/10 µl solution.
   B. Prepare within two hours of use.
4. Lactoperoxidase solution
   A. Prepare a 1 µg/ul solution of lactoperoxidase (grade B, Calbiochem, approximately 20 I.U./mg) in dd H_2O. Activity can be checked by taking the ratio of spectrophotometric O.D. at 412 and 280 nm, which should be approximately 0.75.

B. Store frozen at -70C in small aliquots (20 µl) in 1 ml eppendorf microfuge tubes.

5. hCG solution
   A. Make a 1 µg/µl solution of hCG in dd H_2O (Canfield CR-121, national pituitary agency).
   B. Add 5 µl (5 µg) to 1 ml microfuge tubes.
      1. Make additions to the bottom surface of the vial.
      2. Store frozen at -70C until used.
6. Iodide
   Freshly prepared Na-^{125}I (Amersham Co., Arlington Heights, ILL) is used.
7. Transfer solution (16% sucrose w/v)
   For separation by G-25 sephadex column, add 16 g sucrose to 100 ml PBS-.1% BSA (i.e., column buffer). Store frozen at -20C in 150 µl aliquots.
8. Rinse solution (8% sucrose w/v)
   Add 8 g sucrose to 100 ml PBS-.1% BSA and store frozen at -10C in 100 µl aliquots

Preparation of disposable G-25 sephadex column

1. Bovine serm albumin (.1%) in phosphate buffered saline (.01 M, pH 7.4)
   A. To 950 ml double distilled H_2O add 1.74 g K_2HPO_4 and 8.775 g NaCl. Adjust to pH 7.4 by adding KH_2PO_4. Take volume up to 100 ml with dd H_2O.
   B. Add 1 g bovine serum albumin (BSA fraction 5 powder: Sigma Chemical Co., St. Louis, MO) to 1000 ml PBS. Store at 4C, use within one week.
2. Sephadex stock slurry
   A. Add approximately 5 g of G25-150 sephadex (Sigma) to 40 ml PBS-.1% BSA, swirling until there is an even suspension.
   B. Let swell for at least 12 hours at 4C or 30 minutes at room temperature.

3. Preparation of column
   A. Prepare a 5 ml disposable pipette by attaching a small segment of tubing from the bottom end and sealing the attachment point with clear fingernail polish.
   B. Place a small plug of filtering wool (Pyrex) in the bottom of the disposable pipette. Place firmly but do not stuff. If placed too tightly flow rate will be greatly reduced.
   C. Clamp end and fill pipette half full of PBS-.1% BSA, removing air from wool by tapping.
   D. Swirl the sephadex stock slurry and fill the remainder of the column with suspended sephadex. Let settle until approximately .5 ml of sephadex has accumulated above the filtering wool. Open the clamp and slowly add sephadex slurry until the bed is 1.5 to 2 inches from the top of the pipette.

Lactopenoxidase Radioiodination Procedure

1. Maintain all reagents and reacting solutions at 4C.
2. To one vial of CR-121 hCG (5 µg) add 50 µl of 0.1 M phosphate buffer, pH 7.1, and mix by finger tapping.
3. Add 2 mCi of Na-^{125}I (1.2 nm; 10 µl per mCi) to the buffer-hormone mixture and mix by finger tapping.
   A. Use a 10 µl Hamilton syringe to transfer the iodide. Make additions near buffer surface in the vial.
   B. Determine the amount of ^{125}I actually added to the vial by use of manual gamma counter.
4. To the above solution add either 10, 5, or 0 µl of hydrogen peroxide working solution and 10 µl (1 µg) of lactoperoxidase. Let react for 5 minutes at 4C.
   A. Use a 100 µl Hamilton syringe with a Chaney adaptor only for this purpose.
   B. If the iodine is brownish, do not add or reduce the amount of hydrogen peroxide added, sufficient quantities have been provided by radiolysis of water.

5. Add 100 µl of transfer solution and mix
   A. Use a 1 ml tuberculin syringe with a 1-1/2 inch 25-gauge needle.
   B. Transfer to the column.
6. Rinse the vial with 70 µl of the rinse solution and transfer to the column.
7. Remove the clamp from the column and collect the fluid into a 12 x 75 borosilicate tube until the top of the column is dry. Rinse the column with 1 ml .1% BSA/PBS and collect the fluid fraction in another tube. Repeat the wash 11 times.
   A. 13 aliquots should be collected.
   B. Remove nine 10 µl aliquots from each tube and count.
8. Pool tubes from the first cpm peak and dilute with .1% BSA/PBS to give approximately 5 x 10^6 cpm per 200 µl. Store 200 µl aliquots in two places at -70C to avoid radiation damage of the hormone.
   A. Stored aliquots may be used up to one month after preparation.
   B. Aliquots may be thawed only one time; however, working solution may be stored at 4C and used for LH/hCG receptor determination for approximately one wekk.

APPENDIX D

LH/hCG BINDING ASSAY PROCEDURE

Preparation of phosphate buffered saline-.1% bovine serum albumin (PBS-.1% BSA) buffer (10 mm,pH 7.4)

1. To 950 ml double distilled (dd) H_2O add

K_2HPO_4	1.74 g
NaCl	8.775 g

   Stir until dissolved, then add enough dd H_2O to bring volume up to 1,000 ml. Adjust to pH 7.4 with potassium phosphate monobasic (KH_2PO_4).
2. Add 1 g bovine serum albumin (BSA fraction 5 powder: Sigma Chemical Co., St. Louis, MO) to 1000 ml of phosphate buffered saline for a .1% BSA in PBS solution. Store at 4C, use within one week.

Preparation of trypsin inhibitors solution

1. Prepare fresh before use by dissolving 1 mg trypsin inhibitor (lyophilized from soybean: Sigma Chemical Co., St. Louis, MO) in 9 ml PBS-.1% BSA. Maintain at 4C while in use.

Preparation of CL membrances

1. Weigh the CL and immediately place in 8 ml ice-cold PBS-.1% BSA (buffer)- trypsin inhibitor. Maintain on ice until ready to homogenize.
2. Immediately prior to homogenization, mince the CL. Transfer the CL to a 15 ml dounce homogenizer and homogenize 30 strokes while keeping on ice.
3. Strain the homogenate through three layers of cheesecloth into nine chilled centrifuge tube. Centrifuge (4C) at 30,000 x g for 45 minutes.
4. Discard the supernatant and resuspend the pellet in 3 ml buffer-trypsin inhibitor by homogenizing 10 strokes. Maintain the homogenate on ice until used. Vortex thoroughly prior to use.

LH/hCG inhibition curve

1. Prepare appropriate dilutions of CR-121 hCG (Canfield: National Pituitary Agency) from a stock solution of 1 µg 1 µl buffer (stored at -70C) to give the following concentrations: .25, .5, 1.0, 2.5, 5, 10, and 25 ng hCG/100 µl buffer. Also dilute hCG used for nonspecific binding determination (3000 I.U./mg, Sigma Chemical Co., St. Louis, MO) from a stock solution (stored at -20C) containing 1 mg 1 ml buffer to give a final concentration of 20 µg/100 µl buffer.
2. Add 100 µl of the membrane homogenate to 12 x 75 borosilicate tubes containing 700 µl ice-cold buffer. Then add 100 µl of unlabelled hCG and 100 µl of ^{125}I-hCG containing 50,000 cpm to all assay tubes. Prepare the inhibition curve in triplicate. Vortex and let incubate for 24 hours at 25C.

Separation of bound and free hormone

After incubating for 24 hours, add 3 ml ice-cold buffer to each tube, vortex and centrifuge (4C) at 3000 x g for 20 minutes. Aspirate the supernatant, resuspend the pellet in 3 ml buffer and repeat the centrifugation. Aspirate the supernatant and count the pellet for bound ^{125}I-hCG for one minute in a gamma spectrometer.

APPENDIX E

BRADFORD METHOD OF PROTEIN QUANTITATION

Preparation of protein standard (PBS-.1% BSA)

1. To 950 ml double distilled (dd) H_2O add

K_2HPO_4	1.74 g
NaCl	8.775 g

   Stir until dissolved, then add enough dd H_2O to bring volume up to 1,000 ml. Adjust to pH 7.4 with potassium phosphate monobasic (KH_2PO_4).
2. Add 1 g bovine serum albumin (BSA fraction 5 powder: Sigma Chemical Co., St. Louis, MO) to 1000 ml of phosphate buffered saline for a .1% BSA in PBS solution. Store at 4C, use within one week.

Preparation of protein reagent

To 50 ml of 95% ethanol add:

Brilliant Blue G-250 (90% dye content: Sigma)	100 mg
85% phosphoric acid	100 ml

Mix thoroughly, then dilute to 1 liter. Store at room temperature.

Protein Assay Method

1. To 12 x 75 borosililated tubes add the appropriate amount of protein standard to give the following concentrations: 10, 20, 30, 40, 50, 60, 70, 80, and 90 µg BSA. Normalize the volume of each tube to 250 µl with PBS buffer. Prepare the standard curve in triplicate.
2. Aliquote 50 and 100 µl of each unknown in triplicate to 12 x 75 borosilicate tubes and normalize the volume to 200 µl with PBS buffer. Add 50 µl of .3N NaOH to each tube. Incubate for 10 minutes prior to adding the protein reagent.

3. Add 2.5 ml protein reagent to each tube and let incubate two minutes before measuring the absorbance at 595 nm. Accurate up to one hour.
4. Between unknown samples rinse the curvette with 95% ethanol followed by dd H_2O. Clean the cuvette thoroughly with ethanol and rinse with dd H_2O after use.

VITA

Joan R. Hembree, daughter of Shirley and Ralph Hembree, was born on April 28, 1959 and raised in Knoxville, Tennessee. She graduated from Farragut High School in May 1977.

In September 1977 Joan began her undergraduate studies at The University of Tennessee, Knoxville. She received the degree of Bachelor of Science in Agriculture in June 1981. Joan then entered the graduate program in Animal Science at the same university in September 1979 and was awarded the degree of Master of Science in December 1983.