ENDOCRINE, BEHAVIORAL, AND OVARIAN CHANGES IN HOLSTEIN HEIFERS FROM PUBERTY TO BREEDING SIZE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
LLOYD SWANSON
1970

This is to certify that the

thesis entitled

Endocrine, Behavioral, and Ovarian Changes in Holstein Heifers from Puberty to Breeding Size

presented by

Lloyd Swanson

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Dairy

Harold D Chapman
Major professor

Date 12/17/70

O-7639

ABSTRACT

ENDOCRINE, BEHAVIORAL, AND OVARIAN CHANGES IN HOLSTEIN HEIFERS FROM PUBERTY TO BREEDING SIZE

By

Lloyd Swanson

The endocrine, behavioral, and ovarian changes of 37 Holstein heifers were studied with respect to age before puberty and during estrous cycles up to breeding size.

Beginning during their seventh month of age, heifers were observed twice daily for signs of estrus. Jugular blood was obtained from each heifer at monthly intervals until first estrus. Then, during approximately the first, fourth, and seventh estrous cycles, each heifer was bled on days 0 (estrus), 2, 4, 7, 11, and daily from day 18 until estrus. Reproductive organs were palpated via the rectum twice weekly until each heifer was bred.

Serum levels of LH and prolactin were quantified by radioimmunoassay. The double antibody technique was employed to separate bound and unbound hormones. For the steroid assays about 1000 cpm of ^{3}H-labeled progesterone, corticosterone, cortisol, estrone, estradiol, and estriol were added to 5 ml serum and the steroids in an ether extract were separated from each other on 1 x 32 cm

Sephadex LH-20 column eluted with freshly redistilled reagent grade chloroform:95% ethanol (95:5). The hormones were located in the eluate from the Sephadex column and their recoveries calculated by determining radioactivity of portions of the eluate.

Estrogens were quantified by competitive protein binding assays utilizing uteri of estrous rabbits as a source of estrogen binding protein. Progesterone and the glucocorticoids were quantified by competitive protein binding assays utilizing dog plasma as a source of corticosteroid binding globulin.

Follicle size, number of follicles, and serum LH increased within 20 days of first estrus. Increased quantities of LH are released near the onset of puberty. The average age at first estrus was 43.3 $\pm$ 0.9 weeks, body weight was 252.9 $\pm$ 5.5 kg, and withers height was 110.7 $\pm$ 0.8 cm. Estrous cycles averaged 20.8 $\pm$ 0.3 days and heifers reaching puberty at an older age had significantly longer ($P<0.005$) first and second estrous cycles. The incidence of standing heat increased from 65% at the first estrous cycle to 91% at the ninth estrous cycle.

Follicle size and follicle numbers paralleled each other during the estrous cycle; follicle size was increased at estrus and day -2 while follicle numbers were increased at estrus and day -3. Equal numbers of follicles were palpated on the right and left ovaries and follicles were

about of equal size. But of the corpora lutea, 65% were palpated on the right ovary. CL size increased continuously and significantly ($P<0.005$) from 0.8 ± 0.1 cm 2 days after estrus to 2.2 ± 0.1 cm on day 11, and except for a slight increase one day before estrus, decreased to day 2.

Serum LH decreased continuously and significantly ($P<0.01$) from prepubertal levels of 2.16 ± 0.26 ng/ml to 1.44 ± 0.16 ng/ml during the luteal phase of estrous cycles at breeding size. Levels of serum LH increased from 1.42 ± 0.07 ng/ml on day 7 of the estrous cycle to 2.85 ± 0.29 ng/ml on day -1 and to 5.90 ± 1.87 ng/ml 0.5 days before estrus. The ovulatory surge (11.82 ± 1.24 ng/ml) occurred near the onset of estrus and LH declined to day 2. But only 46% of samples obtained from heifers when first observed in standing heat contained elevated levels of LH.

Levels of serum prolactin declined continuously and significantly ($P<0.01$) from prepubertal levels of 141.9 ± 11.4 ng/ml to 15.3 ± 2.2 ng/ml during the estrous cycles at breeding size. During the estrous cycle, prolactin increased ($P<0.025$) to 45.8 ± 4.2 ng/ml during estrus, then decreased to about 35 ng/ml during metestrus, diestrus and proestrus. Decreased levels of LH and prolactin with advancing age suggest that heifers are not fully mature at first estrus.

Serum estrone and estriol were identified but lack of precision for these assays contributed to large variances.

Levels of estradiol were high during estrus (49.6 ± 25.0 pg/ml) and metestrus and decreased to 14.1 ± 4.0 pg/ml on day 11. Progesterone increased continuously ($P<0.005$) from a low of 0.12 ± 0.05 ng/ml on day 2 to 6.88 ± 1.04 ng/ml 3 days before estrus and then decreased sharply to day 2. LH and prolactin were negatively correlated with progesterone on days -3 and -2 and were positively correlated on day -0.5 and day -1 respectively. Levels of corticosterone decreased significantly ($P<0.005$) from 0.48 ± 0.09 ng/ml during the first estrous cycle to 0.23 ± 0.03 ng/ml during the fourth and seventh estrous cycles. Comparable values for cortisol were 9.89 ± 1.29 ng/ml and 6.05 ± 0.67 ng/ml ($P<0.005$). Neither corticosterone nor cortisol levels varied significantly during the estrous cycle.

A study of endocrine and ovarian changes during estrus revealed ovulation occurred 29 ± 6 hr after standing heat was first observed and 32 ± 6 hr after the LH peak. LH remained elevated about 8 hr during early estrus and the peak concentration was 25.92 ± 3.18 ng/ml. Levels of serum LH and prolactin exhibited diurnal variation; they were low ($P<0.05$) at noon and during the night.

ENDOCRINE, BEHAVIORAL, AND OVARIAN CHANGES IN HOLSTEIN HEIFERS FROM PUBERTY TO BREEDING SIZE

By

Lloyd Swanson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Dairy

1970

"Our chief want in life is somebody who shall make us do what we can."

- Emerson

BIOGRAPHICAL SKETCH

of

Lloyd Swanson

I was born in Isanti, Minnesota on October 16, 1938 and received my elementary education at small 'country schools' in that town and at Rock Creek, Minnesota when my parents moved to a larger dairy farm. Completing my secondary education at Pine City, Minnesota, I entered the University of Minnesota in 1956 and received the B.S. degree in the Department of Dairy in 1960.

I then worked 2 years for the U.S. Naval Air Reserve and saw the world, working as an Aviation Electrician on the USS ESSEX. Upon return, I was employed by Cargill, Inc. from 1962 to 1964 as a production foreman in their oil processing division.

Desiring a career as a scientist-teacher I returned to the University of Minnesota and received the M.S. degree in 1967. During that time I had the privilege of working under Dr. Alan Hunter, completing a thesis entitled, "Serological Studies on Fertility." I was accepted by the Dairy Department, Michigan State University and Dr. Harold Hafs to continue my graduate education, shifting my major emphasis from immunology to endocrinology, both of which I

enjoy working in immensely. I am completing my PhD degree with this thesis in December, 1970 and will begin employment in the Department of Animal Science, Oregon State University as a dairy physiologist with responsibilities for research and teaching.

ACKNOWLEDGMENTS

I should like to thank the Dairy Department of Michigan State University and Dr. Charles Lassiter for providing facilities and funds for my graduate studies. Also to be thanked for providing funds are my wife, Grace, who has been diligently teaching in the elementary schools these past years, and the U.S. Government (PL 480). But to my friend and advisor, Dr. Harold Hafs, must be extended a debt of gratitude for being flexible to provide me a unique educational experience. Truly, he has been the friend who, as Emerson said, has made me reach for my potential.

I also am grateful for the advice and consent given me by my committee members, Drs. S.D. Aust, J.L. Gill, and G.D. Riegle. This thesis, as the reader will realize, was not the output of one person, but involved participation of many individuals. They include Dr. Lee Edgerton and Robert Wettemann who devoted many hours of research to developing the CBG and estrogen protein binding assays, respectively. Providing assistance for detecting estrus and obtaining blood samples were Drs. Ed Convey, Louie Boyd, Allen Tucker, and my student colleagues Dr. Don Pritchard, Linda Miller, Dr. Wayne Oxender, Winston Ingalls, Norman Rawlings, Valdin

Smith and Jim Koprowski. I wish to thank Drs. David Morrow, Lee Edwards, and Richard Koritanski of the Michigan State University Veterinary Clinic for performing the many rectal palpations required in this study.

I would thank my wife, Grace for the moral support provided, for typing the first 2 or 3 drafts of this thesis and, as the reader may observe, for rendering the many fine figures included in this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

As efficiency per animal must increase to meet demands for food in the future, fertility of our domestic animals becomes more important. Many factors conceivably may restrict fertility as geneticists and nutritionists strive to increase production. But one must understand the basic physiology of reproduction before these confounding factors can be considered.

Many studies in the past, including some in this laboratory, have been devoted to understanding the basic concepts of reproduction and thus fertility and infertility. We now are aware of biochemical and hormonal changes within organs during growth, puberty, and the estrous cycle. But as our research technology advances, increasingly refined research tools have been developed with which to study reproduction.

Such a major advance in the area of reproductive endocrinology is the advent of sufficiently sensitive techniques to quantify hormones in the minute concentrations present in blood. This is a major breakthrough in endocrinology. Whereas, in the beginning, researchers quantified hormones crudely into "total gonadotropic activity," it is now possible to identify and quantify specific hormones in most organs or biological fluids where they are present.

Thus a primary objective of this study was the determination of levels of hormones in the blood of dairy heifers to detect changes with age and during the estrous cycle. Furthermore, whereas previous workers have studied only one or two hormones, a second objective of mine was to determine the levels of several hormones so that any meaningful relationships might be established. Previous workers have assumed decreased pituitary levels of hormones to represent release of hormone into the blood. Comparison of blood levels of hormones with pituitary hormone levels would test this hypothesis. Furthermore, estrogens have been implicated in causing the ovulatory surge of LH. Thus, it was of interest to determine whether peripheral blood levels of estrogen rise before the ovulatory LH peak, as has been demonstrated in other species.

While the widely accepted radioimmunoassays for pituitary hormones have been in general use for 3 to 4 years, the competitive protein binding assays for the steroid hormones are relatively new. Therefore I found it necessary to develop some of the methods concerned with the steroid hormone assays.

Problems such as these made this research most intriguing for me. My research ranged from basic (development of separational techniques for steroid hormones) to applied (estrus detection). The overall purpose of this study was to provide better understanding of endocrine

events in the normal heifer; hopefully to assist solving problems of lowered fertility after estrous synchronization, to minimize infertility caused by endocrine imbalance, and to provide bases for future research such as increasing the productive life of cattle by decreasing the age at puberty.

Information from this study will not be final, just as measurement of "total gonadotropic activity" was not the final answer for early endocrinologists. For example, already scientists are looking beyond hormone levels in the blood, to the target organs and to rates of synthesis and degradation of the hormones, realizing that these may provide even more definitive information. Studies such as this thesis should provide the knowledge with which to advance to the next stage of sophistication.

REVIEW OF LITERATURE

A. Puberty

Although puberty is a gradual process, it is usually defined as the age when the reproductive organs become functional (Salisbury and Van Demark, 1961). In the bovine, this is usually considered to be the first estrus, a criteria easily determined.

1. Behavioral Changes

Heifers and cows arrive at puberty at any season. The onset of puberty, estrus, is defined by Webster (1956) as "a vehement desire or impulse; frenzy." These psychological manifestations are attributed largely to estrogen, the female sex hormone. Estrus is the period when the female is receptive to the male and permits mating. In this thesis, standing heat will denote estrus. Other less obvious signs of estrus include attempted mounting of other animals, restlessness, frequent bellowing, and a mucous discharge from the vulva.

Abnormal behavior during estrus includes quiet heat when psychological behavior is unnoticed, and nymphomania, a constant psychological manifestation of estrus.

Morrow (1969) reported that silent estrus accompanied the onset of puberty in 73.6% of Holstein heifers.

The occurrence of standing heat increased significantly with each successive estrus after the first and reached 79.3% at the third estrus. But among heifers or cows Hafez and Schein (1962) reported few, if any, differences in behavioral patterns during estrus.

The average Holstein heifer attains puberty between 9 and 18 months of age and at 260 kg body weight (Cupps et al., 1969). But this is variable, depending upon breed and nutrition. Smaller breeds generally attain puberty at an earlier age than larger breeds. The dramatic influence of nutrition was demonstrated by Sorensen et al. (1959). Heifers on a high level of nutrition reached estrus at 8.5 months of age, 2.8 months earlier than heifers on a medium level of nutrition and 8.1 months earlier than heifers on a low level of nutrition. Withers height and body length are less variable at puberty than age and body weight (Sorenson et al., 1959).

Heifers in a communal lot attained estrus at 31.9 $\pm$ 1.5 weeks (Desjardins and Hafs, 1968) while heifers fed a normal ration and housed in stanchion barns attained estrus about 17 weeks later (Sorensen et al., 1959). Possibly behavior patterns developed in communal housing can hasten puberty. Vandenbergh (1967) observed that immature mice exposed to adult females after weaning reached first estrus at an earlier age than immature mice not exposed to adults, and first estrus was significantly hastened when immature

mice were exposed to adult males. Morton et al. (1963) reported that handling of immature female rats can influence their sexual development.

2. Endocrine Changes

Growth of the pituitary gland in the female bovine is linear through 9 months of age and then plateaus (Desjardins and Hafs, 1968). The same workers also noted a significant increase of follicle stimulating hormone (FSH) potency in the pituitary between birth and 1 month of age, a decrease at 2 months, and a plateau at that level through 12 months of age. In contrast, the pituitary concentration of luteinizing hormone (LH) increased rapidly to 4 months of age and then fluctuated considerably, exhibiting 2-fold changes in concentration from month to month. When the pituitary data were adjusted for stage of estrous cycle, pituitary LH potency was highest at 7 months of age and then decreased almost linearly through 12 months of age. The average age at puberty was 31.9 weeks.

Thus pituitary LH is highest at the time of, or just prior to, puberty and is released at first estrus. But pituitary FSH, although adjusted for stage of estrous cycle, did not change at puberty. Pituitary prolactin concentration (Sinha and Tucker, 1969), like pituitary LH, shows marked changes from birth through 12 months of age. Pituitary prolactin was low at 1 and 2, 5 and 6, and 10 and

11 months of age. It was high at 3 and 4 months and at 7, 8 and 9 months of age when mammary development was also greatest in the same heifers. Apparently pituitary prolactin is more closely associated with mammary development than with occurrence of puberty.

In an earlier study, Parlow et al. (1964) found pituitary LH but not FSH increased with age in prepubertal gilts. Pituitary levels of hormones were much higher just before than after puberty; a conclusion also true for rats (Ramirez and McCann, 1963). In addition, FSH releasing activity (Watanabe and McCann, 1969) and LH releasing activity (Ramirez and Sawyer, 1966) decrease at puberty in the female rat.

3. Ovarian Changes

Desjardins and Hafs (1969) observed an increase in ovarian weight of heifers proportionately greater than the increase in body weight from birth to 5 months, a plateau from 5 to 8 months and an increase from 8 to 12 months of age paralleling the increase in body weight. The number of visible ovarian follicles was maximum at 4 months and then decreased until 8 months of age after which the number remained constant. The ovarian change during puberty is more pronounced in the rat where ovarian growth increases at or near the onset of puberty (Watanabe and McCann, 1969). This is probably a result of the decreased pituitary FSH observed in rats at the same time if one assumes that

11 months of age. It was high at 3 and 4 months and at 7, 8 and 9 months of age when mammary development was also greatest in the same heifers. Apparently pituitary prolactin is more closely associated with mammary development than with occurrence of puberty.

In an earlier study, Parlow et al. (1964) found pituitary LH but not FSH increased with age in prepubertal gilts. Pituitary levels of hormones were much higher just before than after puberty; a conclusion also true for rats (Ramirez and McCann, 1963). In addition, FSH releasing activity (Watanabe and McCann, 1969) and LH releasing activity (Ramirez and Sawyer, 1966) decrease at puberty in the female rat.

3. Ovarian Changes

Desjardins and Hafs (1969) observed an increase in ovarian weight of heifers proportionately greater than the increase in body weight from birth to 5 months, a plateau from 5 to 8 months and an increase from 8 to 12 months of age paralleling the increase in body weight. The number of visible ovarian follicles was maximum at 4 months and then decreased until 8 months of age after which the number remained constant. The ovarian change during puberty is more pronounced in the rat where ovarian growth increases at or near the onset of puberty (Watanabe and McCann, 1969). This is probably a result of the decreased pituitary FSH observed in rats at the same time if one assumes that

decreased pituitary levels reflect release of hormone in the blood.

Morrow (1969) reported follicles of sufficient size to palpate in a majority of prepubertal heifers although follicle size in prepubertal and postpubertal heifers was less than in mature cows. Morrow also observed that follicle numbers and follicle size increased at approximately 20 days before the first ovulation, suggesting prepubertal cyclic activity. The number of heifers which failed to ovulate at the first estrus was significantly greater (13.2%) than at the second estrus (1.9%) or at the third estrus (0%).

B. Estrous Cycle

1. Behavioral Changes

The dominant behavioral event of the estrous cycle in the bovine is estrus as described previously. Nalbandov (1964) states that older cows have a more intense estrus than virginal heifers. This trend was confirmed by Morrow (1969) who observed that the incidence of standing heat increased from the first to the third estrous cycle.

Trimberger (1948) reported the average duration of estrus in cows (17.8 hours) to be significantly longer than that in heifers (15.3 hours). No seasonal effect on duration of estrus was detected in either cows or heifers. Trimberger also noted that cows and heifers first observed

in estrus in the morning have a shorter period of estrus than cows and heifers first observed in estrus in the afternoon. More animals are first observed in estrus in the morning than in the afternoon and evening simply because estrus may begin during the night when it would less likely be observed. Hansel and Trimberger (1951, 1952) showed that heifers remain in estrus for 17 hours, slightly longer than observed by Trimberger (1948).

Endocrinologically, the estrous cycle is divided into proestrus, estrus, metestrus, and diestrus. Proestrus, the period of rapid follicular growth, is the 2 or 3 days before estrus. Mating occurs during estrus and the follicle ovulates during metestrus. The corpus luteum also develops during metestrus and progesterone secretion dominates diestrus (the luteal phase).

The length of the estrous cycle is normally 18 to 24 days (avg 21 days). Generally, the cycle is slightly shorter in heifers than in cows. Rajakoski (1960) observed a mean length of 20.6 $\pm$ 0.2 days for heifers. Similar lengths of 20.5 $\pm$ 0.6 days and 20.3 $\pm$ 2.6 days were reported for heifers by Desjardins and Hafs (1968) and Morrow (1969), respectively. Morrow also reported that the cycle length did not change during the first few cycles following puberty.

Vaginal secretions commonly observed during estrus change in consistency from a thick, viscous mucus during

metestrus and diestrus to a thin and clear secretion during proestrus and estrus.

Bleeding is commonly observed during metestrus, occurring within 5 days of estrus. Trimberger (1941) found a majority of heifers and cows showing a bloody discharge on the second day after estrus. Bleeding occurs more frequently in heifers than in cows (Cupps et al., 1969). The blood originates from the uterine endometrium and most frequently from the caruncles. A discharge of blood, common to most heifers, can be taken as an indication of an estrous period 2 to 5 days previously.

2. Endocrine Changes

The study of pituitary endocrine activity during the estrous cycle until recently has been limited to changes in the anterior pituitary gland. Although early investigators could only assay total gonadotropic hormone activity, the advent of specific assays allowed individual measurements of LH (Karg, 1957 and Parlow, 1961) and FSH (Steelman and Pohley, 1953). Further, the ovarian ascorbic acid depletion assay allowed the quantification of plasma LH (Anderson and McShan, 1966), though not with adequate sensitivity.

a. FSH.--The greatest change in pituitary hormones occurs during estrus. Rakha and Robertson (1965) reported a 27% decrease in pituitary FSH between the onset and the end of estrus. This decrease was confirmed by Desjardins

and Hafs (1968) who also observed decreased pituitary FSH during the period approaching mid-cycle (days 6 to 9). A more detailed study of estrus (Hackett and Hafs, 1969) revealed a 49% loss of pituitary FSH between days 18 and 20 and a further reduction (46%) by the day of estrus. Hackett and Hafs also noted decreased pituitary FSH on day 4. Peripheral blood levels of FSH are unavailable for the bovine because purified bovine FSH with which to develop the necessary radioimmunoassay has not been isolated.

b. LH.--Pituitary LH also changes rapidly during estrus, but 2 or 3 days later than does pituitary FSH. The 61% decrease reported by Rakha and Robertson (1965) compares with a 87.8% decrease observed by Desjardins and Hafs (1968). Hackett and Hafs (1969) divided this decrease as a 71% loss from day 20 to estrus and a further 61% decrease between estrus and day 2. Jubb and McEntee (1955) noted a rapid degranulation of pituitary basophil cells simultaneously with the decrease in pituitary LH. Thus the pituitary loss of LH occurs during and after estrus, whereas pituitary FSH decreases before and during estrus. The decreased pituitary LH during estrus was first substantiated by Anderson and McShan (1966) who observed elevated plasma LH at estrus.

Recently, a more precise picture of this change shows that peak blood levels occur at or shortly before the beginning of estrus (Hansel and Snook, 1970) and the peak persists no longer than 6 hours (Schams and Karg, 1969). Both laboratories also have reported a smaller mid-cycle

peak in serum LH occurring at day 8 to 10. Both rises in serum LH correspond with the decreases previously observed in pituitary LH.

c. Prolactin.--Little data are available on prolactin levels in the bovine. Pituitary levels of prolactin decreased significantly between estrus and day 2 in heifers (Sinha and Tucker, 1969) and then steadily rose until a non-significant decrease occurred at mid-cycle. The function of prolactin during estrus, ovulation and luteal growth in the cow is not known. Schams and Karg (1970) could find no relationship between plasma prolactin and the estrous cycle, but noted marked changes in levels of plasma prolactin within a 24-hour period.

d. Progesterone.--Progesterone was first studied in the corpora lutea of cycling cows, reminiscent of the quantification of gonadotropins in the pituitary before they were detected in peripheral blood. Mares et al. (1962) reported a significant increase in the corpus luteum concentration of progesterone and 20β -hydroxy-pregn-4-en-3-one (20β-ol) from day 7 to day 15 followed by a significant drop at day 17 of the estrous cycle. Total progestins were highest on day 15 and 20β-ol was consistently detected only on days 13 and 15. Hafs and Armstrong (1968) found that the progesterone and 20β-ol concentration in the corpus luteum was higher on day 11 than on day 18 of the cycle. A significant increase of corpus luteum protesterone occurred from day 4 to day 7. These researchers also observed that

progesterone synthetic activity of corpus luteum homogenates was maximal from mid-cycle to day 18 or 20. Because blood levels of progesterone decrease before day 18 or 20, it was concluded that in vivo substrates may be rate limiting to steroidogenesis.

Gomes et al. (1963) quantified progesterone and 20β-ol in corpora lutea, ovarian venous plasma and peripheral blood plasma by a spectrophotometric analysis. Progestins were not detected in ovarian venous plasma at estrus or on day 1 but progestins increased significantly from day 2 to a peak on day 15. Ovarian plasma progestins were significantly correlated with day of cycle and with luteal concentration of progestins. But peripheral plasma progesterone was related neither to day of cycle nor to progestins in the corpora lutea or ovarian plasma. These workers did not detect 20β-ol in the peripheral plasma at any time. With cannulation of the ovarian vein, Dobrowolski et al. (1968) detected progesterone in the ovarian plasma during the entire cycle and similar to Gomes et al. (1963) findings, progesterone was highest on days 14 and 15. Using a double isotope derivative procedure, Plotka et al. (1967) observed the lowest level of peripheral plasma progesterone on day 2 (9.9 ng/ml) and levels significantly higher (20 to 26 ng/ml) on days 12 to 14.

A more sensitive technique for quantifying progesterone, gas-liquid chromatography, showed peripheral

blood levels to vary from about 1 ng/ml during estrus to a high of 8 to 10 ng/ml on days 13 to 16 of the cycle (Stabenfeldt et al., 1969; Pope et al., 1969; Hansel and Snook, 1970). These values agree with results obtained using competitive protein binding assays for progesterone (Karg et al., 1969).

e. Estrogen.--Mellin and Erb (1965) summarized the status of estrogen measurements in the bovine. Assays of sufficient sensitivity to detect estrogens in peripheral plasma recently have been developed. These include radioimmunoassay (Abraham, 1969) and competitive protein binding (Korenman et al., 1970) and they have been applied to estrogens in human plasma but not to estrogens in bovine plasma. The available literature on estrogens in the bovine is largely concerned with urinary values which will be discussed here.

Estrone, 17β-estradiol, and 17α-estradiol, but not estriol, have been identified in the bovine. In vitro studies show that estrogens can be synthesized in the bovine adrenal, ovary, and placenta. Generally, the highest concentration of estrogens are found in the largest follicles. Estrogens do not appear to be stored in the body, and they are excreted as metabolites. The feces are a major route of estrogen excretion (Hunt et al., 1961; cited by Mellin and Erb, 1965), but the feces:urinary ratio of estrogen excretion is quite constant. Therefore estrogen metabolites

are most easily determined in urine. The predominant metabolite in the bovine is estradiol - 17α, but large quantities of estrone are also excreted (Mellin et al., 1965). Estimates of total estrogens excreted in the urine vary from 300 $\pm$ 96 ug per hour/500 kg body weight during early pregnancy to 3,402 $\pm$ 412 ug during late pregnancy (Erb et al., 1968).

Nanogram levels of estradiol (per ml) have been reported in peripheral blood plasma (Ayalon and Lewis, 1961; cited by Mellin and Erb, 1965). Thus it appears that estrogen levels are high in bovine peripheral blood, greater than the picogram levels in the peripheral blood of humans. However, nanogram quantities are present in human peripheral blood during pregnancy (Korenman et al., 1970).

f. Glucocorticoids.--Cortisol and corticosterone are the principal glucocorticoids in bovine plasma. Riegle and Nellor (1967) found plasma levels of 29.8 ng/ml cortisol and 9.5 ng/ml corticosterone in bulls; a cortisol:corticosterone ratio of 3.1:1. Plasma levels did not change with age but younger bulls (2 to 4 years) were more responsive to adrenocorticotrophin (ACTH) infusion. And cortisol responded to a greater degree than corticosterone to the ACTH infusion. Recently, Venkataseshu and Estergreen (1970) reported levels of 73 ng/ml cortisol and 30 ng/ml corticosterone in plasma of lactating non-pregnant cows; a cortisol:

corticosterone ratio of 2.8:1. They also observed cortisol, but not corticosterone, to respond to ACTH injections.

3. Ovarian Changes

A detailed quantitative study of the follicular distribution by size in the ovaries of 36 heifers killed at various stages of the estrous cycle was conducted by Rajakoski (1960). The total number of follicles (normal and atretic) per pair of ovaries ranged from 10 to 266 and averaged 92.4 $\pm$ 9.1. More follicles were present during winter and spring than during fall, but the difference was not significant. Furthermore, the difference was limited to small follicles (1 to 4.95 mm in diameter). Rajakoski concluded that during the estrous cycle, two waves of follicular growth occur in the ovaries. The first growth wave begins during days 3 and 4; a normal follicle from this growth persists until day 11 and then it and smaller follicles undergo atresia. The second growth wave begins on days 12, 13 and 14; a large follicle emerges and ovulates at the subsequent estrus.

This hypothesis was substantiated by Hackett and Hafs (1969) who observed the highest follicular wall weight at estrus and another peak during mid-cycle (days 7 to 11). Rajakoski reported a significantly greater number of large ($\geq$ 5 mm) follicles present in the right ovary than in the left, and the largest follicle was found twice as often on

the right ovary. A similar favoring of the right ovary had been observed by Trimberger (1948) in heifers and cows.

Ovulation occurs 10.2 hours after the end of estrus in heifers (Trimberger, 1948), slightly less than the 10.7 hours observed in cows. A seasonal trend on the interval from end of estrus to ovulation was not evident.

4. Experimental Endocrine Influences on the Estrous Cycle

The length of the estrous cycle, the duration of estrus, and the time of ovulation can be altered by exogenous hormones. Oxytocin, when injected during the first week of the estrous cycle, is luteolytic and shortens the length of the cycle (Armstrong and Hansel, 1959). Heifers similarly injected with oxytocin but hysterectomized on day one, failed to exhibit shortened estrous cycles in response to oxytocin. Hysterectomy alone prolongs the life of the corpus luteum (Wiltbank and Casida, 1956), indicating a luteolytic effect either in the uterus or exerted through the uterus. On the other hand, exogenous LH prolongs the life span of the corpus luteum (Donaldson and Hansel, 1965).

Progesterone, administered early (days 0 through 3) in the cycle or daily injections of estrogen shorten the estrous cycle (Ginther, 1970 and Rahlman and Cupps, 1962). Progesterone is also believed to synergize with estrogen in inducing estrus. Estrogen primed, ovariectomized cows exhibit estrous behavior when progesterone is administered

the right ovary. A similar favoring of the right ovary had been observed by Trimberger (1948) in heifers and cows.

Ovulation occurs 10.2 hours after the end of estrus in heifers (Trimberger, 1948), slightly less than the 10.7 hours observed in cows. A seasonal trend on the interval from end of estrus to ovulation was not evident.

4. Experimental Endocrine Influences on the Estrous Cycle

The length of the estrous cycle, the duration of estrus, and the time of ovulation can be altered by exogenous hormones. Oxytocin, when injected during the first week of the estrous cycle, is luteolytic and shortens the length of the cycle (Armstrong and Hansel, 1959). Heifers similarly injected with oxytocin but hysterectomized on day one, failed to exhibit shortened estrous cycles in response to oxytocin. Hysterectomy alone prolongs the life of the corpus luteum (Wiltbank and Casida, 1956), indicating a luteolytic effect either in the uterus or exerted through the uterus. On the other hand, exogenous LH prolongs the life span of the corpus luteum (Donaldson and Hansel, 1965).

Progesterone, administered early (days 0 through 3) in the cycle or daily injections of estrogen shorten the estrous cycle (Ginther, 1970 and Rahlman and Cupps, 1962). Progesterone is also believed to synergize with estrogen in inducing estrus. Estrogen primed, ovariectomized cows exhibit estrous behavior when progesterone is administered

within 12 hours of estrogen (Melampy et al., 1957). Small doses of progesterone given at the beginning of estrus reduce the duration of estrus and shorten the interval between the end of estrus and ovulation (Hansel and Trimberger, 1952).

The administration of estrogen alone also will induce estrus in ovariectomized cows (Asdell et al., 1945). But large doses of estrogen will cause refractoriness; treatment with progesterone causes ovariectomized heifers to exhibit estrous behavior upon subsequent estrogen injections (Carrick and Shelton, 1969).

Progesterone also delays estrus, as observed by Trimberger and Hansel (1955). They found that cows came into estrus about 4.6 days after chronic progesterone treatment. Chronic progesterone administration is believed to act by inhibiting the action of LH on the ovary, much as it does during the luteal phase of the cycle.

C. Pituitary--Ovarian Relationship

The anterior pituitary hormones, carried by blood to the ovaries, modify the action of structures within the ovary. In turn, ovarian hormones secreted by follicles and corpora lutea and carried directly to the anterior pituitary or indirectly to the hypothalamus, modify the action of the anterior pituitary.

Although FSH will stimulate follicular growth, both LH and FSH are considered necessary for estrogen secretion

by the follicles (Nalbandov, 1964). But the rapid follicular growth during the latter part of the cycle is probably caused by increased FSH release from the pituitary at this time (Hackett and Hafs, 1969). Plasma estrogen levels are also high (Mellin and Erb, 1965) and feed back on the pituitary to positively influence FSH release. Because progesterone administration inhibits estrus, it probably inhibits the ovulatory LH release, and masks the expression of estrogen until the levels of progesterone decrease to a threshold. When progesterone secretion by the corpus luteum decreases to this threshold, it allows the behavioral expression of estrogen (Hansel and Snook, 1970).

The preovulatory LH rise does not begin until after progesterone has decreased (Hansel and Snook, 1970), but it is not known whether this is a permissive effect or if LH release is stimulated by increased estrogens at this time. Because exogenous progesterone can shorten the estrous cycle (Ginther, 1970) or hasten ovulation (Hansel and Trimberger, 1952), it was believed that a preovulatory release of progesterone might occur in the bovine as has been demonstrated in monkeys (Johansson et al., 1968). This idea was supported by evidence of progesterone in preovulatory follicular fluid in cows (Edgar, 1953). But Kazama and Hansel (1970) failed to find any preovulatory change of plasma progesterone in Holstein heifers sampled every 6

hours. However, the progesterone may act within the follicle to influence ovulation.

Perhaps the ovulatory LH surge is a result of the increasing estrogen titer. Upon administration of an anti-estrogen, Labhsetwar (1970) noted that rats neither ovulated nor exhibited an ovulatory discharge of LH. In this species, estrogen probably exerts a positive feedback on the ovulatory release of LH. Recently, release of LH has been demonstrated after injection of estradiol - 17β into anestrous sheep (Goding et al., 1969) or on day 3 of the estrous cycle of sheep (Bolt et al., 1970). But Hobson and Hansel (1970), upon estrogen administration to hysterectomized and ovariectomized heifers, observed a depression in plasma LH.

The ovulatory LH surge occurs shortly before the beginning of estrus or shortly after the onset of estrus (Hansel and Snook, 1970; Henricks et al., 1970) and remains for 8 to 10 hours. Progesterone is very low at this time (Henricks et al., 1970; Kazama and Hansel, 1970).

Following ovulation, a corpus luteum is formed from the ruptured follicle (Cupps et al., 1969), grows rapidly and plasma progesterone increases beginning on day 3 (Stabenfeldt et al., 1969). LH appears to stimulate corpus luteum growth in the cow (Hansel, 1966) but low LH levels during diestrus cast doubt on its ability to stimulate progesterone synthesis during the luteal phase of the estrous cycle. LH enhances progesterone synthesis

of bovine corpus luteum slices incubated *in vitro* (Mason *et al.*, 1962) but not of bovine corpus luteum homogenates (Hafs and Armstrong, 1968), demonstrating that cellular integrity is prerequisite to the steroidogenic action of LH.

A large non-ovulatory follicle develops at mid-cycle (Rajakoski, 1960; Hackett and Hafs, 1969) and increased blood LH also has been noted at mid-cycle (Schams and Karg 1969; Hansel and Snook, 1970). Apparently, high progesterone levels at this time prevent ovulation of the large mid-cycle follicle.

MATERIALS AND METHODS

A. Experimental Design

This thesis was designed to study endocrine, behavioral and ovarian changes in dairy heifers with respect to age before puberty and during selected estrous cycles up to breeding size. Additionally, these criteria were studied to determine changes which occurred within estrous cycles with a view to detecting any changes which evolve as the heifer matures. The third phase was a study of the relatively rapid events surrounding estrus. Blood samples were collected at more frequent intervals during this phase and the heifers were under more frequent observation.

Beginning during their seventh month of age, heifers were observed for signs of estrus daily, morning and late afternoon until inseminated and diagnosed pregnant. The reproductive organs of each heifer were palpated via the rectum twice weekly until each heifer was bred. Jugular blood was obtained from each heifer at monthly intervals until first estrus. Then, during the first, fourth, and seventh estrous cycles each heifer was bled during the cycle. The weight and withers height of each heifer was recorded during each of the three cycles studied.

During the acute study, blood was withdrawn from indwelling cannulae at 6-hour intervals until the day

before expected estrus, at 2-hour intervals until estrus, and at 6-hour intervals thereafter for 3 to 4 days.

B. Experimental Animals

Holstein heifers born during July and August 1968 were purchased from commercial dairy farms in central Wisconsin. They were transported to the Michigan State University dairy barns as calves and raised individually for 3 to 4 months. The heifers were then moved to loose housing and fed communally. At 5 months of age, each of the heifers was hemimastectomized on the left side at the Michigan State University Veterinary Clinic as part of another experiment. The heifers were returned to loose housing and maintained on a ration of hay and corn silage, and at approximately 7 months of age, 2 to 3 lb of grain per day each. They were given access to a grass pasture late in spring and throughout the summer.

Following the conclusion of this study, the heifers were artifically inseminated beginning October 15, 1969, and maintained through the first 90 days of the subsequent lactation. Eight normal herd replacement heifers were included with the hemimastectomized heifers as controls. These heifers were born May through October of 1968 and were maintained with the experimental heifers beginning in March, 1969. Consequently, these 8 heifers received the same ration, housing, and experimental treatment.

C. Estrus Detection

Beginning in March 1969, the heifers were observed for signs of estrus twice daily--between 7:30 and 8:30 a.m. and between 5 and 6 p.m. Observations recorded for each heifer included (1) standing heart, (2) riding others, (3) alert, nervous or bawling, (4) being ridden, (5) mucous discharge, (6) red, swollen vulva, and (7) blood discharge. Criteria 2 through 6 aided in detecting marginal behavioral signs of estrus or silent estrus, while blood discharge confirmed the observed or silent estrus. These twice daily observations were continued throughout the experiment until the heifers were bred (beginning October 15, 1969) and diagnosed pregnant.

D. Bleeding Scheme

Jugular blood (40 ml) was obtained by venipuncture, using a 50 cc plastic syringe and a 1.5 inch 16 ga disposable needle, from each heifer at monthly intervals until first estrus. Then, during the first estrous cycle each heifer was bled on days 0 (estrus), 2, 4, 7, 11, 18 and 20. During the middle and last cycle before being bred (usually the fourth and seventh), heifers were bled on similar days, but bleeding commenced at various days of the cycle and heifers were bled daily from day 18 until estrus. During estrus, each heifer was bled when first observed in standing heat, whether in the a.m. or p.m. All other bleedings were in the a.m. after the heifers had been observed for behavioral

activity. The variable starting time for bleeding during the middle and final cycles was designed to overcome any interaction of the day of commencement of bleeding with hormonal content of the blood.

Blood was transferred from the syringe to polypropylene centrifuge tubes and kept at room temperature for 2 to 4 hours to allow clotting. After clotting, the tube was ringed to free any adherent clot from the wall of the tube and the blood was refrigerated. Within 1 to 2 days the blood was centrifuged at 6500 x g for 15 min and the serum transferred to 7-dram plastic vials and stored at -20 C until assayed for hormone content. Niswender et al. (1968) observed that neither temperature nor the period blood was allowed to clot before centrifugation influenced the LH level of sheep serum. In a similar experiment, Reeves et al. (1970) reported serum prolactin to be unaffected by storage of blood before centrifugation. Thus time and temperature of the blood before centrifugation was not considered critical.

E. Rectal Palpation

The ovaries were examined rectally by veterinarians of the Michigan State University Veterinary Clinic at weekly intervals commencing with estrus detection in March 1969. This was increased to twice weekly in May 1969 when cyclic activity began. The number, size and location of each follicle was recorded for each ovary. Follicles and corpora

lutea were palpable when their diameters were estimated at 0.5 and 0.7 cm and at 0.5 cm increments over 1.0 cm. Follicles $\geq$ 2.5 cm diameter were designated as cystic. Normal and atretic follicles were not differentiated. Ovulation was confirmed by the presence of corpora lutea (CL) and their size and location were recorded. Corpora lutea were designated as cystic when a fluid filled cyst $\geq$ 1.0 cm dia could be palpated within the CL. Frequent palpations aided in predicting estrus by detecting large, growing follicles. Palpations were performed in the mornings after the heifers had been observed for estrus and after blood samples were obtained, but before the heifers were turned out to pasture.

F. Growth Measurements

To determine rate of body growth, withers height and body weight were recorded at monthly intervals until first estrus. They were recorded also on random days during the middle and last estrous cycles, but not during estrus.

G. Acute Study of Estrous

1. Experimental Animals

To obtain a better understanding of the rapid changes in serum levels of LH and prolactin during estrus, six heifers with histories of normal estrous cycles were selected from the group. Each heifer was transported to

the main dairy barn about 7 days before her expected ninth estrus. They were housed in a stanchion barn and received the same ration as at loose housing, but with no access to pasture.

2. Cannulation

Needles and catheters were soaked in Zephiran Chloride Tincture (Winthrop Labs, New York, N.Y.) for 12 hours before use. Before cannulation, the neck region of the heifer was scrubbed with the Tincture. On the morning of the fourth day before expected estrus, the jugular vein was punctured with a 2 inch 13 ga stainless steel needle. Then a 24 to 36 inch length of polyethylene catheter (Intramedic-PE190, Clay Adams, Inc., New York, N.Y.) was introduced through the needle and passed down the jugular vein until 6 inches remained uninserted. The needle was removed and a 3 inch x 10 inch length of adhesive tape, which had branding cement applied to its back side, was applied over the catheter with the adhesive side remaining exposed. The catheter was passed through a small slit in the middle of the tape. A second piece of tape of the same dimensions was applied over the first piece so that the two adhesive surfaces were together with the catheter extending from the cranial edge. After the catheter was flushed with 3.5% sodium citrate it was temporarily clamped while the end was heat sealed. Thus, only about 3 to 4 inches of the catheter remained exposed after it had been inserted.

The catheter was resealed in a similar manner after a blood sample had been obtained except during frequent bleedings, when an 18 ga needle shortened to 1 cm was inserted into the catheter. A plug, improvised from a 1 cc plastic syringe, was affixed to the needle. It was a simple matter to remove the plug, discard the first 5 cc of blood, draw the sample with a 20 cc plastic syringe, flush with 3.5% sodium citrate and replace the plug during the frequent bleedings.

The first 5 cc of blood were discarded also after opening a heat sealed catheter. The catheter was flushed with 3.5% sodium citrate again before resealing. When the exposed catheter became too short from cutting the sealed end, or when the catheter became plugged, the outer adheasive tape was lifted to allow extending the catheter a suitable length. Beginning with a 36 inch length of catheter, there was usually about 12 inches remaining in the jugular vein by completion of the experiment.

3. Bleeding Schedule

Commencing at noon on the fourth day before the expected estrus, 20 cc of blood was withdrawn at 6-hour intervals. Beginning the day before expected estrus, an equal amount of blood was withdrawn every 2 hours until standing heat was observed. Then the 6-hour schedule was re-established for 1 to 2 days after estrus. Each sample of blood was processed as explained previously.

4. Palpation and Behavioral Observations

The ovaries were examined rectally daily until standing heat occurred and then about every 6 hours until ovulation was detected. Later during the cycle, each heifer was palpated again to confirm ovulation.

Behavioral observations were recorded each time a blood sample was taken; vaginal swelling and secretions, and general restlessness were important signs. Because an adjacent outside lot was not available, each heifer could not be turned out more than once or twice per day. It was not possible to leave the heifers unattended in a dry lot because they attempted to remove the adhesive tape.

H. Radioimmunoassays (RIA)

1. Luteinizing Hormone (LH)

The procedure used to quantify serum LH was developed by Niswender et al. (1969). LH antibody was developed by repeated injections of purified bovine pituitary LH into rabbits and supplied by Dr. Niswender (University of Michigan, Ann Arbor). Purified bovine LH used for iodination (LER-1072-2) was supplied by Dr. Leo Reichert (Emory University, Atlanta, Georgia). This preparation had an LH potency of 1.66 NIH-LH-S1 units/mg, showed no FSH activity when tested at 3600 ug in the Steelman-Pohley assay, and had a TSH contamination estimated at 0.021 USP units/mg.

a. Radioiodination.--Purified bovine LH (LER-1072-2) had been previously dispensed into 1 ml vials (2.5 ul of a

1 ug/ul solution in glass distilled water) and stored at -20 C. These vials were thawed immediately before iodination and the iodination procedure was performed at room temperature. Twenty-five ul of 0.5 M sodium phosphate buffer (pH 7.5) (Appendix I.A.1) was added to the hormone and mixed. One mc of ^{125}I (50 mc/ml, Iso-Serve Division of Cambridge Nuclear Corporation, Cambridge, Mass.) was added, and the contents gently mixed. More efficient iodinations were obtained when ^{125}I-iodide was used within 1 week of shipment.

Forty ug chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.) (Appendix I.A.3) was added to the vial, the vial was stoppered, and the contents gently mixed by finger tapping. The reaction was stopped at exactly 2 min by adding 125 ug sodium metabisulfite (Appendix I.A.4). After thorough mixing, 25 ul of 2.5% bovine serum albumin (BSA), (Nutritional Biochemicals, Inc., Cleveland, Ohio) in 0.01 M phosphate buffered saline (PBS) pH 7.0 (PBS-2.5% BSA), was added to diminish the loss of hormone adhering to the glass vial.

One hundred ul transfer solution (Appendix I.A.5) was added and the contents of the vial were layered beneath the buffer on the surface of a 1x12 cm glass column packed with Bio Gel P-60 (Bio Rad Labs, Richmond, Calif.). The column had been equilibrated by passing 0.05M sodium phosphate buffer pH 7.5 (Appendix I.A.2) through and then 2 ml

PBS-2.5% BSA was added and eluted with buffer to reduce non-specific binding of the protein hormone to glass. Seventy ul of rinse solution (Appendix I.A.6) was added to the hormone vial, recovered, and also layered beneath the buffer on the column. The column was eluted under gravity with 0.05M sodium phosphate buffer and fifteen one-ml aliquots were collected from the column in 12 x 75 mm disposable glass tubes containing 1 ml of 2% lyophilized egg white albumin (EWA) (Sigma Chemicals Co., St. Louis, Mo.) in PBS (PBS-2% EWA). The elution profile was determined by quantifying the radioactivity of 10 ul from each of the 15 tubes in an automatic gamma counter (Nuclear Chicago Corp., Des Plaines, Ill.).

An example of an elution curve is presented in Figure 1. The first peak represents iodinated LH and the second peak represents free ^{125}I. The peak ^{125}I-LH tube was used in the LH RIA. Luteinizing hormone is difficult to iodinate; usually only about 38% of the ^{125}I was attached to the LH. Specific activity of the iodinated LH illustrated in Figure 1 was less than 153 uc/ug protein derived by assuming that all the LH iodinated (2.5 ug) is represented in the first peak. The iodinated LH was quite stable and when stored at -20 C, could be used up to one month after preparation. When used after this time, the free ^{125}I and radiation-damaged ^{125}I-LH which develop during storage could be separated on a 1 x 12 cm column

Figure 1. Elution profile of iodinated luteinizing hormone (LH) after passage through Bio Gel P-60. The first peak represents iodinated LH and the second peak represents free iodine.

Figure 2. Dose response curves for NIH-LH-B5 standards and for bovine sera.

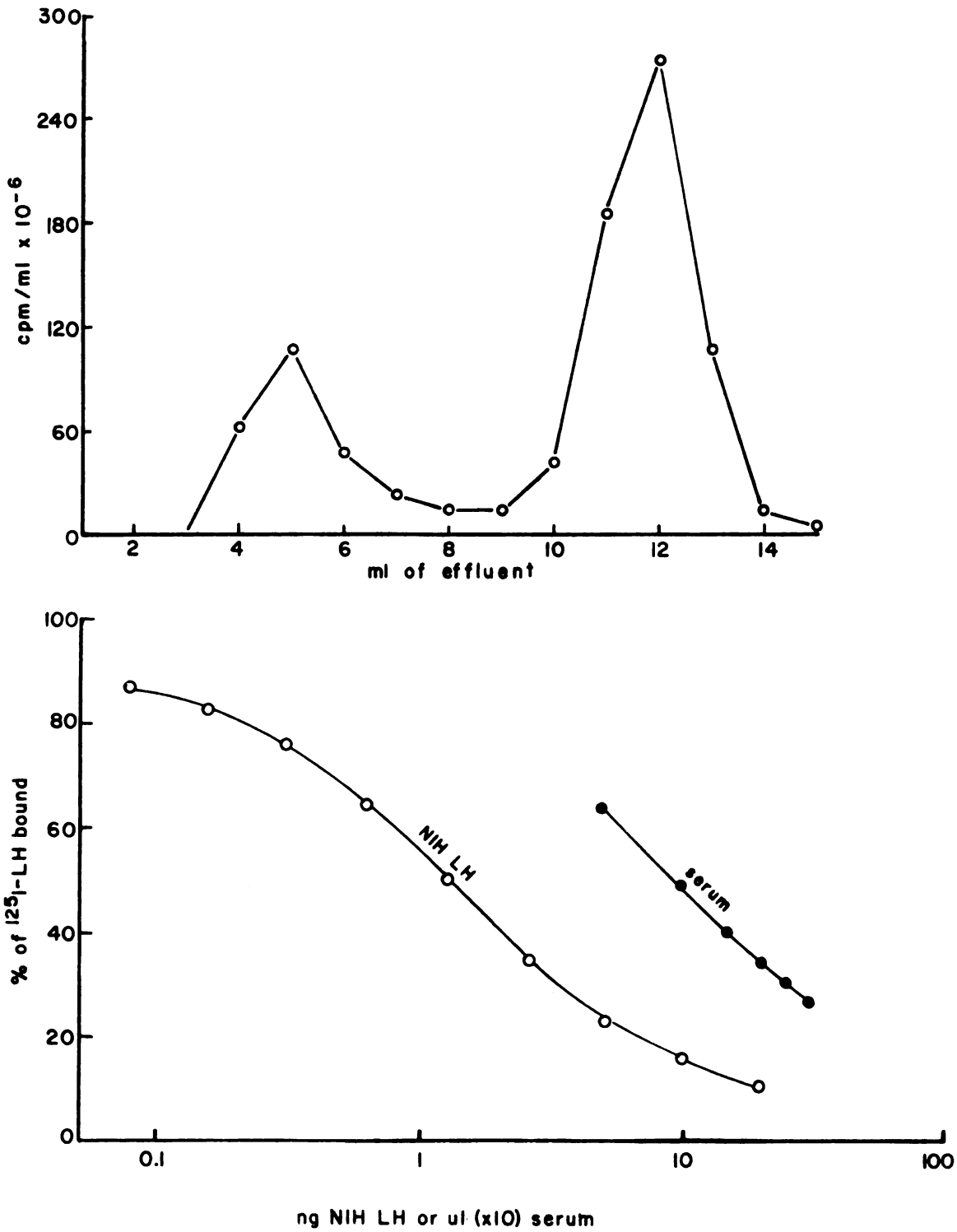
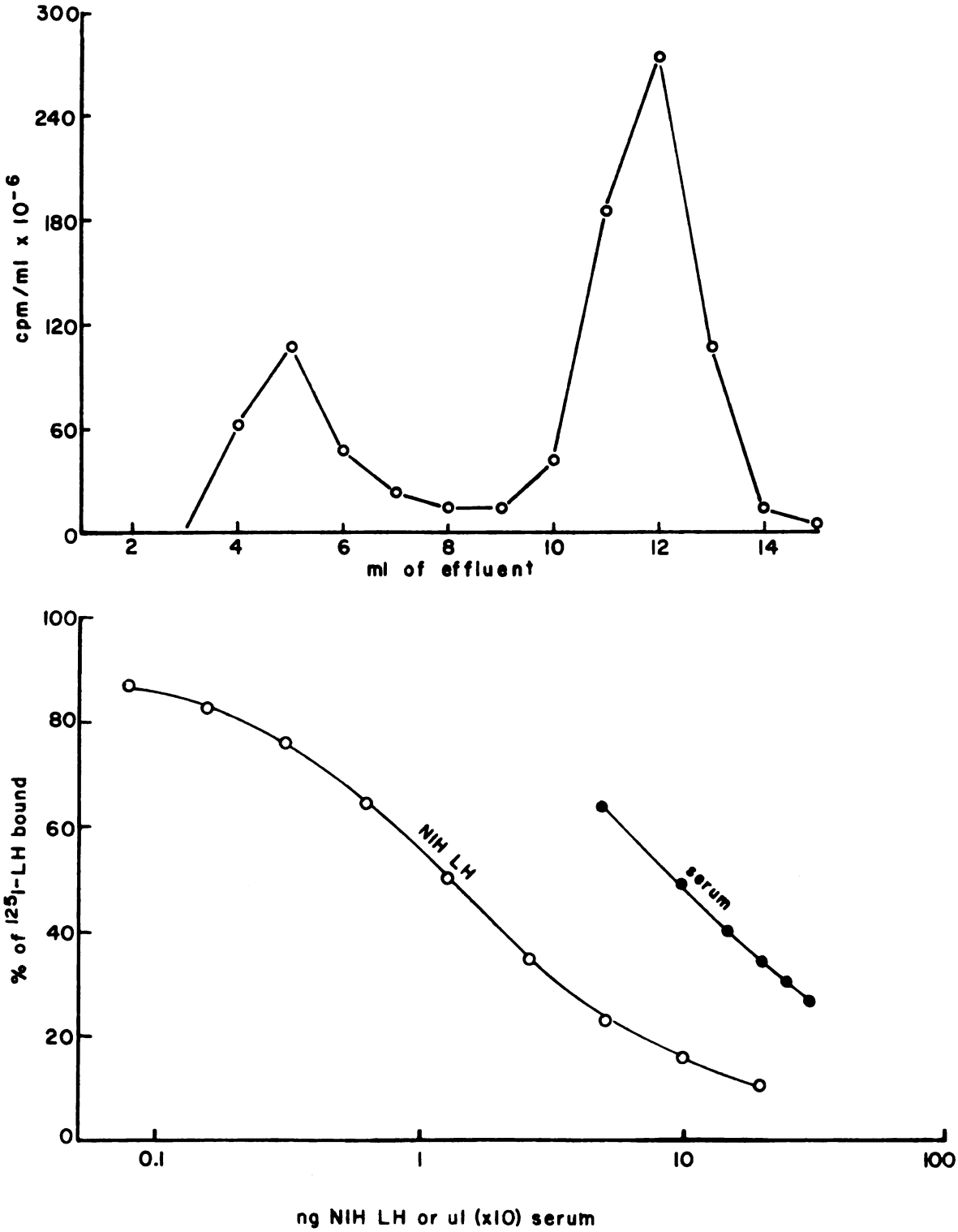

300
240
180
120
60
0
cpm/ml x 10⁻⁶
2
4
6
8
10
12
14
ml of effluent
100
80
60
40
20
0
% of 125I-LH bound
NIH LH
serum
0.1
1
10
100
ng NIH LH or ul (x10) serum

Figure 1. Elution profile of iodinated luteinizing hormone (LH) after passage through Bio Gel P-60. The first peak represents iodinated LH and the second peak represents free iodine.

Figure 2. Dose response curves for NIH-LH-B5 standards and for bovine sera.


300
240
180
120
60
0
cpm/ml x 10⁻⁶
2
4
6
8
10
12
14
ml of effluent
100
80
60
40
20
0
% of 125I-LH bound
NIH LH
serum
0.1
1
10
100
ng NIH LH or ul (x10) serum

of Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.), eluted with 0.05M phosphate buffer (pH7.0).

b. Radioimmunoassay.--Each serum sample was assayed in dilution duplicate. One hundred ul or 200 ul serum was added to disposable glass culture tubes (12 x 75mm) with a Hamilton microliter syringe (Hamilton Co., Whittier, Calif.) and then PBS-1% EWA (Appendix I.B.3) was added to a total volume of 500 ul. As discussed by Hunter (1967), the use of 2 dilutions provides useful evidence of specificity of the assay which is not provided with duplicate determination of the same dilution. Standards were also dispensed in disposable glass tubes with Hamilton microliter syringes. Each lot of 96 tubes included 10 tubes containing 0, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24 or 20.48 ng of standard LH (NIH-LH-B5)[1] (Appendix I.B.4).

Two hundred ul of the LH antibody (Appendix I.B.6) (hereafter referred to as first antibody) was added to each of the culture tubes, and the tubes incubated at 4 C for 24 hours. The first antibody was used at a dilution of 1:80,000. Thus, while the antisera was diluted 1:80,000 with respect to anti-LH, it was diluted 1:400 with respect to normal rabbit serum (NRS). It was important to include this quantity of NRS to provide sufficient mass with which the second antibody could react. ^{125}I-LH, prepared by diluting ^{125}I-LH from the Bio Gel P-60 column with PBS-1%

[1]Supplied by the National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland.

EWA so that 100 ul contained about 20,000 CPM, was then added to each tube. Incubation continued at 4 C for 24 hours.

Goat anti-rabbit gamma globulin (GARGG), later referred to as the second antibody, was purchased lyophilized from Nutritional Biochemicals, Inc. and was reconstituted to original volume with glass distilled water. GARGG was diluted (Appendix I.B.7) to a titer which would optimally precipitate the gamma globulin. The second antibody was necessary to form an antigen-antibody-antibody complex large enough to precipitate. Two hundred ul of GARGG was added to each tube and incubation continued for 72 hours. After each addition, the tubes were vortexed gently and covered to retard evaporation.

Following the final incubation, 3 ml of cold PBS (Appendix I.B.1) was added to each tube to dilute the unbound ^{125}I-LH. The bound ^{125}I-LH was centrifuged at 2500 x g for 30 min in a refrigerated centrifuge with a swinging bucket rotor (Sorvall Model RC-3, Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant was decanted and the tubes allowed to drain for 30 min before any remaining supernatant was removed with absorbant tissue. The bound ^{125}I-LH was then quantified in an automatic gamma counter, usually for 20,000 counts or 10 min, whichever accumulated first. This information was punched on tape by a teletype-writer (Teletype Corp., Skokie, Ill.). The standard curve

was calculated by multiple regression analysis on a CDC 3600 computer. A good fit was obtained with linear, quadratic and cubic components in the regression equation; correlations of fit (R^2) consistently were 0.99 to 1.00. These regression coefficients were entered into an Olivetti computer (Programma 101, Olivetti Underwood, New York, N.Y.) to generate the calculations. The punched tape from the teletypewriter was fed, via a tape editor, (Beckman Model 6912 Tape Editor, Beckman Instruments, Inc., Fullerton, Calif.), through the Olivetti computer which calculated LH concentration in the serum.

Control tubes were included in each assay to determine radioactivity background (tube containing 1:400 NRS in place of the first antibody), total count (tube containing only ^{125}I-LH), and total precipitate (tube containing no unknown or standard). The inclusion of these tubes allowed the later calculation of non-specific binding (background) and of per cent binding (obtained by dividing total precipitate by total count). The triplicate standards were averaged and plotted as the per cent of ^{125}I-LH precipitated at each dose of LH standard compared to the ^{125}I-LH precipitated in the total precipitate tubes (Figure 2).

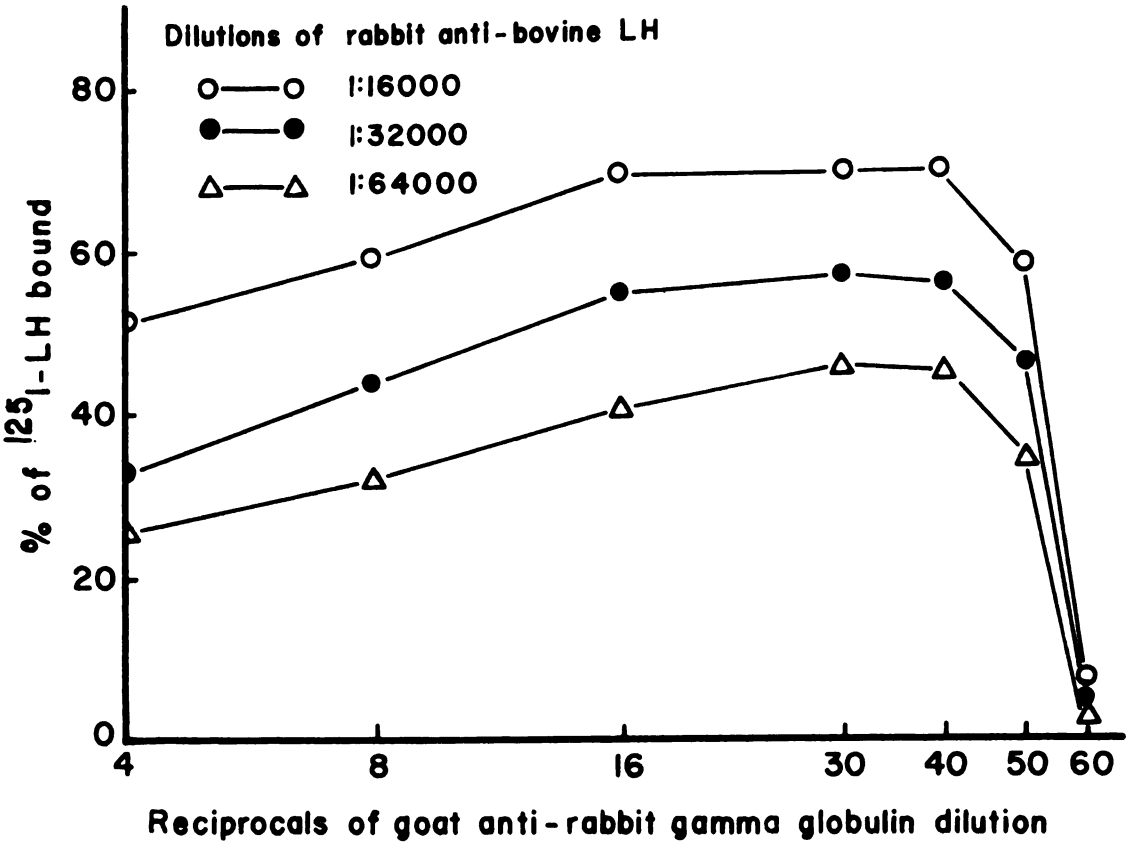
c. Selection of Assay Conditions.--Yalow and Berson (1968) stated that maximum sensitivity of the assay can be obtained when one-third of the labeled hormone is

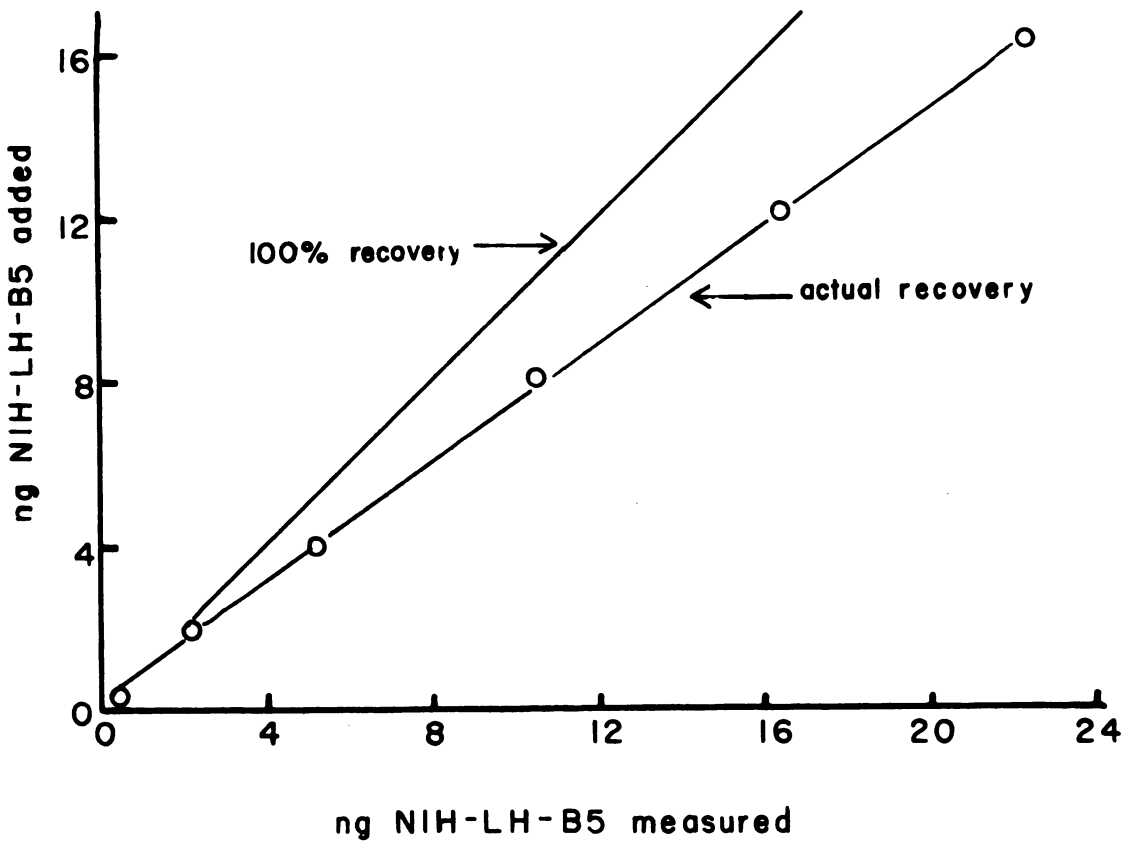
bound. Because binding changed with different iodinated preparations, several dilutions of the first antibody were used in the assay system after each iodination to insure use of the proper dilution. But it usually was diluted 1:80,000, or after all the reagents had been added, at a final dilution of 1:400,000. The second antibody always was used at a dilution which would yield maximum precipitation. Most of the goat anti-rabbit gamma globulin from Nutritional Biochemicals Corp. was of unusually high titer but because it was subject to change, each new shipment was assayed at several dilutions to find one yielding optimal precipitation. An example of a double titration of both first and second antibody is illustrated in Figure 3. On the basis of these data, the GARGG was used at a 1:40 dilution and the first antibody at a 1:64,000 dilution.

d. Validation of Assay.--The high dilution of the first antibody resulted in excellent sensitivity and it was possible to detect levels as low as 0.40 ng/ml. To determine accuracy, varying amounts of NIH-LH-B5 were added to tubes containing 100 ul serum. Accuracy was poor when more than 2 ng exogenous LH was added (Figure 4) but was adequate at levels comparable to those found in serum. For example, serum containing 2 ng LH/ml would contain only 0.4 ng LH in 200 ul of serum. Niswender et al. (1969) obtained excellent recovery of exogenous LH using the same

Figure 3. Per cent iodinated LH bound with varying dilutions of rabbit anti-bovine LH and of goat anti-rabbit gamma globulin.

Figure 4. Recovery of exogenous bovine LH added to 100 ul bovine serum.


Dilutions of rabbit anti-bovine LH
1:16000
1:32000
1:64000
% of 125I-LH bound
0
20
40
60
80
4
8
16
30
40
50
60
Reciprocals of goat anti-rabbit gamma globulin dilution


ng NIH-LH-B5 added
0
4
8
12
16
100% recovery
actual recovery
0
4
8
12
16
20
24
ng NIH-LH-B5 measured

antisera, and the reduced recoveries I obtained are difficult to explain.

Examples of precision of the LH RIA are presented in Table 1. The serum values were analyzed for each dilution duplicate in 3 to 7 different assays and include the range of serum LH concentration usually encountered.

Table 1.--Precision of the determination of LH in analysis of different serum pools.

Serum pool	Results of individual assays	Mean ± SE
	(ng LH/ml)	
No. 1	1.0, 0.7, 1.1, 1.0, 1.0	1.0 ± 0.07
No. 2	2.4, 2.0, 1.7, 2.7, 1.9	2.1 ± 0.2
No. 3	8.4, 8.1, 9.2	8.6 ± 0.3
No. 4	12.0, 14.4, 15.6, 16.7, 11.1, 9.7, 11.9	13.0 ± 1.0

Pooled serum low or high in thyroid stimulating hormone (TSH) did not appear to affect the assay. Niswender et al. (1969) found that this antisera was extremely specific for LH. Preparations representing high levels of TSH, follicle stimulating hormone (FSH), prolactin, or growth hormone (GH) relative to the level of LH did not appear to affect the ability of the antisera to selectively bind LH.

Serum from several species were also analyzed. The bovine LH assay appeared to be specific for LH in the goat and pig, possibly for LH in the horse, but was not specific

for LH in the rabbit. Different quantities of bovine serum were assayed and serum inhibition was not detected (Figure 2). Estimates of serum LH contained in 50 ul to 300 ul serum agreed and the slope of the curve was parallel to the curve for standard LH (Figure 2). Thus the assay appears to be very sensitive and specific, and ideally suited to quantifying low serum LH in luteal phase heifers.

2. Prolactin

The procedure used to quantify bovine prolactin was developed by Tucker (1970). Anti-bovine prolactin was developed in guinea pigs by repeated subcutaneous injections of NIH-Prolactin-B1[2] in the scapular region. For the initial injection, 2 mg prolactin was emulsified in 2 ml 0.85% NaCl and 2 ml Freunds Complete Adjuvant (Difco Lab., Detroit, Mich.). In succeeding injections at monthly intervals, the prolactin was emulsified in 2 ml 0.85% NaCl and 2 ml Freunds Incomplete Adjuvant. Blood was obtained by heart puncture 1 week after the third injection and 1 week after subsequent booster injections. Antisera, recovered by centrifugation, was stored at -20 C.

The second antibody, sheep anti-guinea pig gamma globulin (SAGPGG) was developed in sheep as follows. After dissolving in 2.5 ml distilled water, 50 mg guinea pig

---

[2]Supplied by the National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland.

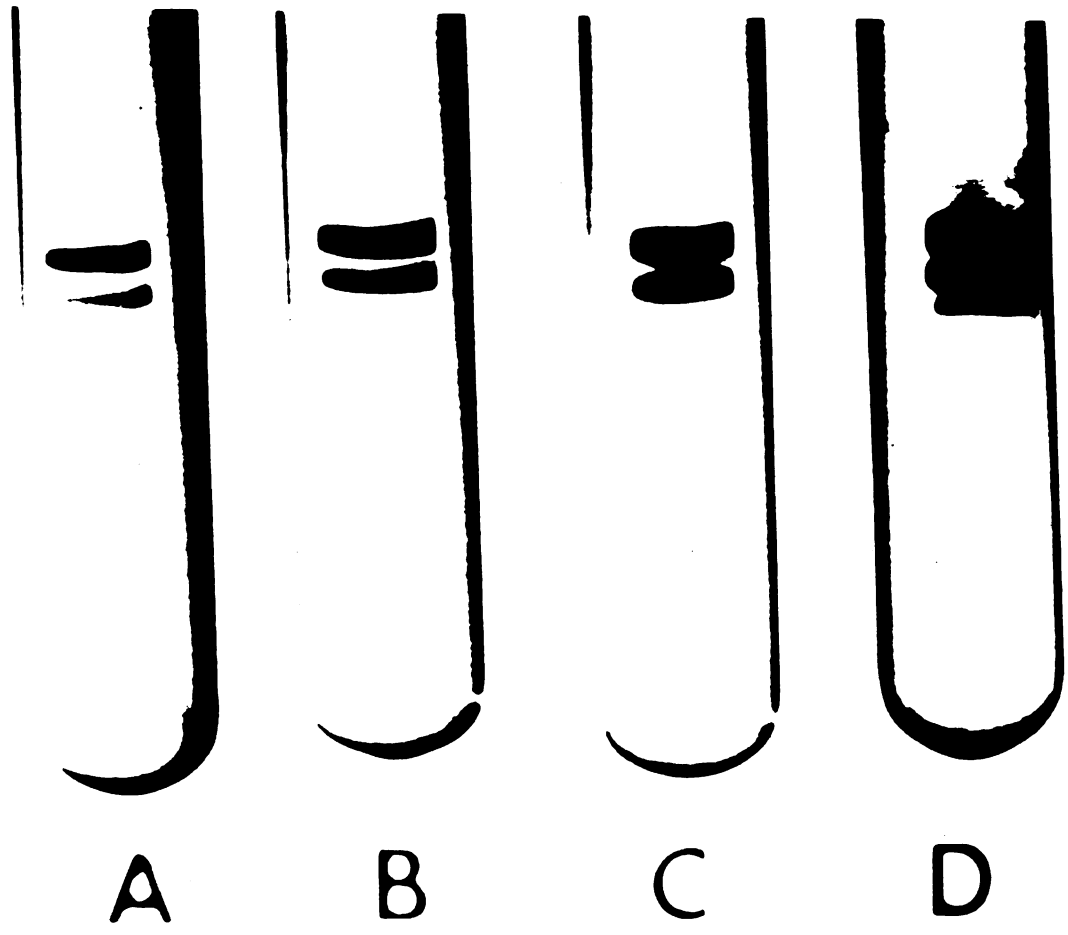
gamma globulin (Fraction II, Pentex Inc., Kankakee, Ill.) was emulsified in an equal volume of Freunds Complete Adjuvant and injected subcutaneously in multiple sites over the scapular region. Succeeding injections at 6-week intervals contained gamma globulin emulsified in Freunds Incomplete Adjuvant. Blood was obtained by jugular venipuncture 10 days after the third injection and 10 days after subsequent booster injections; usually 400 ml blood was withdrawn each time.

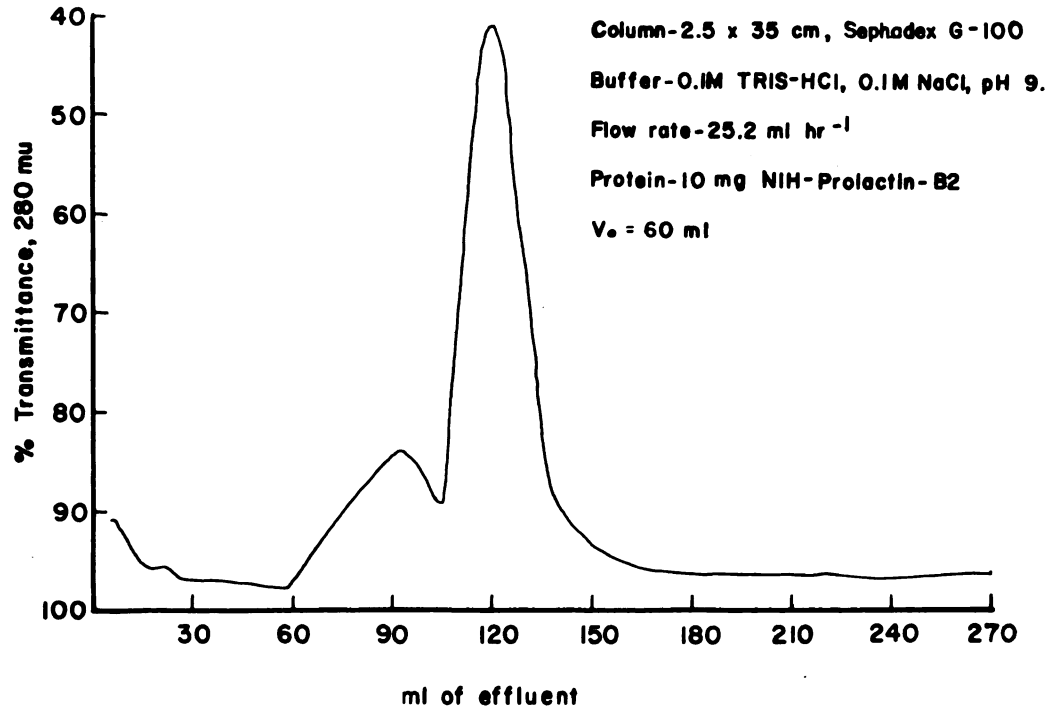
a. Radioiodination.--The procedure followed to iodinate prolactin was very similar to that used to iodinate LH, so only the variations will be pointed out. Because purified bovine prolactin was not available, NIH-Prolactin-B2 was used as a source of prolactin for iodination. As illustrated in Figure 5, the NIH preparation is not a homogeneous protein because 4 bands could be distinguished by disc gel electrophoresis.

The RIA involved competition between a labeled and unlabeled hormone for a limited number of antibody binding sites. Thus the labeled and unlabeled hormones must be identical in every respect save for the label to compete in a dose response fashion. It is impossible to raise a homogenous antibody and it is unnecessary as long as an homogeneous labeled antigen is available. Antibodies other than against bovine prolactin may be present and not interfere in the antigen-antibody reaction of interest. This

Figure 5. Polyacrylamide gel electrophoresis bands of 25(A), 50(B), 75(C), or 100(D) ug NIH-B2-prolactin.

Figure 6. Elution profile of NIH-B2-prolactin after passage through Sephadex G-100.


A
B
C
D


Column-2.5 x 35 cm, Sephadex G-100
Buffer-0.1M TRIS-HCl, 0.1M NaCl, pH 9.1
Flow rate-25.2 ml hr -1
Protein-10 mg NIH-Prolactin-B2
Ve = 60 ml
% Transmittance, 280 mu
40
50
60
70
80
90
100
30
60
90
120
150
180
210
240
270
ml of effluent

was not a problem in the LH RIA because highly purified bovine LH (LER-1072-2) was available.

An attempt to purify the prolactin by passage through Sephadex G-100 failed. The elution profile (Figure 6) showed a small rise before the major peak. The fractions within each peak were dialyzed against distilled water, lyophilized and reconstituted in distilled water before further analysis to remove buffer salt. Subsequent disc gel electrophoresis of this first peak failed to reveal any protein. And the major peak exhibited stained bands identical to the starting material (Figure 5). This purification method was not pursued further, but Ellis et al. (1969) reported that rat prolactin could be purified by passage through DEAE cellulose following Sephadex G-100 chromatography. Additional purification of bovine prolactin by use of DEAE-Sephadex A-50 has been reported by Johke (1969b).

Further attempts to purify NIH-prolactin were explored using the technique of isoelectric focusing (Haglund, 1967). Ten mg NIH-Prolactin-B2 were layered in the middle of an electrofocusing column (Model 9100, LKB-Produkter AB, Sweden) containing a sucrose gradient and 1% ampholine (LKB 8141) with a pH gradient between 3 and 10. The column was maintained at 3 C with coolant and 300 V DC power applied. But the prolactin precipitated within 30 min after the voltage was applied. To increase the

solubility of prolactin, the experiment was repeated with 2M urea, cyanate free, (Urea, Ulta Pure, Mann Research Lab., New York, N.Y.) incorporated throughout the column. But precipitation, though delayed, still occurred. Therefore the project to purify NIH-prolactin was abandoned. Possibly bovine prolactin would not precipitate if it were incorporated into 8M urea as Ellis et al. (1970) reported for bovine GH in electrofocusing.

Five ug of NIH-Prolactin-B2 was reacted with ^{125}I for 2 min in the presence of 25 ug chloramine-T. Other procedures were similar to the LH iodination with the exception that 1-ml aliquots from the Bio Gel P-60 column were collected in tubes containing 1 ml PBS-2%BSA. As illustrated in Figure 7, prolactin was iodinated more efficiently than LH. ^{125}I-prolactin could be used no longer than 2 to 3 weeks after iodination before passage through Sephadex G-100 to separate and remove radiation damaged ^{125}I-prolactin and free ^{125}I which accumulated during storage. The elution profile from a 1 x 12 cm Sephadex G-100 column eluted with 0.05M sodium phosphate buffer was similar to the elution profile from a Bio Gel P-60 column (Figure 7).

b. Radioimmunoassay.--Because of the high levels of prolactin in blood sera, the sera were diluted from 1:2 to 1:10 with PBS-1% BSA (Appendix I.B.3) which was the buffer used in the prolactin RIA. The diluted sera were

then assayed in dilution duplicate. One hundred ul or 200 ul diluted serum was added to disposable glass culture tubes (12 x 75 mm) with a Hamilton microliter syringe and then PBS-1% BSA was added to a total volume of 500 ul. Each lot of 96 tubes included 10 tubes containing 0.1, 0.2, 0.4, 0.8, 1.0, 1.5, 2.0, 3.0, and 4.0 ng of standard prolactin (NIH-Prolactin-B2) (Appendix I.B.4). One hundred ul of the first antibody (GPABP) (Appendix I.B.6) was added on day 1 and incubation periods were similar to the LH RIA. One hundred ul ^{125}I-prolactin was added on day 2 and 100 ul second antibody (SAGPGG) (Appendix I.B.7) was added on day 3. The tubes were vortexed gently, covered, and incubated at 4 C after each addition.

c. Selection of Assay Conditions.--The sensitivity of the pooled antisera obtained from 3 or 4 guinea pigs was determined by using several dilutions of the antisera in the assay. The avidity or binding affinity of the antisera was determined by comparing the slope of the antisera dilution curve; a steep slope indicated high avidity.

The dilution of the second antibody (SAGPGG) was selected to yield optimal precipitation. An example of a double titration of both first and second antibody is shown in Table 2. In this case, the first antibody was used at a 1:25,600 dilution and the second antibody was used at a 1:5 dilution.

Table 2.--Titration of guinea pig anti-bovine prolactin (GPABP) and sheep anti-guinea pig gamma globulin (SAGPGG) for the prolactin radioimmunoassay.

Dilution of GPAB prolactin	Dilution of SAGPGG		
	1:3	1:4	1:5
	(% of ^{125}I - Prolactin bound)		
1:12800	45	46	46
1:25600	36	35	36

d. Validation of Assay.--The prolactin RIA was repeatable and sensitive. A typical standard curve (Figure 8) was sensitive to 0.1 ng prolactin per assay tube. Increasing levels of bovine serum gave a dose response curve parallel to the standard curve (Figure 8). Recovery of exogenous NIH-B2-prolactin added to serum diluted 1:16 was quantitative over the range of the standard curve (Figure 9). Precision of the prolactin RIA is indicated by a standard serum included in all assays. The mean value over 8 assays was 40.3 $\pm$ 2.3 ng/ml when run in dilution duplicate within each assay. The specificity of the assay was discussed by Tucker (1970) and the only bovine hormone which interfered, NIH-GH-B12, did so at levels of 100 ng or more per tube, levels at least 20 times higher than normally found in bovine sera.

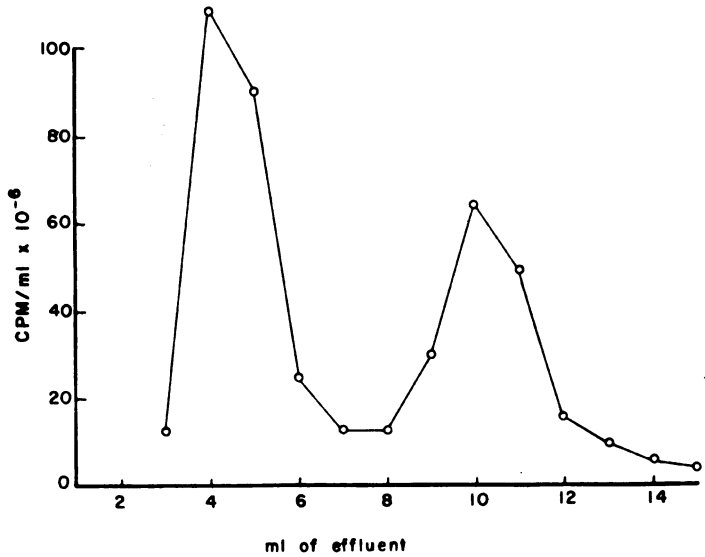
I. Protein Binding Assays

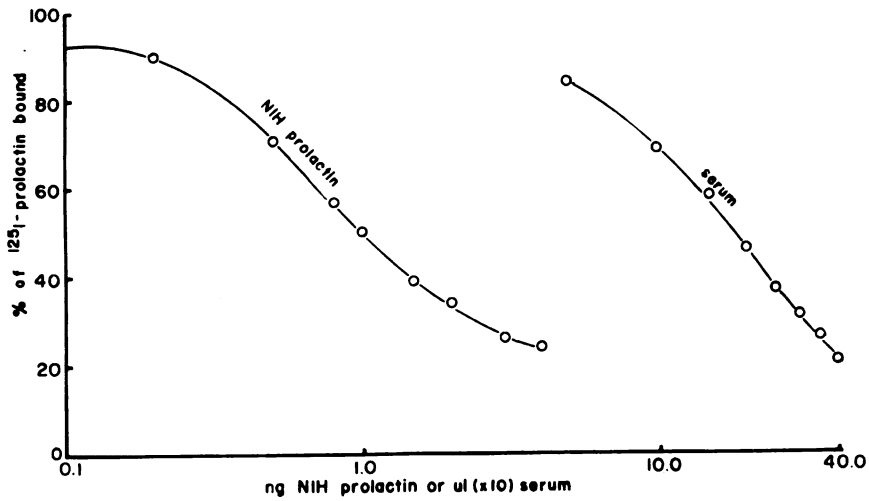
The protein binding assays for steroids, first developed by Murphy (1967), do not have the specificity of

Figure 7. Elution profile of iodinated prolactin after passage through Bio Gel P-60. The first peak represents iodinated prolactin and the second peak represents free iodine.

Figure 8. Dose response curves for NIH-B2-prolactin standards and for bovine sera diluted 1:8.

Figure 9. Recovery of exogenous bovine prolactin added to 100 ul bovine serum diluted 1:16.


CPM/ml x 10^{-6}
100
80
60
40
20
0
2
4
6
8
10
12
14
ml of effluent


% of ^{125}I-prolactin bound
100
80
60
40
20
0
NIH prolactin
serum
0.1
1.0
10.0
40.0
ng NIH prolactin or ul (x10) serum

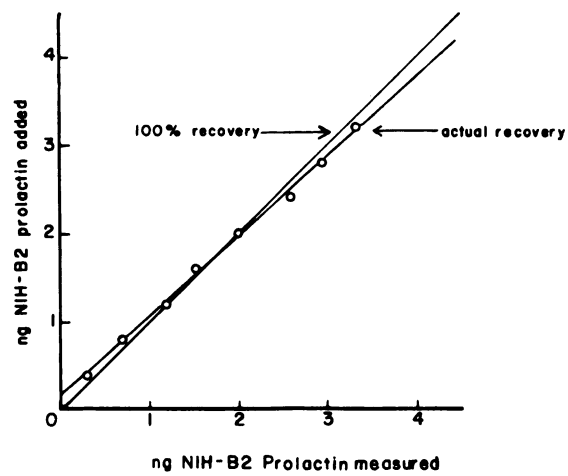
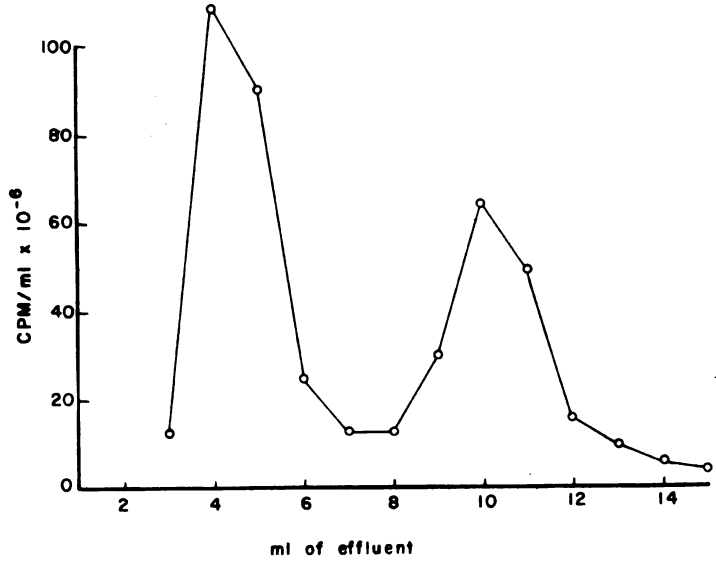
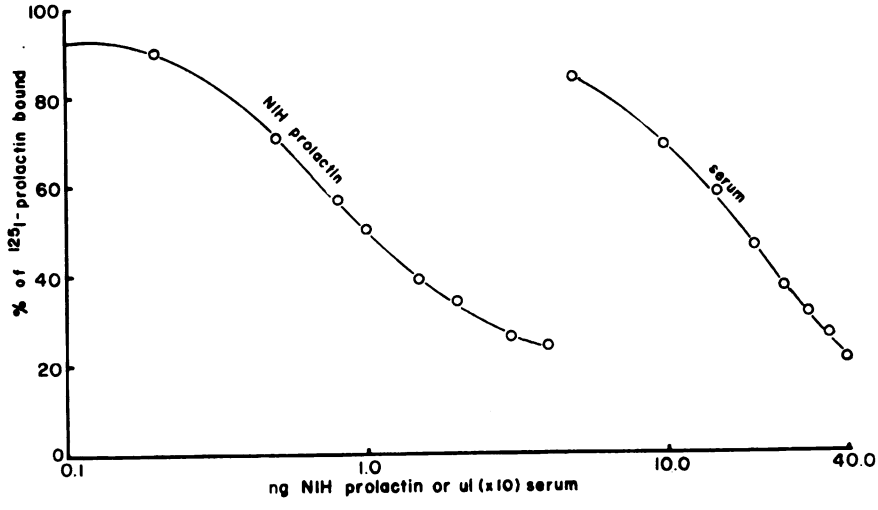

ng NIH-B2 prolactin added
4
3
2
1
0
100% recovery
actual recovery
1
2
3
4
ng NIH-B2 Prolactin measured

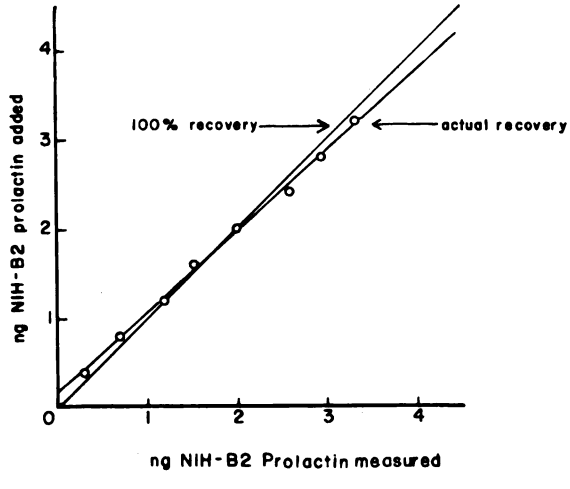
Figure 7. Elution profile of iodinated prolactin after passage through Bio Gel P-60. The first peak represents iodinated prolactin and the second peak represents free iodine.

Figure 8. Dose response curves for NIH-B2-prolactin standards and for bovine sera diluted 1:8.

Figure 9. Recovery of exogenous bovine prolactin added to 100 ul bovine serum diluted 1:16.


CPM/ml x 10⁻⁶
100
80
60
40
20
0
2
4
6
8
10
12
14
ml of effluent


% of 125I-prolactin bound
100
80
60
40
20
0
NIH prolactin
serum
0.1
1.0
10.0
40.0
ng NIH prolactin or ul (x10) serum


ng NIH-B2 prolactin added
4
3
2
1
0
100% recovery
actual recovery
1
2
3
4
ng NIH-B2 Prolactin measured

radioimmunoassays. They are based on competition between a labeled and unlabeled hormone for a limited quantity of binding protein. Because these binding proteins will bind several steroid hormones, the steroids to be assayed in the serum must be separated before quantification.

1. Isolation of Steroid Hormones

I wished to isolate each of the following steroid hormones; progesterone, corticosterone, cortisol, estrone, estradiol, and estriol. For specificity, the separation method should separate the steroid hormones of interest cleanly and efficiently, without interference in the subsequent protein binding assay. Tritiated and ^{14}C-labeled steroids were used in the studies to develop separation methods.

The consensus of opinion at the Second Karolinska Symposium (Diczfalusy, 1970) was that thin layer chromatography, although giving excellent separation, was troublesome in the protein binding assays. Regardless of the procedure used to clean the plates and the gel used with them, blank values in the assays were erratic; usually unacceptably high, presumably because of contaminants from the chromatogram.

a. Techniques.--Separation of steroids was attempted with columns of 60-100 mesh florosil (Fisher Scientific Co.) which had been washed in water and methanol to clean and remove fines. This would be an acceptable system if the

steroid hormones would be eluted individually with a discontinuous solvent system. Various ratios of the following solvent (reagent grade) systems were tried; benzene:ethyl acetate, benzene:acetone, octonol:hexane, chloroform:acetone, chloroform:methanol, and chloroform:methanol:water. Although some of these systems would separate some of the steroids, none isolated all six steroids. However, a solvent system was identified to achieve partial separation. After application to a 1 x 10 cm florosil column the sample was eluted with 40 ml chloroform:acetone (7:3). This fraction contained progesterone, estrone, estradiol and about 75% of the estriol. The column was then eluted with 40 ml chloroform:methanol (9:1) to remove corticosterone, cortisol, and the remaining estriol.

Recently a material suitable for column chromatography with organic solvents (Sephadex LH-20, Pharmacia Fine Chemicals, Inc.), has been made available. This is a lipophilic derivative of Sephadex which is stable and inert. After trying different solvent systems, a solvent combination was identified which would achieve separation of all six steroids. The best combination seemed to be a 96:4 chloroform:ethanol system. Estriol, the final steroid eluted, came off at about 200 ml with the 96:4 system. The other steroids came off in the order; progesterone, corticosterone, estrone, cortisol, and estradiol. To speed the elution of estriol, 9:1 chloroform:ethanol was applied

immediately after elution of cortisol. Estriol then came off at about 125 ml, but slower flow rate and time involved in re-equilibrating the column with the 96:4 system obviated any advantage of switching solvents.

Murphy (1970) reported that reagent grade solvents and even the new "nanograde" solvents could deteriorate and cause problems in the protein binding assays. This problem could be eliminated by redistillation of the solvents. Consequently, use of absolute ethanol (Gold Shield Alcohol, Commercial Solvents Corp., Terre Haute, Ind.) was discontinued and redistilled 95% ethanol was used. With 95% ethanol, the 95:5 chloroform:95% ethanol system gave optimal results.

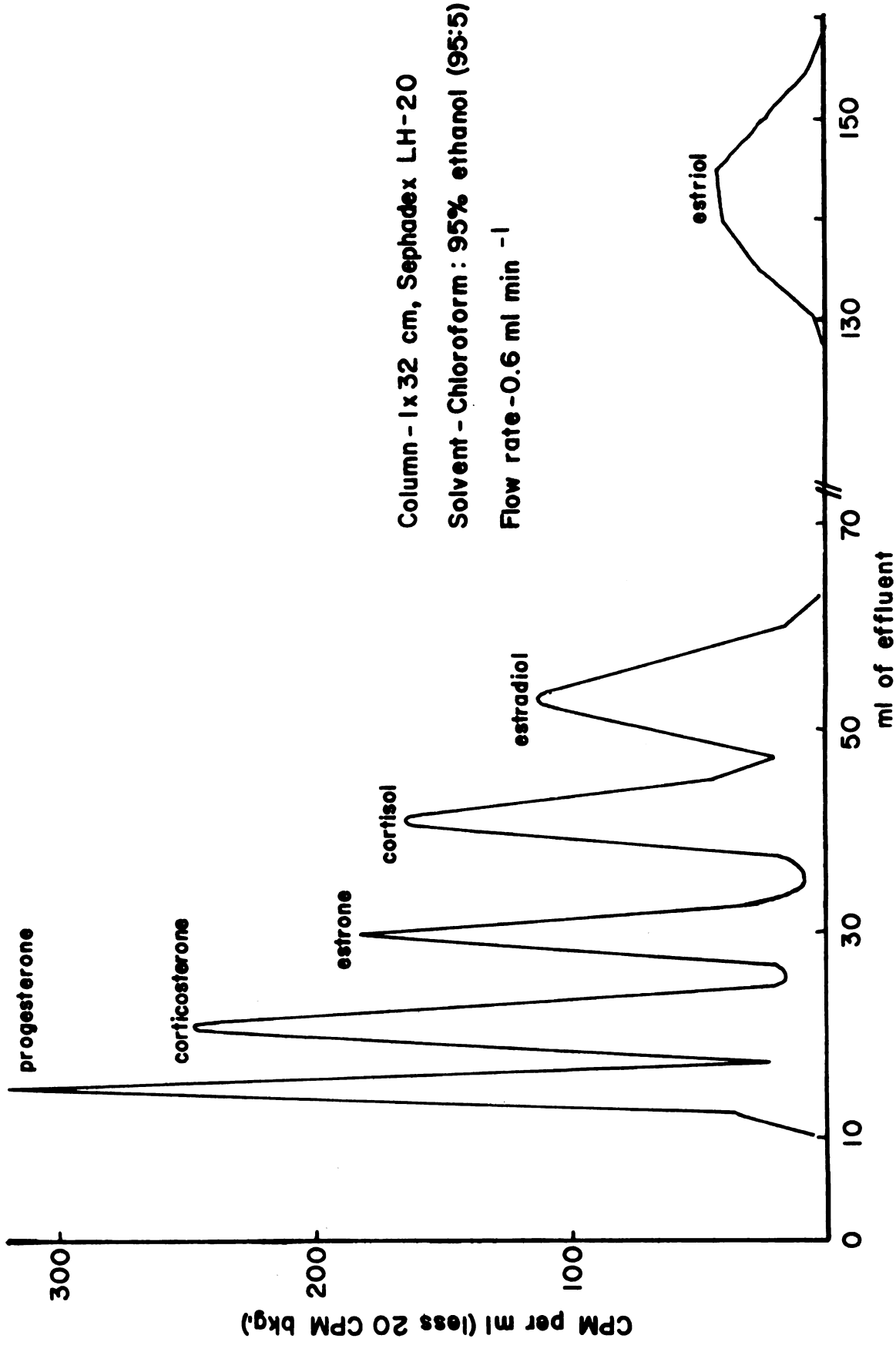
The final variable studied was column size. Two diameters were used, 1.0 and 1.9 cm. Although the 1.9 cm column had a faster flow rate, the steroids were eluted in a larger volume. By increasing the length to 32 cm, 1.0 cm diameter was sufficient to give clear separations. The column eventually used had an internal diameter of 1.0 cm, an overall length of 40 cm, a Teflon stopcock at the discharge end, and a 14/35 glass fitting at the upper end. Sephadex LH-20, equilibrated in chloroform:95% ethanol (95:5), was packed to 32 cm with cotton plugs above and below to prevent the Sephadex LH-20 from floating. A separatory funnel with a Teflon stopcock and 14/35 fitting was attached to the top of the column to provide a solvent

reservoir and a means to adjust flow rate by changing head pressure. This was desirable because six columns were mounted over a fraction collector to collect samples from six columns simultaneously. The fraction collector (Isco Model 567, Instrumentation Specialties Co., Lincoln, Neb.) was equipped with a 11.5-inch reel which would accept 12 x 75 mm disposable glass tubes. One column was mounted over a Volumeter (Isco Model 400) and the elution rates of the other 5 columns were adjusted to that of the first column. Twenty 2.5-ml fractions were collected and then 5-ml fractions were collected until estriol was eluted. The elution profile in Figure 10 was typical and incorporates all the changes studied.

Elution patterns also were established for testosterone, androstenedione and 20α-hydroxypregn-4-en-3-one (20α-ol). Androstenedione eluted with progesterone, 20α-ol between progesterone and corticosterone, and testosterone slightly before corticosterone or superimposed over it.

Reagent grade chloroform and 95% ethanol were redistilled within 3 days of use; columns were flushed for 30 min immediately before use and for 1 to 3 hr after elution of estriol. Flow rates, although dependent on head pressure, were about 0.65 ml/min when freshly packed with Sephadex LH-20. The flow rate tended to slow to 0.4 to 0.5 ml/min after 4 to 8 samples had been eluted. Therefore the columns were repacked every 1 to 2 weeks, re-using the Sephadex LH-20.

Figure 10. Elution profile of an ether extract of bovine serum from a Sephadex LH-20 column.


progesterone
corticosterone
estrone
cortisol
estradiol
estriol
Column - 1 x 32 cm, Sephadex LH-20
Solvent - Chloroform : 95% ethanol (95:5)
Flow rate - 0.6 ml min -1
CPM per ml (less 20 CPM bkg.)
300
200
100
0
10
30
50
70
130
150
ml of effluent

b. Extraction Procedure.--After thorough washing with detergent, all glassware was meticulously rinsed in tap water, distilled water, glass distilled water and redistilled methanol before use in the extraction. About 800 to 1000 cpm (H^3) of each of the 6 steroid hormones was added in ethanol to a 20 ml screw cap vial with a polyethylene liner in the cap. These labeled steroids (estrone -6, 7-^{3}H, 48 c/mM, New England Nuclear, Boston, Mass.; estradiol -6, 7-^{3}H, 31.7 c/mM, Nuclear Chicago Corp.; estriol -6, 7-^{3}H, 14.7 c/mM, New England Nuclear; progesterone -1, 2-^{3}H, 33.5 c/mM, New England Nuclear; corticosterone -1, 2-^{3}H, 49.5 c/mM, New England Nuclear; cortisol -1, 2-^{3}H, 45.3 c/mM, New England Nuclear) were purified on Sephadex LH-20 columns before use.

Serum (5 ml) was added and gently mixed to equilibrate the tracer hormones and then 10 ml anhydrous diethyl ether (Mallinckrodt Chemical Works, St. Louis, Mo.) was added. Each vial was vigorously vortexed 15 sec and then manually agitated for 2 min. After freezing at -20 C, the ether extract was poured into conical tubes. The extract was evaporated under nitrogen to about 0.5 ml and the serum re-extracted with 10 ml ether. The remaining contents in the can of ether was discarded at the end of the day of use. After the sera had frozen, the second ether extract was poured into the same conical tube and evaporated under nitrogen to dryness. The extract was taken up in about

0.2 ml chloroform:95% ethanol (95:5) and was ready for column chromatography.

c. Recovery.--After elution from the column, 1-ml aliquots from selected tubes were evaporated in scintillation vials (Low-Potassium I vial, Packard Instrument Co., Downers Grove, Ill.), 5 ml steroid scintillation fluid (Appendix II.A.) added, and the sample counted for 10 min in a liquid scintillation counter (Nuclear Chicago Model Mark I) to verify the elution peak and to calculate recovery of isotopes. Counting efficiency of the Mark I scintillator using the steroid scintillation fluid averaged 36%. Progesterone was always eluted in one tube so only 0.5 ml was counted for recovery. Recovery estimates (Table 3) and the elution profile (Figure 10) were repeatable.

2. Estrogens

a. Procedure.--Serum estrogens were quantified in a competitive protein binding assay--the estrogen binding protein was isolated from uteri of estrous rabbits. The procedure used was developed by Korenman (1968) and Korenman et al. (1969). The estrone and estriol assays followed the procedure of Tulchinsky and Korenman (1970). Standards were diluted in redistilled 95% ethanol so that 100 ul contained 0, 10, 20, 40, 80, 160, 320 or 640 pg estradiol; 0, 40, 80, 160, 320, 640, 1280 or 2560 pg estrone and 0, 20, 40, 80, 160, 320, 640 or 1280 pg estriol (Appendix III.A).

Table 3.--Recovery estimates of serum steroid hormones after 2 ether extractions and separation on a 1 x 32 cm Sephadex LH-20 column.

Hormone	Recovery (%)
Progesterone	86.2
Corticosterone	97.0
Estrone	76.9
Cortisol	91.9
Estradiol	85.4
Estriol	78.5

The estrogen binding protein (EBP) from rabbit uteri was prepared by homogenizing the uteri of estrous rabbits in 3 volumes buffer A (Appendix III.B.) for two 30-sec pulses at 21,000 RPM in a high speed Waring blender (Waring Products Co., Winsted, Conn.). After centrifugation for 15 min at 5000 x g, the supernatant was centrifuged at 105,000 x g for 90 min (Beckman Spinco Model L). The estrogen binding protein from the ultracentrifugation supernatant is temperature sensitive and all procedures were performed at 4 C. The resulting EBP was packaged in aliquots sufficient for 1 days use and stored at -20 C for no more than 2 weeks.

The unknowns from the columns or standards were pipetted into 12 x 75 mm disposable glass tubes and dried under nitrogen. Because of space limitations in centrifugation, each assay consisted of 48 tubes. Unknowns and standards were prepared in duplicate, and the unknowns in

dilution duplicates. One ml buffer A containing 20% ethylene glycol was added to each tube and the tubes were kept in an ice bath or refrigerated for the remainder of the assay.

Labeled estrogens (for the estradiol assay, estradiol -6, 7-^{3}H, 31.7 c/mM, Nuclear Chicago Corp.; for the estrone and estriol assays, estrone -6, 7-^{3}H, 48.0 c/mM, New England Nuclear) were purified by passing through Sephadex LH-20 and diluted in buffer A so that 10 ul would contain about 12,000 CPM. Ten ul estrogen -^{3}H was added to each tube and the contents of each tube were mixed vigorously using a vortex mixer. The estrogen binding protein was added and gently mixed, and the tubes were covered and incubated overnight (15-18 hr) at 4 C. In the estradiol assay, 25 ul EBP (6.25 ug uterus equivalent) was added, while in the estrone and estriol assays, 50 ul EBP (12.5 ug uterus equivalent) was added. Binding of estrone -^{3}H tracer alone using 25 ul EBP averaged 5 to 10% whereas with 50 ul EBP, binding ranged from 10 to 46% (avg 21.8%).

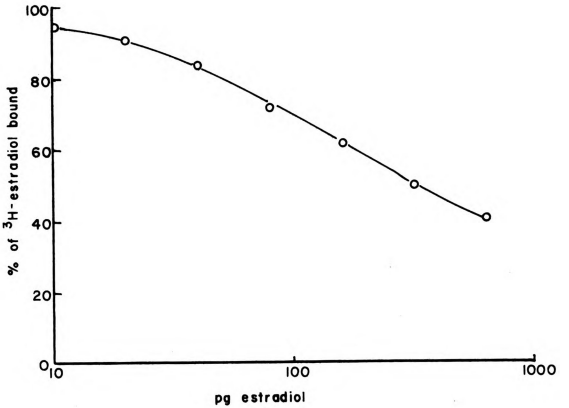
One ml dextran coated charcoal [0.25% Norit A neutral charcoal (Fisher Scientific Co.), 0.025% dextran 150 (Pharmacia Fine Chemicals) in buffer A] was added rapidly and lightly mixed, and the tubes were incubated in an ice bath for exactly 15.5 min from the start of the charcoal addition. Usually charcoal could be added and mixed in 48 tubes within 2 min. The tubes were centrifuged

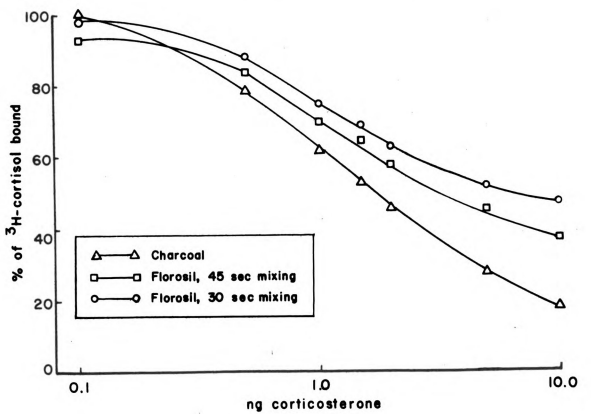
at 4 C in a centrifuge with a swinging bucket rotor (Sorvall Model RC-3) at 2500 x g for 10 min. One-half ml supernatant was removed and added to 10 ml Bray's solution (Appendix II.B.) using an automatic diluter (Model LD-1, York Instrument Corp., Berkeley, Calif.). This was placed in glass scintillation vials (Packard Instrument Co.) and counted for 4 min in a liquid scintillation counter (Nuclear Chicago Model Mark 1). Counting efficiency of the scintillator using Bray's solution was 28%. The duplicate standards were averaged and plotted as the per cent of estrogen $-^3H$ bound at each dose of estrogen compared to the estrogen $-^3H$ bound when tracer alone was added. An example of an estradiol standard curve is presented in Figure 11. Estrone and estriol standard curves were of similar slope and linearity. The unknowns were calculated by interpolation between standards and factors to correct for recovery and volume of serum extracted were applied to arrive at a per ml concentration.

b. Validation of Assay.--Binding of estradiol $-^3H$ tracer alone in the estradiol assay ranged from 14% to 28% (avg 18.7 $\pm$ 1.2%) and binding of estrone $-^3H$ tracer alone in the estrone and estriol assays ranged from 10% to 46% (avg 21.8 $\pm$ 2.3%). Korenman et al. (1970) reported that relative to estradiol, binding affinity of estrone was 66%. The use of 50 ul EBP increased binding of estrone to that of estradiol. The degree of binding did not appear to affect the assays as long as binding was about 10% or more.

Figure 11. Dose response curve for estradiol standards.

Figure 12. Dose response curves for corticosterone standards comparing different methods of removing unbound cortisol-^{3}H.


100
80
60
40
20
0
% of ^{3}H-estradiol bound
10
100
1000
pg estradiol


100
80
60
40
20
0
% of ^{3}H-cortisol bound
Charcoal
Florosil, 45 sec mixing
Florosil, 30 sec mixing
0.1
1.0
10.0
ng corticosterone

Solvent blanks were included in each assay and yielded values which were acceptable for estrone and estradiol, but they were high (238 pg) in the estriol assay (Table 4). The low blank values in the estradiol assay, when considered on a per ml basis (7 pg/ml) are comparable to values reported by Korenman et al. (1969). The high solvent blank values in the estriol assay obviate the usefulness of the assay. The assay of freshly distilled solvents also yielded high blank values (100 pg).

Table 4.--Effect of solvent blanks in the estrogen assays.

Assay		Solvent blank [a,b]	% bound relative to binding of tracer alone[b]
	(n)	(pg)	
Estrone	20	33 ± 10	89.6 ± 3.0
Estradiol	17	22 ± 5	91.0 ± 2.3
Estriol	17	238 ± 36	73.8 ± 2.9

[a]Solvent blanks were collected from the LH-20 column between the elution of estradiol and estriol. They were assayed at 0.5 to 1.0 ml (estrone) and at 2 to 4 ml (estradiol and estriol).

[b]Mean ± SE.

Estimates of recovery and precision for each of the estrogen assays are presented in Table 5. The precision of the estrone assay was satisfactory but recovery of 250 pg or 500 pg estrone was low (50%) and recovery of 1000 pg estrone was greater than 100%. Estimates of estradiol appeared to increase as the volume of serum extracted

Table 5.--Estimates of recovery and precision for the estrone, estradiol, and estriol competitive protein binding assays.[a]

	Estrone			Estradiol			Estriol		
	pg/ml	Recovery pg	Recovery %	pg/ml	Recovery pg	Recovery %	pg/ml	Recovery pg	Recovery %
Serum extracted, ml[b]									
3	26.3 ± 14.2	-	-	13.7 ± 4.4	-	-	271 ± 68	-	-
5	9.8 ± 1.2	-	-	19.4 ± 6.7	-	-	133 ± 33	-	-
7[c]	18.2 ± 2.2	-	-	23.6 ± 11.4	-	-	86 ± 12	-	-
Estrogen added to 5 ml serum, pg[d]									
250	-	116	46.3	-	540	216.0	-	1040	417.0
500	-	314	62.8	-	614	122.8	-	4045	809.0
1000	-	2955	296.0	-	1193	119.3	-	5125	512.5

[a] A common pool of serum was used for all assays.

[b] Mean ± SE of dilution duplicate estimations of 3 replicates.

[c] Mean ± SE of dilution duplicate estimations of 2 replicates.

[d] Average of dilution duplicate estimations for 2 replicates.

increased. Recovery of 500 or 1000 pg estradiol was satisfactory while recovery of 250 pg was 216%. Corker et al. (1970) reported excellent recovery of estradiol in the estradiol competitive protein binding assay and Tulchinsky and Korenman (1970) also reported excellent recovery of estrone in the estrone assay. The recoveries reported here indicate a lack of sensitivity at levels of 50 pg/ml. As with solvent blanks, estriol precision and recovery were unacceptable. The decreasing concentration of estriol as volume of serum increased may indicate very low levels of estriol in bovine serum. If a solvent contaminant is responsible, it is acting in a nonspecific way.

3. Glucocorticoids and Progesterone

a. Procedure.--Serum progesterone and glucocorticoids were quantified by the competitive protein binding method developed by Murphy (1967). These assays use the plasma protein, corticosteroid binding globulin (CBG) as the binding protein. Because this protein has binding affinity for many of the steroid hormones, separation of the steroids is necessary before quantification.

Standards were diluted in redistilled ethanol so that 100 ul would contain 0, 0.1, 0.5, 1.0, 1.5, 2.0, 5.0 and 10.0 ng (Appendix III.A.). Commercial dog plasma (Colorado Serum Co. Labs., Denver, Colo.) was diluted to 2.5% with distilled water and labeled corticoids were added (for progesterone assays, corticosterone -1, 2 -^{3}H, 49.5 c/mM; for corticosterone and cortisol assays, cortisol

-1, 2 -^{3}H, 45.3 c/mM) so that 1 ml would contain about 25,000 CPM (Appendix III.C.).

Prior to use, 12 x 75 mm disposable glass culture tubes were coated with 5% trimethylchlorosilane in toluene. Each assay consisted of 48 tubes, including 2 control tubes containing effluent from the Sephadex LH-20 column. Serum extracts or standards were dried under nitrogen; unknowns and standards were assayed in dilution duplicate. After placing the rack of tubes in crushed ice, 1 ml of the CBG-corticoid -^{3}H reagent was added to each of the tubes. After vortexing 15 sec, tubes were covered and incubated at 4 C overnight (15-18 hr). In the progesterone and corticosterone assay, unbound corticoid -^{3}H was removed by addition of about 80 mg 30 - 60 mesh florosil (Appendix III.D.) and immediately mixed for 45 sec. In the cortisol assay, unbound cortisol -^{3}H was removed by rapid addition of 1 ml dextran coated charcoal (1.25% neutral Norit A charcoal, 0.125% dextran 150 in glass distilled water). The tubes were kept in crushed ice and exactly 5 min after beginning addition of charcoal, they were centrifuged at 2500 x g for 15 min.

In each of the assays, 0.5 ml supernatant was removed, added to 10 ml Brays solution in glass liquid scintillation vials (Packard Instrument Co.) and counted for 4 min in a liquid scintillation counter (Mark 1, Nuclear Chicago Corp.). The duplicates of each standard dose were averaged and plotted as the per cent of corticoid -^{3}H bound at each dose of standard compared to the corticoid

-^{3}H bound when tracer alone was added. Examples of standard curves for corticosterone are presented in Figure 12. Standard curves for progesterone and cortisol were similar. The mass of unknowns were calculated by interpolation between standards, and corrected for recovery and volume of serum extracted.

As discussed by Murphy (1967), florosil is suitable for separating protein-bound and unbound corticosterone-^{3}H. A plastic scoop was devised which would hold 82.8 $\pm$ 0.7 mg (N=10) florosil. The florosil quickly settled after addition to the tubes and centrifugation was unnecessary. Less variation between duplicate standards was observed in the corticosterone assay when florosil was used in place of dextran coated chorcoal. The general shapes of the standard curves for charcoal and florosil were similar (Figure 12) but a steeper slope showed that charcoal removed more of the cortisol-^{3}H. Also illustrated in Figure 12 is a standard curve obtained when the contents of the tube were mixed for 30 sec after addition of florosil. Both procedures (mixing the contents of the tube 30 sec or 45 sec) were satisfactory although florosil was routinely mixed 45 sec.

b. Validation of Assay.--Binding of corticosterone -^{3}H in the progesterone assay ranged from 62% to 91% (avg 81.5 $\pm$ 2.7%). Binding of cortisol-^{3}H in the corticosterone assay ranged from 83% to 90% (avg 87.0 $\pm$ 1.0%) and in the

cortisol assay ranged from 58% to 90% (avg 75.4 $\pm$ 4.5%). The decreased binding in the cortisol assay is a result of using charcoal to remove unbound cortisol-^{3}H.

Solvent blanks from the LH-20 column were uniformly low in each of the assays and averaged about 0.03 ng. Estimates of hormone concentration were in agreement when 3 ml to 7 ml serum was extracted (Table 6). Recovery of progesterone averaged about 100% while recovery of corticosterone and cortisol were about 88%.

J. Statistical Analysis

Prepubertal data were analyzed separately while comparisons during the estrous cycle data were analyzed similarly to the model used by Hackett (1968). The 3-way analysis of variance included 3 main factors; estrous cycles, stage of estrous cycle, and heifers. Blood samples collected after day 18 of the cycle were relabeled days -3, -2, -1, and -0.5 for the statistical analyses. Cycles and stages of estrous cycle were fixed whereas heifers were random. To test for significant differences in levels of hormones due to stage of estrous cycle, I orthogonally partitioned the 8 degrees of freedom for stage of cycle as follows:

1. days -1, -0.5 and 0 versus the others (proestrus and estrus versus metestrus and diestrus.
2. days -1 and -0.5 versus day 0 (proestrus versus estrus).

Table 6.--Estimates of recovery and precision for the progesterone, corticosterone, and cortisol competitive protein binding assays.[a]

	Progesterone			Corticosterone			Cortisol		
	ng/ml	Recovery ng	Recovery %	ng/ml	Recovery ng	Recovery %	ng/ml	Recovery ng	Recovery %
Serum extracted, ml[b]									
3	3.18 ± 0.07	-	-	0.06 ± 0.02	-	-	1.73 ± 0.06	-	-
5	3.15 ± 0.27	-	-	0.07 ± 0.03	-	-	1.76 ± 0.17	-	-
7[c]	3.70 ± 0.60	-	-	0.08 ± 0.005	-	-	1.69 ± 0.03	-	-
Quantity of hormone added to 5 ml serum[d]									
10 ng	-	7.8	78.0	-	8.8	88.5	-	9.0	90.0
25 ng	-	29.3	117.2	-	21.8	87.2	-	21.0	84.0
50 ng	-	50.8	101.6	-	41.9	83.8	-	46.2	92.5

[a] A common pool of serum was used for all assays.

[b] Mean ± SE of dilution duplicate estimations for 3 replicates.

[c] Mean ± SE of dilution duplicate estimations for 2 replicates.

[d] Average of dilution duplicate estimations for 2 replicates.

3. day -1 versus day -0.5 (early proestrus versus late proestrus).

4. days 2 and 4 versus days 7, 11, -3 and -2 (metestrus versus diestrus).

5. day 2 versus day 4 (early metestrus versus late metestrus).

6. day 7 versus days 11, -3 and -2 (early diestrus versus late diestrus).

7. day 11 versus days -3 and -2 (middle diestrus versus late diestrus).

8. day -3 versus day -2 (late diestrus versus end of diestrus).

To test for significant differences in level of hormones due to cycles or ages, I combined the 9 estrous cycles into 3 groups and orthogonally partitioned the 2 degrees of freedom for cycle as follows:

1. cycles 1, 2 and 3 versus the others.

2. cycles 4 and 5 versus cycles 6, 7, 8 and 9.

Other analyses included simple correlations and regressions of levels of pituitary, ovarian, and adrenal hormones. As in all experiments of this kind, a basic assumption of the analysis of variance, that errors be uncorrelated, may be violated in these analyses because repeated measurements were made on individual heifers. To the extent this occurred, the type I error was underestimated.

The prolactin and LH data in the acute estrous study were analyzed two ways. Firstly the two variables, heifers and days, were analyzed to detect serum hormone

changes with days, both before and after estrus. Data from all samples collected within 24-hr periods were pooled for this purpose. Secondly, data from samples collected at specific hours on different days were pooled to analyze for diurnal effects.

RESULTS AND DISCUSSION

A. Prepubertal Phase

1. Growth

Growth, as determined by body weight and withers height, was linear with age (Figures 13 and 14). Postpubertal heifers were slightly larger than prepubertal heifers at similar ages as evidenced by the regression of weight on age for the prepubertal heifers ($b=18.33 \pm 2.12$) and postpubertal heifers ($b=23.97 \pm 1.48$). In general, these heifers had normal body weight but slightly less height compared with Morrison's (1957) standards (Figures 13 and 14).

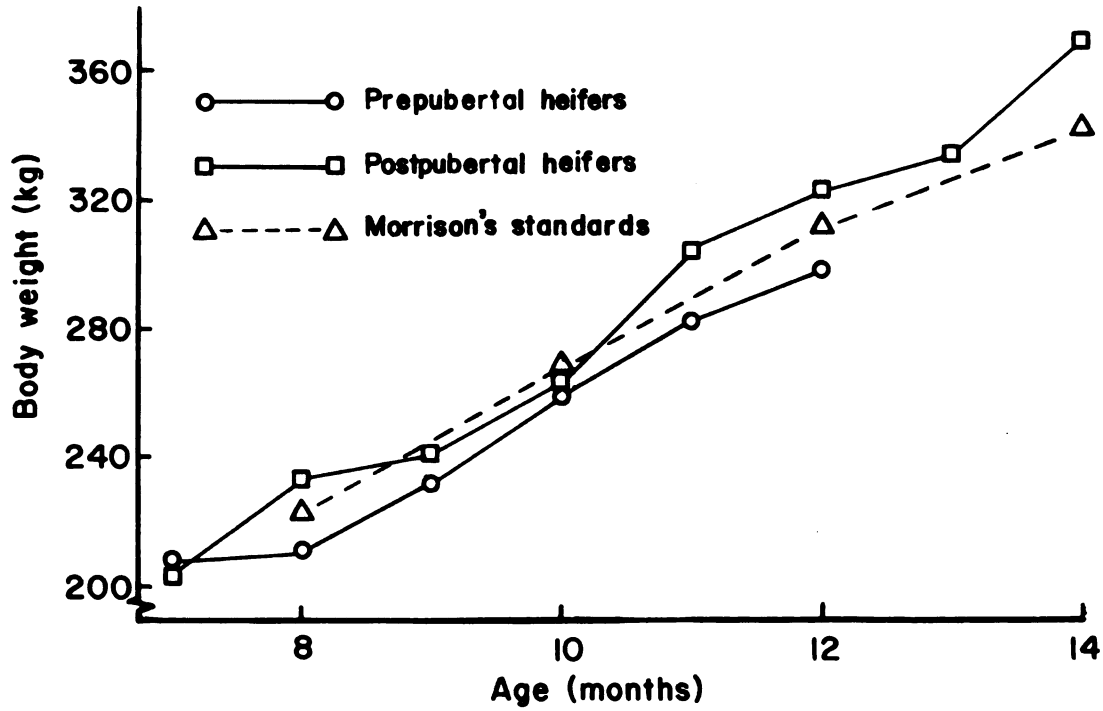
2. Behavior

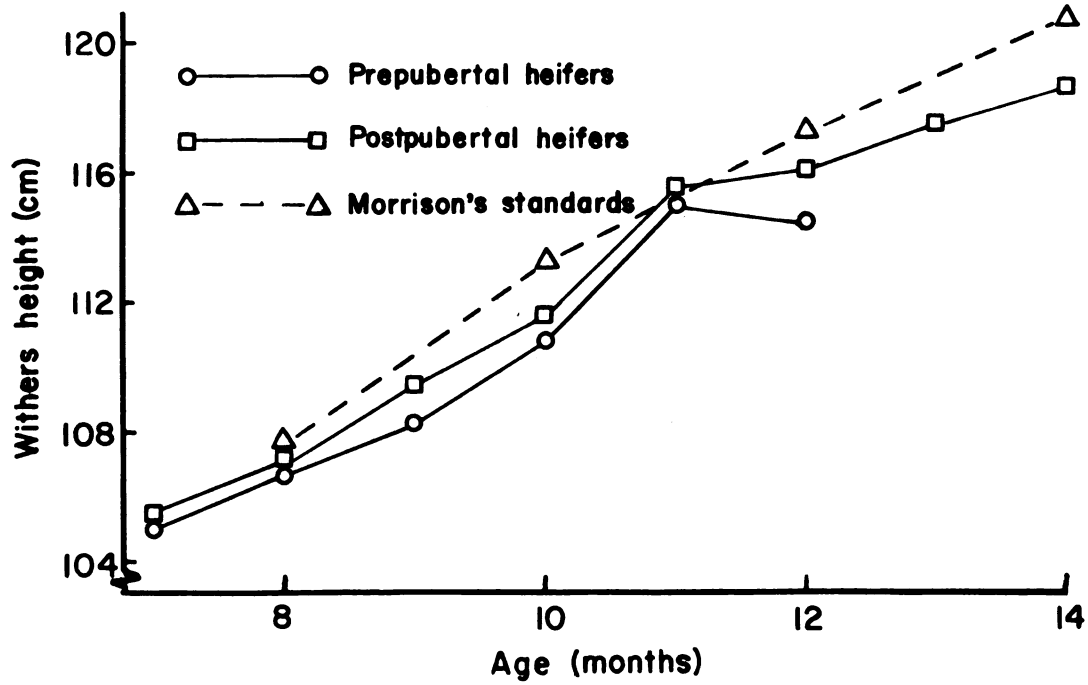
Puberty, the beginning of the first estrous cycle, was considered to occur when a heifer exhibited (1) ovulation, regardless of behavioral activity, or (2) some degree of estrous activity, not necessarily but usually accompanied by ovulation. Of the 37 heifers at puberty, one was anovulatory, 2 had cystic follicles and the remaining 34 ovulated normally.

Thirty-eight observations were recorded of heifers exhibiting some degree of estrous activity before their first estrous cycles. Of these, seven exhibited restlessness and were nervous or bawling and 30 were riding or being

Figure 13. Relationship between age and body weight in prepubertal and postpubertal Holstein heifers.

Figure 14. Relationship between age and withers height in prepubertal and postpubertal Holstein heifers.


Body weight (kg)
360
320
280
240
200
Prepubertal heifers
Postpubertal heifers
Morrison's standards
8
10
12
14
Age (months)


Withers height (cm)
120
116
112
108
104
Prepubertal heifers
Postpubertal heifers
Morrison's standards
8
10
12
14
Age (months)

ridden but not standing. One heifer was in standing heat and had a cystic follicle. But, contrary to Morrow's (1969) results, no evidence of cyclic behavior before the first estrus was observed.

3. Ovarian Changes

The ovaries were palpated per rectum to determine changes in follicular size and number with advancing age. The initial palpation at 7 months of age revealed 2 free-martins; hormone data from these heifers will be presented separately. Diameter of follicles was estimated at 0.5 cm increments, and the smallest size detectable was 0.5 cm. To obtain a more meaningful comparison of changes with physiological age, the data were grouped into 10-day periods before the first estrus (Table 7) and recalculated. Follicle size did not change until the increase at 11 to 20 days before estrus. Follicular size in heifers exhibiting prepubertal estrous activity also increased from 0.9 cm to 1.6 cm during the 10-day period preceding first estrus (Table 8), but this change was not significant.

The number of palpable follicles ($\geq$0.5 cm) increased as heifers approached first estrus (Table 7). Morrow (1969) noticed increased follicular activity in some heifers at 20 and 40 days before the first ovulation. On the basis of my data, although behavioral activity occurred before the first estrus, I did not detect more of it at 20 and 40 days.

Table 7.--Some ovarian and hormone criteria of Holstein heifers at intervals before first estrus.

Days before first estrus	Follicle size[a]		Number of follicles[a]		LH[a]		Prolactin[a]	
	(n)	(cm)	(n)		(n)	(ng/ml)		
101-110	6	0.7 ± 0.1	7	1.9 ± 0.5	7	0.98 ± 0.16	176.5 ± 40.1	
81-90	7	0.5 ± 0.1	8	1.5 ± 0.4	7	1.08 ± 0.11	201.8 ± 53.2	
71-80	7	0.6 ± 0.1	9	1.6 ± 0.4	9	1.14 ± 0.13	151.7 ± 37.6	
61-70	6	0.6 ± 0.1	7	2.1 ± 0.6	7	2.21 ± 1.30	88.4 ± 46.9	
51-60	8	0.7 ± 0.1	9	1.2 ± 0.3	9	1.76 ± 0.29	139.5 ± 28.2	
51-50	8	0.6 ± 0.1	9	2.0 ± 0.3	9	2.13 ± 0.46	145.1 ± 38.2	
31-40	13	0.6 ± 0.1	15	1.7 ± 0.3	15	1.77 ± 0.36	125.1 ± 28.8	
21-30	11	0.7 ± 0.1	12	1.6 ± 0.3	12	2.68 ± 0.50	197.3 ± 44.0	
11-20	8	1.2 ± 0.2	8	1.9 ± 0.2	9	2.47 ± 0.28	98.9 ± 25.6	
1-10	13	0.9 ± 0.1	13	2.7 ± 0.2	13	4.68 ± 1.63	72.6 ± 21.0	

[a]Mean ± SE.

Table 8.--Some criteria of Holstein heifers demonstrating prepubertal estrous activity.

Days before first estrus	Follicle size[a]		Number of follicles[a]		LH[a]		Prolactin[a]	
	(n)	(cm)	(n)		(n)	(ng/ml)		
81-90	2	0.5 ± 0.0	2	1.5 ± 0.5	1	1.90	172.0	
61-70	2	0.7 ± 0.1	2	4.0 ± 0.0	0			
51-60	5	0.7 ± 0.1	5	1.8 ± 0.4	1	1.50	4.9	
41-50	5	1.0 ± 0.2	5	1.8 ± 0.6	1	1.00	12.0	
31-40	1	0.5	1	2.0	1	2.30	35.2	
21-30	5	0.8 ± 0.1	5	2.0 ± 0.0	2	11.20 ± 7.90	103.7 ±	100.7
11-20	3	0.9 ± 0.1	4	2.0 ± 0.7	1	2.10	10.0	
1-10	7	1.6 ± 0.3	8	1.5 ± 0.4	3	4.40 ± 1.59	56.0 ±	21.4
Overall mean	30	0.96 ± 0.1	32	1.9 ± 0.2	10	4.44 ± 1.72	60.9 ±	23.1

[a]Mean ± SE.

4. Endocrine Changes

a. LH.--Serum LH levels were high in prepubertal heifers (Table 7) relative to luteal phase heifers or cows (Hansel and Snook, 1970; Henricks et al., 1970), and were significantly higher ($P<0.01$) than levels during the luteal phase of estrous cycles of heifers after puberty (Table 14). The increase in serum LH with age as the heifers approached puberty agrees with Desjardins and Hafs (1968) conclusion that pituitary LH is released in quantity for the first time near the onset of puberty. This is also evident in the prepubertal heifers showing estrous activity; serum LH levels during the final 3 or 4 weeks before estrus (Table 8) were even higher ($P<0.005$) than the overall prepubertal level. But increased serum LH and increased follicular size in the heifers showing prepubertal estrous activity were not significantly related ($r = 0.22$, $P>0.10$).

Serum LH during 7 monthly bleedings from each of the 2 freemartins averaged 4.6 ± 0.3 ng/ml, twice that of the remaining heifers before first estrus (2.16 ± 0.25 ng/ml). Examination of the reproductive tracts of these 2 heifers at slaughter revealed small seminal vesicles in each. Reduced levels of ovarian steroid hormones may account for the increased serum LH in the freemartins.

An experiment was conducted to determine whether stressful stimuli would influence serum LH. Blood was withdrawn from an indwelling catheter before and after the animals were stressed by repeatedly sticking a needle into

the skin of the neck near the jugular. But there was no response in serum LH (Table 9).

Another experiment was designed to determine whether the venous source of serum influenced LH levels. Blood was obtained simultaneously from an indwelling jugular catheter and from the tail vein by venipuncture. But as listed in Table 10, the site from which blood was taken had no effect ($P>0.10$) on level of serum LH. Serum LH from the tail vein was higher in 1 cow and lower than jugular vein serum LH in 2 cows. The cow with high serum LH was evidently experiencing a preovulatory LH rise.

b. Prolactin.--Serum prolactin (Table 7) was more variable with advancing age than was LH. The marked changes in serum prolactin within animals, as reported by Schams and Karg (1970) also was evident in these heifers. Serum prolactin increased at 7 months and at 10 to 12 months of age, coinciding with a period of rapid mammary growth in heifers (Sinha and Tucker, 1969). Sinha and Tucker (1969) also reported high pituitary prolactin levels at 9 months of age, a period when serum levels were low in my heifers. Perhaps the most interesting observation is the high level of serum prolactin (avg 141.8 ng/ml) in prepubertal heifers. Prepubertal prolactin in this group of heifers was considerably higher than levels of 17.2 ± 2.9 ng/ml observed by Johke (1969a) or levels of 18 ± 2 to 45 ± 7 ng/ml reported by Tucker (1970) in lactating dairy cows. Serum

the skin of the neck near the jugular. But there was no response in serum LH (Table 9).

Another experiment was designed to determine whether the venous source of serum influenced LH levels. Blood was obtained simultaneously from an indwelling jugular catheter and from the tail vein by venipuncture. But as listed in Table 10, the site from which blood was taken had no effect ($P>0.10$) on level of serum LH. Serum LH from the tail vein was higher in 1 cow and lower than jugular vein serum LH in 2 cows. The cow with high serum LH was evidently experiencing a preovulatory LH rise.

b. Prolactin.--Serum prolactin (Table 7) was more variable with advancing age than was LH. The marked changes in serum prolactin within animals, as reported by Schams and Karg (1970) also was evident in these heifers. Serum prolactin increased at 7 months and at 10 to 12 months of age, coinciding with a period of rapid mammary growth in heifers (Sinha and Tucker, 1969). Sinha and Tucker (1969) also reported high pituitary prolactin levels at 9 months of age, a period when serum levels were low in my heifers. Perhaps the most interesting observation is the high level of serum prolactin (avg 141.8 ng/ml) in prepubertal heifers. Prepubertal prolactin in this group of heifers was considerably higher than levels of 17.2 ± 2.9 ng/ml observed by Johke (1969a) or levels of 18 ± 2 to 45 ± 7 ng/ml reported by Tucker (1970) in lactating dairy cows. Serum

Table 9.--Serum levels of luteinizing hormone (LH) and prolactin associated with induced stress.[a,b]

Time before or after stress	LH	Prolactin[c]
(min)	(ng/ml)	
-10	2.56 ± 0.21	15 ± 3
- 5	2.60 ± 0.20	15 ± 0.4
0	2.69 ± 0.22	16 ± 3
+ 5	2.58 ± 0.09	27 ± 7
+10	2.57 ± 0.16	23 ± 7
+15	2.65 ± 0.15	24 ± 7
+20	2.60 ± 0.16	18 ± 5

[a]Stress applied at time 0 by jabbing neck area with needle.

[b]Mean ± SE for 4 lactating cows.

[c]Data from Tucker, 1970.

Table 10.--Luteinizing hormone (LH) in serum taken from two sites.

Cow Number	Site of serum[a]	
	Jugular	Tail vein
	(ng/ml)	
442	2.30 ± 0.10	2.67 ± 0.12
451	1.93 ± 0.13	1.93 ± 0.14
452	20.47 ± 2.73	17.27 ± 2.18
453	2.33 ± 0.12	2.13 ± 0.17

[a]Mean ± SE for 3 bleedings.

prolactin in the 2 freemartin heifers (81.3 ± 23.0 ng/ml) did not differ appreciably from the other heifers.

Tucker (1970) noted that a prolactin response to stressful stimuli is variable in cows (Table 9). However in the present experiment, blood samples were obtained immediately after the heifers were tied and the heifers were not detained in holding chutes or otherwise disturbed prior to bleeding. The data in Table 7 may have an age effect confounded with stress. It is possible that the heifers may have become conditioned to the venipuncture procedure during 5 or less monthly bleedings before puberty. Therefore the data were re-analyzed to determine if prolactin levels changed with the bleedings, fully realizing that the heifers were older at each bleeding. Serum prolactin at the first bleeding (224.8 ± 16.7 ng/ml) was significantly higher ($P<0.01$) than at any of the 4 following bleedings. But the hypothesis of conditioning is difficult to accept because while serum prolactin decreased at the second bleeding (58.7 ± 17.0 ng/ml), it increased significantly ($P<0.01$) to 155.5 ± 16.6 ng/ml at the third bleeding before decreasing to 93.8 ± 24.6 ng/ml and 40.8 ± 9.1 ng/ml at the fourth and fifth bleedings, respectively.

Another indication that increasing age was a more important factor than conditioning to stress is observed in comparing serum prolactin levels during the overall prepubertal period and during prepubertal estrous activity.

Serum prolactin was significantly greater ($P<0.05$) during the overall prepubertal period (Table 7) than during prepubertal estrous activity (Table 8) when the heifers were older but not necessarily conditioned to venipuncture. The lower serum prolactin in the prepubertal heifers showing estrous activity was not significantly correlated with their increased follicular size ($r = 0.03$).

B. Estrous Cycle

1. Growth

Growth, represented in Figures 13 and 14, of these heifers was normal for their breed. First estrus, which I have defined as puberty in this thesis, occurred at 43.3 $\pm$ 0.9 weeks of age (range 33.7 to 60.6 weeks) when the average heifer weighed 252.9 $\pm$ 5.5 kg and had a withers height of 110.7 $\pm$ 0.8 cm. I recognize that puberty is a gradual process involving growth and maturity of the reproductive organs. Heifers were assumed to be capable of reproduction at puberty or at first estrus although reproductive efficiency would probably not be maximized until several estrous cycles after puberty. The body weight of our experimental heifers at first estrus is similar to Holstein heifers fed low, normal, or high levels of nutrition in Sorenson's *et al.* (1959) study. The age at puberty (43.3 wk) is midway between the average age of heifers fed normal and high levels of nutrition. These data substantiate Sorenson's contention that heifers reach puberty at a constant size,

regardless of age. Also, the age at first estrus is similar to 42.3 weeks reported by Morrow (1969) for Holstein heifers but much older than 31.7 weeks reported by Desjardins and Hafs (1968). Although 29 of my heifers were subjected to hemimastectomy at 5 months of age, they were normal in growth rate and age at puberty.

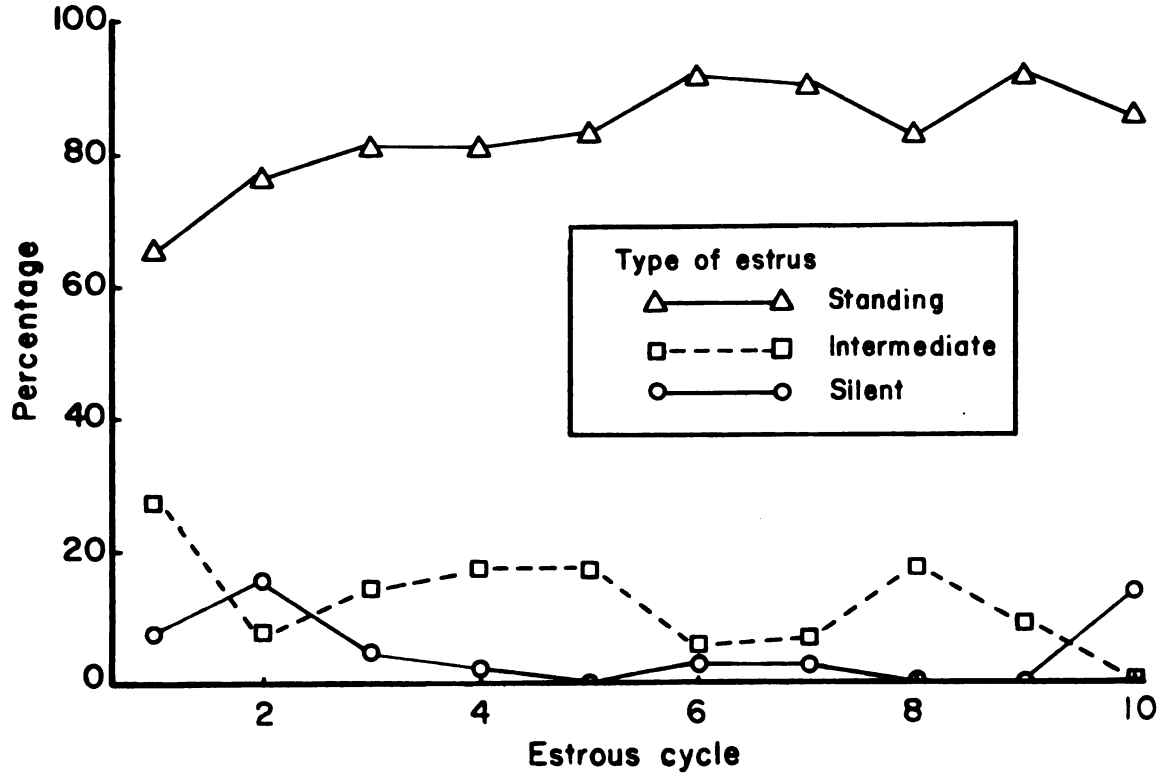
The length of estrous cycles ranged from 9 days to 56 days. Periodic rectal palpations during this study detected silent heats and confirmed abnormal estrous cycle lengths. Heifers with estrous cycles of abnormal lengths usually possessed cystic follicles ($\geq$ 2.5 cm) or cystic corpora lutea (CL with fluid filled cyst $\geq$ 1 cm dia). When only heifers having seven or more estrous cycles were analyzed (Table 11), the average length of the first nine estrous cycles did not differ significantly (avg 20.2 $\pm$ 0.2 days). This agrees with data presented in the Review of Literature and substantiates Morrow's (1969) observation that estrous cycle length does not change with age. The first and second estrous cycles when data from all heifers were included (Table 11) were significantly ($P<0.005$) longer than subsequent cycles. This indicates that heifers attaining puberty at a later age have longer estrous cycles, at least during the first and second cycles.

2. Behavior

The incidence of standing heat increased about 25% (Figure 15) from the first through the sixth estrous cycles.

Figure 15. Incidence of behavioral estrus in Holstein heifers from puberty to the tenth estrous cycle.

Figure 16. Ovarian condition in Holstein heifers from puberty to the tenth estrous cycle.


100
80
60
40
20
0
Percentage
Type of estrus
Standing
Intermediate
Silent
2
4
6
8
10
Estrous cycle

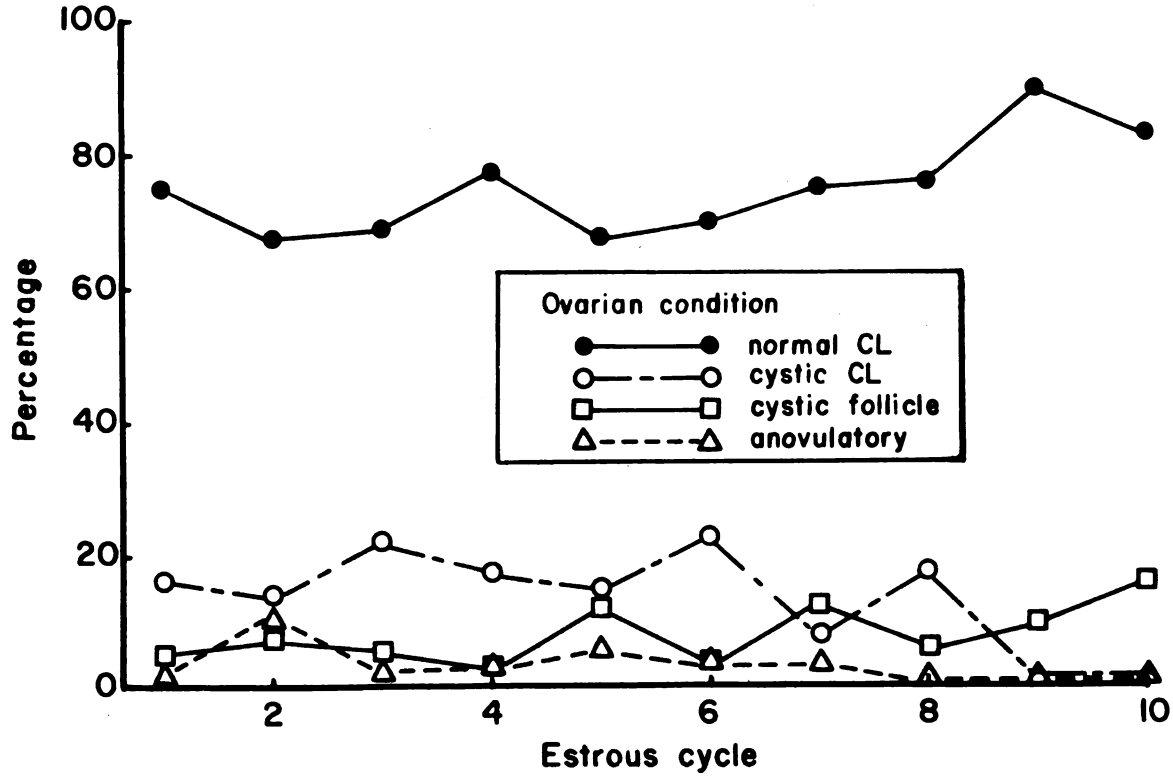

100
80
60
40
20
0
Percentage
Ovarian condition
normal CL
cystic CL
cystic follicle
anovulatory
2
4
6
8
10
Estrous cycle

Table 11.--Mean length of estrous cycle in Holstein heifers.

Estrous cycle	Length of estrous cycle[a]			
	Heifers attaining ≥ 2 estrous cycles		Heifers attaining ≥ 7 estrous cycles	
	(n)	(days)	(n)	(days)
1	37	21.9 ± 1.2	26	19.6 ± 0.4
2	36	21.8 ± 1.0	26	20.5 ± 0.5
3	35	21.1 ± 1.0	26	21.1 ± 1.2
4	35	20.3 ± 0.4	26	20.3 ± 0.6
5	32	20.2 ± 0.4	26	20.2 ± 0.4
6	28	20.9 ± 0.5	26	20.7 ± 0.5
7	17	19.7 ± 0.7	17	19.7 ± 0.7
8	12	19.8 ± 0.3	12	19.8 ± 0.3
9	7	19.0 ± 0.3	7	19.0 ± 0.3

[a]Mean ± SE.

Silent heat (absence of any behavioral signs of estrus) occurred in only 9 of 74 observations during the first and second estrous cycles and only occasionally thereafter. But it never approached the incidence of 74% at first estrus reported by Morrow (1969). Behavioral activity such as restlessness and attempted mounting (but not climaxing in standing heat) occurred during 14% of the estrous cycles and did not change with age.

Of 234 observations, 62% of the heifers were first observed in standing heat in the morning; Trimberger (1948) observed 70% in heat in two morning observations. If heifers came into standing heat without regard to the time

of day, we would expect 62.5% of the occurrences of standing heat to be first observed in the morning, because there was a 15-hour interval between the afternoon observation and the morning observation.

Other observations during the estrous cycle included discharge of mucus and blood. The mucous discharge from the vulva, as noted in the literature review, becomes less viscous during estrus. Mucous discharge during estrus was observed in only 43.4% of the estrous cycles. When heifers or cows are not confined in a barn mucous discharge is more difficult to observe and has less value for determining when cattle are approaching estrus.

Blood discharge following estrus was less frequently observed (27%) than mucous discharge before or during estrus. The incidence of blood discharge increased from about 10% during the first few estrous cycles to about 50% during the latter cycles. The interval between estrus and blood discharge decreased ($P<0.10$) from 2.6 days after estrus during the first 2 estrous cycles to 1.9 days after estrus during the ninth and tenth estrous cycles. As with the mucous discharge, a discharge of blood is more easily detected when cattle are confined to stanchions.

3. Ovarian Changes

The number of follicles ($\geq$ 0.5 cm dia) per pair of ovaries during the first estrous cycle (1.4; Table 12) was less than the 2.7 follicles per pair of ovaries during the

Table 12.--Some ovarian criteria during estrous cycles of Holstein heifers.

Estrous cycle	Follicle size[a]		Number of follicles[a,b]		Corpora lutea size[a]	
	(n)	(cm)	(n)		(n)	(cm)
1	81	0.9 ± 0.1	100	1.4 ± 0.1	50	2.0 ± 0.1
2	20	0.9 ± 0.1	21	1.5 ± 0.2	11	1.8 ± 0.2
3	9	1.0 ± 0.1	10	1.5 ± 0.3	7	1.7 ± 0.3
4	60	1.2 ± 0.1	68	1.5 ± 0.1	49	1.8 ± 0.1
5	57	1.1 ± 0.1	63	1.8 ± 0.1	24	1.3 ± 0.1
6	23	1.2 ± 0.1	31	1.5 ± 0.2	18	2.1 ± 0.2
7	41	1.1 ± 0.1	54	1.2 ± 0.1	38	1.4 ± 0.1
8	8	1.4 ± 0.2	9	1.1 ± 0.2	6	1.6 ± 0.3
9	5	1.1 ± 0.2	5	1.2 ± 0.2	4	1.6 ± 0.4
Overall cycles	304	1.1 ± 0.03	362	1.5 ± 0.1	207	1.7 ± 0.1

[a]Mean ± SE.

[b]Mean per pair of ovaries.

10-day period before first estrus (Table 7) and the difference is probably the result of ovulation at estrus. Comparison of prepubertal (Table 7) and postpubertal (Table 12) ovarian data indicates larger numbers of follicles during the prepubertal period. Similar to the decrease in number of follicles in these heifers, Desjardins and Hafs (1969) noted decreased numbers of follicles at 7 months of age in Holstein heifers, an age coinciding with puberty in those heifers. Follicle numbers, when grouped by cycles were significantly greater ($P<0.01$) during the fourth and fifth cycles than during preceding or succeeding cycles.

Rajakoski (1960) observed a small seasonal effect on the number of follicles but the change was limited to follicles less than 0.5 cm diameter. Histological determination of the number of follicles ≥0.5 cm (1.6 per pair of ovaries) by Rajakoski (1960) agrees with the determination through rectal palpation in this study (1.5; Table 12).

Follicle numbers (≥0.5 cm dia) during the estrous cycle were significantly lower ($P<0.005$) at 2 and 4 days after estrus and then increased to a peak 3 days before the succeeding estrus (Table 13). Follicle numbers again

Table 13.--Some ovarian criteria at various stages of the estrous cycle in Holstein heifers.

Days before or after estrus	Follicle size[a]		Number of follicles[a]		Corpus luteum size[a]	
	(n)	(cm)	(n)		(n)	(cm)
-3	12	1.0 ± 0.1	13	2.0 ± 0.3	9	2.1 ± 0.2
-2	11	1.3 ± 0.3	11	1.5 ± 0.2	9	1.5 ± 0.2
-1	12	1.1 ± 0.1	13	1.5 ± 0.2	9	1.6 ± 0.2
-0.5	9	1.3 ± 0.2	9	1.8 ± 0.2	5	1.0 ± 0.1
Estrus	35	1.3 ± 0.1	37	1.9 ± 0.2	17	1.0 ± 0.1
2	33	1.0 ± 0.1	49	1.1 ± 0.1	13	0.8 ± 0.1
4	39	0.8 ± 0.1	52	1.2 ± 0.1	10	1.0 ± 0.1
7	74	1.1 ± 0.1	87	1.5 ± 0.1	59	1.6 ± 0.1
11	80	1.1 ± 0.1	90	1.5 ± 0.1	76	2.2 ± 0.1

[a]Mean ± SE.

increased at estrus ($P<0.025$) and then decreased following ovulation. The decrease after day -3 may represent atresia of the mid-cycle follicle as suggested by Rajakoski (1960), paralleling the report of 2 large follicles present in ovaries on days 18 and 20 but not on day 0 (Hackett and Hafs, 1969).

Follicular diameter increased with age from 0.9 cm during the first estrous cycle to a size consistently greater than 1.1 cm after the third cycle (Table 12). When the nine estrous cycles were combined into 3 groups, follicular size increased significantly ($P<0.005$) from 0.9 cm during cycles 1, 2 and 3 to 1.2 cm during cycles 4 through 9. The size of follicles during cycles 1 and 2 (Table 12) are similar to follicular size during the 20 day period before first estrus (Table 7). The increase in size before puberty continued for 2 cycles after puberty and then plateaued at the size attained during the third estrous cycle.

Contrary to reports by Trimberger (1948) and Rajakoski (1960) that the right ovary contains significantly more follicles, this study showed both ovaries possessing equal numbers of follicles $\geq$0.5 cm diameter, although in my data, atretic follicles were not differentiated from normal follicles. The average follicle was larger on the right ovary than on the left and 65% of the corpora lutea were detected on the right ovary, similar to Rajakoski's (1960) results.

Within the estrous cycle, follicular size was greatest during estrus ($P<0.005$), decreased significantly ($P<0.025$) in size at days 2 and 4, and then increased at day 7 and day 11 (Table 13). But follicular size from day 7 through day -2 was smaller than during estrus. Although this experiment did not differentiate between normal and atretic follicles, larger size of follicles at days 7 and 11 than at day -3 agree with Rajakoski's theory that the follicles at mid-cycle become atretic and that a new growth of follicles begins about day 12, giving rise to the follicle which ovulates after estrus. Similar to the decrease in follicle size at day -1 in this experiment, Hackett and Hafs (1969) reported a slight decrease in follicle diameter and a larger decrease in follicle wall weight in ovaries of Holstein heifers 1 to 2 days before estrus. Follicle size in that experiment, as determined by direct measurement, was larger than the follicle size in this experiment, as determined indirectly by rectal palpation.

Size of corpora lutea (CL) tended to decrease with age (Table 12). When the 9 estrous cycles were grouped into 3 categories, CL size during cycles 1, 2 and 3 (1.9 cm) was significantly larger ($P<0.005$) than the remaining cycles (avg 1.6 cm). Incidence of cystic CL also remained constant during the first 10 cycles (Figure 16). A greater number of normal corpora lutea tended to develop after the seventh estrous cycle but this increase was small. Cystic

follicles, cystic corpora lutea, or anovulation exhibited little change during the 9 estrous cycles studied. Of the 3 abnormalities, cystic corpora lutea were the most common (15.8% occurrence).

A more dramatic event was the change in CL size during the estrous cycle (Table 13). CL size was lowest 2 days after ovulation and increased linearly and significantly ($P<0.005$) to 2.2 cm on day 11. Following the peak at day 11, CL size decreased significantly ($P<0.01$) to day -2 and then continued a decrease to day 2. The rapid changes in size of the CL is similar to the CL growth pattern established by early researchers and summarized by Cupps et al. (1969). Maximum size was attained by day 11 or shortly thereafter because regression had already begun by day -3. The growth pattern from day 2 to the peak at day 11 was linear. The decline from estrus to day 2 represents the regressing CL from the previous cycle while the increase observed after day 2 probably represents the new CL established after ovulation.

4. Endocrine Changes

Serum LH and prolactin data from the eight control heifers were analyzed separately and compared with data from all heifers to determine differences between the two groups. But analysis between estrous cycles and within estrous cycles revealed no significant effect attributable to hemimastectomy. Therefore the data from all heifers

were combined for analysis. Hereafter, the eight control heifers will not be differentiated from the remaining experimental heifers.

a. LH.--Serum LH, when analyzed over all cycles (Appendix IV) appeared to decrease with age or cycle. But these data were confounded with the time of bleeding on the day of estrus relative to the LH peak in each heifer. The LH peak, as discussed later, is very transient; it remains for only 6 to 8 hours during estrus (Schams and Karg, 1969). Therefore, a better opportunity to obtain a blood sample while serum LH was elevated was afforded during the latter cycles when heifers were bled daily until estrus. Of 164 blood samples taken when heifers were first observed in estrus, only 76 or 46% were $\geq$4.0 ng/ml, and only these could be considered to reflect the ovulatory discharge of pituitary LH.

To accurately determine the effect of cycles or age on serum LH, days -1, -0.5 and 0 were excluded and the 9 estrous cycles were combined into 3 groups (Table 14). It then became apparent that serum LH decreased significantly ($P<0.01$) as the heifers advanced from the first estrous cycle to breeding size. This decrease in serum LH with age coincides with the linear decline in pituitary LH which Desjardins and Hafs (1968) observed in heifers from 7 to 12 months of age. Data from this experiment also substantiate the hypothesis of these workers that synthesis

Table 14.--Levels of serum LH and prolactin in Holstein heifers before puberty and during the first, fourth, and seventh estrous cycles.[a]

Pubertal development	LH[b,c]		Prolactin[c]	
	(n)	(ng/ml)	(n)	(ng/ml)
Prepubertal	103	2.16 ± 0.26[d]	103	141.9 ± 11.4[d]
First estrous cycle	161	1.99 ± 0.06[e]	232	57.0 ± 0.9[e]
Fourth estrous cycle	168	1.69 ± 0.06[f]	245	33.8 ± 2.1[f]
Seventh estrous cycle	125	1.44 ± 0.16[f]	198	15.3 ± 2.2[g]

[a]The first, second, and third estrous cycles are averaged in the first; the fourth and fifth estrous cycles are averaged in the fourth; and the sixth, seventh, eighth and ninth estrous cycles are averaged in the seventh.

[b]LH values for estrous cycles excluded LH surge at estrus (days -1, -0.5, 0).

[c]Mean ± SE.

[d,e,f,g]Values with different superscripts differ significantly ($P<0.01$).

and release of LH is greatest around the time of puberty and declines thereafter.

Within the estrous cycle, serum LH exhibited some dramatic changes (Table 15). From a low of 1.42 ng/ml on day 7, serum LH increased continuously to the surge during proestrus and estrus. This change is accentuated by the significant increase ($P<0.01$) from 2.85 ng/ml on day -1 to 5.90 ng/ml on day -0.5. A further significant increase to 11.82 ng/ml ($P<0.005$) occurred at estrus and serum LH returned to baseline levels on day 2.

Changes in serum LH during the estrous cycle are illustrated in Figure 17. The rapid increase before estrus is more apparent during the fourth and seventh estrous cycles because the heifers were bled daily before the fourth and seventh estrus whereas during the first cycle heifers were only bled on day 18. Figure 17 also illustrates the decrease in serum LH during the luteal phase from the first through the seventh cycles.

The increased serum LH at mid-cycle reported by Schams and Karg (1969) and Hansel and Snook (1970) was not evident in any samples in this experiment. But these heifers were not sampled during days 8 to 10 of the estrous cycle when the elevated mid-cycle levels have been reported. The rapid increase in serum LH during estrus agrees with previously reported changes in pituitary LH (Rakha and Robertson, 1965; Hackett and Hafs, 1969).

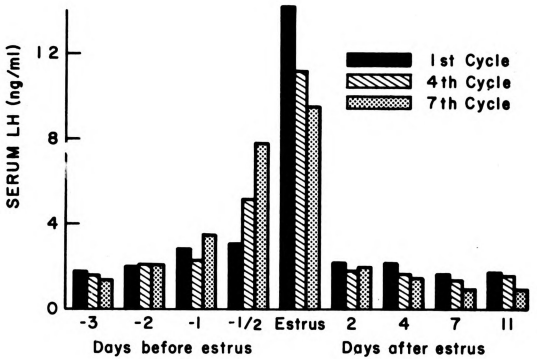
Table 15.--Levels of serum LH and prolactin in Holstein heifers at various stages of the estrous cycle.

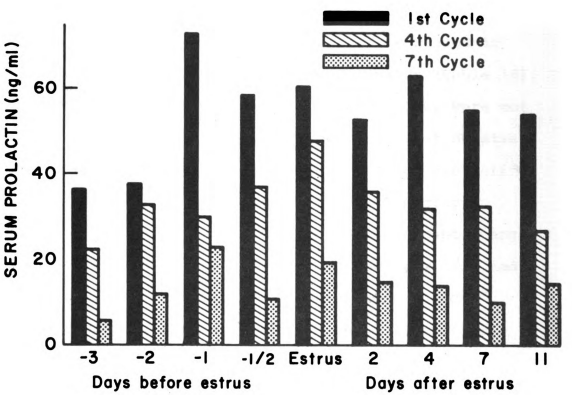
Days before or after estrus		LH[a]	Prolactin[a]
	(n)	(ng/ml)	
-3	25	1.57 ± 0.13	19.9 ± 4.6
-2	47	2.08 ± 0.13	30.1 ± 4.2
-1	65	2.85 ± 0.29	35.8 ± 6.3
-0.5	25	5.90 ± 1.87	25.6 ± 5.5
Estrus	133	11.82 ± 1.24	45.8 ± 4.2
2	96	2.02 ± 0.21	36.4 ± 4.0
4	96	1.83 ± 0.08	38.9 ± 5.0
7	95	1.42 ± 0.07	35.0 ± 4.7
11	94	1.53 ± 0.10	34.0 ± 4.3

[a]Mean ± SE.

Figure 17. Levels of serum LH in Holstein heifers during the first, fourth, and seventh estrous cycles.

Figure 18. Levels of serum prolactin in Holstein heifers during the first, fourth, and seventh estrous cycles.


SERUM LH (ng/ml)
12
8
4
0
1st Cycle
4th Cycle
7th Cycle
-3
-2
-1
-1/2
Estrus
2
4
7
11
Days before estrus
Days after estrus


SERUM PROLACTIN (ng/ml)
60
40
20
0
1st Cycle
4th Cycle
7th Cycle
-3
-2
-1
-1/2
Estrus
2
4
7
11
Days before estrus
Days after estrus

b. Prolactin.--Because serum prolactin did not exhibit pronounced changes comparable to LH during the estrous cycle, the cycle or age effect could be observed without deleting any data. Serum prolactin decreased significantly ($P<0.005$) with age (Appendix V); even more than serum LH. This change is more obvious when the cycles are grouped into 3 age categories (Table 14). The most marked change is the decrease from prepubertal serum levels of 141.9 ng/ml to 57.0 ng/ml during cycles 1, 2 and 3. But the decreases in each group also are significant ($P<0.01$). By the seventh estrus, serum prolactin decreased to levels (15.3 ng/ml) comparable with those reported in lactating cows (Johke, 1969a; Tucker, 1970).

Serum prolactin was significantly higher ($P<0.025$) on the day of estrus (45.8 ng/ml) and then decreased to levels maintained during metestrus and diestrus (Table 15). Changes during metestrus, diestrus, and proestrus were not significant because of variation of serum prolactin between and within heifers. The rapid changes in serum prolactin within heifers will be discussed in the next section. Schams and Karg (1970) failed to note a significant change in prolactin during the estrous cycle. However, they had few cows and large variability obscures these changes unless many samples are analyzed together. Significant increases in serum prolactin during estrus have been reported in sheep (Reeves et al., 1970) and rats (Amenomori

et al., 1970) but these are the first reported for the bovine. Sinha and Tucker (1969) noted a significant decrease in pituitary prolactin during the 2 days following estrus. Serum prolactin in these heifers is higher during metestrus although lower than at estrus.

Changes in serum prolactin during the estrous cycle and between cycles are illustrated in Figure 18. The significant increase at estrus occurs principally during the fourth and seventh estrous cycles. Following estrus, serum prolactin remained at about 35 ng/ml during metestrus and early diestrus and reached a low point at late diestrus. It is difficult to reconcile the significant decrease of pituitary prolactin at day 2 (Sinha and Tucker, 1969) with the changes in serum levels; further information on its function and rates of synthesis and release in the pituitary and rates of uptake by target tissue or organs are necessary.

c. Steroid Hormones.--Serum samples were selected from five heifers with histories of normal estrous cycles. These samples (N=111) were subjected to the steroid isolation and assay procedures described in the Materials and Methods section. Although the assays for estrone and estriol did not appear to be valid, the results will be reported in this section. Possibly, even if the absolute values are incorrect, the relative changes may be of value.

Much discussion has occurred over the relative merits of serum vs. plasma in hormone assays, especially

the steroid hormone assays. Discussions at the Second Karolinska Symposium (Diczfalusy, 1970) indicated little difference between serum and plasma. Serum was preferred by some researchers because it was cleaner, lacking the precipitate which accumulates in plasma. The relative merits of serum, i.e. lack of precipitate, absence of fibrin and ease of preparation, led this author to use serum for all of the assays.

(1) Estrogens: Serum estrone concentration did not change from the first to the seventh estrous cycle (Table 16; Appendix VI). Although further evidence is needed, it would appear that estrone is present in the serum, as well as in follicular fluid, urine and feces (Mellin and Erb, 1965). But any changes in serum level during the first seven cycles were obliterated by the variability within and between heifers. Levels of serum estrone varied as much as several nanograms between samples within a heifer. Samples at the nanogram level were not excluded from the analysis; contaminants, if responsible for these high values, were not identified.

During the estrous cycle, serum estrone tended to increase on the day before estrus (Table 17), but this increase was not significant ($P>0.10$). Another increase occurred at day 7 of the cycle. The relative changes may be meaningful but because of large variance, the absolute levels of serum estrone are unknown. Ayalon and Lewis

Table 16.--Levels of serum estrogens in Holstein heifers during the first, fourth, and seventh estrous cycles.

Cycle	(n)	Estrone[a] (pg/ml)	Estradiol[a] (pg/ml)	Estriol[a] (pg/ml)
1	31	998 ± 314	29.3 ± 4.0	255 ± 30
4	35	629 ± 236	28.1 ± 6.3	226 ± 26
7	38	903 ± 337	41.4 ± 13.6	247 ± 18
Overall	104	839 ± 173	33.3 ± 5.5	243 ± 14

[a]Mean ± SE for 5 heifers.

Table 17.--Levels of serum estrogens in Holstein heifers at various stages of the estrous cycle.

Days before or after estrus	(n)	Estrone[a] (pg/ml)	Estradiol[a] (pg/ml)	Estriol[a] (pg/ml)
-3	3	691 ± 404	19.0 ± 5.6	458 ± 84
-2	4	486 ± 383	25.4 ± 13.4	274 ± 34
-1	13	1021 ± 560	24.5 ± 6.3	271 ± 41
-0.5	6	1178 ± 766	30.0 ± 10.1	106 ± 28
Estrus	18	1134 ± 495	49.6 ± 25.0	198 ± 27
2	15	627 ± 362	48.7 ± 17.7	225 ± 28
4	15	458 ± 204	42.0 ± 11.0	288 ± 40
7	15	1014 ± 667	22.7 ± 6.0	231 ± 33
11	15	735 ± 432	14.1 ± 4.0	257 ± 47

[a]Mean ± SE for 5 heifers.

(1961; cited by Mellin and Erb, 1965) observed increased estradiol-17β equivalent in peripheral blood plasma of cows during the follicular phase.

Levels of plasma estrone in humans, although lower than in my heifers, also increased before ovulation (Tulchinsky and Korenman, 1970). These researchers also observed a circadian rhythm for estrone; low levels at night followed by a sharp morning rise at 8 to 9 a.m.

The concentration of serum estradiol increased slightly from the first cycle to the seventh cycle (Table 16 and Appendix VII) although this increase was not significant ($P>0.10$). Estradiol levels were lower (25 fold) than estrone, but variability between and within heifers obscured significant changes from cycle to cycle. Estradiol increased at estrus (Table 17), but the increase began one day later than the increase in estrone. Estradiol was higher ($P<0.10$) at metestrus than at diestrus but the increase at estrus only approached significance.

A plasma estradiol peak has been shown to precede the ovulatory LH peak by one day (Korenman et al., 1970) and estradiol was higher during the luteal phase than during the follicular phase of humans (Mayes and Nugent, 1970). Recently, Scaramuzzi et al. (1970) reported a rise in ovarian venous plasma levels of estrogen 24 hr before the ovulatory LH rise in sheep.

Estriol did not change with age (Table 16) but decreased slightly ($P<0.10$) during proestrus and estrus (Table 17 and Appendix VIII). Estriol decreased significantly ($P<0.025$) during the day before estrus. These changes may not be meaningful however, because of the poor precision and recovery for the estriol assay (Table 5). Estriol has not been positively identified in the bovine (Mellin and Erb, 1965) but the values reported here, although subject to modification when the assay is validated, indicate that estriol is present in the bovine. Furthermore, subsequent work in this laboratory also has shown the presence of estriol in bovine serum by independent methods (gas-liquid chromatography with electron capture). Estriol has not been identified in other species.

Mellin and Erb (1966) found peak total urinary estrogens prior to ovulation and a smaller peak during days 6 to 11 of the cycle. Urinary estrogens were comprised principally of estradiol-17α and small amounts of estradiol -17β and estrone. Although estradiol-17α was predominant throughout the estrous cycle, estrone was higher during the luteal phase than during the follicular phase of the cycle. The major portion of the urinary estrogens were conjugated to glucuronic acid; the low serum estradiol levels found in my experiment may be due to conjugation in the blood. Conjugated estrogens would not be detected in the protein binding assay unless the estrogens were first hydrolized free of the conjugates.

Norman et al. (1968) identified three estrogens in the plasma of sheep: estrone, estradiol-17β, and 16-ketoestradiol-17β. Total estrogens, determined by a fluorometric assay, were lowest (1.4 ng/ml) 2 days following ovulation and reached a peak of 25.3 ng/ml just prior to ovulation. Of the 3 estrogens detected, 16-ketoestradiol-17β was predominant during the estrous cycle. These estrogen levels reported in sheep are higher than observed in my heifers. It is possible that estrogens other than those I measured may predominate in bovine blood. In sheep, even estradiol-17β reached levels of 9.5 ng/ml prior to ovulation, levels never attained in my study.

Because the competitive protein binding assays for estrogens used in this study lacked precision, the 3 estrogens were not combined. But even total estrogens in this study would not exceed 1 to 2 ng/ml during the estrous cycle.

(2) Progesterone: Serum progesterone did not change from cycles one through seven (Table 18) in heifers and the levels are comparable to those in cows (Henricks et al., 1970; Stabenfeldt et al., 1969). Thus, while CL size decreased significantly with age (Table 12), progesterone synthesis remained constant.

But dramatic changes in serum progesterone occurred during the estrous cycle (Table 19 and Appendix IX).

Table 18.--Levels of serum progesterone and glucocorticoids in Holstein heifers during the first, fourth, and seventh estrous cycles.

Cycle		Progesterone[a]	Corticosterone[a]	Cortisol[a]
	(n)	(ng/ml)		
1	31	1.49 ± 0.35	0.48 ± 0.09[b]	9.89 ± 1.29
4	35	1.42 ± 0.31	0.22 ± 0.04	5.58 ± 1.03
7	38	1.42 ± 0.35	0.24 ± 0.04[c]	6.48 ± 0.85
Overall	104	1.44 ± 0.19	0.30 ± 0.04[d]	7.19 ± 0.62

[a]Mean ± SE for 5 heifers.

[b]29 observations.

[c]35 observations.

[d]99 observations.

Table 19.--Levels of serum progesterone, corticosterone, and cortisol in Holstein heifers at various stages of the estrous cycle.

Days before or after estrus		Progesterone[a]	Corticosterone[a]	Cortisol[a]
	(n)	(ng/ml)		
-3	3	6.88 ± 1.04	0.41 ± 0.29	7.07 ± 3.73
-2	4	2.51 ± 1.27	0.77 ± 0.52[b]	8.55 ± 3.72
-1	13	0.60 ± 0.20	0.27 ± 0.05	6.32 ± 1.30
-0.5	6	0.41 ± 0.21	0.30 ± 0.05[b]	7.55 ± 1.42
Estrus	18	0.19 ± 0.06	0.38 ± 0.10[b]	10.93 ± 1.88
2	15	0.12 ± 0.05	0.19 ± 0.05[b]	6.87 ± 1.75
4	15	0.55 ± 0.09	0.25 ± 0.07[b]	6.66 ± 1.77
7	15	2.70 ± 0.32	0.40 ± 0.14	7.21 ± 1.64
11	15	3.67 ± 0.56	0.21 ± 0.04	3.84 ± 0.93

[a]Mean ± SE for 5 heifers. [b]One observation missing.

Progesterone was low at estrus (0.19 ng/ml) and at day 2, being undetectable in many samples. It began increasing on day 4 and the increase between 4 and 7 days was significant ($P<0.005$). Following a further increase on day 11 (3.67 ng/ml), the peak was reached 3 days before estrus, about day 18. Progesterone declined rapidly ($P<0.005$) to 2.51 ng/ml on day -2 and the decline continued through day 2 following ovulation when presumably, the newly formed corpus luteum began secreting progesterone in increasing quantities. The peak level of progesterone (6.9 ng/ml) is similar to peak levels in non-pregnant dairy cows (6.5 ng/ml; Henricks et al., 1970); these workers noted the peak on day 16 of the estrous cycle. Of course the heifers in this experiment were not sampled between day 11 and day 18 so it is not known when the peak occurred. Stabenfeldt noted the peak to occur from day 11 to day 20 of the cycle. Pope et al. (1969) reported peak levels of 9.0 ng/ml in cows occurring an average of 13 days after ovulation.

The latter two laboratories obtained plasma progesterone levels by gas-liquid chromatography, and their values are comparable to those in this experiment and to those reported by Henricks using competitive protein binding. These comparisons, in addition to the precision and recovery data reported here (Table 6) aid in validating the protein binding assay system used in this experiment.

Stabenfeldt et al. (1969) observed plasma progesterone to begin a rapid decline 1 to 5 days before estrus and Henricks et al. (1970) reported decreased progesterone after day 16. Because blood samples were not taken daily until day -3 in this experiment, it is not known when the rapid decline began. But examination of the pooled data (Table 19) indicated that high levels of progesterone persist to day 19. At estrus, progesterone was undetectable in 6 of the 18 observations and ranged up to 0.98 ng/ml in the other 12 instances. Kazama and Hansel (1970) also reported many of the samples collected during estrus to contain trace or undetectable amounts of progesterone. These researchers failed to observe a preovulatory rise in plasma progesterone even though they sampled every 6 hours. It appears that the cow differs from species in which a preovulatory rise in progesterone has been observed (Johansson et al., 1968).

(3) Corticosterone: Serum corticosterone decreased significantly ($P<0.005$) from 0.48 ng/ml during the first estrous cycle to 0.23 ng/ml during the fourth and seventh cycles (Table 18 and Appendix X). These levels are very low relative to values of 30 ng/ml in cows (Venkataseshu and Estergreen, 1970) and 9.5 ng/ml in bulls (Riegle and Nellor, 1967). However, fluorometric techniques were used to quantify corticosterone in large volumes of plasma in

the earlier experiments. No previous data on corticosterone measured by competitive protein binding are available.

Corticosterone levels are difficult to interpret during the estrous cycle (Table 19 and Appendix X). Low levels occurred on days 2, 4, and 11 following estrus but no more than 2-fold changes occurred between the various days sampled. The author is not aware of other data on corticosterone levels during the estrous cycle.

(4) Cortisol: Serum cortisol, similarly to corticosterone, decreased significantly ($P<0.005$) with age from 9.89 ng/ml during the first cycle to an average of 6.05 ng/ml during the fourth and seventh cycles (Table 18 and Appendix XI). As noted by the standard errors, cortisol levels were variable between and within heifers. Again, these levels are lower than those reported by Venkataseshu and Estergreen (1970) for cows (73 ng/ml) and by Riegle and Nellor (1967) for bulls (29.8 ng/ml). Measuring total glucocorticoids by protein binding assays, Heitzman et al. (1970) reported levels of 5.84 ng/ml in cows and Wagner (1970) found 4.5 ng/ml in non-lactating cows. These values derived by protein binding are very similar to those in this experiment during the fourth and seventh estrous cycles. Furthermore, the levels reported by Wagner were taken from cows by indwelling catheters; the similar levels in this experiment would suggest that any stress to the heifers in collecting the blood did not have a significant effect on the cortisol levels observed.

Wagner also observed a significant diurnal effect, with decreased levels occurring at night. But diurnal variation would have no effect on this experiment because all samples were collected in the mornings. Riegle and Nellor (1967) did not observe any change in cortisol or corticosterone with age in bulls, although young bulls responded to ACTH infusion with higher glucocorticoids than old bulls.

Cortisol tended to increase during proestrus ($P<0.10$) and increased significantly ($P<0.05$) from proestrus to estrus (Table 19). It decreased to preovulatory levels on day 2 and decreased further on day 11. The overall ratio of cortisol:corticosterone was 24:1 and this ratio varied from approximately 11:1 on day -2 to 36:1 on day 2. But in general, the 2 glucocorticoids tended to vary together.

The cortisol:corticosterone ratio of 24:1 is much higher than ratios of 2.4:1 observed by Venkataseshu and Estergreen (1970) and 3.1:1 observed by Riegle and Nellor (1967). Thus, although lower concentrations of both cortisol and corticosterone were obtained by protein binding, corticosterone was detected at much lower levels than cortisol. Recovery and precision were excellent (Table 6) for both hormones. The relatively low levels of corticosterone indicate that separation of the 2 glucocorticoids probably is unnecessary. Total glucocorticoid levels in the literature are comparable to levels reported here when subjected to the same assay technique.

C. Acute Study of Estrus

1. Behavioral Changes

Six heifers were selected for study of the rapid endocrine changes near estrus, and standing heat was observed in five of the six. Mucous discharge, a characteristic at estrus, began 1 to 3 days before onset of standing heat which was first observed in the morning for each of these heifers. Standing heat could not be observed frequently because of the cannulae and, consequently, I did not attempt to determine the duration of estrus. Data from the heifer that failed to exhibit estrus are considered separately.

Other criteria associated with standing heat are presented in Table 20. Confinement did not alter significantly the length of estrous cycles which were noted to be slightly longer ($P>0.10$) than the cycle before the heifers were confined. Bloody discharge was detected in only 3 of the 5 heifers, at 2 or 3 days after estrus.

2. Ovarian Changes

Large follicles were detected by rectal palpation 1 to 2 days before estrus. In some instances, 2 major follicles were present until ovulation. Within 24 hr of ovulation, ovulatory follicles became less turgid and the CL from the previous cycle rapidly declined in size. Assuming that the heifers ovulated midway between the two closest palpations, ovulation occurred 16 to 42 hr after

Table 20.--Some estrual criteria of Holstein heifers.

Criterion	Heifer					
	1	2	3	4	5	Mean ± SE
SH[a] to ovulation (hr)	29	42	17	16	40	29 ± 6
Peak LH to ovulation (hr)	21	50	23	20	44	32 ± 6
Peak LH to SH[a] (hr)	-8	+7	+6	+4	+4	+3 ± 3
Time of peak LH	6 PM	5 AM	4 AM	6 AM	6 AM	-
Relative LH peak (X baseline)	11.7	22.7	20.2	27.5	12.8	19.0 ± 3.0
Cycle length (days)	20	20	20	22	20	20.4 ± 0.4
Previous cycle length (days)	21	19	19	19	18	19.2 ± 0.5

[a]Standing heat.

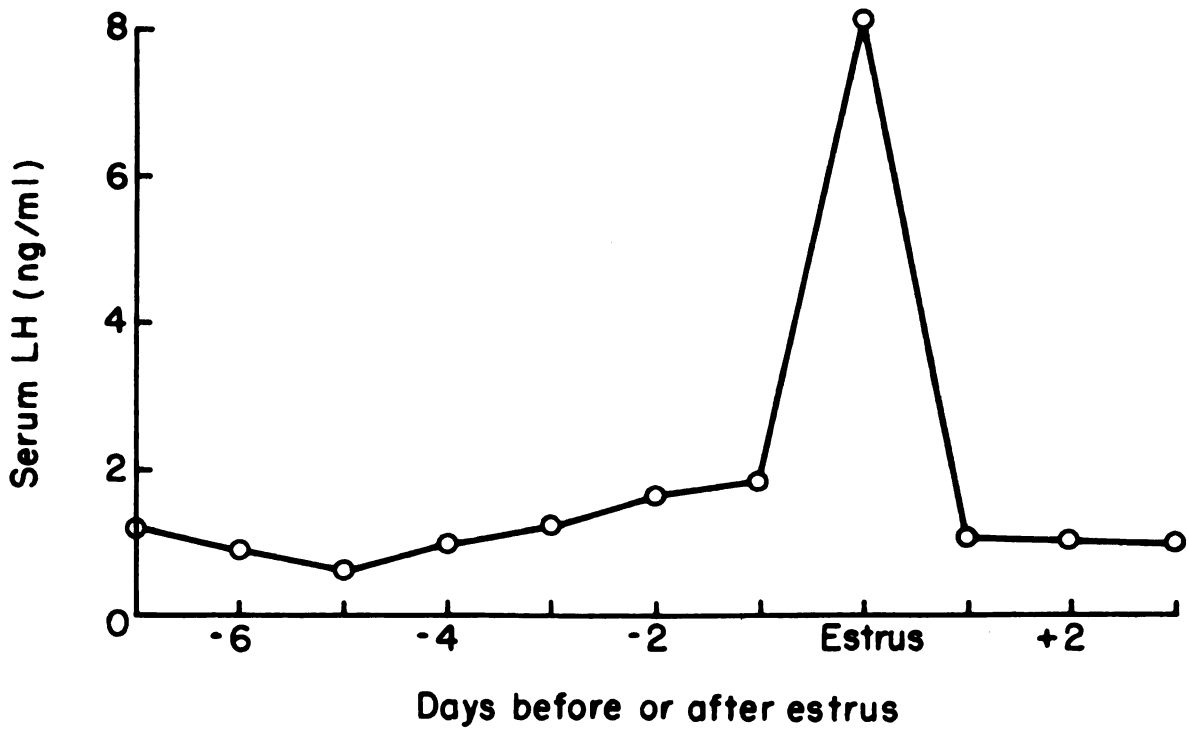
the onset of standing heat (Table 20). A summary of Hansel's early work (Hansel and Trimberger, 1951; 1952) indicates that the average heifer remains in estrus 16.5 ± 1.0 hr and ovulates 12.1 hr later or 28.6 ± 1.0 hr after the onset of estrus. An earlier study by Trimberger (1948) concluded that Holstein heifers remain in estrus 14.8 hr and that ovulation occurs 10.5 hr later or 25.3 hr after the onset of estrus. These results drawn from detailed studies of estrus and ovulation agree with the data in this thesis.

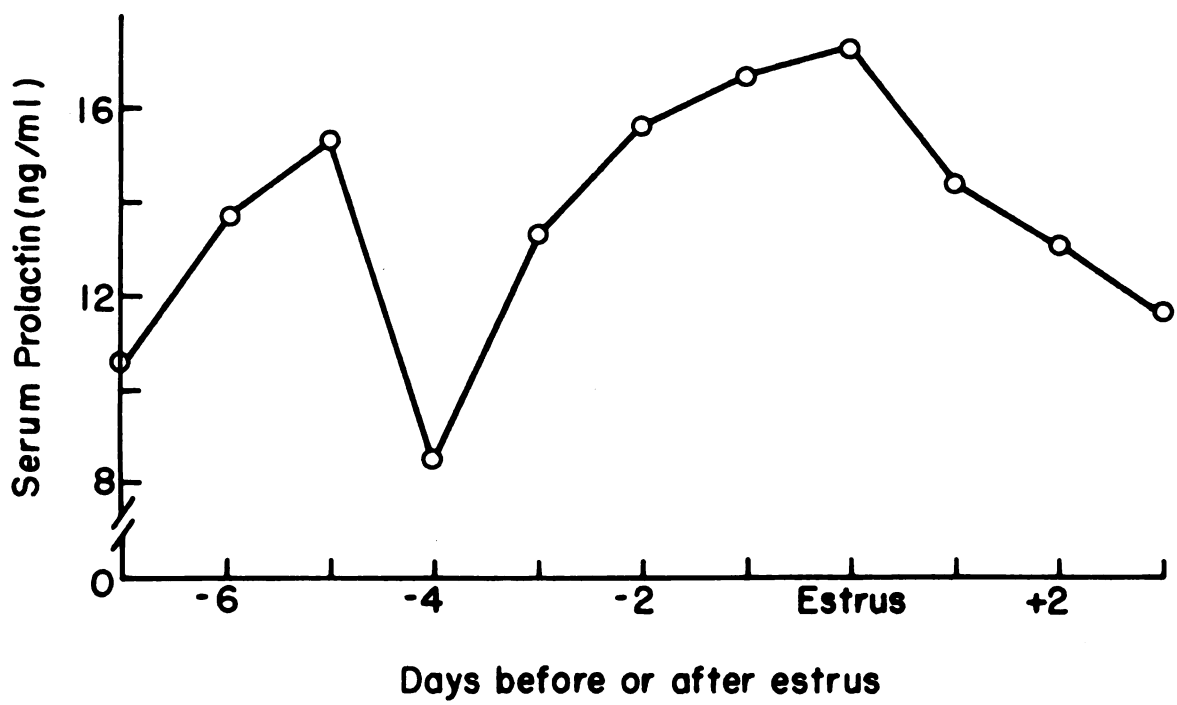
3. Endocrine Changes

a. LH.--Because the ovulatory surge of LH was detected on the day of estrus in each of the 5 heifers, the data were summarized first by averaging all samples collected within 24-hour periods (Figure 19). Serum LH began a 182% linear increase from 0.66 ± 0.05 ng/ml 5 days before estrus to 1.86 ± 0.11 ng/ml the day preceding estrus. It increased significantly ($P<0.005$) to 8.07 ± 1.50 ng/ml on the day of estrus and fell to 1.10 ± 0.08 ng/ml the following day where it remained for the next 2 days. Though serum LH reaches peaks of 20 to 60 ng/ml (Schams and Karg, 1969), it appears low on the day of estrus when averaged over 24-hour periods. Serum LH ranged from 0.25 to 3.89 ng/ml (avg 1.39 ± 0.05 ng/ml) during the period before and after estrus. Variations among heifers during estrus were greater than at any other time.

Figure 19. Daily levels of serum LH through estrus in Holstein heifers.

Figure 20. Daily levels of serum prolactin through estrus in Holstein heifers.


Serum LH (ng/ml)
8
6
4
2
0
-6
-4
-2
Estrus
+2
Days before or after estrus


Serum Prolactin (ng/ml)
16
12
8
0
-6
-4
-2
Estrus
+2
Days before or after estrus

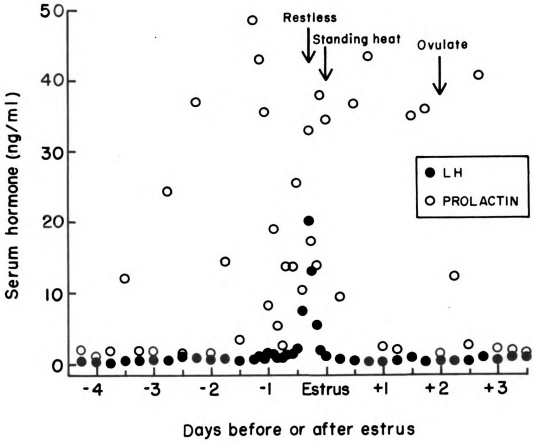
Serum LH was extremely stable before and after estrus, as illustrated for a single representative heifer (Figure 21). The first appreciable change is evident during the day before estrus. The ovulatory peak (20.24 ng/ml) was 23X greater than baseline and LH remained elevated for about 6 hr though the peak was transitory. The heifer began to show signs of estrus at the time of the LH peak and standing heat began 7 hr later. Ovulation occurred 50 hr after the LH peak.

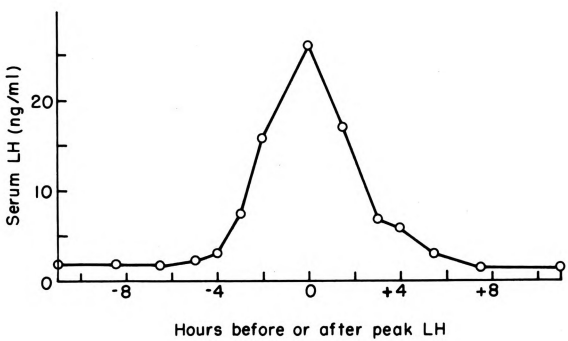
The ovulatory surge of LH on the day of estrus was analyzed further by adjusting serum LH values during estrus to the hour of peak serum LH (Figure 22). The significant ovulatory rise of LH began 3 to 4 hr before the peak and LH levels returned to the baseline 4 to 6 hr later. Thus the ovulatory surge persisted about 8 hr, similar to the 6 hr increase reported by Schams and Karg (1969) and elevations of 8 to 10 hr reported by Henricks et al. (1970).

When expressed in this manner, the average peak value was 25.92 $\pm$ 3.18 ng/ml. The pituitary must release a large amount of LH rapidly during estrus unless the rates of degradation or uptake change drastically. In other terms, serum LH (Table 20) increased 19-fold at estrus. The relatively short burst of serum LH emphasized the importance of frequent sampling and may explain the differences in absolute levels reported by different laboratories. Henricks et al. (1970)

Figure 21. Daily levels of LH and prolactin and signs of estrual behavior in a Holstein heifer.

Figure 22. Levels of serum LH during estrus, adjusted to peak LH at time 0.


Restless
Standing heat
Ovulate
LH
PROLACTIN
Serum hormone (ng/ml)
50
40
30
20
10
0
-4
-3
-2
-1
Estrus
+1
+2
+3
Days before or after estrus


Serum LH (ng/ml)
20
10
0
-8
-4
0
+4
+8
Hours before or after peak LH

observed an average peak of 40 ng/ml while Schams and Karg (1969) reported peaks ranging from 3 to 62 ng/ml.

One heifer reached a serum LH peak after exhibiting standing heat, while 4 heifers had LH peaks 4 to 7 hr before onset of standing heat (Table 20). Other researchers (Henricks et al., 1970) have discerned an LH peak 3 to 6 hr after the onset of estrus in non-lactating cows. In my heifers, ovulation occurred 29 hr after the onset of standing heat and 32 hr after peak LH (Table 20). In other words, standing heat was detected 3 hr after the LH peak. Four of the 5 heifers had LH peaks between 4 and 6 a.m. but it must be realized that the LH peak is related to the onset of estrus which also occurred in the morning in all 5 heifers. These data differ from Henricks et al. (1970) and from Schams and Karg (1969) who reported peak serum LH 15 to 26 hr before ovulation.

Ovulation, as detected by rectal palpation, occurred 2 days prior to the LH peak in the heifer which did not exhibit behavioral signs of estrus. No significant change in serum LH was noted prior to ovulation when blood samples were collected every 2 hr, and the LH rise 2 days later was similar to the ovulatory LH rise in the other heifers.

Hackett and Hafs (1969) reported a 70% loss of pituitary LH during the 24-hr period ending on the day of estrus and another 60% loss during the next 2 days. Jubb and McEntee (1955) observed degranulation of basophilic

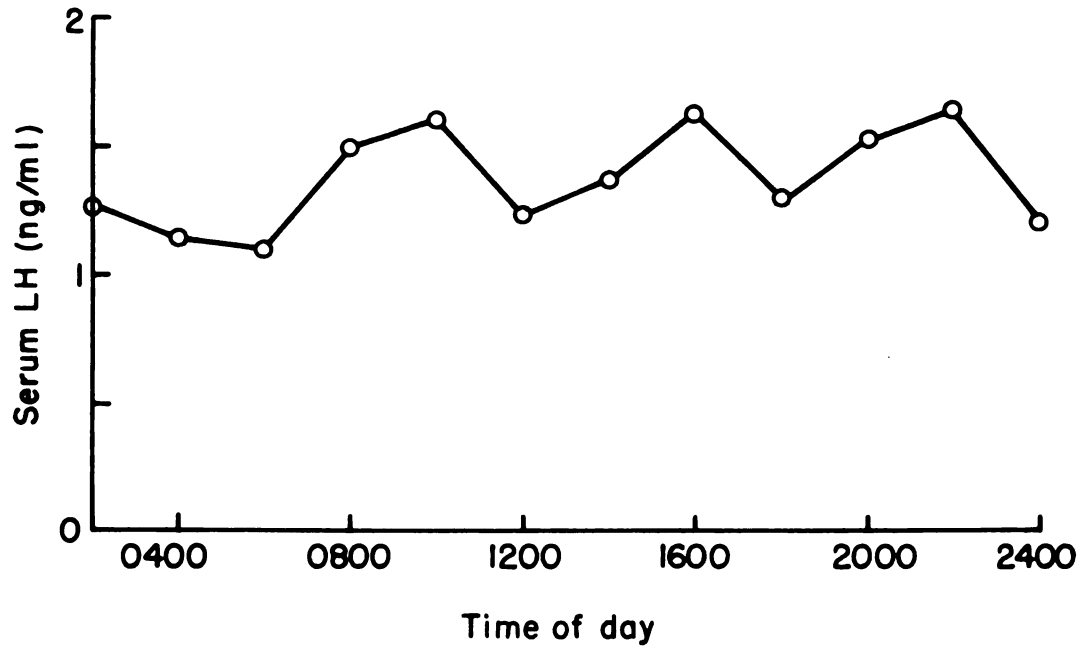
Variations in the interval of time between the increase in serum LH and the onset of standing heat suggest that the prolonged release indicated by Hackett and Hafs (1969) may have resulted from variation in the interval between the time of the LH surge and the time the heifers were killed. The brief period of elevated levels of serum LH (Figure 22) before standing heat (Table 20) suggests that increased serum LH beginning the day before estrus (Table 15) resulted from LH surges before standing heat.

The same data for all heifers in this acute study were re-analyzed to provide information on diurnal changes in serum LH. Each point on Figure 23 represents the average level of LH in all 6 heifers for all blood samples collected (except the ovulatory LH rise) at each time. A significant rise ($P<0.01$) occurred at 8 to 10 a.m., followed by non-significant increases at 4 p.m. and 10 p.m. The low periods occurred at noon and at night. Comparable data, to my knowledge, are unavailable in any species. The cyclic changes probably are not determined by environmental or managemental practices and further research is necessary to understand this phenomena. Average serum LH, excluding the LH peak, varied significantly among heifers ($P<0.01$) and averaged 1.31 ± 0.04 ng/ml (Table 21).

b. Prolactin.--Daily serum prolactin levels for the 5 heifers exhibiting estrus began a general and sustained

Figure 23. Diurnal changes in levels of serum LH in Holstein heifers.

Figure 24. Diurnal changes in levels of serum prolactin in Holstein heifers.


2
1
0
Serum LH (ng/ml)
0400
0800
1200
1600
2000
2400
Time of day

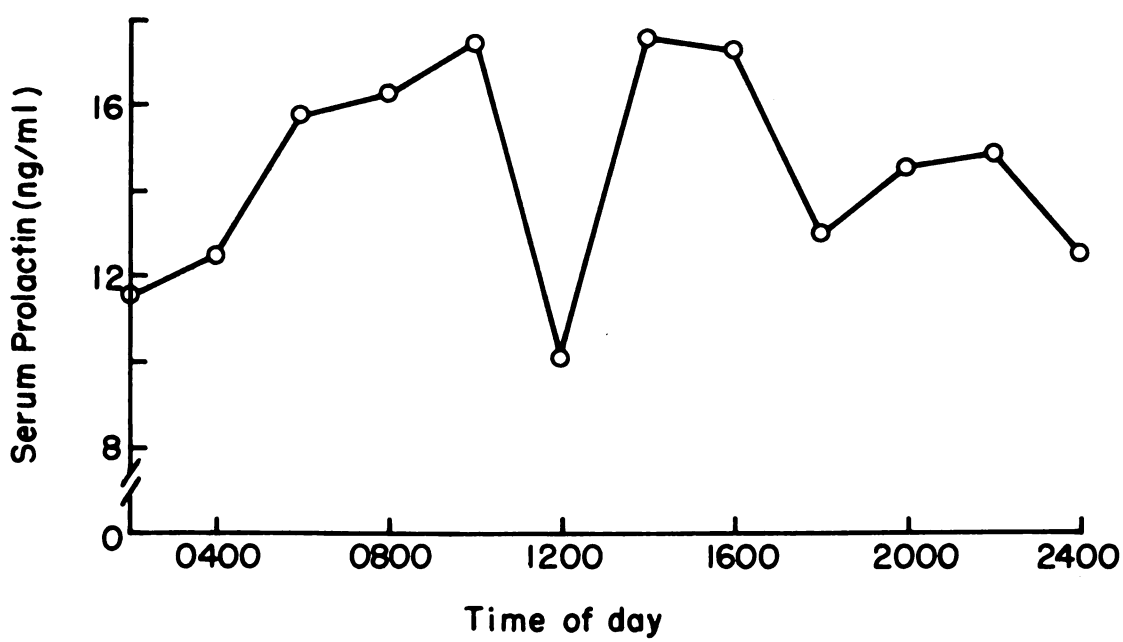

16
12
8
0
Serum Prolactin (ng/ml)
0400
0800
1200
1600
2000
2400
Time of day

Table 21.--Levels of serum luteinizing hormone (LH) and prolactin during the estrous cycle of Holstein heifers.[a,b]

Heifer	LH	Prolactin
	(ng/ml)	
1	2.05 ± 0.17	19.8 ± 3.1
2	0.89 ± 0.07	16.6 ± 2.3
3	1.57 ± 0.11	10.9 ± 2.2
4	1.27 ± 0.08	15.3 ± 1.4
5	1.46 ± 0.10	11.8 ± 1.5
6	0.98 ± 0.07	9.0 ± 0.9

[a]Excluding the ovulatory increase in LH.

[b]Mean ± SE.

rise during the 4 days before estrus, (Figure 20) as did LH. Serum prolactin increased 100% from a low of 8.5 ± 1.7 ng/ml 4 days before estrus to 17.3 ± 2.5 ng/ml on the day of estrus, then decreased. But the change for prolactin only approached significance because of variability among and within heifers. Schams and Karg (1970) also concluded that serum prolactin showed no marked differences during the estrous cycle. In sheep, Reeves et al. (1970) have observed a significant increase of serum prolactin during proestrus and estrus, preceding the ovulatory LH rise.

Average serum prolactin during the entire period varied significantly ($P<0.01$) among heifers from 9.0 ng/ml to 19.8 ng/ml (Table 21). The average for the 6 heifers, 13.7 ± 0.8 ng/ml, is similar to serum prolactin during the

entire estrous cycle of the older heifers (Figure 18) suggesting that the method of sampling (cannulation vs. venipuncture) had no effect on prolactin levels. Data from one heifer (Figure 21) illustrate the variability in serum prolactin. Although prolactin fluctuates considerably, levels of prolactin are generally higher during estrus than before or after estrus.

The data for serum prolactin were recalculated to provide information on diurnal changes (Figure 24). Similar to LH, high prolactin levels were discernable in the morning and afternoon and prolactin was significantly lower ($P<0.05$) at noon and during the night. In an experiment specifically designed to detect diurnal changes, Koprowski et al. (1970) detected a significant decrease in the morning and a significant increase during the evening in lactating dairy cows. Thus further experiments are necessary to determine whether diurnal changes differ between heifers and lactating cows.

GENERAL DISCUSSION

Among observations of behavioral activity, ovarian activity, and levels of serum LH and prolactin before puberty, the only significant changes detected were increases in follicle size and follicle number and an increase in serum LH, all within 20 days before first estrus. These results agree with observations of Desjardins and Hafs (1968) from slaughter data on Holstein heifers before and after puberty.

Prepubertal estrous activity was observed, although the activity did not conform to any 20-day cyclic rhythm as was noted by Morrow (1969). But increased serum LH detected in prepubertal heifers within 20 days before first estrus suggested that pituitary LH may be released at sub-ovulatory levels prior to puberty, similar to elevated serum LH at days 8 to 10 of the estrous cycle of cows (Schams and Karg, 1969). Increased levels of estrogen before puberty may stimulate release of LH, as reported in rats (Miyake, 1969) and sheep (Goding et al., 1969; Scaramuzzi et al., 1970), and also elicit submaximal estrous behavioral activity. But any LH surge before first estrus evidently was not sufficiently large to cause ovulation because of sub-optimal amounts of stimulatory estrogen or immaturity of the cyclic center of the hypothalamus (Gorski, 1966).

Serum prolactin was significantly higher ($P<0.01$) during the prepubertal period (141.9 $\pm$ 11.4 ng/ml) than in the first estrous cycle (57.0 $\pm$ 0.9 ng/ml). Prolactin continued decreasing with age, but until we understand the function(s) of prolactin relative to reproduction we can only speculate on reasons for the decline. Swann (1970) also observed levels of prolactin in blood serum to decrease dramatically with age in dairy heifers. A possible explanation for decreased prolactin with age is a decreasing requirement for prolactin by the mammary gland as it attains mature development. Sinha and Tucker (1969) observed greatest mammary growth (mammary DNA) relative to body growth between 3 and 9 months of age. The month to month fluctuations of pituitary prolactin they noted were similar to fluctuations in serum prolactin observed in my study.

Schams and Karg (1970) reported that prolactin levels do not change during lactation, but preliminary evidence in our laboratory tends to refute this (Koprowski, 1970). A prolactin peak several hours prior to parturition also has been reported (Schams and Karg, 1970). The rapid fluctuations of serum prolactin suggest secretory control mechanisms other than through the hypothalamic hormones alone. These may involve brain catecholamines, or similar to growth hormone, they may involve some nutritional intermediary metabolite such as glucose or fatty acids.

Rapid and dramatic changes in serum levels of hormones during the estrous cycle correspond with earlier reports of changes in levels of pituitary (Hackett and Hafs, 1969) and ovarian (Hafs and Armstrong, 1968) hormones. This substantiates the hypothesis that decreased pituitary levels of LH represent release of LH into the blood. But this was not true for changes in prolactin during the estrous cycle. Nor did this generalization hold for changes in LH or prolactin from before to after first estrus. In general, levels of LH and prolactin were higher in pituitaries and in blood before first estrus.

Hafs and Armstrong (1968) reported highest progesterone content of the CL between days 11 and 18 followed by a precipitous drop at estrus. Mares et al. (1962) found the highest progesterone content in corpora lutea on day 15 of the estrous cycle. These observations agree with the finding in this study of peak serum progesterone 3 days before estrus. Progesterone may have been even higher during the 7-day period before day -3 when no samples were collected.

Progesterone decreased 64% from day -3 to day -2, showed a further 76% decrease by day -1 (Figure 26), and the decline was continuous until day 2 when LH returned to baseline levels. Progesterone increases before ovulation in humans (Johansson and Wide, 1969) and in monkeys (Johansson et al., 1968). And Hansel and Trimberger (1952)

observed that small amounts (5 to 15 mg) of progesterone administered at the beginning of estrus would hasten ovulation. But no such preovulatory increase of progesterone was evident in my heifers, substantiating the earlier observation of Kazama and Hansel (1970).

Hansel and Snook (1970) observed a negative feedback of progesterone on pituitary LH during the early follicular phase of the estrous cycle, and similar correlations ($r=-0.43$ and -0.65) were observed in this study on days -3 and -2 (Table 22). Prolactin appears to be luteolytic ($r=-0.95$ and -0.68) on days -3 and -2, but luteotropic ($r=0.61$) (perhaps to the ovulatory follicle) on day -1. However, prolactin and progesterone levels were not highly correlated between days -0.5 and 2; then they were positively related on day 4 ($P<0.05$), negatively related on day 7 ($P<0.05$) and positively related on day 11 ($P<0.10$). The reasons for these dramatic shifts during the estrous cycle in relationships between levels of prolactin and progesterone are not apparent.

Prolactin had little relationship to size of the CL (Table 23), and there were few significant correlations of progesterone and size of CL (Table 24). This could be construed to mean that sources other than the CL may synthesize progesterone, but this is beyond the scope of this thesis. The important action of LH on progesterone

Table 22.--Some correlations between pituitary and ovarian hormones during the estrous cycle of Holstein heifers.

Days before or after estrus	(n)	Serum LH vs serum progesterone	Serum LH vs serum estradiol	Serum prolactin vs serum progesterone	Serum prolactin vs serum estradiol
-3	3	-0.43	-0.83	-0.95*	-0.65
-2	4	-0.65	0.47	-0.68	0.60
-1	13	0.06	-0.11	0.61*	0.30
-0.5	6	0.98**	0.69	-0.10	-0.06
Estrus	18	-0.08	-0.05	0.19	-0.20
2	15	-0.45	-0.28	0.38	-0.21
4	15	0.04	0.10	0.55*	0.00
7	15	-0.66**	-0.41	-0.53*	-0.45
11	15	0.28	0.11	0.47	-0.08

*$P<0.05$.

**$P<0.01$.

Table 23.--Some correlations between pituitary hormones and follicle size or corpus luteum size during the estrous cycle of Holstein heifers.

Days before or after estrus	Serum LH vs follicle size	Serum prolactin vs follicle size	Serum LH vs corpus luteum size	Serum prolactin vs corpus luteum size
-3	0.26 (12)[a]	-0.13 (12)	-0.27 (9)	0.03 (9)
-2	-0.06 (11)	0.21 (11)	0.08 (9)	0.32 (9)
-1	0.19 (12)	0.29 (12)	0.15 (9)	0.01 (9)
-0.5	0.16 (9)	0.37 (9)	-0.37 (5)	0.26 (5)
Estrus	0.00 (34)	-0.06 (34)	0.39 (16)	0.34 (16)
2	0.03 (32)	-0.18 (32)	-0.34 (13)	0.22 (13)
4	0.00 (39)	-0.25 (39)	0.31 (10)	0.19 (10)
7	-0.14 (74)	0.00 (74)	-0.02 (59)	0.27*(59)
11	-0.26*(79)	0.00 (79)	0.09 (76)	0.23*(76)

[a] r values; numbers in parentheses refer to number of observations.

*P<0.05.

Table 24.--Some correlations between ovarian hormones and cortisol, ovarian follicles or corpora lutea, or estrous intensity[a] in Holstein heifers.

Days before or after estrus	Serum estradiol vs serum cortisol	Serum estradiol vs size of largest follicle	Serum estradiol vs estrous intensity	Serum progesterone vs size of largest CL
-3	-0.06 (3)[b]	-0.27 (3)	0.10 (3)	0.07 (3)
-2	-0.66 (4)	(1)	0.34 (4)	(1)
-1	0.36 (13)	0.42 (3)	-0.21 (13)	0.46 (3)
-0.5	0.40 (6)	(2)	c (6)	(2)
Estrus	-0.03 (18)	-0.90** (6)	-0.67** (18)	0.81* (6)
2	-0.08 (15)	-0.63 (7)	-	-0.20 (7)
4	-0.27 (15)	0.43 (12)	-	0.51 (12)
7	-0.38 (15)	-0.13 (14)	-	-0.14 (14)
11	0.38 (15)	-0.16 (15)	-	-0.45 (15)

[a]Estrous intensity at estrus coded as follows; 1 - silent estrus, 2 - restlessness, 3 - riding activity, and 4 - standing heat.

[b]r values; numbers in parentheses refer to number of observations.

[c]One factor had constant variance.

*P<0.05

**P<0.01

synthetic activity of the CL has been documented in the bovine, and prolactin exhibited no luteotropic activity (Hansel, 1966).

Snook et al. (1969) observed regression of the CL and reduced progesterone content of the CL after administration of LH antiserum to heifers. The changing correlations between LH or prolactin and progesterone (Table 22) indicate that age of the CL may be important in determining the luteotropic or luteolytic properties of the two hormones, as has been observed in rats (Saito et al., 1970). Snook et al. (1969) concluded that luteal regression probably could not be attributed to declining levels of serum LH. Indeed, this study has shown that during the period of rapid CL regression, serum LH actually increases (Figure 27).

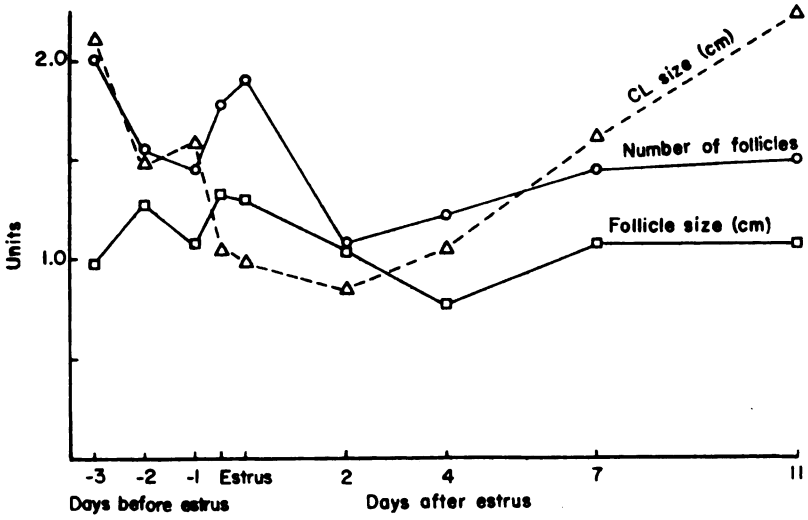
Follicular size and number were significantly greater ($P<0.025$) during proestrus and estrus than during the remainder of the estrous cycle (Figure 25). Increased follicular size and increased estradiol during proestrus (Figures 25 and 26) coincide with the observed decrease in pituitary FSH (Hackett and Hafs, 1969). The decreased number of follicles from day -3 to day -1 may represent loss of midcycle follicles or atresia of large follicles except for the preovulatory follicle.

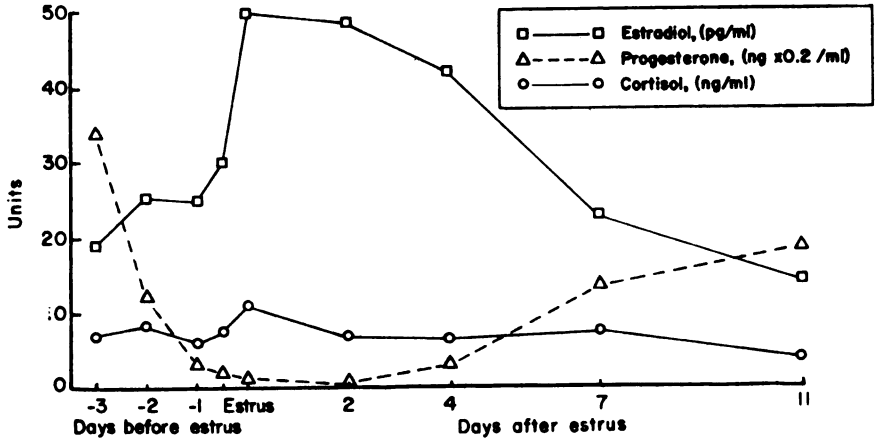
The ovulatory LH surge begins during late proestrus and continues during the early part of estrus (Figure 27). Hackett and Hafs (1969) reported an increase in pituitary

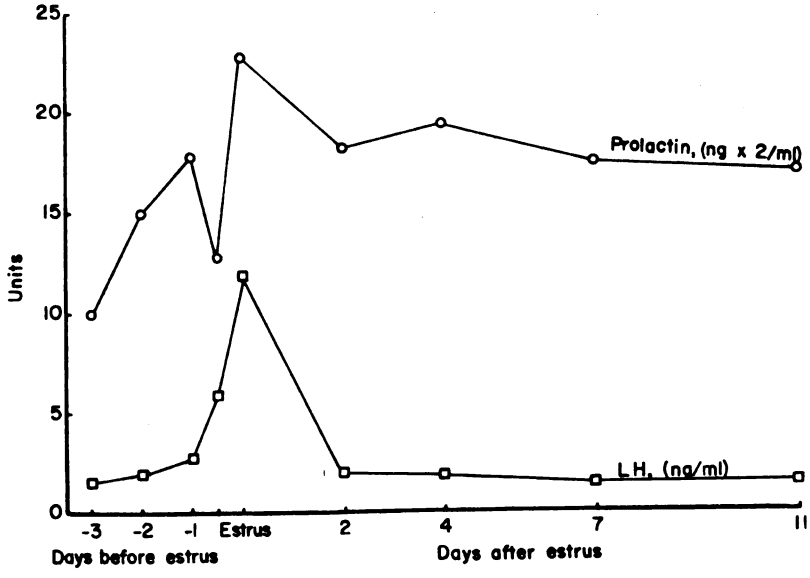
Figure 25. Ovarian activity during the estrous cycle in Holstein heifers.

Figure 26. Levels of ovarian and adrenal hormones in blood serum during the estrous cycle in Holstein heifers.

Figure 27. Levels of LH and prolactin in blood serum during the estrous cycle in Holstein heifers.


2.0
1.0
Units
CL size (cm)
Number of follicles
Follicle size (cm)
-3 -2 -1 Estrus 2 4 7 11
Days before estrus
Days after estrus


50
40
30
20
0
Units
Estradiol, (pg/ml)
Progesterone, (ng x0.2 /ml)
Cortisol, (ng/ml)
-3 -2 -1 Estrus 2 4 7 11
Days before estrus
Days after estrus


25
20
15
10
5
0
Units
Prolactin, (ng x 2/ml)
LH, (ng/ml)
-3 -2 -1 Estrus 2 4 7 11
Days before estrus
Days after estrus

LH at this time; synthesis of LH must exceed release during proestrus. Levels of prolactin in blood serum also peak during estrus (Figure 27) as does the level of estradiol (Figure 26). But any stimulatory effect of estradiol on prolactin secretion is not evident from the correlations between the two (Table 22).

The LH peak at estrus (Figure 27) is lower than levels reported in cows (Henricks et al., 1970), but it is difficult to determine if this is an effect of age or of time of bleeding relative to peak LH. Norman et al. (1968) observed the highest total estrogens just prior to ovulation in sheep, and Goding et al. (1969) elicited release of LH in sheep after injection of estradiol-17β. Mikhail et al. (1970) reported a significant increase in estradiol and a slight increase in estrone on the day of the LH peak in humans. I did not detect that estradiol increased before LH, but a positive relationship existed between estradiol and LH 0.5 days before estrus (Table 22) when levels of both hormones are rising. Possibly estradiol has a positive feedback on LH at this time to stimulate the ovulatory surge of serum LH. But based on these results, the hypothesis that increased estrogens cause the ovulatory LH surge cannot be accepted.

Estrous intensity, rated on a 1 to 4 scale ranging from silent heat to standing heat, was related neither to estradiol levels before or during estrus (Table 24) nor to

serum LH or prolactin levels before or during estrus (Table 25). Perhaps the scale used to rate estrous intensity was based on human values rather than on physiological criteria.

LH levels are low (avg 1.71 ng/ml) during metestrus and diestrus, lower than levels reported by Schams and Karg (1969) and by Hansel and Snook (1970). This discrepancy probably can be attributed to differences in the assays used. Diestrus levels of LH were very stable and no increases were noted in the intervals between two estrous periods. Serum prolactin decreased during metestrus and diestrus though levels at this time were higher than LH. The significant decrease in pituitary prolactin between estrus and day 2 (Sinha and Tucker, 1969) and decreased serum levels (Figure 27) signifies decreased pituitary synthesis as well as decreased release of prolactin. During metestrus the CL from the previous cycle degenerated to a corpus albicans and progesterone synthetic activity begins in the newly formed CL. Serum progesterone (Figure 26) and CL size (Figure 25) increased by day 4.

Estradiol failed to increase at day 11 when increased follicular growth has been reported (Rajakoski, 1960). But I would have missed any transitory rise in estradiol which may occur simultaneously with an LH surge between days 8 and 10 as reported earlier (Schams and Karg, 1969). The high levels of estradiol during metestrus (Figure 26) are difficult to interpret. Results for levels of serum estradiol obtained by competitive protein binding need

Table 25.--Some correlations between pituitary hormones or type of CL[a] and estrous intensity[b] in Holstein heifers.

Estrous cycle	Serum LH[c] vs estrous intensity	Serum prolactin[c] vs estrous intensity	Type of CL vs estrous intensity
1	0.08 (30)[d]	0.13 (30)	0.31 (30)
2	0.18 (22)	-0.17 (22)	-0.69** (22)
3	d (6)	d (6)	d (6)
4	-0.04 (26)	-0.19 (26)	0.08 (26)
5	0.26 (32)	0.13 (32)	0.30 (31)
6	-0.58* (16)	-0.33 (16)	0.04 (15)
7	0.20 (24)	-0.20 (24)	0.13 (20)
8	0.19 (13)	0.15 (13)	0.12 (13)
9	(2)	(2)	(2)
Overall cycles	0.08 (171)	0.02 (171)	0.11 (165)

[a]The CL of the cycle immediately preceding estrus was coded as follows; 1 - anovulation, 2 - normal CL, 3 - cystic CL, and 4 - cystic follicle.

[b]Estrous intensity during estrus was coded as follows; 1 - silent heat, 2 - restlessness, 3 - riding activity, and 4 - standing heat.

[c]Estrous intensity was correlated with hormone concentrations averaged for days -1, -0.5, and 0.

[d]r values; the numbers in parentheses refer to the number of observations.

*P<0.05. **P<0.01.

validation by an independent method but it seems possible that estradiol-17β may not be the major estrogen in cows. Norman et al. (1968) found 16-ketoestradiol-17β to be the major blood estrogen in sheep, and a similar situation may exist in the bovine. Levels of blood estrone equivalent determined by bioassay, increased from 1 ng/ml during the second trimester to 7 ng/ml during the third trimester of gestation in cows (Pope et al., 1965). Most of the blood estrogens were present as water-soluble conjugates and these workers reported undetectable blood levels of estrogen in cows during estrus. The curves for levels of estradiol and progesterone during the estrous cycle are virtual mirror images of each other (Figure 26) and one might speculate that the two hormones act in opposition to each other. However, the changes in progesterone levels during the estrous cycle are highly significant ($P<0.005$), whereas changes in levels of estradiol during the estrous cycle do not approach significance.

Serum LH and prolactin decreased significantly ($P<0.05$) from first estrus to breeding size. And Henricks et al. (1970) found levels of LH during the luteal phase even lower in cows than in these heifers. Significant declines with age were also noted for cortisol and corticosterone. The levels of corticosteroids reported here agree with others using the protein binding assay (Heitzman et al., 1970; Wagner, 1970). Decreased hormone levels with

advancing age may indicate that heifers are not fully mature until several estrous cycles after puberty.

In these heifers, ovulation occurred 29 hr after the onset of standing heat and 32 hr after the LH peak. This differs from reports in the literature for cows (Henricks et al., 1970; Schams and Karg, 1969) to the extent that this study showed that the LH peak, on the average, occurred slightly before the onset of estrus. This may indicate that physiological events occur at slightly different intervals in cows and heifers. For example, Trimberger (1948) observed that relative to cows, heifers had a shorter duration of estrus and a shorter interval between estrus and ovulation.

SUMMARY AND CONCLUSIONS

Endocrine, ovarian, and behavioral changes were studied from prepubertal age to breeding size in 37 heifers.

Estrous behavior before first estrus was associated with increased serum LH ($P<0.005$). Within 20 days before first estrus, follicle size, number of follicles, and serum LH increased while serum prolactin decreased. LH was released in increased quantity near the onset of puberty. Levels of prolactin in the serum were significantly higher ($P<0.01$) before than after puberty; they decreased continuously with age. Prolactin levels were highly variable between and within heifers. Although estrous activity occurred before puberty, it did not follow any specific pattern. Growth rate was linear before and after puberty and similar to Morrison's standards.

The average age at first estrus was 43.3 ± 0.9 weeks when the heifers attained 110.7 ± 0.8 cm withers height and weighed 252.9 ± 5.5 kg. These dimensions resemble those in the literature, showing that heifers reach puberty at a similar size rather than at a constant age. Estrous cycle length was constant from the first to the ninth estrus (avg 20.8 ± 0.3 days) but the first and second estrous cycles of heifers attaining puberty at a

later age were significantly longer ($P<0.005$). The incidence of standing heat increased from 65% at the first estrous cycle to 91% at the ninth estrous cycle, and 62% of the heifers were first observed in standing heat in the a.m.

The number of palpable ovarian follicles per heifer decreased ($P<0.005$) from 2.7 ± 0.2 during the 10-day period before first estrus to 1.4 ± 0.1 during the first estrous cycle. During the estrous cycle, follicle numbers per heifer decreased significantly ($P<0.005$) to 1.1 ± 0.1 at 2 days after estrus and increased to 2.0 ± 0.3 at 3 days before estrus. Follicular diameter increased similarly to number of follicles during the estrous cycle. Follicle size increased significantly ($P<0.005$) from cycles 1, 2 and 3 (avg 0.9 cm) to cycles 4 through 9 (avg 1.2 cm). Equal numbers of follicles were palpated on the right and left ovaries, but those on the right were slightly larger and ovulated more frequently.

The incidence of cystic follicles ($\geq$ 2.5 cm dia) and cystic corpora lutea (fluid filled cyst $\geq$1.0 cm dia) exhibited little change from the first to the ninth estrous cycle. Size of corpora lutea decreased significantly ($P<0.005$) with age from cycles 1, 2 and 3 (avg 1.9 cm) to cycles 4 through 9 (avg 1.6 cm). CL size increased continuously and significantly ($P<0.005$) from 0.8 ± 0.1 cm 2 days after estrus to 2.2 ± 0.1 cm on day 11, and decreased to day 2.

During the luteal phase of the estrous cycle, serum LH decreased significantly ($P<0.01$) from cycles 1, 2 and 3 (1.99 ± 0.06 ng/ml) to cycles 6, 7, 8 and 9 (1.44 ± 0.16 ng/ml). And serum LH during the luteal phase of the first estrous cycle was significantly lower ($P<0.01$) than during the prepubertal period (2.16 ± 0.26 ng/ml).

Levels of serum LH increased from 1.42 ± 0.07 ng/ml on day 7 of the estrous cycle to 2.85 ± 0.29 ng/ml on day -1 and to 5.90 ± 1.87 ng/ml 0.5 days before estrus. The ovulatory surge (11.82 ± 1.24 ng/ml) occurred on the day of estrus and levels of LH returned to baseline on day 2. The duration of the ovulatory LH surge is quite short, because only 46% of the samples taken from heifers when first observed in estrus displayed elevated LH levels. Serum LH levels were very stable during the luteal phase of the cycle; no instances of elevated LH were noted in the interval between estrous periods.

Levels of serum prolactin declined continuously and significantly ($P<0.01$) from 141.9 ± 11.4 ng/ml during the prepubertal period to 15.3 ± 2.2 ng/ml during estrous cycles 6, 7, 8 and 9. During the estrous cycle, prolactin increased ($P<0.025$) to 45.8 ± 4.2 ng/ml at estrus and decreased to about 35 ng/ml during metestrus, diestrus and proestrus. Serum prolactin levels fluctuated considerably during the estrous cycle; between heifers, and between samples from each heifer. Decreased levels of LH and

prolactin with age suggest that heifers are not fully mature at first estrus.

Serum estrone and estriol were identified in the bovine, but inadequate assay conditions contributed to large variances. Levels of serum estrone increased at proestrus, estrus and on day 7 while estriol decreased during estrus. Levels of estradiol were high during estrus (49.6 $\pm$ 25.0 pg/ml) and metestrus and decreased to 14.1 $\pm$ 4.0 pg/ml on day 11. Correlations of estradiol and prolactin, though not high, were negative at all stages of the estrous cycle except for positive correlations on days -2 and -1. The reasons for the negative correlations are not apparent but the positive correlations at proestrus may indicate a positive feedback of estradiol on subsequent prolactin release at estrus.

Levels of serum progesterone did not change from the first to the seventh estrous cycles but closely followed changes in CL size during the cycle. Progesterone increased continuously ($P<0.005$) from a low of 0.12 $\pm$ 0.05 ng/ml on day 2 to 6.88 $\pm$ 1.04 ng/ml 3 days before estrus and then decreased continuously to day 2. LH was correlated negatively with progesterone on days -3 and -2 and then changed to a significant ($P<0.01$) positive correlation 0.5 days before estrus. These, together with the LH increase beginning 3 days before estrus may indicate a luteolytic effect of LH during late diestrus and early proestrus. That

prolactin may also have a role in regression of the CL is indicated by a change from a significant ($P<0.05$) negative correlation on day -3 ($r=-0.95$) to a significant positive correlation of prolactin and progesterone on day -1 ($r=0.61$).

Serum corticosterone decreased significantly ($P<0.005$) from 0.48 ± 0.09 ng/ml during the first estrous cycle to 0.23 ± 0.03 ng/ml during the fourth and seventh estrous cycles. Cortisol also decreased significantly ($P<0.005$) from 9.89 ± 1.29 ng/ml during the first estrous cycle to 6.05 ± 0.67 ng/ml during the fourth and seventh cycles. No significant changes were noted with cortisol or corticosterone during the cycle. These values for cortisol are similar to reports for total glucocorticoids by protein binding assays but both corticosterone and cortisol are much lower than values of 30 to 70 ng/ml cortisol and 10 to 30 ng/ml corticosterone as determined fluorometrically.

A study of the endocrine and ovarian changes during estrus revealed ovulation occurred 29 ± 6 hr after standing heat and 32 ± 6 hr after the LH peak. In other words, standing heat was first observed about 3 hr after the LH peak had occurred. LH remained elevated about 8 hr during estrus and the average peak concentration was 25.92 ± 3.18 ng/ml. In agreement with results from the major estrous cycle study, serum LH began a linear increase 4 days before

estrus. Prolactin also increased from 3 days before estrus to a peak at estrus, but these changes in prolactin were not significant. A re-analysis of the serum LH and prolactin data revealed diurnal changes; levels of both were low ($P<0.05$) at noon and during the night. LH and prolactin levels were increased in the morning and in the afternoon. But these diurnal changes must be proven in another experiment since my experiment was not designed for this purpose.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abraham, G.E. 1969. Solid-phase radioimmunoassay of estradiol-17β. J. Clin. Endocrinol. 29: 866.

Amenomori, Y.; C.L. Chen; and J. Meites. 1970. Serum prolactin levels in rats during different reproductive states. Endocrinol. 86: 506.

Anderson, R.R. and W.H. McShan. 1966. Luteinizing hormone levels in pig, cow and rat blood plasma during the estrous cycle. Endocrinol. 78: 976.

Armstrong, D.T. and W. Hansel. 1959. Alteration of the bovine estrous cycle with oxytocin. J. Dairy Sci. 42: 533.

Armstrong, D.T.; J. O'Brian; and R.O. Greep. 1964. Effects of luteinizing hormone on progestin biosynthesis in the luteinized rat ovary. Endocrinol. 75: 488.

Asdell, S.A.; J. de Alba; and S.J. Roberts. 1945. The level of ovarian hormones required to induce heat and other reactions in the ovariectomized cow. J. Animal Sci. 4: 277.

Bolt, D.J.; H.E. Kelley; and H.W. Hawk. 1970. Variation during the estrous cycle of the ewe in luteinizing hormone release after injection of estradiol. (Abstr.) J. Dairy Sci. 53: 670.

Bray, G. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279.

Carrick, M.J. and J.N. Shelton. 1969. Oestrogen-progesterone relationships in the induction of oestrus in spayed heifers. J. Endocrinol. 45: 99.

Corker, C.S.; D. Exley; and F. Naftolin. 1970. Assay of 17β-oestradiol by competitive protein binding methods. Acta Endocrinol. 64 Suppl. 147: 305.

Cupps, P.T.; L.L. Anderson; and H.H. Cole. 1969. The estrous cycle, p. 217-250. In H.H. Cole and P.T. Cupps, Reproduction in Domestic Animals, 2nd ed., Academic Press, New York.

Desjardins, C. and H.D. Hafs. 1968. Levels of pituitary FSH and LH in heifers from birth through puberty. J. Animal Sci. 27: 472.

Desjardins, C. and H.D. Hafs. 1969. Maturation of bovine female genitalia from birth through puberty. J. Animal Sci. 28: 502.

Diczfalusy, E., (ed.) 1970. Steroid Assay by Protein Binding. Transactions of Second Karolinska Symposia on Research Methods in Reproductive Endocrinology. 366 p.

Dobrowolski, W.; E. Stupnicka; and E. Domański. 1968. Progesterone levels in ovarian venous blood during the oestrous cycle of the cow. J. Reprod. Fert. 15: 409.

Donaldson, L.E. and W. Hansel. 1965. Prolongation of life span of bovine corpus luteum by single injections of bovine luteinizing hormone. J. Dairy Sci. 48: 903.

Edgar, D. G. 1953. The progesterone content of body fluids and tissues. J. Endocrinol. 10: 54.

Ellis, S.; R.E. Grindeland; and P.X. Callahan. 1970. Subunits of bovine and ovine growth hormones: Separation by electrofocusing. (Abstr.) Proc. of 52nd Annual Meeting of The Endocrine Society. St. Louis, Mo. p. 113.

Ellis, S.; R.E. Grindeland; J.M. Neunke; and P.X. Callahan. 1969. Purification and properties of rat prolactin. Endocrinol 85: 886.

Erb, R.E.; R.D. Randel; T.N. Mellin; and V.L. Estergreen, Jr. 1968. Urinary estrogen excretion rates during pregnancy in the bovine. J. Dairy Sci. 51: 416.

Ginther, O.J. 1970. Effect of progesterone on length of estrous cycle in cattle. Am. J. Vet. Research 31: 493.

Goding, J.R.; K.J. Catt; J.M. Brown; C.C. Kaltenbach; I.A. Cumming; and B.J. Mole. 1969. Radioimmunoassay for ovine luteinizing hormone. Secretion of luteinizing hormone during estrus and following estrogen administration in the sheep. Endocrinol. 85: 133.

Gomes, W.R.; V.L. Estergreen, Jr.; O.L. Frost; and R.E. Erb. 1963. Progestin levels in jugular and ovarian venous blood corpora lutea and ovaries of the nonpregnant bovine. J. Dairy Sci. 46: 553.

Gorski, R.A. 1966. Localization and sexual differentiation of the nervous structures which regulate ovulation. J. Reprod. Fert. Suppl. 1: 67.

Hackett, A. J. 1968. Endocrine and Reproductive Changes During the Estrous Cycle of the Bovine. PhD Thesis. Michigan State University.

Hackett, A.J. and H.D. Hafs. 1969. Pituitary and hypothalamic endocrine changes during the bovine estrous cycle. J. Animal Sci. 28: 531.

Hafez, E.S.E. and M.W. Schein. 1962. The behaviour of cattle, p. 274. In E.S.E. Hafez, (ed.), The Behaviour of Domestic Animals, The Williams and Wilkins Company, Baltimore.

Hafs, H.D. and D.T. Armstrong. 1968. Corpus luteum growth and progesterone synthesis during the bovine estrous cycle. J. Animal Sci. 27: 134.

Haglund, H. 1967. Isoelectric focusing in natural pH gradients--A technique of growing importance for fractionation and characterization of proteins. Sci. Tools 14: 17.

Hansel, W. 1966. Luteotrophic and luteolytic mechanisms in bovine corpora lutea. J. Reprod. Fert. Suppl. 1: 33.

Hansel, W. and R.B. Snook. 1970. Pituitary ovarian relationships in the cow. J. Dairy Sci. 53: 945.

Hansel, W. and G. W. Trimberger. 1951. Atropine blockage of ovulation in the cow and its possible significance. J. Animal Sci. 10: 719.

Hansel, W. and G.W. Trimberger. 1952. The effect of progesterone on ovulation time in dairy heifers. J. Dairy Sci. 35: 65.

Heitzman, R.J.; T.C. Adams; and G.D. Hunter. 1970. The assay of corticosteroids in plasma of the dairy cow. J. Endocrinol. 47: 133.

Henricks, D.M.; J.F. Dickey; and G.D. Niswender. 1970. Serum luteinizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. Biol. Reprod. 2: 346.

Hobson, W.C. and W. Hansel. 1970. Plasma luteinizing hormone levels in hysterectomized and ovariectomized heifers treated with estradiol and progesterone. (Abstr.) J. Dairy Sci. 53: 669.

Hunter, W.M. 1967. The preparation of radioiodinated proteins of high activity, their reaction with antibody in vitro: The radioimmunoassay. p. 608-654. In D.M. Wier, (ed.), Handbook of Experimental Immunology, F.A. Davis Co., Philadelphia, Pa.

Johansson, E.D.B.; J.D. Neill; and E. Knobil. 1968. Periovulatory progesterone concentration in the peripheral plasma of the rhesus monkey with a methodologic note on the detection of ovulation. Endocrinol. 82: 143.

Johansson, E.D.B. and L. Wide. 1969. Periovulatory levels of plasma progesterone and luteinizing hormone in women. Acta Endocrinol. 62: 82.

Johke, T. 1969a. Prolactin release in response to milking stimulus in the cow and goat estimated by radioimmunoassay. Endocrinol. Japonica 16: 179.

Johke, T. 1969b. Radioimmunoassay for bovine prolactin in plasma. Endocrinol. Japonica 16: 581.

Jubb, K.V. and K. McEntee. 1955. Observations on the bovine pituitary gland. II. Architecture and cytology with special reference to basophil cell function. Cornell Vet. 45: 593.

Karg, H. 1957. Ascorbin säuredynamik im Ovar als Gonadotropinnachweis. Klin. Wschr. 35: 643.

Karg, V.H.; B. Hoffmann; and D. Schams. 1969. Verlauf der Blutspiegel an Progesteron, Luteinisierungshormon and Prolaktin während des Zyklus bei einer Kuh. Zuchthygiene 4: 149.

Kazama, N. and W. Hansel. 1970. Preovulatory changes in the progesterone level of bovine peripheral blood plasma. Endocrinol. 86: 1252.

Koprowski, J.A. 1970. Personal communication.

Koprowski, J.A.; H.A. Tucker; and E.M. Convey. 1970. Prolactin and growth hormone circadian periodicity. (Abstr.) J. Animal Sci. 31: 224.

Korenman, S.G. 1968. Radio-ligand binding assay of specific estrogens using a soluble uterine macromolecule. J. Clin. Endocrinol. 28: 127.

Korenman, S.G.; L.E. Perrin; and T.P. McCallum. 1969. A radio-ligand binding assay system for estradiol measurement in human plasma. J. Clin. Endocrinol. 29: 879.

Korenman, S.G.; D. Tulchinsky; and L.W. Eaton, Jr. 1970. Radio-ligand binding procedures for estrogen assay in normal and pregnancy plasma. Acta Endocrinol. 64 Suppl. 147: 291.

Labhsetwar, A.P. 1970. Role of estrogens in ovulation: A study using the estrogen-antagonist, I.C.I. 46, 474. Endocrinol. 87: 542.

Mares, S.E.; R.G. Zimbelman; and L.E. Casida. 1962. Variation in progesterone content of the bovine corpus luteum of the estrual cycle. J. Animal Sci. 21: 266.

Mason, N.R.; J.M. Marsh; and K. Savard. 1962. An action of gonadotropin in vitro. J. Biol. Chem. 237: 1801.

Mayes, D. and C.A. Nugent. 1970. Plasma estradiol determined with a competitive protein binding method. Steroids 15: 389.

Melampy, R.M.; M.A. Emmerson; J.M. Rakes; L.J. Hanka; and P.G. Eness. 1957. The effect of progesterone on the estrous response of estrogen in conditioned ovariectomized cows. J. Animal Sci. 16: 967.

Mellin, T.N. and R.E. Erb. 1965. Estrogens in the bovine--A review. J. Dairy Sci. 48: 687.

Mellin, T.N. and R.E. Erb. 1966. Estrogen metabolism and excretion during the bovine estrous cycle. Steroids 7: 589.

Mellin, T.N.; R.E. Erb; and V.L. Estergreen. 1965. Quantitative estimation and identification of estrogens in bovine urine. J. Dairy Sci. 48: 895.

Mikhail, G.; C.H. Wu; M. Ferin; and R.L. Vande Wiele. 1970. Radioimmunoassay of plasma estrone and estradiol. Steroids 15: 333.

Miyake, T. 1969. Causal relationship between ovarian steroid secretion and pituitary luteinizing hormone

release in the rat estrous cycle. Endocrinol. Japonica Suppl. 1: 83.

Morrison, F.B. 1957. Feeds and Feeding, 22nd ed. p. 680. Morrison Publishing Co., Ithaca, New York.

Morrow, D.A. 1969. Estrous behavior and ovarian activity in prepuberal and postpuberal dairy heifers. J. Dairy Sci. 52: 224.

Morton, J.R.C.; V.H. Denenberg; and M.X. Zarrow. 1963. Modification of sexual development through stimulation in infancy. Endocrinol. 72: 439.

Murphy, B.E.P. 1967. Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein-binding radioassay. J. Clin. Endocrinol. 27: 973.

Murphy, B.E.P. 1970. Methodological problems in competitive protein-binding techniques; the use of Sephadex column chromatography to separate steroids. Acta Endocrinol. 64 Suppl. 147: 37.

Nalbandov, A.V. 1964. Reproductive Physiology, 2nd ed., W.H. Freeman, San Francisco. 316 p.

Niswender, G.D.; L.E. Reichert, Jr.; A.R. Midgley, Jr.; and A.V. Nalbandov. 1969. Radioimmunoassay for bovine and ovine luteinizing hormone. Endocrinol. 84: 1166.

Niswender, G.D.; J.F. Roche; D.L. Foster; and A.R. Midgley, Jr. 1968. Radioimmunoassay of serum levels of luteinizing hormone during the cycle and early pregnancy in ewes. Proc. Soc. Exp. Biol. Med. 129: 901.

Norman, R.L.; B.E. Eleftheriou; H.G. Spies; and P. Hoppe. 1968. Free plasma estrogens in the ewe during the estrous cycle. Steroids 11: 667.

Parlow, A.F. 1961. Bioassay of luteinizing hormone (LH, I.C.S.H.) by the ovarian ascorbic acid depletion method, p. 300. In A. Albert, ed., Human Pituitary Gonadotrophins. C.C. Thomas, Springfield, Ill.

Plotka, E.D.; R.E. Erb; C.J. Callahan; and W.R. Gomes. 1967. Levels of progesterone in peripheral blood plasma during the estrous cycle of the bovine. J. Dairy Sci. 50: 1158.

Pope, G.S.; S.K. Gupta; and I.B. Munro. 1969. Progesterone levels in the systemic plasma of pregnant, cycling and ovariectomized cows. J. Reprod. Fert. 20: 369.

Pope, G.S.; H.E.H. Jones; and H.B. Waynforth. 1965. Oestrogens in the blood of the cow. J. Endocrinol. 33: 385.

Rahlman, D.F. and P.T. Cupps. 1962. Effects of estradiol and dehydroepiandrosterone on the reproductive cycle of the cow. I. Estrous cycle length, ovulation, temperature changes, vaginal mucus flow, initiation and maintenance of pregnancy. J. Dairy Sci. 45: 1003.

Rajakoski, E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left-right variation. Acta Endocrinol. 34 Suppl. 52: 1.

Rakha, A.M. and H.A. Robertson. 1965. Changes in levels of follicle-stimulating hormone and luteinizing hormone in the bovine pituitary gland at ovulation. J. Endocrinol. 31: 245.

Ramirez, V.D. and S.M. McCann. 1963. Comparison of the regulation of luteinizing hormone (LH) secretion in immature and adult rats. Endocrinol. 72: 452.

Ramirez, V.D. and C.H. Sawyer. 1966. Changes in hypothalamic luteinizing hormone releasing factor (LHRF) in the female rat during puberty. Endocrinol. 78: 958.

Reeves, J.J.; A. Arimura; and A.V. Schally. 1970. Serum levels of prolactin and luteinizing hormone (LH) in the ewe at various stages of the estrous cycle. Proc. Soc. Exp. Biol. Med. 134: 938.

Riegle, G.D. and J.E. Nellor. 1967. Changes in adrenocortical function during aging in cattle. J. Gerontology 22: 83.

Saito, M.; A. Arimura; S. Sawano; and A.V. Schally. 1970. Luteotropic and luteolytic effects of prolactin in hypophysectomized rats. Endokrinol. 56: 129.

Salisbury, G.W. and N.L. Van Demark. 1961. Physiology of Reproduction and Artificial Insemination of Cattle. W.H. Freeman, San Francisco. 639 p.

Scaramuzzi, R.J.; B.V. Caldwell; and R.M. Moor. 1970. Radioimmunoassay of LH and estrogen during the estrous cycle of the ewe. Biol. Reprod. 3: 110.

Schams, D. and H. Karg. 1969. Radioimmunologische LH-Bestimmung im blutserum vom Rind unter besonderer berücksichtigung des Brunstzyklus. Acta Endocrinol. 61: 96.

Schams, D. and H. Karg. 1970. Untersuchungen über Prolaktin im Rinderblut mit einer radioimmunologischen Bestimmungsmethode. Zbl. Vet. Med. 17: 193.

Sinha, Y.N. and H.A. Tucker. 1969. Mammary development and pituitary prolactin level of heifers from birth through puberty and during the estrous cycle. J. Dairy Sci. 52: 507.

Snook, R.B.; M.A. Brunner; R.R. Saatman; and W. Hansel. 1969. The effect of antisera to bovine LH in hysterectomized and intact heifers. Biol. Reprod. 1: 49.

Sorensen, A.M.; W. Hansel; W.H. Hough; D.T. Armstrong; K. McEntee; and R.W. Bratton. 1959. Causes and prevention of reproductive failures in dairy cattle. I. Influence of underfeeding and overfeeding on growth and development of Holstein heifers. Cornell Univ. Agr. Exp. Sta. Bull. 936.

Stabenfeldt, G.H.; L.L. Ewing; and L.E. McDonald. 1969. Peripheral plasma progesterone levels during the bovine oestrous cycle. J. Reprod. Fert. 19: 433.

Steelman, S.L. and F.M. Pohley. 1953. Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotrophin. Endocrinol. 53: 604.

Swann, H. 1970. Personal communication.

Trimberger, G.W. 1941. "Menstruation" frequency and its relation to conception in dairy cattle. J. Dairy Sci. 24: 819.

Trimberger, G.W. 1948. Breeding efficiency in dairy cattle from artificial insemination at various intervals before and after ovulation. Univ. of Nebraska Agr. Exp. Sta. Research Bull. 153.

Trimberger, G.W. and W. Hansel. 1955. Conception rate and ovarian function following estrus control by

progesterone injections in dairy cattle. J. Animal Sci. 14: 224.

Tucker, H.A. 1970. Hormonal response to milking. J. Animal Sci. In press.

Tulchinsky, D. and S. G. Korenman. 1970. A radio-ligand assay for plasma estrone; normal values and variations during the menstrual cycle. J. Clin. Endocrinol. 31: 76.

Vandenbergh, J.G. 1967. Effect of the presence of a male on the sexual maturation of female mice. Endocrinol. 81: 345.

Venkataseshu, G.K. and V.L. Estergreen, Jr. 1970. Cortisol and corticosterone in bovine plasma and the effect of adrenocorticotropin. J. Dairy Sci. 53: 480.

Wagner, W.C. 1970. Plasma corticoids in the cow. (Abstr.) J. Animal Sci. 31: 233.

Watanabe, S. and S.M. McCann. 1969. Alternations in pituitary follicle-stimulating hormone (FSH) and hypothalamic FSH- releasing factor (FSH-RF) during puberty. Proc. Soc. Exp. Biol. Med. 132: 195.

Webster's New Collegiate Dictionary. 2nd ed. 1956. G. and C. Merriam Co.

Wiltbank, J.N. and L.E. Casida. 1956. Alteration of ovarian activity by hysterectomy. J. Animal Sci. 15: 134.

Yalow, R.S. and S.A. Berson. 1968. General principles of radioimmunoassay, p. 7-39. In: R.L. Hayes, F.A. Goswitz and B.E.P. Murphy, ed., Radioisotopes in Medicine: In Vitro Studies. U. S. Atomic Energy Commission.

APPENDICES

APPENDIX

Appendix I. Composition of reagents used in radioimmunoassay.

A. Reagents for radioiodination

1. 0.5 M sodium phosphate buffer, pH 7.5
   Monobasic (0.5M)
   Add 69.005 g $NaH_2PO_4.H_2O$ to distilled water. Dissolve, dilute to 1 liter.
   Dibasic (0.5M)
   Add 70.98 g Na_2HPO_4 to distilled water. Heat to dissolve, then dilute to 1 liter.
   Mix monobasic and dibasic to give pH 7.5.
   Dispense in 1 ml portions, store at -20 C.
   Store the monobasic and dibasic buffers at 4 C.

2. 0.05 M sodium phosphate buffer, pH 7.5
   Solution A
   $NaH_2PO_4.H_2O$ - - - - - - - - - - 2.78 g
   Merthiolate - - - - - - - - - - 0.01 g
   Dilute to 100 ml with distilled water.
   Solution B
   $Na_2HPO_4.7H_2O$ - - - - - - - - - 26.825 g
   Merthiolate - - - - - - - - - 0.05 g
   Dilute to 500 ml with distilled water.
   Use 16 ml Solution A, 84 ml Solution B, dilute to 400 ml with distilled water.
   Adjust pH to 7.5 with NaOH, if necessary.
   Store at 4 C.

3. Chloramine - T, 1 ug/ul
   Upon receiving chloramine - T, dispense into small, tightly sealed vials, cover with foil, and store at -20 C.
   Dilute 10 mg chloramine - T to 10 ml with 0.05M $NaPO_4$, pH7.5 buffer. Use within 30 min of preparation. Discard chloramine - T remaining in vial.

4. Sodium metabisulfite, 2.5 ug/ul
   Dilute 25 mg $Na_2S_2O_5$ to 10 ml with 0.05M $NaPO_4$, pH7.5 buffer. Use within 30 min of preparation.

5. Transfer solution
   Sucrose - - - - - - - - - - - 1.6 g
   KI - - - - - - - - - - - - - - 0.1 g
   Dilute to 10 ml with distilled water.
   Dispense in 1 ml portions, store at -20 C.

6. Rinse solution
   Sucrose - - - - - - - - - - - 0.8 g
   KI - - - - - - - - - - - - - - 0.1 g
   Bromphenol blue - - - - - - - 0.001 g
   Dilute to 10 ml with distilled water.
   Dispense in 1 ml portions, store at -20 C.

B. Reagents for Radioimmunoassay

1. 0.01 M phosphate buffered saline, pH 7.0 (PBS)
   NaCl - - - - - - - - - - - - - 143 g
   Monobasic phosphate - - - - 100 ml
   (See Appendix A.1)
   Dibasic phosphate - - - - - 260 ml
   (See Appendix A.1)
   Merthiolate - - - - - - - - - 1.75 g
   Dissolve in distilled water and transfer to a large container.
   Dilute to 17.5 liters with distilled water.
   Adjust pH to 7.0, if necessary.
   Store at 4 C.

2. 0.05 M EDTA - PBS, pH 7.0
   disodium EDTA - - - - - - - - 18.612 g
   Add approximately 950 ml PBS.
   Adjust pH to 7.0 with 5 N NaOH while stirring.
   Dilute to 1 liter.
   Store at 4 C.

3. PBS - 1% egg white albumin (PBS - 1% EWA) or PBS -1% bovine serum albumin (PBS - 1% BSA).
   Add 990 ml PBS to beaker.
   Add 10 g EWA (Sigma Chemical Corp.) or 10 g BSA.
   Mix over magnetic mixer.
   Filter through Whatman No. 1 filter paper.
   Store at 4 C.

4. Hormone standards (LH and prolactin)
   PBS - 1% EWA is used for LH and PBS - 1% BSA is used for prolactin; hereafter they will be referred to as buffers.
   Rinse a small screw-cap vial with buffer, dry.
   Weigh 200-400 ug NIH-LH-B5 or NIH-Prolactin-B2 on Cahn Electrobalance and transfer hormone to the screw-cap vial.
   Add 0.85% saline to 1 mg/ml.
   Add buffer to 9 volumetric flasks.
   Using Hamilton microliter syringes, add appropriate volumes of the 1 mg/ml stock hormone to volumetric flasks to obtain the following concentrations:
   LH - 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 20.48 and 40.96 ng/ml.
   Prolactin - 0.2, 0.4, 1.0, 1.6, 2.0, 3.0, 4.0, 6.0 and 8.0 ng/ml.
   Add buffer to final volume in each volumetric flask.
   Dispense each standard in quantities suitable for one assay.
   Freeze in Dry Ice - ethanol, store at -20 C.
   For use, thaw rapidly with warm water.

5. 1:400 normal rabbit serum (NRS) or 1:400 normal guinea pig serum (NGPS).
   Obtain blood from rabbit or guinea pig that has not been used to develop antibodies.
   Allow blood to clot, recover serum and store the serum in convenient quantities at -20 C.
   Add 2.5 ml of appropriate serum to a 1 liter volumetric flask, dilute to 1 liter with 0.05M PBS-EDTA, pH 7.0.
   Divide into 100 ml portions and store at -20 C.

6. Rabbit anti-bovine LH (RABLH) or guinea pig anti-bovine prolactin (GPABP).
   Dilute the antisera to 1:400 with 0.05M PBS-EDTA, pH 7.0.
   Dispense in small quantities, store at -20 C.
   On day of use, dilute the 1:400 antisera to the required concentration using 1:400 NRS as diluent for anti-LH or 1:400 NGPS as diluent for anti-prolactin.

7. Anti-gamma globulin

Use anti-rabbit gamma globulin in LH assay and anti-guinea pig gamma globulin in prolactin assay.

Dilute antisera to required concentration with 0.05M PBS-EDTA, pH 7.0.

Store at 4 C or at -20 C.

Appendix II. Composition of liquid scintillation fluids

A. Steroid scintillation fluid (Armstrong *et al.*, 1964)

Naphthalene	1.20 g
PPO	7.5 g
POPOP	0.075 g

Dissolve in 1 liter xylene:p-dioxane (1:1).

B. Bray's solution (Bray, 1960)

Naphthalene	60 g
PPO	4 g
Dimethyl POPOP	0.2 g
Ethylene glycol	20 ml
Methanol	100 ml
p - dioxane	816 ml

Mix until dissolved.

Appendix III. Reagents for steroid protein binding assays.

A. Steroid standards.

Weigh 600-800 ug high purity estrone ($\Delta^{1, 3, 5 (10)}$-Estratrien-3-ol-17-one), estradiol ($\Delta^{1, 3, 5 (10)}$-Estratriene-3, 17β-diol), estriol ($\Delta^{1, 3, 5 (10)}$-Estratriene-3, 16α, 17β-triol), progesterone (Δ^{4}-Pregnene-3,20-dione), corticosterone (Δ^{4}-Pregnene -11β, 21-diol-3,20-dione) or cortisol (Δ^{4}-Pregnene-11β, 17α, 21-triol-3, 20-dione) on Cahn Electrobalance and transfer the hormone to a small, clean, screw-cap vial.

Add redistilled 95% ethanol to 500 ug/ml.
Add redistilled 95% ethanol to 7 volumetric flasks.
Using Hamilton microliter syringes, add appropriate volumes of 500 ug/ml stock hormone to volumetric flasks to obtain the following concentrations:

Estradiol - 100, 200, 400, 800, 1600, 3200 and 6400 pg/ml.

Estriol - 200, 400, 800, 1600, 3200, 6400 and 12,800 pg/ml.

Estrone - 400, 800, 1600, 3200, 6400, 12,800 and 25,600 pg/ml.

Progesterone, cortisol, and corticosterone - 1, 5, 10, 15, 20, 50 and 100 ng/ml.

Add redistilled 95% ethanol to final volume.
Store at 4 C in screw-cap vials with polyethylene liners in the caps.

B. Buffer A (0.01M Tris-HCl, 0.001M EDTA, 0.25M sucrose, pH 8.0)

Tris - - - - - - - - - - - - -	1.2114 g
disodium EDTA - - - - - - - -	0.3722 g
Sucrose - - - - - - - - - - -	85.85 g

Dissolve in distilled water.
Adjust pH to 8.0 with 1N HCl.
Dilute to 1 liter.
Store at -20 C in quantities suitable for one assay.

C. Corticosteroid binding globulin (CBG)

Upon receiving dog plasma, divide the plasma into 2.5 ml portions and store at -20 C.
Before use, thaw 2.5 ml dog plasma and dilute to 100 ml with distilled water (2.5% solution).
Stir the 2.5% CBG with 8 g 60-100 mesh florosil for 15 min.
Centrifuge the 2.5% CBG - florosil at 5000 x g for 10 min.
Decant supernatant into a flask in which 35 to 40 ng labeled corticoid in solvent has been dried.

1. For progesterone, use corticosterone -1, 2-^{3}H.
2. For cortisol and corticosterone, use cortisol -1, 2-^{3}H.

Solubilize the labeled corticoid by gentle mixing. Store at 4 C and use within 3 days.

D. Florosil

For use in the cortisol CBG assay, fines should be removed from the florosil (30-60 mesh) by washing several times with distilled water. Then wash in reagent grade methanol and place the washed florosil in an oven until dry.

APPENDIX TABLE IV.--Levels[a] of serum LH at various stages of the first through the ninth estrous cycles.

Cycle		Day of estrous cycle									Overall days
		-3	-2	-1	-0.5	Estrus	2	4	7	11	
1	n	3	11	9	1	30	30	30	29	29	172
	$\bar{x}$	1.77	2.09	2.90	3.10	14.72	2.23	2.14	1.64	1.93	4.26
	SE	0.24	0.18	0.37		3.51	0.17	0.12	0.12	0.18	0.71
2	n	1	1	3		22	5	5	5	5	47
	$\bar{x}$	2.10	1.90	3.00	-	15.70	2.54	2.84	2.30	1.58	8.61
	SE			0.42		3.74	0.61	0.11	0.46	0.16	1.99
3	n	1	1	1		5	2	2	3	3	18
	$\bar{x}$	1.70	1.20	2.20	-	6.68	1.00	1.35	1.70	1.77	2.98
	SE					3.40	0.20	0.25	0.25	0.78	1.04
4	n	8	16	23	10	3	6	13	16	24	119
	$\bar{x}$	1.61	2.26	2.39	5.71	2.27	1.61	1.65	1.38	1.67	2.19
	SE	0.20	0.25	0.20	1.96	0.57	0.30	0.20	0.17	0.19	0.20
5	n	4	6	6	2	27	24	19	16	8	112
	$\bar{x}$	1.70	1.75	2.18	2.90	9.17	1.81	1.62	1.41	1.24	3.48
	SE	0.48	0.20	0.37	0.10	1.90	0.15	0.14	0.18	0.21	0.54
6	n	3	4	9	3	7	13	11	10	10	70
	$\bar{x}$	1.77	2.32	3.08	3.60	19.03	3.02	1.89	1.42	1.35	3.92
	SE	0.33	0.56	0.42	0.21	4.02	1.44	0.28	0.17	0.34	0.77
7	n	4	6	12	8	23	14	14	14	13	108
	$\bar{x}$	1.15	1.97	3.30	8.55	8.80	1.27	1.40	0.76	0.79	3.56
	SE	0.33	0.50	1.28	5.41	2.19	0.12	0.16	0.11	0.11	0.69
8	n		1	1		13	1	1	1	1	19
	$\bar{x}$	-	3.00	11.40	-	8.93	1.00	1.10	0.50	0.70	7.04
	SE					2.69					1.99
9	n	1	1	1	1	3	1	1	1	1	11
	$\bar{x}$	0.50	0.90	1.10	2.40	15.00	1.10	1.20	0.40	0.40	4.82
	SE					13.70					3.76
Overall cycles	n	25	47	65	25	133	96	96	95	94	675
	$\bar{x}$	1.57	2.08	2.85	5.90	11.82	2.02	1.83	1.42	1.53	3.95
	SE	0.13	0.13	0.29	1.87	1.24	0.21	0.08	0.07	0.10	0.30

[a] ng/ml.

APPENDIX TABLE V.--Levels[a] of serum prolactin at various stages of the first through the ninth estrous cycles.

Cycle		Day of estrous cycle									Overall days
		-3	-2	-1	-0.5	Estrus	2	4	7	11	
1	n	3	11	9	1	30	30	30	29	29	172
	$\bar{x}$	42.3	42.6	72.3	58.6	61.2	49.6	67.6	60.9	59.1	58.9
	SE	11.1	9.5	18.2		11.5	8.1	12.8	12.4	8.4	4.3
2	n	1	1	3		22	5	5	5	5	47
	$\bar{x}$	19.1	25.8	76.8	-	70.6	90.7	58.9	40.7	43.2	63.7
	SE			29.4		10.9	14.8	19.4	12.1	12.6	6.5
3	n	1	1	1		5	2	2	3	3	18
	$\bar{x}$	100.6	75.8	74.0	-	43.1	6.7	3.7	47.4	27.2	39.5
	SE					18.0	4.6	3.4	39.7	18.2	9.8
4	n	8	16	23	10	3	6	13	16	24	119
	$\bar{x}$	15.1	35.8	29.9	41.9	58.4	30.0	34.1	22.4	30.2	31.0
	SE	2.1	8.2	5.4	10.2	50.4	12.3	8.9	4.4	8.0	2.9
5	n	4	6	6	2	27	24	19	16	8	112
	$\bar{x}$	17.5	17.2	24.1	14.0	47.4	36.7	31.4	36.8	13.2	33.9
	SE	13.8	9.5	12.3	10.6	7.1	7.3	6.3	7.7	3.3	3.1
6	n	3	4	9	3	7	13	11	10	10	70
	$\bar{x}$	5.5	6.8	6.8	4.0	36.1	11.3	6.5	5.5	4.4	9.8
	SE	0.8	2.1	2.1	1.7	15.1	4.2	2.0	0.9	1.1	2.0
7	n	4	6	12	8	23	14	14	14	13	108
	$\bar{x}$	5.8	11.8	13.0	14.0	18.3	14.6	19.4	13.8	9.7	14.6
	SE	1.7	4.4	4.3	5.6	4.9	5.0	5.3	5.1	2.5	1.7
8	n		1	1		13	1	1	1	1	19
	$\bar{x}$	-	38.2	318.6	-	22.7	126.8	17.2	6.9	181.8	51.8
	SE					8.8					18.8
9	n	1	1	1	1	3	1	1	1	1	11
	$\bar{x}$	20.8	32.7	3.2	10.2	19.4	3.0	1.8	6.9	7.2	13.1
	SE					9.0					3.6
Overall cycles	n	25	47	65	25	133	96	96	95	94	675
	$\bar{x}$	19.9	30.1	35.8	25.6	45.8	36.4	38.9	35.0	34.0	36.6
	SE	4.6	4.2	6.3	5.5	4.2	4.0	5.0	4.7	4.3	1.7

[a] ng/ml.

APPENDIX TABLE VI.--Levels of serum estrone at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)
-3	1	567	1	62	1	1440	3	691 ± 404
-2	1	1615	1	306	2	10 ± 11	4	486 ± 383
-1	3	1496 ± 1408	5	1341 ± 1246	5	417 ± 386	13	1021 ± 560
-0.5	0	-	3	1616 ± 1601	3	739 ± 421	6	1178 ± 766
Estrus	6	2401 ± 1337	5	567 ± 510	7	454 ± 201	18	1134 ± 495
2	5	577 ± 210	5	146 ± 94	5	1159 ± 1094	15	627 ± 362
4	5	353 ± 169	5	585 ± 510	5	435 ± 373	15	458 ± 204
7	5	829 ± 508	5	147 ± 112	5	2066 ± 1983	15	1014 ± 667
11	5	217 ± 138	5	575 ± 225	5	1412 ± 1306	15	735 ± 432
Overall days	31	998 ± 314	35	629 ± 236	38	903 ± 337	104	839 ± 173

[a]Mean ± SE for 5 heifers.

APPENDIX TABLE VII.--Levels of serum estradiol at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)
-3	1	14.2	1	12.5	1	30.2	3	19.0 ± 5.6
-2	1	9.7	1	14.9	2	38.6 ± 27.0	4	25.4 ± 13.4
-1	3	46.9 ± 22.2	5	21.6 ± 6.2	5	14.0 ± 4.0	13	24.5 ± 6.3
-0.5	0	-	3	36.7 ± 15.2	3	23.3 ± 15.3	6	30.0 ± 10.1
Estrus	6	39.1 ± 11.6	5	23.9 ± 6.8	7	76.8 ± 65.0	18	49.6 ± 25.0
2	5	29.8 ± 9.0	5	51.0 ± 27.4	5	65.4 ± 48.2	15	48.7 ± 17.7
4	5	31.0 ± 7.9	5	50.4 ± 30.9	5	44.6 ± 14.0	15	42.0 ± 11.0
7	5	17.8 ± 4.7	5	10.8 ± 2.9	5	39.6 ± 15.2	15	22.7 ± 6.0
11	5	23.3 ± 9.3	5	11.3 ± 5.7	5	7.7 ± 4.1	15	14.1 ± 4.0
Overall days	31	29.3 ± 4.0	35	28.1 ± 6.3	38	41.4 ± 13.6	104	33.3 ± 5.5

[a]Mean ± SE for 5 heifers.

APPENDIX TABLE VIII.--Levels of serum estriol at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)	(n)	(pg/ml)
-3	1	326	1	615	1	433	3	458 ± 84
-2	1	224	1	372	2	249 ± 9	4	274 ± 34
-1	3	416 ± 77	5	209 ± 83	5	246 ± 20	13	271 ± 41
-0.5	0	-	3	122 ± 41	3	91 ± 43	6	106 ± 28
Estrus	6	163 ± 52	5	194 ± 69	7	232 ± 25	18	198 ± 27
2	5	209 ± 45	5	257 ± 68	5	209 ± 34	15	225 ± 28
4	5	286 ± 94	5	244 ± 55	5	335 ± 64	15	288 ± 40
7	5	207 ± 66	5	240 ± 71	5	247 ± 45	15	231 ± 33
11	5	326 ± 111	5	171 ± 45	5	276 ± 73	15	257 ± 47
Overall days	31	255 ± 30	35	226 ± 26	38	247 ± 18	104	243 ± 14

[a]Mean ± SE for 5 heifers.

APPENDIX TABLE IX.--Levels of serum progesterone at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)
-3	1	5.16	1	6.74	1	8.74	3	6.88 ± 1.04
-2	1	0.24	1	2.86	2	3.46 ± 2.46	4	2.51 ± 1.27
-1	3	0.47 ± 0.20	5	0.87 ± 0.51	5	0.41 ± 0.09	13	0.60 ± 0.20
-0.5	0	-	3	0.64 ± 0.41	3	0.19 ± 0.06	6	0.41 ± 0.21
Estrus	6	0.18 ± 0.16	5	0.12 ± 0.05	7	0.26 ± 0.07	18	0.19 ± 0.06
2	5	0.19 ± 0.14	5	0.07 ± 0.04	5	0.11 ± 0.05	15	0.12 ± 0.05
4	5	0.40 ± 0.18	5	0.56 ± 0.14	5	0.67 ± 0.15	15	0.55 ± 0.09
7	5	3.06 ± 0.54	5	1.90 ± 0.31	5	3.13 ± 0.67	15	2.70 ± 0.32
11	5	4.04 ± 0.93	5	4.14 ± 0.80	5	2.85 ± 1.22	15	3.67 ± 0.56
Overall days	31	1.49 ± 0.35	35	1.42 ± 0.31	38	1.42 ± 0.35	104	1.44 ± 0.19

[a]Mean ± SE for 5 heifers.

APPENDIX TABLE X.--Levels of serum corticosterone at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)
-3	1	0.98	1	0.12	1	0.12	3	0.41 ± 0.29
-2	1	1.81	1	0.13	1	0.36	3	0.77 ± 0.52
-1	3	0.38 ± 0.04	5	0.09 ± 0.03	5	0.38 ± 0.07	13	0.27 ± 0.05
-0.5	0	-	3	0.25 ± 0.05	2	0.37 ± 0.11	5	0.30 ± 0.05
Estrus	5	0.30 ± 0.11	5	0.50 ± 0.15	7	0.36 ± 0.20	17	0.38 ± 0.10
2	4	0.41 ± 0.10	5	0.08 ± 0.01	5	0.12 ± 0.05	14	0.19 ± 0.05
4	5	0.36 ± 0.17	5	0.22 ± 0.07	4	0.14 ± 0.09	14	0.25 ± 0.07
7	5	0.81 ± 0.36	5	0.16 ± 0.05	5	0.22 ± 0.05	15	0.40 ± 0.14
11	5	0.22 ± 0.03	5	0.28 ± 0.10	5	0.12 ± 0.02	15	0.21 ± 0.04
Overall days	29	0.48 ± 0.09	35	0.22 ± 0.04	35	0.25 ± 0.04	99	0.30 ± 0.04

[a]Mean ± SE for 5 heifers.

APPENDIX TABLE XI.--Levels of serum cortisol at various stages of the first, fourth, and seventh estrous cycles.[a]

Days before or after estrus	Estrous Cycle						Overall cycles	
	1		4		7			
	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)
-3	1	14.00	1	1.20	1	6.00	3	7.07 ± 3.73
-2	1	17.40	1	3.00	2	6.90 ± 5.10	4	8.55 ± 3.72
-1	3	10.60 ± 2.75	5	3.48 ± 1.08	5	6.58 ± 2.23	13	6.32 ± 1.30
-0.5	0	-	3	6.73 ± 2.97	3	8.37 ± 0.73	6	7.55 ± 1.42
Estrus	6	8.23 ± 3.67	5	14.18 ± 3.54	7	10.91 ± 2.82	18	10.93 ± 1.88
2	5	13.26 ± 3.58	5	2.02 ± 0.66	5	5.34 ± 1.44	15	6.87 ± 1.75
4	5	8.16 ± 3.92	5	6.64 ± 3.43	5	5.18 ± 2.15	15	6.66 ± 1.77
7	5	11.58 ± 3.30	5	3.84 ± 2.09	5	6.20 ± 2.27	15	7.21 ± 1.64
11	5	5.80 ± 1.65	5	4.04 ± 2.00	5	1.68 ± 0.51	15	3.84 ± 0.93
Overall days	31	9.89 ± 1.29	35	5.58 ± 1.03	38	6.48 ± 0.85	104	7.19 ± 0.62

[a]Mean ± SE for 5 heifers.