GONADOTROPIN AND GONADAL HORMONES ASSOCIATED WITH FERTILIZATION AND EMBRYOGENESIS IN THE BOVINE

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This is to certify that the

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Robert Paul Wettemann

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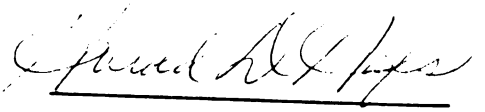

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ABSTRACT

GONADOTROPIN AND GONADAL HORMONES ASSOCIATED WITH FERTILIZATION AND EMBRYOGENESIS IN THE BOVINE

By

Robert Paul Wettemann

Endocrine changes associated with early pregnancy, estrous synchronization and nonfertile inseminations were studied in four experiments with Holstein heifers and cows. The major objectives were to quantify gonadotropin and gonadal hormones during the first 75 days of pregnancy and to determine whether reduced fertility after estrous synchronization is related to altered hormones.

Serum luteinizing hormone (LH) and prolactin were quantified by double antibody radioimmunoassays. Estradiol and progesterone were extracted from 10 ml of blood serum and isolated by column chromatography using a 1 x 32 cm Sephedex LH-20 column eluted with freshly distilled chloroform: 100% ethanol (96:4). Progesterone was quantified by competitive protein binding assay and estradiol was measured by radioimmunoassay.

Jugular blood was obtained from 11 nonpregnant heifers at seven intervals during the estrous cycle after

an infertile insemination and from 26 pregnant heifers at 18 intervals during the first 75 days of pregnancy.

Serum LH concentration in heifers was greatest on the day of estrus (avg. 9.7±1.4 ng/ml) when 51% of the heifers possessed LH greater than 4 ng/ml. Between-heifer variation in LH was not significant ($P>.10$) and LH concentration did not vary significantly ($P>.10$) from day 18 to day 75 of pregnancy. Pregnant heifers had lower serum LH compared to nonpregnant heifers (1.0±.1 vs 1.2±.1 ng/ml, respectively, $P<.10$) during days 2 through 11 after insemination.

Serum prolactin varied significantly ($P<.005$) between heifers. Average prolactin concentration of samples from individual heifers ranged from 7±3 to 56±14 ng/ml. Prolactin did not change significantly with day of the estrous cycle or pregnancy.

Serum estradiol was high for 1 or 2 days before estrus and decreased from 12.6±2.4 pg/ml at estrus to 8.4±.6 pg/ml by day 4 of pregnancy. Estradiol averaged 6 to 8 pg/ml during days 7 to 75 of pregnancy except on day 40, when four of the eight heifers had clearly elevated estradiol. Serum progesterone increased from 0.4±.1 ng/ml at estrus to 6.8±.4 ng/ml on day 11. Maximum progesterone (about 11 ng/ml) during the first 75 days of pregnancy was attained by day 18; then progesterone decreased significantly ($P<.05$) about 20% during the next 5 days and returned to 11 ng/ml by day 35.

Forty-eight cows were treated in a 2 x 2 factorial experiment with and without MGA and with and without HCG given at estrus. Serum LH was not influenced by MGA, HCG or pregnancy. Similar to the heifers, pregnant cows had lower LH than nonpregnant cows during days 2 through 25 after insemination, but difference in cows was not significant. Serum prolactin was not affected by MGA, HCG or pregnancy.

On the last day of MGA treatment in the cows, progesterone averaged 0.7±.3 ng/ml and only 3 cows had greater than 1 ng/ml. Progesterone increased from 0.3±.1 ng/ml at estrus to 6.9±.6 ng/ml by day 11. Pregnant and nonpregnant cows did not differ (P>.10) in progesterone concentration during days 2 through 11 after insemination.

Serum estradiol was greater in MGA treated cows than in control cows (P = .15). On the last day of MGA treatment, 47% of the cows had estradiol concentration comparable to the high values during proestrus in control cows. Within 3 days after MGA withdrawal, estradiol increased as much as 10-fold in some cows. Estradiol concentration was similar in pregnant and nonpregnant cows during days 2 through 11 after insemination. Serum estradiol averaged about 6 pg/ml during days 7 through 75 of pregnancy and, similar to the heifers, 50% of the cows had elevated estradiol between days 30 and 42 of pregnancy.

Serum concentrations of LH, prolactin and progesterone were not altered after estrous synchronization with MGA. But concentrations of estradiol comparable to those at proestrus were present at MGA withdrawal and possibly began during treatment. Prolonged elevations in serum estradiol during and following progestogen treatment may account for the infertility of inseminations at the synchronized estrus.

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By

Robert Paul Wettemann

A THESIS

Submitted to
Michigan State University
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DOCTOR OF PHILOSOPHY

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To My Parents

BIOGRAPHICAL SKETCH

of

Robert Paul Wettemann

I was born on November 12, 1944, in New Haven, Connecticut. I attended public schools in Guilford, Connecticut, graduating in June 1962. In September 1962, I enrolled at the University of Connecticut, majoring in Dairy Science, and received a Bachelor of Science degree in June, 1966.

I accepted a graduate assistantship at Michigan State University in September, 1966, and I was granted an National Institutes of Health Predoctoral Fellowship in March, 1968. In September 1968, I received the Master of Science degree and my thesis was entitled, "Endocrine Changes Influencing Sperm Capacitation". I completed the requirements for the PhD degree in February, 1972, and I shall be employed by Oklahoma State University as an environmental physiologist.

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The thoughtful guidance and willing assistance of my major professor, Dr. H. D. Hafs are invaluable to me. The facilities and assistance provided by Dr. C. A. Lassiter have made graduate school an enjoyable experience.

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I wish to thank the National Institutes of Health for my fellowship and the Endocrinology Study Section of the National Institutes of Health, E. R. Squibb and Sons and The Upjohn Company for gifts of hormones. I am obliged to Dr. S. A. Tillson for an antibody to estradiol.

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INTRODUCTION

Our knowledge of the hormonal changes associated with fertilization and embryogenesis is limited in the bovine. Advances in techniques used in endocrinology within the last 3 years made it possible to measure blood serum luteinizing hormone (LH) and prolactin by radioimmunoassay (RIA). Similarly, progesterone in peripheral serum can be quantified by gas-liquid chromatography, protein binding assay or by RIA, but there were no reports of determination of serum estradiol in cattle. Therefore, I set out to develop techniques to quantify estradiol in blood serum of cattle, and to relate serum estradiol with other steroid and gonadotropin hormones and with fertility in cattle.

More specifically, the major objectives of this thesis were to determine ovarian and gonadotropin hormones during the first 75 days of pregnancy in heifers and cows, and to determine whether reduced fertility after estrous synchronization is related to altered hormones.

In the future, general acceptance of artificial insemination of cattle probably will depend upon high fertility following estrous cycle synchronization because of increased labor costs, larger herds, and fewer farms.

Ovulation can be controlled with progestogens, but presently fertility following ovulation synchronization is usually reduced 10 to 30 percentage points. This reduced fertility makes estrous synchronization economically unfeasible in most commercial herds. Some possible causes of the reduced fertility after estrous synchronization are (1) altered sperm or ova transport (2) unfavorable uterine environment for sperm or ova (3) altered sperm capacitation (4) aged ova or (5) inhibition of implantation.

Possibly, large ovarian follicles known to persist during progestogen treatment may secrete abnormal amounts of estrogen. Or progesterone may be secreted by abnormal luteal or ovarian interstitial tissue. Alteration of sperm or ova transport could be associated with abnormal contractility of the uterus or oviducts caused by altered estrogen secretion or by residual progestogens. An unfavorable uterine environment could be associated with increased estrogen secretion prior to proestrus, higher than normal quantities of progesterone or progestogen present during proestrus. The stimulation of uterine glands by these steroids could alter uterine secretions and also alter sperm capacitation. In other words, most of the hypothetical causes of reduced fertility at the synchronized estrus probably are associated with or caused by altered hormone levels.

Information from this study will characterize hormones during early pregnancy in normal cattle, then altered hormone secretion after estrous synchronization can be determined. These data may suggest hormones which are altered in infertile cattle and possibly help to increase fertility at synchronized ovulation.

REVIEW OF LITERATURE

Pregnancy

Pregnancy begins at fertilization and terminates at parturition or abortion. During this reproductive stage the secretion of endocrine glands is altered compared with the estrous cycle.

Endocrine Changes During the Estrous Cycle

Pituitary luteinizing hormone (LH) decreases during estrus (Rakha and Robertson, 1965; Hackett and Hafs, 1969) and plasma LH increases (Anderson and McShan, 1966). The ovulatory surge of LH, which begins shortly before estrus and is maintained about 6 hours (Swanson and Hafs, 1971), may be initiated by increased serum estradiol since estradiol injection will cause release of LH in cattle (Howland et al., 1971). Recently we demonstrated that the proestrus increase in serum estradiol usually precedes LH release by 1 or 2 days (Wettemann et al., 1972). Hansel and Snook (1970) and Schams and Karg (1969) reported small midcycle peaks of serum LH occurring on days 8 to 10.

Presently, techniques are not available to quantitate FSH in bovine plasma. Pituitary FSH decreases during estrus (Rakha and Robertson, 1965; Desjardins and Hafs, 1968) and Hackett and Hafs (1969) found that this decrease started

between days 18 and 20 of the cycle. Therefore, FSH as well as LH probably participate in estrus and ovulation.

Pituitary prolactin declines from estrus to day 2 of the cycle (Sinha and Tucker, 1969) and Swanson et al. (1972) observed higher serum prolactin at estrus compared with metestrus, diestrus, and proestrus. Raud et al. (1971) also observed elevated serum prolactin during proestrus or estrus; however, Schams and Karg (1970) found no significant changes in serum prolactin during the estrous cycle in milking cows.

Mature nonpregnant cattle exhibit estrus once about every 21 days, or injection of estrogen will induce estrus (Foote and Walker, 1961; Carrick and Shelton, 1969). Estradiol in peripheral blood serum of heifers increases to about 10 pg/ml during the 2 or 3 days before estrus, and remains at about 30 to 40% of the proestrus level throughout the luteal phase of the estrous cycle (Wettemann et al., 1972). The major urinary estrogens during the estrous cycle are estrone, 17α-estradiol and 17β-estradiol (Garverick et al., 1971) and excretion is greatest from 3 days prior to estrus until 3 days after estrus. During the luteal phase of the cycle, estrone is excreted in greatest quantity, whereas 17α-estradiol and 17β-estradiol predominate near estrus.

Progesterone and 20β-hydroxy-pregn-4-en-3-one (20β-ol) in the corpus luteum were greatest from days 9 to 15 of the cycle, however the concentration of 20β-ol

was only 2 to 30% of the concentration of progesterone (Mares et al., 1962). Hafs and Armstrong (1968) confirmed that both progesterone and 20β-ol concentrations of the corpus luteum were greatest at mid-cycle and steroidogenesis also was greatest from days 11 through 20 of the cycle. Although Gomes et al. (1963) could not detect progesterone in ovarian venous plasma at estrus using macro techniques, they found that progesterone concentration increased from low levels on day 2 to a maximum on day 15 and then decreased before the next estrus. At estrus, Plotka et al. (1967), found 10.1 ng/ml of progesterone in peripheral plasma and a maximum concentration of 25.8 ng/ml on day 14. With more sensitive and precise techniques (Stabenfeldt et al., 1969; Swanson et al., 1972), progesterone concentration in peripheral plasma was less than 0.5 ng/ml at estrus and increased to about 7 ng/ml on days 15 to 18 of the estrous cycle.

Ovulation and Fertilization

Luteinizing hormone causes rupture of the graffian follicle and discharge of the ovum. In heifers, ovulation occurs about 10 hours after the end of estrus and cows ovulate slightly later (Trimberger, 1948). Swanson and Hafs (1971) observed that ovulation occurred about 32 hours after the ovulatory surge of LH in heifers. Fertility is maximal when inseminations are performed 7 to 24 hours

before ovulation; reduced fertility results from earlier or later inseminations (Trimberger, 1948).

Regulation of the Corpus Luteum

Factors controlling the life span of the corpus luteum have been studied extensively. An intriguing question is, what causes the corpus luteum of pregnancy to be maintained whereas the corpus luteum of the estrus cycle regresses within about 3 weeks?

The presence of a foreign body which distends the bovine uterus alters the cycle length. If the foreign body is inserted within 2 or 3 days after ovulation, the cycle is shortened but there is no change in cycle length when it is inserted on day 6 or 8 (Yamauchi and Nakahara, 1958). Ginther et al. (1966) observed that an intra-uterine plastic coil had a unilateral inhibitory influence on the corpus luteum. Injection (SQ) of pharmacological doses of oxytocin during the first week after estrus also shortens the cycle (Armstrong and Hansel, 1959) because corpora lutea fail to develop. Although oxytocin shortens the cycle in unicornual heifers with the retained horn adjacent to the corpus luteum, it is without effect when the retained horn is opposite to the corpus luteum (Ginther et al., 1967). This suggests that at least part of the oxytocin effect is through a local uterine-ovarian relationship. Hysterectomy also alters ovarian activity in cattle. Removal

of the uterus 5 to 12 days post estrus maintains the corpus luteum for more than 150 days (Wiltbank and Casida, 1956).

The pituitary gland has an active role in maintenance of the corpus luteum. When cows are injected with purified bovine LH, the inhibitory effect of concurrently injected oxytocin on luteal tissue is abolished (Hansel and Seifart, 1967), but prolactin, FSH or growth hormone are ineffective in overcoming the luteostatic influence of oxytocin. Injection of human chorionic gonadotropin (HCG) into heifers from day 15 through 26 of the cycle prolongs the cycle (Wiltbank et al., 1961).

Exogenous steroid hormones also alter the life expectancy of the corpus luteum. The corpus luteum regresses early if estradiol valerate is injected during days 3 to 9 of the cycle, but if injections are on days 15 or 16 the corpus luteum doesn't regress and large ovarian cysts develop (Wiltbank, 1966). Estrogen injection also will cause regression of corpora lutea in pregnant or hysterectomized heifers. Since injection of human chorionic gonadotropin (HCG) negates the luteolytic effect of estrogen, (Wiltbank, 1966) the estrogen may act by decreasing plasma LH concentration.

Injection of progesterone during the first 10 days of the cycle shortens the cycle (Woody et al., 1967). Ginther (1970) confirmed the shortening effect of progesterone given early in the cycle and found that simultaneous

administration of HCG maintained normal cycle length. Progesterone injections from day 3 to 8 do not alter the cycle but injections after day 8 lengthen the cycle. Thus progesterone administration may inhibit LH release which is necessary for ovulation and corpus luteum growth.

Implantation

Based on studies in the rat, LH and estrogen may have a causative effect on implantation in bovine. Macdonald et al. (1967) demonstrated that LH initiates blastocyst implantation in rats and this effect of LH is probably mediated through estrogen since Yoshinaga and Hosi (1961) found that estrogen caused implantation in lactating rats. Hormonal changes have not been related to embryo development in bovine.

An embryo is considered implanted when it is fixed in one position in the uterus. The bovine embryo remains in the uterine cavity and is only loosely attached before formation of the placenta (Hafez, 1969). During formation of the placenta (syndesmo-chorial type), the uterine epithelium is erroded and the chorionic ectoderm comes in direct contact with the vascular uterine connective tissue (Arey, 1954).

In cattle, the ovum reaches the uterus about 4 days after estrus and the blastocoele begins forming at 7 days (Winters et al., 1942). About the eleventh or

twelveth day, the embryo attaches loosely to the uterine wall and the chorion starts elongating. The placental attachment is gradual, with the first placental plates forming on the chorionic membrane at 30 days and in the body of the uterus at 35 days (Melton et al., 1951). By 35 days the placental attachment is sufficiently developed so that the embryo can receive some of its nourishment through the cotyledons.

Endocrine Activity during Pregnancy

Luteinizing Hormone.--Recently Edgerton and Hafs (1971) observed lower serum LH concentration during the first 18 days post-insemination in lactating cows that conceived than in cows which did not conceive. Henricks et al. (1970) observed that nonpregnant cows had higher LH on day 8 and pregnant cows had higher LH on day 15. Randel and Erb (1971) found that LH concentration in serum was low at day 7 of pregnancy and varied little until day 260. But with more frequent sampling, Schams (1969) observed elevated LH at 52 days of pregnancy in one cow and at 61 days in another.

Prolactin.--Reports of serum prolactin during pregnancy in the bovine are limited. Serum prolactin didn't differ between pregnant and non pregnant lactating cows during the first 18 days after insemination, but there was extreme variation between animals and days

(Edgerton and Hafs, 1971). Serum prolactin decreased with advancing pregnancy in sheep (Arai and Lee, 1967) and Davis et al. (1971) found that serum prolactin in ewes stabilized at the third to fourth month of pregnancy.

Estrogens.--In vitro evidence summarized by Mellin and Erb (1965) indicates that estrogens can be formed from acetate, cholesterol, progesterone or neutral steroids in the bovine adrenal, ovary and placenta. Estrogens are transformed to less active biological forms and these metabolites are excreted in feces and urine. The major excretory forms are estrone, 17α-estradiol and 17β-estradiol, especially conjugated as sulfates and glucuronides.

The study of estrogen activity during early pregnancy until recently has been limited to changes in urinary estrogens. But estrone in peripheral blood during late gestation has been quantified since concentrations at this time are about 1000 fold greater than during early pregnancy (Robinson et al., 1970). Since Randel et al. (1971a) observed higher urinary estrogen excretion during the first 7 days postbreeding in infertile cows as compared with pregnant cows, they speculated that altered estrogen metabolism may influence tubal transport of ova and the uterine environment. Pregnant cows excreted more 17α-estradiol than nonpregnant cows 42 days after breeding (Randel et al., 1971b) and the excretion of 17α-estradiol on day 42 of pregnancy also was significantly greater than

on days 35 and 65 (Randel and Erb, 1971). With advancing pregnancy from day 65 to day 230, excretion of 17α-estradiol and estrone increased 20-fold and 6-fold, respectively. Excretion of 17β-estradiol was less than that of 17α-estradiol and estrone, and it increased only slightly after mid pregnancy.

Ovariectomy on days 111 to 251 of pregnancy does not significantly decrease urinary estrogens (Erb et al., 1968c), so the ovary isn't the major source of estrogen during late pregnancy. Findlay and Cox (1970) found low concentrations of unconjugated estrogens in fetal sheep, but conjugated forms of 17α-estradiol and 17β-estradiol with lesser quantities of estrone were present. Total estrogen concentration in plasma increased in both male and female ovine fetuses from 70 to 125 days of gestation. By perfusion in vivo of the auto-transplanted ovary of the ewe, Rado et al. (1970) determined that 17β-estradiol was the major phenolic steroid synthesized from either testosterone or androstenedione. Though smaller amounts of estrone were also produced, 17α-estradiol, estriol or conjugated estrogens were not isolated.

Progesterone.--Progesterone concentration in corpora lutea and ovarian veins were used as indicators of ovarian function before techniques were available to quantitify peripheral plasma progesterone. Erb et al. (1968b) determined that the corpus luteum contained 94% of the total

progestogens in ovaries and found no relationship between progesterone concentration in jugular plasma, ovarian venous plasma and content of the ovary during pregnancy. Although progesterone concentration in ovarian venous plasma decreases during pregnancy, jugular plasma levels increase (Erb et al., 1968a). Zimbelman et al. (1961) observed a decreased percentage of functional cells in corpora lutea on day 23 of pregnancy compared with days 14 and 18, but Wickersham and Tanabe (1967) concluded that the functional activity of the corpus luteum is constant throughout pregnancy based on luteal weights, progesterone concentration and de novo progesterone synthesis.

Although the corpus luteum is necessary for normal pregnancy, abortions usually do not occur when ovariectomy takes place after 200 days of pregnancy although gestation is usually shortened by about 2 weeks (Estergreen et al., 1967). Early parturition could be caused by the decrease in plasma progesterone observed after ovariectomy performed late in pregnancy (Erb et al., 1968c). Tanabe (1970) reported that more progesterone is needed to maintain pregnancy after the corpus luteum is enucleated during early pregnancy than during mid pregnancy. Apparently extraovarian progesterone in the bovine increases as pregnancy progresses. The source of progesterone could be the placenta (Ainsworth and Ryan, 1967) or the adrenal (Stormshak and Erb, 1961).

Shemesh et al. (1968) first observed an influence of the conceptus on plasma progesterone at 19 days after breeding, and Henricks et al. (1970) reported higher progesterone in pregnant than nonpregnant cows from 10 to 14 days after breeding. In another study, Henricks et al. (1971a) observed higher plasma concentration of progesterone in pregnant heifers by 9 days after estrus than in nonpregnant heifers.

There is disagreement as to the progesterone secretion pattern during pregnancy. Early workers found that progesterone concentration in peripheral plasma was relatively constant from day 32 to day 250 of pregnancy (Short, 1958). Randel and Erb (1971) observed a rapid increase in plasma progesterone concentration from 2 ng/ml at estrus to 9 ng/ml by day 7 of pregnancy, followed by a linear increase until day 42. Then plasma progesterone decreased from 20 ng/ml on day 42 to 10 ng/ml on day 125 and then increased to 24 ng/ml by day 200. Stabenfeldt et al. (1970) found that plasma progesterone concentration was stable from 140 to 200 days of pregnancy but the level of progesterone appeared lower than during 10 to 20 days of pregnancy.

Estrous Synchronization with Progestogens

Soon after progesterone was isolated from corpus luteum tissue (described by Petrow, 1970), Makespeace et al.

(1937) determined that injection of progesterone would inhibit ovulation after mating in estrogen primed rabbits. Willett (1950) injected heifers with progesterone starting on day 14 or 15 of the estrous cycle and inhibited estrus during the 13-to 17-day treatment. Estrus occurred about 5 days after progesterone withdrawal, but only 50% of the heifers conceived. Although fertility decreased after progesterone injections, the infertility was temporary and limited to the estrus following treatment (Trimberger and Hansel, 1955). In a recent review (Petrow, 1970), the structure and biological activity of contraceptive progestogens are described.

Administration and Effectiveness

Progesterone is not used to synchronize estrous cycles because it is only slightly active orally and it is costly to produce relative to alternatives. Many orally active progestogens have been synthesized. Although the effective dose of different progestogens varies, decreased fertility is observed at the first estrus after withdrawal of all progestogens including progesterone. Progestogens have been administered by injection, orally and vaginal pessaries, but use of implants maybe more efficient in most management practices.

Since melengestrol acetate (MGA, The Upjohn Co.) has been studied extensively and is the progestogen used

in my experiments, I shall discuss its biological effects in cattle. To my knowledge, except for potency, biological action of MGA resembles that of most other progestogens. Whether injected or ingested, MGA is a potent synthetic progestogen (Zimbelman and Smith, 1966a). Heifers exhibit estrus 3 to 6 days after the last feeding of MGA, depending partly on the dose. The number of animals with detectable corpora lutea decrease during MGA treatment as the number of animals with follicles increase (Zimbelman and Smith, 1966b). Follicular size increases during treatment and based on proestrus behavior, vaginal mucus and fern patterns in cervical mucus, there appears to be increased estrogenic activity. During MGA treatment, estrus and ovulation can be induced in heifers by injection of estrogens (Smith and Zimbelman, 1968). Although injection of gonadotropins will also cause ovulation during MGA treatment, functional corpora lutea do not always develop.

Induced Ovulation

Graves and Dziuk (1968) used 6α-methyl-17α-acetoxy-progesterone (MAP) to synchronize estrus in cattle, then induced ovulation by HCG injection 60 hrs after the last feeding of MAP. Ovulation occurred about 40 hrs after HCG injection and resultant ova were fertilizable. Although induction of ovulation with HCG after estrus synchronization did not influence incidence of estrus or conception, Baker

and Coggins (1968) found that it eliminated the problem of estrus detection and reduced the time required for restraining and inseminating cows.

Fertility

Progestogens can effectively synchronize estrus and ovulation in cattle but fertility at the first estrus after progestogen feeding usually is reduced significantly. Results from fertility trials using various progestogens are presented in Appendix Table III. A summary of these data indicated reduced fertility at first estrus after synchronization in 16 experiments; 420 control cows averaged 55% conception at first service but 609 treated cows had only 42% conception. Zimbelman et al. (1970) summarized data for 5 years involving 24 studies in which 1853 cows were synchronized with MGA and there were 537 control cows. Conception rate from inseminations at the first synchronized estrus was about 70% of the rate of controls. This reduction of fertility after estrous synchronization greatly limits the usefulness of progestogens for this purpose.

Mode of Action

Rats and Guinea Pigs.--Labhsetwar (1968) observed that when rats were given chlormadinone, a potent synthetic progestogen, ovaries as well as ovarian interstitial tissue atrophied and ovarian compensatory hypertrophy was blocked in unilaterally spayed rats. Both pituitary LH and FSH

concentration were increased by progestogen injection, suggesting that release of these hormones is blocked but synthesis is not altered. Although plasma LH was depressed after progestogen treatment (Schally et al., 1968), administration of luteinizing hormone releasing factor (LRF) caused elevation of plasma LH. Since LRF can overcome the blocking effect of progestogens on LH release, progestogens probably affect blood LH at the level of the hypothalamus or higher brain centers.

Based upon intracranial implants of MAP in female guinea pigs, progestogens can act directly on the anterior pituitary to inhibit ovulation (Malven and Ruiz-Diaz, 1971). While MAP implants into the arcuate nucleus of the hypothalamus were partially effective in inhibiting ovulation, implants in other areas of the hypothalamus had no effect.

Primates.--Ovulation was blocked in cycling Rhesus monkeys by injection of progesterone (Spies and Niswender, 1971). The ovulatory surge of LH was inhibited by progesterone injections but basal LH levels were not altered. Similarly, the ovulatory surge of LH was suppressed during administration of progestogens in women (Mishell and Odell, 1971; Saunders et al., 1971), but basal urinary excretion of LH was not depressed (Larsson-Cohn et al., 1970). Increased urinary excretion of estrogen also was observed when women were treated with progestogens (Larsson-Cohn et al., 1970). Llerena et al. (1969) found a greater

concentration of LH but not FSH in peripheral plasma than in ovarian venous plasma of women during the menstrual cycle. This difference in LH concentration was not apparent when women were on progestogen treatment. Therefore, LH may be metabolized by the ovary during a normal menstrual cycle but not when ovulation is blocked by progestogens.

Sheep.--Injection of female sheep with progesterone for 10 days did not alter blood serum LH as determined by ovarian ascorbic acid depletion assay (McDonald and Clegg, 1967), but elevated serum LH occurred 4 to 6 days after withdrawal in both intact and ovariectomized ewes. Exogenous progestogen did not alter cyclic changes in luteal function of ewes when treatment started after formation of corpora lutea (Smith and Robinson, 1969). However when progestogen was given during formation of the corpus luteum, full luteal size was not attained and early luteal regression occurred.

Pelletier and Thimonier (1969) observed that the ovulatory surge of LH was smaller than normal after treatment of ewes with progestogens and estrogen secretion also was altered. Using fluorimetry, Smith and Robinson (1970) noted decreased estrogen in ovarian venous blood at the synchronized estrus after treatment with a low dose of progestogen but the level of estrogen in plasma of ewes treated with a higher dose was similar to controls. Recently

Smith and Allison (1971) reported that the synchronized estrus was shorter than a normal estrus, and daily maximum cervical mucus secretion occurred before rather than after the onset of estrus. Although the daily maximum secretion of mucus was less than normal after treatment, the total volume produced during proestrus and estrus was similar. This alteration of cervical mucus secretion may be related to changes in gonadal hormone secretion.

Cattle.--Administration of MGA to cattle did not influence follicular activity, in the presence of a corpus luteum, but follicular fluid weights increased up to 3-fold in the absence of a corpus luteum (Zimbelman, 1966). Although pituitary FSH was not altered significantly, pituitary LH was increased by MGA treatment. Hill et al. (1971) also observed more large follicles in heifers treated with MGA and basal plasma LH tended to be higher in treated than control heifers. Plasma progesterone concentrations during MGA treatment were similar to comparable days during the estrous cycle. Limited data (Guthrie et al., 1970) suggest that follicles may luteinize and become atretic during MGA treatment. Lamond et al. (1971a) observed higher progesterone after MGA withdrawal than that usually found in the follicular phase of the estrous cycle.

When heifers were fed MGA for several months, adrenal weight, plasma cortisol, adrenal cortisol concentration and adrenal fasiculata layer widths decreased

compared with controls (Purchas et al., 1971). Average daily gain in carcass weight was greater but plasma growth hormone concentration was significantly lower in MGA treated animals. Heifers also had increased mammary DNA and RNA compared with control heifers after several months of MGA treatment (Pritchard et al., 1972).

MATERIALS AND METHODS

Experimental Animals and Designs

Holstein heifers or cows were housed either in Michigan State University's stanchion barn or in loose housing. In the stanchion barn, cows were observed at least once daily, in the morning, for signs of estrus. Heifers and cows in loose housing were observed for signs of estrus twice daily; between 7:30 and 8:30 am and between 5 and 6 pm.

For estrous synchronization experiments, melengestrol acetate (MGA, The Upjohn Co., Kalamazoo, Michigan) premix was mixed with a concentrate mixture so the daily dose could be administered in 2 pounds of grain mix.

Experiment I: Changes in Gonadotropin and Gonadal Hormones Associated with Early Pregnancy and after Nonfertile Inseminations.

The purpose of this experiment was to quantify endocrine activity during early pregnancy. Twenty-eight heifers, 14 to 15 months of age and during approximately their seventh estrous cycle, were managed in loose housing for this study. Semen from Baron, a bull with high fertility, was used for all artificial inseminations during the breeding period (October 15, 1969 to November 26, 1969). When estrus (standing heat) occurred before noon, heifers

were inseminated in the late afternoon of the same day. Those heifers first observed in estrus after noon were inseminated the next morning. If heifers did not conceive to the first insemination, the above breeding regime was repeated at the next estrus. Heifers not conceiving to the second insemination were removed from the study.

Jugular blood was obtained by venipuncture from all heifers when first observed in standing heat (day 0). Then each heifer was bled in the afternoon on days 2,4,7, 11,18,20,22,25,30,35,40,42,45,50,60,63 and 75 of pregnancy or until she returned to estrus. Blood was transferred from the syringe to polypropylene centrifuge tubes containing 31.7 mg oxalic acid and centrifuged at 650 xg for 15 minutes to remove the blood cells. Plasma was dacented into another centrifuge tube containing 27.8 mg calcium chloride and stored at 5 C to allow clotting. Within 1 to 2 days the clotted plasma was centrifuged and the serum was transferred to 7-dram plastic vials and stored at -20 C until assayed for hormone concentration.

Pregnancy was confirmed by rectal palpations 40 to 60 days postinsemination. Withers height and body weight also were recorded at the beginning of the breeding period.

Unless stated otherwise, these experimental details (insemination time, bleeding regime, preparation of serum and pregnancy diagnosis) were applicable in all subsequent experiments.

Experiment II: Serum Luteinizing Hormone (LH) and Prolactin after MGA Withdrawal

This experiment was conducted to determine when the ovulatory surge of LH occurs after oral administration of MGA and if prolactin is elevated at the synchronized estrus. Beginning February 1, 1970, nine heifers were fed 1.0 mg of MGA daily for 18 days in individual stalls. After the last feeding, the heifers were moved to loose housing, cohabited with 35 heifers of similar age, and observed twice daily for estrous. Jugular blood was obtained each afternoon for 8 days, starting on the last day of MGA administration. Heifers were inseminated at the first estrus after treatment and at the subsequent estrus. Palpations per rectum at 13 days after MGA withdrawal determined if ovulation had occurred.

Experiment III: Induced Ovulation and Ova Recovery

This experiment was designed to estimate when estrus synchronized cows ovulate after injection of Human Chorionic Gonadotropin (HCG) and to determine the fertility of the ova. Twenty-five first calf heifers at 60 to 90 days postpartum were group fed 1.0 mg MGA each, daily for 14 days. They were injected with 2500 IU of HCG (Squibb Chorionic Gonadotropin, E. R. Squibb and Sons, Inc., New York) on the morning of day 17 (3 days after the last MGA feeding) and inseminated on the morning of days 18 and 19. The time of ovulation was determined by twice daily (8 am and 5 pm)

palpations starting on the afternoon of day 17. If a follicle was present at a palpation and absent at the next palpation, ovulation was estimated to occur midway between the two observations. On the morning of day 21, the cows were transported 11 kilometers to a local abattoir and killed; reproductive tracts were recovered and returned to our laboratory. The corpus luteum was measured and ova were flushed from the oviducts and uteri. A blunt 16 ga needle was inserted into the ovarian end of the oviduct or the cervical end of the uterine horn. Ova were collected from the tubouterine end of the oviduct or from the uterus in a watch glass as warm medium TC 199 (Difco, Detroit, Michigan) was flushed through. Ova were observed for cleavage with a dissecting microscope (X35) and by phase contrast or dark field illumination (X250).

Experiment IV: Gonadotropin and Gonadal Hormones after Estrous Synchronization and after Fertile and Nonfertile Inseminations.

The purpose of this experiment was to quantify serum hormones after MGA treatment and to relate hormones with fertility. A second objective was to relate endocrine changes during early pregnancy with fetal development.

Between 30 and 60 days post-partum, the uterus and structures on the ovary were palpated to determine if the uterus was involuted and if the cow was having estrous cycles. Sixty cows were randomly assigned to five groups and a genetic herd classification as to best and worst

sires was blocked across treatments. Cows in groups I, II, and III received 1.0 mg of MGA daily for 18 days starting on the first day of the treatment month. Cows in group I were injected with 2500 IU of HCG at 8 am on the third day after the last MGA feeding, and inseminated approximately 12 and 24 hours after HCG injection. Cows in group II were injected with HCG when first observed in estrus after MGA withdrawal and inseminated about 12 hours later. Group III cows were treated similarly to those in group II, but HCG was omitted.

Cows in groups IV and V were treated similarly to cows in groups II and III, respectively, but estrous cycles were not synchronized with MGA. So that average insemination time post-partum would be the same in synchronized and control cows, cows in groups IV and V were not inseminated until the eleventh of each month (41 to 71 days post-partum).

Jugular blood was obtained on the last day of MGA administration and 2 days later, as well as at the same intervals as heifers in Experiment I. Since cows in group I were not inseminated with regard to estrus, the day-0 blood sample was taken immediately prior to HCG injection. Anestrus controls and cows which failed to exhibit estrus within 7 days after MGA withdrawal were removed from the experiment.

Steroid Hormone Assays

At the initiation of this study, estradiol had never been quantified in bovine peripheral blood. The possibility of using thin layer chromatography and column chromatography for purification of estradiol was investigated. I validated a protein binding assay for estradiol, similar to that described by Korenman (1968) and Korenman et al. (1969) for human plasma, and an estradiol radioimmunoassay using an antibody described by Tillson et al. (1970) to quantify bovine serum estradiol. Progesterone was measured using a protein binding assay described by Murphy (1967) and modified by Swanson et al. (1972).

Isolation of Steroid Hormones

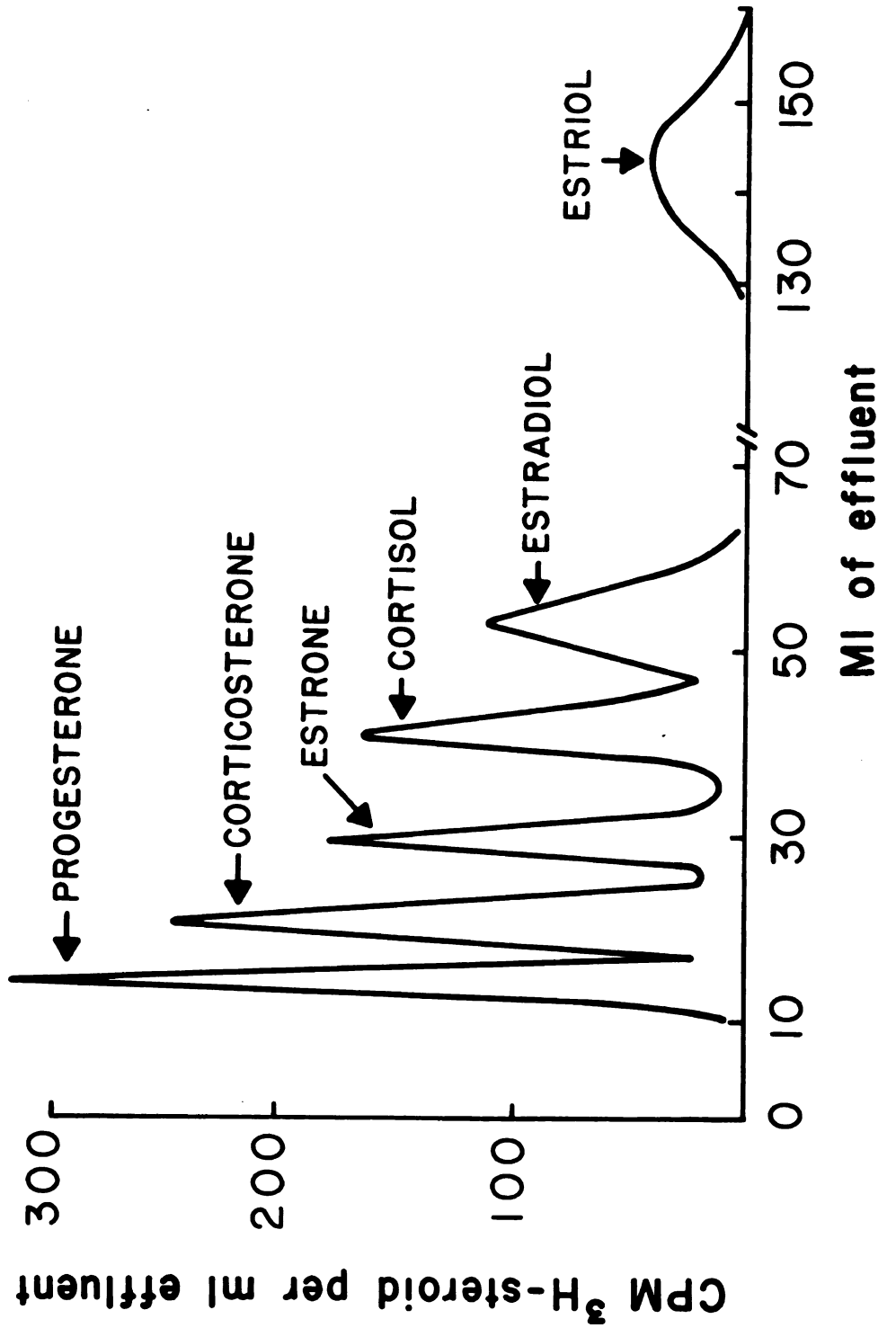
Thin Layer Chromatography.--Satisfactory isolation of standard estrone, estradiol and estriol was achieved by two dimentional chromatography using silica gel thin layer plates or Eastman chromagram sheet 6060 (Distillation Products Industries, Rochester, N.Y.) with cyclohexane: ethyl acetate in the first dimension (1:1) and cyclohexane: ethyl acetate:ethanol (45:45:10) in the second dimension. High solvent blank values were obtained in the protein binding assay for estradiol when both types of chromatograms were eluted with nanograde ethyl acetate, ethanol or methanol. After trying numerous modifications of the techniques, I discontinued using thin layer chromatography

for purification of micro quantities of estradiol because I encountered high solvent blanks by protein binding assay. Evidently, substances from the thin layer chromatogram interfere with the estradiol assay. This observation was confirmed by others at the Second Karolinska Symposium (Diczfalusy, 1970).

Column Chromatography.--Column Chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) in a 1 X 32 cm column gave satisfactory separation of progesterone, estrone, estradiol and estriol and low column solvent blank values using freshly distilled benzene:methanol (85:15). The elution pattern was similar to that described by Mikhail et al. (1970). But corticosterone eluted with estrone and cortisol eluted with estradiol. Since there was slight cross-reaction with cortisol in the protein binding assay for estradiol this solvent system was unsatisfactory.

After testing several ratios of chloroform:ethanol, we determined that 24:1 gave adequate separation of the six steroids of major interest (Figure 1). They were eluted in the following order: progesterone, corticosterone, estrone, cortisol, estradiol and estriol. Although 20α-01 (20α-hydroxypregn-4-ene-3-one) and 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3, 20-dione) are eluted slightly later than progesterone, they were included in the 2.5 ml

Figure 1.--Elution profile of steroids in an ether extract of bovine serum from a Sephadex LH-20 column using chloroform: ethanol (24:1) as the solvent.


CPM ³H-steroid per ml effluent
300
200
100
0
10
30
50
70
130
150
Ml of effluent
PROGESTERONE
CORTICOSTERONE
ESTRONE
CORTISOL
ESTRADIOL
ESTRIOL

progesterone fractions routinely collected. MGA eluted with progesterone in this separational system.

Glass columns (1.0 X 40 cm) were packed with 32 cm of LH-20 which had equilibrated in the solvent system for at least 3 hours. Cotton plugs were placed in the tops and bottoms of the columns to prevent floating of the LH-20. The bottoms of the columns were fitted with teflon stopcocks and the tops had 14/35 ground glass joints so 250 ml separatory funnels with teflon stopcocks could be attached as solvent reservoirs.

Using a fraction collector with an 11.5 inch drum (ISCO model 567, Instrumentation Specialities Co., Lincoln, Nebraska) and 12 X 75 mm disposable test tubes, fractions were collected from six columns simultaneously. Although only one column was mounted over a volumeter (ISCO model 400), 2.5 ml fractions were collected from all six columns because flow rate among the columns differed no more than about 5%.

Between samples, each column was flushed with about 250 ml of solvent. Reagent grade chloroform and 100% ethanol were distilled within 2 days of use. During the course of my research, flow rates of the columns varied from 0.4 to 0.8 ml per min, with freshly poured columns eluting faster. Columns were repacked after 8 to 10 samples, re-using the LH-20, and repacked with new LH-20 after about 20 samples.

Extraction Procedure.--Because of the sensitivity of these assays, glassware was washed with detergent and rinsed in tap water, distilled water, glass distilled water and distilled methanol before use. Ten ml of serum was added to a 30-ml culture tube with a screw cap and teflon liner. About 3,000 dpm estradiol -2,4,6,7-^{3}H (New England Nuclear, 95 c/mM) and 2,000 dpm of progesterone -1,2-^{3}H (New England Nuclear, 50 c/mM, and purified by column chromatography) were added to the serum and mixed gently. The serum was mixed vigorously with about 18 ml of freshly opened anhydrous diethyl ether for 2 min and stored at -20C for 3 hours before the ether extract was decanted. The extraction procedure was repeated and the combined ether extract was dried under nitrogen. Steroids from the ether extracts were dissolved in 0.5 ml of chloroform:ethanol (24:1) and layered on columns. After fractions (2.5 ml) were collected from the column, radioactivity was determined (Scintillation Fluid, Appendix I) in 0.5 ml of each fraction to locate steroids and to calculate procedural losses of steroids. Approximately 85% of the progesterone and 90% of the estradiol tracers were recovered from the column.

Estradiol

Protein Binding Assay.--The estradiol protein binding procedure was developed by Korenman (1968) and Korenman et al. (1969). Uteri were obtained from estrous

rabbits and homogenized for 1 min in three volumes of buffer A (Appendix II) at 4C in a Waring blender. The homogenate was centrifuged at 3,000 xg for 20 min and the supernatant fluid was recentrifuged at 95,000 xg for 90 minutes. The supernatant cytosol was frozen at -20C in aliquots useful for single assays.

Standards were diluted in redistilled 200 proof ethanol so that 100 µl contained 0, 10, 20, 40, 60, 80, 100, 120, 160, or 320 pg of estradiol. Two standard curves were used in each 48 tube assay and the first fraction collected from the LH-20 column was assayed as a column blank to check purity of solvent and cleanliness of reagents and glassware. Standard 17β-estradiol (Sigma Chemical Co.), column blanks and 4 ml (the two fractions containing the maximum ^{3}H-estradiol) of the eluate containing estradiol from the LH-20 column for each unknown were dried under nitrogen in disposable glass test tubes (12 X 75 mm). During drying, the walls of the tubes were rinsed twice were chloroform:ethanol (24:1). After addition of 0.8 ml of buffer A and 0.2 ml of ethylene glycol to each tube, 60,000 dpm of ^{3}H-estradiol (95 c/mM) in 0.01 ml of buffer A was added and the tubes were mixed vigorously for 5 seconds. Then 0.025 to 0.040 ml (depending upon the titer of estrogen binding protein) of cytosol from rabbit uteri was added, the tubes were mixed gently for 5 sec and incubated at 4C for about 15 hours.

To separate free and bound estradiol, 1 ml of .25% Dextran 150 (Pharmacia, Uppsula Sweden) and 2.5% carbon decolorizing neutral Norit (Fisher Scientific Co.) was added in buffer A to each tube. The tubes were shaken gently for 5 sec and incubated for 10 minutes at 4C, then centrifuged at 2,500 xg for 5 minutes. A 0.5 ml sample of the supernatant fluid was mixed with 10 ml of Bray's solution (Bray, 1960; Appendix I) and radioactivity was quantified in a liquid scintillation spectrometer (Nuclear Chicago Corp. Mark I).

The mass of estradiol in unknowns was calculated by interpolation between standards and corrected for procedural losses. Figure 2 illustrates a typical standard curve for 17β-estradiol and cross-reactions with estrone and estriol. Cortisol and corticosterone also competed with estradiol in the protein binding assay (Table 1), but little cross reaction occurred with progesterone and MGA. When 500 or 1,000 pg of 17β-estradiol was added to 10 ml serum samples, 88±13 (n=9) and 60±6 (n=12)% were recovered by the protein binding assay.

Radioimmunoassay (RIA).--For RIA of estradiol, we used an antibody (Antibody SLC-6X generously supplied by Dr. S. A. Tillson and associates, Worcester Foundation for Experimental Biology, Shrewsbury, Mass.) which was obtained by immunization of sheep with the conjugate 1,3,5(10)

Figure 2.--Standard curve for 17β-estradiol and cross-reactions with estrone and estriol using a rabbit uterine cytosol binding protein (n=2).

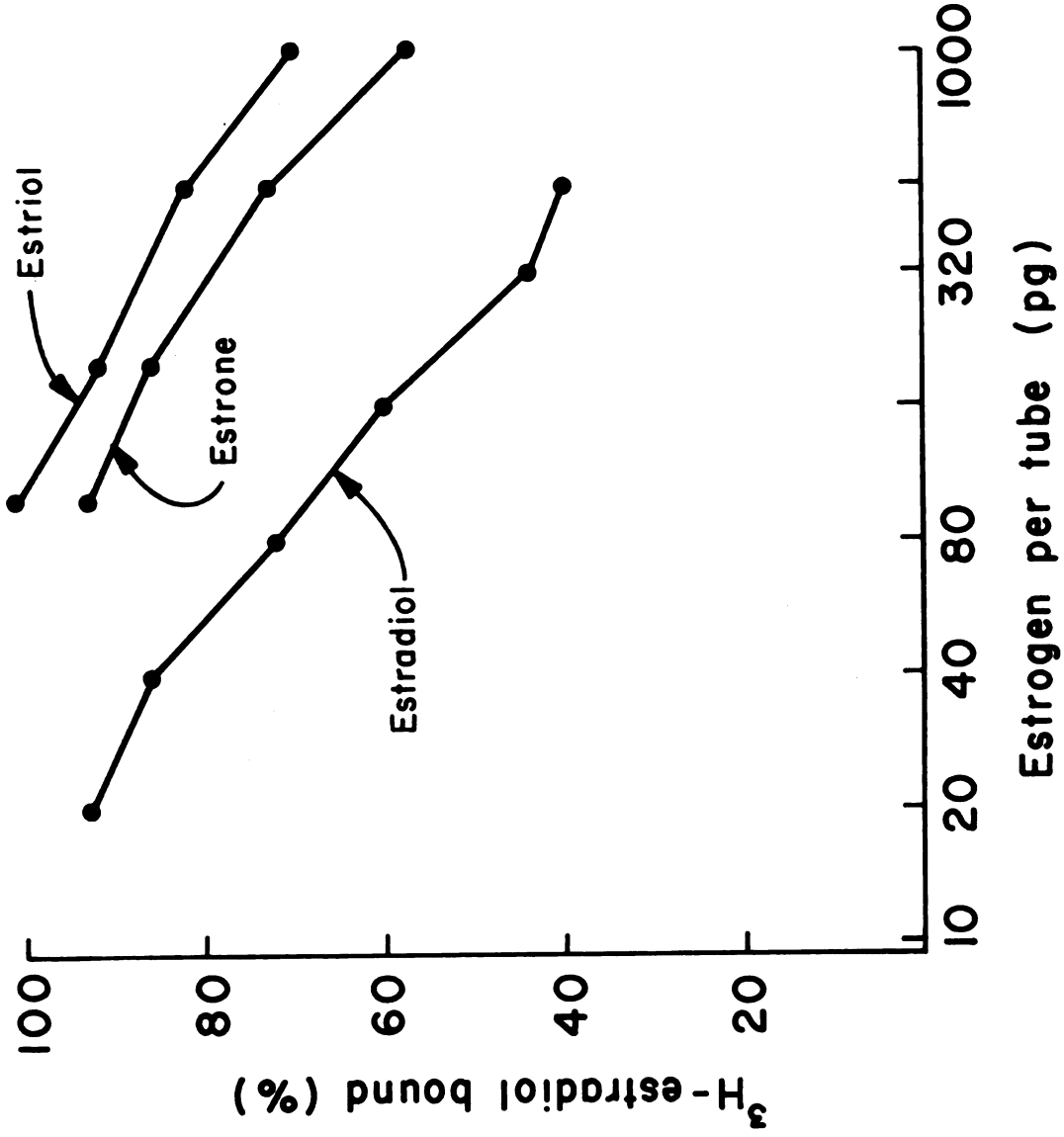

Estriol
Estrone
Estradiol
100
80
60
40
20
^{3}H-estradiol bound (%)
10
20
40
80
320
1000
Estrogen per tube (pg)

TABLE 1.--Relative activity of selected steroids in the estrogen protein binding assay.

Steroid	Relative Activity[a]
Estradiol	1.00
Corticosterone	0.004
Cortisol	0.006
Melengestrol Acetate	<0.0001
Progesterone	0.0004

[a]Relative Activity = $\frac{\text{pg of estradiol bound}}{\text{pg of steroid X bound}}$

estratriene-3,17β-diol,17β-succinyl-bovine serum albumin. Some of the properties of this antibody and its reactions with various steroids have been reported (Tillson et al., 1970).

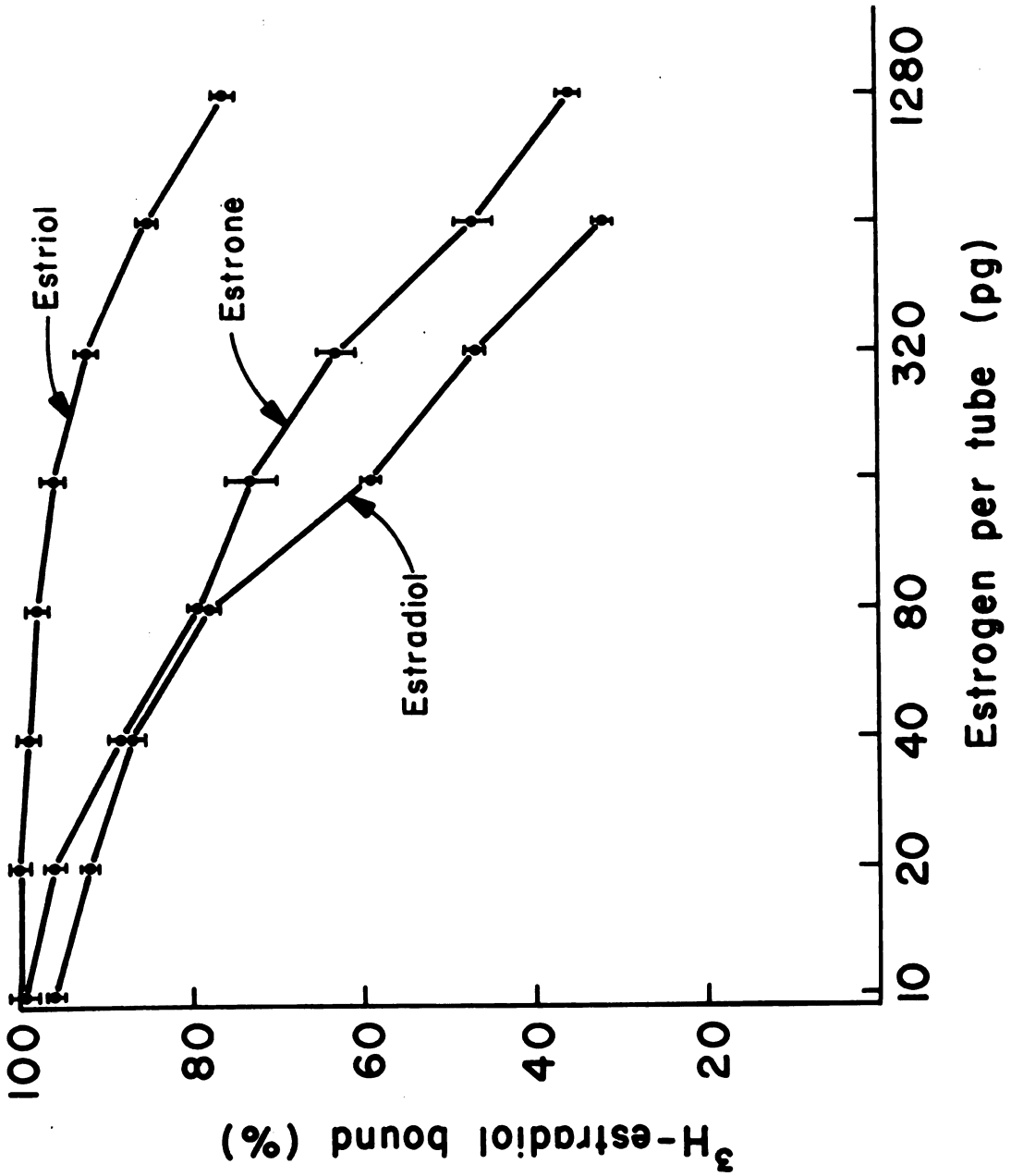
Standard and unknown estradiol were dried in assay tubes as described for protein binding assay. Then 0.1 ml antibody diluted 1:15,000 in buffer AA (Appendix II) was added to each tube. After gentle vortex mixing for 5 seconds, the tubes were incubated for 30 min at room temperature. About 60,000 dpm of ^{3}H-estradiol (95 c/mM) was added to each tube in 0.1 ml buffer AA, the tubes were shaken for 5 sec and incubated at 4 C for 3 to 4 hours. Then, 0.1 ml of buffer AA containing 0.5% gelatin was added to each tube. Free and bound estradiol were

separated, radioactivity was quantified and mass of estradiol was calculated as described for the protein binding assay.

Because of the small quantity of estradiol in 10 ml of serum, only one estimate on each unknown could be performed. For each unknown, the first fraction collected from the LH-20 column was assayed as a column blank to check purity of solvents and cleanliness of reagents and glassware. Column solvent blanks never exceeded 10 pg estradiol, and they were not subtracted from estradiol determined in the 10-ml serum unknowns.

When 40, 80, or 160 pg 17β-estradiol were added to 10-ml serum samples, 72±18 (n=4), 98±9 (n=8) and 110±8 (n=10)%, respectively, were recovered. A serum sample from a steer was assayed with each group of extractions. Twenty-three determinations on the pool of steer serum averaged 5.1 ± 0.4 pg/ml. Ten pg of estradiol was easily distinguished from no hormone with 95% confidence (n=4). Therefore, allowing for procedural losses and for an aliquot to determine recovery, the sensitivity of the assay was about 2 pg/ml serum. Within assay coefficient of variation was 2.3% and the between assay coefficient of variation was 3.8% as determined from ten standards in duplicate in each of eight assays. Figure 3 illustrates a typical standard curve for 17β-estradiol and cross-reactions with estrone and estriol determined by RIA.

Figure 3.--Standard curve for 17β-estradiol and cross-reactions with estrone and estriol using a radioimmunoassay (antibody against 1,3,5(10)-estratriene-3, 17β-diol, 17β-succinyl-bovine serum albumin). Plotted points are means ± standard errors for n=4.


Estriol
Estrone
Estradiol
100
80
60
40
20
^{3}H-estradiol bound (%)
10
20
40
80
320
1280
Estrogen per tube (pg)

Since the antiserum was not specific for estradiol, but crossreacted with estrone and estradiol, isolation of the steroids in blood sera was required.

To determine which column fractions contained radioimmunoactive estrogen, a serum extract was fractionated on an LH-20 column and the first 24 fractions (about 60 ml) were assayed by estradiol RIA. Competition for immunological activity in the RIA occurred in four peaks in the elute from the column. The first was fraction 2, soon after the void volume; there was a small amount of activity in the two fractions (numbers 7 and 8) after progesterone and the other two peaks were associated with estrone and estradiol. I assayed only four fractions eluted from the column after estradiol, so there may be other immunoreactive materials which are eluted after estradiol in serum extracts.

Ether extracts of steer serum were assayed for estradiol by RIA and compared with steer serum extracts purified by column chromatography. Although the values for the ether extracts were about 3.5 times the values obtained after purification of the sample on LH-20, the overestimation (17.7 ± 2.51 pg/ml, n=3) was consistent.

Comparison of Methods.--The RIA resulted in more precise recovery of estradiol added to sera (described in assay method); it also had lower values than protein binding for solvent blanks from LH-20 columns (1.2 ± 0.1, n=70 vs 2.6 ± 0.6, n=27, pg/ml, respectively).

Estradiol was quantified by RIA and by protein binding assay in two different sets of blood sera collected during different estrous cycles from eleven heifers (Table 2). Based upon RIA, estradiol was lowest (3.0 pg/ml) on day 2 and relatively constant during the luteal phase of the estrous cycle, averaging about 3.6 pg/ml from days 2 through 11. Then estradiol increased ($P<.05$) to 4.8 pg/ml at 3 days before estrus, continued to increase ($P<.05$) to 9.7 pg/ml 0.5 days before estrus and remained high on the day of estrus (8.4 pg/ml). Changes in estradiol determined by protein binding assay resembled those determined by RIA but quantities were greater at all times during the estrous cycle. But I do not know if higher estradiol in samples quantified by protein binding assay reflects assay or sample differences. The within day correlation of average estradiol values during the estrous cycle as determined by RIA with averages obtained by protein binding determination was 0.85 ($P<.01$).

Similar to my data, Echternkamp and Hansel (1971) found 8.1 pg/ml of estradiol at estrus when estradiol was purified on Sephadex LH-20 and quantified by RIA. Swanson (1970) in our laboratory, quantified estrone, estradiol and estriol during the estrous cycle but lack of precision for his assays contributed to large variance and very high values.

TABLE 2.--Levels of estradiol in peripheral blood serum from holstein heifers.

Day of Cycle[b]	Estradiol Assay Method[a]			
	Radioimmunoassay		Protein Binding	
	(n)	(pg/ml)	(n)	pg/ml)
-3	4	4.8±1.2[c]	3	5.9±2.9
-2	8	7.7±1.7	4	9.0±2.2
-1	11	8.7±1.1	7	11.6±2.0
-.5	5	9.7±2.2	3	10.5±2.2
0	9	8.4±1.6	7	12.3±3.8
2	10	3.0±0.9	6	5.7±1.5
4	10	3.9±0.4	4	8.5±2.7
7	10	3.8±0.6	4	7.4±1.9
11	10	3.6±0.4	4	7.5±1.9

[a]Different sets of sera were used for the two methods.

[b]Day 0 was day of estrus.

[c]Mean ± S.E.

Measuring total estrogens by RIA, Henricks et al. (1971b) found 2 to 3 times more estrogen in heifers than the quantity of estradiol I found at the time of estrus and Christensen et al. (1971) found 176 pg/ml of estrogen 24 hr before the ovulatory LH surge. Whether the difference represents immunoreactive estrogens other than estradiol, remains to be tested.

Progesterone Assay

Progesterone was isolated by column chromatography as described above (Figure 1) and quantified by the competitive protein binding assay (Murphy, 1967) recently described by Swanson et al. (1972). This assay uses the corticosteroid binding globulin (CBG) which has binding affinity for many steroid hormones.

Dog plasma (Colorado Serum Co. Labs, Denver, Colo.) was used as a source of CBG. To remove endogenous steroids from the CBG, 8 gm of 30 to 60 mesh Florosil was added to 100 ml of 2.5% dog plasma diluted in distilled water. The plasma and Florosil were mixed at room temperature for 30 min, then centrifuged at 6500 xg for 30 min to remove the Florosil. The Florosil-treated dog plasma was diluted to 0.62 - 1.25% depending upon the CBG titer. After drying the solvent from ^{3}H-corticosterone (50 c/mM) it was diluted with CBG until a concentration of 8,000 dpm per ml was achieved.

Standards were diluted in redistilled ethanol so 100 µl would contain 0, 0.1, 0.5, 1.0, 1.5, 2.0, 5.0 or 10.0 ng of progesterone. Standards or unknowns were added to 12 x 75 mm disposable glass test tubes which had been coated with 5% trimethylchlorosilane in toluene. Each assay (72 to 250 tubes) included four to six standard curves and a sample of steer serum for comparison between assays. Dilution duplicates of unknowns or standards were dried under nitrogen. Then 1.4 ml of the diluted CBG with ^{3}H-corticosterone was added to each tube with a pipettor (Oxford Laboratories). After vortexing for 5 sec, the tubes were covered and incubated for 15 to 24 hr at 5 C.

Free corticosterone was removed from bound by vortexing with 80 mg of 30 to 60 mesh Florosil for 45 seconds. Before using, Florosil was washed 3 times with distilled water and twice with methanol to remove the fine particles. I found reduced variations and greater slope for standard curves if the assay tubes were placed in ice water for 30 min before separation of bound and free steroids. One half ml of the supernatant was removed and added to Bray's solution; radioactivity was quantified in a liquid scintillation spectrometer. The mass of progesterone was calculated by interpolation between standards and corrected for procedural losses of ^{3}H-progesterone.

Twenty-seven determinations on a pool of steer serum assayed with each group of extractions averaged

0.44 ± 0.05 ng/ml. Validation of this procedure has been reported by Swanson et al. (1972).

Protein Hormone Assays

Luteinizing Hormone (LH)

The procedure used to quantify LH is similar to that reported by Niswender et al. (1969). The antibody against bovine LH was prepared in our laboratory and validation of the assay has been reported (Oxender et al., 1972). Purified bovine LH used for iodination (LER-1072-2) was supplied by Dr. Leo Reichert (Emory University, Atlanta, Georgia).

Standards were diluted in 1% egg white albumin so 0.5 ml would contain 0, .08, .16, .32, .64, 1.28, 2.56, 5.12, 10.24, or 20.48 ng. Two groups of standards prepared from NIH LH-B5 (supplied by the National Institute of Health, Endocrinology Study Section, Bethesda, Maryland.) were used; one group for Experiments I and II and the other group for Experiment IV. Four sets of standards were included with each set of 300 to 500 assay tubes. Each serum sample was assayed in dilution duplicate and a standard cow serum was included in each assay. Examples of precision of the LH assay and comparison of the two sets of standards are listed in Table 3.

TABLE 3.--Precision of the determination of LH in analysis of different serum pools in different assays.

Serum Pool	Standard	Results of Individual Assays	Mean ± S.E.
1	1[a]	0.93, 1.06, 1.03, 0.68	0.92 ± 0.09
1	2[b]	0.55, 0.98, 0.62, 1.02, 1.11	0.86 ± 0.11
2	1	2.84	2.84
2	2	2.66, 3.09, 2.74, 2.90, 2.88	2.85 ± 0.07

[a]The standard in Experiment I and II was NIH LH-B5.

[b]The standard in Experiment IV was NIH LH-B5.

Prolactin

The procedure used to quantify prolactin was developed by Tucker (1971) and validation of the assay has been reported (Koprowski and Tucker, 1971). Standards were diluted in 1% bovine serum albumin so 0.5 ml would contain 0, 0.1, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, or 4.0 ng of prolactin. Four sets of standards were included in each set of 300 to 500 assay tubes. One group of standards prepared from NIH Prolactin-B1 (Supplied by the National Institute of Health, Endocrinology Section, Bethesda, Maryland) was used for analysis of Experiments I and II. And another group of standards from NIH Prolactin-B2 was used for analysis of samples in Experiment IV.

Because of the high concentration of prolactin in some samples relative to the sensitivity of the assay, sera were diluted 1:2 to 1:20 in 1% bovine serum albumin. Each serum sample was assayed in dilution duplicate and a standard cow serum sample was included in each assay. Examples of precision of the prolactin assay are listed in Table 4.

Statistical Analysis

When repeated measurements are made on animals in each treatment group, a split plot analysis is desirable (Gill and Hafs, 1971). The analysis used for Experiment I

TABLE 4.--Precision of the determination of prolactin in analysis of different serum pools in different assays.

Serum Pool	Standard	Results of Individual Assays	Mean ± S.E.
1	1[a]	472.8, 470.1, 400.0, 647.0	497.5 ± 52.6
2	1	139.8	139.8
2	2[b]	131.5, 94.5, 226.5, 105.5, 90.5	129.7 ± 25.2
3	2	108.0, 60.6, 172.2, 93.6, 89.4	104.8 ± 18.5

[a]The standard in Experiments I and II was NIH Prolactin B1.

[b]The standard in Experiment IV was NIH Prolactin B2.

to determine the influence of pregnancy on serum LH and prolactin is shown in Table 5. Predetermined orthogonal contrasts were used to partition the 5 degrees of freedom for periods. Another set of orthogonal contrasts (Table 6) was used to partition the 17 degrees of freedom for the days of pregnancy. Within-day correlations between LH and estradiol, LH and progesterone, LH and prolactin, estradiol and progesterone, estradiol and prolactin, and prolactin and progesterone after insemination were determined in pregnant and nonpregnant heifers.

A split plot analysis similar to that in Table 5 was used for Experiment IV except there were four between block treatments (genetic classification, MGA, HCG, and pregnancy) and one within block treatment (stage of pregnancy). Orthogonal contrasts listed in Table 6 were used to partition the 17 degrees of freedom for stage of pregnancy.

TABLE 5.--Split plot analysis of hormones during early pregnancy.

Source of Variation	df
Between Heifers	(36)
Treatments	1
Cows Within	35
Within Heifers	(176)
Periods	5
T X P	5
Heifers X P (error)	166

TABLE 6.--Orthogonal contrasts used to partition the 17 degrees of freedom for stage of pregnancy.

Source of Variation	df
Days of pregnancy	17
Days 0,2,4,7,11, vs 18,20,22,25,30,35,40,42,45,50, 60,63,75	1
Days 0,2,4 vs 7,11	1
Day 7 vs 11	1
Day 0 vs 2,4	1
Day 2 vs 4	1
Days 18,20,22,25,30,35,40,42 vs 45,50,60,63,75	1
Days 18,20,22 vs 25,30,35,40,42	1
Among days 18,20,22	2
Among days 25,30,35,40,42	4
Days 45,50 vs 60,63,75	1
Days 45 vs 50	1
Among days 60,63,75	2

RESULTS AND DISCUSSION

Experiment I

Changes in Gonadotropin and Gonadal Hormones Associated with Early Pregnancy and after Nonfertile Inseminations

Size and Behavior at Breeding

The body weight (367 ± 5 kg) of the heifers in this experiment compared closely to Morrison's (1957) standards but their height at the withers (119 ± 1 cm) was slightly less. Standing heat (estrus) was first observed in the morning in 76% of the heifers (n=37). Similarly, Swanson et al. (1972) first observed 62% of their heifers in standing heat in the morning and Trimberger (1948) reported 70% first showing estrus in the morning.

Fertility

Since heifers first observed in estrus in the morning were inseminated in the afternoon by design, 76% of the heifers were inseminated in the afternoon and the remainder in the forenoon. Sixty-eight per cent of the 28 heifers conceived at the first insemination and fertilities of forenoon and afternoon inseminations were similar (71 and 67%, respectively). The interval from onset of standing

heat to time of insemination was similar in heifers that became pregnant and those that did not; 10 ± 2 hours. An average of 1.33 inseminations was performed per conception.

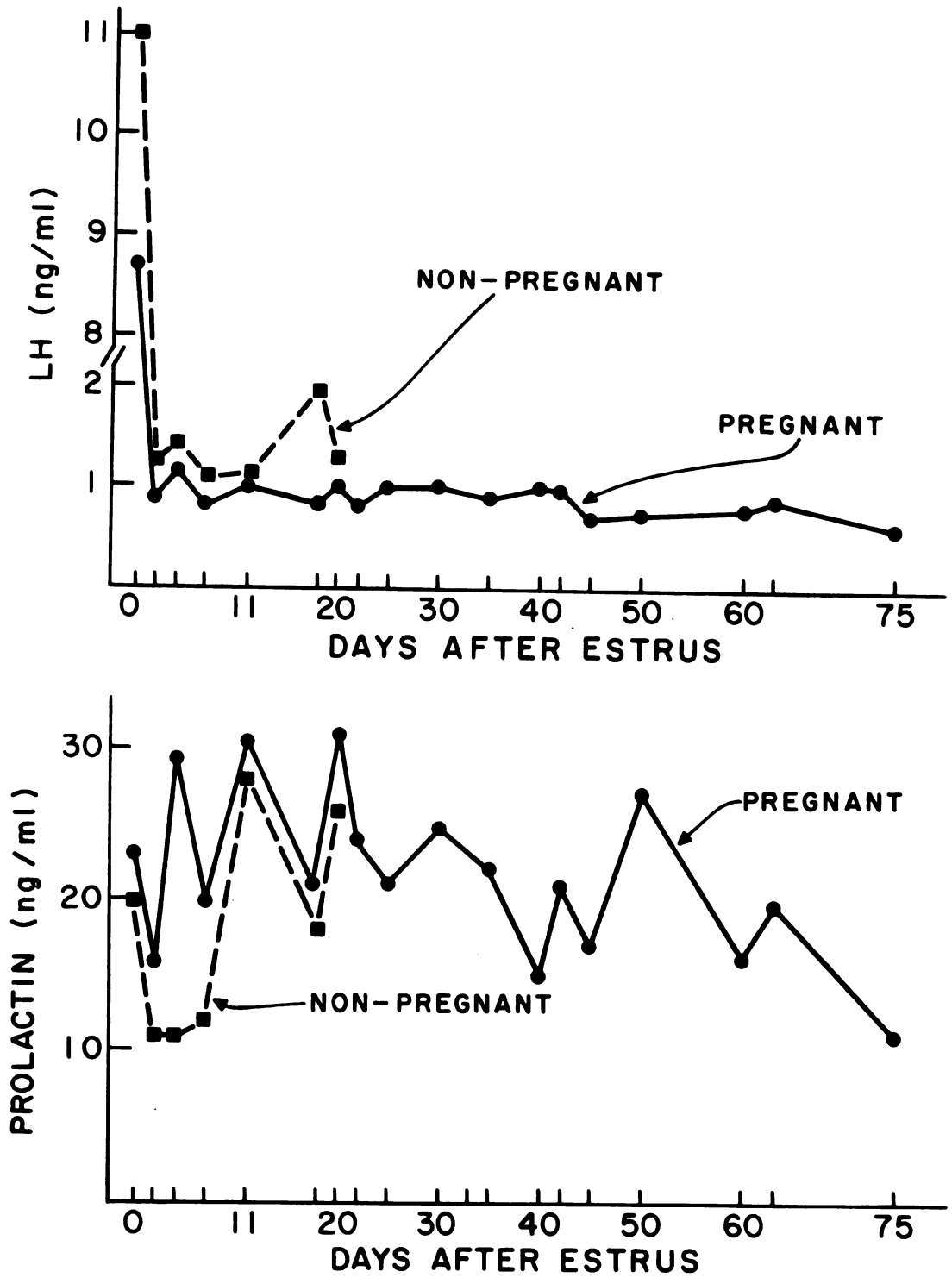
Endocrine Changes

LH.--Serum LH concentration was greatest on the day of estrus, averaging 8.7 ± 1.6 ng/ml in heifers that conceived and 12.0 ± 3.2 ng/ml in heifers that failed to conceive (Figure 4, Appendix Table IV). Since the ovulatory surge of LH in cattle occurs between 3 hr before and 6 hr after the onset of standing heat and persists 6 to 10 hours (Schams and Karg, 1969; Henricks et al., 1970; Swanson and Hafs, 1971), LH concentration on the day of estrus is dependent on the intervals between blood sampling. Nineteen of 37 blood samples taken on the day of estrus contained more than 4.0 ng/ml LH, indicating that part of the ovulatory surge of LH was detected in 51% of the heifers sampled when first observed in estrus. Swanson et al. (1972) detected 46% of the ovulatory discharges of LH when heifers were bled when first observed in estrus.

Although serum LH (Figure 4 and Appendix Table IV) appeared to decrease with advancing pregnancy, LH concentration did not change significantly ($P>.10$) from day 18 to 75. Similarly, Randel and Erb (1971) did not observe any significant changes in plasma LH from day 7 to day 260 of pregnancy. There was little among-heifer variation of

Figure 4.--Serum LH in heifers after insemination.

Figure 5.--Serum prolactin in heifers after insemination.


LH (ng/ml)
11
10
9
8
2
1
NON-PREGNANT
PREGNANT
0
11
20
30
40
50
60
75
DAYS AFTER ESTRUS
PROLACTIN (ng/ml)
30
20
10
PREGNANT
NON-PREGNANT
0
11
20
30
40
50
60
75
DAYS AFTER ESTRUS

LH (P>.10) during early pregnancy and after the day of estrus only three samples contained more than 4 ng/ml; one each on days 11, 30 and 40 and all in different heifers. Schams (1969) found elevated LH in one sample from each of two cows bled three times daily during the first 2 months of pregnancy.

Serum LH concentration was greater in nonpregnant heifers (P<.10), averaging 1.2 ± .1 ng/ml during days 2 through 11 after insemination compared with 1.0 ± .1 ng/ml in pregnant heifers (Figure 4 and Appendix Table IV). Edgerton and Hafs (1971) also observed higher LH in non-pregnant cows compared with pregnant cows during days 2 through 11 after insemination. In a study with five pairs of cows, Henricks et al. (1970) found higher LH in non-pregnant cows 8 days after estrus but higher LH in pregnant cows at 16 days after estrus. Greater concentration of serum LH may be caused by less steroid hormone feedback at the pituitary so more LH is released. On day 18, non-pregnant heifers had elevated LH (2.0 ± .4 ng/ml), possibly associated with proestrus increases described by Swanson et al. (1972) and Garverick et al. (1971).

Prolactin.--Serum prolactin concentration was more variable than LH and differed significantly (P<.005) among heifers. Average prolactin concentration of all samples from individual heifers ranged from 7±3 to 56±14 ng/ml. But milk production during days 30 to 60 of the subsequent

lactation was not related significantly (r=.07) with average serum prolactin during the first 75 days of pregnancy.

Although prolactin tended to decrease with advancing pregnancy (Figure 5 and Appendix Table IV), stages of pregnancy did not differ significantly (P>.10). Stress associated with jugular puncture occasionally may release prolactin from the pituitary (Tucker, 1971; Raud et al., 1971), so sampling blood by a non-stressful method or after uniform stress may be necessary to determine precisely prolactin changes associated with early pregnancy. During the first 3 days of pregnancy in the rat, serum prolactin is higher than during the following 18 days (Amenomori et al., 1970). A gradual decline in prolactin levels during pregnancy also has been observed in sheep (Arai and Lee, 1967; Davis et al., 1971).

In contrast to serum LH, prolactin was higher (P<.10) in pregnant than in nonpregnant heifers during the first 18 days of pregnancy (Figure 5 and Appendix Table IV). From 2 to 11 days after estrus, prolactin averaged 24±3 ng/ml in pregnant heifers and 16±3 ng/ml in nonpregnant heifers.

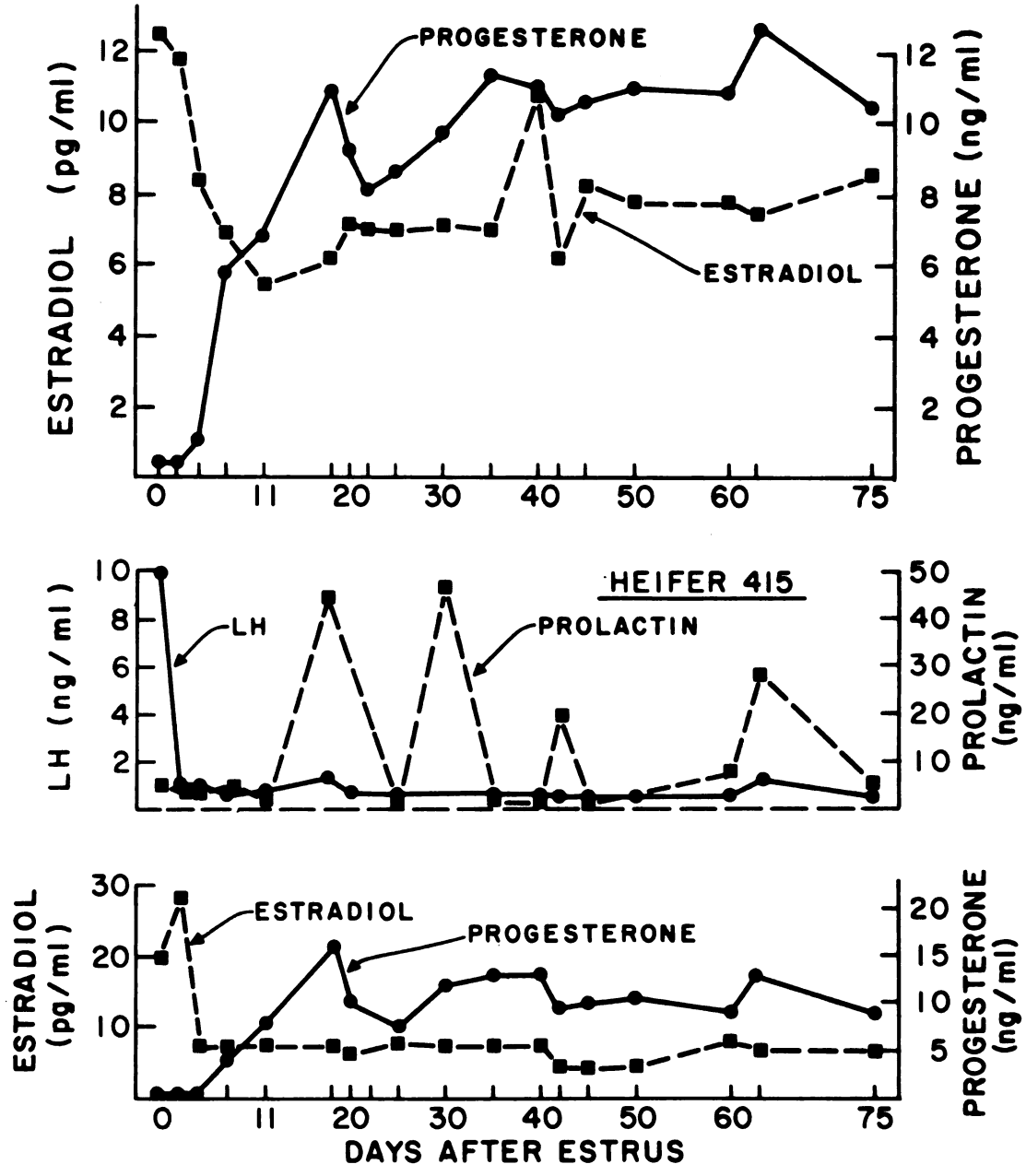
Estradiol.--Sera from 8 of the 26 pregnant heifers were selected to isolate and quantify estradiol and progesterone. Concentration of estradiol in heifers did not differ significantly during early pregnancy (P>.10). Estradiol decreased from 12.6±2.4 pg/ml at estrus to 8.4±.6

pg/ml by day 4 of pregnancy (Figure 6 and Appendix Table V). Although estradiol concentration on day 2 was similar to that at estrus in these pregnant heifers, estradiol was markedly reduced by day 2 of the estrous cycle (Wettemann et al., 1972). During days 18 to 75, estradiol averaged 6 to 8 pg/ml except for day 40 when estradiol averaged 11.1±2.3 pg/ml ($P<.01$) because four of the eight heifers had elevated estradiol on day 40. More frequent sampling of blood near day 40 would be necessary to determine if elevated estradiol occurs in all pregnant heifers near this time. Randel et al. (1971b) observed elevated urinary estrogens at day 42 of pregnancy in cows. Increases in serum estradiol at this stage of pregnancy could be related to growth of the placenta.

Progesterone.--Serum progesterone increased dramatically from 0.4 ng/ml on the day of estrus to 6.8 ng/ml on day 11 (Figure 6 and Appendix Table V). This rapid increase is similar to changes observed during the estrous cycle (Stabenfeldt et al., 1969; Swanson et al., 1972) and during early pregnancy (Henricks et al., 1971a). Progesterone concentration differed significantly among days 18, 20 and 22 of pregnancy (10.8±1.3, 9.2±.9 and 8.1±.9, respectively, $P<.05$). During the estrous cycle, serum progesterone is usually greatest after day 15 but before day 18, (Stabenfeldt et al., 1969; Swanson et al., 1972). Therefore the decrease in serum progesterone from day 18 to day 22 of pregnancy may

Figure 6.--Serum estradiol and progesterone during pregnancy in heifers.

FIgure 7.--Serum LH, prolactin, estradiol and progesterone during pregnancy in a typical heifer.


PROGESTERONE
ESTRADIOL
ESTRADIOL (pg/ml)
PROGESTERONE (ng/ml)
HEIFER 415
LH
PROLACTIN
LH (ng/ml)
PROLACTIN (ng/ml)
ESTRADIOL
PROGESTERONE
ESTRADIOL (pg/ml)
PROGESTERONE (ng/ml)
DAYS AFTER ESTRUS

represent an analagous luteal regression which is terminated by the products of conception in the uterus about day 22 to 25 of pregnancy. On day 18, serum LH and progesterone were highly correlated ($r = .87$, $P<.01$).

Serum progesterone increased slightly from 8.1±.9 ng/ml on day 22 to 11.3±1.6 ng/ml by day 35 and remained at about this level until day 75 of pregnancy except for an elevation on day 63 (12.8±1.1 ng/ml, $P<.10$). Henricks et al. (1971a) reported similar progesterone concentrations on days 30 to 39 of pregnancy and Randel and Erb (1971) observed slightly higher values on days 35 and 42 (17 and 20 ng/ml), but about 13 ng/ml on day 65. During days 45 to 75 of pregnancy, progesterone was significantly higher ($P<.005$) than from day 18 to 42. Wickersham and Tanabe (1967) reported that functional activity of the corpus luteum was constant during pregnancy. Therefore increased progesterone during 45 to 75 days of pregnancy suggests an extra-ovarian source of progesterone. This hypothesis is supported by the finding that less progesterone is required to maintain pregnancy when the CL is enucleated after 60 days than before this period (Tanabe, 1970).

Relationships of Hormones.--During the first 75 days of pregnancy, serum LH was very stable as illustrated for heifer 415 (Figure 7). In this heifer, LH decreased from 10 ng/ml on the day of estrus to less than 1 ng/ml by day 2 and remained at this concentration until day 75. Prolactin

was much more variable, transient elevations occurred on days 18, 30, 42 and 63. Average serum prolactin in this heifer was 13 ng/ml during the first 75 days of pregnancy and it did not appear to vary in a meaningful manner with other hormones measured (Figure 7). Estradiol in heifer 415 increased from 20 pg/ml at estrus to 27 pg/ml at day 2, then decreased to an average of 6 pg/ml for the next 71 days, and elevated estradiol was not detected near day 40. Progesterone increased from 0.5 ng/ml during the first 4 days of pregnancy to a maximum of 16 ng/ml at day 18. Then progesterone varied from 7.2 to 13.4 ng/ml between days 20 and 75. Changes in serum progesterone for this representative heifer are very similar to the averages for the eight heifers (Figure 6).

Within day correlations between estradiol and progesterone were positive at 5 of the 6 days sampled during the first 18 days of pregnancy (Appendix Table VI). But estradiol was negatively correlated with progesterone within day 20 through 75 of pregnancy. Although none of the within day correlations between estradiol and progesterone was significantly different from zero ($P>.05$), this trend suggests a shift in the regulation of steroid hormones at about day 18 to 20 of pregnancy.

Serum LH and prolactin during the first 75 days of pregnancy were not influenced by the sex of the fetus (Appendix Table VII). Also, sex of fetus (5 female and

3 male fetuses) did not influence estradiol and progesterone in the eight animals studied.

Experiment II

Gonadotropin Levels after MGA Withdrawal

Behavior and Fertility

Only three of the nine heifers were observed in standing heat within 6 days after MGA withdrawal (Table 7). This low incidence of estrus could be related to moving the heifers from the stanchion barn to the loose housing barn on the day of MGA withdrawal. Of the three heifers inseminated at the synchronized estrus, only one conceived. Seven of the eight nonpregnant heifers returned to estrus an average of 24.7 ± .8 days after MGA withdrawal. Thus the length of estrous cycle after synchronization appeared to be normal in length. Fertility was normal at the second estrus; of seven heifers inseminated, six conceived.

Endocrine Changes

LH.--The ovulatory surge of LH was detected in six of the nine heifers; it occurred 2 to 7 days after the last MGA feeding (Table 7). The per cent LH peaks detected was similar to 51% of the LH peaks observed by bleeding heifers on the day of estrus (Experiment I). Because of the variation in interval from the last MGA feeding to the ovulatory surge of LH, average LH values by days after MGA withdrawal is not meaningful (Table 8). The ovulatory surge

TABLE 7.--Some estrual criteria of nine Holstein heifers after MGA treatment.

Heifer	MGA[a] to 1st Standing Heat	MGA[a] to LH Peak	Fertility at 1st Estrus[b]	MGA[a] to 2nd Standing Head	Fertility at 2nd Estrus[b]
(No)	(days)	(days)		(days)	
1	ND[c]	ND	NI[d]	24	-
2	ND	7	NI	25	+
3	ND	5	NI	23	+
4	ND	7	NI	25	+
5	ND	ND	NI	ND	NI
6	ND	ND	NI	23	+
7	6	2	+	—— Pregnant ——	
8	5	5	-	29	+
9	5	5	-	24	+
Mean ± SE	5.3±.3	5.2±.7		24.7±.8	

[a]Last day of MGA treatment.

[b](+)=pregnant, (-)=open.

[c]Not detected.

[d]Not inseminated.

TABLE 8.--Serum LH and prolactin in Holstein heifers after MGA treatment.[a]

Days After Last MGA Feeding	LH (ng/ml)	Prolactin (ng/ml)
0[b]	0.30 ± 0.11[c]	52.3 ± 12.8[c]
1	1.02 ± 0.07	40.8 ± 13.2
2	2.09 ± 1.03	30.2 ± 21.1
3	1.06 ± 0.11	11.9 ± 1.8
4	1.22 ± 0.17	48.4 ± 20.7
5	2.57 ± 1.10	25.4 ± 6.2
6	1.19 ± 0.32	32.0 ± 12.3
7	2.75 ± 1.66	6.0 ± 2.2

[a]Nine observations (heifers) at each day.

[b]Last day of treatment.

[c]Mean ± S.E.

of LH was analyzed further by adjusting LH values to the day of peak serum LH (Figure 8). Maximum LH was 8.2 ± 2.3 ng/ml, which is similar to 8.7 ± 1.6 ng/ml observed in fertile heifers (Experiment I). Basal values were about 1 ng/ml. Thus the ovulatory surge of LH as determined by once daily bleeding after MGA treatment appeared normal (Swanson et al., 1972) in magnitude and duration, lasting less than a day.

Prolactin.--All six heifers that had detectable LH peaks after MGA withdrawal also had elevated prolactin on the day before or on the day of the LH peak. Elevated prolactin near the time of estrus has been reported (Swanson et al., 1972; Raud et al., 1971). Serum prolactin increased significantly ($P<.05$) from 29 ± 13 ng/ml 2 days before the LH peak to 83 ± 30 ng/ml the day before the LH peak, then decreased to 18 ± 3 ng/ml by the day after the LH peak (Figure 8). This increase in prolactin at the synchronized estrus appears less variable and of greater magnitude than serum prolactin during estrus (Swanson and Hafs, 1971).

Experiment III

Induced Ovulation and Ova Recovery

Ovulation occurred in 22 of 25 cows when HCG was injected 3 days after the last MGA feeding, but ova were recovered from only 7 cows (Table 9). I feel that ova were recovered from only 31% of the cows because ova

Figure 8.--Serum LH and prolactin in heifers after MGA withdrawal.

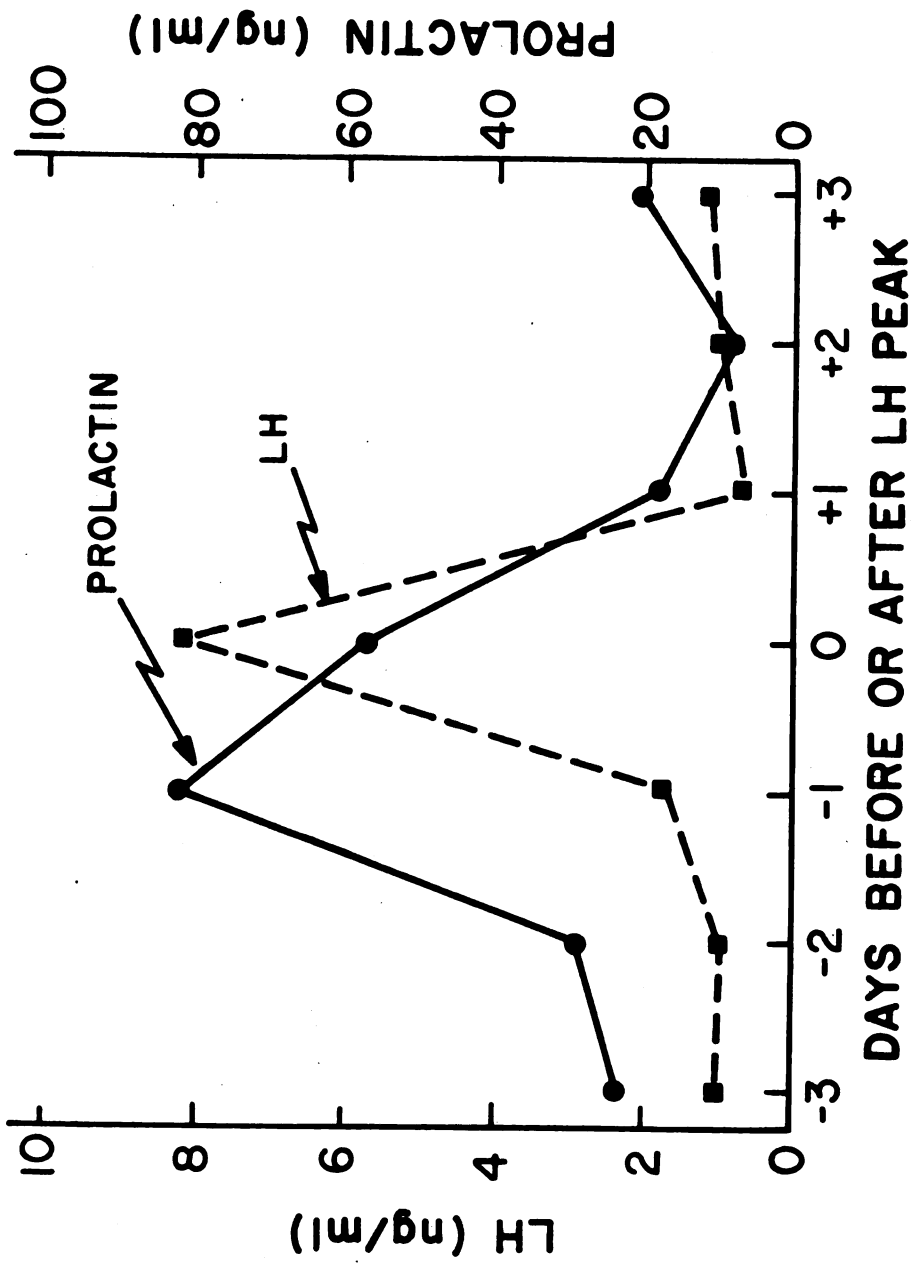

PROLACTIN (ng/ml)
100
80
60
40
20
0
LH (ng/ml)
10
8
6
4
2
0
-3
-2
-1
0
+1
+2
+3
DAYS BEFORE OR AFTER LH PEAK
PROLACTIN
LH

TABLE 9.--Ovulation rate and ovarian characteristics of Holstein cows at induced ovulation.

	Cows		
	Ova Recovered	Ova Not Recovered	Total
Number of cows	7	15	22
Number of ovulations	8	16	24
Ovulation time[a] (hr post HCG)	38±6	35±3	36±3
(hr pre-slaughter)	58±6	61±3	60±3
Size of follicle that ovulated(cm)	1.6±0.1	2.0±0.1	1.9±0.1
Size of CL at slaughter(cm)	1.0±0	1.1±0.1	1.1±0.1

[a]Estimated by twice daily palpation.

recovery was performed too late--at 4 days after HCG injection. Wagner et al. (1968) found 79% of the ova in the oviducts of CAP treated cows 3 to 3.5 days after the onset of estrus. In my hands, ova recovery from the uterus is considerably less efficient than that from the oviduct, and some ova were in the uterus when the cows were slaughtered. However, I cannot exclude rapid ova transport as a possible cause of low ova recovery after MGA treatment.

Ova recovery, but not ovulation rate, may be related to the stage of the estrous cycle at the start of MGA treatment. The data in Table 10 suggest that ova were not recovered when the cows were proestrus or estrus (days 19 through 0) or mid diestrus (days 6 through 14) at the outset of MGA treatment. But ova were recovered from cows when MGA was started during metestrus and late diestrus (60 and 50%, respectively).

Ovulation was synchronized quite well, occurring 36±3 hr after HCG injection or about 60 hr before slaughter (Table 9). Graves and Dzuik (1968) estimate that ovulation occurred in synchronized cows about 40 hr after HCG given 1.5 to 2.5 days after the last MAP feeding. During the estrous cycle, ovulation occurred 20 to 32 hr after the LH peak (Schams and Karg, 1969; Swanson and Hafs, 1971).

Although the ovulatory follicles were slightly larger in cows from which ova were not recovered (2.0±.1 cm,

TABLE 10.--Relation between ova recovery and stage of the estrous cycle cows were started on MGA treatment.

Day of Cycle MGA Started	Cows	Cows Ovulated		Ova Recovered[a]	
	(no)	(no)	(%)	(no)	(%)
19 through 0	3	3	100	0	0
1 through 5	5	5	100	3	60
6 through 14	6	5	83	0	0
15 through 18	9	8	89	4	50
Total	23[b]	21	91	7	33

[a]Per cent recovered based on the number of cows that ovulated.

[b]Stage of the cycle was not available on two cows.

Table 9), they were not significantly different (P>.10) from follicles in cows from which ova were recovered (1.6±.1 cm). Corpora lutea averaging 1.1±.1 cm in diameter were observed at slaughter on all ovaries that had ovulated.

Four of the seven cows from which ova were recovered ovulated between the first and second breeding and had 2- to 8-cell fertilized ova in their oviducts (Table 11). In contrast to rabbits (Pritchard, Wettemann and Hafs, 1970) sperm transport was not inhibited in these MGA treated cows. A fragmented ovum was observed in the oviduct of one cow and in the uterus of another. These fragmented ova may or may not have been fertilized since Chang (1967) demonstrated that fragmented rabbit ova may or may not develop

TABLE 11.--Fertility of ova after controlled ovulation of Holstein cows.

Cow Number	Ovulation		Ova	
	Before Slaughter	Before 2nd Insemination	Location	Fertility
	(hours)	(hours)		
407	55	7	Oviduct	2 cells
408	19	29 after	Oviduct	Unfertilized
409[a]	67	19	Oviduct	Fragmented
	55	7	Oviduct	8 cells
413[b]	67	19	Uterus	Unfertilized
416	67	19	Oviduct	6 cells
430	67	19	Oviduct	6 cells
432	67	19	Uterus	Fragmented

[a]Two ova were recovered from this cow.

[b]Sperm deposited posterior to cervix.

into viable young. The unfertilized ovum recovered from cow 408 could be expected since ovulation occurred about 29 hr after the second insemination, probably well after the end of the fertile life of the sperm. Cow 413 also had an unfertilized ova which may be due to the fact that sperm were deposited posterior to the cervix at the inseminations, because the breeding catheter could not be passed through the cervix. Therefore, fertility appears normal in cows that ova were recovered from.

Experiment IV

Gonadotropin and Gonadal Hormones after Estrous Synchronization and Insemination

Reproductive Performance

This experiment was designed to study endocrine changes after MGA and HCG treatment and it was recognized at the onset that fertility data would be limited by the number of cows available. Cows in this study were started on MGA treatment an average of 55 days postpartum. Anestrus controls and cows which failed to exhibit estrus within 7 days after MGA withdrawal were removed from the experiment. Of the 65 cows assigned at random to the five treatments, 11 to 13 cows per treatment completed the study (Table 12). Ninety per cent of the cows exhibited estrus within 7 days after MGA withdrawal, but few cows conceived at the first insemination; an average of 20% of the treated cows and

TABLE 12.--Reproductive performance of cows after estrous synchronization.

Criterion	Treatment				
	(I) MGA+HCG[a]	(II) MGA+HCG[b]	(III) MGA	(IV) HCG[b]	(V) Control
Cows (no.)	11	11	11	13	12
Cows pregnant (no.)	2	1	2	4	3
S/C[c]	2.5	2.4	3.3	2.6	2.5
Days Open	131	111	131	132	120
Days pp[d]	67	51	52	56	51

[a]HCG given 3 days after last MGA feeding.

[b]HCG given when first observed in estrus.

[c]Services (inseminations) per conception.

[d]Days postpartum MGA was started.

25% of the control cows. Days from parturition to conception (days open) and the number of inseminations per conception were not altered significantly by the treatments (Table 12). Of the 12 cows which conceived to the first insemination, six were in the genetic group inseminated with semen from best sires and the other six were inseminated with semen from worst sires.

Endocrine Changes

By plan, cows in treatment I were not observed for estrus; they were inseminated at 12 and at 24 hr after HCG given 3 days after the last MGA feeding. Therefore they were omitted from the split plot analysis (Appendix Table VIII).

LH.--Serum LH averaged 1.0 ng/ml (n=33) on the last day of MGA treatment, similar to values observed during days 2 through 18 in nonpregnant cows. LH did not differ ($P>.10$) between the last day of MGA treatment and 2 days later. The ovulatory surge of LH ($\geq$ 4.0 ng/ml) was detected in only 23% (n=70) of the samples taken on the day of estrus, whereas I detected 51% of the surges in heifers (Experiment I). Ovulatory surges occur between 3 hr before and 6 hr after the onset of estrus (Schams and Karg, 1969; Henricks et al., 1970; Swanson and Hafs, 1971), so the peak of LH is less likely to be detected if cows are not bled early in estrus. It was more difficult to

determine the onset of estrus in cows at the stanchion barn than in heifers at loose housing. Failure to determine the onset of estrus in the cows may be related to low fertility and to the low proportion of cows in which an LH peak was observed compared to the heifers (Experiment I). The ovulatory surge of LH averaged 13.2 ± 9.0 ng/ml (n=10) in cows that conceived and 2.5 ± .6 ng/ml (n=36) in nonpregnant cows (Tables 13 and 14).

Serum LH was not influenced directly by genetic classification, pregnancy or MGA (Appendix Table VIII). Cows treated with HCG had less serum LH ($P<.05$) than non-treated cows. But this difference was caused by less LH in HCG-treated than non-treated cows prior to HCG injection. Therefore, the HCG could not have caused the difference in LH. A genetic by pregnancy interaction ($P<.05$) resulted; among best cows the nonpregnant had more LH while among worst cows the pregnant had more LH (Table 15). The physiological meaning of this interaction as well as the interaction between genetic classification, pregnancy and HCG ($P<.005$) are not apparent.

The day by pregnancy interaction was significant ($P<.005$); LH was similar in pregnant and nonpregnant cows during proestrus, greater in pregnant than nonpregnant cows at estrus but less in pregnant than in nonpregnant

TABLE 13.--Serum LH (ng/ml) in pregnant cows after MGA and HCG treatment.

Day	Treatment[a]				
	MGA + HCG[b]	MGA + HCG[c]	MGA	HCG[c]	Control
Last MGA	0.7±.1[d]	0.4	1.0±.3		
Last MGA+2	0.8±.1	0.8	1.0±.2		
-6			0.8[e]		
-5			1.3[e]	0.6[e]	
-4			0.7[e]	0.9[e]	
-3	0.7±.1	0.4	1.2[e]	0.6[e]	
-2			2.4[e]	1.0[e]	
-1	0.8±.1	0.8		1.1±0[f]	
Estrus	5.1±3.2	5.1	1.6±.6	3.1±1.2	37.1±28.1
2	0.5±.2	0.6	0.9±.1	0.9±0.2[g]	0.6± 0.2
4	0.4±0	1.0	0.8±.1	0.9±0.2[g]	0.4± 0.2
7	0.2±.1	0.4	0.8±.1	0.6±0.1	0.5± 0.2
11	0.2±.1	0.3	0.9±0	0.7±0.1	0.5± 0.2
18	0.2±.1	0.3	0.7±0	0.8±0.3	0.8± 0[f]
20	0.4±.1	0.2	0.7±.2	0.9±0.3[g]	0.3± 0.1
22	0.3±.1	0.3	0.9±.1	0.6±0.2	0.7± 0.1
25	0.1±0	0.4	1.2±.1	1.3±0.5	0.5± 0.2[f]

30	0.2±0	0.7	0.8±.2	0.6±0.1[g]	0.4± 0.1
35	0.2±.1	0.6	0.7±0	1.6±0.9	0.4± 0
40	0.8±.6	0.8	0.8[e]	0.7±0.1	0.5± 0.2
42	0.2±0	0.4	0.9±.2	0.6±0.2[g]	0.3± 0.2
45	0.4±.2	0.4	0.7[e]	0.6±0	0.8± 0.1
50	0.4±.2	0.3	0.9±0	0.5±0	0.4± 0.1
60	0.3±0	0.4	1.0±0	0.5±0[g]	0.4± 0.1
63	0.4±.1	0.2	0.7±.1	0.6±0.1	1.4± 0.9
75	0.2±0	0.2	0.9±.4	0.4±0.1	1.6± 1.1

[a]MGA + HCG[b] (n=2), MGA + HCG[c] (n=1), MGA (n=22), HCG[c] (n=4), control (n=3).

[b]HCG given 3 days after the last MGA feeding.

[c]HCG given when cows were first observed in estrus.

[d]Mean ± S.E.

[e]One observation.

[f]Two observations.

[g]Three observations.

TABLE 14.--Serum LH (ng/ml) in nonpregnant cows after MGA and HCG treatment.

Day	Treatment				
	MGA + HCG[a]	MGA + HCG[b]	MGA	HCG[b]	Control
Last MGA	0.8±0.1(9)[c]	1.2±0.4(10)	1.1±0.4(9)		
Last MGA+2	1.2±0.1(9)	1.1±0.2(8)	2.6±1.7(8)		
-6		1.8±1.3(3)		1.1±.3(2)	0.2 (1)
-5		1.1±0.4(2)	0.5 (1)		
-4		1.1±0.4(4)	1.2±0.2(2)	2.8 (1)	
-3	0.8±0.1(9)	.9±0.2(4)	1.2±0.6(5)	0.8 (1)	1.0±0.1(2)
-2		1.0±0.1(3)	1.1±0.3(3)	1.4 (1)	
-1	1.2±0.1(9)	1.0±0.4(2)	1.3±0.2(4)	3.6 (1)	1.8 (1)
Estrus	1.4±0.2(9)	2.1±0.3(10)	4.8±2.0(9)	1.3±.4(9)	1.6±0.8(8)
2	1.7±0.4(9)	1.0±0.2(10)	0.7±0.1(8)	1.0±.1(9)	1.0±0.1(9)
4	1.0±0.1(9)	1.0±0.2(10)	0.7±0.1(8)	0.9±.2(9)	1.1±0.2(9)
7	1.1±0.3(9)	0.9±0.2(9)	0.5±0 (7)	0.6±.1(9)	0.8±0.1(9)
11	0.9±0.2(9)	0.7±0.1(10)	0.7±0.3(9)	0.9±.4(9)	0.8±0.1(9)
18[d]	1.0±0.3(3)	1.3 (1)		0.9±.2(3)	1.2±0.5(4)
20[d]	1.0±0.3(3)			1.0±.4(3)	0.7±0.4(4)
22[d]	0.9±0.3(3)	7.1 (1)		0.4±.2(3)	0.8±0.5(4)
25[d]	0.8±0.2(3)	0.5 (1)		0.7±.2(3)	0.5±0.2(4)

-7		0.2 (1)	0.3 (1)		
-6		0.4 (1)		0.4 (1)	
-5	0.4±0.2(2)	1.3±0.8(4)	1.2±0.5(2)	1.0±0 (2)	4.2 (1)
-4	1.7±1.4(3)		0.4 (1)	0.3±0 (2)	
-3	0.6±0 (3)	0.9±0 (4)	0.8±0.2(5)	1.5±.8(2)	0.7±0.1(2)
-2	1.6±0.7(3)	1.6±0.2(3)	1.0±0.1(5)	1.0±.3(3)	
-1	2.3±0.7(2)	1.3±0.4(4)	3.4±1.4(3)	1.3 (1)	7.6±6.4(3)
Estrus	2.0±1.3(6)	4.3±2.8(7)	3.5±2.0(8)	0.5±0 (2)	9.7 (1)

[a]HCG given 3 days after the last MGA feeding.

[b]HCG given when cows were first observed in estrus.

[c]Mean ± S.E.; the numbers in parentheses refer to the number of observations (cows).

[d]Values for nonpregnant cows that were not observed in estrus.

TABLE 15.--Serum LH in cows: Influence of genetic classification, pregnancy and HCG treatment.

	Genetic Classification				
	Best		Worst		
	Pregnant	Nonpregnant	Pregnant	Nonpregnant	Average
	(ng/ml)				
HCG	0.8±.1[a]	1.3±.2	0.8± .1	1.2±.1	1.1±.1
Control	0.8±.1	1.6±.3	3.8±2.7	1.4±.3	1.7±.4
Average	0.8±.1	1.4±.2	2.0±1.0	1.3±.2	

[a]Mean ± S.E.

cows from days 2 through 25 after insemination. This agrees with my finding that nonpregnant heifers also had greater serum LH than pregnant heifers. Edgerton and Hafs (1971) also reported a greater concentration of LH in nonpregnant cows compared with pregnant cows.

Values for serum LH in nonpregnant cows on days 18,20,22 and 25 after estrus are listed in Table 14. These samples were from cows not observed in estrus between days 18 and 25. When cows were sampled daily before estrus, increased LH was detected before estrus (Table 14), probably because we did not detect estrus among the cows as carefully as among the heifers where the LH surge usually occurred after the onset of estrus.

Prolactin.--Serum prolactin was not influenced ($P>.10$) by genetic classification, pregnancy, MGA or HCG. Although days after insemination differed significantly ($P<.01$) no meaningful pattern of prolactin changes could be established (Tables 16 and 17). Prolactin ranged from 20±4 ng/ml on the day of estrus to 37±8 ng/ml on day 22 of pregnancy. Other values were intermediate between these extremes until day 75. Prolactin values were similar in pregnant and nonpregnant cows (Tables 16 and 17). Increases in prolactin near estrus, which have been reported for heifers (Raud et al., 1971; Swanson et al., 1972) were not observed in these cows.

TABLE 16.--Serum prolactin (ng/ml) in pregnant cows after MGA and HCG treatment.

Day	Treatment[a]				
	MGA + HCG[b]	MGA + HCG[c]	MGA	HCG[c]	Control
Last MGA	35±20[d]	54	19± 8		
Last MGA+2	22± 6	26	20± 8		
-6			26[e]		
-5			11[e]	26[e]	
-4			29[e]	52[e]	
-3	35±20	54	13[e]	22[e]	
-2			18[e]		
-1	22± 6	26		25± 2[f]	
Estrus	45± 3	12	31±11	17± 5	20± 6
2	17± 2	36	28± 1	18± 5[g]	33± 8
4	22± 3	42	46±22	18± 3[g]	17± 1
7	29± 5	10	32±15	26± 2	27± 7
11	20± 4	10	27± 1	24±10	19± 7
18	16±14	34	23± 7	35± 8[g]	32±21[f]
20	18± 3	20	50± 5	17± 7[g]	31±10
22	14±11	22	44± 5	46±17	27±11
25	15± 7	27	46± 5	24± 6	34± 5

30	8± 2	23	37± 6	42±20	14± 4
35	22± 3	35	51±12	31± 7	22± 8
40	50±15	57	25[e]	27± 6	15±11
42	16± 2	35	36±17	18± 2[g]	13± 3
45	30±16	57	44±11	22± 3	12± 1
50	38[e]	53	22± 3	45±16	7± 4
60	16± 3	42	38±19	23± 6[g]	18± 9
63	12±12	37	25± 3	32±10	35±24
75	1± 1	46	23± 5	42±14	30±23

[a]MGA + HCG[b] (n=2), MGA + HCG[c] (n=1), MGA (n=2), HCG[c] (n=4), Control (n=3).

[b]HCG given 3 days after the last MGA feeding.

[c]HCG given when cows were first observed in estrus.

[d]Mean ± S.E.

[e]One observation.

[f]Two observations.

[g]Three observations.

TABLE 17.--Serum prolactin (ng/ml) in nonpregnant cows after MGA and HCG treatment.

Day	Treatment				
	MGA + HCG[a]	MGA + HCG[b]	MGA	HCG[b]	Control
Last MGA	29± 6(9)	42±13(10)	39±14(9)		
Last MGA+2	24± 5(9)	26±10(7)	22± 8(8)		
-6		102±42(2)		27± 8(2)	67 (1)
-5		12± 2(2)	15 (1)		
-4		35±10(5)	11± 2(2)	22 (1)	
-3	29± 6(9)	24±15(4)	62±28(4)	12 (1)	38±32(2)
-2		25±17(3)	24±18(2)	22 (1)	
-1	24± 5(9)	29 (1)	34±14(4)	12 (1)	126 (1)
Estrus	29± 8(9)	22± 7(9)	27± 8(8)	23± 5(9)	42±13(8)
2	37±16(9)	42±12(10)	37± 7(8)	24± 5(9)	42±16(9)
4	30± 6(9)	45±11(10)	29± 8(8)	18± 3(9)	33±10(9)
7	31±11(9)	35± 9(10)	24± 9(7)	23± 4(9)	24± 7(9)
11	29± 6(9)	59±16(10)	27± 6(9)	25±13(9)	35±13(9)
18[d]	30±15(3)	23 (1)		22± 9(3)	53±17(4)
20[d]	41± 8(3)			28± 4(3)	33± 9(4)
22[d]	45±10(3)	65 (1)		28± 6(3)	56±23(4)
25[d]	29± 5(3)	1 (1)		25± 5(3)	37±12(4)

18[d]	30±15(3)	23 (1)		25±13(9)	35±13(9)
20[d]	41± 8(3)			22± 9(3)	53±17(4)
22[d]	45±10(3)	65 (1)		28± 4(3)	33± 9(4)
				28± 6(3)	56±23(4)
					42±12(4)
-7		44 (1)	18 (1)	24 (1)	
-6		20± 6(2)		36±23(3)	
-5	19± 7(2)	61±22(4)	47± 3(2)	21 (1)	11 (1)
-4	40±11(3)	12 (1)	38 (1)	18±10(3)	
-3	15± 8(3)	99±47(3)	32± 6(5)	6± 1(2)	31±19(2)
-2	44±12(3)	16± 4(3)	21±11(4)	18± 5(3)	
-1	15± 4(2)	60±24(3)	32±12(4)	9± 3(2)	29± 9(3)
Estrus	15± 4(6)	19± 3(8)	26± 6(8)	15±10(3)	13± 3(2)

[a]HCG given 3 days after the last MGA feeding.

[b]HCG given when cows were first observed in estrus.

[c]Mean ± S.E.; the numbers in parentheses refer to the number of observations (cows).

[d]Values for nonpregnant cows that were not observed in estrus.

The lactating pregnant cows in this experiment had greater prolactin (28±1 ng/ml, n=184) than the nonlactating pregnant heifers in Experiment I (21±1 ng/ml, n=423), and the trend for decreasing serum prolactin with increasing duration of pregnancy observed in heifers was not apparent in cows. Differences in serum prolactin between cows and heifers could be related to lactation, since milking causes a transitory increase in serum prolactin (Tucker, 1971). Similar to heifers, the among cow variation in serum prolactin was large; cow averages ranged from 6±1 to 89±16 ng/ml.

Significant two and three way interactions of days with the four main effects (pregnancy, genetics, HCG, MGA) are difficult to interpret because of much day variation in prolactin concentration.

Estradiol.--Serum estradiol was greater in MGA-treated cows than in control cows (P = .15, Tables 18 and 19). This difference was apparent from proestrus until 2 days after estrus, but at no other time. None of the other main effects, two way or three way interactions approached significance.

Only one of the non-MGA treated cows had greater than 22 pg/ml of estradiol and this elevation occurred on day 42 of pregnancy. But 13 samples from 13 different cows after MGA treatment had greater than 35 pg/ml estradiol (Table 20). During the estrous cycle in heifers,

TABLE 18.--Serum estradiol (pg/ml) in pregnant cows after MGA and HCG treatment.

Day	Treatment[a]				
	MGA + HCG[b]	MGA + HCG[c]	MGA	HCG[c]	Control
Last MGA	10.4±1.2[d]	12.4	7.2±0.7		
Last MGA+2	14.6±1.0	18.3	12.0±3.4		
-6			6.5[e]		
-5			7.9[e]	5.5[e]	
-4			8.5[e]	7.8[e]	
-3	10.4±1.2	12.4	15.4[e]	4.5[e]	
-2				11.7[e]	
-1	14.6±1.0	18.3		14.2±2.0[f]	
Estrus	348.2±342.0	60.5	7.5±0.5	9.6±3.0[g]	10.0±4.3[f]
2	2.8±0.4	10.1	7.6±0.4	5.6±1.0[g]	5.2±0.4
4	6.0±2.8	14.5	7.4±0	7.8±1.7[g]	4.4±1.1
7	4.4±2.4	7.9	8.8±1.4	4.8±1.3	4.1±0.8
11	4.3[e]	10.2	4.4±1.2	4.9±0.6	4.1±0.9
18	3.3±0.9	9.5	5.2±1.0	5.2±0.8	3.6±1.4[f]
20	2.0±0.4	7.8	4.8±1.4	5.3±0.8	3.4±1.0
22	3.0[e]	8.3	7.0±1.4	5.1±2.0	7.1±2.5
25	4.8±2.5		8.1±1.1	8.0±1.9	3.2±0.9

30	3.1±0.2	11.7	10.1±0.3	4.4±1.6	3.5±0.8
35	2.0±0	13.2	7.2±2.0	5.8±1.0	3.5±1.0
40	7.0±4.4	12.2	7.8[e]	6.7±1.0	3.9±0.8
42	1.9±0.2	6.5	6.6±2.8	16.2±11.7[g]	3.1±0.8
45	1.9±0.4	10.6	4.6±1.1	5.4±0.9	4.2±1.7
50	2.6±0	10.7	5.9±2.8	5.7±1.0[g]	4.6±1.9
60	3.1[e]	7.8	7.1±2.6	5.1±1.5[g]	4.5±1.8
63	3.8±1.2	9.9	13.0±8.2	4.6±0.5	4.0±1.3
75	1.8±0.2	8.2	5.2±0.5	4.8±1.3[g]	5.7±1.4

[a]MGA + HCG[b] (n=2), MGA + HCG[c] (n=1), MGA (n=2), HCG[c] (n=4), Control (n=3).

[b]HCG given 3 days after the last MGA feeding.

[c]HCG given when cows were first observed in estrus.

[d]Mean ± S.E.

[e]One observation.

[f]Two observations.

[g]Three observations.

TABLE 19.--Serum estradiol (pg/ml) in nonpregnant cows after MGA and HCG treatment.

Day	Treatment				
	MGA + HCG[a]	MGA + HCG[b]	MGA	HCG[b]	Control
Last MGA	76.9±65.2(9)	9.5± 2.4(8)	11.1± 2.4(8)		
Last MGA+2	14.3± 1.7(9)	11.2± 2.3(8)	11.0± 1.7(8)		
-6		11.2± 8.4(2)		6.0±1.0(2)	4.6 (1)
-5		17.9 (1)	8.4 (1)		
-4		8.8± 4.6(4)	7.6± 0.6(2)	8.2 (1)	
-3	76.9±65.2(9)	11.3± 1.0(4)	15.9± 3.2(3)	8.4 (1)	7.8±0.8(2)
-2		8.0± 2.6(3)	13.4± 4.8(2)	8.3 (1)	
-1	14.3± 1.7(9)	10.3± 7.2(2)	13.4± 3.1(4)	9.3 (1)	10.4 (1)
Estrus	92.5±65.4(9)	40.1±29.9(9)	24.1±12.0(8)	6.9± .9(9)	10.0±2.5(7)
2	9.1± 1.9(9)	40.8±35.0(10)	4.9± 1.1(7)	7.3±1.7(8)	5.8±1.0(8)
4	9.7± 2.1(9)	8.8± 1.3(10)	4.3± 0.7(8)	5.8±0.8(9)	3.3±0.4(8)
7	11.7± 4.2(9)	6.1± 1.0(10)	5.2± 1.0(7)	6.3±0.6(9)	5.3±1.0(8)
11	6.1± 0.8(9)	6.1± 1.2(9)	4.9± 1.0(9)	5.5±1.7(8)	5.2±0.9(8)
18[d]	6.4± 1.6(3)	8.9 (1)		9.2±5.3(3)	7.2±1.7(4)
20[d]	8.0± 2.8(3)			7.4±2.7(3)	6.4±2.5(4)
22[d]	11.7± 8.2(3)	17.2 (1)		2.2±0 (2)	4.8±2.1(3)
25[d]	5.0± 0.4(3)	19.6 (1)		9.1±5.4(3)	1.9±0.6(4)

-7	5.4 (1)	3.3 (1)	4.9 (9)	4.8 (1)	
-6		5.2± 3.0(2)		2.9±0.8(4)	
-5	4.2± 1.0(2)	5.2± 0.7(4)	6.0± 0.8(2)	5.6	8.0 (1)
-4	4.5± 1.3(3)		12.5 (1)	4.9±1.2(3)	
-3	12.6± 8.2(2)	6.4± 1.9(3)	26.7±20.4(4)	6.5±0.2(2)	13.6±8.4(2)
-2	21.0±11.8(3)	10.3± 2.8(3)	9.5± 0.2(4)	8.3±1.8(3)	
-1	8.9 (1)	8.0± 0.2(2)	7.6± 2.0(3)	5.4±1.8(2)	6.8±0.5(3)
Estrus	6.2± 1.1(5)	7.0± 1.6(7)	6.2± 1.0(7)	6.4±2.2(4)	7.9 (1)

[a]HCG given 3 days after the last MGA feeding.

[b]HCG given when cows were first observed in estrus.

[c]Mean ± S.E.; the numbers in parentheses refer to the number of observations (cows).

[d]Values for nonpregnant cows that were not observed in estrus.

TABLE 20.--Cows with high (>35 pg/ml) serum estradiol.

Cow	Treatment	Day	Estradiol
(no)			(pg/ml)
765	MGA + HCG[a]	3 days post MGA	44
851	" "	" " " "	100
957	" "	" " " "	610
958	" "	Last day of MGA	598
977[b]	" "	3 days post MGA	690
1027	" "	Day 7	43
1037	" "	Day 20	44
824[b]	MGA + HCG[c]	Estrus	60
946	" "	Estrus	458
998	" "	Day 2	356
897	MGA	Estrus	38
897	MGA	Day 22	88
1005	MGA	Estrus	104
771[b]	HCG[b]	Day 42	40

[a]HCG 3 days after last MGA feeding.

[b]Pregnant, other cows were nonpregnant.

[c]HCG when first observed in estrus.

estradiol concentration exceeds 10 pg/ml only at proestrus and estrus (Wettemann et al., 1972). On the last day of MGA treatment 47% of the cows had greater estradiol (30.1± 19.6 pg/ml) than control cows at proestrus. When this elevation in estradiol began during MGA treatment is unknown. Most cows had clearly elevated estradiol during the 2 to 3 days after the last feeding of MGA (Table 21), but only 10% of the cows had estradiol greater than 10 pg/ml by 8 days after MGA. This decrease in estradiol with time after MGA treatment may be attributed to LH release from the pituitary after MGA withdrawal or to injections of HCG; both cause ovulation, removing the source of estradiol.

Both pregnant and nonpregnant MGA treated cows had high estradiol during proestrus and estrus compared with controls. The duration of elevated estradiol during MGA treatment could be related to infertility. High concentrations of estradiol for a prolonged period before estrus could alter uterine and oviducal secretions and contractility, creating a hostile environment for gametes. The onset of elevated serum estradiol may be related to the day of the estrous cycle when progestogen treatment is started. For example, if a large follicle is present at the outset, it may be maintained and secrete estrogen during treatment. Similarly, fertility after progestogen treatment also may be related to the day of the estrous

TABLE 21.--Serum estradiol in cows after MGA withdrawal.

Days After MGA	Number of Cows	Avg. ± SE	Per Cent of Samples with ≥10 pg/ml
		(pg/ml)	
0	30	30.1±19.6	47
2	30	12.6± 1.0	67
3	17	98.2±50.8	70
4	6	9.3± 2.1	50
5	20	7.9± 1.1	30
6	10	67.1±42.1	20
7	22	12.6± 4.4	18
8	10	6.7± 1.0	10

cycle when progestogen treatment is started (Wagner et al., 1968; De Bois and Bierschwal, 1970).

During days 2 through 11 after insemination, estradiol concentration was similar in pregnant and non-pregnant cows (Tables 18 and 19). In contrast, on day 2 in pregnant heifers, estradiol resembled that at estrus (Figure 6). Differences in estrus detection between the cows and the heifers, as described previously, may be responsible for the difference in estradiol at day 2.

Estradiol during pregnancy ranged from 4.9±.6 pg/ml on day 20 to 8.6±3.9 pg/ml on day 42. Similar to heifers, 50% of the pregnant cows had elevated estradiol between days 30 and 42. More frequent sampling would be required to test adequately if growth of the placenta and embryo influence estradiol near day 40.

Progesterone.--Serum progesterone averaged 0.7 ng/ml (n=32) on the last day of MGA treatment and only 3 cows had more than ng/ml. The three cows with 4.5 to 6.5 ng/ml progesterone at MGA withdrawal did not exhibit estrus until 6 to 9 days later. Hill et al. (1971) and Britt and Ulberg (1972) found about 5 ng/ml progesterone at the end of 14 days of MGA when MGA was started on day 14 of the estrous cycle, although corpora lutea had regressed. My cows were at all days of the estrous cycle when MGA treatment started. Also, I used column

chromatography to isolate progesterone from other endogenous steroids which might erroneously inflate progesterone values.

Progesterone averaged 0.3±.1 ng/ml at estrus and increased to 6.9±.6 ng/ml by day 11 in both pregnant and nonpregnant cows (Tables 22 and 23). This rapid increase in progesterone concentration is similar to the change I observed in pregnant heifers and similar to that described by Stabenfeldt et al. (1969), Henricks et al. (1971a), and Swanson et al. (1972). Progesterone values in pregnant and nonpregnant cows did not differ during days 2 through 11 after insemination. Henricks et al. (1971a) reported higher progesterone by nine days after insemination in pregnant cows compared to nonpregnant cows. In my cows, progesterone decreased beginning about 5 to 6 days before the nonpregnant cows returned to estrus, and progesterone decreased further to 2 ng/ml by 2 days before estrus.

Progesterone decreased from day 20 to 22 and to day 25 of pregnancy (10.9±1.5, 10.0±1.2 and 8.8±1.0 ng/ml, respectively). This decrease is similar to one detected in heifers (Figure 6) occurring between days 18 and 22 of pregnancy, possibly representing initiation of luteal regression which is terminated by the conceptus in the uterus by about day 25. From day 30 through day 75 of pregnancy, progesterone plateaued and averaged about 9.5 ng/ml.

TABLE 22.--Serum progesterone (ng/ml) in pregnant cows after MGA and HCG treatment.

Day	Treatment[a]				
	MGA + HCG[b]	MGA + HCG[c]	MGA	HCG[c]	Control
Last MGA	0.1±0[d]	0.2	2.7±2.7		
Last MGA+2	0±0	0.2	2.1±2.1		
-6			5.4[e]		
-5			0	7.4[e]	
-4			4.3[e]	4.4[e]	
-3	0.1±0	0.2	0[e]		
-2				0[e]	
-1	0±0	0.2		0.1±0[f]	
Estrus	0.1±0	0.2	0.1±0.1	0.1±0[g]	0±0
2	0.1[e]	0.1	0.5±0.3	0.2±0.1[g]	0.2±0.1
4	0.8±0.5	1.4	2.4±0.6	2.4±0.8[g]	1.3±0.3
7	5.0±4.0	4.4	5.0±0.1	6.9±2.4	3.7±0.6
11	5.1[e]	7.7	6.2±0.1	6.3±1.0	7.0±2.6
18	8.8±0.8	11.7	9.7±0.9	9.2±0.6	8.1±1.3[f]
20	7.9±1.4	6.7	12.7±4.2	11.6±3.0	10.2±3.6
22	6.3±0.8	13.2	15.5±1.6	7.4±0.9	8.9±2.3
25	6.4±1.7		7.3±0.9	9.9±0.3	8.4±3.2

30	6.6±4.5	12.2	9.0±2.5	12.1±0.5[g]	8.2±3.6
35	6.0±3.3	13.8	9.8±3.5	12.3±0.3	7.4±1.7
40	6.0±0.5	13.1	8.6[e]	10.3±0.8	8.2±1.4
42	5.8±1.1	17.8	12.2±3.2	8.9±0.4[g]	9.8±0.9
45	5.4±0.2	14.9	12.7±2.7	9.0±1.5	12.3±1.7
50	7.7±3.2	11.6	10.5±1.2	9.0±1.5[g]	8.9±2.1
60	5.0[e]	11.2	10.2±1.3	7.7±0.6[g]	8.0±2.5
63	10.1±3.2	8.7	9.5±0.8	11.7±2.0	9.9±2.7
75	10.9±7.8	9.2	11.2±1.8	9.1±2.1[g]	9.7±2.5

[a]MGA + HCG[b] (n=2), MGA + HCG[c] (n=1), MGA (n=2), HCG[c] (n=4), Control (n=3).

[b]HCG given 3 days after the last MGA feeding.

[c]HCG given when cows were first observed in estrus.

[d]Mean ± S.E.

[e]One observation.

[f]Two observations.

[g]Three observations.

TABLE 23.--Serum progesterone (ng/ml) in nonpregnant cows after MGA and HCG treatment.

Day	Treatment				
	MGA + HCG[a]	MGA + HCG[b]	MGA	HCG[b]	Control
Last MGA	0.1±0 (9)[c]	0.9±0.6(10)	0.7±0.5(8)		
Last MGA+2	0±0 (9)	0.2±0.1(8)	1.5±1.0(8)		
-6		3.6±2.9(2)		2.0±1.7(2)	10.9 (1)
-5		0.4±0.3(2)	0 (1)		
-4		0.1±0 (5)	0.2±0.1(2)	0 (1)	
-3	0.1±0 (9)	0.3±0.2(4)	0.2±0.1(3)	3.0 (1)	2.1±2.0(2)
-2		0.1±0 (3)	0.3±0 (2)	0 (1)	
-1	0±0 (9)	0.2±0.1(2)	0.7±0.6(4)	0 (1)	0 (1)
Estrus	0.2±0.2(9)	0.2±0.1(10)	0.2±0.1(8)	0.5±0.2(9)	0.8±0.4(7)
2	0.1±0 (9)	0.6±0.2(10)	0.2±0.1(7)	0.8±0.5(8)	0.8±0.4(8)
4	0.4±0.1(9)	1.8±1.0(10)	3.4±2.0(8)	1.5±0.7(9)	1.5±0.8(8)
7	3.3±0.8(8)	4.5±1.3(10)	4.6±0.5(7)	4.2±1.6(9)	4.1±1.2(8)
11	5.8±1.2(9)	7.8±1.6(9)	7.1±1.1(9)	6.7±1.6(8)	6.3±1.6(7)
18[d]	3.4±2.3(3)	5.1 (1)		0.8±0.3(3)	4.2±1.7(4)
20[d]	2.2±2.1(3)			2.7±1.4(3)	5.3±2.2(4)
22[d]	2.9±2.7(3)	5.9 (1)		5.6 (1)	4.5±2.7(3)
25[d]	2.7±2.6(3)	9.0 (1)		1.8±0.2(3)	5.7±2.0(4)

-7	9.6 (1)	9.0 (1)	8.3 (1)	7.4 (1)	
-6		8.3±6.2(2)		5.1±2.0(3)	
-5	5.7±3.8(2)	7.8±1.7(4)	10.6±2.7(2)	3.2 (1)	6.7 (1)
-4	5.0±1.1(3)		0 (1)	3.8±1.9(3)	
-3	1.4±0.8(3)	2.8±1.3(3)	6.6±2.9(4)	3.8±3.4(2)	5.7±5.7(2)
-2	1.5±1.2(3)	0.6±0.4(3)	3.0±2.8(4)	0.7±0.3(3)	
-1	0±0 (2)	7.3±7.1(2)	0.5±0.3(3)	0.9±0.9(2)	2.1±2.0(3)
Estrus	2.0±1.9(5)	0.2±0.1(8)	1.4±1.1(8)	0.2±0 (4)	0.2 (1)

[a]HCG given 3 days after the last MGA feeding.

[b]HCG given when cows were first observed in estrus.

[c]Mean ± S.E.; the numbers in parentheses refer to the number of observations (cows).

[d]Values for nonpregnant cows that were not observed in estrus.

The interaction between genetic classification, pregnancy and HCG was significant ($P<.05$). Nonpregnant cows in all treatment combinations had similar progesterone concentration but best pregnant cows and worst pregnant cows given HCG had greater progesterone than worst pregnant cows without HCG (Table 24). A similar interaction was observed for serum LH (Table 15). Comparing LH and progesterone in the eight treatment combinations, cows with higher progesterone had lower LH and vice versa except for worst pregnant cows given HCG. These same treatment combinations also caused the genetic by pregnancy interaction to be significant ($P<.05$). I do not comprehend the physiological meaning of these interactions.

TABLE 24.--Serum progesterone in cows: Influence of genetic classification, pregnancy and HCG treatment.

	Genetic Classification				
	Best		Worst		
	Pregnant	Nonpregnant	Pregnant	Nonpregnant	Average
	(ng/ml)				
HCG	8.3±.7[a]	3.0±.4	7.9±.7	2.3±.3	4.5±.3
Control	9.4±.7	2.8±.4	5.5±.5	3.5±.5	4.9±.3
Average	9.0±.5	2.9±.3	6.9±.5	2.8±.3	

[a]Mean ± S.E.

GENERAL DISCUSSION

Estrus was first observed in the morning in 76% of the heifers and serum LH was greatest at this time. Elevated LH ($\geq$4 ng/ml) was detected in 51% of the heifers bled when first observed in estrus. Only 23% of the LH surges were detected in cows sampled similarly, probably because of less meticulous estrous detection among the cows. This concurs with observations that the ovulatory surge of LH occurs between 3 hr before to 6 hr after the onset of estrus and persists for 6 to 10 hours (Schams and Karg, 1969; Henricks et al., 1970; Swanson and Hafs, 1971). Because of the short duration of the peak, LH concentration at estrus is dependent on the interval between blood sampling; there is no evidence from my data on heifers or cows that the shape or magnitude of the LH surge is related to fertility.

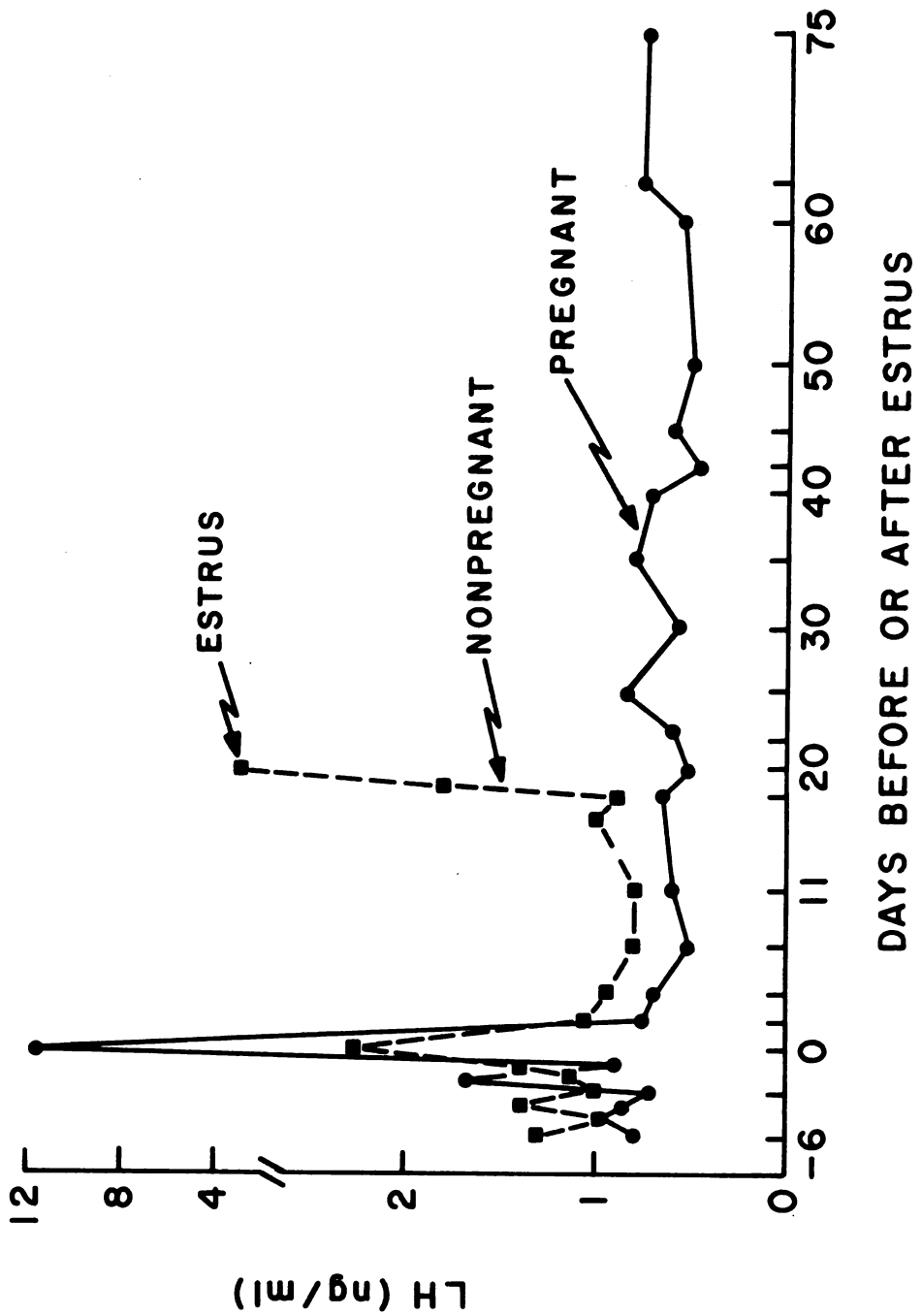
Sera concentrations of LH, prolactin, estradiol and progesterone were not influenced by genetic classification. The majority of the cows in this study were foundation cows in the genetic experiment, so the influence of best or worst sires would not be present until the next generation.

As described earlier, cows in treatment I of Experiment IV were omitted from the split plot analysis because they were not observed for estrus and were inseminated at 12 and at 24 hr after HCG given 3 days after the last MGA feeding. Cows in treatment II (given HCG when observed in estrus) were compared with cows in treatment I (Appendix Table IX). Serum LH, prolactin, estradiol and progesterone were similar after these two treatments. Therefore the graphs in this section (except for estradiol) depict average hormone concentration for cows in all five treatments.

Serum LH varied little among heifers ($P>.10$), and it did not decrease ($P>.10$) with advancing pregnancy in heifers (Figure 4) or cows (Figure 9). None of the pregnant cows had elevated LH ($\geq$4 ng/ml) after estrus and only three samples from three different pregnant heifers were elevated after estrus. Randel and Erb (1971) observed no significant changes in serum LH from day 7 to day 260 of pregnancy, but Schams (1969) detected increases in serum LH at day 52 in one cow and day 61 in another with more frequent sampling.

Nonpregnant heifers had more LH ($P<.10$) than pregnant heifers during days 2 through 11 after insemination, similar to a report for cows (Edgerton and Hafs, 1971). Nonpregnant cows in my experiment also had higher serum LH from days 2 through 11 after insemination, but this difference was not

Figure 9.--Serum LH in cows before and after insemination.


LH (ng/ml)
12
8
4
2
1
0
-6
0
11
20
30
40
50
60
75
DAYS BEFORE OR AFTER ESTRUS
ESTRUS
NONPREGNANT
PREGNANT

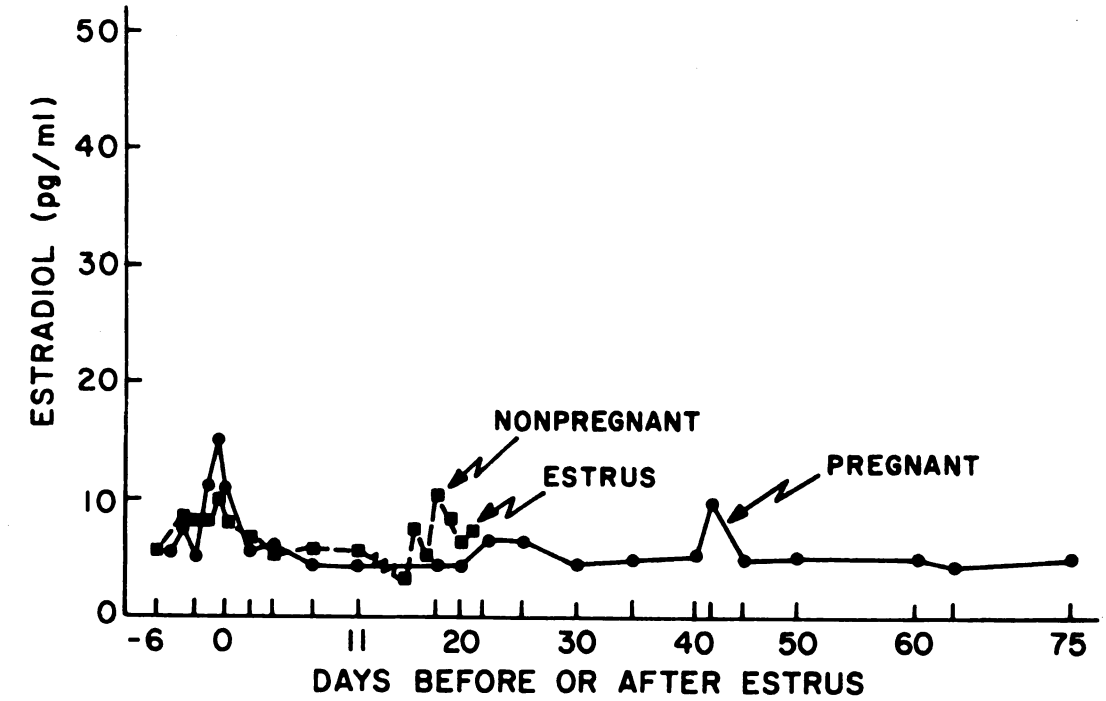
significant (P>.10). Lower LH in pregnant heifers or cows probably is not related to estradiol or progesterone feedback at the pituitary or hypothalamus because I detected no differences in serum estradiol or progesterone between pregnant and nonpregnant cows during days 2 through 11 after insemination (Figures 10, 11, 12).

Prolactin concentration differed among heifers (P<.005). But average prolactin in heifers during the first 75 days of pregnancy was not related significantly to milk production in the subsequent lactation (r=.07). Serum prolactin did not vary significantly with day of the estrous cycle or day of pregnancy in cows or heifers (Figures 5 and 13). However, after heifers were synchronized with MGA, a significant increase in prolactin occurred at the time of the ovulatory surge of LH. Increases in prolactin at estrus have been reported in heifers (Raud et al., 1971; Swanson et al., 1972) and prolactin decreases during pregnancy were reported in rats (Amenomori et al., 1970) and sheep (Arai and Lee, 1967; Davis et al., 1971).

Serum estradiol was lowest during the luteal phase of the estrous cycle and increased beginning about 3 days before estrus to maximal values on the day before or on the day of estrus (Table 2). Estradiol and progesterone were correlated significantly only on the day before estrus (r=-.57, P .05). The ovulatory surge of LH usually occurred 1 or 2 days after the first clear evidence of rising

Figure 10.--Serum estradiol in cows before and after insemination.

Figure 11.--Serum estradiol in MGA treated cows before and after insemination.


ESTRADIOL (pg/ml)
50
40
30
20
10
0
NONPREGNANT
ESTRUS
PREGNANT
-6
0
11
20
30
40
50
60
75
DAYS BEFORE OR AFTER ESTRUS

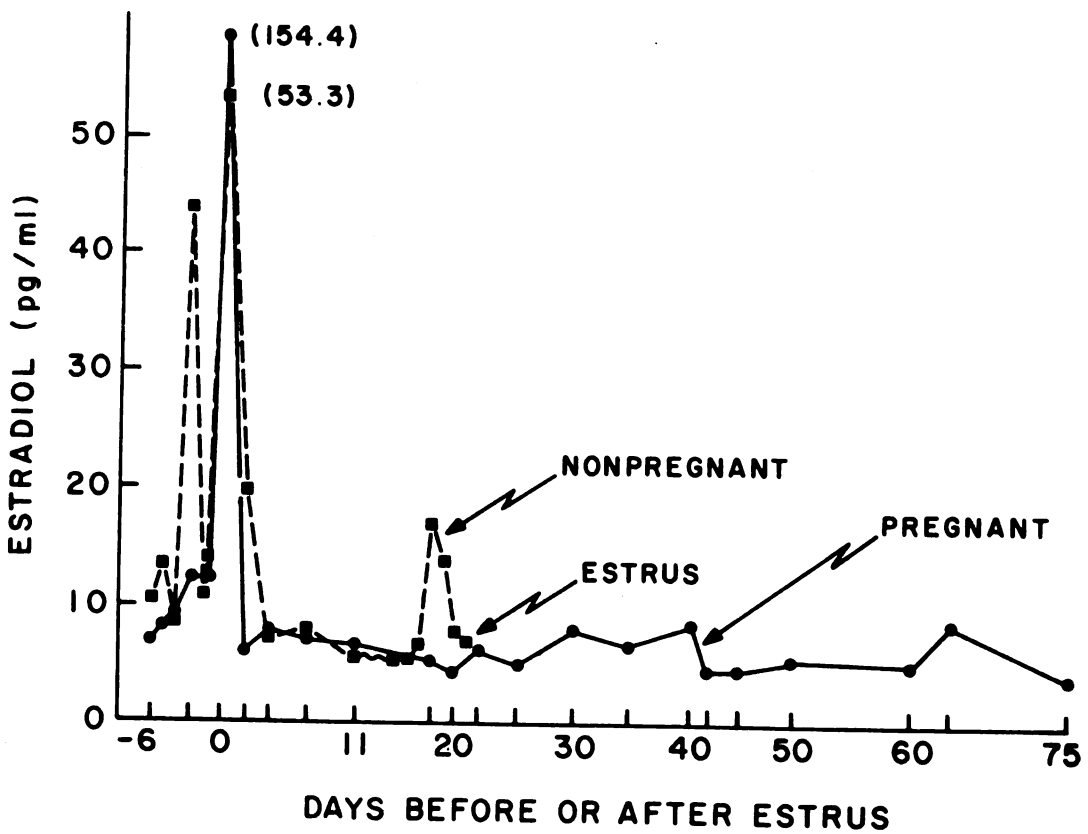
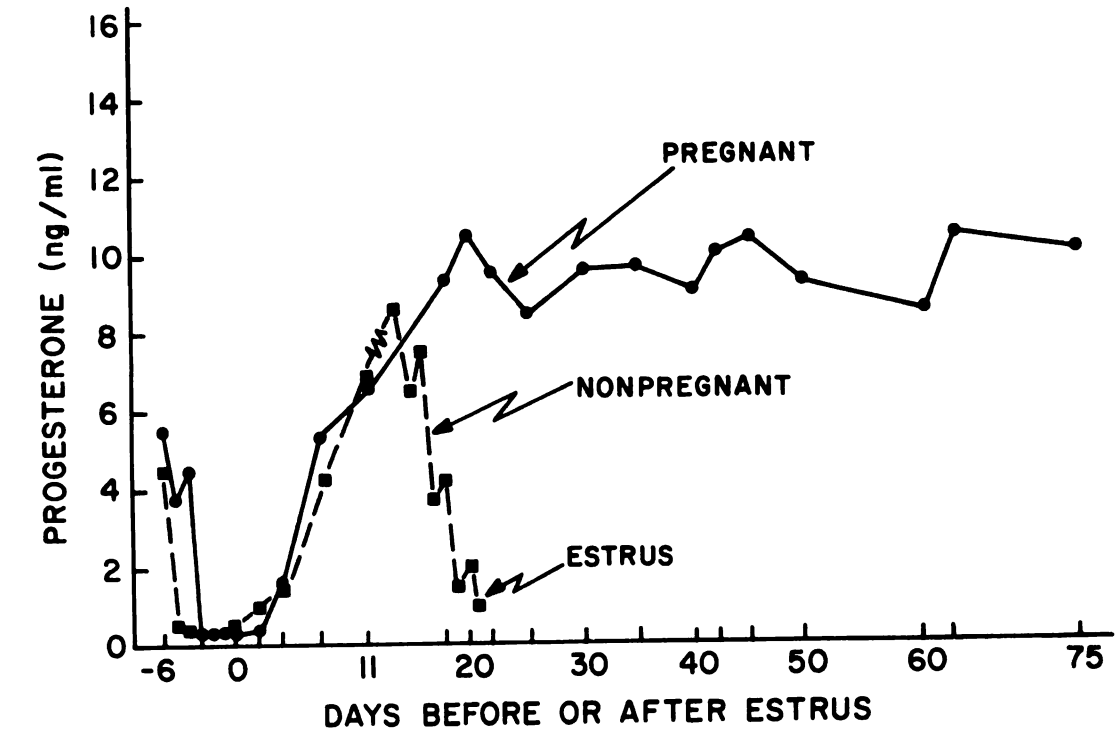
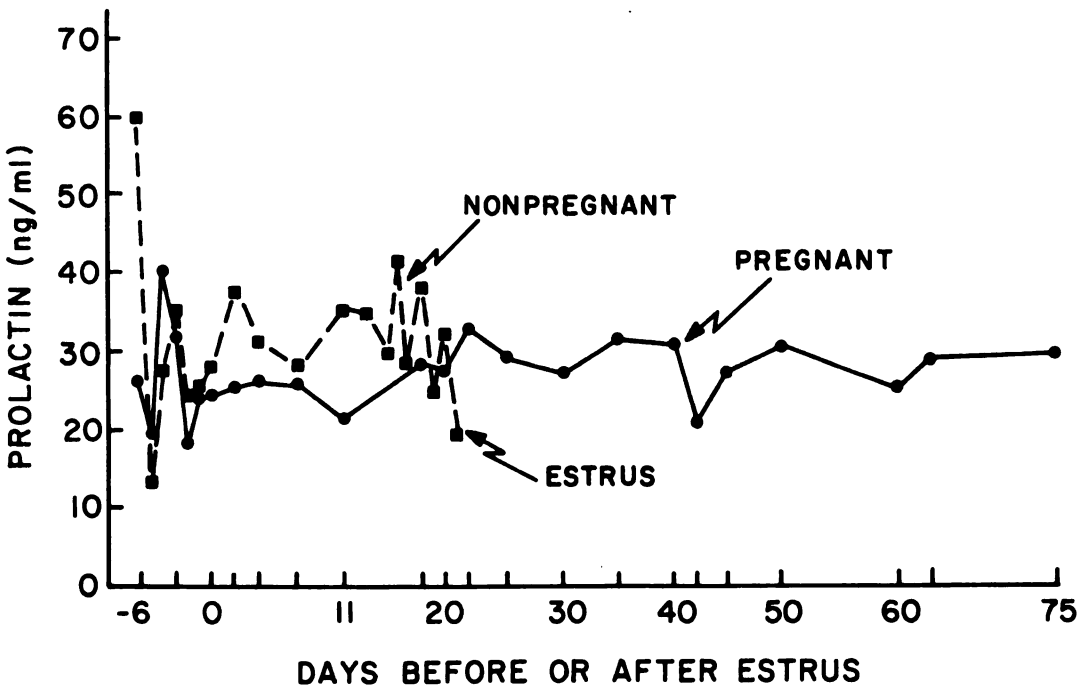

(154.4)
(53.3)
ESTRADIOL (pg/ml)
50
40
30
20
10
0
NONPREGNANT
PREGNANT
ESTRUS
-6
0
11
20
30
40
50
60
75
DAYS BEFORE OR AFTER ESTRUS

Figure 12.--Serum progesterone in cows before and after insemination.

Figure 13.--Serum prolactin in cows before and after insemination.


PROGESTERONE (ng/ml)
16
14
12
10
8
6
4
2
0
PREGNANT
NONPREGNANT
ESTRUS
-6
0
11
20
30
40
50
60
75
DAYS BEFORE OR AFTER ESTRUS


PROLACTIN (ng/ml)
70
60
50
40
30
20
10
0
NONPREGNANT
PREGNANT
ESTRUS
-6
0
11
20
30
40
50
60
75
DAYS BEFORE OR AFTER ESTRUS

estradiol. Injection of 10 mg estradiol caused increased serum LH 20 to 24 hours later in cows (Howlands et al., 1971) and similar data were described in sheep (Goding et al., 1969). Therefore my data suggest that estrogen secretion normally regulate the ovulatory LH surge in cattle.

Pregnancy did not alter serum estradiol in control or MGA treated cows until after day 11 when proestrus increases began only in nonpregnant cows (Figures 10 & 11). Randel et al. (1971) reported altered excretion of urinary estrogens compared with pregnant cows during the first 9 days after breeding in cows which did not conceive. At present, the relationship between serum estradiol and urinary estrogens has not been established. The cause of infertility may determine whether estrogen concentration is altered after insemination. For example, cows with ovarian cysts may have increased serum estradiol and be infertile, but cows that do not conceive because they are inseminated too late may have normal estradiol levels.

During days 18 through 75 of pregnancy, estradiol averaged 7 pg/ml in heifers except for day 40 when four of eight heifers had elevated estradiol (11.1±2.3 pg/ml). Similar to heifers, 50% of the pregnant cows had elevated estradiol between days 30 and 42. Luteinizing hormone initiates blastocyst implantation in rats (Macdonald et al., 1967), probably mediated through estrogen secretion since

Yoshinaga and Hosi (1961) found that estrogen causes implantation in lactating rats. Shaikh (1971) reported increased estrone and estradiol in ovarian venous blood from rats on day 4 after breeding; the day of implantation. Therefore, possibly the increased estradiol I found in some cows may facilitate implantation in cattle. More frequent sampling between days 35 and 45 of pregnancy would be necessary to examine precisely the role of estradiol.

Serum progesterone was similar ($P>.10$) in pregnant and nonpregnant cows through day 11 after insemination (Figure 12). Henricks et al. (1970) reported significantly greater serum progesterone in pregnant cows than nonpregnant cows from 10 to 14 days after insemination, but progesterone was similar in the two groups on day 16. No conclusive differences in progesterone between pregnant and nonpregnant cows have been described until after day 16.

Progesterone began decreasing about 5 to 6 days before estrus in cows. Lamond et al. (1971b) observed greatest progesterone in beef cattle 3.5 to 5 days before estrus. Maximum progesterone (about 11 ng/ml) during the first 75 days of pregnancy was attained by day 18 in heifers and by day 20 in cows, then progesterone decreased significantly ($P<.05$) about 20% during the next 4 or 5 days. This decrease in serum progesterone may represent the initial phases of luteal regression, terminated by the conceptus about day 22 to 25. Heifers had greater

progesterone ($P<.005$) during days 45 through 75 of pregnancy than during days 20 through 42, but this difference was not observed in cows.

Ovulation occurred 36±3 hr after HCG given 3 days after the last MGA feeding. Twenty-two of the 25 cows ovulated. But I recovered ova from only 31% of the cows at slaughter 4 days after HCG injection. Fertility appeared normal in the few cows that ova were recovered from. Wagner et al. (1968) and Hill et al. (1970) recovered a greater percentage of ova 3 to 3.5 days after insemination but fertility was decreased by progestogen treatment.

The ovulatory surge of LH which occurred 2 to 7 days after MGA withdrawal, was not altered by MGA treatment. Basal levels of LH on the last day of MGA treatment were similar to levels during proestrus. Hill et al. (1970) found higher LH during MGA treatment than in controls. In my experiments, serum LH after the synchronized estrus did not differ from control cows.

Estradiol was greater in pregnant and nonpregnant MGA treated cows than in control cows (Figures 10 & 11). On the last day of MGA treatment, 47% of the cows had greater than 10 pg/ml estradiol, similar to levels normally observed during proestrus. About 40% of the cows had estradiol greater than 35 pg/ml after MGA withdrawal.

The major endocrine abnormality I found after estrous cynchronization with MGA is a prolonged proestrus

period (possibly beginning during MGA treatment) typified by high serum concentration of estradiol after withdrawal. This elevated estradiol is accompanied by low progesterone, but this should not be a problem while the cow receives exogenous progestogen. To return a synchronized cow to the normal estrous endocrine state, it may be necessary to remove the influence of the excess serum estradiol; possibly with an antiestrogen to compete with estradiol for binding sites at target tissues.

SUMMARY AND CONCLUSIONS

Endocrine changes associated with early pregnancy and after nonfertile inseminations were studied in 28 heifers and 58 cows. Estrus was first observed in the morning in 76% of the heifers and fertility did not differ between forenoon and afternoon inseminations. An average of 1.33 inseminations were performed per conception.

Serum LH concentration was greatest on the day of estrus averaging 8.7±1.6 ng/ml in heifers that conceived and 12.0±3.2 ng/ml in nonpregnant heifers. There was little among heifer variation in LH and the concentration of LH did not differ ($P>.10$) from day 18 through day 75 of pregnancy. Pregnant heifers had lower serum LH ($P<.10$) during days 2 through 11 after insemination compared to nonpregnant heifers (1.0±.1 vs 1.2±.1 ng/ml, respectively).

Serum prolactin differed significantly ($P<.005$) among heifers. Average prolactin concentration ranged from 7±3 to 56±14 ng/ml for samples from individual heifers. Changes in prolactin could not be related to stage of estrous cycle or pregnancy. In contrast to LH, prolactin was greater ($P<.10$) in pregnant heifers (24±3 ng/ml) than in nonpregnant heifers (16±3 ng/ml) during the first 18 days of pregnancy.

Serum estradiol decreased from 12.6±2.4 pg/ml at estrus to 8.4±.6 pg/ml by day 4 of pregnancy. During days 7 through 75 of pregnancy, estradiol averaged 6 to 8 pg/ml except on day 40 (11.1±2.3 pg/ml) when four of the eight heifers had elevated estradiol. Increased estradiol concentration at day 40 of pregnancy may be related to growth of the conceptus.

Serum progesterone increased ($P<.005$) from 0.4 ng/ml at estrus to 6.8 ng/ml on day 11, and differed signigicantly ($P<.05$) among days 18, 20, and 22 of pregnancy (10.8±1.3, 9.2±.9 and 8.1±.9 ng/ml, respectively). This decrease in progesterone resembles the initial stages of luteal regression about day 18 of the estrous cycle. Possibly the conceptus terminates this luteal regression about day 22 of pregnancy. On day 18 of pregnancy, serum LH and progesterone were highly correlated ($r = .87$, $P<.01$). Progesterone was significantly ($P<.005$) greater during days 45 through 75 of pregnancy than from days 18 through 42, suggesting an extra-ovarian source of progesterone beginning about day 42.

The ovulatory surge of LH in synchronized heifers occurred 2 to 7 days after the last MGA feeding and appeared similar in magnitude and duration to LH peaks observed at control estrus. Serum prolactin was elevated ($P<.05$) on the day before or on the day of the LH peak.

Ovulation was synchronized by HCG injected 3 days after the last MGA feeding. Twenty-two of 25 cows ovulated 36±3 hr after HCG. Only 31% of the ova were recovered, but fertility appeared normal in cows from which ova were recovered.

Endocrine changes after estrous synchronization and insemination were studied in 58 cows. Serum LH was not influenced by genetic classification, pregnancy or MGA. The day by pregnancy interaction was significant ($P<.005$); pregnant and nonpregnant cows had similar LH during proestrus, LH was greater in pregnant cows at estrus, but LH was lower in pregnant than nonpregnant cows during days 2 through 25 after insemination.

Serum prolactin in cows was not affected significantly by genetic classification, pregnancy, MGA or HCG ($P>.10$). Prolactin concentration ranged from 20±4 ng/ml at estrus to 34±8 ng/ml on day 22 of pregnancy, but no meaningful physiological pattern of prolactin changes could be established.

Progesterone averaged 0.7 ng/ml on the last day of MGA treatment and only 3 of 32 cows had greater than 1 ng/ml. Serum progesterone increased significantly ($P<.01$) from 0.3±.1 ng/ml at estrus to 6.9±.6 ng/ml by day 11, both in MGA and in control cows. Progesterone did not differ ($P>.10$) between pregnant and nonpregnant cows during days 2 through 11 after insemination.

The MGA treated cows had greater serum estradiol than control cows (P = .15). During the estrous cycle of control cows, estradiol exceeded 10 pg/ml only at proestrus and estrus. On the last day of MGA treatment, 47% of the cows had estradiol concentration comparable to or greater than that during proestrus and estrus in controls. When this elevation in estradiol began during MGA treatment is unknown. After MGA withdrawal, serum estradiol increased to greater than 100 pg/ml in some cows.

Estradiol concentration was similar in pregnant and nonpregnant cows during days 2 through 11 after insemination. During pregnancy estradiol ranged from an average of 4.9±.6 pg/ml on day 20 to 8.6±3.9 pg/ml on day 42. Similar to heifers, 50% of the pregnant cows had elevated estradiol between days 30 and 42, possibly related to growth of the conceptus.

I conclude that a major endocrine abnormality after estrous synchronization with MGA is prolonged proestrus typified by high levels of estradiol, possibly beginning during MGA treatment and persisting for 2 to 7 days after MGA withdrawal.

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APPENDICES

TABLE I.--Preparation of liquid scintillation fluids.

A. Steroid scintillation fluid (Hafs and Armstrong, 1968).

Naphthalene	480 g
PPO	30 g
POPOP	0.3 g
Xylene	2000 ml
p-dioxane	2000 ml

Mix until dissolved.

B. Bray's solution (Bray, 1960)

Naphthalene	240 g
PPO	16 g
Dimethyl POPOP	0.8 g
Ethylene Glycol	80 ml
Methanol	400 ml
p-dioxane	3264 ml

Mix until dissolved.

TABLE II.-- **Composition of buffers used in estradiol assays.**

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

Tris	1.211 g
disodium EDTA	0.372 g
Sucrose	85.85 g

Dissolve the reagents in 800 ml distilled water and adjust the pH to 8.0 with 6 N HCl. Then dilute to 1 liter. Store buffer at -20 C in quantities sufficient for single assays.

B. Buffer AA.
(0.1M phosphate, 0.15M sodium chloride, 0.015M sodium azide, 0.1% gelatin, pH 7.0).

Sodium phosphate, monobasic	5.38 g
Sodium phosphate, dibasic, heptahydrate	16.35 g
Sodium chloride	9.0 g
Sodium azide	1.0 g
Gelatin (Knox Gelatin, Inc., Johnston, N. Y.)	1.0 g

Dissolve the reagents in distilled water and dilute to 1 liter. Store buffer at 5 C for up to 1 month.

TABLE III.--Fertility of cattle after estrous synchronization with progestogens.

Progestogen[a]	Days treated	Synchronized[b]	Treated Animals		Control Animals		Reference
			Number	Fertility	Number	Fertility	
	(no)	(%)		(%)		(%)	
MAP	11-18[c]	95	19	26	21	81	Fahning, et al., 1966
MAP	18	87	31	39	16	19	Dhindsa, et al., 1967
MAP	18	55	88	31	44	43	" " "
MAP	18	87	15	7	15	47	Drewry, et al., 1968
MAP	18	94	32	59	32	63	" " "
DHPA[d]	9	95	66	54	33	52	Wiltbank and Kasson, 1968
DHPA[d]	9	74	90	60	25	83	" " "
MGA	18	71	24	21	24	33	Tripathi and Howell, 1969
DHPA	9	55	55	24	25	60	" " "
MGA	18	64	28	11	25	60	" " "
MGA	18	NA[e]	26	35	26	31	Boyd, 1970
MAP	18	NA[e]	48	52	53	57	Spahr, et al., 1970
Nor[d]	9	93	14	61	33	65	Wiltbank, et al., 1971
Nor	16	87	15	38	33	65	" " "
Nor[d]	9	100	56	34	49	55	" " "
Nor[d]	9	100	21	10	19	44	" " "
Nor[d]	9	77	17	54	15	67	" " "
Nor[d]	9	94	16	67	14	75	" " "

[a]DHPA (dihydroxyprogesterone acetophenide, E. R. Squibb and Sons)
MAP (medroxyprogesterone acetate, The Upjohn Co.)
MGA (melengestrol acetate, The Upjohn Co.)
Nor (17-ethyl-19-norestosterone, G. D. Searle Co.)
[b]Estrous occurred within 7 days after progestogen withdrawal.
[c]Treatment started on day 14 of the estrous cycle.
[d]Estradiol valerate given with the progestogen.
[e]Information not available.

TABLE IV.--Serum LH and prolactin in pregnant and nonpregnant heifers.

Days after estrus	LH				Prolactin			
	Pregnant		Nonpregnant		Pregnant		Nonpregnant	
	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)
0[a]	24	8.7±1.6[b]	10	12.0±3.2	23	23±5	10	20±4
2	26	0.9±0	10	1.2±0.4	26	16±4	9	11±2
4	24	1.2±0.1	10	1.4±0.3	21	29±6	11	11±2
7	26	0.8±0.1	10	1.1±0.2	23	20±5	11	12±3
11	25	1.0±0.2	11	1.2±0.2	25	30±6	11	28±10
18	26	0.8±0.1	11	2.0±0.4	24	21±4	11	14±4
20	26	1.0±0.1			25	31±9		
22	22	0.8±0.1			20	24±7		
25	26	1.0±0.1			26	21±5		
30	26	1.0±0.2			25	24±6		
35	26	0.9±0.1			24	22±4		
40	26	1.0±0.2			24	15±3		
42	23	1.0±0.1			22	19±4		
45	26	0.7±0.1			21	17±3		
50	26	0.8±0.1			22	27±6		
60	25	0.8±0.1			26	16±5		
63	26	0.9±0.1			24	20±5		
75	26	0.6±0			22	11±2		

[a]Day of estrus
[b]Mean + SE

TABLE V.--Serum estradiol and progesterone in heifers during early pregnancy.

Days after estrus	Estradiol (n)	Estradiol (pg/ml)	Progesterone (n)	Progesterone (ng/ml)
0[a]	8	12.7±2.4[b]	8	0.4±0.1
2	8	11.8±2.4	8	0.4±0.2
4	8	8.4±0.6	8	1.1±0.2
7	8	6.9±0.8	8	5.7±0.9
11	8	5.5±0.7	8	6.8±0.4
18	8	6.2±0.5	8	10.8±1.3
20	7	7.2±0.6	8	9.2±0.9
22	7	7.0±0.3	7	8.1±0.9
25	8	6.9±0.7	8	8.6±0.9
30	8	7.2±0.8	8	9.7±1.1
35	6	7.0±0.4	6	11.3±1.6
40	8	11.1±2.3	8	11.0±1.1
42	8	6.2±0.7	8	10.2±0.9
45	8	8.3±0.9	8	10.6±1.1
50	7	7.8±1.5	7	11.0±0.9
60	8	7.8±0.7	8	10.8±1.0
63	8	7.3±0.8	8	12.8±1.1
75	8	8.6±1.3	7	10.6±0.8

[a]Day of estrus
[b]Mean + SE

TABLE VI.--Some within day correlations between pituitary and ovarian hormones in blood serum during early pregnancy in heifers.

Days after estrus	LH vs progesterone	LH vs estradiol	Prolactin vs progesterone	Prolactin vs estradiol	Estradiol vs progesterone
0	-.21 (8)[a]	.01 (8)	-.15 (8)	.36 (8)	.47 (8)
2	-.22 (8)	.63 (8)	.63 (8)	-.52 (8)	-.15 (8)
4	.04 (8)	-.12 (8)	.94 (5)*	.58 (5)	.67 (8)
7	.50 (8)	.02 (8)	.00 (8)	.52 (8)	.29 (8)
11	.31 (8)	-.26 (8)	-.70 (8)	-.62 (8)	.28 (8)
18	.87 (8)**	.22 (8)	.41 (7)	.36 (7)	.25 (8)
20	.26 (8)	.44 (7)	-.38 (7)	.62 (6)	-.48 (7)
22	-.42 (7)	.77 (7)	-.38 (5)	-.46 (5)	-.26 (7)
25	.54 (8)	.24 (8)	.66 (8)	.00 (8)	-.18 (8)
30	.72 (8)*	-.10 (8)	.21 (8)	-.41 (8)	.00 (8)
35	-.06 (6)	-.37 (6)	-.75 (5)	.28 (5)	-.06 (6)
40	.66 (8)	.26 (8)	-.78 (8)	-.40 (8)	-.13 (8)
42	-.08 (8)	-.34 (8)	-.12 (8)	.47 (8)	-.30 (8)
45	-.04 (8)	.60 (8)	-.40 (7)	.57 (7)	-.23 (8)
50	.45 (7)	.28 (7)	.63 (5)	.38 (5)	-.07 (7)
60	.52 (8)	.25 (8)	.38 (8)	.15 (8)	-.37 (8)
63	.41 (8)	-.58 (8)	-.36 (7)	.45 (7)	-.64 (8)
75	.57 (7)	.26 (8)	-.64 (4)	.66 (5)	-.20 (7)

[a]Numbers in parentheses refer to number of observations.
*P < .05
**P < .01

TABLE VII.--Serum LH and prolactin during early pregnancy in heifers carrying female or male fetuses.

Days after estrus	LH				Prolactin			
	Female fetus		Male fetus		Female fetus		Male fetus	
	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)	(n)	(ng/ml)
0[a]	15	6.1±1.5[b]**	8	14.6±3.3	13	17±3	9	33±11
2	16	0.9±0.1	9	0.9±0.1	16	20±6	9	9±2
4	14	1.2±0.2	9	1.0±0.1	12	28±7	8	22±10
7	16	0.8±0.1	9	0.8±0.2	14	19±5	9	21±10
11	15	1.0±0.3	9	0.9±0.2	15	37±8	9	24±8
18	16	0.9±0.1*	9	0.7±0.1	14	18±5	9	27±8
20	16	1.0±0.2	9	0.8±0.2	16	37±12	8	20±11
22	13	0.7±0.1	8	0.8±0.1	12	19±7	7	34±18
25	16	0.9±0.2	9	1.2±0.2	16	22±6	9	20±7
30	16	1.1±0.3	9	0.7±0.2	15	24±8	9	28±10
35	16	1.0±0.2*	9	0.6±0.1	14	28±6	9	14±4
40	16	1.0±0.2	9	1.0±0.4	14	16±4	9	14±5
42	15	1.1±0.2	7	0.6±0.1	15	16±3	7	26±10
45	16	0.8±0.1	9	0.6±0.1	12	17±2	8	16±8
50	16	0.8±0.1	9	0.6±0.1	14	29±7	7	19±6
60	16	0.8±0.1	8	0.7±0.1	16	14±3	9	22±12
63	16	0.8±0.1	9	1.0±0.2	14	22±7	9	16±6
75	16	0.6±0.1	9	0.6±0.1	14	13±3	7	9±3

*Significantly ($P < .10$) different from that in cows with male fetuses.
**Significantly ($P < .01$) different from that in cows with male fetuses.

[a]Day of estrus.

[b]Mean ± SE.

TABLE VIII.--Split plot analysis of luteinizing hormone for Experiment IV.

Source	df	Mean Square	Probability (type I)
Between cows	(46)		
Genetic (G)	1	15.38	ns
Pregnancy (P)	1	0.13	ns
MGA (M)	1	0.33	ns
HCG (H)	1	43.78	P<.05
GP	1	51.37	P<.05
GM	1	3.09	ns
GH	1	38.90	P<.10
PM	1	34.79	P<.10
PH	1	19.89	ns
MH	1	29.06	P<.10
GPM	1	26.59	ns
GPH	1	93.34	P<.005
GMH	1	-16.02	ns
PMH	1	18.39	ns
GPMH	1	-67.44	ns
Cows / GPMH	31	9.73	
Within cows	(461)		
Day	32	26.51	ns
DG	31	11.42	ns
DP	14	64.30	P<.005
DM	31	4.75	ns
DH	30	12.87	ns
DGP	11	80.97	P<.005
DGM	22	- 0.18	ns
DGH	26	15.49	ns
DPM	10	40.37	P<.05
DPH	11	53.00	P<.01
DMH	23	9.38	ns
Remainder (error)	220	21.83	

TABLE IX.--Split plot analysis of luteinizing hormone for cows in treatments I and II of Experiment IV.

Source	df	Mean square	Probability (type I)
Between cows	(21)		
Pregnancy (P)	1	21.91	P<.01
HCG (H)	1	4.90	ns
PH	1	- 2.18	ns
Cows/PH	18	2.26	
Within cows	(227)		
Day (D)	31	3.84	ns
DP	10	2.19	ns
DPH	9	2.34	ns
DH	25	1.28	ns
Remainder (error)	152	2.98	