OVARIAN STEROID PLASMA CONCENTRATIONS IN THE NORMAL BOVINE ESTROUS CYCLE

by

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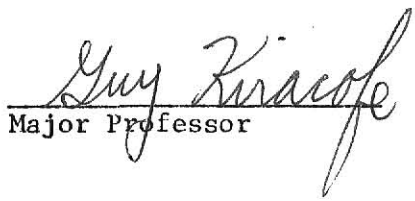

Major Professor

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INTRODUCTION

Ovarian steroids have long been implicated in the physiological processes of growth, development and reproduction. The gross effects of ovarian hormones were established by the classical approach of ablation and replacement techniques. With the advancement of methodology applied to ovarian hormone detection, basic information and interactions began to be assimilated.

Edgar (1953) definitely identified progesterone in the bovine corpus luteum and Gorski (1958) subsequently isolated progesterone from human ovaries, bovine corpus luteum, and adrenals of pregnant cows.

Estrogens have been isolated from follicular fluids (Short, 1962), bovine placenta (Veenhuizen, et al., 1960) and in milk (Lunas, 1963).

Knowledge of ovarian hormones present in the peripheral circulation is essential to complete understanding of the bovine estrous cycle. Plasma levels of progesterone were reported by Short (1957) and Schomberg et al. (1967). Blood levels of estrogen were reported by Szego and Roberts (1946), Saba (1964), and Pope et al. (1965).

Until recently it had not been feasible to measure ovarian steroid hormones on a routine basis. The development of gas liquid chromatography (Stabenfeldt, 1965) and more recently competitive protein binding (CPB) (Murphy, 1969; Korenman, 1969) and radioimmunoassay (RIA) (Abraham, 1969), permitted a re-examination of ovarian hormones present during the estrous cycle.

Progesterone concentrations in bovine blood have been reported by Stabenfeldt (1965), Plotka et al. (1967), Hansel and Echternkamp (1972), Shemesh et al. (1972) and Sprague et al. (1971). Estrogen concentrations

have been reported by Henricks (1971), Shemesh et al. (1972), Wettemann et al. (1970) and Lemon et al. (1975).

Inconsistencies in absolute concentrations of steroid hormones due to laboratory and animal variation have been observed. The purpose of this study was to establish the ovarian hormone concentrations in the apparently normal dairy cow in the Kansas State University Dairy Herd. This study would then serve as a standard for future ovarian hormone investigations.

REVIEW OF LITERATURE

Progesterone

Early research considering progesterone secretion and metabolism was hampered by the lack of accurate chemical techniques sensitive and specific to facilitate the quantitation of the hormone in small plasma samples. Investigators hoping to estimate progesterone levels extracted the steroids from tissues collected clinically or at slaughter (Gomes et al., 1963; Erb and Stormshak, 1961), then attempted to purify the progesterone by chromatographic or selective extraction techniques.

Certain biological assays are reasonably sensitive but lack specificity and have undefined endpoints (Edgar et al., 1959; Miyake, 1962). Such assays were based on such undefinable end points as complete progestational proliferation, uterine granulose-mucosa response, carbonic anhydrase activity, uterine deciduoma, pregnancy maintenance, or parturition delay all of which lack specificity and sensitivity and are extremely time consuming. Local progestational tests designed by McGinty and Hooker, and Forbes are the most sensitive bioassays developed, but these tests also utilized subjective endpoints (Pearlman, 1960; Zarrow, 1964). Bioassays, now replaced largely by chemical methods, played a vital role in progestin research in establishing animal responses in assessment of activity in crude extracts.

Numerous chemical methods have been applied to determination of progesterone in biological tissues. Extraction of steroids from tissues and fluids has generally been accomplished by the use of organic solvents or mixtures of solvents (Zander, 1962). Difficulties involved in solvent extraction include the great quantity of lipid extracted. Zaffaroni et al.

(1953) modified the previously used dialysis procedure and reported 100% recovery of progesterone ^{14}C added to the blood.

Separation of steroids has been successfully accomplished by the application of counter-current distribution (Loy et al., 1960). Column chromatography (Wilson et al., 1958), and thin layer chromatography (TLC) have been used extensively in separation of steroids due to its speed (20-40 min.), distinct resolution (5-500 µg), and the noticeably sharp separation of compounds (Hara et al., 1964).

Zarrow et al. (1957) confirmed that the Δ^4-ene-3-one grouping on the steroid nucleus must be present for progestational activity. This group can be quantitated by its strong absorbence at 240 mµ in alcoholic solutions (Bush, 1961). Although this group is not specific for progesterone, ultraviolet absorption can be used to establish presence and for quantification if suitable separation and purification has been accomplished. Wiest (1959) measured the absorption of progesterone at 240 mµ by direct spectro-photometric scanning of the paper chromatogram.

Other spectro-photometric assays are based on the absorbance of ultraviolet light at wave lengths other than 240 mµ by progesterone derivatives (Zander, 1962). These methods have the advantage of shifting the quantification wave length away from that of interferring materials, but lack specificity and make difficult the identification of isolated material as progesterone.

Fluorometric procedures have been used since 1948 for the quantitation of steroid estrogens (Jailer, 1948); however progesterone exhibits less than 1% as much fluorescence as is shown by estradiol in sulfuric acid (Touchstone and Muravec, 1960). The fluorescence peak could be increased 100-fold (Touchstone and Murawec, 1960) by treating the progesterone with

methanolic potassium hydroxide. By the use of a microcell adaptation of this method, Short and Levitt (1962) were able to detect 0.05 µg of progesterone standard. When samples assayed by this method and the spectrophotometric method of Short and Levitt (1962) were compared, the fluorescence results tended to be higher at low concentrations of progesterone and lower at high concentrations, thus prompting questions as to the specificity of the system reaction.

In 1960, gas chromatography was first applied to steroid separation and qualitative identification (Patti and Stein, 1964). Yannone et al. (1964) reported a highly sensitive gas chromatograph for progesterone with sensitivity to less than 0.02 µg. The development of gas liquid chromatography (GLC) increased the sensitivity, precision and reproducibility of the assay (Van der Molen and Groen, 1965).

The introduction of isotopic reagents for the estimation of progesterone overcame the problem of inadequate sensitivity of conventional chemical methods. The development of sensitive quantitative assays such as double-isotope derivation (Woolever and Goldfein, 1963), competitive protein binding (Murphy, 1967 1969; Neill, 1967), radioimmunoassay (Abraham, 1969) and gas liquid chromatography made possible the study of corpus luteum function in vivo by determining even the lowest progesterone levels in the peripheral plasma.

Edgar (1953) was the first to definitely identify progesterone in the bovine corpus luteum. Following gonadotropin treatment of a calf, he isolated 20 µg of progesterone from 60g of luteal tissue. Gorski et al. (1958) reported the isolation of progesterone and 20β-hydroxy-Δ^4-pregnene-3-one from human ovaries and subsequently reported progesterone present in corpus lutea, ovaries and adrenals of pregnant cows.

Bowerman and Melampy (1962) and Mares et al. (1962) showed that in

beef and dairy cows the weight and progesterone content of corpora lutea is greatest on days 15 to 16 of the estrous cycle. Erb et al. (1971) summarized data on corpora lutea size and progesterone content. The corpus luteum of a cow grows rapidly from days 3 to 6 (day 0 = estrus), slowly on days 7 to 8, followed by an increase (day 13 to 15), and then a 70% decline in weight from days 17 to 20. The luteal content of progesterone follows these changes in weight remarkably well, though luteal function declines in 2 days as compared to 4 days for morphological regression based on decline in corpora luteal weight.

Christensen et al. (1974) reported higher levels of progestins in the three cycles of a 12 year old cow than was obtained from the younger cows (4 years), suggesting that the younger cows may have smaller corpora lutea.

Short (1957, 1961) reported single determinations in each of three cows as 4.0 ng/ml plasma 24 hours before ovulation, 3.8 ng/ml 12 days after ovulation and 9.4 ng/ml during the luteal phase. Schomberg, Courdet, and Short (1957) obtained an average of 3.0, 4.8, 9.6, and 1.5 ng/ml on days 6, 11, 16 and 21 respectively of the bovine estrous cycle. Gupta and Pope (1968) reported progesterone concentrations in the cow varied from 1-2 ng/ml around estrus to 7.5-10.0 ng/ml during the luteal phase. Stabenfeldt et al. (1969) reported luteal phase values (5.2 to 7.9 ng/ml) which are in reasonable agreement with the previously mentioned reports. Plotka et al. (1967) recorded higher values (25 ng/ml) at peak luteal function.

Hansel and Echternkamp (1972) collected plasma from 30 Holstein heifers (2-3 years) and mature cows (3-5 years) throughout the estrous cycle. Progesterone determinations were made by sequential thin layer

chromatography and gas liquid chromatography techniques (Kazama and Hansel, 1970; Snook et al., 1971) and by a modification of competitive protein binding technique of Murphy (1969). They found that progesterone concentrations within jugular plasma on the day 0, 1, and 2 were < 0.25 ng/ml. Then the level rose rapidly from days 4 to a peak of 4.5 ng/ml on day 12, remaining relatively constant until about day 16. The progesterone level then declined rapidly between days -4 and -2 of the cycle (Fig. 1).

Stabenfeldt et al. (1969) reported similar determinations during the bovine estrous cycle. They found progesterone levels in six cows ranged from 0.5 ng/ml plasma during the follicular phase to an average of 6.6 ng/ml at peak luteal phase (Fig. 1). Pope, Gupta and Monroe (1969) reported similar findings during follicular phase (< 2 ng/ml) but 9.0 mg/ml during peak luteal function. Shemesh, Linder and Ayalon (1971) reported 0.7 ng/ml from day -2 to +2, and a mean peak value of 5.4 ng/ml during luteal stage. Donaldson, Bassett and Thorburn (1970) measured progesterone concentrations in peripheral plasma by protein-binding radioassay method. They reported the mean concentration to be lowest at estrus (0.44 ng/ml) and then increase to a maximum of 6.8 ng/ml about day 14. Sprague et al. (1971) measured progesterone, by protein binding method, in peripheral blood of cycling beef cows and determined that progesterone was declining at estrus, reached a low point (1.4 ng/ml) 8 to 20 hours after the LH peak, then increased from days 2 to 3 (3 ng/ml), declined on day 4 (1.9 ng/ml), increased to its highest values at day 8-11 after estrus (3.9 ng/ml), then declined before the next estrus. Similar values were reported by Gupta and Pope (1968), Stabenfeldt et al. (1969) and Snook et al (1971).

Henricks et al (1971) collected blood samples from Angus and Hereford heifers and from non-lactating dairy cows during proestrus and estrus stages.

They reported progesterone levels were highest (5-12 ng/ml) 4 days prior to estrus, then decreased progressively to barely detectable levels on the day prior to estrus (Fig. 1). Plotka et al. (1967) determined higher values (10 ng/ml) by the use of double isotope derivative assay, as did Gupta and Pope (1968), during the same time period.

Ayalon and Shemesh (1974) reported a proestrus surge of progesterone concentration in the cow similar to pre-ovulatory progesterone rises reported in women, monkeys, rats and rabbits (Kazama and Hansel, 1970). They reported a peak in plasma progesterone (2.28± 0.32 ng/ml) occurring approximately 16 hours before the onset of estrus (Fig. 1). They attributed this proestrus surge to adrenal origin. In contrast Dobrowolski et al. (1968) measured ovarian vein progesterone concentrations and reported that lowest values are around estrus. McCracken (1963) measured very low levels of plasma progesterone in ovarectomized cows. By contrast, Lemon et al. (1975) collected blood every two hours from day 18 through estrus, but was unable to detect a proestrus or pre-ovulatory progesterone surge. Katongole, Naftolin, and Younglai (1973) observed what appears to be fluctuations in progesterone levels during estrus. It is doubtful whether these changes are significant since all the values were close to the limit of sensitivity of the method. No pre-ovulatory rise in progesterone was noted.

Henricks, Dickey and Niswender (1970) reported progesterone concentrations were higher from day 10 to 14 in pregnant cows than in non-pregnant cows. Shemesh, Ayalon, and Linder (1968) reported that progesterone concentrations were similar from days 10 to 18 after ovulation in cycling cows and in inseminated cows whether pregnant or non-pregnant. They noted that a striking difference between pregnant and non-pregnant cows occurred on the 19th day after ovulation. Progesterone levels were very low in the non-pregnant in contrast to the maintained high levels in pregnant cows.

Arije et al. (1974) reported progesterone concentrations were fairly constant (6 ng/ml) on the day of parturition and declined to 1.6 ng/ml the day after, fluctuated between 0.2 and 2.0 ng/ml to 3 days before estrus and then fell to 0.2 ng/ml at estrus. Similar observations have been reported by Pope et al. (1969), Donaldson et al. (1970), Henricks et al. (1971), and Erb et al. (1971).

EXPLANATION OF FIGURE 1

AYALON: Blood was collected from nine lactating Holstein cows daily between 7:30 a.m. and 8:30 a.m. In addition, starting 24 hr. before the expected onset of estrus, blood collections were carried out at 4 hr. intervals until the time of ovulation. Progesterone levels in plasma were determined by competitive protein-binding assay.

HANSEL: Blood was collected from 30 Holstein heifers (2-3 yr. of age) and mature cows (3-5 yr. of age). Progesterone determinations were made by sequential thin layer, gas-liquid chromatography, and competitive protein binding techniques.

HENRICKS: Five Angus and Hereford heifers were bleed twice a day four days prior to estrus. Plasma progesterone was assayed using competitive protein binding techniques.

STABENFELDT: Blood was obtained from five Holstein cows daily between 8:00 a.m. and 10:00 a.m., for one complete estrous cycle. Plasma progesterone was determined by a modified gas liquid chromatography technique.

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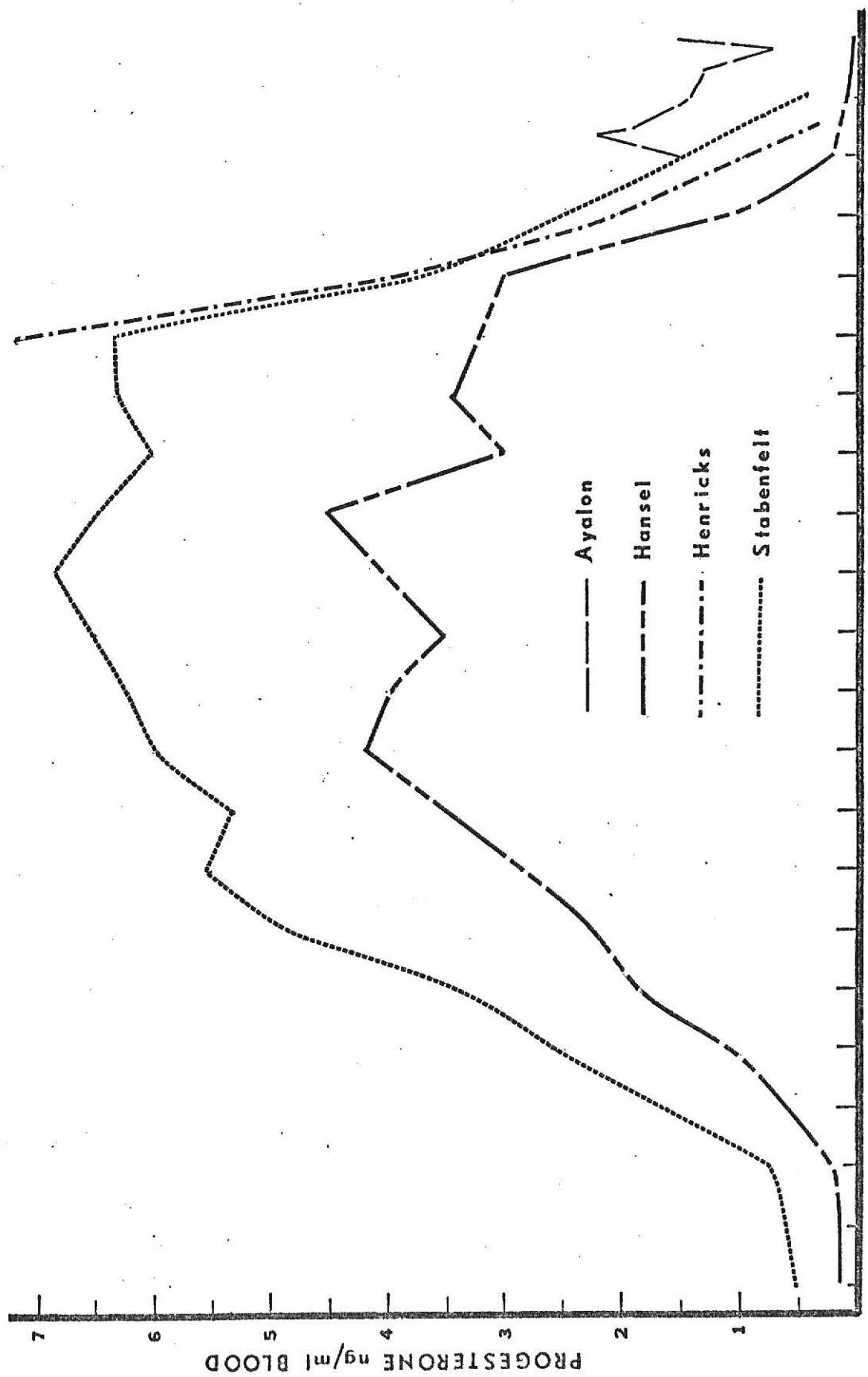

Figure 1. Changes in plasma concentrations during the bovine estrous cycle.

Estrogen

Estrogenic substances are continuously implicated as an important factor of metabolic mechanisms concerning growth, development, and reproductive performance. Gross biological responses to estrogens have been established by the classical technique of replacement therapy following ablation of known sources of endogenous hormones. A number of investigators have examined estrogenic activity in tissues, body fluids, secretions and excretions (Mellin and Erb, 1965).

It is difficult to compare quantitations of estrogens especially from older literature, because assay procedures have continuously improved in sensitivity, specificity and reliability. Early investigations of estrogenic activity via bioassays were hampered by variations of end point measurements, species, strain within a species of test animal and presences of augmenting or inhibiting substances present in unknown extracts (Pincus, 1950; Martin, 1959). Bioassay methods measured total estrogenic activity, usually in relatively impure extracts, and gave no indication of the specific compounds involved. Chemical methods have largely replaced bioassays but are still variable because extraction and measurement procedures are not standardized and a high degree of purification and separation must be attained to achieve reliable quantitative estimates. Chemical methods have been developed for the assay of estrogens in bovine placenta (Veenhuizen et al., 1960), follicular fluids (Short, 1962) and milk (Lunnas, 1963), but these lack the sensitivity for accurate measurements that have been developed for determining estrogens in blood. Although the chemical tests were not totally reliable, preliminary investigations provided substantial foundation for presence and potential effects of estrogens.

Mellin and Erb (1965) documented the presence of estrogens in the ovaries, blood, amniotic and allantoic fluids, placentae, lacteal secretions,

bile, adrenals, urine and feces of the cow. Estrone, estradiol-17α and estradiol-17β have been identified from bovine urine by the use of ^{14}C (Mellin and Erb, 1966; Velle, 1963). The results of these investigations established the outline of the biosynthesis, metabolic and excretory patterns for estrogens in the bovine.

To establish a better understanding of estrogen secretion, metabolism and effect upon reproductive performance, a knowledge of estrogen concentrations in cow blood is necessary. Szego and Roberts (1946) conducted preliminary investigations of circulating estrogens in blood of pregnant cows. They precipitated blood proteins with acetone, then treated supernatant and precipitate fractions separately. The total estrogen content of blood was established to be approximately 0.5 μg/100 ml of β-estradiol equivalents. Two-thirds of the total estrogen was closely associated with the protein fraction. The remaining one-third existed mainly in a hydrophilic form not conjugated with protein.

Saba (1964) measured total estrogenic activity in plasma of pregnant cows after acid hydrolysis (Preedy and Aiken, 1957) but was unable to detect estrogenic activity until the last month of pregnancy. During the last ten days of pregnancy, estrogen was detected in all samples. Estrogen content dropped rapidly after parturition and no estrogen was detected one to ten days post-partum. The highest estrogenic activity observed before parturition was equivalent to 0.2 μg estradiol per 100 ml plasma.

Extracts of jugular vein blood of pregnant cows and of cows in estrus (Pope et al., 1965) was treated by methanolic HCl to prepare phenolic fractions. By the use of paper chromatography, acetone treatments, the Roy and Brown (1960) extraction method, and boiling methanol extraction, they were able to detect an estrogenic activity equivalent to 1 to 10 ng estrone per liter in the blood of cows at estrus up to 7 μg per liter in late preg-

nancy. The estimates of estrogen in blood of cows in late pregnancy by means of the acetone extraction method are in reasonably good agreement with those of Szego and Roberts (1946). The estrogen concentration estimates in the blood of cows in late pregnancy are also in reasonably good agreement with those of Saba (1964).

These preliminary investigations furnished insight to the extremely small quantities of estrogen present in blood. The use of bioassays in conjunction with chemical methods provided a means of obtaining estimates, but have their deficiencies as mentioned earlier. The large quantities of blood needed for analysis and the number of laboratory hours required rendered routine examination of blood estrogen impractical.

With the advent of more sophisticated quantitative techniques, such as competitive protein binding (CPB) (Korenman et al., 1969) and radioimmunoassay (RIA) (Abraham, 1969), it became feasible to measure blood estrogens on a routine basis. Lower limits of estrogen concentrations which were undetectable by bioassay could now be monitored. Estrogen levels determined by RIA and CPB have been reported for the human during the menstrual cycle (Korenman et al., 1969; Abraham, 1969), in sheep (Scaramuzzi, 1970), and in the cow through the estrous cycle (Henricks et al., 1971; Shemesh et al., 1972).

Henricks et al. (1971) measured total plasma estrogen concentrations by radioimmunoassay (Tillson et al., 1970), for the periods of proestrus and estrus. In Trial I, five Angus and five Hereford heifers were bled twice daily for four days prior to estrus. In all heifers, the estrogen level fluctuated within a range of 0.5 to 10 pg/ml for the first 3 days of the 4 day period prior to estrus, then reached a major peak (15 to 25 pg/ml) on the day prior to and during the early part of estrus. Trial II consisted

of four nonlactating dairy cows, which were bled once each day on the two days prior to estrus and at 2 to 4 hour intervals four or five times beginning with the onset of estrus. Estrogen levels prior to estrus and during estrus were as high as the values obtained for the heifers in Trial I. The maximum level detected was 30 pg/ml during the first part of active estrus (Fig. 2).

Shemesh et al. (1972) determined estradiol changes by competitive protein binding assay in peripheral plasma of nine lactating Holstein cows with a normal cycle length (19 to 23 days). Blood samples (100 to 200 ml) were collected once daily through most of the cycle but one day before the expected onset of estrus, blood was taken every four hours until ovulation, which was determined by rectal palpation. Estradiol concentrations gradually increased from 1.5 to 7.5 pg/ml during the 3 days preceding estrus and showed a sharp peak (17 pg/ml) about 4 hours before the onset of estrus. At the time estrual behavior was observed, the estradiol level had begun to decline and reached its nadir (0.8 pg/ml) 12 hours later. A minor rise was noted on day 4 of the cycle and a more substantial increase on days 10 to 13, with an average of peaks on day 11 (8.1 pg/ml) (Fig. 2).

Estradiol concentrations present in blood sera was determined by Wetteman et al. (1972) in eleven Holstein heifers by RIA and CPB (Fig. 2). Blood was obtained by jugular puncture when the heifer was first observed in estrus (day 0) and at 8:00 a.m. on days 2, 7, 11 and daily from day 18 until estrus during their fourth and seventh estrous cycles. Estradiol was quantified in one set of sera by RIA and in a different set by protein binding assay. By RIA, estradiol was lowest (3.0 pg/ml) on day 2 and relatively constant during the luteal phase of the estrual cycle, averaging about 3.6 pg/ml from days 2 through 11. Estradiol then increased to 4.8

pg/ml at 3 days before estrus and continued to increase to 9.7 pg/ml 0.5 days before estrus. During estrus, estradiol remained high (8.4 pg/ml). Values obtained from protein binding assay resembled those determined by RIA, but RIA was more repeatable and had lower blank values. Similar data has been reported by Echternkamp and Hansel (1971). Christensen et al. (1974) reported highest total estrogen (176 pg/ml) prior to the onset of estrus.

Lemon et al. (1975) paid particular attention to the onset and duration of estrus in four cycling multi-parous Holstein cows (Fig. 2). Blood samples were collected from the jugular vein at intervals of 2 hours for a period of five days starting on day 18 of the cycle through estrus (day 0). Estradiol-17 was determined by RIA (Terqui et al., 1973). No clearcut estradiol peak was noted before the onset of estrus, but a series of peaks of fluctuating concentrations occurred from day 2 to onset of estrus with an overall increase in their maxima to approximately 25 pg/ml. During estrus, estradiol-17 levels decreased to an average of 13 pg/ml and continued to decrease on day 1 of the estrous cycle.

Katongole et al. (1973) measured diurnal variations in ovarian steroids in six heifers at estrus by RIA. Blood plasma samples (20 ml) were collected daily by jugular venopuncture for four days prior to onset of estrus, then when estrus was detected, indwelling polyethylene catheters were inserted into a jugular vein, and samples collected every hour on the hour. They reported that the highest estrogen level occurred during the day prior to estrus. There seemed to be fluctuations in estrogen secretion which were more marked in the cow exposed to a bull between 2:00 p.m. and 4:00 p.m. on the day of estrus. The fluctuations were observed at 10:00 p.m. and 6:00 a.m. the next day, and may not be significant.

Glencross et al. (1973) observed that plasma estradiol-17-β concentra-

tions rose slowly during late progestinal phase of the bovine estrous cycle, then rose rapidly to a pre-estrus peak of about 6 pg/ml. The estradiol-17-β level fell during estrus and reached a minimum on days 1 and 2 of the cycle. These investigators also measured estradiol-17-β in the cow during the first 30 days of pregnancy. The pattern of estradiol-17-β was similar to that expected had conception failed. Estradiol-17-β peaks were noted at 21 day intervals each followed by a smaller peak 6 or 7 days later.

Henricks et al. (1971) measured estrogen concentrations for the first 39 days of pregnancy. The mean estrogen concentration increased from 2.5 pg/ml on day 3 to 4.5 pg/ml on day 12. From day 12 through day 39 the mean concentration ranged between 3.7 and 4.8 pg/ml.

The review of literature concerning peripheral blood estrogen concentration indicates that estrogen is at its highest level during or just prior to the onset of estrus, begins to decline during estrus, and reaches a minimum level 1 to 2 days after estrus.

Arije, Wiltbank and Hopwood (1974) reported estrogen levels in postparturient cows. Their data show estrogen values decreased to 500 pg/ml at parturition and decreased postpartum to about 200 pg/ml. Estrogen values peaked (500 pg/ml) two days prior to estrus. During the cycle estrogens fluctuated between 50 and 500 pg/ml with a high on day 5.

Echternkamp and Hansel (1971) reported mean plasma concentrations for estrone and estradiol were 4653 and 7549 pg/ml respectively on the day of parturition. The mean estradiol concentration at estrus was 809 pg/ml while estrone was 254 pg/100 ml. Estradiol values for 3 days before and 3 days after estrus were 147, 422, 546, 179, 176, and 169 pg/100 ml. Estrone concentrations were 98, 147, 162, 128, 95 and 111 pg/100 ml. Similar data has been reported by Henricks et al. (1971), Hansel and Echternkamp (1972), and Wettemann and Hafs (1973).

EXPLANATION OF FIGURE 2

HENRICKS: Five Angus and Hereford heifers were bled twice a day from four days prior to estrus. Four nonlactating dairy cows were bled once every day on the 2 days prior to estrus and at 2 to 4 hr. intervals four or five times beginning with the onset of estrus. Estrogen was determined in plasma samples by RIA.

LEMON: Blood was collected from four cyclic multi-parous Holstein cows, at intervals at 2 hr. for a period of 5 days, starting on Day 18 of the cycle (day of estrus = Day 0). Daily blood collections were made for several days before this and until Day 10 of the following cycle. Estradiol-17-β was measured from plasma samples by RIA.

SHEMESH: Blood was collected from nine lactating Holstein cows daily between 7:30 and 8:30 a.m. In addition, starting 24 hr. before the expected onset of estrus, blood collections were carried out at 4 hr. intervals until the time of ovulation. Estradiol levels in plasma were determined by competitive protein-binding assay.

WETTEMANN: Blood was obtained from eleven Holstein heifers when first observed in estrus (day 0) and at 8 a.m. on days 2, 4, 7, 11 and daily from day 18 until estrus during their fourth and seventh estrous cycles. Serum levels of estradiol were determined by RIA.

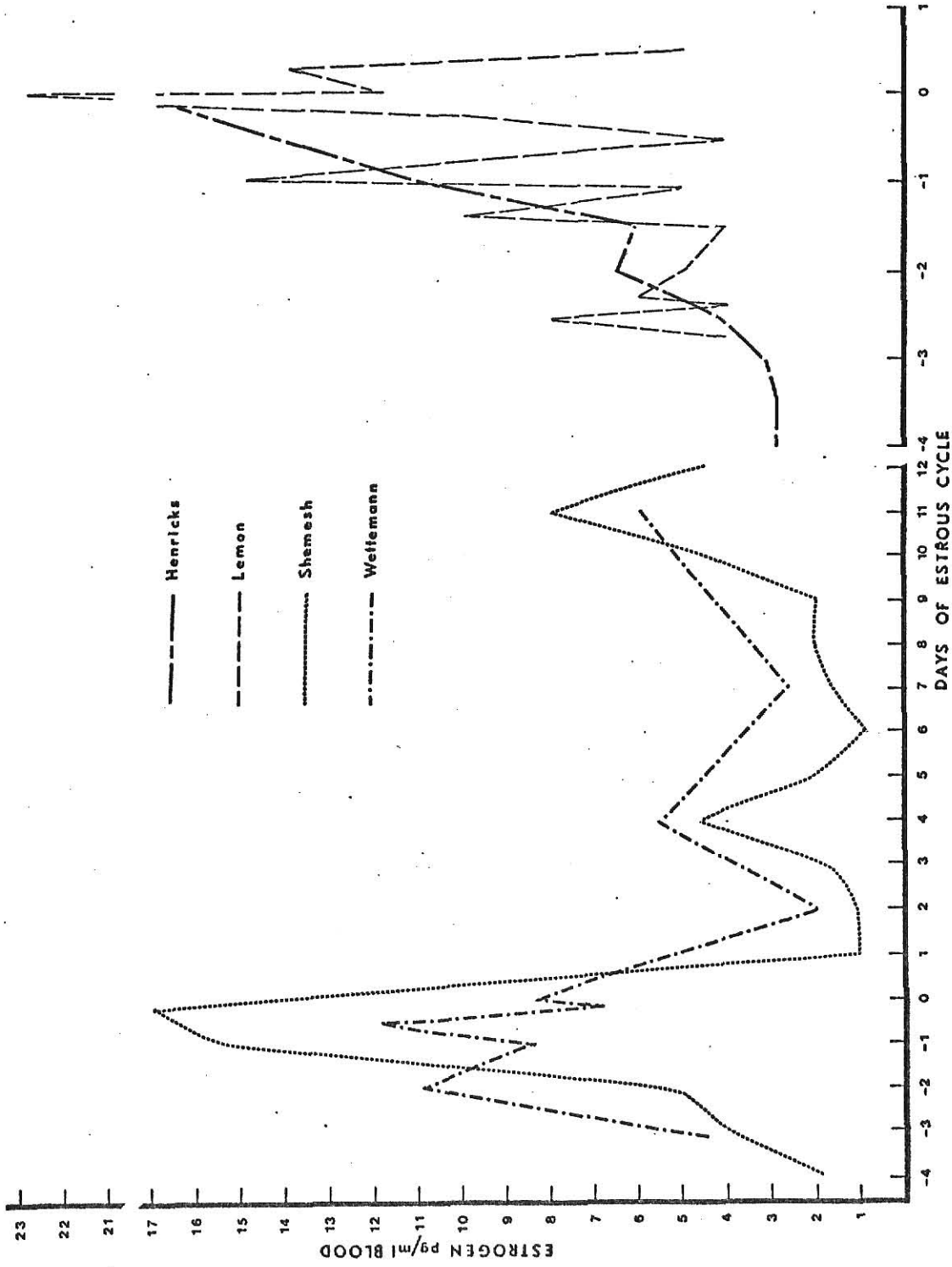

Figure 2. Changes in plasma estrogen concentrations during the bovine estrous cycle.

MATERIALS AND METHODS

The animals used in this study were clinically normal mature Holstein cows, belonging to the Kansas State University Dairy Herd, with known reproductive histories. All cows were milked twice a day, with daily productions recorded. The cows were maintained on a normal dairy ration.

Collection of blood samples was conducted in two groups.

Group I

Consisted of three cows with an average estrous cycle length of 21 days. Sampling was conducted once a day usually following the afternoon milking, for a total of five complete estrous cycles. All samples were obtained via the jugular vein. Blood was collected in centrifuge tubes containing anticoagulent EDTA (ethylenediaminetetracetate), immediately placed on ice, then centrifuged at 1,000 RPM for 20 minutes. Plasma was stored at -10° C until assayed.

Group II

Consisting of two cows with an average estrous cycle length of 22 days. Blood samples were collected through three complete estrous cycles, and was obtained in the same manner as in Group I. Sampling frequency was conducted as follows.

Day of Estrous Cycle	No. of Samples	Time of Collection
5 to 16	1	4 p.m.
17 to 19	2	4 a.m. - 4 p.m.
20 to +4	4	4 a.m. - 10 a.m. - 4 p.m. - 10 p.m.

Observations for estrous were made by herdsman and at the time of sampling. Rectal palpation was conducted on a near daily basis. Position, size, and firmness of follicles larger than 8 mm were recorded. Size

and location of corpora lutea and uterine tone were also recorded. During the Group II study, all psychic behavior was noted before and after blood collection. External genitalia was noted daily for color, puffiness and moisture.

Assay determinations for total estrogen, estradiol-17-β and progesterone was based on the procedures of Abraham (1971) and Chen (personal communication) as modified by Tillson (personal communication). The following outlines describe the procedures for the hormones quantified by RIA.

EXTRACTION % TOTAL ESTROGEN, ESTRADIOL-17-β

1. Two ml plasma Cow #187 Day 15 into centrifuge tube. Add 10 µl labelled *H steroid 12000-13500 CPM, vortex gently, incubate at room temperature, minimum 1 hour.

2. Add 5 ml fresh cold ether, stopper tightly with type "0" stopper, wrapped in saran wrap. Vortex vigorously 1 minute, stopping briefly every 10 seconds, stand 10 minutes, freeze 1 hour.

3. Decant ether into 12 X 75 mm disposable culture tubes. Dry in vacuum oven, adjust to room temperature, reconstitute with 0.5 ml assay buffer, vortex gently, stand 1 hour.

4. Decant to counting vial, add 10 ml counting fluid, shake; count one minute. (The CPM will then be used as the numerator for percent extracts steroid). In order to calculate extraction % two total count tubes are prepared:

   a. Add 0.5 ml assay buffer to 12 X 75 mm disposable culture tube. Add 10 µl labelled *H steroid, vortex gently, incubate at room temperature 1 hour, then refrigerate.

   b. Decant to counting vial. Add 10 ml counting fluid, shake, count 1 minute. The resulting number is "total count per minute."

Calculation of Extraction %: $\frac{\text{Extraction \% CPM}}{\text{Total Count CPM}}$ = % Extracted Steroid

EXTRACTION % PROGESTERONE

1. The same procedure used as in total Estrogen and Estradiol-17-β, except, use 100 µl plasma Cow #187 Day 0.

RIA TOTAL ESTROGEN, ESTRADIOL-17-β PROCEDURE

Standards used for total Estrogen, Estradiol-17-β and Progesterone

A = 1000 pg/0.5 ml

B = 500 pg/0.5 ml

C = 250 pg/0.5 ml

D = 100 pg/0.5 ml

E = 50 pg/0.5 ml

F = 25 pg/0.5 ml

G = 10 pg/0.5 ml

H = 5 pg/0.5 ml

I = 2.5 pg/0.4 ml

Prepare in Duplicates:

1. Pipet 0.5 ml of each standard solution into a 12 X 75 mm disposable culture tubes. Add to each 100 µl antibody (Total Estrogen - Abraham antisera S - 1310 #5 Estradiol-17-β = Abraham antisera S - 1554 #6), vortex gently, stand at room temperature, minimum 30 minutes.

2. Add 100 µl labelled *H steroid 12000 - 13500 CPM. Vortex gently, incubate in refrigerator 16 hours.

3. Place immediately in ice bath, add 200 µl A-70 charcoal solution, vortex gently, stand minimum 20 minutes not more than 45 minutes.

4. Centrifuge 2,000 RPM's for 10 minutes. Decant supernatant to counting vial, add 10 ml counting fluid, shake, count 1 minute.

Plot % Bound vs. pg/tube.

RIA TOTAL ESTROGEN, ESTRADIOL-17-β PROCEDURE

Unknown Samples.

1. Two ml plasma into centrifuge tube. Add 5 ml fresh cold ether, stopper tightly with type "0" stopper wrapped in saran wrap. Vortex vigorously 1 minute, stopping briefly every 10 seconds. Stand 10 minutes, freeze 1 hour.

2. Decant ether into 12 X 75 mm disposable culture tubes. Dry in vacuum oven, adjust to room temperature, reconstitute with 0.5 ml assay buffer, vortex gently, stand 1 hour.

3. Add 100 µl antibody (Total estrogen - Abraham antisera S - 310 #5; Estradiol-17-β - Abraham antisera S - 1554 #6), vortex gently, stand room temperature minimum 30 minutes.

4. Add 100 µl labelled *H steroid 12000-13500 CPM. Vortex gently, incubate in refrigerator 16 hours.

5. Place immediately into ice bath, add 200 µl A-70 charcoal solution, vortex gently, stand minimum 20 minutes <u>not</u> more than 45 minutes.

6. Centrifuge 2,000 RPM for 10 minutes. Decant supernatant to counting vial, add 10 ml counting fluid, shake, count 1 minute.

RIA PROGESTERONE PROCEDURE

Unknown Samples.

1. One hundred µl plasma into centrifuge tube. Add 5 ml fresh cold ether, stopper tightly with type "0" stopper wrapped in saran wrap. Vortex vigorously 1 minute, stopping briefly every 10 seconds. Stand 10 minutes, freeze 1 hour.

2. Decant ether into 12 X 75 mm disposable culture tubes. Dry in vacuum oven, adjust to room temperature, reconstitute with 1 ml assay buffer,

vortex gently, stand 1 hour.

3. Pipette 400 µl *duplicates) to new 12 X 75 mm disposable culture tube. Add 100 µl antibody (Anti-progesterone - Abraham S -83 #5). Vortex gently, stand room temperature minimum 30 minutes.

4. Add 100 µl labelled *H progesterone 10,000-15,000 CPM. Vortex gently, incubate in refrigerator 16 hours.

5. Place immediately into ice bath, add 200 µl A-70 charcoal solution, vortex gently, stand minimum 20 minutes not more than 45 minutes.

6. Centrifuge 2,000 RPM for 10 minutes. Decant supernatant to counting vial, add 10 ml counting fluid, shake, count 1 minute.

RIA PROCEDURE

For each assay Total Count (TC), Non-Specific Binding (NSB) and Total Binding (TB) tubes are prepared in duplicates.

1. Total Binding: Add 0.5 ml assay buffer to 12 X 75 mm disposable culture tube. Add 100 µl anti-total estrogen S-310 #5 or anti-estradiol-17-β S-1554 #6. Vortex gently, stand at room temperature minimum 30 minutes.

2. Total Count and Non-Specific Binding: Add 0.6 ml assay buffer to 12 X 75 mm disposable culture tubes.

3. Add 100 µl labelled *H steroid to all tubes (T.C., N.S.B., and T. B.), vortex gently, incubate in refrigerator 16 hours (do not freeze). Place in ice bath immediately.

4. Add 200 µl assay buffer to Total Count tubes only. Add 200 µl A-70 charcoal solution to Non-Specific Binding and Total Binding tubes. Vortex gently, stand 20 minutes <u>not</u> more than 45 minutes.

5. Centrifuge all tubes 2,000 RPM for 10 minutes. Decant supernatant to counting vial, add 10 ml counting fluid, shake, count 1 minute.

Calculations:

$$\frac{\text{N.S.B. CPM}}{\text{T.C. CPM}} = \%\ \text{N.S.B.}$$

$$\frac{\text{T.B. CPM}}{\text{T.C. CPM}} = \%\ \text{T.B.}$$

$$\frac{\text{Standards CPM - N.S.B. CPM}}{\text{T.B. CPM - N.S.B. CPM}} = \%\ \text{Bound}$$

$$\frac{\text{Unknowns CPM - N.S.B. CPM}}{\text{T.B. CPM - N.S.B. CPM}} = \%\ \text{Bound}$$

RESULTS AND DISCUSSION

Progesterone

Progesterone is low (0.8 to 1.0 ng/ml) during day -2 to day 4, rises steadily from day 5 to day 17, reaching a maximum level on day 17 (12.2 ng/ml). Between days -4 to -2 a rapid decline in progesterone was observed (Fig. 3). Graphic presentations of progesterone concentrations from individual cows are given in figures 4 to 8 and a combined, summarization of data from all cows is in table 1.

The change in peripheral blood progesterone concentrations found in this study are in agreement with the known changes in corpus luteum structure and function in the cow. Progesterone levels begin to increase between days 3 to 6, depending on the individual corpus luteum formation. One cow, No. 006, did not begin to show a progesterone increase until day 9. Ovarian palpation per-rectum indicated that ovulation had occurred normally on the left ovary about 1.5 days after onset of estrus. Subsequent palpation indicated apparent normal corpus luteum development. By day 4 the corpus luteum was approximately 12 mm in diameter, 18 mm on day 8 and 25 mm on day 12. Although the corpus luteum appeared to develop normally, it failed to secrete adequate progesterone to provide significant levels in the peripheral circulation until it was greater than 18 mm in diameter, or after day 8 (Fig. 4).

Progesterone levels were low (1.0 ng/ml) through day 8. Estrogen levels during this time were significantly high (10 to 13 pg/ml). Although these levels exceeded estrogen levels of day -1 and day 0 (estrus), no estrogenic psychic behavior was observed. Progesterone began to increase on day 9 and reached a maximum level on day 12 (9.5 ng/ml). As progesterone increased, estrogen levels decreased concurrently as observed on days 9,

12 and 16 (Fig. 4).

Failure of an apparently normal corpus luteum to produce adequate amounts of progesterone has not been previously reported, so an explanation has not been attempted. Possible explanations for this abnormal situation could be attributed to: 1) failure of proper luteinization of the stratum granulosa cells; 2) insufficient amounts of LH secretion from the anterior pituitary, or 3) insensitivity of ovarian hormonal receptor sites. Further investigation will be necessary to properly explain this abnormal condition.

The cyclic changes in progesterone concentration found in this study are in general agreement with previous reports. Stabenfeldt et al. (1969) reported peak progesterone levels of 5.2 to 7.9 ng/ml. Hansel and Echternkamp (1972) reported 4-5 ng/ml. Henricks et al. (1971) estimated mean values of ten cows to be 7.2 ng/ml. Our results do not agree with those reported by Plotka et al. (1967) from determination by the double isotope method. They determined the concentration at peak luteal function to be 25 ng/ml and 10 ng/ml during the proestrus-estrus period.

Data from two cows (No. 006 and 191-D) sampled four times a day from day -2 to day 3 showed no proestrus increase in progesterone, contrary to what Ayalon and Shemesh (1974) reported.

β-Estradiol: Estradiol data was compiled from four cows and six estrous cycles. The estradiol concentrations from individual cows are given in figures 9 to 11 and a summarization of data from all cows in table 2.

Estradiol concentrations on day -2 was 14.5 pg/ml. A steady increase was observed till a maximum concentration (30 pg/ml) was reached the day before estrus (day -1), then decreased on day 0 and remained low through day 2.

Estrogen levels did not rise significantly until progesterone levels

had decreased below 1 ng/ml. Minor concentration increases were found on days 3-4, 8, and 12-14. Palpation data revealed ovarian follicular activity and increased uterine tone during these periods. Whether the increase in estrogen was due to follicular or luteal activity is not definitely known but the increases in estrogen levels corresponded closely with time of detection of large, palpable follicles.

Total estrogen was determined in one estrous cycle in an attempt to correlate β-estradiol (Fig. 11). Differences between total estrogen and β-estradiol were determined in the same samples, with determined total estrogen usually less than β-estradiol. Such differences could be due to differences in mass reaction of the antibodies used. An explanation of the discrepancies can be found only by the use of the tedious, time-consuming and expensive process of chromatographic separation, then quantitation of each of the fractions.

The levels of total estrogen in jugular plasma found in this study were similar to the concentrations of β-estradiol. Levels on day -3 were about 13 pg/ml and increased to 24.4 pg/ml the day before estrus, then decreased on day 0 and remained low through day 2. These results are within the values (15 to 25 pg/ml) reported by Henricks et al. (1971), and Katongole et al. (1973) of 30 pg/ml, but much less than the 176 pg/ml reported by Christensen et al (1974).

Lemon et al. (1975) reported baseline values of 10 pg/ml estradiol increasing to a maximum of 25 pg/ml the day before estrus. Similar values have been reported by Wettemann et al. (1972) and Glencross et al. (1973). Our determinations are in general agreement with those mentioned. One cow, 006, was determined to be abnormal and therefore was not included in the summary data (Fig. 4).

Table 1. Progesterone Data from the Peripheral Blood Plasma from Holstein Cows During The Estrous Cycle.

Cow No.	Cycle Length (Days)	Peak Plasma Progesterone mg/ml Blood day of progestation stage	Day of Progesterone Decline
002	22	5.6/16	19
003	20	3.99/14	17
004	22	8.0/15	16
191-D	22	15.1/16	18
006	22	17.5/16	21
006*	22	11.5/13	21

*Not included in summary data.

Table 2. Estrogen Concentrations (pg/ml) from Peripheral Blood of Holstein Cows During the Estrous Cycle.

Day of Cycle	002	003	004	006*
-3	20.73	20.07	7.9	11.1
-1	31.3	29.16	-	7.98
Estrus	24.2	20.97	18.7	10.92
1	22.02	13.1	20.9	9.87

*Not included in summary data.

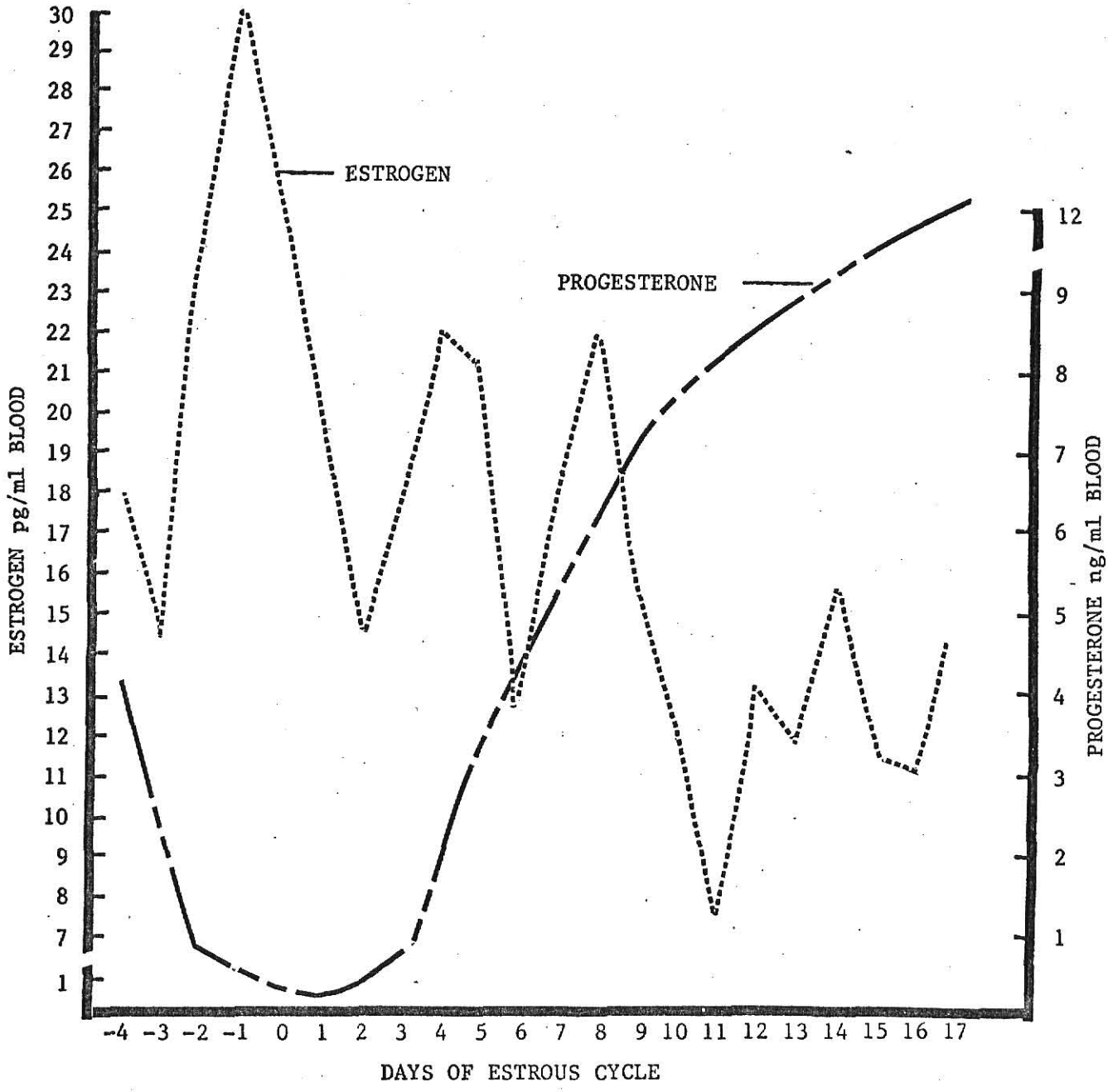

Figure 3. The mean concentrations of progesterone and estrogen during the normal estrous cycle.

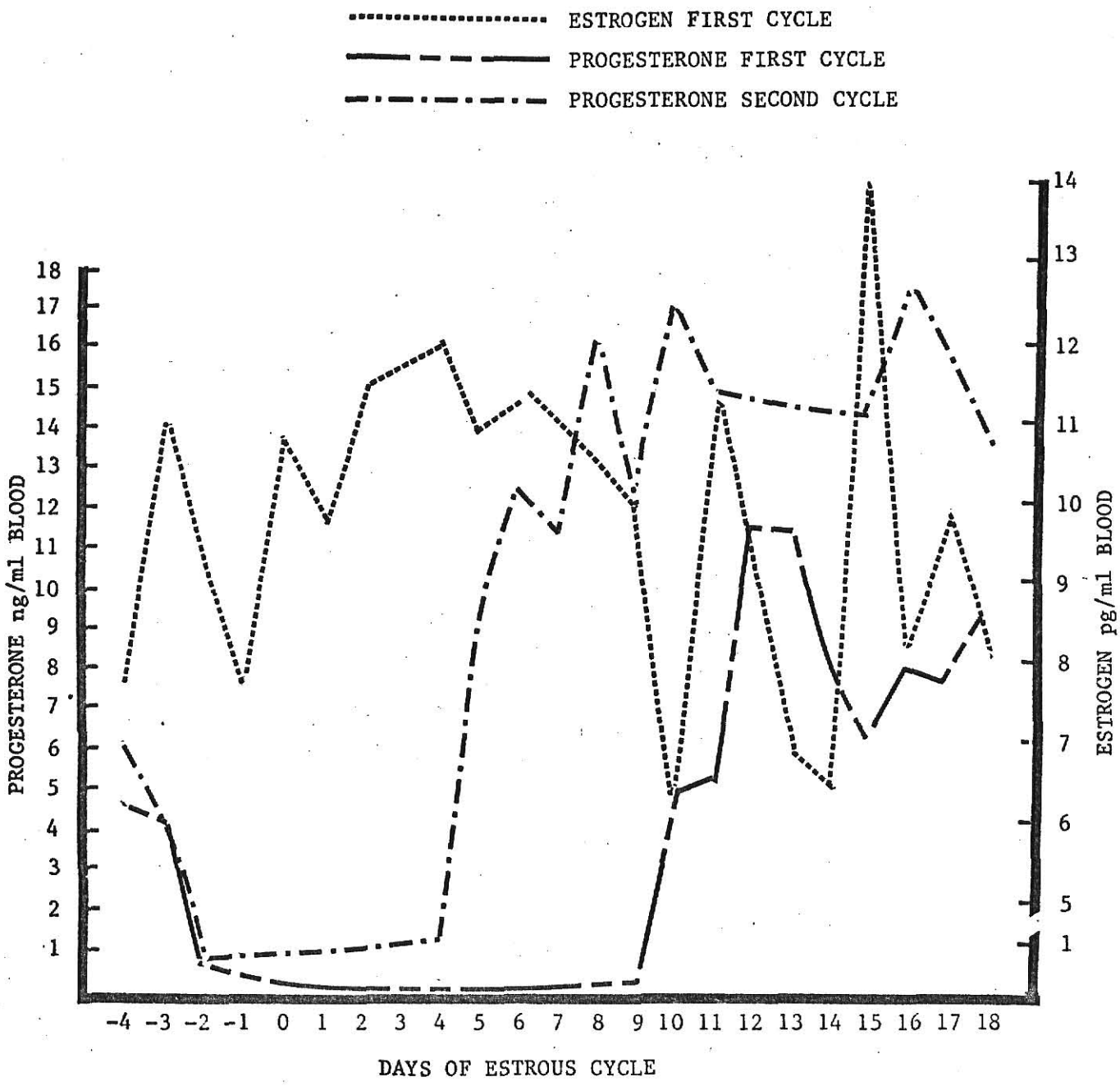

Figure 4. Changes in plasma progesterone and estrogen concentrations in cow No. 006.

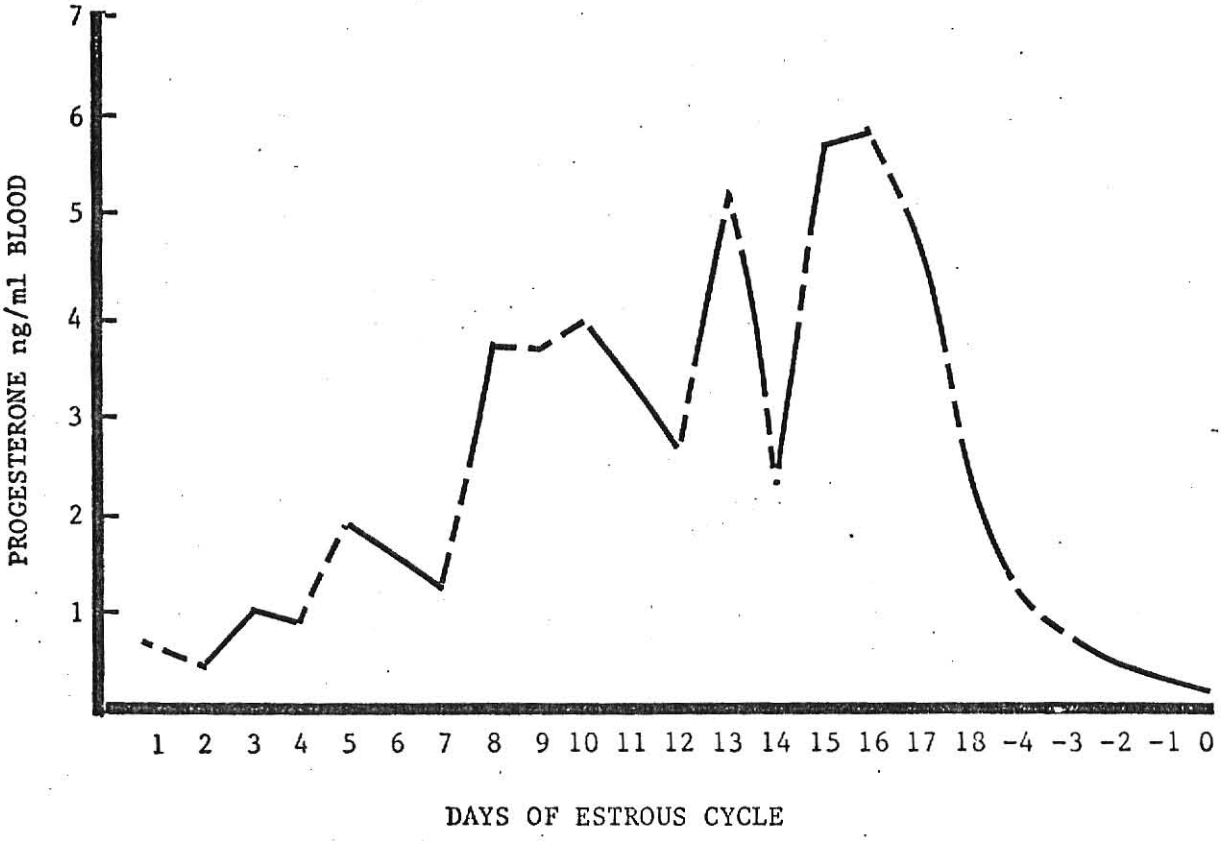

Figure 5. Changes in plasma progesterone concentrations in cow No.002.

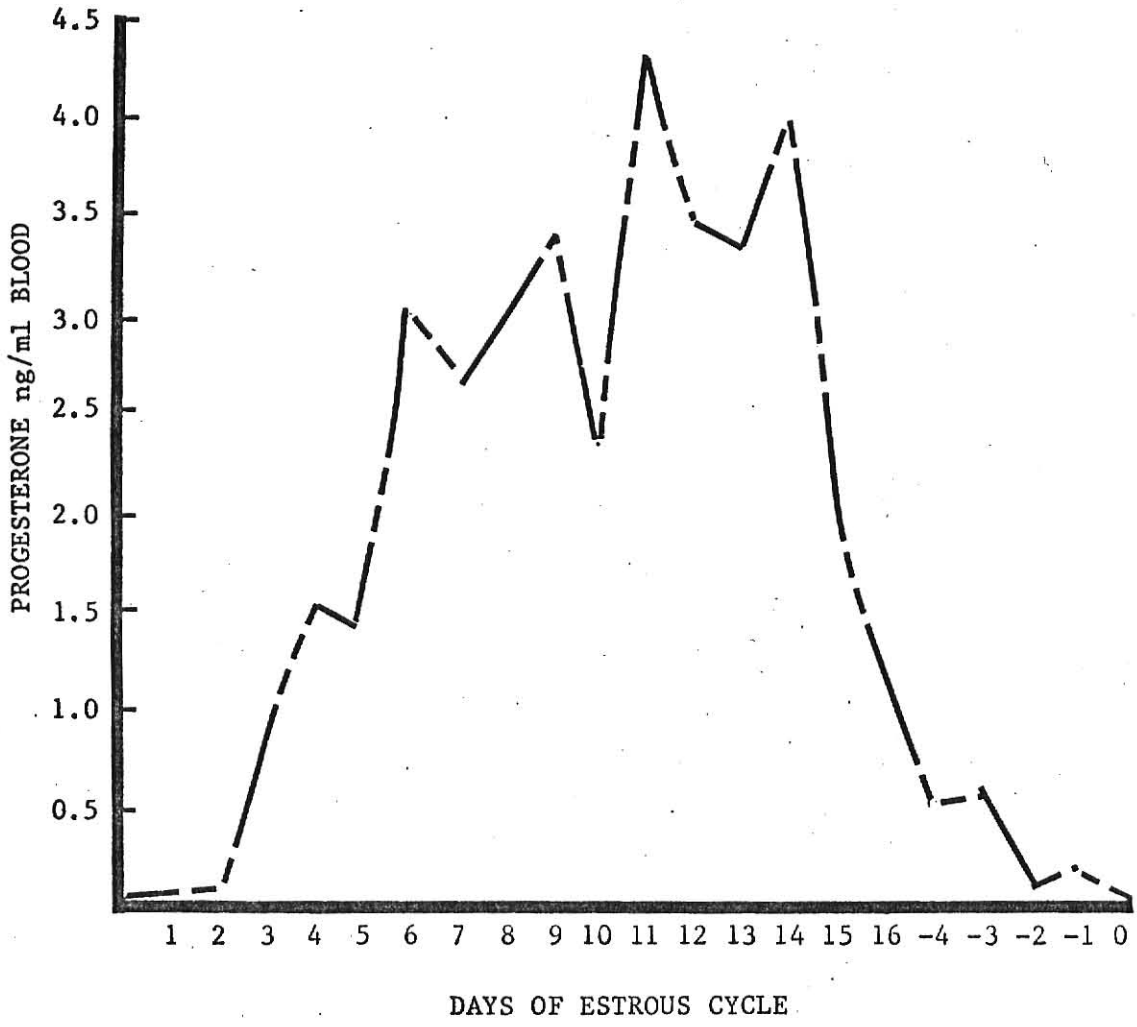

Figure 6. Changes in plasma progesterone concentrations in cow No. 003.

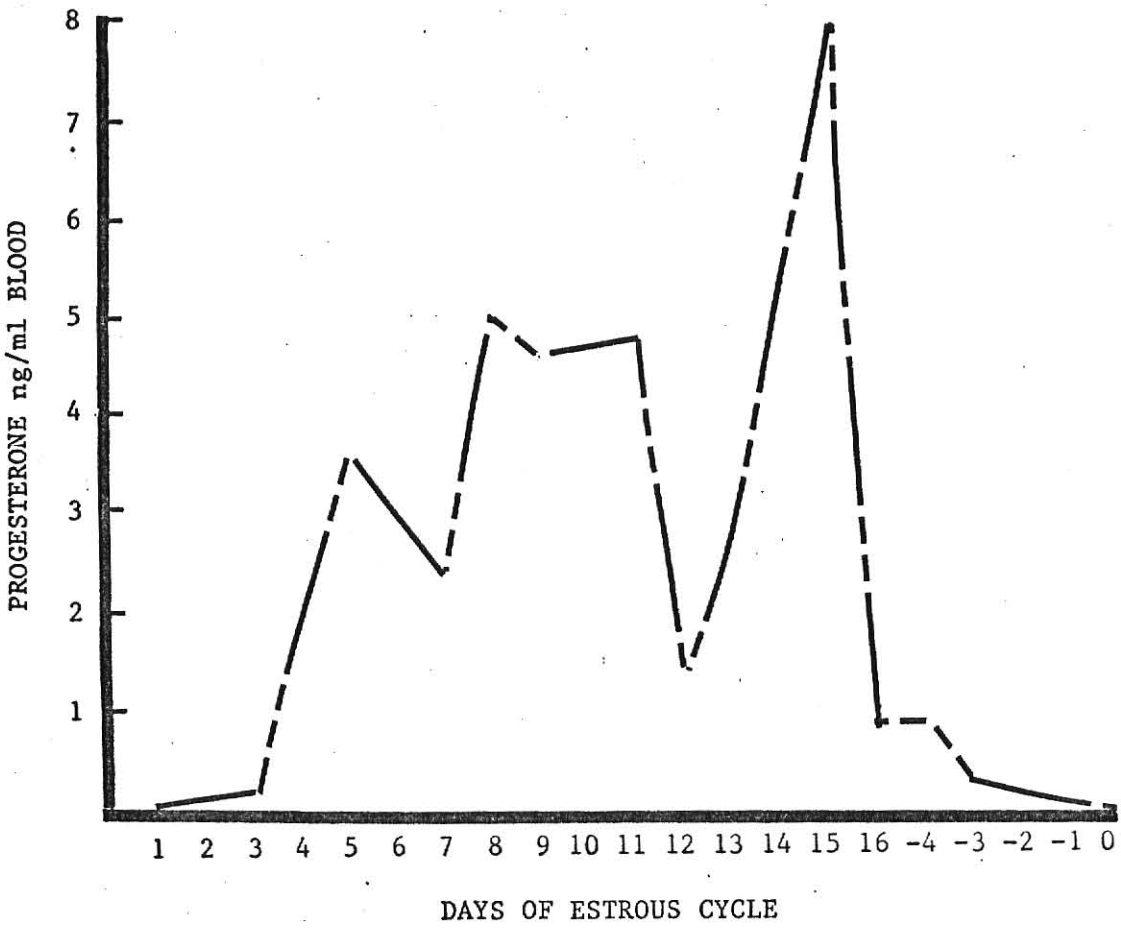

Figure 7. Changes in plasma progesterone concentrations in cow No.004.

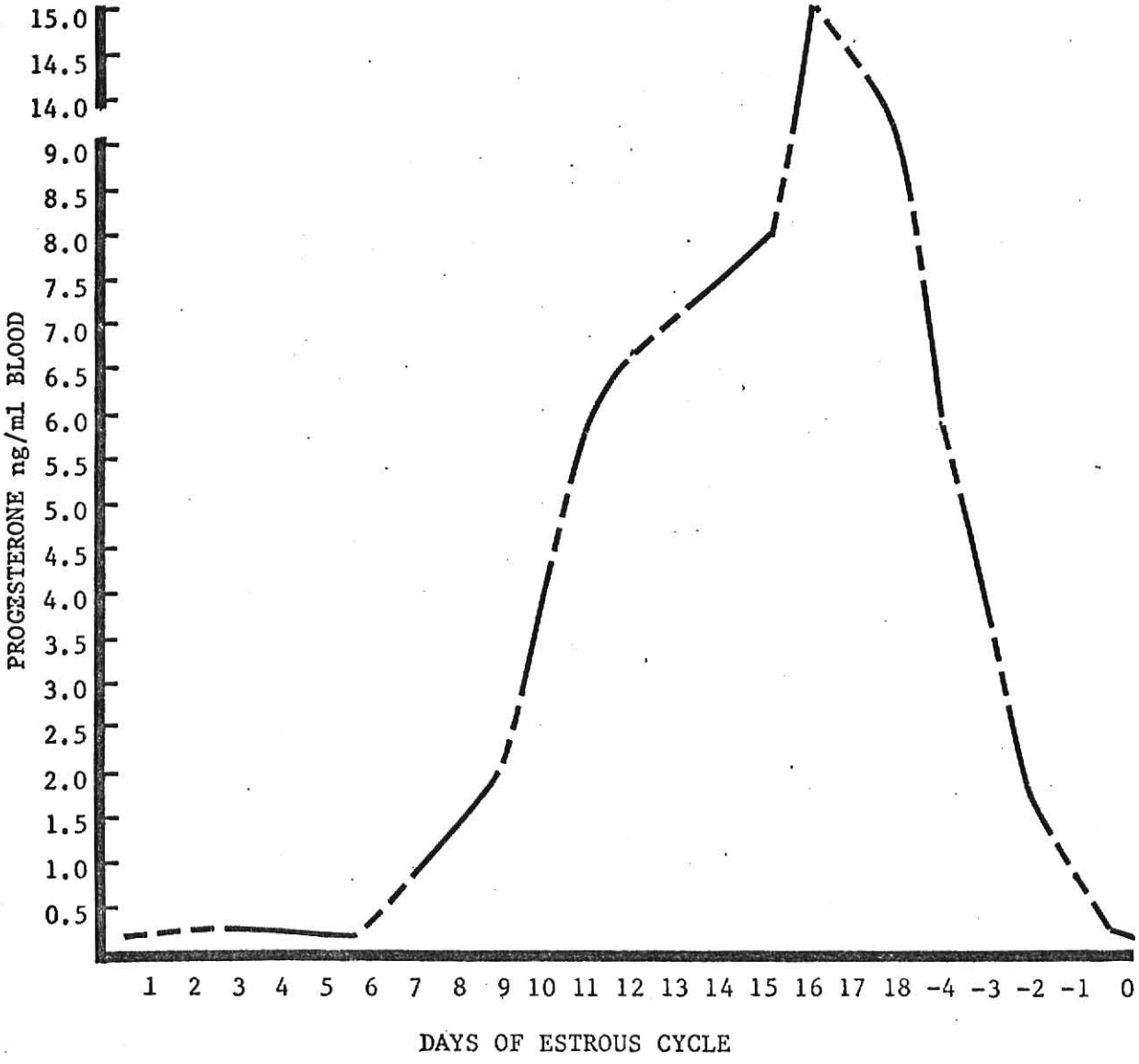

Figure 8. Changes in plasma progesterone concentrations in cow No. 191-

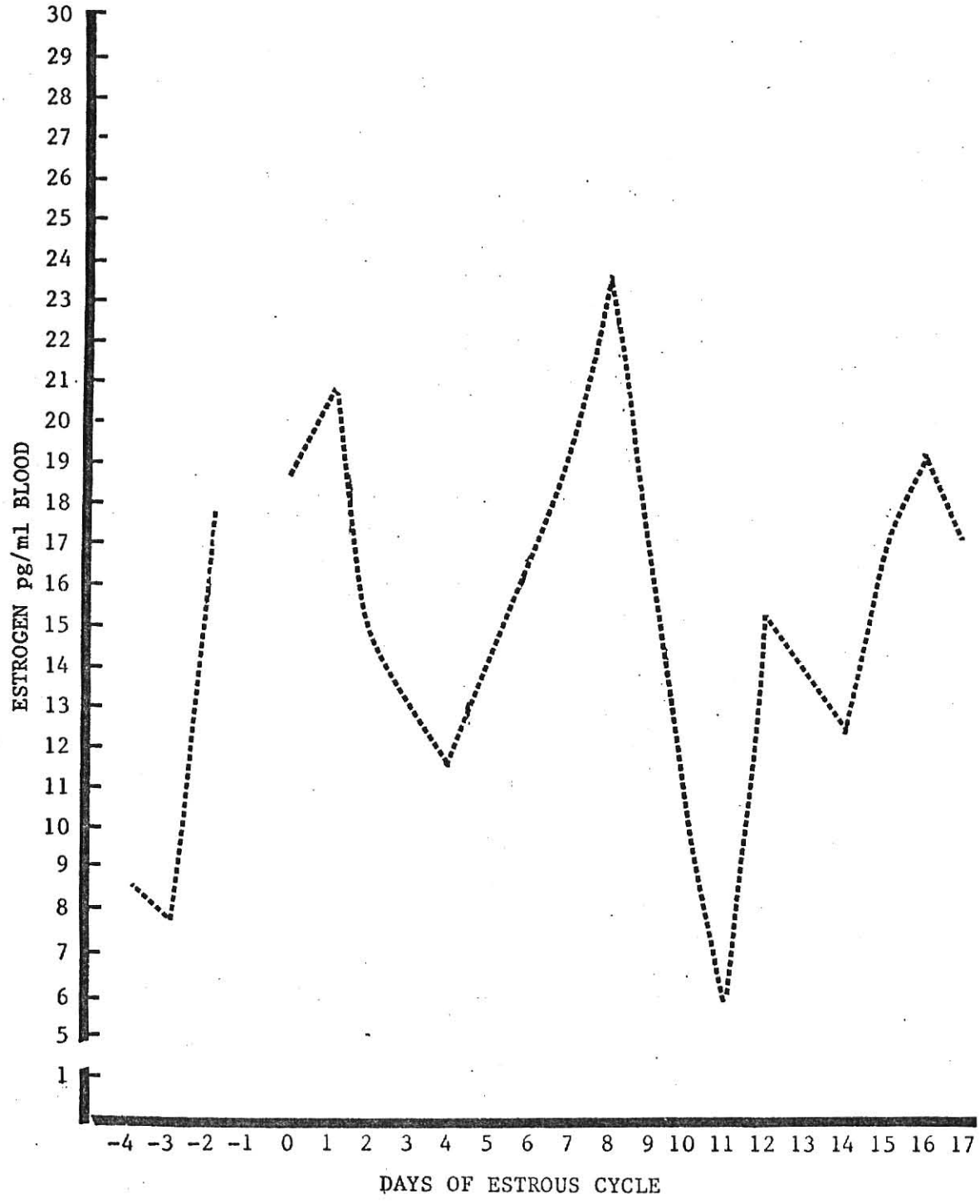

Figure 9. Changes in plasma estrogen concentrations in cow No. 004.

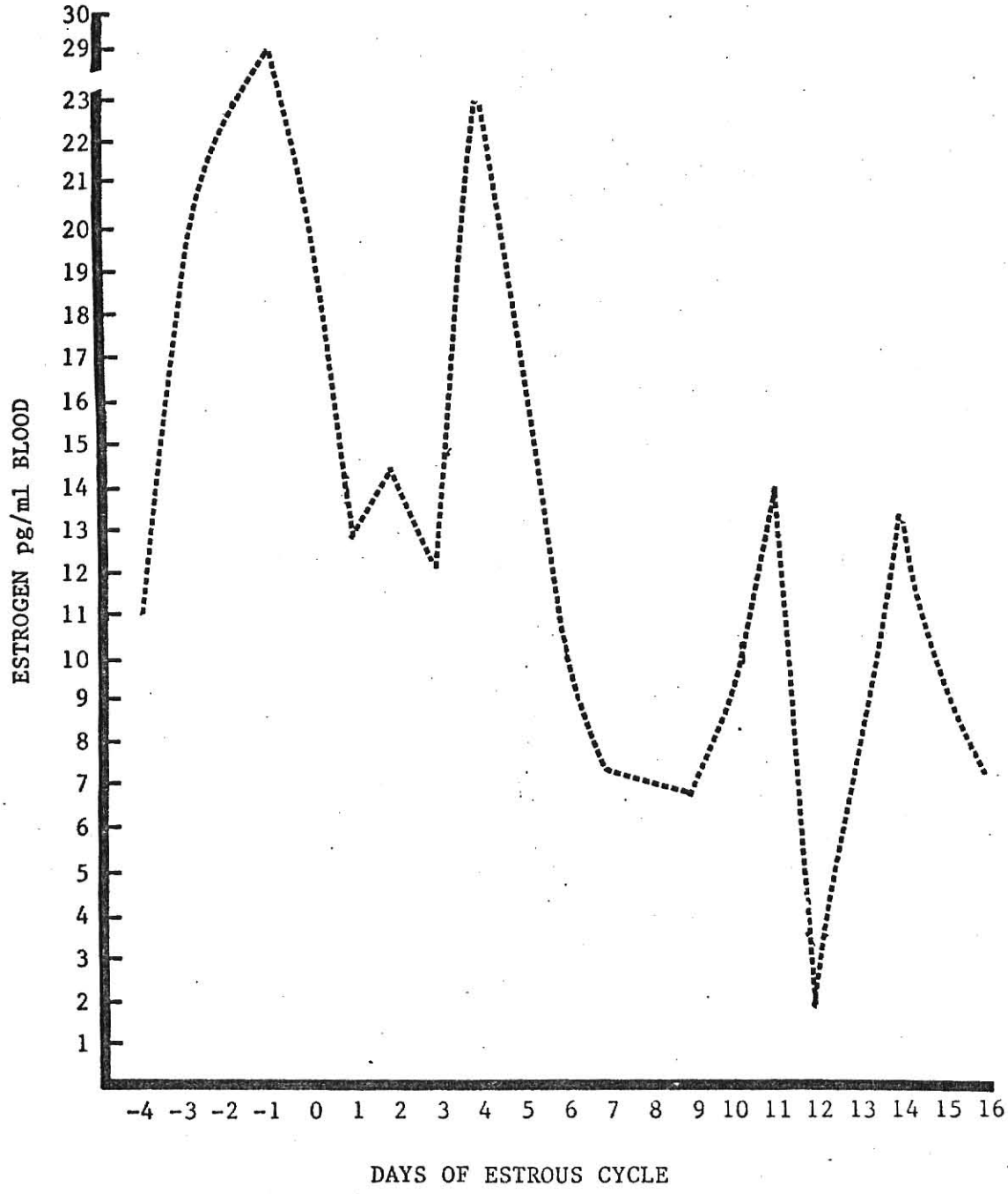

Figure 10. Changes in plasma estrogen concentrations in cow No. 003.

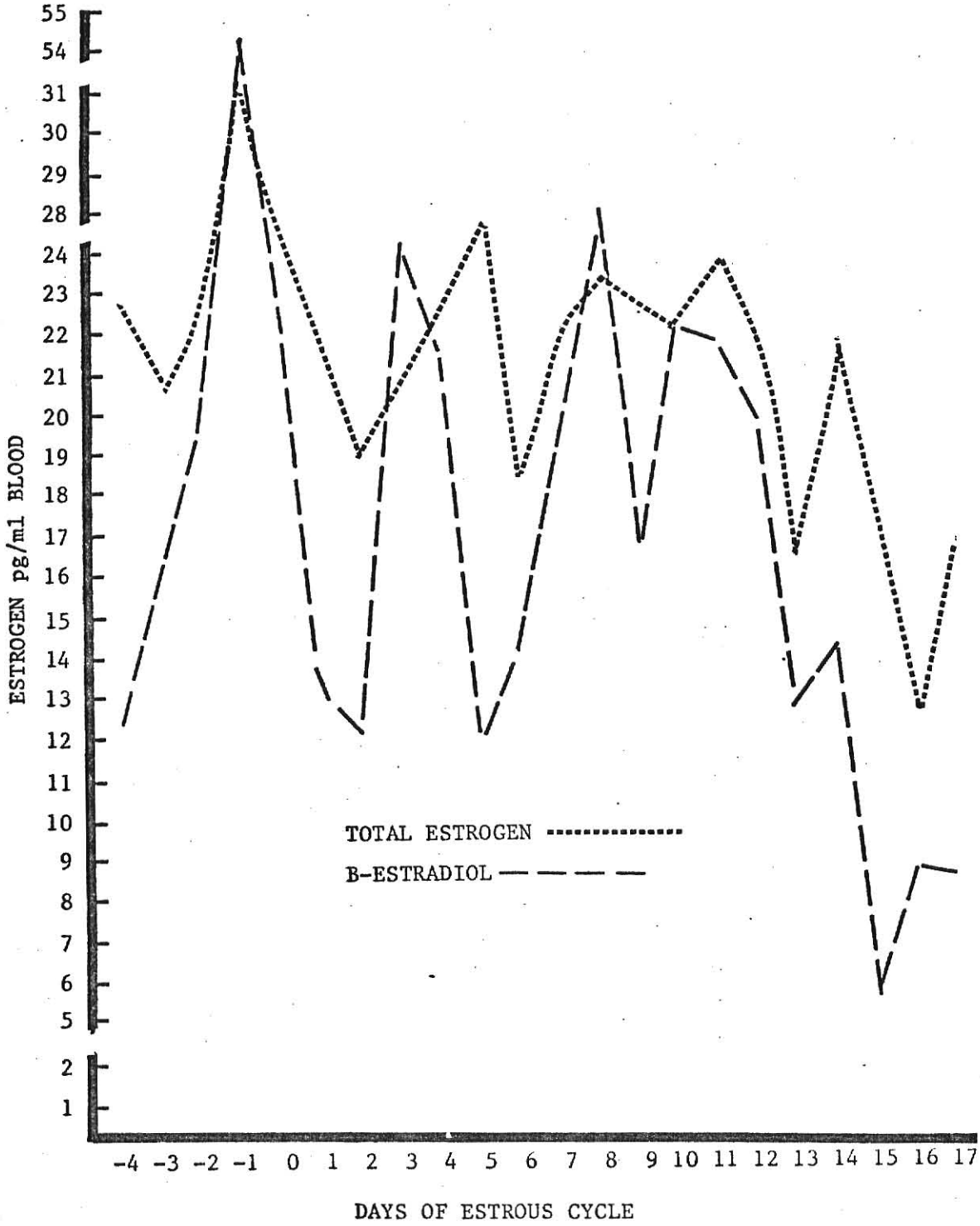

Figure 11. Changes in plasma total estrogen and B-estradiol concentrations in cow No. 002.

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APPENDIX

Solutions:

Assay Buffer

To a two liter flask add:

32.7 grams of sodium phosphate dibasic heptahydrate (M.W. = 26P)
10.8 grams of sodium phosphate monobasic monohydrate (M.W. = 138)
2.0 grams of sodium azide (M.W. = 65)
18.0 grams of sodium chloride (M.W. = 58)

Then add water to a total volume of two liters. The unadjusted pH should be 7.0 + 0.1.

The assay buffer consists of a 0.1% gelatin solution (Knox USP) in the above buffer. Assay buffer was kept refrigerated at 4° C, pH = 6.8 - 7.0.

Charcoal Solution

To a 100 ml flask add:

0.625 grams of Norit A (Matheson, Coleman, and Bell)
0.0625 grams of Dextran T-70 (Mann Research Labs)
100.000 ml of assay buffer

Stopper flask and shake vigorously for 30 seconds. Charcoal solution was kept, capped, and stored at 4° C.

Water: All water used was double glass distilled.

Solvents:

Ether: Anesthesia grade ether was obtained from Squibb Laboratories in copper coated one pint cans. A fresh can of ether was used once and remnant discarded.

Counting Fluid: A quasal-universal LSC cocktail was obtained from New England Nuclear Catalogue No. NEF-934.

Ethanol: Absolute ethanol obtained commercially.

Steroids:

Standards: Standards were prepared in assay buffer in the laboratory.

Concentrations for total Estrogen, Estradiol-17-β and Progesterone are:

Solution A = 20 µl of stock (20 mg) in 10 ml of buffer
(0.5 ml = 1000 pg)
Solution B = 5 ml of solution A + 5 ml of buffer
(0.5 ml = 500 pg)
Solution C = 5 ml of solution B + 5 ml of buffer
(0.5 ml - 250 pg)
Solution D = 4 ml of solution C + 6 ml of buffer
(0.5 ml = 100 pg)
Solution E = 5 ml of solution D + 5 ml of buffer
(0.5 ml = 50 pg)
Solution F = 5 ml of solution D + 5 ml of buffer
(0.5 ml = 25 pg)
Solution G = 4 ml of solution E + 5 ml of buffer
(0.5 ml = 10 pg)
Solution H = 5 ml of solution G + 5 ml of buffer
(0.5 ml = 5 pg)
Solution I = 5 ml of solution H + 5 ml of buffer
(0.5 ml = 2.5 pg)

Antisera: Obtained commercially from Abraham, G. E.

Anti Total Estrogen S-310 #5
Anti Estradiol-17-β S-1554 #6
Anti Progesterone - S-83 #5

Radioactive Steroids: Labelled steroids were obtained from New England Nuclear.

Estradiol (2, 4, 6, 7 - ^{3}H) M.W. 272.4 NET - 317
Specific Activity - 91 Ci/m mole
Solvent - Benzene:ethanol (9:1)
Cal Biochem.

Progesterone (1, 2, 6, 7 - ^{3}H) M.W. 314.5, NET - 318
Lot #773-144
Specific Activity - 105 Ci/m mole
Solvent - Benzene
Sigma #P-0130

Counter: Nuclear Chicago Isocap/300 Liquid Scintillating System

Glassware: All glassware reused was acid-washed. Disposable culture tubes were obtained from Matheson Scientific. Cat. No. 61643-08.

Calculations:

A Wang 600 series advanced program calculator was used to calculate standard curve, by means of polynomial regression using STAT ROM advanced statistics. The concentrations of total estrogen, estradiol-17-β and progesterone in the aliquots of unknown plasma samples were calculated by interpolation of the standard curve. Four assay buffer samples treated like unknowns served as a check on the blank of the system. The average blank value was subtracted from the unknown value, divided by the extraction per cent equalled the corrected amount of hormone present.

$$\frac{\text{Unknown plasma value pg/tube} - \text{Blank pg/tube}}{\text{Extraction per cent}} = \text{hormone concentration}$$

OVARIAN STEROID PLASMA CONCENTRATIONS IN THE NORMAL BOVINE ESTROUS CYCLE

by

EDWARD EUGENE MORRISON

B. S., University of Massachusetts, 1970

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Animal Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1976

ABSTRACT

Jugular blood was collected from mature Holstein cows daily, twice daily and four times daily during different stages of the estrous cycle. Ovarian steroids were quantitated by radioimmunoassay.

Progesterone concentrations begin to increase between days 4 and 5 and continue to increase until a maximum of 12.2 ng/ml on day 17. Between days -4 and -2 progesterone rapidly declined. No proestrus increase in progesterone was observed. One cow failed to secrete adequate amounts of progesterone until day 9 even though an apparently normal corpus luteum was present.

Estradiol concentrations on day -3 was 14.5 pg/ml, increasing to peak concentrations on the day before estrus 30 pg/ml, then decreased to 19 pg/ml on estrus (day 0) and remained low through day 2. Estradiol levels did not rise significantly until progesterone concentrations decreased below 1 ng/ml. Minor estradiol peaks were noted on days 3-4, 8 and 12-14. Both total estrogen and β-estradiol were determined in one cow in an attempt to correlate the concentrations. Determined total estrogen levels were less than estradiol levels due to the lack of chromatographic separations and quantitation of the fractions.

This work establishes actual levels and fluctuations in those levels of progesterone and β-estradiol for the animals in the Kansas State University Dairy herd and will serve as the base for a more extensive study of these hormone levels in cows with abnormal estrous cycles.