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To the Graduate Council:

I am submitting herewith a dissertation written by David Banks Batson, entitled "Progesterone and Estrogen Levels in the Peripheral Plasma of Non-bred, Non-pregnant and Pregnant Cows." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Animal Science.

Don O. Richardson
Major Professor

We have read this dissertation
and recommend its acceptance:

J. T. Miles

Robert R. Shrode

R. L. Murphree

William James Davis

Accepted for the Council:

Vitton A. Smith
Vice Chancellor for
Graduate Studies and Research

PROGESTERONE AND ESTROGEN LEVELS IN THE PERIPHERAL PLASMA OF NON-BRED, NON-PREGNANT AND PREGNANT COWS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

David Banks Batson

December 1972

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ABSTRACT

The first objective of this study was to evaluate a radioimmunoassay technique for the assay of estrogen. Secondly, the interrelated changes in the concentrations of progesterone and estrogen were to be determined in peripheral plasma of non-bred, non-pregnant and pregnant Holstein cows. Progesterone was determined by competitive protein-binding.

Six hundred twenty-nine blood samples were collected from the jugular vein of 14 lactating cows. Sample collection began five days prior to estrus and continued at daily intervals for 25 days post-insemination. Non-bred cows were bled through one or two cycles. The cows were divided into the following groups: A) non-bred (estrus detected), B) non-bred (estrus undetected), C) non-pregnant and D) pregnant. In Groups A, C and D, Day 0 represents the day of estrus. In Group B, Day 0 represents the day of highest estrogen concentration.

The extraction efficiencies for labeled and unlabeled progesterone were 93.58 $\pm$ 0.78 percent (mean $\pm$ standard error) and 90.20 $\pm$ 2.15 percent, respectively.

The extraction efficiencies for 20 pg and 50 pg of estradiol-17B were 91.18 $\pm$ 4.58 percent and 88.57 $\pm$ 5.24 percent, respectively. The extraction efficiency for 4 nanocuries of estradiol-17B-2, 4, 6, 7-H^3 was 94.04 $\pm$ 0.59 percent. The unlabeled and labeled extraction efficiencies were not significantly different ($P > 0.10$).

The estradiol-17B antibody exhibited considerable crossreaction with estrone, while the crossreaction with estriol was negligible.

The coefficients of variation for points on progesterone and estrogen standard curves were less than 10 percent, with the exception of 100 pg of estradiol-17β (12.80 percent). The lower limit of sensitivity for the progesterone standard curve was 0.3 ng. The lower limit of sensitivity for the estradiol-17β standard curve was 6 pg.

The progesterone and estrogen concentrations were inversely related during the follicular phase and the late luteal phase period of the cycle. During the period of metestrus, the progesterone and estrogen concentrations increased simultaneously.

An estrogen peak occurred on Day 0 in all groups. A secondary estrogen surge occurred five or six days post-estrus in Groups A, C and D. Normal follicles or follicles undergoing atresia may have contributed to both of these surges. The estrogen concentration in bred cows was significantly higher ($P < 0.10$) than the concentration in non-bred cows on Days 5, 6 and 10. This suggests that the zygote may be involved in regulating luteal phase estrogen secretion. The elevated estrogen levels on Day 5 and Day 6 may influence ovum transport in the oviducts or development of the uterine endometrium. The high estrogen level at Day 10 may be involved in preparing the uterus for implantation.

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CHAPTER I

INTRODUCTION

Understanding the reproductive processes requires a thorough understanding of the endocrine system which controls them. Our knowledge of the endocrine control of reproduction has been greatly enhanced by the development of very sensitive assay methods such as competitive protein-binding (CPB) assay and radioimmunoassay (RIA). These techniques have made it possible to detect nanogram (ng) and picogram (pg) quantities of steroid and protein hormones in blood plasma and serum.

Progestins and, more recently, estrogens have been quantitated in the uterine and ovarian veins and the peripheral circulation (jugular vein and carotid artery) of a number of different species. In order to understand various reproductive problems and to develop potential solutions it is necessary to know the concentrations of these hormones in normal and abnormal animals. For example, research has indicated that low plasma progesterone and/or high plasma estrogen may be responsible for embryonic death. Blood progesterone levels also have been used to determine pregnancy at very early stages.

The purpose of this experiment was to study the progesterone and estrogen interrelationships in the peripheral blood of non-bred, non-pregnant and pregnant cattle.

CHAPTER II

REVIEW OF LITERATURE

This review includes the methodology of CPB and RIA, as applied to progesterone and estrogen assays. In addition, a brief review of the ovarian sex steroids found in the blood of domestic animals has been included.

Investigators have used varied terminology in referring to hormone assays. The two most frequently used terms are CPB (Murphy, 1964) and RIA (Berson et al., 1956). The former term has been used when the assay protein was naturally occurring in blood or tissue, while the latter term has been used when the assay protein was an antibody. Other terms that have been used are saturation analysis (Ekins and Newman, 1970) and radioligand binding assay (Murphy, 1970). To avoid confusion, only CPB and RIA will be used in this review.

CPB and RIA are based on the competition between a radioactive compound and a non-radioactive compound for binding sites on the assay protein. The amount of compound bound is dependent on the concentration in the reaction mixture.

I. METHODOLOGY

One of the first significant breakthroughs in hormone assays came in 1956 when Berson et al. (1956) demonstrated that increasing quantities of non-radioactive insulin would displace insulin-I^{131} from a fixed antibody dilution. According to Yalow and Berson (1971),

Berson and Yalow first applied this technique to quantitate the disappearance of beef insulin in rabbits. The principles of the CPB method have been applied to the assay of other compounds such as glucagon (Unger et al., 1959), vitamin B_{12} (Rothenberg, 1961), cortisol (Murphy, 1964) and progesterone (Neill et al., 1967).

Extraction

The extraction of progesterone from plasma has been necessary because progesterone is protein bound and because corticosteroids compete for binding sites on the protein (corticosteroid binding globulin). The most commonly used extractant has been petroleum ether (30°C-60°C boiling point). Neill et al. (1967) demonstrated that a 5 milliliter (ml) petroleum ether extraction of 0.5 - 1.0 ml human plasma would extract 90 percent of the progesterone, 90 percent of the 20 α-hydroxypregn-4-ene-3-one and 40 percent of the 17 α-hydroxyprogesterone, and less than 1 percent of the cortisol and corticosterone. Johansson (1969) and Reaves et al. (1970) obtained similar results using petroleum ether, even though their progesterone recoveries were slightly lower (85.5 - 86.5 percent). Other compounds that have been used for extraction are ethyl acetate (Martin, Cooke and Black, 1970), hexane (Demetriou and Austin, 1970) and isooctane (Thatcher, 1970) found that isooctane would extract 93.7 percent of the tritiated progesterone and only 3.3 percent and 1.3 percent of the corticosterone and cortisol, respectively.

Ethyl acetate (Shutt, 1969), dichloromethane (Mayes and Nugent, 1970), methylene chloride (Dufau et al., 1970) and diethyl ether

(Corker and Exley, 1970) have been used to extract estrogens from plasma. Diethyl ether has been the most widely used solvent. Henricks, Dickey and Hill (1971) reported that 4 ml of cow plasma extracted twice with 4 ml diethyl ether resulted in greater than 90 percent recovery of labeled and unlabeled estradiol-17β.

Purification

Purification of the extracted material has been necessary since most solvents are not completely selective and/or the assay proteins are not specific for progesterone or estradiol-17β. However, this purification step may not be necessary in quantitating progestins or total estrogens.

The most commonly used purification methods have been paper chromatography, thin layer chromatography (TLC) and column chromatography. Paper chromatography has been used to purify progesterone (Martin, Cooke and Black, 1970) and estradiol-17β (Mayes and Nugent, 1970). Paper chromatography has been an acceptable method, but it is extremely time-consuming. Thin layer chromatography (silica gel) and column chromatography (Sephadex LH-20) have gained wide acceptance because they have been much faster than paper chromatography. Neill et al. (1967) found pre-coated TLC plates, developed in ether-benzene (2:1), to be satisfactory in separating progesterone from 20 α-hydrozy-pregn-4-ene-3-one and 17 α-hydroxyprogesterone. Thin layer chromatography has fallen into disfavor with some investigators because of high blank values. Thatcher (1970) could not get a workable standard

curve when TLC blanks were added to standards. Because of high blank values, Murphy (1970, 1971) found it necessary to use column chromatography (Sephadex LH-20). This compound was practically inert with respect to steroids. Blank values which occurred in the assay were due to solvents and not to the purification material. Solvent blanks may be reduced by using nanograde or spectro-grade material, using solvents from recently opened containers and redistillation. Sephadex LH-20 appears to be the material of choice for steroid purification because of the ease in handling and the adequate separation between progesterone and estrogen metabolites. This material was found to be very stable and could be used repeatedly at room temperature. If necessary it could be removed from the column, washed with ethanol, dried at 30°C and reused with no detectable change in elution properties (Murphy, 1970). This purification method has been applied to estrogens, androgens, progestins and corticoids with very good separation among metabolites (Carr, Mikhail and Flickinger, 1971; Murphy, 1971).

Assay Proteins

An ideal assay protein should be specific for the compound being assayed. However, such a protein has been difficult to obtain. Corticosteroid binding globulin (CBG), the assay protein for progesterone, also binds with the metabolites of progesterone and corticosteroids (Murphy, 1971). Sex steroid binding plasma protein (SBP), which binds estradiol-17β also binds androgens (Mercier-Bodard, Alfsen and Baulieu, 1970).

The most frequently used source of CBG has been dog plasma, which has been obtained from unanesthesized dogs and diluted to the desired concentration (Neill et al., 1967). Other sources of CBG that have been used are plasma from ovariectomized cow (Rao and Estergreen, 1969); chicken, rabbit and cat plasma (Murphy, 1967); and plasma from pregnant women (Demetrion and Austin, 1970), women on steroid contraceptives (Stone et al., 1971) and ovariectomized women receiving estrogen and dexamethasone (Yoshimi and Lipsett, 1968). Reaves et al. (1970) found that plasma from women treated with estrogen and dexamethasone produced a more sensitive curve than plasma from non-treated women. Dexamethasone suppressed adrenal function, and estrogens increased the concentration of CBG two to three fold (Slaunwhite and Sandbert, 1970).

Some investigators have found it advantageous to remove endogenous steroids that may occupy binding sites on the assay protein. Florisil (Fylling, 1970), charcoal (Plotka, 1971) and Sephadex G-25 (Bassett and Hinks, 1969) have been used to "strip" steroids from the assay protein.

Abraham et al. (1971) and Niswender (1972) have recently been successful in developing an antibody against progesterone. Abraham et al. (1971) conjugated 11-deoxycortisol to human serum albumin (HSA), and produced a sheep-anti-progesterone serum that cross reacts with 17α-hydroxyprogesterone (90 percent), 11-deoxycortisol (90 percent) and 11-deoxycorticosterone (35 percent). Niswender's (1972) anti-progesterone-bovine serum albumin serum was specific for progesterone.

A progesterone specific antibody should permit the assay of extracted progesterone without further purification.

Proteins used in estrogen assays include rabbit uterine protein (cytosol) (Korenman, 1968; Korenman, Perrin and McCallum, 1969; Tulchinsky and Korenman, 1970), sheep uterine protein (Shutt, 1969), a human pregnancy plasma (Dufau et al., 1970; Mayes and Nugent, 1970) and estradiol antibody (Thorneycraft et al., 1970; Wu and Lundy, 1971). Antibodies are advantageous because they are more stable and more specific than the uterine and pregnancy plasma proteins.

Because steroids are non-antigenic, it is necessary to conjugate them to larger protein molecules such as HSA and bovine serum albumin (BSA) to induce antibody formation. Goodfriend and Sehon (1970) have been pioneers in this area. They coupled estrone to HSA or BSA through highly stable carbomido-linkages at the C-17 and C-2 positions. Midgley, Niswender and Ram (1969) coupled estradiol to BSA through its chloro-derivative. They did not determine whether the conjugation was at the 3, the 17 or at both of these carbon atoms.

Separation of Bound and Free Steroid

In CPB assay and RIA it has been necessary to separate the free steroid from the protein bound steroid. This has been accomplished by a number of different methods such as dialysis, electrophoresis, gel filtration, use of ion exchange resins, protein precipitation and adsorption of the unbound fraction to insoluble particles (aluminum silicates, florisil and coated charcoal) (Murphy, 1967).

Florisil has been very convenient to work with because the reaction is negligible until the mixture is shaken, and centrifugation is not required. It has been the primary adsorbent used in CPB assays of progesterone. Dextran-coated charcoal has been used primarily in RIA of estrogens, because of smaller reaction volumes. Charcoal has been found to be less convenient than florisil because it requires centrifugation (Murphy, 1970).

In contrast to the advantages of florisil, there are some disadvantages. The steroid adsorbing ability of this material varies considerably between batches. Johansson (1970) established a criterion for testing different batches of florisil. His testing procedure consisted of determining the ability of the batch of florisil to bind the steroid in water. A batch of florisil was acceptable if it adsorbed 80 percent or more of the labeled steroid. Some batches of florisil bound as little as 30 percent of the tracer.

II. PROGESTERONE AND ESTROGEN LEVELS IN THE BLOOD OF DOMESTIC ANIMALS

This portion of the review has been limited primarily to research conducted with CPB assay and RIA on peripheral blood hormone levels. However, the data obtained by gas liquid chromatography (GLC) have been discussed when pertinent. In addition reference has been made to steroid concentrations in the uterine vein, ovarian vein and corpus luteum (CL).

Bovine

It appears that changes in CL growth, CL progestin content, ovarian venous blood progesterone concentration and jugular venous progesterone concentration are correlated during the estrous cycle.

The CL of the cow appears to have a triphasic growth pattern. Foley et al. (1964) found increased growth from days 3 to 6, 9 to 10 and 13 to 15. The CL weight declined approximately 70 percent from day 15 to day 20. CL progestin content followed a very similar pattern (Erb and Stormshak, 1961). Dobrowolski, Stupnicka, and Domanski (1968) assayed progesterone in the ovarian vein of cycling cows. The changes that occurred in ovarian venous progesterone were not in complete agreement with CL changes. They found that the concentration actually decreased on days 9 and 10 and increased on days 11 and 13. In peripheral blood the concentration increased rapidly from day 3 through 6 and then plateaued through day 12. Between days 12 and 16 there was a slight increase in concentration (Shemesh, Lindner and Ayalon, 1971). Stabenfeldt, Ewing and McDonald (1969) found that the concentration drops very rapidly between days 17 and 20. Part of the discrepancy between CL growth and blood progesterone patterns may be due to the fact that CL retain their capacity to synthesize progesterone in vitro for 18 to 19 days after estrus (Armstrong and Black, 1966).

In pregnant cows the production of progesterone by the CL has been found to be essential for pregnancy maintenance during the first 180 to 200 days (Estergreen et al., 1967). Erb et al. (1968a) found

that even though the CL remains active throughout pregnancy, CL weight and progestin content do not reflect the decline in ovarian venous plasma progesterone or the increasing levels of progesterone in jugular plasma. The increased concentration of progesterone in jugular plasma in the late stages of pregnancy may be due to an extra-ovarian source or a decrease in the metabolic clearance rate.

A difference between jugular venous plasma progesterone levels in pregnant and non-pregnant beef heifers has been detected as early as day 9 after estrus, and possibly as early as day 6 (Henricks et al., 1971). However, Shemesh, Ayalon and Lindner (1968) and Batson (1968) were not able to detect differences until 19 days and 21 days, respectively, in Holstein cows. Part of the discrepancy may be due to the fact that some of Batson's non-pregnant cows had cycle lengths longer than 21 days, and thus the progesterone concentration remained elevated for a longer period of time.

Donaldson, Bassett and Thorburn (1970) found that the progesterone concentration does not change significantly from day 15 until 220 to 240 days of gestation. Their results indicated that the concentration between 100 and 170 days of gestation may actually be lower than the levels during earlier or later stages of pregnancy. Stabenfeldt, Osburn and Ewing (1970) reported that the progesterone concentration in Hereford cows varied insignificantly between 140 and 200 days. They noted that an increase of 2 ng per ml occurred by day 250, which was followed by a decline of 2.8 ng per ml to about 10 days before parturition. The results of Erb et al. (1968a) show a positive

linear correlation between progesterone concentration and days in the reproductive cycle; this indicated a significant upward trend from day 11 to day 284 of pregnancy. Individual means indicated that the concentration decreased between days 14 and 51, increased slightly between days 51 and 204 and then decreased slightly between 204 and 264 days.

Pope, Gupta and Munro's (1969) fitted regression line indicated that the progesterone concentration decreased approximately 50 percent, from 50 days pre-partum to parturition. Twenty to 24 hours before parturition the progesterone concentration began to drop very rapidly (Donaldson, Bassett and Thorburn, 1970; Stabenfeldt, Osburn and Ewing, 1970). Donaldson, Bassett and Thorburn (1970) found that the levels decreased approximately 2 ng per ml at 5 hour intervals, beginning 20 hours before calving. The concentration on the day of calving was less than 1 ng per ml (Donaldson, Bassett and Thorburn, 1970; Stabenfeldt, Osburn and Ewing, 1970).

Early post-partum plasma progesterone values are usually low and non-cyclic in most cows. Ovulations often occur at erratic intervals and the developing CL may or may not secrete progesterone (Pope, Gupta and Munro, 1969). Pope, Gupta and Munro (1969) found that some cows during the early post-partum period may have two to three ovulations, unaccompanied by increased progesterone levels. In other cows they noted cyclic changes in progesterone but no detectable heat periods.

During pre-estrus and estrus, progesterone and estrogen have been found to be inversely related. Henricks, Dickey and Hill (1971)

have demonstrated that peripheral estrogen levels began to rise two to three days before estrus (4-5 pg) and reached a peak concentration (12-30 pg) during the day prior to estrus. This occurred at a time when progesterone was very low (usually less than 1 ng per ml). After reaching the peak concentration, the estrogen level began to fall (within two to five hours) and reached the base level between estrus and 24 hours post-estrus. In another study Henricks et al (1972) showed that the estrogen concentration in pregnant heifers averages 2-4 per ml plasma from 3 to 39 days post-insemination. However, in mated heifers that returned to service the estrogen level on day nine was greater than 12 pg per ml, which was significantly higher than the levels at other days of the cycle. This peak on day nine may have induced luteal regression, and thus the cows returned to heat. Wiltbank (1966) demonstrated that injected estradiol valerate would induce luteal regression on day eight or nine of the cycle.

Hoffman (1972) reported considerably higher estrogen values than Henricks, Dickey and Hill, (1971) and Henricks et al. (1972). Using an antisera that exhibited 100 percent cross-reactivity with estradiol-17β, estradiol-17α and estrone, he found values of 100 pg ml during the luteal phase and 350 pg per ml around the time of estrus. Part of the discrepancy in the above findings may be due to the fact that the antisera used by Henricks, Dickey and Hill (1971) exhibited large cross reactions with estradiol-17β and estrone and only slight cross reaction with estratiol-17α and estriol.

In contrast to the above reports, Wettemann et al. (1972) found values slightly lower than those of Henricks, Dickey and Hill

(1971) and values much lower than those of Hoffman (1972). Their results with chromatographically purified extracts indicated that a peak estradiol concentration of 9.7 pg per ml occurred 0.5 days before estrus.

There has been very little research on blood estrogen levels during the mid-stages of pregnancy. Wettemann and Hafs (1972) found serum estradiol levels in Holsteins to average 6.1 pg per ml during days 7 through 75. They found also elevated estradiol concentrations between days 30 and 42 in 50 percent of the cows.

In the third trimester of pregnancy blood estrogen concentration increased more than 100 fold in comparison with cyclic and early pregnancy levels. Henricks et al. (1972) indicated that the concentration increased from 500 pg per ml at 14 days pre-partum to 2600 pg per ml on the day of calving. The work of Edgerton and Hafs (1972) indicated that the major increase in estrogen concentration was due to an increased estrone concentration. Estradiol increased linearily from 32 pg per ml at 26 days pre-partum to 295 pg per ml at 1.5-2.0 days before calving. Estradiol then dropped to 52 pg per ml at one day post-partum and 20 pg per ml at three days post-calving. Estrone increased from 218 pg per ml at 26 days pre-partum to 2,279 pg per ml at 2.5 days pre-partum. This was followed by a gradual decline to 1,794 pg per ml at 0.5 days pre-partum then to 142 pg per ml at 0.5 days post-partum. By 13 days post-partum the concentration of estrone had dropped to 45 pg per ml. Robinson et al. (1970) reported considerably higher estrone concentrations, as

determined by the Ittrich-Koher fluorometric procedure. At 14 to 16 weeks pre-partum, the estrone concentration increased from 1.2 ng per ml to 2.5 ng per ml at 6-8 weeks pre-partum. The concentration was 4.8 ng per ml at 2 to 4 weeks before calving and 8.3 ng per ml at 5 days pre-partum. At 5 days post-partum the concentration averaged 0.7 ng per ml.

Erb et al. (1968b) found that total estrogen excretion in the urine increases from 650 micrograms (μg) per 500 kilogram (kg) of liveweight at 230 days of pregnancy to 3,402 mg per kg of liveweight at 281 days of pregnancy. The major portion of total estrogen excretion was estradiol-17α, as compared with the high blood levels of estrone.

Blood estrogen patterns have been found to be variable in post-partum cows (Henricks et al., 1972). Some cows have estrogen peaks (15-23 pg per ml) and visible signs of heat, while other cows have estrogen peaks (18-25 pg per ml) but no visible signs of heat (silent estrus). In addition, other cows do not exhibit cyclic estrogen patterns or visible signs of heat (Henricks et al., 1972).

Ovine

Peripheral plasma progesterone levels in the cycling ewe have been found to be considerably lower than the concentration found in cows during the luteal phase of the cycle (Thorburn, Bassett and Smith, 1969). During the first four days of the cycle, the progesterone concentration in ewes (0.1-0.2 ng per ml) was not greatly different from that found in wethers, anestrous ewes or ovariectomized ewes. Between

days 4 and 9 the concentration increased to 1.7 ng per ml plasma. Luteal growth stages corresponded to the changes in peripheral concentration. Howland et al. (1966) found rapid growth from days 3 to 7, followed by static growth through day 15. Luteal regression and the cessation of luteal function occurred simultaneously between days 15 and 16 (Howland et al., 1966; Erb, Randel and Callahan, 1971). Bjersing et al. (1972) found that the progesterone concentration started to fall abruptly on day 15 (16.5 day cycle) after estrus. The concentration (less than 0.2 ng per ml) was low from six hours after estrus to 30 hours after estrus in both pre- and post-ovulatory groups. Obst and Seamark (1970) showed that the cycle length in ewes may be altered when they are grazing Yarloop clover (estrogenic pasture). They found that progesterone levels began to drop rather sharply 13 days after estrus, as compared with 15 days in ewes not grazing estrogenic pasture. The ingested plant estrogens may have had a direct luteolytic effect on the CL or acted in conjunction with a luteolytic agent in the uterus.

In pregnant ewes the progesterone concentration increased about 10 fold, as compared with estrous cycle levels. The peripheral level increased slightly from 20 days to 100 days of pregnancy and then more rapidly to a peak concentration (7-13 ng per ml for single fetuses) at 130-140 days (Bassett et al., 1969; Fylling, 1970; Stabenfeldt, Drost and Franti, 1972). According to Stabenfeldt, Drost and Franti (1972) the progesterone levels in ewes carrying two fetuses was higher than in ewes carrying only a single fetus. The concentration increased

from 3 ng per ml at 55 days to 9.5 ng per ml at 125 days for single pregnancies, as compared with 3 ng per ml at 55 days to 15.5 ng per ml at 130 days for twin pregnancies. Regressions of concentration time for single and twin pregnancies from 60 days to 130 days were significantly different (Stabenfeldt, Drost and Franti, 1972). Gadsby et al. (1972) found a correlation of 0.999 between litter size and peripheral progesterone concentration. However, the realized percent accuracy of prediction of litter size was only 63 to 69 percent.

The CL of the pregnant ewe has been found to be necessary for pregnancy maintenance during the first half of gestation. In the latter half of pregnancy the CL may be removed and pregnancy will not be interrupted (Nalbandov, 1964). It appears that extra-ovarian sources may be producing progesterone during the latter stages of pregnancy. Thorburn and Mattner (1971) showed that the utero-ovarian vein concentration decreased from 90 ng per ml at 100 days to 50 ng per ml at 140 days, while the jugular concentration increased from 2 ng per ml to 7 ng per ml. The increase in jugular concentration was not due to changes in metabolic clearance rate, which remained relatively constant throughout pregnancy (Bedford et al., 1972). The secretion rate into the ovarian vein (3.0-4.0 milligram per day) has been found to be fairly stable between 40 and 120 days. However, between 120 and 139 days the secretion rate decreased to 0.04 mg per day. The concentration of progesterone in the ovarian vein (1000-1600 ng per ml) between 40 and 120 days of gestation has been found to be approximately 23 fold greater than the concentration in the

uterine vein (16-99 ng per ml) (Moore, Varrett and Brown, 1972). This does not agree with the work of Linzell and Heap (1968) who found that the placenta produces progesterone at the rate of 14 milligram (mg) per day as compared to 2 mg per day for the ovaries (119 to 126 days of pregnancy).

As in the cow, the progesterone concentration in peripheral plasma of the ewe began to decline as parturition approached (Fylling, 1970). Fylling (1970) found that by two days pre-partum the concentration had dropped 50 percent. Stabenfeldt, Drost and Franti (1972) found that in one ewe the concentration decreased from 7.4 ng per ml at 7 hours pre-partum to 2.0 ng per ml at 11 minutes post-partum. By 38 minutes post-partum the concentration had dropped to 0.5 ng per ml.

Scaramuzzi, Caldwell and Moor (1970) demonstrated that the ovarian venous estrogen concentration in the non-pregnant ewe began to rise two days before expected estrus and remained high over a three day period (through estrus). On the day of estrus the concentration was significantly higher than concentrations on other days of the cycle. Estrogen peaks were also noted on days two and eight of the cycle, which approached significance (Scaramuzzi, Caldwell and Moor, 1970). Cox, Mattner and Thorburn (1971) reported a post-ovulatory estradiol-17β secretion rate peak on days three and four of the cycle. An increase in secretion occurred between days six and nine of the cycle (Mattner and Braden, 1972). The source or significance of these luteal peaks has not been ascertained, but there has

been some indication that the three and four day peaks may result from follicles undergoing stresia (Holst, Braden and Mattner, 1972). Cox, Mattner and Thorburn (1971) speculated that this early luteal peak (three to four days) may influence ovum transport in the fallopian tube or development of the uterine endometrium.

The estrogen scheme in the pregnant ewe has been found to be similar to the pattern observed in the cow during the latter stages of pregnancy. Obst and Seamark (1972) found that the estrogen concentration in jugular venous plasma increased from 8 pg per ml at 72 hours pre-partum to 155 pg per ml at 8 hours pre-partum. At 8 hours post-partum the concentration had dropped approximately 55 percent. The estrogen concentration remained relatively high until after parturition (Bedford et al., 1972). In the uterine vein and the carotid artery the concentration increased through 32 minutes post-partum. By 2.5 hours the concentration had dropped more than 50 percent in the uterine vein (Bedford et al., 1972).

Caprine

Jugular venous plasma progesterone levels in the cycling goat were intermediate between the ewe and the cow. Thorburn and Schneider (1972) reported a steady increase in concentration from day 2 (0.2 ng per ml) through day 10 (4.0 ng per ml), followed by very little change through day 18. On days 19 and 20 there was a very sharp drop in concentration to 1.8 ng per ml and 0.5 ng per ml, respectively. In contrast to the cow and the ewe, the CL of the goat did not appear to start regression until two days before estrus.

In pregnant goats the progesterone concentration did not decline on day 20 (Irving, Jones and Knifton, 1972). In those goats bearing single fetuses the concentration (2.5-3.5 ng per ml) remained fairly constant from day 10 through day 60; and increased to around 4.5-5.5 ng per ml during the second stage of pregnancy. As in sheep, goats with twin fetuses have higher progesterone values than those with single fetuses (Irving, Jones and Knifton, 1972).

At about six to seven days pre-partum the progesterone concentration began to decrease (Thorburn and Schneider, 1972). According to Irving, Jones and Knifton (1972) the concentration dropped 5.0 ng per ml during the last seven days of pregnancy or about 0.71 ng per ml per day.

Total unconjugated estrogens in pregnant goats have been found to increase from less than 5.0 pg per ml at 130 days before parturition to greater than 600 pg per ml at parturition (Challis and Linzell, 1971). According to Thorburn et al. (1972) the estrogen concentration was fairly stable during the pre-partum week (approximately 500 pg per ml). However, on the day of parturition the concentration may have increased by two to three fold.

Porcine

The circulating levels of plasma progesterone have been found to be higher in pigs as compared with other domestic animals. The results of studies with CPB assay (Edqvist and Lamm, 1971) and GLC (Stabenfeldt et al., 1969) were in good agreement. There was a sharp

increase in concentration from day 1 (0.5 ng per ml) through day 8 (20 ng per ml), followed by a more gradual increase to a peak concentration on day 12 (30 ng per ml). The concentration began to decrease on day 14, with a 30 fold decrease usually occurring within 48 hours. The changes in CL weight, CL progesterone concentration (Masuda et al., 1967) appeared to be closely related to changes in peripheral concentrations during the first eight days of the cycle. During the latter stages of the cycle, the peripheral plasma concentration did not decrease as quickly as CL weight and progesterone content. Tillson, Erb and Niswender (1970) were able to detect differences in progesterone concentrations of pregnant and non-pregnant sows as early as 14-16 days after estrus. In pregnant sows the progesterone concentration was maximum at 12 days and then declined gradually (approximately 0.4 ng per ml per day) through 28 days post-breeding. The progesterone concentration during this time period did not appear to be related to litter size, as was the condition in sheep. Tillson and Erb (1970) were unable to detect differences in the progesterone concentration in sows with less than 9 embryos, when compared with sows with more than 11 embryos. The progesterone concentration of the CL of pregnant sows increased slightly between days 18 and 40 and then declined rather sharply through day 70. Between days 70 and 106 there was practically no change in concentration.

Henricks, Guthrie and Handlin (1972) reported that the estrogen concentration in the peripheral blood of the cycling pig was considerably higher than the levels found in cows. During the luteal phase

of the cycle, the levels fluctuated between 10-30 pg per ml. On the day prior to estrus the concentration peaked at 60-70 pg per ml. At estrus the level declined and was somewhat variable, between 12 and 86 pg per ml.

Equine

There have been very few studies conducted on steroid hormones in mares. Plotka, Witherspoon and Foley (1972) assayed progesterone in cycling mares. The concentration was lowest between 5 days pre-ovulation and 1 day post-ovulation (0.41 ng per ml and 0.09 ng per ml, respectively). On days 2, 3 and 4 post-ovulation the concentration increased to 1.5 ng per ml. On day 5 the concentration increased to greater than 7 ng per ml. The average concentration between days 5 and 13 was 5.49 ng per ml. Psychic signs of estrus occurred soon after the decrease in progesterone concentration.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. OBJECTIVES OF EXPERIMENT

The first objective of this research project was to evaluate the technique of RIA of estrogen, as described by Henricks (1970) and Henricks, Dickey and Hill (1971). The second objective was to determine the interrelated changes in the concentrations of progesterone and estrogen in the jugular venous plasma of non-bred, non-pregnant and pregnant Holstein cows.

The hormonal changes that occur prior to estrus and near the time of implantation were of special interest. Henricks et al., (1971) found that high progesterone levels prior to estrus may hinder ova development and thus reduce fertility in cows. Abubaker and Abraham (1969) demonstrated an estrogen surge, four days post-breeding in pseudopregnant rats, that may be involved in uterine sensitization. In rabbits it has been found that the estradiol-17β secretion rate increased prior to implantation (Hillard and Eaton, 1971). Johansson (1971) found that the average estradiol-17β levels increased in the peripheral plasma of pregnant women and decreased in non-pregnant subjects, beginning 10 days post-ovulation.

II. ANIMALS AND SAMPLING

Six hundred twenty-nine blood samples were collected from the jugular veins of 14 lactating cows. Twelve of the cows, had exhibited

at least one heat period prior to the initiation of sample collection. Estrus was defined as standing for mounting by other cows. Cows were observed for estrus three to four times daily.

Attempts were made to begin collection three to four days prior to estrus. However, this was not always possible, because of variation in cycle lengths. Sample collection was continued, at daily intervals, for 25 days after insemination in bred cows. If the bred cows returned to estrus, sample collection was continued for another 25 days post-insemination. Samples were taken at daily intervals in non-bred cows through one or two cycles. Some cows appeared in both non-bred (first cycle) and bred (second cycle) groups. Six cows did not exhibit estrus at the expected interval. However, blood collection was continued for 30 to 40 days in this group of animals.

The cows were divided into the following groups: A) non-bred (estrus detected), B) non-bred (estrus undetected), C) non-pregnant and D) pregnant. Pregnancy was determined by rectal palpation at 40 to 50 days post-breeding.

III. MATERIALS

Phosphate buffered saline solution (0.1 M, pH 7.0) plus gelatin (PBSG) was prepared by the following procedure:

5.38 grams	$NaH_2PO_4 . H_2O$
16.35 grams	$Na_2H\ PO_4 . 7H_2O$
9.00 grams	Sodium azide
1000.00 ml	Double distilled water
1.00 gram	Knox unflavored gelatin

Dextran-coated charcoal solution was prepared by the following procedure:

250 mg Norit A charcoal

25 mg Dextran T-70

100 ml PBSG

The charcoal (5 grams) was washed seven times with distilled water (40 ml) to remove excessive fines. The removal of excessive fines was necessary to prevent erroneously high counts.

All solvents were reagent grade, with the exception of toluene (spectroquality) and were used without further purification. Maximum purity of toluene was required to attain a high counting efficiency. Petroleum ether and diethyl ether were used from recently opened containers. This was necessary because of the instability of these solvents in repeatedly opened containers. Solvent blank values were minimized by this procedure. Petroleum ether blanks averaged 0.09 ± 0.01 (ng ± standard error) (10 assays). Diethyl ether blanks averaged 2.11 ± 0.81 pg (10 assays).

Progesterone was obtained from Mann Research Laboratories. Estradiol-17β, estrone and estril were purchased from Sigma Chemical Company. These steroids were used without further purification.

Progesterone-1, 2-H^3, corticosterone-1, 2-H^3 and estradiol-17β-2, 4, 6, 7-H^3 were obtained from New England Nuclear and used without further purification. Liquiflour was also obtained from New England Nuclear. Corticosterone-1, 2-H^3 was checked for purity by thin-layer chromatography (Eastman pre-coated plates and a solvent

system of chloroform: methanol: water (188:12:1). All isotopes were obtained in small quantities (0.25 millicuries), because their stability decreases with storage.

IV. PROGESTERONE ASSAY PROCEDURE

Dowlen's (1972) CPB assay procedure for progesterone was modified in the following manner: (1) Progesterone was extracted with petroleum ether by shaking forty 15 ml conical centrifuge tubes, in a test tube rack for two minutes. This extraction procedure was adopted because it was much faster than the mechanical wrist action shaker which contained only 24 extraction tubes. (2) After the addition of florisil, the culture tubes were stoppered (rubber); and all tubes were shaken simultaneously. This modification proved superior to the vortexing of individual samples because it reduced the variation between duplicate samples. (3) A toluene based counting solution was substituted for the dioxane cocktail used by Dowlen, because of higher counting efficiency (35 percent versus 25 percent) and the greater stability of toluene as compared with that of dioxane.

The following CPB assay procedure was used:

1. A progesterone stock solution was prepared in absolute ethanol at a concentration of 100 ng per ml. The stock solution was diluted with absolute ethanol to a concentration of 1 ng per 0.5 ml, 3 ng per 0.5 ml and 6 ng per 0.5 ml. These concentrations, plus 0.5 ml of absolute ethanol for the zero level, were assayed in duplicate to construct a standard curve for each assay.

2. Labeled and unlabeled progesterone recoveries were determined for each assay. One nanocurie of progesterone-1,2-H^3 (50.3 curies per millimole) was added to each of two 15 ml extraction tubes. One-half ml of plasma from the cow being assayed was added to the extraction tubes. Crystalline progesterone (3 ng) was also added to each of two extraction tubes. One-half ml of steer plasma was added to the tubes containing unlabeled progesterone. The extraction tubes were mixed prior to extraction to enhance the solubility of progesterone.
3. One-half ml of cow plasma was pipetted in duplicate into 15 ml conical centrifuge tubes. Five ml of petroleum ether was added to the tubes and the tubes were stoppered with linear high density polyethylene stoppers. Forty tubes were shaken simultaneously in a test tube rack.
4. The tubes were placed in an acetone-dry ice bath and the ether phase was decanted into 13 X 100 millimeter (mm) disposable culture tubes.
5. Petroleum ether was evaporated in a water bath (40°-45°C), under a stream of dry air.
6. After thawing, the plasma was extracted a second time.
7. Progesterone was concentrated in the bottom of the culture tubes by rinsing the tubes with 1 ml of petroleum ether.
8. One ml of CBG-B^3H solution (17.5 ng of corticosterone-1,

$2\text{-}H^3$, 44 curies per millimole and 2 ml of dog plasma per 98 ml of double distilled water) was added to standard and unknown samples.

9. The tubes were vortexed for five seconds, placed in a water bath (40°-45°C) for five minutes and vortexed again for five seconds.

10. The samples were placed in an ice bath for no less than 10 minutes.

11. Eighty mg of florisil was added to each tube to absorb free corticosterone-1, $2\text{-}H^3$.

12. The culture tubes were stoppered (rubber) and all tubes were shaken simultaneously in a test tube rack.

13. One-half ml of the supernatant was pipetted into 10 ml of a toluene counting solution (1000 ml of toluene plus 42 ml of liquiflour).

14. The samples were vortexed vigorously for five seconds and placed in a Nuclear-Chicago liquid scintillation counter one hour prior to counting. The samples were counted to a pre-set count of 10,000 counts per minute.

The counts per minute from the progesterone standards were used to calculate an asymptotic regression line with a Wang computer. The regression line was used in calculating the quantity of progesterone in unknown samples. The amount of progesterone in an unknown sample was inversely related to the counts per minute (quantity of corticosterone-1, $2\text{-}H^3$).

An elaborate discussion of this procedure has been purposefully omitted, because Dowlen (1972) covered the subject thoroughly.

Illustrated in Figure 1 is an asymtotic regression line which represents 10 consecutive standard curves. The standard errors exhibit the daily variation between assays. The coefficients of variation (CV) ranged between 5.33 percent (0 ng) and 9.30 percent (6 pg). A total of 24 assays were performed in this experiment. However, due to the limited capacity of the Wang computer, the number of standard curves used to construct the asymptotic regression line were limited to 10.

V. ESTROGEN ASSAY PROCEDURE

The procedure of Henricks (1970) and Henricks, Dickey and Hill (1971) was modified for the RIA of estrogen in this laboratory. 1) Estrogen was extracted from plasma with 6 ml of diethyl ether, as opposed to 4 ml of diethyl ether. This was done to reduce the incidence of gel formation that occurred when 4 ml of plasma were shaken with 4 ml of diethyl ether. 2) NH_4OH was not added to plasma prior to extraction because it was found to be ineffective in reducing the extraction of yellow pigment. 3) The dilution of estradiol-17β antibody was increased from 1:15,000 to 1:20,000. This modification was instituted to increase the sensitivity of the standard curve in the low range. 4) The ice water incubation period of the reaction mixture was increased from 30 minutes to 3.5 hours. This change was made to increase the recovery of unlabeled estradiol-17β and for

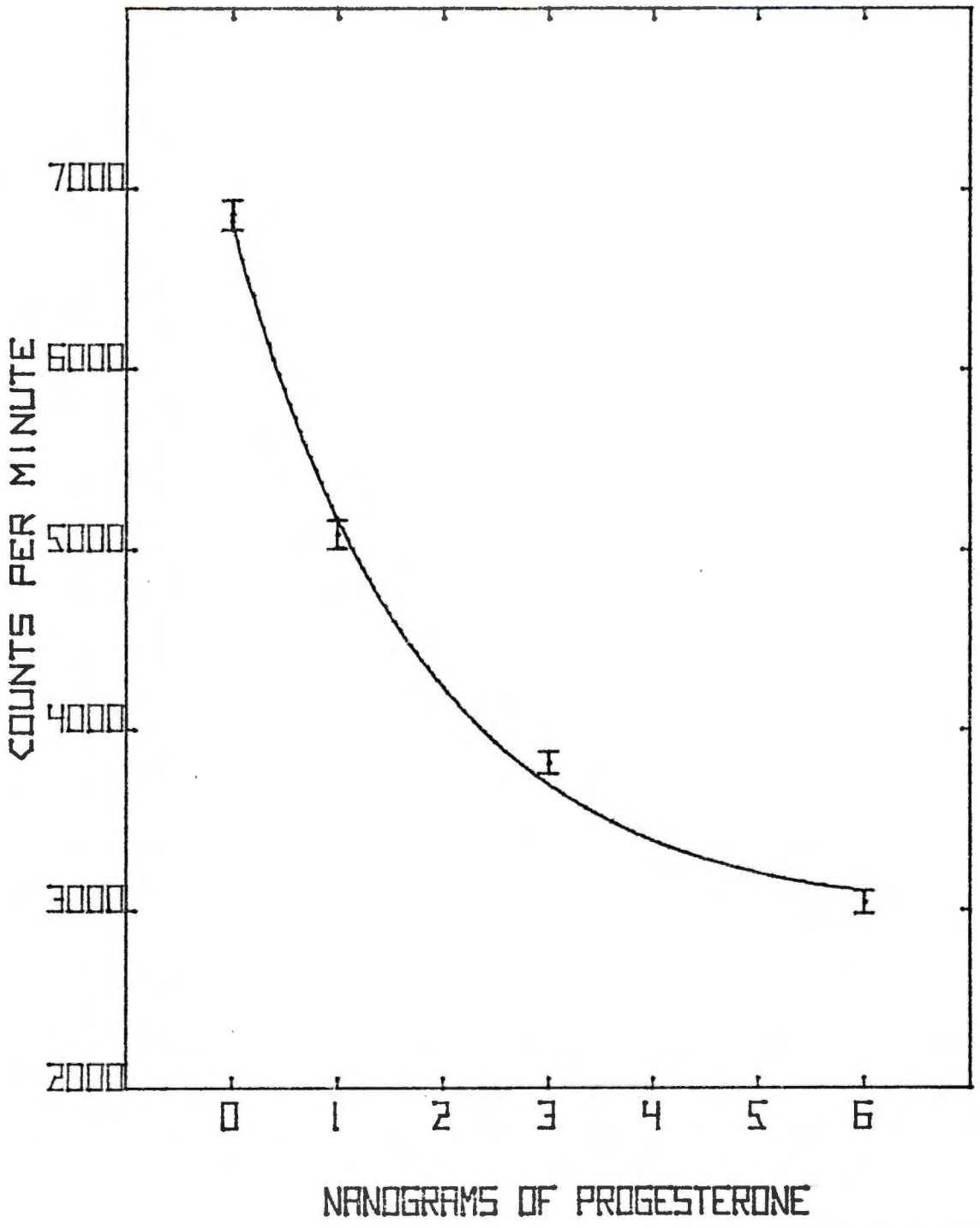

Figure 1. An asymptotic regression line and standard errors for 10 consecutive progesterone standard curves.

convenience in the assay procedure. Between 70 and 80 plasma samples were extracted during one day. The following morning estradiol-17β-2, 4, 6, 7-H^3 and estradiol-17β antibody were added to the tubes containing estrogen. The samples were then incubated for 3.5 hours. During the incubation period preparations were made for the continuation of the assay and for the next assay.

The following procedure was used for the RIA of estrogen:

1. A stock solution of estradiol-17β was prepared in absolute ethanol at a concentration of 100 ng per ml. Portions of the stock solution were diluted with absolute ethanol to concentrations of 10 pg per 0.5 ml, 20 pg per 0.5 ml, 50 pg per 0.5 ml and 100 pg per 0.5 ml. The above quantities of estradiol-17β plus zero pg per 0.5 ml were used for the standard curve.
2. The recovery of estradiol-17β was determined by adding 4 nanocuries of estradiol-17β-2, 4, 6, 7-H^3 to each of two 15 ml conical centrifuge tules and 20 pg and 50 pg of unlabeled estradiol to each of two centrifuge tubes. After the addition of estradiol-17β, 4 ml of plasma from the cow being assayed was added; and the samples were mixed and extracted. Two levels of unlabeled estradiol-17β were used to determine the efficiency of the procedure at low and high concentrations.
3. Four ml of cow plasma was pipetted in duplicate into 15 ml conical centrifuge tubes. Six ml of diethyl ether was

added to each tube, and tubes were stoppered with linear high density polyethylene stoppers. Forty tubes were shaken simultaneously in a test tube rack for two minutes.

4. The ether phase was removed by pipette or an aspiration apparatus attached to a vacuum line and placed in 13 X 100 mm culture tubes. The culture tubes were placed in a water bath (40°-45°C), and the ether was evaporated under a stream of dry air.

5. A second extraction was performed on the plasma. After extraction, the centrifuge tubes were immersed in an acetone-dry ice bath; and the ether phase was decanted into the air-dried culture tubes and evaporated. It was necessary to aspirate the ether after the first extraction to reduce the amount of yellow pigment in the second extraction. The freezing of plasma and decanting of the ether phase was much faster than aspiration.

6. Estrogen was concentrated in the bottom of the culture tubes by rinsing the tubes with 1 ml of diethyl ether.

7. One hundred µl of estradiol antibody[1] (1:20,000) in phosphate buffered saline plus gelatin (PBSG) solution was added to each standard and unknown tube. The tubes were vortexed for five seconds.

8. One hundred µl of estradiol-17β-2, 4, 6, 7-H^3 (49.4 pg - 110 curies per millimole) in PBSG was added to the culture

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[1]The estradiol antibody was obtained from Dr. Burton V. Caldwell (Yale University).

tubes, and the tubes were again vortexed for five seconds.

9. The samples were incubated in an ice bath for 3.5 hours.
10. One ml of dextran-coated charcoal solution was added to each sample, and the tubes were stoppered (rubber) and shaken in a test tube rack for five seconds.
11. The culture tubes were centrifuged for five minutes at 1500 X g.
12. One-half ml of supernatant was placed in 10 ml of toluene-liquiflour counting solution. The samples were mixed and placed in the counter overnight before counting. This was necessary to attain a higher counting efficiency.
13. The samples were counted to a total of 10,000 counts per minute.

The quantity of estrogen was determined in a manner similar to that described for progesterone.

Illustrated in Figure 2 is an asymptotic regression line representing 10 consecutive estradiol-17β standard curves. The CV ranged between 4.53 percent (0 pg) and 12.80 percent (100 pg). As illustrated by CV, the daily variation between assays is similar to that shown by progesterone standard curves.

The estradiol antibody was produced by immunizing sheep with estradiol-17β conjugated to BSA. The antiserum was diluted 1:100 in

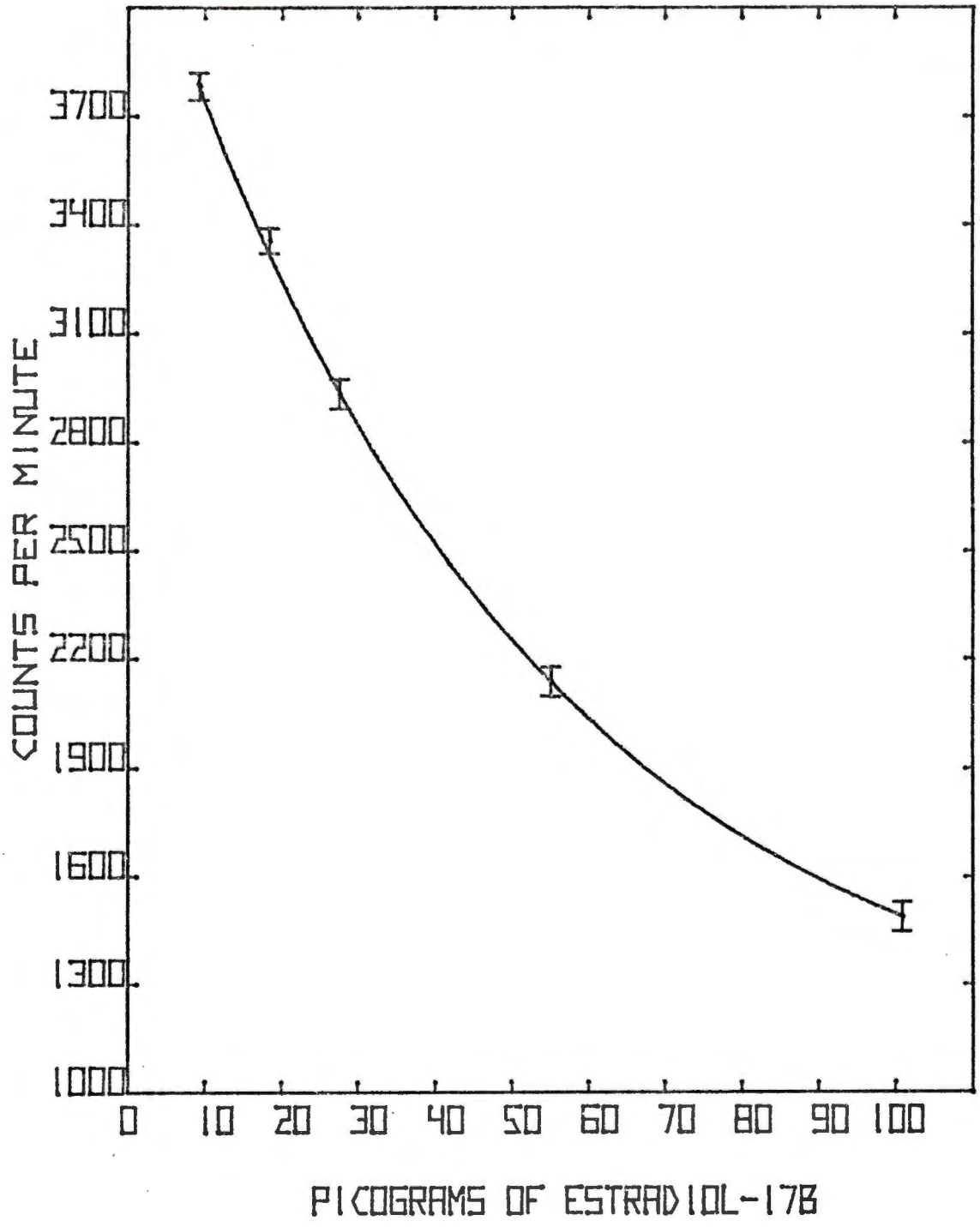

Figure 2. An asymptotic regression line and standard errors for 10 consecutive estradiol-17β standard curves.

PBSG and stored in 1 ml aliquots at -20°C. The final antibody dilution was determined by constructing standard curves with 1:5,000, 1:10,000, 1:15,000, 1:20,000 and 1:30,000 dilutions of antibody (Figure 3). The 1:20,000 antibody dilution was selected because it exhibited the greatest amount of sensitivity over the range, zero to 100 pg.

The antiserum was prepared by thawing a 1 ml aliquot of the 1:100 antiserum and diluting to 1:1,000. Antiserum at 1:1,000 was very stable at 3°C, since no detectable loss in binding efficiency occurred over a two month period. One ml of 1:1,000 antiserum was diluted to 1:20,000 at the time of the assay. Twenty ml of 1:20,000 antiserum was usually a sufficient quantity for two to three assays.

VI. STATISTICAL ANALYSIS

Progesterone and estrogen data were punched on computer cards and orthogonal comparisons were made to test the differences between the groups of cows. The following comparisons were made: 1) non-bred (estrus detected) versus non-bred (estrus undetected), 2) non-pregnant versus pregnant, 3) non-bred (estrus detected and estrus undetected) versus bred (non-pregnant and pregnant). Day differences were tested for significance by Duncan's Multiple Range Test.

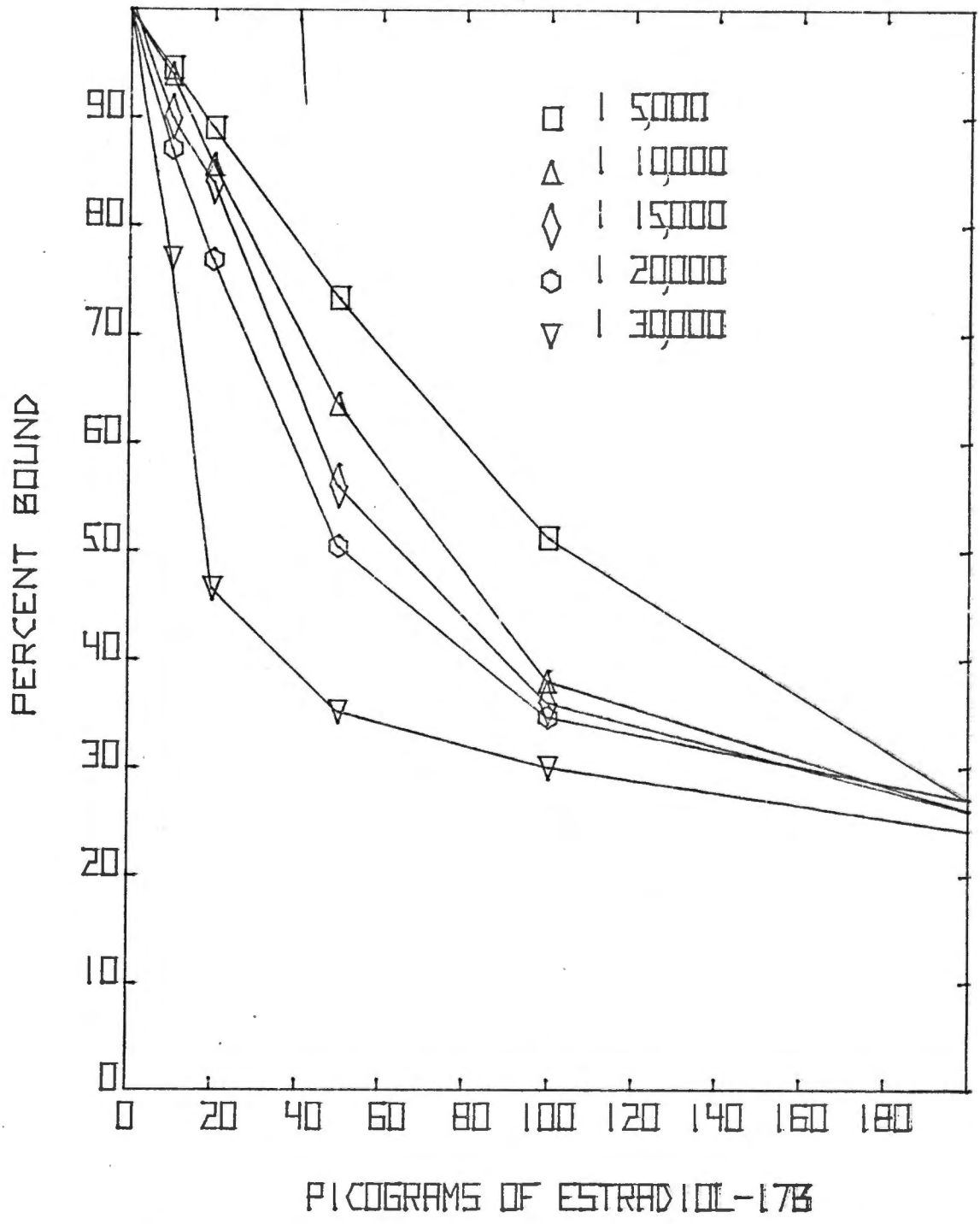

Figure 3. Estradiol-17β standard curves constructed with antibody dilutions of 1:5,000, 1:10,000, 1:15,000 and 1:20,000 and 1:30,000.

CHAPTER IV

RESULTS AND DISCUSSION

I. EVALUATION OF ASSAY METHODS

Extraction Efficiency and Precision

The extraction efficiency of progesterone-1, 2-H^3 averaged 93.58 percent with a standard error of 0.78 percent for 24 assays. The extraction efficiency of unlabeled progesterone was 90.20 ± 2.15 percent (24 assays). There was no significant difference ($P > 0.05$) in extraction efficiencies. The CV for labeled and unlabeled progesterone was 4.54 percent and 14.53 percent, respectively. The unlabeled progesterone extraction efficiency was used to correct for procedural losses.

In the initial stages of establishing the estrogen assay considerable difficulty was encountered in obtaining a high recovery rate of unlabeled estradiol-17β. This problem was alleviated by increasing the incubation time of the reaction mixture in ice water. The extraction efficiency of 20 pg of unlabeled estradiol-17β at one hour incubation (10 assays) and 3.5 hours incubation (25 assays) was 76.08 ± 3.23 percent and 91.18 ± 4.58 percent, respectively. The 3.5 hour incubation resulted in a significantly higher ($P < 0.01$) extraction efficiency. A 16 hour incubation period did not significantly increase ($P > 0.10$) the extraction efficiency (92.13 ± 3.21 percent in 5 assays).

To determine the efficiency of the procedure at 50 pg, this quantity was included in 25 assays. The efficiency of extraction was 88.57 ± 5.24 percent, which was not significantly different from 20 pg recoveries ($P > 0.10$).

The extraction efficiency of 4 nanocuries of estradiol-17β-2, 4, 6, 7-H^3 was 94.04 ± 0.59 percent. There was no significant difference ($P > 0.10$) between the average unlabeled and labeled estradiol-17β recoveries. The CV for 20 pg, 50 pg and 4 nanocuries extraction efficiencies were 25.35 percent, 18.76 percent and 3.79 percent, respectively. Unknown samples were corrected for procedural losses using the extraction efficiency of 20 pg, since most of the unknowns were near this quantity.

Specificity of Estradiol-17β Antibody

Since the estrogen metabolites were not separated after extraction, it was necessary to test the antibody for cross reaction. This was done by preparing standard curves with estradiol-17β, estrone and estriol (Figure 4). Estriol cross reacted only slightly with the antibody. However, the estrone cross reaction was considerably greater. Therefore, this assay procedure measures both estradiol-17β and estrone and estriol to a much smaller degree.

Sensitivity of the Standard Curves

Sensitivity may be defined as the smallest amount of hormone that can be distinguished with confidence from zero or the ability of

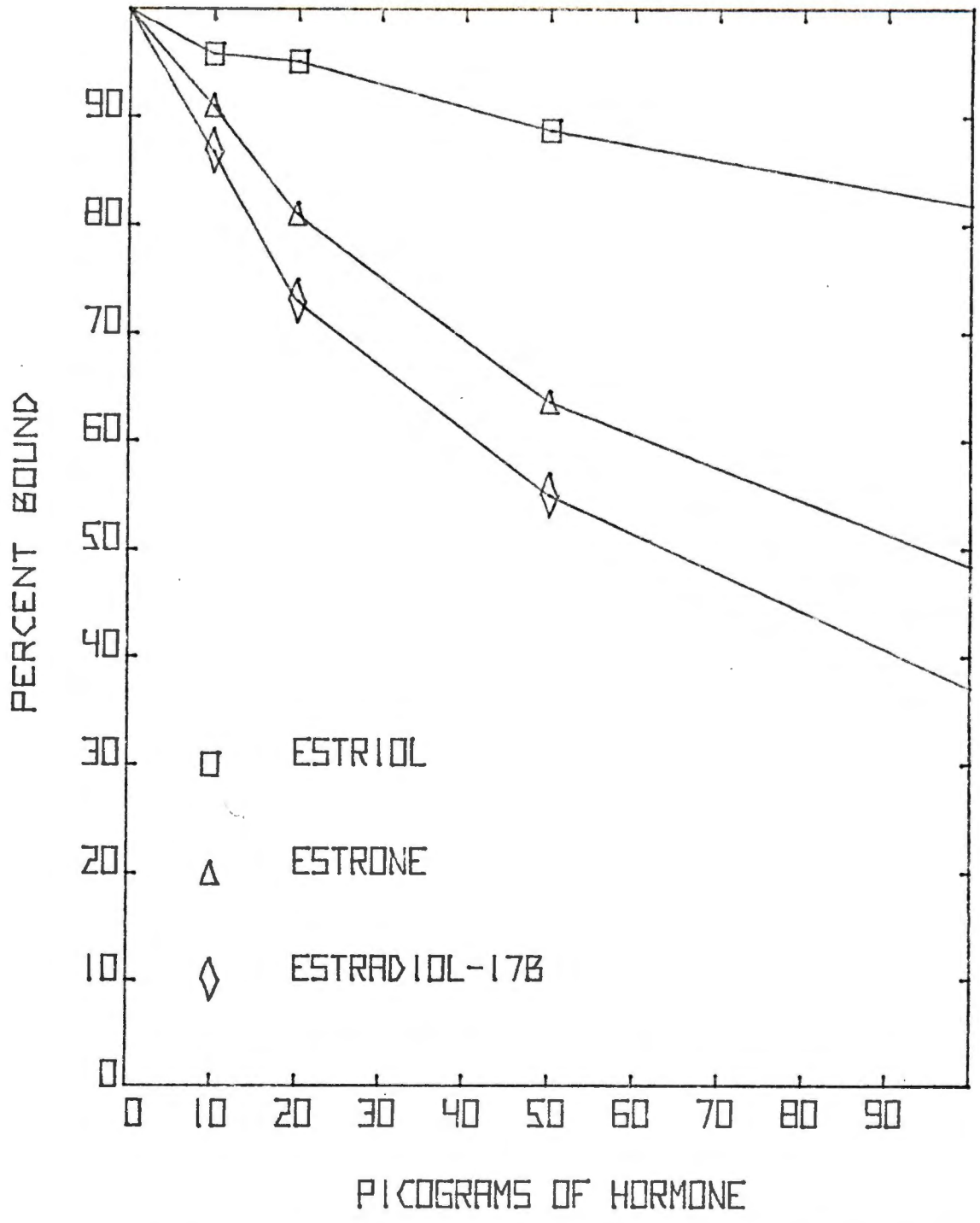

Figure 4. The cross reaction of estradiol-17β, estrone and estriol with estradiol-17β antibody.

the assay system to distinguish between hormone concentrations over its entire applicable range (Reaves and Calhoun, 1970). The former definition is applicable in this particular instance. The sensitivity of the progesterone standard curve was determined by assaying in triplicate 0.1 ng quantities of progesterone (Figure 5). Because 0.3 ng was significantly different ($P < 0.01$) from zero and 0.2 ng was not as significantly different ($P > 0.10$), the lower limit of sensitivity was 0.3 ng. The sensitivity of the estradiol-17β standard curve was determined by assaying in triplicate 2.0 pg quantities of estradiol-17β (Figure 6). Six picograms was significantly different ($P < 0.01$) from zero, while 4.0 pg was not as significantly different ($P > 0.10$). Therefore, 6.0 pg was the lower limit of sensitivity.

II. EVALUATION OF PROGESTERONE AND ESTROGEN DATA

The progesterone and estrogen data (mean, standard error and sample number) for each group of cows are presented in Tables I and II. The mean values are depicted graphically in Figure 7 (non-bred estrus detected = Group A), Figure 8 (non-bred estrus undetected = Group B), Figure 9 (non-pregnant = Group C) and Figure 10 (pregnant = Group D). Day 0 represents the day of estrus in Group A, Group C and Group D. In Group B, Day 0 represents the day of highest estrogen concentration since these animals were not detected in estrus. Cows in Group C were mated and returned to service or palpated non-pregnant.

The levels of progesterone found in this experiment are similar to the values previously obtained in this laboratory (Batson, 1968;

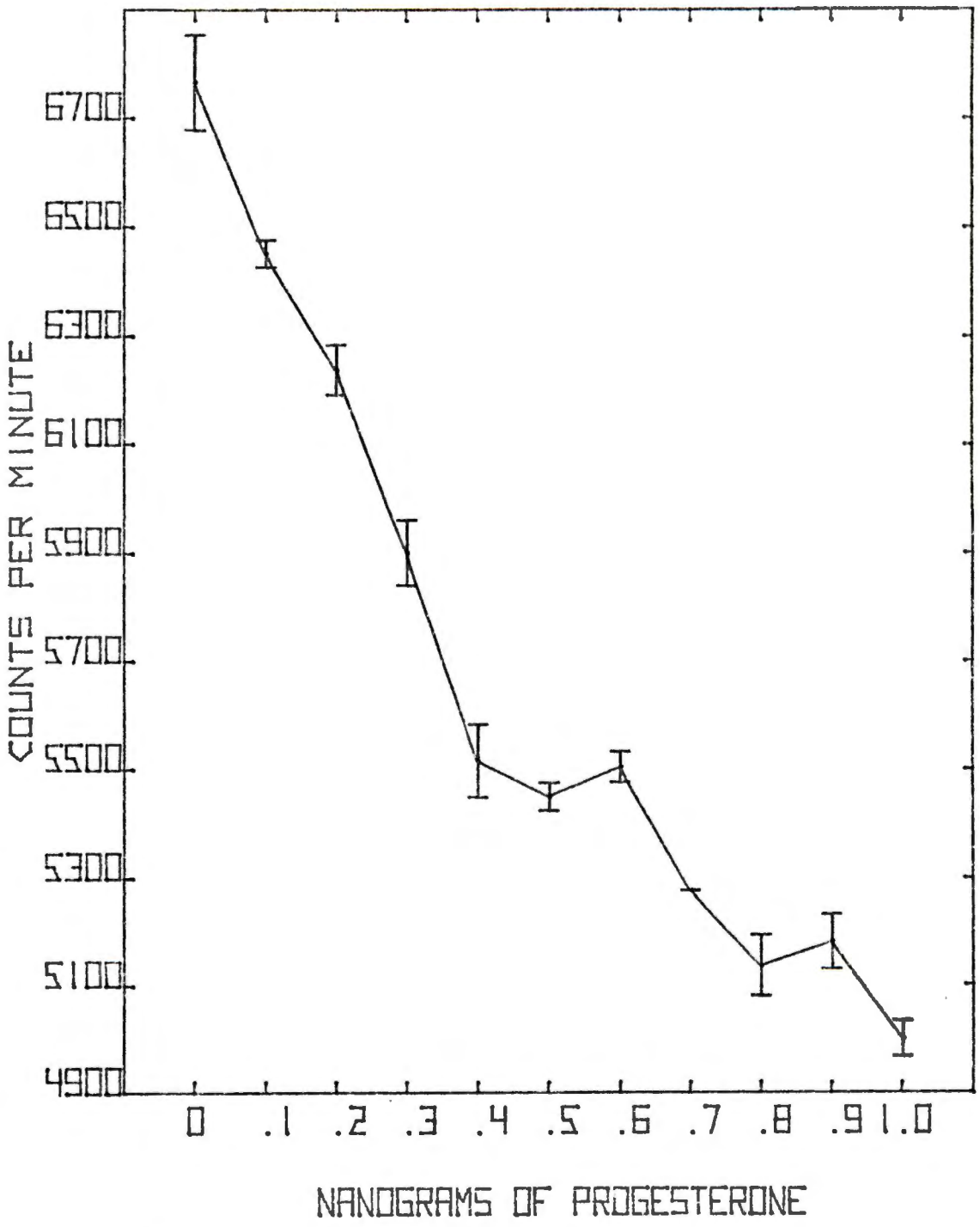

Figure 5. Progesterone sensitivity curve.

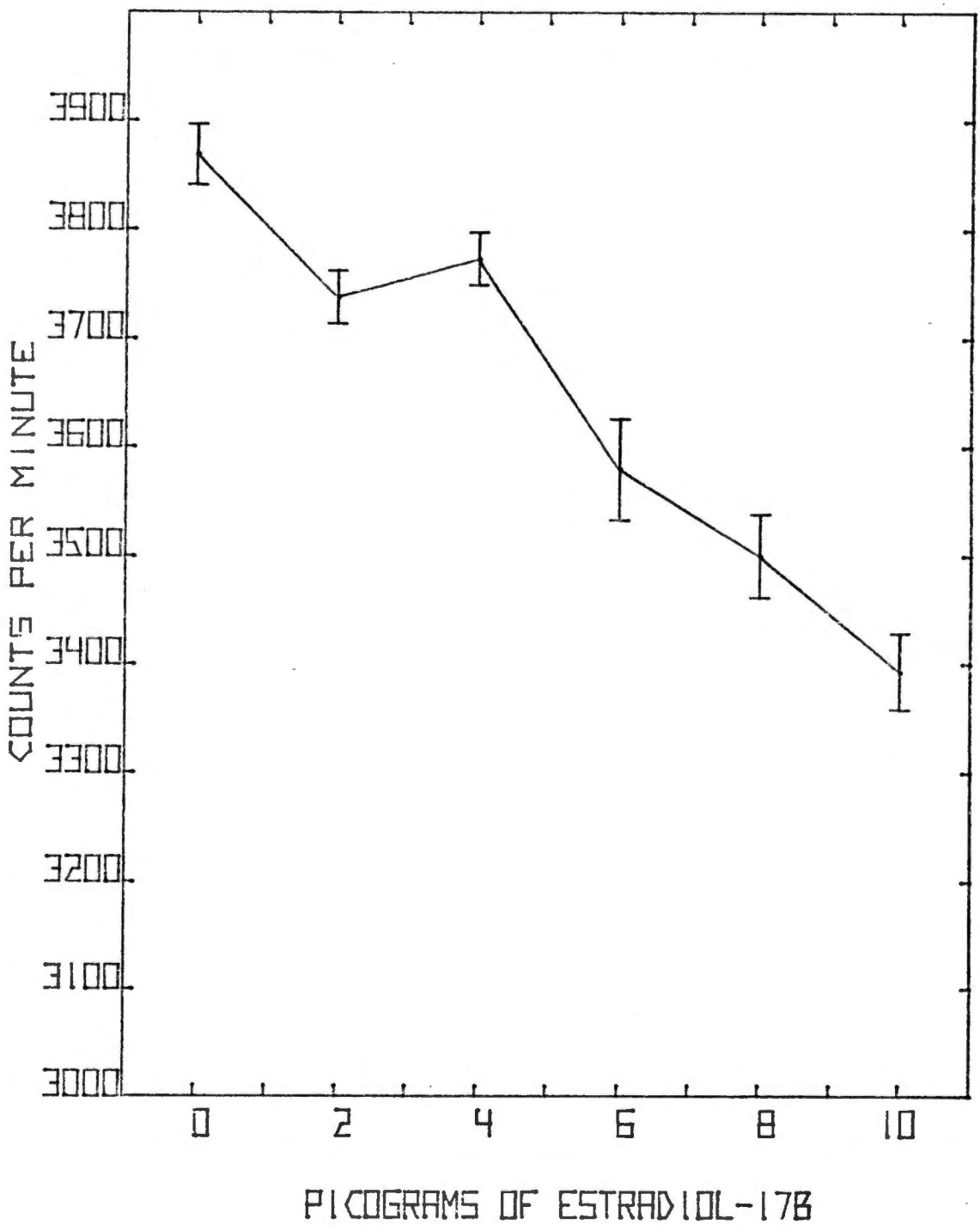

Figure 6. Estradiol-17β sensitivity curve.

TABLE I

PROGESTERONE LEVELS IN THE PERIPHERAL PLASMA OF NON-BRED, NON-PREGNANT AND PREGNANT COWS

Day of Cycle	Mean (ng/ml), Standard Error and Sample Number			
	Group[a]			
	A	B	C	D
-5	7.57±0.40(2)	7.17±0.41(6)	7.68±0.35(3)	10.54±1.81(6)
-4	5.64±0.12(2)	7.83±1.18(8)	8.37±1.05(3)	8.54±0.79(6)
-3	5.23±0.63(3)	5.19±0.61(11)	6.36±0.80(3)	7.37±0.82(6)
-2	2.85±1.21(3)	2.48±0.42(11)	1.39±0.22(3)	1.71±0.32(6)
-1	1.13±0.03(3)	2.00±0.49(12)	0.65±0.06(2)	0.71±0.17(7)
0	0.67±0.14(6)	1.78±0.46(12)	0.18±0.06(4)	0.50±0.14(7)
1	0.64±0.13(8)	2.01±0.64(12)	0.35±0.16(4)	0.30±0.11(7)
2	0.94±0.16(8)	1.85±0.55(12)	0.49±0.25(4)	0.77±0.25(7)
3	1.26±0.18(8)	1.84±0.54(12)	0.99±0.32(4)	0.87±0.24(7)
4	1.88±0.20(8)	2.51±0.59(11)	1.50±0.22(4)	1.85±0.27(6)
5	2.83±0.24(8)	2.83±0.48(11)	2.26±0.31(4)	2.62±0.34(6)
6	4.29±0.23(8)	3.30±0.52(8)	3.05±0.27(4)	3.75±0.31(6)
7	4.94±0.26(8)	4.28±0.50(9)	4.48±0.24(4)	4.61±0.42(5)
8	5.14±0.33(8)	5.54±0.67(10)	4.41±0.41(4)	5.45±0.35(6)
9[b]	6.09±0.37(8)	4.29±0.40(7)	5.50±0.47(4)	6.51±0.82(5)
10[b]	6.76±0.47(8)	4.84±0.49(7)	6.01±0.53(4)	6.09±0.48(6)
11[b]	7.96±1.00(8)	5.16±0.49(7)	6.41±0.36(3)	6.57±0.58(6)
12[b]	9.52±1.19(8)	5.79±0.65(6)	6.81±0.36(4)	6.45±0.55(6)
13[b]	9.21±1.32(8)	5.87±0.53(7)	8.82±1.33(3)	6.48±0.53(6)
14	7.22±0.54(6)	6.29±0.47(6)	6.50±1.09(3)	6.78±0.49(6)
15	6.65±0.21(4)	7.23±0.99(5)	5.37±0.67(2)	7.11±0.43(6)

[a]Group A = non-bred (estrus detected), Group B = non-bred (estrus undetected), Group C = non-pregnant and Group D = pregnant.

[b]Cows in Group A had higher progesterone levels than cows in Group B (day 9 $P < 0.10$), Day 10 ($P < 0.05$), Day 11 ($P < 0.05$), Day 12 ($P < 0.025$) and Day 13 ($P < 0.10$).

TABLE II

ESTROGEN LEVELS IN THE PERIPHERAL PLASMA OF NON-BRED NON-PREGNANT AND PREGNANT COWS

Day of Cycle	Mean (pg per ml), Standard Error and Sample Number			
	Group[a]			
	A	B	C	D
-5	2.88±0.53(2)	4.09±1.13(6)	5.04±1.74(3)	2.93±0.54(6)
-4	2.81±0.27(2)	4.83±1.13(9)	5.52±1.39(3)	3.13±0.53(6)
-3	3.43±0.57(3)	6.28±1.14(12)	7.17±2.80(3)	3.83±0.40(6)
-2	5.32±1.19(3)	8.44±1.28(12)	11.99±3.63(3)	8.23±1.78(6)
-1	7.68±0.73(3)	8.87±1.21(12)	9.55±2.19(2)	6.70±0.51(7)
0	9.30±0.92(6)	11.37±1.18(12)	14.83±3.60(4)	8.40±0.50(7)
1	3.90±0.46(8)	6.36±1.36(11)	4.72±1.13(4)	2.75±0.41(7)
2	3.50±0.41(8)	4.91±0.94(11)	3.52±0.93(4)	3.54±0.53(7)
3	3.36±0.30(8)	4.84±0.87(12)	5.15±1.33(4)	3.51±0.51(7)
4	4.21±0.47(8)	4.48±0.86(10)	6.15±1.54(4)	7.14±1.53(6)
5[b]	4.86±0.58(8)	4.13±0.67(10)	9.51±2.84(4)	6.64±1.24(6)
6[b]	4.47±0.39(8)	4.82±0.71(9)	7.35±1.50(4)	6.86±0.96(6)
7	3.15±0.50(8)	5.11±0.93(8)	4.83±0.87(4)	6.45±1.19(5)
8	3.31±0.55(8)	4.91±1.01(9)	5.78±1.54(4)	4.85±1.10(6)
9	2.75±0.42(8)	4.73±1.44(7)	3.83±1.39(4)	4.48±1.26(6)
10[b]	2.54±0.42(8)	2.77±0.48(7)	5.12±1.46(4)	4.96±1.22(6)
11	2.79±0.52(8)	3.02±0.56(7)	3.93±1.43(4)	3.57±0.83(6)
12	2.62±0.48(8)	2.64±0.37(7)	3.87±1.24(4)	3.18±0.85(6)
13	2.91±0.48(8)	3.43±0.52(7)	2.54±1.25(3)	4.10±1.08(6)
14	2.83±0.48(6)	2.49±).56(6)	3.18±1.36(3)	3.61±0.71(6)
15	3.38±0.49(5)	3.42±0.89(5)	1.17±0.41(2)	3.72±0.80(6)

[a]Group A = non-bred (estrus detected), Group B = non-bred (estrus undetected), Group C = non pregnant and Group D = pregnant.

[b]Mated cows had higher estrogen levels than non-bred cows ($P < 0.10$).

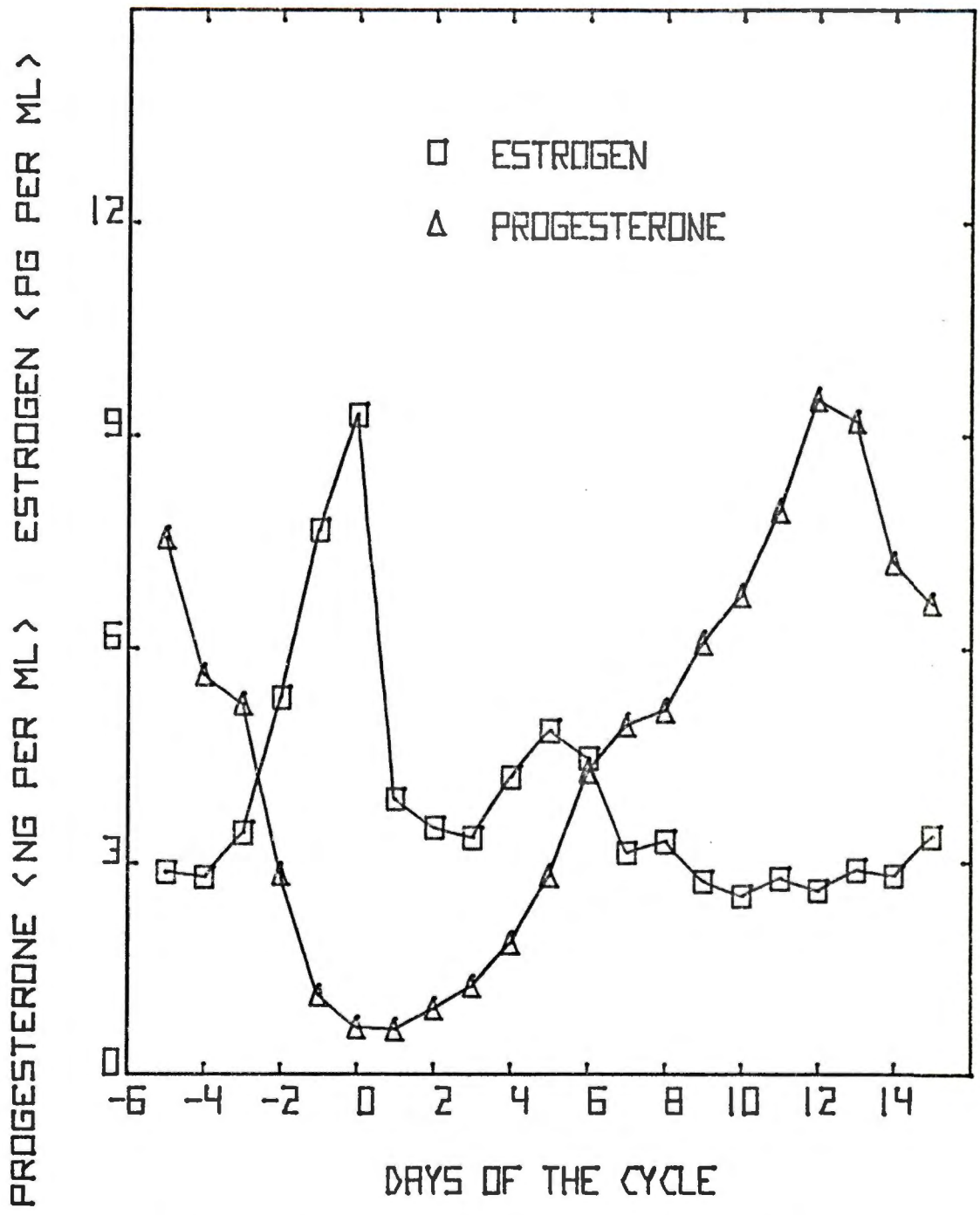

Figure 7. Progesterone and estrogen levels (means) in the peripheral plasma of non-bred cows (estrus detected) (Group A).

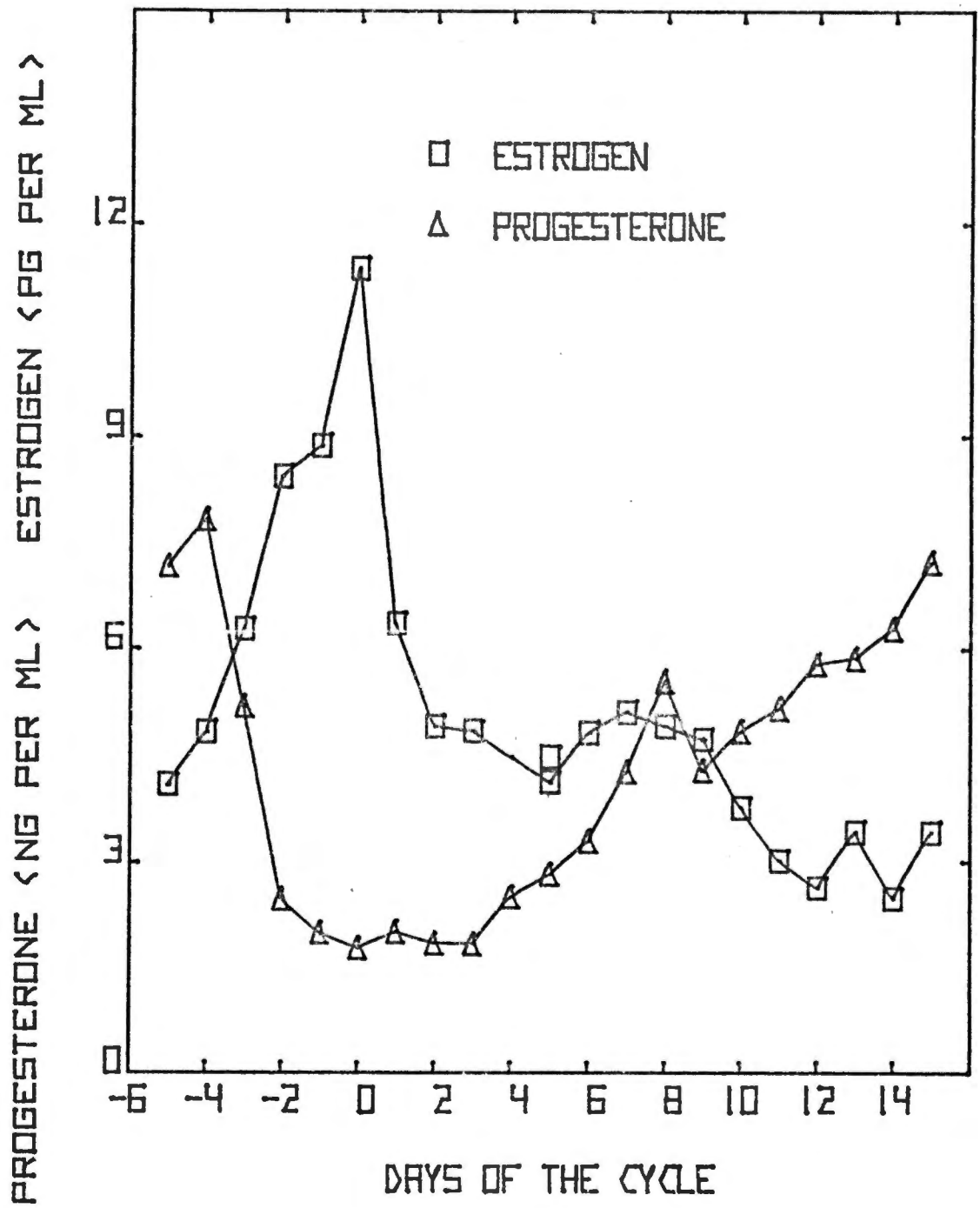

Figure 8. Progesterone and estrogen levels (means) in the peripheral plasma of non-bred cows (estrus undetected) (Group B).

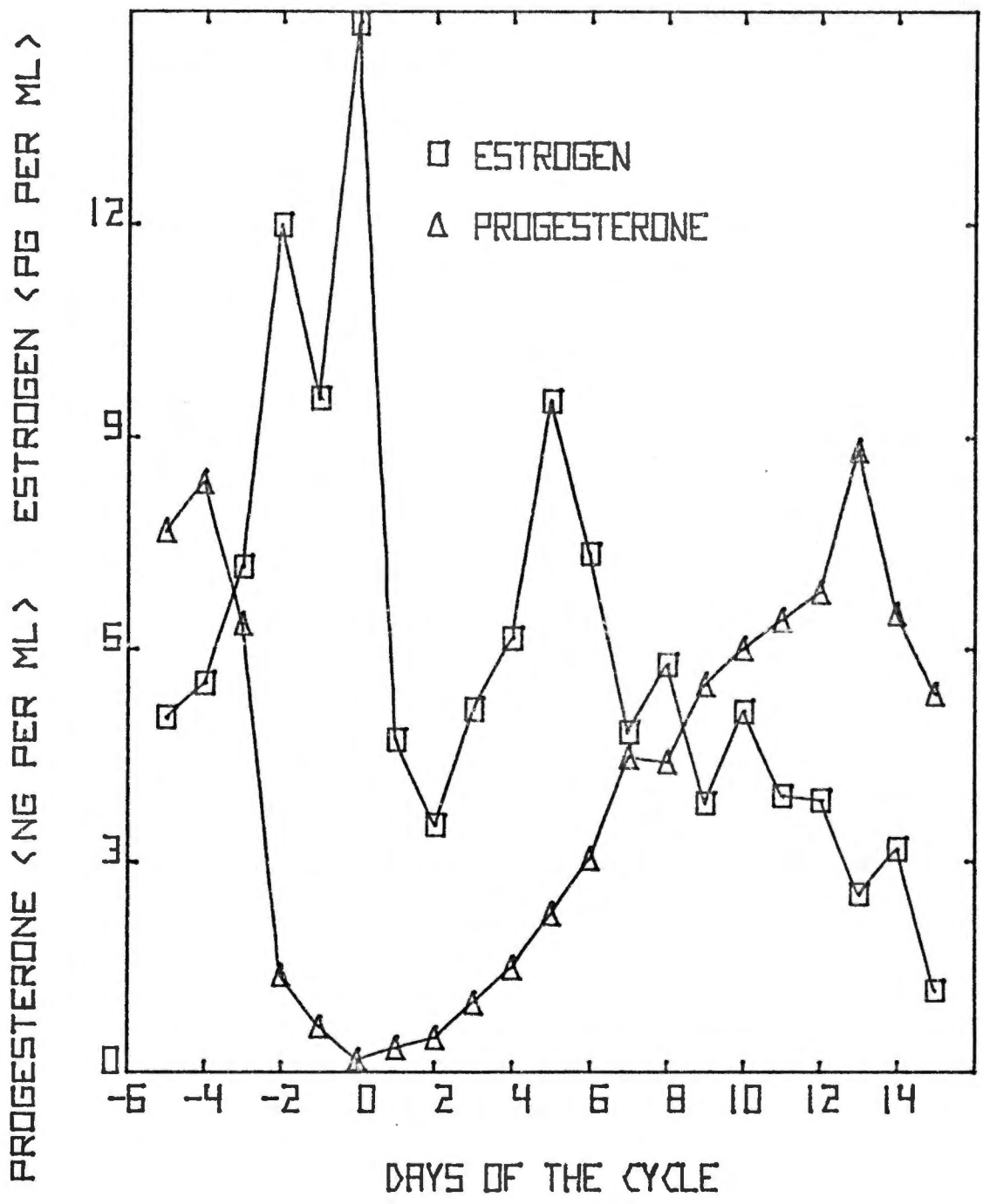

Figure 9. Progesterone and estrogen levels (means) in the peripheral plasma of non-pregnant cows (Group C).

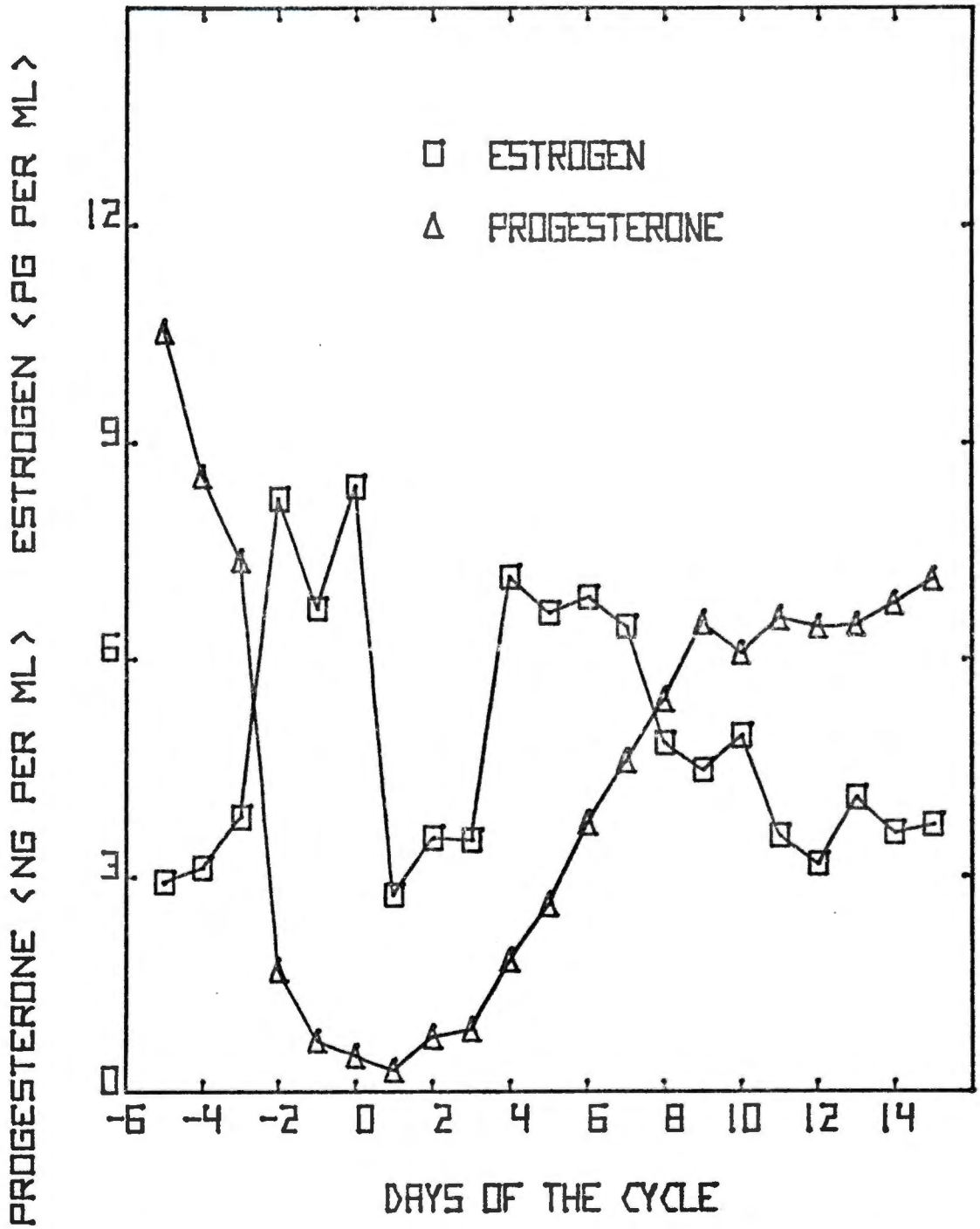

Figure 10. Progesterone and estrogen levels (means) in the peripheral plasma of pregnant cows (Group D).

Dowlen, 1972; Murphree, 1972). There was no significant difference ($P > 0.10$) in progesterone concentration in the compared groups between Day -5 and Day 8. However, on Day 9 ($P < 0.10$), Day 10 ($P < 0.05$), Day 11 ($P < 0.05$), Day 12 ($P < 0.025$) and Day 13 ($P < 0.10$) the progesterone levels in non-mated (estrus detected) cows were higher than the levels in non-mated (estrus undetected) cows. A portion of this difference may have been due to high progesterone levels (greater than 20 pg per ml) that occurred in one cow.

The changes in progesterone concentration that occurred within groups were very similar. The concentration began to decrease on Day -4 or Day -3 and dropped rapidly to the lowest concentration on Day 0 or Day 1 (less than 1 ng per ml in non-bred) (estrus detected) and mated cows and less than 2 ng per ml in non-bred (estrus undetected) cows. The concentration began to increase rapidly on Day 3 and Day 4 and by Day 8 the concentration had reached 5 ng per ml. A small increase in concentration continued through Day 15 in non-bred (estrus undetected) and pregnant cows. In non-bred (estrus detected) and non-pregnant cows the concentration continued to increase and then decreased slightly on Day 14 and Day 15.

The estrogen concentration was inversely related to the progesterone concentration during and prior to Day 0. The estrogen concentration began a significant increase ($P < 0.05$) on Day -2 in all groups. The highest estrogen concentration occurred on Day 0 (9.30 pg per ml in Group A, 11.37 pg per ml in Group B, 14.83 pg per ml in Group C and 8.40 pg per ml in Group D). Other investigators

have reported similar results. Echternkamp and Hansel (1971) reported a peak estradiol-17β concentration of 8.09 pg per ml on the day of estrus. Wettemann et al. (1972) showed that the highest estradiol-17β concentration occurred 0.5 day prior to estrus. However, the concentration remained high during estrus (8.4 pg per ml). Henricks, Dickey and Hill (1971) found that the estrogen concentration reached a peak (12-30 pg per ml) immediately prior to the onset of estrus. Christensen, Wiltbank and Hopwood (1971) reported considerably higher values. Their results indicated that estrogen is highest (176 pg per ml) 15 hours prior to the onset of estrus. The high levels of estrogen during the follicular phase of the cycle, reported in this study and other studies, may have been due to atretic follicles as well as normal follicles (Choudary, Gier and Marion, 1968).

There were no significant differences in estrogen level ($P > 0.10$) in the following comparisons: Group A versus Group B, Group C versus Group D and non-bred (Groups A and B) versus bred (Groups C and D) between Day -5 and Day 0.

During metestrus, or the five-day period subsequent to Day 0 the progesterone and estrogen concentration increased simultaneously in Groups A, C and D. The absence of an increased estrogen concentration during this period in Group B may have been due to abnormal ovarian function. During the diestrus period, or the period subsequent to Day 5, the progesterone estrogen relationship reverses and the levels of the two again become inversely related.

In Groups A and C the estrogen concentration on Day 5 was higher ($P < 0.05$) than the concentrations on Day 1, 2, 3, 7, 8, 9, 10, 11, 12,

13, 14 and 15. In addition the concentration on Day 6 (Group C) was higher ($P < 0.05$) than the concentrations on Days 2, 9, 11, 12, 13, 14 and 15. In pregnant cows the estrogen concentration on Day 4 was greater ($P < 0.05$) than the concentrations on Days 1, 2, 3, 8, 9, 10, 11, 12, 13, 14 and 15. The concentration on Day 7 was higher ($P < 0.05$) than the levels on Days 1, 2, 3, 11, 12, 13, 14 and 15.

An analysis of variance across groups indicated that the estrogen concentration on Day 5, Day 6 and Day 10 in bred cows was greater ($P < 0.10$) than the concentration in non-bred cows.

Elevated estrogen levels in the luteal phase of the cycle in cattle have been reported (Christensen, Wiltbank and Hopwood, 1971) and also in sheep (Scaramuzzi, Caldwell and Moor, 1970); Christensen, Wiltbank and Hopwood (1971), found a secondary estrogen peak on Day 5 and Day 6 in cycling beef cows. Scaramuzzi, Caldwell and Moor (1970) were able to detect elevated levels on Day 2 and Day 8 in the ovarian venous blood of cycling ewes. Cox, Mattner and Thorburn (1971), showed that the estradiol secretion rate increased on Day 3 and Day 4 in cycling ewes. Holst, Braden and Mattner (1972), indicated that the increased secretion rate on Day 3 and Day 4 in ewes may be due to follicular atresia. Follicular atresia in cattle may contribute to the luteal estrogen peak. Choudary, Gier and Marion (1969), have found that large (6-12 mm) atretic follicles are present during the luteal phase of the bovine estrous cycle. However, follicular growth which also occurs during the luteal phase of the cycle may contribute to elevated estrogen levels. According to Cupps, Anderson and Cole (1969), Rajakoski found that bovine follicle diameter increased from

4.5 mm at Day 2 to 9.5 mm at Day 5. On Day 6 the diameter decreased slightly and again increased through Day 11 (12 mm). Cox, Mattner and Thorburn (1971) stated their belief that the increase in estrogen levels may influence ovum transport in the oviducts and development of the uterine endometrium.

The fact that bred cows had higher levels than non-bred cows suggests that the zygote may have been involved in estrogen secretion. If the zygote was involved in regulating estrogen secretion, the elevated estrogen levels in bred cows was probably due to follicular growth, as opposed to follicular atresia. The zygote may have stimulated the hypothalmus by a neural mechanism to stimulate the release of gonadotropins from the anterior pituitary. The fact that a neural mechanism may exist between the uterus and hypothalmus was demonstrated in experiments by Nalbandov, Moore and Norton, as cited by Bland and Donovan (1966). These researchers found that glass beads of varying diameter placed in one uterine horn during the luteal phase of the cycle would change cycle length.

The higher estrogen concentration on Day 10 in mated cows as opposed to non-mated cows suggests that estrogen may be involved in preparing the uterus for implantation. According to Salisbury and Van Demark (1961), the bovine zygote forms a weak uterine attachment 12 days after mating.

Elevated estrogen levels near the time of implantation have been observed in women (Johansson, 1971), pseudopregnant rats (Abubakar and Abraham, 1969) and rabbits (Hillard and Eaton, 1971).

CHAPTER V

SUMMARY AND CONCLUSIONS

The first objective of this study was to evaluate a radioimmunoassay technique for the assay of estrogen. Secondly, the interrelated changes in the concentrations of progesterone and estrogen were determined in peripheral plasma of non-bred, non-pregnant and pregnant Holstein cows. Progesterone was determined by competitive protein-binding.

Six hundred twenty-nine blood samples were collected from the jugular vein of 14 lactating cows. Sample collection began five days prior to estrus and continued at daily intervals for 25 days post-insemination. Non-bred cows were bled through one or two cycles. The cows were divided into the following groups: A) non-bred (estrus detected), B) non-bred (estrus undetected), C) non-pregnant and D) pregnant. In Groups A, C and D, Day 0 represents the day of estrus. In Group B, Day 0 represents the day of highest estrogen concentration.

The extraction efficiencies for labeled and unlabeled progesterone were 93.58 ± 0.78 percent (mean ± standard error) and 90.20 ± 0.78 percent (mean ± standard error) and 90.20 ± 2.15 percent, respectively.

The extraction efficiencies for 20 pg and 50 pg of estradiol-17β were 91.18 ± 4.58 percent and 88.57 ± 5.24 percent, respectively. The extraction efficiency for 4 nanocuries of estradiol-17β-2, 4, 6, 7-H^3 was 94.04 ± 0.59 percent. The unlabeled and labeled extraction efficiencies were not significantly different ($P < 0.010$).

The estradiol-17β antibody exhibited considerable cross reaction with estrone, while the cross reaction with estriol was negligible.

The coefficients of variation for points on progesterone and estrogen standard curves were less than 10 percent, with the exception of 100 pg of estradiol-17β (12.80 percent). The lower limit of sensitivity for the progesterone standard curve was 0.3 ng. The lower limit of sensitivity for the estradiol-17β standard curve was 6 pg.

The progesterone and estrogen concentrations were inversely related during the follicular phase and the late luteal phase period of the cycle. During the period of metestrus, the progesterone and estrogen concentration increased simultaneously.

An estrogen peak occurred on Day 0 in all groups. A secondary estrogen surge occurred five or six days post-estrus in Groups A, C and D. Normal follicles or follicles undergoing atresia may have contributed to both of these surges. The estrogen concentration in bred cows was significantly higher ($P < 0.10$) than the concentration in non-bred cows on Day 5, 6 and 10. This suggests that the zygote may be involved in regulating luteal phase estrogen secretion. The elevated levels on Day 5 and Day 6 may influence ovum transport in the oviducts or development of the uterine endometrium. The high estrogen level at Day 10 may be involved in preparing the uterus for implantation.

LITERATURE CITED

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Abraham, G. E., R. Swerdloff, D. Tulchinsky and W. D. Odell. 1971. Radioimmuneoassay of plasma progesterone. J. Clin. Endocr. 32:619.

Abubakar, A. S. and G. E. Abraham. 1969. Measurement of estrogen surge during pseudopregnancy in rats by radioimmunoassay. Biol. Reprod. 1:378.

Armstrong, D. T. and D. L. Block. 1966. Influence of lutemizing hormone on corpus luteum metabolism and progesterone biosynthesis throughout the estrous cycle. Endocr. 78:937.

Bassett, J. M. and W. T. Hinks. 1969. Micro-determination of corticosteroids in ovine peripheral plasma: Effects of venipuncture, corticotrophic, insulin and glucose. J. Endocr. 44:387.

Bassett, J. M., T. J. Oxborrow, J. D. Smith and G. D. Thorburn. 1969. The concentration of progesterone in the peripheral plasma of the pregnant ewe. J. Endocr. 45:449.

Batson, D. B. 1969. Progesterone levels in bovine plasma as determined by competitive protein-binding assay. M.S. Thesis. University of Tennessee, Knoxville.

Bedford, C. A., J. R. G. Chellis, F. A. Harrison and R. B. Heap. 1972. The role of estrogens and progesterone in the onset of parturition in various species. J. Reprod. Fert. Suppl. 16:1.

Berson, S. A., R. S. Yalow, A. Bauman, M. A. Rothschild and K. Newerly. 1956. Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin-treated subjects. J. Clin. Invest. 35:170.

Bjersing, L. M., M. F. Hay, G. Kann, R. M. Moor, F. Naftolin, R. J. Scaramuzzi, R. V. Short and E. V. Younglai. 1972. Changes in gonadotrophins, ovarian steroids and follicular morphology in sheep at estrus. J. Endocr. 52:465.

Bland, K. P. and B. T. Donovan. 1966. The uterus and the control of ovarian function, p. 187. In A. McLaren (ed.) Advances in Reproductive Physiology. Academic Press, New York.

Carr, B. R., G. Mikhail and G. L. Flickinger. 1971. Column chromatography of steroids on Sephadex LH-20. J. Clin. Endocr. 33:358.

Challis, J. R. G. and J. L. Linzell. 1971. The concentration of total unconjugated estrogens in the plasma of pregnant goats. J. Reprod. Fert. 26:401.

Choudary, J. B., H. T. Gier and G. B. Marion. 1969. Cyclic changes in bovine vesicular follicles. J. Animal Sci. 27:468.

Christensen, D. S., J. N. Wiltbank and M. L. Hopwood. 1971. Blood hormone levels during the bovine estrous cycle. J. Animal Sci. 33:251. (Abstr.).

Corker, C. S. and D. Exley. 1970. The determination of plasma estradiol-17β by competitive protein binding radioassay. Steroids. 15:469.

Cox, R. I., P. E. Mattner and G. D. Thorburn. 1971. Changes in ovarian secretion of estradiol-17β around estrous in the sheep. J. Endocr. 49:345.

Cupps, P. T., L. L. Anderson and H. H. Cole. 1969. The estrous cycle, p. 227. In H. H. Cole and P. T. Cupps (ed.) Reproduction in Domestic Animals. Academic Press, New York.

Demetrion, J. A. and F. G. Austin. 1970. A rapid competitive protein-binding assay for plasma progesterone. Clin. Chim. Acta. 33:21.

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

Donaldson, L. E., J. M. Bassett and G. D. Thorburn. 1970. Peripheral plasma progesterone concentration of cows during puberty, estrous cycles, pregnancy and lactation, and the effects of undernutrition or exogenous oxytocin on progesterone concentration. J. Endocr. 48:599.

Dowlen, H. H. 1972. Progesterone levels in cows suspected of embryonic death. M.S. Thesis. University of Tennessee, Knoxville.

Dufau, M. L., A. Dulmanis, K. J. Catt and B. Hudson. 1970. Measurement of plasma estradiol-17β by competitive binding assay employing pregnancy plasma. J. Clin. Endocr. 30:351.

Echternkamp, S. E. and W. Hansel. 1971. Plasma estrogens, luteinizing hormone, and corticoid in post-partum cows. J. Dairy Sci. 54:800. (Abstr.).

Edgerton, L. A. and H. D. Hafs. 1972. Blood serum estrogens around parturition in cows. J. Animal Sci. 35:240. (Abstr.).

Edqvist, L. E. and A. M. Lamm. 1971. Progesterone levels in plasma during the estrous cycle of the sow measured by a rapid competitive protein beinding techniniques. J. Reprod. Fert. 25:447.

Ekins, R. and B. Newman. 1970. Theoretical aspects of saturation analysis. In E. Diczfalusy (ed.) Karolinska Symposia on Research Methods in Reproductive Endocrinology, Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Erb, R. E., V. L. Estergreen, Jr., W. R. Gomes, E. D. Plotka and O. L. Frost. 1968a. Progestin levels in corpora lutea and progesterone in ovarian venous and jugular vein blood plasma of the pregnant bovine. J. Dairy Sci. 51:1.

Erb, R. E., R. D. Randel and C. J. Callahan. 1971. Female Sex steroid changes during the reproductive cycle. J. Animal Sci. Suppl. 1:80.

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

Erb, R. E. and F. Stormshak. 1961. Progestins in corpora lutea, ovaries and adrenals after estrus and breeding of normal and abnormal cows. J. Dairy Sci. 44:888.

Estergreen, V. L., Jr., O. L. Frost, W. R. Gomes, R. E. Erb and J. F. Bullard. 1967. The effect of ovariectomy on pregnancy maintenance and parturition in dairy cows. J. Dairy Sci. 50:1293.

Foley, R. C., D. L. Block, W. G. Black, R. A. Damon and R. G. Howe. 1964. Ovarian and luteal tissue weights in relation to age, breed and liveweight in non-pregnant and pregnant heifers and cows with normal reproductive histories. J. Animal Sci. 23:752.

Fylling, P. 1970. The effect of pregnancy, ovariectomy and parturition on plasma progesterone level in sheep. Acta Endocr. 65:273.

Gadsby, J. E., R. B. Heap, D. G. Powell and D. E. Walters. 1972. Diagnosis of pregnancy and of the number of fetuses in sheep from plasma progesterone concentrations. Vet. Rec. 90:339.

Goodfriend, L. and A. Sehon. 1970. Early approaches to production, analysis and use of steroid-specific antisera. In F. G. Peron and B. V. Caldwell (ed.). Immunologic Methods in Steroid Determination. Meredith Corporation, New York.

Henricks, D. M., J. F. Dickey, J. R. Hill and W. E. Johnston. 1972. Plasma estrogen and progesterone levels after mating, and during late pregnancy and post-partum in cows. Endocr. 90:1336.

Henricks, D. M., J. F. Dickey and J. R. Hill. 1971. Plasma estrogen and progesterone levels in cows prior to and during estrus. Endocr. 89:1350.

Henricks, D. M., H. D. Guthrie and D. L. Handlin. 1972. Plasma estrogen, progesterone and lutenizing hormone levels during the estrous cycle in pigs. Biol. Reprod. 6:210.

Henricks, D. M., D. R. Lamond, J. R. Hill, and J. F. Dickey. 1971. Plasma progesterone concentrations before mating and in early pregnancy in the beef heifer. J. Animal Sci. 33:450.

Henricks, D. M. 1970. Personal communication. Dept. Food Sci. and Biochem. Clemson University, Clemson.

Hillard, J. and L. W. Eaton, Jr. 1971. Estradiol-17β, progesterone and 20α-hydroxypregn-4-ene-3-one in rabbit ovarian venous plasma. II. From mating through implantation. Endocr. 80:552.

Holst, P. J., A. W. H. Braden and P. E. Mattner. 1972. Estradiol-17β secretion from the ewe ovary and related ovarian morphology on days two and three of the cycle. J. Reprod. Fert. 28:136.

Hoffmann, B. 1972. Determination of estrogens in bovine peripheral plasma by radioimmunoassay. J. Endocr. 2:xvi. (Abstr.).

Howland, B. E., R. L. Kirkpatrick, A. L. Pope and L. E. Casida. 1966. Pituitary and ovarian function in ewes fed on two nutritional rations. J. Animal Sci. 25:716.

Irving, G., D. E. Jones and A. Knifton. 1972. Progesterone concentration in the peripheral plasma of pregnant goats. J. Endocr. 53:447.

Johansson, E. D. B. 1969. Progesterone levels in peripheral plasma during the luteal phase of the normal human menstrual cycle measured by a rapid competitive protein binding technique. Acta Endocr. 61:592.

Johansson, E. D. B. 1970. A simplified procedure for the assay of progesterone, p. 191. In E. Diczfalusy (ed.) Karolinska Symposia of Research Methods in Reproductive Endocrinology, Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Johansson, E. D. G. 1971. Plasma levels of estradiol-17β and progesterone around the time of implantation in women. Acta Endocr. Suppl. 155:74.

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

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

Linzell, J. L. and R. B. Heap. 1968. A comparison of progesterone metabolism in the pregnant sheep and goat: Sources of production and an estimation of uptake by some target organs. J. Endocr. 41:433.

Martin, B. T., B. A. Cooke and W. P. Black. 1970. Evaluation of a rapid method for the measurement of plasma progesterone by competitive protein binding. J. Endocr. 46:369.

Masuda, H., L. L. Anderson, D. M. Henricks and R. M. Melampy. 1967. Progesterone in ovarian venous plasma and corpora lutea of the pig. Endocr. 80:240.

Mattner, P. E. and A. W. H. Braden. 1972. Secretion of estradiol-17β by the ovine ovary during the luteal phase of the estrous cycle in relation to ovulation. J. Reprod. Fert. 28:136. (Abstr.).

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

Mercier-Bodard, C. A. Alfsen and E. E. Baulieu. 1970. Sex steroid binding plasma protein (SBP), p. 216. In E. Diczfalusy (ed.) Karolinska Symposia on Research Methods in Reproductive Endocrinology. Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Midgley, R. A., Jr., G. D. Niswender and J. S. Ram. 1969. Hapten-radioimmunoassay: A general procedure for the estimation of steroidal and other haptenic substances. Steroids. 13:731.

Moore, N. W., S. Barrett and J. B. Brown. 1972. Progesterone concentrations in maternal and fetal blood plasma of ewes. J. Endocr. 53:187.

Murphree, R. L. 1972. Unpublished results. Dept. Anim. Sci. University of Tennessee, Knoxville.

Murphy, B. E. P. 1964. Application of the property of protein-binding to the assay of minute quantities of hormones and other substances. Nature. 201:679.

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

Murphy, B. E. P. 1970. Methodological problems in competitive protein-binding techniques; the use of Sephadex column chromatography to separate steroids, p. 37. In E. Diczfalusy (ed.) Karolinska Symposia on Research Methods in Reproductive Endocrinology. Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Murphy, B. E. P. 1971. Sephadex column chromatography as an adjunct to competitive protein binding assays of steroids. Nature New Biol. 232:21.

Nalbandov, A. V. 1964. Reproductive Physiology. W. H. Freeman and Co., San Francisco.

Neill, J. D., E. D. B. Johansson, J. K. Dalta and E. Knobil. 1967. Relationship between the plasma levels of lutenizing hormone and progesterone during the normal menstrual cycle. J. Clin. Endocr. 27:1167.

Niswender, G. W. 1972. Personal communication. Dept. Anim. Sci. Colorado State University, Fort Collins.

Obst, J. M. and R. F. Seamark. 1970. Plasma progesterone concentrations during the reproductive cycle of ewes grazing Yarloop clover. J. Reprod. Fert. 21:545.

Obst, J. M. and R. F. Seamark. 1972. Plasma estrogen concentrations in ewes during parturition. J. Reprod. Fert. 28:161 (Abstr.).

Plotka, E. D., D. M. Witherspoon and C. W. Foley. 1972. Luteal function in the mare as reflected by progesterone concentrations in peripheral blood plasma. Am. J. Vet. Res. 33:918.

Plotka, E. D. 1971. Personal communication. Marshfield Clinic Found. Med. Res. Educ., Marshfield, Wisconsin.

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

Rao, C. V. and V. L. Estergreen, Jr. 1969. Studies on competitive protein binding radioassay for progesterone. Sixty-fourth Annual Meeting, ADSA. University of Minnesota, Minneapolis and St. Paul.

Reaves, B. D. and D. W. Calhoun. 1970. Reliability criteria for saturation analysis of steroids by competitive protein binding, p. 63. In E. Diczfalusy (ed.). Karolinska Symposia on Research Methods in Reproductive Endocrinology. Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Reaves, B. D., M. L. A. de Souza, I. E. Thompson and E. Diczfalusy. 1970. An improved method for the assay of progesterone by competitive protein binding. Acta Endocr. 63:225.

Robinson, R., R. D. Baker, P. A. Anestassiadia and R. H. Common. 1970. Estrone concentrations in the peripheral blood of pregnant cows. J. Dairy Sci. 53:1592.

Rothenberg, S. P. 1961. Assay of serum vitamin B_{12} concentration using Co^{57}-B_{12} and intrinsic factor. Proc. Soc. Exp. Biol. Med. 108:45.

Salisbury, G. W. and N. L. VanDemark. 1961. Physiology of Reproduction and Artificial Insemination of Cattle. W. H. Freeman and Co., San Francisco.

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

Shemesh, M., N. Ayalon and H. R. Lindner. 1968. Early effect of conceptus on plasma progesterone level in the cow. J. Reprod. Fert. 15:161.

Shemesh, M., H. R. Lindner and W. Ayalon. 1971. Competitive protein-binding assay of progesterone in bovine jugular venous plasma during the estrous cycle. J. Reprod. Fert. 26:167.

Shutt, D. A. 1969. Measurement of estradiol-17β in plasma by competitive protein-binding radioassay. Steroids. 13:69.

Slaunwhite, W. R., Jr., and A. A. Sandbert. 1970. Analysis of Corticosteroids, p. 144. In E. Diczfalusy (ed.) Karolinska Symposia on Research Methods in Reproductive Endocrinology. Second Symposium: Steroid Assay by Protein Binding. Bogtrykkeriet Forum, Copenhagen.

Stabenfeldt, G. H., E. L. Akins, L. L. Ewing and M. C. Morrissette. 1969. Peripheral plasma progesterone levels in pigs during the estrous cycle. J. Reprod. Fert. 20:442.

Stabenfeldt, G. H., M. Drost and C. E. Franti. 1972. Peripheral plasma progesterone levels in the ewe during pregnancy and parturition. Endocr. 90:144.

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

Stabenfeldt, G. H., B. J. Osburn and L. L. Ewing. 1970. Peripheral plasma progesterone levels in the cow during pregnancy and parturition. Amer. J. Physiol. 218:571.

Stone, S., R. M. Nakamura, D. R. Mishell, Jr. and I. H. Thorneycroft. 1968. A modified technique for the assay of progesterone in blood using celite column chromatography. Steroids. 17:411.

Thatcher, W. W. 1970. Personal communication. Dept. of Dairy Sci. University of Florida, Gainesville.

Tillson, S. A., R. E. Erb and G. D. Niswender. 1970. Comparison of lutenizing hormone and progesterone in blood and metabolites of progesterone in urine of domestic sows during the estrous cycle and early pregnancy. J. Anim. Sci. 30:795.

Tillson, S. A. and R. E. Erb. 1970. Relation of number of embryos in domestic sows to sex steroid hormone changes during the first 28 days of pregnancy. J. Anim. Sci. 31:1178.

Thorburn, G. D., J. M. Bassett and I. D. Smith. 1969. Progesterone concentration in the peripheral plasma of sheep during the estrous cycle. J. Endocr. 45:459.

Thorburn, G. D. and P. E. Mattner. 1971. Anastomosis of the utero-ovarian and anterior mammary veins for collection of utero-ovarian venous blood; progesterone secretion rates in cyclic ewes. J. Endocr. 50:307.

Thorburn, G. D., D. H. Nicol, J. M. Bassett, D. A. Shutt and R. I. Cox. 1972. Parturition in the goat and sheep: Changes in corticosteroids, progesterone, estrogens and prostoglandin F. J. Reprod. Fert. Suppl. 16:61.

Thorburn, G. D. and W. Schneider. 1972. The progesterone concentration in the plasma of the goat during the estrus cycle and pregnancy. J. Endocr. 52:23.

Thorneycraft, I. H., S. A. Tillson, G. E. Abraham, R. J. Scaramuzzi and V. B. Caldwell. 1970. Preparation and purification of antibodies to steroids, p. 63. In. Peron, F. G. and Caldwell, B. V. (eds.) Immunologic Methods in Steroid Determination. Meredith Corp., New York.

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

Unger, R. H., A. M. Eisentraut, M. S. McCall, S. Keller, H. C. Lanz and L. L. Madison. 1959. Glucagon antibodies and their use for immunoassay for glucagon. Proc. Soc. Exp. Biol. Med. 102:621.

Wettemann, R. P., H. D. Hafs, L. A. Edgerton and L. V. Swanson. 1972. Estradiol in blood serum during the bovine estrous, cycle and relationships with progesterone, LH and Prolactin. J. Anim. Sci. 34:1020.

Wettemann, R. P. and H. D. Hafs. 1972. Gonadal and pituitary hormones after insemination. J. Anim. Sci. 35:257 (Abstr.).

Wiltbank, J. N. 1966. Modification of ovarian activity in the bovine following injection of estrogen and gonadotrophin. J. Reprod. Fert. Suppl. 1:1.

Wu, C. and L. E. Lundy. 1971. Radioimmunoassay of plasma estrogens. Steroids. 18:91.

Yalow, R. S. and S. A. Berson. 1971. Introduction and general considerations, p. 2. In W. D. Odell and W. H. Daughaday (ed.). Principles of Competitive Protein-Binding Assay. J. B. Lippincott Co., Philadelphia.

Yoshimi, T. and M. B. Lipsett. 1968. The measurement of plasma progesterone. Steroids. 11:527.

VITA

David Banks Batson was born in Dickson County, Tennessee on April 14, 1944. He attended Montgomery County elementary and secondary schools. In 1962 he graduated from Montgomery Central High School and enrolled at Austin Peay State College, Clarksville, Tennessee.

He received the Bachelor of Science degree with a major in agriculture and chemistry in 1966. While at Austin Peay, he was selected to be a member of Delta Tau Alpha, honorary agricultural fraternity.

In the Fall of 1966 he was granted an assistantship in the Dairy Department at the University of Tennessee. He obtained his Master of Science degree in Dairying in December of 1968 and is presently completing the requirements for the Doctor of Philosophy degree in Animal Science. He is a member of the American Dairy Science Association and the honorary fraternities, Gamma Sigma Delta and Phi Kappa Phi. He is an alumnus of Alpha Zeta.

After the completion of his graduate work, he will be employed by the Food and Drug Administration.