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I am submitting herewith a thesis written by David Banks Batson entitled "Progesterone Levels in Bovine Plasma as Determined by Competitive Protein-Binding Assay." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree Master of Science, with a major in Dairying.

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PROGESTERONE LEVELS IN BOVINE PLASMA AS DETERMINED BY  
COMPETITIVE PROTEIN-BINDING ASSAY

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A Thesis  
Presented to  
the Graduate Council of  
The University of Tennessee

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In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

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by  
David Banks Batson  
December 1968

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## ABSTRACT

A competitive protein-binding assay procedure with certain modifications was used to measure progesterone in peripheral blood of cows for a 21 day period following insemination. This assay procedure is based upon the ability of progesterone to displace corticosterone-1,2-<sup>3</sup>H which is bound to the protein, corticosteroid binding globulin.

A total of 19 cows was used in the experiment. The cows were divided into the following groups, according to the day on which they cycled: (A) 19-27 days, (B) 42-43 days, (C) 73-101 days and (D) pregnant. Blood samples were drawn from the jugular vein every third day beginning on the day of heat and continuing for 21 days. The progesterone determinations were made on 0.5 ml. aliquots of plasma.

Since the lower limit of sensitivity of this assay procedure appears to be 0.30 ng., it is fairly reliable in measuring progesterone from Day 3 through Day 21. The coefficient of variation for recovery rates and pooled plasma was 6.1 percent and 22.6 percent, respectively.

Cow differences on a within-group basis were non-significant ( $P>0.05$ ). However, there was a significant difference in progesterone concentration across days in two of the groups; Group A ( $P<0.05$ ) and Group D ( $P<0.05$ ). There was also a significant difference ( $P<0.05$ ) across groups on Day 21. The cows in the pregnant group exhibited significantly ( $P<0.05$ ) higher progesterone levels than did the other groups on Day 21.

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## CHAPTER I

### INTRODUCTION

Reproductive failures have been a major problem area for the reproductive physiologist for a long time. The primary cause of these failures has not been ascertained but it is believed that failure of ovulation, anatomical abnormalities and infections, fertilization failure, failure of implantation and abortions all contribute to some extent to return to service after insemination. Each of these facets has been studied but knowledge is still insufficient to establish corrective or preventative programs.

Failure of implantation and abortion could possibly be due to an endocrine imbalance, such as might be caused by an improperly functioning corpus luteum. If the corpus luteum does not produce a sufficient amount of progesterone, the uterus will not be suitably conditioned to receive the fertilized egg and thus implantation may not occur. Even if implantation does occur, the presence of an adequate amount of progesterone is required for the maintenance of pregnancy.

The progesterone concentration of corpus luteum tissue and ovarian venous blood has been measured by both bioassay and physico-chemical techniques. However, the levels of progesterone in peripheral blood were not known until recently. The minute levels in the blood have been measured by very sensitive and specific physico-chemical

methods. The importance of knowing the peripheral blood levels lies in the fact that the hormone travels to its site by action of the blood.

The purpose of this experiment was to adapt a relatively new competitive protein-binding assay procedure to bovine plasma and to measure the progesterone levels in peripheral plasma for a 21 day period following insemination of cows which cycled at different intervals.

## CHAPTER II

### REVIEW OF LITERATURE

#### 1. PROGESTERONE ASSAY

##### Bioassay Methods

The discovery of progesterone is attributed to Corner (11) who demonstrated that the hormone produces characteristic changes in the uterine endometrium in preparation for ova implantation and is responsible for the maintenance of pregnancy. Some of the bioassay methods are based upon end-point changes that occur in the uterus under progestin influence.

Generally speaking, the bioassay methods that have been developed may be categorized as endometrial proliferation, stromal nuclear hypertrophy, carbonic anhydrase estimation, deciduomata formation and pregnancy maintenance.

Endometrial proliferation. Corner and Allen (12) were the first investigators to describe a test based on endometrial proliferation. They used adult female rabbits which had been spayed after mating and injected the material to be assayed subcutaneously. The minimum amount which would cause complete progestational proliferation was in the order of 1-2 milligram (mg.) of progesterone. McPhail (53) and, according to Miyake (54), Clauberg revised the Corner-Allen test and used estrogen-primed immature female rabbits. This revision increased the sensitivity and enabled detecting a minimum of 0.75 mg. of progesterone.

A local progestational test was developed by McGinty et al. (52). The compound to be tested was introduced into isolated loops of rabbit uteri. Three days later the rabbits were killed and the progestational response measured. The lower limit of sensitivity for this procedure was in the range of 0.125 to 0.500 microgram (ug.).

The usefulness of the progestational proliferation test has been expanded by objectively measuring the ratio of glandular proliferation to total mucosa (64, 65) and measuring the diameter of the flushed ovum (65).

Stromal nuclear hypertrophy. Probably the most widely used of the bioassay methods is that procedure set forth by Hooker and Forbes (30). The test is performed on an ovariectomized adult female mouse and is based on the response of the stromal nuclei to progesterone. The minimum amount of progesterone required to produce a positive reaction is 0.0002 ug. These investigators found the test to be not only very sensitive but also specific, because low concentrations of deoxycorticosterone acetate, testosterone and estrogen would not give a positive reaction in the treated uterus.

More recent investigators have not substantiated the results obtained by Hooker and Forbes. Zarrow et al. (100) found that 17- $\alpha$ -hydroxyprogesterone was 60 times more potent than progesterone in a different strain of mice. Olsen et al. (60) in their investigation found that 17- $\beta$ -estradiol in the ratio of 1:200 with progesterone would hinder progesterone activity.

Carbonic anhydrase estimation. The most recently developed bioassay method for protestin determination is the measurement of

endometrial carbonic anhydrase of the rabbit uterus. This procedure was first used by Lutwak-Mann (46) on estrogen-primed, albino, immature female rabbits. The measurement of the enzyme can be performed by a manometric procedure (59) which measures the CO<sub>2</sub> liberated or by a colorimetric procedure (64).

The results obtained by Lutwak-Mann have been confirmed using Clauberg rabbits and the procedure has been adapted as a standard assay technique (55, 64). Even though this procedure is relatively new and has been used as a standard assay technique, it may be undesirable because of lack of specificity and the minimal effective dose is 0.5 mg. (54).

Deciduomata formation. The idea of assaying for progestins by using the deciduomata reaction was first investigated by Astwood (2). This technique is based upon the swelling which occurs in a maternal placental tumor under progestational influence. The deciduoma may be initiated by various types of stimulation (physical, chemical or electrical). Histamine (chemical stimulation) was used by Zarrow et al. (101) to produce a decidual reaction and they obtained satisfactory results. Steroids such as testosterone and estrogen have been shown to hinder progesterone activity while pregnenolone and pregnandione will not affect the tumor (88).

Pregnancy maintenance. Investigators (70, 81) have taken advantage of the fact that progestins are necessary to maintain pregnancy and have established assay procedures based on this principle.

In ovariectomized animals, exogenous progestins have been shown to maintain pregnancy if administered with or without estrogen (9, 63). However, it has also been demonstrated that estrogen greatly enhances the ability of progesterone to maintain pregnancy (47). McGinty (51) found that a 1 mg. subcutaneous dose of progesterone would maintain pregnancy in 90 percent of mice that had been ovariectomized at mid-pregnancy.

In the procedure developed by Saunders and Elton (70) the spayed and injected animals are sacrificed on Day 18. Then the uteri are weighed, the implantation sites are counted and finally the fetuses are removed and weighed. As can be seen from this procedure, the big disadvantage is that it would require a large number of experimental animals and would be time-consuming.

In general, we may sum up the bioassay methods as lacking specificity and sensitivity. However, there are exceptions such as the Hooker and Forbes (30) test. Their minimal effective dose response levels readily compare with the more recent physico-chemical techniques. In spite of the disadvantages of the bioassay methods in quantitating progesterone, they are advantageous for identifying unknowns and clarifying synthetic compounds.

#### Physico-Chemical Assay Methods

The changeover in assay procedures from biological to physico-chemical began with the development in the steroid field of such quantitative detection techniques as spectrophotometry (14, 23, 72, 73,

74, 98), fluorescence (26, 75, 84), isotope labeling (68, 93), gas chromatography (17, 44, 45) and competitive protein-binding (56, 57, 96). These techniques have been explored extensively and some of them have proven very successful in measuring progesterone in minute amounts.

The steps that precede the actual measurement of progesterone are very important, because the crude sample cannot be measured before extraction and some purification. The degree of purification, needless to say, is dependent upon the detection method to be used.

Extraction methods. The choice of a proper solvent in extracting the steroid is necessary to minimize extraneous material and other contaminating steroids which might be contained in the extract. Since progesterone in the blood does not occur in the conjugated form and is bound by a weak bond to protein, a solvent should be selected for its efficiency and specificity for progesterone. Because progesterone is of low polarity, it can be easily dissolved and extracted with a non-polar solvent such as petroleum ether. With non-polar solvents, compounds with higher polarities will not be extracted and thus will not interfere with the detection (86).

One of the big problems encountered in extraction is the presence of contaminating lipids. Allen (1) found that by using 70 percent methanol and petroleum ether he could separate the progesterone into the alcoholic phase. Zander (99) found that by freezing the alcoholic extract and centrifuging he could recover 96 percent of added labeled progesterone. The addition of a strong alkali (NaOH) to plasma samples

has been shown to reduce the fatty material in the ether extract (61, 73, 76).

Purification of the isolated extract. As stated above, the sample can be partially purified by selecting the proper solvent and by adding alkali or freezing. However, these steps are not sufficient in cleaning up the sample and it becomes necessary to revert to other purification techniques. Some of these are countercurrent distribution, paper chromatography, column chromatography and thin-layer chromatography.

Countercurrent distribution has been used on both tissue (42, 61) and blood extracts (62). A solvent system of equal volumes of petroleum ether and 34.5 percent ethanol with a 24-transfer distribution was found by Diczfalussy, according to Zander (99), to be a satisfactory purification method. However, this procedure has not been accepted widely because it is rather tedious and time-consuming. Loy et al. (42) are the only investigators who have used this procedure in routine determinations.

Paper chromatography has found tremendous application in the purification of various steroid compounds for microdeterminations. This analytical tool is advantageous because it is relatively simple and remarkably sharp separations are possible from other relatively weak polar steroids which can be readily recognized on the paper. One of the requirements that must be met before paper chromatography can be applied to extracted material is that the extract must be relatively free of lipid material (99).

Monophasic solvent systems in paper chromatography have not proven too successful for the isolation of progesterone (23). However, biphasic systems have proven useful. Bush (7) used a system of light petroleum as the mobile phase and 80 percent methanol as the stationary phase. If the alcohol concentration is lowered, clearer separations may be obtained because the impurities and other steroids will run with the solvent front (99). Systems using stationary phases such as nonaqueous solvents (6, 97) and water (14) have not proven successful because of difficulties in removing the sample or excessive trailing.

"Open column" chromatography or thin-layer chromatography as it is more often referred to is becoming widely accepted as a steroid purification tool. Its wide acceptance is based on a relatively short developing time, clear separation and the fact that fluorescent indicators and various spray reagents can be applied to identify the compound in question (19). Waldi (89) discussed various solvent systems, luminous indicators and spray reagents which are applicable to steroid purification.

Lurie et al. (45) used a solvent system of chloroform-acetone (9:1) on silica gel plates to purify progesterone and obtained an RF value ( $RF = \frac{\text{distance of center sample spot from starting point}}{\text{distance of solvent front from starting point}}$ ) of 0.64.

Neil et al. (57) also used silica gel plates but their solvent system was composed of ether-benzene (2:1). By using this system they could effectively separate progesterone from other contaminating steroids.

Spraying of coated plates with phosphoric acid and heating for 7-15 minutes at 110-120 centigrade (C.) will cause progesterone to

fluoresce under ultraviolet (UV) light at 365 millimicron ( $\mu$ ). If the progesterone does not appear distinctly after phosphoric acid treatment, a 1.5 percent alcoholic phosphomolybdic acid solution may be sprayed on the plate followed by heating to enhance the color development (89).

Some investigators (8, 45, 76) have devised column chromatographic procedures as a step in isolating progesterone. This technique has proven very successful in removing large amounts of extraneous material. The big disadvantage with this tool is the time required and difficulty in packing the column, especially the partition (dry) columns.

Butt et al. (8) and Lurie et al. (45) used a Hyflo Supercel column in their routine progesterone determination procedures. Both groups of investigators used aqueous methanol as the stationary phase. However, Butt et al. (8) used n-hexane as the mobile phase while the latter found 2,2<sup>4</sup>-trimethylpentane to be a very satisfactory mobile phase.

Quantitative methods. Detection methods for progesterone have progressed significantly from the earlier polarographic and spectrophotometric procedures to the current gas chromatographic, isotope labeling and competitive protein-binding (CPB) techniques.

Polarographic estimation of progesterone has not found wide acceptance in the field of progesterone assay. This is primarily due to the fact that this procedure is not sensitive enough for the detection of progesterone in blood (99).

Spectrophotometric applications have been widely used in steroid detection. Ultraviolet absorption at 240 mu. was used by a number of investigators (14, 23, 61, 72, 73). Progesterone-bis-dinitro-phenyl-hydrazone derivatives have been prepared and measured at 380 mu. (28). Oertel et al. (58) measured the absorption of progesterone in 80 percent ethanol and 66 percent sulphuric acid at a wave length of 290 mu.

The use of alkali fluorescence has greatly increased the sensitivity of fluorometric determinations (75, 84, 92). Touchstone and Murawec (84) found that if progesterone was heated in 2 N KOH before dissolving in sulphuric acid, they could get a 100 fold increase in sensitivity. By enzymatically converting progesterone to 20- $\beta$ -hydroxy-pregn-4-en-3-one and using a sulphuric acid-ethanol reagent the sensitivity of the fluorometric method may be extended to 3 nanogram (ng.) (26).

Since 1960, gas chromatography has been widely applied in the measurement of steroids and notably progesterone. Various types of gas chromatographic detection systems such as electron capture, flame ionization and argon ionization have been used for progesterone.

To measure progesterone with an electron capture detector, it is necessary to prepare a derivative that is capable of capturing electrons, since progesterone alone will not capture a sufficient amount to measure. Landowne and Lipsky (40) prepared a monochloro-acetate derivative of progesterone and found the sensitivity to increase 100 fold as compared with standard argon or flame ionization detectors.

Stabenfeldt et al. (79) used an electron capture detector and a halogenated derivative in following the technique of van der Molen and Groen (87) in analyzing progesterone in the peripheral blood of pregnant cows.

Flame ionization has proven to be a very acceptable detection device for minute amounts of progesterone if a solid injector device is used. Goldfien et al. (18) using this method could detect as little as 10-20 ng. of progesterone. Lurie et al. (45) found the low limit of sensitivity to be 20 ng. However, their method tended to overestimate progesterone in the 20 to 80 ng. range.

Collins and Sommerville (10) using an argon ionization detector were able to detect progesterone in plasma of the order of 10 ng.

The isotope labeling principle is a fairly recent venture in the steroid field. Riondel et al. (68) were the first group to use this principle for the assay of progesterone. The lower limit of sensitivity for this method is in the range of 1.0 to 2.0 ng. According to van der Molen and Aakvaag (86), however, this technique tends to overestimate progesterone at a level of 4 to 5 ng. per sample for samples in the range of 0-200 ng. Woolver and Goldfien (93) prepared their radioactive progesterone derivative using sodium-<sup>3</sup>H-borohydride, while Riondel et al. (68) preferred to use <sup>35</sup>S-thiosemicarbazide. This technique is advantageous because of the ease in derivative formation and it readily permits the determination of recovery rates by using labeled progesterone.

Yalow and Berson (94) were two of the first investigators to

use the binding capacity of proteins in blood as an assay procedure. Their radioimmunoassay technique was developed for the measurement of human chorionic gonadotrophin. Since then, competitive protein-binding assay procedures have been devised for steroids such as corticoids (56), testosterone (31) and progesterone (57). The procedure as outlined by Neil et al. (57) is based upon the displacement of labeled corticosterone from a protein molecule by progesterone. This technique is relatively specific and very sensitive. The lower limit of sensitivity appears to be 0.1 to 0.2 ng.

Other physico-chemical methods have been developed to measure steroid compounds such as the immunochemical technique developed by Beiser et al. (4). Koreman (37) has developed a procedure for the measurement of estradiol which resembles the CPB assay procedure to some extent. However, the binding molecule is located in the pregnant rabbit uterus. The assay procedure was linear over the dose range of 17 to 133 picogram ( $10^{-9}$  mg.).

Some physico-chemical detection methods and their sensitivities are summarized in Table I.

## 2. PROGESTERONE LEVELS IN THE BOVINE

Progesterone levels in the peripheral blood of cows have been determined during pregnancy for normal gestations (5, 15, 16, 21, 22, 71, 74) and prolonged gestations (29). More recently, the levels have been determined during the estrous cycle (20, 41, 48, 66, 67, 78). Table II illustrates the considerable variation in progesterone levels

TABLE I  
METHODS AND ACCURACY OF VARIOUS PHYSICO-CHEMICAL ASSAYS<sup>a</sup>

| Detection                                                                 | Sensitivity (ng.) | Reference                                                                          |
|---------------------------------------------------------------------------|-------------------|------------------------------------------------------------------------------------|
| Absorption of Progesterone                                                | 500               | Zander (98)                                                                        |
| Absorption of Isonicotinic Acid Hydrazide Derivative                      | 500               | Sommerville and Desphande (76)                                                     |
| Absorption of Sulphuric Acid-Ethanol Chromogen                            | 300               | Oertel <u>et al.</u> (58)                                                          |
| Absorption of Thiosemi-Carbazide Derivative                               | 200               | Sommerville <u>et al.</u> (77)                                                     |
| KOH-Sulphuric Acid Fluorescence                                           | 50-100            | Touchstone and Murawec (84)                                                        |
| Conversion to 20- $\beta$ -dihydroprogesterone and Sulphuric Fluorescence | 3-5               | Heap (26)                                                                          |
| Isotope Labeling                                                          | 2.5-100           | Woolver and Goldfien (93)                                                          |
| Isotope Labeling                                                          | 1-2               | Riondel <u>et al.</u> (68)                                                         |
| Gas Chromatography Argon Ionization                                       | 10-100            | Collins and Sommerville (10)                                                       |
| Gas Chromatography Flame Ionization                                       | 5-100             | Luisi <u>et al.</u> (44)<br>Lurie <u>et al.</u> (45)<br>Yannone <u>et al.</u> (95) |
| Gas Chromatography Electron Capture                                       | 1                 | van der Molen and Groen (87)                                                       |
| Competitive Protein-Binding                                               | 0.1               | Johansson <u>et al.</u> (32)<br>Yoshimi and Lipsett (96)                           |

<sup>a</sup>Taken in part from H. J. van der Molen and A. Aakvaag, Progesterone. In Hormones in Blood, Vol. II. Academic Press, London and New York, 1967.

TABLE II  
 PROGESTERONE LEVELS IN PERIPHERAL PLASMA AT VARIOUS DAYS  
 OF THE ESTROUS CYCLE<sup>a,b</sup>

| Day<br>of<br>Cycle | Shemech<br><u>et al.</u><br>(71) | Long<br>(41) | Plotka<br><u>et al.</u><br>(67) | Plotka<br><u>et al.</u><br>(66) | McCracken<br>(48) | Gomes<br><u>et al.</u><br>(20) |
|--------------------|----------------------------------|--------------|---------------------------------|---------------------------------|-------------------|--------------------------------|
| 0                  |                                  | 2.8          | 10.1                            | 10.6                            |                   | 3.2                            |
| 1                  |                                  | 0.9          |                                 |                                 | 1.1               | 18.0                           |
| 2                  |                                  | 1.7          | 9.9                             | 10.5                            |                   |                                |
| 3                  |                                  |              |                                 |                                 |                   | 8.2                            |
| 4                  |                                  |              | 17.7                            | 18.8                            |                   | 6.1                            |
| 5                  |                                  |              |                                 |                                 |                   |                                |
| 6                  |                                  |              | 10.6                            | 13.6                            |                   | 2.7                            |
| 7                  |                                  |              |                                 |                                 |                   | 3.1                            |
| 8                  |                                  |              | 17.7                            | 21.7                            |                   | 8.5                            |
| 9                  |                                  |              |                                 |                                 |                   | 21.6                           |
| 10                 |                                  |              | 18.4                            | 20.4                            |                   |                                |
| 11                 | 4.3                              |              |                                 |                                 |                   | 22.0                           |
| 12                 | 4.3                              |              | 20.4                            | 21.6                            |                   |                                |
| 13                 | 4.3                              |              |                                 |                                 | 10.6              |                                |
| 14                 | 4.3                              |              | 25.8                            | 32.9                            |                   | 12.1                           |
| 15                 | 4.3                              |              |                                 |                                 |                   | 11.6                           |
| 16                 | 4.3                              |              | 17.1                            | 19.4                            |                   |                                |
| 17                 | 4.3                              |              |                                 |                                 |                   |                                |
| 18                 | 4.3                              |              | 19.4                            | 20.6                            |                   |                                |
| 19                 | 4.7                              |              |                                 |                                 |                   |                                |
| 20                 | <1.5                             |              | 13.3                            | 13.4                            |                   |                                |

<sup>a</sup>Ng. per ml. plasma.

<sup>b</sup>Taken in part from D. W. Long, Variation in peripheral plasma progesterone levels and its association with ovulation in the bovine. M.S. Thesis, Ohio State University, Columbus, Ohio, 1968.

among investigators that exist during the cycle. Some of this variation may be due to the assay procedure or the presence of other progestins. The values obtained by Plotka et al. (66, 67) may be open to question because the double isotope derivative method is believed to overestimate samples by 4 to 5 ng. if they fall within the range of 0 to 200 ng. (86). It is also possible that the differences in the values obtained by Shemesh et al. (71) using the gas chromatography and McCracken (48) and Gomes et al. (20) using spectrophotometry may be due to a lack of sensitivity in the detection methods. It also appears that some of the differences among investigators may be due to sampling, because considerable variation exists among animals (41).

From the work conducted by McCracken (48), it appears that the corpus luteum is the primary source of progesterone and it should reflect the levels present in peripheral blood. However, Gomes et al. (20) did not substantiate this with their findings. Their results indicate that there is no relationship between peripheral and ovarian venous plasma concentrations.

### 3. EFFECTS OF EXOGENOUS PROGESTERONE

The effects of exogenous progesterone have been studied during the estrous cycle (35, 43, 85, 90) and during pregnancy (13, 24, 27, 33, 34, 49, 50, 82, 91). Loy et al. (43) gave progesterone injections to open heifers and found a significant decrease in corpus luteum weight and progesterone concentration. Johnson and Ulberg (35) noted estrus and ovulation inhibition when they injected progesterone.

Exogenous progesterone has been shown to increase the fertility of repeat breeding cows. Wiltbank et al. (91) found that cows receiving 50 mg. of progesterone had 44.4 percent normal embryos compared to 33.3 percent with normal embryos in the untreated group. Johnson et al. (34) found that progesterone increased the reproductive efficiency of normal cows 28 percent for first service conceptions. They believed that the progesterone made the uterus more compatible by increasing the secretions of the endometrium.

The effects of exogenous progesterone can be further demonstrated by the work of McDonald and Hays (49). They found that 500 mg. of repositol progesterone injected once a day every 10 days would maintain pregnancy in animals without a corpus luteum for as long as two to five months.

Contrary to the above results, Hawk et al. (24) found that by comparing ovariectomy and hormone treatment in first service and repeat breeder cows that the fertility level of the repeat breeders did not reach that of the first service animals. They agree with Wiltbank et al. (91) in that embryonic death in repeat breeders occurred before regression of the corpus luteum.

#### 4. INFERTILITY IN THE BOVINE FEMALE

The exact cause of reproductive failure in the bovine female is not known. It has been postulated that causes of infertility may be due to lethal genes, immunological causes, anatomical abnormalities,

diseases and infections and finally, hormonal imbalances. It is the latter of these in which we are primarily interested.

The presence of the corpus luteum is necessary because it produces progesterone which prepares the uterus for implantation and maintains pregnancy (69). So it seems reasonable to assume that if the corpus luteum malfunctions, embryonic death may occur. The incidence of embryonic death has been studied in both normal and repeat-breeder cows. Laing (38, 39) in an experiment with heifers was able to determine two types of early embryonic death: (1) the fertilized egg died before interfering with the next estrous cycle, and (2) the fertilized egg survived along with the corpus luteum to delay the next estrous period. In normal cows 15 percent of potential embryos die before 30-34 days (3, 36). However, in repeat-breeder cows the percent embryonic death is considerably higher. From summarized data Salisbury and Vandemark (69) found an embryonic death rate of 20.7 percent for hard to settle heifers and 32.5 percent for repeat-breeder cows. However, Hawk et al. (25) and Tanabe and Casida (83) reported embryonic death rates for repeat-breeder cows to be 51.7 percent (16-34 days) and 65.1 percent (before 34 days), respectively.

## CHAPTER III

### EXPERIMENTAL PROCEDURE

#### Objectives of Experiment

The two primary goals of this research project were to adapt the competitive protein-binding assay procedures used by Neil et al. (57) and Johansson et al. (32) to the analysis of progesterone in bovine peripheral plasma and to compare levels of progesterone in cows for a 21 day period following insemination. It was originally planned to make this comparison on first service cows and cows cycling four or more times. However, this idea was abandoned because of the relatively small number of cows recycling at 21 day intervals. Consequently, the cows were grouped, disregarding the number of services, according to cycle length. The cows were placed in one of the following groups: (A) 19-27 days, (B) 42-43 days, (C) 73-101 days, and (D) pregnant.

#### Animals

Holstein cows selected from the University of Tennessee milking herd were used throughout the experiment. The blood samples were obtained at approximately 11:00 A.M. every third day, beginning on the day of heat and continuing for 21 days. Estrus was determined by visual observation of the animals. The blood was drawn from the jugular vein into heparinized flasks. The samples were then

centrifuged and the plasma was withdrawn and stored at  $-23^{\circ}$  C. The plasma samples were usually ready for storage within one hour after collection.

#### Assay Procedure

The competitive protein-binding assay procedure as set forth by Neil et al. (57) and Johansson et al. (32) is based upon the ability of progesterone to displace corticosterone- $1,2-^3\text{H}$ , which is bound to the assay protein, corticosteroid binding globulin (CBG). The assay procedure used in this experiment is very similar to the procedures used by the above investigators, with the exception of three modifications: (1) it was found necessary to increase tracer (corticosterone- $1,2-^3\text{H}$ ) concentration to 52.2 ng. per 100 milliliter (ml.) of corticosteroid binding globulin-corticosterone- $1,2-^3\text{H}$  (CBG- $\text{B}^3\text{H}$ ) solution; (2) the number of plasma extractions was increased from one to two; (3) the thin-layer step was omitted. The primary reasons for these changes are discussed in Chapter IV.

The modified CPB assay procedure as used in this experiment is as follows:

1. Standards were prepared in duplicate by adding a measured amount of progesterone (0,1,2,3,4,6 and 8 ng.) to 12 x 75 millimeter (mm.) culture tubes.
2. Plasma (0.5 ml.) was extracted twice with  $30-60^{\circ}$  C. (boiling point) petroleum ether (4 and 3 ml.) with 60 minute shakings.
3. The ether phases were transferred to 12 x 75 mm. culture

tubes and evaporated to dryness under nitrogen gas at 45° C.

4. One ml. of CBG-B<sup>3</sup>H solution, which contained 52.2 ng. of corticosterone-1,2-<sup>3</sup>H and 2.5 ml. of dog plasma diluted to 100 ml. with distilled water, was added to each tube (standard and unknown).

5. The tubes were shaken and placed in a 45° C. water bath for 5 minutes and shaken again after removal from the water bath.

6. The samples were placed in an ice bath for 10 minutes.

7. Eighty mg. of florisil was added to each tube and the tubes were shaken immediately for exactly 30 seconds on a Vortex mixer and returned to the water bath.

8. One-half ml. of the supernant solution from each tube was added to 15 ml. of scintillation fluid and the samples were counted in a liquid scintillation detector.

The amount of progesterone present in the unknown samples was determined by comparing its displacement of corticosterone-1,2-<sup>3</sup>H with that of known amounts of progesterone. A standard curve was prepared each time unknown samples were analyzed. All samples were counted three times for 2 minutes. With two-minute countings, the difference between triplicate countings could be reduced to 2 percent or less. Figure 1 illustrates a typical standard curve that was used in the determination of unknowns. A linear relationship may be obtained by plotting the reciprocal of the percent bound corticosterone-1,2-<sup>3</sup>H against added progesterone. Calculations may be omitted if time required to reach a pre-set count is plotted against added progesterone as illustrated in Figure 2.

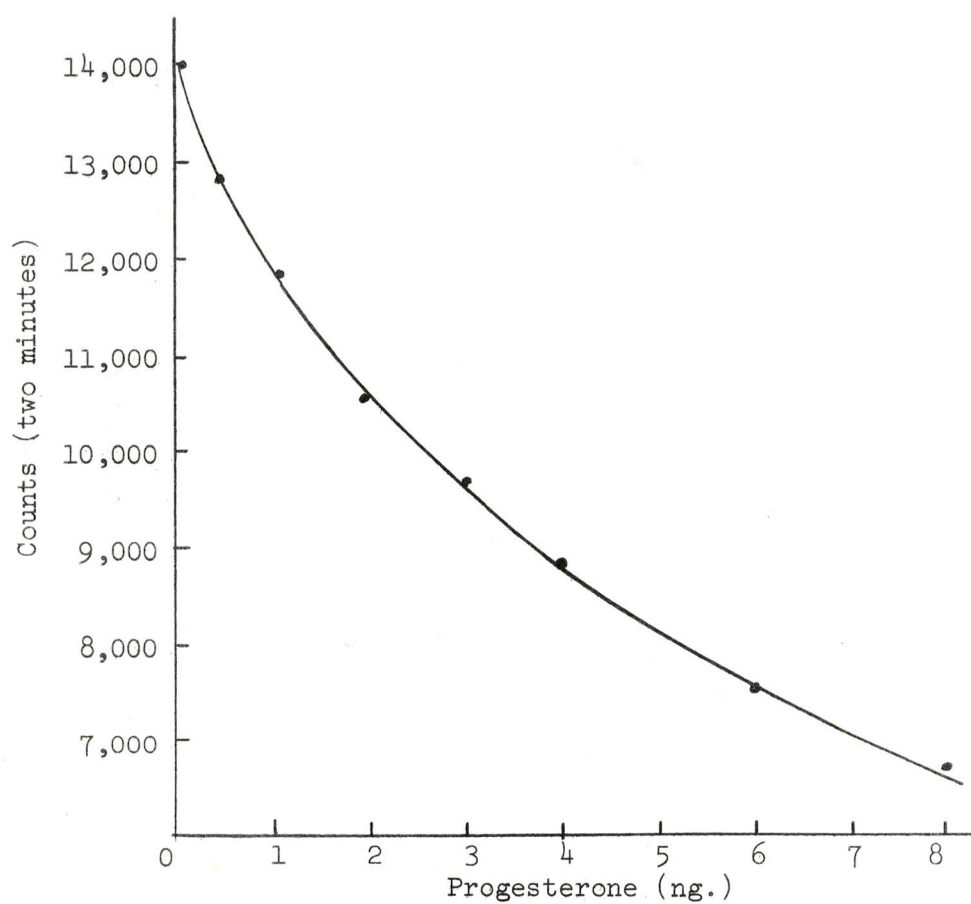


Figure 1. A typical standard curve for determining progesterone in blood plasma.

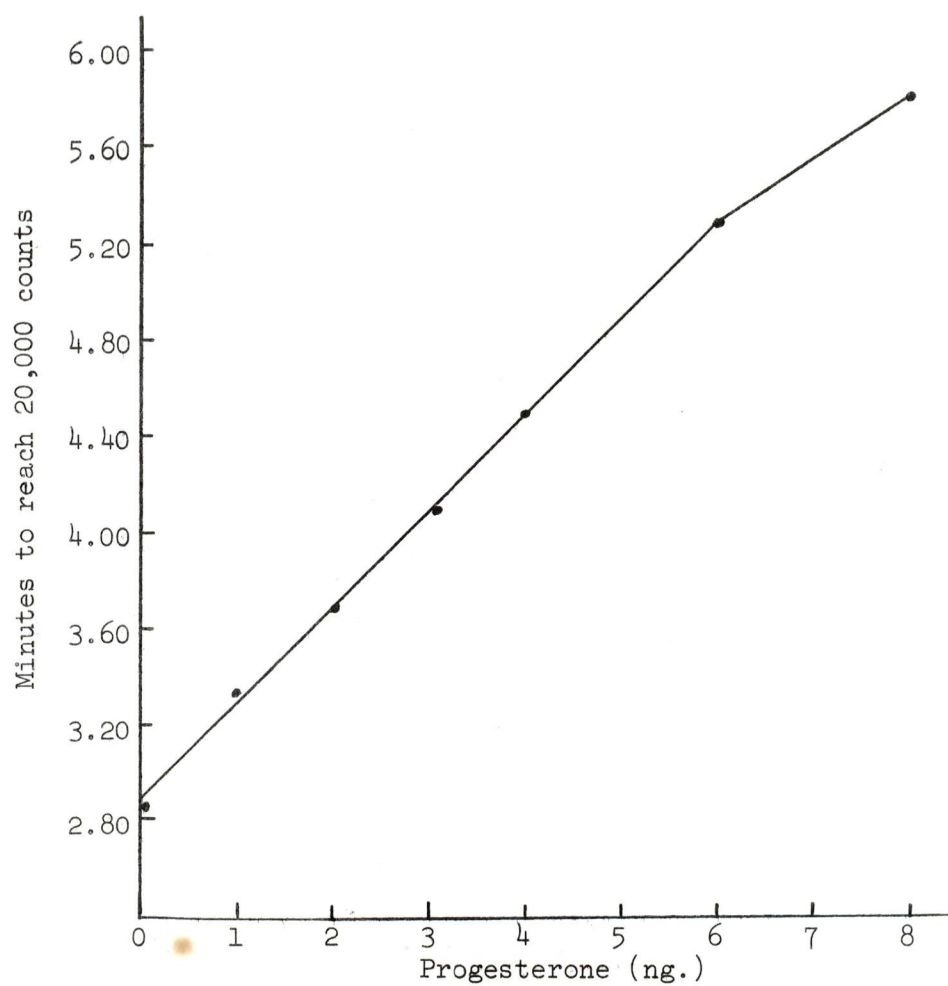


Figure 2. A typical linear relationship for determining progesterone in blood plasma.

The labeled corticosterone was obtained from the New England Nuclear Corporation and the labeled progesterone was obtained from Schwartz Biochemical Research, Inc. These compounds plus the other chemicals (reagent grade) were used without further purification.

The scintillation fluid was composed of 100 gram (g.) naphthalene, 7.0 g. of 2,5-diphenyl-oxazole and 0.3 g. 1,4-bis-2 (5-phenyl-oxazolyl)-benzene per liter of dioxane. This solution was prepared on the day of use because it appeared to be unstable (color change).

The dog plasma was obtained from the femoral vein of three male dogs and pooled. Three ml. aliquots of the pooled plasma were stored at  $-23^{\circ}$  C. The pooled cow plasma which was used to check the procedure and technique, was obtained from four cows between days 10 and 16 of the estrous cycle.

Statistical analysis was performed according to the procedures outlined by Steel and Torrie (80).

## CHAPTER IV

### RESULTS AND DISCUSSION

#### 1. PROCEDURE EVALUATION

##### Tracer Concentration

Corticosterone-1,2-<sup>3</sup>H concentration was increased from 15 ng. (32) and 25 ng. (57) to 52.2 ng. This increase in concentration was necessary because of a low specific activity of 25 curies per millimole (c. per m. mole) as compared to specific activities of 57.2 c. per m. mole used by Johansson et al. (32) and 33.4 c. per m. mole used by Neil et al. (57). Figure 3 illustrates the relationship between tracer concentration and the shape of the standard curve. At lower concentrations the curve flattens too quickly over the anticipated range of progesterone levels.

##### Extraction

Under the conditions of this experiment, two extractions with 4 ml. and 3 ml. portions of petroleum ether were used to obtain recovery rates of approximately 90 percent. The recovery rate of crystalline progesterone from steer and cow plasma was 92.3 percent with a standard error (SE) of 1.41 percent. A coefficient of variation (CV) of 6.1 percent was calculated for samples assayed on 16 different occasions. Neil et al. (57) reported that by using a single extraction (5-10 volumes) with petroleum ether, they obtained a recovery rate

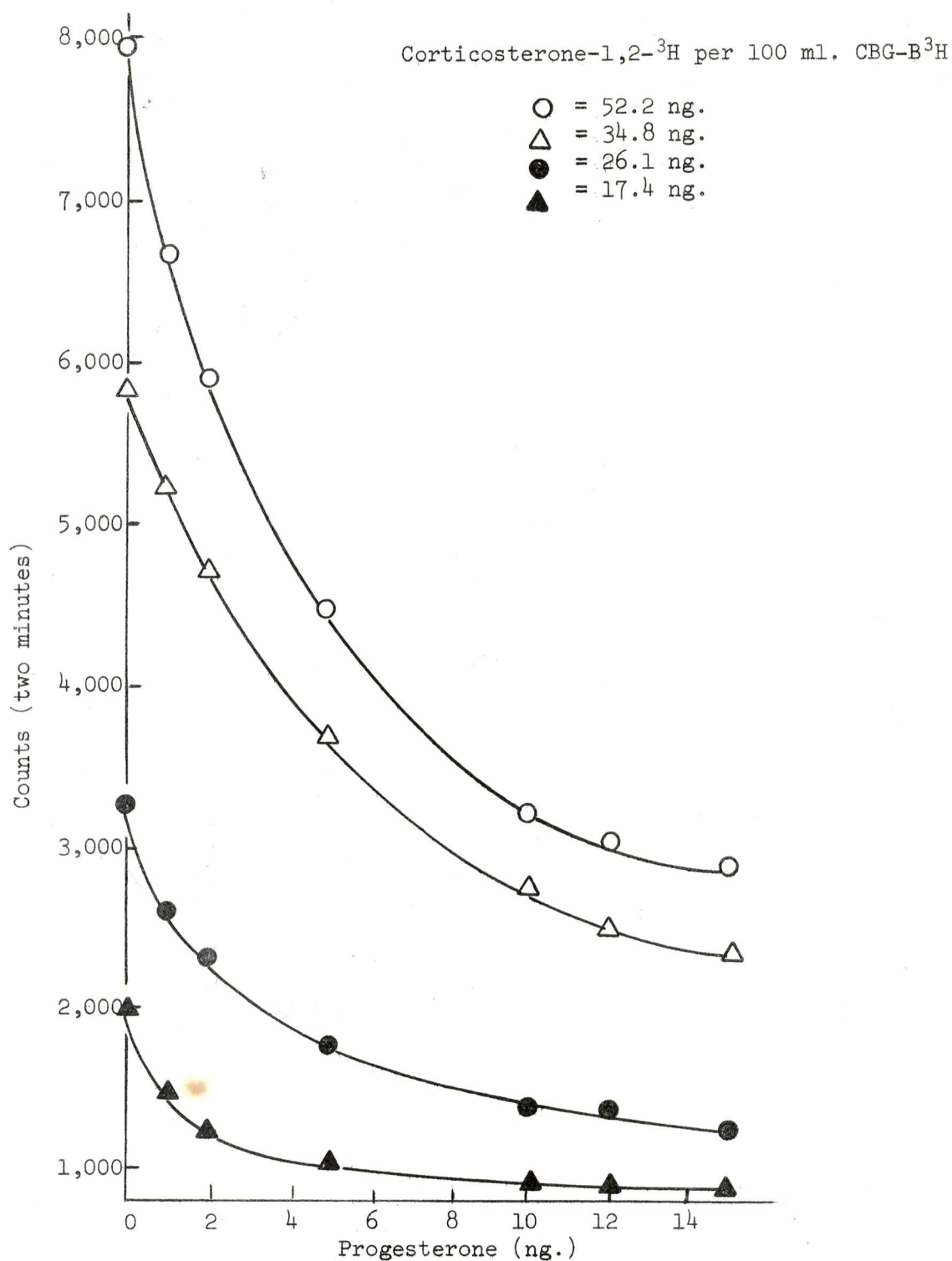


Figure 3. Tracer concentration and slope of the standard curve.

of approximately 90 percent. However, Long (41) found that two 5 ml. extractions with petroleum ether were necessary in obtaining a mean progesterone recovery of 83.1 percent with a standard deviation of 6.13 percent.

#### Thin-Layer Chromatography

The chromatographic step was eliminated from this procedure primarily because of the time and labor involved and the difficulty encountered in evaluating the results. Long (41) reported that reasonable standard curves could not be obtained if the progesterone standards were chromatographed and corrected for losses. The thin-layer step may not be necessary in the analysis of bovine plasma. Neil et al. (57) used this purification step to separate progesterone from 17- $\alpha$ -hydroxyprogesterone (17- $\alpha$ -OH) and 20- $\alpha$ -hydroxypregn-4-ene-3-one (20- $\alpha$ -OH) which are present in the plasma of the rhesus monkey. Neither of the above compounds nor  $\Delta^4$ -pregnen-20- $\beta$ -ol-3-one (5), which has been measured in bovine corpora lutea tissue, have been identified in peripheral bovine plasma. According to Johansson et al. (32), the only steroids in the petroleum ether extract which significantly displace corticosterone-1,2- $^3$ H are progesterone and 17- $\alpha$ -OH. Since 17- $\alpha$ -OH has not been found in peripheral bovine plasma, it appears that progesterone is the primary compound being measured.

#### Time Exposure Before and After Florisil Addition

The purpose of florisil in this procedure is to remove bound

corticosterone-1,2-<sup>3</sup>H. The shaking time is critical and should be rigidly standardized for both unknown and standard samples. Murphy (56) found that shaking over long time periods increased the uptake by the adsorbents, thus causing the curve to shift. She also noted that if the samples were allowed to stand for various lengths of time over a ten minute period after shaking, there was no increased uptake by florisol. However, no mention was made on the effect of the length of time in the ice bath before florisol was added. It was found in this experiment that if the samples were allowed to remain in the ice bath longer than one hour, the counts became higher. It appears that over this extended period of time that more of the corticosterone became bound to the protein and the displaced progesterone became adsorbed by the florisol, thus causing higher counts and giving the appearance of less progesterone in the sample. When 12 samples containing 3 ng. of progesterone were allowed to stand one hour and twenty minutes to two hours before the addition of florisol, the progesterone content of the samples appeared to decrease approximately 50 percent.

#### Quantity of Plasma Extracted

One-half ml. of plasma was used throughout this experiment for the determination of progesterone in unknowns. However, a test was conducted to determine if the assay procedure was equally effective over a range of plasma levels (0.125, 0.250, 0.50 and 1.00 ml.). If time to reach a pre-set count is plotted against amount of plasma, the relationship is linear (Figure 4). Therefore, it appears from

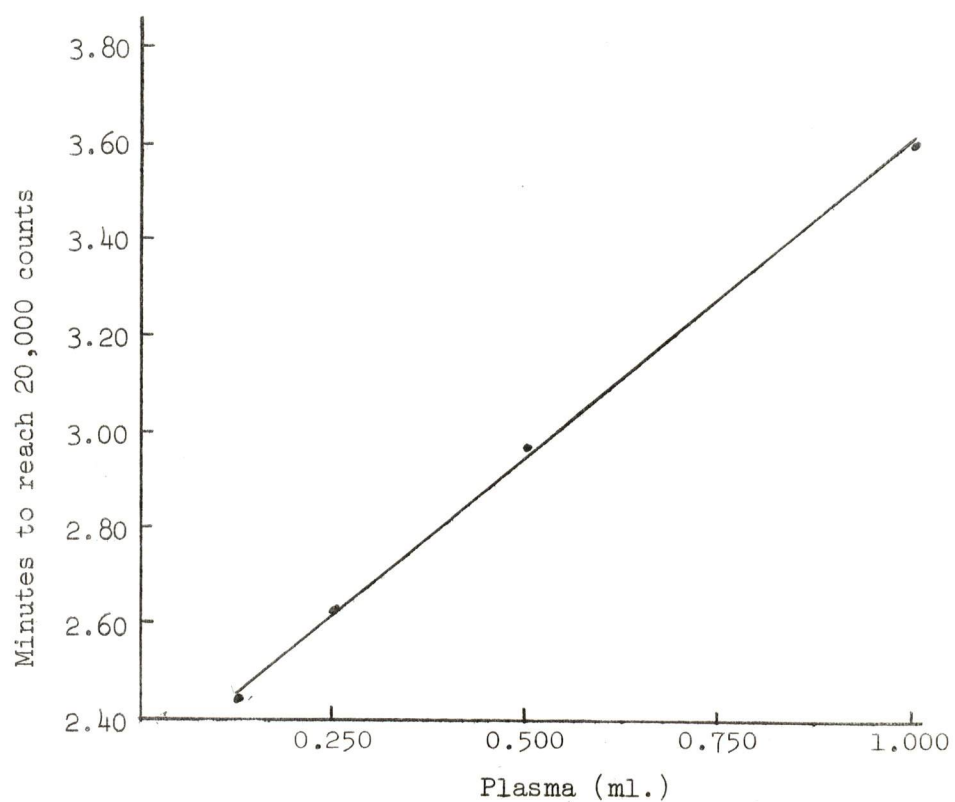


Figure 4. Effectiveness of the procedure over a range of plasma levels.

the limited amount of data (6 observations per sample) that the assay procedure is effective for plasma levels from 0.125 ml. to 1.00 ml.

#### Sensitivity and Accuracy

The sensitivity of the modified procedure is slightly lower than that found by Neil et al. (57) and Johansson et al. (32). Figure 5 illustrates the lower limit of sensitivity was approximately 0.30 ng. The reduced sensitivity of the modified procedure is believed due to the lower specific activity of the corticosterone-1,2-<sup>3</sup>H (25 c. per m. mole) as compared to the specific activities used by Neil et al. (33.4 c. per m. mole) (57) and Johansson et al. (57.2 c. per m. mole) (32).

A check on the reliability of the technique and the procedure was conducted by analyzing 0.5 ml. pooled cow plasma. These samples were run in triplicate on 14 separate occasions and had a mean progesterone content of  $5.35 \pm 0.33$  ng. per ml. (SE) and a CV of 22.6 percent. The primary reason for the difference between the CV's of pooled plasma and plasma containing added progesterone is due to the fact that the pooled samples were based on individual values, while the recovery rate determinations were an average of triplicate values, thus, giving rise to less variation and a smaller CV.

## 2. PROGESTERONE DATA EVALUATION

The mean progesterone values, standard errors, sample numbers and results of statistical analysis are presented in Table III. The

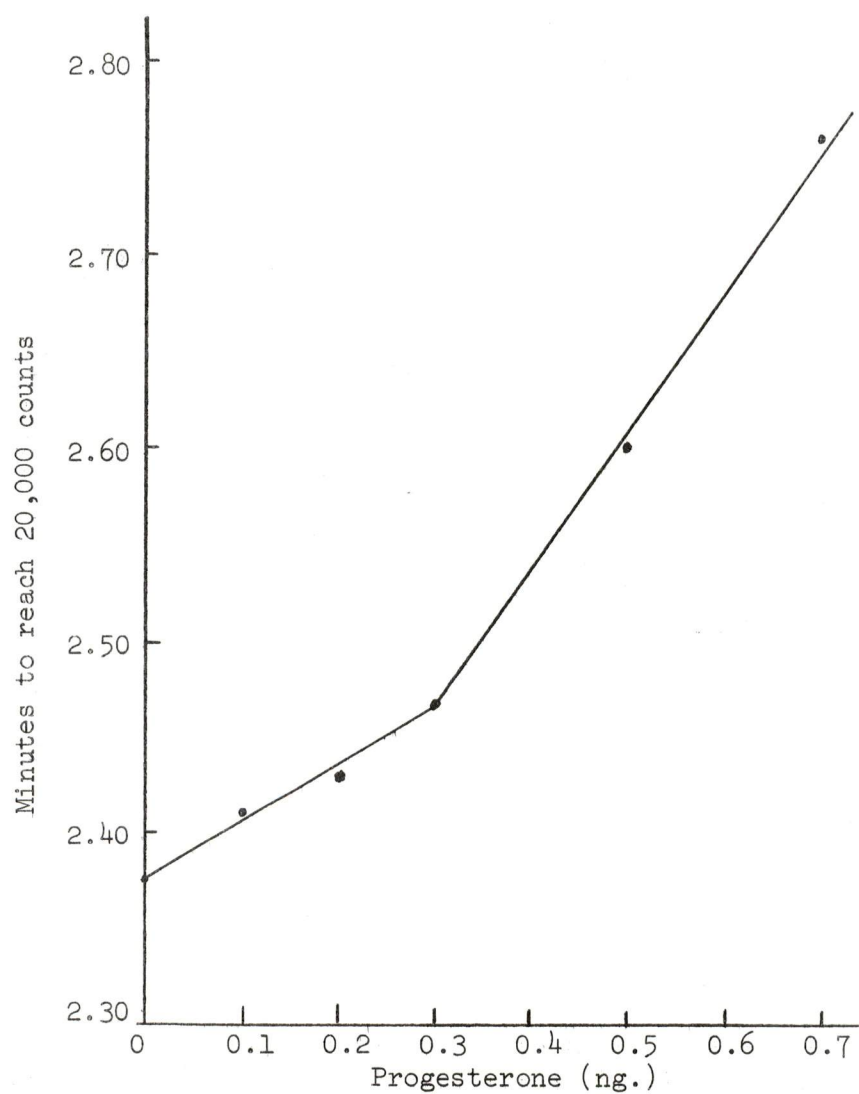


Figure 5. Sensitivity of the assay procedure.

TABLE III  
 PROGESTERONE IN BLOOD PLASMA OF COWS TWENTY-ONE  
 DAYS AFTER INSEMINATION

| Day after<br>Insemination | Progesterone mean $\pm$ SE (ng. per ml.) and number of samples |                    |                    |                                    |
|---------------------------|----------------------------------------------------------------|--------------------|--------------------|------------------------------------|
|                           | A <sup>1</sup>                                                 | B                  | C                  | D                                  |
| 0                         | 0.71 $\pm$ 0.09 <sup>a*</sup> (4)                              | 0.20 $\pm$ 0.06(3) | 0.67 $\pm$ 0.63(2) | 0.64 $\pm$ 0.07 <sup>a</sup> (4)   |
| 3                         | 1.80 $\pm$ 0.23 <sup>b</sup> (5)                               | 0.75 $\pm$ 0.27(4) | 1.58 $\pm$ 0.21(2) | 1.21 $\pm$ 0.13 <sup>a</sup> (6)   |
| 6                         | 1.87 $\pm$ 0.14 <sup>b</sup> (4)                               | 3.19 $\pm$ 0.41(4) | 3.46 $\pm$ 0.43(3) | 2.92 $\pm$ 0.22 <sup>b</sup> (7)   |
| 9                         | 4.87 $\pm$ 0.57 <sup>c</sup> (4)                               | 5.37 $\pm$ 0.52(4) | 4.76 $\pm$ 0.39(3) | 5.40 $\pm$ 0.36 <sup>c</sup> (6)   |
| 12                        | 6.04 $\pm$ 0.45 <sup>d</sup> (5)                               | 7.51 $\pm$ 0.84(4) | 6.77 $\pm$ 0.43(3) | 6.13 $\pm$ 0.22 <sup>cd</sup> (7)  |
| 15                        | 6.39 $\pm$ 0.45 <sup>d</sup> (5)                               | 8.39 $\pm$ 1.12(4) | 5.52 $\pm$ 0.16(1) | 6.62 $\pm$ 0.32 <sup>de</sup> (7)  |
| 18                        | 4.50 $\pm$ 0.63 <sup>c</sup> (5)                               | 6.73 $\pm$ 1.02(4) | 6.02 $\pm$ 0.47(3) | 7.12 $\pm$ 0.43 <sup>e</sup> (7)   |
| 21                        | 3.14 $\pm$ 0.60 <sup>e</sup> (4)                               | 3.12 $\pm$ 0.57(4) | 5.69 $\pm$ 0.50(2) | 8.49 $\pm$ 0.60 <sup>f**</sup> (7) |

\*Means with different superscripts are significantly different (P<0.05) within groups.

\*\*Pregnant group means were significantly higher (P<0.05) than other groups on Day 21.

<sup>1</sup>Group A cycled 19-27 days, Group B cycled 42-43 days, Group C cycled 73-101 days and Group D became pregnant.

progesterone values were not corrected for losses. The possibility does exist that other progestins in addition to progesterone may be present in bovine peripheral plasma. If other progestins are present they have not been identified and measured in this study.

In Group A there were four cows (five cycles) which cycled on the following days: cow 335 (19 days), cow 295 (22 days), cow 70 (25 days) and cow 225 (25 and 27 days). In this group of cows, the progesterone level on Day 21 did not drop to the Day 0 (estrus) level (Figure 6), apparently because of the extended cycle length. There was no significant difference ( $P>0.05$ ) in progesterone levels among cows. However, there was a significant difference among days ( $P<0.05$ ). The progesterone concentration exhibited a moderate increase from Day 0 to Day 3 and no increase from Day 3 to Day 6, which occurs at about the same time as the decrease in progesterone concentration reported by Plotka et al. (66, 67). From Day 6 the progesterone concentration increased sharply through Day 9 and then increased moderately to the peak level at Day 15. This agrees with Plotka et al. (66, 67) who found the progesterone concentration to peak on Day 14 with samples taken every two days.

There were a total of four cows that cycled 42-43 days after insemination (Group B). From Day 6 through Day 18 the progesterone concentration in these cows was higher than in Group A (Figure 7). However, the concentration at Day 21 was very similar for both groups. The high levels between Day 6 and Day 18 may be due to individual cow differences or the increased production of progesterone by the corpus

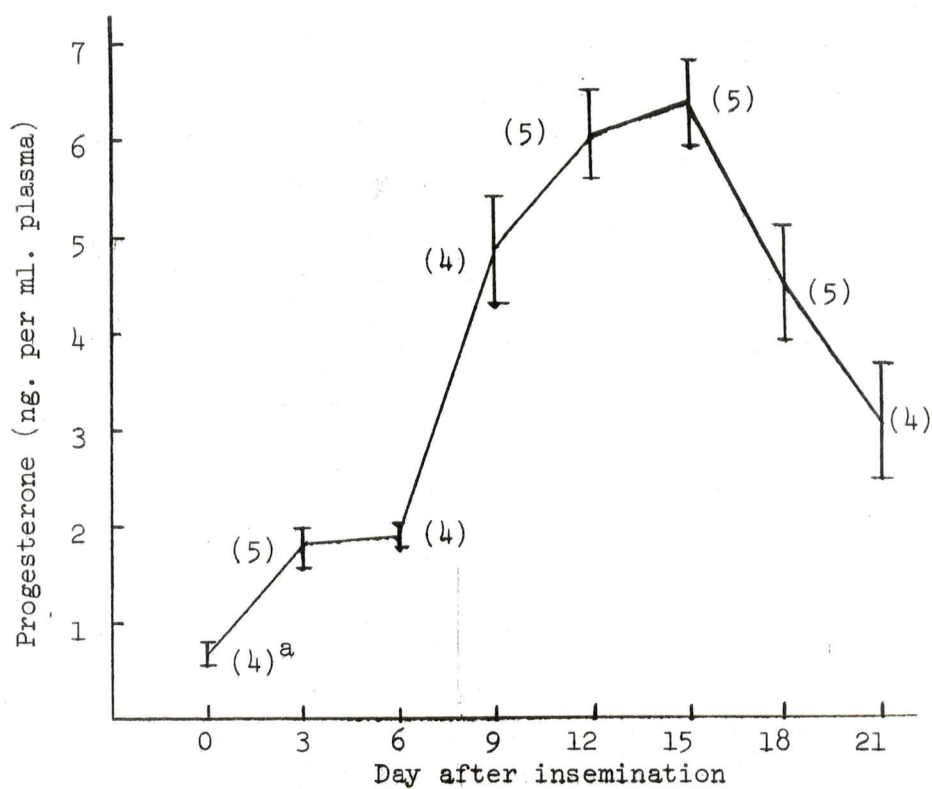


Figure 6. Progesterone concentration and standard error (ng. per ml. plasma) for cows cycling 19-27 days (Group A) after insemination. Day 0 equals estrus.

<sup>a</sup>Number of samples.

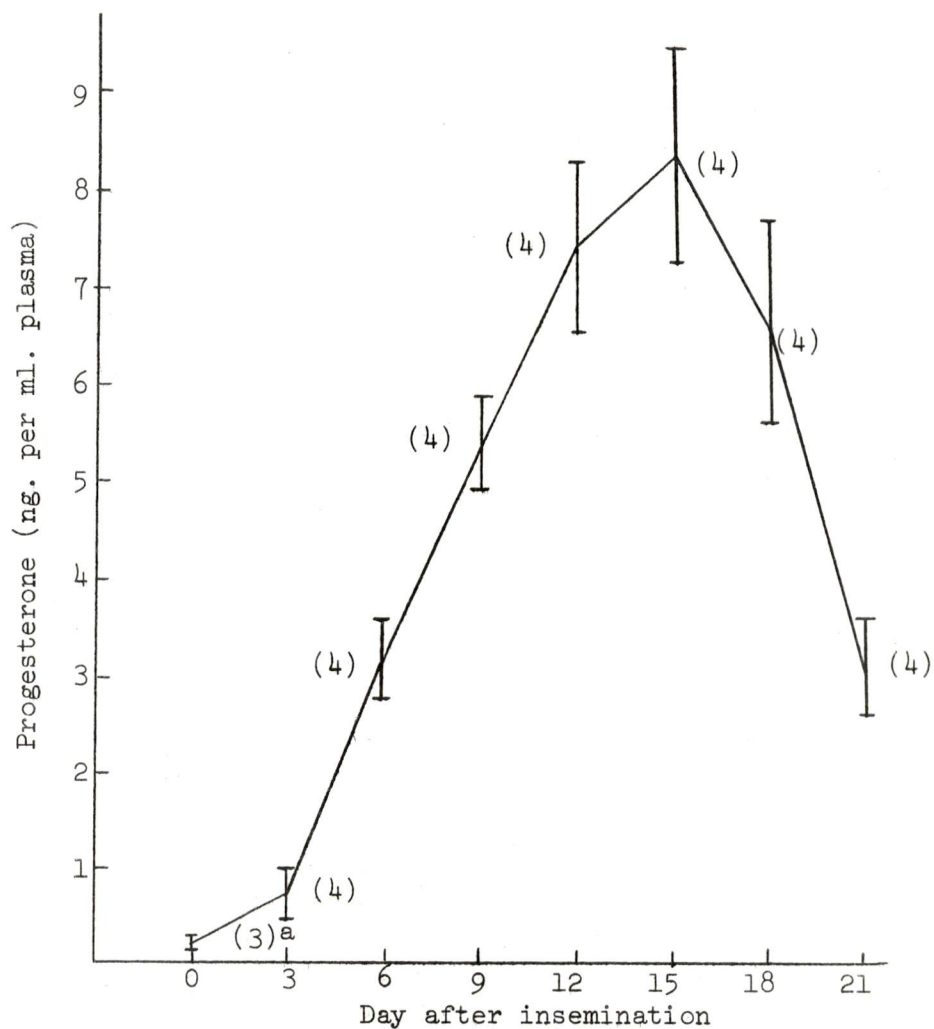


Figure 7. Progesterone concentration and standard error (ng. per ml. plasma) for cows cycling 42-43 days (Group B) after insemination, Day 0 equals estrus.

<sup>a</sup>Number of samples.

luteum in response to the embryo. Alternatively, the cows may have exhibited silent heats or the heats were undetected, and thus the regressing corpus luteum led to a decrease in progesterone output causing the levels of progesterone to drop as they did on Day 21. In this group of cows there was no significant difference ( $P>0.10$ ) in progesterone concentration among days and among cows. However, there was a significant ( $P<0.005$ ) cow-day interaction.

There were three cows which did not show signs of heat until 73-101 days after insemination (Group C): cow 168 (73 days), cow 391 (75 days) and cow 303 (101 days). The progesterone concentration in this group increased steadily to Day 12 and then dropped slightly at Day 15. This curve (Figure 8) is similar to the curve for Group D (Figure 9) through Day 12. The reasons for the lower progesterone concentrations from Day 15 through Day 21 could be due to individual animal differences or possibly due to a reduced output of progesterone from a malfunctioning corpus luteum. There was no significant difference ( $P>0.10$ ) in progesterone concentration among cows or among days. The non-significant difference, as in the previous group, is probably due to insufficient numbers. However, there was a significant ( $P<0.005$ ) cow-day interaction.

In Group D (Figure 9), the progesterone concentration remains elevated and does not show the expected drop exhibited in Group A. However, the increase from Day 18 through Day 21 was unexpected. It was thought that progesterone concentration would reach its peak around Day 15 and then increase gradually as the placenta grew. Erb et al.

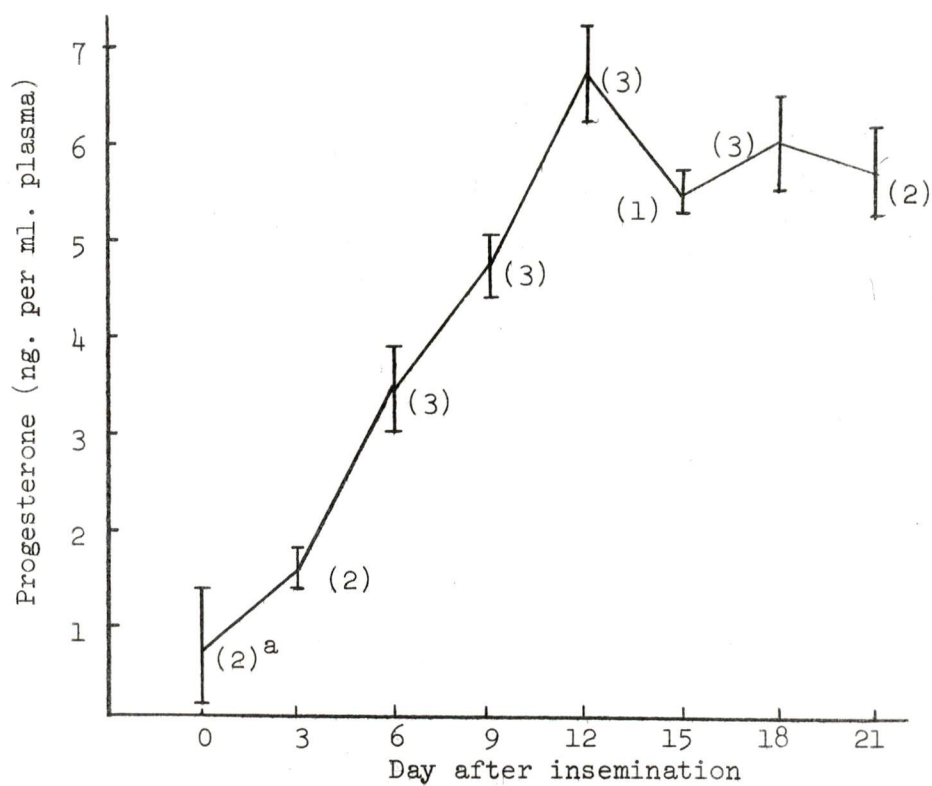


Figure 8. Progesterone concentration and standard error (ng. per ml. plasma) for cows cycling 73-101 days (Group C) after insemination. Day 0 equals estrus.

<sup>a</sup>Number of samples.

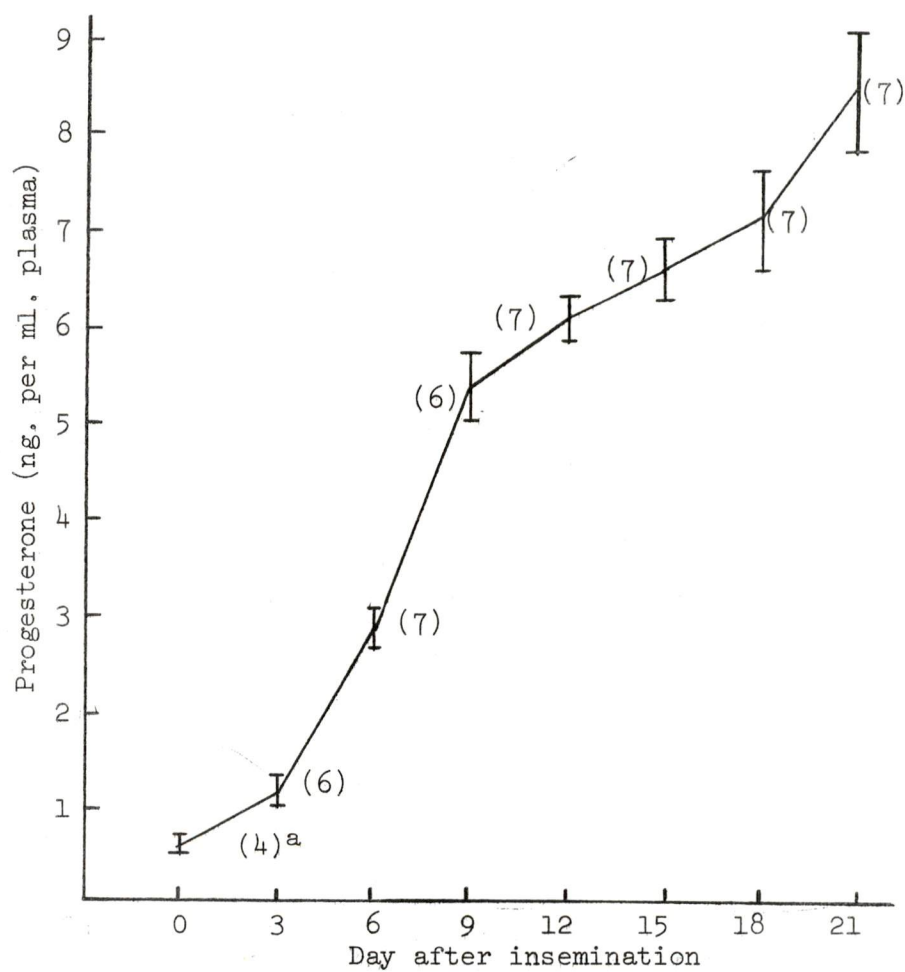


Figure 9. Progesterone concentration and standard error (ng. per ml. plasma) for pregnant cows (Group D). Day 0 equals estrus.

<sup>a</sup>Number of samples.

(15) found progesterone concentration to increase in normal pregnancies through approximately 100 days.

An analysis of variance on the above group of cows showed that there was a significant difference ( $P < 0.05$ ) among days but no significant difference among cows ( $P > 0.10$ ) for progesterone concentration. As in the previous groups, there was also a significant cow-day interaction.

When an analysis of variance was applied across groups there was no significant difference ( $P > 0.05$ ) for Day 0 through Day 18. However, there was a significant difference ( $P < 0.05$ ) across groups on Day 21. The progesterone concentration in the pregnant group was significantly higher than the other groups in Day 21.

## CHAPTER V

### SUMMARY AND CONCLUSIONS

The results of this experiment indicate that the competitive protein-binding technique as modified is suitable for the detection of progesterone in the peripheral blood of the female bovine. The coefficient of variation for recovery rates (6.1 percent) and pooled cow plasma (22.6 percent) are acceptable.

The analysis of variance within groups demonstrated that there was no significant difference ( $P > 0.10$ ) among cows. However, there was a significant difference among days in two of the groups, Group A ( $P < 0.05$ ) and Group D ( $P < 0.05$ ). When an analysis of variance was applied across groups, there was a significant difference ( $P < 0.05$ ) only on Day 21. The cows in the pregnant group exhibited significantly higher progesterone levels than the other groups. It appears from these data that this assay procedure may be a satisfactory method of determining pregnancy as early as Day 21. Shemesh et al. (71) were able to detect a difference in progesterone levels in the peripheral blood of cows 20 days after estrus. However, their procedure, which utilizes paper and gas chromatography, is time-consuming and lacks sensitivity in comparison with the CPB assay.

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## VITA

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