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Progesterone Levels in Cows Suspected of Embryonic Mortality

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To the Graduate Council:

I am submitting herewith a thesis written by Henry Hamilton Dowlen entitled "Progesterone Levels in Cows Suspected of Embryonic Mortality." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Don O. Richardson, Major Professor

We have read this thesis and recommend its acceptance:

R.L. Murphree, J.T. Miles

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

March 3, 1972

To the Graduate Council:

I am submitting herewith a thesis written by Henry Hamilton Dowlen entitled "Progesterone Levels in Cows Suspected of Embryonic Mortality." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

Don O. Richardson
Major Professor

We have read this thesis
and recommend its acceptance:

R. L. Murphree

J. T. Miles

Accepted for the Council:

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PROGESTERONE LEVELS IN COWS SUSPECTED OF EMBRYONIC MORTALITY

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Henry Hamilton Dowlen

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ABSTRACT

The competitive protein binding assay without chromatography was used to quantitate progesterone in the peripheral plasma of cows. The objective of this experiment was to estimate progesterone concentrations in cows suspected of experiencing embryonic mortality. Samples from 19 cows were assayed. The cows were divided into the following groups according to their estrous cycle length: (1) 20-22 days, (2) 26-32 days, (3) 40-69 days, and (4) pregnant.

Jugular vein blood samples were taken beginning 12 days after insemination. The blood sampling schedule was as follows: 12, 15, 17, 19, 21, 23, 26, 29, 32, 35, 38, 41, 44, 47, 58, 61, and 64 days post-breeding. Plasma was separated and stored at -20°C until assayed. The progesterone determinations were performed on 0.5 ml aliquots of plasma.

To moniter the effectiveness of each assay, steer plasma and steer plasma fortified with 3 ng of progesterone were assayed for progesterone. The progesterone content of steer plasma could not be distinguished from zero and the fortified steer plasma averaged 2.75±0.09 ng per ml. Tritiated progesterone was used to estimate the recovery percentage from cow plasma. The average recovery rate for all assays was 95.6±0.6 percent.

Analysis of variance on all groups through Day 21 indicated that Group 1 was different ($P<0.05$) from Groups 2, 3, and 4. A significant ($P<0.10$) day difference was found in Group 1 and no group-day interactions ($P>0.05$) were found in all groups. Group 2 had greater ($P<0.05$) progesterone concentrations than Group 1 indicating that the cows in the delayed estrus group (Group 2) may have been pregnant but experienced embryonic death shortly after implantation. This group was not different ($P>0.05$) from Group 4 (pregnant) at 21 days.

Comparison of Groups 3 and 4 through 47 days indicated that the progesterone level of Group 3 was significantly ($P<0.01$) less than the level of Group 4. From this analysis it was postulated that the cows in Group 3 may have had less progesterone than required for the maintenance of pregnancy.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The repeat-breeder cow is one of the most important problems facing dairy cattle herdsmen today. The failure of the cow to conceive to the first service after an appropriate period following parturition causes the dairyman loss of income due to lowered milk production and lengthened calving interval.

The repeat-breeder cow is one that is clinically normal, as far as can be determined by gross examination of the genital tract, but fails to conceive on first service. Many opinions have been offered on how many failures to conceive a cow must experience to be classed as a repeat-breeder; actually it is evident that loss of income occurs if she fails to conceive to the first service.

Two major contributing factors to return to service are fertilization failure and embryonic mortality. The purpose of this study was to investigate embryonic mortality using competitive protein binding assay techniques to measure peripheral plasma progesterone levels in cows that returned to service at abnormal intervals. These cows were compared to cycling and pregnant cows to determine if there was a progesterone deficiency in those cows suspected of embryonic mortality.

CHAPTER II

REVIEW OF LITERATURE

1. FERTILIZATION RATE

Normal Cows

In order to investigate embryonic mortality a knowledge of the number of potential embryos available is necessary. Using cows of normal fertility and various fertility bulls, Kidder et al. (25) found a fertilization rate of 85.9 percent. Boyd et al. (5) reported a fertilization rate of 85 percent using 112 cows selected for high fertility and inseminated under optimum conditions. Another study using heifers reported that greater than 90 percent of ova are fertilized (3).

Repeat-Breeder Cows

When repeat-breeder cows and heifers are studied reports indicate much lower fertilization rates (6, 14, 16, 45, 46). In all experiments the repeat-breeders were carefully examined for genital abnormalities capable of causing sterility and for the presence of infectious diseases. Casida (6) reported that 54.7 percent of 129 repeat-breeder cows had fertilized eggs at slaughter three days after insemination. Tanabe and Casida (45) used 104 cows to determine rate of fertilization failure

and the reason for failure and reported a 66.1 percent fertilization rate. At slaughter 16 days after the beginning of estrus embryos were recovered from the uteri of 58 percent of 50 cows (16).

Graden et al. (14) recovered 104 ova from 150 repeat-breeders (69.3%), and 58 of them were fertilized (55.8%). These workers tested early breeding (first eight hours of estrus) against late breeding (16 hours after noticed in estrus) and found that the fertilization rate was significantly ($P<0.05$) lower (48.5%) for cows bred during the first eight hours of heat than for those bred 16 hours later (68.6%). They also found that breeding twice during the heat period did not improve the fertilization rate.

The only report with greater than 66 percent fertilization rate in repeat-breeders was by Christian et al. (8) in which 88.5 percent of 42 cows had fertilized ova at three days; however, their previous years work on 104 cows had shown only 66.1 percent fertilization which was comparable to the percentage found on 63 repeat-breeding heifers (66.7% fertilized) by Tanabe and Almquist (46).

Fertility Studies

In a massive fertility study of normal Friesian herds in Great Britain, the conception rate for 5,744

first inseminations was 60.6 percent (4). In the United States the fertilization rate for 14,771 inseminations was 51.1 percent (1). Completed pregnancy was the measure of conception in the British study and in the U. S. study palpation of the amnionic vessel was the indication of pregnancy. In both studies the conception rate declined as the order number of insemination increased.

2. CAUSES OF FERTILIZATION FAILURE

Careful observation of the data in the previous section indicates that even in high fertility cows and heifers 100 percent fertilization is not achieved. There are some abnormalities which cause failure that cannot be diagnosed in the live animal. Tanabe and Almquist (46) studied 96 repeat-breeding heifers and found at slaughter that 13 (13.5%) showed gross genital abnormalities. Casida (6) estimated the incidence of repeat-breeding which appeared to be due to tubal obstructions and ovulation failure at 6 percent. More recently it was found that 8.7 percent of 150 cows failed to ovulate and 6.7 percent had oviduct obstructions (14). The same study showed 8.6 percent of the fertilization failures were due to abnormal ova, ovarian adhesions and endometritis.

Lost ova are reported in fertilization studies and these may be a result of the tedious recovery process

(14, 25, 45, 46). The startling aspect of fertilization studies with repeat-breeder cattle is the high percentage in which no explanation can be given for the failure to conceive. Casida (6) reported 39.3 percent failed to conceive for no apparent reason; other reports are somewhat lower, 31 percent (45) and 24.7 percent (14). These values represent a large source of loss to dairymen when compared to unexplained fertilization failure in normal cattle of 12.2 percent reported by Kidder et al. (25). It has been theorized that this sterility is of a functional type and involves endocrine imbalances and various physiological malfunctions of the genital tract (7).

3. EMBRYONIC MORTALITY

Embryonic mortality occurs for unexplained reasons in cows of high fertility and shows a greater incidence in repeat-breeder animals (3, 7, 16). Casida (6) listed several causes of embryonic death such as inherited lethals, infections, nutritional deficiencies, hormonal imbalances and aging of gametes. Nearly 20 years have passed since the previous listing was made, and following an extensive review of dairy herd fertility studies Olds (33) could not attribute the large embryonic death rate to any one cause.

In heifers of normal fertility the embryonic death rate has been estimated at 21 percent (25) and 15 percent (3). Using cows Boyd et al. (5) found an embryonic death rate of 15 percent by 26 days after breeding.

Embryonic Mortality in Repeat-Breeder Cows

When repeat-breeders are studied the situation is much more dismal. Tanabe and Almquist (46) found an embryonic death rate of 54.1 percent within the first month of pregnancy. Tanabe and Casida (45) estimated embryonic death in cows at 65.1 percent at 34 days post-breeding; Christian et al. (8) reported a value of 69.8 percent at the same stage of gestation and Casida (6) found a similar value of 59.4 percent on a greater number of cows. Hawk et al. (16) slaughtered 50 cows 16 days after breeding and found 58 percent with normal embryos; however, at 34 days only 28 percent of 50 cows had normal embryos. The estimate of embryonic mortality in these 100 repeat-breeders was 51.7 percent. Calculating embryonic death loss as a percentage of total repeat-breeders inseminated, Olds (33) summarized the data from 337 animals and reported that 28.5 percent had embryonic death.

Time of Embryonic Death

Laing (26, 27) and Hanly (15) indicate two types of embryonic death: (1) the fertilized egg died so early that the next estrus is not delayed, and (2) the fertilized egg survives long enough to delay the next estrus. Hawk et al. (14) indicate that the great majority of embryonic deaths actually occur between the sixteenth day of gestation and the day the cow returns to heat. They found that 77.8 percent of the cows that did not have a normal embryo at 34 days either returned to estrus or had a quiet ovulation by 25 days post-breeding.

In a study which incorporated measurement of plasma progesterone levels, Boyd et al. (5) observed that cows with short 16-day blastocysts tended to have low plasma progesterone levels and that most of the embryonic loss that was going to occur before 26 days had already occurred by the twelfth day of gestation. The period 12 to 16 days of gestation in the bovine is a critical time in corpus luteum function since it is during this time that implantation of the embryo is occurring within the uterus (5, 19, 40).

Not all embryonic death occurs by the sixteenth day. Observation of abnormal returns to estrus indicate that loss may occur at any time during the developmental stage of the embryo. Randel et al. (36) found that cows which exhibited plasma progesterone concentrations

indicative of pregnancy on Day 19 after breeding returned to estrus over a period of 20 days. The hypothesis among endocrinologists is that insufficient output of progesterone may decrease the survival rate of the embryo (17).

4. NECESSITY OF PROGESTIN

Several important factors about the corpus luteum (CL) of pregnancy are known to endocrinologists. The CL is necessary for the maintenance of pregnancy throughout gestation in the rabbit, rat, sow, goat, and dog, but for less than full term in the guinea pig, mare, and primate (13). Progesterone is the principle hormone secreted by the corpus luteum. This hormone is functional in providing a suitable environment in which the fertilized ovum can develop. By the previous results reported we can see that fertilization failure is not the major cause of reproductive failure but these failures may be attributed to an improper uterine environment in which the developing embryo cannot survive. This loss may be due to an insufficient output of progesterone during gestation. Many reports indicate that the corpus luteum or exogenous progestin is essential in cattle until 200 days of gestation since ovariectomy always induces embryonic death (11, 13, 17, 24, 30, 31, 47, 48, 49).

Progestin Therapy

Naturally occurring hormonal imbalances in ovarian hormone secretion may have harmful effects on embryo survival in infertile cattle (18). Henricks et al. (19) and Boyd et al. (5) indicate that lowered progesterone concentrations are found in non-pregnant animals and in animals with short embryos (60 millimeter (mm) or less at 16 days). Cattle fertility has been improved to some extent by treatments with progestins (9, 21, 23, 35, 51). This improvement was believed to be a result of a more compatible uterus due to increased secretions of the endometrium (23).

When progestin is administered to ovariectomized cows, pregnancy can be maintained from the first week until parturition (13, 17, 24, 30, 47, 48, 49). However, Hawk et al. (17) found that fertility was not improved for ovariectomized repeat-breeders by administration of progestin at the levels necessary to support pregnancy in normal animals. They indicate that administration of exogenous progestin in amounts greater than used on normal cattle might improve fertility in ovariectomized repeat-breeders.

5. PROGESTERONE LEVELS IN THE BOVINE

This review will be limited to more recent reports due to the numerous studies involving the use of current

and more sensitive assay techniques (2, 10, 12, 18, 19, 20, 28, 34, 36, 38, 39, 40, 41, 42, 43, 44, 50). The levels in peripheral and ovarian venous blood have been assayed for non-pregnant cycling and pregnant cows. Table I summarizes a portion of progesterone levels reported to date.

Peripheral plasma progesterone is low at estrus, begins to rise three to four days after cessation of heat and reaches a peak at 12 to 15 days and begins to decline if the cow is not pregnant. The progesterone concentration remains fairly constant in pregnant animals after reaching a peak 15 to 19 days of gestation. Individual animals exhibit much fluctuation and variation during the estrous cycle and during gestation (10, 28).

There has been general agreement among investigators who have reported progesterone concentrations in cows. Summary of the reports indicate that the level of cows in estrus is less than one nanogram (ng) per milliliter (ml). A peak of 5 to 7 ng is reached at 12 to 15 days post-estrus in cows that did not conceive and the concentration then declines before the ensuing estrus. Cows that became pregnant maintained the peak level and continued to rise slowly to greater than 10 ng 25 to 30 days after breeding. This concentration is maintained throughout gestation with a sharp decrease just prior to parturition.

TABLE I

PROGESTERONE LEVELS IN PERIPHERAL PLASMA[a]

Day of Cycle	Henricks[b] et al. (19)	Henricks[c] et al. (19)	Shemesh[c] et al. (41)	Batson[c] (2)	Randel[d] et al. (36)	Garverick[c] et al. (12)	Stabenfeldt[c] et al. (44)
0	<1	<1	0.6	0.7	2.0	6.2	<1
6	4.5	2.0	3.7	1.87	5.2 (7)*	9.0	3.5
12	9.9	7.0	5.1	6.04	10.5 (14)	12.0	6.5
18	11.0	4.5	3.1	4.5	11.0 (19)	3.5	2.5
21	11.0	1.2	0.6	3.14		4.0	<1
24	11.0				6.5 (28)		
30	11.0				9.5 (35)		
39	13.9				12.0 (42)		

[a]Ng per ml plasma

[b]Pregnant cows

[c]Non-pregnant cows

[d]Cow cycling 29-49 days

*Numbers in parentheses indicate days

Pregnancy Diagnosis

Use of the rapid progesterone determination to diagnose pregnancy has been examined (19, 39, 40). The abrupt fall in the progesterone level near estrus is not seen in the pregnant animal (40). Henricks et al. (19) reported that as early as the ninth day after mating, progesterone concentrations in heifers that returned to estrus 18 to 20 days after mating were significantly ($P<0.025$) lower than in pregnant heifers. Pregnant heifers had about 1.7 times as much progesterone in peripheral plasma during the first 15 days after mating as those that returned to estrus (19). The rate of increase in progesterone levels in pregnant heifers could have been due to the development of a viable blastocyst which stimulated progesterone secretion by the CL or caused a change in utilization or degradation of progesterone (19, 36).

CHAPTER III

EXPERIMENTAL PROCEDURE

Objectives of Experiment

The goal of this experiment was to seek insight into the problem of embryonic mortality in dairy cattle. Peripheral plasma progesterone was measured using the competitive protein binding (CPB) assay introduced by Murphy (29) and modified by Neill (32) and Johansson (22). The assay in this laboratory was also modified by Batson (2). Cows suspected of embryonic death were identified by abnormal returns to estrus. The peripheral plasma progesterone levels in these animals were compared to the levels found in both normal pregnant and cycling cows. Many of the cows were repeat-breeders.

Animals and Sampling

Eighty-seven cycles of 60 Holstein cows from the University of Tennessee milking herd were used to omit any inter-breed variation. Blood was collected in heparinized tubes from the jugular vein and centrifuged at 2500 revolutions per minute (rpm) for 30 minutes to separate the plasma. Sampling followed the schedule as follows: 12, 15, 17, 19, 21, 23, 26, 29, 32, 35, 38, 41, 44, 47, 58, 61, and 64 days post-breeding. The samples were stored at -20^{o}C until assayed.

Assay Procedure

The CPB assay as adapted for this laboratory by Batson (2) was used to quantitate progesterone. The method involves the competition between progesterone and corticosterone for binding sites on the corticosteroid binding globulin (CBG) of dog plasma. Tritiated corticosterone (B^3H, 44 curies per millimole) was bound to CBG and subsequently displaced by unlabeled progesterone when the CBG-B^3H was added to the unknown samples in the system. An estimate of the progesterone was made by counting the remaining B^3H bound to the CBG. The procedure as used by Batson (2) was modified as follows: (1) Tracer concentration (corticosterone-1,2-^3H) and percent dog plasma in the binding solution were reduced to 17.5 ng and 2 percent respectively, (2) the shaking time for two plasma extractions with petroleum ether was increased to five minutes each, and (3) the plasma extract was transferred to culture tubes for drying by freezing the plasma in a dry ice-acetone bath and decanting the ether phase. These changes will be discussed in greater depth in Chapter IV.

The steps followed in the CPB assay are outlined as follows:

1. Progesterone standards were prepared in duplicate by pipetting the desired quantity of progesterone (0, 1, 3, and 6 ng) to 13 x 100

mm culture tubes. The progesterone standard was serially diluted from a stock solution (100 ng per ml) in order that each tube would have one-half ml of ethanol containing the progesterone.

2. Three ng of progesterone was added to each of two extraction tubes and 20 microliters (µl) of tritiated progesterone (50.3 curies per millimole) was added to one extraction tube. Steer plasma was added to the tubes containing 3 ng of progesterone and an aliquot of the cow's plasma being assayed was added to the tube containing tritiated progesterone after the ethanol was evaporated. These samples were mixed on a Vortex mixer.
3. Plasma (0.5 ml) was pipetted into each extraction tube and 5 ml of petroleum ether (30-60^{o}C boiling point) was added to each tube with a repeating pipet. The tubes were capped with linear high density polyethylene caps and shaken for five minutes with a wrist-action shaker.
4. After shaking the caps were loosened and the tubes immersed in a dry ice-acetone bath to freeze the plasma. The ether phase was decanted easily into 13 x 100 mm culture tubes.

The plasma was allowed to thaw and was extracted again for five minutes with 5 ml of petroleum ether.

5. The ether phases were evaporated to dryness under nitrogen gas or filtered air. A 45°C water bath was used to speed the drying process.
6. When all samples were dry 1 ml of petroleum ether was used to wash the sides of the tube thus concentrating the extracted progesterone in the bottom of the tube. The tubes were again evaporated to dryness using the water bath and air or nitrogen. If it was impossible to complete the assay that day, the culture tubes were covered and placed in a refrigerator until the next day.
7. One ml of CBG-B^{3}H solution containing 17.5 ng of corticosterone-1,2-^{3}H and 2 ml of dog plasma per 100 ml of double distilled water was added to each tube. A Repipet was used if many samples were done.
8. The tubes were mixed on a Vortex mixer and placed in a 45°C water bath for five minutes and mixed again after removal from the water bath.

9. The samples were incubated in an ice bath for at least 10 minutes. This incubation period was a critical step as the ice bath increases the affinity of the binding protein for the steroids.
10. Eighty milligrams (mg) of washed florisil was added to each tube from a calibrated volumetric funnel using a cork to fill one end of the stopcock hole. The tubes were shaken immediately for exactly 30 seconds on a Vortex mixer and returned to the ice bath.
11. One-half ml of the supernatant containing protein bound progesterone was removed with an automatic pipet (using disposable polyethylene tips) and added to 10 ml of scintillation fluid. The samples were mixed and counted in a liquid scintillation counter.

The scintillation counter counts the remaining corticosterone-1,2-^{3}H in the solution. Quantitation of the amount of progesterone present in the sample was made by comparing its displacement of corticosterone-1,2-^{3}H with that of the known amounts of progesterone in the standard curve. The samples were counted to 10,000 total counts which reduced counting error to approximately 2 percent.

The sample containing the tritiated progesterone was decanted into a scintillation vial after freezing the plasma portion and the ether phase was evaporated. Scintillation fluid was added to the vial along with another vial containing a 20 µl standard. These were counted at the same time as the unknown samples and used to determine recovery percentage.

Steer plasma and fortified steer plasma along with petroleum ether blanks were carried out as qualitative checks on the assay procedure.

Steer plasma was taken from a steer treated with Flucort (5 mg per day) to reduce endogenous corticoid production. Male dog plasma was obtained locally at a veterinary clinic from a St. Bernard dog accustomed to blood donor procedure. All plasma was stored at -20°C. The tritium labeled corticosterone and progesterone were purchased from New England Nuclear Corporation and the unlabeled progesterone from Mann Research Laboratory. These chemicals were diluted with absolute ethanol and stored in a 5°C refrigerator. Use of petroleum ether that had been previously opened led to high blank values; therefore, the petroleum ether used in this assay was purchased in one pint bottles because this amount could be used in one day.

The scintillation fluid was composed of 50 grams (gm) of naphthalene, 4 gm of 2,5-diphenyl-oxazole and

0.5 gm of 1,4-bis-2(5-phenyl-oxazolyl)-benzene per liter of dioxane. Enough of this solution for one week's use was prepared and stored in a brown glass bottle to prevent decomposition.

An asymptotic regression analysis was performed on each standard curve using a Wang Computer. The computer print-out contained the progesterone value of each unknown sample and the counts per minute reading from the scintillation counter.

CHAPTER IV

RESULTS AND DISCUSSION

1. EVALUATION OF ASSAY PROCEDURE

Extraction Methods

The CPB assay technique utilizes relatively simple extraction and purification methods in comparison to the older assay techniques for progesterone. The extraction procedure, needless to say, must not produce a solvent blank containing spurious progesterone values.

During the initial assays in this experiment, high petroleum ether blanks produced erroneous progesterone values. Originally the solvent was purchased in one gallon cans. After being opened for a time the solvent underwent some type of degradation which produced erratic unknown progesterone values and high extraction blanks. This problem was solved by the utilization of the solvent procured in one pint brown glass bottles which could be fully utilized in one day with little wastage.

Two 5 ml extractions with reagent grade petroleum ether were used. Recovery rates averaged 95.6 percent with a standard error of ±0.6 when tritiated progesterone was added to a sample of cow plasma. The extraction efficiency of 3 ng of crystalline progesterone from steer plasma was 2.75±0.09 ng of progesterone. Unfortified

steer plasma had a progesterone content that could not be distinguished from zero. Petroleum ether blanks were below the level of sensitivity of the assay.

In preliminary work the extraction phase was found to be both tedious and time consuming. To overcome these problems a Burrell Wrist-Action shaker was adapted to simultaneously shake 24 samples. A plexiglass platform with slots cut to fit 13 ml centrifuge tubes was bolted to the shaker. Linear high density polyethylene stoppers were used to seal the tubes which had ground glass mouths. These stoppers provided an effective seal during the shaking process and did not effect the estimate of the progesterone.

Batson (2) used two 60 second shakings to extract one-half ml of plasma. The shaker used in this experiment was not as vigorous as the Vortex mixer previously used in this laboratory (2). Two five minute shakings gave recovery rates comparable to those found previously (2, 28, 32).

A dry ice-acetone bath was used to speed removal of the ether phase from the plasma. The tubes were immersed in the bath to quick freeze the plasma. The ether phase was decanted into culture tubes. This procedure eliminated the tedious removal of the ether by aspiration with Pasteur pipets. The freezing did

not have an adverse effect on the plasma or the estimate of the progesterone value.

Tracer Concentration

Corticosterone-1,2-^{3}H of higher specific activity than used by Batson (2) was obtained for this assay. As a result of the higher specific activity (44 curies per millimole) the tracer concentration was decreased to 17.5 ng per 100 ml of binding solution. This concentration gave a standard curve similar to that obtained earlier in this lab (2).

Florisil Treatment

Considerable difficulty was encountered with the florisil used in early assays. The appearance of fines in the florisil caused poor agreement between duplicate samples. A rigorous washing procedure was devised to remove the fines. This procedure consisted of washing 10 to 15 times with distilled water and decanting all particles that did not settle within 30 seconds after mixing. After washing the florisil was dried in an oven and stored in a desiccator. Before each assay the amount to be used that day was again heated for at least four hours. This reheating plus the washing procedure eliminated the erratic values caused by the appearance of fines in the supernatant.

Binding Procedure

The binding solution was prepared fresh each day the assay was performed. No difference was noticed when the binding solution was added to the culture tubes with a Repipet or a one ml volumetric pipet. Previously a rigorous time schedule was adhered to during the incubation and florisil addition period (2). During this assay all tubes were heated for five minutes and cooled in an ice bath at one time. After 10 minutes in the ice bath 80 mg of florisil was added to each tube and the tube was shaken immediately for 30 seconds and returned to the ice bath. One-half ml of the supernatant was then removed with an automatic pipettor (with disposable plastic tips) and added to the scintillation fluid. The samples were allowed to stabilize for at least one hour before counting was initiated.

Elimination of Chromatography

Batson (2) and Long (28) experienced difficulty in performing the assay when thin-layer chromatography was employed to separate progesterone from the progestin metabolites and other steroids that might be extracted by the petroleum ether. Johansson et al. (22) reported that petroleum ether extracts 90 percent of the progesterone and only minimal amounts of the progesterone metabolites while leaving behind over 99 percent of the

cortisol and corticosterone. The only steroids in the extract which significantly displace corticosterone from the binding protein are progesterone and 17-α-hydroxy-progesterone (17-α-OH) (22). This latter compound has not been identified in peripheral bovine plasma; thus progesterone is the primary hormone measured by this assay. Shemesh et al. (41) examined 65 samples by competitive protein binding with the chromatographic step and competitive protein binding without chromatography and found a regression coefficient of 0.87 ($P<0.001$). The standard deviation of the difference between values obtained for the same sample by the two methods was estimated as 0.80 ng per ml. Donaldson et al. (10) chromatographed petroleum ether extracts on thin layer plates and eluted one centimeter (cm) sections along the plate which were assayed by the CPB assay, and found that material with a mobility similar to progesterone accounted for virtually all the progesterone found when the crude extract was assayed. Alumina column chromatography on 12 samples ranging in concentration from 0.1 to 16 ng per ml revealed that the progesterone concentration was 90.3±6.8 percent of the value obtained when the crude extract was assayed without purification (10).

These results indicated that the chromatographic step could be eliminated especially when the objective

was to estimate progesterone during the luteal phase of the cycle and during gestation.

2. EVALUATION OF STANDARD CURVE

The progesterone concentrations used in the standard curve were prepared with each assay and subjected to the binding step in the same manner as the unknown samples. A typical standard curve and a composite standard curve for 20 assays are presented in Figure 1. These standard curves are the actual counts per minute (cpm) plotted on ng of progesterone. The unknown progesterone values were obtained by performing an asymptotic regression on the standard curve cpm readings rather than an average of the two values for each point on the curve.

Although there was considerable drift on the cpm axis, the shape of the standard curve remained constant. Progesterone values in steer plasma and steer plasma fortified with 3 ng of progesterone were estimated with each assay. The steer plasma had a progesterone concentration that was not different from zero and the average progesterone concentration in fortified steer plasma was 2.75±0.09 ng. An assay was not used if the fortified steer plasma value for the duplicate values was below 2.5 ng or above 3.5 ng.

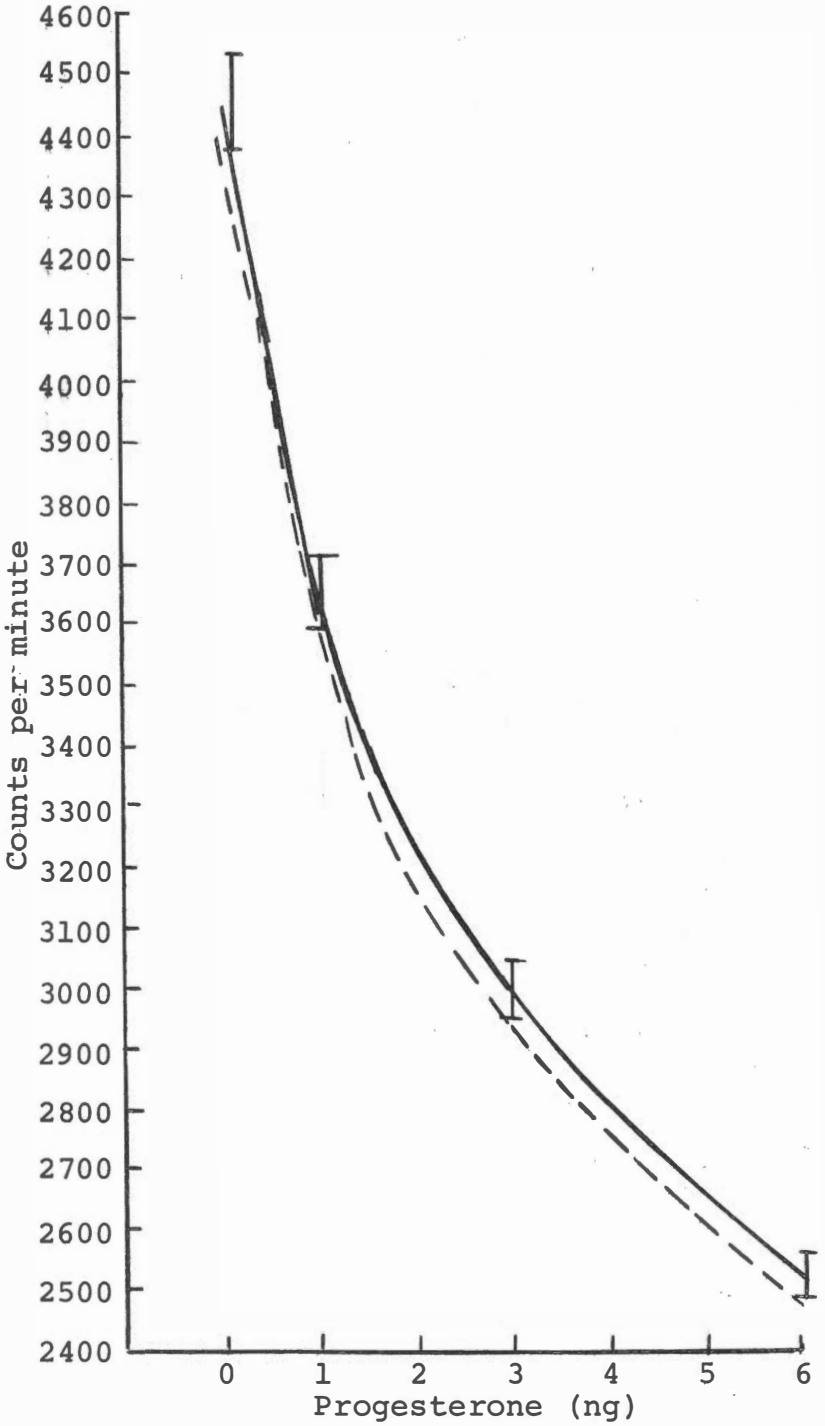

Figure 1. A typical standard curve (-----) and a composite standard curve for 20 assays (_____).

3. EVALUATION OF PROGESTERONE DATA

Ten of the 60 cows that were bled had abnormal cycle lengths indicative of embryonic mortality. These 10 cows were compared to a sample of the non-pregnant and pregnant cows. The cows assayed were divided into three groups according to cycle length with a fourth group consisting of pregnant cows. Group 1 was made up of three cows that cycled at 20 to 22 days; four cows with delayed returns to estrus (26-32 days) were placed in Group 2; Group 3 consisted of six cows suspected of embryonic death as a result of their abnormal cycle intervals (40-69 days); and Group 4 consisted of six pregnant cows. All cows were inseminated with high quality semen by experienced personnel. The mean progesterone concentrations with standard errors for each day are presented in Table II. For the statistical analysis all groups were compared through Day 21 and Groups 3 and 4 were also compared through Day 47. There was no significant ($P>0.05$) group-day interaction in either comparison.

Group 1 was composed of three cows that cycled at 20 to 22 days. Since this was the same length as expected in the normal estrous cycle these cows were considered non-pregnant. The mean progesterone concentrations with standard errors are presented in Figure 2.

TABLE II

PROGESTERONE IN PERIPHERAL PLASMA OF COWS

Day After Insemination	Progesterone Mean (ng per ml) and Standard Error Group[1] 1	2	3	4
12	4.93±1.23	5.67±0.69	5.01±0.59	6.02±0.80
15	5.10±1.32	5.71±0.93	5.91±0.51	6.35±0.42
17	5.53±0.09	7.42±0.79	5.67±1.42	5.65±0.43
19	2.78±1.28	5.94±0.63	4.76±1.04	8.17±1.59
21	0.38±0.36	4.64±1.58	5.41±0.97	6.69±0.92
23		3.12±1.24	5.55±0.96	8.20±0.66
26		2.32±2.25	6.08±0.76	8.59±0.87
29			6.05±0.50	7.82±0.76
32			7.01±0.67	8.14±0.90
35			6.70±0.73	7.74±0.92
38			6.41±1.28	8.26±0.84
41			4.52±1.31	8.28±0.96
44			4.66±1.26	7.93±1.09
47			4.98±1.92	11.10±1.73
Group Means* 21 Days	3.98^{a}	5.94^{b}	5.35^{b}	6.58^{b}
Group Means* 47 Days			5.66^{c}	7.70^{d}

*Means with different superscripts are significantly different ($P<0.05$).

[1]Group 1 cycled 20-22 days, Group 2 cycled 26-32 days, Group 3 cycled 40-69 days, and Group 4 were pregnant.

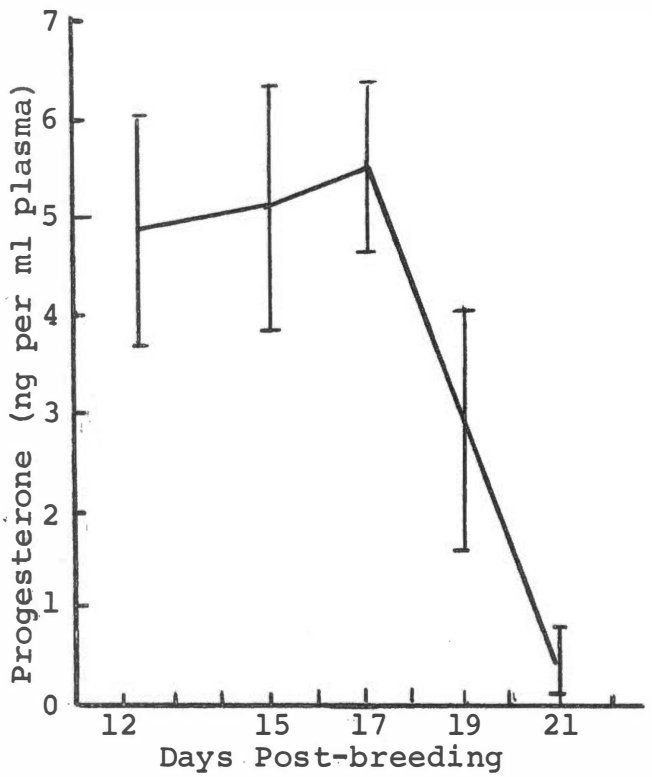

Figure 2. Progesterone concentration and standard error for cows cycling at 20-22 days (Group 1) post-breeding.

These are in close agreement with the values reported by several other researchers (2, 10, 19, 41, 43) but are somewhat less than the values found in other reports (12, 36, 37) when the same assay technique was used. Four cows that cycled at longer intervals (26-32 days) were assigned to Group 2. Group 3 contained six cows suspected of experiencing embryonic death with returns to estrus ranging from 40 to 69 days. The fourth group was composed of six pregnant cows. The mean progesterone concentrations for these groups are presented in Figures 3, 4, and 5.

All groups were statistically compared through Day 21 and Group 1 was significantly different ($P<0.05$) from the other groups with the difference first becoming detectable ($P<0.10$) on Day 19. Henricks et al. (19) found that a difference between cycling cows and cows known to be pregnant could be observed ($P<0.025$) by Day 9 post-breeding. This difference could not be detected until Day 19 in this study. Within group analysis for day differences showed that only Group 1 had a difference between days ($P<0.10$) by Day 21 post-breeding.

Comparison of Groups 1 and 2 showed a difference ($P<0.05$) between groups and between days ($P<0.05$). These results indicate that the delayed estrus cows in Group 2 may have had active CL's and developing embryos

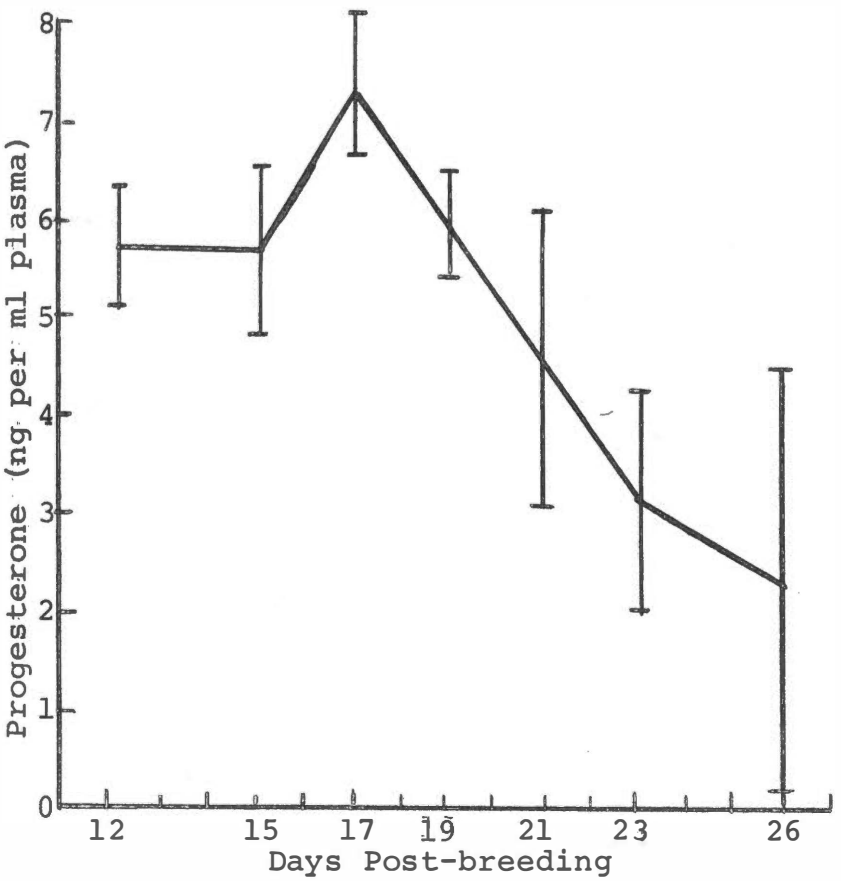

Figure 3. Progesterone concentration and standard error for cows cycling 26-32 days (Group 2) post-breeding.

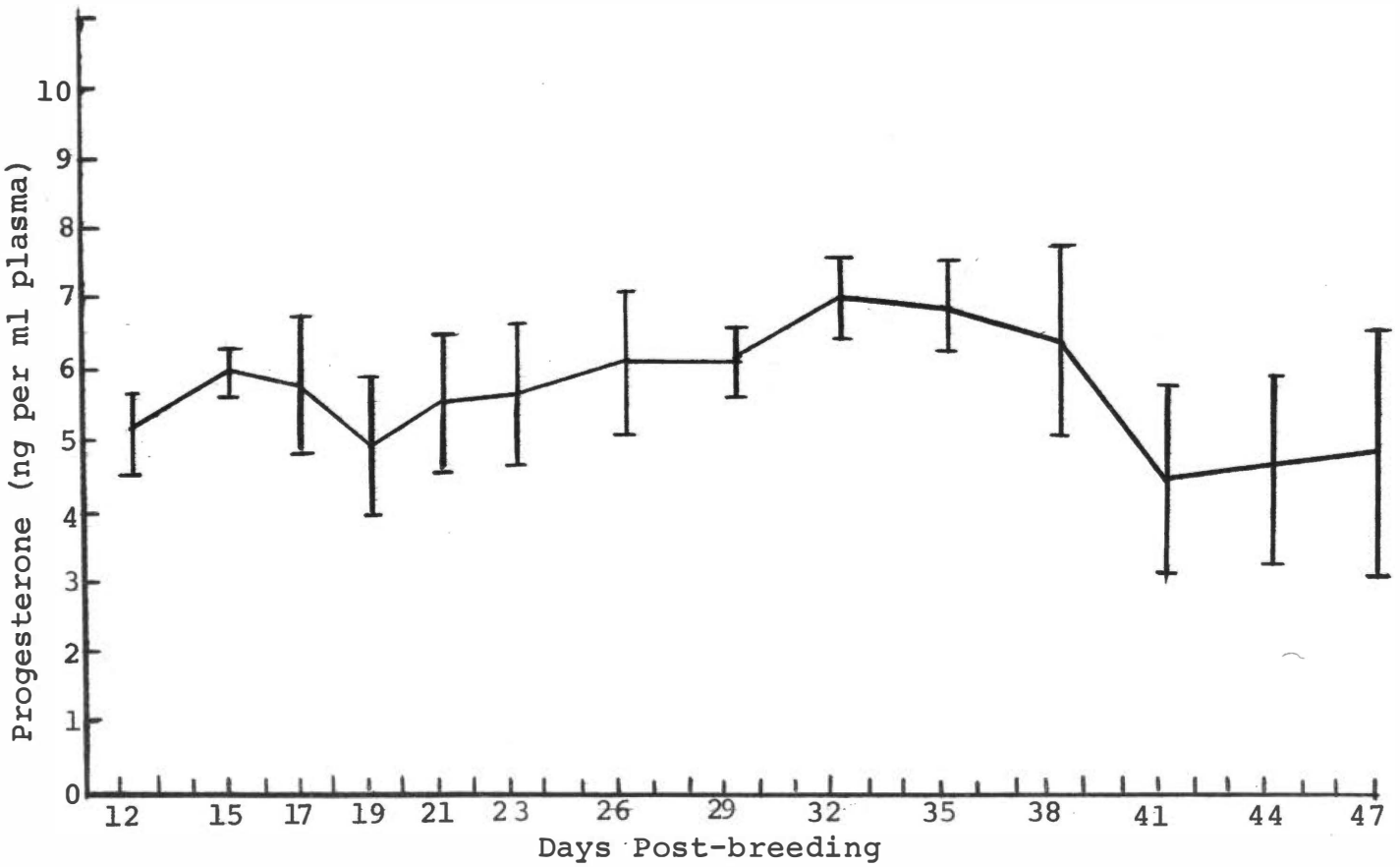

Figure 4. Progesterone concentration and standard error for cows cycling 40-69 days (Group 3) post-breeding.

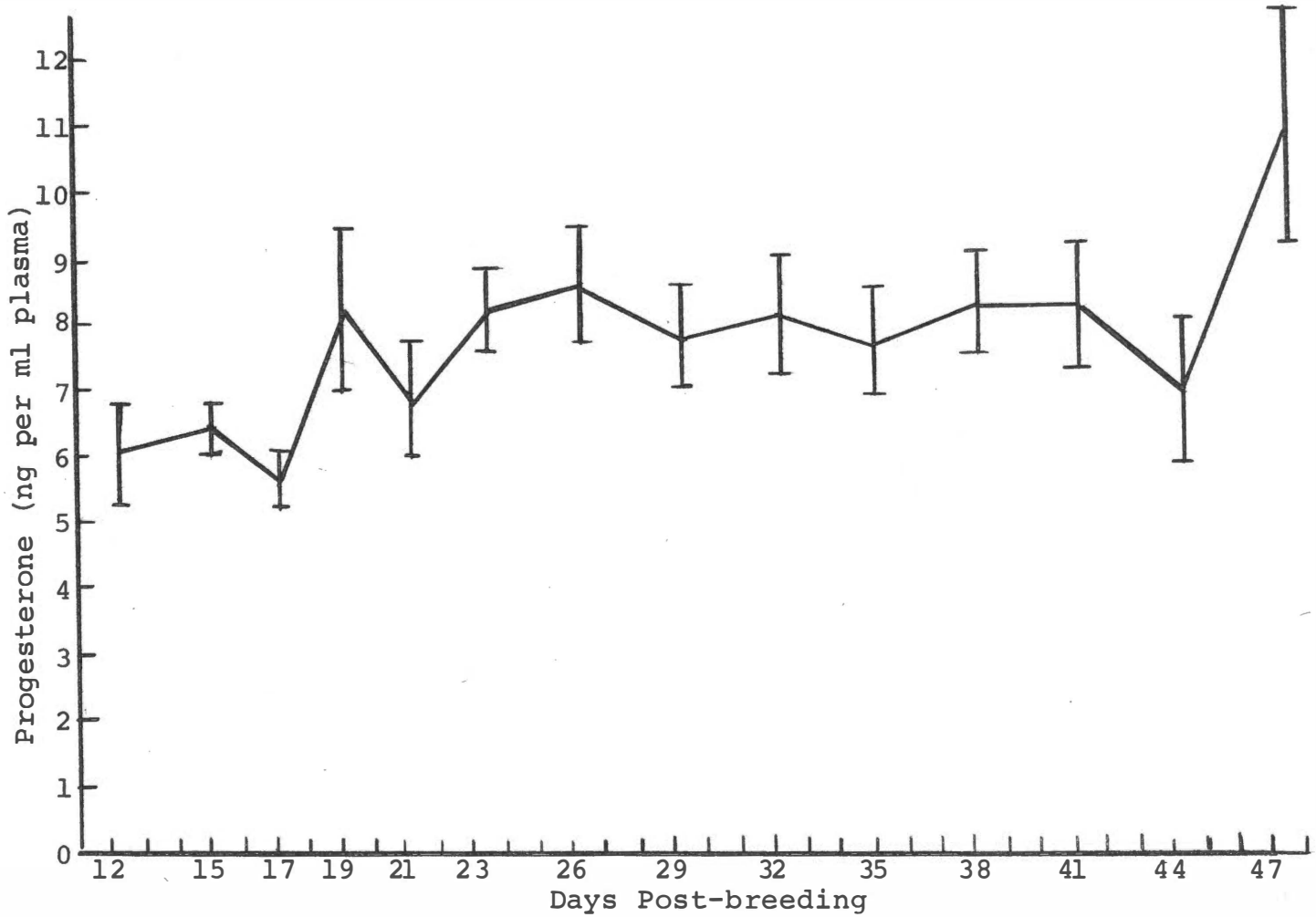

Figure 5. Progesterone concentration and standard error for pregnant cows (Group 4).

which died but their presence caused the cycle to be lengthened. Hanly (15) reported that prolonged estrous cycles were most frequently due to persistence of a CL that had been associated with pregnancy. Henricks et al. (19) postulated that pregnant heifers had faster rates of increase in progesterone concentrations due to the presence of a viable blastocyst. The hypothesis that Group 2 experienced early embryonic death is strengthened when all groups were compared and Group 2 was not different ($P>0.05$) from Groups 3 and 4 through 21 days.

Groups 3 and 4 were compared through 47 days post-breeding and Group 3 had progesterone levels significantly lower ($P<0.01$) than Group 4. Day differences were not significant ($P>0.05$); however, the progesterone concentrations in Group 3 were never as high as the pregnant group with the exception of Day 17 when the levels were equal. From this analysis it is possible that the cows in Group 3 had lowered progesterone which caused slow development and eventual death of the embryo. Boyd et al. (5) found that short 16 day embryos were associated with low progesterone concentrations in pregnant animals. Studies on ovariectomized cows indicate that the progesterone concentration must remain above a critical level during early gestation or the developing embryo will be lost (17, 49). In this study it is possible that the cows in Group 3 were near this

critical level and could not maintain pregnancy. The progesterone concentrations in the pregnant group were in close agreement with the values reported by Donaldson et al. (10).

CHAPTER V

SUMMARY AND CONCLUSIONS

The competitive protein binding assay without chromatography was used to quantitate progesterone in the peripheral plasma of cows. The objective of this experiment was to estimate progesterone concentrations in cows suspected of experiencing embryonic mortality. Samples from 19 cows were assayed. The cows were divided into the following groups according to their estrous cycle length: (1) 20-22 days, (2) 26-32 days, (3) 40-69 days, and (4) pregnant.

Jugular vein blood samples were taken beginning 12 days after insemination. The blood sampling schedule was as follows: 12, 15, 17, 19, 21, 23, 26, 29, 32, 35, 38, 41, 44, 47, 58, 61, and 64 days post-breeding. Plasma was separated and stored at -20°C until assayed. The progesterone determinations were performed on 0.5 ml aliquots of plasma.

To moniter the effectiveness of each assay, steer plasma and steer plasma fortified with 3 ng of progesterone were assayed for progesterone. The progesterone content of steer plasma could not be distinguished from zero and the fortified steer plasma averaged 2.75±0.09 ng per ml. Tritiated progesterone was used to estimate

the recovery percentage from cow plasma. The average recovery rate for all assays was 95.6±0.6 percent.

Analysis of variance on all groups through Day 21 indicated that Group 1 was different ($P<0.05$) from Groups 2, 3, and 4. A significant ($P<0.10$) day difference was found in Group 1 and no group-day interactions ($P>0.05$) were found in all groups. Group 2 had greater ($P<0.05$) progesterone concentrations than Group 1 indicating that the cows in the delayed estrus group (Group 2) may have been pregnant but experienced embryonic death shortly after implantation. This group was not different ($P>0.05$) from Group 4 (pregnant) at 21 days.

Comparison of Groups 3 and 4 through 47 days indicated that the progesterone level of Group 3 was significantly ($P<0.01$) less than the level of Group 4. From this analysis it was postulated that the cows in Group 3 may have had less progesterone than required for the maintenance of pregnancy.

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VITA

Henry Hamilton Dowlen was born in Hamilton County, Tennessee on July 29, 1945. He attended Falling Water Elementary School for eight years and graduated from Hixson High School in 1963. In the fall of 1963, he enrolled in the University of Tennessee at Knoxville.

He received the Bachelor of Science degree with a major in Dairy Production in December, 1967. At graduation he had the highest scholastic average in the College of Agriculture. Following graduation he was granted an assistantship to do graduate work in the Dairy Department at the University of Tennessee.

Upon completion of one year of graduate study, he was called to serve in the United States Army. He served a tour in the Republic of South Vietnam and following his discharge from active duty, he returned to the University in January, 1971, to complete his studies.

He is a member of the American Dairy Science Association and the honorary fraternities, Alpha Zeta and Gamma Sigma Delta.

He is the son of Mr. and Mrs. Earl Dowlen of Chattanooga, Tennessee, and is married to Patricia Ann Dowlen.