The effects of blood preparation for radioimmunoassay and competitive protein binding assay on the stability of progesterone and estrogen concentrations

Vaughan A. Wenzel

To the Graduate Council:

I am submitting herewith a thesis written by Vaughan A. Wenzel entitled "The effects of blood preparation for radioimmunoassay and competitive protein binding assay on the stability of progesterone and estrogen concentrations." 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.

R. L. Murphree, Major Professor

We have read this thesis and recommend its acceptance:

D. O. Richardson, J. B. McLaren

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

November 21, 1972

To the Graduate Council:

I am submitting herewith a thesis written by Vaughan A. Wenzel entitled "The Effects of Blood Preparation for Radioimmunoassay and Competitive Protein Binding Assay on the Stability of Progesterone and Estrogen Concentrations." 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 Animal Science.

R. L. Murphree
Major Professor

We have read this thesis
and recommend its acceptance:

Don O. Richardson

J. B. McLaren

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
Graduate Studies and Research

THE EFFECTS OF BLOOD PREPARATION FOR RADIOIMMUNOASSAY AND

COMPETITIVE PROTEIN BINDING ASSAY ON THE STABILITY

OF PROGESTERONE AND ESTROGEN CONCENTRATIONS

———————

A Thesis

Presented to

The Graduate Council of

The University of Tennessee

———————

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

———————

by

Vaughan A. Wenzel

December 1972

ACKNOWLEDGMENTS

ABSTRACT

Competitive protein binding (CPB) assay for progesterone and radioimmunoassay (RIA) for estrogen were used to evaluate the effects of various blood handling techniques from the time of collection to assay on the stability of progesterone and estrogen concentrations in blood. Eight 40 ml samples were collected consecutively in polycarbonate centrifuge tubes from the jugular veins of eight cows and subjected to various treatments.

Treatments consisted of collecting samples in pre-cooled heparinized (1000 U/ml) tubes maintained in an ice bath, centrifuging immediately (2400 rpm for 30 minutes) and storing the plasma fraction at $-15°C$; and collecting samples in heparinized or non-heparinized, pre-cooled and warm (room temperature) tubes, and holding as whole blood either at $4°C$ for 24 hours or at room temperature for 4 or 24 hours prior to centrifutation and storage of the plasma or serum fraction at $-15°C$. The last of the eight samples collected from each cow was handled identically to the first sample to evaluate the effects of animal stress during bleeding and volume of blood collected on hormone concentration.

Significant differences in the progesterone concentrations existed between treatments ($P < 0.001$). Plasma and serum samples held at $4°C$ for 24 hours were significantly lower in progesterone than plasma samples centrifuged. There was no difference in progesterone concentrations of plasma and serum samples subjected to similar treatment

effects. Samples collected in warm (room temperature) tubes and held as whole blood at room temperature until centrifuged were significantly lower in progesterone content ($P < 0.001$) than those collected in cool tubes. Warm tube samples held as whole blood at room temperature for 24 hours prior to centrifugation were significantly lower in progesterone than those held as whole blood for 4 hours ($P < 0.001$). Serum samples held at room temperature for 4 hours and 24 hours prior to centrifugation were 38 percent and 89 percent lower in progesterone content, respectively, than the plasma samples centrifuged immediately. Animal stress or volume of blood collected had no effect on progesterone concentration.

Radioimmunoassay for estrogen was conducted on blood from the same cows and subjected to the same treatments used for CPB assay of progesterone. There was no significant difference in estrogen concentrations between any of the treatments evaluated in this study.

Aliquots of pooled plasma samples from a number of cows were subjected to alternate freezing and thawing to a maximum of 6 and 7 times prior to the assay for progesterone and estrogen, respectively. No significant effect of repeated freezing and thawing on the concentration of either progesterone or estrogen was detected.

The average recovery of labeled progesterone added to cow plasma was 93.3 ± 0.64 percent. Following the addition of 3 ng of unlabeled progesterone to steer plasma, the average recovery was 96.9 ± 0.45 percent or 2.9 ng of progesterone. RIA was carried out using one 6 ml extraction and 2 hours ice water incubation. Recovery of 20 pg and 50 pg of estradiol-17β added to pooled cow plasma averaged 91.0 ± 0.35 percent and 81.0 ± 0.60 percent, respectively. Average recovery of

estradiol-17β ^{3}H from a cow being assayed was 89.0 ± 1.5 percent.

Based on the data obtained in this study, samples used for CPB assay of progesterone should be centrifuged and the plasma fraction of whole blood removed as soon as possible after collection and frozen until assayed. Estrogen concentrations in samples prepared for RIA of estrogen are apparently not as susceptible to the effects of various sample preparation methods as is progesterone. However, it is recommended that samples of estrogen assay also be centrifuged and stored at subzero temperatures as soon as possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The development of radioimmunoassay and competitive protein binding assay procedures have enabled investigators to quantitatively measure steroids in several species at such minute levels that they are expressed in units of picograms and nanograms. A procedure of such sensitivity must certainly be under the influence of numerous factors that can affect the results of the assay. One such factor may be the methods with which a pheripheral blood sample is prepared for assay.

Most investigators use plasma obtained by relatively standardized methods of collection, while some report the use of serum. In several cases investigators have made observations concerning blood handling techniques that were incidental to the experiments they were conducting and this has led to minor revisions in sample preparation. However, no comparative study on the effects of various blood sample preparation techniques has been reported. An in-depth comparison dedicated to the evaluation of blood handling methods used to prepare samples for steroid assay was felt to be necessary. Such an evaluation was conducted in this study.

CHAPTER II

REVIEW OF LITERATURE

Numerous studies using competitive protein binding assay and radioimmunoassay techniques to quantitate the levels of progesterone and estrogen in the blood have been reported. The most widely accepted form of blood used in either of the steroid assays for numerous species is plasma (Thorburn and Schneider, 1972; Moore, Barrett, and Brown, 1972; Robertson and Sarda, 1971; Martin, Cooke, and Black, 1970; Giorgi and Crosignon, 1969; Keller, Sendelbeck, Richardson, Moore, and Yates, 1966). The procedure used to obtain plasma as reported by most investigators involves collecting blood in heparinized tubes, usually pre-cooled, centrifuging as soon as possible, and storing the plasma fraction at -10° to -29°C until assayed.

Snook, Saatman, and Hansel (1971) used serum for progesterone quantitation via gas liquid chromotographic techniques and obtained estimates that were 41 percent lower at peak progesterone levels than Stabenfeldt, Ewing, and McDonald (1969) using plasma and similar assay techniques. Murphy, in the discussion following a paper by Lipsett, Doerr, and Bermudez (1970) presented at the Karolinska Symposium (1970), asked if anyone at the symposium had ever found any differences in values between plasma and serum. Murphy contended that serum was more conven-ient and cleaner to use, and eliminated the accumulation of precipitates. Abraham stated that recovery of estradiol-17β-^{3}H was higher from serum than from plasma using three different volumes of ether for each of

three extractions. Abraham also noted fibrin clots after freezing and thawing of plasma and gel formation during the extraction of plasma, neither of which were observed in the use of serum.

Blood serum apparently contains lower concentrations of progesterone than plasma, according to Erb, Randel, and Callahan (1971). This may be due to the fact that blood constituents have a catabolic effect on progesterone (Henricks, Dickey, and Hill, 1971) or there may be a conversion of progesterone to 20β-dihydroprogesterone (Short, 1958), or to 6β-hydroxyprogesterone (Palmer, Blair, Ericksson, and Diczfalusy, 1966) during the period of clot contraction. In any case a considerable uptake of progesterone by erythrocytes in vitro has been observed (Sandberg, Slaunwhite, and Antoniades, 1957; DeVenuto, 1967). Van der Molen and Groen (1968) contend that the binding of progesterone to plasma proteins also occurs and the affinity of the plasma for progesterone exceeds that of the red cells; it is generally accepted that following centrifugation the progesterone is present only in the plasma fraction.

Similar findings have also been reported in relation to estrogens. The conversion of estrone to estradiol-17α and estradiol-17β to estrone has been reported to occur in whole blood of cows (Axelrod and Werthessen, 1960). It was suggested by Werthessen, Baker and Field (1950) that this conversion may have been due to an enzyme in bovine blood that was capable of inducing oxidation of estrone. This effect was not observed in steers and bulls (Axelrod and Werthessen, 1960). Of interest with respect to the radioimmunoassay, is the possibility that a considerable portion of the immunoassayable estrogen in cow plasma may

not be biologically active due to such conversions (Henricks, Dickey, and Hill, 1971).

Wall and Migeon (1959) added estrone-16-C^{14} to human whole blood and recovered 75.0 to 88.4 percent of the radioactivity in the plasma while the remaining 12 to 25 percent was recovered from the red blood cells. The length of time or temperature of incubation had no apparent influence on C^{14} distribution. Baird (1968) reported that 7 to 10 percent of labeled steroid remained with the human red cells following centrifugation, while Sandberg, Slaunwhite, and Antioniades (1957) reported as much as 30 percent of C^{14} remained with the red cells in similar studies. The fact that the labeled steroid could be recovered by washing the red cells in 0.9 percent saline solution indicated that the steroid had adsorbed to the red cells (Baird, 1968; Korneman, Perrin, McCallum, 1964). Wall and Migeon (1959) state that red cells must play a rather important role in transport of the hormone since 12 to 25 percent on the labeled estrone appears to be associated with the red cells; and that red cells cause conversion of estradiol to estrone, while plasma alone does not cause such conversion.

Although it has been established that the use of plasma or serum may affect the concentration of estrogen or progesterone in the blood sample, or that one form may be more convenient for assay than the other, no quantitative comparisons have been reported in the literature to this date. Borth summed up the discussion after Lipsett's presentation (1970) on the use of plasma or serum by stating that no comparisons between plasma and serum aliquots of the same blood sample have been made and that the reasons for using plasma are traditional and throetical ones.

Robertson and Sarda (1971), in an attempt to devise a test which could be used under normal husbandry conditions, stored the whole blood at 4°C for a period of 24 to 72 hours prior to centrifugation and freezing of the plasma. Progesterone levels obtained from blood maintained in ice, centrifuged, and the plasma fraction frozen (-20°C) within one hour after collection were comparable to those centrifuged after 24 to 72 hours storage. The serum progesterone levels reported by Snook, Saatman, and Hansel (1971) were obtained from whole blood allowed to clot overnight (apparently at room temperature) and the serum fraction centrifuged and removed the following morning. Standard laboratory procedure for quick serum collection requires only 4 hours storage at room temperature (Campbell, Garvey, Crewer, and Scessdorf, 1964). Keller, et al. (1966), in a study of cortisol in rat plasma, reported that the levels in plasma or serum samples collected in chilled centrifuge tubes and centrifuged at 4°C were similar to those which were centrifuged at room temperature without prior chilling of tubes. Wall and Migeon (1959) reported that neither length of incubation (0 to 4 hours) nor temperature(5°, 22° and 37°C) appeared to influence the distribution or recovery of labeled esterone in plasma or erythrocytes.

One step in sample preparation agreed upon by all investigators when samples were not assayed immediately, was the final storage of either plasma or serum at subzero temperatures (-10° to -29°C). Steroids contained in samples of plasma appeared to remain essentially unaltered for months to years if frozen (Murphy, 1970). However, Martin, Cooke, and Black (1970) reported a 50 percent decrease in progesterone levels of pregnant humans after the first 4 weeks of storage at -20°C. After

6 months storage, however, non-pregnant human plasma progesterone levels remained unaltered. They concluded that repeated freezing and thawing had no effect on the steroid level although no details were given concerning the method used to reach this conclusion. Dog plasma used as a source of binding protein by Martin, Cooke, and Black (1970) was stored at -20°C for three years with no effect on the binding capacity for corticosterone and progesterone.

No published data of any kind was found concerning the effects of animal stress during bleeding or the volume of blood collected on the concentration of estrogen or progesterone in the sample or their concentration in peripheral blood.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. OBJECTIVES OF EXPERIMENT

This study was designed to evaluate the effects of various methods used to prepare samples for competitive protein binding (CPB) assay of progesterone and for radioimmunoassay (RIA) of estrogen. A quantitative comparison of estrogen and progesterone levels in both plasma and serum, subjected to the same handling techniques, was made to evaluate the hormones' stability in either form. Various holding times and holding temperatures prior to centrifugation were evaluated to determine the feasibility of plasma or serum sample collection when it is necessary to collect samples at locations other than close proximity of laboratory facilities and under normal husbandry conditions. An investigation into the effects of animal stress and blood volume collected during bleeding on hormone levels was made. The effects of repeated thawings and re-freezings of bovine plasma on both estrogen and progesterone were also evaluated.

II. ANIMALS

Blood samples were collected from four 13-year old nonpregnant Hereford cows from the UT-AEC Agricultural Research Laboratory herd, and from four cycling dairy cows (3 Holsteins, 1 Jersey) from the Knoxville dairy herd. The cows were at various stages of the estrous cycle at the time blood samples were taken. Cows were coded as shown in Table I.

7

TABLE I

COW ESTRUS IN RELATION TO BLEEDING DATE

Cow No.	Code No.	Date of Bleeding	Observed Estrus	
			Days Since Last Estrus	Days to Next Estrus
687[1]	1	12 July, 1972	1	20
403[1]	2	"	1	20
442[1]	3	"	17	4
2430[1]	4	"	17	6
502[2]	5	7 August, 1972	6	17
553[2]	6	"	15	22
341[2]	7	"	15	28
333[3]	8	"	20	2

[1] Hereford

[2] Holstein

[3] Jersey

Stress during bleeding was one of the treatment effects being evaluated. Although there was no effort to quantitate behavior during sample collection, notation of observable stress factors was made. Cow 1 was subjected to rectal palpation immediately prior to bleeding and had to be forced into the chute and was extremely nervous during sample collection. Cows 2, 3, and 4 all had to be forced into the chute, but were relatively quiet during sample collection. All cows had previously

been subjected to blood collection under restraint, but the dairy cows
(numbers 5, 6, 7, and 8) in all cases exhibited much more resistance
than the beef cows during the entire procedure to the point of being
considered hostile.

III. SAMPLING

Blood Treatment Phase

Eight blood samples (40 to 45 ml each) were collected consecu-
tively from each of the eight animals. Each sample was collected in a
50 ml polycarbonate centrifuge tube. Four drops of sodium heparin
(1000 U/ml) were added to the tubes in which plasma was to be collected
using a syringe with a 20 gauge hypodermic needle. Tubes designated as
"cool tubes" were placed in storage at 4°C overnight and were maintained
in an ice bath at all other times except during the actual filling of
the tube.

Bleeding was accomplished by restraining the animal in a squeeze
chute equipped with a headgate and collecting the samples from the
jugular vein via a sterilized California bleeding needle. During a
single insertion of the needle all eight blood samples that were to
receive various treatments were collected from each cow, filling the
next centrifuge tube as shown as the preceding tube was full. In this
manner of continuous bleeding all eight blood samples were collected
within 3 to 4 minutes. Bleeding proceeded in the order of treatment
number (i.e., beginning with treatment tube number one and ending with
treatment tube number eight). Those samples to be centrifuged without
prior storage were centrifuged within 1 hour after collection.

Treatment of each blood sample and the order of bleeding was as follows (see Figure 1):

Treatment I. Blood was collected in pre-chilled tubes containing heparin to permit separation of plasma. The sample was centrifuged at 2400 rpm for 30 minutes at 4°C. The plasma was removed immediately and stored at -15°C until assayed for estrogen and progesterone.

Treatment II. Treatment II was handled identically to Treatment I except that it was stored as whole blood at 4°C for 24 hours prior to centrifugation at 4°C.

Treatment III. Treatment III was identical to Treatment II except heparin was not added thus yielding serum.

Treatment IV. Treatment IV was collected in warm (room temperature) tubes containing heparin and was held as whole blood at room temperature for 4 hours before centrifugation in a non-refrigerated centrifuge at 2400 rpm for 30 minutes.

Treatment V. Treatment V was identical to Treatment IV except that heparin was not added to permit the collection of serum.

Treatment VI. Treatment VI was identical to Treatment IV except the sample was held as whole blood at room temperature for 24 hours prior to centrifugation and removal of the plasma.

Treatment VII. Treatment VII was identical to Treatment VI except
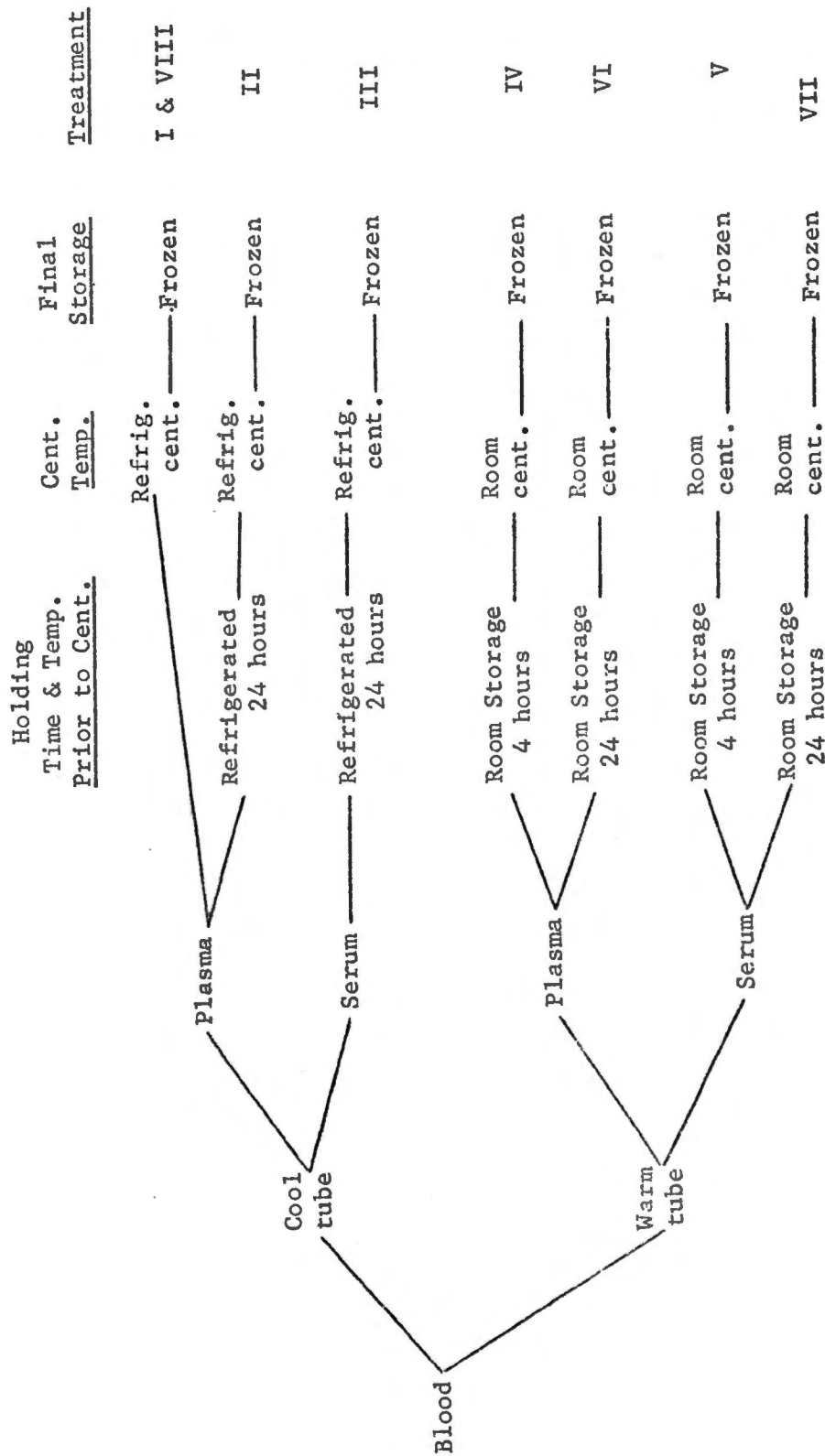
Figure 1. Flow-Diagram of Blood Treatment Phase

that heparin was <u>not</u> added to the tube, thus yielding <u>serum</u>.

<u>Treatment VIII</u>. Treatment VIII was identical to Treatment I.

Ten ml of plasma or serum was transferred from each tube to 15 ml glass vials using a separate plastic syringe for each sample. These were used for radioimmunoassay of estrogen. Then 2 ml of plasma or serum was transferred to an 8 ml glass vial for use in the competitive protein binding assay of progestins. These volumes were sufficient for duplicate assays of each hormone. The samples were stored at -15°C until assayed.

Upon thawing of plasma samples fibrin strands and clots were observed. It was necessary to remove these as they interferred with accurate pipetting. This was accomplished by aggregating the fibrin with an applicator stick and removing them or by swirling the sample bottle and extracting the clump with an applicator stick. Slow mixing on a Vortex mixer was found to speed up fibrin aggregation. It was noted that serum clot retraction was not as extensive as expected in plastic tubes. Due to this, glass centrifuge tubes are recommended for future preparation of serum samples. The fact that clot retraction was not extensive, however, did not affect the volume of serum yielded. This was true in both cool and warm (room temperature) tubes.

Freeze-Thaw Phase

In this phase, plasma samples were assayed for progesterone and estrogen to determine the effects of repeated freezing and thawing on hormone stability. Enough whole blood was collected to provide at least 200 ml of plasma following the collection procedure of Treatment I. The

pooled plasma was divided into 12 ml aliquots and transferred to each of fifteen 15-ml vials for storage at -15°C.

Treatments consisted of successive thawings of five sets of triplicate plasma samples to room temperature and then refreezing the samples. Thawing began with the three samples in set I. Upon refreezing set I, sets I and II were both thawed and refrozen. Then sets I, II and III were thawed to room temperature and refrozen. This process continued until sets IV and V were subsequently thawed and refrozen. Recalling that all plasma samples had been frozen originally, the total number of freezings for set I was six; for set II, five; set III, four; and for sets IV and V, a total of three and two freezings, respectively. The last thawing took place when the samples underwent preparation for progesterone assay. Estrogen assay samples received one additional freezing and thawing for a total ranging from three to seven. Fibrin particles tended to reappear with each thawing and were removed just prior to pipetting samples for assay.

IV. PROGESTERONE ASSAY PROCEDURE

Peripheral plasma and serum progestins were measured using the competitive protein binding (CPB) assay as introduced by Murphy (1967) and modified by Johansson, Neill and Knobil (1968) and Neill, Johansson, Datta, and Knobil (1967). Further modifications of the assay in this laboratory were made by Dowlen (1972) and Batson (1972) and are integrated into the description of this study.

The following competitive protein binding assay procedure was used in this study.

1) A progesterone stock solution of 100 ng per ml of absolute ethanol was used to prepare progesterone standards. Progesterone standards were diluted with fresh absolute ethanol to yield 25 ml at a concentration of 1, 2, 3, 4, 5, and 6 ng per 0.5 ml of absolute ethanol. A 0.5 ml aliquot of absolute ethanol served as a zero ng standard. These concentrations were pipetted in duplicate into 13 x 100 mm culture tubes, using a 0.5 ml volumetric pipette. The ethanol was evaporated in a 40°C water bath using dry-filtered air. The standards were prepared at the beginning of each assay to provide a standard curve.

2) Recovery rates or extraction efficiencies were estimated for each assay using both labeled and unlabeled progesterone. Labeled progesterone recoveries were prepared by adding 1 nanocurie of progesterone-1,2-^{3}H (specific activity 50.3 curies per millimole) in 20 microliters (μl) of ethanol to each of two 15 ml extraction tubes (conical centrifuge tubes), evaporating the ethanol dilutant, and adding 0.5 ml of the unknown plasma or serum to be assayed. To each of two scintillation counting vials 20 μl of the tritiated progesterone solution were added to provide the denominator of the recovery ratio (expressed as a percent).

The extraction efficiency or recovery rate of unlabeled progesterone was estimated by adding 3 ng of unlabeled progesterone in 0.5 ml of absolute ethanol to each of two extraction tubes, evaporating the ethanol, and adding 0.5 ml of steer plasma. The counts per minute obtained from these samples were compared to the counts per minute of the 3 ng standard solution. Two more tubes containing 0.5 ml of steer plasma alone were prepared as a further check of extraction. Its values were compared to the zero ng standard.

3) The unknown plasma or serum was pipetted in duplicate 0.5 ml aliquots into 15 ml extraction tubes. Five ml of petroleum ether was added to all extraction tubes (unknown samples and recovery samples) and stoppered with linear high density polyethylene stoppers. Two petroleum ether blanks were also prepared as a check of the petroleum ether solvent. Should erroneous blank values occur the assay would be rejected. All extraction tubes were then vigorously shaken simultaneously in a test rube rack for two minutes by hand. The polyethylene stoppers were loosened, but not removed.

4) The tubes were immersed in a dry-ice acetone bath to quick freeze the aqueous phase. Polyethylene stoppers were removed and the ether phase was decanted into 13 x 100 mm disposable culture tubes; the polyethylene stoppers were then replaced in their respective tubes. The plasma or serum was allowed to thaw and an additional 5 ml of petroleum ether added for a second two minute extraction.

5) Before the second extraction was began the culture tubes were placed in a 40°C water bath and the ether evaporated under dry-filtered air. Then the second ether phase was decanted into the culture tubes and evaporated again.

6) The ether phase in the extraction tubes containing the tritiated progesterone used for labeled recovery was decanted into counting vials after each freeze and evaporated to total dryness prior to the addition of toluene scintillation fluid.

7) The walls of the culture tubes were rinsed down with 1 ml of petroleum ether to flush all progesterone to the bottom of the tubes.

They were replaced in the 40°C water bath and evaporated to total dryness.

8) A 2 percent dog plasma (CBG-B^{3}H) binding solution was prepared by adding 17.5 ng of corticosterone-1,2-^{3}H (specific activity 44 curies per millimole) in 50 μl of ethanol to 98 ml of triple glass distilled water and vigorously mixing. Two ml of dog plasma were added and mixed by swirling the flask in order to prevent foaming. One ml of CBG-B^{3}H solution was added to the evaporated standards and unknown samples using a repipette.

9) The tubes were mixed on a Vortex mixer at a moderate speed for 5 seconds, placed in a 40°C water bath for 5 minutes, and mixed again for 5 seconds.

10) The culture tubes were incubated in an ice bath for no less than 10 minutes to increase the affinity of the binding protein for the steroids.

11) Eighty milligrams (mg) of previously washed and dried florisil (Dowlen, 1972; Murphy, 1969) was added to each tube and mixed on a Vortex mixer at a moderate speed for precisely 15 seconds and returned to the ice bath. The addition of florisil was speeded up by placing a small plug in one end of the stop-cock lumen of a separatory funnel in such a position that it would dispense 80 mg of florisil when rotated (de Sonza, Williamson, Moody and Diczfalusy, 1970).

12) One-half ml of the supernatant containing CBG-B^{3}H bound progesterone was removed from each evaporation tube with a semi-automatic pipette (using disposable polyethylene tip) and added to counting vials containing 10 ml of toluene scintillation solution (1000 ml toluene plus

42 ml Liquifluor which contained 4 gm PPO + 5 mg POPOP per liter). Counting solution was also added to the labeled recovery vials.

13) The counting vials were mixed on a Vortex mixer at maximum speed for 5 seconds and the outsides wiped clean of any deposits or finger prints that might interfere with the path of photons.

14) The vials were then placed in a Nuclear-Chicago Mark I, liquid scintillation counter and allowed to equilibrate to 10°C. The samples were counted to a total of 10,000 counts. A ^{3}H-quenched standard was maintained in the counter and placed at the end of each series of counting vials.

The counts per minute from the standard curve were used to calculate an asymptotic regression line with a Wang computer. Using this data the computer would then calculate the corresponding level of progesterone in an unknown sample when supplied the counts per minute for that sample.

V. ESTROGEN ASSAY PROCEDURE

Peripheral plasma and serum estrogen was estimated using the radio-immunoassay (RIA) procedure described by Henricks (1970) and Henricks, Hill and Dickey (1971) and modified by Batson (1972). The procedure was further modified in this study by increasing the incubation period in an ice water bath from 30 minutes (Henricks, 1970) to 2 hours. This facilitated an increase in the recovery of unlabeled estradiol-17β since only one extraction was conducted.

Each assay consisted of the standard solutions, unknown samples from 4 cows, labeled and unlabeled recoveries. Because as many as 50

duplicate standards and samples were run in each assay, extraction was completed on one day and the balance of the assay on the next day. A detailed description of the preparation, dilution, purification and storage of materials and solutions used for assay in this laboratory is discussed by Batson (1972).

The following radioimmunoassay procedure was used in this study:

1) An estradiol-17β stock solution of 100 ng per ml of absolute ethanol was used to prepare estradiol-17β standards. Estradiol standards were diluted to yield 25 ml at a concentration of 10, 20, 30, 40, 50, 60, 80, and 100 pg per 0.5 ml of absolute ethanol. These concentrations plus 0.5 ml of absolute ethanol serving as a zero pg standard were counted to construct the standard curve for each group of unknown samples. Five tenths ml of the standard solution were pipetted in duplicate into 13 x 100 mm disposable culture tubes using 0.5 volumetric pipette and evaporated to total dryness in a 40°C water bath under dry filtered air.

2) Recovery rates or extraction efficiencies were determined for each assay using both labeled and unlabeled estradiol with an additional unlabeled recovery made on each individual cow. Labeled recoveries were prepared by adding 100 microliters (μl) of estradiol-17β-2,4,6,7-^{3}H solution containing 4 nanocuries to each of two 15 ml extraction tubes (conical centrifuge tubes) and to each of two scintillation counting vials. The ethanol was evaporated in a 40°C water bath under a dry filtered airstream and 4 ml of pooled cow plasma was added to each tube. To each of two additional 15 ml extraction tubes 4 ml of the same pooled cow plasma was added.

Unlabeled recovery rates were determined by adding 0.5 ml of the 20 pg standard solution to each of two extraction tubes and 0.5 ml of 50 pg standard solution to two more extraction tubes. These tubes were evaporated to dryness and 4 ml of pooled cow plasma added to each tube. To obtain the recovery rate or extraction efficiency these tubes were compared to each of two other tubes containing 0.5 ml of ethanol evaporated to dryness and 4 ml of pooled cow plasma.

3) Four ml of the unknown cow plasma or serum was added in duplicate to 15 ml extraction tubes. Two drops of ammonium hydroxide solution were added to each sample containing plasma or serum. Six ml of anhydrous diethyl ether was added and the tubes stoppered with linear high density polyethylene stoppers. All tubes were vigorously shaken by hand in a test tube rack for two minutes and then centrifuged at 2400 rpm for 10 minutes at 4°C. The stoppers were loosened but not removed.

4) The tubes were immersed in a dry ice acetone bath to freeze the aqueous phase. The stoppers were removed and the ether phase decanted into 13 x 100 mm disposable culture tubes. The culture tubes were placed in a 40°C water bath and the ether evaporated under a stream of dry filtered air. Labeled recovery samples were decanted into counting vials and evaporated.

5) Estrogen was concentrated in the bottom of the culture tubes by rinsing with one ml of diethyl ether and evaporating to total dryness. Tubes were covered and stored overnight at 4°C.

6) Estradiol antibody (1:20,000) in a 0.1 M, pH 7.0 phosphate buffered 0.1 percent gel solution (PBGS) was added to each standard and sample in 100 μl aliquots. The tubes were mixed on a Vortex at a

moderate speed for 5 seconds.

7) Forth-nine and four tenths pg estradiol-17β-2,4,6,7-^{3}H (110 curies per millimole specific activity) in PBGS was added to each tube in 100 μl aliquots and mixed on a Vortex at a moderate speed for 5 seconds.

8) The samples were covered and incubated for 2 hours in an ice bath.

9) Dextran coated charcoal solution (250 mg charcoal 25 mg dextran per 100 ml of phosphate buffer solution) was constantly stirred using a magnetic stirrer and 1 ml was added to each tube using a semi-automatic pipette (disposable polyethylene tip). Rubber stoppers were inserted and all tubes were very gently shaken by hand in a test tube rack for 5 seconds and the stoppers removed.

10) The tubes were then centrifuges at 1500 rpm for 5 minutes.

11) Five tenths ml aliquots of the supernatant were transferred with a semi-automatic pipette into counting vials containing 10 ml of toluene-liquifluor counting solution (1000 ml toluene and 42 ml liquifluor). Counting vials were mixed on a Vortex at maximum speed and wiped clean.

12) Counting vials were placed in a Nuclear-Chicago, Mark I liquid scintillation counter and allowed to equilibrate at 10°C overnight in order to improve the counting efficiency.

13) The samples were counted to a total of 10,000 counts. A Wang computer was used to determine estrogen content in a manner similar to that described in the progesterone assay.

Tritium labeled progesterone, estradiol-17β and corticosterone were procured from New England Nuclear Corporation. Unlabeled progesterone and estradiol-17β were purchased from Mann Research Laboratory. Liquifluor was purchased from New England Nuclear Corporation in pint bottles. Estradiol-17β antibody was obtained from Dr. B. C. Caldwell of Yale University.

VI. STATISTICAL ANALYSIS

Replicated determinations of all samples were averaged to provide sample means which were subjected to analysis of variance. Orthogonal comparisons were made to test for significant differences between treatment means (Ostle, 1954). The sources of variance in the analysis were treatment, cow type, treatment x cow type interaction, and cows within cow type.

CHAPTER IV

RESULTS AND DISCUSSION

I. PROGESTERONE ASSAY PROCEDURE

The procedure used in this study involved the competition between progesterone and corticosterone-^{3}H for binding sites on the cortico-steroid binding globulin (CBG). Tritiated corticosterone (1,2-^{3}H) was bound to the CBG and subsequently displaced by unlabeled progesterone when the CBG-B^{3}H binding solution was added to the unknown samples. Unbound labeled corticosterone was adsorbed to the florisil and the remaining B-^{3}H bound to CBG counted via liquid scintillation methods. From the resulting counts per minute an estimate was made of the progesterone present. Since the amount of progesterone from the unknown samples is inversely related to the amount of corticosterone-B^{3}H present in the sample (as determined by the counts per minute), the amount of progesterone could be quantitated by comparing its displacement of corticosterone-B^{3}H with that of known progesterone levels in the standard curve.

Two 5 ml extractions with reagent grade petroleum ether were used. High blank values producing erroneous progesterone values were encountered by Dowlen (1972) in this laboratory when the solvent was purchased in one gallon cans. He noted that the solvent underwent some type of degradation which produced erratic progesterone values and high extraction blanks. To avoid similar problems the solvent was procured

22

in one pint brown glass bottles for use in this procedure. This volume could be fully utilized in one day's assay with little waste and eliminated high extraction blank values.

Extraction efficiency of 3 ng of unlabeled progesterone from steer plasma when compared to the 3 ng standard solution was (mean $\pm$ s.e.) 96.9 $\pm$ 0.45 percent or 2.9 ng of progesterone. The recovery rates of tritium labeled progesterone when added to a sample of cow plasma averaged 93.3 $\pm$ 0.64 percent.

Steer plasma was used to evaluate extraction efficiency since it theoretically contains no endogenous progesterone that would interfere with the recovery of added progesterone. The steer plasma without added progesterone could not be distinguished from the zero ng standard solution and the petroleum ether blanks were below the levels of sensitivity.

A dry ice-acetone bath was used to speed removal of the ether phase of plasma or serum. This eliminated the removal of ether by aspiration with Pasteur pipettes and reduced the chance of procedural losses in transferrence of the ether phase. Freezing the samples did not have any adverse effects on the estimate of progesterone values.

It was determined that, if necessary, samples could be covered and stored at 4°C overnight after the ether phase had been evaporated to total dryness. This was done with no apparent harm to the results of the assay.

In the preparation of the standard solutions used to construct the standard curve, a larger number of concentrations than usual were used in order to improve the accuracy of quantitation. A higher degree of accuracy was felt to be necessary since this study was not only

estimating the level of hormone present but any differences in levels as an effect of sample treatment. A typical standard curve is presented in Figure 2.

The source of CBG used in this laboratory was male St. Bernard dog plasma obtained locally at a veterinary clinic from an animal accustomed to blood donor procedure. Therefore high endogenous cortisol levels reported to appear in plasma of excited animals by Murphy (1971) was not a problem. The dog plasma was stored in 2.5 ml aliquots for long periods at -15°C with no apparent loss of binding affinity for progesterone. Martin, Cooke and Black (1970) stored dog plasma at -20°C for 3 years with no apparent loss of binding capacity with respect to corticosterone and progesterone. Murphy (1970) also noted that binding proteins can be frozen for long periods with no apparent degradation.

Although Neill, et al. (1967) reported that 2.5 percent dog plasma CBG-B^{3}H would remain stable for at least two weeks when stored at 5 to 8°C, an appropriate amount of 2.0 percent dog plasma CBG-^{3}H binding solution was prepared fresh for each assay in this laboratory. This was done to assure that the assay would not be affected by the possibility of using degradated CBG-B^{3}H.

II. ESTROGEN ASSAY PROCEDURE

The procedure used in this study involved the immunologic activity of 17β-estradiol antibody and the competition of estradiol-17β-2,4,6,7-^{3}H with unlabeled 17β-estradiol (E^2) for binding sites on the antibody molecule. All unbound labeled estradiol was adsorbed with charcoal and the remaining estradiol-^{3}H bound to the antibody counted via liquid
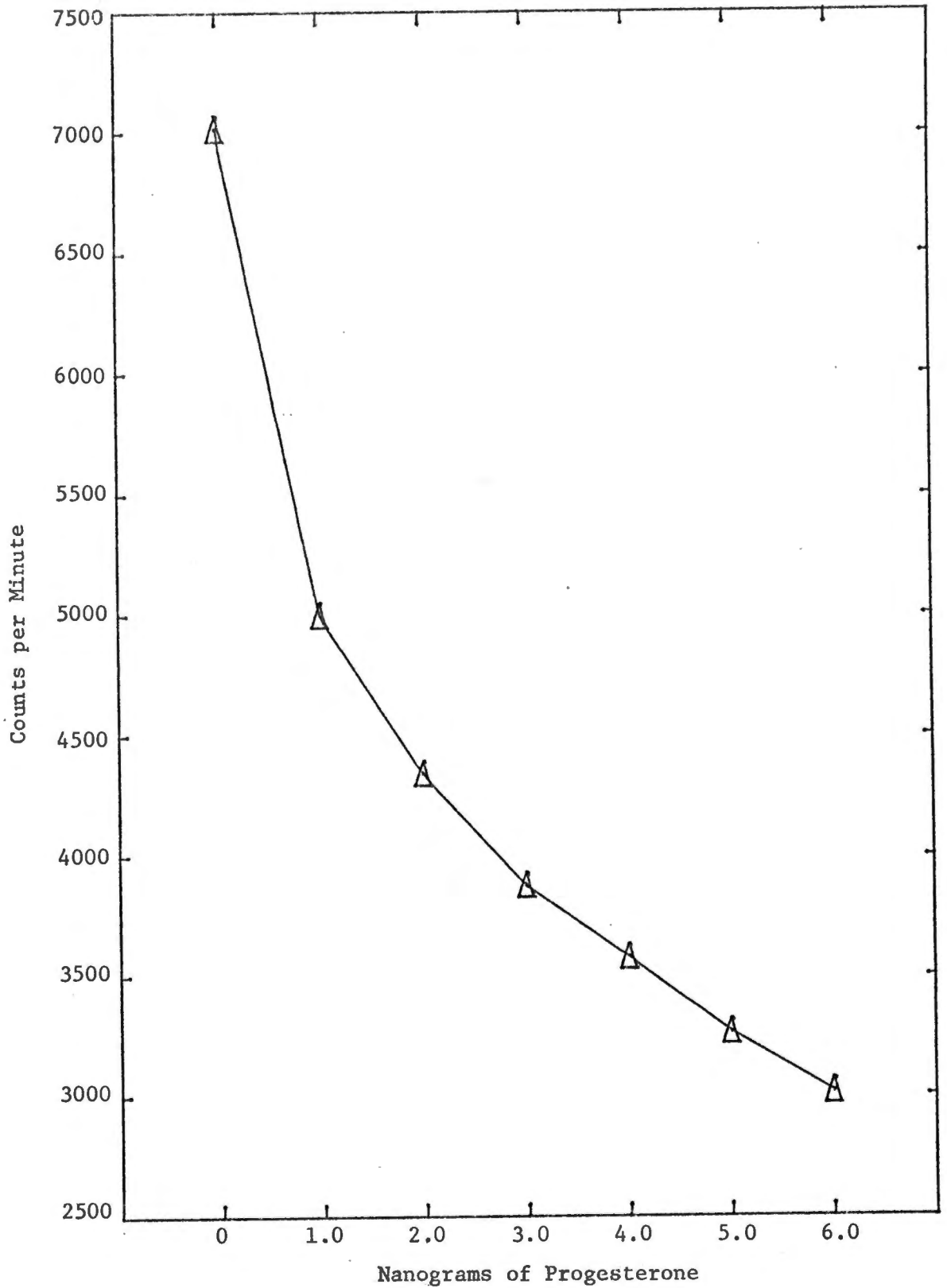
Figure 2. A typical progesterone standard curve.

scintillation methods. The more unlabeled estradiol that was present the less binding sites on the antibody molecule were available to the estradiol-[3]H. By quantitating the amoung of estradiol-^{3}H bound to the antibody an estimate of the unlabeled estradiol present in the sample could be made when compared to the standard curve.

One 6 ml extraction with anhydrous diethyl ether was carried out. An evaluation of the extraction efficiency was made at 20 pg where a majority of the 4 ml samples should read and at 50 pg which was expected to be the maximum any 4 ml samples would read. Extraction efficiency at the 20 pg level averaged 91.0 $\pm$ 0.35 percent and at the 50 pg level 81.0 $\pm$ 0.60 percent. Extraction efficiency of estradiol-^{3}H was 89 $\pm$ 1.5 percent.

Batson (1972) used the same procedure as in this study was the exception of a 3.5 hour incubation and two 6 ml extractions. He obtained an extraction efficiency of 91.19 $\pm$ 4.58 percent at 20 pg and 88.57 $\pm$ 5.24 percent at 50 pg, and reported a 94.04 percent extraction efficiency of E^2-[3]H. Pooled cow plasma was used in determination of extraction efficiency of unlabeled zero ng samples and fortified samples; while an unknown sample from one of the cows being assayed was used to determine extraction efficiency of tritiated estradiol.

A larger number of concentrations of standard solutions than usual were used to construct the standard curve for each assay in order to improve accuracy. A typical standard curve is shown in Figure 3.

Separation of the ether phase was carried out in a dry ice acetone bath which froze the aqueous phase, thus allowing the ether phase to be decanted into culture tubes. This procedure saved a great deal of time
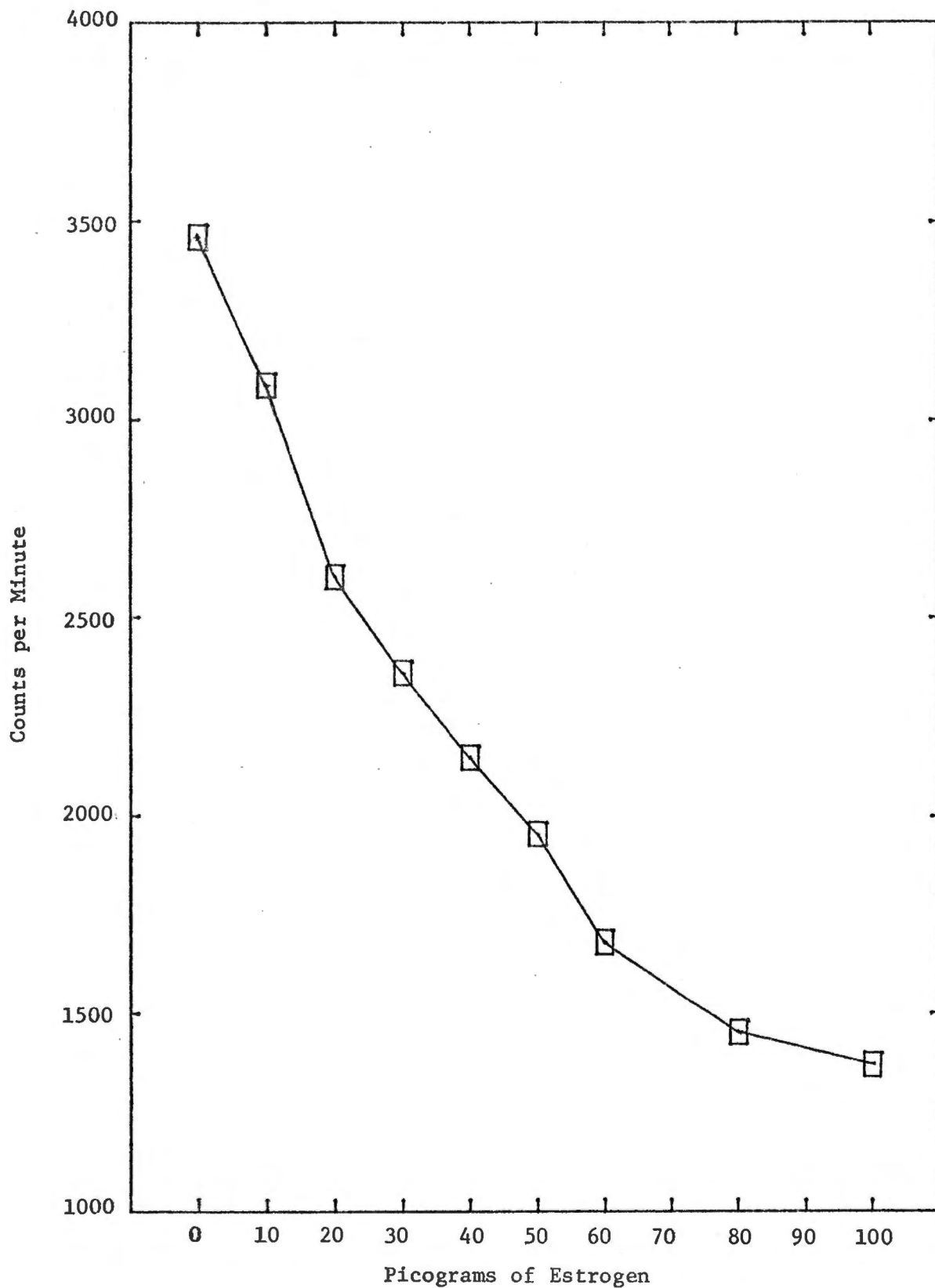
Figure 3. A typical estrogen standard curve.

and probable loss of the sample in the use of Pasteur pipettes.

III. PROGESTERONE DATA

The blood from each of eight cows was subjected to eight treatments to evaluate the stability of progesterone under the influence of these treatment effects. Treatment means were determined for all eight animals and statistically analyzed for treatment effect, cow type (beef or dairy), individual cows overall and within cow type, and for interaction between treatment and cow type. The individual cow, cow type and treatment means with standard errors are presented in Table II and Figure 4.

By analysis of variance there were significant differences between treatments when considered overall, between individual cows overall, and individual cows within a cow type ($P < 0.001$). There was no difference between the two cow types. The differences in cows were easily understood since individual differences existed between cows, and cows were at various stages of the estrous cycle when bled (Stabenfeldt, Ewing, and McDonald, 1969). It was determined that cow type did not have any influence on treatment effect since a test for interaction between cow type and treatment was nonsignificant. Both cow types responded the same to each treatment effect (Table III).

Based on orthogonal comparisons there was no significant difference bewteen Treatments I and VIII (treatment mean difference 0.01 ng/ml) which indicated that the volume of blood collected (up to 350 ml) or animal stress during bleeding has no effect on the concentration of progesterone in plasma. There was significant difference ($P < 0.001$),

TABLE II

TREATMENT MEAN NANOGRAMS OF PROGESTERONE PER ML

| Treatment* | Cow Number | | | | | | | | Tmt Mean + S.E. | | |
	1	2	3	4	5	6	7	8	Beef Cows 1 – 4	Dairy Cows 5 – 8	All Cows
I	0.79	0.78	6.36	5.69	3.25	7.88	5.65	8.92	3.40±1.51	6.42±1.26	4.91±1.08
II	0.64	0.35	5.60	4.73	3.13	7.48	4.17	7.34	2.83±1.36	5.53±1.11	4.18±0.96
III	0.68	0.13	4.95	4.82	2.67	7.11	4.04	7.82	2.64±1.30	5.41±1.23	4.03±0.98
IV	0.15	0.09	3.97	3.63	2.19	6.05	2.06	6.12	1.96±1.06	4.10±1.14	3.03±0.83
V	0.27	0.13	4.86	4.20	2.15	2.72	5.00	5.66	2.36±1.26	3.88±0.85	3.12±0.76
VI	0.04	0.04	1.21	0.63	0.93	3.37	0.59	1.98	0.48±0.28	1.72±0.63	1.09±0.39
VII	ND	0.35	1.21	1.38	1.10	1.65	2.43	3.19	0.73±0.33	2.09±0.46	1.41±0.37
VIII	0.74	0.74	6.77	6.37	3.32	6.74	5.89	8.83	3.65±1.68	6.14±1.39	4.92±1.14

ND = Non Detectable.

*I Plasma collected in chilled tubes, centrifuged immediately.
II Plasma collected in chilled tubes, held as whole blood for 24 hours prior to cent.
III Serum handled identically to Treatment II.
IV Plasma collected in warm tubes, held as w.b. 4 hours prior to centrifugation.
V Serum handled identically to Treatment IV.
VI Plasma collected identically to IV, held 24 hours as w.b. prior to centifiguration.
VII Serum handled identically to IV.
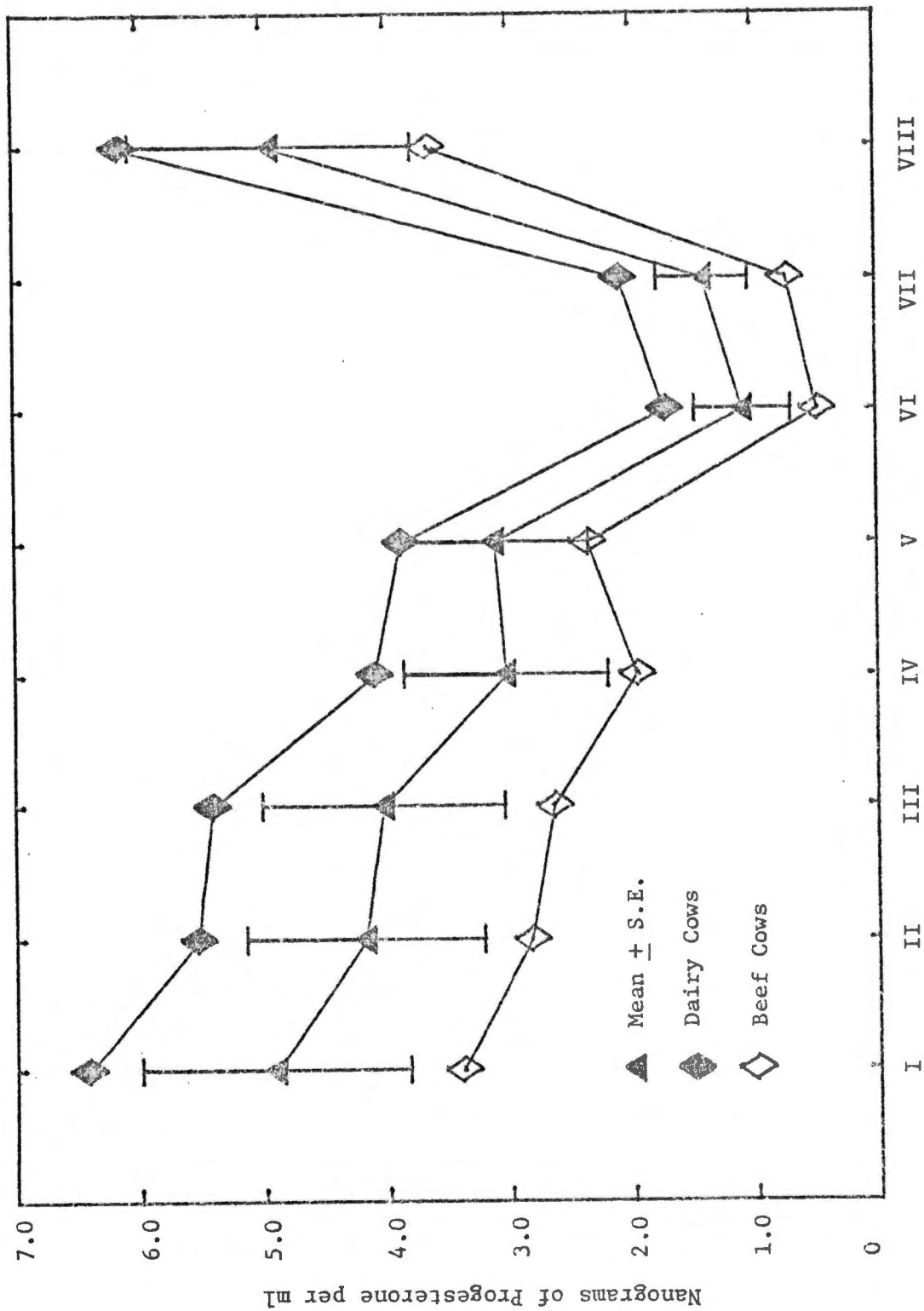VIII Plasma handled identically to I.

Figure 4. Effect of treatment on progesterone concentration.

TABLE III

STATISTICAL ANALYSIS OF PROGESTERONE DATA

Source	Degree Freedom	Sum of Squares	Mean Square	F Value
Total	63	439.92		
Treatments	7	120.50	17.21	14.22***
Contrast 1	1		5.26	4.32*
2	1		88.20	72.89***
3	1		0.09	0.074 NS
4	1		26.54	21.93***
5	1		0.09	0.074 NS
6	1		0.31	0.26 NS
7	1		0.0004	0.0003 NS
Cow Type	1	74.89	74.89	2.41 NS
Cows/Cow Type	6	186.54	31.09	25.69***
Treatment x Cow Type	7	6.95	0.99	0.82 NS
Error	42	51.04	1.21	

*(P < 0.05)

***(P < 0.001)

NS - Non-Significant

Contrast 1 Cool samples held at 4°C for 24 hrs vs. 0 hrs holding time prior to centrifugation.

2 Samples collected in cool tubes vs. warm tubes.

3 Plasma samples vs. serum samples (excluding Treatments I and VIII).

4 Samples collected in warm tubes and held at room temperature for 4 hrs vs. 24 hrs.

5 Interaction of holding time x plasma and serum collected in warm tubes.

6 Interaction of holding temperature x plasma and serum (excluding Treatments I and VIII).

7 Treatment I vs. Treatment VIII.

however, between Treatments I and VIII, and all other treatments. A comparison made within cool tube and refrigerator storage samples held for zero hours (Treatments I and VIII), and 24 hours (Treatments II and III) indicated a significant difference (P < 0.05). There was a 16 percent difference in the treatment means. Treatments II and III were similar to the procedure described by Robertson and Sarda (1971).

Within similar treatments (II vs. III, IV vs. V, VI vs. VII), there were no differences between plasma or serum assays. Therefore Treatment II yielded similar results to Treatment III.

Samples collected in warm tubes and held at room temperature, and cool tubes held at 4°C prior to centrifugation were significantly different (P < 0.001) with the treatment mean of the warm tubes being 53 percent lower than that of the cool tubes. Within the warm tube samples held at room temperature for 4 hours (Treatments IV and V) and 24 hours (Treatments VI and VII) there appears to be a negative relationship between the length of time and the level of progesterone. Samples held for 24 hours contained significantly less progesterone than those held for 4 hours (P < 0.001). There was no significant interaction between the treatment effects with either plasma or serum.

Treatment V was similar to serum collection procedures described by Campbell, Garvey, Crewer, and Sussdorf (1964), and Treatment VII was similar to methods used by Snook, Saatman and Hansel (1971). A comparison of the treatment means of Treatment I with V indicated a 38 percent decrease in the progesterone content of Treatment V, while the progesterone content of Treatment VII was 89 percent lower than that of Treatment I.

In general, any blood preparation procedure other than the one described as Treatment I in this study resulted in lower levels of progesterone at the time of assay and its validity as a method of blood preparation for the CPG assay measurement of progesterone should be questioned. These data indicate that samples should be prepared for assay immediately and stored at sub-zero temperatures until the assay can be conducted. Serum collection procedures described by Campbell, et al. (1964) and Snook, Saatman and Hansel (1971) were reproduced in this study. These procedures resulted in a 38 and 89 percent loss of progesterone content, respectively. Although it was not the purpose of this study to establish the causes of lowered progesterone concentrations, several possibilities were cited in the review of literature. Animal stress or volume of blood collected has no effect on plasma progesterone levels.

IV. ESTROGEN DATA

The blood from each of eight cows was subjected to eight treatments to evaluate the stability of estrogen concentration under the influence of these treatment effects. Treatment means were determined for all eight animals and statistically analyzed for treatment effect, cow type (beef or dairy), cows within cow type, and for interaction between treatment and cow type. The individual cow, cow type, and treatment means with standard error have been presented in Table IV and Figures 5, 6 and 7.

By analysis of variance there was no significant difference

TABLE IV

TREATMENT MEAN PICOGRAMS OF ESTROGEN PER ML

| Treatment* | Cow Number | | | | | | | | Tmt Mean ± S. E. | | |
	1	2	3	4	5	6	7	8	Beef Cows 1 - 4	Dairy Cows 5 - 8	All Cows
I	7.85	5.34	5.96	8.64	3.93	9.90	3.40	9.24	6.95±0.78	6.62±1.71	6.78±0.87
II	10.75	5.24	4.73	7.56	4.92	7.07	2.90	6.78	7.16±1.37	5.42±0.96	6.29±0.84
III	11.49	8.08	5.40	7.81	5.21	3.38	3.65	6.21	8.19±1.25	4.61±0.67	6.40±0.94
IV	10.51	7.14	4.27	6.53	5.14	2.97	4.74	5.75	7.11±1.29	4.65±0.60	5.88±0.81
V	9.95	6.31	4.45	6.70	5.18	2.99	3.12	5.20	6.85±1.19	4.12±0.62	5.48±0.79
VI	10.67	8.31	7.22	6.87	10.60	2.74	5.83	5.07	8.27±0.86	6.06±1.65	7.16±0.96
VII	9.51	9.23	6.63	6.59	10.27	4.65	8.00	5.99	7.99±0.80	7.34±1.32	7.66±0.73
VIII	8.96	7.93	6.98	7.28	8.53	3.83	6.92	5.54	7.79±0.44	6.20±1.00	6.99±0.59

*I   Plasma collected in chilled tubes, centrifuged immediately.
II   Plasma collected in chilled tubes held as whole blood for 24 hours prior to cent.
III Serum handled identically to Treatment II.
IV   Plasma collected in warm tubes, held as w.b. 4 hours prior to centrifugation.
V   Serum handled identically to IV.
VI   Plasma collected identically to IV, held 24 hours as w.b. prior to centrifugation.
VII Serum handled identically to VI.
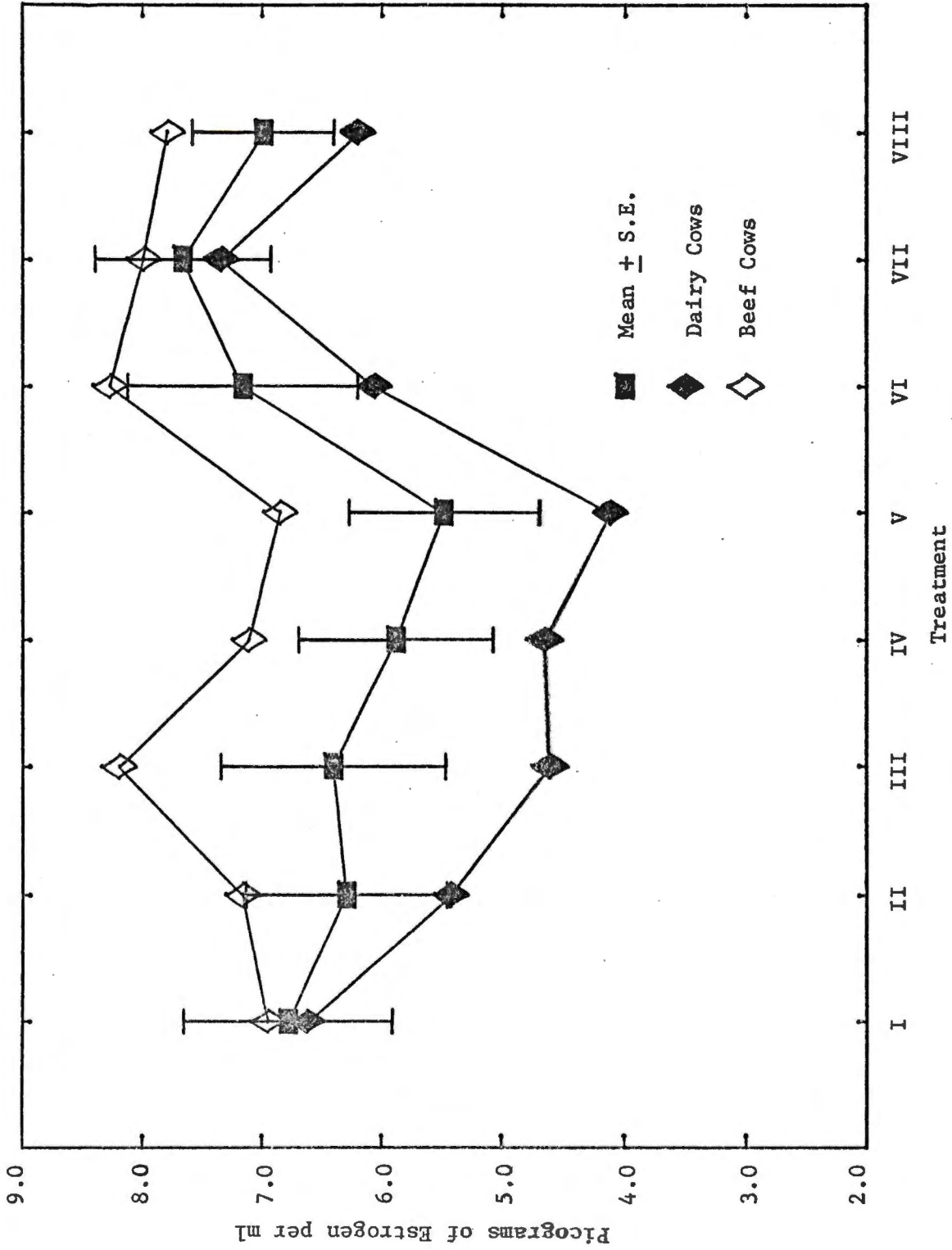VIII Plasma handled identically to I.

34
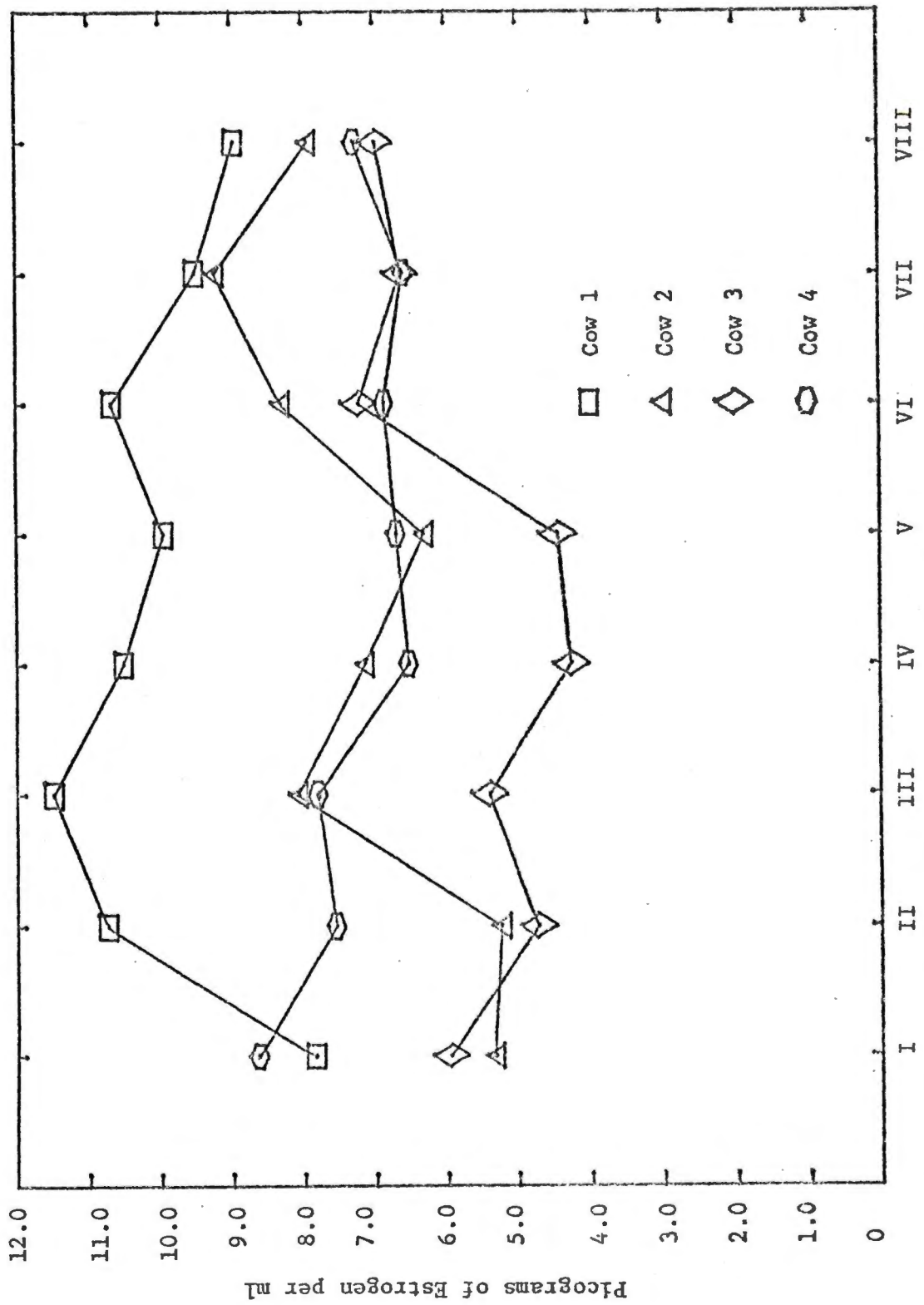
Figure 5. Effects of treatment on estrogen concentration.

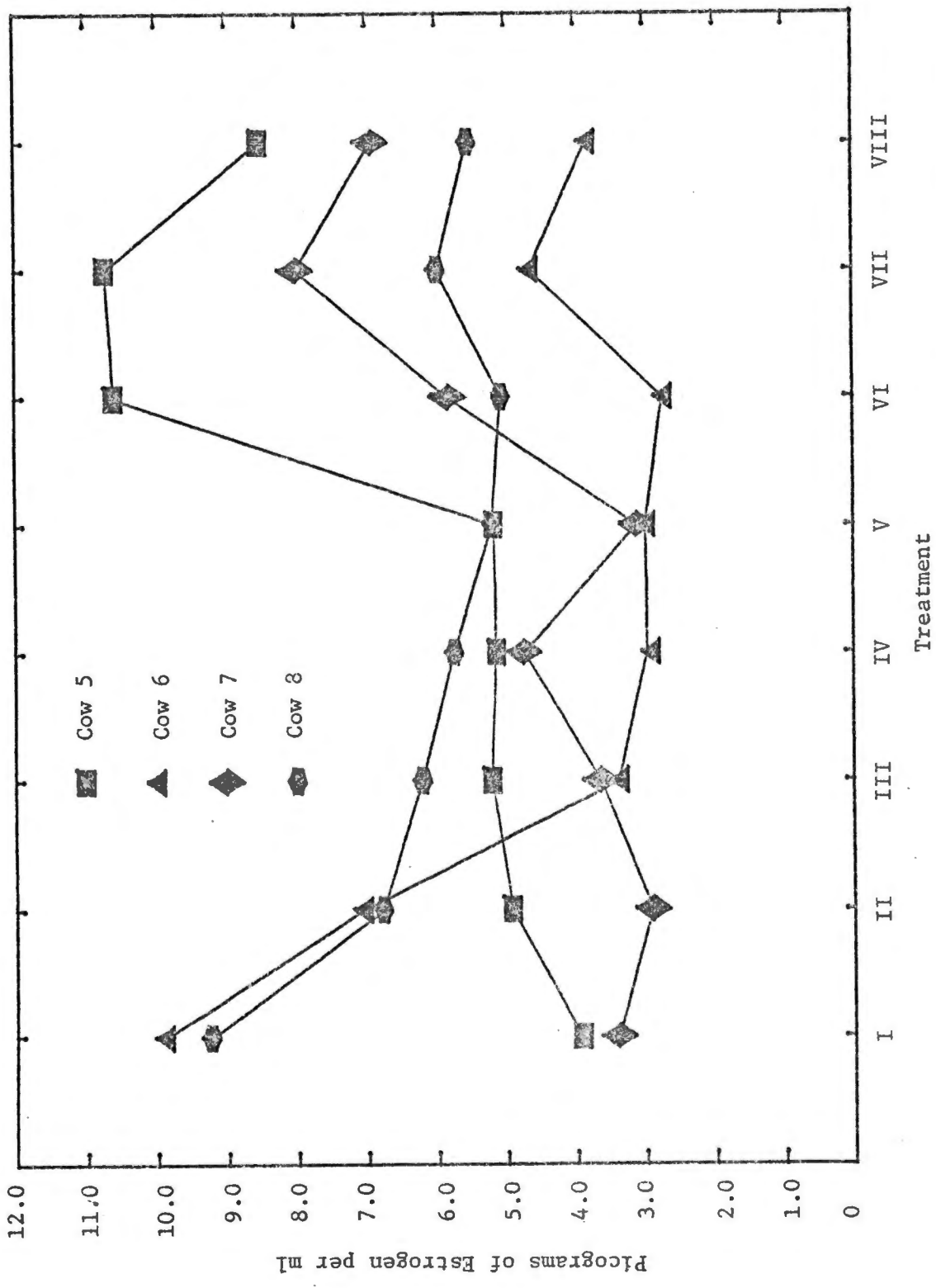Figure 6. Effect of treatment on estrogen concentration in individual beef cows.

Figure 7. Effect of treatment on estrogen concentration in individual dairy cows.

between treatments when considered overall. There was significant difference (P < 0.025) between individual cows due to individual and cyclic difference between cows. There was no significant difference between beef and dairy cow types. A test for interaction determined that cow type did not have any influence on treatment effect.

Based on orthogonal comparisons of the estrogen data the only effect tested which was significantly different (P < 0.01) was a comparison of warm tubes held at room temperature for 4 hours (Treatments IV and V) and 24 hours (Treatments VI and VII) with a difference of 34 percent in treatments means (Table V). The biological significance of this is not understood.

Although there generally was no significant difference in treatments, certain trends in response to treatment were noted. Cows that were in the diestrus stage of the estrous cycle (cows 5 and 7) responded very much the same to each of the treatments in terms of estrogen levels present. Cows in metestrus (cows 1 and 2) also responded similarly to treatment, although their response was different from any other cows. Still again, cows in early proestrus (cows 3 and 4) exhibited near identical response to each treatment, yet different from cows in either metestrus or diestrus. Cows thought to be in proestrus or exhibiting high estrogen concentrations due to a diestrus surge in estrogen (cows 6 and 8) (Batson, 1972) both responded in like manner to the effects of the treatments (Figures 6 and 7).

The significance of this observed relationship between stage of estrous cycle and response to treatment is not know. It has been shown

TABLE V

STATISTICAL ANALYSIS OF ESTROGEN DATA

Source	Degrees Freedom	Sum of Squares	Mean Square	F Value
Total	63	331.76		
Treatment	7	28.41	4.06	1.33 NS
Contrast 1	1		2.57	0.84 NS
2	1		0.05	0.02 NS
3	1		0.09	0.03 NS
4	1		23.89	7.83**
5	1		1.59	0.52 NS
6	1		0.03	0.01 NS
7	1		0.18	0.06 NS
Cow Type	1	57.78	57.78	3.43 NS
Cows/Cow Type	6	101.13	16.86	5.53*
Treatment x Cow Type	7	16.21	2.32	0.76 NS
Error	42	128.26	3.05	

*($P < 0.025$)

**($P < 0.01$)

NS - Non-Significant

Contrast 1 Cool samples held at 4°C for 24 hrs vs. 0 hrs holding time prior to centrifugation.

2 Samples collected in cool tubes vs. warm tubes.

3 Plasma samples vs. serum samples (excluding Treatments I and VIII).

4 Samples collected in warm tubes and held at room temperature for 4 hrs vs. 24 hrs.

5 Interaction of holding time x plasma and serum collected in warm tubes.

6 Interaction of holding temperature x plasma and serum (excluding Treatments I and VIII).

7 Treatment I vs. Treatment VIII.

that in presence of red cells, there is a conversion of estrone to
estradiol-17α and estradiol-17β to estrone (Axelrod and Werthessen,
1960) and that there is an adsorption of estrone to red cells (Wall and
Migeon, 1959). Such a conversion does not take place in the presence of
plasma alone (Wall and Migeon, 1959). The fluctuations in estrogen con-
centration observed in this study occurred in treatments that were held
as whole blood for 4 hours and 24 hours, and at 4°C and room temperature.
Baird (1968) has established that the proportion of estrogen bound to
the red cells varies with the concentration present. Therefore, the
adsorption and subsequent conversion of the constituents of total estro-
gen could be influenced by the state of cycle due to the various levels
of estrogen present at various stages.

Unfortunately, although the antibody used in this study is
specific for estradiol, it also shows a high binding affinity for estrone.
For that reason the two could not be distinguished and valid conclu-
sions drawn concerning the relationship of stage of cycle and response
to treatment. Should a study capable of measuring this relationship be
devised it may result in a re-evaluation of certain variables needed to
be taken into consideration for RIA and provide more insight into the
endrocrinology of reproduction.

V. FREEZE-THAW DATA

Five sets of pooled cow plasma in triplicate were assayed for both
progesterone and estrogen to evaluate their stability in plasma samples
subjected to repeated thawing and refreezing. There was no significant

effect of freezing and thawing on either estrogen or progesterone concentration. Maximum differences in set means were not more than 0.75 pg/ml of estrogen nor more than 0.5 ng/ml of progesterone (Table VI). Graphic depiction of the set means are presented in Figure 8.

This phase of the study revealed that a single plasma sample could be reused numerous times for either estrogen or progesterone assay if the sample was refrozen after each assay. Murphy (1970) contends that steroids contained in plasma samples can be frozen for months or years with essentially no alteration or decomposition. These results suggest that a single plasma sample can be maintained in the frozen state and assayed numerous times over long periods without fear of consequential changes in steroid value estimates.

With this knowledge an investigator might consider preparing large numbers of aliquots of steroid fortified, pooled plasma to serve as standards to be used with each assay. This would provide a valuable aid in the investigator's evaluation of the validity of each assay, giving him a benchmark from which assays could be compared.

TABLE VI

FREEZE-THAW PHASE DATA

Sample No.	Nanograms of Progesterone/ml				
	Set				
	I	II	III	IV	V
1	3.36	2.90	2.68	2.92	2.48
2	2.58	2.78	3.21	2.81	2.85
3	2.71	3.02	2.88	2.64	2.35
Set mean	2.88	2.90	2.92	2.79	2.56
S.D.[1]	± 0.42	± 0.12	± 0.27	± 0.14	± 0.26

Sample No.	Picograms of Estrogen/ml				
	Set				
	I	II	III	IV	V
1	8.36	7.20	7.17	6.86	7.68
2	5.16	7.44	7.86	7.70	6.31
3	7.07	6.05	7.67	6.94	7.10
Set mean	6.86	6.89	7.57	7.17	7.03
S.D.	± 1.61	± 0.74	± 0.36	± 0.46	± 0.69

[1]Standard deviation.
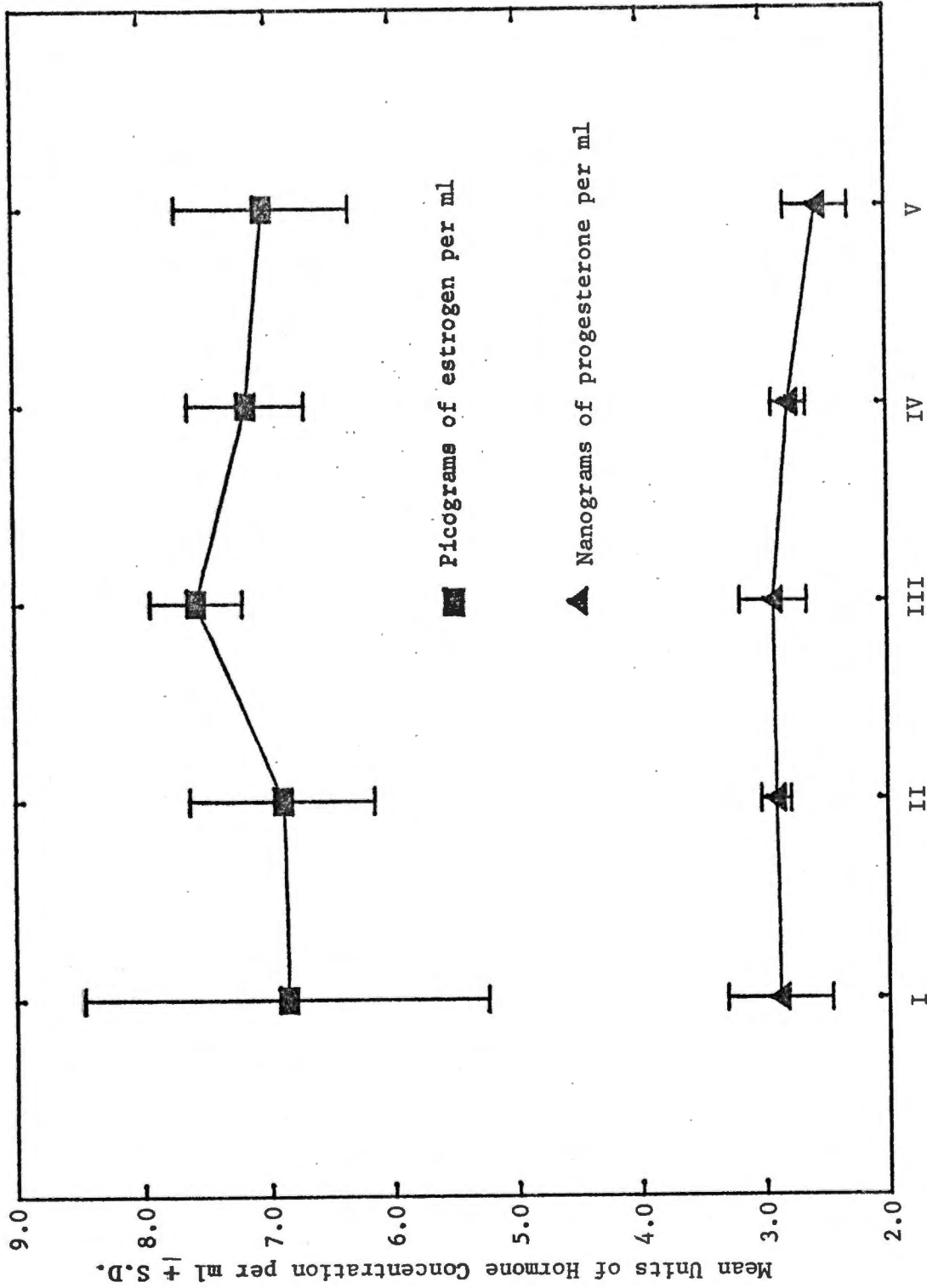
Figure 8. Effect of repeated freezing and thawing on estrogen and progesterone concentration.

CHAPTER V

SUMMARY AND CONCLUSIONS

Competitive protein binding assay of progesterone and radio-immunoassay of estrogen were used to evaluate the effects of various blood handling techniques on the stability of estrogen and protesterone concentrations in peripheral blood. In one phase of the study, eight 40 to 45 ml samples were collected consecutively from each of eight cows (4 beef type and 4 dairy type). These eight samples were subjected to methods of preparation that might be employed by an investigator collecting either plasma or serum for hormone assay.

Treatments consisted of collecting samples in pre-cooled heparinized (1000 U/ml) tubes maintained in an ice bath, centrifuging immediately (2400 rpm for 30 minutes) and storing the plasma fraction at $-15°C$; and collecting samples in heparinized or non-heparinized, pre-cooled and warm (room temperature) tubes. These were held as whole blood either at $4°C$ for 24 hours or at room temperature for 4 or 24 hours prior to centrifugation and storage of the plasma or serum fraction at $-15°C$. The last of the eight samples collected from each cow was handled identically to the first sample to evaluate the effects of animal stress and volume of blood collected on hormone concentration.

In another phase of this study, assay aliquots of pooled cow plasma were subjected to repeated freezing and thawing in order to evaluate the effects of this treatment on estrogen and progesterone

44

concentrations. Plasma used for progesterone assay received six subsequent freezings and thawings while plasma used for estrogen assay received seven.

The average recovery of labeled progesterone added to cow plasma was 93.3 ± 0.64 percent. Following the addition of 3 ng of unlabeled progesterone to steer plasma, the average recovery was 96.9 ± 0.45 percent or 2.9 ng of progesterone. RIA was carried out using one 6 ml extraction and 2 hours ice water incubation. Recovery of 20 pg and 50 pg of estradiol-17β added to pooled cow plasma averaged 91.0 ± 0.35 percent and 81.0 ± 0.60 percent, respectively. Average recovery of estradiol-17β^3H from a cow being assayed was 89.0 ± 1.5 percent.

In the progesterone assay, treatment means of all treatments were significantly different as were differences between individual cows ($P < 0.001$). Cow type had no influence on the response to the various treatments. There was significant difference between plasma centrifuged immediately and the plasma or serum samples held at 4°C for 24 hours prior to centrifugation ($P < 0.05$). There was no difference between plasma and serum progesterone concentrations in samples that were subjected to the same handling methods.

Samples collected in warm (room temperature) tubes and held as whole blood at room temperature until centrifuged were significantly lower in progesterone content than those collected in cool tubes ($P < 0.001$). Warm tube samples held as whole blood at room temperature for 24 hours were significantly lower ($P < 0.001$) than warm tube samples held for 4 hours. When compared to the control in this study, the serum

samples held as whole blood for 4 hours at room temperature were 38 percent lower in progesterone content, while serum samples collected after 24 hours at room temperature were 89 percent lower in progesterone than the control.

In the estrogen assay there was significant difference between cows ($P < 0.025$) but no difference between cow type. There was no cow type x treatment interaction. By analysis of variance, there was no difference between treatment means. Certain trends were observed, however, that may indicate there is a relationship between stage of the estrous cycle and response to treatment; but it was not possible in this study to correctly evaluate this possibility.

It was concluded that progesterone concentration in a whole blood sample is susceptible to the effects of time and temperature from the interval of sample collection to centrifugation for separation of plasma or serum. Estrogen concentrations in samples prepared for RIA of estrogen are apparently not influenced by the effects of time or temperature from the interval of collection to centrifugation as is progesterone. It is suggested, however, that samples for RIA of estrogen be centrifuged and stored at sub-zero temperature as soon as possible. Neither stress of bleeding nor volume of blood collected had any apparent effect on estrogen or progesterone concentration in peripheral blood. It was determined that plasma samples may be frozen and thawed repeatedly with no effect on the estrogen or progesterone concentration in the sample.

LIST OF REFERENCES

LIST OF REFERENCES

1. Axelrod, L. R., and N. T. Werthessen. 1960. The metabolism of estrone-16-C^{14} in bovine blood. Arch. of Biochem. Biophys. 86:53.

2. Baird, D. T. 1968. A method for the movement of estrone and estradiol-17β in peripheral human blood and other biological fluids using 35SPipsyl chloride. J. Clin. Endocr. 28:422.

3. Batson, D. B. 1972. Progesterone and estrogen levels in the jugular venous plasma of non-bred, non-pregnant and pregnant cows. Ph.D. Thesis. University of Tennessee, Knoxville, Tennessee.

4. Campbell, D. H., J. S. Garvey, N. E. Crewer, and D. H. S. Scessdorf. 1964. Methods in Immunology. W. A. Benjamin, Inc., New York.

5. de Sonza, M. L. A., H. O. Williamson, L. O. Moody, and E. Diczfalusy. 1970. Further assessment of the reliability of progesterone assays by competitive protein binding. In E. Diczfalusy (Ed.) 2nd Symposia: Steroid Assay by Protein Binding. Transactions of the Second Symposium in Geneva on March 23-25, 1970. Karolinska Symposia on Research Methods in Reproductive Endocrinology. Bogtrykkeriet Forum, Copenhagen.

6. De Venuto, F. 1967. Interaction of progesterone and aldosterone with red blood cells of the rat. Proc. Soc. Exp. Biol. (N.Y.) 124:478.

7. Dowlen, H. H. 1972. Progesterone levels in cows suspected of embryonic mortality. Masters Thesis, University of Tennessee, Knoxville, Tennessee.

8. Erb, R. E., R. D. Randel, and C. J. Callahan. 1971. Sex steroid changes during the reproductive cycle. J. Ani. Sci. 32:80.

9. Giorgi, E. P., and P. G. Crosignani. 1969. Competitive binding of free and conjugated estrogens to plasma proteins. J. Endocr. 44:219.

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

11. Henricks, D. M., J. F. Dickey, and G. O. Nieswender. 1970. Serum lutenizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. Biol. of Reprod. 2:346.

12. Johansson, D. R. B., J. D. Neill, and E. Knobil. 1968. Preovulatory progesterone concentration in the peripheral plasma of the Rhesus monkey with a methodologic note on the detection of estrus. Endocr. 82:143.

13. Keller, N., L. R. Sendelbeck, U. I. Richardson, C. Moore, and F. E. Yates. 1966. Protein binding of corticosteroids in undiluted rat plasma. Endocr. 79:884.

14. Korneman, S. G., L. E. Perrin, and T. P. McCallum. 1964. Radio-ligand binding assay system for estradiol measurement in human plasma. J. Clin. Endocr. 29:879.

15. Lipsett, M. D., P. Doerr, and J. S. Bermudez. 1970. Saturation assays for plasma progesterone and 17-hydroxyprogesterone. In E. Diczfalsy (Ed.) 2nd Symposia: Steroid Assay by Protein Binding. Transactions of the Second Symposium in Geneva on March 23-25, 1970. Karolinska Symposia on Research Methods in Reproductive Endocrinology. Bogtrykkeriet Forum, Copenhagen.

16. Martin, B. T., B. A. Cooke, and W. P. Black. 1968. Evaluation of rapid method for measurement of plasma progesterone by competitive protein binding. J. Endocr. 46:369.

17. Martin, B. T., B. A. Cooke, and W. P. Black. 1970. Further studies on the measurement of plasma progesterone by competitive protein-binding. J. Endocr. 48:XIX (Abstract).

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

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

20. Murphy, B. E. P. 1969. Protein binding and the assay of non-antigentic hormones. Recent Progr. Hormone Res. 25:563.

21. Murphy, B. E. P. 1970. Methodological problems in competitive protein-binding techniques: The use of sephadex column chromotography to separate steroids. In E. Diczfalusy (Ed.) 2nd Symposia: Steroid Assay by Protein Binding. Transactions of the Second Symposium in Geneva on March 23-25, 1970. Karolinska Symposia on Research Methods in Reproductive Endocrinology. Bogtrykkeriet Forum, Copenhagen.

22. Murphy, B. E. P. 1971. Chapter V. Hormone assay using binding proteins in blood. Principles of Competitive Protein Binding Assays (Ed.). W. D. Odell and W. H. Daughaday, J. B. Lippincott Co., Philadelphia and Toronto.

23. Neill, J. D., E. E. B. Johansson, J. K. Datta, and E. Knobil. 1967. Relationship between the plasma levels of lutenizing hormone and progesterone during the normal menstral cycle. J. Clin. Endocr. Metab. 27:167.

24. Ostle, B. 1954. Statistics in Research. Iowa State College Press, Ames, Iowa.

25. Palmer, R., J. A. Blair, G. Ericksson, and E. Diczfalusy. 1966. Studies on the metabolism of C-21 steroids in the human foeto-placenta. III. Metabolism of progesterone and 20α- and 20-β-dihydro-progesterone by mid term placentas profused in situ. Acta. Endocr. (Kbh) 53:407.

26. Robertson, H. A., and I. R. Sarda. 1971. A very early pregnancy test for mammals: Its application to the cow, ewe, and sow. J. Endocr. 49:407.

27. Sandberg, A. A., W. R. Salunwhite, Jr. and H. N. Antoniades. 1957. The binding of steroids and steroid conjugates to human plasma proteins. Recent Progr. Hormone Res. 13:209.

28. Short, R. B. 1958. Progesterone in blood. I. The chemical determination of progesterone in peripheral blood. J. Endocr. 16:415.

29. Snook, R. B., R. R. Saatman, and W. Hansel. 1971. Serum proges-terone and luteinizing hormone levels during the bovine estrous cycle. Endocr. 88:678.

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

31. Thorburn, G. D. and W. Schnieder. 1972. Progestin concentration in the plasma of the goat during the oestrus cycle and pregnancy. J. Endocr. 52:23.

32. Van der Molen, H. J. and D. Groen. 1968. Interconversion of pro-gesterone and 20α-hydroprogesterone and of androstenedione and testosterone in vitro by blood and erythrocytes. Acta. Endocr. (Kbh) 58:419.

33. Wall, P. E., and C. J. Migeon. 1959. In vitro studies with 16-C^{14} estrone: Distribution between plasma and red blood cells of man. J. Clin. Invest. 38:611.

34. Werthessen, N. T., C. F. Baker, and N. S. Field. 1950. Conversion of esterone on incubation in blood. J. Biol. Chem. 184:145.

VITA

Vaughan A. Wenzel was born in Jonesboro, Arkansas on September 14, 1948. He attended elementary schools in Ashya, Japan; Abilene, Texas; Memphis, Tennessee; and Bossier City, Louisiana. He completed junior high school in Bossier City and entered high school there, and then graduated from George Washington High School in 1966 on Agana, Guam.

He received his Bachelor of Science degree with a major in Animal Husbandry in December, 1970 from the University of Tennessee, and upon graduation entered graduate school in the Animal Husbandry Department. While at the University of Tennessee, he participated in the Reserve Officers Training Corps and was commissioned a Second Lieutenant in the United States Air Force on June 8, 1972. During his ROTC training he was presented the Staff Officer Award and was a member of both the Arnold Air Society and Scabbard and Blade.

During his graduate work he was a member of Phi Sigma Society of Biological Sciences and the American Association of Laboratory Animal Science.

He is the son of Lt. Col. and Mrs. Alfred E. Wenzel (USAF Ret.) of Memphis, Tennessee, and is married to the former Donna Lynn Tate of White House, Tennessee.