Progesterone levels in the jugular vein and posterior vena cava of normal cycling Holstein heifers
by Dennis Paul Davis

Abstract:
Four normally cycling Holstein heifers were cannulated with Silicone rubber tubing in the jugular vein and posterior vena cava. The blood samples were analyzed for progesterone with a competitive proteinbinding assay which included a progesterone-internal standard. The study included five normal and four abnormal estrous cycles, ranging from 18 to 21 days and 14 to 30 days in length, respectively.

A comparison of 142 plasma progesterone pairs (jugular -vs- vena cava) showed a significantly higher concentration in the vena cava (P<.001). Average daily progesterone concentration in vena cava plasma of three heifers during five normal estrous cycles ranged from a low of 0.98 + 1,30 ng/ml at estrus to a high of 8.62 + 2.83 ng/ml at Day 15. Complete daily progesterone concentrations for jugular plasma were obtained for two normal estrous cycles. At the day of estrus a mean of 1.25+ 0.35 ng/ml was observed followed by a low of 0.35 + 0.50 ng/ml at Day 3 and a high of 3.00 + 0.60 ng/ml at Day 16.

A t-test of 10 progesterone sample-pairs (jugular -vs- vena cava) for the day of estrus found no significant differences; however, the ratio (jugular/vena cava) was 1.0 or greater for seven of the 10 pairs with a mean ratio of 7,29 + 5.06. Ratios for the three days before estrus starting with the day preceding estrus. were 0.81 + 0.52, 0.49 + 0.12 and 0.47 + 0.14, respectively. The progesterone levels in the jugular vein exceeded that in the vena cava during four of the five surgery days. These data suggest that blood sampling from several sources within an animal may be useful in a compartmental analysis approach to. hormone binding and metabolism in vivo.

In presenting this thesis in partial fulfillment of the requirements for an advanced degree of Montana State University, I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by my major professor, or, in his absence, by the Director of Libraries. It is understood that any copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Signature _Dennis Paul Davis_

Date _July 23, 1970_

PROGESTERONE LEVELS IN THE JUGULAR VEIN AND POSTERIOR VENA
CAVA OF NORMAL CYCLING HOLSTEIN HEIFERS

by

DENNIS PAUL DAVIS

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree
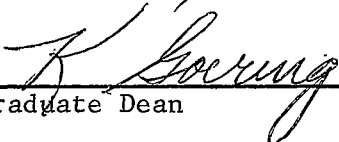
of

MASTER OF SCIENCE

in

Animal Science

Approved:

_____
Head, Major Department

_____
Chairman, Examining Committee

_____
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 1970

iii

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

INDEX TO APPENDIX

x

LIST OF FIGURES

ABSTRACT

Four normally cycling Holstein heifers were cannulated with Silicone rubber tubing in the jugular vein and posterior vena cava. The blood samples were analyzed for progesterone with a competitive protein-binding assay which included a progesterone-^{14}C internal standard. The study included five normal and four abnormal estrous cycles, ranging from 18 to 21 days and 14 to 30 days in length, respectively.

A comparison of 142 plasma progesterone pairs (jugular -vs- vena cava) showed a significantly higher concentration in the vena cava (P < .001). Average daily progesterone concentration in vena cava plasma of three heifers during five normal estrous cycles ranged from a low of 0.98 ± 1.30 ng/ml at estrus to a high of 8.62 ± 2.83 ng/ml at Day 15. Complete daily progesterone concentrations for jugular plasma were obtained for two normal estrous cycles. At the day of estrus a mean of 1.25 ± 0.35 ng/ml was observed followed by a low of 0.35 ± 0.50 ng/ml at Day 3 and a high of 3.00 ± 0.60 ng/ml at Day 16.

A t-test of 10 progesterone sample pairs (jugular -vs- vena cava) for the day of estrus found no significant differences; however, the ratio (jugular/vena cava) was 1.0 or greater for seven of the 10 pairs with a mean ratio of 7.29 ± 5.06. Ratios for the three days before estrus starting with the day preceding estrus were 0.81 ± 0.52, 0.49 ± 0.12 and 0.47 ± 0.14, respectively. The progesterone levels in the jugular vein exceeded that in the vena cava during four of the five surgery days. These data suggest that blood sampling from several sources within an animal may be useful in a compartmental analysis approach to hormone binding and metabolism in vivo.

INTRODUCTION

The isolation of progesterone was achieved almost simultaneously by four groups of workers. The hormone was isolated in 1934 by the extraction of sows' ovarian tissue, and later the same year its structure was determined. It has been reported in bovine CL, ovaries, adrenals, placenta and blood.

Progesterone appears important in preparation of the uterus for implantation of the blastocyst, maintenance of pregnancy, as a conditioning agent in estrus, normal cyclic functions as a factor affecting tubal and uterine transport of sperm and ova, development of the alveolar system in the mammary gland, and hypophyseal-gonadal interrelationships.

Until recently studies relating to the metabolism and secretion of progesterone have been hindered by the lack of techniques sufficiently sensitive and specific enough for the quantitation of the hormone in small volumes of plasma. In the past it has been necessary to obtain reproductive tissue during surgery or slaughter for the estimation of progesterone.

There is not a great deal known about the hormone interrelationships involved in reproduction and any research providing basic information about these processes is worthwhile.

The present study was designed to quantitatively determine progesterone concentrations in the blood during the cycle to provide knowledge beneficial in future research. Some of the important phases of research

that may utilize conclusions drawn from this work are the estrus

synchronization of cattle and attempted super ovulation leading to mul-

tiple births.

REVIEW OF LITERATURE

Cannulation Materials.-

A procedure using a medical grade of silicone rubber tubing for vascular cannulation was developed by Hull (1967). The tubing was physiologically inert, flexible and could be autoclaved many times. It was found that inherent rigidity was necessary for patency in blood collection. However, the tubing should have sufficient flexibility to not traumatize the vascular endothelium and induce thrombophlebitis as does the stiffer tubing. Christison and Curtin (1969) utilized silicone rubber for a catheter in the cranial vena cava of the pig. Venous blood samples could be drawn, or fluid injected into unanesthetized and unrestrained animals. Hamilton (1965) introduced silicone rubber catheters into the descending aorta of dogs and cats.

Robinson et al. (1969) devised an improved polyethylene cannulation technique. The method was developed to prevent the accidental removal of cannulae. A small bead was formed in the tubing which makes it possible to secure the cannula in place. A small section of the polyethylene tubing is heated momentarily which causes the plastic to contract lengthwise. This causes the formation of a small bead on the outside of the tube, but does not alter the lumen. Sutures can be secured immediately behind the bead or it can be passed into a vessel and the incision closed tightly around the tubing. Popovic and Popovic (1960) described a technique whereby the aorta and the vena cava of rats and ground squirrels are permanently intubated using polyethylene tubing. Shoemaker

et al. (1965) utilized polyethylene tubing for catheterization of the major hepatic vessels of dogs. Conner and Fries (1960) siliconized the inside and outside of polyethylene tubing for catheterization of portal and mesenteric veins of Holstein-Friesian calves. Conrad et al. (1958) fitted a polyethylene catheter in the gastrosplenic vein of a calf. Lumb et al. (1966) collected adrenal venous blood from cattle with polyethylene cannulae.

Blair-West et al. (1962) collected adrenal venous blood from sheep fitted with cannulae made of poly-vinyl tubing. Ralston et al. (1949) described a method whereby poly-vinyl chloride catheters were placed in the external jugular veins of cows. Katz and Bergman (1969) employed cannulae consisting of medical grade poly-vinyl tubing with attached "cuffs" for simultaneous collection of blood entering or leaving the liver and portal bed of the unanesthetized sheep.

Moodie et al. (1963) used two flexible polyamide (nylon) tubes, one of which fitted inside the other for the collection of portal and hepatic venous blood from conscious sheep. The authors found nylon to be less reactive than polyethylene and poly-vinyl chloride catheters, although within half an hour after insertion the nylon was coated with a fiberous coating. Ultimately, the presence of the nylon catheter resulted in the development of a low grade phlebitis.

Cannulation and Catheterization Sites in Various Animals.-

Ralston et al. (1949) inserted catheters in both external jugular

veins of cows. Radioactive substances were introduced into one jugular vein and blood samples were withdrawn from the other. The functional range of this catheter was 2.1 hours to 14.5 days. Popovic et al. (1968) surgically placed cannulae in the jugular veins of 50 mice. The polyethylene cannulae remained patent for drug injection as long as an animal lived. However, when blood samples were drawn, some cannulae were not functional longer than 10-15 days.

Popovic and Popovic (1960) exposed the right jugular vein of rats and ground squirrels. An incision was made in the anterior portion of the jugular to allow a cannula to be pushed into the body of the vein until the tip laid in the junction of the right superior and inferior vena cava. The cannulae would remain functional for 40 days with proper care. Christison and Curtin (1969) described a surgical technique whereby a catheter was inserted in the facial and external jugular veins of the pig and passed into the cranial vena cava. The catheters were placed in 11 pigs and remained patent for 0-47 days.

Popovic and Popovic (1960) made incisions in the neck of rats and ground squirrels in order to expose the left carotid artery. A small incision not larger than one-third the diameter of the carotid was made in the anterior part of the artery. A polyethylene tube was inserted through the incision and passed down the vessel until the tip reached the aorta. The cannula floating in the aortic blood stream seemed to have a longer life than those cannulae ending in the carotid. Hamilton

(1965) surgically implanted arterial catheters which allowed blood sampling and injection of fluids into unanesthetized and undisturbed dogs and cats. The silicone tubing is inserted into the descending aorta by way of the carotid artery and the free end passed through the skin at the back of the neck.

Conner and Fries (1960) described a technique for collecting portal vein blood from unanesthetized calves. The cannulae were used successfully for obtaining portal blood samples up to 73 days after an operation. Moodie et al. (1963) fitted catheters in the portal vein of sheep. The tip of the catheter laid in the vessel at the entrance to the liver (porta hepatis). Venous blood samples were collected from the region for an average period of 83 days (range 41-161 days). Katz and Bergman (1969) developed a procedure for cannulation of the hepatic vein of sheep. Cannulae remained patent up to five months, but the average functional period was five weeks. Shoemaker et al. (1965) described a surgical method for catheterization of the common hepatic vein, portal vein and splenic artery of dogs. The method allowed simultaneous blood sampling from all three major hepatic vessels in an unanesthetized dog.

Hume and Nelson (1954) developed a cannulation procedure for adrenal venous blood collection from conscious dogs. Blair-West et al. (1962) devised a surgical method for the successful cannulation of the adrenal vein in the sheep. Blood was allowed to flow from the left adrenal vein through a poly-vinyl tube, which passed outside the body and

was inserted in the jugular vein. Samples were collected from the conscious animal for a period of 2-4 days by breaking a Teflon joint inserted in the tubing. Lumb et al. (1966) reported a technique for obtaining adrenal venous blood from the conscious, as well as the anesthetized calf. The adrenal vein was cannulated and a loop of the polyethylene tubing brought through a stab wound in the skin and passed back into the body through another stab wound. Then the tip of the cannula was inserted in the posterior vena cava to maintain blood flow and allow free access to the cannula for venous effluent collection.

Hull (1967) described a procedure for the successful cannulation of the posterior vena cava of cattle. The cannulae were surgically placed in 12 Holstein-Friesian heifers and remained patent in blood sampling for 4 to 90 days (average functional life of 35 days).

Assay Methods for Progesterone.-

Studies concerned with the quantitative determination of progesterone levels in small volumes of plasma have been hindered in the past by the lack of sufficiently sensitive and specific chemical techniques. Gomes and Erb (1965) summarized all the available assay methods in a review of progesterone involvement in bovine reproduction.

Bush (1952) devised a paper chromatographic method for the effective isolation of small amounts of steroids in body fluids and biological media. Edgar (1953a) and Edgar (1953b) utilized ultra-violet absorption spectroscopy to determine progesterone content of body tissues and fluids.

Loy et al. (1957) developed a modified spectrophotometric technique which would permit estimation of the progesterone content of luteal tissue. Short (1958) described the successful quantitation of progesterone in large volumes of peripheral blood.

The general procedure for this widely used spectrophotometric method was an ether or hot alcohol extraction, column chromatography, countercurrent distribution, paper chromatography, ultra-violet absorption at 240 mu and the use of progesterone -4-^{14}C (Staples and Hansel, 1961 and Stormshak et al. 1961).

The spectrophotometric methods have a limit of sensitivity ranging from 0.5 to 1.0 ug. if micro-cells are used in the spectrophotometer. Otherwise, five times as much hormone is required. Modification of the spectrophotometric techniques have been made to improve their sensitivity in progesterone measurements. Sommerville et al. (1963) quantitated progesterone in 5-10 ml of human plasma with a modification of the thiosemicarbazide reaction. The method was commonly used for 8-10 years in estimating progesterone content of tissues and yielded satisfactory progesterone determinations to 0.1 ug, but lacked the sensitivity necessary for blood analysis.

Short and Levitt (1962) described a fluoremetric procedure that was quite sensitive, but required purification and extreme care with reagents and paper to provide the necessary specificity. However, when the method was compared with the spectrophotometric method of Short

(1958), the fluorescence gave higher results at low concentrations of progesterone and lower results at high concentrations, indicating a lack of specificity in the fluorescence reaction. Riondel et al. (1962) applied the double-isotope-derivative procedure to the estimation of progesterone using progesterone^{3}H with thiosemicarbazide^{35}S as the derivative forming agent. Woolever and Goldfein (1963) later reported quantitation by the conversion of progesterone to 20 B-ol using tritiated sodium borahydride and progesterone^{14}C as the isotope sources. This method allowed effective measurements to slightly less than 0.01 ug of progesterone. Excellent techniques in gas chromatography have been reported by Yannone et al. (1964) and Stabenfeldt et al. (1970). Such methods afford sensitivity and specificity, but require a great deal of training to successfully apply them to steroid determination.

It has been proven recently that radio-immunoassays are sensitive and accurate in assaying the polypeptide hormones (Everett, 1969). The radio-immunoassay technique has been extended to steroid measurements and has the advantage that purification of the steroid is not necessary. However, Abraham (1969) does stress the importance of highly purified radio-labelled steroids.

Ferin et al. (1968) reported a radio-immunoassay technique for estradiol which employed an antisera raised in a ewe against estradiol-17B conjugated to bovine serum albumin. The assay has allowed detection of as low as 10 picograms estradiol, with dose response curves proven to

be linear between 10 and 200 picograms. Abraham (1969) has described a procedure for the radio-immunoassay of estradiol-17B. Plastic test tubes are coated with a standard amount of specific antibody and the quantity of radioactively labelled estradiol-17B that remains unbound or bound is determined in the presence of the test solution. Midgley et al. (1969) has demonstrated that the hapten-radio-immunoassay can be utilized for the quantitative determination of low molecular weight substances which can be made immunogenic by conjugation to a protein. This procedure has been employed for the quantitation of non-conjugated estrogenic steroids by the radioiodination of the protein portion of the conjugate.

Hendricks et al. (1969) employed gas-liquid chromatography and the protein-binding method to determine progesterone levels in systemic plasma collected from cows during the estrous cycle. Blood plasma analyzed with gas chromatography for Days 8, 12, 16 and 20 after estrus had progesterone levels at 4.4 ± 1.2, 7.9 ± 1.2, 7.1 ± 0.5, 1.4 ± 0.2 ng/ml, respectively. Determinations made with the protein-binding assay were 0.6 ± 0.2, 5.2 ± 0.7, 4.9 ± 0.3, 5.8 ± 0.9, 1.6 ± 0.6 and 0.4 ± 0.1 ng progesterone per ml of plasma for 0, 10, 15, 17, 19 and 21 days after estrus. The results of this study indicated that plasma progesterone could be effectively measured with the less laborious protein-binding assay.

Competitive Protein-Binding Radioassay.-

The competitive protein-binding assay is a relatively simple, fast procedure for determining steroid levels in small volumes of plasma. The technique is specific in that the corticosterone-binding globulin (CBG, transcortin) has a binding affinity for only cortisol, corticosterone and progesterone.

This steroid binding protein was designated as CBG (cortisol-binding globulin) by Daughaday (1958) and transcortin by Slaunwhite and Sandberg (1959). Later, Murphy et al. (1963) termed the ∝ globulin fraction as corticosteroid-binding globulin, which she used in a simple method for determining plasma corticoids. The assay employing this binding globulin has been named by Murphy (1964) as the "competitive protein-binding (PB) analysis" and by Barakat and Ekins (1961) as a "saturation analysis".

Murphy et al. (1963) utilized the steroid-binding properties of plasma to estimate levels of cortisol and corticosterone in human plasma. When increasing amounts of unlabelled cortisol were added to an equilibrium dialysis system containing plasma and a constant amount of cortisol-4-^{14}C, there was a proportional decrease in the percentage of cortisol-4-^{14}C bound to the plasma protein. These findings suggested that the binding depression induced by added steroid might provide a process for determining plasma levels of cortisol and corticosterone. Murphy (1967) found it possible to routinely measure cortisol in 0.1 ml of plasma or

cerebrospinal fluid and progesterone in 0.3 ml of plasma. She was also able to determine levels of compound S(11-desoxycortisol), corticosterone, cortisone, and 17-hydroxyprogesterone with the protein-binding assay. Neill et al. (1967a) analyzed the daily relationship between the concentrations of luteinizing hormone (LH) and progesterone in the peripheral plasma during the normal human menstrual cycle. Progesterone levels were determined in 0.5 ml aliquots of plasma by utilizing a modification of the protein-binding method reported by Murphy (1967). Neill et al. (1967b) determined the plasma progesterone concentrations throughout the menstrual cycle of normal Rhesus monkeys by employing the competitive protein-binding technique. Johansson et al. (1968) used the protein-binding assay to measure the peripheral plasma progesterone concentrations in normal cycling Rhesus monkeys at the day of ovulation.

Bassett and Hinks (1969) utilized the assay for the determination of corticoids in 0.1 ml or less of ovine plasma. Bassett et al. (1969) quantitated progesterone levels in the peripheral plasma of pregnant ewes throughout gestation with the protein-binding assay.

Before progesterone or other steroid levels can be quantitatively determined with the protein-binding assay they must be effectively isolated. Neill et al. (1967a) reported that progesterone could be extracted from 0.5-1.0 ml of plasma with 5 ml reagent grade Petroleum Ether (boiling 30°-60°C). This procedure extracted 90 percent of the progesterone from the plasma, and left behind over 99 percent of the

cortisol and corticosterone. After extraction, progesterone was isolated from its metabolites in a single chromatographic step. Samples were spotted on silica gel thin layer sheets and developed in ether-benzene 2:1 by ascending chromatography.

The manner in which the competitive protein-binding globulin is prepared is an important consideration in the assay. Murphy (1967) investigated the effectiveness of plasma from various species as a binding globulin in the competitive protein-binding assay. One ml of dog plasma could be used satisfactorily over the range 0.2-4 ng cortisol, monkey plasma for the range 0.2-4 ng corticosterone, cat plasma was effective over the range 0.2-4 ng 11-desoxycortisol, rabbit plasma over the range 0.2-4 ng cortisol and chicken plasma was satisfactory for 0.2-4 ng cortisol, 11-desoxycortisol, progesterone, or cortisone. Murphy (1967) concluded a 100-fold increase in the sensitivity of the assay could be obtained by selecting the proper species for the source of the binding protein and the use of tritiated steroids of higher specific activity than the previously used ^{14}C labeled steroids. Neill et al. (1967a) prepared CBG-corticosterone -1, 2-^{3}H by diluting 2.5 ml of dog plasma to 100 ml with distilled water. Then 25 ng of corticosterone -1,2-^{3}H (100 ng/ml ethanol) was added and the solution mixed gently. Johansson et al. (1968) enhanced the sensitivity of the assay by increasing the activity of the tritiated corticosterone from 33.4 to 57.2 c/mmole. The higher specific activity of the steroid allowed the mass of corticosterone

-1,2-^{3}H to be reduced from 25 to 15 ng for a solution containing 2.5 ml

of dog plasma dispersed in 97.5 ml of distilled water.

In order to quantitatively determine the steroid levels in plasma

it is necessary to establish a standard curve for every group of samples

analyzed. Neill et al. (1967a) pipetted a standard curve representing

1, 2, 5, and 10 ng of progesterone from aliquots of a progesterone solu-

tion in absolute ethanol (100 ng/ml). One ml of CBG saturated with

corticosterone -1,2-^{3}H was added to each sample tube containing isolated

progesterone and every member of the standard curve. All tubes were

placed in a 45°C water bath for five minutes to bring the progesterone

into solution. The tubes were transferred to an ice bath and allowed to

remain there for 10 minutes to increase the affinity of the CBG for the

steroids. The steroids establish a dynamic equilibrium and compete for

the binding sites on the protein.

A variety of procedures have been used to separate the unbound

steroids from the fraction bound to the protein. Murphy et al. (1963)

developed a dialysis method for estimating cortisol and corticosterone

in human plasma. The chief disadvantage of dialysis from the clinical

point of view is the 40 hours required for the process. Murphy and

Pattee (1964) used corticosteroid-binding globulin in a gel filtration

procedure to determine plasma corticoids. It was similar to the di-

alysis method employed earlier by Murphy et al. (1963) and retained the

specificity, precision, and sensitivity. However, gel filtration could

be carried out in approximately two hours. De Moor et al. (1962) determined the amount of transcortin-bound corticoids in human plasma by using gel filtration on Sephadex columns. At a later time, De Moor et al. (1963) used this procedure to study the influence various steroids have on the cortisol-binding ability of human plasma in vitro. Murphy (1967) examined the effectiveness of several insoluble adsorbing agents (Fuller's earth, Florisil, coated charcoal) in separating protein-bound and unbound steroids. Murphy (1967) concluded that Fuller's earth, coated charcoal, and Lloyd's reagent were satisfactory for separating protein-bound and unbound cortisol, while Florisil and coated charcoal were suitable for the separation of protein-bound and unbound corticosterone. Neill et al. (1967a) reported that 80 mg of Florisil (activated magnesium silicate, 60-100 mesh) effectively adsorbed to all of the unbound labelled corticosterone in the solution when mixed for exactly 30 seconds in a Vortex mixer.

Neill et al. (1967a) had established that 0.5 ng of progesterone could be measured with a high degree of precision using the protein-binding assay. The lower limit of sensitivity in the technique appeared to be approximately 0.2 ng of progesterone. Johansson et al. (1968) found that saturation of the binding-globulin in dog plasma with high specific activity (57.2 c/mmole) corticosterone, in addition to the rigid standardization (exactly 30 seconds) of the shaking in the Vortex

mixer allowed detection of as little as 0.1 ng progesterone in 0.5 ml monkey plasma.

Progesterone Levels in the Non-pregnant Cow.-

Corpus Luteum and Ovary.- Several research workers concerned with various aspects of reproduction have measured progesterone levels in luteal tissue from cows and heifers at different stages of the estrous cycle.

Erb et al. (1962) collected 42 corpora lutea from 31 cows and heifers throughout the estrous cycle and analyzed them for progesterone content. Three cows had a small amount of progesterone in regressing corpora lutea (CL) during estrus or one day after, but there was no detectable progesterone at Day 2. However, from three to nine days after estrus, the average progesterone content increased from 16 to 158 ug per corpus luteum. The mean progesterone content of the corpus luteum reached a maximum of 570 ug at Day 14, which was followed by a sharp decline for Days 15 through 20. The considerable variation among animals in luteal tissue progesterone content was apparently related to the body weight and age of the animal. The mean concentration of progesterone was found to be 19 ug/g at Day 3 and reached a peak of 38 ug/g at Day 5, declining to 25 ug/g at Day 9, with a corresponding rapid increase in the concentration to a peak of 73 ug/g at Day 14.

Erb and Stormshak (1961) concluded the developing corpus luteum has a high progestin concentration (progesterone and $\triangle$ 4-pregnene-20B-ol-

3-one) per gram of luteal tissue on the second and third days of the cycle, but the total quantity was less than 100 ug until after Day 6. At a later stage of the estrous cycle (Days 14-16), the average progestin content rose to 251 ug. They also reported that progestins in the corpus albicans ranged from 0 to 24 ug before ovulation.

Armstrong and Hansel (1959) concluded that administration of natural or synthetic oxytocin to cycling heifers during the first week after estrus resulted in a shortened estrous cycle of 8 to 12 days. Corpora lutea formed during the oxytocin treatment period were smaller than the normal CL. The effect of exogenous oxytocin on the progestin content of the CL was investigated by Mares and Casida (1963). Normal cycling heifers were injected intravenously with (110 I.U.) oxytocin twice daily on Days 12 and 13, and the corpus luteum removed on Day 14, 24 hours after the last injection. The treated corpora lutea had a significantly ($P < .01$) greater content of both △ 4-pregnene-20B-ol-3-one) and progesterone than control CL.

Ovarian Vein.- The progestin levels in venous plasma collected through laparotomy and sectioning one of the veins draining the ovary containing the active CL or ripened follicle in three non-pregnant cows and heifers was analyzed by Gomes et al. (1963). The plasma levels were not detectable at estrus or one day following. However, there was a significant increase in the progestin concentrations in the ovarian venous plasma from Day 2 of the estrous cycle to a peak level of 6.2 ug/ml on

Day 15. Dobrowolski et al. (1968) determined the progesterone content

of ovarian venous blood for nine cycling cows, three of which rose from

5.6 ug/100 ml plasma on Day 1 of the estrous cycle, to approximately

125 ug/100 ml at Day 8, and to about 180 ug/100 ml on Days 14 and 15 of

the cycle. After this, there was a rapid decline (10 to 20 ug/100 ml)

as the animals approached ovulation.

Jugular Vein.- Gomes et al. (1963) reported progesterone levels

ranging from non-detectable to 0.0540 ug/ml of plasma in the jugular

blood of 31 non-pregnant, cycling dairy cows and heifers. Stabenfeldt

(in a discussion by Kaltenbach and Cook, 1968) reported a rapid rise in

jugular vein plasma progesterone levels after estrus in four cows start-

ing about Day 4 and continuing through about Day 16, followed by a mark-

ed decline at approximately Day 17 or 18.

Stabenfeldt et al. (1969) determined the daily progesterone levels

in the jugular plasma of six cows during seven complete estrous cycles.

The progesterone levels extended from less than 0.5 ng/ml plasma during

the follicular phase to 6.6 ng/ml plasma at peak luteal phase. The

study indicated that progesterone levels measured throughout 21-day

cycles increased markedly from Day 3 to Day 8 (estrus = Day 1) with a

much slower rate of increase from Day 8 to Day 17.

Progesterone Levels in the Pregnant Cow.-

Corpus Luteum and Ovary.- Gorski et al. (1958) determined the

progesterone content of corpora lutea (five assays each containing three

CL) taken from pregnant cows. The luteal tissue contained an estimated

90 percent of the progesterone found in the ovaries of cows with levels

ranging from 6.4 to 15.2 ug/g of tissue. The ovaries of a Jersey heifer

pregnant 259 days contained an estimated 50 ug of progesterone.

The progesterone concentration in bovine corpora lutea throughout

gestation were investigated by Melampy et al. (1959). They reported a

mean of 2.3 ± 0.7 ug progesterone/g of luteal tissue for Days 10-49 of

pregnancy, 3.6 ± 1.5 ug/g for Days 50-89, 5.0 ± 1.2 ug/g for Days 90-

129, 3.8 ± 0.9 ug/g for Days 130-169, 3.9 ± 0.7 ug/g for Days 170-209,

1.5 ± 0.5 ug/g for Days 210-249, and 1.1 ± 0.3 ug/g for Days 250-280.

The residual ovarian tissue was also investigated for progesterone con-

tent, and found to contain 1.1 ± 0.5 ug/g at Days 10-49 of gestation,

1.0 ± 0.4 ug/g for Days 50-89, 2.7 ± 0.6 ug/g for Days 90-129, 1.6 ± 0.5

ug/g for Days 130-169, 1.9 ± 0.5 ug/g for Days 170-209, 0.7 ± 0.1 ug/g

for Days 210-249, and 0.8 ± 0.5 ug/g for Days 250-280 of the gestation

period.

Erb et al. (1960) determined the progestin content of corpora lutea

taken from 41 pregnant cows. The progestins (progesterone +Δ 4-preg-

nene-20B-ol-3-one) averaged 25.5 ± 3.1 ug/g of luteal tissue for cows

from 65 to 275 days of gestation. However, the concentration was great-

er (P < 0.005) for 11 cows between 50-109 days of gestation (49.0 ± 8.5

ug/g) than for cows in the late stages of pregnancy (23.5 ± 1.8 ug/g).

The corpora lutea at the early stages of gestation were approximately 43

percent larger and did not change appreciably in concentration after 110 days of pregnancy. A study performed by Erb et al. (1961) indicated that the progesterone content in the CL of pregnancy was at a peak of about 270 ug at Days 13-15 and reached a low average of about 150 ug for Days 37-75, which was followed by an increase in content to Day 212 and a gradual decline to parturition.

Stormshak and Erb (1961) assayed 89 CL and 54 pairs of ovaries to determine progestin levels in cows pregnant 16 to 275 days. The progestin concentration was significantly higher (P<0.01) in corpora lutea collected during Days 16 to 89 of gestation (33.6 ug/g) than at Days 90 to 179 (20.4 ug/g) or after 180 days (27.5 ug/g). There was significantly less progesterone per gram of luteal tissue at 90 to 179 days (12 ug/g) than after 180 days (18.7 ug/g) or for 16 to 89 days (20.4 ug/g). It was generally found that the progesterone content of the ovaries without corpora lutea was less than 0.1 ug/g.

Erb and Stormshak (1961) reported that seven pregnant cows had an average progestin (progesterone and 20B-ol) content of 161 ug/CL on Days 25-34, and six cows had 250 ug/CL at 37-42 days. Erb et al. (1968) reported the progesterone content (P<0.025) of the pregnant bovine CL raised significantly as the age of the animal increased, but progesterone concentration did not. The average values for the study were: progesterone content of the CL 199 ± 9.9 ug and CL concentration,

31.1 $\pm$ 1.4 ug/g during Days 11 to 284 for cows which averaged 54 $\pm$ 2.2 months of age.

Staples and Hansel (1961) utilized the effectiveness of oxytocin injections in repressing corpus luteum development to study the relationship between embryo survival at 15 days after insemination and the progesterone content of luteal tissue. The 20 control heifers with normal embryos had a progesterone content in the CL of 270.2 ug. Eighteen of the heifers that received subcutaneous oxytocin (7 u.s.p. units per hundred pounds of body weight per day) had normal embryos and the average total progesterone content of their corpora lutea was 310.6 ug. Ten of the oxytocin treated heifers were not pregnant and had significantly less (P $<$ 0.05) total progesterone (154.1 ug) than did the treated heifers having embryos. The data suggests that a threshold level of 100 ug progesterone in the CL is necessary for embryo survival at the 15th day.

Johnson and Erb (1962) reported that the progesterone concentration and the total progesterone content in the corpus luteum of pregnant dairy cattle was significantly increased (P $<$ 0.005) by the administration of exogenous progesterone 1 to 12 days before ovariectomy. The results of the study indicated that pregnancy could be maintained if ovariectomy was performed at no earlier than 88 days after conception and exogenous progesterone treatment was begun at least eight days before surgery.

The progesterone levels in the corpora lutea of beef cattle bred at estrus after being treated with PMS on Day 16 and HCG at Day 20 were determined by Estergreen (1964). The progesterone content in luteal tissue differed significantly ($P < .01$) among those studied at three days, 30 to 45 days, and 60 to 90 days after ovulation occurred, with the highest levels being determined for the heifers pregnant 30-45 days.

Niswender et al. (1965) studied the effect low levels of estradiol-17B had on the ovarian activity of cycling beef heifers. Estradiol-17B injections were administered at various dosage levels on Day 6 of the estrous cycle. The CL weight, progestin content, progestin concentration and follicular fluid weight at Day 12 was generally lower in the heifers treated with higher levels of estrogen than in the control heifers. Estrogen injections given daily from Day 6 to 18 resulted in a reduction of luteal and follicular activity, with higher levels of estradiol showing the greatest decrease. It was concluded that six levels of estrogen (20, 40, 80, 160, 320 and 640 ug) administered to the heifers resulted in a reduction of all measures of ovarian activity.

Adrenal Gland.- The cortex of the mammalian adrenal gland had been found to contain nearly 50 steroids (Turner, 1966). Among these are androgens, progestogens and estrogens which are essential to reproductive processes.

Gorski et al. (1958) found the adrenal glands of a Jersey heifer pregnant 258 days to contain less than 10 ug of progesterone. The pro-

gesterone content of the left adrenal gland was investigated by Melampy et al. (1959). The steroid was non-detectable for Days 10-49 of pregnancy. However, the mean progesterone concentration was 1.5 ug/g of adrenal tissue for Days 50-89, 1.7 ± 0.04 ug/g for Days 90-129, 1.5 ± 0.09 ug/g for Days 130-169, 2.1 ± 0.09 ug/g for Days 170-209, 0.4 ug/g for Days 210-249 and 0.7 ug/g for Days 250-280.

Erb et al. (1960) reported the progestin content in adrenal glands collected during gestation to be 23.4 ug. Stormshak and Erb (1961) concluded that the progesterone content of 54 pairs of adrenal glands taken from pregnant cows was generally less than 1 ug/g. Erb and Stormshak (1961) described the adrenals of four cows 8-22 days post-partum to be large (average 46 g) and with progestin levels about four times greater than is generally observed (66 ug).

Placenta.- The placenta is a source of estradiol and progesterone in most farm animals (Hafez, 1968). However, the endocrine activity of the placental tissue varies a great deal among species. The progesterone concentrations in the placenta are low and are not always readily detected.

Gorski et al. (1958) found negative results for progesterone determination in six assays of cow placentae. Melampy et al. (1959) analyzed the bovine placenta during gestation for the presence of progesterone. The placental tissue contained an average of 0.08 ± 0.04 ug progesterone/g during Days 10-49 of pregnancy, 0.08 ± 0.03 ug/g for

Days 50-89, 0.16 ± 0.09 ug/g for Days 90-129, 0.20 ± 0.06 ug/g for Days

130-169, 0.20 ± 0.07 ug/g for Days 170-209, 0.05 ± 0.02 ug/g for Days

210-249 and 0.03 ± 0.01 ug/g for Days 250-280. Williams et al. (1964)

characterized the distribution of progesterone in the uterine endomet-

rium and myometrium. Centrifugal fractionation and chromatography on

silica gel were used to study the presence of progesterone, 20B-ol and

hydroxyprogesterone, in both uterine endometrium and myometrium.

Ovarian Vein.- Erb et al. (1961) reported that the progesterone

concentration in the ovarian venous plasma from the CL bearing ovary was

four times greater than the levels in the plasma from the opposite ovary

(P<0.005). Progesterone levels in the ovarian vein of cows pregnant

250 to 282 days were estimated by Gomes et al. (1962). Whole blood from

ovaries (with corpora lutea) was obtained from the sectioned ovarian

vein after laparotomy of cows pregnant 250-254 days averaged 2.2 ug pro-

gesterone/ml. However, during this same period, two plasma samples

averaged 3.9 ug/ml. Blood collected from the ovarian vein of a cow

pregnant 282 days had 1.2 ug/ml of whole blood and 1.4 ug/ml of plasma.

Erb et al. (1968) described a significant (P<0.01) decline in the pro-

gesterone values of ovarian venous plasma during pregnancy. The pro-

gesterone concentration declined from the 14 day level, 4.5 ± 0.77 ug/ml

to 2.6 ± 0.29 ug/ml, during the second and third month of pregnancy.

This was followed by a rise in progesterone level during the fourth

month only to decline to the lowest levels during 199 to 237 days of pregnancy.

Jugular Vein.- Short (1960) found that progesterone levels in the peripheral blood of pregnant cows remained relatively constant throughout the gestation period, but approximately 10 days before parturition it starts to decline. Gomes et al. (1962) reported that two cows pregnant 250 and 251 days had an average of 0.0092 ug progesterone per ml of peripheral plasma. Holm and Short (1962) examined progestrone levels in the peripheral blood of five cows pregnant up to 92 days beyond the normal gestation period (285 days). The levels during mid-pregnancy were the same as in normal cows, but there was no decline in progesterone level just prior to the expected date of parturition which is characteristic of normal cows. The progesterone content of jugular plasma was investigated by Erb et al. (1968) and found to increase significantly ($P < 0.05$) during pregnancy, with an average of 30.3 ± 1.7 ng/ml throughout gestation. Shemesh et al. (1968) concluded that the progesterone levels in jugular plasma were similar in cycling cows (mean = 0.43 ± 0.35 ug/100 ml) and inseminated cows during Days 10 to 18 after ovulation, regardless of whether the bred cows were pregnant (0.48 ± 0.43 ug/100 ml) or not. However, a significant difference between non-pregnant (cycling) and pregnant cows was observed 19 days after ovulation. Only two of 12 non-pregnant cows had measurable blood levels of progesterone (0.16 to 0.22 ug/100 ml); whereas, eight pregnant

cows had plasma progesterone levels greater than 0.34 ug/100 ml, on Day 19. The pregnant cows averaged 0.47 ± 0.03 ug/100 ml at that stage of gestation.

Erb et al. (1961) collected blood from the jugular vein of pregnant cows before and after ovariectomy to investigate the influence removal of the ovaries has on the concentration of progesterone in plasma. The levels in 115 plasma samples taken from intact cows, averaged 35 ± 1.1 ng/ml and did not alter significantly from 181 days of pregnancy through 12 hours post-partum.

A peak concentration of 44 ± 2.7 ng/ml was achieved at 261 days. In ovariectomized animals there was no apparent difference in the plasma progesterone levels following surgery in comparison with presurgery concentration until after seven days post-ovariectomy. These levels declined significantly thereafter and were maintained at a low concentration through one day post-partum. Four cows pregnant 250-254 days, and ovariectomized by Gomes et al. (1962) had no detectable progesterone in 1400 ml of peripheral plasma taken one hour after ovariectomy. Shemesh et al. (1968) detected a low concentration of progesterone (0.17 ug/100 ml) in jugular venous plasma collected from a cow at seven days post-ovariectomy, but was undetectable (≤ 0.15 ug/100 ml) three weeks after the surgery had been performed.

Stabenfeldt et al. (1970) determined progesterone concentrations in peripheral plasma of pregnant cows from Day 140 through parturition.

From Days 140-200 of gestation there was little increase in levels, with an average of 4.6 ng progesterone/ml plasma for the interval. The average progesterone concentrations had increased to 6.8 ng/ml plasma by Day 250. This was followed by a decline to 4 ng 10 days before parturition and an abrupt drop to less than 1 ng/ml approximately 24 hours prior to parturition. The authors (Stabenfeldt et al., 1970) concluded that the preparation for parturition begins 10 days pre-partum in the cow.

MATERIALS AND METHODS

Normal cycling, 600 pound Holstein heifers were selected from the Montana State University Dairy herd. Cannulations were made in the jugular vein and posterior vena cava of five heifers. The vena cava cannulae were surgically inserted by laparotomy and palpation, ventral and slightly lateral to the spinal column as described by Hull (1967).

Silastic rubber tubing manufactured by Dow Corning Corporation[1] was utilized as a cannulation material. The size of tubing was not the same for every cannula. Two sizes were introduced into the veins, the first had an inside diameter (I.D.) of 0.062" and an outside diameter (O.D.) of 0.095" and a thicker walled tubing, I.D. 0.062" and O.D. 0.125".

Prior to collection of the blood sample, the heparinized cannula was gently flushed with 2 to 3 ml of saline. Approximately 5 ml of saline, heparin, and blood was drawn into the syringe and the mixture discarded. A sample of 15 ml venous blood was drawn into the syringe and transferred to a heparinized centrifuge tube. Parafilm was placed securely over the tube and the blood mixed gently to distribute the heparin. Approximately 2 ml of heparin was injected onto each cannula to prevent clotting until the next blood collection.

Each morning after the blood samples had been collected, the heifers were watched to detect estrus. If an animal was due to come in estrus and no characteristic behavior was noticeable in the morning, observations were made in the afternoon, and all estrus dates recorded.

---

[1] Distributed by Biddle Crowther Co., 1801 Broadway, Seattle, Wash.

The blood samples were brought into the laboratory and immediately placed in refrigeration. Three to four hours later the samples were centrifuged at approximately 1200 RPM for two hours. This yielded 6-7 ml of straw-colored plasma. Disposable Pasteur pipettes were used to transfer the plasma to plastic Whirl-Pak containers. These containers were labelled and stored in the freezer at -10°C.

Progesterone Analysis.-

The quantitative determination of progesterone was made with the competitive protein-binding analysis of Murphy (1963). Specific developments for progesterone determination made by Neill et al. (1967a) were utilized in the assay.

The procedure is based on the ability of cortico-steroid binding globulin (CBG) in dog plasma to bind a select group of steroids, i.e., cortisol, corticosterone and progesterone. High specific activity (57.2 c/mmole) corticosterone -1,2-^{3}H is used to saturate all the binding sites on the protein. When isolated progesterone is added to the system, a dynamic equilibrium is established with progesterone and corticosterone -1,2-^{3}H competing for the binding sites. The amount of progesterone is quantitated by counting the ^{3}H labelled corticosterone which remains bound to the CBG. The greater the concentration of progesterone in the system, the lower the counts of ^{3}H due to a lower amount of bound corticosterone.

Plasma samples were allowed to thaw at room temperature prior to

extraction. In order to determine losses of endogenous progesterone incurred through extraction and isolation from the plasma, an interval standard of ^{14}C progesterone was prepared. The standard solution contained 1ng of ^{14}C progesterone (sp. act. 58.5 mc/mmole) per 10 ul of ethanol. Before pipetting the plasma into the 12 ml extraction tubes, a 10 ul aliquot of the activity was added to each tube with a microliter syringe #725[1]. At the same time, six standards of ^{14}C progesterone (for each group of 40 samples) were pipetted into 18 X 56 mm, low potassium counting vials.

Plasma volumes ranging from 0.25 to 2.5 ml were added to the extraction tubes. The plasma was extracted twice, with five times its volume of petroleum ether (30°-60° C Ligroine) as described by Neill et al. (1967a). The tubes were shook for approximately 30 seconds in each extraction and the extract transferred with Pasteur pipettes to 15 ml disposable culture tubes. They were placed in a 52°C salt bath under the hood and dried with a light stream of nitrogen. Two ml of pet ether were used to rinse the walls of each culture tube. After the extract had dried sufficiently under nitrogen, the rinsing procedure was repeated with 0.5 ml pet ether, further concentrating progesterone in the bottom of the tube.

In order to quickly and effectively isolate progesterone from other steroids and plasma pigments that were in the pet ether extract, a

[1] Manufactured by Hamilton Co., Inc., Whittier, Calif.

process of thin layer chromatography was adopted. Spotting single samples with a Pasteur pipette proved to be time consuming, so a spotting device was built to accommodate two chromatogram sheets at one time. (Figure 1).

A plexiglass overlay with seven evenly spaced slots was placed upon the 250 u 20 X 20 cm silica gel chromatogram[1]. The metal tip of a dissecting pith was used to mark six lanes into the silica gel utilizing the overlay as a guide (Figure 2). Then the sheets were placed on the spotting device in preparation for spotting.

Two hundred ul of chloroform was added to each culture tube to serve as a spotting medium. The solution was taken up in Pasteur pipettes and added to the plastic syringes. The tip of each 1", 30 guage needle was suspended approximately 1.0 cm above the center of every lane and 2.2 cm from the bottom of the chromatogram. As the chloroform solution dripped from the needles onto the sheets, a stream of warm air was passed across the silica gel with an electric hair dryer[2]. The warm air hastened the drying of the chloroform spot and thus prevented excessive spreading of the solution. When the spotting was complete, syringes and needles were thoroughly rinsed to cleanse them of any progesterone residue. First 2 ml of anhydrous methanol were pipetted into each syringe and expelled through the tip of the needle. This procedure was then repeated with 1-2 ml of chloroform.

[1] Eastman Chromatogram sheet No. 6060 with fluorescent indicator.
[2] Model 38100, McGraw-Edison Co., Bersted Mfg. Div., Boonville, Mo.