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I am submitting herewith a thesis written by Carol Ann Collyer entitled "The Effects of Method of Handling Blood and Saliva on Progesterone Concentration in the Dairy Cow." I 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

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8

THE EFFECTS OF METHOD OF HANDLING BLOOD AND SALIVA ON PROGESTERONE CONCENTRATION IN THE DAIRY COW

A Thesis

Presented for the

Master of Science

Degree

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Carol Ann Collyer

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ABSTRACT

The radioimmunoassay for progesterone was used to determine the effects of holding blood for increasing lengths of time (0, 6, 12 hours) prior to serum separation on the concentration of progesterone. The radioimmunoassays for progesterone in whole blood and whole saliva were also evaluated as possible applications for progesterone testing under field conditions. Five consecutive 10 ml blood samples and two 10 ml saliva samples were collected from each of the ten Holstein cows in this study and were subjected to various handling treatments.

Significant differences in progesterone concentrations existed between serum and whole blood samples and between serum and saliva samples ($P<.001$). Blood samples held for 6 or 12 hours at $4^{o}C$ prior to centrifugation and separation of serum from cells resulted in lower serum progesterone concentrations than blood centrifuged immediately upon return to the laboratory ($P<.05$). Significant loss of progesterone was also determined in serum from blood held for 12 hours at $4^{o}C$ when compared to serum from blood held for 6 hours ($P<.05$). The use of serum separation tubes (SST; Becton-Dickinson, Rutherford, N.J.) gave comparable results to serum collected from blood samples centrifuged immediately upon return to the laboratory.

The determination of progesterone in whole saliva samples showed a positive correlation ($r=.76$) ($P<.001$) to the concentration of progesterone in serum samples. The concentration of progesterone was 23 ± 5.3 percent (range 19-49 percent) of the progesterone levels determined in serum. No loss of progesterone was observed in saliva samples held at room temperature for 24 hours prior to storage at -20^{o}.

The average recoveries of unlabeled progesterone added to serum and saliva samples were 88 $\pm$ 7 percent and 64.7 $\pm$ 9 percent, respectively. When increasing volumes of serum and saliva were analyzed, measured progesterone concentrations differed from expected concentrations by 15.9 $\pm$ 3 percent and 30.0 $\pm$ 11 percent. The interassay variation for serum samples in this study was 6.3 percent, while intraassay variation ranged from 5.8 to 21.3 percent. The interassay variation for saliva samples was 32.7 percent, while intraassay variation ranged from 16 to 36 percent.

An intravenous does of 250 mg progesterone resulted in a peak of 3400 ng/ml and 800 ng/ml in the plasma and saliva of a lactating non-pregnant dairy cow at five minutes post infusion. The progesterone concentrations in 15 matched plasma and saliva samples taken over a 24-hour period were highly correlated ($r=.96$) ($P<.001$).

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Efficient reproductive management is the key to success for all livestock operations. This is especially true of the dairy industry since maximum production relies heavily on the ability to determine the reproductive status of each cow in the herd. In particular, the accurate determination of pregnancy at a very early stage of gestation would enable the dairyman to identify open cows before time and profits are lost. The accurate measurement of progesterone is one method of determining pregnancy or reproductive status in the bovine.

Abraham et al. (1971) first described the measurement of human plasma progesterone by radioimmunoassay (RIA), following the successful production of an antiserum highly specific for progesterone. Numerous refinements and modifications have been made to develop a rapid, convenient and economical procedure that is valid for most species. Early investigators suggested the possibility of measuring progesterone concentration in the peripheral blood to assess corpus luteum function and as a possible early test for pregnancy. Since then, the radioimmunoassay has been applied to peripheral blood and milk progesterone sampling for early pregnancy determination in the bovine as well as in other species. A great deal of emphasis has been placed on the validation of radioimmunoassay procedures for progesterone determination with concern for the sources of high variation in results reported within and among laboratories. The radioimmunoassay for progesterone is highly sensitive, enabling the measurement of picogram

levels of the hormone. There is now strong evidence that external factors such as time and temperature may indeed affect the stability of progesterone in blood samples when samples must be stored between the time of collection and prior to plasma or serum separation and frozen storage.

This study was designed to evaluate the effects of different methods of collecting and handling blood samples prior to radioimmunoassay for progesterone in the dairy cow. Frequently, blood samples collected in the field must be held for varying lengths of time prior to reaching the laboratory, centrifugation and plasma or serum separation. This study compared serum samples held under refrigeration for increasing lengths of time and explored the possibility of analyzing heparinized whole blood samples for progesterone. In addition, whole saliva was concurrently examined as a matrix for the measurement of progesterone in the dairy cow.

CHAPTER II

REVIEW OF LITERATURE

The literature is saturated with studies on the measurement and function of progesterone in the bovine. However, prior to the introduction of a competitive protein binding assay for progesterone by Murphy (1967) and the radioimmunoassay for progesterone by Abraham (1971), studies related to the secretion and metabolism of progesterone were hampered by the lack of accurate, sensitive testing methods. Early bioassay and physicochemical methods for progesterone quantitation lacked specificity and sensitivity (Orczyk et al., 1979). For the most part, the literature reviewed here will primarily deal with the relevance of progesterone determination in the bovine, the radioimmunoassay for progesterone, factors affecting progesterone concentration in bovine blood, the metabolism of progesterone in the bovine and the measurement of steriods in saliva.

I. EARLY PREGNANCY DIAGNOSIS

Estergreen et al. (1967) demonstrated that progesterone secretion by a functional corpus luteum was essential for the maintenance of pregnancy in the cow during the first 200 days of gestation. Removal of the corpus luteum after 200 days may result in a viable fetus but progesterone levels are not adequate to suppcrt normal gestation length and normal parturition (Salisbury et al., 1978). Stabenfeldt et al. (1969) found a relatively high correlation between corpus luteum function and peripheral plasma progesterone.

The possibility of an early pregnancy test in cattle based on the measurement of plasma progesterone was first suggested by Shemesh et al. (1968). Robertson and Sarda (1971) pursued the possibility of measuring progesterone 20 to 24 days following estrus and insemination. When plasma progesterone concentrations >1.0 ng/ml were considered indicative of a functional corpus luteum during this period, 90-100 percent correct pregnancy diagnoses were reported. Later reports show accurate results in the diagnosis of non-pregnant cows based on the method of Robertson and Sarda (1971) but variable high false positive results (18-24 percent) (Pennington et al., 1976; Pope et al., 1976; Thibier et al., 1977). Laing et al. (1979) recommended progesterone testing for pregnancy diagnosis at 38 and 46 days following insemination to increase the accuracy of positive diagnosis primarily by accounting for early embryonic mortality. Shemesh et al. (1978) have reported a comparable reduction in false positive pregnancy diagnoses from 22 percent at 21 days post insemination to 10 percent at 40 days and 7 percent by testing progesterone concentrations 44 days after insemination. Although the measurement of plasma progesterone for early pregnancy diagnosis has been almost totally replaced in dairy cattle by milk progesterone testing, progesterone determination in the peripheral blood is applicable to reproductive management of beef cattle (Laing et al., 1979; Thirapatsukun et al., 1978).

II. RADIOIMMUNOASSAY FOR PROGESTERONE

Abraham et al. (1971) first described the radioimmunoassay of progesterone in human plasma, following extraction with ether

(10x sample volume) and chromatography of the extracted residue. The use of a steroid conjugate, 4-preg-11α-ol-3, 20 dione-11 hemisuccinate:BSA described by Niswender (1973), to elicit the production of a highly specific antibody to progesterone in the rabbit permitted the direct measurement of progesterone in ether extracts of blood plasma, since cross reaction by other steroids is minimal (Gordon and Sherwood, 1978). The radioimmunoassay for progesterone is now considered a relatively simple, highly specific, sensitive and accurate method for the determination of progesterone (Orczyk et al., 1979). However, a very specific progesterone binding globulin from the pregnant guinea pig (PBG) produces comparable results between competitive protein binding and radioimmunoassay analyses for progesterone (Serio et al., 1974).

Van der Molen and Aakvaag (1967) stated that the affinity of the plasma fraction of peripheral human blood for progesterone was much greater than that of the blood cells, recommending the use of plasma for progesterone determinations. In the early literature, the most widely accepted form of blood used for progesterone analysis in all species has been plasma. Most investigators collected blood into heparinized tubes, which were held on ice or under refrigeration prior to centrifugation and plasma stored at -20^{o}C prior to analysis (Herriman et al., 1979; Schams et al., 1978).

III. METHODS AND EFFECTS OF BLOOD SAMPLING

During a discussion at the 1970 Karolinska Symposium on Steroid Assay by Protein Binding (Diczfalusy, 1970), Murphy reported a preference for the use of serum samples over plasma samples for

progesterone analysis, claiming serum was more convenient, cleaner to use and had fewer precipitates. Abraham noted the formation of fibrin clots when plasma was frozen and thawed and the occasional presence of emulsions and gel formation with plasma extractions. Borth concluded that the preference for the use of plasma over serum for steroid analyses was traditional and not based on any quantitative comparisons. However, Erb et al. (1971) reported lower concentrations of progesterone in serum than in plasma from the peripheral blood of cattle.

Wenzel (1972) reported no differences in progesterone concentration measured by competitive protein binding when serum and plasma samples from the same cow were subjected to similar handling and storage. These findings have been confirmed by Vahdat et al. (1979) when bovine blood was incubated at either $4^{o}C$ or $40^{o}C$ for 30 minutes or for 24 hours at $4^{o}C$ after collection. However, Vahdat et al. (1979) found plasma progesterone concentrations significantly lower ($P<.05$) than serum concentrations (1.7 ng/ml vs 3.9 ng/ml) when blood samples were held at $40^{o}C$ for 6 hours prior to centrifugation. In matched samples incubated at $40^{o}C$ for 24 hours, plasma progesterone again was significantly lower than serum levels (2.8 ng/ml vs 4.4 ng.ml) (Vahdat et al., 1979).

Several recent reports involving the radioimmunoassay for progesterone in the bovine have reported the use of serum, but details on the exact method of collection and preparation were not specified (Morris et al., 1976; Kamimura, 1978; Seguin et al., 1978). Gowan and Etches (1979) analyzed serum from dairy cows for progesterone using a solid-phase radioimmunoassay. Blood samples were stored for 24 hours at $4^{o}C$ prior to centrifugation and storage of serum at $-20^{o}C$.

Robertson and Sarda (1971) first attempted to collect blood samples under field conditions prior to progesterone assay where blood was refrigerated at 4°C for 24-72 hours prior to centrifugation and storage of serum at -20°C. Progesterone concentrations detected in stored samples were reported comparable to those cooled in ice, centrifuged and frozen immediately. In 1974, Ginther et al. conducted a study where heparinized whole blood was stored 3-4 days at 4°C prior to separation of the plasma with no apparent loss of progesterone.

However, recent studies have shown that the method of handling of blood samples from the time of collection to the time of storage at subzero temperatures does affect progesterone concentration and stability. The rate of progesterone loss appears to be both time and temperature dependent.

Wenzel (1972) reported an average of 53 percent loss of progesterone from samples collected in cold tubes and held under refrigeration (4°C) and samples collected and held at room temperature. Plasma and serum samples from blood held at room temperature for 24 hours were determined to have lower ($P<.001$) concentrations of progesterone than samples from blood incubated for only 4 hours at room temperature. Whole blood samples that were refrigerated for 24 hours also yielded serum and plasma with progesterone levels lower ($P<.05$) than samples that were centrifuged immediately following collection.

IV. PROGESTERONE METABOLISM IN BOVINE

The extent that progesterone is bound to plasma proteins in bovine blood has only recently been determined. Kanchev (1976) reported no differences in the percent of progesterone bound in bovine blood by equilibrium dialysis during the different stages of the estrous

cycle (mean 94.5 $\pm$ .15 percent bound). However, during gestation, Kanchev (1976) detected an increase in the percent of progesterone bound to plasma proteins (mean 96.12 percent). The fractions of bovine serum protein found to bind progesterone were: albumin 98.97 percent; α globulin .04 percent; β globulin 2.64 percent; and γ globulin .08 percent.

Ganjam et al. (1975) indicate that progesterone in the blood can be loosely bound to low affinity, high capacity and non-specific proteins (like albumin) or to high affinity, low capacity proteins such as corticosteroid binding globulin (CBG). In the human, 95-98 percent of plasma progesterone is bound to plasma proteins (primarily albumin) while CBG has a three times greater affinity for progesterone than cortisol (Van der Molen and Aakvaag, 1967). Since progesterone is highly soluble in non-polar organic solvents, such as petroleum ether, 90 percent or better extraction is expected from blood samples (Neill et al., 1967; Runnebaum, 1975). Rash et al. (1980) found that altered serum lipid can interfere with the radioimmunoassay of steroids but can be controlled by thorough extraction of samples with petroleum ether.

It is generally agreed that once progesterone enters the peripheral circulation it is subject to metabolism or inactivation (Gomes and Erb, 1965). Like most steroids, progesterone is rapidly metabolized primarily by the liver, although kidney and mammary tissue also appear to play a role in the fate of circulating progesterone (Goldman and Zarrow, 1973). According to earlier studies, progesterone in the bovine moves readily into and out of body tissues, maintaining equilibrium with the blood (Estergreen et al., 1977). Fat, on the other hand, appears to act as a depot for progesterone in the dairy cow,

accumulating 5-10 times the progesterone concentration of the blood (McCracken, 1964). Progesterone has also been found to be primarily associated with milkfat of the dairy cow (Bulman, 1979).

In the bovine, excretion of metabolites of progesterone is primarily via hepatoenteric pathways into the feces (Miller et al., 1956). This has been demonstrated by Williams (1962) where, 12 hours after an intravenous dose of [^{3}H]progesterone, 45 percent of the labeled metabolites were recovered from the feces, 1.7 percent from the urine and .03 percent in the milk of a pregnant lactating dairy cow. A similar study conducted by Estergreen et al. (1977) detected 49.9 percent of the radioactive label in the feces, 1.96 percent in the urine and .25 percent in the milk of a lactating non-pregnant cow.

Through the use of radioactively labeled progesterone, the biological half-life of exogenous progesterone has been determined for several species. Goldman and Zarrow (1973) report a range of 3-29 minutes for the biological half-life of progesterone in the human. Half-lives were greatest for non-pregnant women and shortest for pregnant women (Thijssen and Zander, 1966). Tsang and Hackett (1979) have determined an increase in mean clearance rate near term in pregnant ewes, in contrast to the earlier report by Short and Rowell (1962) that no change in rate of progesterone metabolism occurred in the ewe near term.

Miller et al. (1963) determined the half-life of an exogenous dose of progesterone to be 36.3 minutes in a non-pregnant dairy cow. In a detailed study of the metabolism of progesterone in the pregnant dairy cow, Heap et al. (1975) found the mean clearance rate for progesterone from the blood was 22.5 liter/minute. Mammary uptake of

3.1 ug/minute progesterone was lower than reported earlier by Darling et al. (1972). Heap et al. (1975) concluded that higher levels of progesterone found in milk samples do not necessarily indicate increased mammary uptake of progesterone and are probably due to the high lipid solubility of progesterone. Heap et al. (1975) determined that progesterone accounted for more than 88 percent of the radioactive label found in the milk samples, indicating a low level of progesterone metabolism by the mammary gland.

The disappearance of an exogenous dose of progesterone from the blood appears to follow a biphasic curve. According to Goldman and Zarrow (1973), the early, rapid decline in progesterone from the blood reflects the equilibration of progesterone in the blood with body fluids and tissues. The later portion of the curve reflects the slower removal of progesterone through metabolism and excretion. Evans et al. (1975) reported a similar biphasic clearance of progesterone from the peripheral blood of mares. A rapid decrease in plasma progesterone occurred within 15.7-16.2 minutes following progesterone administration. A second, slower decrease in progesterone was detected 61.5-69.5 minutes after dosage. Ganjam et al. (1975) also determined the biological half-life of progesterone in the mare, but observed three components to the disappearance curve: a rapid clearance of progesterone within 2.5 minutes; a slower decline in progesterone within 20 minutes; and a very slow component that was not measured.

The short half-life of progesterone in the blood may account for some of the variability seen in the measurement of progesterone in the peripheral blood (Short, 1958). In 1962, Axelrod and Werthessen postulated that peripheral blood itself may serve as a site for the

conversion of steroid hormones, to help account for the rapid rate of removal of steroids from the circulation. The in vitro metabolism of progesterone has also been cited as a possible explanation for the variability of reported values for serum and plasma progesterone concentrations (Henricks et al., 1971),.but the exact causes or mechanisms are still speculative.

Hooker and Forbes (1949) reported measurable losses in progesterone activity when plasma was kept at body temperature prior to bioassay. As discussed earlier in this review, more recent studies have shown significant losses in progesterone concentration when blood is incubated for varying lengths of time (Wenzel, 1972; Vahdat et al., 1979; Owens et al., 1980). Short (1958) incubated progesterone with whole ox blood at 37°C for 3 hours and determined that progesterone undergoes a slow breakdown to 20 β-dihydroprogesterone, estimating the in vitro half-life of progesterone in ox blood to be about 7 hours. Short (1958) also determined that the plasma fraction contained greater than 90 percent of the added progesterone following incubation. DeVenuto (1967) found that 70-85 percent of the progesterone in contact with the red blood cells in whole rat blood is adsorbed to the cell surface during equilibrium dialysis. No evidence of metabolism of progesterone by the red blood cells in an in vitro system was reported. Rather, DeVenuto (1967) postulated that the steroid was associated with the phospholipid layer of the red blood cell membrane. DeVenuto (1967) also determined that there was no significant uptake of aldosterone by the red blood cells, concluding that progesterone was more lipophilic with fewer polar groups, resulting in a higher affinity to serum proteins and cell constituents. The

uptake of progesterone by human red blood cells was demonstrated by Sandberg et al. (1957). Short (1958) concluded that variable recovery rates for progesterone added to blood in vitro was due to the association with protein constituents of blood, although fairly constant recovery rates over a wide range of progesterone concentrations indicates a reversible combination of some form.

In 1971, Philip and Marotta (1971) demonstrated that the uptake of cortisol by canine erythrocytes was also greater than explainable by the equilibration between the plasma and red blood cell fractions of blood when incubated for 15 minutes.

V. STEROIDS IN SALIVA

Backstrom et al. (1976) identified the passive transport of progesterone, estradiol and testosterone across the blood-cerebrospinal fluid (CSF) barrier in human patients. A transfer of approximately 10 percent of the endogenous plasma progesterone to the CSF was determined. Since plasma proteins do not cross the blood-CSF barrier, Backstrom et al., (1976) have concluded that the progesterone levels in the CSF correspond to the level of "free" or unbound progesterone in the blood.

In 1978, a similar phenomenon was reported where the direct radioimmunoassay of cortisol in human saliva revealed cortisol levels comparable to unbound, physiologically active levels of cortisol in the blood (Walker et al., 1978). Walker et al. (1978) concluded that saliva sampling is a non-invasive, stress-free method of determining adrenal status in the human. Cortisol titer was found to be independent of

saliva flow rate and no significant differences were found between cortisol concentrations in parotid fluid and whole mixed saliva samples.

Smith et al. (1978) and Luisi et al. (1980) reported a precise radioimmunoassay for testosterone in human saliva samples. Smith et al. (1978) found a high correlation between testosterone levels measured in the saliva, indirectly calculated free (unbound) testosterone levels and free testosterone measured by equilibrium dialysis. Horning et al. (1977) reported that most therapeutic agents were transferred rapidly from the blood to the saliva and reflect the free or unbound plasma drug concentrations. Apparently, human saliva does not contain any corticosteroid binding globulin (Walker et al., 1977) or permit the transfer of plasma proteins from the blood to the saliva.

In 1979, Walker et al. (1979) described a precise radioimmunoassay for progesterone in human saliva. Mixed whole saliva samples stored at -20^{o}C were stable for longer than 6 months. Regression analysis of progesterone concentration in matched plasma and saliva samples revealed high correlation in all patients (r=.91-.97). Extractions of progesterone from saliva samples were performed with petroleum ether, similar to the method of Gordon and Sherwood (1973) for plasma samples. Walker et al. (1979) reported that the recovery of labeled progesterone from saliva samples was consistently greater than 95 percent. Elatter (1975) incubated whole saliva from human patients with radioactively labeled progesterone and found no apparent metabolites or evidence of progesterone metabolism.

VII. SUMMARY OF LITERATURE REVIEW

The radioimmunoassays for progesterone in bovine blood and milk have been well-defined and are available as a tool for the early pregnancy diagnosis, assessment of corpus luteum function and analysis of infertility in cattle (Thibier et al., 1977). The factors affecting the concentration of progesterone in the blood from the time of sampling to analysis are still poorly defined, although time and temperature appear to have a major effect. The radioimmunoassay of whole saliva for progesterone, testosterone and other steroids and drugs has been well-defined in the human, but has not been reported in the bovine.

CHAPTER III

MATERIALS AND METHODS

I. ANIMALS

Blood and saliva samples were collected from ten lactating Holstein cows randomly selected from the University of Tennessee-Knoxville dairy herd. The cows used in this study were all in the first trimester of gestation with the exception of Cow number 9 (open) and Cow number 10 (second trimester). Table I includes the herd number, randomly assigned experiment number, and number of days since last insemination for each cow.

II. BLOOD SAMPLING

Five blood samples (10 to 12 ml each) were consecutively drawn from each of the ten cows in this experiment. Each blood sample was collected in a pre-labeled evacuated blood collection tube (Vacutainer; Becton-Dickinson, Rutherford, N.J.). The blood tubes used for Treatment II contained an inert barrier material to facilitate serum separation (SST; Becton-Dickinson, Rutherford, N.J.). All blood collection tubes were stored at $4^{o}C$ the night prior to collection and held on ice during the collection period.

Bleeding was accomplished by restraining each cow in a chute with a headgate and by tying the cow's head to one side to expose the jugular vein and further restrain the cow. Venipuncture with a 20 gauge 1-1/2-inch sterile multiple sample needle (Vacutainer; Becton-Dickinson, Rutherford, N.J.) facilitated the collection of all five blood samples

TABLE I

STAGE OF GESTATION DURING SAMPLING

Experimental Cow No.	Herd No.	No. Days Since Last Insemination
1	41	52
2	43	58
3	50	10
4	83	67
5	33	87
6	984	97
7	972	49
8	928	63
9	973	Open
10	902	185

within 2 to 3 minutes. For each cow, blood tubes were filled in the order of treatment number (i.e. I-V). Once filled, tubes were immediately returned to a covered ice chest.

Treatments of each blood sample for each cow and the order of collection were as follows:

Treatment I. Blood was collected into a cold evacuated blood tube and stored on ice until returned to the laboratory (no more than two hours). This sample was centrifuged immediately in a refrigerated centrifuge at $4^{o}C$ for 30 minutes at 2500 rpm. Serum was drawn off immediately and divided into a minimum of five 1 ml aliquots. All samples were labeled and stored at $-20^{o}C$ prior to radioimmunoassay.

Treatment II. Blood was collected into a cold SST tube, gently inverted five times and centrifuged upon return to the laboratory (2 hours). Serum was poured off, divided into aliquots and stored at $-20^{o}C$.

Treatment III. Blood was collected into a cold blood tube and stored at $4^{o}C$ for 6 hours prior to centrifugation, serum separation and storage at $-20^{o}C$.

Treatment IV. Blood was collected into a cold blood tube and stored at $4^{o}C$ for 12 hours prior to centrifugation, serum separation and storage at $-20^{o}C$.

Treatment V. Blood was collected into cold blood tubes containing three drops (1000 U/ml) heparin and gently inverted. Two hematocrit tubes were prepared from this sample for determination of packed cell volume for each cow. Whole blood was held on ice and divided into five aliquots upon return to the laboratory and stored at $-20^{o}C$.

III. SALIVA SAMPLING

Prior to blood collection, the mouth of each cow was rinsed with approximately 1 liter of water using a stainless steel dose syringe. Immediately following the collection of all blood samples, saliva samples were collected as follows:

A. Four sterile 4 x 4-inch 8-ply gauze sponges (Johnson and Johnson, New Brunswick, N.J.) were rolled and tied together using approximately 20 inches of heavy package string. Prepared sponges were stored in a plastic bag.

B. The gauze sponges were determined to have no significant effect on progesterone concentration. Sponges were tested by soaking in assay buffer, extraction with petroleum ether and the radioimmunoassay for progesterone.

C. Using separate latex gloves for each cow, the cow's lips were parted and the rolled sponge was placed in the back of the mouth using a pair of 14-inch stainless steel forceps. In most cases, the sponges were held in place with the forceps throughout collection. The sponge was removed periodically and saliva was collected by squeezing the sponge over a separate, clean 100 ml plastic beaker until approximately 30 ml of saliva was collected.

D. Immediately following collection, the saliva was poured into two clean evacuated blood tubes.

E. Saliva samples were held at room temperature throughout and treated as follows:

Treatment I. Saliva held at ambient temperature (21^{o}C) and centrifuged at 2500 rpm for 30 minutes to remove debris upon return to

the laboratory. Clear saliva was divided into a minimum of five 3 ml aliquots. Saliva samples were stored at -20°C until analyzed.

Treatment II. After return to the laboratory, saliva was held at room temperature for an additional 24 hours prior to centrifugation and storage at -20°C.

IV. RADIOIMMUNOASSAY PROCEDURE

Progesterone from peripheral whole blood, serum and saliva was measured according to the radioimmunoassay described by Gordon and Sherwood (1978). Following is a description of the characteristics of the RIA prccedure including any modifications made by this laboratory.

Preparation of Assay Buffer

All dilutions of unknown samples, progesterone standards, labeled progesterone tracer, and antiserum were made using a phosphate-buffered saline solution containing .1 percent gelatin and .01 percent merthiolate preservative (PBS-gel). Fresh PBS-gel was prepared approximately every month as follows:

Solution A - 5.36 g $Na_2HPO_4 \cdot 7H_2O$
16.36 g NaCl
Dissolved in 2 liters distilled H_2O

Solution B - 1.38 g $NaH_2PO_4 \cdot H_2O$
8.18 g NaCl
Dissolved in 1 liter distilled H_2O

Solutions A and B were mixed with a stirring magnet until a pH of 7.00 was reached. Gelatin (.1%) (275-Bloom; Fischer Scientific

Co., Fairlawn, N.J.) and preservative (.01% Ethylmercurithiosalicylic Acid Sodium Salt) (Fischer Scientific Co., Fairlawn, N.J.) were added. PBS-gel was stored at $4^{o}C$.

Preparation of Dextran-Coated Charcoal

A suspension of charcoal was prepared each time new assay buffer was made as follows:

100 ml assay buffer, gelatin omitted

.625 g charcoal (Norit A; Matheson Coleman and Bell, Norwood, Ohio)

.0625 g dextran (Dextran T-70; Pharmacia Fine Chem, Sweden)

Charcoal and dextran were added to the buffer in a flask, shaken vigorously, stoppered and stored at $4^{o}C$.

Charcoal-dextran suspension was always used at $4^{o}C$ under continuous mixing using a magnetic stirring bar.

Labeled Progesterone - Tracer

The label used in this assay was [1,2,6,7-^{3}H(N)] - Progesterone (NET-381) obtained from New England Nuclear Corp. (Boston, MA). An intermediate stock solution of tracer was prepared by the addition of PBS-gel until 100 ul counted approximately 200,000 counts per minute (cpm). A one to ten dilution of this stock solution was used to prepare tracer for each assay with a total count of approximately 18-20,000 cpm/100 ul.

Progesterone Standards

A progesterone stock solution of 1 ug/ml (Lot #1-26) was obtained from Radioassay System Lab, Inc. (Carson, CA). From this stock

solution, a 1000 pg/.1 ml solution was prepared by dilution with PBS-gel. The seven progesterone standard concentrations used in this assay were prepared as follows:

	Standard Concentration
10 ul stock solution + 9.99 ml PBS-gel	1000 pg/.1 ml
5 ml of 1000 pg/0.1 ml Std + 5 ml PBS-gel	500 pg/.1 ml
5 ml of 500 pg/0.1 ml Std + 5 ml PBS-gel	250 pg/.1 ml
5 ml of 250 pg/0.1 ml Std + 5 ml PBS-gel	125 pg/.1 ml
4 ml of 125 pg/0.1 ml Std + 6 ml PBS-gel	50 pg/.1 ml
5 ml of 50 pg/0.1 ml Std + 5 ml PBS-gel	25 pg/.1 ml
4 ml of 25 pg/0.1 ml Std + 6 ml PBS-gel	10 pg/.1 ml

Progesterone Antiserum - Antibody

The progesterone antiserum used in this experiment was provided by Dr. O. D. Sherwood, Reproductive Physiology Laboratory, University of Illinois (Lot 253-10, 5-6-77). The high specificity of this antibody for progesterone is shown in Table II.

Sensitivity

Sensitivity refers to the smallest detectable concentration of progesterone which will result in a significant change in the binding of the labeled hormone. According to Gordon and Sherwood (1978), and confirmed in this study, the sensitivity of this assay is 10 picograms.

Antibody Titration

Serial dilutions of the antibody were periodically performed to determine the appropriate dilution to obtain 50 percent binding of the

TABLE II

SPECIFICITY OF PROGESTERONE ANTISERUM[1]

Steroid	Cross-Reactivity (%)	Steroid	Cross-Reactivity (%)
Progestins:		Corticosteroids:	
Progesterone	100.00	Desoxycorticosterone	1.78
11α-hydroxyprogesterone	22.00	Corticosterone	1.00
5α-pregnan-3, 20-dione	3.98	Hydrocortisone	.13
17α-hydroxyprogesterone	1.78	Androgens:	
20α-hydroxyprogesterone	.79	Testosterone	.13
5α-pregnan-3β-ol-20-one	.25	5α-dihydrotesterone	.13
20β-hydroxyprogesterone	.13	Cholesterol	.13
5-pregnen-3β-ol-20-one	.13		

[1]Lot 253-10, 5-6-77 (University of Illinois)

total labeled progesterone added. A dilution of 1:2000 was found to bind approximately 50 percent of the total cpm in the final analyses.

Pipetting

All pipetting was performed with set volume micropipettes (Oxford Sampler Micropipette, Foster City, CA).

Protocol

Each assay was performed according to the following protocal. Standards, unknown samples and pooled samples were prepared in triplicate for each assay.

Tube No.	I.D.	Sample Volume	Reagents			
			PBS-gel	Ab	Tracer	Charcoal
1-2-3	Total cpm	---	+	-	+	-
4-5-6	Background	---	+	-	+	+
7-8-9	Bound (Bo)	---	+	+	+	+
A-B-C	Standard	100 ul	+	+	+	+
X-Y-Z	Unknown	100 ul	+	+	+	+

Sample Extractions

1. All unknown samples were maintained at -20^{o}C until immediately prior to analysis. Sample aliquots were thawed once at room temperature.

2. Samples less than 300 ul were pipetted directly into 12 x 75 mm disposable culture tubes (RTU; Becton-Dickinson, Rutherford, N.J.) and samples 300 ul or larger were pipetted into 15 ml disposable glass test tubes.

3. Petroleum ether (Fisher Scientific Co., N.J.) was added at the rate of ten times the sample volume.

4. Each tube was vortexed for 1-2 minutes.

5. The aqueous layer was separated from the solvent layer by freezing in liquid nitrogen.

6. The solvent phase was decanted into a clean 12 x 75 mm culture tube.

7. Tubes containing the frozen aqueous layer were immediately rinsed twice with approximately 200 ul of petroleum ether and the solvent was collected.

8. The assay tubes were placed in a 40°C water bath under nitrogen gas to evaporate the solvent from the hormone residue.

9. Dried samples were reconstituted to 100 ul by adding 100 ul of fresh PBS-gel.

10. Each tube was vortexed, covered and held overnight at 4°C.

Incubation of Samples with Antibody and Tracer

Labeled progesterone tracer (18-20,000 cpm/100 ul) and diluted antibody (100 ul of 1:2000) were added to all tubes containing standard and unknown samples (see Sect I. Protocol). PBS-gel assay buffer was added to bring all tubes to a constant volume (700 ul). All tubes were vortexed, covered and incubated at 4°C for approximately 24 hours.

Separation of Bound from Free Progesterone

1. Following incubation, 200 ul of charcoal-dextran suspension was rapidly added to each tube (except those designated "Total cpm"), working in a cold room at 4°C. Each tube was vortexed, placed in a cold centrifuge carrier and incubated for 15 minutes.

2. Tubes were centrifuged in a 4°C refrigerated centrifuge (TJ-6 model; Beckman, Palo Alto, CA) at 3,000 rpm for 10 minutes.

3. As rapidly as possible, 500 ul of supernate was pipetted from each assay tube directly into a 4 ml scintillation vial (Bio-Vial; Beckman, Palo Alto, CA).

4. Three ml of scintillation fluid (Aquasol-2; New England Nuclear, Boston, MA) was added to each vial. All scintillation vials were capped and shaken vigorously.

Counting

The radioactivity of the bound steroid was counted using a liquid scintillation counter (ISOCAP/300/Searle Analytic., Des Plaines, Ill.). Each sample was counted for one minute and counts per minute (cpm) were recorded by a teletype printer.

Calculations

Calculations of all standard curves and determination of progesterone concentrations of all unknown samples were made using a Hewlett-Packard calculator (HP-9815A) and printer (9871A) with a taped radioimmunoassay program (09815-14254) from Hewlett-Packard Calculator Products Division (Loveland, Colo.) according to the method of Rodgers (1974).

An unweighted least squares regression of logit (% bound) VS $\log_{10}$ (concentration) was calculated, unknown samples compared to the transformed standard curve and a mean progesterone concentration was printed for each set of triplicate samples.

VI. PROGESTERONE RADIOIMMUNOASSAY VALIDATION

The immunological validation for bovine blood and saliva was performed using pooled serum and saliva samples from three cows used in a preliminary study.

Antibody Specificity and Cross-Reactivity

The specificity of the antibody used in this study was further tested in this laboratory by reacting the progesterone antiserum with sets of Estradiol-17β and testosterone standards (10, 25, 50, 125, 250, 500 and 1000 pg/.1 ml).

Recovery of Unlabeled Progesterone

The accuracy of the extraction procedure was tested by adding 0, 25, 50 and 125 pg of progesterone (100 ul of each respective progesterone standard solution) to 100 ul of pooled serum and 300 ul of pooled saliva in triplicate. These combined samples were extracted, incubated and counted in the same manner as all unknown samples. Percent recovery was determined by dividing the measured concentration of progesterone by the expected progesterone concentration.

Parallelism

Parallelism of this assay was tested by measuring progesterone in 100, 200 and 300 ul samples of pooled serum and 300, 500 and 1000 ul of pooled saliva to test for proportionate increases in progesterone concentration.

Precision

The precision of the radioimmunoassay performed in this laboratory was determined as the coefficient of variation of identical samples (triplicates) analyzed in the same assay (intraassay) and successive (interassay) radioimmunoassays. Interassay variation was determined by analyzing pooled serum (100 ul) and pooled saliva (300 ul) samples with each assay performed (n=6). Intraassay variation was calculated for each assay by analyzing each pooled serum and saliva sample in triplicate (n=3). Coefficient of variation was calculated according to the method of Steel and Torrie (1960):

$$CV = 100\% \times s/\bar{x} \text{ , } s = \text{standard deviation}$$

Biological Validation

Due to the low and variable progesterone concentration in bovine saliva samples in a preliminary study, a biological validation was performed using a single animal and monitoring the disappearance of an exogenous dose of progesterone. A mature lactating dairy cow (in mid-luteal phase of estrous cycle) was samples over a 24-hour period (at 0, 5, 10, 15, 20, 25, 30, 60, 120, 180, 240, 360, 480, 720, 1440 minutes) following an intravenous dose of 250 mg progesterone in 5 ml corn oil. (The cow was restrained in a chute with a headgate for all sampling.)

An indwelling cannula was prepared from polyethylene tubing 1.270 mm ID x 2.286 mm OD with a blunt 18-gauge needle in the external end that was capped with a male Luer-Lok cap (Becton-Dickinson, Rutherford, N.J.). The cannula was flushed with heparinized saline (2% sodium heparin) and inserted into the right jugular vein by feeding

it through a 4-inch 12-gauge stainless steel needle. Following insertion of approximately 50 cm of tubing, the 12-gauge needle was removed and the cannula was secured in place by 4 skin sutures through an adhesive tape flap on the tubing at the point of insertion. To minimize the stress of frequent blood samples, the free end of the cannula was fed up the neck to the withers and securely taped in place.

Blood samples were collected after withdrawing and discarding the heparinized saline in the cannula and approximately 5 cc of blood using a 60 cc syringe. Each 15 cc blood sample was then collected in a sterile 20 cc syringe containing three drops of sodium heparin (1000 U/ml). Saliva (20 ml) was collected immediately following each blood sample in the same manner as outlined previously. Blood and saliva were placed in pre-labelled cold 15 ml centrifuge tubes and immediately centrifuged at 2500 rpm for 20 minutes. Plasma and the clear saliva fractions were drawn off and divided into a minimum of 5 aliquots and frozen at -20^{o}C until assayed for progesterone.

Several preliminary trials were performed to determine the appropriate dilution of all samples. The final assay included all plasma and saliva samples in triplicate over the 24-hour period. The correlation between plasma and saliva progesterone concentrations over time was determined.

Adherence of Progesterone to Test Tube Walls

The possible adherence of progesterone to the walls of the glass blood collection tubes used in this study was investigated. Labeled progesterone was added to a series of blood tubes containing 5 ml assay buffer, and the tubes were incubated for 0, 6 or 12 hours at 4^{o}C.

Following incubation, a 500 ul sample from each tube was counted in 3 ml scintillation fluid to determine any loss in progesterone concentration. Following incubation, the test tubes were emptied, rinsed and the residual radioactivity counted by adding 3 ml scintillation fluid. No significant adherence of labeled progesterone above background levels of radioactivity was detected following incubation for 0, 6 or 12 hours.

VII. STATISTICAL ANALYSIS

Triplicate determinations of each sample were averaged and the assay of all final blood and saliva samples was repeated three times within a three-week period. Treatment means were determined for each blood and saliva treatment for each cow and tested by analysis of variance and orthogonal comparisons. Where differences appeared, data was also subjected to Duncan's multiple range test.

The correlation coefficients between serum and saliva samples and serum and whole blood samples were calculated.

CHAPTER IV

RESULTS AND DISCUSSION

I. PROGESTERONE RADIOIMMUNOASSAY VALIDATION

Cross-Reactivity

No cross-reactivity between the progesterone antiserum and testosterone or Estradiol-17β was detected. Refer to Table II, p. 22, for the complete characterization of this antiserum.

Recovery of Unlabeled Progesterone

The results of the recovery phase as shown in Table III were 96, 83 and 85 percent recovery when 25, 50 and 125 pg of unlabeled progesterone were added to 100 ul of pooled serum containing 330 pg of progesterone. When 25, 50 and 125 pg of progesterone were added to 300 ul of pooled saliva containing 46 pg of progesterone, 74, 62 and 58 percent of the total progesterone was recovered.

While many investigators have used labeled progesterone to test recovery efficiency, those reporting results following the addition of cold hormone report 88-100 percent recovery (Runnebaum, 1975). The recoveries of 96, 83 and 85 percent reported here for the addition of 25, 50 and 125 pg progesterone to 100 ul serum samples are within the expected range. However, recoveries of 74, 62 and 58 percent when 25, 50 and 125 pg progesterone were added to 300 ul saliva samples are lower than those reported by Walker et al. (1979), where recoveries of tritiated progesterone added to human saliva samples exceeded 95 percent. While saliva sampling is a stress-free, simple and

TABLE III

RECOVERY OF PROGESTERONE (P_4) FROM SERUM AND SALIVA

	P_4 added (pg)	P_4 in pooled sample (pg)	Total P_4 expected (pg)	P_4 recovered (pg)	% Recovery[1]
Pooled serum (.1 ml/sample)	25	330	355	341	96
	50	330	380	315	83
	125	330	455	387	85
Pooled saliva (.3 ml/sample)	25	46	71	53	74
	50	46	96	60	62
	125	46	171	99	58

[1] $\% \text{ Recovery} = \frac{P_4 \text{ recovered}}{P_4 \text{ expected}}$

apparently highly reliable procedure in the human for most biologically active compounds, (Walker et al., 1977), data on bovine saliva sampling for similar compounds is not available.

Parallelism

Table IV shows the mean progesterone concentrations measured when increasing volumes of identical serum and saliva samples were analyzed. When 100, 200 and 300 ul samples of pooled serum were tested in triplicated, 345, 592 and 853 pg of progesterone were measured, showing a difference of 14.2 and 17.6 percent from concentrations expected in the 200 and 300 ul samples. Increasing concentrations of 42, 51 and 94 pg were also measured when 300, 500 and 1000 ul samples of pooled saliva were analyzed, but the percent differences from expected concentrations were higher for saliva samples, 27.1 and 32.9 percent for the 500 ul and 1000 ul samples respectively.

Precision

Six complete assays were performed on blood and saliva samples testing for handling effects where identical antibody dilutions, tracer, standard solutions and reagent were used. Pooled serum (100 ul) and pooled saliva (300 ul) samples run in triplicate with each of these assays were used to calculate the precision of this procedure in the laboratory. Table V gives the mean value, standard error of the mean and coefficient of variation for these samples. For pooled serum samples, intraassay variation ranged from 5.83 to 21.28 percent (mean 11.87) and interassay variation was 6.3 percent. The intraassay variation for saliva samples was higher, ranging from 16.0 to 36.5 percent with an average of 22.3 and interassay variation of 32.7 percent.

TABLE IV

PROGESTERONE (P_4) MEASURED IN INCREASING VOLUMES OF SERUM AND SALIVA

	Sample size (ul)	P_4 Measured (pg)	P_4 Expected (pg)	% Difference[1]
Pooled serum	100	345		
	200	592	690	14.2
	300	853	1035	17.6
Pooled saliva	300	42		
	500	51	70	27.1
	1000	94	140	32.9

[1]Percent difference from expected.

TABLE V

VARIATION WITHIN AND BETWEEN PROGESTERONE RADIOIMMUNOASSAYS

Assay No.	Serum progesterone (ng/ml)			Saliva progesterone (pg/ml)		
	Mean[1]	SEM	CV	Mean[1]	SEM	CV
1	3.02	.102	5.83	114.3	13.51	20.5
2	3.45	.130	6.55	139.9	14.55	17.9
3	3.15	.144	13.75	167.6	25.11	25.9
4	3.58	.142	11.87	96.6	20.32	36.5
5	3.27	.402	21.28	58.8	5.71	16.8
6	3.19	.221	11.96	149.8	13.86	16.0
Overall[2]	3.28	.085	6.3	121.2	16.17	32.7

[1]n = 3

[2]n = 6

Biological Validation

The disappearance of an intravenous dose of 250 mg progesterone from the plasma and saliva of a lactating dairy cow over time is represented in Figure I.

Five minutes following the intravenous administration of 250 mg of progesterone, progesterone concentrations in the plasma and saliva peaked at 3400 ng/ml and 800 ng/ml, respectively. The progesterone concentration in 15 matched plasma and saliva samples taken over a 24-hour period were highly correlated ($r=.96$) ($P<.001$). An apparent plateau in progesterone level of 5.4 ng/ml was reached in the plasma by 180 minutes and .40 ng/ml in the saliva by 240 minutes following infusion.

An estimate of the peak progesterone concentration expected was made by calculating and total blood volume for a 545.5 kg cow, assuming an average of 65 ml blood/kg body weight (Stahr, 1977). The dilution of 250 mg progesterone in 35,454 ml blood would be approximately 7051 ng/ml. It is probable that the actual peak in progesterone concentration may have occurred prior to the five minute samples, since exogenous progesterone is rapidly metabolized (Goldman and Zarrow, 1973). Miller et al. (1963) reported the half-life of exogenous progesterone in the blood of a non-pregnant dairy cow was 36.3 minutes.

II. EFFECTS OF BLOOD HANDLING

Five blood samples from each of the ten cows used in this study were subjected to different treatments prior to serum collection, storage and radioimmunoassay for progesterone. Three treatments were

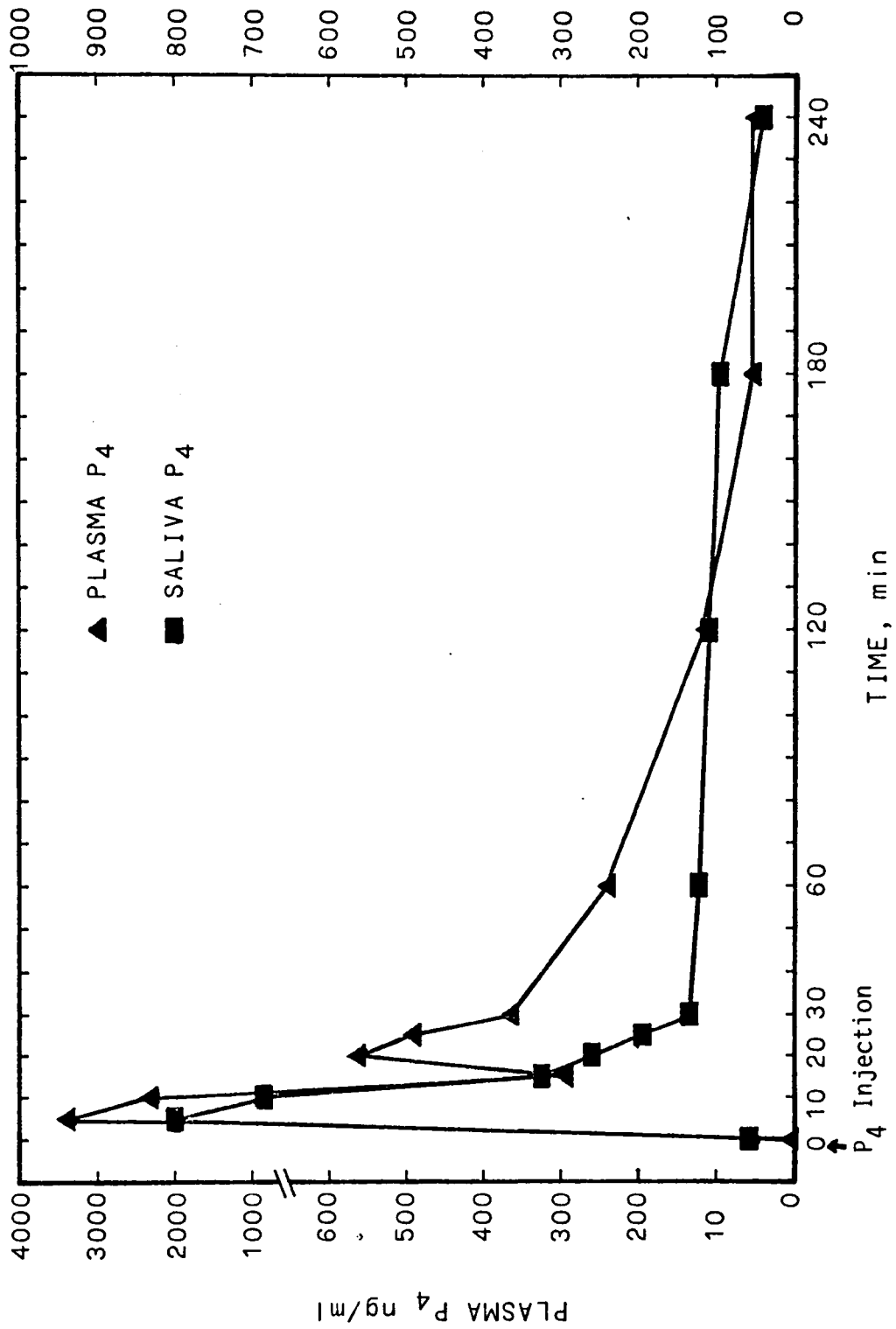

Figure I. The Disappearance of 250 mg Exogenous Progesterone (P_4) from the Blood and Saliva of a Dairy Cow.

designed to compare the effects of holding blood at 4°C in the laboratory for increasing lengths of time (0, 6, 12 hours). A fourth serum sample was prepared using special serum separation tubes (SST; Becton-Dickinson, Rutherford, N.J.) containing an inert barrier to facilitate serum collection. The fifth blood sample was heparinized whole blood, frozen immediately after collection. Treatment means for each cow were statistically analyzed for treatment effect and the effect of individual cow on progesterone concentration. Treatment means for each cow and overall treatment means are shown in Table VI. Treatment means were also subjected to Duncan's multiple range test.

By analysis of variance and orthogonal comparisons, significant differences were observed due to the method of handling blood prior to analysis (P .001) and to the individual cows in this study ($P<.001$). Although nine of the ten cows sampled were confirmed pregnant at the time of sampling, the differences in progesterone concentrations between cows have been reported elsewhere (Bowerman and Melampy, 1961; Laing et al., 1979; Stabenfeldt et al., 1970).

There was a significant loss of progesterone when blood samples were held at 4°C for either 6 or 12 hours prior to serum separation, when compared to samples centrifuged immediately upon return to the laboratory ($P<.05$). Progesterone concentration was lower in samples held for 12 hours than in samples incubated for 6 hours ($P<.05$).

The use of the serum separation tubes (Treatment II) resulted in no detectable loss of progesterone. The use of the SST tube proved to be an efficient, convenient method for serum collection in this study. Following centrifugation, serum was simply poured into storage

TABLE VI

MEAN PROGESTERONE CONCENTRATION IN BLOOD SAMPLES[1]

Cow no.	Treatments[2] I	II	III	IV	V
1	4.18 ± .15^{a}	3.79 ± .07^{b,c}	3.87 ± .07^{b}	3.53 ± .09^{c,d}	3.43 ± .07^{d}
2	4.78 ± .09^{a,b}	5.09 ± .38^{a}	4.87 ± .22^{a,b}	4.43 ± .08^{b}	2.97 ± .17^{c}
3	6.23 ± .03^{a}	6.12 ± .08^{a}	6.16 ± .16^{a}	4.99 ± .12^{b}	3.29 ± .12^{c}
4	3.59 ± .19^{a}	3.52 ± .17^{a}	3.45 ± .07^{a}	3.27 ± .05^{a}	2.20 ± .09^{b}
5	4.55 ± .20^{a}	4.23 ± .07^{a}	4.22 ± .11^{a}	3.36 ± .14^{b}	2.79 ± .08^{c}
6	4.04 ± .18^{a}	3.73 ± .11^{a}	3.66 ± .08	3.70 ± .11^{a,b}	2.90 ± .08^{c}
7	3.77 ± .07^{a}	3.69 ± .11^{a}	3.62 ± .16^{a}	3.45 ± .11^{a}	2.53 ± .10^{b}
8	3.66 ± .11^{a}	3.48 ± .07^{a}	3.48 ± .09^{a}	3.01 ± .14^{b}	2.39 ± .13^{c}
9	1.20 ± .14^{a,b}	1.31 ± .05^{a,b}	1.31 ± .06^{a,b}	1.41 ± .08^{a}	1.09 ± .09^{b}
10	3.90 ± .13^{a}	3.92 ± .02^{a}	3.84 ± .20^{a}	3.34 ± .09^{b}	2.83 ± .08^{c}
Overall treatment means	3.99 ± .34^{a}	3.88 ± .51^{a}	3.85 ± .27^{a}	3.45 ± .40^{b}	2.64 ± .53^{c}

[1]Values are mean ± SEM (n = 3) ng/ml

[2]Treatment I Serum - no incubation
Treatment II Serum separation tube
Treatment III Serum - 6 hr. incubation 4°C
Treatment IV Serum - 12 hr. incubation 4°C
Treatment V Whole blood

^{a,b,c,d}Treatment means in a row with different superscripts are different ($P<.05$) (Duncan's multiple range test)

vials, since the inert barrier forms a plug between the serum and the clot.

The analysis of heparinized whole blood for progesterone resulted in significantly lower progesterone concentrations ($P<.001$) than serum samples of the same volume (Treatment I). This was expected, due simply to the dilution effect of the red blood cells. To account for this dilution, the packed cell volume was determined for each cow as presented in Table VII. The concentration of progesterone in whole blood samples ranged from 52.8 to 90.8 percent of the progesterone concentrations determined in serum samples of the same volume, with a mean of 66.2 percent.

As represented in Table VII, a value for serum progesterone was calculated from measured whole blood concentrations by multiplying by a factor representing the ratio of serum:cells (1-PCV/PCV) for each cow. When compared to the overall mean serum concentration measured in Treatment I (3.99 ng/ml), this calculated value for serum progesterone (4.78 ng/ml) using whole blood samples was 16.5 percent higher.

This calculation is based on the assumption that 100 percent of the progesterone appears in the serum or plasma of peripheral blood in cattle following the separation from the blood cells, although the uptake or association of progesterone with red blood cells has been postulated or observed (DeVenuto, 1967; Sandberg et al., 1957). According to Short (1958) the plasma fraction of bovine blood should contain greater than 90 percent of the circulating progesterone in peripheral blood.

TABLE VII

WHOLE BLOOD PROGESTERONE (P_4) ANALYSIS

Cow no.	PCV[1]	Serum P_4 measured[2]	Whole blood P_4 measured[3]	Serum P_4 calculated[4]	Percent of expected serum concentration[5]
		ng/ml			
1	36	4.18	3.42	6.08	68.8
2	35	4.78	2.97	5.52	86.6
3	37	6.23	3.29	5.60	111.3
4	36	3.59	2.20	3.91	91.8
5	34	4.55	2.79	5.42	83.9
6	37	4.04	2.90	4.94	81.8
7	35	3.77	2.53	4.69	80.4
8	35	3.66	2.39	4.44	82.4
9	37	1.20	1.09	1.86	64.5
10	34	3.90	2.83	5.49	71.0
Overall mean	35.6	3.99	2.64	4.78	83.5

[1]Packed Cell Volume

[2]Mean value for Treatment I (Serum-0 Hr.)

[3]Mean value for Treatment V (Whole Blood)

[4]Whole Blood Concentration x $(\frac{1-PCV}{PCV})$

[5]Serum P_4 measured/serum P_4 calculated

III. PROGESTERONE IN BOVINE SALIVA

Two whole saliva samples were collected from each cow and analyzed for progesterone. The mean progesterone concentrations for samples incubated for 24 hours at room temperature and samples frozen immediately upon return to the laboratory as well as overall treatment means and standard errors of the means are shown in Table VIII. Saliva data was statistically analyzed in the same manner as blood handling data.

Based on orthogonal comparisons, the progesterone concentration in saliva (mean .93 ng/ml) is significantly lower than the concentration in whole blood or serum ($P<.001$). The low concentration of steroids in saliva from human patients has been previously reported (Smith et al., 1979; Walker et al., 1978; Walker et al., 1979). The concentration of progesterone in bovine saliva in this study (.93 ng/ml) was 23 percent (range 19-49 percent) of the concentration observed in serum samples from Treatment I (3.99 ng/ml). The progesterone concentration in the matched serum and saliva samples from the ten cows were tested for correlation ($r=.76$) ($P<.05$).

Other investigators report a high correlatior between the "free" or unbound levels of steroids in the peripheral blood and hormone levels determined from human saliva samples (Smith et al., 1979; Walker et al., 1979). However, the salivary progesterone levels measured in this study are higher than those expected, if 96 percent of the progesterone in the peripheral blood of pregnant cattle is indeed bound to plasma proteins, as reported by Kanchev (1976). This discrepancy may indicate the interference of feed matter or other

TABLE VIII

MEAN PROGESTERONE CONCENTRATION IN SALIVA SAMPLES[1]

Cow no.	Treatments[2] I	II
1	1.13 ± .09	1.22 ± .09
2	1.26 ± .06	1.25 ± .16
3	1.25 ± .14	1.09 ± .09
4	.78 ± .14	.71 ± .06
5	1.02 ± .06	1.06 ± .08
6	.78 ± .07	.96 ± .06
7	.77 ± .10	.77 ± .10
8	.54 ± .16	.51 ± .07
9	.55 ± .03	.62 ± .05
10	1.12 ± .04	1.13 ± .06
Overall treatment means	.92 ± .31	.93 ± .26

[1]Values are mean ± SEM (n = 3), ng/ml

[2]Treatment I-Saliva - no incubation
Saliva - 24-hour incubation

enzymes or proteins in the saliva or digestive secretions of the cow at the time of sampling with the radioimmunoassay for progesterone. Differences in progesterone metabolism between the bovine and human species may also account for some of the differences observed.

The incubation of saliva samples for 24 hours at room temperature (Saliva Treatment II) had no effect on measurable progesterone concentration. The loss of progesterone from serum samples stored for 6 or 12 hours at $4^{o}C$ in this study, and reported by others in bovine serum and plasma samples (Owens et al., 1980; Vahdat et al., 1979; Wentzel, 1972) warrants further analysis of the use of saliva as a matrix for the determination of progesterone in the bovine. Saliva samples collected in the field would require minimal special handling to insure accurate determination of progesterone concentration. In addition, if it could be determined that salivary progesterone concentrations in the bovine reflect the level of "free" or unbound circulating progesterone, a more accurate measurement of physiological activities of progesterone in the bovine could be made.

CHAPTER V

SUMMARY

The determination of progesterone concentration in the peripheral blood is an effective indicator of reproductive status of the dairy cow. This study confirms earlier reports that the method of handling blood samples from the time of collection to the time of frozen storage significantly affects the stability of progesterone in the blood. The use of whole blood samples frozen immediately following collection would reduce the equipment and time required to process blood samples collected in the field or under normal husbandry conditions.

Progesterone concentrations in serum and saliva samples in the bovine are correlated (r=.76-.96). The progesterone concentration in whole saliva samples is 23 percent of the concentration found in serum samples. However, progesterone appears to be stable in saliva samples stored at room temperature for up to 24 hours.

The radioimmunoassay for progesterone is a sensitive, precise method for determination of progesterone in bovine blood, milk and saliva samples in the dairy cow. The effects of method of handling and storage on progesterone stability are especially critical for blood samples and care must be taken tc avoid significant progesterone losses. Time and temperature should be controlled to insure accurate measurement of progesterone concentrations as a guide to the control of reproduction in the bovine.

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