BOVINE PLASMA ANALYSIS FOR PROGESTERONE.

A QUANTITATIVE METHOD EMPLOYING GAS LIQUID CHROMATOGRAPHY

by

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

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ABSTRACT

A technique was developed that could quantitate sub-microgram amounts of progesterone from bovine blood plasma. Using ether extraction, thin-layer chromatography and gas chromatography, nanogram amounts of progesterone were measured with excellent precision and reproducibility.

Six cows were assigned to one of two groups. One group of three cows had their ovaries removed to eliminate a possible source of progesterone. The other three were left intact and used as controls. All six cows were bled from the jugular vein on days 1, 5, 10, 15, 20 (day 1 is the day of estrus) prior to being heat stressed or ovariectomized. In effect, each cow acted as her own control. Allowing a 30 day period for the ovariectomized cows to heal, they were then placed in a controlled environment chamber for a 24 hour period. The cows were again bled at 5 day intervals for 30 days, starting the day they came out of the heat barn. The control cows were also bled at five day intervals for 30 days after heat stressing. This was done to determine if there was an increase in progesterone due to the stress effect and not directly contributable to the ovaries. All cows, both control and ovariectomized, showed the effect of heat stress. Some cows had a delayed response to the elevated temperature.
Evidence suggests that heat stress does increase the production of adrenal progesterone, and the possibility of an adrenal relationship to lowered breeding efficiency may exist in areas where high ambient temperatures are common.
INTRODUCTION

Considerable research has shown that cattle are influenced in their reproductive behavior by seasonal and temperature changes. A wide range of information is available on different aspects of male bovine fertility related to ambient temperature. Recent investigations, however, indicate that the cow, rather than the bull, is the major cause of lowered seasonal fertility in Arizona.

Basic knowledge on the exact physiological cause of this lowered fertility is limited. Such information is necessary for an understanding of the problem, and as a means of determining how to improve reproductive efficiency in areas of the world where high temperatures are common.

A recent investigation has shown a significant increase in progestin content of the adrenal glands of bovine under conditions of thermal stress. It seems evident that if the higher levels of adrenal progesterone are being secreted into the blood, this could alter the normal reproductive activity and be responsible for low breeding efficiency of cattle during hot weather. However, it is essential to demonstrate this relationship of heat stress and high blood levels of progesterone to reproductive failure, if this is to be used as a logical explanation.
Studies related to the secretion and metabolism of progesterone in the peripheral blood have been hampered by the lack of chemical methods sufficiently sensitive and specific to quantitate the hormone with relative ease and reproducability.

With this in mind, the present study was designed to:

1. Develop a technique, using gas chromatography, to measure quantitatively peripheral blood levels of progesterone in bovine.

2. Determine if induced thermal stress in bovine will effect peripheral blood progesterone.

3. Determine the endocrine source of peripheral blood progesterone under stress conditions.
REVIEW OF LITERATURE

Seasonal Variation in Breeding Efficiency

Considerable research has shown that cattle are influenced in their reproductive behavior by seasonal and temperature changes (3, 4, 10, 12, 23, 25, 26, 28, 30, 34, 39, 40, 47, 48, 50, 53, 54, 57, 61, 62, 63, 64, 65, 66, 67, 70). A wide range of information is available on different aspects of male bovine fertility related to ambient temperature (12, 39, 67). It would appear from recent investigations by Stott and co-workers (61, 62, 63, 64, 70) that the cow rather than the bull is the major contributor to low seasonal fertility. Knowledge of the basic physiological causes of this lowered fertility of the female is limited (17, 18, 22, 61, 62).

Some workers have shown that during critical periods of early pregnancy, excessive progesterone in proportion to estrogen can cause a disruption of cleavage (14), too rapid passage of the fertilized ovum through the oviduct (2, 17), lack of implantation (63), and finally, abnormal placentation (56).

Howarth et al. (38) felt that the sperm were affected by the higher body temperature of the female causing a smaller number of sperm to be
transferred up the oviduct and fewer sperm in the zona pellucida of the recovered eggs.

Most research workers agree that the major source of progesterone in the early stages of pregnancy (up to placentation) is the corpus luteum (24, 32, 33, 51, 59). Gomes (31) reported the major source of progesterone throughout pregnancy in the cow is the corpus luteum, but that ovaries and adrenals might contribute a significant amount, and body fat may store the hormone to be used as needed. However, it has been established that the adrenal glands secrete significant amounts of progesterone (5, 6, 7, 13, 15, 33, 51, 59). Moody (50) found that under periods of heat stress the adrenal glands contained up to twice their normal level with no increase in progesterone from the corpus luteum. It might be expected that the adrenal glands contain more progesterone, since progesterone is an intermediate of cortisone production. Cortisone is produced in the cortex of the adrenal under stress conditions. It is, therefore, necessary to demonstrate that this added progesterone finds its way into the peripheral blood and is responsible for the reproductive failures found in Arizona during the summer months.

Assay Methods for Progesterone

Studies related to the secretion and metabolism of progesterone in the peripheral blood have been hampered by the lack of chemical methods
sufficiently sensitive and specific to quantitate the hormone with relative ease and reproducability. Present methods require the use of large quantities of blood plasma (58).

**Bioassy:** Some biological assays are very sensitive, but lack specificity, quantitation, and have undefined limits of error (21, 37). The assays are based on a subjective end point such as complete gestational proliferation, uterine granulosa/mucosa area, carbonic anhydrase activity, pregnancy maintenance, or parturition delay. These methods have indicated progesterone levels in biological fluid that cannot be verified by chemical assay (20, 21, 58).

**Chemical Methods:** In recent years considerable progress has been made in the development of chemical methods to determine progesterone (19, 20, 44, 58).

Extraction of progesterone from plasma has generally been accomplished by the use of organic solvents such as ether, methylene chloride, acetone, or ether-chloroform (29, 46, 52, 68, 69, 73). Zander (74) has shown that pre-treatment of plasma samples with sodium hydroxide as proposed by Short (58) improves the precision of the final progesterone quantitation. In addition, sodium hydroxide decreases the formation of solvent-plasma emulsion during ether extraction.

One of the difficulties involved in solvent extraction is the great quantity of lipid extracted. To obtain a more nearly pure extract from plasma several techniques have been used. A. O. Lurie et al. (46)
purified the extract from plasma by chromatography through miniature Celite columns using trimethyl pentane as the mobile phase and 90% methanol as the stationary phase. This step removed a large quantity of pigmented material and cholesterol. Niell et al. (52) further cleaned the ether extract with washings of 1/10 volume of water before evaporating to dryness. Another clean-up procedure proposed by Williams (69) was to take the dried, crude lipid sample into solution in benzene and chromatograph on silic acid columns.

Separation and additional purification of the steroids by countercurrent distribution has been utilized in only one routine method (45). Application of this method appears to be limited by the relatively long time required to separate and purify a single sample.

Use of paper chromatographic techniques in the separation and identification of adrenal steroids (8, 9) has evident advantages, but again is limited by the time involved.

Thin-layer chromatography has recently come on the scene as a technique that will supplement or even replace paper chromatography. The speed (20-40 min.) compared to that of paper chromatography (4-24 hr.), the wide range of distinct resolutions (5-500 ug.), and the noticeably sharp separation of compounds are distinct advantages of thin-layer chromatography (36). The method has been tested for a great number of steroids in various solvent systems and complete data are available for the pregnane series (41, 42, 43).
Warner-Chilcott (68) dissolve the ether extraction residue in chloroform and apply it to a thin-layer plate coated with Silica Gel G containing fluorescein. The plate is developed using Benzene-ethyl acetate (32). Williams (69) uses the same type of plates, but has found a saturated chamber of chloroform: absolute ethanol (9:1, v/v) useful for steroid separation. A wide range of solvent systems are available (for specific solvents see references 9, 27, 29, 36, 41, 42, 43, 45, 46, 52, 68, 69, 73).

Quantitation of Progesterone

The Δ4 - ene-3-one grouping of the steroid nucleus may be quantitated by its strong absorbance of UV light at 240 μm in alcoholic solutions (10, 11, 16).

Other spectrophotometric assays have been reviewed by Zander (74) and are based on absorbance of UV light at wave lengths other than 240 μm by progesterone derivatives. These include the progesterone bis-thiosemicarbazone at 300 μm, the progesterone bis-dinitrophenyl-hydrazone at 380 μm, and the sulfuric acid-ethanol chromagen at 290 μm. As Zander (74) pointed out, these methods have the advantage of shifting the Quantitation wave length away from that of interfering materials, but lack specificity and make it difficult to identify the isolated material as progesterone. The limit of sensitivity of the spectrophotometric
methods range from 0.5 to 1.0 ug if micro-cells are used. The levels of progesterone in peripheral blood are expected to be in the 0.1 ug range, much below the range of the spectrophotometer.

The double isotope derivative (DID) principle generally involves the addition of an isotopically labeled steroid (C$^{14}$) to the unknown sample. The unknown steroid is then extracted and purified along with the labeled steroid and are both converted to a derivative using a reagent labeled with a second isotope, Tritium (H$^{3}$). The amount of H$^{3}$ in the final derivative quantitates the amount of derivative formed and the C$^{14}$ measures procedural losses.

Riondell (55) first applied the DID procedure to progesterone determination using progesterone-H$^{3}$ with thiosemicarbazide-S$^{35}$ as the derivative forming agent. Woolever and Goldfein (72) converted progesterone to $\Delta^{4}$ pregnene-20B-ol-3-one (20B-ol) using tritiated sodium borohydride and C$^{14}$-progesterone as the isotope source. This method is reported sensitive to 0.01 ug of progesterone, but it was found that the time (8-19 days) and per cent recovery were the big drawbacks with this method (71).

Gas Chromatography

As recently as 1962 Futterweit, et al. (29) reported the first use of gas chromatography for the quantitative assay of progesterone. However, it was a difficult, time consuming method with less sensitivity than
available methods. In 1964, Yannone, M.E. et al. (73) reported the first highly sensitive gas chromatography assay for progesterone. This method is reported sensitive to less than 0.02 ug of progesterone. Since that time many workers have reported excellent use of gas chromatography for the quantitation of progesterone (29, 46, 52, 60, 68, 69). Gas chromatography seems to offer specificity that cannot be shown by any of the before mentioned techniques.

A wide range of column packings and temperatures have been used for the quantitation of progesterone. Estergreen (27) reported resolutions of progesterone at 0.05 ug with the use of 6-foot glass columns packed with Anakrom ABS 80-100 mesh for the support and 1% SE-30 liquid phase, and a column temperature of 210° C. Lurie (46) reported detection of 0.02 to 0.22 ug added to male pooled plasma with columns packed with 3% XE-60 on Gas Chrom P of 80-100 mesh (Perkin-Elmer), but added that the method tends to over-estimate the amount of progesterone in the 20-80 nanogram range. Neill (52) reported the smallest amount of progestin that could be detected with gas chromatography was near 0.01 ug on 1% SE-30 on 60-80 mesh Chromport 3X (Micro Tek Instrument Inc.). Coiled pyrex glass columns 6 feet x.125" O.D. were used with a column temperature of 250° C. Warner-Chilcott (68) used columns 33" x.125" O.D. packed with 1% XE-60 on Chromosorb G (100-120 mesh) DMCS treated but didn't give the lower range of detection.
EXPERIMENTAL PROCEDURE

General

One of the main purposes of this study was to determine the endocrine source of progesterone under conditions of stress. Six cows, 4 Holstein and 2 Gurnsey's, were assigned to one of two groups. One group of 3 cows had their ovaries removed to eliminate this as a possible source of progesterone. This unilateral ovarectomy was accomplished by a simple laparotomy, clamping off the ovaries for approximately 30 minutes, and then teasing away with the fingers. The other three, all of the Holstein breed, were left intact and used as controls. The three ovarectomized cows (G-254, G-266, H-710) were bled from the jugular vein on days 1, 5, 10, 15, 20 (day 1 is the day of estrus) prior to having their ovaries removed. Allowing a 30 day period for healing, the cows were then placed in a controlled environment chamber (temperature \( \times 101^\circ C \) humidity \( \times 44\% \)) for a 24 hour period. They were bled at 5 day intervals for 30 days, starting the day they came out of the heat chamber, to determine if there was an increase in progesterone due to the stress effect and not directly contributable to the ovaries.

The control cows (H-564, H-706, H-708) were also bled from the jugular vein on days 1, 5, 10, 15, 20 (day 1 is the day of estrus) prior
to heat stress. On the day of next estrus they were placed in the controlled environment chamber (temperature $103^\circ C$ humidity $44\%$) for a 24 hour period to heat stress.

Peripheral blood was collected from the jugular in clean glass bleeding bottles. The bottles contained Sodium Heparin (NBC), 1 unit per 1 ml of blood, dried down to reduce hemolysis. Approximately 20 ml of blood was collected at each bleeding, spun down within one hour, and the plasma drawn off. No attempts were made to cool the blood while being transported from the farm to the laboratory. From the 200 ml of blood, approximately 80-90 ml of plasma was obtained, placed in plastic vials, and frozen until needed for analysis.

**Progesterone Determination**

It was essential to develop a technique for measuring submicrogram amounts of progesterone, before the remaining portion of the experiment could be carried out. The general procedure used for determination of progesterone was a composite of several published techniques (58, 68, 69, 72, 74).

The frozen plasma samples were thawed at room temperature. Twenty ml of the plasma was placed in stoppered centrifuge tubes, and 500 lambda of progesterone $4-C^{14}$ (0.1 microcuries) was added to the sample. Also, 1 ml of 5N aqueous Sodium Hydroxide, and 1 ml of toluene
were added and mixed thoroughly. The plasma was then washed 8 times with an equal volume of ether. The ether extraction was poured into a round bottom flask, placed in a 70°C water bath and dried under a vacuum. The sides of the round bottom flask were then washed down with ether which was evaporated. The process was repeated twice in order to concentrate the extract at the bottom of the flask. The residue was then put into solution with .5 ml of methanol: chloroform 1:1 (v:v) and spotted on thin-layer plates. After the original .5 ml had been spotted, an additional .5 ml of methanol:chloroform was added to the round bottom flask and spotted.

Because of the sensitivity of progesterone determinations to almost any kind of contaminant, all solvents were redistilled before use.

**Thin Layer Chromatography (TLC)**

The thin-layer plates were pre-coated with Silica Gel F254 (E. Merck AG * Darmstadt Germany and distributed by Brinkman Instrument Co.). The plates were divided into five equal lanes and scored to prevent over-lap of the samples. The plasma extract was spotted on 3 lanes (the two outer and the middle), and a progesterone standard spotted on the remaining two.

The chromatogram was then developed in an equilibrated tank containing Benzene:Ethyl acetate 4:1 (v:v). The chromatograph was completed in about 45 minutes. The plate was then taken out of the tank, allowed to
dry, and then replaced in the tank another 30 minutes to further move the pigments. The plate was then removed, allowed to dry, and the position of the progesterone standards were determined under UV light (Minera-light, Ultra-Violet Products, Inc., San Gabriel, California). The corresponding areas in the lanes containing the sample extract were marked, scraped into a heap, and placed in a 15 ml centrifuge tube. After the Silica Gel was transferred to the tube, 1 ml of ethanol was added, shaken thoroughly for one minute, and then centrifuged for 15 minutes at 25,000 rpm. The eluate was collected in a 2.5 ml stoppered, tapered centrifuge tube and dried under a stream of nitrogen. The process was then repeated one more time to be sure that all the free progesterone had been recovered from the Silica Gel. The sides of the small tube were then washed 2 times with ethanol and the solvent was evaporated each time to concentrate the residue at the bottom of the tube. The plasma extract was then ready for gas chromatography.

Gas Chromatography (GLC)

After extraction and purification, progesterone was identified and quantitated, using a Micro-Tek model DSS 170 Gas Chromatography with a dual hydrogen flame detector. This model was further modified by the installation of a glass injection system. The columns were packed with 3% S. E. 30 on Aeropack 30 of 80/100 mesh prepared in bulk by Wilkens
Instrument and Research Inc., Walnut Creek, California. For the purpose of obtaining the data for this study, a 3' pyrex glass column with an OD of 1/8" were packed with 3% SE 30 on Aeropack 30 80/100 mesh and conditioned overnight at 260° C and a nitrogen carrier pressure of 30 psi. When running samples, the oven was kept at 255° C, the detector at 255° C, and the vaporization chamber at 315° C. Nitrogen flow was 20 psi, Hydrogen 25 psi, and Oxygen .2CFH.

Samples from TLC separation were taken into solution in 20 ul of chloroform and 1-5 ul injected onto the column. The samples were introduced through a rubber diaphragm with a Hamilton Micro-syringe onto the column. The progesterone peak was recorded at the optimal attenuation to produce an easily measured peak height. Before and after each group of samples had been analyzed, known quantities of progesterone were chromatographed and the standard curve derived was used to quantitate the peak heights recorded in the plasma extract (Figure 1).

**Per Cent Recovery**

Before quantitating the plasma extract by GLC, 100 lambda of each sample was placed in counting vials to determine per cent recovery of progesterone. The samples in the counting vials were dried, and 15 ml of PPO-POPOP was added before placing in the counting chamber. The
scintillator fluid was prepared by adding 5 grams of PPO (2, 5-Diphenyloxazole) and 0.3 grams of Dimethyl POPOP (1, 4-5is-2-(4-methyl-5-Phenyloxazolye)-Benzene) to one liter of toluene. Liquid scintillation counting was done in a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co.) for 10 minutes. The CPM of the sample was divided by the CPM of the Standard to determine procedural loss.
RESULTS AND DISCUSSION

The lack of an acceptable method to determine bovine systemic blood progesterone for comparative purposes necessitated first the development of a dependable procedure that could be used routinely for analysis.

Evaluation of Methods

Following the procedure of Wooley and Goldfein (71), 0.1 microcuries of progesterone-4-C\textsuperscript{14} was added to the plasma in order to measure recovery. The addition of 1 ml 5N aqueous Sodium Hydroxide to plasma greatly improved the recovery of labeled progesterone. The addition of 1 ml of toluene to plasma containing Sodium Hydroxide usually resulted in a gummy mass that was easier to extract with ether. Extraction with ether did not lead to formation of emulsions and this gave the best per cent recovery of progesterone-4-C\textsuperscript{14} added to the samples. Several methods were tried, but these amounts which gave the greatest recovery of steroid were adopted for routine use.

An additional purification step was found necessary since pigments were visible and gas chromatography of the residue revealed very large peaks and a considerable amount of non-specific background material.
which required a prolonged period to elute from the gas chromatography column. Thin-layer chromatography was decided on for this additional clean-up because of the speed and sharp separation of compounds.

The small residue obtained after ether extraction was efficiently handled by the Silica Gel F254 with little evidence of overloading. In the few cases that showed evidence of overloading, the samples were re-chromatographed. The system of Benzene:Ethyl acetate 4:1 (v:v) not only separated progesterone from cholesterol, but also proved highly successful in removing the non-specific background material.

Several methods for recovering the steroid from thin-layer silica gel were tried. Lurie et al. (46) proposed scrapping the silica gel into a heap, taking it up by vacuum into a tapered glass tube plugged with glass wool. After the silica gel was transferred to the glass tube, 5 ml of chloroform/ether 1:1 was eluted through it to recover the progesterone. The method decided on, that of scrapping the silica gel off into a centrifuge tube and eluting the steroid with ethanol, compared favorably with the method of taking the silica gel up by vacuum and proved to be less time consuming.

It was optimistically hoped by some workers in the past that some simple and rapid extraction method of steroids from biological fluids would suffice to prepare the samples for application to chromatographic columns. This, however, has not proved to be the case, and it is now
evident that the small quantities of steroid present in these fluids are completely obscured by the accompanying material.

Gas chromatography was decided on as a method of quantitation since it gave excellent specificity and reproducibility using a progesterone standard. However, the G.L.C. had to be further modified with a glass injection system to measure sub-microgram amounts of progesterone. The smallest amount of progesterone standard that could be satisfactorily measured with a flame ionization detector was 10 nanograms. The measurement of smaller amounts were not reproducible. Estimates of progesterone in the samples can be obtained by using peak height alone and was found to be as accurate as peak height x peak width at half-height (Figure 1). The retention time of progesterone on successive injections was highly reproducible and this added confidence to the identification of each peak. Successful quantitation of sub-microgram amounts of progesterone on the gas chromatograph requires the use of a short column to obtain sharp measurable peaks. Since the efficiency of a short (3') column is limited, the hormone to be assayed had to be isolated with a minimum amount of interfering substance.

Three per cent SE 30 was found to be the most satisfactory stationary phase for the chromatography of plasma extracts since most of the non-specific background material was eluted after the pergesterone peak. The height of the peak could be measured with confidence when sub-microgram amounts were present.
The several steps of this method have been examined and its overall results indicate that it is suitable for measurement of progesterone in the plasma. The method is simple and rapid enough to quantitate many samples in one day. The greatly increased sensitivity in the measurement of progesterone by the use of gas chromatography has been a notable advantage of this method. The use of gas chromatography for the measurement of progesterone in plasma required careful extraction and purification of the steroid.

**Peripheral Progesterone in Experimental Cows**

After a method for measuring nanogram amounts of progesterone had been developed, it was then necessary to determine if induced thermal stress in bovine would effect peripheral blood progesterone. It was also essential to demonstrate the endocrine source of peripheral blood progesterone under stress conditions.

To do this, experimental cows were exposed to high temperature and relative humidity in the controlled environment chamber. Response was noted in the way of elevated body temperature, apparent discomfort and increased respiration rates. Cows placed in the heat chamber by themselves went off feed for the 24 hour period, while those cows placed in the chamber with another cow seemed to consume the regular amount of feed. This might possibly be a stress effect not anticipated in the experimental design.
It was found that there was less hemolysis of the blood plasma if it was collected in glass bottles containing dried sodium heparin, 1 unit per 1 ml of blood. The less the blood was shaken, and the sooner it was centrifuged after collection, the more it aided in reducing hemolysis.

Ovarectomized Cows - Pre-heat stress, Pre-ovarectomy (Figures 2 and 3): It has been shown in the past that most species of animals reach a peak of progesterone about day 15, when the corpus luteum is fully developed.

Cow 254 seemed to show an early abnormally high level of progesterone early in her cycle. Cow 266 shows a peak of progesterone on day 20 towards the end of her cycle. These results are contrary to what has been assumed in the past, that day 15 is when the progesterone peak is reached. These two cows seem to show their peak of progesterone production around day 1 or estrus. This could be normal since our knowledge of the cow's progesterone secretion during a cycle is limited. Cow 710 shows a progesterone production closer to that seen in other species.

Ovarectomized Cows - Post-heat stress, Post-ovarectomy (Figures 2 and 3): Cows in the ovarectomized and heat stressed group all showed a response to the heat stress. Cow 254 and cow 266 showed an obvious immediate response and a gradual tapering to 20 days. Cow 710 didn't show an immediate increase in progesterone until day 15. It is interesting to note that on day 30 all cows seem to be much higher, indicating a residual or delayed effect of the heat stress.
Control Cows - Pre-heat stress (Figures 4 and 5): The control cows, pre-heat stress, on the average showed the cycle (levels of progesterone) that has been seen in other species of farm animals. Again, there are deviations from the expected 15 day high level of progesterone and is most probably due to sampling error. Significant is the fact that again two cows (564 and 708) showed high levels of peripheral plasma progesterone early in the cycle. Also, the controls and ovariectomized cows pre-heat stress, show a higher level of progesterone on day 5, than they do on day 1 or 10.

Control Cows - Post-heat stress: Cows 564 and 706 again show an immediate effect of heat stress with cow 708 showing a delayed effect. Again note that on day 30 all cows seem to have a higher progesterone level than normal.

All cows, both control and ovariectomized, showed the effect of heat stress. Some cows had a delayed response. A possible explanation of these varied results is that since the blood samples were taken every five days, the peak of progesterone production was reached somewhere between one of these five day intervals. Every cow's cycle is of a different length, and 17-25 days is considered normal. So it is possible that every cow's corpus luteum matures earlier or later than day 15.

Cows culled from the herd were used in the ovariectomized group. They
were hard breeders or repeat breeders, which may account for this wide variance in results.

The averages for the six cows pre-heat stressed and pre-ovarectomized is as follows:

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The limited data in this study suggests that progesterone concentration is increased in the peripheral blood by thermal stress. Since the ovaries were removed in half of the experimental cows, it seems evident that this increase had to come from the adrenal glands. This is in agreement with Moody (50) who found that heat treated animals had, on the average, about twice the progesterone concentration in the adrenals as the corresponding control group.

Several conclusions can logically be built from these observations that would possibly explain reduced breeding efficiency during hot weather.

One theory is that high levels of progesterone at the time of estrus seems to speed up the time of ovulation and shortens the duration of estrus (Hansel et al., 35). This gives two possible reasons for lowered fertility during times of high ambient temperature: (a) shortened duration of estrus results in failure of observation of estrus, (b) the time
of ovulation is hastened, resulting in immature fertilized ovum reaching the uterus too early, resulting in embryonic death.

Alliston and Ulberg (2) by the use of ova-transfer technique have illustrated the inability of normal fertilized ova from temperature-stressed ewes to develop in the uterus of a non-stressed ewe. They suggest that the speed the embryo travels through the oviduct may be increased in the case of the stressed ewe and the embryonic mortality may be due to "immature" embryos reaching the uterus. This is in agreement with Dutt (17) that the critical period is the time when the fertilized ova is in the oviduct.

A study by Williams (70) of ovulation time with cows in central Arizona during the first two weeks in September found no evidence of abnormally early ovulation. However, he did observe that the cows were very inactive during the heat of the day. If we assume that the increased levels of progesterone observed in the blood plasma in this study came from the adrenal glands during periods of thermal stress, we could conjecture a shortened duration of estrus as observed by Hansel et al.,(35). Correlating the inactivity of cows during the heat of the day with the possibility of a shortened duration of estrus, the failure of detection of estrus seems plausible.
SUMMARY

An essential part of this research was to develop a technique for measuring quantitatively peripheral blood progesterone. The several steps of this method have been examined and its over-all results indicate that it is suitable for measurement of progesterone in the plasma. The method is relatively simple and rapid enough to quantitate many samples in one day. The greatly increased sensitivity in the measurement of progesterone by the use of gas chromatography has been a notable advantage of this method.

The present study was designed to investigate the possibility of a hormonal imbalance due to elevated body temperatures during the time of estrus and shortly thereafter. Peripheral plasma progesterone in non-heat stressed animals was compared to that of heat stressed animals. One group of cows was ovariecotomized to eliminate the ovaries as a possible source of progesterone. Both groups of cows were heat stressed and plasma progesterone compared.

Progestion concentration in the plasma were on the average higher for the two groups of heat stressed animals. The mean recovery of all samples was 41.9% with a range of 16.8-88.0%.

Evidence suggests an increased progesterone production by the adrenals under stress conditions.
APPENDIX
Figure I. Standard Curve for Determination of Progesterone.
<table>
<thead>
<tr>
<th>Day</th>
<th>Cow No. 254</th>
<th></th>
<th>Cow No. 266</th>
<th></th>
<th>Cow No. 710</th>
<th></th>
<th>( \bar{X} ) ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Recovery(^a)</td>
<td>ng/ml(^b)</td>
<td>% Recovery(^a)</td>
<td>ng/ml(^b)</td>
<td>% Recovery(^a)</td>
<td>ng/ml(^b)</td>
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**Pre-Ovarectomized and Pre-Heat Stressed**

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<th>Cow No. 266</th>
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<th>Cow No. 710</th>
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</table>

\(^a\)Per cent recovery of progesterone 4-C\(^{14}\) from plasma

\(^b\)Nanograms (10\(^{-9}\)) of progesterone/ml of plasma

Figure 2. Ovarectomized cows.
Figure 3. Ovarectomized Cows.
<table>
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<td>% Recovery(^a) ng/ml(^b)</td>
<td>% Recovery(^a) ng/ml(^b)</td>
<td>X ng/ml</td>
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</table>

\(^a\)Per cent recovery of progesterone 4-Cl\(^{14}\) from plasma

\(^b\)Nanograms (10\(^{-9}\)) of progesterone/ml of plasma

Figure 4. Control cows.
Figure 5. Control Cows.
Figure 6. Averages of Control Cows and Ovarectomized Cows.
BIBLIOGRAPHY


