Periparturient estrogen levels in the plasma of beef cows
by John Norman Stellflug

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Animal Science
Montana State University

Abstract:
Continuous TLC, with petroleum ether (BPT 30-60°C) anydrous ether and formic acid (100:100:2), separated estradiol epimers .65 cm and estradiol-17α and estrone 3.31 cm. The TLC elutants exhibited contamination of 0.2, 2 and 5 ng for estradiol-17α estradiol-17β and estrone, respectively, when assayed in a CPBA. These TLC elutants also resulted in contamination greater than 300 pg when they were assayed with RIA, however, purification with a 10 cm Sephadex LH-20 microcolumn lowered the contaminants to 53, and 90 pg for estradiol-17α and estrone, respectively, but did not influence the estradiol-17β contaminants. Total estrogens were measured with a RIA in the plasma of six beef cows at four hour intervals from 158 hours prepartum to 46 hours postpartum. The RIA, developed with antisera which had 60 and 59.7% binding for estradiol-17,5 and estrone, respectively, had a work sensitivity of 12 pg. Dextran-coated charcoal was used to separate the unbound from the bound estra*-diol-17β -6,7-3H. An assay contained a complete set of samples from one cow. Precaution was taken in comparing separate assays since the standard curves moved on the γ axis (P<.01), however, the same line of regression was observed for each standard curve. A linear regression equation (P<.01) and a quadratic equation (P<.05) define an increase of postpartum estrogen levels and a marked decrease of estrogen postpartum, respectively. The dramatic discrepancy between the theoretical values calculated for parturition (hour 0) from the regression equations (188 pg/ml) and the dramatic drop between the least-squares means estrogen level two hours before and after parturition (275 pg/ml) described the rapid decrease of estrogen at this time. Another hour which was nonsignificant but of importance occurred at -90 hours and was speculatively related to the initiation of prolactin release. Blood estrogens for individual cows showed that blood collection will have to be at closer intervals than four hours to adequately define the estrogen fluctuation which suggested a homeostatic control and triggering mechanisms for physiological events. The dramatic estrogen level decrease started by 14 hours prepartum in one cow while the average time for the estrogen decrease occurred between two hours pre- and postpartum. This decrease was related to the initial loss of circulation between the maternal and fetal . environment and finally the loss of fetal cotyledons. The direct relationship of gestation length (P<.05) and inverse relationship of birth weight (P<.01) to prepartum estrogen levels appear to contradict each other; however, a larger number of cows would be necessary to elucidate this phenomenon. It was speculated that these relationships may be true and not contradictory. If the fetus metabolized estrogens in proportion to size and all of the estrogen sources combined, synthesized estrogen at a relatively constant rate then the latter relationship would be true, while the former wpuld be true if one or more of the sources of estrogen levels above the amount the fetus could metabolize.

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ABSTRACT

Continuous TLC, with petroleum ether (BPT 30-60°C), anydrous ether and formic acid (100:100:2), separated estradiol epimers .65 cm and estradiol-17α and estrone 3.31 cm. The TLC elutants exhibited contamination of 0.2, 2 and 5 ng for estradiol-17α, estradiol-17β and estrone, respectively, when assayed in a CPBA. These TLC elutants also resulted in contamination greater than 300 pg when they were assayed with RIA, however, purification with a 10 cm Sephadex LH-20 microcolumn lowered the contaminants to 53, and 90 pg for estradiol-17α and estrone, respectively, but did not influence the estradiol-17β contaminants. Total estrogens were measured with a RIA in the plasma of six beef cows at four hour intervals from 158 hours prepartum to 46 hours postpartum. The RIA, developed with antisera which had 60 and 59.7% binding for estradiol-17β and estrone, respectively, had a work sensitivity of 12 pg. Dextran-coated charcoal was used to separate the unbound from the bound estradiol-17β -6,7-^{3}H. An assay contained a complete set of samples from one cow. Precaution was taken in comparing separate assays since the standard curves moved on the Y axis ($P<.01$), however, the same line of regression was observed for each standard curve. A linear regression equation ($P<.01$) and a quadratic equation ($P<.05$) define an increase of postpartum estrogen levels and a marked decrease of estrogen postpartum, respectively. The dramatic discrepency between the theoretical values calculated for parturition (hour 0) from the regression equations (188 pg/ml) and the dramatic drop between the least-squares means estrogen level two hours before and after parturition (275 pg/ml) described the rapid decrease of estrogen at this time. Another hour which was non-significant but of importance occurred at -90 hours and was speculatively related to the initiation of prolactin release. Blood estrogens for individual cows showed that blood collection will have to be at closer intervals than four hours to adequately define the estrogen fluctuation which suggested a homeostatic control and triggering mechanisms for physiological events. The dramatic estrogen level decrease started by 14 hours prepartum in one cow while the average time for the estrogen decrease occurred between two hours pre- and postpartum. This decrease was related to the initial loss of circulation between the maternal and fetal environment and finally the loss of fetal cotyledons. The direct relationship of gestation length ($P<.05$) and inverse relationship of birth weight ($P<.01$) to prepartum estrogen levels appear to contradict each other; however, a larger number of cows would be necessary to elucidate this phenomenon. It was speculated that these relationships may be true and not contradictory. If the fetus metabolized estrogens in proportion to size and all of the estrogen sources combined, synthesized estrogen at a relatively constant rate then the latter relationship would be true, while the former would be true if one or more of the sources of estrogen levels above the amount the fetus could metabolize.

INTRODUCTION

Estrogens, the first of the steroid hormones isolated, were crystalized from human pregnancy urine in 1929. Isolation directly from ovarian tissue was accomplished in 1935.

Estrogens, found in ovaries, testes, adrenals, placental and even small amounts in spermatozoa, play a very important role in reproduction which is one of the primary reasons they are subject to extensive study. They produce a great variety of effects on specific target organs and on the chemistry of the body as a whole.

Some factors limiting the research on estrogens have been the lack of rapid isolation methods and sensitive assay methods. There is also a great deal of evidence expressing the need for the separation of the different estrogens allowing qualitative quantitation of estrogens in blood throughout the reproductive cycle.

The objectives of this study were to (1) develop a separation scheme which would allow measurement of individual estrogens in the sensitive assays of today, (2) to define the blood estrogen level changes surrounding parturition.

REVIEW OF LITERATURE

Separation of Estrogens

The chromatographic characteristics of steroids have been studied extensively as a possible means to help define their biological importance. A small portion of this research has included separation techniques for estrone, estradiol-17α and estradiol-17β. However, many of the procedures developed for other steroids are applicable to the separation and recovery of these estrogens.

Many different chromatography techniques have been investigated. Maugras, Robin, and Gay (1962) reported the separation of 17α - and 17β -estradiol with continuous chromatography, while Schott, et al. (1964) obtained partial separation of these epimers by a 1 x 25 cm silica gel column using ethyl acetate with benzene (1:20) as a solvent. Knuppen, Roa and Breuer (1968) separated 17α and 17β -estradiol with methylcychohexane:chloroform (4:1) on paper, and Preedy, and Aitken (1961) achieved separation of the free estrogens estradiol-17β, estrone and estriol from each other and from the large quantities of interferring material present in extracts through column partition chromatography. Gas chromatography has also been utilized by Wotiz and Martin (1961). Therefore, almost every chromatographic device known has been experimented with, including the extensive variations and modifications of thin-layer systems. For example, multiple runs in the same or a different solvent have been successful for separating some polar steroids, Lisboa (1963), while ascending and descending

chromatography are some other possibilities with ascending chromatography being the most widely used.

There are two different systems which employed ascending TLC (thin-layer chromatography) with continuous development. Zoellner and Wolfram (1962) developed a system which is dependent on evaporation of the solvent from the top of the plate. Lisboa (1969) presents a list of references for these techniques along with the description of another system which consists of using a cotton layer or a metal trough containing dry absorbent which was attached on the upper portion of the plate to absorb the solvent.

Some intricate modifications that were utilized within the various TLC systems include activated plates, basic plates, and numerous absorbents and solvents. The separation of hydrophobic compounds was best achieved by the use of activated plates, therefore, Duthie, Simmons and Urey (1969) heated their TLC plates at 120°C for one-half hour prior to use. Another method the latter authors employed in their chromatographic techniques involved use of the "basic plate" developed by Skipski, Peterson and Barclay (1962). These "basic plates", prepared from silica gel G slurry made with .01 M sodium acetate or sodium carbonate solutions, corrected the trailing problem of several estrogens including the estradiol epimers and estrone. Another method used to decrease the trailing of estrogens was developed by Flickinger and Touchstone (1968). They found that the trailing of estrogen sulfates

which occurred in the neutral and acidic systems was overcome completely when ammonia was included in the solvents.

Different absorbents and solvents play important roles in obtaining maximum separation. An extensive literature review on available absorbents for TLC has been compiled by Lisboa (1969) and Kannen and Sunshine (1970) have compiled information on the comparison of TLC absorbents with a review on supports and developing units. An exhaustive number of solvent systems have been used for the separation of estrogens. Two of the important systems used to separate the three estrogens of interest in this particular instance were ethyl acetate alone and petroleum ether:diethyl ether:formic acid (100:100:2). Touchestone, Murawec and Brual (1968) obtained Rf (x 100) values of 93, 78, and 70 for estrone, estradiol-17α and estradiol-17β, respectively, with the first solvent while Duthie, Simmons and Urey (1969) separated estradiol-17α and estradiol-17β with the latter.

The identification of estrogens has been another area of extensive research. Much of the research on detection of the phenolic compounds has been summarized and referenced by Lisboa (1969). One of the more current reagents used for detection included iodine as a non-destructive location reagent for steroids in thin-layer chromatography (Stevens and Turner, 1969). Spraying a mixture of concentrated sulfuric acid:water (1:1) on a thin-layer plate and heating it at 120°C until the steroids charred was the system used to stain estradiols-17α -

and 17β -, estrone and other steroids by Duthie, Simmons and Urey (1969). Wright (1971) found that by diluting 1 ml of primuline (.1 gm in 100 ml of water) with 100 ml of a mixture of acetone:water (4:1) he produced a reagent capable of locating ultraviolet non-absorbing steroids on silica gel with conservation of the detected material for further investigation. Crocker and Lodge (1971) identified several estrogens with 50 percent sulfuric acid in ethanol and heat. They also discovered that silica gel G plates gave more distinct colors for estrone and the estradiol epimers than silica gel H plates or silica gel H silver nitrate-impregnated plates.

Many researchers have stated that the recovery of very small amounts of steroids from silica gel is a serious problem. Lisboa (1969) states that the choice of the solvent to be used for elution depends upon the polarity of the steroids concerned and that the use of highly polar solvents for the extraction of free steroids from thin-layer plates is limited by the methods used for further quantitation since these solvents extract fine particles of interferring absorbent. Lisboa (1969) has compiled a review of literature to support these views.

Several publications on the development of steroid recovery have helped in the development of techniques for separating estrogens. Frankel and Nalbandov (1966) found that the solvents commonly used in extraction, transfer, and general manupulation of adreno-corticoids

contained impurities capable of converting the steroid into a derivative of differing chromatographic behavior and even capable of eliminating part of the ring structure. Low recovery monitored by labeled steroids due to the loss of radioactivity was found to be identical with C^{14} (ring label) and ^{3}H labeled steroids indicating that part of the loss of activity was from a breakage and deletion of part of the steroid ring structure and not just the exchange of ^{3}H. The authors used the Peterson and Bush methods of purification for ethanol and methanol, respectively, to overcome this problem. Idler, Kimball and Truscott (1966), working with cortisol, corticosterone and testosterone, report good recovery in the first elutate from thin-layer plates. Therefore, they believe the major destruction occurred during concentration and drying of the eluate. They used a Gellman Metricel Filter GA-9, pore size of .01μ, and reported better recovery. Destruction, in this instance, seemed to be due in part to soluble substances from the silica gel. They found reduction of the eluting solvent to be the most effective and simplest means of minimizing destruction of cortisol. Elution with ethanol resulted in no serious loss, while dichloromethane:methanol (9:1) also gave good recovery for these steroids with little variation. This last observation was supported by evidence reported by Idler and Horne, (1968) who found recovery of cortisol to be greatly improved with the latter solvent in comparison to elution with methanol even when the methanol was highly purified. It appeared

that the radioactivity was lost as a volatile product upon the evaporation of the methanol eluate.

Attal et al. (1967) and Doerr (1971) reported some experimentation with the recovery of estrogens specifically. Attal et al. (1967) devised a method to elute estrogens from silica gel, using a microcolumn, to obtain 89.6 percent and 95.4 percent recovery for estrone and estradiol, respectively. The eluting solvents were a critical factor in obtaining high purification of the estrogens. Ethyl acetate was a suitable solvent for estrone and methanol-toluene gives optimum recovery of estradiol-17β. It was also very important that these solvents be highly purified as pointed out by Attal et al. (1967) and Doerr (1971). Doerr (1971) developed a system which allows artifact-free TLC elution of picagram (pg) amounts of estradiol-17β and yields blank values in the end point determination by a hapten radioimmunoassay system below 10 pg. He also utilized a microcolumn consisting of a Pasteur pipette, as did Attal et al. (1967). However, Doerr (1971) eluted the estrogens with 100 μl of 10 percent methanol in dichloromethane. Doerr (1971) found that the longer the exposure time of estradiol-17β to air at room temperature after being spotted on the thin-layer plate, the greater was the extent of artifact formation. Artifact formation was prevented by adding a small amount of a mild reducing agent, phenol or mercaptoethanol, to the chromatographic system.

The most recent chromatography system which allowed separation of many different steroids, including estrogens, androgens, progestins and corticoid was the Sephadex LH-20 column (Murphy, 1971). The unique characteristics of Sephadex LH-20, unlike other forms of Sephadex, is that most of its hydroxyl groups are alkylated. This feature makes it possible to elute with organic and aqueous solvents. The column sufficiently purifies estradiols and estrone to allow quantitation of picagram amounts of these estrogens in the plasma of humans (Mikhail, Wu and Ferin, 1970 and Wu and Lundy, 1971). Cattle will be discussed in another section of this literature review with respect to this matter.

Biosynthetic and Metabolic Pathways of Estrogens

Mellin and Erb (1965) reviewed the literature on estrogens in the bovine, including the biosynthesis and metabolism of these steroids. Separate portions of the biosynthetic pathway for estrogens were combined into a single pathway by Ryan and Smith (1961). The sequence was acetate $\longrightarrow$ cholesterol $\longrightarrow$ 20α hydroxy-cholesterol $\longrightarrow$ pregnenolone $\longrightarrow$ progesterone $\longrightarrow$ 17-hydroxyprogesterone $\longrightarrow$ androstenedione (or testosterone) $\longrightarrow$ estrone or estradiol. An alternative pathway is adrenal tissue documented by Meyer (1955) was pregnenolone $\longrightarrow$ 17α-hydroxypregnenolone $\longrightarrow$ dehydroepiandrosterone $\longrightarrow$ androstenediene. A study of the metabolic scheme in the cow suggested that estrogen metabolism proceeds irreversibly from estradiol-17β to estrone and reversibly from estrone to estradiol-17α, (Mellin and Erb, 1966).

Cellular Activity of Estrogens

As reviewed by Mellin and Erb (1965), two general theories have evolved on the actions estrogen exhibit at the cellular level. The first proposed enhancement of the mobility of metabolites into or within the uterine cell by altering the permeability of cellular membranes of vascular or cytoskeletal structures. The second concept stated that the estrogens affect enzyme function by altering the activity of an enzyme or by stimulating the de novo synthesis of critical enzymes.

Since enzymes play such an important role in metabolism, the second concept has been the favored hypothesis resulting in several main points of view with respect to how steroid hormones in general alter enzyme activity. According to Lang (1971) the five main concepts that have been discussed are: (1) Hormones act as an enzyme, co-enzyme or apo-enzyme; (2) hormones act as allosteric effectors; (3) hormones act on cyclic AMP; (4) hormones lead to new enzyme formation at the transcriptional or post-transcriptional level; and (5) hormones act by decreased degradation of enzymes.

Considering the first three concepts, Lang (1971) expressed a view that concept 1 has never been proven despite some promising approaches such as the disproven hypthesis that estrogens act as co-enzymes for the soluble pyridine nucleotide transhydrogenase in human placenta. Concept 2 has not been very well accepted either

because some of the allosteric actions capable of estrogens could be mimicked by other factors such as ATP and DPNH. Another example Lang (1971) gave for his views on concept 2 was that no transhydrogenase activity could be found in the uterus making it hard to rationalize that direct activation of transhydrogenase could be the mechanism of estrogen action. Looking at concept 3, Lang (1971) noted that the stimulation of adenylcyclase does not play an important role concerning steroid hormones.

To discuss concepts 4 and 5, Lang (1971) covers in depth the experiments conducted on ecdysone and cortisol by him and others to support his viewpoint. The author concluded that sufficient data had been compiled on these hormones to extend the concept of gene activation as the initial step of hormone action to other steroid hormones acting in mammalian systems. However, there were other studies suggesting that the post-transcriptional level was the site of hormone action and the concept on degradation inhibition was upheld here. Therefore, to summarize Lang (1971) stated that there was a primary action in the nucleus at least for the two hormones mentioned, and RNA polymerase activity was stimulated by some unknown mechanism. There was an increase in RNA synthesis part of which was known as messenger RNA that was assumed to control enzyme synthesis in the cytoplasm. From here Lang (1971) left this area of uncertainty open as to whether other regulatory mechanisms are involved and suggested that it was possible

for one hormone to have more than one site of action.

Another proposal, which Mueller (1971) felt was in accord with the studies on ectdysone, was estrogen action on genetic expression. By putting together the segments of information from separate experiments on estrogen action that Mueller (1971) reviews, he documented an argument for estrogen receptors and proposed a role for these receptors in the hormone response. The estrogen receptors seemed to act as a building block for the formation of macromolecular aggregates. The stability and character of this aggregate was regulated by estrogen presence or absence on the receptor. The absence of the hormone resulted in formation of complexes by the receptor that restrict the availability of some essential protein in the genetic scene. Introduction of the hormone caused disassociation of the existing complexes and reassociation with new complimentary sub-units. Then Mueller (1971) proposed that the new association of sub-units makes some limiting proteins available for the genetic expression system that may be more supportive in nature than specific in effecting the genetic expression. However, this explanation did not exclude specific induction or repression effect in the genetic scene since each sub-unit may exert its effects either genetically or catalytically as individual macromolecules. Mueller (1971) noted that in this research on estrogen action it was important to remember that the uterus has been depicted so that growth response and an increase of genetic expression was a dominant

aspect of the response. However, he felt that estrogen receptors could also affect the function of macromolecular factors which were important elsewhere in the cell and exert extra genomic effects. By using these estrogen studies as a model, the author proposed a concept that may serve for other steroid hormones. The main points of the concept are: "(1) That cells, which are responsive to a steroid hormone, contain a receptor protein (or protein complex) which interacts specifically with certain structural features of the steroids; (2) that the receptor protein is further characterized by a remarkable propensity to enter into distinctive associations or aggregations with other macromolecules of the cell; (3) that these associations or complexes may play a role in the control of cell function by regulating the availability or functionality of certain macromolecules; (4) that the character, composition, and stability of these receptor complexes is modified by the binding of the steroid to the receptor protein; (5) that the metabolic response which ensues is determined by the way in which the altered availability or activity of a specific component of the complex affects limiting cell processes, either metabolically or genetically (Mueller, 1971)."

Assay Methods of Estrogens

Quantitative and qualitative evaluations of estrogens have been hindered by the lack of rapid, sensitive and specific assays. Bioassays were the principal tool utilized to develop the initial stages of

estrogen research by establishing the sources of this biological activity and by making possible the measurement of total estrogens in some tissues and body fluids, Mellin and Erb, (1965). However, determination of individual estrogens was not possible until such chemical procedures as colorimetric and fluorimetric methods were developed, Svendsen (1960). Fluorimetry was characterized with greater sensitivity but less specificity than colorimetry. This lack of specificity was avoided by the development and utilization of several purification techniques. However, this method and various modifications of it did not provide the sensitivity required to measure estrogen levels in blood, Mellin and Erb (1965).

The next stage of development was initiated by Leegwater (1956) who utilized the isotope derivative principle, originally developed by Keston, Undenfriend and Levy (1960) for amino acids, in an attempt to measure blood estrogens in cattle. Then Svendsen (1960) developed a double isotope derivative system capable of analyzing plasma estrone and estradiol-17β with the limit of sensitivity of approximately two nanograms. Baird (1968), by modifying the double isotope derivative method, measured unconjugated estrone and estradiol-17β in ovarian and adrenal venous blood of human subjects with a sensitivity of .4 ng. However, he reported overall recovery of approximately 10 percent for both the estrogens mentioned and that one technician was able to analyze only eight samples for both steroids in five days. The constant

search for rapid assay systems with good sensitivity and specificity requiring minimal purification of extracts continued due to these restrictions. A suitable system was discovered during this time period for quantitative and qualitative evaluation of estrogens in the urine. Urinary estrogen levels obtained by these methods established the fundamental guidelines for the role of estrogens in reproduction, (Mellin and Erb, 1965, and Mellin, Erb, and Estergreen, 1965).

Carrier mechanisms for the transport of estrogens throughout the body became a popular area of research as time progressed. Albumin was expected to be the major binding protein for estrogens. However, Tavernette et al. (1967), through observations on patients with liver cirrhosis discovered that the differences in albumin concentration could not be correlated with differences in estradiol concentration. Therefore, they postulated the existence of an additional estrogen binding factor in plasma. Rosenbaum, Christy and Kelly (1966) used electrophoresis to identify a β globulin in human plasma and reported its ability to bind estradiol. Murphy (1964) outlined the general procedure for a protein-binding assay and credits its first use to Ekins (1960) for the determination of thyroxine. Murphy (1967) developed a competitive protein-binding radioassay for corticoids using corticosteroid-binding-globulin (CBG, transcortin). Murphy (1968) investigated the properties of the β globulin , demonstrating how this protein could be utilized to

provide a sensitive radioassay for both estradiol and testosterone. Mayes and Nugent (1970) further developed the competitive protein in increasing quantities especially during late pregnancy. Considerable data has been reported on a rat estradiol-binding plasma protein that is quite specific for estrogens with very little specificity for testosterone, Raynaud, Mercier-Bodard and Baulieu (1971). Other estrogen-binding proteins were also considered during the period of development for a protein binding assay with plasma globulins. Korenman (1968) utilized a soluble uterine macromolecule from rabbits. Shutt (1969) reported a similar assay method with a soluble macromolecular fraction from ovariectomized ewe uteri.

The most recent development for estrogen assays has been modifications of the radioimmunoassay (RIA) utilized for polypeptides. The assay was developed for compounds of small molecular weight by the conjugation of steroid compounds to a protein to obtain an immunological response, (Abraham, 1969 and Midgley, Niswender, and Ram, 1969). Most of the antisera produced by this response has been obtained by immunizing sheep and rabbits with steroid derivatives conjugated at various positions to bovine or rabbit serum albumin, Liebermann et al. (1959), Ferin et al. (1968), and Erlanger et al. (1967).

One recent antiserum was the estradiol-17β -6- (O-carboxymethyl) oxime-bovine serum albumin conjugate prepared by Dean, Exley and Johnson (1971). These researchers, aware that structural differences

in the steriod molecule distal to the conjugation has the greatest influence on relative activity (Midgley and Niswender 1970), coupled the bovine serum albumin via the 6th position, obtaining a highly specific antibody. The cross-reactions for this antibody were 2 percent or less with all other steroids (estradiol-17α 2 percent and 1 percent for estrone and estriol) in comparison with serious cross-reactions of 35-100 percent to estrone and estradiol-17α for most of the antibodies conjugated at the functional groups 3 or 17. Consequently, this newly-discovered antiserum avoids the need for chromatographic separation, shortening the number of time-consuming steps and reducing the chances for the introduction of high background through various solvents, Dean, Exley and Johnson (1971).

Once antisera became available, extensive research has developed several assay systems. Abraham (1969) evolved the solid-phase radioimmunoassay for estradiol-17β incorporating the procedure of Catt and Tregear (1967) for coating tubes and estradiol-17β -succinyl-bovine serum albumin conjugate. Abraham (1969) recorded the practical sensitivity of this assay to be from 20-30 picagrams per ml (pg/ml) of plasma. He obtained results comparable to the double isotope derivative method of Baird (1968) by analyzing the same blood samples. However, there was an indication that a necessary purification step beyond the ether extract should be included. Abraham and Odell (1970) experimented further with this assay system and combined the equilibration and

separation of free from bound steroids in a single step.

Midgely, Niswender and Ram (1969) reported another general procedure known as the double antibody method for the estimation of steroids and other haptenic substances. They accomplished separation of the bound and unbound fractions by precipitation of bound antibody-steroid with a second antibody, however, this required incubation for several days. Mikhail et al. (1970) separated free from bound estradiol by polymerizing the antibody to estradiol-17β and centrifuging at high speeds. The necessary purification and separation step was accomplished with a Sephadex LH-20 column. Tillson et al. (1970) summarized the processes in treating antisera for the development of new radioimmunoassays by describing the effects of certain physiochemical factors which must be examined.

Hotchkiss, Atkinson and Knobil (1971) modified the procedures of Abraham (1969) and Mikhail et al. (1970) by employing dextran-coated charcoal to separate the bound from unbound estradiol. Then, Wu and Lundy (1971) combined the Sephadex LH-20 microcolumns and the dextran-coated charcoal procedures into a single assay. They also experimented with a rivanol-treated antibody and an untreated antibody. The water blanks for estrone and estradiol were negligible for both antibodies. However, a water blank problem was found with the rivanol-treated antibody for estradiol and the binding capacity of the untreated antibody was much greater than the treated antibody. Wu and Lundy (1971) like

Mikhail et al. (1970) found it important to use the same amount of solvent when pippetting the standard curve along with including a similar aliquot of column elutant to all tubes to standardize effects of the solvent.

Reproductive Role of Estrogens in Relation to Bovine Urinary Estrogen Data

Although estrogenic biological activity is also exhibited in blood throughout the reproductive cycle in the cow, amniotic and allantoic fluid, colostrum, milk, ovaries, follicular fluid, corpora lutea, placenta and feces (Mellin and Erb, 1965), much of the foundation work for evaluating the physiological importance of estrogens in reproduction has been based on urine analysis. Mellin and Erb (1965) published an extensive review article on the preliminary research that went into development of the separate sections of the assay system such as the extraction scheme described by Mellin, Erb and Estergreen (1964) which has since been modified slightly by Garverick et al. (1971). Some of this preliminary work resulted in a limited amount of data available on the excretion of estrogens in cows, Mellin and Erb (1966) and Varman, Smith and Hull (1964). The latter group compared blood estrogens to urinary estrogens and found two peaks in both body fluids during the estrous cycle. The first estrogen peak occurred at six to eight days after estrus simultaneously in blood and urine. The second peak was identified on the fourteenth to sixteenth days and on the eighteenth to twentieth days after estrus in blood and urine, respectively. Excretion of estrone was higher

on the sixth day of the cycle than estradiol-17α and estradiol-17β, with a reverse in this order occurring toward the start of estrus. Mellin and Erb (1966) found two urinary estrogen peaks, one during the three days immediately preceding ovulation and a second six to ten days following ovulation. On day 16, they monitored an estrogen ratio of 86, 2 and 8 percent for estradiol-17α, estradiol-17β and estrone, respectively, after injection of radioactive estrogens.

To supplement this limited information, Garverick et al. (1971) made a comparison study of the reproductive steroids, including various estrogen levels in the cow during the estrous cycle. Average urinary excretion values were 5.2, 6.7, 7.4 and 19.2 ng/mg creatine for estradiol-17α, estradiol-17β, estrone and total estrogen, respectively, in the non-pregnant cow, contrary to estradiol-17α being the major urinary metabolite in the pregnant cow, Erb, Randel and Callahan (1971). Garverick et al. (1971) relates the variation in rates of excretion and proportions of the three estrogens he observed to the triphasic growth of Graafian follicles as primarily occurring on days 3 to 5 and 9 to 11 after estrus and 3 to 4 days preceding estrus. The fluctuation of estradiol-17β coincided closer to the first two Graafian follicle stages than estradiol-17α. Estrone remained fairly constant throughout the estrous cycle and displayed a positive correlation to corticosterone. Garverick et al. (1971) explained the latter relationship by suggesting an adrenal source of estrone as postulated by Beall

(1939) or a different metabolic pathway than bioconversion of the two estradiol epimers. Another hormone interrelationship was the positive correlation between estradiol-17β excretory levels and progesterone plasma levels.

Garverick et al. (1971) found that heifers excreted more estradiol-17α and less estradiol-17β for the first estrus in conjunction with higher average levels of plasma LH and corticosterone in comparison to cows. The highest estrogen levels in each group were measured during a seven day interval of time, surrounding estrus. However, estradiol-17β, estradiol-17α (nonsignificant) and total estrogen levels were higher while estrone and plasma steroids (progesterone and corticoids) remained nearly the same if the LH surge occurred simultaneously with sample collection than if the samples missed the LH surge. Garverick et al. (1971) related this characteristic to the same situation reported in the sow, ewe and human, and references this material. The author in discussing the work of Short (1962) explained the higher estradiol-17α excretion in comparison to total estrogen excretion in the heifer, unlike the cow, as a possible indication that estrogen metabolism is generally more complete in the heifer at most stages during the estrous cycle if estradiol-17β is the major estrogen synthesized by the ovaries of the nonpregnant cow. Research by Plotka and Erb (1969) and Plotka, Erb and Harrington (1970) on the female sex steroid relationships during the estrous cycle of the ewe is in agreement with the general

information given by Garverick, et al. (1971). The negative correlation of estrone to plasma progesterone and the positive correlation to estradiol 17β was also reported.

Some research by Wiltbank (1966) showed that injection of exogenous estrogen on days 3, 4, 8 or 9 after estrus caused luteal regression. Menge and Verille (1969) found the same effect of oxogenous estrogen injected approximately 4 to 7 days before implantation. These factors encouraged Randel, et al. (1971) to measure urinary estrogen levels during early pregnancy. Randel et al. (1971a) conducted an experiment on fertile and non-fertile cows which were grouped in relation to the number of days they required to return to estrus after breeding (Group 1 - less than 19 days, Group 2 - 19 to 24 days, Group 3 - 34 to 49 days and Group 4 - pregnant). Relatively large fluctuation of high and low daily rates were observed in Groups 1-3 in comparison to Group 4, with the pregnant cows excreting significantly more estradiol-17α on the day of breeding and on day 9 than Groups 1 or 2. Based on these observations Randel et al. (1971a) postulated that the large variations seen in the nonpregnant cow versus slight fluctuation in the pregnant cow may cause a synchronization between tubal transport in the ova and progesterone changes required in the uterus to support zygotes. Abnormal levels may have caused a luteolytic effect which Wiltbank (1966) obtained by exogenous estrogen induction while normal endogenous levels may have a luteotrophic role. The last concept was supported by the positive

correlation of estradiol-17β to progesterone reported by Garverick et al. (1971). Forty cows, grouped the same as the fertile and non-fertile cows arranged by Randel et al. (1971a), were observed for a difference in excretion levels of estrogen up to implantation (Randel et al., 1971b). They observed the same alternating high and low rates of excretion of urinary estrogen in conjunction with decreased levels of plasma progesterone on day 7 in the nonpregnant groups. Groups 1 to 3 excreted proportionately more estrone than Group 4, and the excretion ratios of different estrogen were very similar to ratios observed in the nonpregnant cow by Garverick et al. (1971).

Randel and Erb (1971) looked at changes and interrelationships of reproductive steroids from 0-265 days of gestation. The first significant excretion rate change was an increased estradiol-17α level from day 35 to day 42, followed by a slight decrease from day 42 to day 65. After day 65, the estradiol-17α and estrone levels increased progressively to a level 20 fold and 6 fold high, respectively, by day 230. The rapid increase of estrogen excretion around day 42, according to Randel and Erb (1971), may be a characteristic associated with completion of implantation. Randel and Erb (1971) used partial regression analysis to identify hormone interrelationships. Partial regressions for estradiol-17α and estrone, positive and negative, respectively, were significant during 7 to 260 days gestation when related to plasma cortisol and may indirectly indicate an effect of these estrogens on

the amount of cortisol bound to proteins in plasma. However, these authors felt that postulation of a probable mechanism would need information that compares blood levels to excretion levels of separate estrogens. They also found a positive relationship of progesterone with estradiol-17β from 7 to 42 days and a negative one from progesterone with LH from 42 to 260 days.

Hunter et al. (1970), working with 46 Red Danish and 43 Holstein females studied the relationship of several reproductive hormones during late gestation. They distributed the cows into groups: A (cows with less than 280-day gestation periods), B (280-284-days) and group C (more than 284-days). These groups differed in the last 34 days of gestation such that group A showed a rapid decline in plasma progesterone combined with little change in excretion of estradiol-17α or total estrogens, Group B exhibited a minor change in plasma progesterone levels and a rapid increase of urinary excretion of estradiol-17α . Group C was characterized by a steady decline of progesterone within 2 days of parturition followed by an increased decline up to parturition. Estrogens in Group C increased steadily up to parturition but at a slower rate than observed in Group B. The proportion of total estrogen made up by estradiol-17α and estrone were 50 and 44 percent one month before parturition, 76 and 21 percent at parturition and 93 and 5 percent at one-half day after calving, respectively. By eight days after calving, the ratios were approximately the same as one month

prior to calving. Hunter et al. (1970) suggests that, since the excretion rate of estradiol-17α was different for group B in comparison to groups A and C during the last seven days before calving but the proportion of 17α was similar at comparable periods before parturition, there is a change in estrogen metabolism and estradiol-17α may be more influential in controlling the time of parturition than estrone or estradiol-17β . They noted that the rate at which estrogen overdominance occurred differed depending on the length of gestation but this concept does not distract from the theory that parturition may occur when a condition of decreasing levels of progesterone and increasing estrogen excretion is obtained. Also, Holstein females excreted more estrogen than the Red Danish indicating a breed difference, and cows that twinned excreted significantly more urinary estrogen, as was reported earlier by Randel et al. (1968).

Urinary excretion data surrounding parturition was procured and analyzed by Mellin, Erb and Estergreen (1966). They collected samples at 8-hour intervals from six cows beginning three days before expected parturition and continuing for 52 hours postpartum. The average excretion of estradiol-17β , estradiol-17α and estrone was 39±6, 386±23 and 226±10 mcg/hr per 100 kg of live weight, respectively. Total estrogen excretion was significantly higher during the 8-hour interval surrounding parturition than 32, 40 and 48 hours after calving and 40 hours before calving. They also noted that although the estrogen quantities

excreted varied considerably between cows, breeds and intervals, the individual estrogen proportions as part of the total tended to remain constant between intervals and remained characteristic for individual cows. The averages were 6.3, 58.6 and 35 percent, respectively, for estradiol-17β , estradiol-17α and estrone. Mellin, Erb and Estergreen (1966) related the increases of estrogen excretion in urine and feces during late gestation to increases in the estrogen content of bovine fetal cotyledons reported by Veenhuizen, Erb and Gorski (1960). Another paper that related increased estrogen excretion rates to late gestation and fetal cotyledon estrogen content was published by Erb et al. (1968). Mellin, Erb and Estergreen (1966) reported excretion rates of estrone to be equal to or higher than estradiol-17α through 273 days with a marked 10-fold increase of estradiol-17α compared to a 4-fold increase of estrone from 237 days to term.

Reproductive Role of Estrogens in Relation to Bovine Plasma and Serum Data

It becomes obvious through the review of literature on urinary estrogens that more information was required to discover and develop the physiological roles of total and separate estrogens. One of the big disadvantages of urinary excretion data was that more than half the estrogen excretion is through the feces (Hunt, Legault, and Herrick, 1961), rendering it not the most ideal source of information. Therefore, assay systems for monitoring the blood levels of estrogens have been and are being developed and modified to fit the needs, Mellin and Erb

(1965) compiled a thorough review on the limited amount of information on blood estrogen levels available prior to 1965. At this time, no endogenous estrogens had been unequivocably identified in the peripheral blood of cattle. However, estrogenic activity had been observed in the blood at different reproductive stages (Mellin and Erb, 1965).

Henricks, Dickey and Hill (1971), using a modification of an assay described by Tillson et al. (1970), were able to measure low estrogen levels in bovine plasma during the estrous cycle. They found a 3-10 pg/ml level during the first three days of proestrous followed by a 15-25 pg/ml concentration on the day prior to estrus and on the day of estrus with the plasma level starting to decrease within 2-5 hours after the onset of estrus and remaining low throughout the rest of the cycle. They found that the estrogen levels did not exceed 10 pg/ml until the progesterone level had fallen to 2 ng/ml or less. Also, the increase in estrogen preceded the rise in serum LH by 3-15 hours, and because the progesterone was at a low level for 1-3 days prior to estrus, Henricks, Dickey and Hill (1971) suggested that the increase in estrogen level may be responsible for LH release which supports the evidence Scaramuzzi, et al. (1971) obtained using ovariectomized ewes administered exogenous estrogen and progesterone. These authors recognized that determination of separate estrogens will be very difficult since the total estrogen levels are so low in the cow. It may also be that a considerable amount of the measurable estrogens

might be biologically inactive, (Henricks, Dickey and Hill, 1970).

Christensen, Wiltbank and Hopwood (1971), through research on blood hormone levels during the estrous cycle in the cow, reported varying estrogen concentrations between 98 and 133 pg/ml throughout the estrous cycle except for a peak of 176±31.6 pg/ml 24 hours before the LH peak and an increased level of 141±44.2 pg/ml on days 5 and 6. The authors defined the LH surge as occurring 24.0±2.5 hours prior to ovulation and 9.17±8.7 hours after the onset of estrus. Progesterone reached a low on day 0 and began to increase on day 4 and 5, reaching its peak on day 15. Wettermann, et al. (1972), measured estradiol and progesterone in blood serum during the estrous cycle of the cow with a radioimmunoassay and a protein binding assay. Estradiol analyzed with the radioimmunoassay averaged about 3.6 pg/ml from day 2 through day 11 with a low of 3 pg/ml on day 2. At 3 days before estrus, estradiol increased to 4.8 pg/ml, then increased to 9.7 pg/ml 5 days before estrus and remained at 8.4 pg/ml on the day of estrus. The protein binding assay showed similar results, however, it was felt that the radioimmunoassay was more repeatable and gave lower blank values. Since the estrogen peak precedes the LH peak by 1 to 2 days, it suggests that this surge may regulate the LH surge in cattle. Wettermann et al. (1972) also found that freemartins in comparison to normal heifers have twice the expected levels of LH during diestrus, progesterone was close to expected levels during estrus and estradiol

equaled that during diestrus.

The other general areas of bovine reproduction that have received considerable attention are the important phenomena leading up to, during, and after pregnancy. Robinson et al. (1970) measured estrone levels in the peripheral blood of pregnant cows by an Ittrich-Kober fluorometric procedure. The average estrone concentration was 1.2 ng/ml at 16 to 14 weeks prepartum, progressed slowly to 2.5 ng/ml at 8 to 6 weeks prepartum, and then more rapidly to 4.8 ng/ml at 4 to 2 weeks before parturition. The highest average was 8.3 ng/ml measured at 5 days prepartum. However, samples were not taken closer to parturition. Robinson et al. (1970) concluded from this study that estrone concentrations in blood increase rapidly during the last month of gestation, that this assay method was inadequate to measure estradiol-17β , and that separate estrogen concentrations in blood before and after parturition to correspond with urine data published by Mellin, Erb and Estergreen (1966) would be of interest.

Echternkamp and Hansel (1971) measured plasma estrogens with a RIA and corticoids with a CPBA. The mean plasma estrone concentration at estrus and on the day of parturition were 25.4 pg/ml and 465.3 pg/ml, respectively. Concurrently, estradiol-17β levels were 80.9 pg/ml and 754.9 pg/ml, respectively. The mean values for plasma corticoids were 7.6 ng/ml on the day of parturition and decreased to 3.1 ng/ml 4 days later, after which time the values ranged from 1.0 to 24.6 ng/ml.

Some recent data on plasma estrogen and progesterone levels after mating, during late gestation and postpartum in cows was by Henricks, et al. (1972). The experimental design consisted of two trials. The first trial was made up of 18 beef heifers that were bled every 3 days after mating until they either returned to estrus or until day 39 of pregnancy. Trial II consisted of 10 dairy cows bled daily from 7 to 14 days prior to calving and then bled twice a week for 60 days postpartum. In cows that failed to conceive, the mean estrogen level was below 7 pg/ml on days 3, 6, 12 and 15, with a minor peak of 13 pg/ml on day 9, and a major peak occurring just before estrus, averaging 25 pg/ml. The estrogen levels in pregnant cows remained below 5 pg/ml from day 3 to day 39, while plasma progesterone started with 1.2 ng/ml on day 3 in each group and increased to 8.2 ng/ml and 9.9 ng/ml on day 12, respectively. Beyond day 12, the progesterone levels fell in the nonpregnant cows and increased in the pregnant group to 13.9 ng/ml. Henricks et al. (1972) related the minor estrogen peak which occurred on day 9 in only the nonpregnant heifers to the luteolytic effect exhibited by exogenous estrogen injected on day 8 or 9 of the cycle but not on day 15. However, the major peak in plasma estrogen signified a miximum level of ovarian secretion the day before estrus, and in general the estrogen levels show close correlation with follicular development during the bovine estrous cycle as reported by Rjakowski (1960). During the first part of pregnancy estrogen levels were noted

as being lower than during the estrous cycle which suggested to the authors a lack of follicular development at this time, and brought to their minds the question of whether or not estrogen is required during early pregnancy. Also, Henricks et al. (1972) did not find a surge of estrogen required for implantation (Randel et al. 1971b) but rationalized that with 3 day intervals for sampling, this surge could have been missed unless the slight rise seen on days 6 to 12 accomplished this.

In Trial II from 14 days prior to parturition up to day 0 (calving) estrogen levels progressed from 500 pg/ml to 2660 pg/ml with an average increase of 248 pg/day for the last 5 days. Progesterone remained steady at approximately 4.6 ng/ml until it decreased to .7 ng/ml one day before parturition. Estrogen levels after parturition ranged from nondetectable concentrations to 40 pg/ml while progesterone ranged from nondetectable to 5.6 ng/ml. Comparing cows having postpartum intervals less than 35 days to cows with a postpartum period greater than 60 days, Henricks et al. (1972) found that estrus for the first group coincided with the major peak in estrogen while progesterone followed a low level cyclic pattern as seen in the estrous cycle. The other cows displayed no estrogen peak and for the most part progesterone was nondetectable. Cows with a short gestation period (less than 281 days) exhibited a progesterone level decrease, the same as for animals with a long gestation period

(greater than 285 days), however, their estrogen concentrations increased more rapidly just prior to parturition.

In conclusion, Henricks et al. (1972) supported the theory that a rise in plasma estrogen in the presence of a declining progesterone level is required for initiation of parturition based on their concurrent plasma estrogen and progesterone study.

MATERIALS AND METHODS

Six pregnant cows (see history appendix #1) on a nutritional plane of 100 percent NRC requirement for prepartum and postpartum cows (National Research Council, 1962) were bled at four hour intervals via jugular cannula for approximately 6 days before and 2 days after parturition. Cows, cannulated with silicone rubber tubing, were restrained in relatively stress-free collection stantions. All cows had been familiarized with the stantions prior to cannulation and sample collection.

Approximately 5 ml of fluid, blood and heparin was drawn from the cannula and discarded prior to collection of a 30 cc blood sample with a 50 cc syringe. Injection of 2-3 ml of heparin solution into the cannulas before resealing them helped prevent blood clots. Meanwhile the samples were transferred to heparinized centrifuge tubes which were covered with parafilm and inverted to distribute the heparin. Blood samples were refrigerated at $4^{o}C$ until the following morning before centrifugation at 2500 rpm for 30 minutes in a model HNS centrifuge[1/]. Plasma was transferred from the samples into labeled, plastic whirl-pack containers and stored at $-10^{o}C$ until assayed by RIA. All samples for each cow were assayed with the same standard curve to lower the assay variation within each cow.

1/ Manufactured by International Equipment Co., Needham Heights, Mass.

Estrogen Separation

The following procedure was developed to obtain separation of estrone, estradiol-17α and estradiol-17β [2]. Glass plates (31x20 cm) were coated with a 750μ layer of silica gel H[3]. A Chromatofilm spreader[4] was used to spread the slurry solution which consisted of a .01 M sodium carbonate solution to silica gel H (2:1) (Duthie, Simmons and Urey, 1969). The thin-layer chromatrography plates (TLC), allowed to dry at room temperature overnight, were activated at 120°C for at least one-half hour prior to spotting the estrogens on them. The estrogens diluted to 100,000 ng/ml in double distilled ethanol (ETOH) were spotted by using a modified version of the thin-layer spotting device described by Davis (1970). Two samples, applied equidistant from the center and 3.5 cm from the bottom of the plate, consisted of either two known estrogen samples represented by 5 mg, 10 mg and 10 mg aliquots of estradiol-17α, estradiol-17β and estrone, respectively, or one known and an unknown sample. The TLC plates were allowed to dry 15 minutes at room temperature, once the samples had been spotted. Meanwhile, a 32x18 cm chromatography jar[5] fitted with a ground-glass cover to provide a sealed system was filled to a .5 to .6 cm level from the bottom with solvent and allowed to

2/ Sigma Chemical Co., St. Louis, Mo.
3/ EM Reagents Div., Brinkmann Instruments, Inc., Westbury, N.Y.
4/ Kensington Scientific
5/ Purchased from Van Waters and Rogers Scientific, Seattle, WA.

equilibrate at 17±1°C in an incubator[6] for at least one-half hour before use. The solvent, petroleum ether (Bpt 30-60°C):anhydrous ether:formic acid (100:100:2) (Duthie, Simmons and Urey (1964), was prepared fresh each time to reduce contamination. Precaution was taken to clean all glassware and the troughs thoroughly for this reason also. The troughs for continuous chromatography (Lisboa, 1969) were made out of stainless steel shaped to fit over the top of a TLC plate to provide support for two small glass slides which held the absorbent. The troughs were attached to the top of the plates and filled with silica gel H for absorbant after the spots dried. Then the apparatuses were placed in the tank to develop for 6 hours.

The developed plates were dried for five minutes before the known estrogens were identfied with a spray atomizer[7]. The lanes of the known spots were sprayed with sulfuric acid and water (1:1) and heated with a hair dryer[8] until the estrogens charred (Figure 1). Unknown estrogen areas were protected from spray by covering their lane with a glass plate. The hot air stream from the dryer was localized on the known estrogen areas to protect the unknowns. Consequently, unknown estrogens were isolated by drawing parallel lines across the plate with respect to the known areas. The silica gel was scraped from the

6/ B.O.D. Incubator #31213. Percision Scientific, Chicago, Ill.
7/ Van Waters and Rogers, Scientific, Chicago, Ill.
8/ Model 38100 McCraw-Edison Co., Berstod Mfg. Div., Bobnville, Mo.

plates into microcolumns (disposable pastuer pipettes plugged with glass wool). The estrogens were eluted six times with 1 ml of ethyl acetate as described by Attal et al. (1967). The eluates were dried under nitrogen in a 45°C sand bath, redissolved in double distilled ETOH, transferred to 12x75 mm disposable culture tubes and concentrated in the bottom of them. Next the samples were either included directly into one of the two assay systems or dissolved in 0.1 ml of manograde benzene and methanol (90:10) and stored until layered on a Sephadex LH-20 mcirocolumn. A preliminary check was also made on recovery from TLC by using radioactive labeled estrogen.

The Sephadex LH-20 microcolumn, a glass pipette with a 0.7 cm inside diameter and a 15 cm barrel, was plugged with a glass bead and filled to 0.5 cm height with acid washed sand before the Sephadex was added. Meanwhile, approximately 1.5 mg of Sephadex LH-20 per column was washed three times with 15 ml of nanograde methanol for each 1.5 gm of Sephadex. The methanol and fine particles were decanted after each wash had settled for 10 minutes. Then, the gel was washed three times with 20 ml of benzene:methanol (90:10) per 1.5 gm of Sephadex to equilibrate the gel with the solvent used for elution. Nanograde chemicals were used for elution to lower blank values. The prepared gel was poured slowly into the columns to prevent air pockets. An 11 cm column was poured originally to make a 10 cm column since the gel settled with time. The columns were intially washed with 20 ml of fresh solvent

before use. A calibrated 25 ml burret equipped with a telfon stopcock and needle valve[9] served as a reservoir and a flow volume estimator.

Estrone-14-^{14}C (SA 52 mc/mM) and estradiol-17β -4-^{14}C (SA 45.2 mc/mM)[10] were used to identify the elution pattern of estrogen through the microcolumns. The elution volume of estradiol-17β -4-^{14}C provided an estimate for both estradiol epimers because radioactive estradiol-17α was not available. The microcolumns were washed with 5 ml of solvent, after the radioactive estrogens were eluted. The blank samples corresponding to the radioactive estrogen elution volumes were collected before layering unknown samples on the microcolumns. Also, column elutant blanks of 4 ml and 2 ml for the estradiol epimers and estrone, respectively, were collected after the samples were eluted and collected. A different column was used for each estrogen which had been separated by TLC.

The general procedure for evaluating radioactive tracers was standard throughout this research project. Samples were evaporated in 18x56 mm, low potassium counting vials[11] if they were dissolved in a solvent other than aqueous solution. A 250 ml Packard fluid dispenser (Pyrex No. 4984) was used to add 15 ml of scintillation fluid (100 gm napthaline, 7 gm ppo, .3 gm popop and 1 liter of 1,4-Dioxane to dried samples and aqueous solutions. Samples were counted with a

9/ Van Waters and Rogers Scientific, Seattle WA.
10/ New England Nuclear, Boston, Mass.
11/ Packard Instrument Co., Inc.

Beckman liquid scintillation counter[12/] on the channels programmed with the appropriate isoset for the ^{3}H or ^{14}C spectra above ^{3}H determined by the isotopes for each particular instance. The counter analyzed samples for 10 minutes or .2 percent error which ever occurred first.

The TLC separation data was recorded by measuring the distance each estrogen moved from a common origin with a metric rule. The average of two spots from each plate was used to calculate the arithmetic means and standard error between plates (Ostle, 1962). The same parameters were calculated for the diameter of each spot. The column elution values were determined by subtracting the column background from the counts per minute (CPM) for the ^{14}C labeled estrogens. These values were graphed according to elution volumes to depict the elution patterns of estrone and estradiol-17 .

Estrogen Assays

The competitive protein-binding assay (CPBA) developed by Murphy (1967) and improved by Mayes and Nugent (1970) was tested. The theory of the protein binding assay is based on the ability of the plasma globulin, found in increased amounts in human late pregnancy plasma (LPP), to bind both testosterone and estrogens. Therefore, testosterone-1,2-^{3}H (specific activity, 46.5 (ci/mM) is used to saturate the binding sites. The estrogens compete with the radioactive testosterone for the

12/ Model LS-100 Liquid Sintillation System

binding sites when they are introduced into the system. Consequently, the greater the amount of estrogen introduced into the system, the lower are the ^{3}H counts in an aliquot of supernatant, since the unbound radioactivity is precipitated.

Human LPP was donated by subjects in the third trimester and frozen at -10°C in 2.5 ml aliquots to avoid repeated freezing and thawing. The two percent LPP solution used in the assay for the estradiol-17β standard curve was prepared by pipetting 2 ml of LPP and 150 ul testosterone-1,2-^{3}H into a 100 ml volumetric flask which was then filled to volume with double distilled water. Several different proportions of protein and radioactive testosterone were experimented with to develop a suitable standard curve for estradiol-17β and estrone. The standard curve for each of the three estrogens was prepared from a solution of 100 ng/ml of double distilled ETOH. Corresponding Lambda pipettes were used to deliver 0.2, 0.6, 1.0, 2.0, 3.0, 5.0, 10.0 and 20.0 ng aliquots to 14x100 mm dispassable culture tubes. Empty tubes were included for controls.

The rest of the assay procedure was very similar to the protein binding assay for progesterone used by Davis (1970). The standard curves were evaporated to dryness under nitrogen prior to the addition of 1 ml of the corresponding LPP solution to all of the samples including the controls. The samples were incubated at 45°C for 5 minutes

while agitating in a Metabolic Shaking Incubator[13] adjusted to 80 revolutions per minute. Then the samples were cooled to 10°C in an ice bath for at least 10 minutes before the next treatment and remained in this bath throughout the duration of the assay. Each sample, one at a time, received 40 mg of washed Florsil and agitation for 30 seconds at speed two in a Vari-Whirl mixer[14] after the 10 minute cooling interval. The washed Florisil was prepared by washing it twelve to fifteen times with double distilled water and drying it at 100°C. Another 10 minute interval requirement elapsed on each sample before 0.5 ml aliquots of supernatant were pipetted with care to avoid contamination with Florisil. All aliquots were scintillated and counted for ^{3}H by the same procedure discussed earlier.

The assay values for standard curves were graphed and observed for regression. The data was also analyzed by a Hewlett Packard computer programmed with the same program used for progesterone analysis by Davis (1970). An analysis of variance and a regression equation was calculated by the computer. The undesirable standard curves due to lack of regression for estrone and estradiol-17β were one reason for adoption of the radioimmunoassay. Also, the blanks and aliquots of column elution solvent were assayed with the corresponding curves.

13/ Dubnoff Precision Scientific C., Sub. of GCA Corp., Chicago, Ill.
14/ Catalog No. 58810, distributed by Van Waters and Rogers Scientifc, Seattle, WA.

Their assay values indicated high contamination of estrone and estradiol-17β when compared to standard curve values.

The theory behind the radioimmunoassay is the immunological response of the sheep, in this instance, to build an antibody to the injected foreign protein-estrogen complex. These antisera collected and treated as described by Mikhail (1970) serves as a highly specific binding protein. Antisera, saturated with a radioactive estrogen serves the same role as the protein does in a CPBA. Unlabeled, known amounts of estrogens can be measured which in turn allow unknowns to be estimated through comparison to this standard curve.

The antiserum to an estradiol-17β -succinyl-bovine serum albumin conjugate was prepared, treated and donated by Dr. Randel (1971). Cross reaction studies by Randel (1971) showed 4.0, 59.7, 60.0, 2.3, and 3.2 percent binding of 0.01μc of ^{3}H labeled cortisol, estrone, estradiol-17β , testosterone and progesterone, respectively for an antiserum dilution of 1:6000. This antiserum was diluted to 1:100 in Buffer A, a 0.1 M phosphate buffer (PH 7) containing 5.38 gm NaH_2PO_4, 16.35 gm Na_2HPO_4, 9 gm NaCl, 1 gm Na Azide and 1 gm Knox Gelatin in 1 liter double distilled water. Two ml aliquots of this 1:100 dilution were stored at -10^{o}C in one-half gram vials with screw caps, to avoid repeated freezing and thawing. Then a second stock solution of 1:30,000 was made and stored at -10^{o}C in 50 ml polyethylene vials and in turn diluted to the final concentration (approximately 1:45,000). The final

concentration determination for the antiserum consisted of a dilution study comparing zero aliquots and zero standards over a wide range of dilutions. The zero aliquot represents total counts and the zero standards represent bound counts. Therefore, the dilution, binding approximately sixty percent of the total counts, could be identified for the assay procedure. This dilution study and the ether check referred to in the extraction procedures were analyzed with the assay procedure which will be discussed after the section on extraction methods.

The extraction of 1 ml blood samples and 1 ml aliquots of water for blanks was accomplished by extracting two times with 3 ml anesthetic grade ether by vigorous agitation of each sample for 1 minute on a vortex. Each sample, prior to extraction, had been injected with approximately 1,000 cpm estradiol-17β -6, 7-^{3}H (SA 46.6 C/mM) dissolved in buffer A for recovery. The radioactive estradiol-17β was pipetted with a 250μl Hamilton syringe[15] adjusted to 0.1 ml with a Cornwall pipetor[16]. Three 0.1 ml aliquots of radioactivity were put directly into clean counting vials to estimate total counts at the same time the samples were injected. Also, several 3 ml aliquots from two, one pound cans of anesthetic grade ether were assayed along with zero aliquots and zero standards to determine ether contamination.

15/ Manufactured by Hamilton Co., Inc. Whittier, CA.
16/ Purchased from Van Waters and Rogers Scientific, Seattle, WA.

The can of ether exhibiting the least contamination was used for extractions.

Both ether extracts from each sample were pipetted into counting vials and evaporated in a 45°C water bath. The extracts were redissolved in 1 ml double distilled ETOH taking care to rinse the sides of the vial. Then two .3 ml aliquots were pipetted with an Ependorf pipette[17/] into two different 12x75 mm disposable culture tubes per sample for duplicates. These samples were covered with parafilm and stored at room temperature until assayed. The remainder of the samples were evaporated to dryness in a 45°C water bath, scintillated and counted for ^{3}H along with the total count for recovery vials.

Quadruplicate standard curves for estradiol-17β were prepared by dilution from a 10,000 mg/ml solution to obtain the correct concentrations so a 10μl pipette could be used to aliquot 10, 20, 30, 50, 50, 100, 200 and 300 pg concentrations. The zero aliquots and zero standards were included as part of each standard curve; however, 10 μl of double distilled ethanol was pipetted into them instead of estrogen. The unknown samples, water blanks and standard curves were evaporated under nitrogen in a 45°C sand bath. All samples excluding the standard curve were also concentrated in the bottom of the tubes by rinsing the sides of the tubes with decreasing volumes of double distilled ETOH.

17/ Catalog #53512 purchased from Van Waters and Rogers Scientific, Seattle, WA.

The samples were arranged in small test tube racks such that the unknown samples and blanks had duplicate standard curves before and after them. The sequence of treatments in the assay progressed in the same direction throughout the assay to allow more uniformity. All additions, depending upon their volumes, were delivered via 250μ l Hamilton syringes or 3 ml Cornwall syringes regulated with 1 and 3 ml Cornwall pipetors, respectively. First each sample received a 1 ml aliquot of dilute antisera, followed by gentle agitation for 2 seconds on a Vortex set at 1.5, then a 30 minute incubation period at room temperature, allowed the estrogen and antisera to equilibrate. This incubation period was omitted as unnecessary in the dilution study mentioned earlier. Next, 0.1 ml estradiol-17β 1, 2-^{3}H (approximately 10,000 cpm) dissolved in Buffer A was added, preceded by another gentle 2 second agitation, the samples covered with parafilm were placed into the cold room at 4^{o}C for two and one-half hours. It was critical to keep the assay tubes cold throughout the rest of the assay so the following additions were distributed in the cold room along with all additives being chilled to 4^{o}C. One-tenth ml of Buffer B (same as Buffer A except 5 percent Knox Gelatin) was pipetted to each tube followed by the addition of 1 ml of dexron-coated charcoal (100 ml Buffer A, 0.025 gm dextrane[18] and 0.25 mg charcoal "Novit a" [19]

18/No. D4751, Sigma Chemical Co., St. Louis, MO
19/Purchased from Matheson Coleman and Bell, Los Angeles, CA.

with fines removed) to all samples except the zero aliquots. Charcoal fines were removed by washing it six times with double distilled water and decanting after the charcoal settled for 5 minutes. The washed charcoal was dried in an oven at 100°C. After the final additions, all samples were agitated at Vortex dail setting 5 for 2 seconds each and arranged in four centrifuge buckets[20] which were placed in a refrigerated centrifuge[21] at 4°C. A 5 minute time interval elapsed before samples were centrifuged at 2500 rpm for 10 minutes. One bucket at a time was removed from the cold and 0.5 ml aliquots were transferred into counting vials. A 0.5 ml Eppendorf was used to aliquot samples, being careful to avoid contamination with charcoal. Fifteen ml of scintillation fluid was distrubuted to all counting vials containing supernatant, and to six empty vials for background. Tritium content was counted as discussed earlier.

The raw data (CPM) from the counter print-out tape was first corrected for background by subtracting the average background CPM for each group of samples.

The average for each estrogen level of the standard curve run in quadruplicate were calculated and plotted against their corresponding picagram level. A hand drawn curve through the average means served as a guidline to estimate the amount of estrogen in 0.3 ml of each

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20/ Catalogue 2528 R40 distributed by Arthur Thomas Co., Philadelphia.
21/ Model PR-2 centrifuge manufactured by Internation Equipment Co., Boston, Mass.

extract, including water blanks. Since each plasma or water sample was represented in duplicate in the assay, the value used to estimate the amount of estrogen in 0.3 ml was the mean of each sample. The pg/ml concentration was calculated from the 0.3 ml estimate after the 0.3 ml water blank value was substracted. Then the pg/ml estrogen levels were corrected for recovery from extraction, providing a total estrogen value for each sample. The percent recovery was calculated from the .5 ml aliquot of extractant.

The total estrogen values were analyzed by a least-squares program (Harvey, 1968) on the computer for two groups of data, before and after parturition. The least-squares means, an analysis of variance and the regression coefficients for an orthogonal polynomial were calculated for each group. Another least-squares analysis was run to calculate the ANOV and the regression coefficients for gestation and birth weight before parturition while the orthogonal option was used for these coefficients after parturition. The least-squares program with the orthogonal polynomial to the fifth power was used to obtain regression coefficients and the ANOV for the standard curve of each assay and for an overall standard curve. F-tests were calculated according to Ostle (1963) to evaluate the standard curves. The first F-test tested simultaneously for variation in the intercepts and the regression coefficients for all degrees of the polynomial. The second F-test tested for variation of the intercepts or means alone and the third F-test was

used to test for a difference in the regression coefficients alone.

RESULTS

Estrogen Separation

Thin-layer chromatography provided a means for separating estrone and the two estradiol epimers. The distance each estrogen moved from the origin was 21.34±.19, 16.75±.31 and 14.79±.28 cm for estrone, estradiol-17α, and estradiol-17β, respectively (Figure I). Correspondingly, the average spot diameters for 10 TLC plates were 1.33±02, 1.24±.02 cm, 1.34±.03 cm, respectively. Therefore, the critical separation between the estradiol epimers was .65 cm.

The elution patterns of estrone-4-^{14}C and estradiol-17β-4-^{14}C, as listed in Tables I and II, respectively, were accomplished with a Sephadex LH-20 microcolumn. Radioactive estrone was eluted from the microcolumn with 3 to 5 ml of solvent in column #1 (Figure 2) and approximately between the 3.75 and 6 ml volume with column #2 (Figure 3). The peak of radioactivity occurred in both instances at the 4.5 ml elution volume. Estradiol-17β 4-^{14}C elution required from 6.5 to 12.5 ml and 6.0 to 12.5 ml of effulent to flow through column #1 and column #2 (Figures 4 and 5), respectively.

Estrogen Assay

A marginal standard curve was developed for estradiol-17α with the competitive protein binding assay. The usable portion of the standard curve extended from 0.2 ng to 3 ng. The TLC blanks compared to the estradiol-17α standard curve showed contamination of the 0.2-0.6 ng level when double distilled ETOH and methanol was used for extraction

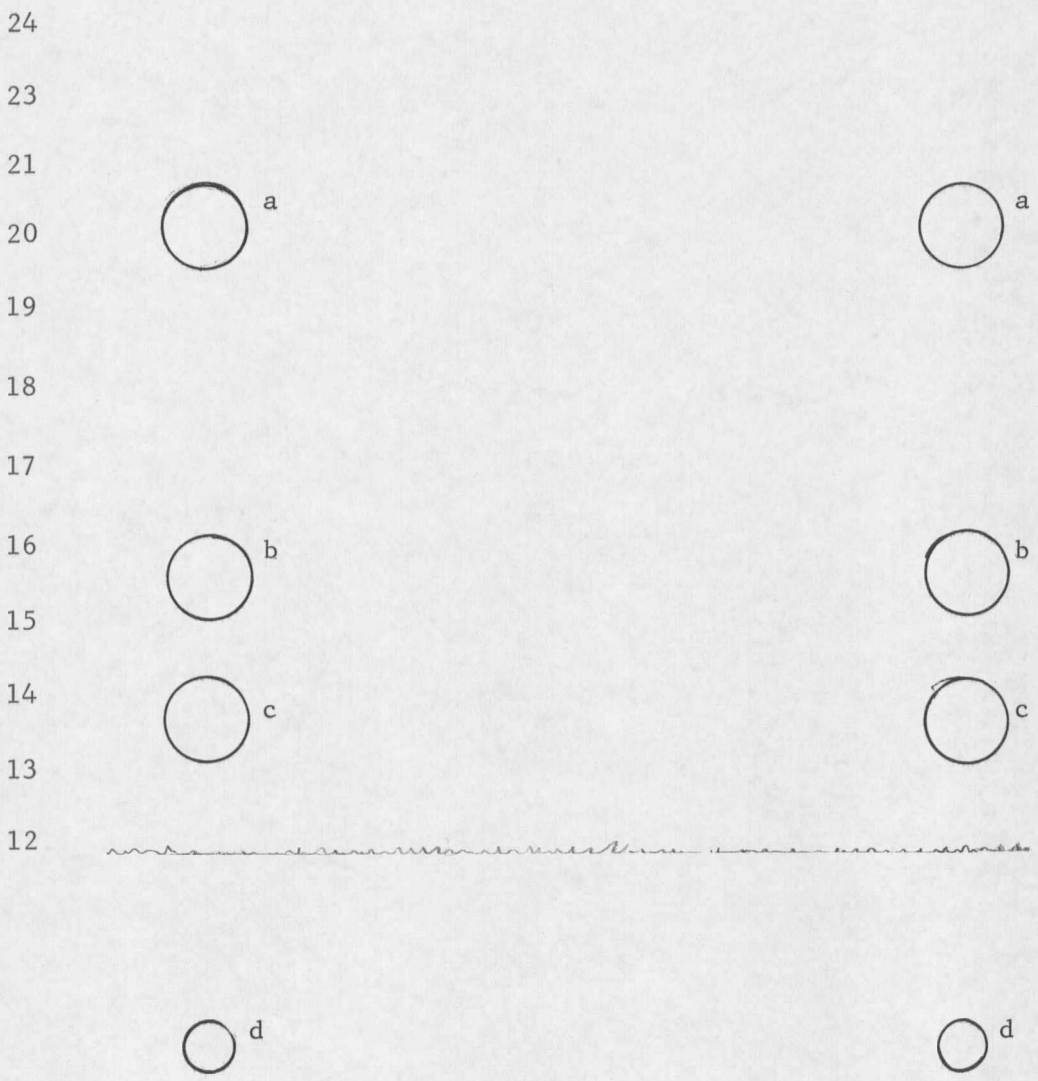

Figure 1. Continuous TLC separation of estrone[a], estradiol-17α[b], and estradiol-17β[c]. Origin = d.

TABLE I. SEPHADEX LH-20 COLUMN ELUTION PATTERN FOR ESTRONE-4-^{14}C

Effulent Column #1 ml	Column #1 CPMs	Effulent Column #2 ml	Column #2 CPMs
0	0	0	0
1.75	9	1.75	9
2.00	7	2.00	8
2.25	8	2.25	9
2.50	16	2.60	0
3.00	5	3.00	8
3.75	98	3.50	16
4.00	200	4.00	183
4.50	234	4.50	239
4.75	16	5.00	87
5.00	6	6.00	3
6.00	0		

TABLE II. SEPHADEX LH-20 COLUMN ELUTION PATTERN FOR ESTRADIOL-17β -4-^{14}C

Effulent ml	Column #1 CPMs	Column #2 CPMs
6.0	0	0
6.5	2	5
7.0	17	11
7.5	66	60
8.0	66	64
8.5	64	65
9.0	68	74
9.5	61	63
10.0	45	42
10.5	34	31
11.0	23	20
11.5	13	14
12.0	16	10
12.5	3	1
13.0	0	1

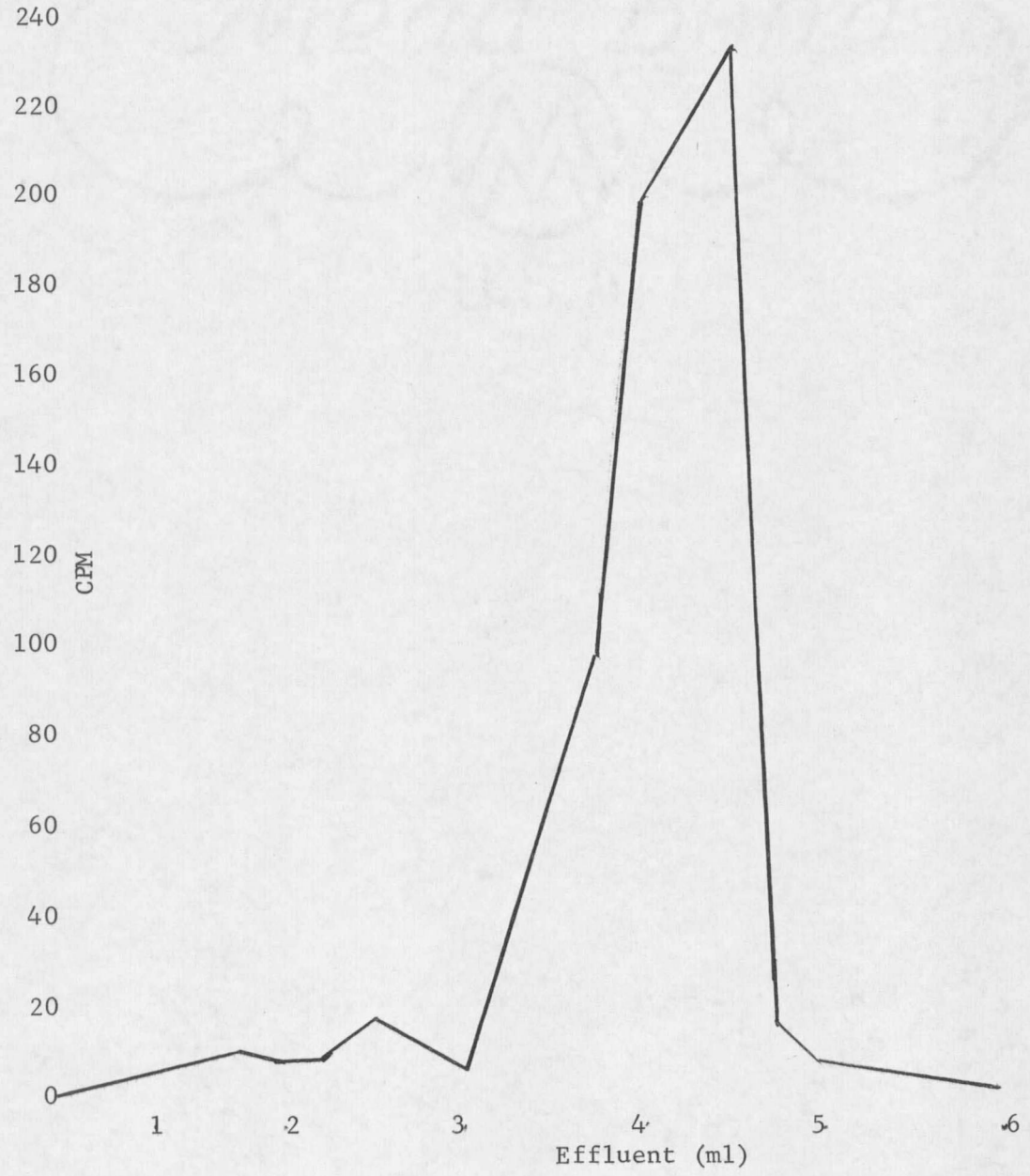

Figure 2. Sephadex LH-20 column #1 elution pattern for estrone-4-^{14}C.

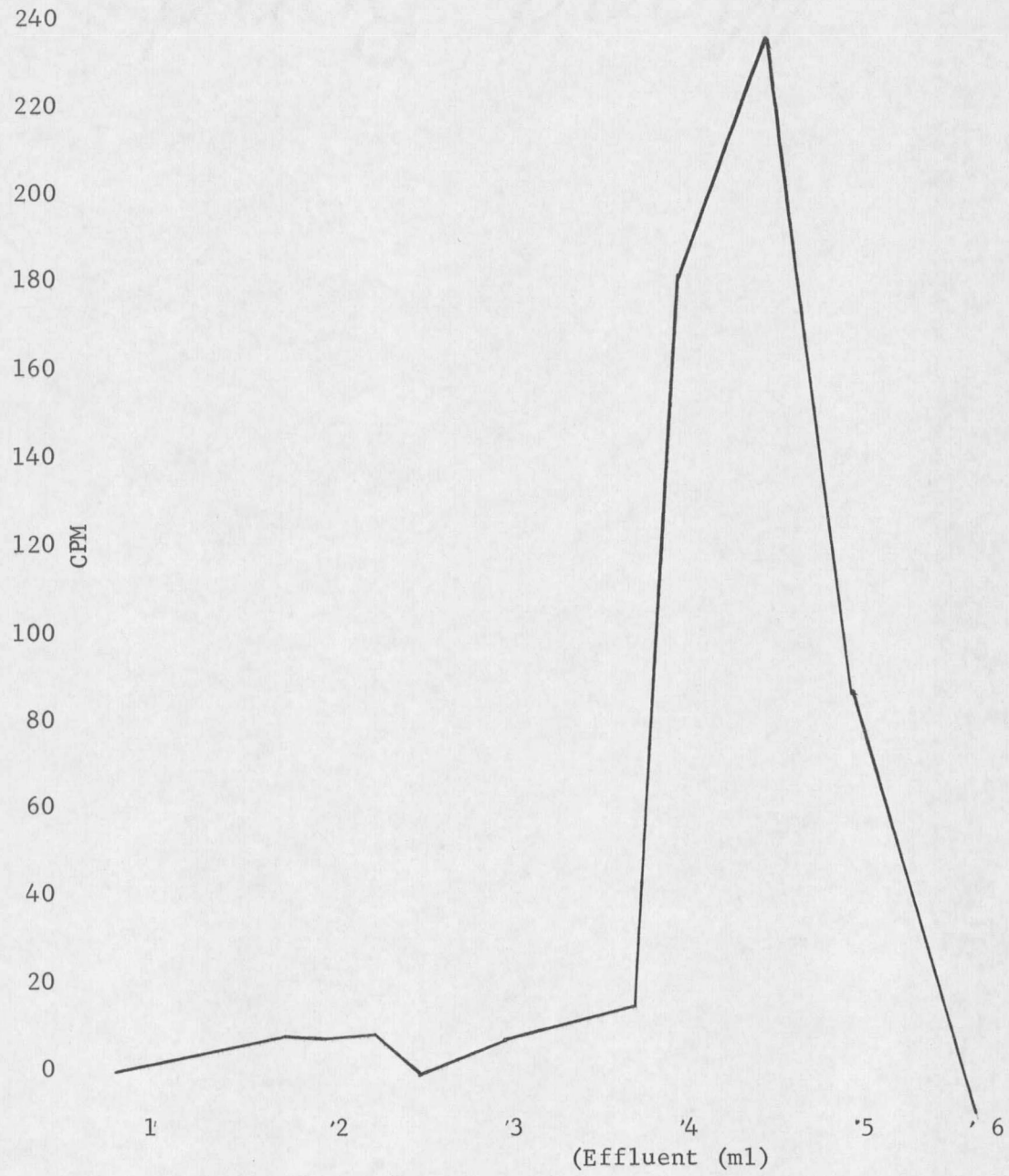

Figure 3. Sephadex LH-20 column #2. Elution pattern for estrone-4-^{14}C.

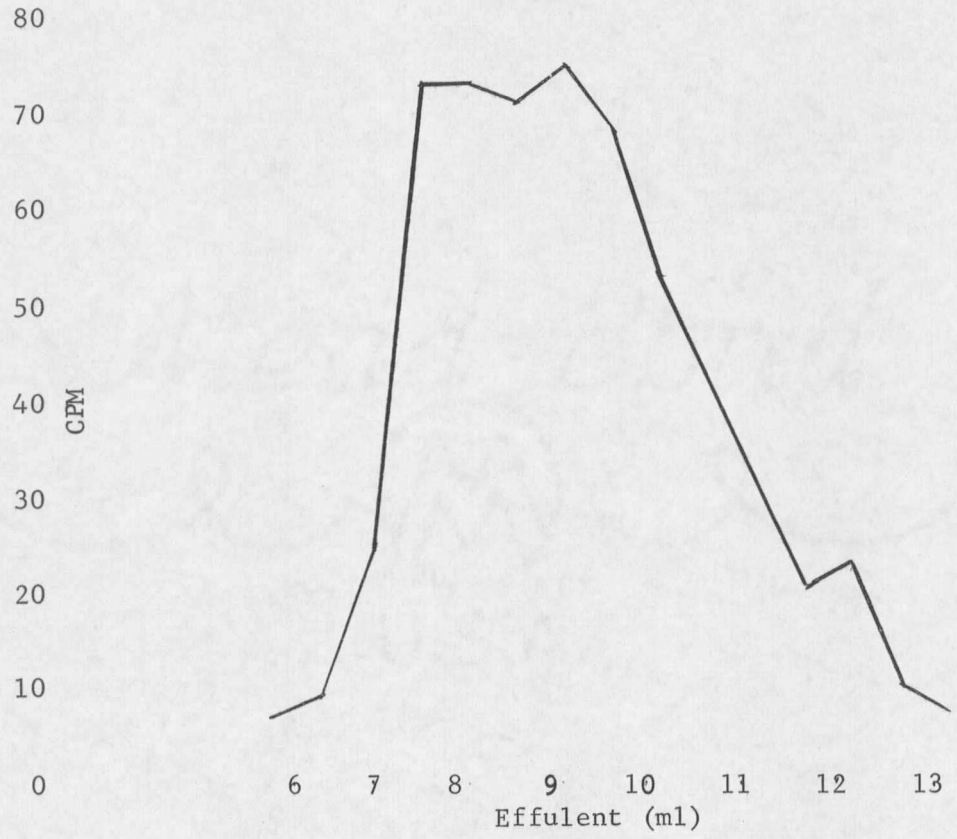

Figure 4. Sephadex LH-20 column #1 elution pattern for estradiol-17β-4-^{14}C.

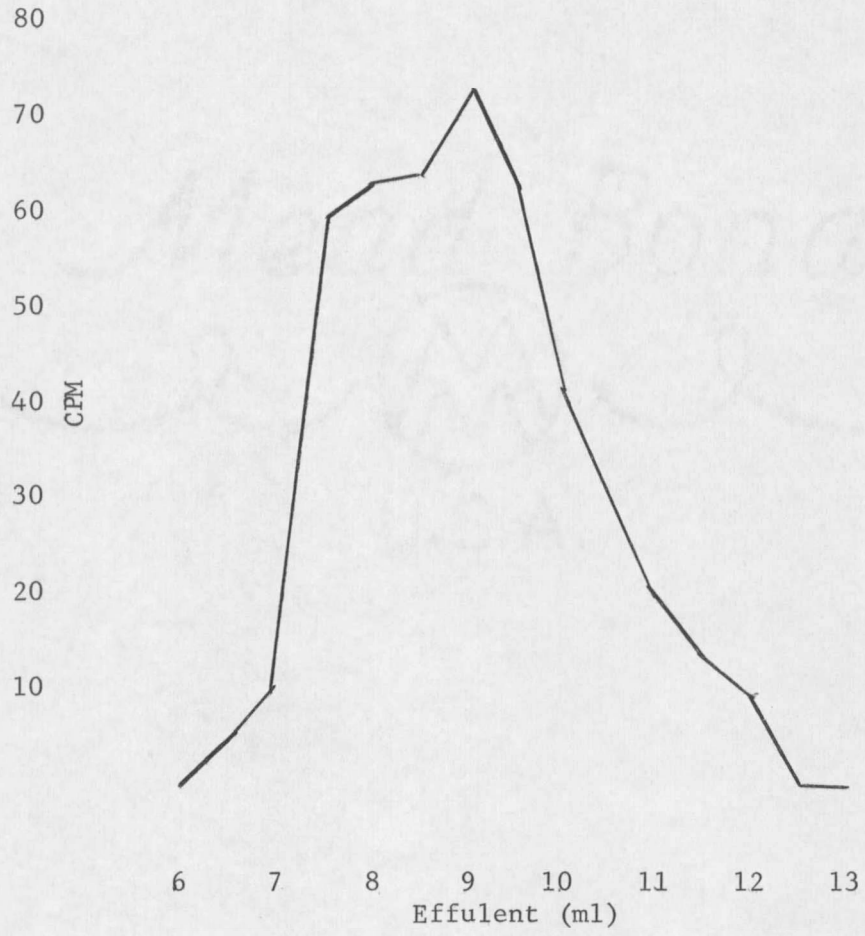

Figure 5. Sephadex LH-20 column #2 elution pattern for estradiol-17β-4-^{14}C.

Table III). An ether extractant exhibited comtamination slightly higher than the ETOH extractant. The standard curve for estrone and estradiol-17β were found unsuitable due to lack of regression. Experimentation with various proportions of radioactive testosterone and LPP solutions to solve this problem gave limited success. Contamination from thin-layer blanks was found to be above 2 ng for estradiol-17β and 5 ng for estrone as designated by the standard curve concentrations even when the blanks were eluted several times with ethanol and methanol (Tables IV and V).

The radioimmunoassay used to assay the plasma estrogens, had a working sensitivity of greater than 12 pg based on the water blanks which never exceeded 12 pg. The RIA also exhibited contamination problems. The elution blanks from the columns for the individual estrogens were 22, 24 and 54 pg for estrone estradiol-17α and estradiol-17β, respectively. When the TLC blanks were eluted, the corresponding contamination levels were 90 pg for estrone, 53 pg for estradiol-17α and 300 pg for estradiol-17β, respectively. The contamination for all of the individual estrogens was greater than 300 pg if the TLC estrants were assayed before they were eluted from the column.

The standard curves for each RIA (Figure 6) and a composite standard curve were defined as discussed in the materials and methods section and evaluated with three F-tests defined in Appendix Table III according to Ostle (1963). The values from the F-tests Nos. 1, 2, and

TABLE III. ESTRADIOL-17α STANDARD CURVE AND BLANKS (2% LPP, 150 μl TESTOSTERONE-1,2-^{3}H)

	Estradiol-17α ng	CPMs
Controls	0	9211
	0.2	8865
	0.6	7907
	1.0	7910
	2.0	7217
	3.0	6971
	5.0	6717
	10.0	6798
	20.0	6477
Blank Treatments		
ETOH 1st wash		8096
ETOH 2nd wash		8634
ETOH 3rd wash		8399
Methanol 4th wash		8591

TABLE IV. ESTRADIOL-17β STANDARD CURVE AND BLANKS (0.5% LPP, 100 μl TESTOSTERONE-1,2-^{3}H)

	Estradiol-17β ng	CPMs
Controls	0	5650
	0.2	5608
	0.6	5687
	1.0	5547
	2.0	5091
	3.0	4526
	5.0	4330
	10.0	3945
	20.0	3818
Blank Treatments		
ETOH 1st wash		4332
ETOH 2nd wash		4517
ETOH 3rd wash		4823
Methanol 4th wash		4719

TABLE V. ESTRONE STANDARD CURVE AND BLANKS (0.5% LPP, 100 μl TESTOSTERONE-1,2-^{3}H)

	Estrone ng	CPMs
Control	0	5552
	0.2	5799
	0.6	5737
	1.0	5771
	2.0	5410
	3.0	5280
	5.0	5038
	10.0	4483
	20.0	4123
Blank Treatments		
ETOH 1st wash		4432
ETOH 2nd wash		4696
ETOH 3rd wash		4861
Methanol 4th wash		4707

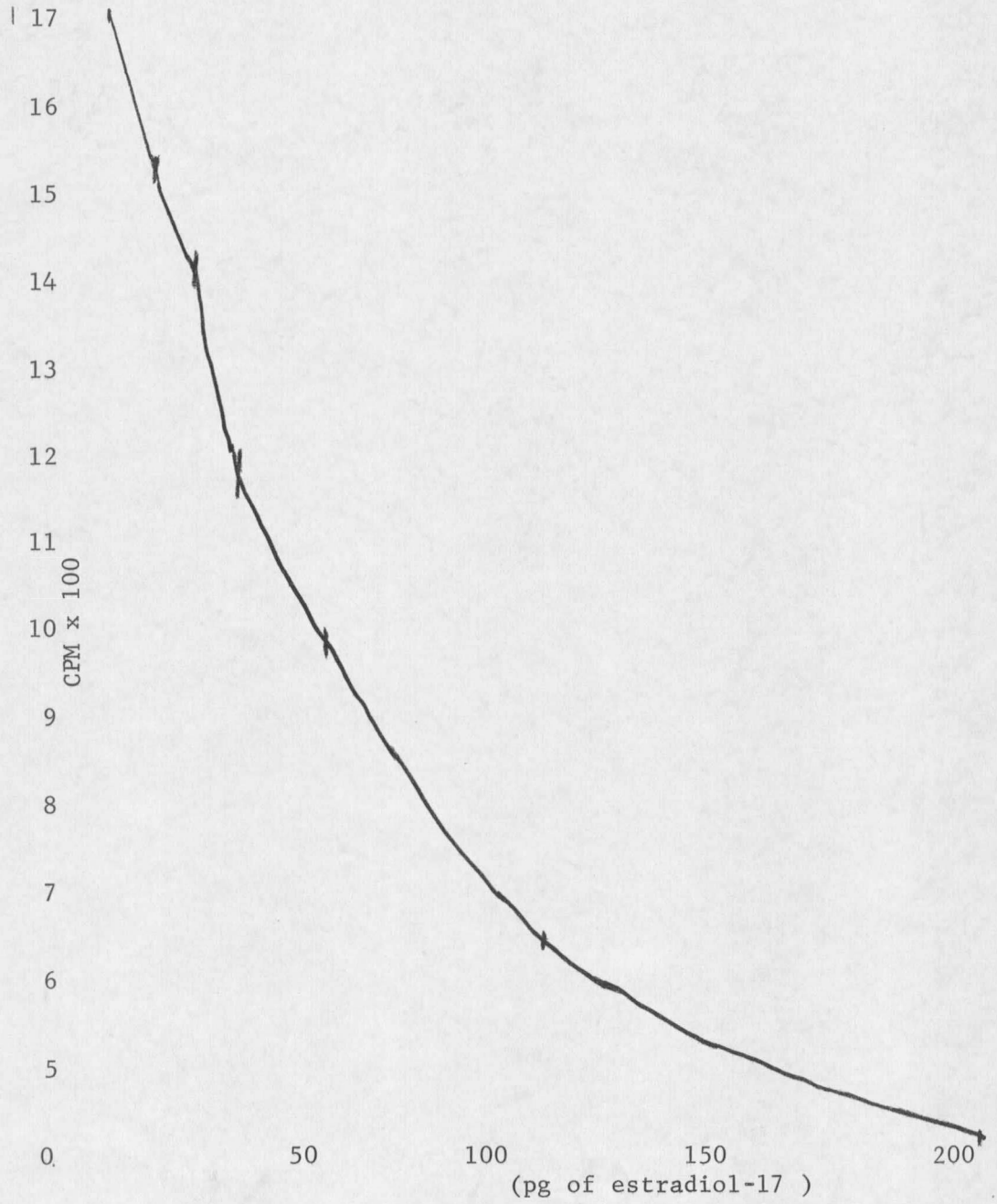

Figure 6. An example of a RIA standard curve and standard errors from points 10, 20, 30, 50, 100, and 200 pg run in quadruplicate.

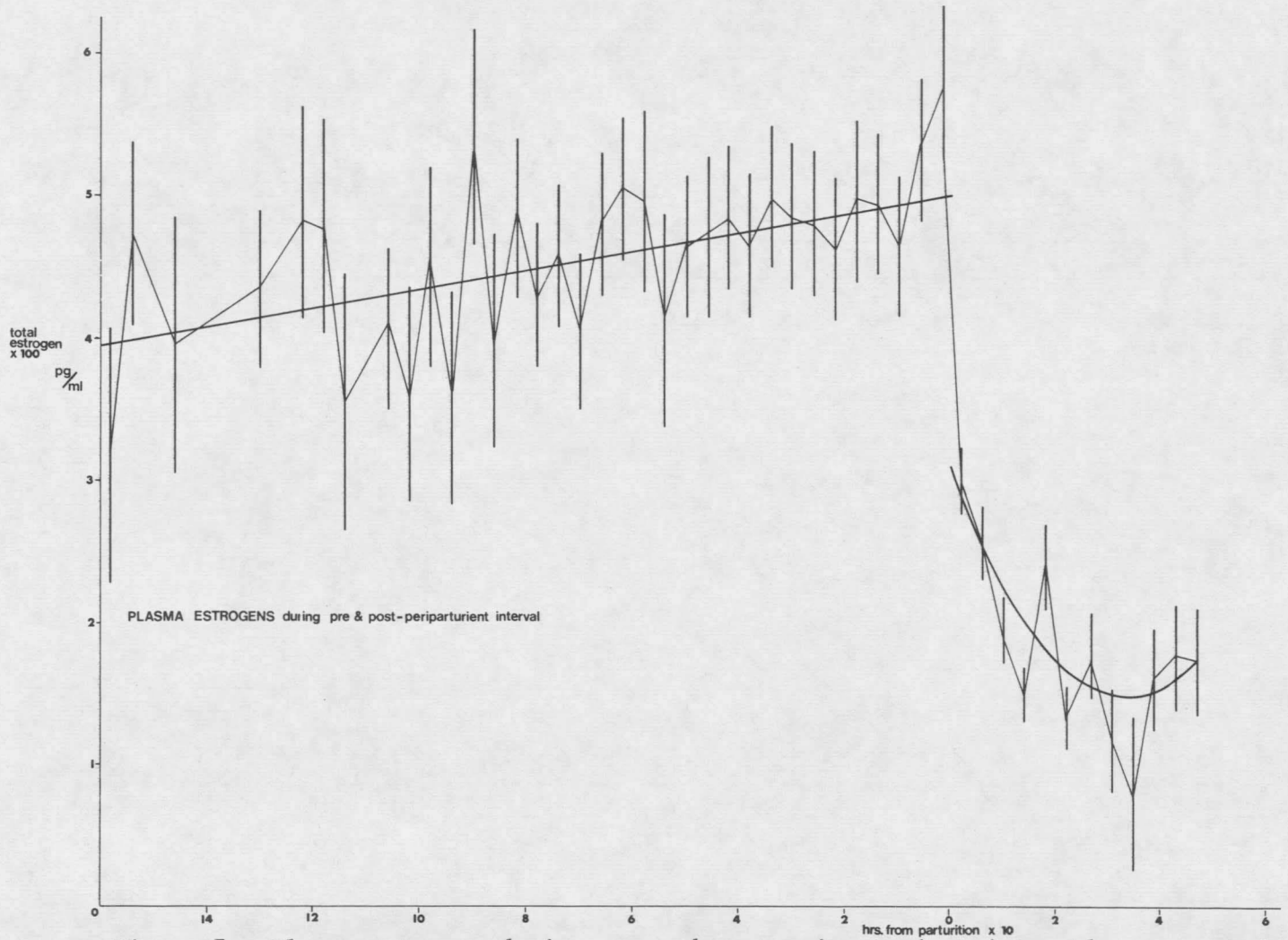

Figure 7. Plasma estrogen during pre and postperiparturient interval represented by least-squares means and standard error, a linear regression ($Y = 454.37 + .66(Xi - 472.23)$ and a quadratic regression ($Y = 162.21 + (-2.67)(Xi - 179.82) + (.15)(Xi - 179.82)^2$.

3 were 2.99, 11.91 and 1.71, respectively. F-test No. 2 which compared the means was significant ($P < .01$) while F-tests Nos. 1 and 3 on the total reduction and for all regression coefficients except the slope, respectively, were nonsignificant.

Periparturient Estrogen in Plasma Using a Radioimmunoassay

The total estrogen concentration in plasma collected from six cows at four hour intervals periparturient were determined with the RIA and analyzed with a least-squares program (Harvey, 1968). The least-squares means and the standard error of estrogen levels for each hour were computed and graphed in Figure 7 with the regression lines for each group of hours before and after parturition (hour 0). The least-squares means were defined by a significant ($P < .01$) linear regression equation ($Y = \beta o + \beta_1 X$) as samples progressed from hour 0 to 158 hours before calving (Table VI). A quadratic regression equation ($P < .05$) ($Y = \beta o + \beta_1 X + \beta_2 X^2$) defined the line of regression of estrogen levels up to 46 hours after hour 0 (Table VII). There was a theoretical estrogen level calculated for hour 0 with each equation. The linear equation estimated an hour 0 value of 501 pg/ml compared to a 313 pg/ml estrogen level estimated by the quadratic equation. The estrogen represented by the linear regression line increased approximately 15.7 pg/ml/day from 399 to 501 pg/ml. After the abrupt 188 pg/ml decrease between theoretical zero values (parturition) estrogen levels corresponding to the quadratic continued to decrease more gradually from 313 to 150 pg/ml from hour 0

TABLE VI. REGRESSION OF TOTAL ESTROGEN BEFORE GESTATION

Source	D.F.	Least-Squares Analysis of Variance Sum of Squares	Mean Squares	F
Total	39	417451.00		
Total Reduction	17	360469.81	21204.11	8.59*
MU-Y	1	38146.82	38146.82	15.46**
Hour	11	103920.56	9447.32	3.83
Line	1	53836.30	53836.30	21.82**
Quad	1	24929.53	24929.53	10.10*
Cubi	1	460.84	460.84	.19
Quar	1	2036.73	2036.73	.82
Quin	1	950.52	950.52	.38
Residual	6	21706.75	3617.79	1.47
Cows	5	127510.25	25502.05	10.34*
Remainder	22	54281.19	2467.33	

* F-test is significant at the ($P < .05$) level.
** F-test is significant at the ($P < .01$) level.

TABLE VII. REGRESSION OF TOTAL ESTROGEN AFTER GESTATION

Source	D.F.	Least-Squares Analysis of Variance Sum of Squares	Mean Squares	F
Total	39	414751.00		
Total Reduction	17	360469.81	21204.10	8.59*
MU-Y	1	38146.82	38146.82	15.46**
Hour	11	103920.56	9447.32	3.82
Line	1	53836.29	53836.30	21.82**
Quad	1	24929.53	24929.53	10.10*
Cubi	1	460.84	460.84	.19
Quar	1	2036.73	2036.73	.82
Quin	1	950.52	950.52	.38
Residual	6	21706.75	3617.79	1.47
Cows	5	127510.25	25502.05	10.34*
Remainder	22	54281.19	2467.33	

* F-test is significant at the $P < .05$) level.
** F-test is significant at the $P < .01$) level.

to +34 hours, respectively, followed by an increase to 176 pg/ml by hour +46.

Observation of the least-squares means with their standard error indicated a significant difference ($P < .05$) between two hours before and after parturition. Correspondingly the values were 577.60±57.26 and 302.14±22.50 pg/ml. This significance was estimated by not observing an overlap when two times the standard errors of the means were graphed. An observation which was not significantly different by the above definition but worth noting occurred at hour -90 where a peak of 538-95±74.50 pg/ml occurred in relation to low values of 358.95±74.50 pg/ml 4 hours before and 393.95±74.50 pg/ml 4 hours afterward.

The general trend of least-squares means estrogen values over the whole periparturient interval observed was marked by substantial fluctuation between hours -158 and -98 followed by slight variations up to -6 hours. Then the estrogen level increased approximately 45 pg/ml between hours -6 and -2, followed by a very rapid drop to the +2 hour. From +2 hours onward, the trend decreased with some fluctuations and appeared to start leveling out at 22 hours after calving. The range of estrogen levels extended from 578 pg/ml on hour -2 to 106 pg/ml on hour +38.

The total estrogen levels for individual cows (670, 678, 254, 908, 82 and 02) are listed in Appendix Table II and displayed in Figures 8-14. Cow 670 (Figure 8) upon commencement of bleeding had a blood

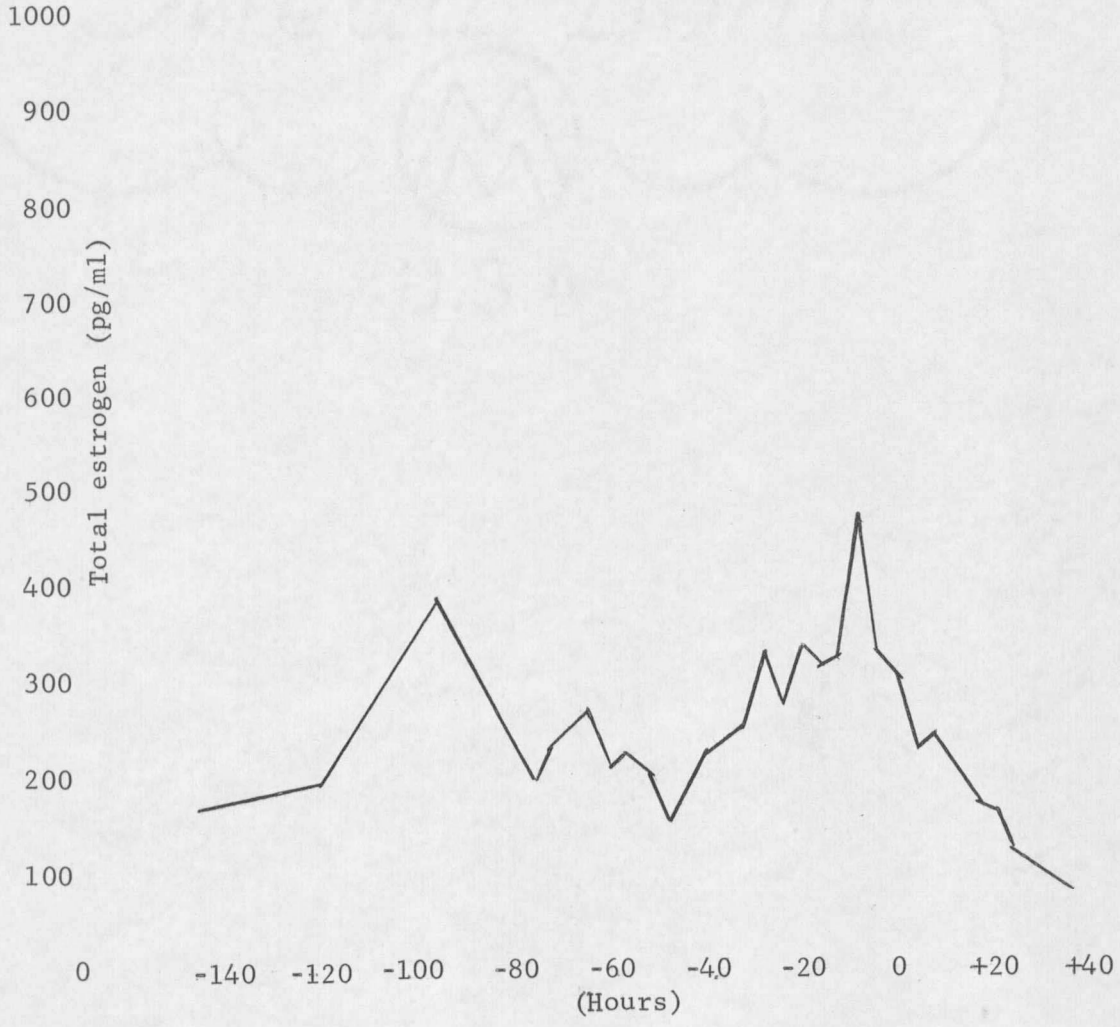

Figure 8. Total estrogen levels in plasma of cow No. 670 for four hour intervals from hours -146 to +34. 0 = parturition.

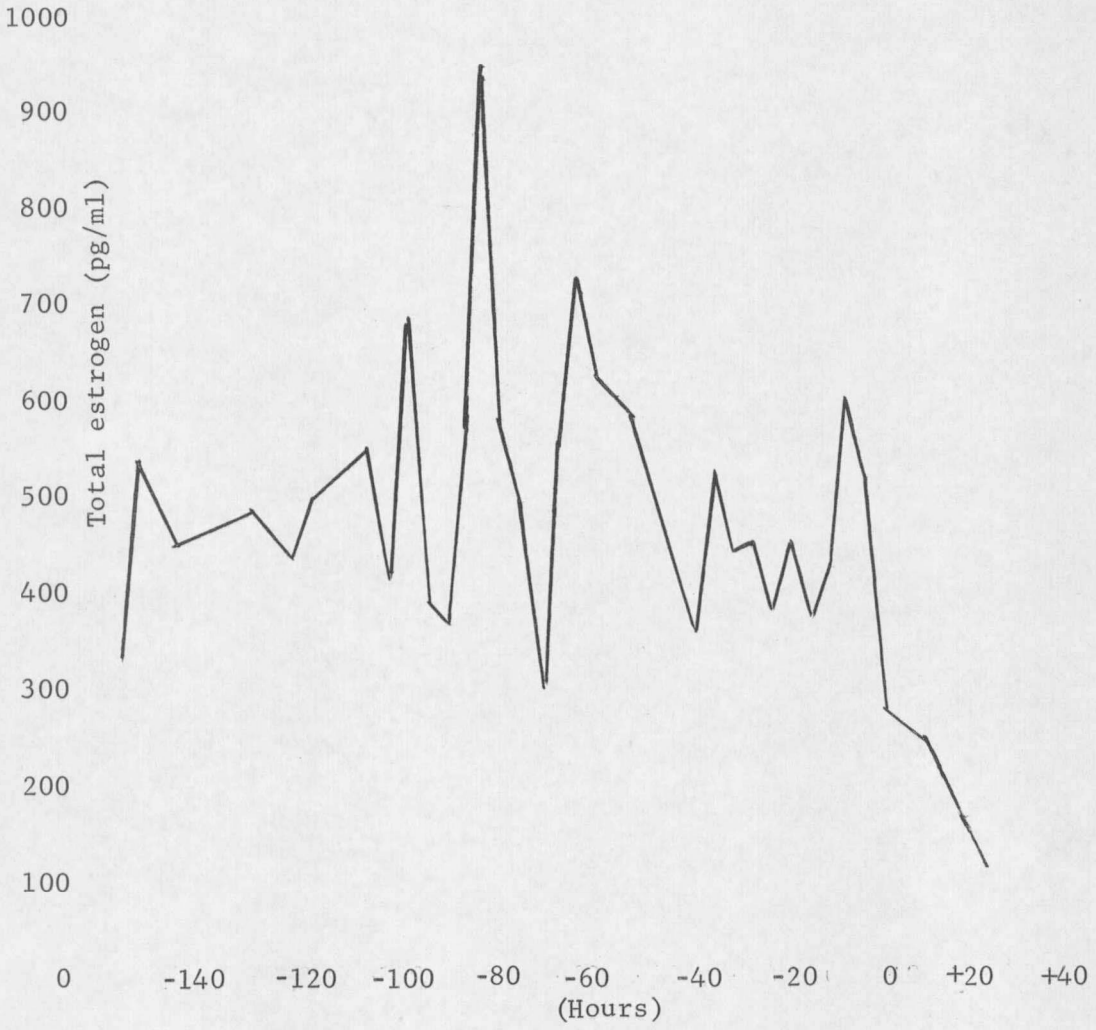

Figure 9. Total estrogen levels in plasma of cow No. 678 for four hour intervals from -158 to +18. 0 = parturition.

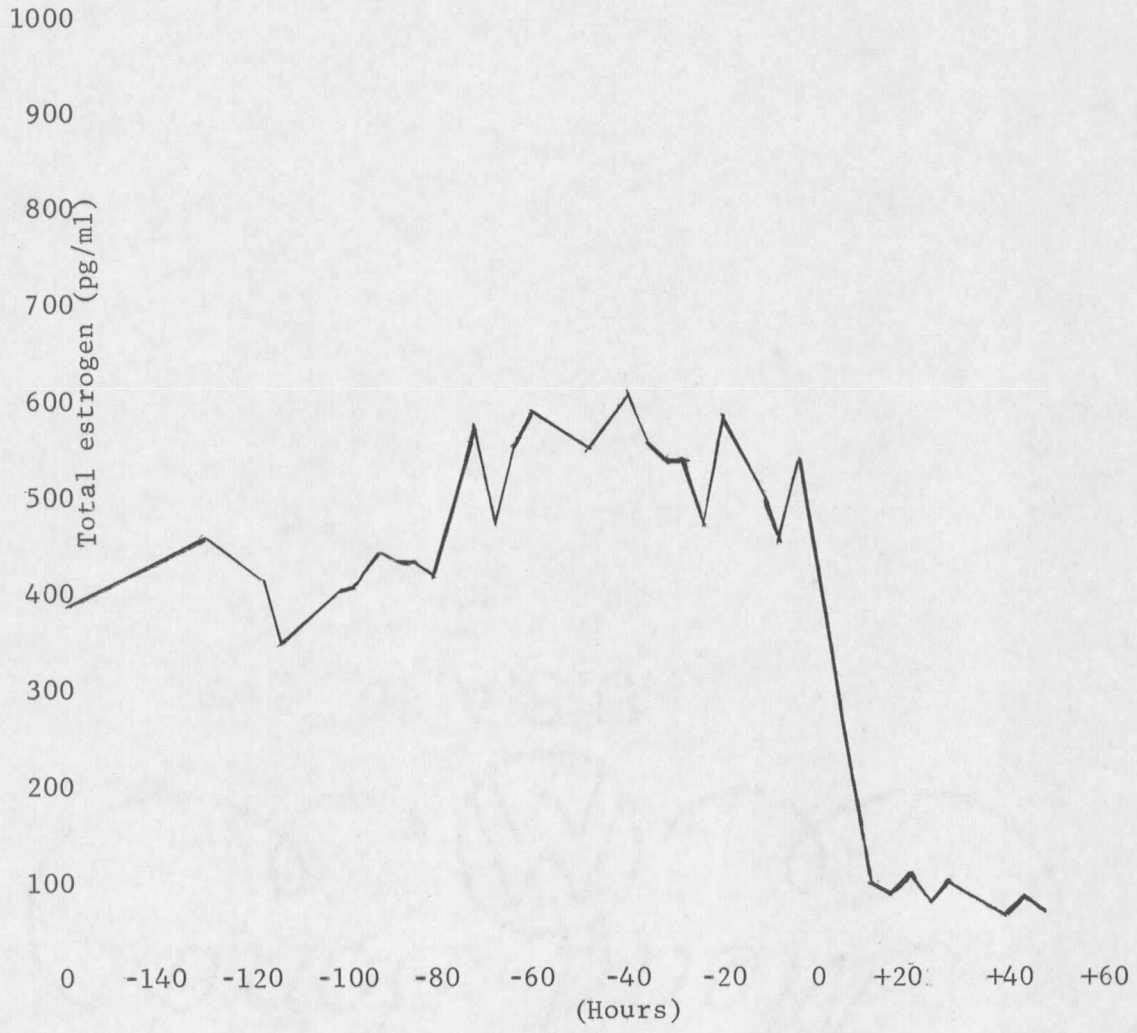

Figure 10. Total estrogen levels in plasma of cow No. 254 for four hour intervals from -158 to +46. 0 = parturition.

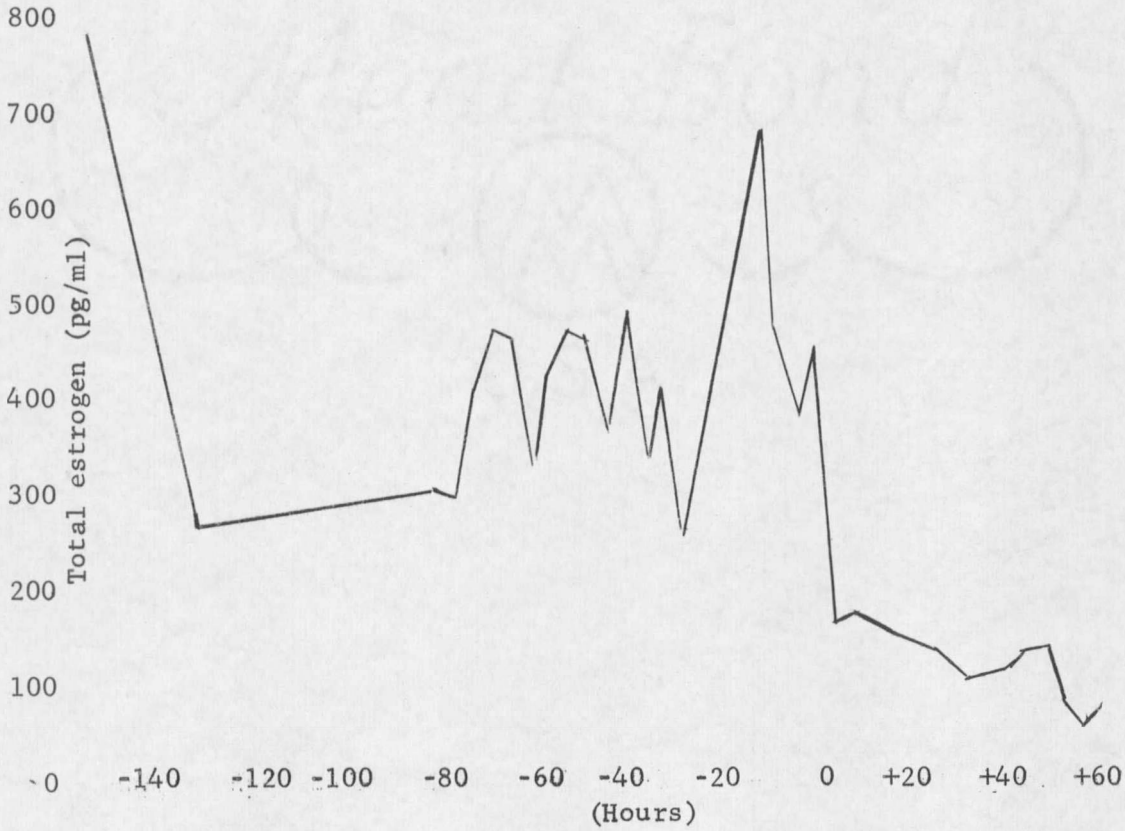

Figure 11. Total estrogen levels in plasma of cow No. 908 for four hour intervals from -154 to +58. 0 = parturition.

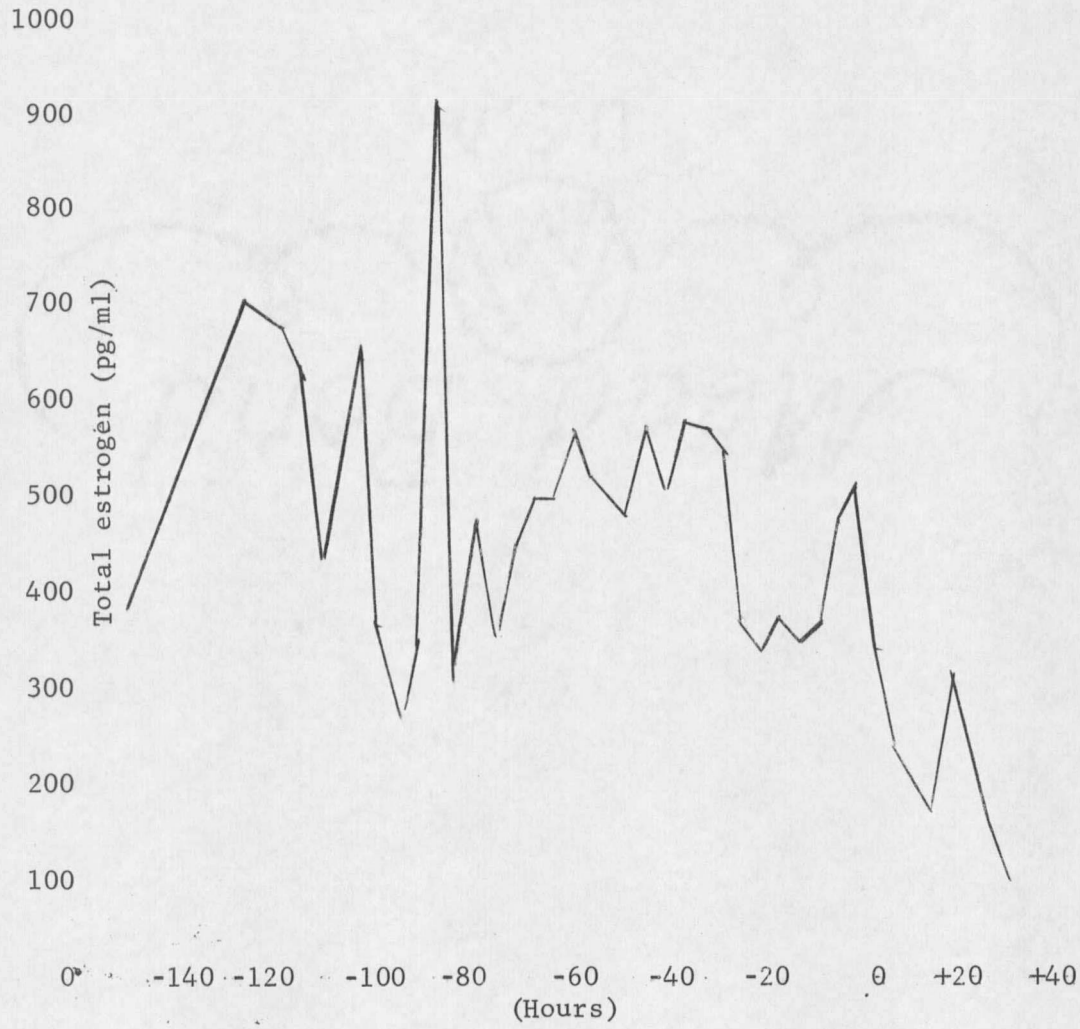

Figure 12. Total estrogen levels in plasma of cow No. 82 for four hour intervals from -154 to +30. 0 = parturition.

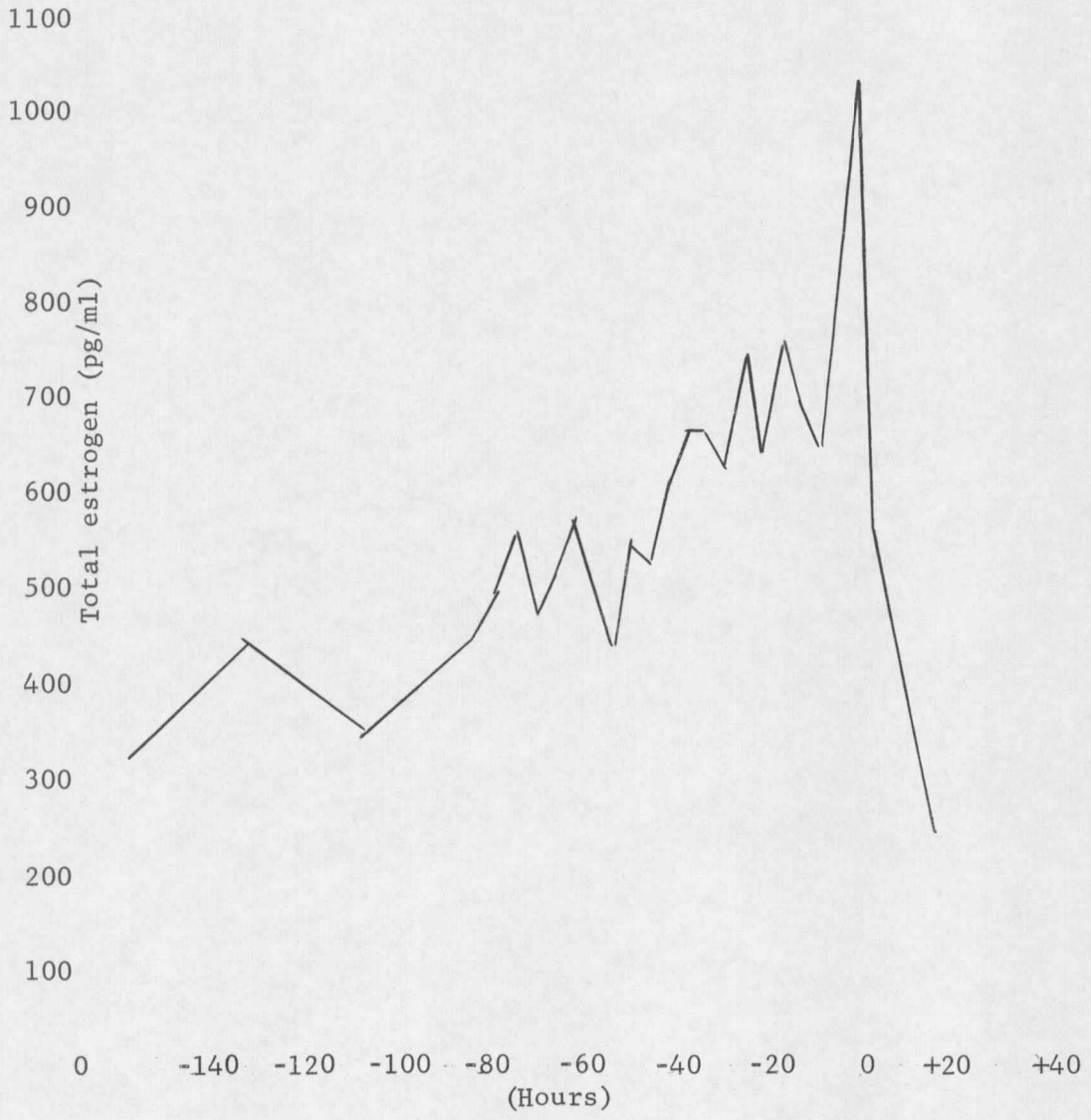

Figure 13. Total estrogen levels in plasma of cow No. 02 for four hour intervals from -154 to +14. 0 = parturition.

estrogen level of 174 pg/ml and reached a peak of 348 pg/ml at hour -98 and dropped to a reduced level abruptly and fluctuated between 273 and 160 pg/ml from hours -78 to -50. Cow 670 by hour -42 had increased to 238 pg/ml of estrogen and the level continued to increase to the major peak of 481 pg/ml at hour -10 followed by a sharp decrease to 333 pg/ml 4 hours later. Cow 670 displayed a steady decrease in estrogen to a low of 77 pg/ml at hour +30.

Cow 678 had a 351 pg/ml estrogen concentration at hour -158 followed by rapid increase to 570 pg/ml surge measured 4 hours later. The estrogen levels fluctuated around 500 pg/ml until hour -98 where a 715 pg/ml peak occurred and then fell to 419 and 394 pg/ml, 4 and 8 hours afterwards, respectively. At hour -82, estrogen levels were at the highest level reached (987 ng/ml) only to drop markedly to 329 pg/ml 12 hours later. The secondary peak (759 pg/ml) occurred at hour -62 and decreased gradually to 382 pg/ml by hour -38 and fluctuated between this level and 480 pg/ml before starting to increase by hour -10. At hour -6 a 626 pg/ml peak marked the end of increased estrogen levels as they fell dramatically to 300 pg/ml at hour +2 and progressed downward to 128 pg/ml by hour +18.

Cow 254 (Figure 10) was at 389 at hour -158, increased to 462 pg/ml on hour -130, decreased to 348 pg/ml by hour -114 and then started a gradual increase to 584 pg/ml by hour -74. Then fluctuations between 617 pg/ml (the highest peak at hour -42) and 409 pg/ml continued until

hour - 6. A dramatic decrease of estrogen occurred between hours -6 and + 10 after which time it remained at approximately 95 pg/ml and reached a low of 71 pg/ml on hour +38.

The first sample from cow 908 (Figure 11) collected 154 hours before parturition was measured at 768 pg/ml followed by a decrease to 248 pg/ml on hour -130. The next abrupt increase to 451 pg/ml was recorded on hour -70 after starting to increace at hour -78. This peak was relatively flat for a 4-hour interval before decreasing to 305 pg/ml on hour -62. Another similar flat peak occurred between hours -62 and -46 followed by jagged fluctuations until the levels began to increase rapidly from 232 pg/ml on hour -30 to the peak of 671 pg/ml on hour -14. After hour -14 total estrogen decreased abruptly to 155 pg/ml by hour +2 except for a slight peak of 438 pg/ml recorded at hour -2. Then estrogen levels decreased to a low value of 39 pg/ml on hour +54 with slight variations.

Cow 82 (Figure 12) exhibited substantial fluctuation of estrogen levels between approximately 300 and 700 pg/ml from hours -154 to -94. The highest estrogen peak of 929 pg/ml occurred on hour -90 surrounded by immediate low values of 356 and 316 pg/ml. Then the estrogen levels increased to 594 pg/ml by hour -38 in a sawtooth fashion until they decreased to 382 pg/ml on hour -26 and fluctuated around this concentration until hour -10. An estrogen peak of 535 pg/ml was monitored on hour -2 and the levels had decreased to 184 pg/ml by hour +14

followed by a slight peak of 330 pg/ml on hour + 18. This increased level decreased to 107 pg/ml the lowest estrogen value for cow 82 on hour +30.

Cow 02 (Figure 13), first monitored at hour -154 (320 pg/ml) had levels of 438, 348 and 449 pg/ml for hours -130, -110 and -86, respectively, for the first 38 hours. After these samples, taken at 24-hour intervals, the estrogen increased gradually in sawtooth fashion before increasing rapidly from 642 pg/ml at hour -10 to the peripartur-ient interval peak of 1031 pg/ml on hour -2. The estrogen levels after the hour -2 peak decreased rapidly to 236 pg/ml on hour +14 which was the last sample collected due to death from severe metritis.

A significant linear regression of gestation length, hours ($P < .05$) and birth weight ($P < .01$) on estrogen levels before parturition was calculated. However, the F-tests calculated for the linear regression of gestation and birth weight on estrogen levels after calving were nonsignificant.

DISCUSSION

Estrogen Separation

The necessity for a system which would allow rapid qualitative and quantitative evaluation of individual estrogens is well documented by urinary estrogen data (Hunter et al. 1970). This methodology could help elucidate the physiological roles of estrogens during the reproductive cycle, Randel and Erb (1971). It appears that highly specific antibodies for the radioimmunoassay such as the one developed by Dean, Exley and Johnson (1971) may well fulfill these requirements in the future. Another method proposed, prior to the development of such antiserum, was a TLC separation system combined with a Sephadex LH-20 microcolumn to purify the TLC extracts which have been found to interfere with several assay systems, Lisboa (1969).

First, an attempt was initiated to separate estrone, estradiol-17α and estradiol-17β with ascending TLC systems reported in the literature by Touchstone, Murawec and Brual (1968) and Duthie, Simmons and Urey (1969). Adequate separation of the two estradiol epimers was not possible with either system. The solvent system developed by the latter authors, a continuous TLC method described by Lisboa (1969) and extra long plates (31 cm) were combined to provide sufficient separation. The distances estrone, estradiol-17α and estradiol-17β moved from the origin were 21.34$\pm$.19, 16.75$\pm$.31 and 14.79$\pm$.28 cm, respectively, for 10 plates resulting in separation of .65 cm between the estradiol epimers. Other adaptations which were required for acceptable

separation were the "basic plates" developed by Skipski, Peterson and Barclay (1962) and activation of the TLC plates which were both methods also used by Duthie, Simmons and Urey (1969). The "basic plates" reduced the trailing of estrogen spots very well producing spot diameters of 1.33±.02, 1.24±.02 and 1.34±.03 cm for estrone, estradiol-17α and estradiol-17β , respectively. Temperature control was a critical factor and had to be maintained at 17°± 1°C for satisfactory results. The combination of these factors into a single system provided separation sufficient to allow qualitative analysis of these estrogens after separation.

Precaution was found necessary to avoid spraying and heating the unknown estrogen areas during the identification procedures because it lowered the recovery. Recovery of estrogen from TLC plants in such manner that high recovery was coupled with minimum interference in assay systems from the silica gel (Liboa, 1969) proved to be the main problem. A preliminary study showed recovery of radioactive estrogens to be slightly higher from silica gel H versus silica G. This corresponds to the statement made by Idler and Horne (1968), that recovery was affected by the types of absorbent. The solvents for extraction of estrogens from the silica gel were the most critical factor affecting recovery. The purity of solvents used for recovery was not the only important criteria but selection of solvents was important as well (Attal, et al. (1969), Idler and Horne (1968),Doerr (1971), and

Liboa (1969). These concepts became reality when TLC blanks washed with ethanol and methanol were over 2 and 5 ng when assayed in the CPBA with standard curves of estradiol-17β and estrone, respectively. The low contamination, less than 0.6 ng from TLC gel extracted with methanol or double distilled ETOH, was observed when these extractants were assayed with an estradiol-17α standard curve and suggest that high blanks may not be a factor in this system.

TLC blanks eluted with ethyl acetate from microcolumns (Attal, et al. 1969) also caused high contamination problems in the RIA. Therefore, an attempt was initiated to combine the elution scheme of Attal et al. (1969) with the Sephadex LH-20 microcolumns. The blank values of 90 pg for estrone and 53 pg for estradiol-17α were a substantial improvement in comparison to just the TLC elutant blank values which were over 300 pg. The level of contamination for estradiol-17β was recorded at approximately 300 pg, however, this was partially credited to the high column elutant contaminants. The column elution blanks collected before the estrogens were layered on the microcolumns showed 22, 24 and 54 pg of estrogen activity in the assay for estrone, estradiol-17α and estradiol-17β , respectively. However, blanks collected after estradiol-17α and estradiol-17β elution had contamination over 300 pg/ml. This suggested that the contaminants which eluted just after the estradiols could cause a problem unless the elution volumes are well defined and observed during the collection of samples.

Further development of the system was not pursued but it appears quite promising. These results were supported by some other encouraging results reported by Doerr (1971). He measured only 10 pg blank values with a RIA when estradiol-17β was eluted with dichloromethane and methanol (90:10) from silica gel in a microcolumn. The author also noted that artifact formation increased with the amount of time the estrogen was exposed to the air while on TLC plates. Doerr (1971) was able to perfect his system to such a degree that 40 pg of radioactive estradiol-17β could survive TLC, elution and evaporation.

In conclusion, incorporation of the techniques of Doerr (1971) with the continuous TLC may provide a suitable system for individual measurement of estrone and estradiol-17β and estrdiol-17α. If this proves unsatisfactory, it would appear from preliminary results, that the addition of Sephadex LH-20 column after TLC to the scheme will make the measurement of individual estrogens possible. A system like this which would provide high recovery and low contamination is needed to pursue the role of the individual estrogen in the reproductive cycle. Hopefully highly specific antibodies for each estrogen will be developed in the near future which would be a less laborious system, however, the other system would allow a check on the specificity of the antibodies.

It appears possible to measure estradiol-17α in the CPBA after separation and purification according to preliminary results, however, the RIA looks like the most promising assay system due to the higher

sensitivity and a more positive capability of allowing measurement of estrone and estradiol-17β especially if a volume of blank column elutant corresponding to each estrogen elution volumn is added to the standard curve to remove the column background from the system.

Estrogen Assays

The competitive protein binding assay utilizing human late pregnancy plasma was not operational for several reasons. The standard curves for estrone and estradiol-17β lacked regression and a limited regression was obtained with estradiol-17α. The sensitivity of the CBA appeared to be approximately 0.2 ng for the standard curves in comparison to a working sensitivity of 12 pg for the RIA. Therefore, the RIA was developed and used to measure total estrogen levels in the plasma samples.

The antiserum was diluted at 1:45,000 to obtain a 60 percent binding efficiency and produce a useable standard curve. Analysis of the standard curves as described earlier proved that the same regression line was obtained in each of the six assays. However, the significance ($P < .01$) between the means indicated that the complete curve moved up and down on the y axis. This variation between the standard curves signified that caution should be taken when comparisons between cows were made because all of the samples from one cow were analyzed with the same standard curve.

Periparturient Estrogen Levels in Plasma

A radioimmunassay was used to measure total estrogen in plasma samples collected at four hour intervals 158 hours before to 58 hours after parturition (hour 0). Then, the total estrogen levels on the six cows were analyzed with a least-squares program obtaining least-squares means and regression equations for the two time intervals surrounding hour 0. A linear regression equation for estrogen levels 158 hours before hour 0 defined the increasing estrogen levels leading up to calving time. The increase of estrogen concentration prior to parturition was the expected response in relation to blood estrogen data reported by Robinson et al. (1970), Arjie, Wiltbank and Hopwood (1971) and Henricks et al. (1972). The urinary estrogen data reported by Randel and Erb (1971), Hunter et al. (1970) and Mellin, Erb and Estergreen (1966) also support the prepartum estrogen rise.

The total increase of estrogen corresponding to the line of regression before parturition was 102 pg/ml over the approximate 6.5 day period or an increase of 15.7 pg/ml/day with the highest level observed equal to 501 pg/ml. This daily increase and the high level were considerably lower than other levels reported with an Ittrich-Kober Fluorometric method (Robinson et al. 1970) and with RIA (Arjie, Wiltbank and Hopwood, 1971 and Henricks et al. 1972). Arjie, Wiltbank and Hopwood (1971) reported estrogen ranged from 870 to greater than 1,300 pg/ml over a 20 day period before parturition in beef cows. Henricks et al.

(1972) experimenting with dairy cows, measured an increase of estrogen from 500 pg/ml to 2660 pg/ml over a 14-day interval before calving and an average increase of 248 pg/day for the last 5 days.

The discrepancy between levels reported at the Montana State University Endocrinology Laboratory and the last two references were attributed in part to assay variations. Assay variations could be caused by different antibodies with a different percentage of cross reaction for other steroids or the individual estrogens not to mention procedural differences between laboratories.

The antibody used to analyze the pre- and postperiparturient plasma possessed a 59.7 and a 60.0 percent binding efficiency for estrone and estradiol-17β, respectively. This binding efficiency was observed at a 1:6,000 (antiserum:buffer A) dilution when estradiol-17β-6-7-^{3}H was utilized to make the standard curve. Although the cross reaction for estradiol-17α cannot be obtained due to lack of its radioactive isotope, the very close binding efficiencies for estrone and estradiol-17β make this antiserum quite ideal for the measurement of total estrogens. Some other factors to which discrepency could be credited, were breed differences as observed by Hunter et al. (1970) in urinary estrogen excretion levels and individual cow variation (Mellin, Erb and Estergreen 1966).

The significant increase in estrogen levels during late gestation has been related to the increasing estrogen levels in fetal cotyledons

reported by Veenhuizen, Erb and Gorski (1960). Mellin, Erb and Estergreen (1966) and Erb et al. (1968) both referred to this phenomenon when they discussed increased urinary estrogen excretion during late gestation.

The least-squares means representing estrogen concentration after parturition were defined by a quadratic equation. The quadratic line defines these estrogen levels quite well after hour +2 as decreasing until the line started increasing at hour +34 to form a parabola which according to the literature and personal observation is not true. Arjie, Wiltbank and Hopwood (1971) described the estrogen levels as falling to 100 pg/ml at parturition followed by an increase slightly postpartum to remain at 200 pg/ml until reaching 500 pg/ml at 2 days before estrus. In comparison, Henricks et al. (1972) reported estrogen concentration to range from nondetectable to 50 pg/ml after parturition. Personal observations tend to support the findings of Henricks et al. (1972) because cow 908 for hours +50, +54 and +58 had estrogen levels of 66, 39 and 64 pg/ml, however, this was very limited data. From this evidence it would appear necessary to use a different equation to define the estrogen levels after parturition correctly.

The dramatic 188 pg/ml estrogen decrease between the two theoretical hour 0 values for each regression equation was another vivid example of the inadequacy of the equations to define a realistic graphic representation of estrogen levels after parturition. This was credited

to the fact that estrogen levels dropped so fast at parturition that samples would have to be collected at intervals closer than every four hours to correctly define this decrease. The reason that estrogen decreased so rapid at parturition could be associated to the initial loss of the circulation between the maternal and fetal environment at parturition and finally the loss of the fetal cotyledons. This concept would be harmonious with the increase of estrogen content in the fetal cotyledons effecting the increased estrogen level during the last month of pregnancy. The rapid decline at or just prior to parturition observed during natural conditions would be an interesting phenomenon to consider with corticoid induced parturition since retained placentas occur at such high incidence (Jöchle, 1971).

The four hour bleeding intervals allowed closer obervation of the blood estrogen changes in the pre- and postpartum cow. Some of the observations of interest were the ranges of both groups before and after hour 0 and the least-squares means of hours -90 and the two hours before and after hour 0. The increase of estrogens before parturition was from 137 pg/ml on hour -58 to 577 pg/ml on hour -2 or an increase of approximately 67 pg/ml/day. The estrogen levels decreased from 577 pg/ml on hour 0 to a low of 106 pg/ml by hour +34. The characteristic increase and decrease, before and after parturition described by the least-squares means are a better definition of the same estrogen level trends described by the lines of regression

mentioned previously. The noticeable peak of 538 pg/ml at hour -90 for the composite least-squares means is in close association with a well defined peak in cows 678 and 82. The importance of this peak may indicate an estrogen surge of distinct physiological importance such as the concept that estrogen is required to stimulate the release of prolactin; however, this supportive evidence was on rats in which prolactin is luleolytic unlike in the cow. Sharr, and Clemens (1971) concluded in a study with rats that estrogen was necessary for the activation of neurons which either inhibited prolactin inhibiting factor secretion or stimulated a prolactin stimulating factor. Further evidence for this concept from experimentation with rats was reported by Chen and Meites (1970). They found that contrary to some earlier experiments, no evidence could be discovered for large doses of estrogen inhibiting prolactin release and even referenced material which suggested prolactin increases with increasing estrogen doses. They also indicated that large doses of progesterone could partially counteract the stimulating action of estrogen on anterior pituitary prolactin release.

Another reason the approximate hour -90 estrogen surge could effect prolactin release is that prolactin has been reported as increasing from 50 to 100 ng/ml to 200 ng/ml before paturition by Ingalls, Hafs, and Oxender (1971). Arije, Wiltbank and Hopwood (1971) reported prolactin as increasing from below 50 ng/ml to above 300 ng/ml from two days before calving to 20 days postpartum. They also reported progestin as

declining to 3 ng/ml a week before and 1 ng/ml at parturition. Therefore, the timing of this approximately -90 hour surge could correspond with the stimulation of prolactin release. Progestrogens decreasing levels observed in conjunction with increasing urinary estrogen excretion (Hunter et al. 1970) as well, do not contradict this concept.

The significant difference between the least-squares means at 2 hours before and after parturition identfied the major phenomenon during the periparturient interval. The values for the corresponding hours were 577.60±57.26 and 302.14±22.50 pg/ml, respectively. The particulars of this phenomenon were discussed in regard to the theoretical zero values earlier.

The birth weight, hours and length of gestation were regressed on estrogen levels before and after parturition. The linear regression of hours on estrogen levels before ($P < .05$) and after ($P < .01$) parturition were not used because a better description of the curve had already been obtained with the two equations discussed previously. The regression of birth weight on estrogen levels ($P < .01$) indicated that as the birth weight increased, estrogens decreased while the regression of gestation length on estrogen ($P < .05$) showed a positive relationship of gestation length to estrogens. These two phenomenon appear to be contradictory to each other, however, one way is speculated that both could be true. The first relationship would be true if the calf metabolized estrogens in proportion to his size and the combined

source of estrogen (maternal, placental and fetal) produced estrogen at a relatively constant rate independent of calf size, correspondingly the latter would be true if one or more of the estrogen sources gained in its capability to produce estrogen faster than the calf could metabolize the estrogens. The inverse relationship of birth weight to estrogen levels contradicted reports of higher urinary estrogen excretion in a cow that has twins versus a single calf (Randel et al. 1968 and Hunter et al. 1970). The comparison of these two factors were somewhat limited to the emphasis which could be placed on this contradiction. The number of cows observed was very limited and the comparison of single birth to twinning in regard to birth weight was complicated with the different affect two fetal endocrine systems could have on the estrogen levels in comparison to one.

The positive relationship of gestation length to estrogen could be related to the data reported by Hunter et al. (1970). He compared urinary estrogen excretion with plasma progesterone concentration and reported that the rate which estrogen overdominance occurred differed with the length of gestation. This difference was observed in the last 34 days of gestation. Cows that calved prior to 280 days gestation exhibited a rapid decline in plasma progesterone in conjunction with little change in excretion of estradiol-17α or total estrogens, while cows calving between 280 to 284 days gestation showed a minor change in plasma progesterone levels and a rapid increase in urinary

estrogen excretion. In comparison cows with a gestation period longer than 284 days displayed a steady decline of progesterone within 2 days of parturition followed by an increased decline of progesterone while estrogen levels increased steadily up to parturition but at a slower rate than the latter group. However, the small number of cows used to obtain the positive relationship of gestation length to estrogen levels and the indirect relationship between estrogen levels for 6.5 days and 34 days makes it impossible to draw concrete analogies. Therefore, a larger number of cows over about a 30 day prepartum interval combined with estrogen and progesterone levels appear necessary to relate the overdominance of estrogens in blood to the urinary scheme reported by Hunter et al. (1970).

Individual cows

Close observation of the individual cows was very advantageous because the least-squares means and the regression lines tend to obscure individual characteristics. The high values for each cow were 481, 978, 617, 768, 929, 1031 pg/ml for cows 670, 678, 254, 908, 82 and 02, respectively, (Figures 8-13). Cows 678 and 82 had their highest peak on hours -82 and -90, respectively, which corresponded with the increased level noted at hour -90 for the least-squares means while cows 02, and 670 reached the highest peaks at hours -2 and -10, respectively, which corresponded to the theoretical hour 0. Cow 908 hit a high peak at hour -158 which does not correspond to any

other cow however, a secondary peak of 671 pg/ml occurred at hour -14 which relates to cows 02 and 670. Cow 254, unlike the others, showed a plateau of estrogen levels starting on hour -74 and extending to hour -6 with a slight peak above the plateau occurring at hour -42. Cow 254 experienced the typical rapid decline of estrogens after hour -6, therefore, a consistent observation in most of the cows was a peak just prior to the rapid decrease in estrogen levels close to parturition. This consistency could be related to the common reference point (parturition) and as the cows got further away from parturition, the individual characteristics would deviate further from other cows.

One other consistency signifying importance, noted throughout cows, was the sawtooth fashion displayed by fluctuating estrogen levels. This sawtooth affect was contributed to the homeostatic control of estrogens. This control appears to act as a thermostat which lacks close precision and overcorrected the increase or decrease in estrogen in search of an equilibrium. It was apparent in the individual graphs that in order to accurately measure the estrogen fluctuation, it would be necessary to analyze samples at closer intervals than four hours due to the sharp spikes. Supportive evidence for close hour sampling was offered by the research on defining the short duration of the dynamic LH peak prior to ovulation in the cow. Elevated LH levels have a 12.4 ± 1.56 hour duration according to Christensen, Wiltbank and Hopwood (1971) while very close sampling was required to define the dynamic LH

peak itself. With close identification of estrogen fluctuation, an insight may be gained to the times estrogen influence physiological events in individual cows. Since some of the dramatic surges of estrogen appeared to be triggering mechanisms while the less substantial fluctuation appeared to occur when a constant level was being searched for.

Cow 670

Hereford cow number 670, after 274 days gestation, gave birth to a 90 pound bull calf. One hundred forty-six hours prior to calving, collection of blood samples began at twenty-four hour intervals until hour -78. Therefore, the first three samples assayed did not correctly define the early estrogen samples in this time interval, however, one level worth mentioning occurred at hour -98. This hour was represented by an estrogen peak of 348 pg/ml and corresponds with the noticeable peak of hour -90 of the composite of least-squares means. Observation of four hour intervals produced the sawtooth pattern mentioned previously. This homeostatic control appeared to regulate the estrogen quite effectively until the control seemed to loose some of its precision as hours -98 and especially hour -10 approached and suggested the triggering of a physiological event. After the peak of 481 pg/ml at hour -10, estrogen declined rapidly at first followed by a more gradual decline to the low of 77 pg/ml at hour +34. This phenomenon marked the time of parturition, however, the more gradual decrease

could have indicated some retained placenta although actual observation of this fact was not recorded.

Cow 678

The long gestation length of 289 days and about average calf birth weight (67 lb) for cow 678 in regard to the positive and negative relationship defined by their regression on estrogen, respectively, suggest that the long gestation length should increase estrogen levels along with a slight affect from birth weight. Comparing this observation to the graphs of other cows, it did not indicate a distinct affect in estrogen levels.

The homeostatic control of cow 678 showed precision by slight fluctuation before hour -102 but several of the four hour intervals were absent (hours -150, -142, -138, -134, -126, -114 and -110) and tended to bias this observation. Precision of regulation seemed to decrease for intervals surrounding hour -82 where the high peak of 978 pg/ml of estrogen occurred marking the speculated triggering of prolactin release. The fluctuation became limited again after hour -38 until a surge of estrogen resulted in a peak of 626 pg/ml of at hour -6. After hour -6, the dramatic drop indicating parturition occurred and estrogen levels fell to +128 pg/ml at hour +8.

Cow 254

Angus female number 254, after 276 days gestation, gave birth to a heifer calf weighing 58 pounds and exhibited a lesser degree of

fluctuation in the four hour estrogen concentration pattern than the other five cows. The reason for the estrogen level graph resembling a plateau would suggest that cow 254 had a more precise control for regulating her estrogen levels unless the samples were not at close enough intervals to distinguish the difference between the control and triggering action. Cow 254 still exhibited the characteristic decrease close to parturition, along with remaining at low levels (approximately 90 pg/ml) after parturition.

Cow 908

Angus cow number 908 gave birth to a 58 pound heifer calf after 274 days gestation. This cow was the only one out of six that had the highest level of estrogen (768 pg/ml) at hour -154. This could suggest that this sample was in error, or that it was characteristic of cow 908. It was impossible to distinguish what happened close to this time due to irregular sampling while the suspected surge around hour -90 could have been missed due to the 24-hour sampling until hour -82. This early estrogen surge could have been missed in other cows due to the sharp estrogen spikes that can also occur. Vivid examples of peaks being missed were observed at approximate hours -70 and -54. It is obvious that samples were collected on each side of the peaks indicating that more frequent sampling would be necessary to correctly identify estrogen levels.

An estrogen peak of 671 pg/ml at hour -14 showed resemblance of

the characteristic peak that occurs at parturition, however, in this cow, another small peak (438 pg/ml) occurred at hour -2. Followed by another dramatic decrease. This phenomenon could be related to the gradual decrease observed in cow 670 and indicated the need for closer observation. Both of the peaks for cow 908 and 670 occurred on hours -14 and -10, respectively, which was further from hour 0 than any of the other estrogen surges which decreased dramatically at parturition. It would appear that the triggering mechanism either occurred too soon before parturition or that the rapid decline at parturition may well be in an erratic manner.

Cow 82

Angus female number 82 showed an estrogen pattern very similar to cow 678, however, cow 82 gave birth to a 75 pound bull calf after 280 days gestation. The relationships obtained by regressing birth weight and gestation length on estrogen decreased due to an approximate average gestation length. This would indicate that cow 82 should have lower estrogen than cow 678, however, they showed the opposite result, if any affect. This contradiction gave indication that either the relationships need further investigation or what affected the discrepancy was assay variation between the cows.

Individual cow difference was obvious in cows 678 and 82. For example, the high estrogen peak in cow 82 was 929 pg/ml and occurred on hour -90 to correspond exactly with the estrogen surge observed with

the least-squares means while cow 678 reached 978 pg/ml at hour -82. This example indicated one of the short-comings of grouping.

Cow 82 also followed the pattern of 678 by indicating close regulation of control of estrogen levels between hour -90 and the peak at time of parturition at which time the triggering mechanism overcame the control. The peak just before parturition in cow 82 occurred at hour -2 and was followed by the characteristic decrease. Cow 82 had another distinct peak of 330 pg/ml after parturition at hour +18 which was not characteristic of other cows, therefore, it could have been in error or an indication of early follicular development in this cow.

Cow 02

Angus female number 02, after hour -82 showed increase in a saw-tooth pattern up to 1031 pg/ml on hour -2 before birth was given to a 68 pound bull calf at 273 days gestation. Parturition was marked by the characteristic decrease after hour -2 to a low estrogen concentration of 236 pg/ml on hour +14. Hour +14 was the last sample collected before death due to severe metritis.

The early four hour interval estrogen levels before parturition were not collected and was the expected reason for not observing the estrogen surge to correspond with hour -90 referred to in the least-squares means graph. The homeostatic control mechanism could have limited this estrogen surge also since estrogen levels prior to the

hour -2 surge appeared to have close regulation.

The rapid increase of estrogen up to parturition observed in cow 02 similated the description Hunter et al. 1970 designated for the group of dairy heifers that calved between 280-284 days gestation. However, the 273 days gestation would put cow 02 in the short gestation group since the average gestation length for Angus is 279 days. Caution must be observed in relating these two sources of data due to several variations such as 3 day sample intervals versus four hour intervals, breed differences and urinary versus plasma estrogens.

SUMMARY

This study was initially conducted to obtain separation and measurement of estrone, estradiol-17α and estradiol-17β in plasma to help elucidate the physiological importance of these estrogens in reproduction. This led to the development of a continuous thin-layer system for separation and a Sephadex LH-20 microcolumn for purification of the TLC extract. The TLC extract contained contaminants which interferred with the CPBA for estrone and estradiol-17β even though their standard curves lacked regression. The CPBA for estradiol-17α produced a marginal standard curve and was not hindered by TLC contaminants, however, the CPBA did not meet the sensitivity requirements.

The RIA proved to be a more sensitive assay for estrogens and less sensitive to interference by TLC contaminants if the TLC extracts for estrone and estradiol-17α were eluted from a 10 cm Sephadex LH-20 microcolumn. Estradiol-17β TLC extracts were still contaminated after elution from the microcolumn; however, it is speculated that slight modification of this system could produce measurement of the individual estrogens however, the procedure appeared too laborious to make a large number of measurements feasible.

The RIA was utilized to pursue the physiological importance of total estrogens in six beef cows that were bled at four hour intervals 158 hours before and 46 hours after parturition. The total estrogen levels were estimated from an estradiol-17β standard curve and the estimates were analyzed with a least-squares program and the orthogonal

polynomial option. The major findings of this particular section are as follows:

1. The antibody used for the RIA was considered suitable for the measurement of total estrogens due to a 60 and 59.7 percent binding efficiency for estradiol-17β and estrone, respectively, and minimum cross reaction with other steroids.

2. Although the same line of regression was observed for each standard curve, precaution must be taken in comparing estrogen values between assays, and cows, in this instance, due to the movement of the standard curves on the y axis ($P < .01$).

3. The least-squares means of blood estrogen levels increased ($P < .01$) from 158 hours prepartum up to parturition and decreased ($P < .05$) for 34 hours after parturition.

4. Speculation is made that the sawtooth pattern, characteristic of the prepartum total estrogen levels for all six cows, displayed the effects of a homeostatic control mechanism when fluctuations were slight.

5. It is speculated that the dramatic surges observed close to hour -90 and hour -2 signified a triggering action of estrogen. The estrogen surge near hour -90 could initiate the release of prolactin.

6. In view of the short duration of some of the estrogen surges, it will be necessary to measure estrogen levels closer than four hour intervals to correctly identify the estrogen levels during the

pre- and postparturient interval.

7. The direct relationship ($P < .05$) obtained by the regression of gestation length on estrogen levels and the correspondingly inverse relationship of birth weight ($P < .01$) to prepartum estrogen levels appear to be in constradiction but would be worth investigating in a larger number of cows. One speculated concept which clarifies the suggested contradiction would be if the fetus metabolized the estrogens in proportion to size and all of the sources of estrogen combined, synthesized estrogen at a relatively constant rate then, as birth weight increased the estrogen levels would decrease. Correspondingly, the estrogens could increase as days of gestation increased if one or more of the sources of estrogen increased in its ability to produce estrogen raising the estrogen levels above the amount the fetus could metabolize.

APPENDIX

APPENDIX TABLE I. HISTORY OF COWS

Cow No.	Breed of Cow	Gestation Length (days)	Sex of Calf	Birth Wt. of calf (lbs)	Date of parturition 1971
908	Angus	274	Female	59	29 May
254	Angus	276	Female	68	31 May
002	Angus	273	Male	68	29 May
082	Angus	280	Male	75	4 June
670	Hereford	274	Male	90	29 May
678	Hereford	289	Male	67	4 June

APPENDIX TABLE II. TOTAL ESTROGEN LEVELS IN PLASMA FROM SIX COWS (pg/ml)

Hours from Parturition	Cow Number					
	670	678	254	908	82	02
-158	*	351	389	*	*	*
-154	*	570[a]	*	768	393	320
-150	*	*	*	*	*	*
-146	174	481	*	*	*	*
-142	*	*	*	*	*	*
-138	*	*	*	*	*	*
-134	*	*	*	*	*	*
-130	*	514	462	248	721	438
-126	*	*	417	*	*	*
-122	199	464	*	*	695	*
-118	*	526	*	*	636	*
-114	*	*	348	*	441	*
-110	*	*	*	*	*	*
-106	*	576	389	267	675	348
-102	*	435	406	*	380	*
- 98	348	715	412	*	287	*
- 94	*	419	447	*	356	*
- 90	*	394	439	*	929	*
- 86	*	576	435	*	316	*
- 82	*	978	425	289	490	449
- 78	202	595	505	270	366	492
- 74	240	510	584	393	467	551
- 70	*	329	476	451	514	463
- 66	273	589	550	448	512	505
- 62	216	759	595	305	584	568
- 58	234	648	*	410	541	*
- 54	211	*	555[a]	450	*	431
- 50	160	604	*	447	493	539
- 46	*	530	585	346	584	520
- 42	238	456	617	476	520	600
- 38	248	382	514	399	594	655
- 34	264	551	542	396	589	658
- 30	333	471	545	232	564	618
- 26	287	480	479	365	382	740
- 22	347	399	589	443	359	642
- 18	322	481	*	552	387	752
- 14	331	391	521	671	367	688
- 10	481	449	409	440	379	642

APPENDIX TABLE II. (CONTINUED)

Hours from Parturition	Cow Number 670	678	254	908	82	02
- 6	333	626	548	377	488	820
- 2	306	538	*	438	535	1031
- 0	*	*	*	*	*	*
+ 2	239	300	*	155	357	552
+ 6	249	*	196	163	255	*
+ 10	212	264	105	148	*	364
+ 14	182	216	94	136	184	236
+ 18	175	128	116	*	330	*
+ 22	124	*	85	127	*	*
+ 26	*	*	108[a]	196	167	*
+ 30	*	*	*	89	107	*
+ 34	77	*	*	*	*	*
+ 38	*	*	71	101	*	*
+ 42	*	*	91	115	*	*
+ 46	*	*	75	124	*	*
+ 50	*	*	*	66	*	*
+ 54	*	*	*	39	*	*
+ 58	*	*	*	64	*	*

* It was not possible to obtain a measurement.

a Serum samples.

APPENDIX TABLE III. VALUES FROM LEAST-SQUARES ANALYSES FOR F-TESTS ON RIA STANDARD CURVES

Source	Mean (CPM)	Sum of Squares (Total Reduction)	Mean Squares (Remainder)
Cow 670	1027.00	7513800.00	1324.67
Cow 678	1039.22	7504063.00	2507.17
Cow 254	1047.94	6555099.00	3245.04
Cow 908	1053.56	7069158.00	1191.75
Cow 82	981.75	6371111.00	1923.04
Cow 02	1015.28	6756777.00	948.75
Composite (all cows)	1027.46	41548256.00	2658.43

F-Test No. 1:

$$F = \frac{/\sum_{\text{assays}}^{6} (\text{S.S.T.R.}) - \text{S.S.T.R. composite})/ \div DF}{\text{Mean of M.S.R.}} = 2.99$$

F-Test No. 2

$$F = \frac{/\ \text{S.S.M.}\ /}{\text{Mean of M.S.R.}} \div DF = 11.91^{**}$$

F-Test No. 3

$$F = \frac{\sum_{\text{assays}}^{6} (\text{S.S.T.R.}) - (\text{S.S.T.R. composite}) - \text{S.S.M}) \div DF}{\text{Mean of M.S.R.}} = 1.71$$

S.S.T.R. = Sum of squares (total reduction) from Table IV.
M.S.R. = Mean squares (remainder) from Table IV.
S.S.M. = Sum of squares of the means = $6\ 32\ (\bar{Y}i - \bar{\bar{Y}})^2$
$\bar{\bar{Y}}$ = Mean of composite standard curve.
$\bar{Y}i$ = Mean of each standard curve.
M.S.R. = Mean square remainder.
** Significant at ($P < .01$) level.

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